3 Pharmaceuticals in sewage systems and surface waters – status quo

3.1 Introduction

3.1.1 Background

This Chapter summarises new findings and insights relating to the occurrence of pharmaceuticals in the environment. With the introduction of the 'Watch List', which now features several pharmaceuticals (Table 3.1), a quantitative understanding of sources, available dilution and resulting concentrations of pharmaceuticals occurring in the aquatic environment remains important. Surface water measurement campaigns in the partner countries provide a useful 'snapshot' of levels of pharmaceuticals found in environmental waters, whereas waste water treatment plant (WWTP) influent and effluent concentrations, especially in combination with flow data, offer insights into the load discharged into the environment and dilution required to keep environmental concentrations below target levels, should these be set in the future. Sewage sludge is in some countries spread on agricultural land in the interest of nutrient cycling. Effects of pharmaceuticals on grazing animals have been established by Bellingham et al. (2012). Section 3.2 reports on concentrations and loads encountered in the course of our sampling campaigns in WWTP, rivers and sludges, including on the effect of stabilisation treatments on concentrations and partitioning of pharmaceuticals in sludge.

Name of substance/group of substances	CAS number(1)
17-Alpha-ethinylestradiol (EE2)	57-63-6 200-342-2
17-Beta-estradiol (E2), estrone (E1)	50-28-2, 53-16-7
Diclofenac	15307-86-5
Macrolide antibiotics: erythromycin, clarithromycin, azithromycin	114-07-8, 81103-11-9, 83905-01-5

Table 3.1: Pharmaceuticals on the 'Watch List', adapted from EC (2015)

Whilst the introduction of environmental quality standards for single substances, such as via the Directive on Environmental Quality Standards (Directive 2008/105/EC), offers some protection for environment, it does not fully account for the complexity of ecosystems and toxicity effects. Whole sample toxicity testing is complementary to pharmaceutical analysis; it can flag up mixture effects such as concentration additivity and take into account toxicity of unknown metabolites. Section 3.3 reports on ecotoxicity analysis of wastewater and surface water samples. Section 3.4 concerns antibiotic resistant bacteria (ARB); subsequent to our findings in the PILLS project (PILLS, 2012), concerns over ARB have received considerable attention in the press and in public policy. Wastewater, and in particular hospital wastewater, can be a significant source of multi-resistant bacteria (Stalder et al. 2013) and as such constitute a pathway for such organisms into the natural environment.

3.1.2 The sampling campaigns

This section focuses on the sampling campaigns in conventional wastewater treatment plants and surface waters. Hospital sampling campaigns were also conducted; these are mentioned below but reported on in full in Chapter 6.

In Germany, sampling took place at the influent and effluent of centralised WWTP Dülmen on 8 occasions, as well as upstream and downstream from the WWTP in the receiving water, the Tiberbach. A separate sampling campaign was carried out at the dedicated hospital wastewater treatment plant (HWWTP) Marienhospital, which is also described in full in Chapter 6.

In France, the participating HWWTPs is dedicated to Hospital Center of Alpes-Leman (CHAL France), whereas the WWTP treats effluent from the nearby urban area (Figure 3.1). The WWTP and HWWTP are on the same site and have a combined discharge into the river Arve. Influent samples were taken at the discharge of the hospital, from the effluent outlet of the HWWTP, in the urban sewer and after the urban WWTP. In addition, samples were collected from the River Arve upstream and downstream of the treated effluent discharge pipe. Samples were collected on three separate occasions: November 2013, and March & September 2014.

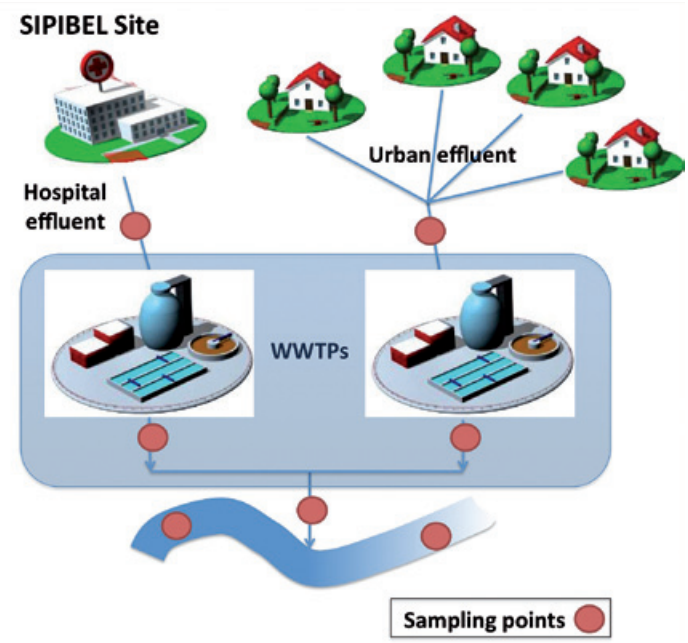

Figure 3.1: Location of sampling points at SIPIBEL Site

In Luxembourg, monitoring of wastewater at the partner hospital Centre Hospitalier Emile Mayrisch (CHEM) and the downstream municipal WWTP Schifflange took place over the time period of 28th April 2014 to 8th June 2014. It was implemented in parallel to a urine separation campaign in radiology department of the CHEM (see chapter 5). The time period was chosen because it was exclusively out of school holiday periods and standard working conditions were expected on the level of the radiology department involved in the urine separation campaign.

In Scotland, sampling took place at the influents and effluents of two WWTP, one using mainly trickling filter technology (TF) and one using mainly conventional activated sludge technology (CAS), and upstream and downstream in the receiving waters. For each WWTP, two 4-day sampling campaigns were undertaken, one in a dry week and one in a wet (rainy) week. In addition, samples were taken from 7 locations in the River Almond catchment on 4 consecutive days to gain an understanding of spatial variation in the catchment.

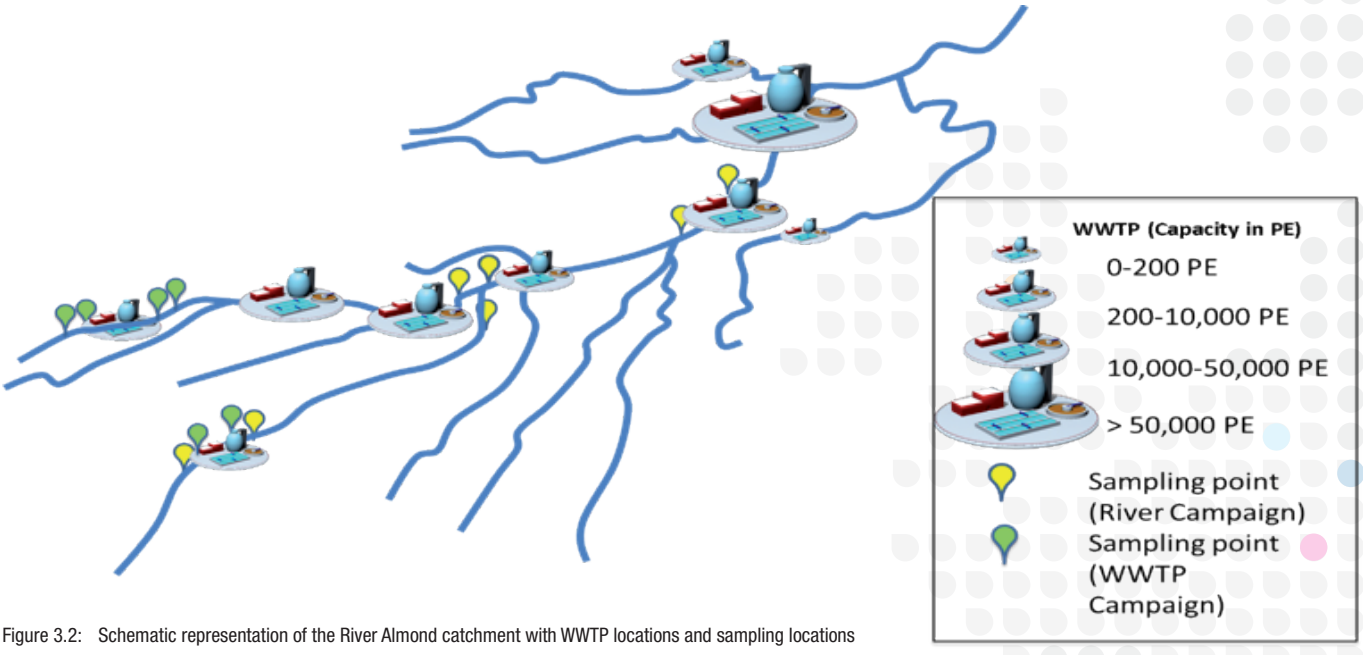

Figure 3.2: Schematic representation of the River Almond catchment with WWTP locations and sampling locations

Table 3.2 gives an overview of participating conventional treatment works.

Participating treatment works	Treatment technology
Luxembourg – WWTP Schifflange	CAS
Germany- WWTP Dülmen	CAS
Scotland – WWTP 1	TF (+ CAS as tertiary treatment for 20% of effluent)
Scotland – WWTP 2	CAS (+ TF as tertiary treatment)
France – WWTP SIPIBEL	CAS

Table 3.2: Participating conventional treatment works

3.2 Loads and concentrations in wastewater, treated effluent, surface water and sludge

3.2.1 WWTP influent and effluent concentrations

A number of pharmaceutical compounds were selected for transnational comparison of occurrence in various environments: atenolol, carbamazepine, ciprofloxacin, clarithromycin, diclofenac, erythromycin, ibuprofen, naproxen and sulfamethoxazole.

Comparing the range of concentrations found at influent and effluent (Figure 3.3), it can be observed that whilst in the influent the analgesics naproxen and ibuprofen dominate, in the effluent erythromycin and diclofenac are found in the highest concentrations. These two compounds also showed the most variation in removal efficiency between the investigated treatment plants. Most of the compounds investigated are present in effluent in ecotoxicologically relevant concentrations. The Predicted No Effect Concentration (PNEC) is a measure of aquatic toxicity and indicated by a red line for each compound in Figure 3.3. It should be noted that PNEC is not the only factor to be considered in determination of safe levels; other issues such as the potential to bioaccumulate and persistence in the environment are also relevant.

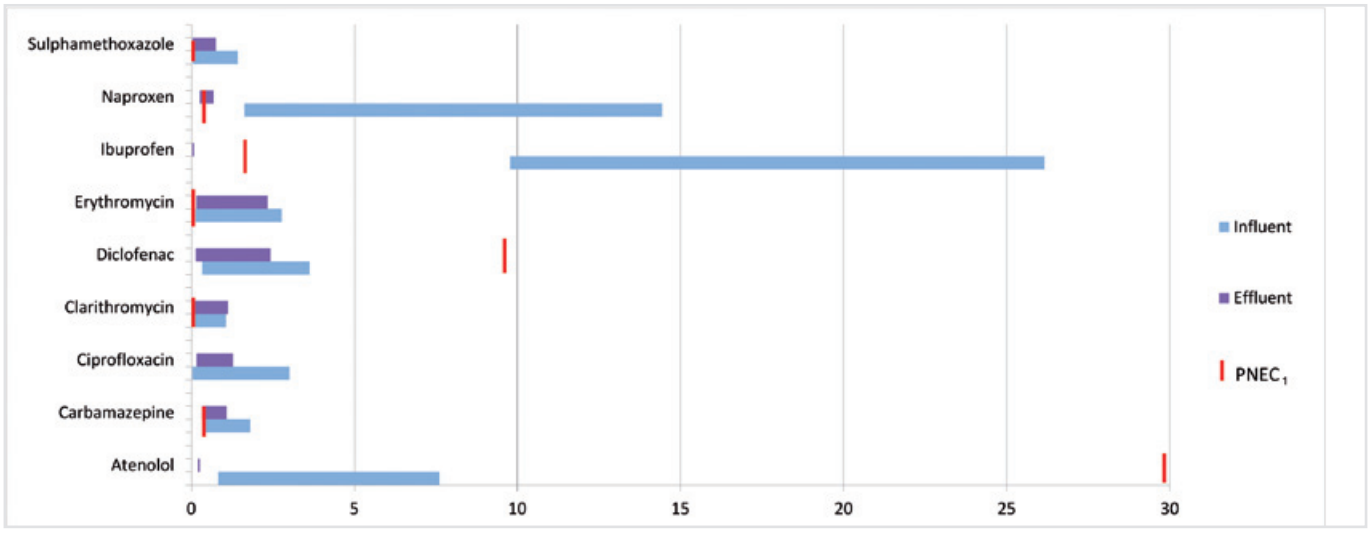

Figure 3.3: Range of influent and effluent mean concentrations (based on mean values at WWTPs in Germany, Luxembourg and Scotland) (µg/l), with indication of Predicted No Effect Concentration (PNEC). PNEC $_1$ values were taken from literature: Atenolol, Clarithromycin and Erythromycin from Boillot (2008), in Verlicchi et al. (2012); Diclofenac from Ra et al. (2008), in Verlicchi et al. (2012); Ibuprofen from Quinn et al. (2008), in Verlicchi et al. (2012); Naproxen and Sulfamethoxazole from FASS Allmänhet (2013); Carbamazepine from Ferrari et al. (2003); Ciprofloxacin from Halling-Sørensen et al. (2000).

A number of other interesting findings emerged:

- Investigating diurnal variation via analysis of two-hour composite samples over a 24 hour period, peaks in the load of specific pharmaceuticals received at a Scottish trickling filter plant (approx. 5000 population equivalent (PE)) appeared to correlate with the pattern of drug administration. A peak load was visible between 8:00 and 10:00 for atenolol, normally taken once a day, whilst three distinct peaks were observed for erythromycin, normally taken three times a day. Untreated, such diurnal variation in discharge rate could lead to short term peaks in river concentrations. However, unless combined sewer overflows are active, the treatment plant will act as a buffer and less variation is expected in effluent. Work on measuring diurnal variation in effluent is ongoing.

- In Luxembourg, amoxicillin, ciprofloxacin, clarithromycin, sulfamethoxazole, lidocaine, diclofenac, naproxen, carbamazepine, iobitridol and iodixanol were all found in every influent and effuent sample at WWTP Schifflange. Similarly, in Scotland, during the 4-day campaign, atenolol, carbamazepine, erythromycin, clarithromycin, lidocaine and Ranitidine were found in all influent and effluent samples at WWTP 1.

- In Scotland, cyclophosphamide, a cytostatic used in the treatment of cancer, was found in influent and effluent samples on one day of the sampling period, despite the fact that no hospital effluent is treated at the WWTP. Although cyclophosphamide is usually administered in hospital, patients will normally go home after treatment and therefore excrete the drug into community wastewater. Cyclophosphamide was not detected in the Scottish hospital wastewater samples during the PILLS project.

- In Luxembourg, for all the substances on the common partner list (amoxicillin, ciprofloxacin, clarithromycin, erythromycin, sulfamethoxazole, diclofenac, naproxen, carbamazepine) significant daily variations of concentrations were observed at all monitoring locations. For carbamazepine, the daily concentrations culminate in the highest concentrations at the end of the week. This is also the case for diclofenac on the level of the WWTP inflow. Although for the other substances clear daily variation of concentrations were observed, they have no recognizable recurring weekly pattern. The widest ranges from maximum to minimum concentration were observed for clarithromycin, diclofenac and naproxen in hospital samples and for amoxicillin and ciprofloxacin for the WWTP influent and effluent samples.

- Of the selected compounds, carbamazepine, lidocaine and clarithromycin are hardly removed in the WWTPs in the study. Erythromycin was moderately removed in the German WWTP but poorly in Luxembourg and Scotland. Diclofenac was moderately removed in Luxembourg and Germany, but somewhat better in France and Scotland. The common analgesics (paracetamol, ibuprofen, naproxen) were all well removed. Comparing removal efficiencies with values in a review paper by Verlicchi et al. (2012), values were generally in good agreement with the literature; however, atenolol and diclofenac were removed better than suggested by the literature whilst clarithromycin and amoxicillin were not removed as well as in previous studies. An overview is provided in Table 3.3, with literature values for comparison.

Poorly removed (<30%)	Moderately removed (30-70%)	Well removed (>70%)
Carbamazepine (18%)	Bezafibrate (61%)	Atenolol (38%)
Clarithromycin (40%)	Ciprofloxacin (70%)	Naproxen (73%)
Erythromycin (26%)	Diclofenac (29%)	Ibuprofen (87%)
Lidocaine	Sulfamethoxazole (52%)	Paracetamol (93%)

Table 3.3: Removal of selected pharmaceuticals in the investigated conventional WWTP (literature value in brackets; from Verlicchi et al., 2012)

Summary:

- Analgesics are generally well removed but, due to their high concentrations in raw sewage, may pose a problem in CSO situations where they bypass treatment.
- A number of other pharmaceuticals are not effectively removed by conventional treatment.

Policy pointers:

- Monitoring of sewage discharges, including those from CSO in wet weather situations, is recommended.
- Current levels of several pharmaceuticals, including macrolide antibiotics, in WWTP effluents in our study were well in excess of Predicted No Effect Concentrations and may pose ecotoxic situations in surface waters unless significant environmental dilution is available.

3.2.2 Concentrations in surface waters

The available dilution by the flow in the receiving water can have a critical effect on whether a discharge results in toxic situations in the river. In Germany, the concentrations downstream from the river were almost the same as the effluent concentrations, indicating the stream has a very low dilution capacity (around 1.2): the Dülmen plant is not the only source of pharmaceuticals in the Tiberbach and many compounds were detected upstream from the WWTP; hence, its capacity to dilute the concentrations in the effluent is limited. However, Erythromycin and Clarithromycin, two of the 'Watch List' compounds, were only detected downstream from the WWTP and for most other compounds downstream concentrations were at least an order of magnitude higher than upstream. Only Ciprofloxacin was not detected in the river at all.

In France, all pharmaceutical compounds analysed were found both upstream and downstream from the WWTP; as expected, concentrations downstream were higher than upstream. The data do not indicate the dilution factor as the ratio between measured effluent and river concentrations varies per compound.

In Scotland, the available dilution for WWTP 1 is low, but higher than in Germany; during dry weather, the dilution factor in Scotland was between 2 and 6. Mean (treated) effluent concentrations in wet weather were around half of those during dry weather and a higher dilution rate was also observed.

The dilution available at the investigated sites in Germany and Scotland is much lower than the default dilution factor of 10, used in the risk assessment method published by the European Medicines Agency (EMEA).

Most pharmaceuticals in rivers, measured in France, Germany and Scotland, are in the high nanogram range, but some – notably Erythromycin and Diclofenac – are present in higher concentrations (Figure 3.4). It is important to consider concentrations in the context of toxicity; especially antibiotics can be toxic at very low (0.05 µg/l) concentrations.

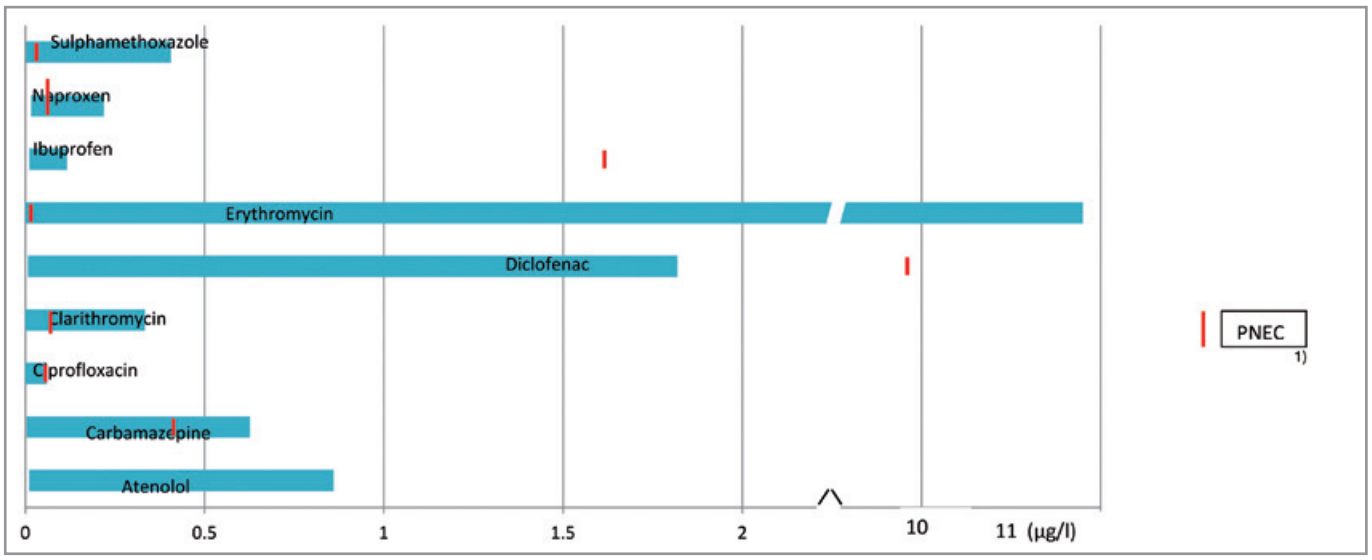

Figure 3.4: Range of mean concentrations in surface waters (based on mean values at single locations in Germany, Scotland and France; total 11 locations)
1) For PNEC value references, see figure 3.3.

The most extensive river monitoring work was carried out in Scotland. The River Almond (West Lothian) catchment is highly urbanised; the river and its tributaries receive effluent from multiple WWTP as well as numerous smaller discharges such as from septic tanks. To investigate spatial variation, daily grab samples were taken at seven locations in the upper and middle sections of the catchment. Eleven investigated compounds were detected at all but one locations, at concentrations mostly in the high ng/l range but up to 14 µg/l (erythromycin), indicating these compounds are ubiquitous in the catchment. Four of these, ciprofloxacin, ibuprofen, and the two macrolide antibiotics erythromycin and clarithromycin recently added to the Watch List were consistently found at toxicologically relevant concentrations in several locations. Some compounds were detected in a small tributary upstream from any WWTP input, and, comparing two locations 10km apart with no WWTP effluent inputs in between, several compounds were detected at similar or even higher concentrations at the location 10 km downstream. Although further research is necessary, these results suggest that non-WWTW discharges (e.g. septic tanks, veterinary sources) may not be negligible as contributors to overall levels of pharmaceuticals in this small stream.

For one location in Scotland, the daily load was calculated from measured concentration and flow, using NHS prescription data, taking excretion and removal efficiencies from literature (Table 3.4). Despite some limitations (removal values from literature were not available for TF technology so CAS removal efficiencies were used; measured values were based on grab samples only), measured values were within a factor 3 of predicted values.

Of all the WWTP discharging into the investigated parts of the catchment, only the furthest downstream receives hospital effluent. Despite this, there was no clear change in the range or concentrations of pharmaceuticals detected downstream from this WWTP compared to those detected in locations further upstream, which contain effluent from non-hospital sources.

	Expected daily load in river in the Breich Water tributary (downstream of WWTP), Scotland (mg/day)	Measured daily load (mg/day)
Atenolol	4404	3802
Bezafibrate	285	133
Carbamazepine	195	462
Clarithromycin	916	503
Lidocaine	nd[a]	216

Table 3.4: Comparison with predicted concentrations.
a: due to uncertainty over both the route of administration and the amount sold over the counter for Lidocaine, no predicted value could be calculated

Summary:

- Pharmaceuticals are ubiquitously present in the environment.
- Some, including macrolide antibiotics, are present in ecotoxicologically relevant concentrations.
- A clear increase in concentrations is observed after sewage effluent enters the river.
- The available environmental dilution is an important factor in the risk ensuing from effluent concentrations; where multiple discharges enter the same surface water the dilution capacity can be less than suggested by flow volumes.

Policy pointers:

- There are indications that non-WWTP sources may contribute significantly to pharmaceutical loads in the aquatic environment. Further research is needed to verify this and to determine the relevance of other sources, as actions to upgrade WWTP may not always be sufficient to protect the environment.
- As our measurements indicate that some of the macrolide antibiotics on the 'Watch list' are present in sufficient quantities to pose an actual environmental risk, more extensive monitoring of these compounds is recommended.
- Risk assessments should where possible consider realistic available dilution and take account of multiple inputs as cumulative loads.

3.2.3 Concentration in biological sludge and impact of stabilization treatment on the fate of pharmaceutical compounds in hospital sludge

Removal pharmaceutical in biological processes could be due to volatilisation, biodegradation and sorption on sludge. In this last case, pharmaceuticals are still present at variable concentration and could contaminate soils in case of agricultural application. Via soils, compounds could furthermore enter groundwater or surface waters (Lachassagne, 2014). It is then important to know the concentrations and the stability of pharmaceuticals during sludge stabilisation processes, before land spreading.

The behaviour of 11 pharmaceutical compounds was investigated during the treatment of sludge from hospital wastewater (SIPIBEL France): carbamazepine* (CBZ), ciprofloxacin* (CIP), sulfamethoxazole* (SMX), salicylic acid (SAL), ibuprofen (IBU), paracetamol (PAR), diclofenac* (DIC), ketoprofen (KTP), econazole (ECZ), atenolol (ATN) and propranolol (PRP). Thickened activated sludge was subjected to two different stabilisation treatments: anaerobic digestion and liming, before lab scale agricultural application (Figure 3.5). Modification of biochemical properties of sludge after stabilization are reported in Table 3.5.

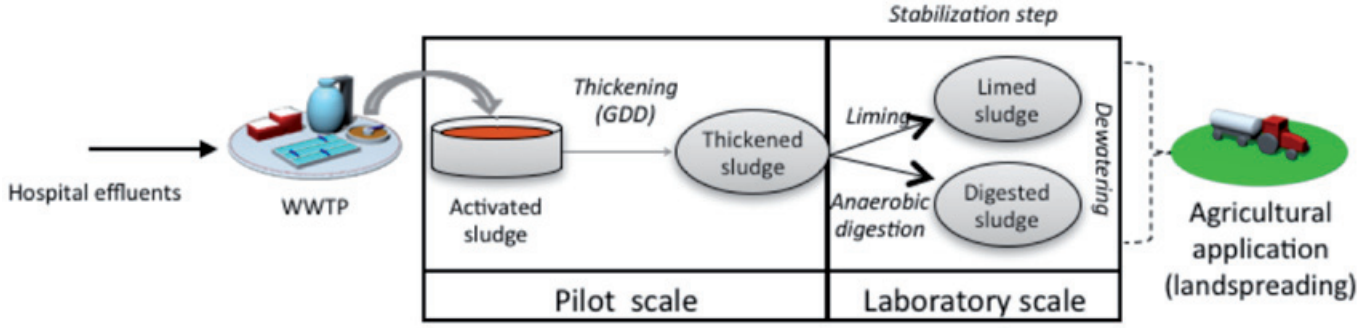

Figure 3.5: Different stages of hospital sludge treatment, stabilization and application (GDD: drip grid)

Liming	Anaerobic digestion
• The protein concentration is higher in the soluble fraction of the limed sludge, probably due to cell lysis of the microorganisms present in the sludge due to the pH increase taking place during the liming.	• Digested sludge was mainly constituted of humic-like substances. • The soluble fraction was mainly composed of carboxyl groups and the particulate fraction of phosphoric and amine groups. • Phase distribution of pharmaceutical compounds showed that carbamazepine and ibuprofen were mainly in the soluble fraction, so could be more available after landspreading. • Sulfamethoxazole was the only compound removed during anaerobic digestion.

Table 3.5: Summary of the effects of stabilization steps on biochemical composition of hospital sludge.

1 * noPills substances

Figure 3.6 shows that the concentrations of pharmaceutical compounds in the sludge after stabilization by liming or anaerobic digestion were very different depending on the specific compound. Whatever treatment applied, among these molecules, Ciprofloxacin had the highest concentration in the sludge, whilst econazole had the second highest concentrations. Ciprofloxacin concentrations are not shown; they vary between 4.05 and 1.5 during liming and between 4.05 and 1.0 during anaerobic digestion)

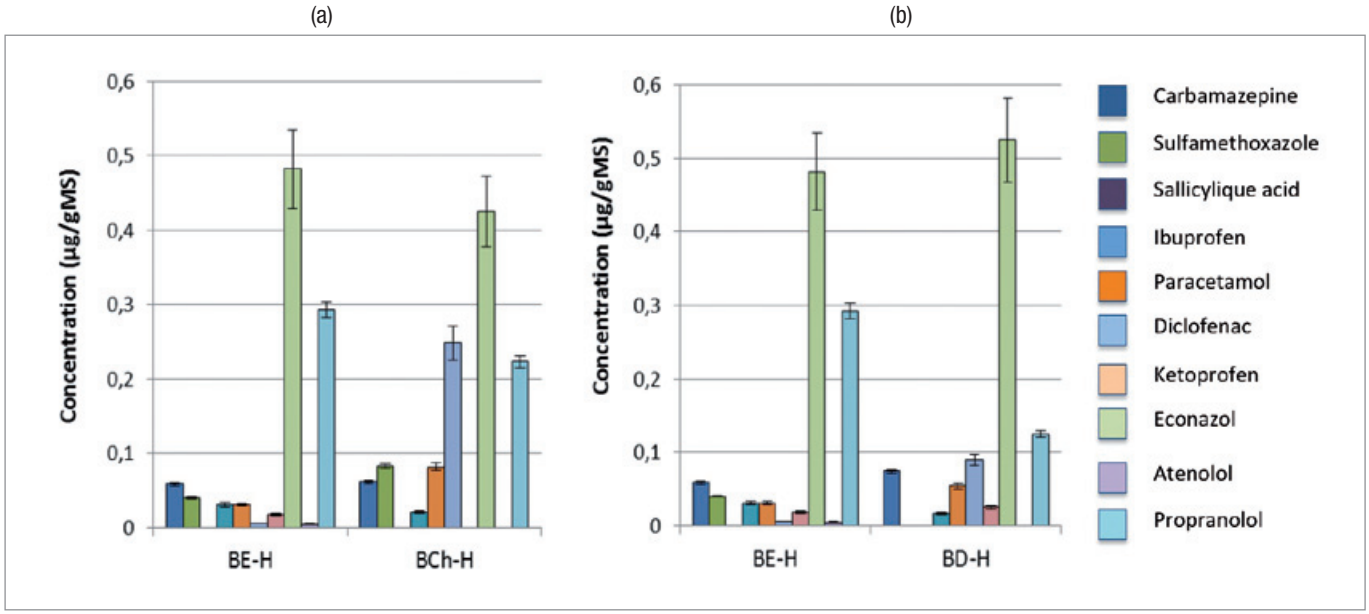

Figure 3.6: Evolution of pharmaceutical compounds concentrations during hospital sludge stabilization processes: liming (a) and anaerobic digestion (b). BE-H : hospital Thickened Sludge, BCh-H : Hospital limed Sludge, BD-H: Hospital Digested Sludge. The concentrations are expressed in µg/gTS.

Organic micropollutants behaviour during sludge treatment is linked to specific interactions between functional groups of sludge structure and those of the compounds. The pKa of functional groups such as carboxyl, amine, phosphate and hydroxyl characterises these interactions, which are partially responsible for the sorption of pharmaceutical compounds onto sludge.

Proton binding site concentrations and corresponding pKa values were assessed in soluble and particulate fractions by a combination of potentiometric tritrations. Activated, thickened, limed and digested sludges, showed four groups of pKa values in particulate and soluble fractions, which can be attributed to the following functional groups of components: pKa1 and pKa2 to carboxylic group, pKa3 to phosphoric group and pKa4 can be attributed to amine and/or hydroxyl groups.

The functional group distribution in the particulate fraction of activated, thickened and digested sludges was similar, except for the carboxyl group distribution which was lower for the particulate fraction of digested sludge. In the soluble fraction, the distribution of each group of components was different between the three kinds of sludge. Indeed, the distribution of carboxyl groups was less important for thickened sludge (10 %) than for activated (50 %) or digested (65 %) sludge. Regarding digested sludge, the distribution of carboxyl groups was more important in the soluble fraction. Carboxyl groups can be linked to proteins, humic-like substances and uronic acids. Amine groups were mainly present in proteins whereas hydroxyl groups originate essentially from polysaccharides and humic-like substances.

The two different stabilisation treatments have different effects on the partitioning of the pharmaceutical compounds in the sludge. The phase distribution of pharmaceutical compounds in soluble and particulate fractions of hospital sludge after stabilization was determined and presented in table 3.6. Sludge stabilization treatment (liming or anaerobic digestion) processes did not lead to a complete elimination of pharmaceutical compounds; only phase distribution of compounds changed between the two parts of the sludge during the treatment.

Compound	Limed hospital sludge			Digested Hospital Sludge		
	% particulate	% soluble	$Kd_{sorption}$ (L/kg)	% particulate	% soluble	$Kd_{sorption}$ (L/kg)
Carbamazepine	51.2	48.8	36.9	47	53	50.4
Ciprofloxacin	80.3	19.7	143	92.5	7.5	698
Sulfamethoxazole	100	0	8265[b]	N.d.[a]	N.d.[a]	N.d.[a]
Salicylic Acid	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]
Ibuprofen	0	100	0	0	100	0
Paracetamol	100	0	4065[b]	100	0	2643[b]
Diclofenac	70.5	29.5	84	0	100	0
Ketoprofen	N.d.[a]	N.d.[a]	N.d.[a]	0	100	1193[b]
Econazole	100	0	42 465[b]	100	0	52 443[b]
Atenolol	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]	N.d.[a]
Propranolol	100	0	743 298[b]	57.7	42.3	77.6

Table 3.6: Particulate-soluble pharmaceutical compounds repartition and Kdsorption values for limed and anaerobically digested hospital sludge
a: N.d = Not determined, because the compound was not detected in the total sludge
b: $Kd_{sorption}$ is maximum (even infinite). In those cases where the concentration in the soluble phase is less than the detection limit, the value of the detection limit was used for calculation.

Regarding phase distribution and stabilization process, different behaviours for all compounds are summarized table 3.7.

Liming	Anaerobic digestion
Pharmaceutical compounds were present at concentrations less than 0.5 µg/gTS with the exception of ciprofloxacin. Overall, liming causes a reduction of the drug content, except for sulfamethoxazole, diclofenac (hospital sludge) and econazole . Regarding phase distribution, differences in behaviour between all these compounds was observed. Carbamazepine was equally distributed in the soluble and particulate fractions of sludge. Paracetamol, econazole, propranolol and sulfamethoxazole were mainly in the particulate fraction, whereas ibuprofen was mainly in the soluble fraction.	The drug concentrations are less than 0.5 µg/gTS, except for salicylic acid which is present at a concentration of 1.2µg/gTS in urban sludge. Sulfamethoxazole was the only compound that was completely disappearing after anaerobic digestion while carbamazepine was still present after treatment. In digested sludge, all the ibuprofen was present in the soluble fraction. This compound could be more likely desorbed into the soil if the sludge is used for landspreading. Carbamazepine and propranolol were equally distributed between the particulate and soluble fractions, Ciprofloxacin, paracetamol and econazole were mostly in the particulate fraction, and ibuprofen, Diclofenac and ketoprofen were mainly in the soluble fraction.

Table 3.7: Impact of sludge stabilization treatment on pharmaceutical phase distribution

The organic compounds (in this case pharmaceuticals) are sorbed to sludge partly by hydrophobic type interactions, but mainly by electrostatic interactions. Microorganisms present in the sludge have a negative surface charge and act as cation exchangers, which causes a strong interaction between the micro-organisms' surface and positively charged compounds at the typical pH of sludge, such as carbamazepine or atenolol. However, it appears that hydrophobic interactions play a role for the positively charged compounds. In addition, at a typical pH for wastewater, compounds having a high log Kow, such as diclofenac and ketoprofen, are mainly negatively charged (ionized form) and will tend to be present in the aqueous phase, whereas compounds having a low logKow are mainly present in the particulate phase (Lachassagne, 2014).

In conclusion, hydrophobicity (log Kow) cannot by itself explain the sorption behaviour of sludge and the soluble / particle distribution of micropollutants. The functional groups present in sludge at each stage of processing also play an important role in the interactions.

Summary:

- Pharmaceuticals are partly sorbed to sludge by hydrophobic type interactions, but mainly by electrostatic interactions (Lachassagne, 2014).
- Stabilisation processes during sludge treatment could modify these interactions depending on the process. Molecules can then become available and can reach water bodies.

Policy pointers:

- Potential contamination of sludge during biological treatment and stability of sorption has to be considered in the overall balance of removal and in decision making on the use of sludge.

3.3 Environmental ecotoxicity evaluation

3.3.1 Introduction

When chemical compounds are developed to enter the EU market, their potential fate and effect in the environment is assessed under the EU REACH regulation. The testing is focused on evaluating the toxic effects on humans and ecosystems, and their fate in the environment: persistence and bioaccumulation in the food chain. When chemicals are very toxic, or are not degraded in the environment, leading to increasing environmental concentrations, or when they accumulate in the food chain, leading to high concentrations in the top predators, measures to prevent release of the chemicals into the aquatic environment may be required or the marketing authorisation can be denied. As some of the most potent pharmaceuticals may be used in low doses, total tonnage may be below REACH thresholds. Furthermore, if an environmental risk for pharmaceuticals is identified, certain mitigation proposals may be required, but a marketing authorisation will not be denied (BIO Intelligence Service, 2013). Pharmaceutical residues enter the environment, either as a result of excretion from the human body, or as a result of discharge of medicine waste, and can include very toxic (e.g. cytostatics) or very persistent (e.g. X-ray contrast agents) compounds.

Although pharmaceuticals are produced to heal humans, Paracelsus knew already in the 15th century that "Dosis facit venenum", "The dose makes the poison". If the concentration of a medicinal compound in a body is too high, it will act as a toxic compound. This is the same in the environment where the wide range of creatures exposed will respond differently, thus it is important to evaluate the toxicity of pollutants or polluted environments with a range of test organisms. We know that the toxic dose of one compound for different environmental organisms may vary by more than a factor 1000; in general smaller organisms are more sensitive than bigger organisms due to their larger surface-to-volume ratio. When determining the environmental toxicity of a drug the mode of action should also be considered as the target receptors and enzymes may affect different species in different ways. Furthermore, the effects of long term exposure to a compound may appear at lower concentrations than a one-off exposure to a high environmental concentration that disappears quickly.

Whole sample ecotoxicity testing exposes test organisms to the mixture of all chemicals present in the sample. Toxicities of individual compounds may be synergistic or antagonistic; whole effluent toxicity is almost impossible to predict as an ever-changing mixture of thousands of compounds is present in sewage effluent. Mixture toxicity has been investigated for few compounds only (e.g. Christensen et al., 2007; Cleuvers, 2004). Ecotoxicity testing as described below therefore offers vital complementary data to the chemical analytical data on single pharmaceutical concentrations.

3.3.2 Ecotoxicity testing

The ecotoxicity of collected wastewater samples was assessed using a battery of tests (Table 3.8).

Country/ Evaluation	Scotland		France	
Acute toxicity	Bacteria Algae Fish	*Aliivibrio fischeri* (ISO 1348-3) *Raphidocelis subcapitata* *Danio rerio*	Crustacean	*Daphnia magna* (ISO 6341)
Chronic toxicity	Fish	*Danio rerio*	Algae Crustacean Rotifer	*Pseudokirchneriella subcapitata* (ISO 8692) *Heterocypris incongruens* (ISO 14371) *Brachionus calyciflorus* (ISO 20666)
Genotoxicity			Bacteria Mammalian cells	SOS chromotest single cell comet assay
Mutagenicity	Fish	*Danio rerio*	Fish	*Danio rerio*
Endocrine disruptors			Human cell line	Estrogenic activity (MELN cell line)

Table 3.8: Test organisms utilized during the evaluation of wastewater samples plus relevant ISO standard followed or in-house protocols followed.

3.3.3 Outcomes

Scotland

Of the 99 samples evaluated using the inhibition of *Aliivibrio fisheri* luminescence, 45 % were defined as acutely toxic and 55 % as not acutely toxic (i.e. where there was no decrease in relative light units after 30 mins). The maximum inhibition recorded was 28 %, in WWTP influent. Thirty five percent of the samples were considered as toxic to *Danio rerio* embryos as judged by mortality (Table 3.9). Pre-concentration of the samples utilizing freeze-drying as the enhancement step continues to be investigated. Toxicity evaluation utilizing algae is still on-going.

Location	**Luminescent bacteria** *(Aliivibrio fischeri)*	**Zebrafish** *(Danio rerio)*
River	21.9	21.9
WWTP Influent	72.7	45.5
WWTP Effluent	36.4	36.4

Table 3.9: Percentage of samples defined as being toxic to the test organism. Collated data for 2 treatment works, $n_{total} = 11$.

Of the two WWTP's monitored, the trickling filter treatment facility yielded the largest number of acutely toxic samples compared with the activated sludge treatment facility (Table 3.10). This observation can partially be accounted for the increased toxicity of the influent samples reaching the trickling filter facility compared to those entering the activated sludge treatment facility.

Primary sewage treatment	**Sampling Location**	**Number of samples (n)**	**Luminescent bacteria** *(Aliivibrio fischeri)*	**Zebrafish** *(Danio rerio)*
	Weather condition: low rainfall (total 5.6mm TF; 5.5mm AS during campaign)			
Trickling filter	Influent	4	100.0	25.0
	Effluent	4	50.0	50.0
Activated sludge	Influent	3	33.3	0.0
	Effluent	3	0.0	0.0
	Weather condition: high rainfall (total 9.1mm TF during campaign)			
Trickling filter	Influent	4	75.0	75.0
	Effluent	4	50.0	50.0

Table 3.10: Effect of treatment within WWTP and of rainfall on samples defined as being toxic to the test organism (percentage of samples).

France

A range of ecotoxicity assays were used to characterize the environmental impacts of a samples entering and leaving the WWTP associated with the monitored hospital (Table 3.11). The toxicity of the hospital effluent changed with time, with the spring 2014 sample being considered the most toxic. The whole organism toxicity (either acute or chronic) and the endocrine disruptor evaluation appeared to be the useful measures, however, to characterize the environmental impacts of a sample of water a battery of assays are required. A major reduction in the ecotoxicity of the effluent was noted after treatment.

Assessment	Outcome measure	Hospital effluent	After WWTP	Hospital effluent	After WWTP	Hospital effluent	After WWTP
		November 2013		March 2014		September 2014	
Acute toxicity							
Crustaceans *Daphnia magna*	EC_{50} (%)	56.6	>90	8.3	>90	56.6	>90
Chronic toxicity							
Freshwater Algae *Pseudokirchneriella subcapitata*	EC_{20} (%)	19.9	>80	15.7	68.7	19.9	>80
Rotifer *Brachionus calyciflorus*	EC_{20} (%)	61.5	100	6.8	100	61.5	100
Ostracode *Heterocypris incongruens*	Growth inhibition (%)	39.9	0	59.0	0	39.9	0
Genotoxicity & Mutagenicity							
Comet assay	Tail DNA (%)	NS	NS	NS	NS	NS	NS
SOS chromotest	Induction factor	2.0	1.5	1.7	1.8	2.0	1.5
Micronucleus	number of nuclei	14.0	1.7	25.0	4.0	14.0	1.7
Endocrine disruptors							
Thyroid hormone	ng/l EqT3	NS	NS	NS	NS	NS	NS
Estrogens	ng/l EqE2	30.5	0.14	14.0	0.12	30.5	0.14
Estrogens	ng/l EqE2	30.5	0.14	14.0	0.12	30.5	0.14

NS:- not significant

Table 3.11: EC50 concentrations indicating ecotoxicity of the hospital effluent before and after the WWTP (as percentage of the concentration measured in the sample)

The tests used were not sensitive enough to measure neither the background toxicity nor the impact of the effluent in the river Arve due to dilution of toxic compounds. Only the assessment of the chronic ecotoxicity using ostracode and rotifer and the evaluation of the endocrine disruptors yielded measurable results during two of the three monitoring periods.

Summary:

- Conventional WWTP are effective in reducing ecotoxicity levels but some toxicity remains.
- The most toxic WWTP effluent was that of the Trickling Filter plant. This may be partly ascribed to high influent concentrations.
- Over 20% of Scottish river samples were acutely toxic to aquatic organisms, indicating high pollution levels. However, it must be noted that it is not certain that the toxicity is due to pharmaceutical content.

Policy pointers:

- Research into the pharmaceutical contribution to toxic effects in surface waters is recommended.
- Research on ecotoxicological tests has to be improved to define the most relevant environmental impact(s) for monitoring.
- It is recommended that ways to assess whole effluent ecotoxicity (such as e.g. via biomarkers), should be considered for possible future standards, in order to account for full complexity of the mixture.

3.4 Antibiotic Resistance

3.4.1 Introduction

The discovery and use of antibiotics in modern medicine has undoubtedly contributed to the increase in life expectancy observed in the latter part of the 20th century. However, from the 1940s, the first cases of resistant strains were identified (sulfonamides 1939, penicillin 1941). The occurrence of these strains has resulted in the design of new molecules, but this forward march reaches its limit with the increase of resistant bacteria. The consequences are increased morbidity and mortality (estimated 25,000 deaths / year in Europe) but also the associated costs (additional cost 1.5 billion € / year) (Chomarat et al. 2014). Thus, control of antibiotic resistance in hospitals as well as in the community, has become a priority issue in public health in many industrialized countries and a priority for the World Health Organisation (WHO, 2015).

The emergence of antibiotic resistance phenomena is related to adaptive pressure process of germs to the presence of antibiotics. These phenomena are mostly due to horizontal transfer of genes, by exchange of mobile elements (plasmids, transposons, integrons) (Stokes and Gillings, 2011; Buckley, 2009) and via different phenomena (transformation, conjugation, transduction). This horizontal gene transfer probably occurs in all terrestrial ecosystems colonized by bacteria.

In recent years, particularly since the end of the European research program "Pills", the consideration of resistant bacteria carried by wastewater effluent, even treated, or hospital effluents, increased, with concern about the dissemination of bacterial resistance, and gene transfers that may accompany it. A significant number of publication states the presence of the Antibiotic Resistant Bacteria (ARB) along an aquatic continuum or watershed (Allen et al., 2010; Baquero et al., 2008; Wright et al., 2007; Schwartz et al., 2003; Novo et al., 2010).

The results in this study come from French locations. Antibiotic consumption in France remains above average in Europe and the United States. Between 2000 and 2013, antibiotic consumption declined by 10.7%, but increased by 5.9% since 2010 with 32.3 Defined Daily Doses /1000 Inh/Day. In terms of volume, over 90% of consumption of antibiotics is in the community and slightly less than 10% in the hospital. Exposure to antibiotics is high hospitals; on any given day about 4 out of 10 patients receive a dose of antibiotics (ANSM- French National Agency for Medicines Safety, 2014).

3.4.2 Determination of Antibiotic Resistant Bacteria

One of the difficulties in the analysis of antibiotic resistance is the choice of the method of determination, and, especially as the matrix in which occurs this research is complex (e.g. effluents, manure, soil). It is now recognized, and Pills program has contributed to this, that the search for Resistance Integrons (RI) is an approach contributing to an overall reliable and relatively simple estimation of antibiotic resistance. RI are genetic elements involved in acquisition, storing, and expression of antibiotic resistance genes embedded within a gene cassette, composed of a intI gene encoding an integrase protein, a specific recombination site attI, and a promoter, Pc. These RI are not self-transposable elements but are often located on plasmids or transposons, which promote their dissemination among bacteria.

Thus, the assessment of the amount of integration (concentration or relative abundance) is able to quantify and/or qualify the occurrence of antibiotic resistance, by molecular biology methods. The quantification of integrons was done in the same manner and with the same developed method as in Pills project (PILLS, 2012; Stalder et al., 2014).

All results are expressed either in concentration, representing the prevalence of RI in a given bacterial population, or in relative abundance, corresponding to the RI concentration divided by the estimated number of bacteria (calculated by dividing the number of 16S-rRNA-encoding gene per the average quantity of 16S-rRNA-encoding-gene per bacteria (4.1 gene per bacteria)).

The different samples collected from the different sites during the Pills and noPills programs clearly showed the specificity of hospital effluents compared to urban effluent, to other anthropic effluent, and to natural water (figure 3.7B). This is especially true if we consider the Relative Abundance (figure 3.7A).

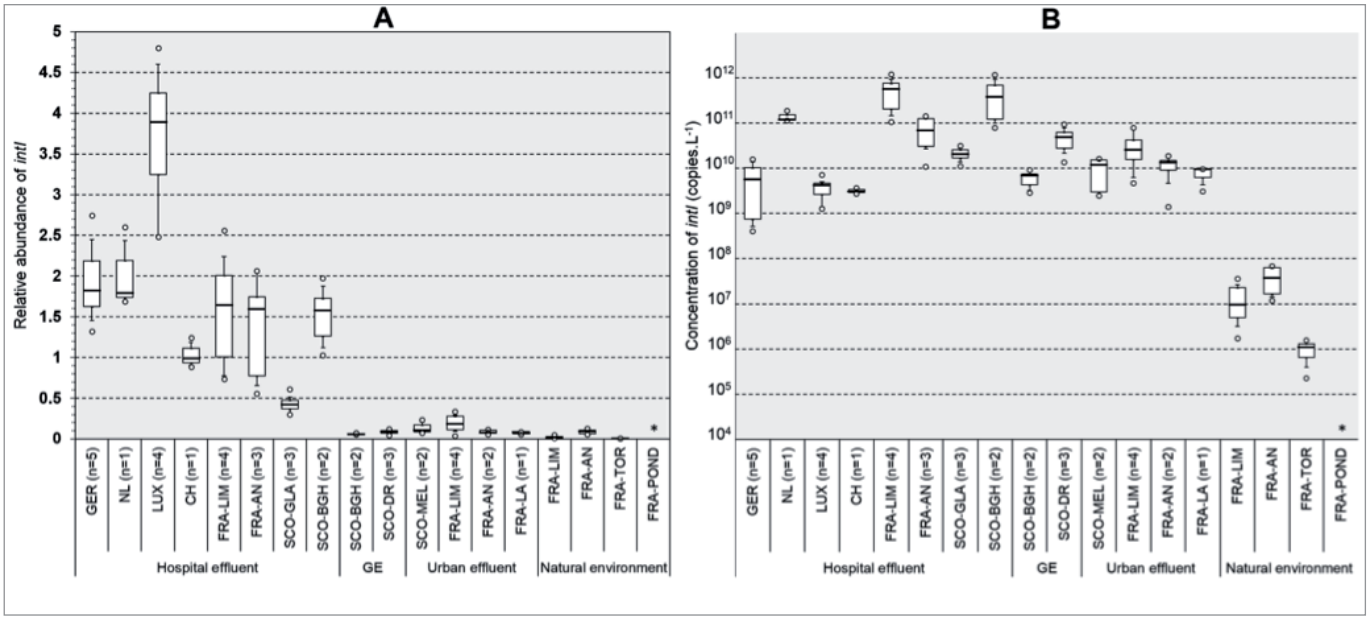

Figure 3.7: Relative abundance and concentration of Resistance Integrons in various samples (GER Germany, NL Nederland, SCO Scotland, FRA France, LIM Limoges, AN Annemasse, GLA Glasgow, TOR River.

3.4.3 Monitoring ARB

The pilot site of Bellecombe (SIPIBEL)
Located on the department of Haute-Savoie (Figure 3.8), near the Swiss border, the pilot site (described in Chapter 3.1) consists of:

- The Hospital Center of Alps Leman (CHAL) commissioned in February 2012, with a capacity of 450 beds;
- A wastewater treatment plant (WWTP) of Bellecombe with two separate processing lines one for the urban effluent, one for the CHAL, closed to the WWTP;
- A receiving water: Arve River, which supplies water for human consumption in Geneva.

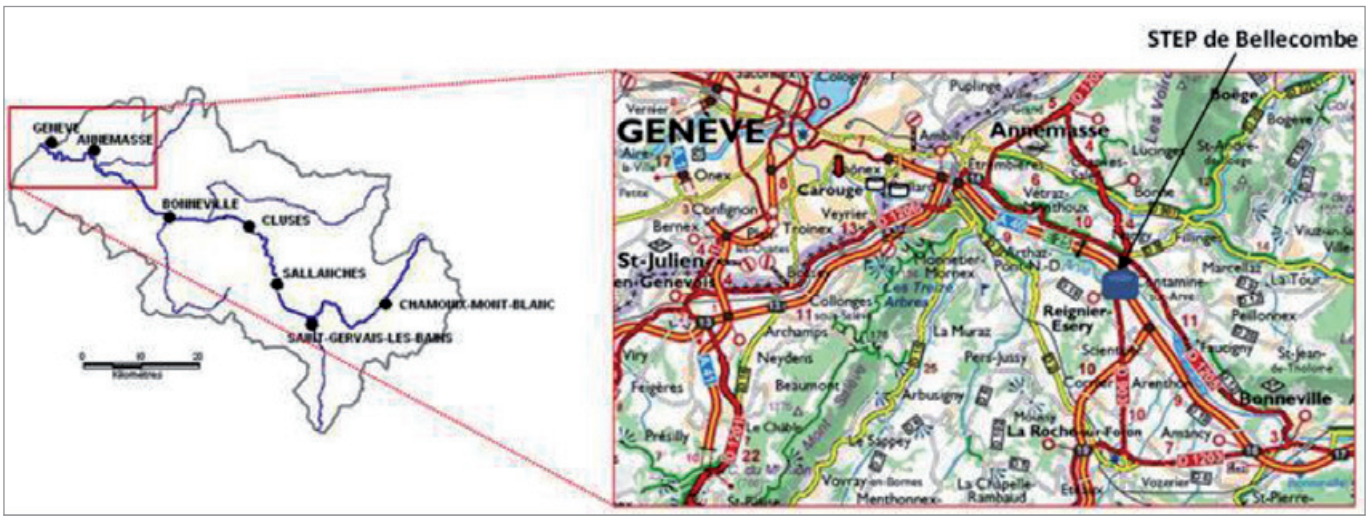

Figure 3.8: Localisation of SIPIBEL

A biological treatment system of activated sludge for 5400 population equivalent (PE) is dedicated exclusively to the treatment of hospital wastewater.

Prior to the opening of the facility in 2012, effluent samples discharging into the river were analysed.

Dynamic evolution on the investigated catchment area

Resistance Integrons (RI) were monitored and Relative abundance (RA) calculated during 3 years on SIPIBEL. Regarding the relative abundance, the cumulative results showed that:

The RA in the effluent discharged by the hospital was significantly higher than those of the urban effluent (figure 3.9), however the data was highly variable.

As in the last study, RA in urban wastewater was very low and statistically equal to those of the river, even downstream.

The wastewater treatment plant treating the hospital effluent showed a significant decrease in RI. This is likely due to a conventional removal of the number of bacteria (2-3 log), but for hospital effluent, these bacteria were multi-resistant.

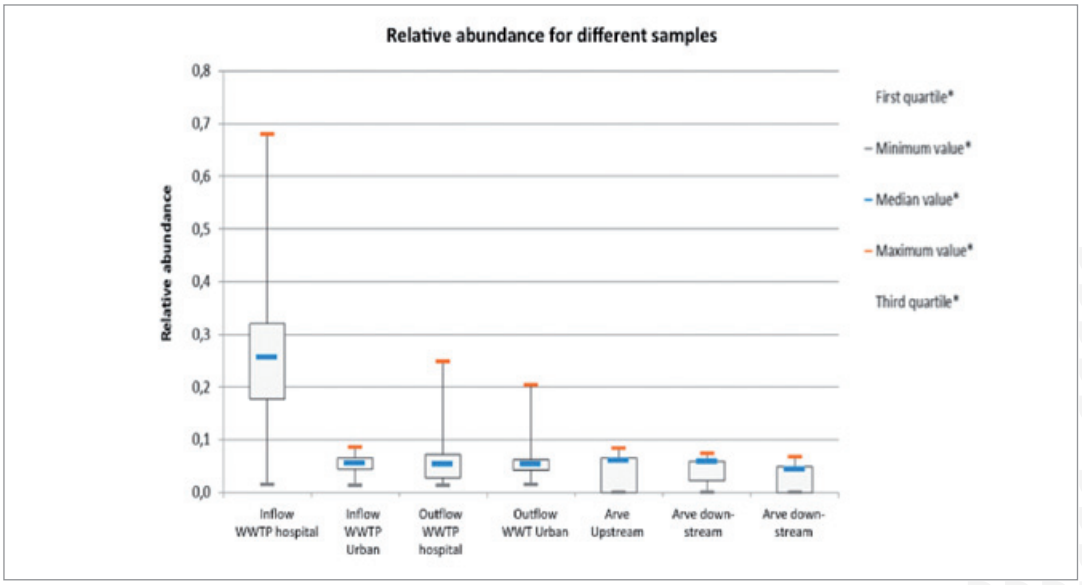

Figure 3.9: RA in different samples: influent and effluent of the urban and hospital WWTPs and Arve river.

The Bray-Curtis similarity index was used to analyse qualitatively the similarity between samples in terms of both gene cassette diversity and gene cassette arrays. We found (Figure 3.10) that the urban effluent and WWTP influent were most similar, while the hospital effluent and the recirculation sludge exhibited very specific patterns, showing the specificity of hospital effluent in term of resistance to antibiotics.

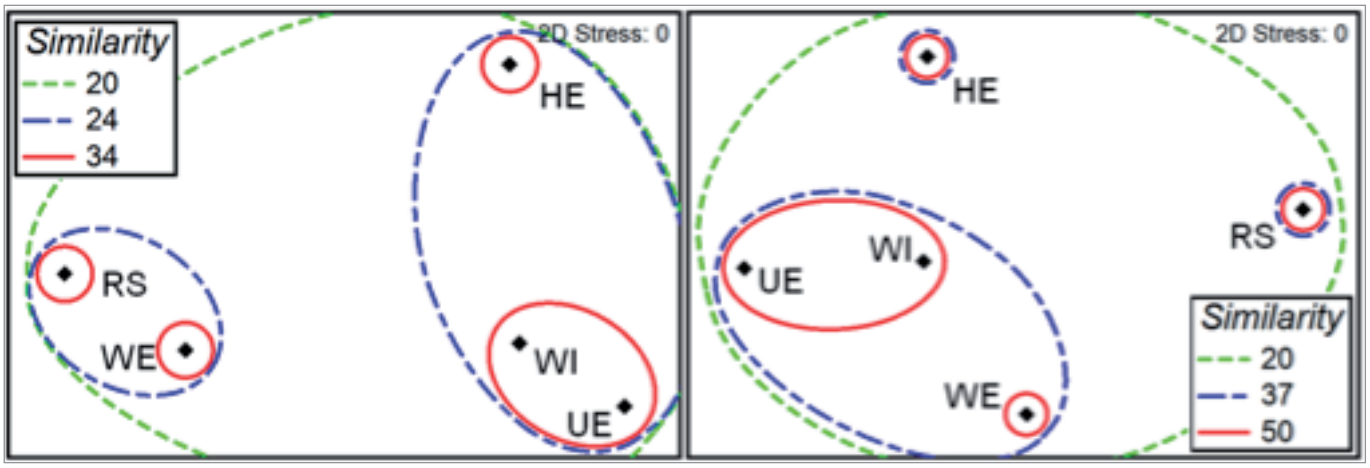

Figure 3.10: Index of Bray-Curtis for (A) the gene cassettes diversity, and (B) the gene cassettes pool. UE, urban effluent, HE hospital effluent, WI, influent WWTP, WE effluent WWTP, RS, sludge.

The evolution of RI and RA in the hospital effluent before and after treatment is reported in Figure 3.11 and compared to the urban effluent at the same time, and over a three year period.

It is noted that the evolution of RI and especially of the RA is constantly higher in the effluent from the hospital than in the urban effluent. It is confirmed that the output values of the two treatment plants, urban or hospital, are

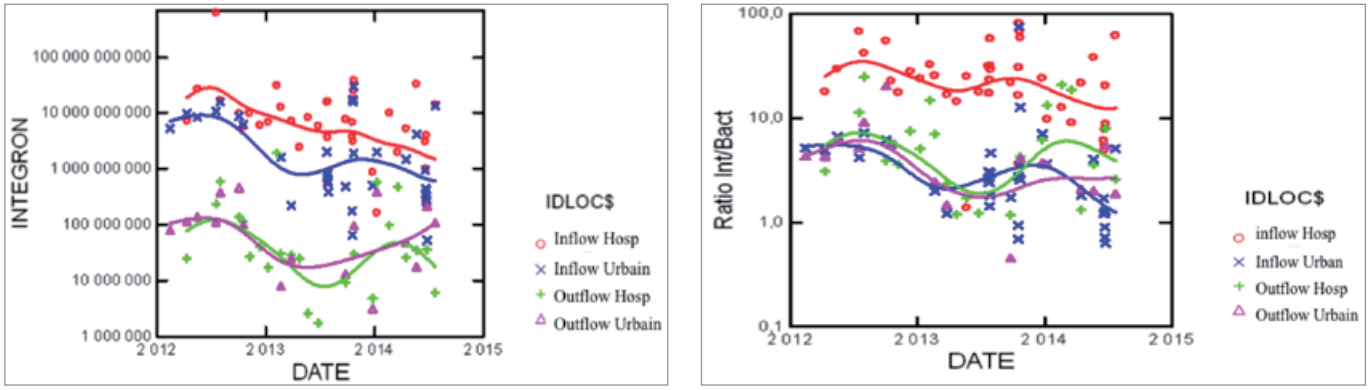

Figure 3.11: Concentration of RI (2) and RA (1) during the noPills Programme

statistically comparable during the entire time of the experiment. One diminution is relatively standard compared to the bacterial elimination in a WWTP (2 to 3 log). The number of RI spread into the environment from a wastewater treatment plant is approximately proportional to the bacterial content and similar between hospital and urban effluents.

Summary:

- Sewers collect wastewater, which comes from homes or care centres, and may contain a resistant bacteria load. The relative abundance of resistant bacteria in a hospital effluent is higher than in an urban effluent.
- The quantification of integrons and relative abundance could be a method to evaluate an overall resistance before a specific identification with molecular technique.

Policy pointers:

- The fight against antibiotic resistance requires a range of approaches, which could include:
  - The standardization of quantification methods
  - The definition of indicators to monitor ARB –such as integrons used in this study
  - The definition of a methodology for risk assessment
  - The evaluation of gene transfers in anthropic systems
- Control of resistant bacteria at source could play a role in maintaining effectiveness of antibiotic treatments.
- Fundamental research of resistant bacteria and gene transfer is recommended.

3.4.4 Concluding remarks

Worldwide, national governments have embarked on numerous initiatives to reduce risks from antibiotic resistance, e.g.:

- French 'Roadmap 2015' of the Ministry of Ecology "…on reducing health risks by assigning a expert mission to ANSES (French Agency for Food, Environmental and Occupational Health & Safety)
- French Ministry of Health coordinated the preparation of a technical guide "for waste management (from drugs – liquids) by the health and social service institutions" to be published in 2015.
- UK Department of Health Antimicrobial stewardship initiative (DOH, 2011)
- Key measures proposed by the European COST TD 0803 (see Berendonk et al, 2015)
- The United States of America proposed "a national action plan for combating antibiotic-resistant bacteria" (TWH, 2014)
- At EU level, macrolide antibiotics have been added to the 'Watch list' (erythromycin clarithromycin, azithromycin) (European Commission, 2015)

Areas of research and development include the development of rapid diagnostic techniques, the development of new antibiotic drugs, improvements in waste and wastewater management, and understanding and control of pathways of resistance. Many initiatives have been undertaken, but given the potential crisis to come, much research and development remains to be done to protect public health.