



Compartmentalization of class 1 integrons and IncP-1 plasmids in the Orne river (France), an aquatic ecosystem impacted by urban and industrial anthropogenic pressures

Magali de La Cruz Barrón

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par Magali DE LA CRUZ BARRÓN

**Compartmentalization of class 1 integrons and IncP-1 plasmids in
the Orne river (France), an aquatic ecosystem impacted by urban
and industrial anthropogenic pressures**

20 décembre, 2018

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
ARG	Antibiotic-Resistance Gene
ARB	Antibiotic Resistant Bacteria
CFC	Continuous Flow Centrifugation
CV	Coefficient of Variation
DDD	Defined Daily Doses
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DREAL	Regional Department of the Environment, Planning and Housing
DOC	Dissolved Organic Carbon
EC	Electrical Conductivity
EEA	European Economic Area
EDX	Energy Dispersive X-ray Spectroscopy
EPA	Environmental Protection Agency
EU	European Union
FCM	Flow Cytometry
FSC	Forward Scatter Light
HGT	Horizontal Gene Transfer
ICE	Integrative Conjugative Element
IME	Integrative Mobilizable Element
<i>intl 1</i>	Class 1 integrons marker
LIEC	The Interdisciplinary Laboratory for Continental Environments
MGE	Mobile Genetic Elements
MOBISED	Modelling the remobilization of sediments and the release of associate contaminants
N-PAC	Nitrogen Polycyclic Aromatic compounds
OD	Optical Density

O-PAC	Oxygenated Polycyclic Aromatic compounds
<i>oriT</i>	Origin of Transfer
PAC	Polycyclic Aromatic Compounds
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal Component Analysis
qPCR	Quantitative Polymerase Chain Reaction
Rif	rifampicin
rpm	revolution per minute
SIERM	Système d'Information sur l'Eau Rhin-Meuse
SPM	Suspended Particulate Material
SSC	Side Scatter light
Tet	Tetracycline
<i>trfA</i>	IncP-1 plasmid marker
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

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INTRODUCTION

INTRODUCTION

1. ANTIBIOTIC RESISTANCE, MORE THAN A HEALTH ISSUE

Since the discovery of penicillin by Alexander Fleming in 1928, and its introduction in the 1940s, antibiotics have been massively used in medicine, leading to a significant decrease of human mortality and morbidity. As a matter of fact, the development and use of antibiotics were not solely expanded for controlling and preventing infections in human, they also have been used to prevent and treat animal and plants infections (Carvalho and Santos 2016). Additionally, antibiotics have been used in some countries as additives promoting animal growth (Carvalho and Santos 2016). Although this practice is no more allowed in Europe, some antibiotics are still being used indirectly as feed additives mainly for metaphylaxis and therapeutic purposes (De Briyne et al. 2014).

In 2016, the mean antibiotic consumption in the European Union and the European Economic Area (EU/EEA) for systemic use in the community (outside hospitals) was 21.9 defined daily doses (DDD) per 1000 inhabitants, while for the same year the mean of antibiotic consumption in the hospital sector was 2.1 DDD per 1000 inhabitants (Figure 1) (European Centre for Disease Prevention and Control, Annual epidemiological report, 2016). Although the global trend corresponding to these two values for the 2012-2016 period is a 5% increase and a 1% decrease, respectively, it is estimated that for developing countries, the antibiotic consumption for human medicine will increase by 200% by 2030 (Sutherland 2018), while a 67% increase is expected for food-production for the same period (Van Boeckel et al. 2015).

Today, it is believed that the ever-increasing use/overuse and misuse of antibiotics have resulted in the ever-increasing emergence of antibiotic resistant bacteria (ARB) and their subsequent dissemination (Figure 2). The consequences of the global increase of antibiotic resistance are not only linked to the reduction of antibiotic effectiveness to treat common infections, but predictions of a return to the pre-antibiotic era are also being debated regarding several domains of modern medicine (J. Davies and Davies 2010).

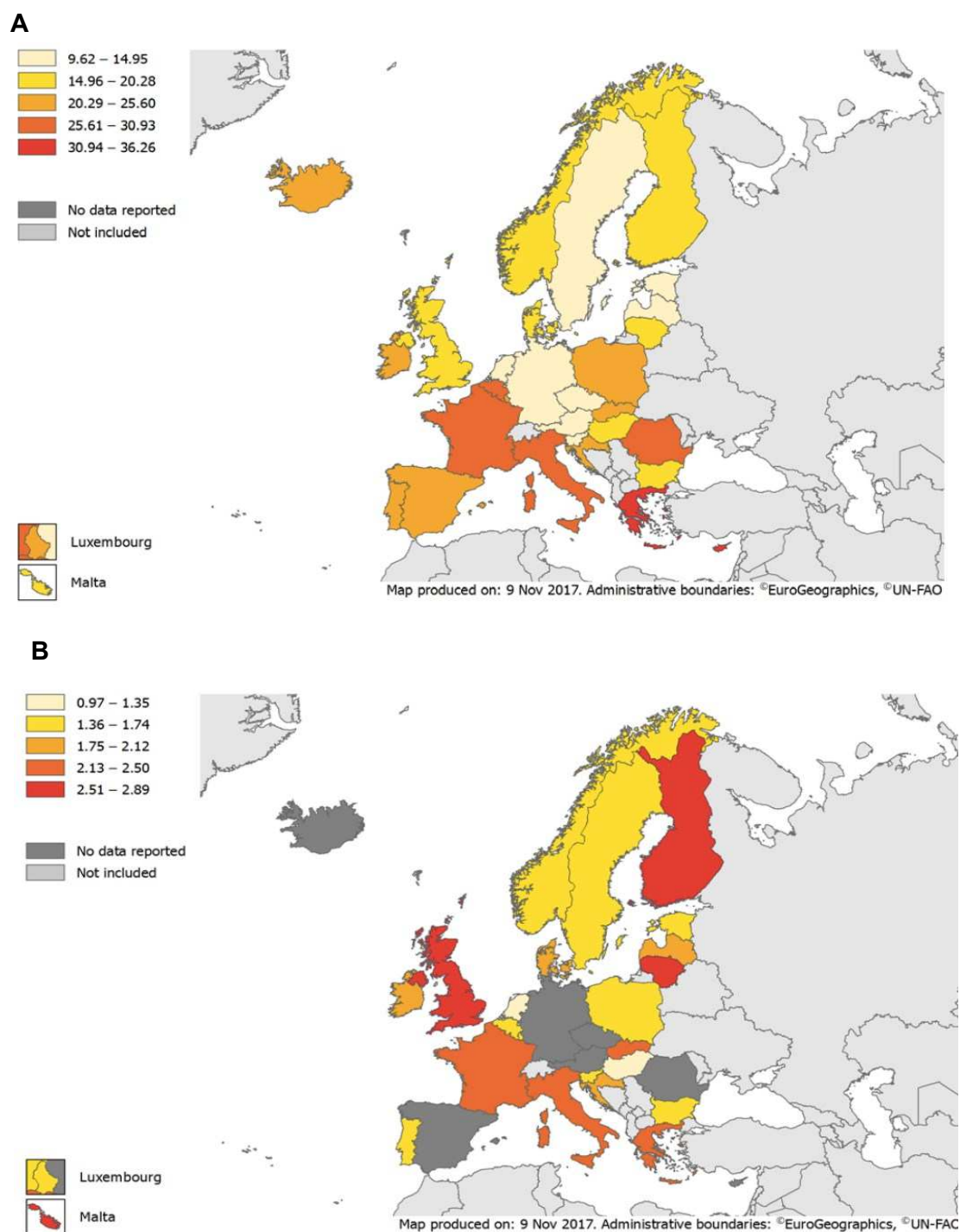


Figure 1. Consumption of antibiotics for systemic use in the European Union and the European Economic Area (EU/EEA), 2016, expressed as DDD per 1 000 inhabitants (A) in the community (outside hospitals); (B) in the hospital sector (source: European Centre for Disease Prevention and Control, Annual epidemiological report, 2016).

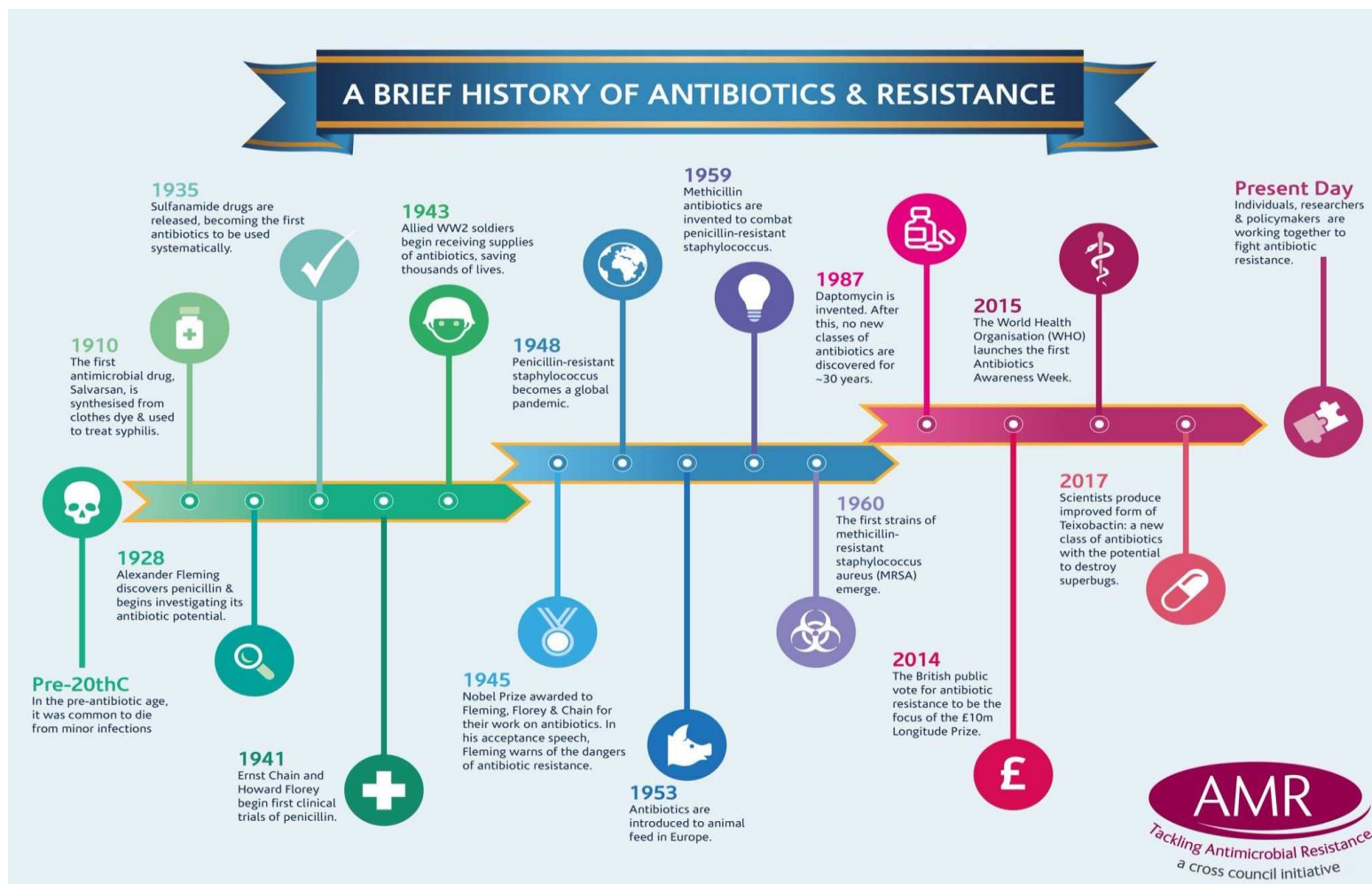


Figure 2. Timeline of key events for antibiotic resistances and antibiotic introduction (source: <https://www.ukri.org/>).

Indeed, some medical developments as such associated to invasive surgery or organ transplantation may become compromised, without talking of the increasing cost of health expenditures that is also mentioned as an important consequence of the increasing occurrence of antibiotic resistance. The progressive increase of multidrug resistance around the world has been identified as a public health priority according to the World Health Organization (WHO, Global Action Plan on Antimicrobial Resistance, 2015), as it is estimated to cause around 10 millions premature deaths by 2050, with a loss of up to \$100 trillion to the global economy (O'Neill 2014). If the way to evaluate the health and economic burdens of antibiotic resistance is still controversial (Abat et al. 2018; de Kraker et al. 2016), there is no debate on the fact that urgent actions need to be taken to reduce the emergence and dissemination of resistant pathogens in human and veterinary medicine ("Antimicrobial-Resistance-in-G7-Countries-and-Beyond", 2015; "Resistance Ascends the Political Summit", 2016). With this respect, several programs have been developed and implemented to better control the spread antibiotic resistance, such as the "One health initiative" (<http://www.onehealthinitiative.com/>), for instance. This also led to series of recommendations at national and international levels, intending to mitigate the spread of antibiotic resistances (Ministère du Travail, de l'Emploi et de la Santé, France, plan national d'alerte sur les antibiotiques 2011-2016, Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2016 (MARAN, 2017), Council on veterinary medicinal products (COM(2014)0558–C8-0164/2014 – 2014/0257(COD))). Considering the correlation between antibiotic consumption and occurrence of resistances in bacteria (E. Y. Furuya and Lowy 2006; Julian Davies 2007), most recommendations proposed to take action in the public health and veterinary/farming by limiting the inappropriate exposure of bacteria to antibiotics in order to slow down a natural evolution toward resistance. But would that be enough? Recently the LCPME demonstrated that sub-inhibitory concentrations of antibiotics from the MLS group (Macrolides, Lincosamides, Streptogramins) could induce the dissemination of tetracycline resistance genes between *Enterococcus faecalis* strains (Scornec et al. 2017). This point out that antibiotic selection is not the only driver of the resistance dissemination, and that antibiotics are not solely involved in the dissemination of their own

resistance (collateral effects). Clearly, the dissemination of antibiotic resistance is a complex matter involving different mechanisms by which bacteria can become resistant. Not less important is the question of the origins of antibiotic resistance in clinical and non-clinical context and the role of the environment in their persistence and dissemination.

2. MECHANISMS OF ANTIBIOTIC RESISTANCE

Antibiotic resistance happens when an antibiotic fails to stop the growth and/or kill a particular bacteria. The acquisition of antibiotic resistance can result from different phenomenon, either intrinsic mechanisms, mutations leading to target modification or decrease drug entry, for instance, or the horizontal acquisition of new genes for drug efflux or drug modification, for instance (Blair et al. 2015). Actually, the type of mechanism involved in antibiotic resistance also depends of the mode of action of the antibiotic considered.

Antibiotics act selectively on microbial functions to kill (bactericidal) or inhibit (bacteriostatic) bacterial growth, with minimal effects or without affecting host functions. The different modes of action of antibiotics are presented in Figure 3 these include: (i) inhibition of the cell wall synthesis; (ii) alteration of the cytoplasmic membrane integrity; (iii) inhibition of protein synthesis; (iv) inhibition of DNA metabolism; (v) others.

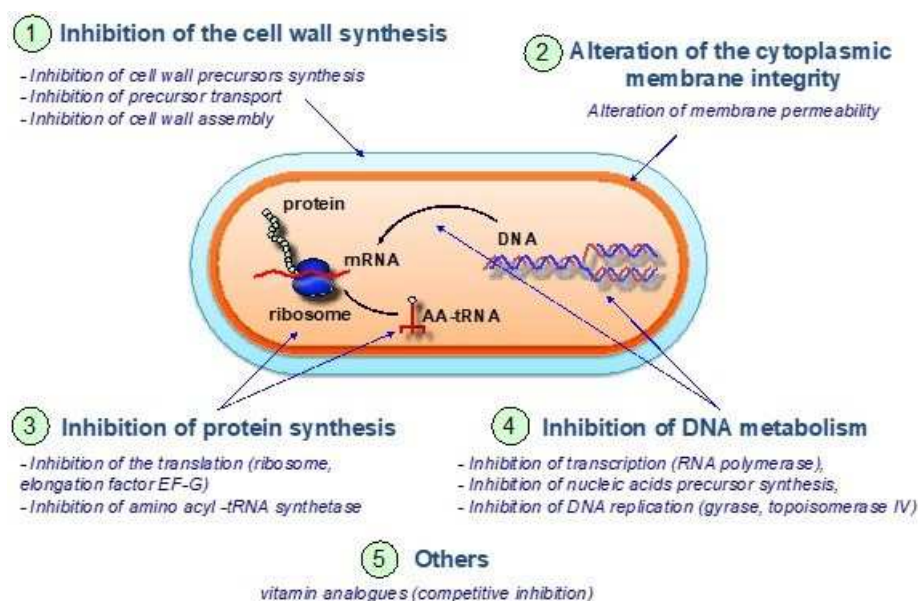


Figure 3. Antibiotics: modes of action and targets (source: Merlin C.).

The intrinsic resistance to an antibiotic is the ability of bacteria to resist the action of that antibiotic as a result of inherent structural or functional characteristics (Blair et al. 2015). Some bacteria are “naturally” resistant to certain antibiotics because they have either an impermeable membrane or do not possess target sites for the antibiotic (Figure 4, (1)). Bacteria can also acquire or develop resistance by minimizing the intracellular concentrations of an antibiotic thanks to efflux pumps secreting the drug from the cell, either directly outside (Figure 4, (2)) or, into its periplasm. Other mechanisms are the inactivation of the antibiotic by covalent modifications (Figure 4, (3)); and the modification of the antibiotic target, by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact (Allen et al. 2010), for instance. The mode of action of some antibiotics, their targets in bacterial cells and the known corresponding resistance mechanisms are summarized in Table 1.

On the other hand, antibiotic resistance acquired via horizontal gene transfer (HGT), results from changes in the bacterial genome and it is considered as the major mechanism responsible of the dissemination of antibiotic resistance and will be discussed in the next section.

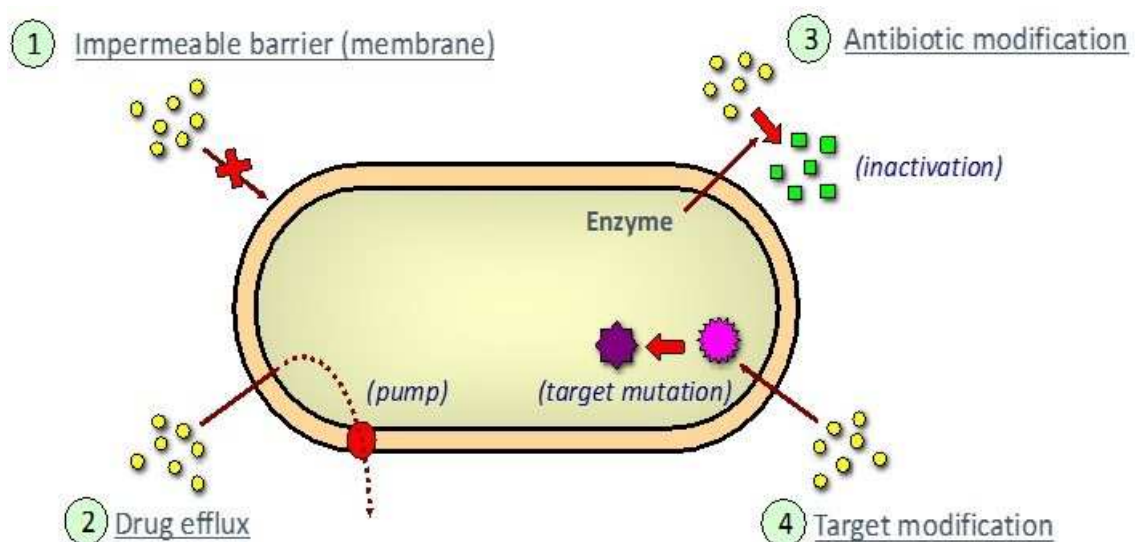


Figure 4. Mechanisms of antibiotic resistance in a Gram-negative bacterium. (source: Merlin C).

Table 1. Mode of action and resistance mechanisms of commonly used antibiotics (source: Morar and Wright 2010).

Antibiotic class	Examples	Target	Mode(s) of resistance
β-lactams	Penicillins (ampicillin), Cephalosporins (cephamycin), Penems (meropenem), Monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, Streptomycin, Spectinomycin,	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, Teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracycline's	Minocycline, Tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, Azithromycin	translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicol	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C ₁ metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C ₁ metabolism	Efflux, altered target
Rifamycin	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target

3. DISSEMINATION OF ANTIBIOTIC RESISTANCE GENES BETWEEN BACTERIA BY HORIZONTAL TRANSFER

The dissemination of antibiotic resistance genes (ARGs) can be understood as the movement of resistant genes between genetic, biological and physical locations (Stokes and Gillings 2011). With this respect, HGT is defined as any process where DNA is physically transferred from one cell to another without cell division (Stokes and Gillings (2011)). This DNA transfer can be done thanks to cell-cell contacts, bacteriophages or free DNA that can either be actively secreted by living bacteria or be released by dead cells. Thus, HGT can occur by three principal mechanisms of transfer (Figure 5), namely conjugation, transduction and transformation (Frost et al. 2005).

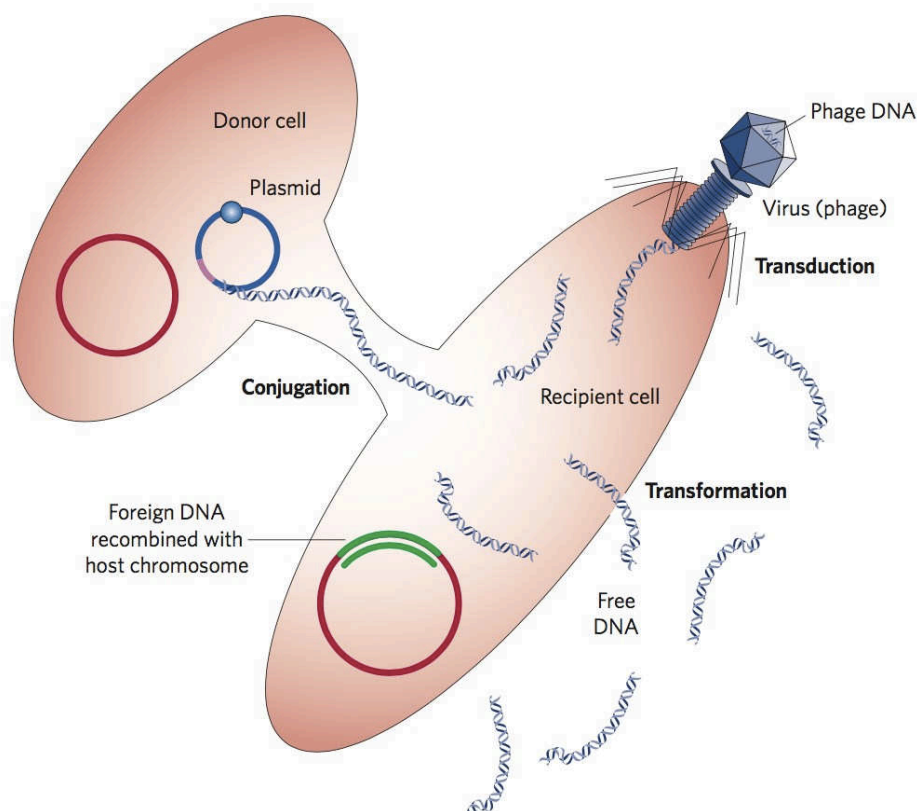


Figure 5. Transfer DNA mechanisms between bacterial cells. Conjugation requires cell to cell contact *via* cell surface pili or adhesin, transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA, finally, specialized or generalized transduction involved bacteriophages transferring bacterial DNA from a previously infected donor cell to a recipient cell. (source: Stewart 2013).

3.1 Bacterial transformation

Bacterial transformation was the first HGT mechanism discovered for prokaryotic (Frost et al. 2005). In such a case, gene transfer is mediated by the uptake of free DNA. In naturally transformable bacteria, chromosomally encoded proteins allow the uptake of free extracellular DNA before this DNA is integrated into the bacterial genome, more often by homologous recombination (Figure 6). There is evidence indicating that transformation provides a broad capacity for the horizontal spread of resistance genes between different species (von Wintersdorff et al. 2016). Moreover, Mao et al. (2014) showed that extracellular DNA is relatively stable in environments such as sediments due to its binding to clay and organic matter, which decreases susceptibility to degradation. As such, sediments can act as a reservoir of DNA that may be accessible for transformation and could eventually facilitate antibiotic resistance dissemination as far as ARGs are concerned.

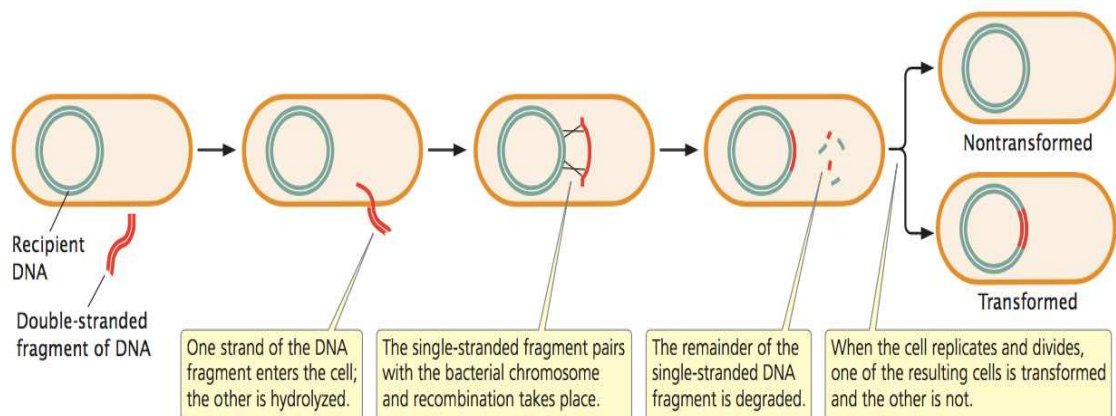


Figure 6. Gene transfer by transformation mechanism (source: Pierce 2012).

3.2 Transduction

Transduction is the horizontal transfer of DNA between bacteria mediated by bacteriophages (phages). Phages infect bacteria by introducing their genome into the host cell, from which phage DNA can either remain silent, in a “stasis” period, and be transmitted to the host progeny as full part of its genome (lysogenic cycle), or directly replicate and be encapsidated in order to form new virions (lytic cycle) (Pierce 2012).

There are two types of DNA exchange by transduction: generalized and specialized (Figure 7). Generalized transduction concerns the transfer of any portion of the donor genome to the recipient cell by lytic or lysogenic (temperate) phage cycle. Basically it refers to an error of encapsidation in the phage particle. On the other hand, specialized transduction solely involves temperate phages, and just a few specific donor genes are transferred to the recipient cell.

The carriage of ARGs by bacteriophages has been documented in samples from diverse environments. For instance Colomer-Lluch et al. (2014) used qPCR to show that the β -lactam ARGs *bla*_{TEM}, *bla*_{CTX-M} and *mecA* were present in bacteriophages from the river and urban sewage water samples. Even if ARG transduction has never been formally demonstrated, these findings as well as those from other works (Balcazar 2014; Quirós et al. 2014; E. Marti, Variatza, and Balcázar 2014), suggest that bacteriophages may play an important role in the spread of ARGs in various bacterial communities and environments.

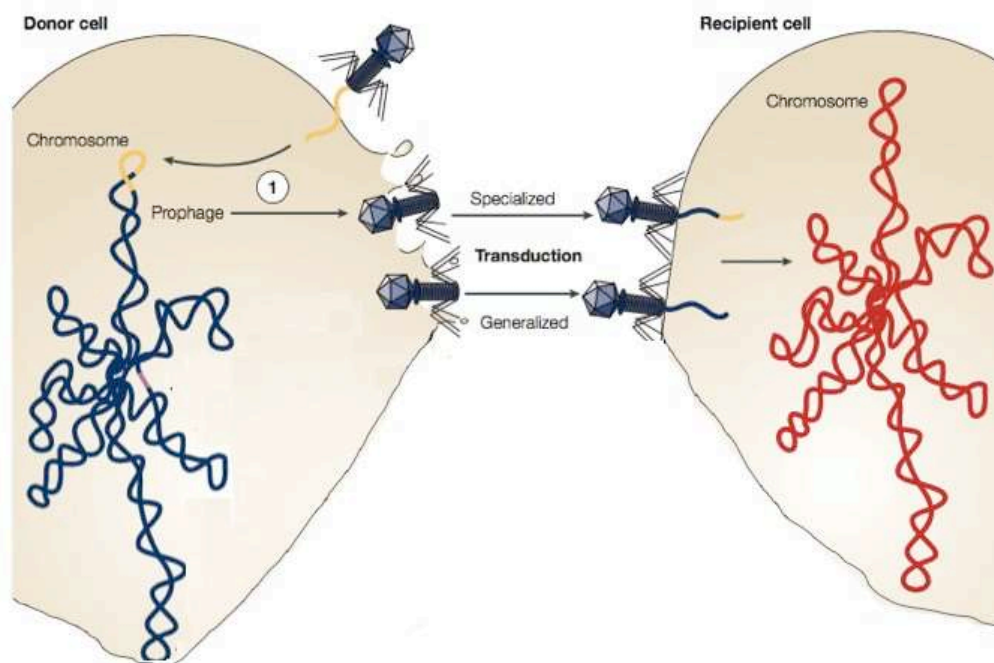


Figure 7. DNA transfer between bacteria mediated by phages. A phage infects a bacterial cell and inserts its DNA (yellow) into the chromosome (dark blue) as a prophage; it later replicates, occasionally packaging host DNA (generalized transduction) or specific genes (specialized transduction), lyses the cell, and infects a recipient cell in which the novel DNA recombines into the recipient host cell chromosome (red). (Source: Frost et al. 2005).

3.3 Conjugation

Bacterial conjugation is the most commonly studied mechanism of HGT. Cell-cell contact is required for conjugation *via* cell surface pili or adhesins (Thomas and Nielsen 2005). Different elements can be transferred by conjugation such as integrative conjugative elements (ICEs), integrative mobilizable elements (IMEs), part or, in some instances, the totality of bacterial chromosomes, and finally plasmids. In the case of plasmid transfer, the mechanism involves two main steps: first, the donor cell establishes contact with the recipient cell (Figure 8). In Gram negative bacteria, this is made possible by a particular type IV secretion apparatus encoding a pilus which allows connecting donor and recipient bacteria and establishing a mating pore through which the plasmid DNA and some donor-encoded proteins can be transported to the recipient. At this stage, it is worth noting that conjugative plasmids also promote changes in cell surface properties, since conjugative pili have been also defined as a substrate for cell adhesion (Ghigo 2001). The second step, involves the transfer and processing of DNA. One strand of the plasmid DNA is nicked at a specific site named origin of transfer (*oriT*) by a plasmid-encoded relaxase homodimer, while one of these monomers remains covalently attached to the DNA 5'-end generated. Then, the relaxase monomer associated to the nicked strand is transferred from donor to recipient cell through the pilus channel. In the donor cell, replacement strand DNA synthesis reconstitutes the double-stranded plasmid DNA. After one round of DNA transfer, the relaxase attached to the 5' terminus of the transferred-strand DNA binds the second *oriT* copy entering in the recipient cell and catalyzes DNA ligation allowing reconstituting the plasmid circular structure (N. Furuya and Komano 2000; Thomas and Nielsen 2005).

The importance of this DNA transfer lies in the fact that plasmids can confer resistance to the several classes of antibiotics, including β -lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides and quinolones (Carattoli 2013), and that plasmids can promote the horizontal transfer of resistance of genetic determinants among a broad host range, including pathogenic bacteria. Additionally, at least *in vitro*, some

plasmid can transfer at a relatively high frequency (up to 100%) making them good candidate for promoting ARGs dissemination in the environment.

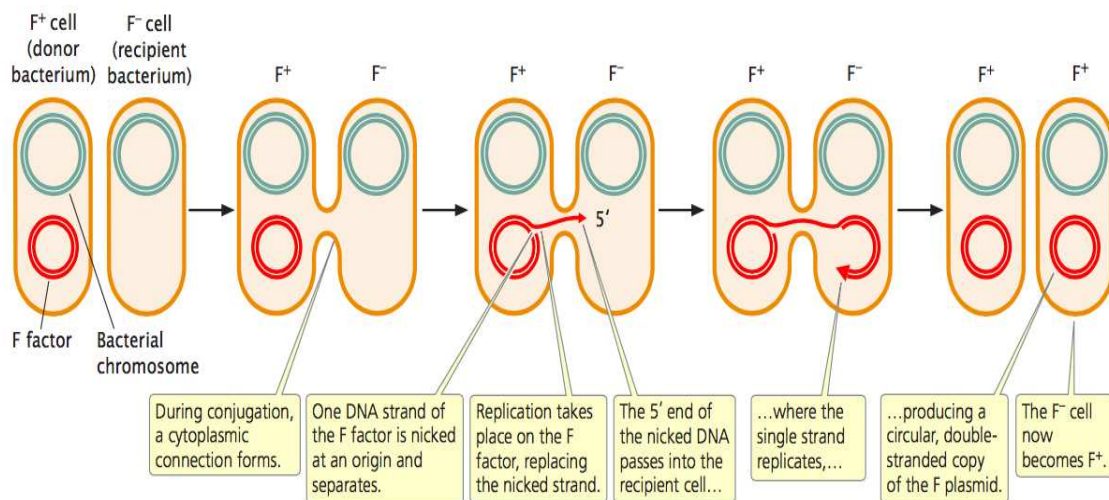


Figure 8. Horizontal gene transfer between bacterial cells by conjugation (source: Pierce 2012).

4. MOBILE GENETIC ELEMENTS, AGENTS OF DNA MOBILITY

Mobile genetic elements (MGEs) are DNA sequences that allow the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility) (Frost et al. 2005). The movement of resistance genes and in general the intercellular movement of DNA is facilitated by a variety of MGEs such as transposons, ICEs, genomic islands, integrons and plasmids. It is worth noting, that MGEs do not act alone and that HGT should be reasoned in its complexity where several MGEs can act together. For instance, ARGs can be captured by integrons, jump onto a conjugative plasmid (by transposition) before being transferred toward a new host (conjugation). As such, MGEs responsible for intracellular mobility are also important contributors of HGT between cells and of bacterial genomic plasticity.

4.1 Transposons

The transposable elements consist at least of one gene encoding a transposase (enzyme ensuring the transposition of the mobile element) and,

most of the time, repeated inverted sequences flanking the element. The transposase recognizes inverted repeats at the ends of the element and catalyzes the transposition of the transposon ends to a target site. In addition to the sequences involved in transposition, transposons may carry accessory genes such as ARGs (Kazazian 2004). There are two main modes of transposition, the conservative (non-replicative) and the replicative transposition (Figure 9). In the first one, the transposon is physically disconnected from the genome and self-relocated somewhere else in the same genome. In the replicative mode of transposition, a new copy of the element is generated, finally leading to a second copy of the transposon in the target site, while an original copy remains in the former donor site (Hallet and Sherratt 1997). However, to be transferred from one cell to another, these elements must be associated with genetic elements capable of intercellular mobility, such as certain plasmids, phages, or ICEs, or have to transpose into a new replicon after their carrier DNA being transferred by transformation.

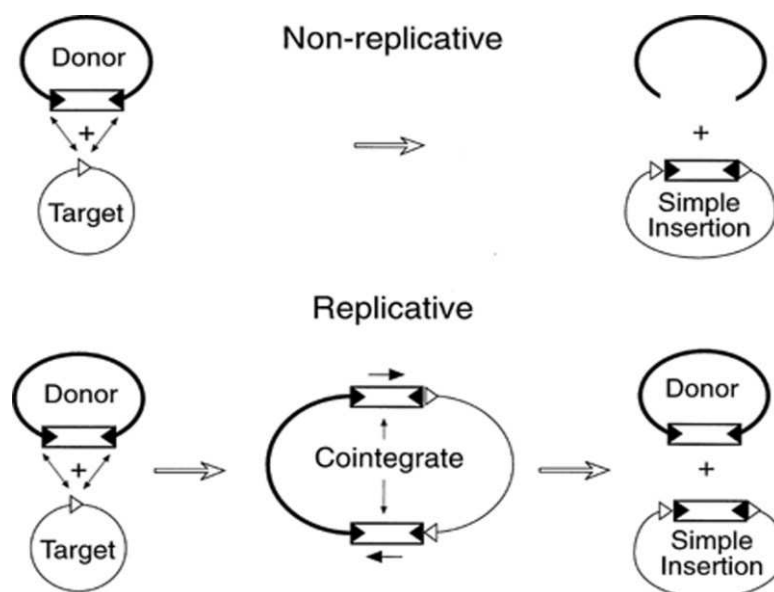


Figure 9. The two common modes of transposition. In non-replicative transposition, the element (rectangle) is excised from the donor genome and relocated on the target site of the same genome. In the replicative mode of transposition, the donor and target backbones are fused and a new copy of the element is generated, and each copy remains attached to the donor backbone at one end, whereas the other end is joined to the target (source: Hallet and Sherratt 1997).

4.2 Integrations

Integrations are genetic structures commonly found in bacterial genomes, on chromosomes and/or other MGEs, that can efficiently capture and express exogenous genes as part of DNA fragment named cassettes (Gillings 2014; Gillings et al. 2015). Although there are different classes of integrations (see below), the basic structure of these MGEs includes a stable platform and a variable cassette array (Figure 10). Three essential elements form the conserved platform: i) the gene *intl* which encodes an integrion integrase (IntI), a type of tyrosine recombinase catalyzing the integration and excision of gene cassettes; ii) a recombination site (*attI* site) for the integration/excision of cassettes to take place; iii) once a gene cassette is integrated, it is expressed by an integrion-associated promotor, Pc (Gillings 2014). Integrion gene cassettes are acquired in their circular form. Basically, they often consist in a single open reading frame (ORF) and a cassette-associated recombination site *attC* joining the two extremities of the cassette (Figure 10). The ORFs of gene cassettes can encode various functions such as catabolic genes, stress response genes, antibiotic resistance genes.

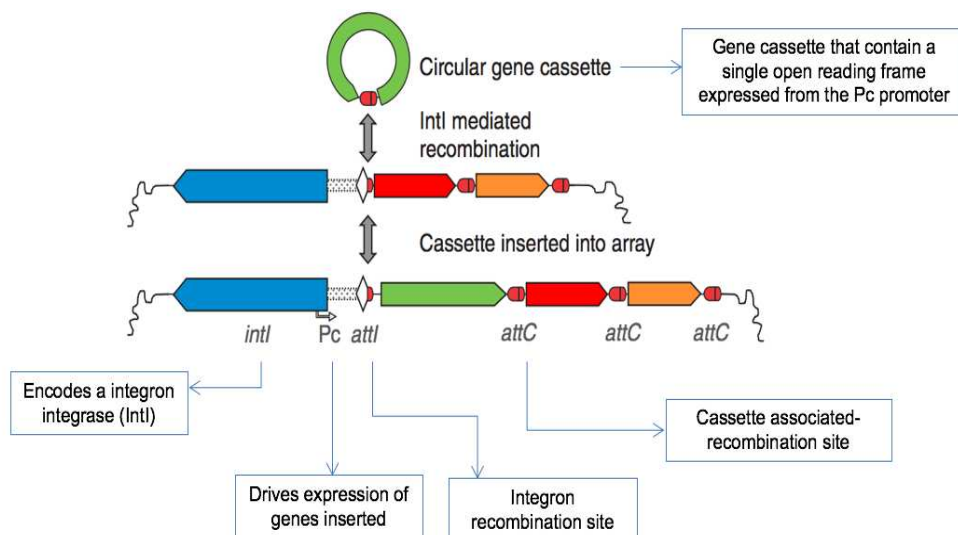


Figure 10. General structure of integrions (Gillings et al. 2015).

Integrions were first classified in two main categories: the chromosomal integrions and the mobile integrions. Chromosomal integrions, also called “superintegrions”, can carry up to 200 cassettes, most of them encoding proteins

with unknown functions, have homogenous *attC* sites and are generally carried by bacteria belonging to the *Vibrio* genus (Stalder et al. 2012). On the other hand, mobile integrons are genetic elements that cannot be transferred by themselves but that are located on other MGEs such as transposons and plasmids. Different classes of mobile integrons can be distinguished based on the relative homology of the *intl* genes and their surrounding sequences (Gillings et al. 2015). Integrons from classes 1, 2, and 3 are the most commonly detected. Among them, class 1 integrons and the clinically relevant antibiotic resistance cassettes they carry are recognized to have been horizontally transferred in a wide range of environmental and pathogenic bacteria, and therefore have been extensively studied. They are commonly found among clinical isolates and are associated with transposons deriving from *Tn402*, which can be embedded in plasmids or larger transposons that could support their dissemination.

Class 1 integrons usually carry 1 to 4 resistance gene cassettes among the more than 130 different ones that have been described to date (Partridge et al. 2009). In recent years, class 1 integrons are gaining attention because of their apparent role in the acquisition of antibiotic resistance genes. Because they can carry many different ARGs and also because they tend to present an increasing abundance in human impacted environments, some authors started to use this marker as a proxy for evaluating anthropogenic ARG pollution (Gillings et al. 2015). With this respect, several studies have correlated the occurrence of class 1 integrons with anthropogenic activities. They are found in relatively high abundance in hospital effluents and, in some instance, their occurrence even correlates with the level of metal and antibiotic pollution (Gaze et al. 2011; Koczura et al. 2016; Wright et al. 2008; Stalder et al. 2014).

4.3 Plasmids

For a long time, plasmids were described as small, circular, double-stranded DNA molecules that were distinct from a cell chromosomal DNA, and that were capable of autonomous replication. The minimal structure of plasmids includes the gene(s) that encode(s) for replicative functions and a sequence acting as a

DNA replication origin (Figure 11) (Frost et al. 2005). The plasmid classification is based on their incompatibility between each other. Incompatibility groups (Inc) are defined as the inability of two plasmids to be stably replicated and maintained in a same bacterial cell because their plasmid replication and segregation systems are too similar and interfere between each other. In brief, this is not possible for plasmids with the same replication machineries, *i.e.* from the same incompatibility group, to co-exist in the same cell. The plasmids from the incompatibility groups A/C, F, HI2, I1, L/M, P and Q have been identified as some of the most frequent ARG carriers (Partridge et al. 2018). Among them, plasmids of the incompatibility group IncP-1 (according to the *Pseudomonas* nomenclature) are conjugative plasmids of special interest as they are widely distributed in Gram-negative bacteria, and their presence have been demonstrated in different environments such as soils, surface water, wastewaters, and clinical environments (Popowska and Krawczyk-Balska 2013). They display a broad host range allowing them to maintain in many bacterial species, coupled to an efficient transfer machinery supporting the large possibility of dissemination (Popowska and Krawczyk-Balska 2013). Based on the sequence of the protein TrfA involved in the initiation of plasmid replication, the group of IncP-1 plasmids was divided into six subgroups: $-\alpha$, $-\beta$, $-\gamma$, $-\epsilon$, $-\delta$, and $-\zeta$. The different groups have more or less been shown to be widely involved in ARG mobilization. With this respect it is believed that all clinically relevant ARG have been found associated to this family of plasmids mobilize (Popowska and Krawczyk-Balska 2013; Schlüter et al. 2007).

When a plasmid is transferred to a new cell host, its replication and the expression of its carried genes produce a fitness cost (a reduction of the host to compete successfully with the other members of the community) (San Millan 2018). It means that plasmid can transfer to a new host but to be maintained, selection for some trait encoded in the plasmid is often needed such as resistance to antibiotics, heavy metals or quaternary ammonium compounds used as disinfectants (San Millan 2018). Plasmids can also encode for so called addiction systems, as toxin-antitoxin systems, that may improve maintenance by killing daughter bacteria that would lose plasmids upon cell division. In other words, this means that even with an efficient transfer machinery, the success in ARGs dissemination with plasmids will also rely on the ecological context in

which it takes place, where fitness cost has to be balanced by the selective advantages provided (Werisch, Berger, and Berendonk 2017). With this respect, antibiotics may play a significant role in maintaining these plasmids in the community against the energetic burden they are associated.

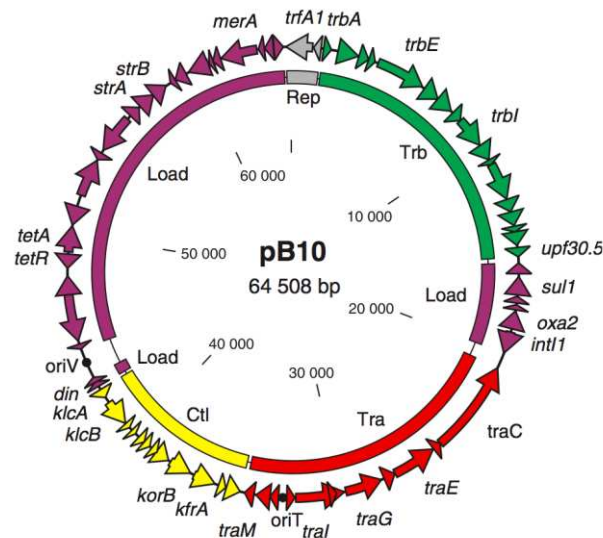


Figure 11. Example of plasmid structure: map of pB10 belonging to the IncP-1 β plasmid incompatibility group. Genes are represented by arrows colorized by functional groups: the plasmid backbone modules for replication initiation, (Rep grey color), for mating-pair-formation (Trb– in green), for conjugative DNA-transfer (Tra–in red) and plasmid control and stable maintenance and inheritance (Ctl–yellow), insertions of accessory genetic elements are shown as lilac regions (load) (source: Schluter 2003).

4.4 Integrative Conjugative Elements

ICEs form a group of conjugative elements that cannot be self-maintained by replication as plasmids do. Thus, ICEs maintain in host bacteria by integrating into the chromosomes but can subsequently excise, circularize and transfer (through conjugation) to neighboring cells (Böltner and Osborn 2004; Frost et al. 2005). On a functional point of view, ICEs share common features, with distinct regions encoding excision and integration functions, conjugative transfer functions, and, most of the time, regulation. Because of their lack of autonomous replication, ICEs have long been perceived as chimeric elements sharing features with both temperate bacteriophage (site-specific recombination system) and conjugative plasmids. Actually, with the availability of metagenomic

data, it became clear that ICEs are probably the most abundant type of MGEs (Guo et al. 2017). ICEs are largely involved in the mobilization of antibiotic resistance genes they carry as cargo genes. They also carry genetic determinant for resistance for heavy metals or degradation to aromatic compounds, for instance (Guo et al. 2017). Last but not least, several ICEs involved in the dissemination of ARGs (e.g. Tn916, SXT, CTnDOT) have been demonstrated as having their mobility induced by sub-inhibitory concentration of antibiotics (Beaber, Hochhut, and Waldor 2004; Salyers, Shoemaker, and Li 1995; Su, He, and Clewell 1992).

5. ANTHROPIC SOURCES OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

Considering selective processes occurring during antibiotic therapies, it is clear that the medical use of antibiotics leads to an enrichment of antibiotic resistant bacteria/genes (ARB/ARGs) in human and animal microbiomes, but the origin of these resistances remains an open question. In recent years, non-clinical environments have begun to receive special attention, as most antibiotics are produced by environmental microorganisms (Martinez 2008) and there are evidence that the selection of ARB can occur even a low antibiotic concentration as they may be found in soil and aquatic environments (Marti, Variatza, and Balcazar 2014). The role of the environment as an important source of antibiotic resistance has been increasingly recognized but the understanding of its contribution is still limited. In the meantime, being the final receiving environment of human activities, the environment is also a dissemination route of ARB and ARGs, where environmental pollution of anthropogenic origin also feed human and animals with ARB and ARGs (Figure 12) (Davies and Davies 2010). Indeed, ARB and ARGs can enter into the soil and the surface or groundwater environments by different pathways. The antibiotics used for animal husbandry (either as growth promoters or for infection treatments) provide selective advantages for ARB to develop in animal gut, that end up in the manures (Xie, Shen, and Zhao 2018). Then, ARB and ARGs can enter into the soil compartment via animal manure used for

fertilization. Lately, some authors as Sandberg and LaPara (2016) suggested that the subtherapeutic use of antibiotics in agriculture could lead to long-term increases in ARGs in the soils treated with manure. Indeed, other works evidenced high levels of ARGs in several agricultural soil types treated with swine, chicken or dairy manures (J. Li et al. 2017; Zhang et al. 2017; Sandberg and LaPara 2016).

The constant use of antibiotic in aquaculture for prophylactic and/or metaphylactic purposes, mainly through food and water additives, could also apply a selective pressure for the emergence and dissemination of ARB and ARGs in fish intestines (Santos and Ramos 2018) and the surrounding bacteria. These ARB and ARGs can enter into the environment by the direct release of wastewater/sludge from closed ponds into water ecosystems or can even be present in the environment since the beginning when open pond systems are considered (Cabello 2006).

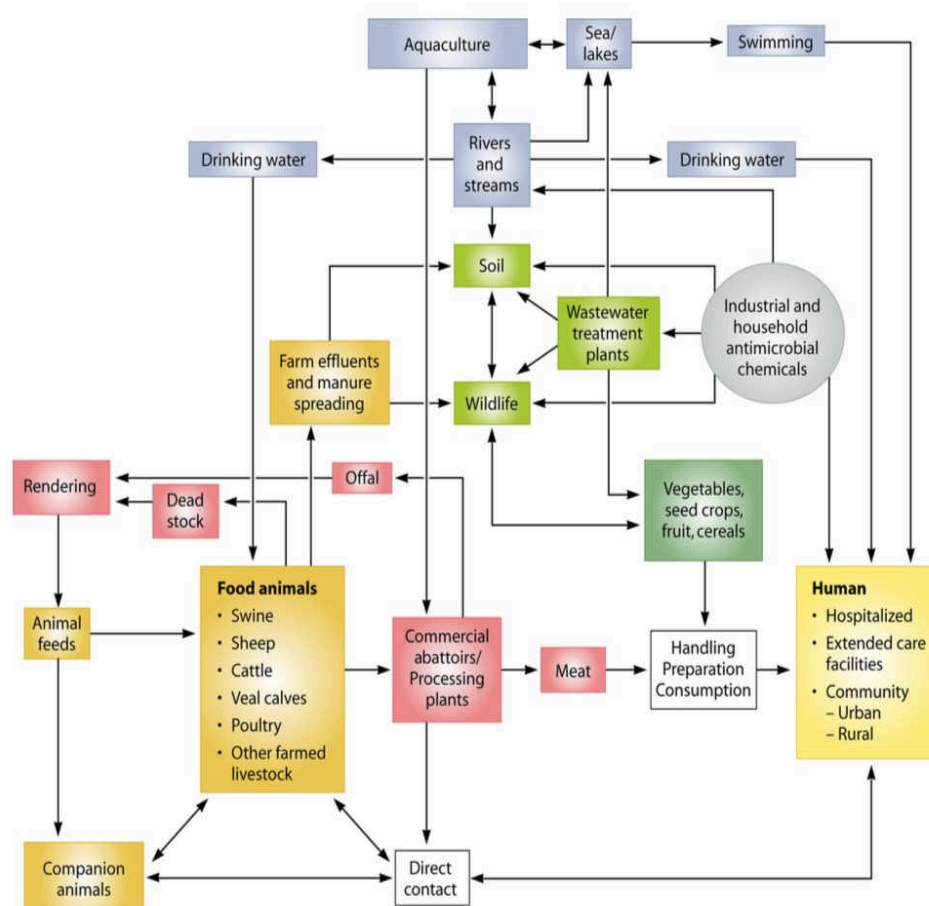


Figure 12. Dissemination routes of ARB and ARGs in the environment influenced by anthropogenic activities (source: Davies and Davies 2010).

Wastewater treatment plants (WWTPs) are designed for the removal of conventional pollutants, such as suspended solids, organic matter, nutrients (nitrogen and sometimes phosphorus), *etc.* However, traditional WWTPs, that are devoid of advance tertiary oxidation processes of treated water like ozonation, UV-treatment or chlorination, are not dedicated to the removal of antibiotics, ARB or ARGs (Pruden et al. 2013). Therefore, WWTPs are likely to be one of the main sources through which ARB and ARGs are released into the environment as they collect wastewaters from different origins including hospitals, residential and industries (Barancheshme and Munir 2018). With this respect, WWTPs are proposed to be hotspots of ARG dissemination between bacteria as they combine several permissive conditions including high cell density and nutrient richness (Rizzo et al. 2013).

Although antibiotic resistances have been present in the environment even before the discovery and use of antibiotics, the exacerbated selection pressure exerted by the antibiotics used in clinical and non-clinical contexts has contributed to a rapid increase of the occurrence of ARB and their ARGs in human and animal microbiome but also in the environment (Allen et al. 2010; Pruden et al. 2006). Several works, such as that accomplished by Knapp et al. (2010), have evidenced this phenomenon by demonstrating a global increase in some ARGs in archived agricultural soils mainly fertilized with manure from 1940 to 2008 (Figure 13). This is of particular interest because it suggests that environments impacted by human activities become reservoirs of ARB, which could be critical points of control to prevent the dissemination of antibiotic resistance.

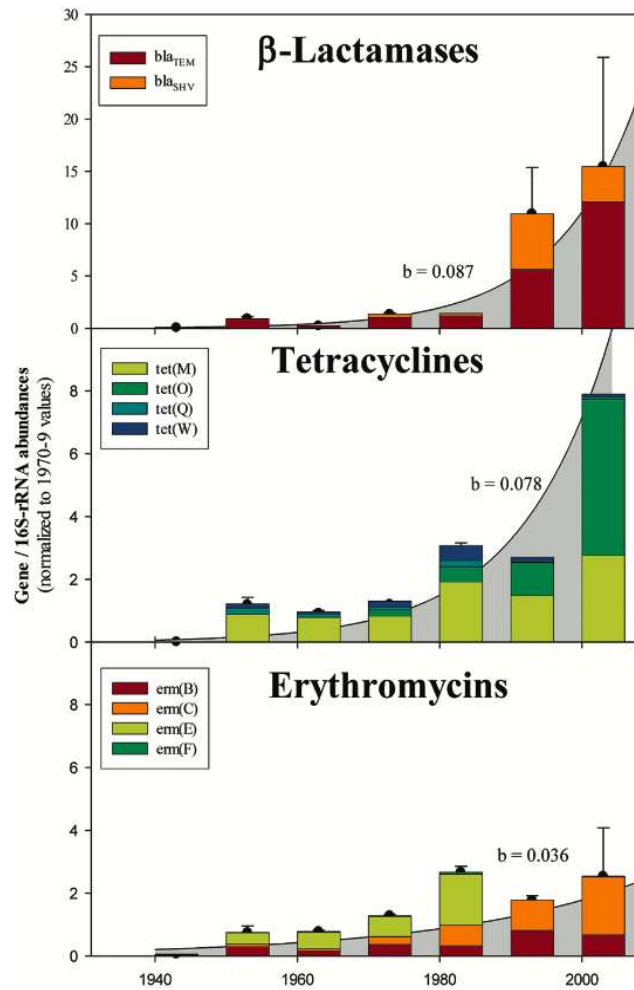


Figure 13. Evidence of ARG accumulation in archived soils from 1940 to 2008 (source: Knapp et al. 2010).

6. ROLE OF AQUATIC ECOSYSTEMS IN THE SPREAD OF ARBs AND ARGs

Aquatic ecosystems are the final receiving environments of many anthropogenic wastes and it is believed that WWTP effluents, as well as the increasing population density along rivers, industrial facilities, agricultural and/or aquacultural activities have contributed to the increase of ARGs in this environment (Marti et al. 2014). Although the fate of ARGs once released in the environment is not well understood, different scenarios of their persistence in the water column have been suggested. For example, Sabri et al. (2018) monitored the occurrence of ARGs along the Dutch river Grote Beerze.

Evaluating the effect of WWTP effluent discharge as the principal source of ARGs, they found that once the ARGs enter the river, they persist in water and sediments along the 20 km of the river section studied. Additionally, Chen et al. (2013) reported that the persistence and the increasing occurrence of tetracycline resistance genes in water samples correlates with the level of anthropogenic pressure (urbanization, industrial, agricultural and aquacultural activities).

The sediment compartment of aquatic ecosystems is increasingly being considered as an important reservoir of ARB that could facilitate the transfer, maintenance, dissemination and accumulation of ARGs (Elisabet Marti, Variatza, and Balcazar 2014). Works such as those reported by Devarajan et al. (2015) demonstrated the accumulation of *bla* and *aadA* genes in lake sediment cores (Lake Geneva) contaminated by hospital and urban wastewaters, supporting the hypothesis that sediments can act as reservoirs for ARGs. Similarly, Kristiansson et al. (2011) demonstrated high levels of ARGs and MGEs (including integrons, transposons and plasmids) in river surface sediments strongly impacted by wastewater from an India antibiotic production plant. By doing so, they highlighted the role of antibiotics at concentrations close to therapeutic levels in selecting for ARGs and MGEs as well as the role of the sediment compartment as a reservoir for them.

Using a metagenomic approach and networking analysis, B. Li et al. (2015) determined the ARG diversity and abundance in 50 samples from Hong Kong, including waters, soils, sediments, sludges, biofilms and feces (from human and livestock), and found common resistance gene patterns between all the samples. These resistance genes were related with the antibiotics locally used in human medicine or veterinary medicine including growth promotion (aminoglycoside, bacitracin, β -lactam, chloramphenicol, macrolide lincosamide streptogramin, quinolone, sulphonamide and tetracycline). Even if the ARG concentrations varied according to the origin of the sample, this work demonstrated that the environment is the final receiving environment of ARGs where they can persist and accumulate in the soil and sediment compartments (Figure 14). Once in the environment, the main problem with biological pollution such as ARB is that they can persist, multiply and transfer their ARGs (Berendonk et al. 2015), contrary to other pollutants which concentration

decrease due to biotic and abiotic factors. On the principle, ARB from the guts reaching a river ecosystem should not persist very long because of a lack of competitiveness against indigenous bacteria. However, this is without counting on the fact that gut ARB reaching the environment could transfer their ARGs in indigenous environmental bacteria by HGT, eventually allowing ARGs to persist longer than the bacteria initially carrying them before being outcompeted in new ecological niches. Even if the mechanisms, conditions and preferential places/niches involved in the ARG dissemination in the environment are not well understood or known, HGT has been recognized as a fundamental process in bacteria evolution, playing an important role in the dissemination of antibiotic resistance (Stokes and Gillings 2011).

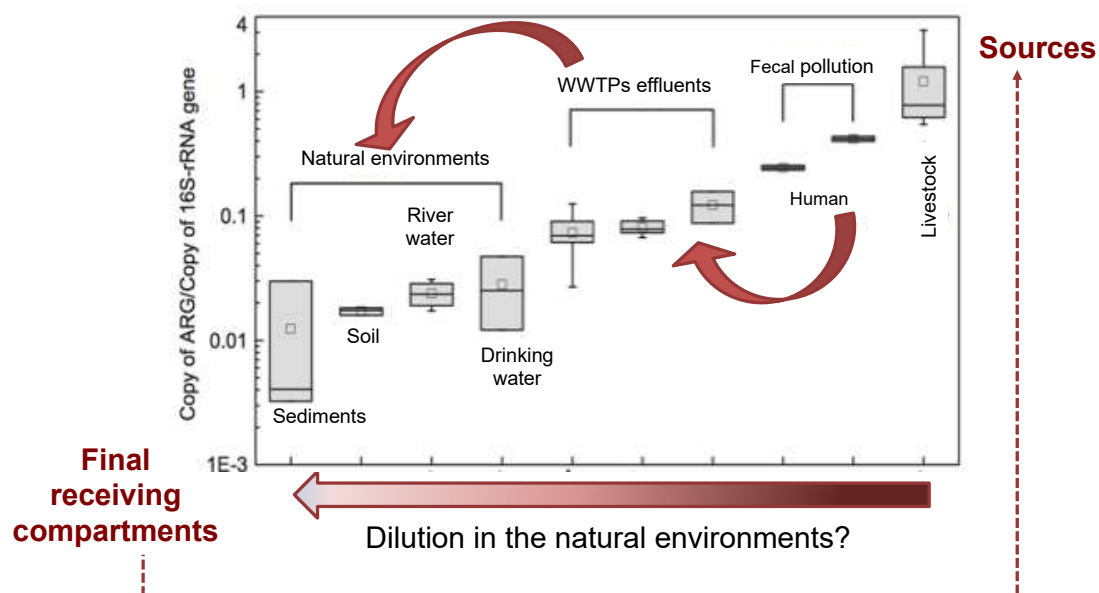


Figure 14. Comparison of the ARGs quantified in different environmental samples and the contribution of anthropogenic activities to the presence of ARGs in environmental samples (source: modified from B. Li et al. 2015)

7. CO-SELECTION OF ANTIBIOTIC RESISTANCE

Although selection for antibiotic resistance was hypothesized for a long time to result from the sole antibiotic positive pressure, recent works strongly suggest that it is not always the case. Indeed, there is growing evidences that tend to demonstrate that selection of ARB and ARGs by heavy metals can also occur (Baker-Austin et al. 2006). Moreover, other organic contaminants seem to be able to exert positive selective pressures for ARGs such as polychlorinated biphenyls (Lo Giudice et al. 2013), pesticides (Anjum and Krakat 2016), disinfectants (Karatzas et al. 2007) and polycyclic aromatic hydrocarbons (PAHs) (Chen et al. 2017), as well. The relationship between metal resistance or metal tolerance and antibiotic resistance in the environment have been established in terms of shared mechanisms for resistance (Table 2), and also several co-processes that can take place due to the presence of metals (Di Cesare et al. 2016):

- *Co-selection*, which refers to the simultaneous selection of two or more genes, even when exposed to a single selective stressor. The co-selection of ARGs with metal resistance genes by metals has been described as major path for the spread and persistence of ARGs in different environments.
- *Co-resistance*, which occurs when the resistance genes (i.e. ARGs and metal resistance genes) are located together on the same genetic element such as a plasmid, transposon or integron.
- *Cross-resistance*, which refers to a dual function selectable by several stressors (e.g. antibiotics and metals). Usually, in the context of antibiotic resistance, it is frequently implemented by efflux pumps, which eject the antibiotics and metals from the bacterial cell.
- *Co-regulation* occurs when the gene expression is influenced by the same factor.
- *Co-localization*, when the ARGs can be maintained in the environment by co-selection with metal resistance genes in polluted areas.

In summary, the presence of one stressor (e.g. metals) is likely to select for the resistance genes to the other (e.g. ARGs); however, the extent to which the

metal levels in the environment affect the selection of resistant bacteria and ARGs is not well elucidated (Knapp et al. 2011, 2017a).

Table 2. Examples of shared structural and functional characteristics for antibiotic and metal resistance (source Baker-Austin et al. 2006).

Resistance mechanism	Metal ^A ions	Antibiotics
Reduction of membrane permeability	As, Cu, Zn, Mn, Co, Ag	Ciprofloxacin, tetracycline, chloramphenicol, β -lactams
Drug and metal alteration	As, Hg	Chloramphenicol, β -lactams
Drug and metal efflux	Cu, Co, Zn, Cd, Ni, As	Tetracycline, chloramphenicol, β -lactams
Alteration of cellular target (s)	Hg, Zn, Cu	Ciprofloxacin, β -lactams, trimethoprim, rifampicin
Drug and metal sequestration	Zn, Cd, Cu	Coumermycin

^A Symbol of metals according to Mendeleev's periodic table

8. THE CASE OF THE INHERITED POLLUTION IN THE ORNE RIVER

With this work, we wanted to understand the fate of ARGs, using two MGEs as proxies, in a river ecosystem impacted by anthropogenic activities so as to identify possible reservoirs, and parameters/driving forces affecting their occurrences. Our choice focused on the Orne River (Lorraine, France), which is the focal point of a research consortium from Nancy University to study the fate of pollutants in sediments. The Orne River, located in northern Lorraine, is a tributary of the Moselle River of special interest because it has been highly impacted by urban, agricultural and industrial activities. During the 20th century, steel-making industries and related activities, such as iron mining, coke and gas production, were very intense in the area surrounding the river. During this period, dams were built as water reservoirs for industrial purposes modifying the riverbed. Several blast furnaces were installed and they were fed with ore

extracting from the nearby iron mines. The blast furnace activities were active at different time periods according to their location, one set at the exit of Joeuf city, active from 1882 to 1970, another at Moyeuvre-Grande city from 1918 to 1975, the last two blast furnaces installed where located within the limits of the Moyeuvre-Grande city and were very active from 1960 to 1988 (Kanbar et al. 2017). Their activity resulted in waste products such as furnace smokes, dust and residual sludge that were released in the river.

Nowadays, the steel production has ended, but the passed steel production and related activities resulted in a strong contamination of the surrounding soils and water, with the accumulation of materials enriched in iron and other metals, and persistent organic pollutants such as PAHs. Reflecting the high pollution levels of the river, it was denominated by the “Ministère de l’écologie, du développement durable et de l’énergie”, and by the “Ministère du logement, de l’égalité des territoires et de la ruralité” as a “Territory in mutation exposed to risk”. In addition, with the actual selective pressures (agricultural and urban), all these conditions could favor the accumulation, dissemination and compartmentalization of ARGs, due to the local anthropogenic inputs (e.g WWTP) and possible co-selection processes as described above, and their remobilization as well as the other pollutants could cause socioeconomic, ecological and health problems (Figure 15).

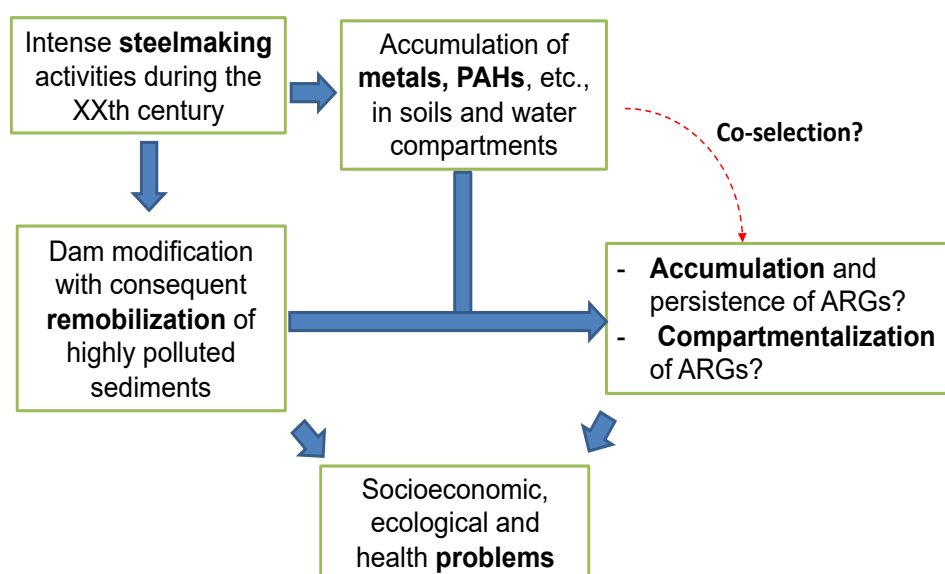


Figure 15. Consequences of mining and metallurgical activities on the Orne river.

9. OBJECTIVES OF THIS WORK

The MOBISED project was developed for studying the fate of pollutants in the river and river sediments, as well as better understanding the processes favoring their compartmentalization in sediment and eventually their remobilization. Its main objective was to better understand the process occurring during the remobilization of sediments and their consequences. This naturally led to the implementation of an extensive multidisciplinary approach aiming at characterizing the studied area, and collecting numerous global and geochemical parameters (e.g. organic and elemental composition) which were analyzed by different research teams. The Environmental Microbiology team of LCPME was involved in the analyzes of molecular microbiological markers to report on possible ARG pollution. In this context, two molecular markers from MGEs were monitored as proxies for ARGs, namely class 1 integrons (*intI1* gene) and IncP-1 plasmids (*trfA* gene). In addition, 16S rDNA gene was also monitored for estimating the global bacteria population and for data normalization, and finally the relative abundance of α -, β -, and γ -proteobacteria 16S rDNA were used as indicators of changes in the bacterial community structures. Combined with the analysis of nearly 100 geochemical and global parameters, the goal of this work was to conduct an exhaustive study aiming at localizing the possible accumulation of MGEs in the different compartments of the Orne River ecosystem (water, suspended material and sediment) and sought for their possible origins based on their correlation with either (i) the local geochemistry, (ii) the local anthropic pressures such as WWTPs, or (iii) the co-occurrence of particular pollutants and their effect on MGE disseminating activity.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MICROBIOLOGICAL ASSAYS

1.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this work are presented in Table 3. All bacteria were routinely grown aerobically in LB broth (Difco™) at 30°C, with agitation at 160 rpm for liquid cultures.

Solid and top agar medium were prepared by adding 15 and 7 g/L of agar before autoclaving, respectively. When required, antibiotic selection was applied (ampicillin at 50 µg/mL, kanamycin at 50 µg/mL, rifampicin at 20 µg/mL, tetracycline at 10 µg/mL and chloramphenicol 15 µg/mL).

Table 3. Bacteria and plasmids used in this study

Name/Species	Genotype/Characteristics	References
<i>Plasmids</i>		
pB10	Wild type IncP-1 plasmid isolated from active sludge	(Schluter 2003)
pBELX	pEX-A derivative (pUC18-based) containing qPCR target sequences for <i>trfA</i> of IncP-1 plasmids and the Eubacterial 16S rDNA gene, Amp ^R	(Bellanger, Guilloteau, Breuil, et al. 2014)
pNORM1	pEX-A derivative (pUC18-based) containing the qPCR target sequences for <i>intI</i> of class 1 integron and the Eubacterial 16S rDNA gene, Amp ^R	(Gat et al. 2017)
pSEVA236	pBBR1 derivate containing the <i>luxCDABE</i> genes, a multiple cloning site, Km ^R	http://seva.cnb.csic.es
<i>Bacillus subtilis</i>		
LMG 7135 ^T	ATCC 6051, Type strain	BCCM/LMG ^b
CM291	Rif ^R derivative of LMG 7135 ^T	LCPME
CM295	CM291(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME
<i>Delftia acidovorans</i>		
CM122	<i>Delftia acidovorans</i> , Rif ^R Kan ^R	Eva Top lab strain collection
CM294	CM122(pB10), Rif ^R , Kan ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME

Name/Species	Genotype/Characteristics	References
<i>Cupriavidus metallidurans</i>		
AE815	plasmid free and Rif ^R derivative of wild type strain CH34	(Springael, Kreps, and Mergeay 1993)
CM124	AE815(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	(Bellanger, Guilloteau, Bonot, et al. 2014)
<i>Escherichia coli</i>		
DH5 α	ϕ 80/lacZ Δ M15 <i>recA1 endA1 gyrA96</i> (Nal ^R) <i>thi-1 hsdR17(r_K⁻m_K⁺) supE44 relA1 deoR</i> Δ (lacZYAargF) U169	Sambrook et al., 1989
DH10B	F- <i>endA1 recA1 galE15 galK16 nupG rpsL</i> Δ lacX74 ϕ 80/lacZ Δ M15 <i>araD139</i> Δ (<i>ara, leu</i>)7697 <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) λ -	Invitrogen
MG1655	Sequenced λ^- and F-derivative of strain K-12	Blattner et al., 1997
CM408	= MG1656; Δ lacZ derivative of MG1655	Mazel et al, 2009
CM102	DH5 α (pB10), Nal ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	Schlüter et al., 2003
CM125	Nal ^R derivative of MG1655	Bellanger et al, 2014a
CM278	CM125(pB10), Nal ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME
CM448	= DH10B(pBELX)	LCPME
<i>Pseudomonas fluorescens</i>		
CIP 69.13 ^T	ATCC 13525, Type strain	ATCC ^c
CM292	Rif ^R derivative of wild type strain CIP 69.13 ^T	LCPME
CM297	CM292(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME
<i>Pseudomonas putida</i>		
SM1443	KT2442 (Rif ^R) with a mini-Tn5- <i>lacI^q</i> insertion	Christensen et al., 1998
CM236	SM1443(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME
<i>Shewanella oneidensis</i>		
CM87	Rif ^R derivative of wild type strain MR-1 ^T	Schwalb et al., 2003
CM293	CM87(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	LCPME

^a Antibiotic resistance phenotype: ampicillin, Amp^R; amoxicillin, Amx^R; kanamycin, Kan^R; nalidixic acid, Nal^R; rifampicin, Rif^R; streptomycin, Str^R; sulfamides, Sul^R; tetracycline, Tet^R.

^b Belgian coordinated collections of microorganisms.

^c American Type Culture Collection

1.2 Crystal-violet staining of adhering biomass

Two milliliters of LB was inoculated with a single colony picked from a fresh plate containing tetracycline (if pB10 is supposed to be carried by the bacteria), and then incubated for 16 h. One hundred microliters of this culture were mixed with 10 mL of fresh medium and placed in a 55 mm petri dish containing a 47 mm-diameter flat polyethylene disk (Kaldnes Biochip Media) that are originally dedicated to the colonization of bacteria in moving bed biofilm reactors in wastewater treatment processes. The plates were incubated at 30°C under agitation (80 rpm) for 18 h. The quantity of biomass adhering to Kaldnes polyethylene disks was determined by classical crystal-violet staining (Chavant et al. 2007). First, the total amount of planktonic bacteria surrounding the polyethylene disks was estimated by measuring broth turbidity (OD_{600nm}). Second, the polyethylene disks were transferred into new petri dishes where they were successively washed 3 times with 10 mL of sterile water for removing poorly adherent bacteria. The polyethylene disks were then air-dried for 1.5 h before being incubated in 10 mL of a 0.1% v/v crystal violet solution in H₂O (Biomérieux) for 45 min at room temperature (20°C±1°C). The stained disks were then gently washed 6 times with sterile water to remove excess of crystal violet. Finally, the biomass-associated crystal violet was dissolved with 10 mL of glacial acetic acid and quantified by UV-Vis spectrophotometry at 540 nm (OD_{540nm}). Polyethylene disks without exposure to bacterial cells were used as a control.

1.3 Bacterial mating assays

Mating assays were performed in liquid medium either containing or not a 47 mm polyethylene disk (Kaldnes Biochip Media). Donor (CM102) and recipient bacteria (AE815) were grown for 16 h in broth, supplemented or not with antibiotics, then washed by centrifugation and re-suspended in one volume of sterile MgSO₄ (10 mM). For mating assays with a polyethylene disk, 10 mL of LB broth containing a 1:100 dilution of both strains, prepared from the washed cell suspensions, were introduced in an empty 55 mm petri dish and incubated

for 18 h at 30°C under agitation (80 rpm). At the end of the incubation period, broth and polyethylene disks were recovered separately for bacterial strain enumeration. Beforehand, the polyethylene disks were washed 3 times with sterile water and adhering bacteria were re-suspended by vigorous vortexing in 20 mL of MgSO_4 (10 mM). Donor (CM102), recipient (AE815), and transconjugant bacteria (AE815(pB10)) were enumerated on selective plates containing either tetracycline (Tet), rifampicin (Rif), or both antibiotics (Tet Rif), respectively. For the control mating assays between planktonic cells, the incubations of donor and recipient cells were performed as described above but the polyethylene disks were omitted.

1.4 Flow cytometry

Bacterial cell concentration in water samples containing particles of different sizes was determined by flow cytometry thanks to a BD Accuri™ C6 flow cytometer (BD Biosciences, USA) equipped with two lasers (a blue one at 488 nm and a green one at 552 nm). Commercial ultrapure water (Aqua B. Braun, Braun, France) was used as sheath fluid. The total cell number was determined after two sonication treatments (1 min at 12 W) and bacterial staining with SYBR™ Green I Nucleic Acid Gel Stain (S7563, Invitrogen, France) at a final concentration 1X for 15 min in the dark. Samples without staining and without sonication were also analyzed as a control (Figure 16). Scatter lights, forward (FSC) and side scatter (SSC), were used to distinguish between cell populations based on size and granularity of the cells, respectively (Givan 2001). Fluorescence intensity of stained bacteria was collected at FL2 channel (λ_{em} : 510 ± 15 nm). Relevant events were enumerated based on a gating using the forward scatter parameter with a threshold 8000 and on FL2 with a threshold 2000 in order to narrow the signal of fluorescent cells and to separate positive signals (stained cells) from background. Event counts provide an estimation of the total cell amount (damaged plus live cells) while the stability of the mean fluorescence reflects the integrity of the intracellular nucleic acid content. The obtained data were analyzed using the BD Accuri™ C6 software (BD Biosciences).

2. STUDY SITE

For a better understanding of the choice of the Orne River as a studied site, it is necessary to have a global view of its history and geography.

The Orne River is located in north-eastern France. It is an 86-km long river that is tributary of the Moselle and sub-tributary of the Rhine Rivers. It crosses 3 from the 4 departments of the Lorraine region: Meuse, Meurthe-et-Moselle and Moselle. It rises in the town of Ornes in the Meuse department and it flows through 35 towns until Richemont, where it joins the Moselle River (Picon 2014). The watershed land uses are mainly urban and industrial (6 %), agricultural (67 %) and forest (27 %) (Corine Land Cover 2006), but these areas are not homogeneously distributed in the watershed. Indeed, the watershed upper part is agricultural, forested and little urbanized whereas the downstream section, that is part of the north Lorraine ferriferous basin, is widely urbanized and industrialized.

The Orne watershed was intensively impacted by steelmaking activities during the last two centuries, (Kanbar et al. 2017). The iron ore mining was divided into several zones in the Lorraine region, among them the Orne Valley with the mines of Joeuf, Moyeuvre, Homécourt and Auboué. The riverbed was strongly modified for industrial purposes, and two important dams were built in the 1958-1965 period in order to create artificial water reservoirs for cooling blast furnaces of the steelmaking facilities (Picon 2014).

Nowadays, the steel production has ended, but the accumulation over the time of diverse organic and inorganic pollutants in river sediments could have an impact not only in river water quality, but also in the occurrence of ARGs in the different compartments of the river water column.

To study the possible impact of the anthropogenic pressure and the accumulation of pollutants in the occurrence of ARGs, different sampling sites were selected along the Orne River course (Table 4). During a 15-month period, several sampling campaigns were performed along the last 23 km of the Orne River: November 4th 2014, February 2nd 2015, March 3rd 2015, March 11th 2015, March 30th 2015, May 5th 2015, October 6th 2015, November 2015 and February 2016.

Table 4. Description of sampling sites along the course of the Orne River (modified from Kanbar, 2017).

Site^A	Abbreviation	Description
Auboué	AUB	Site located upstream of the industrialized section of the Orne River. Sampling campaigns were performed just before the mouth of one the Orne River tributary, the Woigot River.
Woigot	WOI	Site located upstream of the urban area of Homécourt and dominated on the right side by the slag heap of Auboué.
Homécourt Barbusse	BARB	Site surrounded by urban areas (left and right sides of the river) and located upstream of the Homécourt urban area. The site is located in the river section in which hydrodynamics is impacted by the Homécourt dam
Homécourt dam upstream	HOMUP	Site immediately upstream of the Homécourt dam. Forest area on the left side and industrial/mining area on the right side. This site was used as water reservoir by steelmaking industries, mainly between the 1870s and the 1950s.
Joeuf Abattoir	JOAB	Site boarded by forest areas on the left side of the river, located at the entrance of Jœuf city near a former slaughterhouse.
Joeuf Haropré	JOHA	Site located in the very beginning of the Beth dam hydrodynamic influence zone, with a marked decrease of water flow in comparison with the previous site, JOAB.
Joeuf Sainte Anne	JOSAN	Site located in the Beth dam hydrodynamic influence zone. The left shore of the river includes forests whereas the right shore includes former sport/leisure facilities
Joeuf Médiathèque	JOMED	Site located in the Beth dam hydrodynamic influence zone. The right shore of the river contains forests, while the left shore includes industries. The site adjoins the upper area of the former Europipe site
Joeuf Europipe	JOEP	Site located in the Beth dam hydrodynamic influence zone downstream of the former Europipe site
Joeuf wastewater treatment plant	JOWWTP	Effluents from the local and active wastewater treatment plant (51000 PE ^B)
Beth dam upstream	BETHUP	Site immediately upstream of the Moyeuve-Grande dam, surrounded by industrial area
Richemont	RICH	Site located at the end of urbanized and industrial areas just before the river mouth

^A From upstream to downstream

^B PE= Population equivalent or unit per capita loading

2.1 Sample collection

- Raw water

Several sampling campaigns were performed to collect water samples along the Orne River at representative sites (Table 4). Water samples were collected within 3 amber-glass bottles of 2.5 L, transported to the laboratory, then distributed in small 0.5-L bottles and stored at -20°C until use.

- Suspended particulate matter (SPM)

For the purpose of this work, SPM samples were collected by using either a field continuous flow centrifuge or serial filtrations of water samples.

During field continuous flow centrifuge-based sampling, river water was pumped and sent to a continuous flow field CEPA Z-41 centrifuge (20,000 RPM, equivalent to 17,000 $\times g$, with a flow rate of 600 L.h⁻¹) to obtain a representative SPM-enriched fraction from [ca.] 1 m³ of raw water. The cut-off particle size of the field centrifuge was around 5 μm . SPM samples were recovered by scraping the internal part of the centrifuge rotor, transported to the laboratory in an ice-box and then stored at -80 °C until use.

The SPM collection was also performed through a water sample fractionation approach based on serial filtrations, and using a succession of polycarbonate membranes (Milipore Isopore™) with pore sizes: 10, 5, 1.2 and 0.2 μm . A fraction (150 mL) of the filtrate obtained from each of the different pore membranes was subject to flow cytometry and molecular analyses (Figure 16). In brief, 650 mL of raw water was filtered first on a 10 μm pore membrane; from the filtrate obtained, 100 mL was recovered for extracting DNA, then used for qPCR quantifications while others 30 mL was used for flow cytometry analysis (described below). Then, the remaining filtrate (around 500 mL) was filtrated on the 5 μm membrane, and so on until the smaller membrane of 0.2 μm . At each step, 150 mL of the filtrate was recovered for the analyses mentioned above.

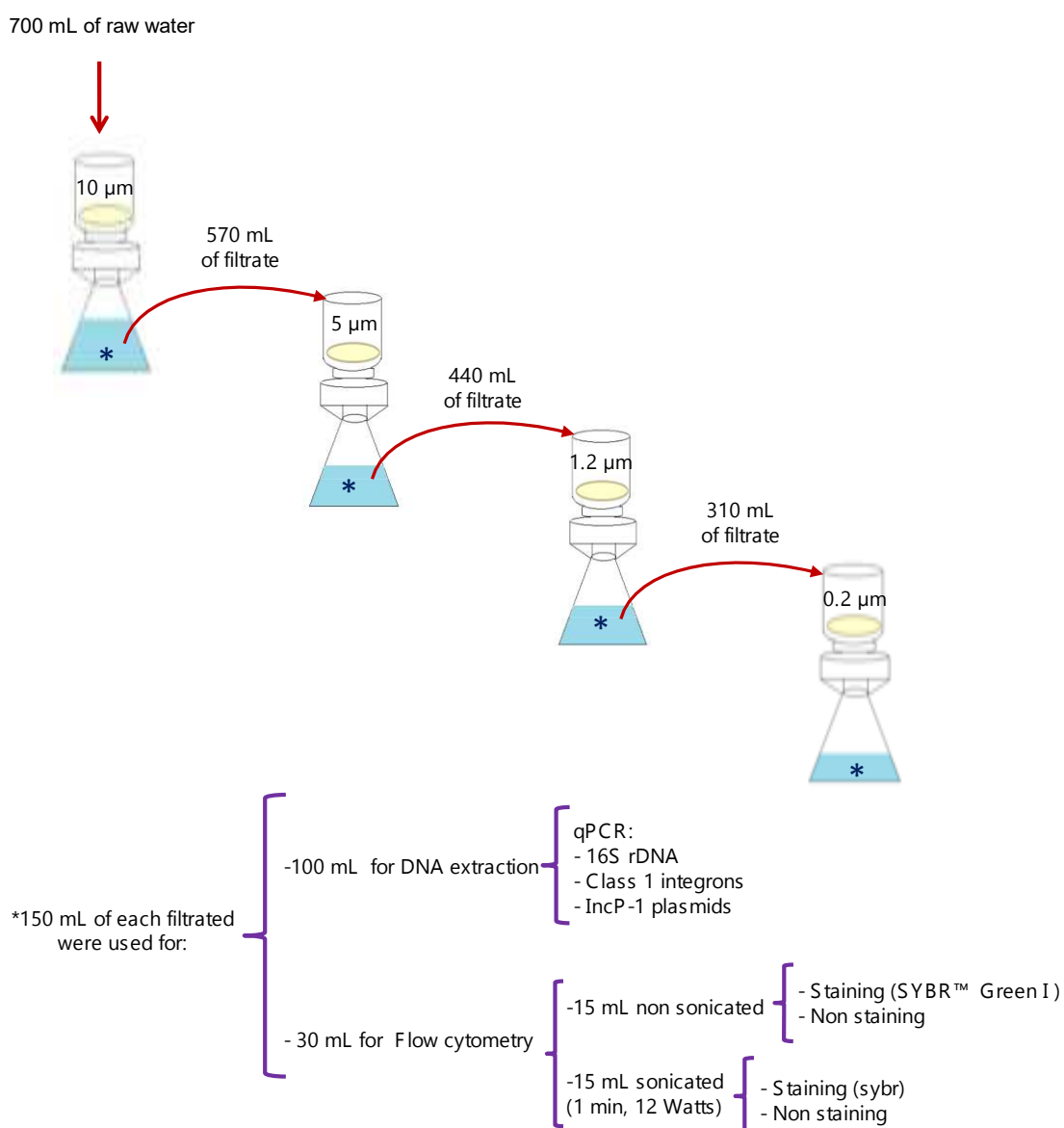


Figure 16. Experimental strategy of the serial filtrations dedicated to recovery of water samples fractions containing SPM of different sizes. The volumes indicated for filtrates are theoretical, without considering possible losses upon performing.

- Sediments

Core sediments were sampled on February 2014 (BETH core) and February 2015 (JOSAN core) following the method described by Kanbar et al. (2017) and using a piston corer (Beeker corer) directly from sediment bank. Sediment cores were sealed from air and transported back to the laboratory in a vertical position where they were sliced into layers of approximately 2 or 3 cm into an N₂-filled glove bag to avoid oxidation and stored at -80°C until use.

2.2 Geochemical analysis, global parameters and elemental content

The geochemical characterization of water, SPM and sediment samples from the Orne River were performed by the research group of the Interdisciplinary Laboratory for Continental Environments (LIEC).

During sampling, global parameters such as water temperature, electrical conductivity (EC), dissolved oxygen concentration, pH and turbidity were measured *in situ* using a portable multiparameter device (Hach®). The elemental content of the river water was also determined. In brief, major elements were detected by inductively coupled plasma optical emission spectrometry (ICP-OES). These analyses were performed at SARM (Service d'Analyse des Roches et des Minéraux – CRPG, Vandœuvre-lès-Nancy, France) and all analytical methods were subject to QC/QA procedures using certified reference materials (Carignan et al. 2001). Trace elements were quantified by inductively coupled plasma mass spectrometry (ICP-MS, X7 ThermoFisher) and total carbon and loss on ignition were calculated by the procedures developed at the SARM (Carignan et al. 2001). Grain size distribution of SPM and sediments were obtained using laser diffraction (SYMPATEC) with two distinct lenses corresponding to two distinct size ranges (0.45-87.5 µm and 4.5-875 µm). Samples were systematically ultrasonicated for 20 s before measurement, and each measurement was duplicated or triplicated. Mineralogy of sediment and SPM samples was investigated using X-ray Diffraction and Transmission Electron Microscopy as mentioned in Kanbar et al (2017) (Kanbar et al. 2017)). The organic characterization in terms of polycyclic aromatic compound and dissolved organic carbon contents were performed as described by Abuhelou et al. (2017). All the physico-chemical parameters analyzed in the different samples of the Orne River are listed in Table 5.

Table 5. Geochemical parameters determined on water, suspended material and sediments from the Orne River

Global parameters

Water temperature^A, pH^A, electric conductivity (EC)^A, turbidity^A, dissolved organic carbon^A, total organic carbon, grain size distribution

Elemental content^B

Major elements	Trace elements	Rare earth elements
Al, Ca, Fe, K, Mg, Mn, P, S, Si and Ti	As, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu, Ga, Ge, Hf, In, Mo, Nb, Ni, Pb, Rb, Sb, Sn, Sr, Ta, Th, U, V, W, Zn and Zr	- Sc and Y - light lanthanides (Ce, La, Eu, Nd, Sm and Pr) - heavy lanthanides (Dy, Er, Gd, Ho, Lu, Tb, Tm and Yb)

Polycyclic aromatic compounds (PACs)

Polycyclic aromatic hydrocarbons (PAHs)	Oxygenated PACs (O-PACs)	Nitrogen PACs (N-PACs)
Naphtalene, Acenaphtylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo(ah)anthracene Benzo[ghi]perylene (BghiP)	Dibenzofuran, 9H-fluorenone, Perinaphtenone, Anthraquinone, Cyclopenta(def)phenanthrene, Methylanthracene-9,10-dione, Benzo(a)fluorenone, Benzanthrone, Benzoanthracenedione, Naphtacene-5,12-dione, Benzo(cd)pyrenone	Quinoline, Benzo(h)quinoline, Acridine, Carbazole, Benzo(c)acridine, Nitropyrene

^A Parameters determined just for water samples

^B Symbol of metals according to Mendeleev's periodic table

3. MOLECULAR BIOLOGY ASSAYS

3.1 Total DNA extraction from environmental samples

The raw waters, SPM and sediment samples were thawed on ice the shortest possible time to collect 100 mL of water or 50 mg of SPM or sediments in triplicate and were then kept at 4°C until dispersion and/or filtration. Sediments or SPM were dispersed in 100 mL of non-pyrogenic sterile water (Aqua B-Braun) by vortexing for 30 s followed by 15 min agitation at 160 rpm and 25°C for recovering particle-bound bacteria and improving their lysis. Then, the 100 mL of raw water, dispersed SPM or dispersed sediment samples were filtered on polycarbonate filters (Whatman Nuclepore filter, pore size 0.22 µm, diameter 47 mm) using a filtration system (Combisart 6-branch Manifold, Sartorius). Total community DNA was extracted from the filters using the PowerWater® DNA Isolation Kit (MO BIO laboratories Inc) according to manufacturer instructions (see annex 1.1 for the detailed protocol) and was eluted from silica columns with 100 µL of PCR grade water (RNase-Free Water, Qiagen) and stored at -20°C until use.

Total community DNA from 250 mg of the sediment samples was also extracted by duplicated using the PowerSoil® isolation kit (Qiagen) according to manufacturer instructions (*i.e.* without dispersion and filtration steps) and was eluted from silica columns with 100 µL of PCR grade water (RNase-Free Water, Qiagen) and stored at -20°C until use (see annex 1.2 for the detailed protocol).

3.2 Determination of DNA concentration and purity

The concentration and purity of the total DNAs extracted were determined spectrophotometrically by measuring optical density at 320, 280, 260 and 230 nm (Spectrophotometer Eppendorf BioPhotometer). The measurement at 260 nm allows the calculation of the DNA concentration (one optical density unit per centimeter corresponding to 50 µg/mL of double-strand DNA). The measurement at 230 nm allows determining possible contamination due to organic compounds. The measurement at 320 nm is an indicator of the presence of particles in the solution (values should be <0.03). The ratio of the

measurements at 260 nm and 280 nm provides an estimation of the purity of the nucleic acid. This ratio is expected to be between 1.8 and 2 for pure DNA. The ratio between values obtained at 260 nm and 230 nm is used as a secondary measure of nucleic acid purity. The 260/230 ratio values for “pure” nucleic acid are supposed to be in the range of 2.0-2.2.

3.3 Quantitative PCR assays

The abundance of eubacterial 16S rDNA, class 1 integrons, IncP-1 plasmids, α -, β - and γ - proteobacteria were quantified in triplicate by qPCR using the “Power SYBR® Green PCR Master Mix” (Applied Biosystems) with the primers listed in Table 6. qPCRs were performed using a “Step One Plus Real-Time PCR System” (Applied Biosystems, driver: StepOne Software v2.2). Each reaction comprised 1.25 μ L of each primer at 10 μ M, 12.5 μ L of SYBR green master mix and 10 μ L of DNA template solution. The thermocycling conditions were set for 16S rDNA, class 1 integrons and IncP-1 plasmids, as follows: 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. For α - and β -proteobacteria, the conditions were: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 56°C. Finally, for γ -proteobacteria, the cycles were: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The quality of the PCR products was subsequently checked by melting curve analyses, for which the temperature was ramped between 60°C and 95°C in increments of 0.3°C. The absence of qPCR inhibitors in the DNA extracts was verified by qPCR by comparing amplification from serially diluted DNA templates. The detection limits were determined to be 1 x10³ gene copies per reaction for 16S rDNA and the 3 proteobacteria, and 1x10¹ gene copies per reaction for class 1 integrons and IncP-1 plasmids.

Serial dilutions of plasmid DNA containing a cloned target sequences were used for obtaining standard qPCR amplification curves. Recombinant plasmid DNAs pBELX and pNORM1, used as qPCR standards, were extracted using the « Wizard® Plus SV Minipreps DNA Purification System » (Promega) according to the recommendations delivered by the manufacturer. The plasmids

were linearized by *Bam*HI (Promega) before being purified with the « QIAquick PCR DNA purification kit » (Qiagen) and spectrophotometrically analyzed.

Table 6. List of primers and conditions used for qPCR and DGGE-related PCR

Name	Sequence	Targeted gene	Amplicon size (bp)	References
331f 518r	5'-TCCTACGGGAGGCAGCAGT-3' 5'-ATTACCGCGGCTGCTGG-3'	16S rDNA	197	Hunter et al., 2002; Muyzer et al., 1993
inti1-lc1 inti1-lc5	5'-GCCTTGATGTTACCCGAGAG-3' 5'-GATCGGTCTGAATGCGTGT-3'	<i>intI1</i> (class 1 integron integrase)	196	Barraud et al., 2010
trfa2-1 trfa2-2	5'-CGAAATTCRTRTGGGAGAAGTA-3' 5'-CGYTTGCAATGCACCAGGTC-3'	<i>trfA</i> (replication initiation of IncP-1 plasmids)	241	Götz et al., 1996
338f 685r	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-TCTACGRATTTACCCYCTAC-3'	α -proteobacteria 16S rDNA	365	Fierer et al., 2005
338f 680r	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-TCACTGCTACACGYG-3'	β -proteobacteria 16S rDNA	360	Fierer et al., 2005
1080f 1202r	5'-TCGTCAGCTCGTGTGTGA-3' 5'-CGTAAGGGCCATGATG-3'	γ -proteobacteria 16S rDNA	170	Bacchetti De Gregoris et al., 2011
968F-GC	5'- CGCCCGCCGCGCCCCGCGCCCGTC CCGCCGCCCCCGCCCGAACGCGAA GAACCTTAC-3'	16S rDNA*	433	Quéméneur et al., 2010
1401R	5'- CGGTGTGTACAAGACCC-3'			

*: for Denaturing Gradient Gel Electrophoresis (DGGE) purpose

3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Essentially, touch-down PCRs were performed using total community DNA to obtaining DNA fragments of about the same size. A GC clamp was added to primer 968F-GC (Table 6) to prevent complete strand separation in denaturing conditions. Each reaction of 50 μ L comprised: 2.5 μ L of each primer at 10 μ M, 5 μ L of Taq buffer 10X, 1 μ L of dNTP mix at 10 mM, 4 μ L of MgCl₂ at 25 mM,

33.75 μL of water, 0.25 μL of Taq DNA polymerase and 1 μL of DNA template (0.3 ng).

The thermocycling conditions were modified from Quéméneur et al., 2010 and consist in a first step of 5 min at 95 °C, followed by 18 cycles of [30 s at 95°C; 45 s from 63 °C to 54 °C (-0.5 °C/cycle) and 30 s at 72 °C], then, 15 cycles of [30 s at 95°C; 45 s at 54 °C and 30 s at 72 °C] and finally, 10 min at 72 °C.

Then an 8% polyacrylamide gel was prepared in a 45% to 60% urea-formamide denaturant gradient using a DCode™ Universal mutation detection system (Bio-Rad). The electrophoresis was carried at a uniform temperature of 60 °C during 16h at 85 V. Thereafter, the gel was stained in an ethidium bromide solution for 1 hour before to be washed for 30 minutes, two times in TAE 1X buffer and a last once in water, and finally imaged using a Gel doc™ XR⁺ (Bio-Rad) System and the Image Lab™ Software.

The resulting band profile, representing the community structure, was analyzed with the Quantity One software (Bio-Rad) thanks to the unweighted pair group method using average linkages (UPGMA).

4. STATISTICAL ANALYSIS

The different statistical tests used for the data analysis of this work are listed in Table 7. They were independently chosen according to the circumstances and performed using the R software. Two tests/approaches needing an exhaustive presentation (Cochran's test) or unusually used (comparison of coefficients of variation) were further detailed in section 4.1 and 4.2, respectively, whereas ANOVA tests especially designed for this study are presented in the results part I, section 2.

Table 7. Statistical tests and their application

Statistical test	Application
Data distribution test	
Shapiro- Wilks W	Verification of data normality
Cochran*	Verification of homogeneity of variances
Spearman's rank	To assess the relationship between two variables

Statistical test	Application
correlation coefficient	(whether linear or not)
Principal Component Analysis (PCA)	To identify patterns in data and to reduce the dimensionality of it
Parametric comparison test (normal distribution of data verified)	
Student	To determine if two set of data differ significantly between them
ANOVA** - Nested (dependent groups) - Mixed (independent groups)	To determine if two or more groups of data (dependent or independent) differ significantly between them
Nonparametric comparison test (non-normal distribution of data)	
Wilcoxon signed-rank	To determine if two related (paired) set of data differ between them
Wilcoxon sum-rank	To determine if unpaired set of data differ between them
Kruskal–Wallis	To determine if two or more independent groups of data differ significantly between them
Friedman	Similar to ANOVA test, but for non-normal distribution of data

*: More details about Cochran's test can be found bellow

**: Specific information for the design of the ANOVA tests can be found in results part I, section 1.

4.1 Cochran's test

Cochran's test is based on the null hypothesis (H_0) that the variances of all compared data groups are equal and the alternative hypothesis (H_a) that at least one variance value is significantly larger/smaller than the others. The test takes into account the largest standard deviation of the data set divided by the sum of the standard deviations of the data set to the square:

$$C \text{ value} = \frac{\text{largest } s^2}{\sum s^2}$$

The resulting C_{value} was then compared with a Critical C_{value} obtained from tables (annex 3) where, usually, the level of significance α was 0.05, the number of data groups was named k , and the degrees of freedom were equal to $n-1$, where n was the number of qPCR replicates (at least 2) (Figure 17). For results interpretation: **if $C_{\text{value}} > \text{Critical } C_{\text{value}}$, the H_0 hypothesis is rejected.**

As a practical example of the use of this test, the data from the transect experiment (results part I, section 1.1) were ordered in terms of sampling dates (3rd, 11th and 30th March 2015), and the homogeneities of variances were verified from each sampling site of the river transect (JOHA and BETHUP) and for each molecular marker (16S rDNA, class 1 integrons, IncP-1 plasmids and their relative abundances), independently. By doing so, each DNA extraction with its qPCR replicates was considered as a group. Thus, for the BETHUP site, we considered 6 sampling points from which total DNA was extracted in triplicate reaching 18 groups of qPCR values. When, for technic reasons, one of the qPCR value was missing, it was necessary to remove the whole corresponding group (*i.e.* a sampling point) to keep the symmetry of the data, otherwise the implementation of the ANOVA test could not be possible even if variances appeared as homogeneous. The homogeneity of variances was evaluated at both the transect level and the molecular level.

Level of significance $\alpha=0.05$

Number of groups k	Degrees of freedom= $n-1$							
	1	2	3	4	5	6	7	8
2	0.9985	0.9750	0.9392	0.9057	0.8772	0.8534	0.8332	0.8159
3	0.9669	0.8709	0.7977	0.7457	0.7071	0.6771	0.6530	0.6333
4	0.9065	0.7679	0.6841	0.6287	0.5895	0.5598	0.5365	0.5175
5	0.8412	0.6838	0.5981	0.5441	0.5065	0.4783	0.4564	0.4387
6	0.7808	0.6161	0.5321	0.4803	0.4447	0.4184	0.3980	0.3817

Figure 17. Example of table for critical C values for the Cochran's test.

4.2 Coefficient of variation

Calculating a coefficient of variation (in %) by dividing the standard deviation (SD) of a value set by the mean value is a way for depicting the dispersion/distribution of these values around the mean. We used coefficients of variation (CV) during statistical analyses in order to compare the dispersions of values corresponding to the relative abundance of class 1 integrons from DNA extracts obtained with two different extraction kits. During this approach, we faced the case where SDs were calculated for sets of 2 values. When $n < 10$, the usual way for calculating SD is not accurate (Gurland and Tripathi 1971). In such case, an unbiased SD correction factor could be applied when the data (global population) has a normal distribution (Soka and Rohlf, 1995).

4.3 Box plot

A box plot allows to show the distribution of data based on the five number summary: minimum, first quartile, median, third quartile, and maximum (Figure 18). This type of graph was frequently used to represent the different results of this work.

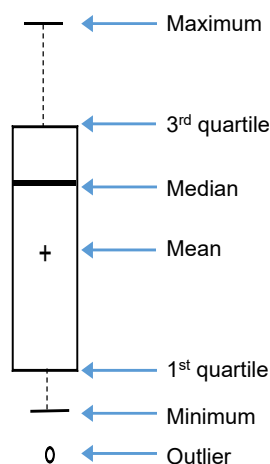


Figure 18. Box plot representation

RESULTS

RESULTS

FOREWORD: FROM ANTIBIOTIC RESISTANCE TO THE ORNE RIVER

The persistence and dissemination of bacterial antibiotic resistance in the environment is relatively complex as it involves multiple environmental parameters and mechanisms. It is well known that the abundance of ARB is greatly magnified by human activities and antibiotic therapies, but the fate of ARB, once reaching the environment, remains less understood. As a matter of fact, the chance of intestinal ARB to persist after being released in the environment is relatively poor and therefore, their ARGs are not supposed to persist very long unless they are transferred into bacteria adapted to the environmental lifestyle. With this respect, the HGT mechanisms involved and the permissivity of the environment in which they take place are key factors for the persistence of ARGs, but is that all? For instance, several reports emphasized the importance of co-selective processes where pollutants such as metals can contribute to enrich ARGs locally. When we started this work, we had the ambition to understand and describe how ARG/ARB abundances fluctuate in time and space in a complex environment, here a river ecosystem, and what is the contribution of the local anthropic pressures to their persistence and enrichment. To do so, the abundance of antibiotic resistance genes was studied using two MGEs as proxies, namely the class 1 integrons and IncP-1 plasmids, in the Orne River ecosystem (Moselle) chosen as being impacted by multiple sources of anthropic pressures. At present, the Orne River watershed has a contrasted land use with urban and industrial (6%), agricultural (67%) and forest areas (27%) (Corine Land Cover, 2006). Apart from expected urban and agricultural pollution, the Orne River ecosystem also inherited pollution originating from industrial activities related to mining (iron, coal) and metallurgical activities (Zn, Fe) that were very active during the 20th century. As a consequence, several plants, blast furnaces and slag heaps were installed at different sites close to the river resulting in pollution caused by the accumulation of organic and inorganic persistent pollutants such as metals and polycyclic aromatic hydrocarbons (PAHs) in water and soil (Kanbar et al. 2017; Abuhelou

et al. 2017). With this respect, it should be noted that the riverbed has also been physically altered for industrial purposes through the construction of dams in order to be used as water supplies. Such constructions also resulted in localized accumulation of heavily contaminated sediments (Kanbar et al. 2017). The river section and sampling sites selected for this study, as well as the cartography of possible sources of anthropogenic pressure, are presented in Figure 19.

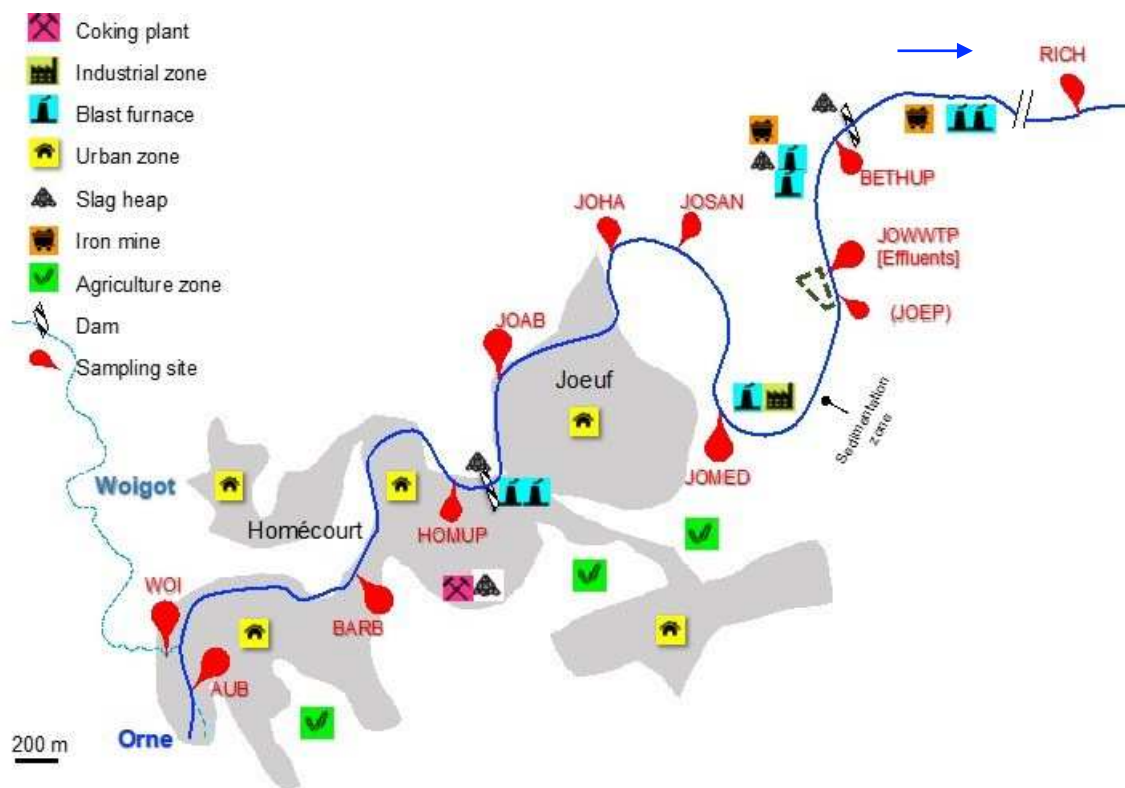


Figure 19. Distribution of the sampling sites and anthropogenic pressures along the studied section of the Orne River. From upstream to downstream: Woigot (WOI), Auboué (AUB), Homécourt Barbusse, (BARB), Homécourt (upstream of the dam; HOMUP), Joeuf Abattoir (JOAB), Joeuf Haropré (JOHA), Joeuf Sainte Anne (JOSAN), Joeuf Mediathèque (JOMED), Joeuf Europe (JOEP), Joeuf wastewater treatment plant (JOWWTP), Beth (upstream of the dam; BETHUP) and Richemont (RICH). Grey area indicates the urban zone of Homécourt and Joeuf cities. The light yellow line over the river between JOHA and BETH represents the zone of influence of the Beth dam on the hydrodynamics of the river (increased sedimentation zone).

The area between the sampling sites of Barbusse (BARB) and the Beth dam (BETH) was very active in iron metallurgy and steelmaking during the XIXth and XXth century. Two dams located in that area, namely Beth (at Moyeuvre-Grande) and Homécourt, were used to create artificial water reservoirs for cooling blast furnaces of the steelmaking facilities. The Richemont (RICH) sampling site marks the Orne River and the end of approximately 23 km of the urbanized area (starting from BARB). The RICH site lies about 1.3 km before the confluence with the Moselle River (Kanbar 2017). Details of sampling sites can be found in Table 4 (materials and methods section 2).

As part of the MOBISED project, whose objective was to evidence the mechanisms involved in sediment remobilization and the consequences regarding water quality, an exhaustive characterization of the samples collected at each sampling site was performed, including global and geochemical parameters (Table 5, materials and methods section 2.2). In the context of this work, this gave us the opportunity to evaluate whether the fluctuation of MGE abundances correlates specific global/geochemical parameters, thus providing information on possible common origins or co-selective processes when relevant.

On a global point of view, the river ecosystem can be perceived as two contrasted compartments, the river water, in which ARGs/ARB transit, and the sediments, where ARGs/ARB can settle, accumulate and form reservoirs over time. In this work, we studied the fluctuation of ARG/ARB abundances overtime, and over space in the two different compartments, and how one could feed the other. These will be the subjects of the three different parts of the following Results section. In each case, we will try to describe how MGE abundances fluctuate in space and time and how do they relate to important global and geochemical parameters.

PART I: UNDERSTANDING MGE ABUNDANCES IN THE ORNE RIVER WATER

When this work was initiated, the main idea was to quantify two types of MGEs, class 1 integrons (*intI1* gene marker) and IncP-1 plasmids (*trfA* gene marker), at different sites over time along the studied river section, and establish possible scenarios concerning their origin and persistence in the water column. The experimental strategy was set based on series of sampling of river water, followed by the extraction of total microbial community DNAs and quantification of class 1 integrons and IncP-1 plasmids by qPCR. On the other hand, the abundance 16S Eubacterial RNA gene (16S rDNA) was also to be quantified by qPCR so as to be used as an indicator of the global bacterial population and to monitor changes in relative abundance of MGEs (copies / 16S rDNA).

Before studying the fluctuation of the two MGEs in space and time, it appeared fundamental to properly define at what level an environmental fluctuation of MGEs could be observed. In other words, what is the contribution of the experimental procedure (DNA extraction, qPCR), and the sampling (depth, position) on the abundances measured? To answer this question, the first part of this experimental work was devoted to evaluating the contribution of any possible source of variation in the quantification of class 1 integrons and IncP-1 plasmids by qPCR.

1. DEFINING THE SOURCES OF VARIABILITY IN THE QUANTIFICATION OF THE MGE ABUNDANCES IN THE WATER COLUMN OF THE ORNE RIVER

On the principle, the sources of variation in the abundance of MGEs of the Orne River water could have been multiple: date of sampling, site of sampling, sampling position relative to the river bank, depth of sampling, DNA extraction, quantification by qPCR. Our initial concern was that the real environmental fluctuation of MGEs has to be higher than technical-based fluctuation to be properly assessed. To address this question, it was decided to study two river

transects over time. Two different sampling sites, localized approximately 4.3 km apart (Joeuf Haropré (JOHA) and Beth (BETHUP); (Figure 19), were sampled at different positions relative to the river bank and different depths during three sampling campaigns carried out in March 2015 (the 3rd, 11th, and 30th). Depending on the level of water in the river at the moment of the sampling, water samples were collected at 4 or 6 sampling points (relative to the river bank and depth) at each site (Figure 20). The experimental sources of variations were evaluated at each step of the experimental strategy implemented, considering that each water sample was subject to three total DNA extractions (using the PowerWater® DNA isolation kit) and that each DNA was used to quantify the abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids by qPCR in triplicates. In the best cases, 9 qPCR values were obtained per sampling point. Nevertheless, sometimes some qPCR values were not considered because they were out of the limits of the standard curve or qPCR inhibitors were present.

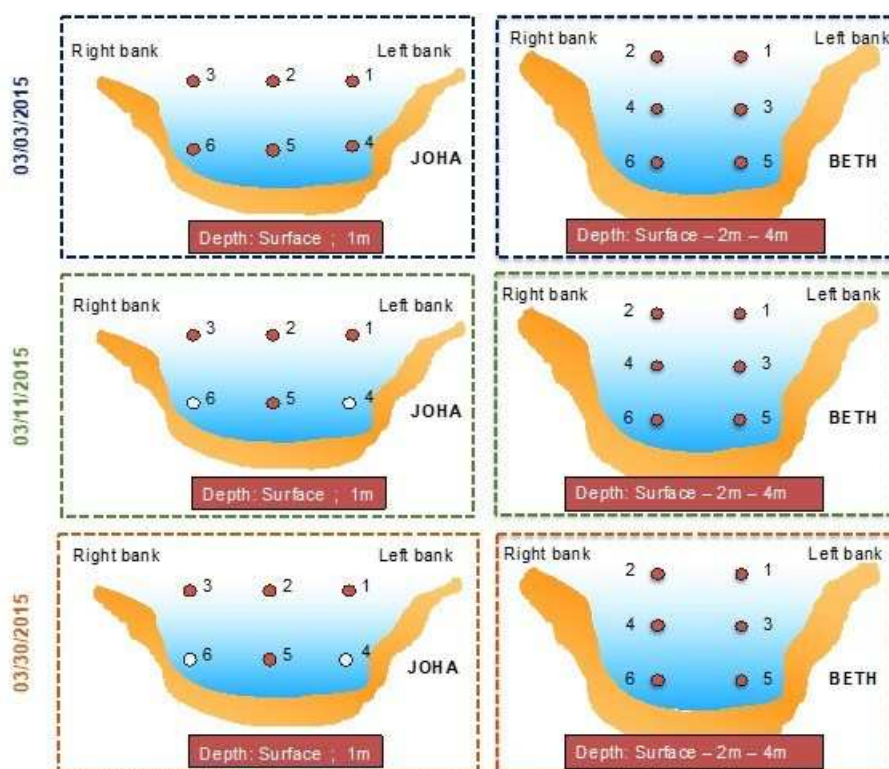


Figure 20. Sampling strategy of the Orne River transects to evaluate environmental fluctuation as opposed to the technical fluctuation of MGE abundances. Sampling points are indicated by red circles. White circles indicate the absence of sample collected for a given date when the level of the water column was too low. Sampling dates are indicated on the left side.

Absolute abundances of MGEs and 16S rDNA (copies/mL of water) in raw water samples are presented in Figure 21. Whatever the sampling site, each individual marker appeared to vary over a wide range of values, covering about 3 orders of magnitude, depending on the date of sampling: from 3.0×10^4 to 1.4×10^7 , 2.3×10^2 to 1.1×10^5 and 6.4×10^1 to 9.4×10^3 copies/mL of water for 16S rDNA, class 1 integrons and IncP-1 plasmids, respectively, at the JOHA site; and from 9.2×10^3 to 1.2×10^7 , 8.0×10^1 to 7.4×10^4 and 4.4×10^0 to 4.4×10^3 copies/mL of water for 16S rDNA, class 1 integrons and IncP-1 plasmids, respectively, at the BETH site. This already strongly suggests that the biggest source of variation is associated with the sampling dates even though they were relatively close to each other (within a month). On the other hand, when comparing MGE and 16S rDNA abundances not that many differences were observed between sampling sites (JOHA *versus* BETH) and between sampling points (transects) for a given sampling date. In other words, MGE and 16S rDNA absolute abundances at JOHA and BETH sites are more or less in the same range when they were compared for the same date. Still, the visual interpretation of the graphs does not allow getting evidence as to know whether sampling sites, sampling position or technical variability could influence (even slightly) the absolute abundances of MGEs and 16S rDNA within the range of values measured. To go further, it became clear that statistical analyses were necessary (i) to determine if the abundances of the different variables considered were significantly different within a sampling date, and (ii) to determine and rank the contribution of each environmental and technical parameter influencing the fluctuation of MGE and 16S rDNA absolute abundances.

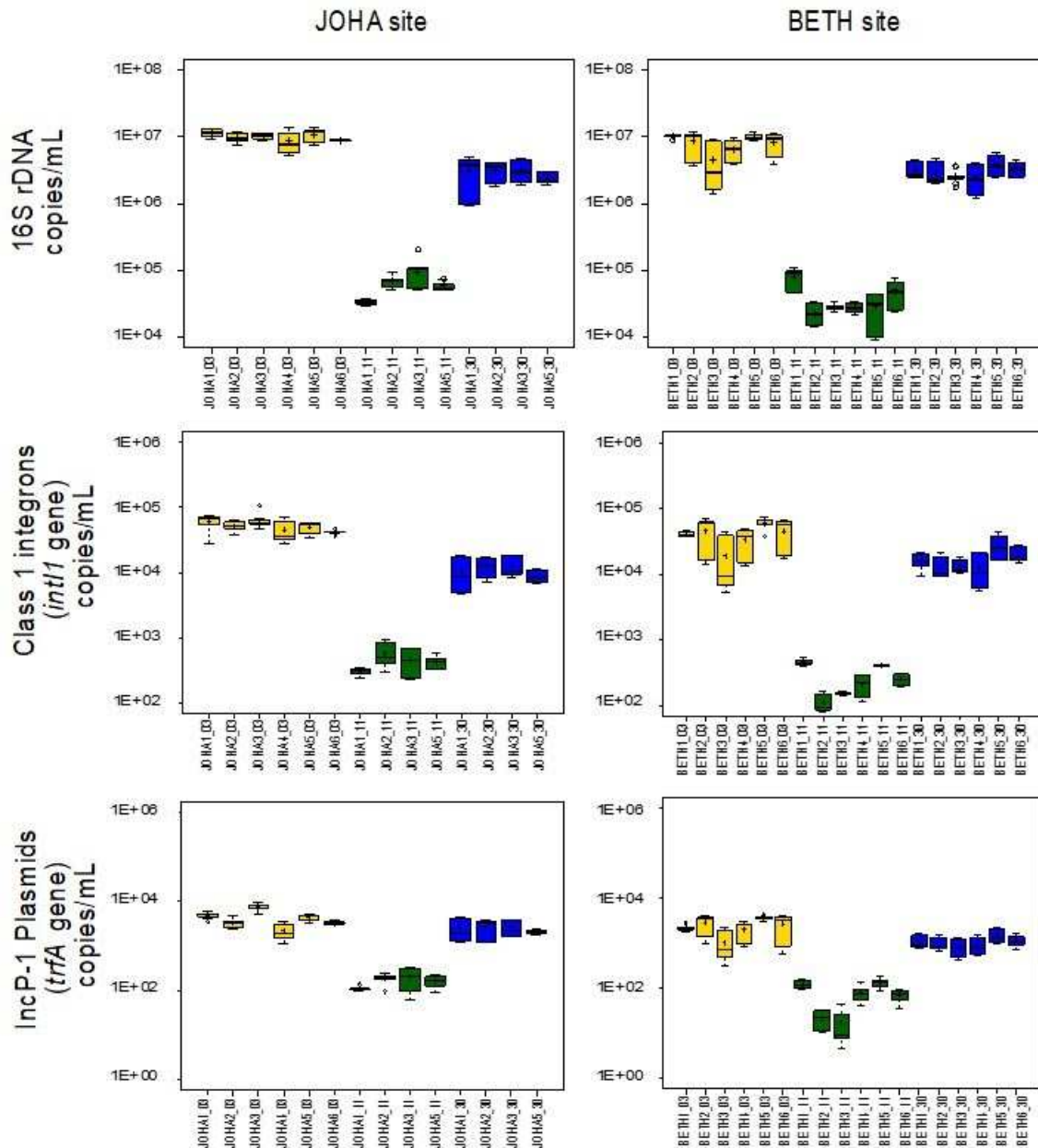


Figure 21. Variability of the absolute abundances of the three molecular markers (*int11*, *trfA*, 16S rDNA) according to sampling position and sampling site (JOHA: left graphs, BETHUP: right graphs). Each color indicates a sampling date (yellow: 03/03/2015, green: 11/03/2015, blue: 30/03/2015). On the “X” axis, samples are named according to the following rational: “siteY_Z”, where “Y” and “Z” refer to the sampling position and day of sampling according to Figure 20, respectively. Boxplot: upper and lower part of the box represent 3rd quartile and 1st quartile; band inside box, the median; (+),mean; upper whisker, maximum value, lower whisker, minimum value; points, outliers.

2. RELATIVE CONTRIBUTION OF ENVIRONMENTAL AND TECHNICAL PARAMETERS TO THE FLUCTUATION OF MOLECULAR MARKERS IN THE ORNE RIVER: A STATISTICAL APPROACH

Considering the large amount of data that we obtained from the transect experiment, almost 1000 values from the qPCR quantifications of the three molecular markers, it became necessary to figure out the best way to analyze them in a robust manner. Ideally, we wanted to use a statistical test that enables the identification of the real sources of variation of the molecular markers quantified among the environmental and technical parameters considered, and eventually rank them according to their influence in terms of coefficient of variation (CV). Depending on the case, we initially thought about using one-way ANOVA tests or Student's T-tests (for unpaired data) to compare the sets of data, in such a way that each comparison could highlight a driving parameter (sampling site, date position, DNA extraction, qPCR) when responsible for a statistical difference. This approach quickly became too complex and not really appropriate, especially because one-way ANOVA is normally used to compare independent groups of data, while in our case, some of the data are dependent to each other (only the three series of data obtained from the three different sampling dates are independent). To go further, we sought help from a biostatistician, Prof. Armand Maul (LIEC, University of Lorraine), who drove our path into a more complex approach considering the interrelationship between the data (i.e. nested ANOVA tests), which requires a hierarchization of the sources of variation (Figure 22).

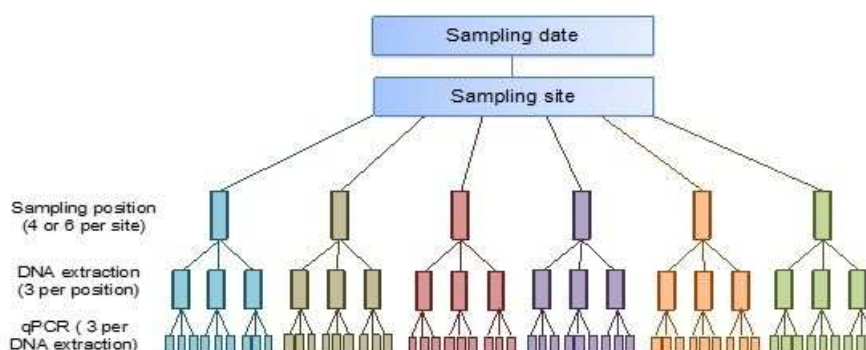


Figure 22. Hierarchization of the possible sources of variation of the abundances of molecular markers in the Orne River. The higher level in the hierarchy corresponds to the sampling date and the lowest level is for the qPCR quantifications.

2.1 Verification of the homogeneity of variances by Cochran's test

Before applying any statistical association tests, it was mandatory to verify the homogeneity of variance of the data at the lowest level of the hierarchy (qPCR) using Cochran's tests. This was made on log transformed data in order to stabilize the variances and normalize the distribution of the data, as usually performed in similar analyses. The Cochran test is based on the null hypothesis that all variances are equal and the alternative hypothesis that at least one variance value is significantly larger than the others. For each group of data (arranged by date, by site and by molecular marker), a C_{value} was calculated and compared to a Critical C_{value} obtained from tables (see section 4.1, materials and methods). In total, the homogeneity of variances was evaluated at the transect level and at molecular level, using 18 Cochran's tests (3 dates x 2 sites x 3 molecular markers). In all cases, the null hypothesis was accepted, $C_{value} < \text{Critical } C_{value}$. Indeed, at the level of the molecular markers, the coefficients of variation corresponding to the residual variability (lower level of the hierarchy, i.e. qPCR repetition) were determined as 36.89 %, 51.50 % and 19.38 % for 16S rDNA, *int1* (class 1 integrons), and *trfA* (IncP-1 plasmids), respectively. Such percentages, allow to considering that the qPCR repetitions are homogeneous (in terms of variances) for a same molecular marker ($p > 0.05$).

Once the homogeneity of variance was verified, Prof. A. Maul designed specific statistical tests aiming to evaluate the relative contribution of all possible sources of variation within our dataset. With this respect it was proposed to use a nested ANOVA when the groups of data were dependent (sampling positions for a given date and site linked as a transect), followed by a mixed ANOVA when the data become independent (when sampling sites and dates are dissociated). It is worth noting that the design of the nested ANOVA tests allowed calculating the different components of variances, expressed as coefficient of variation, that correspond to the variability given by each of the factors: qPCR repetitions, DNA extractions, distribution of samples within the transect and the transect by itself (date + sampling site linked). On the other hand, mixed ANOVA tests allowed us to identify the effect of the factors:

sampling date, sampling site, and the date-site interaction in the fluctuation of the molecular markers.

2.2 Hierarchization of the sources of variation using nested ANOVA

Nested ANOVA tests were performed for the 16S rDNA, *int11*, and *trfA* markers in order to hierarchy the effect of the following factors: transect (date + site), sample distribution (position relative to the bank and depth), DNA extraction and qPCR repetitions. The design of the nested ANOVA test is presented in Table 8. In practice, the calculation begins by performing the sum of squares values from the lowest level of the hierarchy and finishes with the calculation of the variance for the factor transect. Because the symmetry of data is required to run such test, only the positions in the river presenting the same amount of data for all the campaigns were considered. For the nested ANOVA of 16S rDNA, the values from positions 2, 3 and 5 from JOHA were considered, and 2, 4 and 5 from BETHUP site (Figure 20), while the nested ANOVA for class 1 integrons and IncP-1 plasmids were performed with the values from positions 1, 2, 3 and 5 from JOHA site, and 1, 2, 4 and 5 from BETHUP site (Figure 20). The results obtained for the markers are summarized in Table 9, Table 10, and Table 11. Globally, 16S rDNA and *int11* markers appeared influenced by the same parameters and in the same order of magnitude. In both cases, the qPCR factor appeared to play a moderate role in the fluctuation of the abundances measured (CV = 36.4 and 36.9 %, respectively), while the DNA extraction factor had no influence at all (CV = 0.0 %), thus limiting the influence of the technical parameters in the global output. Regarding the environmental factors, the relative position of sampling also moderately influenced the fluctuation of the abundances measured, and slightly more for *int11* (CV = 42.7 %) than for 16S rDNA (CV = 13.1 %). Still, all these coefficients of variation appeared relatively limited compared to what observed for the transect (date + site) influence with CV = 2618 % and 1858 % for 16S rDNA and *int11*, respectively.

Table 8. Nested ANOVA test designed by Prof. A. Maul

Source	Sum of squares (Q)	Degrees of freedom (v)	Expected mean square E[Q/ v]
Transect (Factor A)	$Q_A = pqr \sum_l (\bar{x}_{l...} - \bar{x})^2$ $Q_A = pqr \left[\sum_l \bar{x}_{l...}^2 - t \bar{x}^2 \right]$	(t - 1)	$\sigma_{qPCR}^2 + r \sigma_C^2 + qr \sigma_B^2 + pqr \sigma_A^2$
Sample distribution (Factor B)	$Q_B = qr \sum_l \sum_i (\bar{x}_{li..} - \bar{x}_{l...})^2$ $Q_B = qr \left[\sum_l \sum_i \bar{x}_{li..}^2 - p \sum_l \bar{x}_{l...}^2 \right]$	t (p - 1)	$\sigma_{qPCR}^2 + r \sigma_C^2 + qr \sigma_B^2$
DNA extraction (Factor C)	$Q_C = r \sum_l \sum_i \sum_j (\bar{x}_{lij.} - \bar{x}_{li..})^2$ $Q_C = r \left[\sum_l \sum_i \sum_j \bar{x}_{lij.}^2 - q \sum_l \sum_i \bar{x}_{li..}^2 \right]$	tp (q - 1)	$\sigma_{qPCR}^2 + r \sigma_C^2$
Error qPCR	$Q_R = \sum_l \sum_i \sum_j \sum_k (x_{lijk} - \bar{x}_{lij.})^2$ $Q_R = \sum_l \sum_i \sum_j \sum_k x_{lijk}^2 - r \sum_l \sum_i \sum_j \bar{x}_{lij.}^2$	tpq (r - 1)	σ_{qPCR}^2
Total	$Q_T = \sum_l \sum_i \sum_j \sum_k (x_{lijk} - \bar{x})^2$ $Q_T = \sum_l \sum_i \sum_j \sum_k x_{lijk}^2 - tpqr \bar{x}^2$	tpqr - 1	

Notations

t: number of transects (t=6)	(l= 1, ..., 6)
p: number of samples (p= 2, 3 or 4)	(i = 1, ... , p)
q: number of DNA extractions (q=3)	(j = 1, ... , q)
r: number of replicates (qPCR) (r= 2 or 3)	(k = 1, ... , r)

Table 9. Results of nested ANOVA for 16S rDNA

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Transect (date + site)</i>	$s_A^2 = 1.2319$	2618.0
<i>Sample position</i>	$s_B^2 = 0.0032$	13.1
<i>DNA extraction</i>	$s_C^2 = 0.0000$	0.0
<i>qPCR</i>	$s_{qPCR}^2 = 0.0235$	36.4

Table 10. Results of nested ANOVA for class 1 integrons

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Transect (date + site)</i>	$s_A^2 = 1.1029$	1858.0
<i>Sample position</i>	$s_B^2 = 0.0316$	42.7
<i>DNA extraction</i>	$s_C^2 = 0.0000$	0.0
<i>qPCR</i>	$s_{qPCR}^2 = 0.0240$	36.9

Table 11. Results of nested ANOVA for IncP-1 plasmids

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Transect (date + site)</i>	$s_A^2 = 0.5024$	365.4
<i>Sample position</i>	$s_B^2 = 0.0291$	40.9
<i>DNA extraction</i>	$s_C^2 = 0.0269$	39.2
<i>qPCR</i>	$s_{qPCR}^2 = 0.0057$	17.5

Contrary to what observed with 16S rDNA and *int11*, for the *trfA* marker (IncP-1 plasmids), the qPCR parameter appeared to play a slight role in the fluctuation of the abundances measured (CV = 17.5 %). While the DNA extraction parameter had no influence at all for 16S rDNA and *int11* markers, it was found to have a moderate influence for *trfA* (CV = 39.2 %). The relative

position of sampling also moderately influenced the fluctuation of the abundance measured (CV = 40.2 %), but it was higher than the determined for 16S rDNA and *int11* markers. Finally, the influence of the transect (date + site) is also predominant in the fluctuation of the abundances measured (CV = 365.4 %), although it is not as pronounced as for the other markers. We hypothesized that the differences observed between the behavior *trfA* and that of the two other markers could be due to its lower absolute abundances, sometime close to the qPCR quantification limit, which by themselves may generate stochastic variability in the parameters qPCR, DNA extraction, sampling position. Whatever the origin(s) of the *trfA* marker specific fluctuations, we can say in conclusion that the nested ANOVA tests demonstrated that most of the variation observed in the three marker abundances is linked to the date and/or the site of sampling.

2.3 Studying the spatiotemporal variability of sampling date vs sampling site (mixed ANOVA)

The respective influences of the sampling site and sampling date on the abundances measured for the markers (16S rDNA, *int11*, and *trfA*) were studied using a mixed ANOVA test, following the design presented in Table 12, and using the same set of data as the one considered for the nested ANOVA tests presented above. For its implementation, it was necessary to calculate a mean for the data obtained at each sampling position, thus combining the data obtained at the qPCR and DNA extraction hierarchy levels. In other words, this means that solely one value was associated with each sampling position for a given combination of date and site. In this model, the last variability to be calculated was the factor date (time).

Table 12. Mixed ANOVA design

Source	Sum of squares	Degrees of freedom (v)	Expected mean square: E[Q/ v]
Factor A Time	$Q_A = qr \sum_i (\bar{x}_{i..} - \bar{x})^2$ $Q_A = qr \left[\sum_i \bar{x}_{i..}^2 - p \bar{x}^2 \right]$	p - 1	$\sigma_R^2 + r \sigma_{AB}^2 + qr \sigma_A^2$
Factor B Site	$Q_B = pr \sum_j (\bar{x}_{.j.} - \bar{x})^2$ $Q_B = pr \left[\sum_j \bar{x}_{.j.}^2 - q \bar{x}^2 \right]$	q - 1	$\sigma_R^2 + r \sigma_A^2 + pr \sigma_B^2$
*Interaction AxB	$Q_{AB} = r \sum_i \sum_j (\bar{x}_{ij.} - \bar{x}_{i..} - \bar{x}_{.j.} + \bar{x})^2$	(p-1)(q-1)	$\sigma_R^2 + r \sigma_{AB}^2$
Error (qPCR)	$Q_R = \sum_i \sum_j \sum_k (x_{ijk} - \bar{x}_{ij.})^2$ $Q_R = \sum_i \sum_j \sum_k x_{ijk}^2 - r \sum_i \sum_j \bar{x}_{ij.}^2$	pq(r-1)	σ_R^2
Total	$Q_T = \sum_i \sum_j \sum_k (x_{ijk} - \bar{x})^2$ $Q_T = \sum_i \sum_j \sum_k x_{ijk}^2 - pqr \bar{x}^2$	pqr - 1	

Notations

p: number of sampling dates (p=3)

(i = 1, ... , p)

q: number of sites (q=2)

(j = 1, ... , q)

r: number of replicates (r=2, 3 or 4)

(k = 1, ... , r)

*Interaction: for evaluating if factor date and factor site act together (additive effect) on the variability of the markers.

The results corresponding to the spatiotemporal variability of the three molecular markers are presented in Table 13, Table 14, and Table 15 for 16S rDNA, *int1*, and *trfA*, respectively. Once again, both 16S rDNA and *int1* markers appeared influenced by the same parameters, here the lowest level of variation (factor qPCR + DNA extraction) appeared to play a moderate role in the fluctuation of the abundances measured (CV = 17.7 and 46 % for 16S rDNA and *int1*, respectively), while the factor site of sampling had no influence at all (CV = 0.0 %). The possible interaction between the factors “site of sampling” and “date of sampling” moderately influenced the fluctuation of the 16S rDNA abundance (CV = 40.2 %) but for the *int1* marker there is no interaction (CV = 0.0 %). Conversely, the factor date of sampling is highly predominant compared to the other sources of variation, with CV = 5308 and 3879 % for 16S rDNA and *int1*, respectively. For the *trfA* marker, the sources of variation observed are distributed as before, with CV = 48.0 % for the factor qPCR + DNA extraction and CV = 0.0 % for the interaction of factors date and site of sampling. But, a site effect was identified in the fluctuation of IncP-1 plasmid abundance (CV = 58.9 %), and the effect of the date of sampling was as high as observed for the other makers (CV = 128 %). As before, those differences could be explained by the low *trfA* abundances. Additionally, it may also suggest that the two MGEs quantified do not fluctuate in the same way if, for instance, they are not carried by the same bacteria.

Table 13. Mixed ANOVA results for 16S rDNA

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Time (date)</i>	$s_A^2 = 1.4983$	5308.0
<i>Sampling site</i>	$s_B^2 = 0.0000$	0.0
<i>Interaction date x site</i>	$s_{AB}^2 = 0.0283$	40.2
<i>Error (qPCR+ DNA)</i>	$s_R^2 = 0.0058$	17.7

Table 14. Mixed ANOVA results for class 1 integrons

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Time (date)</i>	$s_A^2 = 1.3801$	3879.0
<i>Sampling site</i>	$s_B^2 = 0.0000$	0.00
<i>Interaction date x site</i>	$s_{AB}^2 = 0.0000$	0.00
<i>Error (qPCR + DNA)</i>	$s_R^2 = 0.0361$	45.9

Table 15. Mixed ANOVA results for IncP-1 plasmids

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Time (date)</i>	$s_A^2 = 1.2796$	128.0
<i>Sampling site</i>	$s_B^2 = 0.0562$	58.9
<i>Interaction date x site</i>	$s_{AB}^2 = 0.0000$	0.0
<i>Error (qPCR + DNA)</i>	$s_R^2 = 0.0361$	48.0

At this stage, it became evident that the main parameter affecting the absolute abundances of 16S rDNA, *int11*, and *trfA* markers was the date of sampling, while either no effect or a slight effect of the sampling site factor could be observed. This was rather surprising as, despite being positioned only 4.3 km apart, the two sampling sites happen to flank a municipal wastewater treatment plant (WWTP) discharging in the river, which is often considered as a source of microbial pollution, as we shall discuss it later (see section 3.2). The absence of a significant difference between the two sampling sites clearly indicates that this WWTP cannot be considered as a major source of bacteria and MGE-bearing bacteria in the Orne ecosystem. Nevertheless, the mere fact that the markers are influenced by the same temporal parameter does not necessarily mean that they all depend on a unique alternative source. If this is true, the temporal parameter should influence the different sources of microorganism in a different extent, which should be visible at the level of the relative load of MGEs. To test this hypothesis, we started to analyze the relative

abundance of MGEs to report on any changes in the microbial community structure, at least related to MGE-carrying bacteria, that could reflect the different input of microbial pollutants.

2.4 Fluctuation of the proportion of MGE-bearing bacteria in the river transects

The absolute abundances of class 1 integrons and IncP-1 plasmids in copies/mL represent the global content of MGEs, while relative abundances of MGEs in copies/16S rDNA copy number rather represent the proportion of bacteria carrying MGEs. Here again, the question was to know whether these proportions could be influenced (or not) by environmental factors. The relative abundances of class 1 integrons and IncP-1 plasmids were determined by normalizing the absolute abundances of MGEs to the corresponding 16S rDNA absolute abundances (Figure 23). At a first glimpse, the variation of relative abundances of both MGE markers according to sampling date appear to be minimized compared to what observed with the absolute abundances (Figure 21). In addition, the relative abundances of both MGEs appeared to increase when both absolute abundances were dropping (Figures 21 & 23) for sampling date March 11th 2015). Finally, it is worth noting that the relative abundances of class 1 integrons and IncP-1 plasmids span in different ranges. From these simple observations, we can already conclude that even if both are sampling date-dependent, absolute abundances and relative abundances can evolve independently, the latter reflecting changes in microbial community structures with respect with MGEs. Here again, and apart from the date effect, the relative contribution of different environmental and technical parameters to the fluctuations observed remains to be elucidated.

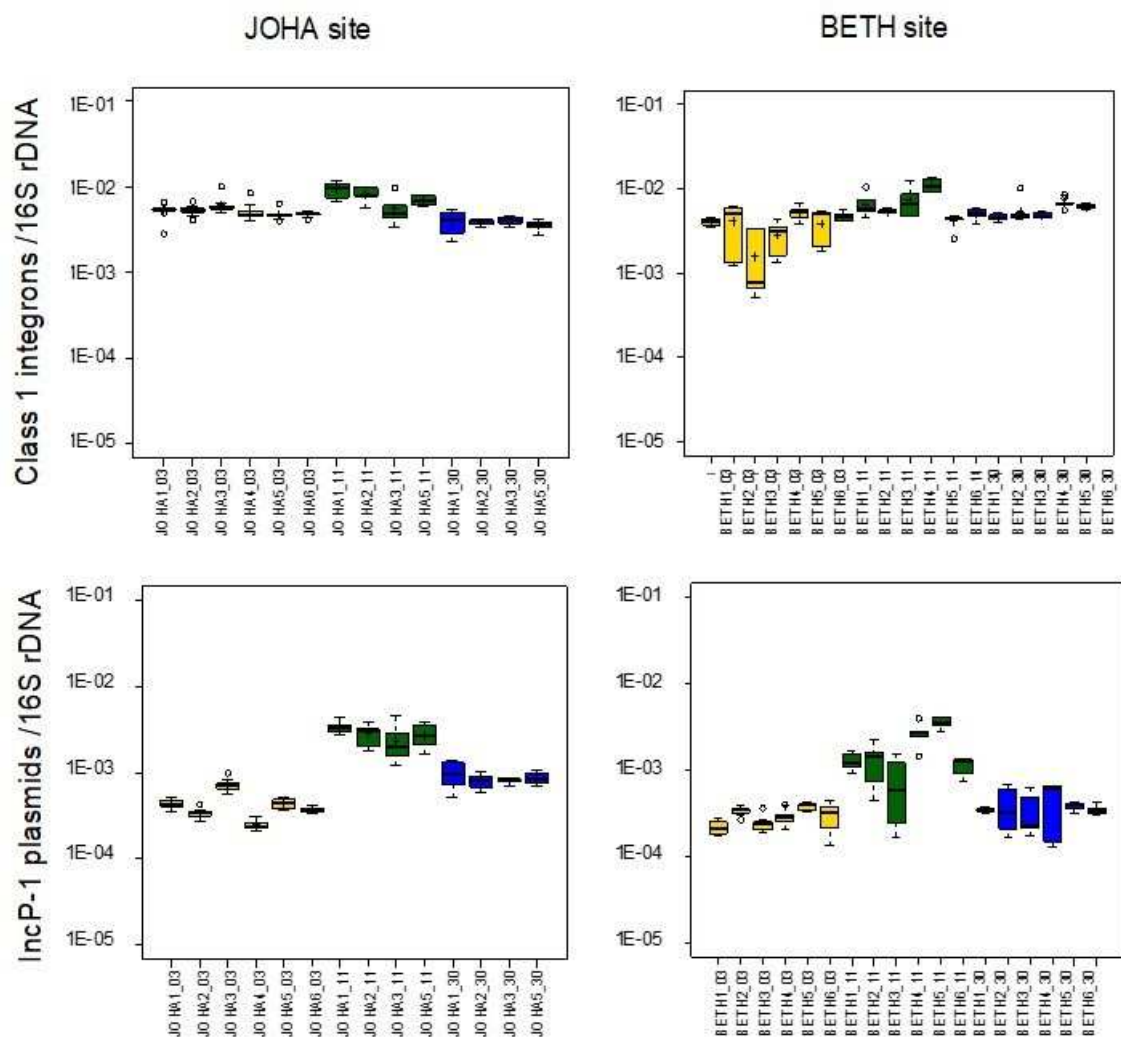


Figure 23. Variability of MGE relative abundances according to sampling position and sampling site (JOHA left graphs, BETH right graphs). Each color indicates a sampling date (yellow: 03/03/2015, green: 11/03/2015, blue: 30/03/2015).

A nested then a mixed ANOVA test was applied as described above to determine whether there was a significant effect of these environmental and technical parameters in driving the fluctuation of the relative abundance of class 1 integrons and IncP-1 plasmids. The results obtained are presented in Tables 16 and 17, for nested ANOVAs (*int11* and *trfA* markers, respectively), and in Tables 18, and 19 for mixed ANOVAs (*int11* and *trfA* markers, respectively). Globally, the nested ANOVA tests revealed again that the transect factor (date + site of sampling) was still dominating the other factors (sampling position relative to the river bank and depth, DNA extraction and qPCR). As relative

abundances are ratios of two fluctuating parameters, it is important to note that CVs calculated upon performing ANOVAs may vary within in a different range than what was observed for the corresponding absolute abundances. Anyhow, when mixed ANOVAs were performed to distinguish the relative contributions of the sampling date and sampling site factors, similar conclusions as before could be drawn. The sampling date is the main parameter influencing the relative abundances of class 1 integrons and IncP-1 plasmids, and these relative abundances are apparently not influenced by the WWTP located in between the two sampling sites.

All in all, the series of ANOVA tests carried out on the MGE abundance data rigorously demonstrated that, above all, if technical parameters are slightly responsible of the variations observed in the abundances measured (essentially the qPCR factor), they are far from dominating over the environmental parameters (mainly associated to the sampling date). All in all, these series of tests indicate that if the date is associated to the global change in both absolute and relative abundances, meaning that both the abundance in bacteria and the structure of the community varies in a date basis. In addition, and on a more practical point of view, the very little influence of the sampling position in the river transects (depth and position relative riverbank) authorized us to pursue the rest of this study by limiting the sampling to one position per sampling site.

Table 16. Results of nested ANOVA for relative abundances of class 1 integrons

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Transect (date + site)</i>	$s_A^2 = 0.4094$	278.7
<i>Sample position</i>	$s_B^2 = 0.0119$	25.5
<i>DNA extraction</i>	$s_C^2 = 0.0000$	0.0
<i>qPCR</i>	$s_{qPCR}^2 = 0.3889$	261.9

Table 17. Results of nested ANOVA for relative abundances of IncP-1 plasmids

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Transect (date + site)</i>	$s_A^2 = 0.4094$	100.8
<i>Sample position</i>	$s_B^2 = 0.0119$	38.2
<i>DNA extraction</i>	$s_C^2 = 0.0000$	21.9
<i>qPCR</i>	$s_{qPCR}^2 = 0.3889$	40.8

Table 18. Results of mixed ANOVA for relative abundances of class 1 integrons

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Time (date)</i>	$s_A^2 = 0.6009$	481.6
<i>Sampling site</i>	$s_B^2 = 0.0000$	0.0
<i>Interaction date x site</i>	$s_{AB}^2 = 0.0051$	16.6
<i>Error (qPCR)</i>	$s_R^2 = 0.01306$	26.8

Table 19. Results of mixed ANOVA for relative abundance of IncP-1 plasmids

<i>Factor</i>	<i>Component of variance</i>	<i>Coefficient of variance %</i>
<i>Time (date)</i>	$s_A^2 = 0.6009$	52.4
<i>Sampling site</i>	$s_B^2 = 0.0000$	0.0
<i>Interaction date x site</i>	$s_{AB}^2 = 0.0051$	0.0
<i>Error (qPCR)</i>	$s_R^2 = 0.01306$	96.5

3. FLUCTUATION OF MGE ABUNDANCES IN THE CONTINUUM OF THE RIVER

The previous section was dedicated to the identification of the different sources of variation that could influence the abundances measured for 16S rDNA and MGEs. Surprisingly for us, no site effect could be observed although

a WWTP is located in between the two sampling sites compared. If we accept that the WWTP effluents do not significantly alter the apparent microbial quality of the river water, we should then conclude that the 4.3 km separating the sampling sites were not distant enough to evidence the possible effects of the inherited and current sources of pollution of the river, as well as the consequences of the riverbed modification regarding the presence of a dam at BETH site that modifies the hydrodynamics of the river. Aiming to evaluate the impact of the different anthropogenic pressures along the Orne River, several sampling sites were selected along an approximately 10 km of distance. This river section from AUB to BETH sites encompasses urbanized and former industrial sectors (Figure 19), including the local WWTP discussed above, and a sampling site on one tributary of the Orne River, the Woigot River (WOI). This Orne River tributary drains an area about 85 km² and flows through the districts of Audun-le-Roman, Briey, and Homécourt. In addition to the previous transect study described above, three sampling campaigns were performed between November 2014 and May 2015 (November 4th and 5th, 2014, February 2nd and 3rd, 2015 and May 5th, 2015). For each of these three campaigns, water samples were collected from the 6 to 8 different sampling sites distributed along the 10 km long river section considered. The WWTP effluents were also collected so as to evaluate the input provided by the WWTP activity. The experimental strategy was the same as before, starting by total DNA extractions from the water samples followed by qPCR quantifications of the molecular markers, 16S rDNA, *intI1* (class 1 integrons) and *trfA* (IncP-1 plasmids).

The quantification of the three markers shows absolute abundances in a wide range of values, about 3 orders of magnitude, with a dependence on the date of sampling, as previously observed in the river transect experiment. On the other hand, when the absolute abundances were compared among the different sampling sites but for the same sampling date (i.e., the same sampling campaign), they appeared relatively stable over the 10 km river section considered, including the WOI site, and significantly differed from the effluents of the WWTP (JOWWTP), which was expected considering the origins of the microbial communities (Figure 24). Whatever the campaign considered and as

previously observed, the WWTP did not seem to increase the absolute abundance of MGEs in the downstream site BETHUP.

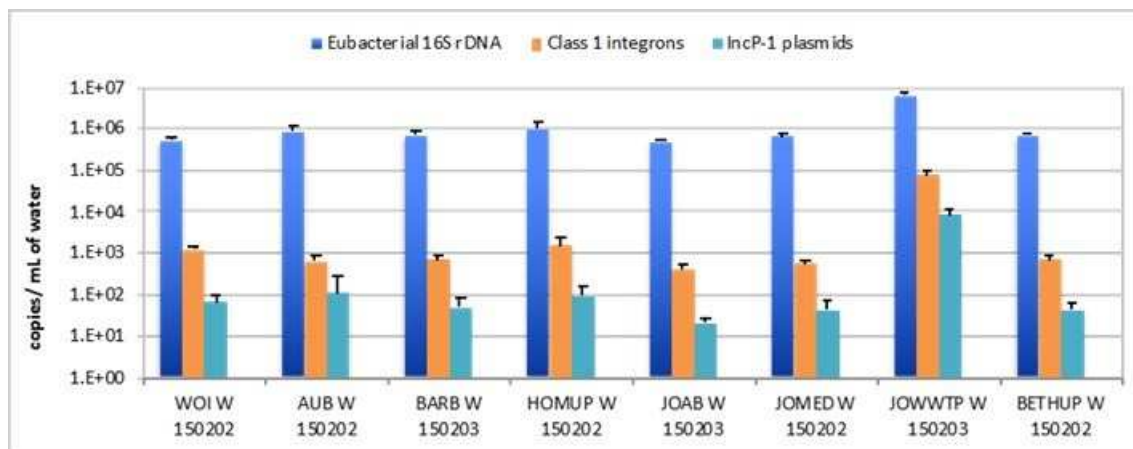


Figure 24. Example of absolute abundances of the markers 16S rDNA, *intI1* (class 1 integrons) and *trfA* (IncP-1 plasmids) (copy/mL) from water collected at different sampling sites along the Orne River during a two days sampling campaign in February 2015. Sampling stations are arranged in the order of appearance (from upstream to downstream) in the 10 km river section studied.

The different sampling sites and campaigns were also analyzed in terms of MGE relative abundances in order to consider possible changes in MGE carriage and therefore changes related to the structure of the microbial community. Here again, the proportions of MGEs appeared slightly different from one campaign to another (less for IncP-1 plasmids than class1 integrons) but no tendency could be drawn according to the sampling site (Figure 25). The absence of symmetry in the whole dataset (different sampling sites depending on the sampling campaign) and the fact that some data were collected during a two-days campaign did not authorize using the same statistical approach as for the transect analysis to identify an eventual slight site effect (“hidden behind error bars”). Nevertheless, to go further, we choose the sole May 2015 campaign, during which all samples were collected the same day, in order to perform statistics for testing the hypothesis of significant differences in MGE abundances between sampling sites. The absence of homogeneity of variances in the data led us to use the non-parametric Friedman’s test, which did not reveal any significant difference between the Orne River sites in terms of absolute ($p > 0.05$) or relative abundance ($p > 0.1$) for both MGEs. Although it is not very robust since only one campaign was used for statistics, it clearly

confirmed what was observed visually in several occasions. Absolute abundances and proportion of MGEs in communities could change over time but not over the 10 km of the Orne River section studied within the same day, therefore limiting the apparent effect of the local anthropic pressures.

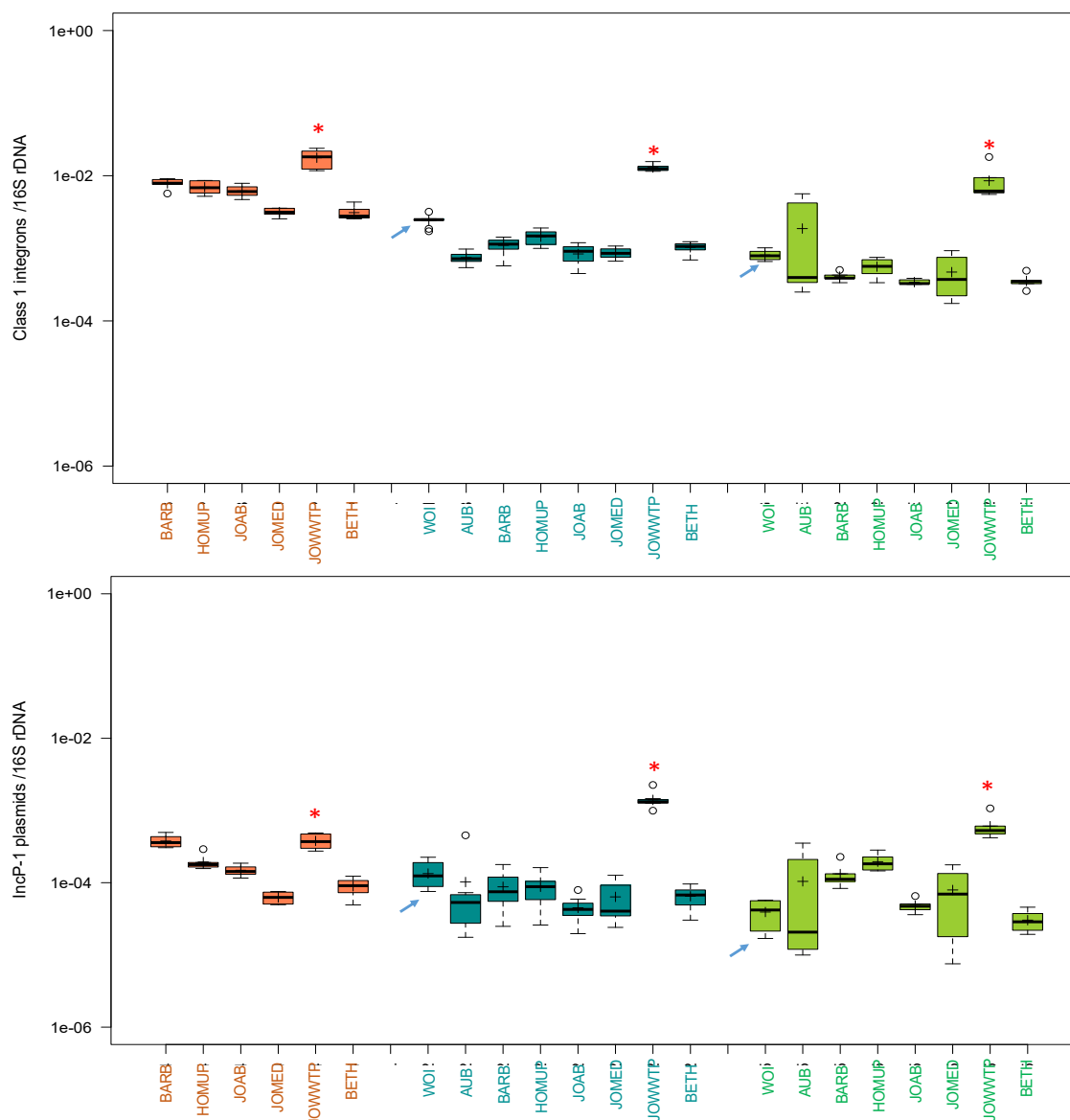


Figure 25. Relative abundance of class 1 integrons and IncP-1 plasmids (normalized to 16S rDNA) according to sampling dates over three sampling campaigns. Each color indicates a different sampling campaign, orange: November 4th and 5th 2014, dark green: February 2nd and 3rd 2015, and light green: May 5th 2015. The red asterisks indicate samples corresponding to the effluents of the WWTP and the blue arrows samples from the Woigot River, a tributary of the Orne River.

3.1 Changes in bacterial community structure in the water column of the Orne River

Several metagenomic studies revealed the predomination of the phylum Proteobacteria in most of the aquatic environments (Araya et al. 2003; Huerta et al. 2013; Stewart 2013b). Thus, in this work we decided to assess changes in the bacterial community structure by evaluating the variation in the relative abundance of certain indicators, namely α -, β -, and γ -proteobacteria. Their quantifications were used to determine their relative proportions with the hypothesis that these proportions should change if the community structure was to be “altered” from one sampling site to another along of the Orne River, therefore pointing out the effect of increasing anthropogenic pressure for instance. The quantification of α -, β -, and γ -proteobacteria by qPCR were done on the same sampling campaigns described above for the continuum of the river (section 3, results part I), and resulted in a poor variability between the sampling sites (as illustrated in Figure 26), except for the effluents from the local wastewater treatment plant where the abundances of the markers were higher, as we already saw for MGEs. Figure 26 represents just the results corresponding to the campaign of February 2015, but the same pattern was observed for the other sampling campaigns (not shown). The small variability between the different classes of proteobacteria according to the sampling sites does not allow to put in evidence of possible changes in the bacterial community structures, and confirm what was understood from the relative proportion of MGEs. For a given sampling date, the microbial communities do not significantly evolve along the 10 km section of the river.

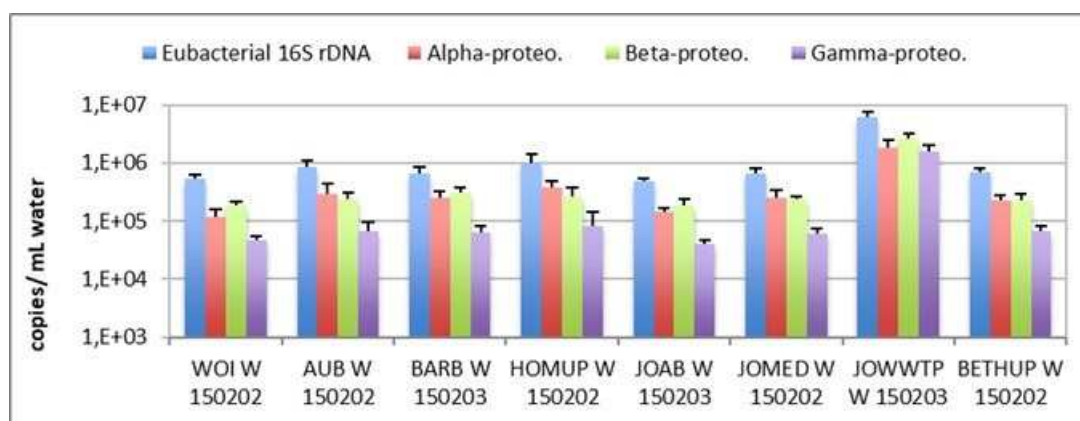


Figure 26. Abundances of 16S rDNA, α -, β -, and γ -proteobacteria per mL of water from different sampling sites across the Orne River during the sampling campaign of February 2015.

3.2 Influence of local anthropogenic pressure on MGE abundances

In this study, the absence of evident anthropic impact was well illustrated by the case of the WWTP. Although WWTP effluents exhibit a significantly higher absolute and relative abundance of both MGEs (Figure 24 & 25), they did not seem to influence the absolute and relative abundances of MGEs in the river water as they remain comparable upstream and downstream of the WWTP. Actually, this phenomenon can be attributed to a dilution effect of the effluents by the river water itself. Indeed, according to the data from the DREAL database (DREAL, 2018) the Orne River in the area studied has an average flow rate of 6.5 m³/s for 2015, which is about two orders of magnitude higher than the one of the WWTP effluents (0.069 m³/s) according to the SIERM data (SIERM, 2018). Considering that, at most, the absolute abundances of MGE were two orders of magnitude higher in the effluents compared to the river, the dilution rate imposed by the river keeps the input of WWTP effluents at an undetectable level. In the meantime, these results show that, in regions that are not affected by water scarcity, the direct effect of one WWTP on the MGE abundances is not so evident to demonstrate.

4. SEEKING FOR VARIATIONS IN MGE ABUNDANCES IN LONGER DISTANCES OF THE CONTINUUM OF THE RIVER

Until this point, it has not been possible to demonstrate any change in MGE abundances from one sampling site to another. In a last effort to evidence a site effect, we decided to compare 3 sampling sites spread over a 23 km distance, between Auboué and Richemont, the latter one being much more urbanized and industrialized at the confluence between of the Orne River and the Moselle River. In addition to the sampling campaigns of May 5th, 2015 and October 6th, 2015, two more sampling campaigns at AUB, BETH and RICH sites were performed on July 17th, 2018 and September 3rd, 2018. From this new dataset, the absolute abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids were compared (Figure 27) and we noticed that the 16S rDNA marker seems to be stable across sampling sites for a given campaign; the same behavior was observed for IncP-1 plasmids (except for the sampling campaign of May 2015). However, a small increase in the absolute abundances of class 1 integrons from AUB to RICH could be observed, whatever the sampling date. As variances were not homogenous within the dataset (verified by a Cochran's test), the significances of those observations were evaluated using the non-parametric Friedman's test. The test on class 1 integrons was significant ($p=0.018$), meaning that there is an increase on the absolute abundance of *intI1* marker from Auboué to Richemont, whereas no significant differences were found between the sampling sites for 16S rDNA and IncP-1 plasmids ($p=0.778$ and $p=0.1053$, respectively).

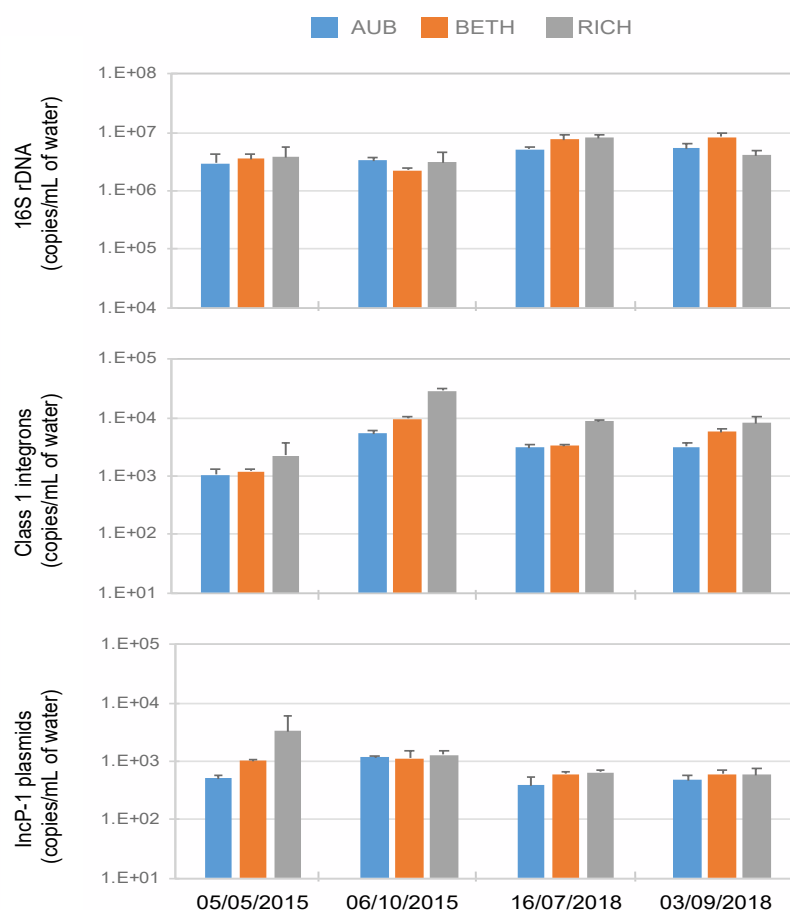


Figure 27. Absolute abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids/mL in River water collected at different sampling sites along the Orne River. Sampling stations are arranged in the order of appearance (from upstream to downstream) in the 23 km river section studied; each site is represented by a different color, blue: AUB, orange: BETH, and grey: RICH.

As before, MGE relative abundances were also analyzed in order to consider possible changes in the community structure of MGE-bearing bacteria (Figure 28). The relative abundances of class 1 integrons increase over the 23km river section, which was expected considering the fact that the absolute abundances of integrons seem to increase faster than the abundances of 16S rDNA. The apparent increase in the relative abundance of class 1 integrons was observed according to the sampling site, independently from the sampling campaign. This was verified by a Friedman's test ($p=0.039$). On the other hand, no significant difference could be found between sampling sites (for a given sampling campaign) for the relative abundance of IncP-1 plasmids ($p=0.47$). These results suggest that the sources of the class 1 integrons- and IncP-1 plasmids-bearing bacteria are probably not the same. Additionally, it also shows that

variation in MGEs relative abundance (here class 1 integrons), from one sampling site to another, can be evidenced only when sampling sites are distant enough, as no significant differences were observed along the 10 km-long section (section 3, results part I). If anthropogenic pressures were to play a role in the abundances of class 1 integrons, this rather means that the local impact of the urban pressure in the Homécourt area is relatively low. On the other hand, the fact that significant differences are only visible when a long distance is considered tends to show that, in the context of the Orne River, the sources of class 1 integrons are rather diffuse and cannot be attributed to given local input in particular. If this is to be the case, the urban and/or industrial pressures cannot be incriminated directly and the abundances of MGEs should fluctuate independently from typical urban and/or industrial pollutants or associated geochemical parameters.

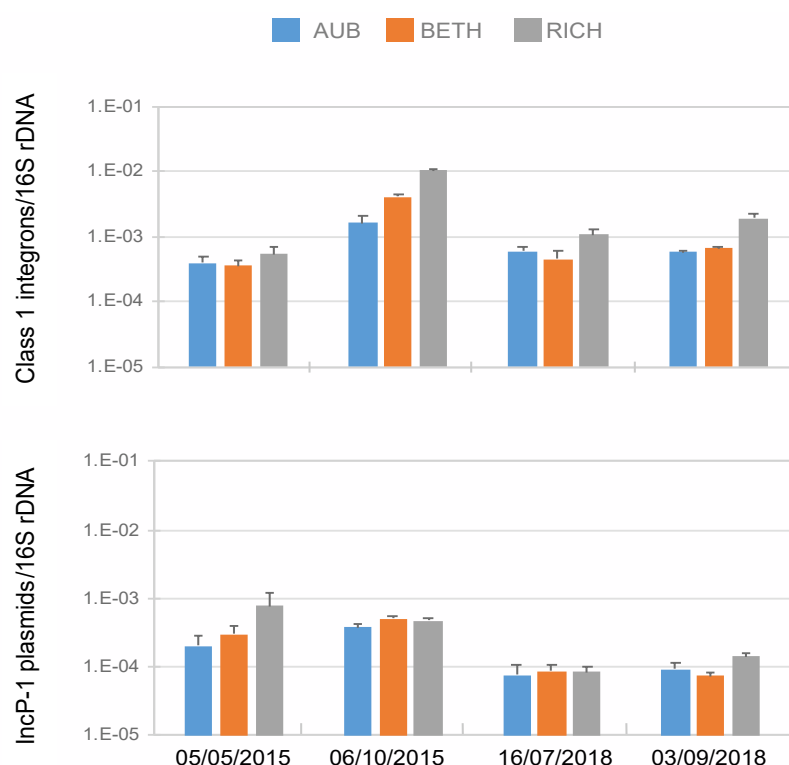


Figure 28. Relative abundance of molecular markers *intI1* (class 1 integrons) and *trfA* (IncP-1 plasmids) (normalized to 16S rDNA) at different sampling sites along the Orne River. Sampling stations are arranged in the order of appearance (from upstream to downstream) in the 23 km river section studied, each site is represented by a different color, blue: AUB, orange: BETH, and grey: RICH.

5. CORRELATION OF MGE ABUNDANCES WITH BIOLOGICAL AND PHYSICOCHEMICAL PARAMETERS

Considering the multidisciplinary of the MOBISED project, several global and geochemical parameters were also characterized by different partners in order to fully characterize the different samples collected from the Orne River. This gave us a good opportunity to attempt understanding why MGE abundance variations are so strongly linked to the sampling date and poorly linked to the sampling site, as seen above. We hypothesized here that statistical correlations between MGE abundances and global or geochemical parameters could help in defining the conditions globally affecting the abundances of MGE-carrying bacteria in the river, or even pinpointing the sources of MGE-carrying bacteria.

In total, nearly 50 parameters including global, elemental and organic analyses were determined for the water samples, (Table 5, materials and methods). Although polycyclic aromatic compounds were quantified for water samples, they were not considered for the following statistical analysis since, due to their hydrophobicity, their quantification always resulted in low concentrations or concentrations below the detectable limits. The remaining global/geochemical data and the MGE abundance data obtained throughout this study were combined and analyzed by principal component analysis (PCA), as a preliminary exploratory tool. Figure 29 shows PCA results in two different plots, the variables factor map and the individuals map. The variables factor map (Figure 29 A) evidences the elements that contribute more or less to the variability of the data (the closer to the external circle, the more significant it is), and the correlation between the parameters analyzed. With this PCA, we can observe that elements such as K, Cr, Cu, V, Co, F, Cs and Ga and parameters such as pH and DOC (dissolved organic carbon) do not contribute to the variability of data. Others like Dy, Er, Eu, Gd, Ho, La, Nd, Ni, Pb, Pr, Rb, Sm, Sr, Tb, Th, Tm, Y and Yb are better represented in the correlation circle as they fluctuate in the same direction and are correlated between them. In the individual factor map (Figure 29 B), the distance between the dots, representing water samples from the different sampling campaigns, indicates the degree of similarity between them.

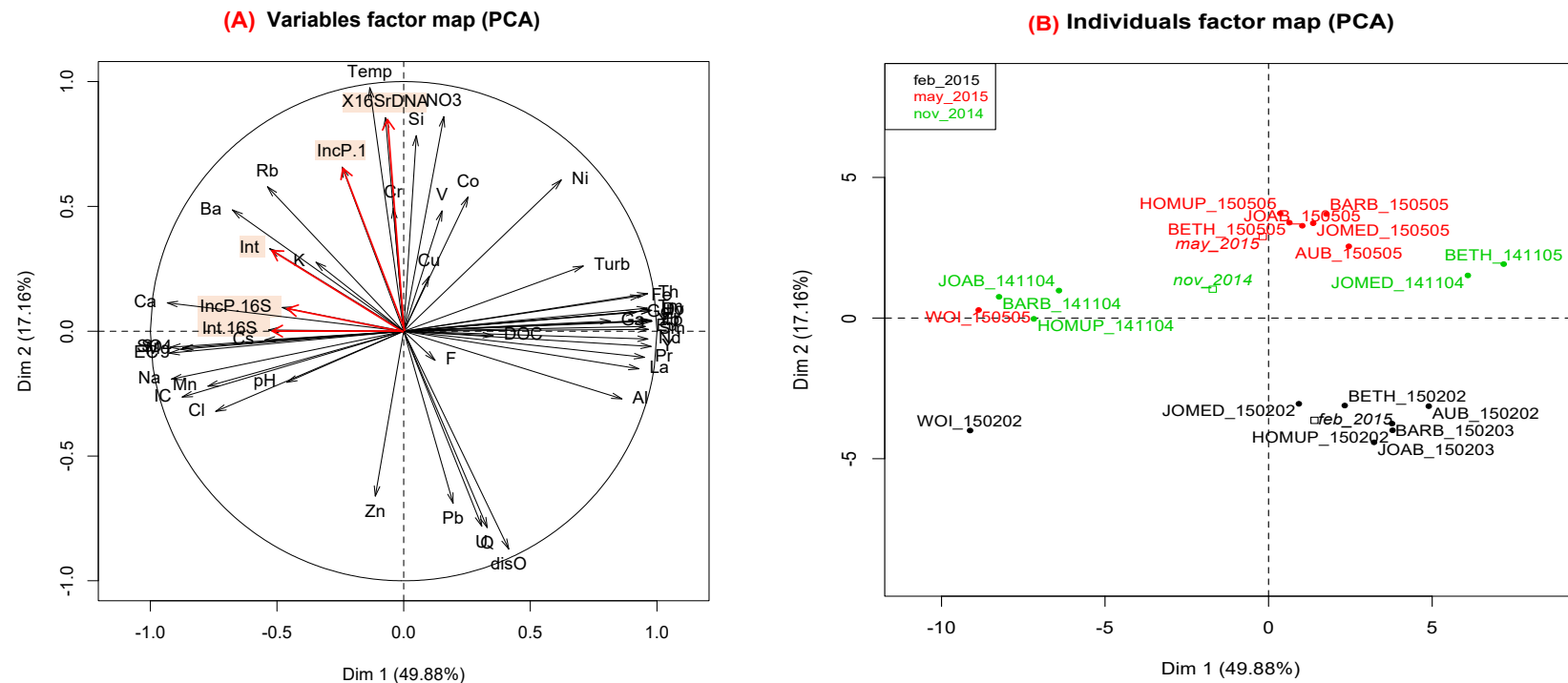


Figure 29. Principal component analysis combining 47 parameters from 19 samples. (A) Variables factor map: flow rate (Q), pH, temperature (Temp), electrical conductivity (EC), dissolved oxygen (disO), turbidity (Turb), dissolved organic carbon (DOC), inorganic carbon (IC), nitrate (NO₃), sulfate (SO₄), fluoride (F), chloride (Cl), aluminum (Al), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), cesium (Cs), copper (Cu), gallium (Ga), nickel (Ni), strontium (Sr), lead (Pb), rubidium (Rb), thallium (Th), uranium (U), vanadium (V), zinc (Zn), cerium (Ce), lanthanum (La), europium (Eu), neodymium (Nd), samarium (Sm), praseodymium (Pr), dysprosium (Dy), erbium (Er), gadolinium (Gd), holmium (Ho), lutetium (Lu), terbium (Tb), thulium (Tm), ytterbium (Yb), scandium (Sc), yttrium (Y); 16S rDNA, absolute abundance of class 1 integrons and Incp-1 plasmids (Int and IncP.1, respectively); and their relative abundance (Int.16S, IncP.16S). (B) Clusterization based on date of sampling, with the exception of WOI samples that cluster apart from the samples of the same sampling date.

neighborhoods, each presenting a similar data profile, whereas dots far away have dissimilar profiles (related with their physicochemical and microbiological characteristics). Here, we observed that the clusterization is based on sampling dates, with the exception of samples from WOI that cluster apart from the samples of the same sampling date. This is not completely surprising since the Woigot (sampling site WOI) is a tributary of the Orne River and therefore exhibits different characteristics. It is worth noting that, regarding the molecular markers analyzed, absolute and relative abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids are not very well represented as indicated by the short length of their vectors on the Variables factor map (Figure 29 A), resulting in weak correlations with other environmental parameters, which then render difficult any kind of interpretation from such statistical test.

When performing a PCA analysis, it is assumed that the relationships between variables, if any, are linear, which is not necessarily the case when dealing with environmental parameters. As a matter of fact, when non-linear correlations are concerned, PCA analyses become non-relevant and alternative statistical approaches should be considered. Possible non-linear correlations between MGEs and geochemical parameters were considered by using the Spearman's rank correlation tests. Significant correlations (positive and negative) were found by this approach for the different molecular markers as reported in Table 20. We observed that the correlation patterns change from one MGE to another, and from absolute abundances to relative abundances, therefore indicating independent changes in both quantity and proportion in the water microbial communities.

Both positive and negative correlations were found between metals and MGE abundances, depending on the element and the nature of the metal. Although the correlation between metals and ARGs in water samples is sometimes hypothesized as the consequence of potential selective pressure (Garner et al. 2016), it is rather difficult to draw a conclusion at our level. Indeed, when we consider Pb and Zn, two metals well represented in the Orne ecosystem, it appears that the highest metal concentrations measured (9.5×10^{-4} and 2.5×10^{-6} mM for Zn and Pb, respectively) were 1,000 to 1,000,000 times below known MIC for *E. coli* in minimal medium, for instance.

Table 20. Significant correlations between the abundances (relative and absolute) of molecular markers (16S rDNA, *intl1*, *trfA*), elemental content (in black) and global parameters (in blue).

Marker	Positive correlations	Negative correlations
16S rDNA	Ba ^{**} , Co ^{**} , Cr ^{**} , Rb ^{**} , Si ^{***} , Ca [*] , NO ₃ ^{***} , Temp ^{A***}	Pb ^{***} , U ^{***} , Zn ^{**} , DisO ^{B***} , Q ^{C**}
Class 1 integrons	Ba ^{***} , Co [*] , Rb ^{***} , Si ^{**} , Sr [*] , V ^{***} , Mn ^{**} , Ca ^{**} , K ^{***} , SO ₄ ^{**} , Temp ^{A**} , EC ^{D*}	DisO ^{B***}
IncP-1 plasmids	Ba ^{***} , Ca ^{**} , Co ^{**} , Rb ^{**} , Si ^{**} , V ^{**} , K ^{**} , Mg [*] , NO ₃ ^{**} , Sr [*] , Temp ^{A***} , EC ^{D*}	Pb ^{**} , U ^{***} , Zn [*] , DisO ^{B***} , Q ^{C*}
Class 1 integrons/16S rDNA	Cs ^{**} , Pb ^{**} , U [*] , Mn ^{**}	Cr ^{**} , Ni [*] , NO ₃ [*]
IncP-1 plasmids/16S rDNA	No correlations	Turb ^{E*}

Significances were tested using Spearman's rank correlation test. *: $p \leq 0.05$;
: $p \leq 0.01$; *: $p \leq 1 \times 10^{-3}$.

^ATemp = temperature

^BDisO = dissolved oxygen

^CQ = flow rate

^DEC = electrical conductivity

^ETurb = turbidity

As such environmental concentrations, toxic or selective effects of the metals are unlikely. Although the literature is relatively scarce on the subject, some authors have suggested the positive correlation between metals and ARGs as a co-selective effect, even at low concentrations (minimum co-selective concentration; MCC) (Seiler and Berendonk, 2012), as the observed in the Orne River. Nevertheless, no mechanism has been demonstrated by the authors, and no real hypothesis has been proposed to explain the negative correlation sometimes observed between metals and ARGs. In anyway, it seems to us that

an alternative explanation could be the common origin of bacteria and metals that could work as a co-selector or a toxic, depending on their concentration, directly at the source of both metal and resistant bacteria (or MGE-bearing bacteria). In such a case, only the visible consequence of the toxicity/selective process can be perceived in the river water, together with the leached metals. To our point of view, determining the origin of such source will probably be a key point for elucidating the nature of the metal-ARG/MGE correlations in the river.

So far, all our results point out the important role of sampling date in the fluctuation of the molecular markers, as the global and elemental parameters do according to the results of the PCA (Figure 29). For this reason, we push forward the analysis in order to see whether the molecular markers and the global parameters monitored were also correlating each other. During each sampling campaign, the global parameters monitored were the water temperature, the electrical conductivity (EC), the dissolved oxygen concentration, the pH and the turbidity, which were measured *in situ*, while measures of daily flow rate were obtained from the DREAL database. In this last statistical analysis, we compared the fluctuation of our molecular markers (absolute and relative abundances) to the fluctuation of the global parameters, somehow indicators of seasonality and river hydrology. Spearman's rank correlation tests (Table 20) demonstrated that absolute abundances of both MGEs and 16S rDNA positively correlate with water temperature and negatively correlate with the dissolved oxygen concentration in parallel trends (Figure 30). This tends to show that the absolute abundance of bacteria and MGE bearing-bacteria increased during the warm season (an increase of water temperature and a decrease of the level of dissolved oxygen), which signs a seasonal effect globally affecting the bacterial content of the river. When considering the flow rate of the river, it also affects the absolute abundance of 16S rDNA and IncP-1 plasmids, this can be explained by dilution effects associated to particular rainfall events (and even flood events see below); but it is not the case for class 1 integrons that do not correlate the flow rate. Once more, this clearly indicates that the two MGEs behave independently from each other. When the relative abundance of MGEs is concerned, no correlation with global parameters could

be found, indicating that the proportion of MGE-bearing bacteria is not affected by rain events. On the other hand, we suggest that the positive correlations found between both MGEs and the electroconductivity (EC) of the water could indicate the source of MGE-bearing bacteria since EC is related with rainfall, where high EC values can be observed during high surface runoff from e.g., agricultural lands or geology of the zone (Edokpayi et al. 2015).

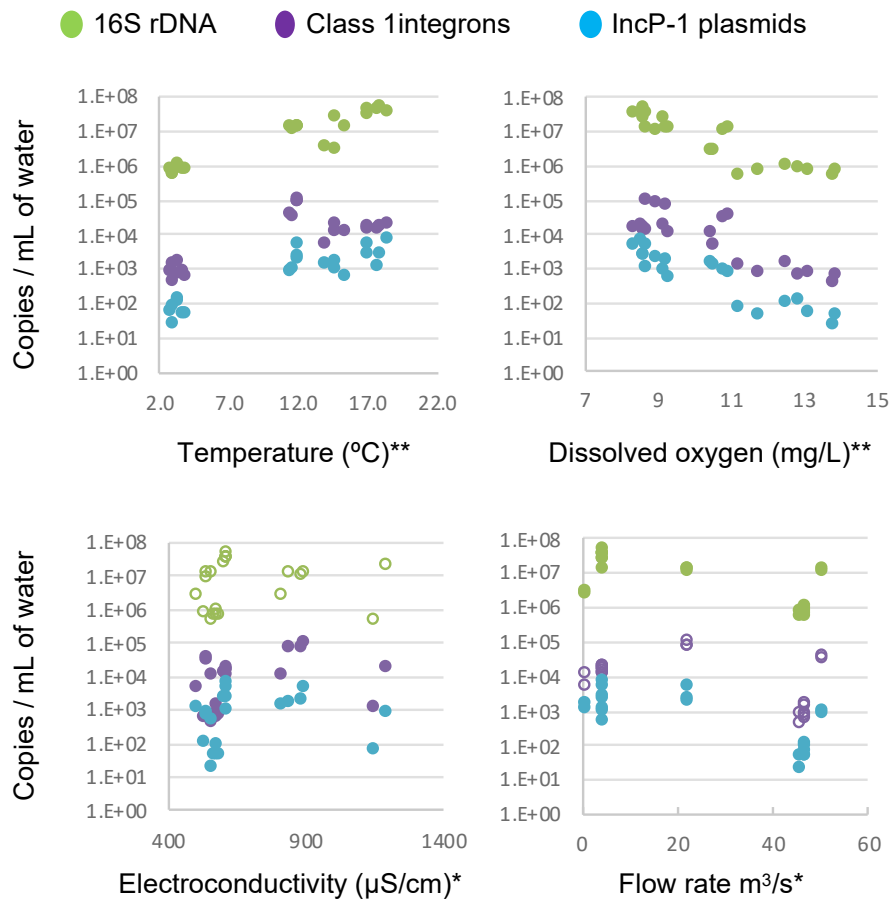


Figure 30. Relationship between seasonality/river hydrology indicators and the absolute abundance of 16S rDNA, class 1 integrons and IncP-1 plasmids. Full and empty circles indicate the presence and absence of correlation between the marker and the variable, respectively. Asterisks indicate level of significance. *, $p < 0.05$; **, $p < 1 \times 10^{-3}$.

6. FLUCTUATION OF MGES DURING HYDROCLIMATIC EVENTS

In the previous section, we saw that the flow rate probably influences the abundances of MGEs in the Orne River, highlighting hydroclimatic events as possible driving parameters of the MGE content in the Orne River water. Flood events are complex and sometimes lead to extreme situations, where sediments and sediment-associated bacteria can be remobilized in the water column, surface bacteria from the watershed can be drained to the river and, in the meantime, the massive input of water should contribute the global dilution of the bacterial load. During this work, we were able to monitor two flood events, in November 2015 and February 2016, to elucidate how MGE fluctuates in the Orne River water during these particular hydroclimatic events. To do so, sampling campaigns were set according to the weather forecast and the flood risk alerts of major rivers in France (Vigicrues, 2018). Samples were collected at the BETH sampling site days before, during and after a predicted flood event. As before, total DNA was extracted using the Powerwater® DNA extraction Kit (MO BIO laboratories) and the three markers 16S rDNA, *int11* and *trfA* were quantified by qPCR. The November 2015 sampling campaign lasted from November 20th to November 30th, with a flood event ending on the 25th of November. Similarly, the February 2016 campaign lasted from February 3rd to February 19th with the end of the flood event on the 12th of February. The daily mean flow rate was obtained from the DREAL database (DREAL, 2018), and indicated a moderate flood event during the November 2015 campaign (flow rate: 2.16 to 10.10 m³/s), and a stronger flood event in February 2016 (Flow rates: 19.01 to 115.90 m³/s). The absolute abundances of the molecular markers quantified for both sampling campaigns are presented in Figure 31. The concentrations of the molecular markers vary from 1.05×10^7 to 1.49×10^3 for 16S rDNA, from 7.07×10^4 to 2.44×10^1 for class 1 integrons, and from 9.87×10^3 to 1.82×10^1 for IncP-1 plasmids. In both cases, the flood peaks are preceded by a slight increase of the 16S rDNA and MGE absolute abundances before decreasing markedly during the flood itself. At the highest flow rate, the two MGE markers were even below the detection limit for the February 2016 flood.

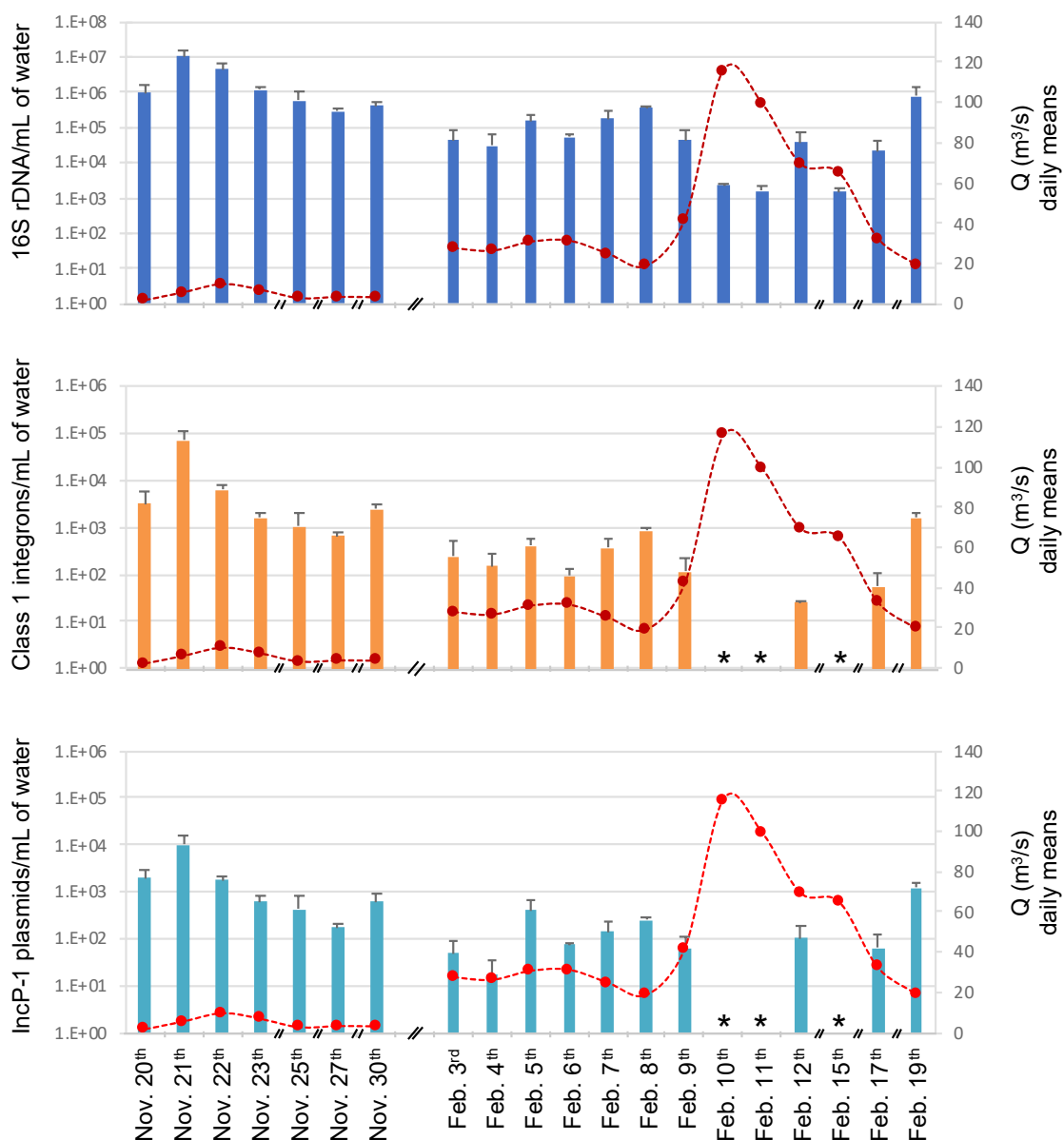


Figure 31. Fluctuation of the absolute abundances (copies/ mL of water) of 16S rDNA, class 1 integrons and IncP-1 plasmids during flood events. Red dots indicated the daily mean flow rate (Q) in m³/s. Asterisks indicated quantification below the detection limit.

Although two flood events are probably not enough to draw a robust conclusion, we can speculate that the early phase of the rainfall events mobilize the bacteria from the watershed, including MGE-bearing bacteria, which then reached the Orne River with the water runoff. This mobilization results in an early increase of bacteria and MGE-bearing bacteria in the River. This flush

effect is then followed by a subsequent decrease of MGE abundance probably resulting from the fact that (i) probably less bacteria are to be mobilized from the watershed and (ii) more rainfalls are increasing the River flow rate. The decrease bacterial mobilization as rainfall goes on is otherwise confirmed by the fact the flux of the three markers (in copies per s.) tend to decrease during the flood (Figure 32). When the relative abundances of the markers were analyzed, no clear tendency related to the flow rate was observed. This signifies that the MGE content in the communities is relatively stable, which can be explained by the fact that community structure in the water did not change significantly during the hydroclimatic event itself (Figure 33).

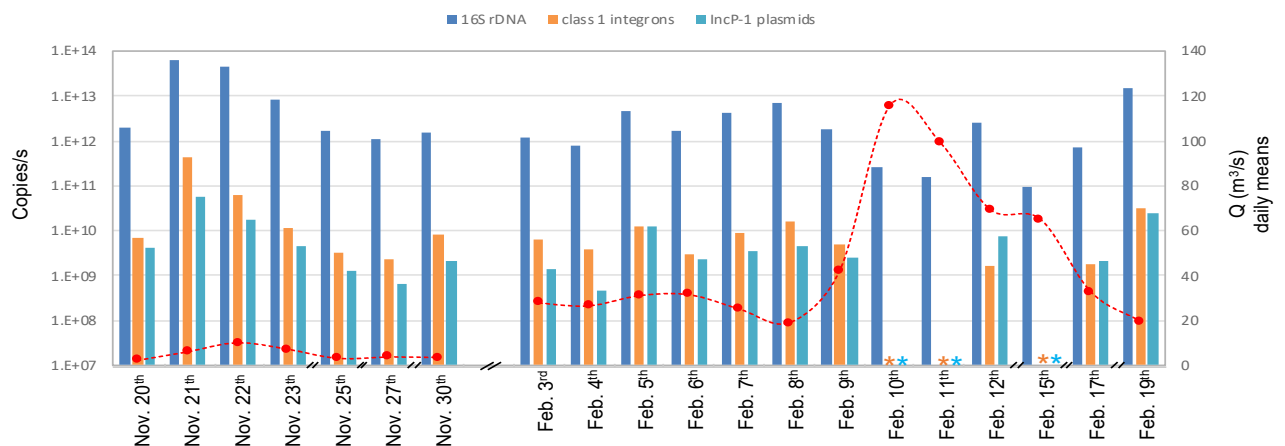


Figure 32. Mobilization and flush effects on the load in copies/s of 16S rDNA, class 1 integrons and IncP-1 plasmids during flood events. Red dots indicated the daily mean flow rate (Q) in m³/s. Asterisks indicated possible dilution of the markers during the flood.

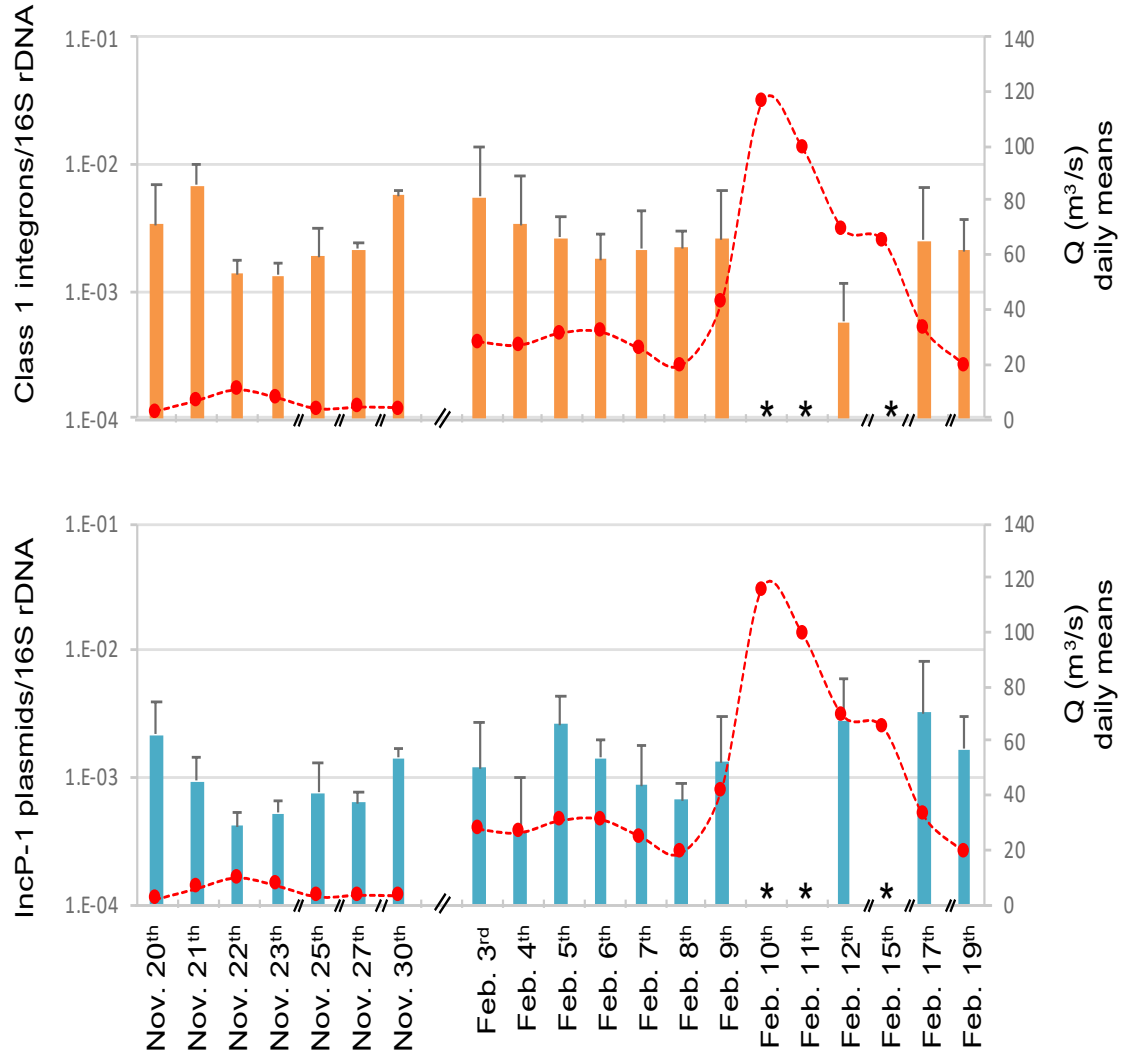


Figure 33. Fluctuation of the relative abundance of class 1 integrons and IncP-1 plasmids during flood events (copies/16S rDNA). Red dots indicated the daily mean flow rate (Q) in m³/s. Asterisks indicated quantification below the detection limit.

7. CONCLUDING REMARKS

This part of the work was dedicated to evaluating the fluctuation of 16S rDNA, class 1 integrons, and IncP-1 plasmids abundances in the water compartment. First, the relative contribution of environmental and technical parameters as sources of variation in their quantification was assessed by statistical analyses, based on nested and mixed ANOVA tests. Importantly, this showed that the main source of variation is given by environmental parameters,

specifically the sampling date, rather than technical sources, which then validate the experimental strategy used. Additionally, this complete statistical analyses also revealed that (i) WWTP effluents had no apparent effect on 16S rDNA, class 1 integrons, and IncP-1 plasmids abundances, which reduces its apparent contribution as local anthropogenic pressures, and (ii) that the two MGE did not fluctuate in the same way thus already revealing probable different sources of MGE-bearing bacteria or different MGE-carrying bacteria. Further analyses of the water compartment suggested that the main sources of MGE-bearing bacteria in the Orne River are diffuse along the watershed rather than localized, and seasonal parameters (as seen by correlation with temperature and river flow rate) are likely to be important in driving the molecular markers fluctuation.

PART II: ROLE OF SUSPENDED MATERIAL IN THE COMPARTMENTALIZATION OF CLASS 1 INTEGRONS AND INCP-1 PLASMIDS

When this PhD work was initiated, we were interested in studying both, the impact of the anthropogenic pressure and the compartmentalization of MGEs to understand their fate in the Orne ecosystem. We have just seen that the effect of the anthropogenic pressure is not so easy to evidence within the scale of the river section studied, where only temporal variation could be detected. Regarding the compartmentalization of MGEs, our initial objective was to demonstrate whether MGEs in water could accumulate in sediments and form reservoirs (or not). But, before studying the stratification of MGEs in sediments (part III), we felt important to consider the link between the water and sediments, i.e., the suspended particulate material (SPM). Indeed, by settling, the SPM plays an important role in the formation and the composition of sediments. As such, the SPM can be considered as shuttle vector between water and sediments, but what is their contribution to the sedimentation and accumulation of MGE-carrying bacteria in sediments was the question we were interested in working on in this part. On an experimental point of view, the idea was to quantify MGEs (class 1 integrons, IncP-1 plasmids) by qPCR in SPM samples and compare it to what obtained from the corresponding water compartment. As we shall see, SPM was first tentatively fractionated by serial filtration so as to include different sizes of particles. However, the difficulties encountered led us to pursue our study using SPM collected by a continuous flow field centrifuge.

1. EXPLORING THE PARTITIONING OF PARTICLE-ATTACHED BACTERIA AS A FUNCTION OF SPM SIZES

Ganesh et. al. (2014) recently demonstrated that the structure of bacterial communities could vary from free-living bacteria and particle-attached bacteria, and we hypothesized that this could be the case for MGE-bearing bacteria. In addition, Ortega-Retuerta et al. (2013), suggested that the structure of SPM

associated bacterial community in oceans also varies according to the size fraction, the quantity, and the quality (organic vs. mineral particles) of SPM. Considering these findings, we decided to evaluate the preference of bacteria for specific particle size fractions before dealing with the community structure involved.

Serial filtration experiments of Orne River water sampled on January 11th, 2016 were performed to obtain water samples (filtrates) containing SPM of different sizes, successively using four filtration membranes characterized by pore sizes of 10, 5, 1.2 and 0.2 μm (Figure 16; materials and methods section). Then, each filtrate fraction was first analyzed by flow cytometry (FCM) to estimate the quantity of bacteria collected in each filtrate. Samples were sonicated so as to disperse aggregates (both particles and bacteria) and the dispersed fractions were stained with the fluorescent dye SYBR green I in order to distinguish between particles and cells. The total number of cells was then determined by FCM. When comparing the different size fractions, it clearly appeared that the concentrations of SPM-associated cells were getting lower when the SPM size was decreasing, somehow suggesting that bacteria are preferentially attached to or be contained into “bigger” particles (Figure 34).

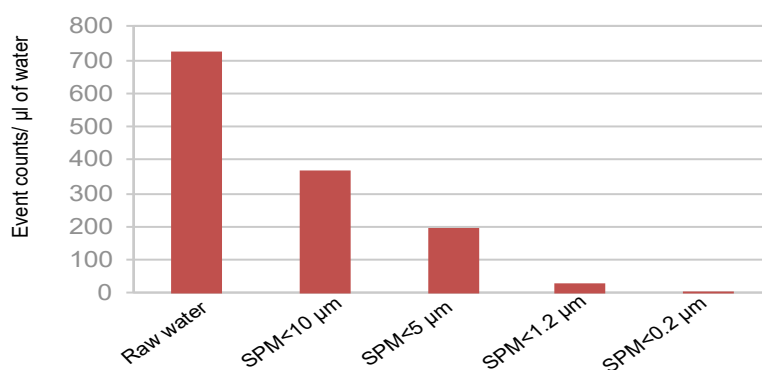


Figure 34. Total cell counts in raw water and water filtrates. Cell count was estimated in different filtrates by flow cytometry (event counts / μl of water) after SYBR I staining and gating set on control bacterial suspension. Proposed SPM sizes in filtrates rely on membrane pore size (n=1).

1.1 Distribution of MGEs in the different fractions of SPM

To explore the distribution of MGEs in different SPM size fractions, the filtrate (100 mL) from the different fractions, the same as those used for the FCM analysis, were used to extract total DNA using the PowerWater® extraction kit, and determine the abundances of 16S rDNA, class 1 integrons, IncP-1 plasmids, and α -, β -, and γ - Proteobacteria by qPCR. As in section 3.1, results part I, the quantifications of α -, β -, and γ - Proteobacteria were used for comparison purposes in order to reveal the differences in community structures, if any. It turned out that their quantification was not fully satisfying as sometimes we detected absolute abundances of the proteobacteria higher than the apparent global bacteria population (Figure 35). These inconsistencies could be caused by qPCR efficiency, specificity and/or selectivity biases resulting in skewed qPCR amplifications. For instance, it is obvious that the absolute abundance of α -proteobacteria in the $<10\ \mu\text{m}$ fraction should not be higher either the one determined for 16S rDNA in the same fraction, or than the absolute abundance of α -proteobacteria in the non-filtrated raw water. Additionally, contrary to what observed with the FCM results where the abundance of stained cells decreased with the fraction size, 16S rDNA quantification appeared relatively stable over the three first fraction sizes considered (Figure 35). Surprisingly, even the filtrate obtained after filtration on $0.2\ \mu\text{m}$ membrane appeared positive with an abundance of 16S rDNA about two orders of magnitude lower than raw water. At this stage, we hypothesized this 16S rDNA detected to come from either free extracellular DNA in water and/or from DNA/bacterial contamination associated to the filter membranes or the material used during the serial filtration experiment. With this respect, control experiments carried out by H  l  ne Guilloteau in the laboratory clearly indicated that the commercial DNA extraction kit used in this study, also contributes partially to the background detection of 16S rDNA (about 2×10^3 - 5×10^3 copies per extraction thus already contributing to approximately 1 % of what is detected).

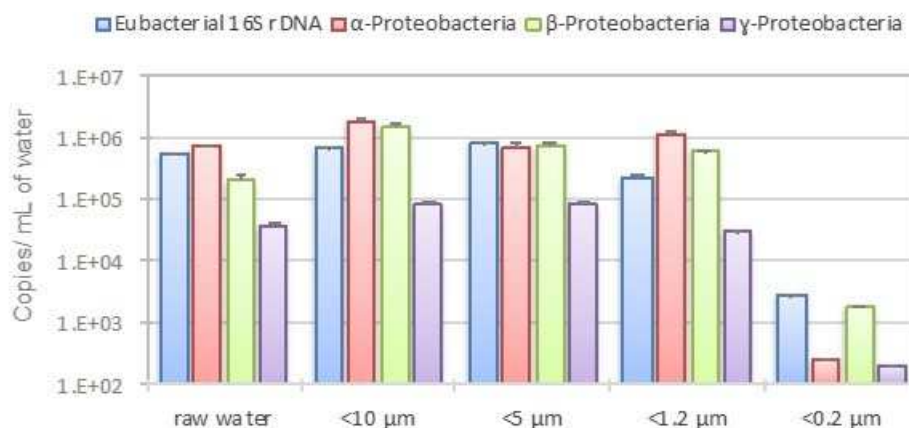


Figure 35. Abundances of 16S rDNA, α -, β -, and γ -proteobacteria per mL of water from the different filtrates with different SPM sizes. On the abscissa are indicated the size of particles expected in the filtrates. Error bars indicate the standard deviation of the mean values ($n=1$; technical qPCR triplicate).

Similarly, although the qPCR amplification of the class 1 integrons is usually robust, the quantification of the MGEs showed a slight increase of absolute abundances from raw water to the fractions SPM < 10 μm or SPM < 5 μm (Figure 36). For IncP-1 plasmids, we observed a small decrease from the raw water sample to the fraction where SPM < 1.2 μm (Figure 36). Considering that just one sample per filtrate was used and the apparent inconsistencies with 16S rDNA quantification, the results obtained were not robust enough to be taken into account. We also noticed that, on the fraction where SPM < 0.2 μm , the quantification of both MGEs were impossible to determine as they were below the detection limit (around 10 copies per tube). The absence of MGE detection is consistent with the 2 order of magnitude decrease of the 16S rDNA marker from the fractions SPM < 1.2 μm to SPM < 0.2 μm (Figure 35).

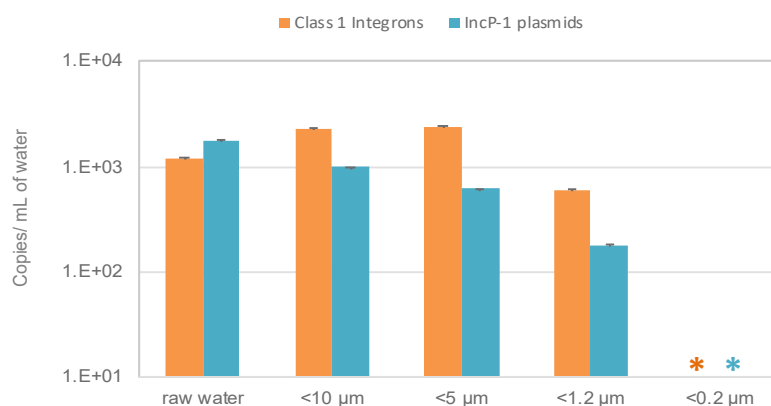


Figure 36. Abundances of class 1 integrans and IncP-1 plasmids per mL of water from the different filtrates with different SPM sizes. Sizes of particles expected in the filtrates are indicated on the X-axis. The asterisks indicated quantities below detection limit (10 copies per mL of water). Error bars indicate the standard deviation of the mean values (n=1; technical qPCR triplicate).

When the relative abundances of MGEs were compared, we observed the same behavior as for their absolute abundances (Figure 37). It seems that there is not a clear tendency that allows us to determine if the particle size could play a role in the preference of MGE-bearing bacteria for their attachment to them.

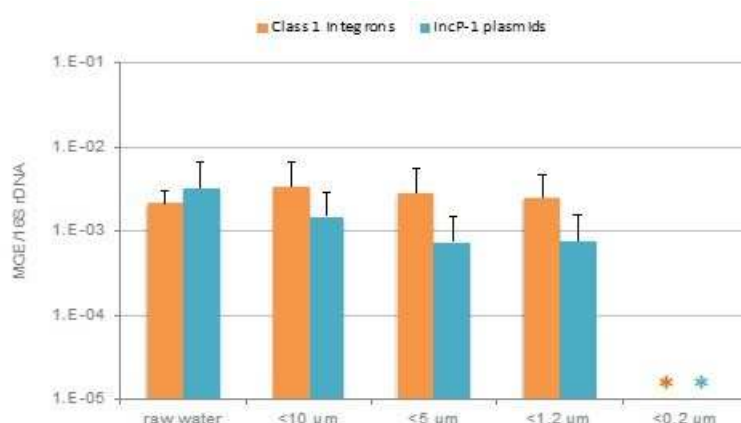


Figure 37. Relative abundances of class 1 integrans and IncP-1 plasmids from the different filtrates with different SPM sizes. Sizes of particles expected in the filtrates are indicated on the X-axis. The asterisks indicated below detection limit. Error bars indicate the standard deviation of the mean values (n=1; technical qPCR triplicate).

After analyzing these preliminary results, where no specific enrichment of MGEs was observed in a particular fraction of SPM, and considering the

apparent preference of bacteria for being attached to bigger particles that are present in raw water (based on the FCM results), the suitability of serial filtration experiment was questioned, at least for two reasons. First, we especially wondered if the abundances of the molecular markers determined could be biased as the fraction of SPM retained on the membranes used for the serial filtration were not analyzed. Second, the approach was clearly difficult to implement, clearly compromising our ability to get multiple repeats and thus significantly representative data. These reasons drove us to consider another approach for the recovery of SPM.

1.2 Enrichment of suspended particles by field continuous flow centrifugation

Several methods have been implemented to separate SPM from water, such as sediment traps and field continuous flow centrifugation (CFC); the latter being the most used when a representative amount of SPM is required (Abuhelou et al. 2017). The characterization of SPM from the Orne River was also one of the goals of the MOBISED project, the idea behind it was to model the flow of materials between the sediment and the water compartments. Interestingly, CFC allows obtaining representative SPM samples as they result from the centrifugation of 500-1000 L. Still, if CFC was already routinely used for the geochemical analyses of the MOBISED project, the same could not be said for our microbiology analysis. A first series of analyses were carried on SPM samples collected during a November 2014 campaign. SPM community DNA was extracted using the PowerWater® extraction kit, and the three markers 16S rDNA, *int11* and *trfA* were quantified by qPCR. As fully explained below (Figure 41), MGE relative abundances in SPM appeared significantly different from the MGE content of the corresponding raw waters. The SPM content in MGE appeared then as an interesting lead to explore for regarding MGE compartmentalization. However, at this stage, we were a bit concerned by the functioning of the CFC. With the kind of CFC used, the speed of centrifugation cannot be set and is fixed at 17,000 x g. With a regular centrifuge, such centrifugation speed would pellet both free-living and particle-associated microorganisms. Was it the case with the CFC, considering a

relatively short residence time in the centrifuge? and, can we control it? were two questions we try to answer here. The only controllable parameter in the CFC setup used is the flow rate of inflow pump connected to the CFC, which then influences the residence time of water/particles in the centrifuge, which in turn possibly influences the size distribution of the collected particles. In the first series of experiments, we evaluated the functioning of the CFC, and specifically the influence of the inflow flow rate, on the abundance of the MGE-carrying bacteria collected. This was the purpose of the September 29th 2015 campaign. Globally, the CFC was run at 17,000 x g, corresponding to an expected cut-off threshold for particle collection close to 5 μm (Abuhelou et al. 2017), and three different flow rates were applied at the level of the inflow pump: 500 L/h, 250 L/h and 180 L/h. SPM and water samples from the inlet and outlet of the CFC were sampled in order to examine differences in particle sizes and thus determine the cut-off particle threshold of the CFC (experiments carried out by the LIEC team). In the meantime, microbiological analyses were carried out to study the influence of the CFC setting on the absolute and relative abundances measured with respect with free-living and particle-attached microorganisms, and their MGEs.

The propensity of the CFC to pellet both particle-associated and free bacteria was first assessed by comparing the inlet and outlet content for 16S rDNA, *int1* and *trfA*. It turns out that the difference between the two kinds of samples was not spectacular (Figure 38). Absolute abundances of 16S rDNA and class 1 integrons differ slightly but significantly from the outlet to inlet samples for the three flow rates tested (Student's t-test for paired data $p=0.012$ and $p=0.024$, respectively). The same tendencies were observed for IncP-1 plasmids although no significant difference was revealed using statistical tests ($p=0.16$). The significant differences for 16S rDNA and *int1* markers, and the tendency for plasmids suggest that a fraction of the total bacterial population is effectively collected by the CFC together with the SPM. Additionally, the relatively weak differences between the inlet and outlet also show that the CFC just partially "sampled" the water as most bacteria go through.

The previous comparison intended to show differences between inlet and outlet water pairs. Next, we wondered whether the residence time (inflow pump flow rate) in the CFC could have an effect on the amount of material collected.

To address this question, three flow rates were compared with respect with the abundances of 16S rDNA, *intI1* and *trfA* within each group of samples (inlet, outlet and SPM samples), independently. At a first glimpse, the flow rate did not seem to have a big effect on the amount of marker measured (Figure 38), only statistical tests could reveal minor differences:

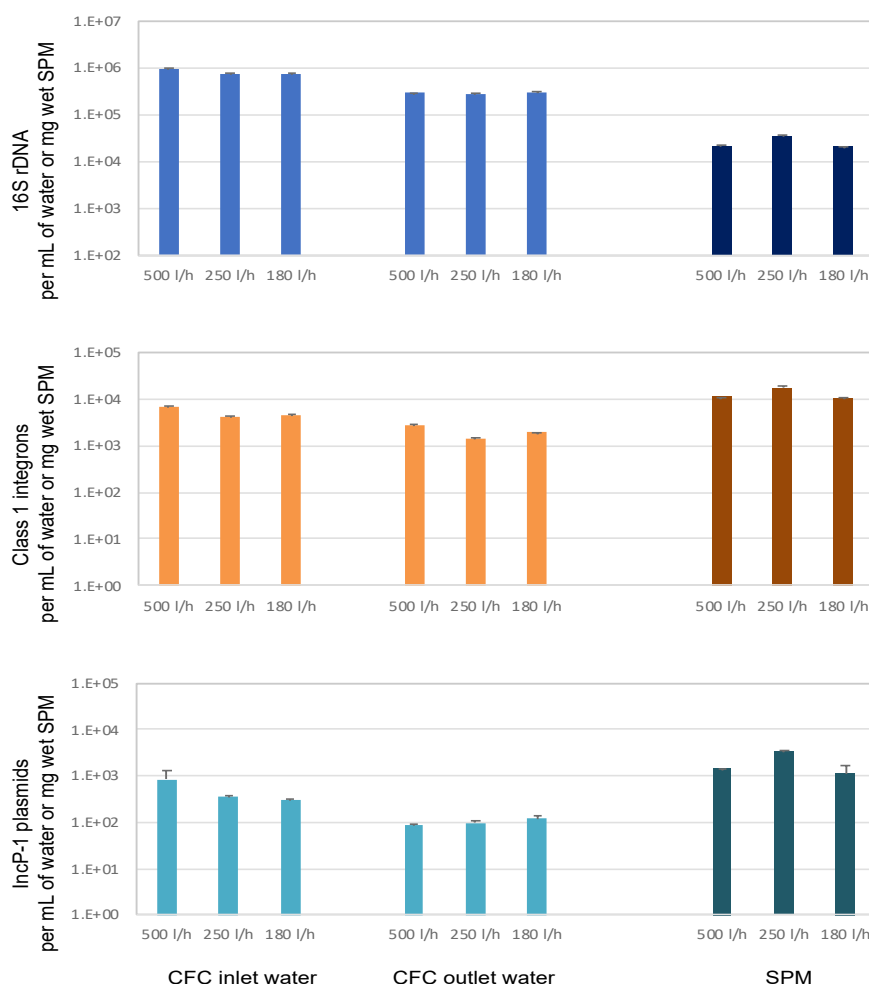


Figure 38. Effect of different input water flow rates upon field centrifugation on the absolute abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids in water and SPM samples. The bars at the left correspond the water samples of CFC inlet, bars in the middle correspond to water samples of CFC outlet and the darkest bars at the right correspond to SPM samples. Error bars indicate the standard deviation of the mean values (n=1 series of sample; technical DNA triplicate, technical qPCR triplicate).

- First, 16S rDNA and *trfA* absolute abundances in CFC inlet or CFC outlet were quite similar (ANOVA tests, $p > 0.05$) showing that there is no apparent effect of the input flow rate on these markers within each group of samples.

An apparent effect was (statistically) detected for the *int11* abundances within both CFC inlet and outlet samples (ANOVA test, $p < 0.05$).

- Second, the effect of the inflow water flow rate was also evaluated in the abundances of the markers within the SPM series of samples. It seems that for SPM, the absolute abundances of 16S rDNA, *int11* and *trfA* markers are all influenced by the flow rate (ANOVA test $p < 0.05$). Indeed, although the abundances corresponding to the higher and lower flow rates are comparable, higher absolute abundances of the three markers were systematically observed for the intermediate flow rate at 250 L/h (Figure 38).

In conclusion, the results obtained from statistics sporadically pointed out an effect of the flow rate on the abundance of marker measured. However, when looking at the data presented in Figure 38, it should be noted that even if detected statistically, these differences are not so obvious and cannot be considered as very important (if any). Now, when it comes to comparing the abundance of the markers in the water sample and what is retained by the CFC in the SPM samples, things are getting slightly more difficult since one is expressed in copies/mL and the other one in copies/mg. However, if CFC was to selectively collect a sub-fraction of the microbial community present in water (the one associated to SPM for instance), it can be argued that differences could be seen when comparing the relative abundance of MGEs if both compartments (SPM and water) are not colonized by the same bacteria (as initially seen on the November 2014 campaign, Figure 41). The relative abundances of MGEs (copies/16S rDNA) were thus key parameters to obtain for pushing forward our study regarding MGE compartmentalization.

Finally, to evaluate if the CFC had an effect on the “partitioning” of the population of MGE-bearing bacteria, in other words, if the two compartments (water and SPM) differ in MGE relative content, the relative abundances of MGE were compared (Figure 39). Here again, no apparent effect of input water flow was observed and the relative abundances from water samples, CFC inlet and outlet, and SPM appeared to be on the same range. The significance of the possible differences between (i) inlet water samples vs. SPM; (ii) outlet water samples vs. SPM; and (iii) inlet water samples vs. outlet water samples, were

also determined by Student's t-test for paired data. The relative abundance of IncP-1 plasmids did not appear significantly different for any of the three comparisons ($p= 0.16, 0.88$ and 0.31 , respectively). Conversely, differences in the relative abundance of class 1 integrons appeared significant in all cases but not in the comparison inlet vs. outlet water samples ($p= 0.005, 0.028$ and 0.39 , respectively).

At this stage, we were a bit puzzled by the contrasting results we obtained. On the one hand, it appeared that the abundance measured did not change easily according to the water input flow rate in the CFC even if so statistical differences were identified. This tends to show that the way SPM are collected by the centrifuge does not alter that much their content microbiologically. However, as we noticed upon sampling that the higher is the flow rate, the better are the SPM stuck within the CFC and the recovery of SPM (Le Meur et al. 2016b), we decided to subsequently use the flow rate of 500L/h. On the other hand, this series of experiment did not really point out any significant enrichment of MGE-bearing bacteria as observed previously in the November 2014 campaign (Figure 41). With this respect, it should be mentioned that the series of experiments presented here were carried out on a single water sample, which is a bit short to draw any conclusion. Considering the differences observed between this campaign and the November 2014 one, it was clear to us that more sample may be necessary to be analyzed to report on putative fluctuation associated with particle-associated bacteria.

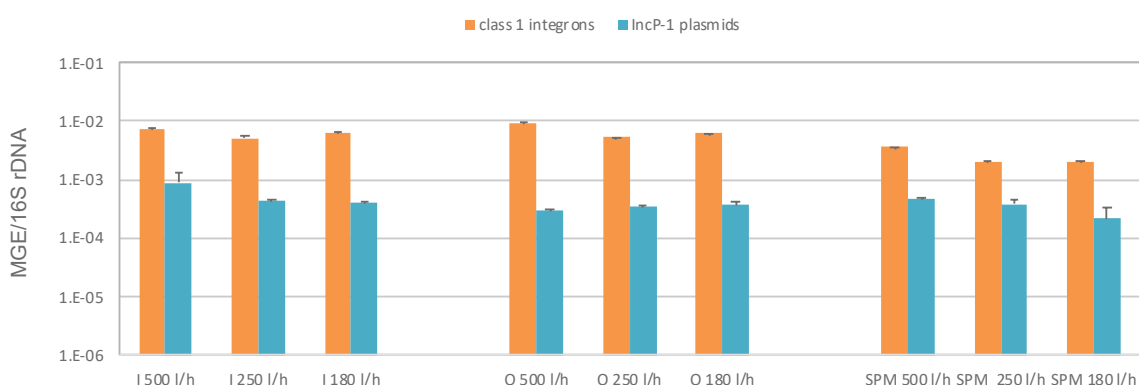


Figure 39. Comparison of the relative abundances of class 1 integrons and IncP-1 plasmids between SPM and water samples from inlet (I) and outlet (O) of the CFC at the different input water flows. Error bars indicate the standard deviation of the mean values ($n=1$; technical DNA extraction triplicate, technical qPCR triplicate).

2. OCCURRENCE OF CLASS 1 INTEGRONS AND INCP-1 PLASMIDS IN SPM SAMPLES VS. RAW WATER SAMPLES

The usefulness of the CFC for collecting SPM of the Orne River has been discussed by our geochemist colleagues, already (Abuhelou et al. 2017). Having demonstrated the feasibility of measuring the abundance of our markers in the SPM fraction collected, it was decided to get an overview of the difference between the water and the SPM microbiological content across a series of 4 sampling campaigns (November 2014, February, May and October 2015), carried out outside rising flood periods and spread along the BARB-RICH continuum of the River. Interested in identifying a possible compartmentalization of MGE-carrying bacteria, we collected series of raw water and their corresponding SPM for comparison using our classical molecular markers: *int11* for class 1 integrons, *trfA* for IncP-1 plasmids, Eubacterial 16S rDNA for normalization, and α -, β -, and γ -proteobacteria for assessing changes in microbial community structure. At the end, 15 pairs of water and their corresponding SPM were compared.

The quantification of the absolute abundances, per mL of water samples or per mg of wet weight for SPM samples, are presented in Figure 40. As it has been presented before for water samples (section 3, results part I), evident fluctuations of the 16S rDNA, *int11* and *trfA* markers were observed according to the date of sampling. We noticed that the fluctuation of these markers in SPM samples in terms of sampling date is less evident (Figure 40), suggesting that they are not influenced by the same parameters or if so, not in the same proportions. The same behavior was observed for the α -, β -, and γ -proteobacteria (data not shown). The 16S rDNA, *int11* and *trfA* absolute abundances did not allow performing any comparison between the two compartments as they are not expressed in the same units. Therefore, we decided to work with the relative abundances of the molecular markers for addressing the question of a putative compartmentalization of the studied MGEs.



Figure 40. Absolute abundances of 16S rDNA, class 1 integrons (*intl1*) and IncP-1 plasmids (*trfA*) in water samples (graphs on the left) and SPM samples (graphs on the right). The data correspond to four sampling campaigns at different sampling sites along the continuum of the river (23 km-section). BARB, JOAB, JOMED and BETH on November 4th, 2014; AUB, BARB, JOAB, JOMED and BETH on February 2nd and 3rd, 2015; JOAB, BETH, RICH on May 5th, 2015; AUB, BETH and RICH on October 6th, 2015. Error bars indicate the standard deviation of the mean values.

When the MGE relative abundances were compared (Figure 41), we observed that, whatever the sampling campaign, IncP-1 plasmid relative abundances and, in a lesser extent, class 1 integrons relative abundances seemed to be higher in SPM samples than in the corresponding raw water. Considering SPM as sub-fraction of the raw water content, this tend to show that both compartments probably exhibit differences in community structures, at least with respect with MGEs-carrying bacteria (the enrichment of MGEs in the SPM compartment will be extensively discussed in the next section). These likely differences were further confirmed when analyzing the relative

abundances of α -, β -, and γ -proteobacteria (Figure 42). Additionally, it seems that the relative abundances of α - and β - proteobacteria sometimes are higher in SPM samples than in raw water samples, while values for γ -proteobacteria remain almost equal for both type of samples, thus further suggesting a differential compartmentalization of the free-living and attached bacteria from these 3 taxonomic groups. Nevertheless, as the relative abundances of these proteobacteria are frequently greater than 1, the obtained values have to be cautiously used and commented.

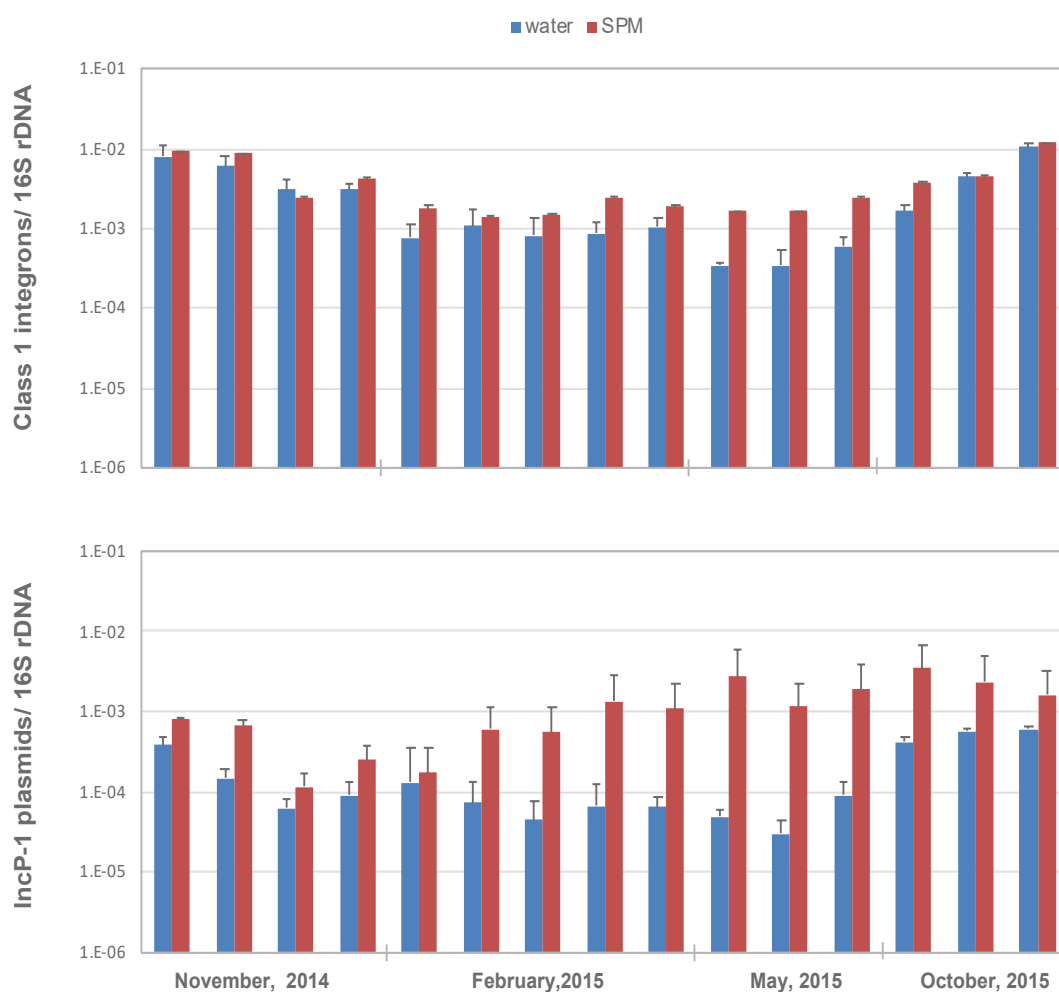


Figure 41. Comparison of the relative abundances of class 1 integrons and IncP1 plasmids in raw water samples and SPM during different sampling campaigns. BARB, JOAB, JOMED and BETH on November 4th, 2014; AUB, BARB, JOAB, JOMED and BETH on February 2nd and 3rd, 2015; JOAB, BETH, RICH on May 5th, 2015; AUB, BETH and RICH on October 6th, 2015. Error bars indicate the standard deviation of the mean values.

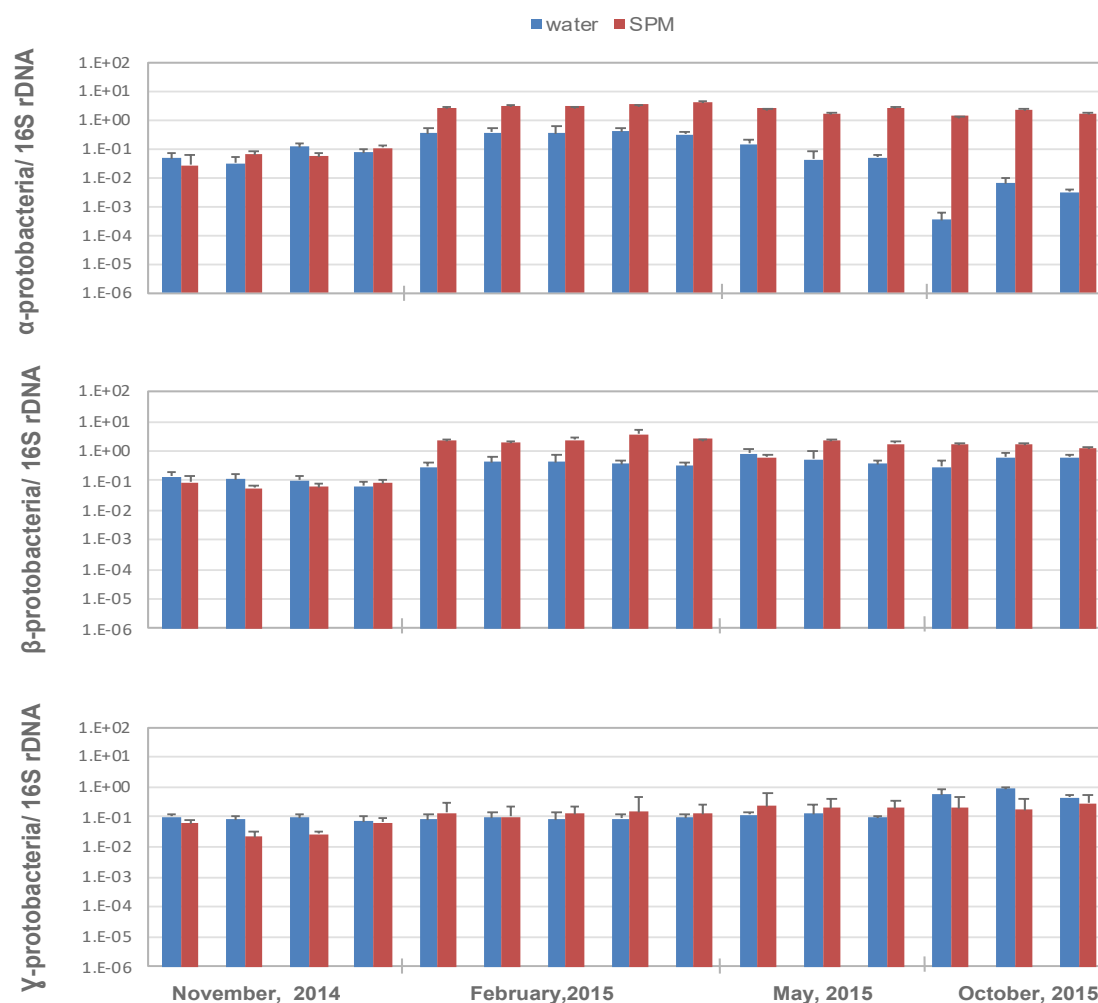


Figure 42. Relative abundances of α -, β -, and γ -proteobacteria in water samples and corresponding SPM samples. The data corresponds to four sampling campaigns at different sampling sites along the continuum of the river (23 km-section). BARB, JOAB, JOMED and BETH on November 4th, 2014; AUB, BARB, JOAB, JOMED and BETH on February 2nd and 3rd, 2015; JOAB, BETH, RICH on May 5th, 2015; AUB, BETH and RICH on October 6th, 2015. Error bars indicate the standard deviation of the mean values. Relative abundances greater than 1 have to be cautiously used, and probably reflect different efficiencies and/or selectivity of the qPCR designs used.

Despite our doubts about the proteobacteria related values, all the data obtained during the 4 campaigns (relative abundances of class 1 integrons, IncP-1 plasmids, α -, β - and γ -proteobacteria) were finally combined in a single Principal Component Analysis (PCA). Based on the correlations observed in the variable factor map of Figure 43, it is tempting to hypothesize that integrons would be more frequently carried by γ -proteobacteria, and IncP-1 plasmids by α -, and β -proteobacteria. On the other hand, there is a clear separation

between the two kinds of samples (SPM and raw water), which form two different clusters (Individuals factor map, Figure 43). In other words, and considering that SPM is part of the raw water, SPM-associated bacteria are clearly a specific sub-fraction of the water communities, with a specific fingerprint. This necessarily implies that, upon settling, SPM should enrich sediments with this specific sub-fraction of the raw water microbial communities. How do MGE-carrying bacteria fit into this bacteria-specific enrichment of sediments, this is what we are going to develop in part III of results.

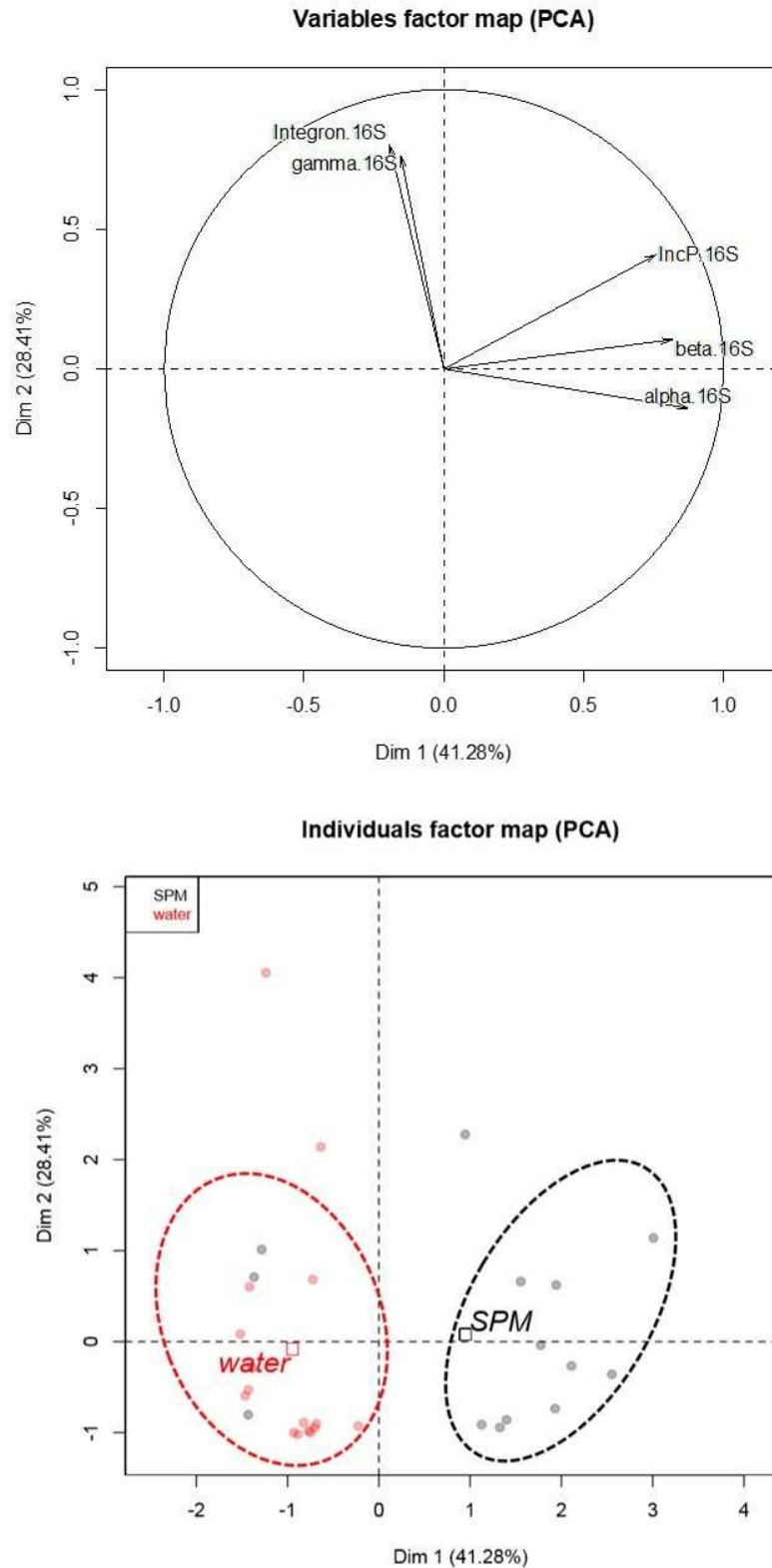


Figure 43. Contrast between free living and attached bacteria in raw water vs. SPM samples. Selected variables: relative abundances of α -, β -, γ -proteobacteria, class 1 integrons and IncP-1 plasmids. The red cluster mainly corresponds to raw water samples whereas the black cluster only contains SPM samples.

3. ENRICHMENT OF CLASS 1 INTEGRONS AND INCP-1 PLASMID-CARRYING BACTERIA IN SUSPENDED PARTICLES

We previously observed that SPM communities differed from raw water communities, with an increase in the relative abundances of MGEs in SPM and site. In exception of 1 of the 15 sample pairs, the relative abundance of class 1 integrons and IncP-1 plasmids appeared higher in SPM than in the corresponding raw water samples, with mean ratios of 2.2 ± 0.4 and 13.3 ± 4.0 , respectively (Figure 44). Statistical analyses, based on Wilcoxon Signed-Rank test for paired data were used for the data comparison. The tests confirmed the statistical significance of the MGE enrichment on particle-attached bacteria (SPM samples) with p-values of 3.1×10^{-4} and 5.8×10^{-4} for class 1 integrons and IncP-1 plasmids, respectively. Although the enrichment of both MGEs was significant, it remains higher for IncP-1 plasmids than for class 1 integrons (Wilcoxon signed rank test; $p = 2 \times 10^{-4}$). This difference points out the fact that these 2 MGEs are probably not carried by the same bacterial population, as the data presented in the PCA Figure 43 suggested (variables factor maps).

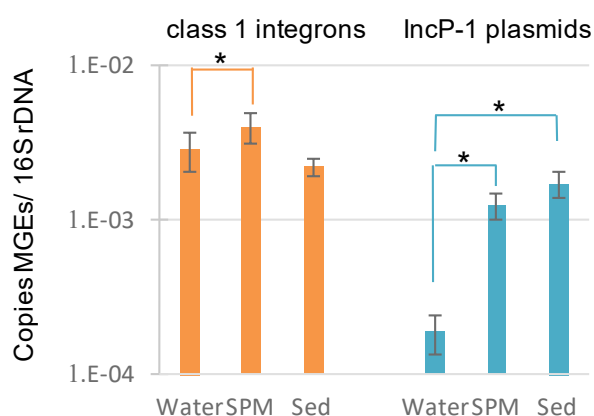


Figure 44. MGE content in the different compartments of the Orne River. Each bar represents the mean of the relative abundances of class 1 integrons and IncP-1 plasmids in the three Orne River compartments. Water: raw water; SPM: suspended particles; Sed: surface sediments. Asterisks indicate a significant difference between the relative abundances ($p < 10^{-3}$). Error bars indicate the standard error of the mean values ($n = 15$ for waters and SPM; $n = 9$ for sediments).

Assuming that the SPM enriched in MGEs eventually settle to form river sediments, we hypothesized that the sediment compartment could be an

important reservoir of ARGs that could eventually be disseminated thanks to the MGEs. Attempting to determine the fate of the MGE-bearing bacteria on SPM after settling, surface sediment samples were also collected and their content in MGEs measured as before (Figure 44). Statistical tests carried out on relative abundances were also performed to compare the MGE relative abundances obtained for sediments to those obtained for the two other compartments. For this statistical comparison, Wilcoxon rank-sum test was used as sediments were sampled independently from raw waters and SPM, and as data were not normally distributed. The statistical test shows that the relative abundance of class 1 integrons in surface sediments did not seem to be significantly different from those of raw waters or SPM ($p=0.39$ and $p=1$, respectively). Conversely, the relative abundance of IncP-1 plasmids was 9.1 and 1.4 higher in sediments than in raw waters and SPM samples, respectively. Additionally, these relative abundances were statistically different between sediments and raw waters ($p=5 \times 10^{-4}$), but it was not the case between surface sediments and SPM ($p=0.18$). The similarity in the relative abundances of IncP-1 plasmids between surface sediments and SPM raises the question of the role of plasmid in their selective enrichment in particle-attached microorganisms rather than free-living microorganisms.

To explain the enrichment of IncP-1 plasmids in SPM, two hypotheses were formulated based on the IncP-1 plasmid characteristics as conjugative elements. First, it is frequently reported that horizontal gene transfer in biofilm communities occurs at higher frequencies than within planktonic bacteria (Madsen et al. 2012). It can be argued that bacterial adhesion to SPM leads to an higher promiscuity between bacteria, which favors plasmid transfer and, therefore, results in an increase in its relative abundance (since conjugative transfer is a replicative process). On the other hand, JM. Ghigo (2001) showed that conjugative plasmids themselves could promote the formation of biofilms as they encode for conjugative pili, a cell appendage also considered as an adhesion substrate. Therefore, the IncP-1 plasmid-bearing fraction of the bacterial population should be more likely to bind to the organo-mineral particles constituting SPM than the plasmid-free bacteria, which should lead to a compartmentalization of the plasmid-bearing bacteria over time. These two hypotheses were further tested using *in vitro* grown biofilms.

3.1 Transfer efficiency of plasmid pB10 in planktonic bacteria and surface-associated cells

The first step in biofilm formation is the adhesion of planktonic cells to a surface, then these adhering cells grow and the adhesion of additional cells increases the biomass of the biofilm (Cook and Dunny 2014). It is generally accepted that cell-to-cell contact provided by biofilms promotes horizontal gene transfer through mechanisms such as conjugation. As such, an increased frequency of transfer in the biofilm may explain the enrichment of IncP-1 plasmid on SPM as each round of conjugative transfer generates a new copy of the plasmid. This hypothesis was tested by comparing the transfer efficiency of the model IncP-1 plasmid pB10 in biparental mating with cells grown either as planktonic bacteria or surface-associated bacteria. The donor bacteria *Escherichia coli* DH5 α (pB10) and the recipient strain *Cupriavidus metallidurans* AE815 were mixed and incubated for 16 h in LB medium in the presence or not of a synthetic support, a Kaldnes polyethylene disk, as a matrix for biofilm development. Selective plates (containing antibiotics) were used for the enumeration of donor, recipient, and transconjugant cells from (i) the disk-free mating experiment, for assessing pB10 transfer in planktonic conditions, and (ii) the Kaldnes polyethylene disks, for assessing pB10 transfer in the biofilm. Figure 45 gives a comparison between the two mating conditions (planktonic vs. biofilms). The corresponding transfer frequencies expressed either as transconjugant per recipient or transconjugant per donor did not appear significantly different from condition to another (Kruskal-Wallis rank sum test; $p>0.05$). Moreover, from previous experiments, we knew that the transfer of pB10 could also take place in liquid cultures. Thus, it was decided to enumerate donor, recipient, and transconjugant cells from the liquid medium surrounding the Kaldnes polyethylene disks. We observed, that the corresponding pB10 transfer frequencies were similar to that observed for the bacteria growing as a biofilm on the disks (Wilcoxon signed rank test; $p>0.1$). This similarity highlights the fact that there were no biases in calculating the transfer frequencies due either to transconjugants accumulating from the liquid to the biofilm or to

transconjugants standing out from the disks to the liquid. Seeing the results obtained, it seems that, under the conditions provided by the tests carried out on polyethylene disks, development as biofilm does not promote a higher transfer level of pB10.

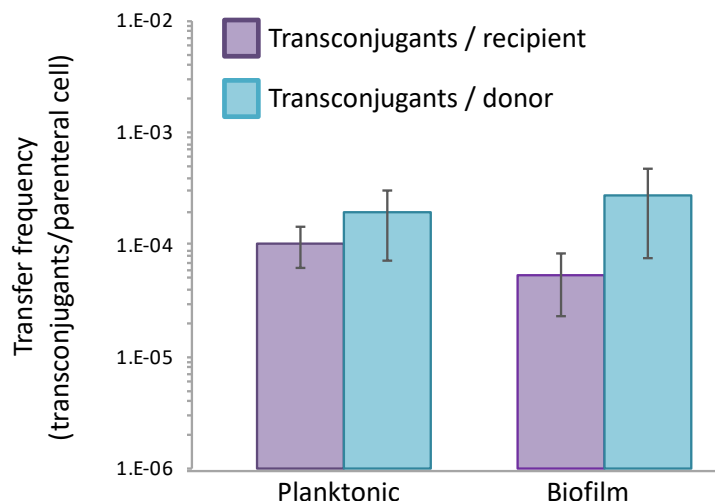


Figure 45. Transfer efficiency of plasmid pB10 in planktonic bacteria and surface-associated cells. Error bars indicate the standard error of the mean values (n=4).

3.2 Role of IncP-1 plasmids in promoting cell adhesion to particles

Considering that the ability to form a biofilm is often dependent on the production of adhesins (such as conjugative pili) that could mediate adhesion to abiotic surfaces (Ong et al. 2009), we wondered if bearing IncP-1 plasmids was sufficient to increase cell adhesion to surfaces compared to plasmid-free bacteria. To evaluate this hypothesis, a series of isogenic bacterial strains from various species, carrying or not the natural IncP-1 β plasmid pB10, were used to evaluate the impact of the plasmid on bacterial adhesion. For this experiment, bacteria were allowed to grow as a biofilm on Kaldnes polyethylene disks immersed in LB medium for 18 h, resulting in a large excess of planktonic cells. The abundance of planktonic bacteria ([P.B.]) was estimated at 600 nm (OD₆₀₀) by spectrometry, while the abundance of the adhering biomass was measured using a classical crystal violet staining method ([C.V.]; OD₅₄₀) (Table 21).

Table 21. Effect of pB10 carriage on bacterial adhesion properties

Strains/Species*	Planktonic biomass (OD _{600nm})		Adhering biomass (OD _{540nm})		Relative biofilm amount (arbitrary unit: OD _{540nm} /OD _{600nm})		
	without pB10	with pB10	without pB10	with pB10	without pB10	with pB10	fold increase
<i>E. coli DH5α</i>	3.205	3.686	0.323	1.101	0.10	0.30	3.18
	±0.032	±0.038	±0.072	±0.129	±0.02	±0.03	±1.04
<i>E. coli MG1655</i>	4.669	4.960	0.254	0.309	0.05	0.06	1.15
	±0.219	±0.388	±0.047	±0.065	±0.01	±0.02	±0.09
<i>C. metallidurans</i>	3.251	3.271	0.530	0.898	0.16	0.28	1.70
	±0.136	±0.313	±0.152	±0.483	±0.04	±0.16	±1.04
<i>B. subtilis</i>	3.988	3.700	0.372	0.864	0.10	0.23	2.53
	±0.357	±0.056	±0.045	±0.222	±0.02	±0.06	±0.64
<i>P. putida</i>	5.787	5.047	2.361	2.838	0.41	0.56	1.44
	±0.211	±0.220	±0.405	±0.092	±0.08	±0.03	±0.33
<i>P. fluorescens</i>	3.401	2.715	0.513	0.591	0.17	0.30	1.64
	±1.238	±1.522	±0.047	±0.026	±0.06	±0.15	±0.38
<i>S. oneidensis</i>	4.985	4.719	0.503	0.419	0.12	0.11	0.82
	±1.317	±1.142	±0.150	±0.200	±0.08	±0.08	±0.09
<i>D. acidovorans</i>	4.501	3.634	2.664	2.294	0.60	0.67	1.09
	±0.488	±0.915	±0.384	±0.928	±0.12	±0.21	±0.14

*: *E.*, *Escherichia*; *C.*, *Cupriavidus*; *B.*, *Bacillus*; *P.*, *Pseudomonas*; *S.*, *Shewanella*; *D.*, *Delftia*.

Values are expressed as a mean ± standard error (n=3).

The propensity to form biofilms was finally estimated as the relative abundance of adhering cells compared to planktonic bacteria, here given in arbitrary units ([C.V.]/ [P.B.]). The results obtained (Table 21) showed that the carriage of pB10 significantly increased the relative amount of adhering bacteria on polyethylene surfaces (Student's T-test for paired data, $p=5 \times 10^{-3}$). Apart from the outlier strains *E. coli* MG1655 and *Shewanella oneidensis* MR-1, that seem to have peculiar behaviors, the effect of pB10 carriage on adhesion is stronger when the basal level of biofilm formation is initially low in the absence

of plasmid (Spearman correlation without considering strains MG1655 and MR-1: $r=-0.943$, $p=0.02$) (Figure 46).

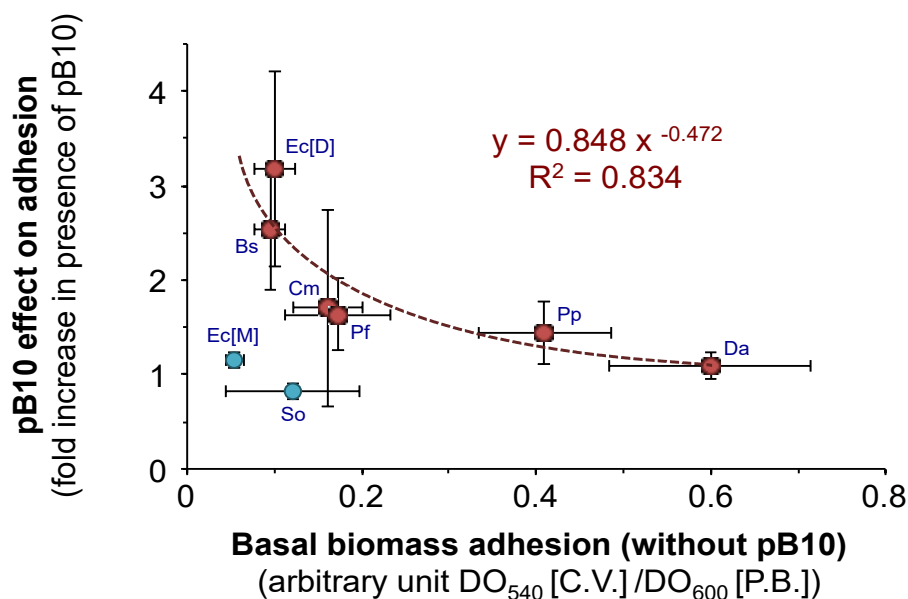


Figure 46. Gain in biofilm formation for pB10-bearing bacteria. Biofilm formation was compared for isogenic strains with and without the IncP-1 β plasmid pB10: *B. subtilis* CM291 (Bs); *C. metallidurans* AE815 (Cm); *E. coli* DH5 α (Ec[D]), *E. coli* MG1655 (Ec[M]); *P. fluorescens* CM292 (Pf); *P. putida* SM1443 (Pp); *S. oneidensis* CM87 (So). Error bars indicate the standard error of the mean values (n=3).

Importantly, it should be noted that the different adhesion behaviors observed for the different strains tested could not be attributed to a strain-dependent instability of the plasmid. This was estimated by plating overnight cultures grown without antibiotics on plates selective or not for pB10-bearing bacteria. Plasmid pB10 appeared to be highly stable as no significant loss could be observed in all the bacterial hosts used (Kruskal-Wallis rank sum test, $p=0.99$), and as it had already been thoroughly assessed before for most of the bacteria and/or strains used in this work (De Gelder et al. 2007). Putting these observations back in the context of the plasmid enrichment observed on the SPM and sediments of the Orne River, it tends to demonstrate as plausible the fact that (i) IncP-1 plasmid carriage is sufficient to promote the compartmentalization of plasmid-bearing bacteria on SPM and thus on freshly deposited sediments, and (ii) the gain in adhesion is not equally distributed

among bacterial strains, which should also contribute to specifically compartmentalize bacteria according to their nature.

4. PHYSICOCHEMICAL CHARACTERIZATION OF SPM AND ITS RELATIONSHIP WITH SEDIMENTS

In section 2.1, it was described that there was no significant difference between SPM and surface sediments when the relative abundances of class 1 integrons and IncP-1 plasmids were compared. If SPM settles to form fresh sediments they should share common characteristics, such as particle size distribution, mineral constituents, and elemental composition. Taking advantage of the MOBISED project consortium, we could dispose of the SPM and sediment characterization, made by Emmanuelle Montargès-Pelletier and collaborators (LIEC), to establish the link between the two compartments. The elemental composition of SPM and surface sediments is presented in Table 22. The element contents are rather close between surface sediments and SPM, with a moderate difference though. SPM is richer in aluminosilicate particles, inducing higher Al, Mg and K contents, and sediments are richer in calcium carbonates inducing a higher Ca content. The contents in organic carbon are similar but SPM displays a higher variability due to the presence of autochthonous organic matter during the warm seasons (spring and summer).

Table 22. Elemental composition of SPM and surface sediments of the Orne river.

Sample	Element (%)								
	C _{organic}	Si	Al	Fe	Ca	Mg	K	P	Fe/Al
Sediment (n=8)	4.9 ± 0.7	16.4 ± 1.2	4.2 ± 0.6	4.1 ± 0.4	11.6 ± 1.6	0.6 ±0.08	1.0 ± 0.1	0.2 ±0.04	1 ±0.07
SPM (n = 54)	4.2 ± 2.1	16.8 ± 3.2	6.9 ± 2.3	5.3 ± 1.3	4.4 ± 1.6	0.8 ±0.18	1.5 ±0.38	0.3 ± 0.2	0.79 ±0.08

The grain size distribution reveals that SPM particles are relatively fine with a grain size distribution centered below 10 μm , and the decile (D50) of Orne River suspended particles was estimated to be $12 \pm 3 \mu\text{m}$. The grain size distribution measurements evidenced that sediments are coarser than SPM, suggesting a size sorting upon settling and/or a post-settlement modification of the grain size distribution. Moreover, in the context of the MOBISED project, about 78 SPM particles and 42 sediment particles were analyzed using the nanoprobe and the EDX detector. The EDX analysis provided the atomic percentages of major elements for each particle (O, Mg, Al, Si, P, S, K, Ca and Fe). Figure 47 represents the results of this analysis following two atomic ratios, that are representative of clay particle mineralogy. The superimposition of the sediments and SPM results, strongly suggest that the clay minerals, which are the predominant constituents, have the same geological origin. Mineralogy, particle size distribution and element contents clearly draw a parental-like link between sediments and SPM.

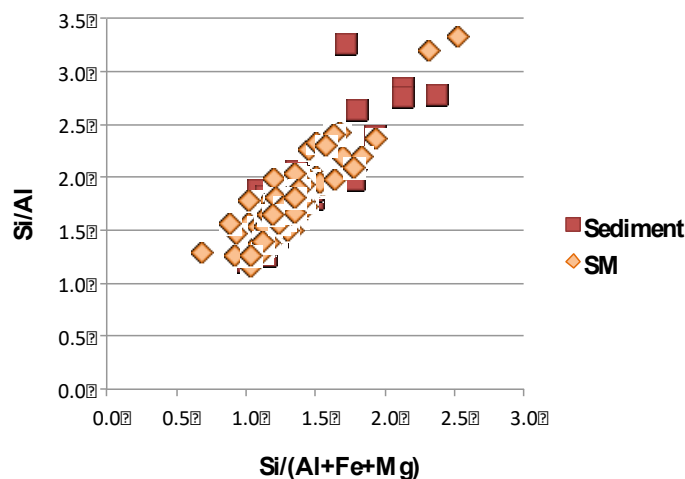


Figure 47. Mineralogy of Orne River suspended materials and sediments by EDX analysis. The atomic ratios Si/Al and Si/(Al+Fe+Mg) are representative of clay particle mineralogy.

5. CONCLUDING REMARKS

To conclude, we considered that SPM is playing an important role in feeding both the water and sediment compartment with particle-associated bacteria. In low flow rate conditions (dry seasons) particles tend to settle and conversely, under specific hydroclimatic events such as floods, remobilization of sediments could happen and formerly settled SPM could return back to the water compartment. Using synthetic biofilms, we strongly suggested that the specific enrichment of plasmid-bearing bacteria on/within suspended particles (river samples) would be mainly due to the improved adhesion properties brought by conjugative plasmids. Once associated to particles, plasmid-bearing bacteria could reach the sediment after settling, and thus contribute to the formation of ARG reservoir. Considering the specificity of the bacterial enrichment on particle, this means that the particle settling process necessarily enrich the sediment with plasmid-bearing bacteria. The occurrence of MGEs in the sediment, as a final receiving compartment, will be addressed in the next chapter.

PART III: SEDIMENTS AS FINAL RECIPIENT **COMPARTMENT OF MGEs**

From the previous section, we learned that the SPM of the Orne River is selectively enriched in MGE-carrying bacteria, especially IncP-1 plasmid-carrying bacteria. If SPM eventually settles to form river sediments, then MGEs and their associated ARGs can accumulate and form reservoirs in the sediment compartment, as far as they persist. The persistence of MGEs or/and MGE-carrying bacteria in river sediments is still a subject of investigation, especially regarding co-selection processes. In a few occasions, the co-occurrence of antibiotic resistance genes and metal resistance genes have been reported in metal-polluted sediment, therefore suggesting such co-selection processes (Seiler and Berendonk 2012; Knapp et al. 2017). In the Orne River context, it has been established that material originating from the steel production and related activities, including several pollutants such as metals and PAHs, have been released into the river, transported by suspended particles and stored in sediments (Le Meur et al. 2016b). The possibility that such pollutants could have an incidence on the fate of MGE persistence in the Orne River sediment remains an open question. Contrary to what observed in water, the occurrence of MGEs in sediments has to be understood in a time scale, where selection/co-selection processes on MGE-carrying bacteria can operate. In this section, we focused our investigations on the stratification of MGEs in the different layers of sediment cores, aiming at better understanding their accumulation and persistence in sediments. Once more, the exhaustive geochemical analyses available gave us a unique opportunity to investigate if MGE accumulation/persistence is related to the pollutant contents in the sediments or not.

This section is dedicated to the detailed analysis of two sediment cores with respect with the occurrence of class 1 integrons and IncP-1 plasmids. The sediment cores were sampled relatively close to each other, at JOSAN and BETH sites, so as to consider the possible impact of the WWTP located in between as well. In parallel, particular attention was given to the molecular methods used, and more precisely to the DNA extraction protocols, that appeared to have a deep impact on the output results, while each protocol provides specific information.

1. DESCRIPTION OF THE SEDIMENT CORES STUDIED

The two sediment cores studied were collected at 4 km apart on the Orne River bank, one from the sampling site Joeuf Sainte Anne (JOSAN), upstream of the WWTP (collected on February 2015), and the second one from BETH site, downstream of the WWTP but upstream of the dam (collected on February 2014)(Figure 19). Both sites are under the influence of the Beth dam regarding the sedimentation of SPM, but with some difference though: JOSAN is at the beginning of the sediment zone while BETH is close to the dam. Once reaching the laboratory after sampling, each core was sliced every 2 or 3 cm under N₂-filled glove bag, while each slice was distributed in aliquots and stored at -80°C. Each team of the MOBISED consortium finally received a set of aliquots to carry out various physicochemical or microbial analyses.

The JOSAN core is 51.5 cm long, its sediment layers are characterized by a fine granulometry (D_{50} , 32 μm) with local zones exhibiting different sand content ($D_{50} > 63 \mu\text{m}$) and accumulations of leaves and other plant debris were observed along the core. Clay mineral predominated its composition, the elements and pollutants related to the iron and steel industry (Fe, Pb, Zn, PAHs) were relatively absent (Table 23) and the core contents vary slightly with depth. On the other hand, the BETH core is 131 cm long and its sediment layers showed a particle size, D_{50} values, ranging from 11 to 34 μm ; no clear zones with defined textures were visually identified in the core (*i.e.* sandy zone), sediments were mostly black and fine. Different concentration profiles (strong variations with depth) were observed according to the determined pollutant content (MOBISED project), but an increase in the concentration of pollutants was observed in the sediment layers from 10 cm depth, including elements such as Fe, Pb, Zn and PAHs (Table 23).

There is a clear contrast in the pollution levels, related to metal and organic compounds between the two sediment cores, suggesting that they have gone through different histories. The dating of BETH core was carried out by the LIEC team, and based on the measures of ¹³⁷Cs and ²¹⁰Pb contents it was determined that BETH sediments have settled during part of the steel-making period, from 1960 to 1988 (Kanbar et al. 2017). Even if the dating of JOSAN

core was not performed, it was assumed that these sediments are “younger” due to the presence of dead leaves in the top of the core, and the lower level of pollutants, reflecting their formation after the end of steel-making period. At this stage, we already hypothesized that these characteristics might influence the occurrence of class 1 integrons and IncP-1 plasmids in the cores by interfering with the persistence of their bacterial host. This was further studied in this section.

Table 23. Range of fluctuation for parameters related to the iron and steel industry in the sediment cores from the Orne River

Parameters	Sediment core (min - max values)	
	JOSAN	BETH
Depth (cm)	51.5	131
Water content (%)	26.5 - 74.3	47 - 73.1
Particle size (D_{50} μm)	32 - 251	11 - 34
Fe_2O_3 (%)	5 - 7	7 - 40
Pb (mg/kg)	15 - 111	68 - 3887
Zn (mg/kg)	263 - 592	348 - 9151
Σ PAHs ($\mu\text{g/g}$) ^A	1.3 - 6.7	17 - 505
Σ O-PACs ($\mu\text{g/g}$) ^B	0 - 0.6	4.9 - 70.5
Σ N-PACs ($\mu\text{g/g}$) ^C	0 - 0.8	0.4 - 2

Σ = sum of the polycyclic aromatic compounds (PACs) based on their global characteristics:

^A PAHs= polycyclic aromatic hydrocarbons

^B O-PACs= oxygenated polycyclic aromatic compounds

^C N-PACs= nitrogen polycyclic aromatic compounds

2. MICRO-RESERVOIRS OF MGES IN SEDIMENT CORES

2.1 Distribution of class 1 integrons in the JOSAN sediment core

Several methods have been developed for extracting the total DNA of microbial communities from different environments; each of them with

advantages and disadvantages in the extraction yield, the purity of the extracted DNA, and also the composition of the bacterial community extracted when considering the susceptibility of the microorganisms to cell lysis method (Daniel 2005). Aiming to evaluate the compartmentalization of MGEs in the Orne River ecosystem, we initially made the technical choice of using the same DNA extraction procedure whatever the compartment considered in order for the different kind of sample to be subject to the same technical biases and thus to keep comparable results. For this reason, the PowerWater® DNA Isolation Kit was used for the DNA extraction of sediment samples, as it was the case for water samples. In brief, for water samples, the DNA extraction starts with the filtration of the sample onto a filter membrane so as to retain bacteria, which are further lysed directly on filters and to proceed with DNA recovery; in the case of the sediment samples from the Orne River, 50 mg fractions of frozen material were resuspended in non-pyrogenic sterile water before proceeding with the protocol as recommended by the manufacturer. We initially thought that, for a better filtration, it would be easier to start with well-dispersed sediment particles and cells. To do so, two dispersing conditions were tested. In the first tested condition, the 50 mg sediment samples were solely resuspended in water by a 30 s vortexing before the filtration step. In the second tested condition, the 30 s vortexing was followed by a 15 min stirring at 160 rpm and 25°C before filtration in case the sole vortexing was not enough. The incidence of the initial dispersion of the sediment was evaluated in two series of DNA extraction from the 25 samples (25 layers) collected in the JOSAN core using each the 2 dispersion methods. The DNA extracts E1 (from method 1) and E2 (from method 2) were further used to quantify 16S rDNA, and class 1 integrons (this part of the work was carried out in collaboration with Lorine Derongs, a Master 2 student from the University of Rennes). As observed in Figure 48, the absolute abundances of class 1 integrons and 16S rDNA present important fluctuations from one layer to another and, importantly, also between extracts from the same layers. The yields of DNA extractions obtained for the E1 series are significantly lower for both markers (Friedman's test, $p=3.10^{-5}$ and $p=3.10^{-5}$). The differences in DNA recovery efficiency were so important that a third series of extraction (E3) was carried out using the "vortex + agitation" dispersion method (method 2). Here again, the absolute abundances (Figure 48 A & 48 B)

appeared variable from one extract to the other, with a difference that could reach up to 2 orders of magnitude. Although tendencies were relatively hard to draw, it seems that DNA extracts collected with the “vortex + agitation” dispersion method tend to globally produce a higher level of 16S rDNA and class I integrons for the same layer. At this stage, we hypothesized that the 15 min agitation may favor the dispersion of cells from aggregates, which then could become more susceptible to cell lysis in the next step compared to aggregates. All in all, this experiment points out a very important variability of the abundances measured from a same layer, even when using the same dispersion protocol.

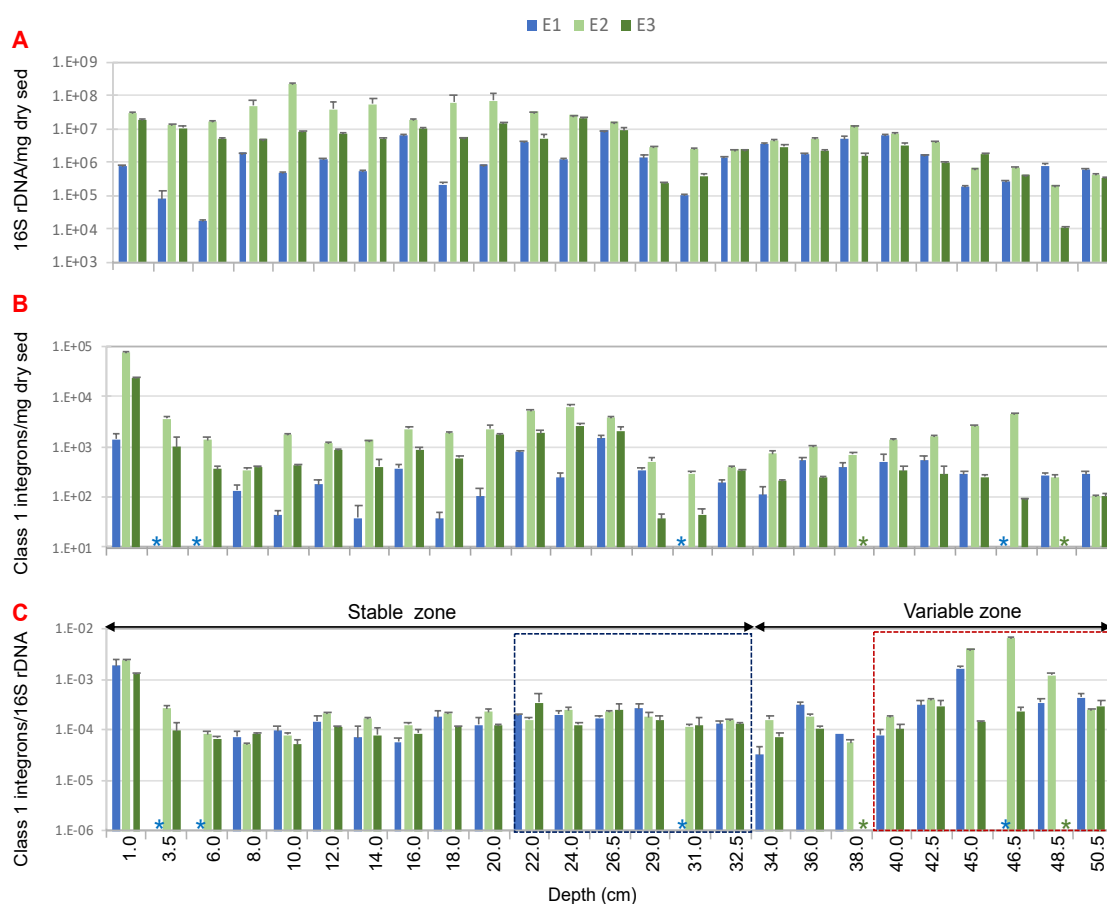


Figure 48. Occurrence of 16S rDNA and class 1 integrons in the sediment layers of JOSAN core. (A) absolute abundances of 16S rDNA and (B) class 1 integrons; (C) relative abundance of class 1 integrons (copies /16S rDNA). Asterisks indicate below detection limit. E1, E2 and E3 correspond to quantification made from DNA extracts obtained with (E2 and E3) or without (E1) a 15 minutes agitation before filtration and DNA extraction. Error bars indicate the standard deviation of the mean values (n=1; technical qPCR triplicate). The squares (dotted lines) delimitate the samples selected for further analysis by DGGE (section 2.2 below).

If the level of particle disaggregation before DNA extraction could alter the yield of DNA extraction, and therefore the amount of marker quantified *in fine*, it should not change the relative abundance of the markers as far as the same community is concerned. This was further explored by analyzing the relative abundance of integrons (absolute abundance of integrons normalized to 16 rDNA abundance). Figure 48 C shows that variations in relative abundances of class 1 integrons in the sediment core are relatively moderate thus confirming partially our hypothesis. However, two zones could be distinguished along the core, denominated “stable” and “variable” zones (with respect with the intra-layer variability between the three repeats). First, a long stable zone could be observed from the top layer to the layer at 32.5 cm depth, where the relative abundances among the three DNA extracts are more or less homogeneous within a given layer and whatever the dispersion method used. Then a variable zone can be observed from 34 to 50.5 cm depth (with some exceptions), with a great variation of relative abundances between the different DNA extracts from a same layer, where local differences of up to two orders of magnitude can be seen (*i.e.* for 45 cm depth). Such fluctuation in the relative abundances between replicates tends to demonstrate that MGE or MGE-carrying bacteria accumulate or persist in discrete “micro-reservoirs” rather than being homogeneously distributed in sediment, at least within a given layer.

2.2 Analysis of the bacterial community structure in the stable and variable zones of JOSAN core

The results described above suggested that disaggregation of bacteria form particles may impact the absolute abundance of the markers 16S rDNA and class 1 integrons, but this effect was less evident when the relative abundance of class 1 integrons was compared (Figure 48). With this respect, the difference in absolute abundances initially observed can be explained by variations in DNA extraction yields from one repetition to another (even if the same protocol was performed) rather than a certain selectivity on the extracted DNAs. However, for the so called “variable zone” we could see that the relative abundance was also variable from one repetition to another, suggesting either

that microbial communities differed among DNA extracts from a same layer or, even if unlikely, that solely the abundance of MGE specifically changed within the conserved community from one extract to another (of the same layer). To evaluate these hypotheses, the bacterial community structures from the stable (samples from depths 22 cm to 32.5 cm; blue square of Figure 48 C) and variable zones (samples from depths 40 cm to 50.5 cm for the variable zone, red square of Figure 48 C) of JOSAN core were independently compared using Denaturing Gradient Gel Electrophoresis (DGGE) on Eubacterial 16S rDNA amplicons. Briefly, 16S rDNA were PCR amplified from the different extracts of 6 layers of the stable zone and 6 layers of the variable zone of the JOSAN core. The amplicons were separated by DGGE and the banding patterns were compared using an “Unweighted Pair Group Method with Arithmetic Mean” (UPGMA) so as to identify the similarities between the samples analyzed.

The dendrogram in Figure 49 A shows the comparative analyses of the bacterial community structures of the stable zone. It could be observed that the community structures from this zone are relatively similar from one extract to another of a same layer (extractions (1), (2) and (3)), and even from one sample to another for sediment layers from different depths, with an overall percentage of similarity greater than 60%. It should be noted, that for the sediment layer at 22.5 cm depth, or for the layer at 29 cm depth, the high community similarities (90 and 80% respectively) observed for DNA extracts obtained with and without agitation prior filtration, tend to confirm that the agitation treatment solely impact the yield of DNA extraction, probably by acting on the level of cell dispersion from aggregates. It seems therefore possible to argue that the agitation preceding the DNA extraction, mostly affects the absolute abundance of the measured markers, but it does not appear to affect the apparent structure of the bacterial communities from which DNA is extracted.

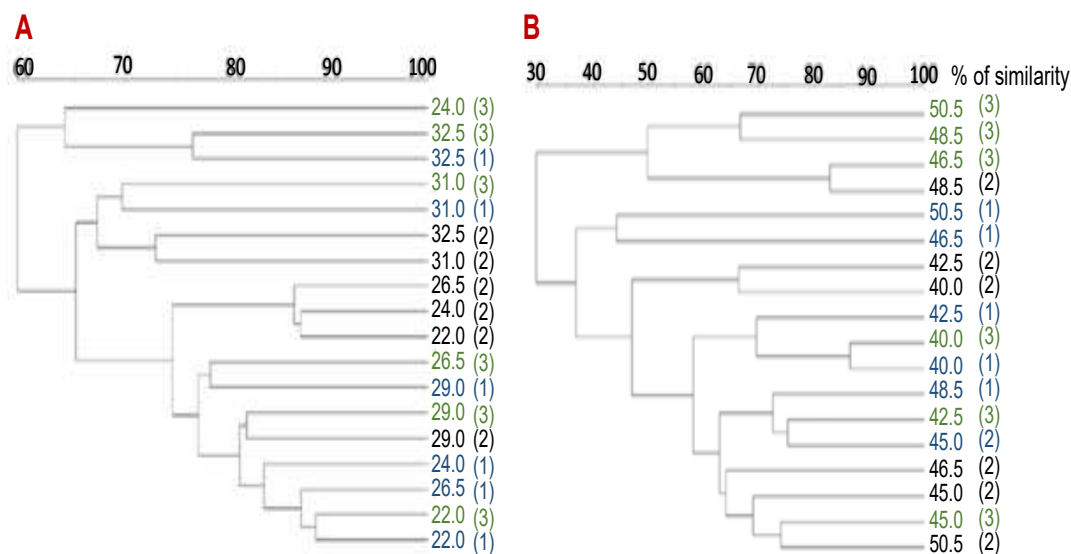


Figure 49. Comparison of bacterial community structures within the stable and the variable zones of JOSAN core based on DGGE profile analyses. (A) Dendrogram for samples of the stable zone (from 22 cm to 32.5 cm depth). (B) Dendrogram for samples of the variable zone (from 40 cm to 50.5 cm depth). The numbers between parenthesis indicate the DNA pre-extraction method: (1) only vortexing, (2) and (3) with vortexing and agitation.

When the bacterial community structures of the variable zone were analyzed, the percentages of similarity between the samples from a same layer were lower than those for the stable zone, with an overall similarity reaching 30% (Figure 49 B). The detailed analysis of the band profiles shows that microbial community structure and the relative abundance of class 1 integrons are not necessarily related. For example, in the extracts of the layer of 40.0 cm depth, the relative abundances in class 1 integrons are similar from one extract to another, with similar community structures for extractions 1 and 3 (similarity close to 85%), but different for the extraction 2 (similarity of 47%). This shows that similar relative abundances in integrons do not necessarily mean similarity of community structures. Conversely, at the 45.0 cm depth layer, noticeable differences in the relative abundances of class 1 integrons were observed between the three extracts (Figure 48 C), but they present similarities in their bacterial community structures, which reached 63 %. In conclusion, this variable zone is particular in the sense that it exhibits a great variability of relative abundances of class 1 integrons among DNA extracts from the same layers.

Second, this variability appeared relatively independent from the bacterial community structure, demonstrating that class 1 integrons can accumulate in very discrete areas to form “micro-reservoirs”, but that the corresponding MGE-bearing bacteria probably represent a minority of the community members, which can explain that related communities may or may not have the same relative abundance of integrons. In any case, such variability represents differences in the evolution paths of microbial communities that occurred at a very small scale in the three dimensions of the core.

2.3 Distribution of MGEs in the BETH sediment core

We have just seen that the distribution of integrons in the JOSAN core was not always homogenous, since defined layers of the core exhibit discrete micro-reservoirs of elevated relative abundance. A question was asked to know whether the existence of micro-reservoirs was particular to the JOSAN core or if it could be observed in other sediment cores as well, and if the distribution of MGEs in sediments could be impacted by anthropic pollution. Considering the fact that JOSAN core has a low level of anthropogenic pollution, it was decided to develop a similar analysis on the BETH core for which elevated levels of PAHs, zinc and lead have been measured in defined layers of the sediment core (Table 23).

Seeking for MGE micro-reservoirs, three series of DNA extractions from the 44 layers of the BETH core were performed in order to assess the fluctuation of the molecular markers along the sediment core. As for JOSAN sediments, the samples were re-suspended in water, then they were vortexed 30 s followed by 15 min stirring to maximize cell disaggregation, before proceeding with DNA extraction using the PowerWater® DNA isolation kit. The different DNA extracts were then subject to qPCR to quantify 16S rDNA, class 1 integrons and IncP-1 plasmids. As before, elevated levels of variation could be observed for absolute abundances of all the markers between repeats of the same layers (Figure 50). Considering our previous observation regarding the

easiness to alter the DNA extraction yield, we preferred focusing on the relative abundance of MGEs at this stage.



Figure 50. Absolute abundances of 16S rDNA, class 1 integrons and IncP-1 plasmids in the different sediment layers of the Beth core. Asterisks indicate absence of values or values below the detection limit. Error bars indicate the standard deviation of the mean values (n=1;technical qPCR triplicate).

The relative abundances of class 1 integrons and IncP-1 plasmids along the BETH core are presented in Figure 51. Unlike JOSAN core, long defined sections of stable zones could not easily be distinguished in BETH core due to the high fluctuation in the relative abundance of MGEs from one extract to another, which makes appear the cores as a “variable zone” from top to bottom. For the relative abundance of integrons, only a few layers may appear to form small “stable zones” while incP-1 plasmids fluctuate all along the core. Interestingly, localized increases at depths of 81.5 cm, 105.5 cm, 126.5 cm and

129.5 cm can be identified for both MGEs, which may suggest that certain depths contain richer MGE micro-reservoirs. IncP-1 plasmids are particular, they were always detected on the first 27 cm of the core, then depending on the extraction series, their detection varied without a clear pattern (in many cases they were below the detection limit), thus limiting further analysis.

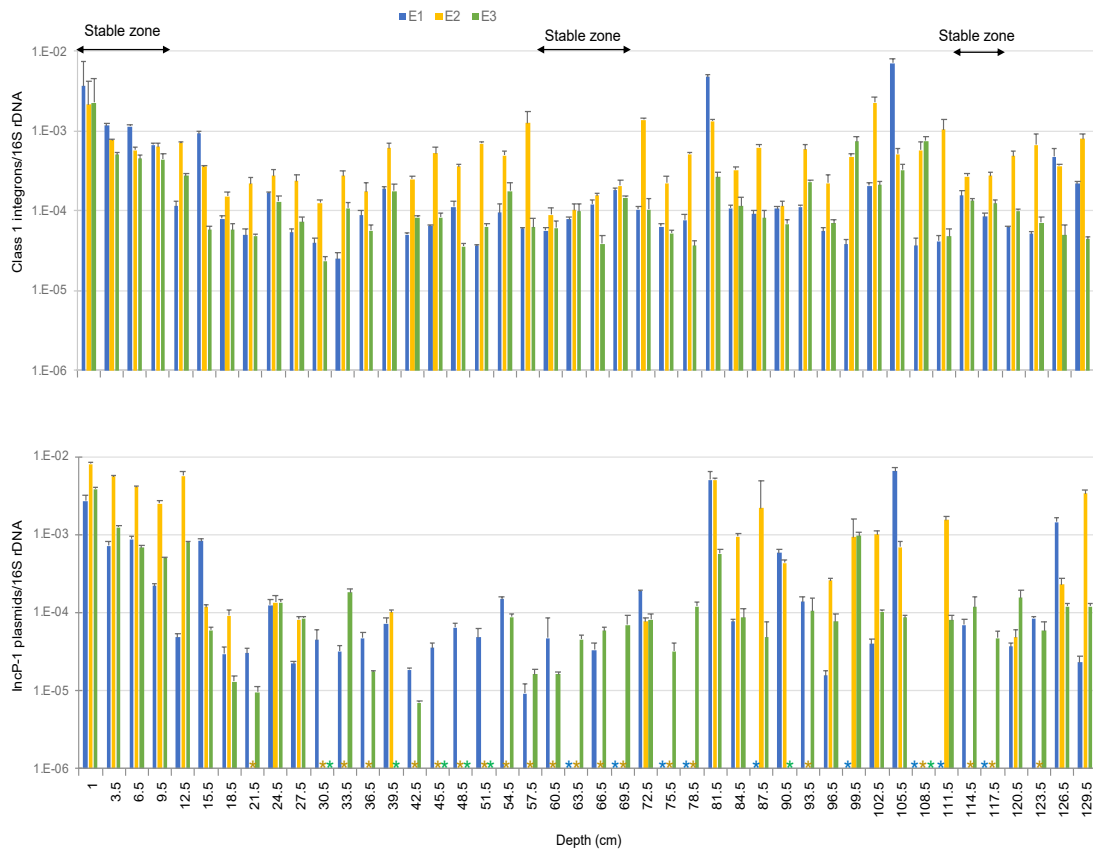


Figure 51. Relative abundances of class 1 integrons and IncP-1 plasmids in the sediment layers of the BETH core. Each color represents an extraction series (E1, E2 and E3). Asterisk indicate absence of values or values below the detection limit. Error bars indicate the standard deviation of the mean values (n=1; technical qPCR triplicate).

Although distributed differently, the analysis of BETH core confirms that the existence of micro-reservoirs was not a particular case of JOSAN. In both sediment cores, we observed that the MGE relative abundance on the top sediment layers is more or less homogenous compared to the rest of the core, this led us to hypothesize that MGE micro-reservoirs could result from the differential evolution and/or adaptation of bacterial community to the sediment

characteristics (physical and chemical), at a very small-scale level. On the other hand, it is interesting to note that the JOSAN and the BETH cores differ by the extent of the stable zone in the first core and the extent of the variable zone in the second one, as far as the relative abundance in class1 integrons is concerned. The reason for such variability (or not) is not known but, considering the fact that the BETH core displayed elevated levels of pollutants, we thought it would be interesting to establish whether these pollutants participate in promoting variability in the relative abundance of class 1 integrons and the integron-bearing bacteria.

2.4 Influence of geochemical parameters in the variability of class 1 integrons relative abundance in BETH core

Several parameters could influence the occurrence of MGEs in environmental systems, and the correlation between metal concentrations and the occurrence of antibiotic resistance genes has been highlighted in several works (Baker-Austin et al. 2006; Knapp et al. 2017b; Seiler and Berendonk 2012; Berkner, Konradi, and Schonfeld 2014). Considering the fact that ARGs are often associated to MGEs, we hypothesized that pollutants could have an influence on the occurrence of MGEs as well. This was further explored with statistical analyses aiming at establishing possible relationships between global parameters, elemental and metal content (including major, trace, and rare earth elements) with the relative abundance of class 1 integrons and more importantly to its evolution/variability in the BETH core. With this respect, when the coefficient of variation of the relative abundance of integrons was plotted as a function of depth, two successive zones of elevated fluctuation could be delimited from 12.5 to 57.5 and 69.5 to 1295 cm (Figure 52). Interestingly these also roughly correspond to zones of elevated and fluctuating concentration of zinc and lead (Figure 52). This observation led us to use the coefficient of variation of the relative abundance as a parameter by itself to identify its correlation with other geochemical/global parameters.

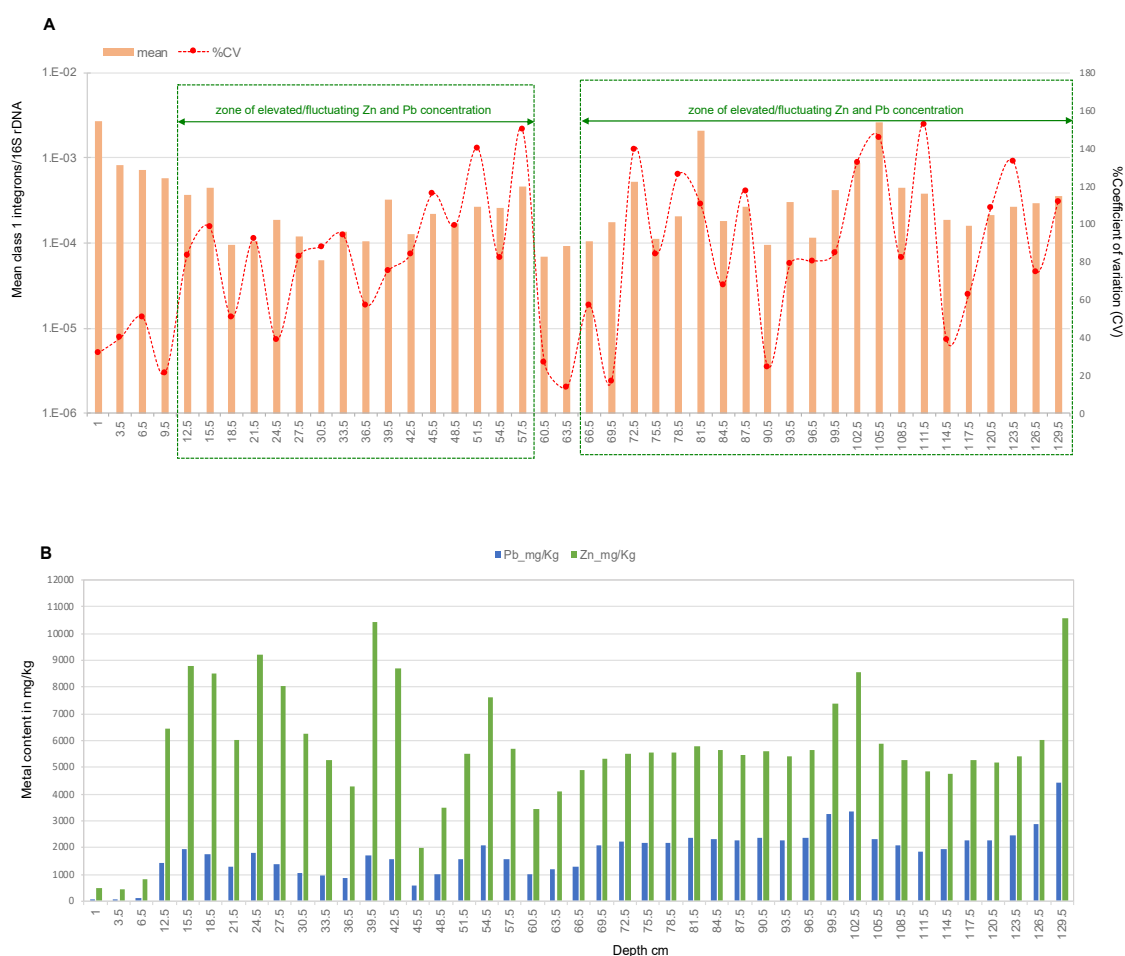


Figure 52. Comparison of class 1 integron relative abundance to zinc and lead contents in the sediment layers of the Beth core. (A.) Mean values of the relative abundances of class 1 integrons. Fluctuations observed between repeats of DNA extractions from a same layer are given by the coefficient of variation (CV). (B.) Zinc and lead contents. The squares (dotted lines) delimitate zones of elevated fluctuation of zinc and lead content in the sediment samples.

We first explored the existence of possible relationships between the geochemical parameters and the coefficient of variation of the class 1 integron relative abundances, using a classical PCA. Nevertheless, even if there are no restrictions in using an elevated number of parameters (variables), the more variables are included the more difficult it can be to interpret the principal components (Jolliffe and Cadima 2016). Then, in order to reduce the complexity of the PCA, a selection of variables was performed based on the availability of data, and also based on their significance related to the principal sources of pollution of the Orne River such as iron, zinc, lead, and physical parameters such as water content and depth. The resulting PCA is presented in Figure 53.

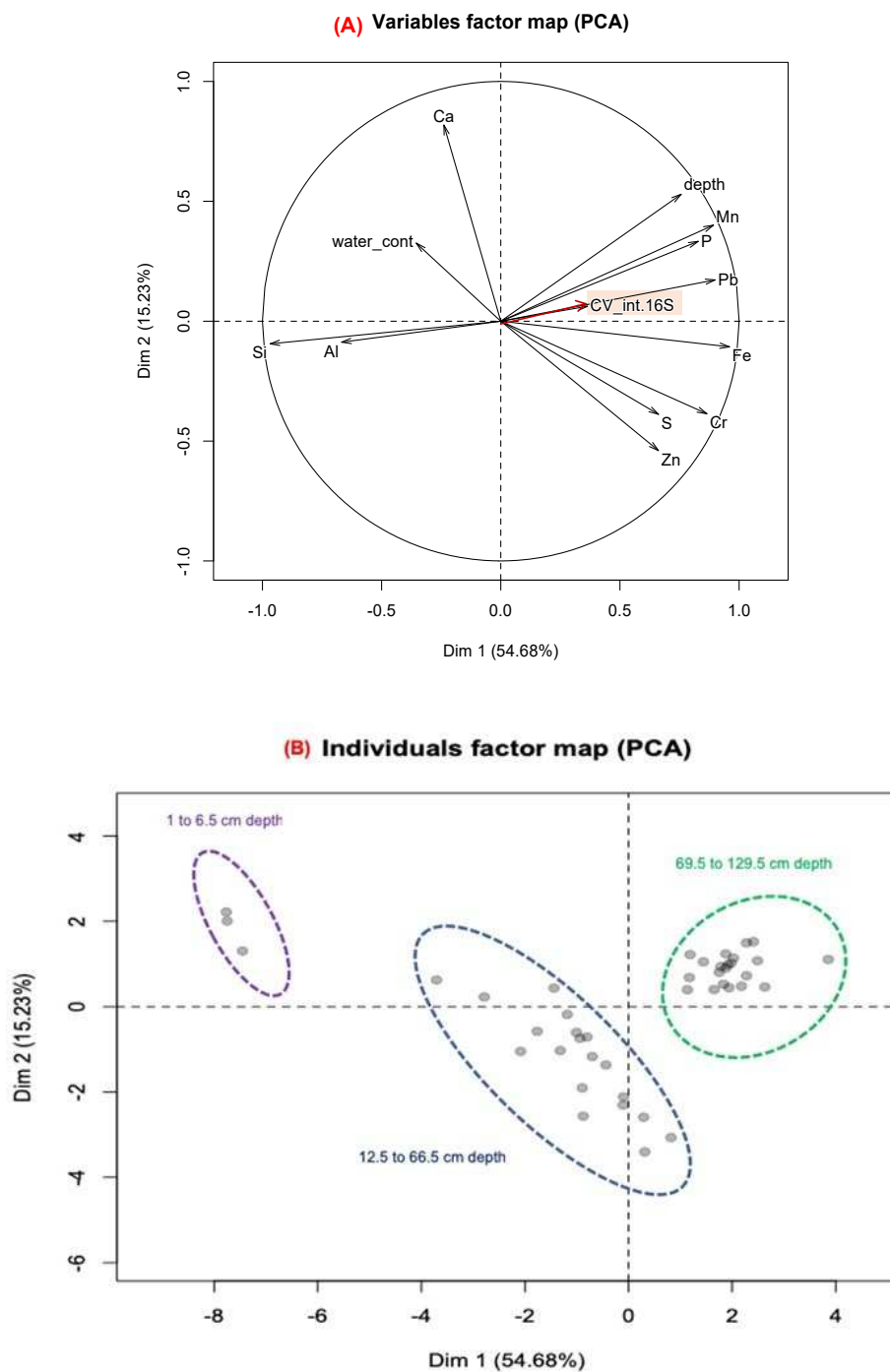


Figure 53. Principal component analysis combining 13 parameters from 43 sediment samples of the BETH core. Variables: coefficient of variation between replicates for the integron relative abundances (CV_int/16S), depth, water content % (water_cont), Fe (%), Pb (mg/Kg), Zn (mg/Kg), Ca (%), P (%), S (%), Si (%), Al (%), Mn (%) and Cr (mg/Kg). Three main clusters were identified corresponding to the top, middle and bottom layers of the core (purple, blue and green circles, respectively).

In the variables factor map (A), we can observe that, if several parameters clearly evolve in the same dimension, the coefficient of variation, here taken as an independent variable, is not well represented in the two dimensions map. Moreover, there is no apparent correlation between the variability of the relative abundance of integrons (%CV) and the selected variables used for the PCA. On the other hand, in the individuals factor map (B), three main clusters can be identified according to the depth of sediment layers, top, middle and bottom of the core, meaning that the history of the sediment, or at least the period represented by the different depths of the core, is an important parameter for sample clusterization. Interestingly, these clusters also roughly correspond to the different zones of variability observed for the integron relative abundance even if no correlation could be established (Figure 52). Any correlation with the level of pollution in the sediment remains to be determined.

At this stage, the statistical analyses of MGE abundances appeared compromised by several technical difficulties. First, the absolute abundances could not be used as they are biased by the variable yield of DNA extraction, influenced by what we believed to be the initial dispersion of the sample in water. Second, as seen with the JOSAN core analysis, the relative abundance can be used to report on the proportion of MGEs in the community but, on the other hand, these relative abundances may appear quite variable between replicates for some layers (variable zones), which probably reflect the local variation of the community. This variation could not be correlated to any evident geochemical parameter. However, it should be reminded that most geochemical parameters were assessed from 250 to 500 mg sediment extract, which should average most of the values obtained. In other words, if using the PowerWater® DNA extraction kit and 50 mg of sediments was efficient to point out the existence of micro-reservoirs of MGEs and/or differential evolution of communities in a small scale, it is plausible that it remains limited to establish a correlation with the corresponding local geochemistry that has not been evaluated from the same amount of sediment sample.

3. INCREASING SEDIMENT SAMPLE QUANTITY FOR MINIMIZING THE IMPACT OF EXTRACTION BIASES IN DETERMINING STATISTICAL CORRELATIONS WITH GEOCHEMICAL PARAMETERS

Aiming at minimizing the biases associated to DNA extraction from the small sample sized (section 2, results part III), it was decided to balance the quantity of sample used for DNA extraction as the one used for the analyses of geochemical parameters. DNA extraction from a higher amount of sample using the PowerWater® DNA Isolation Kit was technically not be possible. Actually, using this DNA extraction kit implies the filtration of resuspended material in water, for which the filter membrane gets quickly saturated/clogged passed 50 mg of sediments. To avoid this limitation, it was decided to use the DNeasy PowerSoil® DNA isolation Kit from the same manufacturer, that allows the extraction of DNA from 250 mg of sediment sample by direct lysis (no filtration step). Before performing any statistical analysis, we decided to compare the two extraction kits (PowerWater® and PowerSoil®) in terms of (i) DNA yield and purity (ii) variations of MGE content between replicates and (iii) bacterial community structures extracted. This part of the work was carried out in collaboration with Arthur Royer, a Master 2 student from the University of Lorraine.

3.1 Comparison of DNA extraction kit performances

Considering the limited amount of sediment sample available, the community DNAs from the 44 layers of the BETH core were solely extracted twice (from 250 mg each) using the PowerSoil® kit and compared to those obtained previously in triplicate for the same layers using the PowerWater® kit (extraction from 50 mg each). For each series, the DNA extraction yields were calculated in ng of DNA/mg of dry sediment (ng/mg). Figure 54 shows the contrasting distribution of the DNA concentrations obtained for all the layer samples and for both extraction kits. Globally, the distribution of the DNA concentration obtained with the PowerSoil® kit seems to be more homogenous with the exception of some outliers corresponding to top layers of the sediment

core that are otherwise known to be richer in bacteria (fresh deposit). On the other hand, the PowerSoil® kit also appears to produce lower yields in DNA extraction compared to what was obtained with the PowerWater® kit; median values were determined at 5.7 ng/mg and 16.2 ng/mg respectively. The significance of this contrasting distribution was verified by the Student's T-test for paired data, that confirm a significant difference between the DNA extraction yields from both DNA isolation kits ($p = 6.4 \times 10^{-13}$).

The purity of the DNA extracts was analyzed spectrophotometrically, and the ratio A_{260}/A_{280} was compared between the two DNA extraction kits to report on the DNA purity. No matter the extraction kit used, the DNA purity appeared homogenously distributed with mean values of 1.62 and 1.42 for the PowerSoil® kit and the PowerWater® kit, respectively; however, no clear pattern of high or low DNA purity along the core was identified as represented by the disperse distribution in the boxplot of Figure 55.

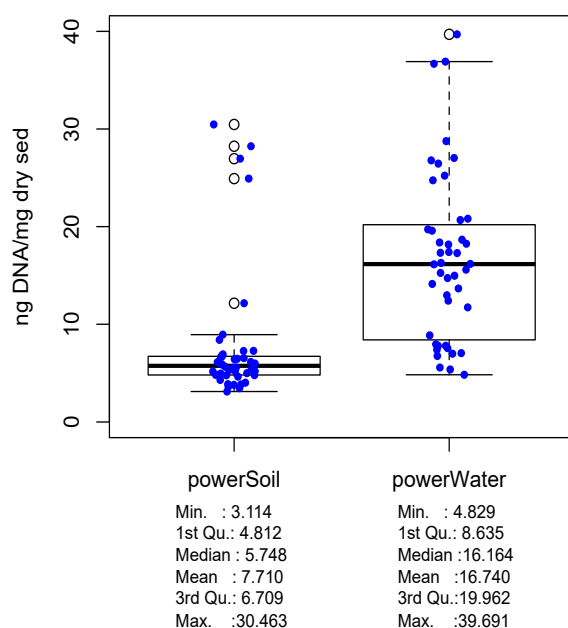


Figure 54. Distribution of DNA extraction yields (in ng of DNA/mg of dry sediment) obtained for the series of extraction the PowerWater® kit and the DNeasy PowerSoil® kit, respectively. Blue points correspond to the mean values of DNA extraction yields from triplicates and duplicates for defined layers, obtained with the PowerWater and the PowerSoil kits, respectively.

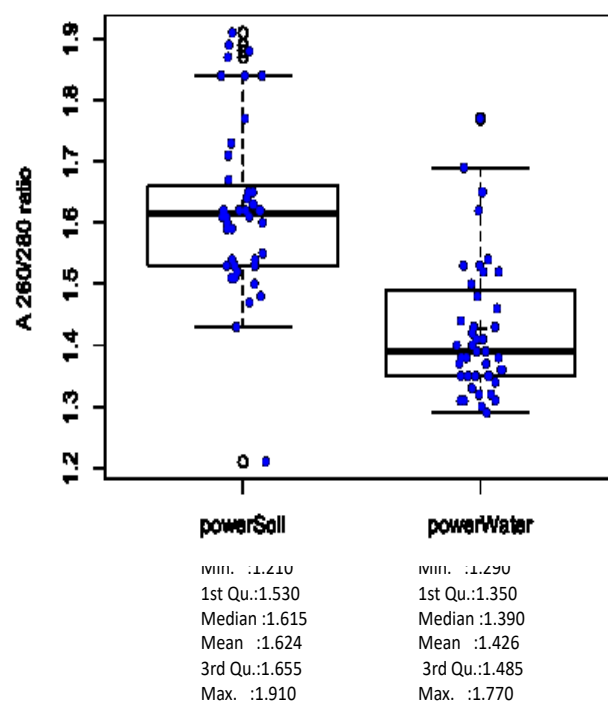


Figure 55. Comparison of the DNA quality, reported as the ratio A_{260}/A_{280} , obtained with the DNA extraction kits, PowerWater® kit and PowerSoil® kit, the blue points correspond to the mean values from triplicates and duplicates DNA extracts from defined layers, obtained with the PowerWater and the PowerSoil kits, respectively.

3.2 Variability of MGE abundances between replicates of DNA extracts from PowerWater® kit and PowerSoil® kit

Following with the comparison of the DNA extraction kits performances, the two new series of DNA extracts obtained with the PowerSoil® kit were used for the quantification of 16S rDNA, class 1 integrons, and IncP-1 plasmids, before being compared with the abundances obtained with the PowerWater® kit. As mentioned before, the quantification of 16S rDNA was performed first with three main objectives: to serve as an indicator of the global bacterial population, for the normalization of class 1 integrons and IncP-1 plasmids, and to assess the absence of inhibitors in the DNA extracts by comparing amplification profiles (qPCR) from serially diluted DNA. Regarding this last point, when the DNA extracts from the PowerWater® kit were assessed, apart from the first sediment

layers (up to 11 cm depth) with high DNA concentrations, DNA dilutions of 1:10 was adequate for further qPCR quantifications as the absence of inhibitors was verified. Conversely, at dilution 1:10, qPCR inhibitors were still detected in DNA extracts from the PowerSoil® kit, and it was necessary to use 1:50 dilutions for further quantifications. Thus, even if the A_{260}/A_{280} ratios were slightly better with the DNA extracts obtained using the PowerSoil® kit, their amplification were not as good as those obtained with the PowerWater® kit. Once the absence of inhibitors verified, DNA extracts were further used for class 1 integron and IncP1 plasmid quantification. The latter marker turned out to be mostly below detection level for a reason that we attributed to its lower abundance and, concomitantly, to the higher dilution level applied to the DNA extracts from PowerSoil® kit for the qPCR to work properly. Focusing on integrons, the relative abundance (*int1*/16S rDNA) were calculated and further compared to what was obtained for DNA extracts from the PowerWater® kit (Figure 56). Whatever the DNA extraction kit used, the variations between their minimum and maximum values are in the same range, 4.1×10^{-5} - 3.8×10^{-3} and 6.3×10^{-5} - 2.7×10^{-3} class 1 integrons/16S rDNA for the PowerSoil® kit and the PowerWater® kit, respectively. In addition, the mean relative abundances as a function of depth roughly follow the same evolution regardless of the kit used. However, it is worth noticing that the coefficients of variation obtained for the series of relative abundances from PowerWater® kit were significantly higher than those obtained from the PowerSoil® kit (Wilcoxon signed-rank test, $p=4.4 \times 10^{-5}$), thus confirming a better reproducibility of the quantification among the extraction replicates for the latter kit.

In summary, we previously show that using the PowerWater® DNA extraction kit appeared relatively interesting to report on micro-reservoirs of community with variable contents in MGEs but, in the meantime, too variable to authorized a proper statistical analysis. This problem was apparently solved with the PowerSoil® DNA extraction kit, which proposed a better reproducibility thanks to extracts from bigger sample (250 mg), therefore authorizing seeking correlations between relative abundances of MGEs and geochemical parameters quantified from the same sample size.



Figure 56. Comparison of the mean relative abundances of class 1 integrons in DNA extracts from (A) the PowerWater® kit and (B) the PowerSoil® kit and their corresponding coefficients of variation.

3.3 Significance of the variability of MGE abundances in sediment

The distribution of relative abundances in class 1 integrons as a function of depth displays two levels of heterogeneity, a mean relative abundance that varies from layer to layer, and a relative abundance that varies within layer (or not). At this stage, we wondered whether the variability of relative abundances observed within a sediment layer was resulting from a divergence of microbial community structure or if, somehow, it was resulting from a differential genetic dissemination of integrons within homogenous community. These hypotheses were explored by evaluating the extent of the divergence of the community structured within layer and comparing it to the extent of the variation in integron content within the same sediment layers. When this experiment was initiated, it

was expected to show that the more the community structures differ between repeats (within layers), the more the integron content varies too. The approach was implemented for a selection of 15 sediment layers, and repeated for each of the DNA extraction series obtained with the PowerWater® and the PowerSoil® kits, respectively.

The divergence of community structures was evaluated by DGGE on 16 rDNA amplicons as previously described for the JOSAN core (see section 2.2 of this chapter). For each of the DNA extraction method used, DGGE profiles were compared layer by layer. The dendrograms resulting from the DGGE analyses (using the UPGMA method) allowed obtaining the percentage of similarity between community profiles (between DGGE profile) of the DNA extracts from the same sediment layer, thus reflecting the proximity of microbial community structures. On the other hand, an estimation of the fluctuation in class 1 integron content was provided by the coefficient of variation (CV; expressed as a percentage) of the relative abundances between replicates within each sediment layer.

For each DNA extraction kit, the percentage of similarity between the microbial communities of replicates from a same sediment layer was plotted against the corresponding CV for relative abundances obtained from the DNA extracts (Figure 57). Against all odds, we could not observe an increasing divergence of class 1 integron content concomitantly to decreasing similarity of community structures, as anticipated. To the contrary, we noticed, that the variation of integron content between the replicates (CV) from the same kit and the same sediment layer, was not correlated with the percentage of similarity of its bacterial community structure, which remains relatively stable from one layer to another. The same observation could be done whatever the DNA extraction kit used. However, the level of similarity within community structures is higher among replicates obtained with the DNA extracts from the PowerSoil® kit ($73.9 \pm 8.7\%$) than for those obtained with the PowerWater® kit ($57.8 \pm 10.8\%$). All in all, these observations demonstrated that (i) as expected, DNA extraction using the PowerSoil® kit clearly allows the recovering of average community DNA which presents less variation in community structure among repeats, (ii) the fluctuation of class 1 integrons is not linked to the similarity level of the

community structure. The latter could be explained by the fact that class 1 integron-bearing bacteria are probably minor members of the community for which the presence or absence do not significantly affect the structure of the community.

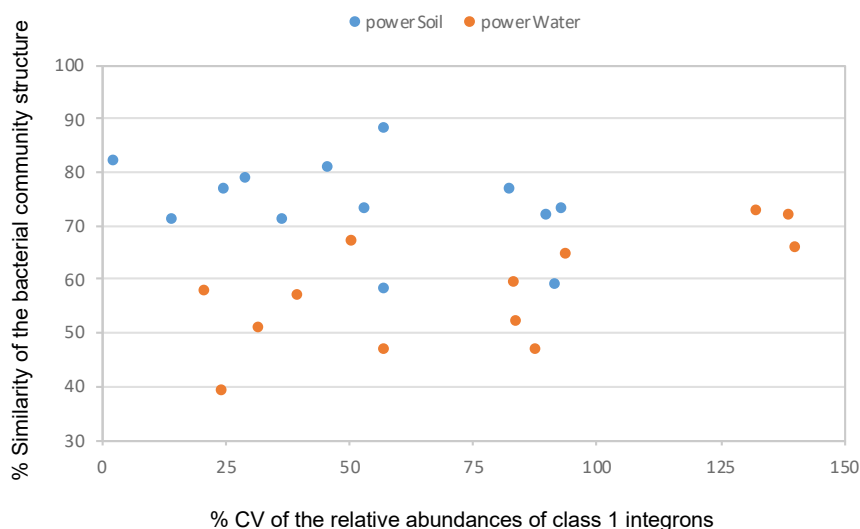


Figure 57. Relationship between the coefficient of variation (%CV) of the relative abundance of class 1 integrons and the percentage of similarity of the bacterial community structure, obtained from PowerSoil® DNA extraction kit and PowerWater® DNA extraction kit, respectively.

4. COMPARTMENTALIZATION OF CLASS I INTEGRONS AND ANTHROPIC POLLUTION

In Part III (section 2.4) we discussed our interest in studying the correlation between global parameters, elemental and metal content (including major, trace, and rare earth elements) and polycyclic aromatic compounds, and the absolute and relative abundances of class 1 integrons. We mentioned before that possible biases could arise as the amounts of sediment sample initially used for DNA extraction were lower than those used for the geochemical analyses. With the DNA extracts made using the PowerSoil® DNA extraction kit, attempting such correlation became possible since DNA was extracted from the same amount of sediment (approximately 250 mg). For correlating absolute and relative abundances of class 1 integrons to the sediment geochemistry, we

first used a classical PCA approach, but it was not very informative and solely applies to linear correlations. For this reason, it was decided to explore the correlation of the class 1 integron abundances to the sediment geochemistry using the Spearman's rank correlation test. Globally, 21 samples from different sediment layers along the BETH core were used to perform the statistical correlation test with global and elemental analysis, on the other hand, just 15 samples were used when polycyclic aromatic compounds (PACs) were considered (limited by the availability of geochemical data). Significant correlations, positives and negatives, for global and elemental parameters can be found in Table 24. Positive correlations between major elements K, Na, Ti, light lanthanides (La, Ce, Nd, and Pr) and 16S rDNA with class 1 integron absolute abundance were found. These major elements and light lanthanides are the main components of the mineral particles present in the sediment core, and their presence suggested mineral particles to be originated from soil weathering in the watershed (Kanbar et al. 2017). On the other hand, trace elements such as Pb, which is not "trace" element considering its high concentration in the Beth core, were negatively correlated with both the absolute abundances of integron and 16s rDNA. The negative correlations with lead could suggest a global toxicity of this compound against bacteria including class 1 integron-bearing bacteria. The same applies for iron and vanadium, two well represented elements in the Orne River sediments as a consequence of former industrial activities and coke production. Other correlations were found but they apparently are not meaningful, in the sense of anthropogenic signatures for the Orne River, and considering that many of the major, trace and rare elements are commonly found in all the environments.

For both Zn/Pb and HAPs, the sediment core seems to be heavily contaminated apart from the top layers of fresh deposit. Below, the two kinds of contaminants fluctuate according to the depth but not necessarily in a parallel way (Figure 52, Figure 58). The level of PAHs is relatively low for the top layers and increase to reach maximum between 12.5 cm to 36.5 cm depth, after what HAP remains at an intermediate concentration (Figure 58). From the 31 PACs considered for statistical analysis, no significant correlations between those organic compounds with either the 16S rDNA or the class 1 integron absolute

abundances were found, apart from the negative ones with benzoanthracenedione (BAdione) (Table 24). Conversely, several negative correlations were found between the relative abundances of class 1 integrons and PCAs (Table 24). Thus, five polycyclic aromatic hydrocarbons considered as priority pollutants by the EPA (Flt, BbF, BkF, IP, and Bghi) from the 16 quantified; and five oxygenated PACs (Flone, Aone, MAone, BAdione, and Ndione) from the 11 quantified negatively correlate with *int11* relative abundance.

Table 24. Significant correlations between molecular markers and global parameters, metal content and polycyclic aromatic compounds

Marker	Positive correlations	Negative correlations
16S rDNA	Particle size (D ₅₀) ^{***} , Ba ^{**} , Ce [*] , Hf ^{***} , La ^{***} , Nb ^{***} , Nd ^{**} , Pr ^{**} , Rb ^{***} , Ta ^{***} , Zr, Si ^{***} , Al ^{**} , Na ^{***} , K ^{***} , Ti ^{***}	Depth ^{**} , As ^{**} , Be ^{***} , Cd ^{***} , Dy [*] , Er [*] , Eu ^{**} , Ga [*] , Ho [*] , Lu [*] , Pb ^{***} , Sc ^{**} , Sb [*] , Sm [*] , Sn ^{***} , Th [*] , Tm ^{**} , V ^{**} , W [*] , Yb ^{**} , Fe ^{**} , Mn ^{***} , Mg ^{**} , P ^{**}
Class 1 integrons	Particle size (D ₅₀) ^{***} , Ba [*] , Hf ^{**} , La [*] , Nb ^{**} , Nd [*] , Pr [*] , Rb ^{**} , Ta ^{**} , Zr [*] , Si ^{**} , Na ^{**} , K ^{**} , Ti [*]	Depth ^{**} , As ^{**} , Be ^{**} , Cd ^{**} , Eu [*] , Ga [*] , Lu [*] , Pb ^{**} , Sc ^{**} , Sn ^{**} , Tm ^{**} , V ^{**} , W [*] , Yb ^{**} , Fe [*] , Mn ^{**} , Mg [*] , P ^{**} , BAdione [*]
Class 1 integrons/16S rDNA	Hf [*] , Nb [*] , Zr [*] , Na [*] , K [*]	As [*] , V [*] , Mn [*] , P [*] , ^A Flt [*] , BbF [*] , BkF [*] , Bghi [*] , IP [*] , Flone [*] , Aone [*] , MAone [*] , BAdione [*] Ndione ^{**}

^AFluoranthene (Flt), Benzo[b]fluoranthene (BbF), Benzo[k]fluoranthene (BkF), Benzo[g,h,i]perylene (Bghi), Indeno[1,2,3-c,d]pyrene (IP), 9H-fluorenone (Flone), Anthraquinone (Aone)^{*}, Methylanthracene-9,10-dione (MAone), Benzoanthracenedione (BAdione), Naphtacene-5,12-dione (Ndione).

Significance of the correlations determined using the Spearman's rank correlation test: $P \leq 0.05$, *; $P \leq 0.01$, **; $P \leq 1 \times 10^{-3}$, ***

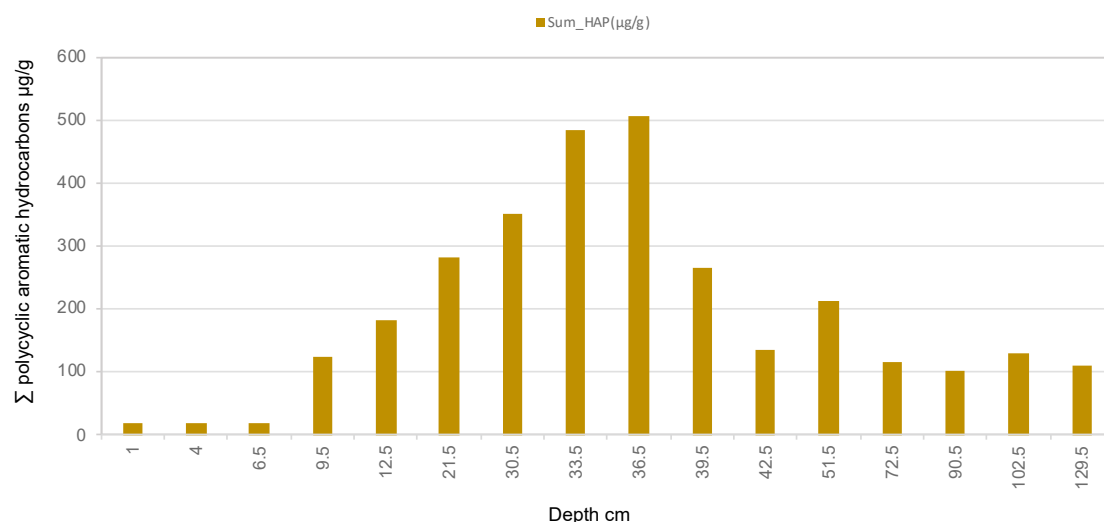


Figure 58. Total content in polycyclic aromatic hydrocarbons along the BETH core.

We hypothesized several scenarios that may explain the highlighted correlations. First, the negative correlations could be due to the toxicity of the compounds, and it would be reflected in a decrease of the abundance of both the global bacterial population and class 1 integrons-bearing bacteria, as we observed for lead (Figure 59). Second, when a pollutant could have a specific effect on the proportion of MGE-bearing bacteria, a shift in the bacterial community would be observed, reflecting a significant correlation (positive or negative) between the pollutant and the relative abundance of class 1 integrons, as the observed for the PAC benzoanthracenedione (Figure 59).

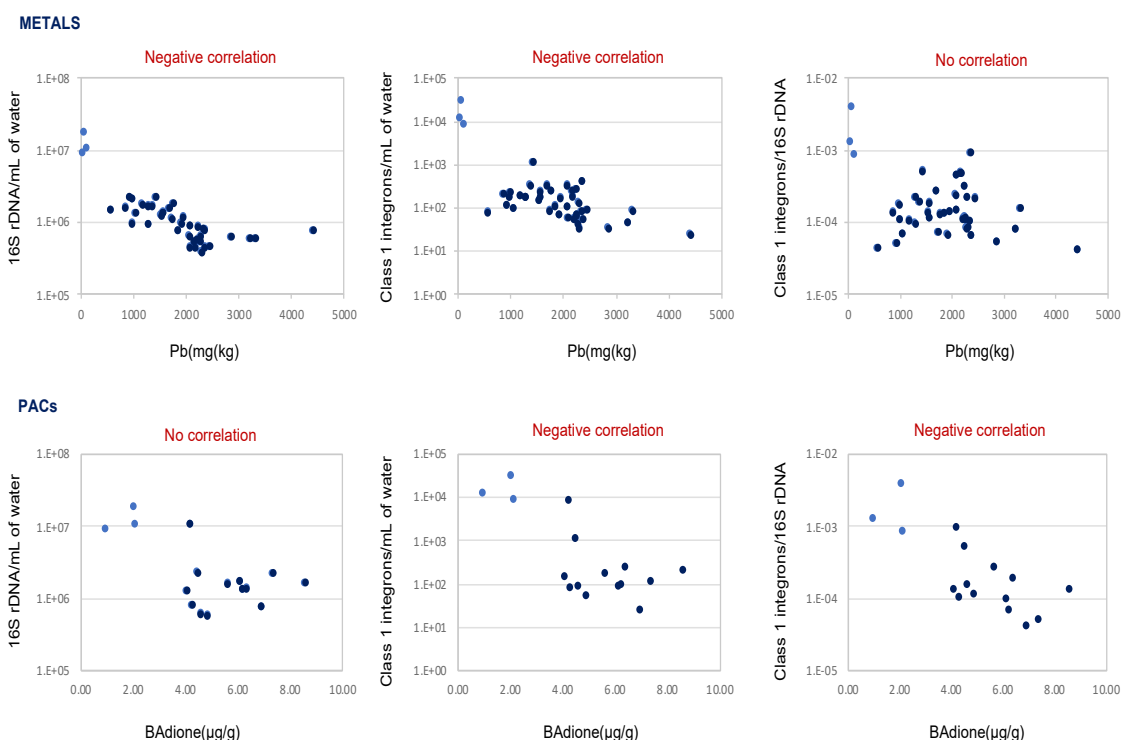


Figure 59. Example of significant correlations (spearman's rank correlation test, $p < 0.05$). between geochemical parameters and molecular markers, absolute and relative abundances. Points in light blue corresponds to the first layers of the sediment core where the level of pollutants is lower than the deeper layers.

5. CONCLUDING REMARKS

This section was dedicated to the analysis of two sediment cores so as to characterize the “evolution” of MGEs distribution as a function of depth and sediment geochemistry. The absolute abundance of 16S rDNA and the relative abundance of MGEs tend to decline quickly after a few centimeters below the recently deposited sediments. Nevertheless, very localized and significant increases (up to two orders of magnitude) in MGE relative abundance could be observed in discrete areas where MGE-bearing bacteria seem to form micro-reservoirs. The presence of these micro-reservoirs was not easy to correlate with the presence of pollutants as they also reflect some local heterogeneity in MGE content. However, with the analysis of “average” samples (extracts from a bigger quantity of sediment), negative correlations were found between the

relative abundance of class 1 integrons and pollutants such as Pb and PAHs. At this stage, it is tempting to propose that bacterial communities reach the sediment compartment with SPM, and evolve differently over the time in function of the presence and the level of pollutants (in addition to the overall characteristics of the sediment). The presence of pollutant may result in a decrease of bacteria (toxic effects) or shift of the bacterial community structure (adaptation/evolution). We also thought about comparing surface sediments between the two cores in order to see whether the WWTP located in between the two sampling sites could have an effect on the accumulation of MGE in the downstream sediments (through SPM). Actually, both surface sediments exhibit relatively similar relative abundances of both MGEs, thus ruling out an evident quantitative effect. With this respect, to go further with the analysis regarding the impact of the WWTP on the sediment compartment, it could be interesting to compare, the gene cassette content of the class 1 integrons in the two cores to differentiate the origin of this MGE quantified (clinical vs environmental).

DISCUSSION/ PERSPECTIVES

DISCUSSION/ PERSPECTIVES

1. MONITORING THE DISSEMINATION OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

The main objective of this work was to participate in a global effort to understand the fate of antibiotic resistance genes once reaching the environmental compartments. It is understood that the quality of our environment is interconnected with the quality of human and veterinary health (the One health concept). In this context, understanding where ARGs/ARB accumulate in the environment (reservoirs) and what are the factors affecting their maintenance in “natural ecosystems” is of prime importance to fight back the increasing difficulties associated to the emergence and dissemination of ARB/ARGs (Pruden et al. 2013; Martinez 2008).

Anthropogenically impacted environments are often described as hot spots for the dissemination of antibiotic resistance (Berendonk et al. 2015). There are probably several reasons for that, first because they are the receiving environment of human waste including ARB and ARGs (e.g. WWTP effluents; Marti, Variatza, and Balcazar 2014), and second because they come with other pollutants that may favor the persistence of ARGs/ARB (e.g. co-selection of resistances by metals; Seiler and Berendonk 2012). For these reasons, this work was focused on a river ecosystem, the Orne River, that has been impacted by past and present anthropogenic pressures, with the idea of establishing relationships (or not) between the fate of ARGs/ARB and these anthropogenic pressures. The experimental strategy we proposed was clearly based on a molecular ecology approach.

Studying the abundance of ARGs in the environment is not as easy as it seems considering the large variety of ARGs that are putatively released with human waste in a river ecosystem. Instead of monitoring a large list of clinically relevant ARGs, more than 373 according to the Antibiotic Resistance Genes Database (ARDB, 2018), we decided to focus on two recognized proxies based on their association with ARGs, namely class 1 integrons (with the *int11* marker) and IncP-1 plasmid (with the *trfA* marker). Because both are MGEs, their

quantifications could also serve as an indicator of the capacity of bacterial communities to transfer/accumulate ARGs.

Obviously, ARG abundances are important markers to monitor and to evaluate the global fluxes of genes in different environmental compartments and this work also give us the opportunity to explore the occurrence of selected ARGs. This selection was based on previous studies of the COST (The European Cooperation in Science and Technology) Action DARE group (Detecting Evolutionary Hotspots of Antibiotic Resistance in Europe, TD 0803). The main criteria for this selection were: clinical relevance, their prevalence in the environment and their association with MGEs. Thus, we quantified six ARGs in samples from the Orne River ecosystem (including raw water, WWTP effluents and sediments). We noticed that all the ARGs quantified, but *vanA* in the river water, was detected in the different kind of samples. Additionally, apart from *sul1*, they were poorly correlated with class 1 integrons or do not fluctuate in the same way (e.g. when the relative abundance of *bla*_{TEM} decrease, the relative abundance of class 1 integrons increase) (Figure 60). However, the frequent choice of class 1 integron for being used as a proxy is based on the fact that this MGE usually carry 1-4 of the more than 130 different resistance gene cassettes described until now (Partridge et al. 2009; Stalder et al. 2012). These apparently different abundances/maintenances of class 1 integrons and many ARGs raise the question of the relevance of the usage of this sole MGE as a proxy for all ARGs. As we demonstrated in this work that class 1 integrons and IncP-1 plasmids behave differently, we could suggest to enlarge the number of MGEs to be monitored as proxies and could propose IncP-1 plasmids but also other widespread MGEs such as IncA/C or IncF plasmids (Partridge et al. 2018) as the members of a panel of indicator MGEs for rendering about the complexity of antibiotic resistance in the environment.

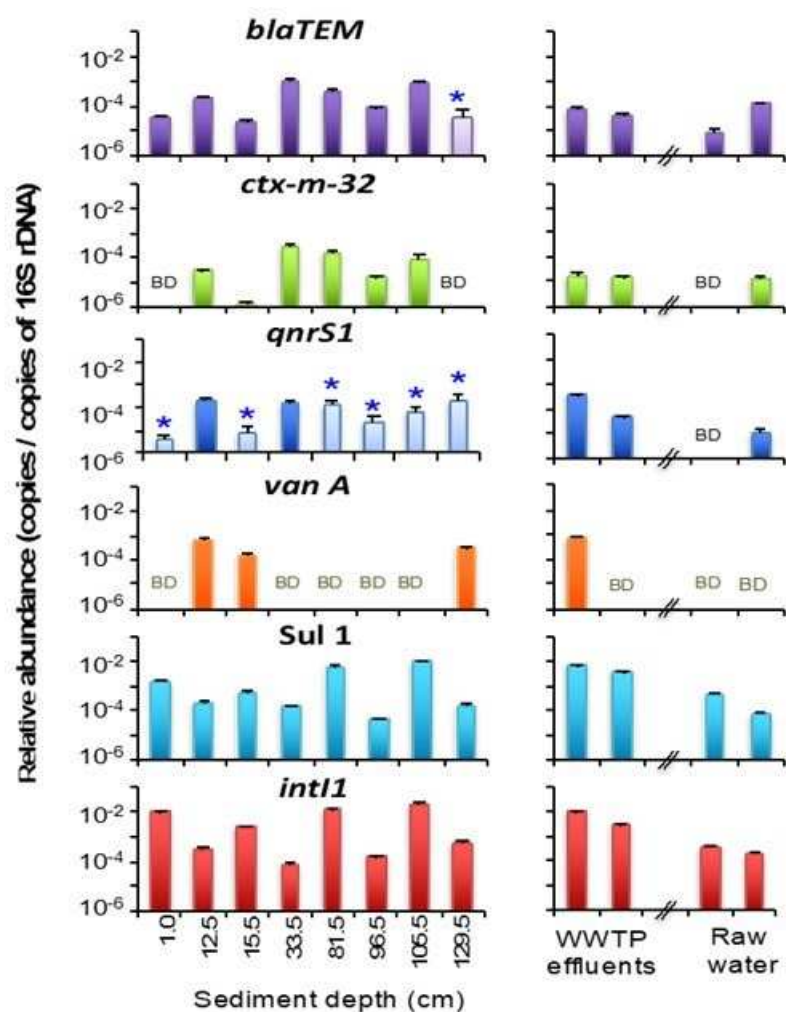


Figure 60. Relative abundance in gene copies/16S rDNA of representative ARGs quantified in sediment, WWTP effluents (February and May 2015) and water samples from the Orne River ecosystem (BETH site, February and May 2015). Asterisks indicate values close to detection limit (1×10^1 copies per reaction), BD= below detection limit.

2. MONITORING CHANGES IN THE BACTERIAL COMMUNITY STRUCTURE

Antibiotic resistance can disseminate in two ways, by horizontal and vertical gene transfer, meaning that ARB expansion by growth should also be considered. Such expansion should lead to a change in microbial community structure. This is why, in this work, MGE relative abundances were monitored together with approaches aiming at comparing the evolution of community structure using DGGE or at quantifying the molecular markers 16S rDNA as an

indicator of global bacterial population and α -, β -, and γ -proteobacteria as indicators of changes in bacterial community.

The quantification of α -, β -, and γ -proteobacteria we made in order to report about changes in the bacterial community (results parts 1 and 2) always resulted in slightly biased results, as their abundances frequently were higher than the corresponding total eubacterial 16S rDNA. This is likely to be due to a difference in priming / qPCR efficiency between the different PCR designs. Still, using α -, β -, and γ -proteobacteria as indicators was not fully satisfactory in a context where nearly no change was observed within one campaign. For these reasons, and probably because of qPCR for α -, β -, and γ -proteobacteria as indicator 16S rDNA were a bit too delicate to implement, the quantification of those indicators was no longer used. However, regarding the related PCA we made in Figure 43 (result part 2), it is interesting to note that the relative abundances of both class 1 integrons and γ -, proteobacteria tend to fluctuate in the same dimension. It is tempting to hypothesize that it reflects the host range of class 1 integrons that may principally be the γ - proteobacteria. This makes sense because class 1 integrons have primarily been characterized from γ -proteobacteria of clinical origin, even if subsequent studies demonstrated that they have been detected in environmental bacteria from diverse taxon (Wright et al. 2008). With this respect, it might be interesting to push forward the characterization of the MGE host range in the Orne ecosystem so as to reveal any new possible integron hosting-taxon.

3. TECHNIC-ASSOCIATED FLUCTUATION OF MGE ABUNDANCES

An important goal of this work was to identify the sources and fate of our two MGE proxies in a river ecosystem that was subject to past and present anthropogenic pressures. Practically speaking, this means understanding the variations in the quantification of the MGE markers *int1* and *trfA*, as well as the 16S rDNA for assessing fluctuation of the eubacterial population. For this purpose, we studied river transects on a short section of the Orne River and we carried out a complex and exhaustive statistical analysis (set by Prof. A. Maul)

aiming to measure the relative contribution of different technical and environmental factors on the variation of the abundances measured. Based on series of nested ANOVA tests we determined that experimental manipulations, including DNA extraction and qPCR quantifications, had no significant effect in the quantification of the molecular markers measured. In other words, most of the fluctuations observed were driven by environmental parameters, e.g. date of sampling. Knowing this, it is now possible to propose a new experimental design where a reduction of the repetitions (for DNA extraction and qPCR) should contribute to reduce the experimental cost and save time. An example of such optimization (for 16S rDNA) is presented in Table 25, the parameters considered were the sampling position related to the river bank (p), DNA extraction (q) and qPCR repetitions (r). Using the transect data obtained in this work, the coefficient of variation was calculated for different combinations of p, q and r repetitions. Even with the worst case scenario, *i.e.* just one sampling at one sampling position, one DNA extraction and one qPCR, the CV reached only 39.0 %, which is very close to 36.4% we obtained using experimental repeats (p = 6, q = 3, r = 3; p q r = 54) (Table 9; Table 25) and still very far from the 5308% previously obtained for the CV associated to the date effect (see section 2.3, results part I). In other words, the same conclusion would have been drawn without technical repeats, fluctuation of MGEs in river transects is mainly driven by the date of sampling.

Table 25. Predicted effects of optimization of the experimental protocol on the 16S rDNA abundance measurements accuracy

Design	Sampling positions related to the river bank (p)	DNA extraction repetitions (q)	qPCR repetitions (r)	<i>Var</i> [\bar{X}] Coefficient of variation (%)
p q r = 1	1	1	1	39.0
	1	1	6	19.6
p q r = 6	2	1	3	17.2
	3	1	2	16.4
	6	1	1	15.5

4. FATE OF ARG PROXIES AND SEASONAL EFFECTS

The occurrence of ARGs in the environment has been reported repeatedly as following the level of anthropogenic pollutants such as metal (Baker-Austin et al. 2006), polychlorinated biphenyls (Lo Giudice et al. 2013), pesticides (Anjum and Krakat 2016), disinfectants (Karatzas et al. 2007) or polycyclic aromatic hydrocarbons (PAH) (Chen et al. 2017). In this work, we also tried to correlate the abundance of MGEs with specific global/geochemical parameters, keeping in mind that, depending on the circumstances, a co-occurrence between MGE abundances and pollutant for instance can be explained either by a common source or by co-selective processes when relevant.

As far as the Orne River water is concerned, of the correlations observed the one between MGE abundances and global parameters were the easiest to understand. We found a positive correlation between the occurrence of MGE-bearing bacteria and temperature. In our case, this correlation is rather explained by the simple decrease of global bacteria abundance during the cold season. However, this kind of trend is not fully elucidated, on one hand some authors have reported a higher abundance of some ARGs during warm seasons (Sabri et al. 2018; Mao et al. 2015), on the other, Yang et al. (2013) identified a higher increase of ARG abundances during the winter period. These observations demonstrate that the parameters driving the fluctuation of ARGs or MGEs in river water cannot solely be reduced to a simple seasonal effect, and that other parameters related to the receiving environment by itself or hydroclimatic changes such as pluvial precipitations should be considered. Regarding this last point, the influence of rainfall, Di Cesare et al. (2017) demonstrated a significant increase on the absolute abundances of several ARGs quantified during moderate rainfall events (with rain intensity peaking between 2.5 and 7.6 mm rain.d⁻¹). In the context of this work, we could show that the entry of class1 integron in the Orne ecosystem is probably diffuse as their abundance gradually increases over a long river section. In the meantime, we could show that the WWTP effluents do not significantly alter the level of class 1 integron in water because of a dilution effect. These two observations

led us to conclude that, at least for the 23 km river section considered, most of the class 1 integron input is made through runoff. With this respect, rain falls can have significant but sometimes contradictory effects: (i) rainfalls are responsible for the mobilization of bacteria toward the river, nevertheless (ii) too much rain falls or heavy hydroclimatic events lead to a washout of the bacteria, and thus a decrease in abundance, while (iii) rainfalls also contribute to reducing the abundance of bacteria and MGE-bearing bacteria by a dilution effect. To go further, we saw that seasonality affect the global abundance of bacteria including the MGE-bearing ones. On the other hand, the negative correlations with flow rate solely concern total bacteria and IncP-1 bearing bacteria. This observation is in favor of different origins for IncP- 1 plasmids and class1 integrons. Spearman correlation tests showed that IncP-1 plasmid-bearing bacteria are negatively correlated to the river flow rate, which suggests a dominance of the rain fall-associated dilution effect. For class 1 integron bearing bacteria, no correlation could be established with the river flow rate. We hypothesize here that, although the seasonal effect is effective, the absence of a clear correlation, in this case, may reflect a balance between mobilization by runoff and dilution effect. Clearly, the different fates observed of the two MGEs indicate two distinct origins where one of them, more sensitive to runoff (class 1 integrons), enters the river ecosystem by rainfall mobilization, while the other one (IncP-1 plasmids), more sensitive to flow rates and not displaying any relevant site effect (no change in abundance over a 23 km section of the river for a given sampling date) is likely to reflect indigenous bacteria already present in the ecosystem.

5. FATE OF ARG PROXIES DURING EXTREME HYDROCLIMATIC EVENTS

The abundance of class 1 integrons and IncP-1 plasmids was monitored during two flood events. For one of them, we lost track of both MGEs while the rain intensities reaching approximately 12.5 mm rain.d-1, with an important increase in the river flow rate up to 50 times more compared with the lowest flow rate monitored during the flood sampling campaigns. This suggested that a dilution effect had occurred. Our results fit more with the work of Garner et al.

(2016) that found that the total ARGs per mL bulk water decreased from pre- to post-flood events at the Cache La Poudre River in Fort Collins Colorado. They also attributed this ARG abundance decrease to a dilution effect. Nevertheless, once the water flow goes back to normal, the levels of MGEs went back to normal as well, immediately in the day(s) after the flood event. In the case of the Orne River, it is interesting to note that the two flood events exhibit some differences regarding the levels of absolute abundances monitored. The absolute abundance (copies/mL) of both 16S rDNA and MGEs were approximately 10 times higher in November 2015 than February 2016 at the beginning and at the end of the flood events. It is tempting to speculate that this difference is associated to the wash out of the markers operated during the winter season, where the November 2015 flood marks the beginning of the raining season and the February one the end. However, calculations were made to evaluate the flux of markers (copies/s) showing that the global amount of markers carried by the river is relatively stable outside the boundaries of the flood peaks (Figure 32, results part I). This observation favors the idea of the dilution effect, while the loss of markers (in abundance and in flux) during the February 2016 flood peak is in favor of a transient excess wash out.

6. MGE ABUNDANCES AND ANTHROPOGENIC PRESSURES

In this work, we could determine that the fluctuation of MGE abundances in the water compartment of the Orne River was dominated by seasonality and river hydrology rather than local anthropic pressures. Indeed, even when the effluents of the local WWTP exhibit a significant a higher abundance of MGE, the effect of effluent release remains unseen in the river because of a too high dilution rate. As a consequence, sites upstream and downstream WWTP have comparable levels of MGEs whether in absolute or relative abundances. Clearly, such comparable levels between sampling sites demonstrate that the WWTP is not a major source of MGEs in the Orne context. This does not necessarily mean that that WWTP has no effect at all. First, because the MGE-carrying bacteria released may preferentially accumulate in a distinct compartment of the river (sediments for instance) and that instant measurement

of abundances do not consider long terms accumulation of MGEs. In addition, WWTP effluent may also have direct or indirect effects on the structure of the river microbial community without significantly affecting its content in MGE that remains a small fraction of the community. A possible way to determine the contribution of the effluents of the WWTP to the autochthonous bacterial community could be the comparison of the bacterial community structure from upstream, WWTP effluents and downstream sites by global approaches such as DGGE or metagenomics.

WWTP effect put on the side, we tried to consider the effect of the anthropogenic pressure in the vicinity of Joeuf and Homécourt by comparing the abundance of MGEs between sampling sites taken along the continuum of the River. When the effect of the WWTP was considered, the distance between the sampling sites was relatively too short (around 4.3 km distance), to observe an influence of the sampling site. We also studied the fluctuation MGE abundances along the continuum of the river for sites spread over 10 km and again no statistical differences could be observed. Only in the last attempt, within a 23 km distance between the first and last sampling site we could detect a significant increase in the abundance of class 1 integrons. The last sampling site, Richemont, is a much more urbanized and industrialized area than the AUB and BETH sites, therefore it could be suggested that is precisely this high anthropogenic pressure (actual pressures) that impact the MGE abundances. However, when the 23 km river section is considered we could also see some differences between AUB and BETH site as well as between BETH and RICH site, thus ruling out an increase in integrons solely attributed to the RICH site effect. To go further, it would be interesting to develop a series of sampling campaigns with sites covering entirely the continuum of the river at regular intervals, including rural areas as well, so as to precise the diffuse aspect of class 1 integron input.

7. COMPARTMENTALIZATION OF MGES

With the global increase in the occurrence of ARGs in non-clinical environments (but impacted by human activities), ARGs started to be

considered as pollutants of emerging concern for which strategies are necessities to prevent their dissemination (Pruden et al. 2006). Even if several studies have shown that the environment can act as a reservoir of ARGs, to best of our knowledge, most of them focus on a specific compartment of the water column, mostly to demonstrate the specific effect of anthropic settings such as WWTP on the occurrence and fate of ARB and ARGs in aquatic environments. As matter of fact, fewer works is dedicated to the evaluation of water and sediment compartment together, forgetting the in-between compartment *i.e.* suspended particulate material (SPM). SPM is composed of various organic and inorganic compounds, such as clay, sand, silts, silicates, polycyclic aromatic compounds, metals, etc., and of course bacteria and other microorganisms (Abuhelou 2016). Considering that SPM can both deposit and form sediment and be remobilized during high hydroclimatic events, it seems that SPM could play a significant role in feeding both water and sediments with ARB and ARGs depending on the hydroclimatic conditions (dry vs. wet periods). This may be particularly relevant when considering the enrichment observed of specific MGE-bearing bacteria on SPM.

Aquatic ecosystems have a greater bacteria diversity and richness. Indeed, they are constantly cluster in two categories: free-living bacteria and particle-attached bacteria (Mohit et al. 2014). Both of them can be different not only in their bacterial community structure, they can also differ physiologically. Mohit et al. (2014) mentioned some of these differences, including more metabolic activity in particle-attached bacteria than in free-living bacteria, differences in their abundance, as well as their growth efficiency, etc. Late studies have shown that the relative abundance of MGEs in particle associated microorganisms is higher than in free-living microorganism, suggesting a compartmentalization of MGE-bearing bacteria in the environment (Ganesh et al. 2014). The reason of this specific enrichment and the role that these compartments could play in the transport of ARB and their ARGs (through MGEs) between the water and sediment compartment is not well understood and becomes a subject of concern. This drove us to study the occurrence of MGEs in the SPM compartment and to compare it with the MGE content in the water column, so as to evaluate if MGE-bacteria can be enriched in the SPM fraction. SPM were collected using a field centrifuge and a significant

enrichment of MGEs, especially of IncP-1 plasmids could be demonstrated therefore confirming a compartmentalization process in the Orne aquatic ecosystem. So far, there are not too many works focused on the enrichment of MGEs or IncP-1 plasmids attached to river particles. Proia et al. (2018) observed an enrichment of several ARGs in particle- attached bacteria by comparison to free-living bacteria, they just hypothesized the higher levels of ARGs in particle attached bacteria as the fact of close contact between cells, consequently increasing the probability for HGT. We also assumed this possibility, but also based on the observations made by Ghigo (2001) where conjugative plasmids by themselves could promote cell adhesion to surface for the formation of biofilms, mediated by conjugative pili, we decided to explore both hypotheses using *in vitro* grown biofilms. Here, we demonstrated that IncP-1 plasmids selectively promote adhesion of bacteria to particle surfaces rather than increasing HGT (De la Cruz Barrón et al. 2018). In the case of class 1 integrons, the differences between the relative abundance of class 1 integrons of surface sediments and those from raw water or SPM were hardly visible. This similarity could be related to the simple fact that class 1 integrons by themselves do not confer any adhesion properties as plasmids do, therefore no specific enrichment of class 1 integron-bearing bacteria on SPM.

Interestingly, we noticed that class 1 integrons and IncP-1 plasmids do not compartmentalize in the same way, making them complementary proxies for ARGs. Even if class 1 integrons are frequently associated with IncP-1 plasmids (Popowska and Krawczyk-Balska 2013), in complete genome database only 12 % of integrons (mostly class 1) appeared associated with plasmids (Cury et al. 2016). Therefore, if 12 % of integrons-bearing bacteria are supposed to behave as plasmid-bearing bacteria do (and even less for IncP-1 plasmid-bearing bacteria specifically), the majority of class 1 integrons are not supposed to be linked to plasmid-induced modifications of bacterial surface adhesion properties. Jiang et al. (2018) also found a different behavior between the two kinds of MGEs, where bacteria found in water harbored higher diversity and abundance of integrons than surface sediment samples, while the abundances of plasmids were higher in sediment samples than water, but with less diversity. In any case, considering that in the Orne River SPM settle to form sediment, here demonstrated by the mineral relationship between sediment and SPM

predominated by clay minerals, this work pinpoints the role of SPM in selectively enriching the sediments with particular MGEs, which could then form MGEs reservoirs. In future work, it would be interesting to know whether the same selectivity applies to ARGs as some may be more prone to be carried by plasmid than other.

8. FATE OF MGE IN SEDIMENTS AND ANTHROPOGENIC POLLUTANTS.

The sediment compartment was of special interest since it is the final receiving compartment of diverse pollutants in the aquatic ecosystems, where ARB can eventually persist, accumulate and transfer their ARGs. With this respect, sediment characteristics play an important role in the accumulation of pollutants (organic, inorganic and/or biological). Considering the frequently reported association between anthropic pollutants and ARGs in different environments, and considering the pollution history of the Orne River, we decided to study this compartment, by analyzing two sediment cores.

When we started working on sediment cores, we first faced a technical issue for the recovery of total DNA from the sediment samples (JOSAN core). As we wanted to maintain the same technical biases associated with DNA extraction, we decided to use the same DNA extraction kit used for water samples. Possible protocol-based biases, fits well with several works mentioning that the DNA extracted could vary according to the method used, where microorganism are more or less susceptible to the cell lysis methods, thus affecting the microbial community profiles (Daniel 2005; Morgan, Darling, and Eisen 2010). Using the PowerWater® DNA extraction kit, we noticed that a 15 min pre-agitation step before the DNA extraction improves the global abundance of total bacteria population determined by the quantification of 16S rDNA. We assumed that the agitation of the re-suspended sediment allows a partial detachment of cells from particles, making more prone to the lysis process. Nevertheless, we did not use any buffer and or detergent for cell detachment as it is sometimes used in improvement of protocols dedicated to the recovery of DNA from sediment samples (Lavergne et al. 2014; Heß, Berendonk, and Kneis 2018),

therefore our quantifications could be underestimated as the complete cell detachment cannot be ensured.

Even using the same extraction kit, the DNA extracts, from JOSAN core, for the same sediment layer did not always result in the same recovery abundances of 16S rDNA or MGEs. On the other hand, this problem seems to be partially minimized when the relative abundance of MGE was used (depending on the layer). For some layers, the absolute abundance appeared quite variable while the relative abundance was relatively stable. We attributed this phenomenon to variability in DNA extraction yield as explain above. On the other hand, some sediment layers appeared variable for the relative abundance as well, thus indicating that the community greatly differed from a repeat to another, which was confirmed by the analysis of the bacteria community structure using DGGE approaches. This finally led us to the concept of micro-reservoirs when a local increase of MGE was observed (Figure 48). The existence of MGE micro-reservoirs was not exclusive of the JOSAN core, we could also identify them in the other core analyzed, the BETH core. In the meantime, the local variation observed, measurable by the coefficient of variation, became a new parameter to test when performing statistical comparisons. However, to date, we could not establish any robust correlation between the local variability of MGE content and the geochemical parameters.

BETH core is completely different from JOSAN core. First, according to its geochemistry and texture, the formation of BETH core involved a combination of natural sedimentation and the direct deposition and accumulation of solid waste from the former steel making industries. Conversely, the JOSAN core seems to have been formed by natural sedimentation. The difference between the cores also lies in the pollutant content, where the highest level of pollution was observed for BETH core. We compared the concentration of several metals quantified in the different layers of the BETH core with consensus-based guidelines for sediment quality (MacDonald, Ingersoll, and Berger 2000; de Deckere et al. 2011). For all the metals compared, As, Cd, Cu, Pb, Ni, and, Zn, their concentration was high enough for a potential or certain risk of decreasing the number of living cells in the system (Table 26).

Table 26. Sediment quality guidelines for metals in aquatic ecosystems

<i>Metal</i>	<i>Concentrations below which no harmful effect is observed^A</i>	<i>Concentrations above which harmful effect is observed^B</i>	<i>Concentrations in JOSAN core^C</i>	<i>Concentrations in BETH core^C</i>
<i>As</i>	<9.79	>21	17.8-25.9	24.6-209.0
<i>Cd</i>	<0.99	>3.6	41.2-89.8	0.6-17.5
<i>Cr</i>	<43.4	>77	46.5-86.1	94.5-192.3
<i>Cu</i>	<31.6	>82	23.8-49.2	35.7-148.6
<i>Ni</i>	<22.7	>31	19.8-36.6	38.4-121.5
<i>Pb</i>	<35.8	>129	23.4-111.2	60.0-4430.0
<i>Zn</i>	<121	>609	319.8-584.0	440.0-10580

All concentrations are expressed in mg kg⁻¹ (dry weight)

^A Values from MacDonald et al., (2000)

^B Values from de Deckere et al., (2011)

^C Minimum and maximum values

Importantly, even if we used to consider the JOSAN core as poorly contaminated by pollutants compared with BETH core, according to those guidelines, the metal concentrations in JOSAN core were higher than the minimum acceptable for a normal “biological availability”, thus highlighting the pollution in the different studied areas of the Orne River. Still, the higher level of pollutants found in BETH core, raised the question of possible toxicity and/or co-selection processes operated in the sediment. At this stage, we faced again our initial technical issue. Keeping the PowerWater® DNA extraction kit protocol from 50 mg of sediment was fine for keeping the same procedure as for the water sample but, on the other hand, geochemical analyses were carried out on larger samples (200-250 mg), which may be of prime importance when considering the variability observed with the molecular markers monitored. To solve this problem, new series of DNA extraction using the PowerSoil® kit were made so as to work with comparable sample size with geochemistry analyses, which, in the meantime, would minimize the variation of molecular marker abundance observed within layers. Beginning by the comparison of the two DNA extraction kits, we expected to have higher DNA yields with the PowerSoil® kit as the quantity of sample was 5 times more than the used with

the PowerWater® kit. Nonetheless, even if the sample quantity was higher for the PowerSoil®, in general, we did not observe a higher DNA extraction yield. Considering that DNA extraction can be impaired by the presence substances such as metals, organic matter, etc., that interfere with the effectivity of the extraction reagents (Fang et al. 2015), the presence of metals and organic compounds in our samples may explain such low yield (metals and organic compounds which were otherwise washed out with the PowerWater® DNA extraction procedure). However, the relative abundances of class 1 integrons between replicates were more homogenous allowing using the mean values for statistical analyses.

Selective pressures exerted by pollutants such as metals are often described as promoting of ARG dissemination by several co-selection processes (described in the Introduction section) (Di Cesare et al. 2016); nevertheless, the relationship between metal levels in the environment and the presence of ARGs has not been fully determined (Knapp et al. 2017a). The works of Stepanauskas et al. (2005, 2006) and Xu et al. (2017) and those of Zhao et al. (2017) evidenced that metals are environmental stressors that select for ARGs, in microcosms and field experiments, respectively. Conversely, we did not observe this kind of trend. Even if several positive correlations between the absolute abundances of 16S rDNA, class 1 integrons and metals were found (Spearman's rank correlation tests), most of the correlating elements, such as rare earth elements, are ubiquitous in the environment (Kanbar, 2017) and the biological meaning of these correlations is not well understood. On the other hand, negative correlations between the absolute abundance of both 16S rDNA and class 1 integrons were found with metals, among others As, Pb, and Cd (Table 24, results part III). Knapp et al. (2017) also identified negative correlations between ARGs and some of the metals quantified in soil samples. They attributed those results to possible toxic effects of these metals. They suggest that the metals not correlating ARGs are present in too low concentrations in the studied environment to have a visible effect. Nevertheless, in the BETH core, we did not find any correlation between the absolute abundance of 16S rDNA, MGEs (absolute and relative abundance) and Zn whereas this metal is one of the most abundant metals found in this core, Other scenarios such as possible cellular stress responses (gene expression),

community adaptation, community shift or cell death (toxic effect) seemed to be more likely to occur at the high metal concentration found in the BETH core, thus explaining the negative correlations observed. Supporting this hypothesis, Hölzel et al. (2012) found that Hg and Pb inhibited bacterial population growth rather than promoting resistance development. We also found a negative correlation of 16S rDNA and the absolute and relative abundance of class 1 integrons with Mn, but it is known that large amounts of Fe oxides and Mn oxides are frequent in urban and industrial streams, and have been reported as common carriers of trace metal (Le Meur et al. 2016a). Could that be the reason for the negative correlation found, remains an open question.

Additionally, Seiler and Berendonk (2012) determined the minimum co-selective concentration (MCC) of a few metals in different environments such as water, sediment, soil and manure. The different MCCs varied according to the metal, the environment and the units (referring to fresh or dry weight in the case of sediment and soils). They assumed that the concentrations of metals higher than the MCCs are considered to have a potential for driving the co-selection of antibiotic resistance in the environment (resulting frequently in positive correlations between ARGs and metals). Nevertheless, it is not mentioned until which metal concentration there is a co-selection process and from which metal concentration results in the opposite case (negative correlation) or none correlation at all (toxicity). The determined MCC for Zn in sediment samples was estimated at 42.5 mg.kg⁻¹ (dry weight) while the concentration of Zn in BETH core is 440 mg.kg⁻¹ at the lowest and reaches 10580 mg.kg⁻¹ (dry weight) at the highest level, but we did not find any correlation between Zn concentration and 16S rDNA and class 1 integrons. However, it should be noted that the bioavailability of the metals in sediment is not discussed. The lack of MCC data available for other metals limited our exploration of the “MCC effect”.

The correlation of our occurrence data with PACs is more complex, as there are not a lot of studies in the field but it is more or less the same scenario as for metal pollution. Indeed, Chen et al. (2017) suggested that PAHs could serve as selective agent for enriching ARG abundances. On the other hand, Kang et al. (2015) suggested that small-sized PAHs reduce the *in vitro* HGT of ARGs, but their study did not demonstrate whether or not PAHs co-select for ARGs. In our

case, we found only a few correlations between class 1 integron absolute abundances and PACs whereas most correlations were found between class 1 integron relative abundance and PACs. Further works need to be developed for evaluating the possible contribution of PACs in the spread of ARGs in the environment.

Although several works have demonstrated the occurrence of ARGs/MGEs in different environments, the understanding of the relationship between antibiotic resistance and the environmental conditions involved in its dissemination/persistence remains unanswered. Additionally, to our knowledge, there is not enough information about MGE activities (facility to be disseminated) in complex ecosystems. We proposed to use light emitting whole cell biosensors to evaluate the activity of MGEs in the sediments of the Orne River and to address the question do MGEs persist better when they are active? Thus, a biosensor was developed by cloning the promoter of the integrase gene from a class 1 integron in a plasmid containing a promoterless *lux* operon (Figure 61 A). This strain allows us to report the integrons activity by monitoring light emission (Figure 61 B). Then, we started to test the effects of pollutants likely to influence the recombinogenic activity of the integrons *via* the induction of the SOS response such as PAHs, metals and pesticides.

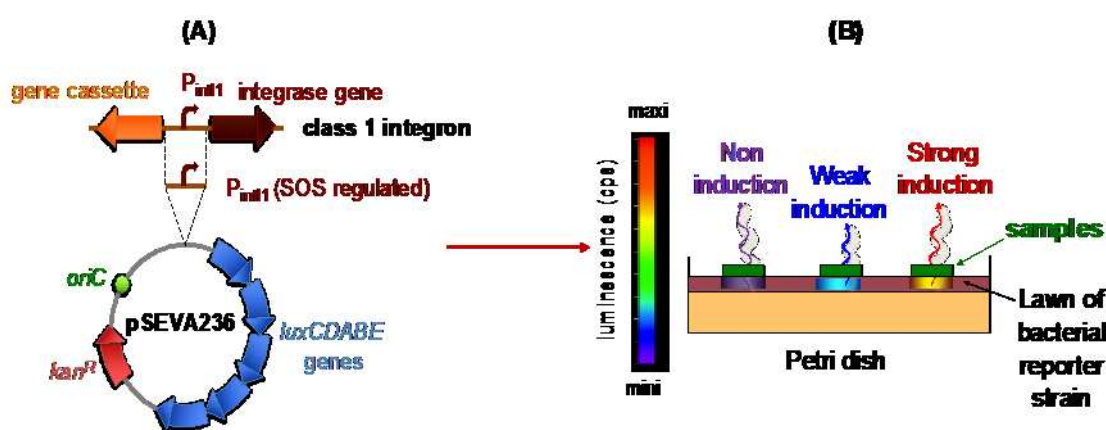


Figure 61. Design of the biosensor test for reporting of the class 1 integron recombination activity. (A) Construction of the reporter plasmid by cloning the Pint1 promoter in the pSEVA236 vector. (B) Light emission assay where Pint1 inductions by pollutants are monitored with a CCD camera. (Source: Bellanger X., Merlin C.)

Preliminary tests of light emission assays using sediment sample deposits showed first that sediments can sometimes induce, slightly, the *PintI1* promoter but that this induction is not correlated to the sediment class 1 integron content (Figure 62 A). Second, sediments were centrifuged in order to separate the interstitial water and tested on the whole cell biosensor. We determined that the integron activity is not related to sediment chemistry, as interstitial water did not trigger the *PintI1* promoter (Figure 62 B), but, unexpectedly, after 24h of incubation, bacteria started to grow and bioluminescence induction was observed in presence of sediment bacteria (artefactual) (Figure 62 C). Finally, the biosensor was also used to study the effect of 6 PAHs on the activity of the promoter of the integrase gene leading to the detection of weak PAH-related weak light emission inductions (Figure 62 D). Even if these experiments are preliminary, they demonstrate the feasibility of this biosensor approach for screening the effect of various pollutants on the activity of integrons, and they will be continued.

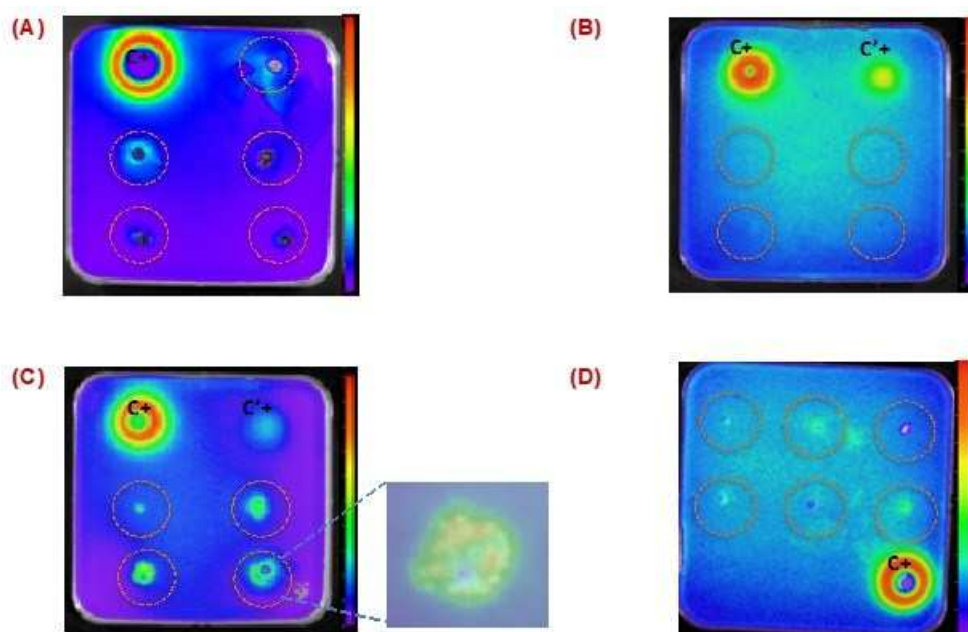


Figure 62. Implementation of biosensor to detect the activity of class 1 integron promoter. The experiment was carried out as described in Figure 61, by exposing the biosensor lawn to different samples: (A) sediments; (B) interstitial water from sediment samples; (C) sediment bacteria; (D) six polycyclic aromatic hydrocarbons (PAHs). C+ and c'+ (dilution of C+) correspond to the positive controls (mitomycin C). The sample deposit is indicated by red dotted lines.

In summary, with this work, we demonstrated the compartmentalization of two different families of MGEs in the Orne River system. Our results allowed us to hypothesize that the sources of integrons in the Orne River are not punctual but their input is “diffuse” since their quantity increases slowly from upstream to downstream. On the other hand, IncP-1 plasmids seem to be endogenous to the Orne ecosystem since their quantity is quite stable from upstream to downstream and vary with hydroclimatic conditions, *e.g.* river flow rate. We detected an enrichment of IncP-1 plasmids in the SPM compartment that we determined to be related to a plasmid-related increase of cell adhesion-properties rather than on a dissemination of the plasmids in the bacteria aggregated within SPM.

Additionally, the Orne River is a complex environment where there was no easy way to demonstrate and to explain the possible co-selective processes due to anthropic pressures, if presents, in driving the occurrence of the MGE abundances in the sediment compartment.

All in all, we have shown that class 1 integrons, frequently used as a proxy for antimicrobial resistance, behave differently from the IncP-1 plasmids in the three compartments studied. Therefore, the relevance of using only class 1 integrons as proxy for ARGs can be questioned, and the use of other MGEs as complementary proxies could allow drawing a better picture of the different phenomena related to the dissemination of ARGs in the environment.

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ANNEXES

ANNEXES

1. DNA extraction protocols

1.1 PowerWater® kit



Experienced User Protocol

Please wear gloves at all times

Warm Solution PW1 prior to use at 55°C for 5-10 minutes. Use Solution PW1 while still warm. Check Solution PW3 and warm at 55°C for 5-10 minutes if necessary. Solution PW3 can be used while still warm.

1. Filter water samples using a reusable or disposable filter funnel attached to a vacuum source. Disposable filter funnels, containing 0.22 µm or 0.45 µm filter membranes, can be ordered from MO BIO Laboratories (see page 3). The volume of water filtered will depend on the microbial load and turbidity of the water sample. **(Please see Types of Water Samples in the Hints and Troubleshooting Guide section of the Instruction Manual).**
2. If using a reusable filter funnel, remove the upper portion of the apparatus. If using a MO BIO Laboratories filter funnel, remove the 100 ml upper portion of the filter cup from the catch reservoir by snapping it off.
3. Using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward.
Note: Do not tightly roll or fold the filter membrane. To see a video of this technique, please visit the PowerWater® DNA Isolation Kit product page on www.mobio.com.
4. Insert the filter into the 5 ml PowerWater® Bead Tube.
5. Add 1 ml of Solution PW1 to the PowerWater® Bead Tube.
Note: Solution PW1 must be warmed to dissolve precipitates prior to use. Solution PW1 should be used while still warm. For samples containing organisms that are difficult to lyse (fungi, algae) an additional heating step can be included. See **Alternate Lysis Method in the Hints and Troubleshooting Guide**.
6. Secure the PowerWater® Bead Tube horizontally to a MO BIO Vortex Adapter, catalog number 13000-V1-15 or 13000-V1-5.
7. Vortex at maximum speed for 5 minutes.
8. Centrifuge the tubes ≤ 4000 x g for 1 minute at room temperature. The speed will depend on the capability of your centrifuge. **(This step is optional if a centrifuge with a 15 ml tube rotor is not available, but will result in minor loss of supernatant).**
9. Transfer all the supernatant to a clean 2 ml Collection Tube (provided). Draw up the supernatant using a 1 ml pipette tip by placing it down into the beads.
Note: Placing the pipette tip down into the beads is required. Pipette more than once to ensure removal of all supernatant. Any carryover of beads will not affect subsequent steps. Expect to recover between 600-650 µl of supernatant depending on the type of filter membrane used.
10. Centrifuge at 13,000 x g for 1 minute.
11. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided).
12. Add 200 µl of Solution PW2 and vortex briefly to mix. Incubate at 4°C for 5 minutes.
13. Centrifuge the tubes at 13,000 x g for 1 minute.
14. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided).
15. Add 650 µl of Solution PW3 and vortex briefly to mix.
Note: Check Solution PW3 for precipitation prior to use. Warm if necessary. Solution PW3 can be used while still warm.
16. Load 650 µl of supernatant onto a Spin Filter and centrifuge at 13,000 x g for 1 minute. Discard the flow through and repeat until all the supernatant has been loaded onto the Spin Filter.
Note: A total of two loads for each sample processed are required.
17. Place the Spin Filter basket into a clean 2 ml Collection Tube (provided).



18. Shake to mix Solution PW4 before use. Add **650 μ l of Solution PW4** and centrifuge at 13,000 x g for 1 minute.
19. Discard the flow through and add **650 μ l of Solution PW5** and centrifuge at 13,000 x g for 1 minute.
20. Discard the flow through and centrifuge again at 13,000 x g for 2 minutes to remove residual wash.
21. Place the Spin Filter basket into a clean 2 ml Collection Tube (provided).
22. Add **100 μ l of Solution PW6** to the center of the white filter membrane.
23. Centrifuge at 13,000 x g for 1 minute.
24. Discard the Spin Filter basket. The DNA is now ready for any downstream application. No further steps are required.

We recommend storing the DNA frozen (-20°C to -80°C). Solution PW6 contains no EDTA. To concentrate the DNA, see the Hints and Troubleshooting Guide.

Thank you for choosing the PowerWater® DNA Isolation Kit Sample!

1.2 PowerSoil® kit

Protocol: Experienced User

Important points before starting

- Solution PW1 must be warmed at 55°C for 5–10 minutes to dissolve precipitates prior to use. Solution PW1 should be used while still warm.
- If Solution PW3 has precipitated, heat at 55°C for 5–10 minutes to dissolve precipitate.
- Shake to mix Solution PW4 before use.

Procedure

1. Filter water samples using a filter funnel attached to a vacuum source. The volume of water filtered will depend on the microbial load and turbidity of the water sample.
Note: Please see Appendix A: Types of water samples.
2. If using a reusable filter funnel, remove the upper portion of the apparatus.
3. Using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward.
Note: Do not tightly roll or fold the filter membrane. To see a video, please visit the DNeasy PowerWater Kit product page at <https://mobio.com/powerwater-filter>.
4. Insert the filter into a 5 ml PowerWater DNA Bead Tube.
5. Add 1 ml of Solution PW1 to the PowerWater DNA Bead Tube.
Note: For samples containing organisms that are difficult to lyse (e.g. fungi, algae) an additional heating step can be included. See Alternative Lysis Methods in the Troubleshooting Guide.
6. Secure the tube horizontally to a Vortex Adapter (cat. no. 13000-V1-5/13000-V1-15).
7. Vortex at maximum speed for 5 min. Centrifuge the tubes $\leq 4000 \times g$ for 1 min at room temperature. (This centrifugation step is optional if a centrifuge with a 15 ml tube rotor is not available, but will result in minor loss of supernatant).
8. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Draw up the supernatant using a 1 ml pipette tip by placing it down into the beads.

Note: Placing the pipette tip down into the beads is required. Pipette until you have removed all the supernatant. Expect to recover 600–650 μ l of supernatant.

9. Centrifuge at 13,000 $\times g$ for 1 min at room temperature.
10. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided).
11. Add 200 μ l of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min.
12. Centrifuge the tubes at 13,000 $\times g$ for 1 min.
13. Avoiding the pellet, transfer the supernatant to a clean 2 ml Collection Tube (provided).
14. Add 650 μ l of Solution PW3 and vortex briefly to mix.
15. Load 650 μ l of supernatant onto an MB Spin Column. Centrifuge at 13,000 $\times g$ for 1 min. Discard the flow-through. Repeat until all the supernatant has been processed.
16. Place the MB Spin Column Filter into a clean 2 ml Collection Tube (provided).
17. Add 650 μ l of Solution PW4 (shake before use). Centrifuge at 13,000 $\times g$ for 1 min.
18. Discard the flow-through and add 650 μ l of ethanol (provided) and centrifuge at 13,000 $\times g$ for 1 min.
19. Discard the flow-through and centrifuge again at 13,000 $\times g$ for 2 min.
20. Place the MB Spin Column into a clean 2 ml Collection Tube (provided).
21. Add 100 μ l of Solution EB to the center of the white filter membrane.
22. Centrifuge at 13,000 $\times g$ for 1 min.
23. Discard the MB Spin Column. The DNA is now ready for downstream applications.

Note: We recommend storing DNA frozen (–20°C to –80°C) as Solution EB does not contain EDTA. To concentrate DNA, see the Troubleshooting Guide.

2. Scientific publications



Suspended Materials in River Waters Differentially Enrich Class 1 Integrin- and IncP-1 Plasmid-Carrying Bacteria in Sediments

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Aquatic ecosystems are frequently considered as the final receiving environments of anthropogenic pollutants such as pharmaceutical residues or antibiotic resistant bacteria, and as a consequence tend to form reservoirs of antibiotic resistance genes. Considering the global threat posed by the antibiotic resistance, the mechanisms involved in both the formation of such reservoirs and their remobilization are a concern of prime importance. Antibiotic resistance genes are strongly associated with mobile genetic elements that are directly involved in their dissemination. Most mobile genetic element-mediated gene transfers involve replicative mechanisms and, as such, localized gene transfers should participate in the local increase in resistance gene abundance. Additionally, the carriage of conjugative mobile elements encoding cell appendages acting as adhesins has already been demonstrated to increase biofilm-forming capability of bacteria and, therefore, should also contribute to their selective enrichment on surfaces. In the present study, we investigated the occurrence of two families of mobile genetic elements, IncP-1 plasmids and class 1 integrons, in the water column and bank sediments of the Orne River, in France. We show that these mobile elements, especially IncP-1 plasmids, are enriched in the bacteria attached on the suspended matters in the river waters, and that a similar abundance is found in freshly deposited sediments. Using the IncP-1 plasmid pB10 as a model, *in vitro* experiments demonstrated that local enrichment of plasmid-bearing bacteria on artificial surfaces mainly resulted from an increase in bacterial adhesion properties conferred by the plasmid rather than an improved dissemination frequency of the plasmid between surface-attached bacteria. We propose plasmid-mediated adhesion to particles to be one of the main contributors in the formation of mobile genetic element-reservoirs in sediments, with adhesion to suspended matter working as a selective enrichment process of antibiotic resistant genes and bacteria.

Keywords: class 1 integrons, IncP-1 plasmids, environmental reservoirs of antibiotic resistance genes, river water column, suspended matter, sediments, biofilm, horizontal gene transfer

INTRODUCTION

Predictions of future scenarios regarding the consequences associated with the burden of antibiotic resistant bacteria (ARB) in the forthcoming years are still a matter of debate (de Kraker et al., 2016), but there is no doubt that this burden is already impairing our ability to treat common infectious diseases (Cosgrove, 2006). Natural environments, especially soils and aquatic ecosystems, are believed to play a key role in the emergence and dissemination of ARB. Indeed, being the final recipient of wastewater discharges or solid wastes from animal husbandry, such environments are often exposed to anthropogenic chemical pollutants such as antibiotics and biocides, as well as microbial pollutants such as ARB (Baquero et al., 2008; Knapp et al., 2010; Heuer et al., 2011). Considering the ever-increasing occurrence of ARB in the anthropogenic-impacted environments, these bacteria and their antibiotic resistance genes (ARGs) are now considered as environmental contaminants of emerging concern (Pruden et al., 2006).

Several studies have shown that, globally, the environment can act as a reservoir of ARGs and that many Gram-positive and Gram-negative pathogens have acquired new resistance traits originating from this huge gene pool (Martinez, 2009; Wright, 2010; Perry and Wright, 2013). Part of the problem arises from the association of ARGs with so-called mobile genetic elements (MGEs) that allow them to be horizontally transferred between environmental and/or clinical bacteria. MGEs represent a rather large variety of elements including conjugative plasmids, integrative and conjugative elements, transposons, and integrons (Frost et al., 2005), some of them being more often associated with ARGs than others. As a consequence, instead of monitoring the occurrence of a large panel of clinically relevant ARGs, some authors prefer focusing on selected MGEs frequently associated with ARGs. Among them, class 1 integrons, and to a lesser extent, IncP-1 conjugative plasmids, have been widely studied as proxies to assess the global ARG content of environmental matrices (Stalder et al., 2012; Rizzo et al., 2013; Gillings et al., 2015; Jechalke et al., 2015). Class 1 integrons are genetic platforms able to capture and express resistance gene cassettes, thus promoting genotypic/phenotypic diversity and adaptation of bacteria (Mazel, 2006). They are minimally constituted of an *intI1* gene encoding a site-specific recombinase and of a recombination site *attI* where IntI1 catalyzes the gene cassette insertions. Class 1 integrons usually carry 1–4 of more than 130 different resistance gene cassettes that have been described to date, which makes them integrated indicators of ARGs (Partridge et al., 2009; Stalder et al., 2014; Cury et al., 2016). The plasmids of the IncP-1 incompatibility group are broad host range conjugative elements (Adamczyk and Jagura-Burdzy, 2003). These plasmids, found in clinical and environmental contexts, often carry multiple antibiotic resistance determinants suggesting that they also play a significant role in ARG dissemination (Popowska and Krawczyk-Balska, 2013).

It has been demonstrated that ARGs tend to accumulate over time in environmental microbial communities as a probable consequence of anthropogenic pressure (Knapp et al., 2010). Their association with MGEs can explain the persistence of ARGs

in the environment as MGEs allow them to be transferred from unfit bacteria originating from the animal/human microbiome to more locally adapted bacteria (Frost et al., 2005; Martinez, 2009). Surprisingly, MGEs are not equally distributed in heterogeneous matrices, with specific enrichment of some MGEs on suspended particles in marine waters, as demonstrated by Ganesh and collaborators (Ganesh et al., 2014). This suggests that MGE-bearing bacteria can selectively and locally be enriched. The reason for such local and selective enrichment of MGEs is not fully elucidated but it has been known for some time that, besides their ability to transfer genes, MGEs, such as conjugative plasmids, also influence surface properties of bacterial cells (Van Houdt and Michiels, 2005). Indeed, the conjugation of plasmids implies the synthesis of external cell appendages (conjugative pili or adhesins in either Gram-negative or Gram-positive bacteria) that have been demonstrated *in vitro* to promote cell adhesion to solid surface and biofilm formation (Ghigo, 2001; Bhatti et al., 2015, 2017). On the principle, bacterial adhesion thanks to plasmid pili and conjugative transfer of plasmids are two phenomena that might contribute to the formation of local reservoirs of enriched plasmid occurrence, but their relative contributions in a context as complex as a river remain to be elucidated.

In the present work, we investigated the relative occurrence of two MGE proxies for ARGs, namely class 1 integrons and IncP-1 plasmids, in the water column and surface sediments of the Orne River (France) by analyzing raw waters, suspended materials (SMs), and sediments. The results obtained tend to show that bacteria carrying IncP-1 plasmids, and to a lesser extent class 1 integrons, selectively attached on SMs that finally settle to form sediments. *In vitro* experiments were further used to assess the contribution of IncP-1 plasmids to the binding of plasmid-bearing bacteria to artificial supports, and the contribution of bacterial adhesion to plasmid dissemination. All in all, our results demonstrate the major contribution of plasmid-mediated adhesion to particles to the formation of MGE reservoirs in sediments.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this work are presented in **Table 1**. Bacteria were grown aerobically in LB medium (LB Broth Miller, Difco™) at 30°C, with agitation at 160 rpm for liquid cultures. Solid medium was prepared by adding 15 g/L of agar. When required, antibiotic selection was applied at the following concentrations: ampicillin at 100 mg/L, rifampicin at 20 mg/L, and tetracycline at 10 mg/L.

Study Site and River Material Sampling

The Orne is an 86 km long river flowing in Lorraine, tributary of the Moselle in northeastern France, and sub-tributary of the Rhine. The Orne is one of the rivers of the area strongly impacted by ancient mining and iron- and steel-making plants during the 20th century. The Orne River takes its source in a forest area, flows through countryside consisting of fields and meadows, then

TABLE 1 | Bacteria and plasmids used in this study.

Name/species	Genotype/characteristics ^a	References
Plasmids		
pB10	Wild type IncP-1 plasmid isolated from activated sludge	Schlüter et al. (2003)
pBELX	pEX-A derivative (pUC18-based) containing qPCR target sequences for <i>trfA</i> of IncP-1 plasmids, the Eubacterial 16S rRNA gene	Bellanger et al. (2014b)
pNORM1	pEX-A derivative (pUC18-based) containing the qPCR target sequences for <i>int1</i> of class 1 integron, the Eubacterial 16S rRNA gene	Gat et al. (2017)
<i>Bacillus subtilis</i>		
LMG 7135 ^T	ATCC 6051, Type strain	BCCM/LMG ^b
CM291	Rif ^R derivative of LMG 7135 ^T	This work
CM295	CM291(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work
<i>Cupriavidus metallidurans</i>		
AE815	plasmid free and Rif ^R derivative of wild type strain CH34	Springael et al. (1993)
CM124	AE815(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	Bellanger et al. (2014a)
<i>Delftia acidovorans</i>		
CM122	<i>Delftia acidovorans</i> , Rif ^R , Kan ^R	Eva Top lab strain collection
CM294	CM122(pB10), Rif ^R , Kan ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work
<i>Escherichia coli</i>		
DH5α	φ80lacZΔacZerecA1endA1gyrA96 (Nal ^R) <i>thi-1 hsdR17(r_K⁻ m_K⁺) supE44 relA1 deoR Δ(lacZYAargF) U169</i>	Sambrook et al. (1989)
MG1655	Sequenced λ ⁻ and F ⁻ derivative of strain K-12	Blattner et al. (1997)
CM102	DH5α(pB10), Nal ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	Schlüter et al. (2003)
CM125	Nal ^R derivative of MG1655	Bellanger et al. (2014a)
CM278	CM125(pB10), Nal ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work
<i>Pseudomonas fluorescens</i>		
CIP 69.13 ^T	ATCC 13525, Type strain	ATCC ^c
CM292	Rif ^R derivative of wild type strain CIP 69.13 ^T	This work
CM297	CM292(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work
<i>Pseudomonas putida</i>		
SM1443	KT2442 (Rif ^R) with a mini-Tn5- <i>lacI</i> ^q insertion	Christensen et al. (1998)
CM236	SM1443(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work
<i>Shewanella oneidensis</i>		
CM87	Rif ^R derivative of wild type strain MR-1 ^T	Schwalb et al. (2003)
CM293	CM87(pB10), Rif ^R , Amx ^R , Str ^R , Sul ^R , Tet ^R	This work

^aAntibiotic resistance phenotype: Amx^R, amoxicillin; Kan^R, kanamycin; Nal^R, nalidixic acid; Rif^R, rifampicin; Str^R, streptomycin; Sul^R, sulfamides; Tet^R, tetracycline. ^bBelgian coordinated collections of microorganisms. ^cAmerican Type Culture Collection.

a highly urbanized and ancient industrialized area before joining the Moselle River. The latter part represents about one fourth of the river linear and, based on geographical data, it is assumed that the river waters drain between 86 and 100% of the watershed surface (1276 km²), depending on the sampling site considered in this study (see Supplementary Figure 1). The riverbed was strongly modified for industrial purposes. Indeed, two dams were built in the 1958–1965 period and the river was calibrated and channeled in the very last kilometers (Abuhelou et al., 2017; Kanbar et al., 2017). Fifteen pairs of river raw water and SM samples were collected along the last 23 km of the Orne River and over an 11-month period from November 2014 to October 2015 (four pairs on 2014/11/04, three pairs on 2015/02/02, two pairs on 2015/02/03, three pairs on 2015/05/05, and three pairs on 2015/10/06). As previously described (Le Meur et al., 2016), the river water was pumped and sent to a continuous flow field CEPA Z-41 centrifuge (20,000 RPM, equivalent to 17,000 × g, with a 600 L/h flow rate), which was used to collect a representative

SM-enriched fraction from [ca.] 1–2 m³ of raw water. Raw water samples were collected from the pumping outlet (excess flow). The top surface sediments were collected along the same Orne River section at five different places from January 2014 to July 2015. Sediment samples were collected (on 2014/03/18 and 2015/02/19) as short cores using a piston corer or simple coring tubes (diameter of 6 cm or 9 cm). Coring was preferred to grabbing in order to preserve the layered structure of sediments. The collected sediments were sealed from air and transported in a vertical position. The upper 2 cm layer, referred to as surface sediments, was separated from the core using a single use spatula and into an N₂-filled glove bag to avoid oxidation. The surface layer is highly hydrated, and loosely attached to the underneath material. Different aliquots were prepared for distinct purposes. For geochemical analyses, an aliquot was freeze-dried and ground using an agate mortar and pestle. For DNA extraction, all samples were kept frozen at –20°C (short term storage of SMs and raw waters) or –80°C (long term storage of sediments).

Geochemical Analyses on Sediments and Suspended Materials

Major elements were detected by inductively coupled plasma optical emission spectrometry (ICP-OES). These analyses were performed at SARM (Service d'Analyse des Roches et des Minéraux – CRPG, Vandœuvre-lès-Nancy, France) and all analytical methods were subject to QC/QA procedures using certified reference materials (Carignan et al., 2001). Grain size distribution of SMs and bottom sediments was obtained using laser diffraction (SYMPATEC) with two distinct lenses corresponding to two distinct size ranges (0.45–87.5 μm and 4.5–875 μm). Samples were systematically ultrasonicated for 20 s before measurement, and each measurement was duplicated or triplicated. Mineralogy of sediment and SM samples was investigated using X-ray Diffraction and Transmission Electron Microscopy (like in Kanbar et al., 2017). A synthesis of those analyses is available in Supplementary Table 1 and Supplementary Figures 2, 3.

DNA Extraction

Recombinant plasmid DNAs pBELX and pNORM1, used as qPCR standards, were extracted using the “Wizard® Plus SV Minipreps DNA Purification System” (Promega) according to the recommendations provided by the manufacturer. The plasmids were linearized by *Bam*HI (Promega) before being purified with the “QIAquick PCR DNA purification kit” (Qiagen). Total environmental DNAs were extracted using the “PowerWater DNA Isolation Kit” (MO BIO laboratories Inc). Briefly, 50 mg of sediments or SMs were thawed before being dispersed in 100 mL of non-pyrogenic sterile water (Aqua B-Braun) by vortexing for 30 s followed by 15 min stirring at 160 rpm and 25°C. These sediment/SM suspensions (or 100 mL of sample for raw waters) were filtered on polycarbonate filters (Whatman Nuclepore filter, pore size 0.22 μm , diameter 47 mm) using a filtration apparatus (Combisart 6-branch Manifold, Sartorius). Total DNAs were directly extracted from the filters according to the recommendations provided by the manufacturer and were eluted from silica columns with 100 μL of PCR grade water (RNase-Free Water, Qiagen). Plasmid and total DNA concentration and purity were estimated by spectrophotometry according to standard procedures, and all DNAs were stored at -20°C until use.

Quantitative PCR Assays

The abundance of class 1 integrons and IncP-1 α/β plasmids were quantified by qPCR in total environmental DNAs using “Power SYBR® Green PCR Master Mix” (Applied Biosystems) with the primers listed in Table 2. Quantitative PCRs were performed in triplicate using “Step One Plus Real-Time PCR System” (Applied Biosystems, driver: StepOne Software v2.2) in a 25 μL reaction volume with 1 μM of each primer, and with thermocycling conditions set as follows: 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The quality of the PCR products was subsequently checked by melting curve analyses, for which the temperature was ramped between 60 and 95°C in increments of 0.3°C. IncP-1 α/β plasmid and class 1 integron quantitative

results were normalized to the amount of eubacterial 16S rRNA gene, also quantified by qPCR with the 331F/518R universal primers and using the cycling conditions described above. For quantifications of 16S rRNA gene and IncP-1 α/β plasmids and of class 1 integrons, plasmids pBELX and pNORM1, linearized with *Bam*HI, were used as standards, respectively (Table 1). The absence of residual inhibitors in the DNA extracts was checked by qPCR by comparing amplifications from serially diluted DNA templates.

Crystal-Violet Staining of Adhering Biomass

Two milliliters of LB was inoculated with a single colony picked from a fresh plate, containing tetracycline for pB10-carrying bacteria, and then incubated for 16 h. One hundred microliters of this culture was mixed with 10 mL of fresh medium and placed in a 55 mm petri dish containing a 47 mm-diameter flat polyethylene disk (Kaldnes Biochip Media) that are originally dedicated to the colonization of bacteria in moving bed biofilm reactors in wastewater treatment processes. The plates were incubated at 30°C under agitation (80 rpm) for 18 h. The quantity of biomass adhering to Kaldnes polyethylene disks was determined by classical crystal-violet staining (Chavant et al., 2007). First, the total amount of planktonic bacteria surrounding the polyethylene disks was estimated by measuring broth turbidity ($\text{OD}_{600\text{nm}}$). Second, the polyethylene disks were transferred into new petri dishes where they were successively washed three times with 10 mL of sterile water for removing poorly adherent bacteria. The polyethylene disks were then air-dried for 1.5 h before being incubated in 10 mL of a 0.1% v/v crystal violet solution in H_2O (Biomérieux) for 45 min at room temperature ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$). The stained disks were then gently washed six times with sterile water to remove excess of crystal violet. Finally, the biomass-associated crystal violet was dissolved with 10 mL of glacial acetic acid and quantified by UV-Vis spectrophotometry at 540 nm ($\text{OD}_{540\text{nm}}$). Polyethylene disks without exposure to bacterial cells were used as control.

Bacterial Mating Assays

Mating assays were performed in liquid medium either containing or not a 47 mm polyethylene disk (Kaldnes Biochip Media). Donor (CM102) and recipient bacteria (AE815) were grown for 16 h in broth, supplemented or not with antibiotics, then washed by centrifugation and re-suspended in one volume of sterile MgSO_4 (10 mM). For mating assays with a polyethylene disk, 10 mL of LB broth containing a 1:100 dilution of both strains, prepared from the washed cell suspensions, were introduced in an empty 55 mm petri dish and incubated for 18 h at 30°C under agitation (80 rpm). At the end of the incubation period, broth and polyethylene disks were recovered separately for bacterial strain enumeration. Beforehand, the polyethylene disks were washed three times with sterile water and adhering bacteria were re-suspended by vigorous vortexing in 20 mL of MgSO_4 (10 mM). Donor (CM102), recipient (AE815), and transconjugant bacteria [AE815(pB10)] were enumerated on selective plates containing either tetracycline (Tet), rifampicin

TABLE 2 | Primers used in qPCR.

Name	Sequence	Targeted gene	Product size (bp)	Referencec
331F	5'-TCCTACGGGAGGCAGCAGT-3'	16S rRNA	197 bp	Muyzer et al., 1993; Nadkarni et al., 2002
518R	5'-ATTACCGCGGCTGCTGG-3'			
intl1-LC1	5'-GCCTTGATGTTACCCGAGAG-3'	<i>intl1</i> (class 1 integrons)	196 bp	Barraud et al., 2010
intl1-LC5	5'-GATCGGTGCAATGCGTGT-3'			
trfA2-1	5'-CGAAATTCTRTTGGGAGAAGTA-3'	<i>trfA</i> (IncP-1 plasmids)	241 bp	Götz et al., 1996
trfA2-2	5'-CGYTTGCAATGCACCAGGTC-3'			

(Rif), or both antibiotics (Tet Rif), respectively. For the control mating assays between planktonic cells, the incubations of donor and recipient cells were performed as described above but the polyethylene disks were omitted.

Statistical Analyses

Statistical analyses were performed using the R software and presented according to Cumming and collaborators (Cumming et al., 2007). The normality of the data was verified using the Shapiro-Wilks test before performing other statistical tests. The potential pairing between data was also taken into account before carrying out the analyses. For statistical analyses of the data, Wilcoxon signed-rank test, Wilcoxon rank-sum test, Kruskal-Wallis rank-sum test, Student's *T*-test, and Spearman correlation test were independently chosen according to the circumstances, as justified in the corresponding Result subsections.

RESULTS

Occurrence of Class 1 Integrons and IncP-1 Plasmids in the Orne River

The distribution of class 1 integrons and IncP-1 α/β plasmids in the different physical compartments of the Orne River was first investigated so as to point out any selective/specific partitioning. Total community DNA was extracted from 15 raw water samples, 15 corresponding SM samples, and eight surface sediment samples collected over an 11-month period. Their contents in class 1 integrons and IncP-1 α/β plasmids were determined by qPCR and then further normalized to the corresponding 16S rRNA gene content, also determined by qPCR. The relative abundance of each MGE in the three compartments is presented in **Figure 1**.

Globally, the relative abundance of class 1 integrons and IncP-1 α/β appeared to be 2.2 ± 0.4 and 13.3 ± 4.0 times higher in SMs than in the corresponding raw water samples, respectively. This trend was observed in all but 1 of the 15 paired samples. Wilcoxon signed-rank test further confirmed the statistical significance of the MGE enrichment on SM-associated microbial communities with *p*-values of 3.10^{-4} and 6.10^{-5} for class 1 integrons and IncP-1 α/β plasmids, respectively. Despite being both significant, the enrichment effect observed for MGE-bearing bacteria on SMs remains higher for IncP-1 α/β plasmids than for class-1 integrons (Wilcoxon signed rank test; *p* = 2.10^{-4}), which already points out two different bacterial populations with different MGE content characteristics.

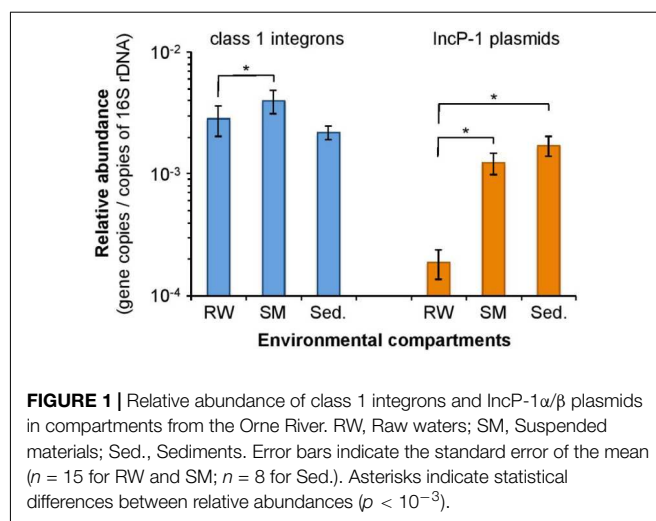


FIGURE 1 | Relative abundance of class 1 integrons and IncP-1 α/β plasmids in compartments from the Orne River. RW, Raw waters; SM, Suspended materials; Sed., Sediments. Error bars indicate the standard error of the mean (*n* = 15 for RW and SM; *n* = 8 for Sed.). Asterisks indicate statistical differences between relative abundances (*p* < 10^{-3}).

The statistical comparison of the MGE abundances obtained for top surface sediments with those obtained for the other compartments requires using a Wilcoxon rank-sum test as sediments were sampled independently from raw waters and SMs, and data were not normally distributed. In this respect, the relative abundance of class 1 integrons in surface sediments did not seem to differ significantly from those of raw waters or SMs (*p* = 0.39 and *p* = 1, respectively). For IncP-1 α/β plasmids though, the relative abundance was 9.1 and 1.4 higher in sediments than in raw waters and SMs, respectively. If these relative abundances were statistically different between sediments and raw waters (*p* = 5.10^{-4}), they remained non-significantly different between surface sediments and SMs (*p* = 0.18). The latter result raises the question of whether plasmid-bearing bacteria from raw waters could selectively be enriched on SMs, which could then settle in the riverbed to form fresh sediments that exhibit a similar IncP-1 plasmid richness, as we shall discuss in subsection 3.2.

The different distributions observed between IncP-1 plasmid- and class 1 integron-bearing bacteria show that MGEs influence their own compartmentalization in aqueous media. Two hypotheses can be made to explain the specific enrichment of IncP-1 plasmids on SMs. First, IncP-1 plasmids are conjugative elements for which it has been suggested that the promiscuity encountered in biofilms favors gene transfer compared to the planktonic bacteria life style (Molin and Tolker-Nielsen, 2003). Considering the fact that conjugative transfer is a replicative event (Lanka and Wilkins, 1995), it can be argued that bacterial

adhesion to SMs leads to biofilm formation, which then favors plasmid transfer and, therefore, results in an increase in its relative abundance. Alternatively, Ghigo (2001) showed that conjugative plasmids themselves could promote the formation of biofilms as they encode for a conjugative pili, a cell appendage also considered as an adhesion substrate. In such a case, the IncP-1 plasmid-bearing fraction of the bacterial population should be more likely to bind to the organo-mineral particles constituting SMs than the plasmid-free bacteria, which should lead to a compartmentalization of the plasmid-bearing bacteria over time. The two hypotheses, “adhesion-promoted plasmid transfer” and “plasmid-promoted adhesion” are not exclusive, but their relative contributions remain to be elucidated.

Mineral Relationship Between Sediments and SMs of the Orne River

If IncP-1 plasmid-bearing bacteria were to be selectively enriched on SMs before particles settle and form sediments, a parent-like relationship between SMs and surface sediments is to be expected. In such a case, SMs and top surface sediments should share common mineral properties demonstrating their relationship in terms of size distribution, element composition, and mineral constituents (Supplementary Table 1 and Supplementary Figures 2, 3). SMs and sediments are predominated by silicates, in particular clay minerals. SM particles are relatively fine with a grain size distribution centered below 10 μm , and the decile (D50) of Orne River suspended particles was estimated to be $12 \pm 3 \mu\text{m}$. The grain size distribution measurements evidenced that sediments are coarser than SMs, suggesting a size sorting upon settling and/or a post-settlement modification of the grain size distribution, as reported elsewhere (Lartiges et al., 2001; Droppo et al., 2009). This modification can be linked to a slightly higher carbon content in sediments ($4.3 \pm 2\%$ and $4.9 \pm 0.7\%$ for SMs and sediments, respectively) as organic matter is known to enhance particle aggregation. All in all, the element composition and the mineralogy clearly draw a parent-like relationship between SMs and surface sediments. Although slight differences remain, they can be attributed to both successive settling events and post-settlement modifications. Yet, a parental-like relationship between SMs and sediments can be interpreted as particle settling, as sediment mobilization, or as both.

Due the locations of the sampling stations (Supplementary Figure 1), the SMs transported by the Orne River are likely to be alternatively predominated by runoff-generated particles during rain events (or high water discharge periods), and by urban inputs and primary production in the water column during low-water discharge periods. The relative contribution of sediment remobilization as SMs is driven by different parameters including hydroclimatic conditions and river characteristics (class, width, flood bed status, sediment flow rate, etc.). This contribution is considered to be significant during specific periods of hydrological sequences, i.e., during the early stages of flood events occurring after a long low-flow period (typically during the fall season). In the studied case, most of the samples were collected in the middle or late stages of flood events (see Supplementary

Figure 1), thus it can be considered that the origin of SMs sampled in the water column was predominated by particles resulting from soil leaching. In the Orne watershed, 67% of the surface is dedicated to agriculture, enhancing the generation of suspended particles during rain events (Le Meur et al., 2016). Thus, although we cannot fully demonstrate the absence of sediment remobilization, its contribution is considered to be minor in such water discharge conditions. Mineralogy, particle size distribution, and element contents clearly draw a parental-like link between sediments and SMs. Thus, these similarities are explained by the SM settling process, which mostly occurs at the end of the flood events, i.e., when water flow is decreasing.

The Carriage of an IncP-1 Plasmid Has Contrasted Effects on Bacterial Adhesion

Considering the selective enrichment of IncP-1 plasmid-bearing bacteria on SMs and sediments, we wondered if the mere fact of bearing this kind of MGEs was sufficient to increase cell adhesion to surfaces compared to plasmid-free bacteria. The phenotypic effect of bearing an IncP-1 plasmid was investigated using a series of isogenic bacterial strains from various species carrying or not the natural IncP-1 β plasmid pB10 (Schlüter et al., 2003). Bacteria were allowed to grow as biofilm on Kaldnes polyethylene disks immersed in LB medium for 18 h, resulting in a large excess of planktonic cells. The abundance of planktonic bacteria (PB) was estimated at 600 nm (OD_{600}) by spectrometry, while the abundance of the adhering biomass was measured using a classical crystal violet staining method [(CV); OD_{540}] (Table 3). The propensity to form biofilms was finally estimated as the relative abundance of adhering cells compared to planktonic bacteria, here given in arbitrary units [(CV)/(PB)]. The results obtained (Table 3) showed that the carriage of pB10 significantly increased the relative amount of adhering bacteria on polyethylene surfaces (Student's *T*-test for paired data, $p = 5.10^{-3}$). Apart from the outlier strains *Escherichia coli* MG1655 and *Shewanella oneidensis* MR-1 that seem to have peculiar behaviors, the effect of pB10 carriage on adhesion is stronger when the basal level of biofilm formation is initially low in the absence of plasmid (Spearman correlation without considering strains MG1655 and MR-1: $r = -0.943$, $p = 0.02$) (Figure 2). It should be noted that the different adhesion behaviors observed for the different strains could not be attributed to a strain-dependent instability of the plasmid. This was estimated by plating overnight cultures grown without antibiotics on plates selective or not for pB10-bearing bacteria. Plasmid pB10 appeared to be highly stable as no significant loss could be observed in all the bacterial hosts used (Kruskal-Wallis rank sum test, $p = 0.99$), and as it had already been thoroughly assessed before for most of the bacteria and/or strains used in this work (De Gelder et al., 2007). Putting these observations back in the context of the plasmid enrichment observed on the SMs and sediments of the Orne River, it tends to demonstrate that (i) IncP-1 plasmid carriage is sufficient to promote the compartmentalization of plasmid-bearing bacteria on SMs and thus on freshly deposited sediments, and (ii) the gain in adhesion

TABLE 3 | Effect of pB10 carriage on bacterial adhesion properties.

Strains/species	Planktonic biomass (OD _{600nm})		Adhering biomass (OD _{540nm})		Relative biofilm amount (arbitrary unit: OD _{540nm} /OD _{600nm})		
	Without pB10	With pB10	Without pB10	With pB10	Without pB10	With pB10	Fold increase
<i>E. coli</i> DH5α	3.205 ± 0.032	3.686 ± 0.038	0.323 ± 0.072	1.101 ± 0.129	0.10 ± 0.02	0.30 ± 0.03	3.18 ± 1.04
<i>E. coli</i> MG1655	4.669 ± 0.219	4.960 ± 0.388	0.254 ± 0.047	0.309 ± 0.065	0.05 ± 0.01	0.06 ± 0.02	1.15 ± 0.09
<i>C. metallidurans</i>	3.251 ± 0.136	3.271 ± 0.313	0.530 ± 0.152	0.898 ± 0.483	0.16 ± 0.04	0.28 ± 0.16	1.70 ± 1.04
<i>B. subtilis</i>	3.988 ± 0.357	3.700 ± 0.056	0.372 ± 0.045	0.864 ± 0.222	0.10 ± 0.02	0.23 ± 0.06	2.53 ± 0.64
<i>P. putida</i>	5.787 ± 0.211	5.047 ± 0.220	2.361 ± 0.405	2.838 ± 0.092	0.41 ± 0.08	0.56 ± 0.03	1.44 ± 0.33
<i>P. fluorescens</i>	3.401 ± 1.238	2.715 ± 1.522	0.513 ± 0.047	0.591 ± 0.026	0.17 ± 0.06	0.30 ± 0.15	1.64 ± 0.38
<i>S. oneidensis</i>	4.985 ± 1.317	4.719 ± 1.142	0.503 ± 0.150	0.419 ± 0.200	0.12 ± 0.08	0.11 ± 0.08	0.82 ± 0.09
<i>D. acidovorans</i>	4.501 ± 0.488	3.634 ± 0.915	2.664 ± 0.384	2.294 ± 0.928	0.60 ± 0.12	0.67 ± 0.21	1.09 ± 0.14

Values are expressed as a mean ± standard error ($n = 3$).

is not equally distributed among bacterial strains, which should also contribute to compartmentalize bacteria according to their nature.

Adhesion to Polyethylene Disks Does Not Increase the Transfer Frequency of Plasmid pB10

Considering the fact that cell-cell contacts, required for conjugation, may be favored in biofilm and that conjugation is an intercellular mode of DNA replication, an increased frequency of transfer in biofilm may also explain enrichment of IncP-1 plasmid on SMs. This hypothesis was tested by comparing the transfer efficiency of plasmid pB10 among both planktonic bacteria and surface-associated cells. The donor bacteria *E. coli* DH5α(pB10) and the recipient strain *Cupriavidus metallidurans* AE815 were mixed and incubated for 18 h in LB medium in the presence

or absence of Kaldnes polyethylene disks. Donor, recipient, and transconjugant cells were then enumerated independently from (i) the disk-free mating experiment for assessing pB10 transfer in planktonic conditions, and (ii) directly from the Kaldnes polyethylene disks for assessing pB10 transfer in biofilm. When comparing the mating conditions (planktonic versus biofilms), transfer frequencies, expressed either as transconjugant per recipient or transconjugant per donor (Figure 3), did not appear significantly different (Kruskal–Wallis rank sum test; $p > 0.05$). Moreover, donor, recipient, and transconjugant cells were also enumerated in the liquid medium surrounding the Kaldnes polyethylene disks. The corresponding pB10 transfer frequencies were similar to that observed for the bacteria growing as a biofilm on the disks (Wilcoxon signed rank test; $p > 0.1$). Therefore, it is unlikely that the calculation of transfer frequencies were biased by transconjugants accumulating from the liquid to the biofilm or standing out from the disks to the liquid. All in all, it thus appears that, under the conditions provided by the tests carried out on polyethylene disks, development as biofilm does not promote a higher transfer level of pB10. Therefore, between the two formulated hypotheses, the enrichment of plasmid-bearing bacteria on SMs and sediments is likely to be dominated by the adhesion properties brought by conjugative plasmids and their conjugative pili.

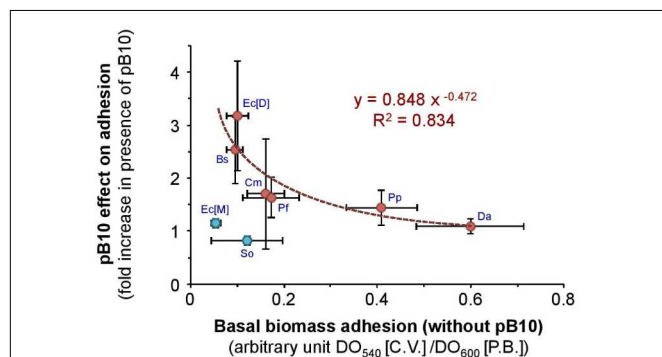


FIGURE 2 | Gain in biofilm formation for pB10-bearing bacteria. Biofilm formation was compared for isogenic strains with and without the IncP-1β plasmid pB10: *B. subtilis* CM291 (Bs); *C. metallidurans* AE815 (Cm); *D. acidovorans* (Da); *E. coli* DH5α (Ec[D]), *E. coli* MG1655 (Ec[M]); *P. fluorescens* CM292 (Pf); *P. putida* SM1443 (Pp); *S. oneidensis* CM87 (So). Error bars indicate the standard error of the means ($n = 3$). The dotted line represents a regression curve related to a Spearman correlation without considering strains MG1655 and MR-1 showing that the effect of pB10 carriage on adhesion is stronger as the basal level of biofilm formation is initially low in the absence of plasmid ($r = -0.943$, $p = 0.02$). The equation of the regression curve is indicated.

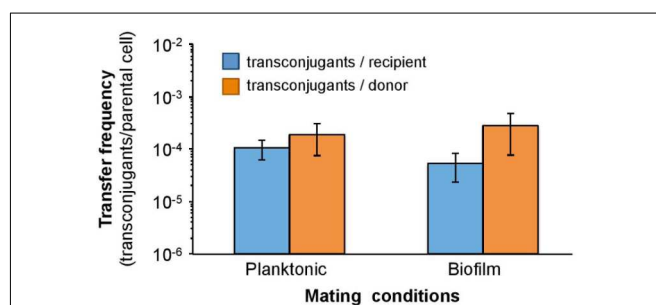


FIGURE 3 | Transfer of pB10 among planktonic and polyethylene disk-adhering bacteria. Error bars indicate the standard error of the means ($n = 4$). There is not any statistical difference between the presented transfer frequencies.

DISCUSSION

With their very close association with ARGs, MGEs undoubtedly play a central role in the dissemination of antibiotic resistance (Frost et al., 2005). Yet, demonstrating such dissemination in environmental settings remains relatively challenging as there is no easy way to evaluate the relative contribution of well-known mechanisms when combined to the complexity and heterogeneity of the environment (Elsas et al., 2002; Bellanger et al., 2014a). Occurrence-based studies have brought to light the existence of environmental reservoirs of ARGs and MGEs, suggesting the existence of environmental hotspots of ARG dissemination (Martinez, 2009; Wright, 2010; Perry and Wright, 2013). Without questioning the simple fact that ARGs can disseminate thanks to MGEs, we demonstrated with plasmid pB10 that physico-chemical interactions of bacteria with particle surfaces could preferentially promote the adhesion of IncP-1 plasmid-bearing bacteria without increasing the plasmid transfer frequency. This also means that, in the environmental context, the biofilm mode of life or adhesion to particles may also act as a “selective enrichment process” for ARGs associated with conjugative plasmids, leading to local reservoirs, even in the absence of any selective antibiotics.

The compartmentalization of IncP-1 plasmid-bearing bacteria observed in the context of the Orne River fits well the spatial clustering reported in a few metagenomic studies showing that numerous genes encoding functions associated with horizontal gene transfer and MGEs, among which type IV secretion systems (conjugation machineries), are statically overrepresented in marine bacteria attached to organo-mineral particles or algae (Burke et al., 2011; Allen et al., 2013; Ganesh et al., 2014). The authors of these works suggest that this enrichment could be due to peculiar conditions encountered in sediments and on SMs (high cell density, probable nutrient richness, shelter effect, etc.) that would promote horizontal gene transfer. In this respect, bacterial biofilms have frequently been described as hot spots of conjugative transfers (Molin and Tolker-Nielsen, 2003; Aminov, 2011; Skippington and Ragan, 2011; Madsen et al., 2012). However, to the best of our knowledge, this alternative has never been challenged experimentally by possible enrichment of plasmid-bearing bacteria due to their adhesion to mineral particles or organo-mineral aggregates.

Slight enrichment in class 1 integrons of environmental bacteria attached on the organo-mineral particles forming SMs from the Orne River by comparison to bacteria from raw waters has also been reported. To our knowledge, such a spatial clustering of class 1 integrons in an environmental compartment had never been reported. Class 1 integrons are frequently described as being hosted by IncP-1 plasmids (Popowska and Krawczyk-Balska, 2013) and as such may then show a similar compartmentalization. However, in complete genome databases, while 67% of integrons could be described as mobile (i.e., integrons associated with MGEs), only 12% of integrons (mostly class 1 integrons) appeared to be plasmid borne (Cury et al., 2016). This moderate association with plasmids could explain why class 1 integron-bearing bacteria of the Orne River do not follow the same distribution as that of the IncP-1 plasmid-bearing

bacteria if the adhesion to particles was to play a central role in the compartmentalization of MGEs.

Here, we described one situation, in which the transfer of plasmid pB10 is not significantly enhanced in biofilm compared to planktonic cells. This observation is in line with a few studies showing that the transfer of conjugative plasmids in bacterial populations structured as biofilm is often limited to the donor-recipient interfaces, where bacteria are most physiologically active (Stalder and Top, 2016 and references therein). Thus, even if highly efficient in biofilm, the ability of a plasmid to transfer to the neighboring cells can appear very localized and may go unnoticed if the active recipient-donor interface represents a minute part of a well developed biofilm as it is often the case (Stalder and Top, 2016 and references therein).

With this study, we showed that IncP-1 plasmid-bearing bacteria are significantly enriched on SMs and surface sediments of the Orne River, and we provided a case study with pB10 where the carriage of plasmid can promote the adhesion of bacteria, while the adhesion to surfaces does not seem to promote significantly the transfer of the plasmid. Future efforts will focus on investigating the occurrence of class 1 integrons and IncP-1 plasmids in deeper sediment layers in order to get an insight into their abundance as a function of the local physical chemistry of the sediments, in order to determine the driving parameters associated with local MGE enrichment.

Whatever the main driving phenomenon, i.e., selective attachment of plasmid-bearing bacteria or increase in plasmid transfer in adhering bacteria, enrichment of MGEs on SMs likely means ARG/ARB enrichment as well. Considering that river SMs participate in sediment formation, this work pinpoints the role that sediments could play as ARG, ARB, and MGE reservoirs. Indeed, in the studied case presented here, SMs can be assumed as the finest fraction of sediments, predominated by clay minerals. Clay and other minerals confer to those river materials high potential to bind to organic matter and microorganisms. Thus, studying the ability of various SM physicochemical surfaces such as phyllosilicates or carbonates in being differentially colonized by ARG/MGE-free or -bearing bacteria would be of interest to predicting whether a given environment is likely to be permissive for the enrichment of MGEs and their ARGs. Ultimately, river sediments can be considered as a compartment where ARGs can accumulate and disseminate by means of MGEs to environmental and pathogenic bacteria. Numerous European rivers have been polluted, channeled, and fragmented by dams, sills, hydroelectric power stations, mills, and other obstacles. This leads to a dramatic reduction in biodiversity and water quality as well as massive disappearance of the associated wetlands, essential for the prevention of floods, and excessive sediment accumulation on riverbeds. The EU Water Framework Directive promotes river restoration and improvement of both chemical and biological water quality, notably by removing dams and other hindrances. These removal operations associated with more and more frequent hydroclimatic events and severe floods resulting from climate change will lead to remobilization of sediments and their associated content in chemical and biological pollutants such as ARGs, ARB, and MGEs. The consequences

on public health of these sediment remobilizations remain an open question that should be addressed in future works/research programs.

AUTHOR CONTRIBUTIONS

XB, EM-P, and CM contributed substantially to the conception, the design, and the supervision of the study. MCB and HG performed experiments. MCB, HG, CM, and XB interpreted the results. XB and CM prepared the manuscript with input from the other co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.01443/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3. Cochran's table

k	Level of significance $\alpha = 0.05$														V_x
	1	2	3	4	5	6	7	8	9	10	16	36	144	∞	
2	0.9985	0.9750	0.9392	0.9057	0.8772	0.8534	0.8332	0.8159	0.8010	0.7880	0.7341	0.6602	0.5813	0.5000	
3	0.9669	0.8709	0.7977	0.7457	0.7071	0.6771	0.6530	0.6333	0.6167	0.6025	0.5466	0.4748	0.4031	0.3333	
4	0.9065	0.7679	0.6841	0.6287	0.5895	0.5598	0.5365	0.5175	0.5017	0.4884	0.4366	0.3720	0.3093	0.2500	
5	0.8412	0.6838	0.5981	0.5441	0.5065	0.4783	0.4564	0.4387	0.4241	0.4118	0.3645	0.3066	0.2513	0.2000	
6	0.7808	0.6161	0.5321	0.4803	0.4447	0.4184	0.3980	0.3817	0.3682	0.3568	0.3135	0.2612	0.2119	0.1667	
7	0.7271	0.5612	0.4800	0.4307	0.3974	0.3726	0.3535	0.3384	0.3259	0.3154	0.2756	0.2278	0.1833	0.1429	
8	0.6798	0.5157	0.4377	0.3910	0.3595	0.3362	0.3185	0.3043	0.2926	0.2829	0.2462	0.2022	0.1616	0.1250	
9	0.6385	0.4775	0.4027	0.3584	0.3286	0.3067	0.2901	0.2768	0.2659	0.2568	0.2226	0.1820	0.1446	0.1111	
10	0.6020	0.4450	0.3733	0.3311	0.3029	0.2823	0.2666	0.2541	0.2439	0.2353	0.2032	0.1655	0.1308	0.1000	
12	0.5410	0.3924	0.3264	0.2880	0.2624	0.2439	0.2299	0.2187	0.2098	0.2020	0.1737	0.1403	0.1100	0.0833	
15	0.4709	0.3346	0.2758	0.2419	0.2195	0.2034	0.1911	0.1815	0.1736	0.1671	0.1429	0.1144	0.0889	0.0667	
20	0.3894	0.2705	0.2205	0.1921	0.1735	0.1602	0.1501	0.1422	0.1357	0.1303	0.1108	0.0879	0.0675	0.0500	
24	0.3434	0.2354	0.1907	0.1656	0.1493	0.1374	0.1286	0.1216	0.1160	0.1113	0.0942	0.0743	0.0567	0.0417	
30	0.2929	0.1980	0.1593	0.1377	0.1237	0.1137	0.1061	0.1002	0.0958	0.0921	0.0771	0.0604	0.0457	0.0333	
40	0.2370	0.1576	0.1259	0.1082	0.0968	0.0887	0.0827	0.0780	0.0745	0.0713	0.0595	0.0462	0.0347	0.0250	
60	0.1737	0.1131	0.0895	0.0765	0.0682	0.0623	0.0583	0.0552	0.0520	0.0497	0.0411	0.0316	0.0234	0.0167	
120	0.0998	0.0632	0.0495	0.0419	0.0371	0.0337	0.0312	0.0292	0.0279	0.0266	0.0218	0.0165	0.0120	0.0083	
∞	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

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RÉSUMÉ

Introduction :

L'usage et mésusage des antibiotiques en médecine humaine et vétérinaire ont conduit à l'émergence et à la dissémination de bactéries résistantes aux antibiotiques (BRA) et de leur contenu en gènes de résistance aux antibiotiques (GRA). Ce phénomène a atteint une telle ampleur que la perte du potentiel thérapeutique qui lui est associé est devenu un vrai problème de santé publique à l'échelle mondiale, et certains auteurs parlent déjà d'un quasi-retour à une ère pré-antibiotique.

Alors que les stations de traitement d'eau usée (STEP) ont été identifiées comme des sources majeures de BRA et de GRA vers l'environnement, le devenir de ces polluants biologiques une fois rejetés reste méconnu bien que plusieurs études démontrent leur accumulation dans des écosystèmes tels que les sols et les rivières. Cependant, comprendre le devenir des GRA dans les différents compartiments d'un écosystème donné est crucial pour identifier les réservoirs environnementaux, et ainsi améliorer la gestion des environnements récepteurs afin de limiter le risque de dissémination des GRA.

La dissémination des GRA dans les communautés microbiennes environnementales est facilitée par leur association avec des structures génétiques, nommées éléments génétiques mobiles (EGM), leur permettant d'être transférés horizontalement entre bactéries. Puisqu'un même EGM peut être impliqué dans la dissémination de très nombreux GRA, certains EGM spécifiques ont été utilisés en tant que « proxy » permettant de suivre l'abondance globale en GRA par des méthodes moléculaires. Les intégrons de classe 1, jusqu'ici impliqués dans la capture de plus de 130 GRA différents, et les plasmides conjugatifs IncP-1 à large spectre d'hôtes sont de tels proxys.

Plusieurs études ont montré que les ARG s'accumulent également dans des environnements pollués par des métaux et/ou des contaminants chimiques, et des hypothèses reposant sur des processus de co-sélection ont dès lors été proposées. Que les EGM puissent effectivement conférer un avantage sélectif aux bactéries envers d'autres polluants, ou bien que d'autres polluants puissent induire la dissémination d'EGM restent des questions ouvertes.

Objectifs :

Cette étude se focalise sur le devenir de deux EGM, les intégrons de classe 1 et les plasmides conjugatifs IncP-1, dans différents compartiments de l'écosystème de la rivière Orne. Cette rivière, située au Nord de la Lorraine (France), est l'environnement récepteur de divers polluants d'origine urbaine (ex : effluents de STEP), industrielle (métallurgie/sidérurgie : métaux, hydrocarbures aromatiques polycycliques, ...) et agricoles (pesticides, ...). Combinée à une analyse de près de 100 paramètres géochimiques et globaux réalisée par nos partenaires scientifiques, nous avons initié une étude détaillée ayant pour but de localiser les possibles zones d'accumulation d'EGM dans différents compartiments de l'écosystème rivière (eau, matières en suspension et sédiments) et de corréliser leur présence avec (i) la géochimie locale, (ii) les pressions anthropiques locales telles que les STEP, ou (iii) la présence concomitante de polluants particuliers et de leurs effets modulateurs sur les fonctions de dissémination des EGM.

Matériels et Méthodes :

L'Orne est une rivière de 85,8 km, affluent de la Moselle et sous-affluent du Rhin, coulant dans le Nord-Est de la France, en Lorraine. Durant une période de 15 mois, plusieurs campagnes d'échantillonnage ont été réalisées le long de tronçon de rivière de 4,3, 10 et 23 km. Ainsi, de l'eau brute, des matières particulaires en suspension (MES), des sédiments de surface, des carottes de sédiments et les effluents d'une STEP locale ont été prélevés.

Les échantillons d'eau ont été collectés en différents points de prélèvement répartis le long de la rivière et en surface de la colonne d'eau, sauf lors de trois campagnes dédiées à des analyses de transects lors desquelles de l'eau a également été prélevée à différentes profondeurs et différentes positions par rapport à la berge et au lit de la rivière. Les échantillons de MES ont été collectés sur site grâce à l'utilisation d'une centrifugeuse de terrain à flux continu ($17000 \times g$, $600 \text{ L} \cdot \text{h}^{-1}$) à partir d'environ 500 L d'eau. Deux carottes de 50,5 et 129,5 cm de long ont été prélevées directement des bancs de sédiments grâce à un carotteur à piston avant d'être protégées de l'air par scellement et transportées au laboratoire pour découpage en tranches d'environ 2-3 cm en boîte à gants remplies d'azote. Des sédiments de surface

supplémentaires ont été collectés par raclage de la couche superficielle de sédiments (0-0,5 cm) fraîchement déposée.

Les caractérisations générale et physicochimique de tous les différents échantillons ont été réalisées au Laboratoire interdisciplinaire des environnements continentaux (LIEC, UMR7360 CNRS-UL) et inclues plus de 100 paramètres, dont : la teneur en eau, la granulométrie, le pH, les analyses élémentaires incluant la teneur en métaux (majeurs, traces et terres rares), la teneur en hydrocarbures aromatiques polycycliques, ...

Les abondances absolues (nombre de copies / mL) en intégrons de classe 1 et plasmides de type Incp-1 ont été estimées par PCR quantitative (qPCR) sur des extraits d'ADN totaux de communautés microbiennes. L'abondance absolue en gène de l'ARN ribosomique eubactérien 16S (ADNr 16S) a également été quantifiée par qPCR afin de pouvoir exprimer les quantités d'EGM en abondance relative (copie d'EGM / copie d'ADNr 16S) et pour avoir un estimateur de la taille globale de la population bactérienne dans les échantillons.

Résultats et Discussion :

Les abondances en intégrons de classe 1 et en plasmides de type IncP-1 dans les eaux de l'Orne ont été déterminées par qPCR à partir d'échantillons collectés en différents sites répartis le long de l'Orne. Les abondances absolues et relatives en EGM et ADNr 16S ont tout d'abord été suivies à différent temps dans deux transects de rivière (durant 3 campagnes de prélèvement effectuées en mars 2015) afin de déterminer quelle est la contribution de la procédure expérimentale (extraction d'ADN, qPCR) et celle de l'environnement naturel (date de prélèvement, site de prélèvement, position de prélèvement par rapport à la berge/au lit de la rivière, profondeur de prélèvement) sur la fluctuations de ces abondances en marqueurs moléculaires. Des analyses statistiques basées sur des ANOVA nichées et mixées ont permis de démontrer que pour les abondances absolues et relatives des EGM : (i) aucune des variations observées n'implique de biais méthodologiques (extraction d'ADN ou qPCR) et que (ii) la position de prélèvement par rapport à la berge/au lit de la rivière, la profondeur de prélèvement ou le site de prélèvement n'impactent pas

significativement les résultats obtenus. Seule la date de prélèvement semble infléchir les abondances (absolues et relatives) des EGM et les populations globales de bactéries (ADNr 16S), suggérant que l'hydrodynamique de la rivière pourrait avoir une influence importante.

Dans cette étude, l'absence d'impact anthropique évident sur notre écosystème a été bien illustrée par le cas de la STEP. Bien que les effluents de STEP peuvent présenter des abondances absolues et relatives des deux EGM significativement plus élevées que celles mesurées dans l'eau de rivière en amont de la STEP, ils ne semblent pas influencer les abondances en EGM de la rivière puisque celles-ci demeurent comparables en aval et en amont de la STEP. Cette absence d'effet a été attribuée à la dilution des effluents dans l'eau de rivière elle-même, montrant ainsi que, dans des régions non-affectées par la rareté en eau, l'effet des STEP sur l'abondance des EGM n'est pas facile à démontrer (d'un point de vue quantitatif). Plus généralement l'effet de pressions anthropiques n'a pas pu être ponctuellement aux abords de sites urbains ou industriel. Ce n'est que sur de longs tronçons de rivière (23 km) qu'une augmentation de l'occurrence en intégrons a pu être démontrée. Ceci tend à montrer que l'entrée des intégrons de classe 1 dans l'écosystème de l'Orne survient plutôt de manière diffuse. Les plasmides IncP-1, quant à eux, ont une abondance qui fluctue indépendamment des intégrons, démontrant donc une origine probablement différente.

Afin d'étudier la compartimentalisation des EGM dans l'Orne, 15 paires d'échantillons d'eau brute et de MES correspondant ont été prélevées sur une période de 11 mois sur différents sites répartis le long de la rivière. Une analyse statistique, basée sur le test des rangs signés de Wilcoxon, a démontré que les abondances relatives des deux EGM étaient significativement plus élevées dans les MES que dans l'eau brute, soient $2,2 \pm 0,4$ fois plus pour les intégrons de classe 1 et $13,3 \pm 4,0$ fois plus pour les plasmides IncP-1 (moyenne \pm erreur standard). Afin de déterminer le devenir des bactéries portant des EGM lorsqu'elles adhèrent aux MES après dépôt dans le lit de la rivière, le contenu en EGM a également été mesuré dans des échantillons de sédiments de surface. Des analyses statistiques ont aussi été effectuées pour comparer les abondances relatives en EGM des sédiments à celles de l'eau brute et des MES. Le test de la somme des rangs de Wilcoxon a montré que l'abondance

relative en intégrons de classe 1 dans les sédiments de surface n'était pas différente de celles de l'eau brute ou des MES ($P=0,39$ et $P=1$, respectivement). A l'inverse, l'abondance relative en plasmides IncP-1 était respectivement 9,1 et 1,4 fois supérieure dans les sédiments de surface que dans l'eau brute et les MES. Ces différences sont statistiquement significatives entre les sédiments de surface et l'eau brute ($P=5 \times 10^{-4}$), mais ne le sont pas entre les sédiments de surface et les MES ($P=0,18$).

La différence entre les abondances relatives en plasmides IncP-1 des eaux brutes et des MES et leur similarité entre les sédiments de surface et les MES soulève les questions (i) du rôle des plasmides dans leur enrichissement dans les bactéries associées aux MES par rapport aux bactéries libres des eaux brutes et (ii) du lien entre cet enrichissement et la similarité de MES et des sédiments de surface. Le fort enrichissement en plasmides IncP-1 dans les MES pourrait être expliqué par le fait que les plasmides conjugatifs codent pour la synthèse d'un pilus conjugatif qui peut aussi avoir un rôle d'adhésine de surface. En effet, en utilisant *in vitro* des cultures pures de diverses espèces bactériennes, nous avons pu montrer qu'une capacité d'adhésion accrue des cellules portant un plasmide IncP-1 β naturel (pB10) prévaut sur l'hypothétique augmentation de la fréquence de transfert conjugatif de ce plasmide biofilm. En supposant raisonnablement que les MES enrichis en EGM se déposent dans la rivière pour former des sédiments, nous avons émis l'hypothèse que le compartiment sédimentaire pourrait être un important réservoir de GRA qui pourraient éventuellement être disséminés par les EGM.

Deux carottes de sédiments (nommées JOSAN et BETH, en accord avec leurs sites de prélèvement), présentant différentes origines et niveaux de pollution, ont été analysées en fonction de la profondeur des couches sédimentaires (épaisses d'environ 2cm) et de leurs caractéristiques géochimiques. Les carottes de sédiments ont été échantillonnées relativement proche l'une de l'autre (4 km) afin d'évaluer le possible impact de la STEP localisée entre les deux sites de prélèvement. En parallèle, une attention particulière a été portée aux méthodes moléculaires utilisées, plus précisément aux deux protocoles d'extraction d'ADN, dont une différence notable est la quantité d'échantillon sédimentaire utilisé pour extraire l'ADN de communautés (50 vs. 250 mg). L'utilisation de l'un ou l'autre de ces protocoles est apparue

avoir un fort impact sur la nature des résultats obtenus puisque, même si certaines conclusions peuvent être communes, chacun d'entre eux fournit également des informations spécifiques. Si les plasmides IncP-1 ont toujours été quantifiés dans les premières couches de sédiments, ensuite, en fonction de la série d'extraction considérée, leur quantité varie sans tendance nette observable (quantité parfois faible et souvent sous la limite de détection), réduisant donc fortement la possibilité d'analyses plus poussées. Le travail a donc été poursuivi avec les intégrons de classe 1 seuls. Nous avons noté, lorsque le protocole basé sur de petites quantités de sédiment a été utilisé, que les replicata de quantifications absolues en ADNr 16S et intégrons de classe 1 dans une même couche de sédiment présentaient une grande variabilité. Même si ces variations entre replicata sont apparues réduites lorsque les intégrons de classe 1 ont été considérés par leur abondance relative, des augmentations très localisées et significatives ont pu être observées dans des zones discrètes où les bactéries porteuses de ces EGM semblent former de riches microréservoirs. Par ailleurs, dans les deux carottes de sédiments, nous avons observé que les abondances relatives en EGM dans les premières couches de sédiments sont relativement homogènes en comparaison du reste des carottes. Cela nous a amené à formuler l'hypothèse que les microréservoirs de EGM pourraient résulter d'une évolution et/ou adaptation différente(s) des communautés bactériennes aux conditions physico-chimiques des différentes couches sédimentaires et à une échelle très fine. La raison d'une telle variabilité (ou absence de variabilité) des abondances relatives n'est pas réellement connue mais, considérant que la carotte de BETH présente des niveaux élevés en polluants, il serait intéressant d'établir si ces polluants promeuvent l'apparition de la variabilité constatée entre des abondances relatives en intégrons de classe 1 et en bactéries portant ces EGM.

Les variations observées ont été explorées plus avant grâce à des analyses statistiques ayant pour objectifs d'établir les éventuelles relations entre les abondances relatives en intégrons de classe 1 et des paramètres globaux et contenus élémentaires (éléments majeurs, traces et terres rares). Afin de chercher ces éventuelles corrélations, les valeurs utilisées ont été celles issues du protocole extrayant 250 mg de sédiments dans le but de réduire la variabilité des mesures de quantification (effet microréservoirs) et de se rapprocher le plus

possible des quantités de sédiments utilisées pour les caractérisations physico-chimiques. D'une part, des corrélations positives entre les éléments majeurs K, Na, Ti, les lanthanides légers (La, Ce, Nd, et Pr) et l'abondance relative en ADNr 16S avec l'abondance relative en intégrons de classe 1 ont été mises en évidence. Ces éléments chimiques sont les constituants principaux des particules minérales présentes dans les carottes de sédiments, et leur présence suggèrent que ces particules minérales sont issues de l'érosion du sol du bassin versant. D'autre part, les concentrations en certains éléments traces tel que Fe, V et aussi Pb, qui en fait ne sont pas présents sous forme de « trace » si l'on considère ces hautes teneurs dans la carotte de BETH (pollution sidérurgique), se sont révélées négativement corrélées avec les abondances absolues des intégrons de classe 1 et de l'ADNr 16S. Ces corrélations négatives avec le plomb suggèrent une toxicité globale de cet élément envers les bactéries porteuses ou non d'intégrons de classe 1. D'autres corrélations ont été révélées avec des éléments majeurs, traces et terres rares mais déterminer leur signification n'est pas évidente dans la mesure où il est difficile d'en faire des marqueurs anthropogéniques et/ou spécifiques de la rivière Orne.

Évaluer si du transfert horizontal de gène *in situ* pourrait être impliqué dans la formation des microréservoirs de EGM détectés dans les deux carottes de sédiments est actuellement très difficile. Quoiqu'il en soit, l'existence de tels réservoirs est alarmant dans le contexte de la modification des lits de rivières (utilisation des sols) ou du changement climatique (fréquence des crues en augmentation) qui peuvent tous deux engendrer une forte mobilisation de dépôts sédimentaires avec le risque d'exposer davantage les installations et populations plus en aval à des bactéries riches en EGM.

Conclusions :

Ce travail avait pour objectifs de déterminer le devenir de deux EGM utilisés comme proxys des gènes d'antibiorésistance, les intégrons de classe 1 et les plasmides conjugatifs IncP-1, dans différents compartiments de l'écosystème de la rivière Orne.

Tout d'abord, nous avons notamment montré que la STEP se déversant dans l'Orne entre Joeuf et Moyeuve-Grande n'avait pas d'impact mesurable en terme d'abondances relative ou absolues en nos deux EGM. Nous avons également vu que les sources d'intégrons dans l'Orne semblent diffuses puisque leur quantité s'accroît globalement d'amont en aval et apparaît sensible au lessivage des sols durant les pluies. Les plasmides conjugatifs IncP-1, quant à eux, semblent être endogènes à l'écosystème Orne puisque leur quantité n'est que très peu sensible aux conditions hydroclimatiques.

Ensuite, les deux EGM étudiés sont apparus comme étant plus abondants dans les bactéries adhérant aux matières en suspension par comparaison aux bactéries libres de l'eau de l'Orne. Les fonctions accessoires d'adhésine des pilus conjugatifs semblent intervenir dans cet enrichissement spécifique. Le dépôt très probable des particules enrichies en EGM dans le lit des rivières conduit apparemment à la formation, au moins dans la couche de sédiment frais, d'un réservoir de bactérie porteuses d'EGM.

Enfin, nous avons montré, grâce à l'analyse de carottes, que l'abondance en nos deux EGM évolue différemment dans les sédiments profonds. De plus, de grandes variations d'abondances en intégrons de classe 1 très localisées ont été mises en évidence, ce qui suggère que ces éléments, et les bactéries qui les portent, sont concentrés à l'intérieur de microréservoirs dans les sédiments. Si certaines corrélations négatives ou positives entre les abondances en EGM et les paramètres globaux et physico chimiques (HAP, Pb, Fe, ...) ont pu être démontrées, établir si celles-ci peuvent résulter d'un effet de toxicité sur les bactéries porteuses et/ou non-porteuses de EGM reste une question ouverte.

Finalement, si l'on considère l'ensemble des compartiments de l'Orne étudiés, nous avons montré que les intégrons de classe 1, fréquemment utilisés comme un proxy de l'antibiorésistance, se comportent différemment des plasmides conjugatifs IncP-1 eux aussi reconnus comme vecteurs d'antibiorésistance et utilisés, mais dans une moindre mesure comme proxy. La pertinence de l'utilisation des seuls intégrons de classe 1 peut donc être soulevée. L'utilisation complémentaire d'autres EGM comme proxys apparaît d'un intérêt certain car elle permettrait une approche plus globale des différents phénomènes liés à la dissémination des gènes d'antibiorésistance.

Abstract:

Mobile genetic elements (MGEs) are genetic structures frequently associated to the dissemination of antibiotic resistance genes (ARGs). In this work, we use two of them as proxies, class 1 integrons and IncP-1 plasmids, to better understand (i) the possible fate of ARGs once released in a river ecosystem (Orne, France), as well as (ii) the effect of anthropogenic pressures on their persistence. From river water analyses, we could show that the two MGEs do not behave the same way. The entry of class 1 integrons in the river system appeared to be diffuse rather than punctual, while the abundance of IncP-1 plasmid is relatively stable along the river section studied (23 km) thus indicating a rather indigenous origin. Anthropogenic inputs such as wastewater treatment plant did not seem to affect the abundance of MGEs because a too high level of effluent dilution. Interestingly, MGE-bearing bacteria appeared to be enriched on suspended material, which is likely to serve as vehicle to drive MGE-rich communities of bacteria toward the sediments. The analysis of two sediment cores clearly indicates that only the top layers displayed an elevated level of MGE-bearing bacteria. These abundances decrease in deeper layers where only localized zones display microreservoirs of elevated MGE abundances. For one sediment core at least, we could show that the relative abundance of MGE negatively correlate pollutant such as lead or certain PAHs.

Key words: Antibiotic-resistance-genes; class-1-integrons; environmental-reservoirs; IncP-1-plasmids; mobile-genetic-elements

Résumé

Les éléments génétiques mobiles (EGM) sont des structures génétiques fréquemment associées à la dissémination de gènes de résistance aux antibiotiques (GRA). Dans ce travail, nous avons utilisé deux EGM comme « proxies », les intégrons de classe 1 et les plasmides IncP-1, afin de mieux comprendre (i) le devenir possible des GRA une fois relargués dans un écosystème fluvial (l'Orne, France), ainsi que (ii) l'effet des pressions anthropiques sur leur persistance. À partir d'analyses de l'eau des rivières, nous avons pu montrer que les deux EGM ne se comportaient pas de la même manière. L'entrée des intégrons de classe 1 dans le système fluvial semblait être diffuse plutôt que ponctuelle, tandis que l'abondance du plasmide IncP-1 est relativement stable le long de la section de la rivière étudiée (23 km), indiquant ainsi une origine plutôt indigène. Les intrants anthropiques tels que les stations d'épuration des eaux usées ne semblent pas affecter l'abondance des EGM en raison d'un niveau trop élevé de dilution des effluents. Par ailleurs, il est intéressant de noter que les bactéries porteuses d'EGM semblaient être enrichies sur les matières en suspension, susceptibles de servir de véhicule pour amener des communautés de bactéries plus riches en EGM vers les sédiments. L'analyse de deux carottes de sédiment indique clairement que seules les couches supérieures présentent un niveau élevé de bactéries porteuses d'EGM. Ces abondances diminuent dans les couches plus profondes où seules des zones ponctuelles présentent des microréservoirs avec des abondances d'EGM plus élevées. Pour une carotte sédimentaire au moins, nous avons pu montrer que l'abondance relative d'EGM corrèle négativement la présence de polluants tel que le plomb ou certains HAP.

Mots clés: gènes de résistance aux antibiotiques; les intégrons de classe 1; réservoirs environnementaux; Plasmides IncP-1; éléments génétiques mobiles