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Camila Ayelén Knecht

Fate of antibiotic resistant bacteria and antibiotic resistance genes in constructed wetlands

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Helmholtz Centre for Environmental Research – UFZ
Department of Environmental Biotechnology

Fate of antibiotic resistant bacteria and antibiotic resistance genes in constructed wetlands

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“La madre tierra militarizada, cercada, envenenada,
donde se violan sistemáticamente derechos elementales, nos exige actuar.
Construyamos entonces sociedades capaces de coexistir
de manera justa, digna y por la vida.”

Berta Cáceres

Mother earth, militarized, fenced, poisoned,
where elementary rights are systematically violated, demands us to act.
Let us then build societies capable of coexisting
in a fair and dignified manner and for life.

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Summary

The World Health Organization (WHO) reported that because of antibiotic resistance, diseases that were previously essentially under control may soon pose a severe global risk to human health. The current extensive release of antibiotic resistant bacteria (ARB) and their respective antibiotic resistance genes (ARG) into the environment is of significant concern. The knowledge about which factors favour the development of antibiotic resistance in clinical settings has increased in the last decades but little is known about its driving forces in the environment. The most common approaches to assess antibiotic resistance in the environment is either to isolate resistant colonies on agar plates or quantify some indicator ARG by means of quantitative PCR (qPCR). As biological contaminants, ARG exhibit different behaviour and fate in various environmental media, since their abundance and composition may change depending on the physiological status of their hosts. Therefore, knowing the ARG hosts is of interest in order to understand the mechanisms underlying the spread of ARG in the environment. Among all phenomena related to the hosts taking place in the environment, horizontal gene transfer (HGT) of ARG is of great concern because bacteria that are pathogenic but susceptible can become resistant and bacteria that were already resistant may become multi-drug resistant.

Wastewater treatment plants (WWTP) represent an interface between human activities and the environment and therefore play a key role in the prevention of ARB and ARG spreading. While the total abundance of ARG decreases during wastewater treatment, an increase in the relative abundance of some ARG usually takes place in WWTP. Although quantities of some ARG significantly decrease during wastewater treatment process, the concentrations of these resistance genes in the effluent are still high. The incomplete removal of antibiotics and ARG in WWTP severely affects the receiving river, where both types of emerging pollutants are found at higher concentration in downstream waters than in samples collected upstream from the discharge point.

Constructed wetlands (CW) constitute a near-natural wastewater treatment technology in operation around the world. CW are viewed both as alternative wastewater treatment option for removal of ARB and ARG and as a near natural habitat to investigate their environmental fate. Horizontal subsurface flow constructed wetlands (HSSF-CW) are the most common type of CW built worldwide due to its technical simplicity, low costs of implementation, and low energy and maintenance requirements. As oxygen is one of the main limiting factors in the removal of organic matter and ammonium through HSSF-CW, aeration of these systems constitutes an improvement for

wastewater treatment. In this work, the effects of aeration on antibiotic resistance in CW was addressed. For that, two HSSF-CW at the Ecotechnology Research Facility in Langenreichenbach (UBZ), Germany were compared: one standard HSSF-CW (H50p) and an aerated HSSF-CW (HAp).

With the aim of evaluating the performance of the aerated CW regarding removal of ARB and ARG in comparison with an standard CW and to study HGT of ARG, the antimicrobials sulfamethoxazole (SMX) and trimethoprim (TMP) were chosen. Five genes were selected: 16S rRNA gene which is useful to quantify total bacterial abundance; *sul1*, *sul2* and *dfrA1* which are ARG that give resistance to the aforementioned antibiotics and *intI1*, a gene which is related to ARG and mobile genetic elements (MGE). A multiphasic approach was applied, making use of culture- dependent and independent methods to analyse samples from in- and outflows as well as from internal points in both CW. Comparing in- and outflows, both CW showed higher reductions of ARG/ARB than what was observed for HSSF-CW before, and aeration was beneficial for the total reduction of ARB and ARG. ARB were identified as members of *Enterobacteriaceae*, *Pseudomonas*, *Aeromonas* and *Enterococcus*, whereas ARG hosts belonged to members of *Enterobacteriaceae* and *Aeromonas*, the other two groups rarely carried them. As *Escherichia coli* and *Aeromonas* were common hosts of the selected ARG, additional analysis were carried out for this bacteria.

The investigated CW showed different internal behaviour. While the trend in reduction was gradual in H50p, at the beginning of HAp high aerobic metabolism took place and an increase in intI1 and *sul* genes abundances was seen. Copy numbers of 16S rRNA gene remained almost constant and *dfrA1* decreased, indicating that the increase in *intI1* and *sul* genes abundances was specific. Cells carrying more than one type of class 1 integron in the first half of HAp showed higher diversity than in the inlet and the rest of HAp, accounting for a higher integrase activity. Because of their very low concentrations and the absence of statically significant differences between the CW, the presence of SMX/TMP in the media was unlikely the cause for the difference in antibiotic resistance observed between the CW, even though a synergistic effect can’t be discarded. It is known that reactive oxygen species (ROS) can increase HGT through activation of the SOS system and they are more abundant in HAp than in H50p. Based on the data from this study and on what was known from other studies, oxidative stress is proposed as an important factor for increasing HGT of ARG in wastewater treatment plants.

Zusammenfassung

Die Weltgesundheitsorganisation berichtete, dass Krankheiten die zuvor im Wesentlichen unter Kontrolle standen, aufgrund von Antibiotikaresistenzen bald ein schwerwiegendes globales Risiko für die menschliche Gesundheit darstellen können. Die derzeitige weitreichende Freisetzung von Antibiotika-resistenten Bakterien (ARB) und ihrer jeweiligen Antibiotika-Resistenzgene (ARG) in die Umwelt gibt Anlass zu großer Sorge. Das Wissen darüber, welche Faktoren die Entwicklung von Antibiotika-Resistenzen im klinischen Umfeld begünstigen, hat in den letzten Jahrzehnten zugenommen, während über die treibenden Kräfte in der Umwelt wenig bekannt ist. Der gebräuchlichste Ansatz zur Bewertung der Antibiotikaresistenz in der Umwelt besteht in der Quantifizierung bestimmter Indikator-ARG mittels quantitativer PCR (qPCR). Als biologische Schadstoffe weisen ARG in verschiedenen Umweltmedien ein unterschiedliches Verhalten auf, da sich ihre Häufigkeit und Zusammensetzung mit dem physiologischem Zustand ihrer Wirte ändern kann. Daher ist es wichtig, die ARG-Wirte zu kennen, um die Mechanismen zu verstehen, die der Verbreitung von ARG in der Umwelt zugrunde liegen. Unter allen in der Umwelt stattfindenden Phänomenen die sich auf die Wirte beziehen, ist der horizontale Gentransfer (HGT) von ARG der bedeutendste. Dadurch können Bakterien die pathogen, aber anfällig sind, resistent und bereits resistente Bakterien multiresistent werden.

Kläranlagen bilden die Schnittstelle zwischen menschlichen Aktivitäten und der Umwelt und spielen daher eine Schlüsselrolle bei der Verhinderung der Ausbreitung von ARB und ARG. Während die Gesamtmenge an ARG in der Abwasserbehandlung abnimmt, findet in Kläranlagen normalerweise eine Zunahme der relativen Häufigkeit von einigen ARG statt. Obwohl die Mengen einiger ARG während der Abwasserbehandlung deutlich abnehmen, sind die Konzentrationen dieser Resistenzgene im Abwasser immer noch hoch. Die unvollständige Entfernung von Antibiotika und ARG in der Kläranlage wirkt sich schwerwiegend auf den aufnehmenden Fluss aus. Beide Arten von Schadstoffen kommen in höherer Konzentration in flussabwärts gelegenen Gewässern vor als in Proben, die stromaufwärts von der Einleitstelle entnommen werden.

Pflanzenkläranlagen (PKA) bilden eine nahezu natürliche Abwasserbehandlungstechnologie, die weltweit eingesetzt wird. PKA werden sowohl als alternative Abwasserbehandlungsoption zur Entfernung von ARB und ARG, als auch als natürlicher Lebensraum zur Untersuchung ihres Umweltverhaltens betrachtet. Horizontal durchströmte Pflanzenkläranlagen (HDS-PKA) sind aufgrund ihrer technischen Einfachheit, niedrigen Implementierungskosten und niedrigen Energie- und Wartungskosten die weltweit am häufigsten genutzte PKA. Da Sauerstoff einer der

Begrenzungsfaktoren für die Entfernung von organischem Material durch HDS-PKA ist, stellt die Belüftung dieser Systeme eine Verbesserung für die Abwasserbehandlung dar. In dieser Arbeit wurden die Auswirkungen der Belüftung auf Antibiotikaresistenzen in PKA untersucht. Dafür wurden eine standard HDS-PKA (H50p) und eine belüftete HDS-PKA (HAp) miteinander verglichen.

Mit dem Ziel, die Leistung der belüfteten PKA hinsichtlich der Entfernung von ARB und ARG zu bewerten und die HGT von ARG in der PKA zu untersuchen, wurden die antimikrobiellen-Stoffe Sulfamethoxazol (SMX) und Trimethoprim (TMP) ausgewählt. Fünf Gene wurden ausgesucht: das 16S-rRNA-Gen, ein Gen die Gesamtbakteriengemeinschaft quantifiziert, *sul1*, *sul2* und *dfrA1* welche ARG sind die Resistenzen gegen die zuvor genannten Antibiotika geben; und *intI1*, ein Gen das mit ARG und mobilen genetischen Elementen (MGE) verwandt ist. Eine mehrphasige Methode wurde eingesetzt, die kulturabhängige und -unabhängige Methoden zur Analyse von Proben beim Ein- und Ausstritt, sowie von internen Punkten in beiden PKA verwendet. Beim Vergleich von Ein- und Ausströmseite zeigten beide untersuchten PKA eine stärkere Reduktion von ARG/ARB als zuvor für HDS-PKA beobachtet wurde und die Belüftung schien für den gesamten Abbau von ARB und ARG vorteilhaft zu sein. ARB wurden als Bestandteil von *Enterobacteriaceae*, *Pseudomonas*, *Aeromonas* und *Enterococcus* identifiziert. Während ARG-Wirte zu *Enterobacteriaceae* und *Aeromonas* gehörten, trugen sie die anderen beiden Gruppen nur selten. Da *E. coli* und *Aeromonas* häufige Wirte des ausgewählten ARG waren, wurden zusätzliche Analysen für diese Bakterien durchgeführt.

Die PKA zeigten ein unterschiedliches internes Verhalten. Während die Tendenzen zum Abbau bei H50p konstant waren, trat in dem ersten Bereich von HAp ein starker aerober Metabolismus auf und es wurde eine Zunahme der *int11* und *sul* Gene beobachtet. Das 16S rRNA Gen blieb nahezu konstant und *dfrA1* nahm ab, was darauf hindeutet, dass die Zunahme der *intI1* und *sul* Gene Abundanzen spezifisch war. Zellen, die mehr als eine Klasse-1-Integron-Kopie trugen, zeigten im ersten Bereich von HAp eine größere Diversität als im Einlass und im Rest von HAp auf, was auf einer erhöhten Integrase-Aktivität hindeutet. Aufgrund ihrer sehr niedrigen Konzentration und des Ausbleibens der statistisch signifikanten Unterschiede zwischen den PKA, war die Ursache für den beobachteten Unterschied in der Resistenz zwischen den PKA um Vorhandensein von SMX/TMP in den Medien unwahrscheinlich, obwohl ein synergistischer Effekt nicht ausgeschlossen werden kann. Reaktive Sauerstoffspezies (ROS) können HGT durch Aktivierung des SOS-Systems erhöhen und sie sind häufiger in HAp als in H50p vorhanden. Basierend auf den Daten aus dieser Studie und auf dem, was bekannt war, wird oxidativer Stress als wichtiger Faktor für die Erhöhung der HGT von ARG in Kläranlagen vorgeschlagen.

Contents

List of acronyms and abbreviations

AIA	Aeromonas isolation agar
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene(s)
AMR	Antimicrobial resistance
APS	Ammonium persulfate
BLD	Below the level of detection
bp	Base pairs
C-BOD_5	5-day carbonaceous biochemical oxygen demand
CFU	Colony forming units
CFU^R	Antibiotic resistant colony forming units
CFU^{SMX}	Sulfamethoxazol resistant colony forming units
CFU^{TMP}	Trimethoprim resistant colony forming units
CW	Constructed wetland(s)
DHF	Dihydrofolate
DO	Dissolved oxygen
EA	Endoagar
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia:* for example
epicPCR	Emulsion paired isolation and concatenation PCR
HAp	Horizontal aerated planted
H50p	Horizontal 50 cm deep
HGT	Horizontal gene transfer
HLR	Hydraulic loading rate
HRT	Hydraulic retention time
HSF	Horizontal surface flow
HSSF	Horizontal sub-surface flow
ICE	Integrative and conjugative element(s)
i.e.	*id est:* that is
IS	Insertion sequence(s)
kb	Kilo base pairs
k_{ow}	Octanol-water partition coefficient
$LOD_{i/m}$	Instrumental/methodological limit of detection
LMIC	Low and middle income countries

LRB	Langenreichenbach
MDR	Multi-drug resistance
MGE	Mobile genetic element
MH	Müller-Hinton
MIC	Minimal inhibitory concentration
N^4-Ac-SMX	N^4-acetylsulfamethoxazole
NGS	Next generation sequencing
OCR	Oxygen consumption rate
OECD	The Organisation for Economic Co-operation and Development
OLR	Organic loading rate
Orf	Open reading frame
OTU	Operational taxonomic unit(s)
PABA	*para*-amino benzoic acid
PIA	Pseudomonas isolation agar
PKA	Pflanzenkläranlage(n)
ROS	Reactive oxygen species
rpm	Revolutions per minute
SF	Surface flow
SMX	Sulfamethoxazole
SPE	Solid-phase extraction
spp.	*species pluralis:* multiple species
TEMED	Tetramethylethylenediamine
THF	Tetrahydrofolate
TKN	Total kjeldahl nitrogen
TMP	Trimethoprim
TSS	Total suspended solids
UBZ	Environmental and Biotechnology Centre of the UFZ
VSF	Vertical surface flow
WHO	World health organization
WWTP	Wastewater treatment plant

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Glossary

Antibiotic: Antimicrobial substance active against bacteria. In the original definition, an antibiotic was produced by one microorganism that selectively inhibits the growth of another. Synthetic antibiotics, usually chemically related to natural antibiotics, have since been produced that accomplish comparable tasks. Antibiotics can be bactericidal or bacteriostatic.

Antimicrobial: Agent that kills microorganisms or stops their growth. Includes antibiotics, antifungals, antiprotozoals, and antivirals.

Cometabolism: Ability of a microorganism to biodegrade a pollutant without using it as a growth-substrate (non-growth-substrate), while sustaining its own growth by assimilating a different substrate (growth-substrate).

Conjugation: Horizontal gene transfer that involves the physical contact of two cells. Often mediated by plasmids.

Co-resistance: Concomitant resistance to different antibiotics. It occurs when antibiotic resistance genes are located in the same cell. Furthermore, these genes can be located on the same mobile genetic element (MGE) (e.g. plasmid pB10 encodes for resistance against amoxicillin, streptomycin, sulfonamides and tetracycline).

Cross resistance: Single resistance mechanism confers resistance to several antibiotics of the same class (e.g. efflux pump MexAB-OprM confers resistance against sulfonamides and trimethoprim).

Dormant state: Reversible state of low metabolic activity after facing with unfavourable environmental conditions.

Enrichment: Favouring the growth of a particular microorganism or group of microorganisms over others.

***gyrB*:** Gene that codifies for the subunit B of the DNA gyrase. The DNA gyrase is an enzyme that reduces topological strain while double-stranded DNA is being unwound.

Integrative and conjugative elements (ICE): Selftransmissible mobile genetic elements. Similar to conjugative plasmids, ICE transfer via conjugation but, unlike plasmids, do not replicate independently from the chromosome.

Metagenome: All the genetic material present in an environmental sample.

MexAB-OprM: Multidrug efflux pump. MexA is the membrane fusion protein; MexB is the inner membrane transporter; and OprM is the outer membrane channel.

Microbiota: The entire collection of microorganisms in a specific environment. It often implies microorganisms living in the guts of animals and when the open environment is referred, the adjective environmental is added.

Micropollutant: Anthropogenic chemicals that occur in the (aquatic) environment above a (potential) natural background level due to human activities but with concentrations remaining at trace levels (i.e. up to the microgram per litre range).

Mobile genetic element: Segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility). Examples: plasmids, insertion sequences (IS), transposons, integron-associated gene cassettes and bacteriophages.

Mobilome: The total pool of mobile genes in the metagenome.

Multi-drug resistance: Resistance against at least three classes of antibiotics.

One health approach: Is a term used to describe a principle which recognises that human and animal health are interconnected, that diseases are transmitted from humans to animals and vice versa and must therefore be tackled in both. The One Health approach also encompasses the environment, another link between humans and animals and likewise a potential source of new resistant microorganisms.

Resistance gene: A gene that confers resistance to antibiotics when it is present and that increases susceptibility to antibiotics when it is absent.

Resistome: Collection of all the antibiotic resistance genes in both pathogenic and non-pathogenic bacteria.

Selection: Inhibition of the growth of certain bacteria.

SOS system: A generalized response to DNA damage that is exhibited by many bacteria.

SXT: Integrative and conjugative mobile element (ICE) with more than 1000 kb which encodes resistance to multiple antibiotics, including trimethoprim and sulfamethoxazole.

Transformation: Horizontal gene transfer mediated by the uptake of naked DNA.

Transduction: Horizontal gene transfer mediated by phages.

***uidA*:** Gene that codifies for the enzyme beta-glucuronidase, which catalizes the breakdown of complex carbohydrates.

1. Introduction

1.1. Antibiotic resistance as a major worldwide health threat

Antibiotics are one of the most important discoveries of modern medicine and, together with vaccines, have played a key role in the increase of human life expectancy. In the early 1900s, the scientist Paul Ehrlich developed the concept of selective toxicity, the ability of a chemical to inhibit or kill pathogenic microorganisms without adversely affecting the host. In the past century a variety of antibiotics with different modes of action have been discovered and synthesised (Figure 1). Some of them, like penicillins, interfere with the cell wall synthesis while others inhibit the enzyme DNA gyrase which in turn stops DNA replication. Other antibiotics like chloramphenicol and kanamycin interfere with ribosomal function, which is the synthesis of proteins. This work focuses on sulfonamides and trimethoprim, which inhibit the synthesis of tetrahydrofolate, a compound necessary for the synthesis of DNA, RNA and proteins.

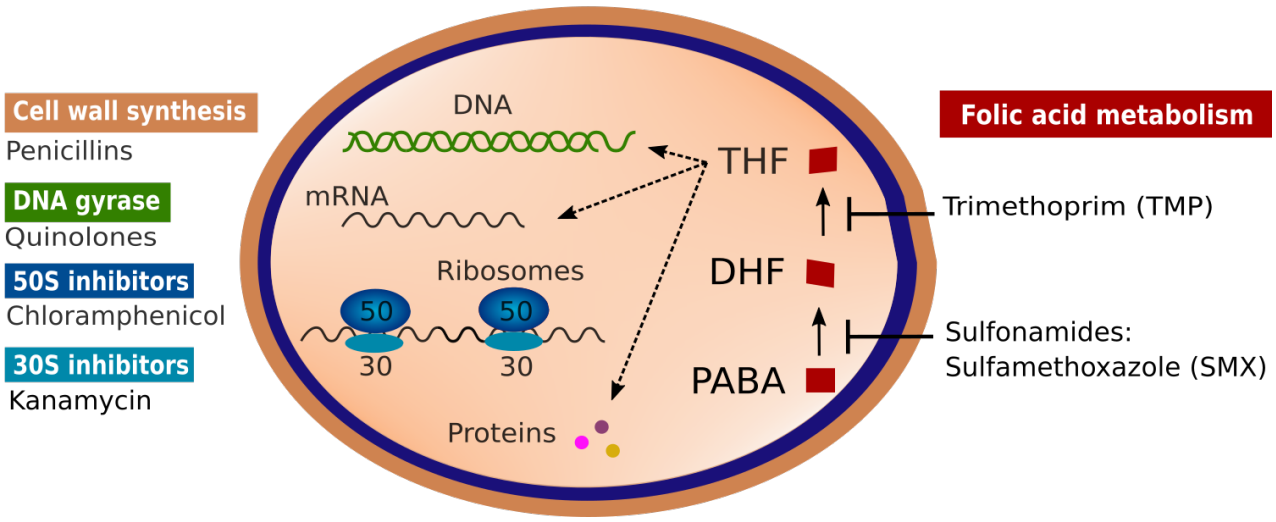


Figure 1| Antibiotics mode of action. Antibiotics can hinder bacterial growth by inhibiting nucleic acid or protein synthesis, interfering with cell wall or cytoplasmic membrane synthesis. PABA: *para*-amino benzoic acid, DHF: dihydrofolate, THF: tetrahydrofolate. Adapted from Gullberg et al., 2011.

However, life struggles to prevail in every being, and bacteria are not an exception. These have developed strategies to escape from the effects of antimicrobial agents, and bacteria that survive and multiply in the presence of antibiotics are becoming more and more common. Some of the mechanisms of antibiotics resistance are shown in Figure 2. Some mechanisms like a decreased

uptake and efflux pumps can be unspecific and therefore protect the cells against diverse damaging substances. Other mechanisms are specific and act against a particular class of molecules. One example of this type of resistance is based on the synthesis alternative enzymes with a higher affinity for the actual substrate than for the antibiotic, which is the mechanisms this work focuses is on.

Although resistance was present before the use of antibiotics in medicine, the rates at which it develops and spreads have increased in the last decades (Xie et al., 2018). In a recent report, The Organisation for Economic Co-operation and Development (OECD) estimated that 2.4 million people in Europe, North America and Australia will die from infections with resistant microorganisms in the next 30 years (OECD, 2018). In low and middle income countries (LMIC) the situation does not look better. In Brazil and Indonesia 40–60% of infections are already caused by resistant microorganisms. The World Health Organization (WHO), that has been warning about antibiotic resistance as a major global health issue since 2014, reported that diseases that were previously essentially under control may soon pose a severe global risk to human health (World Health Organization, 2014). Particularly alarming is the rise and the spreading of multi-drug resistant pathogenic bacteria, which pose a major challenge for the treatment of infections (Aslam et al., 2018).

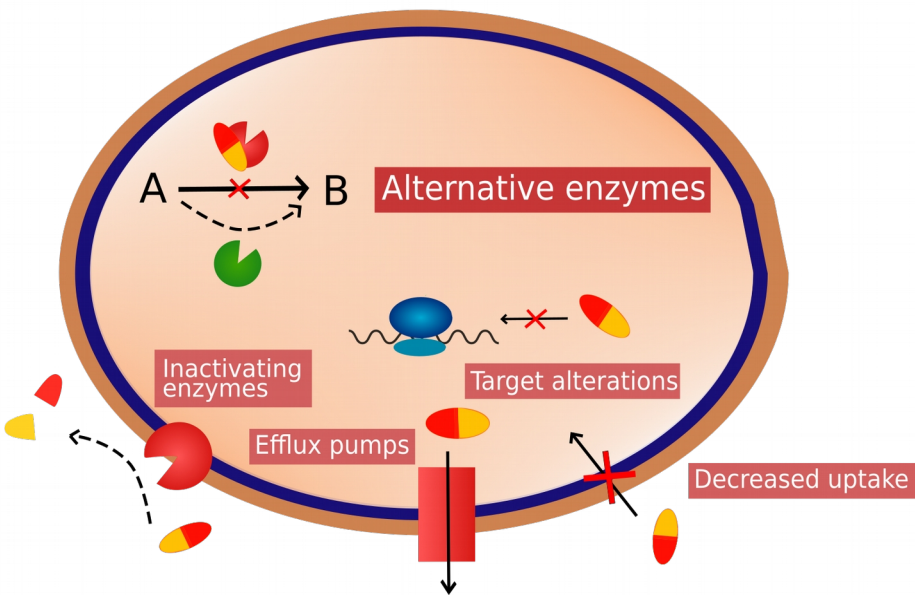


Figure 2| Mechanisms of antibiotics resistance. Antibiotics can hinder bacterial growth by inhibiting nucleic acid or protein synthesis and interfering with cell wall or cytoplasmic membrane synthesis. Resistance mechanisms are efflux pumps, target alterations, inactivating enzymes and a decreased uptake of antibiotics. Adapted from Madigan et al., 2012.

The first antimicrobials used systemically in human medicine were sulfonamides. The antimicrobial effect of sulfonamides was first discovered in 1935 by Gerhard Domagk, and since then diverse sulfonamides have been used to treat animal and human infections. Sulfamethoxazole (SMX) is a widely used sulfonamide (Figure 3) and is often taken in combination with trimethoprim (TMP) in a mass proportion 5:1 (co-trimoxazole) in order to increase their effectiveness (Bushby and Hitchings, 1968). This combination is part of the WHO core list of essential medicines, which presents a myriad of minimum medicine needs for a basic health care system, listing the most efficacious, safe and cost–effective medicines for priority conditions. In a long term project called ANTI-Resist in Dresden, Germany this combination made up 10-20% of the antibiotics prescribed for infections in human medicine. Both antibiotics and other sulfonamides have been detected in several rivers in Europe (Johnson et al., 2015) and are introduced into the environment through farmyard manure and domestic wastewater (Hannappel et al., 2016).

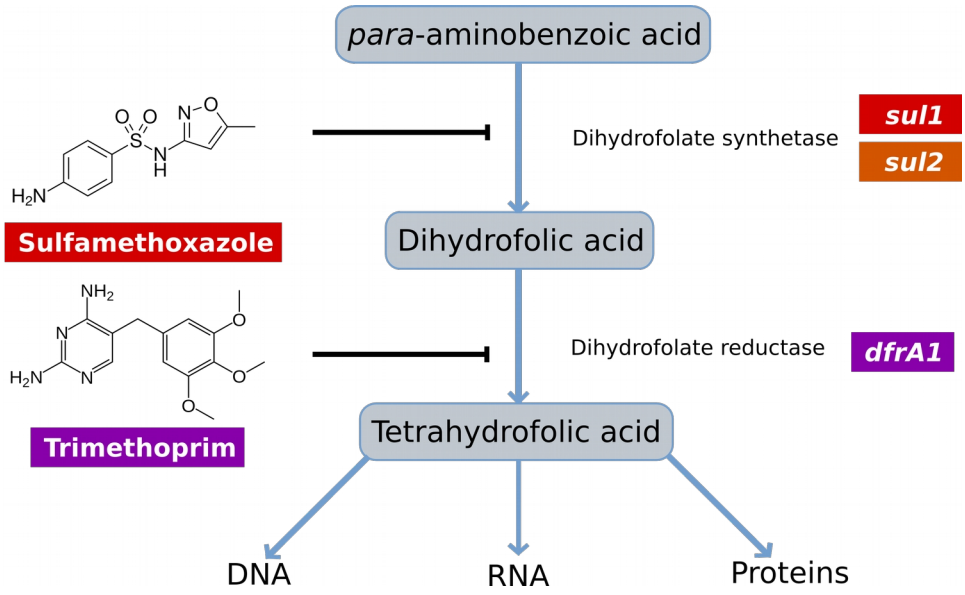


Figure 3| Antibiotics and antibiotic resistance genes selected. Sulfamethoxazole and trimethoprim inhibit the metabolic pathway of tetrahydrofolic acid. On the left side of the figure the chemical structure of the antibiotics SMX and TMP are given and on the right, the names of their respective resistance genes *sul1*, *sul2* and *dfrA1*.

Some years after the introduction of sulfonamides, sulfonamide resistant bacteria were reported for the first time (Davies and Davies, 2010). Since then, different resistance genes for sulfonamides as well as for TMP have been identified. The specific resistance mechanism relies on genes that encode for alternative enzymes with an increased affinity for the target (Jahne et al., 2015). Three of these genes were found for sulfonamide resistance (*sul1*, *sul2* and *sul3*) (Perreten and Boerlin, 2003) and more than 20 for TMP resistance (Kehrenberg and Schwarz, 2005). The genes *sul1* and

sul2 are often found at roughly the same frequency (50% each) among sulfonamide resistant Gram-negative clinical isolates. One reason for this could be that they are both located on very efficient mobile genetic elements (MGE) for dissemination (Sköld, 2000). In the case of *dfrA* gene alleles, their relative frequencies varies and it greatly depends on the origin of the isolates (Seputiene et al., 2010).

Knowledge about which factors favour the development of antibiotic resistance in clinical settings has increased in the last decades but little is known about its driving forces in the environment. While these uncertainties hamper a quantitative risk assessment, the consequences of a transfer of antibiotic resistant bacteria (ARB) from environmental reservoirs into the human population can be severe. Fighting antibiotic resistance needs urgent global concerted action. The concept "One Health Approach" was created in order to emphasize that antibiotics like sulfonamides are used in human and animal medicine. Both sources of antibiotics and antibiotic resistant bacteria (ARB) should be taken into account by researchers and policy-makers.

1.2. The role of horizontal gene transfer in antibiotic resistance

The mechanisms underlying bacterial resistance to antimicrobial agents reside in the ability of bacteria to quickly modify their genomes. When antibiotic resistance was first discovered, multi-drug resistance was not anticipated. The co-appearance of multiple mutations conferring resistance was considered to be beyond the evolutionary potential of a given bacterial population (Mazel, 2006). However, bacterial genome's plasticity is a consequence not only of spontaneous mutations or genome rearrangements that can occur during the bacterial life cycle, but also of exogenous gene acquisition through genetic exchange between bacteria and gene capture in integrons (Baharoglu et al., 2013). Genetic exchange of antibiotic resistance genes (ARG) take place through different horizontal gene transfer (HGT) mechanisms (Figure 4). While HGT indicates the exchange of genetic material between already existing bacteria, vertical gene transfer refers to the propagation of genetic material from a mother cell into daughter cells during cellular division.

Transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA. Through specialized or generalized *transduction*, bacteriophages may transfer bacterial DNA from a previously infected donor cell to the recipient cell. *Conjugation* is a process requiring cell to cell contact via cell surface pili or adhesins, which help to keep the cells close enough to ensure DNA is transferred from the donor cell to the recipient cell. Even though the transfer of ARG through conjugation doesn't necessarily involve plasmids or integrative and

conjugative elements (ICE), when it does the probabilities that more bacteria in the community become resistant increases substantially (Derbyshire and Gray, 2014). Horizontal gene transfer through conjugation doesn't need the recombination of the incoming DNA in the new host in order to be further functional and transferable. Necessary conditions for the transmission of antibiotic resistance through conjugation are:

1) Cell-to-cell contact (connectivity). That means HGT will be favoured in environments with high cell density, like biofilms (Balcázar et al., 2015).

2) Time. 3.5-4 minutes are sufficient for HGT of a plasmid to take place (Andrup and Andersen, 1999).

3) Donor-recipient compatibility. HGT is most likely to occur between individuals of the same species but transfer between rather distantly related bacteria is also possible (Matsushita et al., 2018).

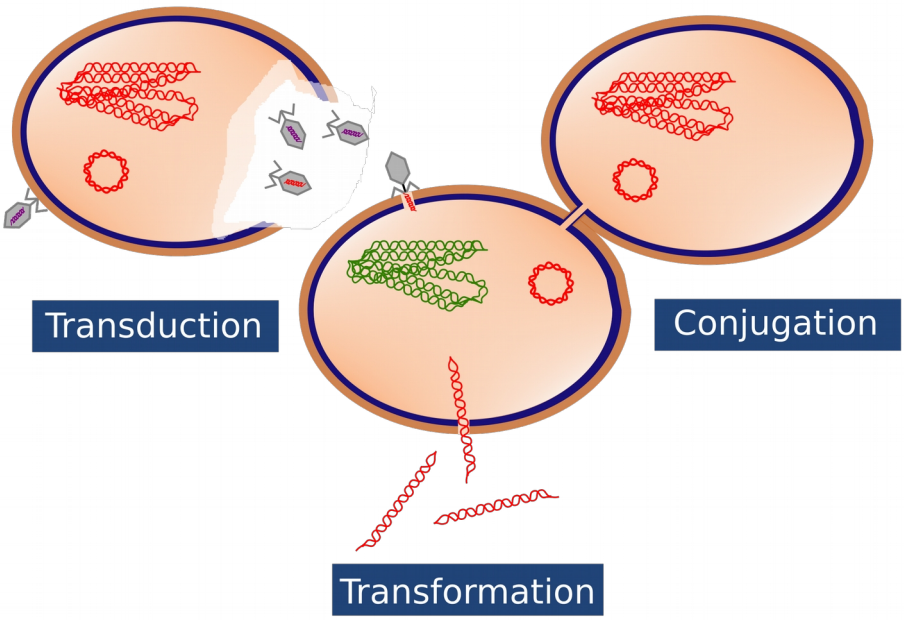


Figure 4| Different mechanisms of horizontal gene transfer. Conjugation, transformation and transduction. Adapted from von Wintersdorff et al., 2016.

Furthermore, it is known that many of the individual processes contributing to HGT are responsive to environmental stress. Some stress sources like antimicrobials (Jutkina et al., 2018), heavy metals (Zhang et al., 2018) and DNA damaging agents (Beaber et al., 2004) can enhance HGT. The physical linkage of numerous and functionally diverse groups of genes within a mobile genetic element (MGE) has implications for their persistence in the environment. Selection that is imposed

towards even a single component/gene of an MGE will automatically select for the whole MGE (co-selection) (Aminov, 2011).

Horizontal gene transfer of ARG is of concern as bacteria that are pathogenic but susceptible can become resistant and bacteria that were already resistant may become multi-drug resistant. The extent to which HGT is affected by environmental stressors is uncertain but conditions in wastewater treatment plants (WWTP) represent a hotspot for it, as both a high concentration of microorganisms and different sources of stress are present. In fact, wastewater was addressed as one of the four genetic reactors in antibiotic resistance, together with human and animal microbiota, hospitals and farms and soil and surface groundwater (Baquero et al., 2008).

Class 1 integrons and horizontal gene transfer of antibiotic resistance genes

Integrons constitute genetic platforms that incorporate open reading frames, known as gene cassettes, by site-specific recombination and convert them to functional genes by inserting them downstream a promotor (Mazel, 2006). In that way, they can be transcribed and later expressed as an active protein. A defining feature of all integrons is a gene coding for a site-specific tyrosine recombinase called integrase, which can excise and integrate gene cassettes into the variable region of the integron (Partridge et al., 2009). It can also reshuffle the order of gene cassettes which affects the relative rate of expression of the individual cassettes (Berglund, 2015). There are over 100 different integron classes and this classification is based on the aminoacids sequence of the integrase (Deng et al., 2015). Most of the integrons classes are present on bacterial chromosomes (chromosomal superintegrons) and are found in approximately 10% of sequenced bacterial genomes (Rapa and Labbate, 2013). Five classes of integrons are present on MGE like plasmids and transposons (mobile integrons) and are known to play a role in the dissemination of ARG (Mazel, 2006). The high diversity of mobile integrons on plasmids in WWTP was already pointed out by Tennstedt et al. (2003). Mobile integrons have been detected mainly in Gram-negative isolates, although they have also been observed in some Gram-positive genera like *Staphylococcus* (Xu et al., 2011) and *Enterococcus* (Nandi et al., 2004).

Among all the integrons, integron class 1 is the most abundant and it belongs to the mobile type. Because integron class 1 is often on MGE, *intI1* abundance can be used as an indicator of the presence of MGE in a bacterial community (Chen et al., 2015a). The structure of a typical class 1 integron is depicted in Figure 5.

Upstream of the gene cassettes the gene *intI1* can be found, which codifies for the integrase. There are several alleles of the integrase and different sets of primers have been developed which are specific for some of them. For example Mazel et al. (2010) designed primers which detect the majority of *intI1* alleles and Gillings et al. (2015) designed primers which can be used to selectively amplify clinical alleles. The clinical allele originated recently under anthropogenic selective pressure and has already spread into diverse pathogenic and commensal bacteria of humans and their domestic animals. Downstream of the gene cassettes, a typical class 1 integron possesses a 3′ -conserved region with the genes *qacEΔ1* and *sul1*, encoding resistance to quaternary ammonium salts and sulfonamides, respectively. The function of Orf5 is still unkown (Deng et al., 2015). The recombination sites are called attI and attC on the integron and on the gene cassette respectively. The class 1 integron's capacity to operate in different hosts is likely the reason why this particular integron has been successful in its mobilization across different bacteria. This trait and possibly a great capacity to integrate cassettes with diverse attC sites (Biskri et al., 2005) make this integron an exception rather than the rule.

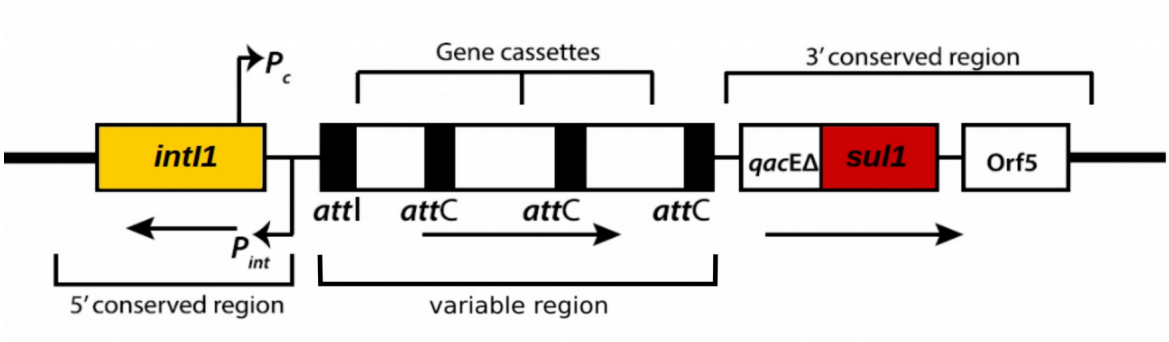


Figure 5| General structure of integrons class 1. On the 5' conserved region *intI1* is found (yellow) which codifies for an integrase. Pc is the promotor from which gene cassettes are transcribed and P_{int} is the integrase promotor. Resistance gene *sul1* (red), *qacEΔ1* and Orf5 are part of the 3' conserved region. attC are recombination sites where ARG can be inserted, a reaction catalysed by the integrase. attI is the recombination site on the integron. The function of Orf5 is unknown. Adapted from Ravi et al., 2015.

The abundance of *intI1* can quickly change in response to environmental pressures, as the class 1 integron is hosted by bacterial species that have rapid generation times and it is often located on MGE that can be transferred among bacteria. Its abundance has been therefore proposed as a proxy for anthropogenic pollution (Gillings et al., 2015). The integrase gene is upregulated under cellular stress, thus increasing the rate at which gene cassettes are mobilized (Michael et al., 2016). This response of the integrase towards stress is mediated by the SOS system (Guerin et al., 2009). A bacterium harbouring a class 1 integron is more prone to become multi-drug resistant as it can easily incorporate gene cassettes which codify for resistance against different classes of antibiotics

(Ghaderpour et al., 2015). This potential increases if the bacteria carries two or more class 1 integrons, which might even be placed on the same MGE in a cell (Cambray et al., 2011).

1.3. Antibiotic resistant bacteria and antibiotic resistance genes in the aquatic environment

The current extensive release of ARB and their ARG into the environment is of significant concern. ARG can be seen as special contaminants in the environment, due to their unique property of being replicative and the big risk they represent to human and animal health (Pruden et al., 2006). Because ARG are harboured within microbes and are selected in the presence of antibiotics, they have the potential to be amplified in response to treatment.

Wastewater treatment plants represent an interface between human activities and the environment and therefore, play a key role in the prevention of ARB and ARG spreading. Figure 6 shows the main processes affecting antibiotic resistance in the water cycle.

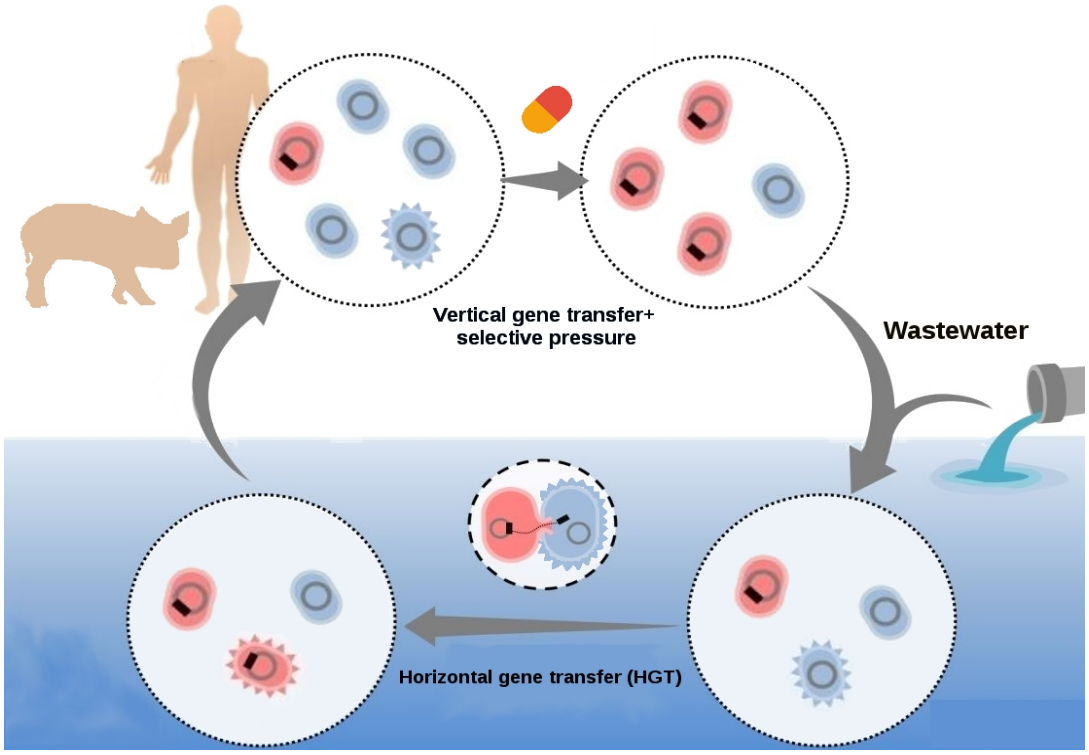


Figure 6| Antibiotic resistance and water cycle. In clinical settings and livestock farming, antibiotics exert selective pressure in the guts of humans and animals, which can increase the prevalence of ARB in the gut microbiota. The ARB can end up in the open environment via wastewater, where ARB can transfer their ARG to environmental bacteria. Environmental bacteria can act as a resistance reservoir. Finally, ARB can end up in drinking water. Bacteria that carry ARG are shown in red and those which don't in blue. Pointy edges represent pathogenic bacteria and smooth edges non-pathogenic bacteria. Adapted from Hernandes et al., 2013.

Even though a clear risk assessment for the whole water cycle can't be done yet, it has been observed that the quality of the WWTP effluents affects receiving waters in terms of antibiotic resistance. Rodriguez-Mozaz et al. (2015) found that the incomplete removal of antibiotics and ARG in WWTP severely affected the receiving river, where both types of emerging pollutants were found at higher concentration in downstream waters than in samples collected upstream from the discharge point. It was noted that the quantities of *tet* and *sul* genes significantly decreased during the wastewater treatment process, but the concentrations of these resistance genes in the effluent were still high (Jahne et al., 2015). It has been indicated that the abundance of background bacteria affects the spread of *tet* genes and *sul* genes (Liu et al., 2014). This implies that the abundance of different types of *sul* genes in the environment would be mainly affected by the content level of those in pollution sources. Koczura et al. (2016) demonstrated that the frequency of *intI1* gene carriage among total heterotrophic bacteria was significantly higher downstream from the WWTP.

As biological contaminants, ARG exhibit different behaviour and fate in various environmental media, since their abundance and composition may change with biological activities. For example, in terms of dissolved oxygen (DO) the lower relative abundance of ARG in the aerobic lagoons with respect to activated sludge could be simply attributed to a higher abundance of bacterial groups that do not carry or rarely carry target ARG, such as autotrophic ammonia oxidizing bacteria (Yi et al., 2017). Whereas the total abundance of ARG decreases during wastewater treatment, an increase in the relative abundance of at least some ARG usually takes place in WWTP (Guo et al., 2017, Rodriguez-Mozaz et al., 2015; Liu et al., 2014; Yi et al., 2017). Relative increase might occur through selection of ARB, acquisition of ARG through HGT or a combination of both (Rizzo et al., 2013).

1.4. Constructed wetlands as a near-natural approach to treat wastewater

Constructed wetlands (CW) constitute a near-natural wastewater treatment technology in operation around the world (Hickey et al., 2018; Zhang et al., 2014). They consist of beds that are usually dug into the ground, lined, filled with a granular medium and planted with emergent macrophytes which can be fixed to a substrate or floating (Afzal et al., 2019). In the case of fixed macrophytes, wasteflow paths through the granular medium and comes into contact with biofilms and plant roots and rhizomes. Compared to conventional treatment systems, CW are low cost, are easily operated and maintained, and have a strong potential for application in developing countries, particularly by small rural communities (Kivaisi, 2001).CW are divided into three main types depending on the

pattern of wasteflow pathing through the CW: free surface flow (FSF), horizontal subsurface flow (HSSF) and vertical subsurface flow (VSSF). In FSF systems some flow paths above the substrate. In a typical HSSF system (Figure 7), wastewater is maintained at a constant depth and flows horizontally below the surface of the granular medium. HSSF-CW are the most common type of CW built worldwide due to their technical simplicity, low costs of implementation, and low energy and maintenance requirements.

In vertical flow systems, wastewater is distributed over the surface of the wetland and trickles downwards through the granular medium. Vertical systems can be sorted in at least four types depending on the hydraulic regimes: unsaturated flow (like conventional trickling filters), permanently saturated flow, intermittent unsaturated flow, and flood and drain wetlands. With the exception of permanently saturated flow systems, the mode of functioning of the other vertical systems improves the aeration of the bed in comparison to horizontal flow wetlands. Consequently, vertical beds operate generally under more oxidized conditions than horizontal beds and are more efficient, as they can treat higher contaminant loads. Nevertheless, HSSF-CW are often preferred because they are easier to design than vertical systems (Hoffmann, H. et al., 2011).

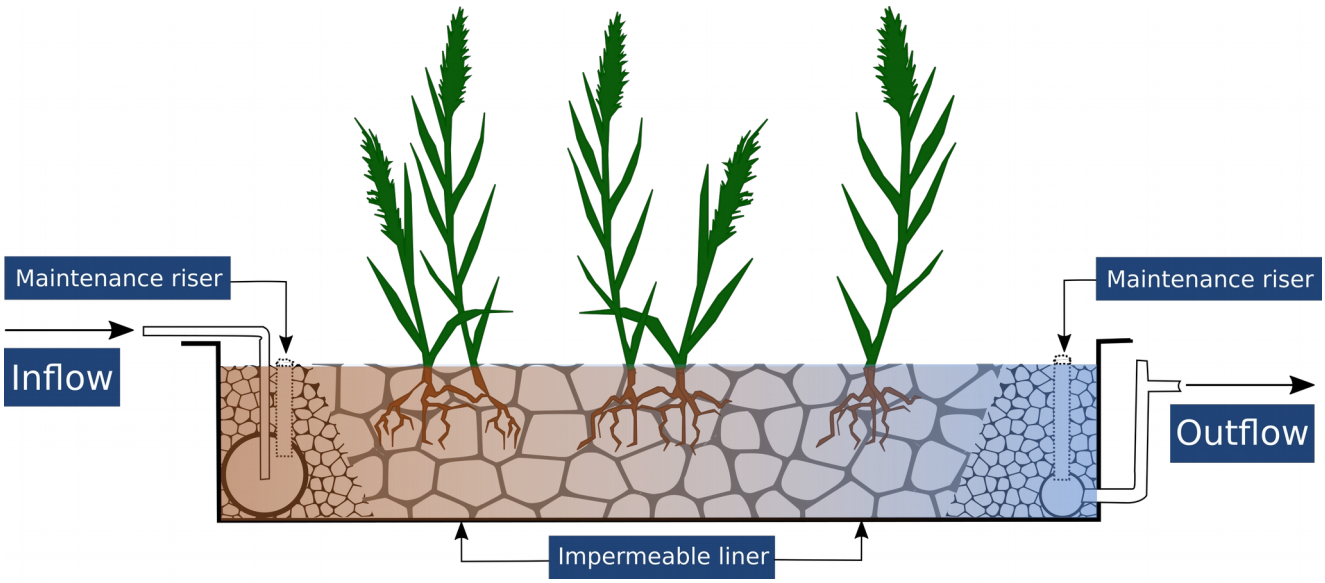


Figure 7| Schematic representation of a horizontal sub surface flow constructed wetland. The main components of a CW are the watertight membrane, the filter media and the helophytes. Adapted from Nivala et al., 2013.

Pilot-scale HSSF-CW have been tested for the removal of organic load (García et al., 2005), *Escherichia coli* (Ibekwe et al., 2016), pathogenic microorganisms (Reinoso et al., 2008), nitrogen and phosphorus (Akratos and Tsihrintzis, 2007), and organic micropollutants (Matamoros and Bayona, 2006) from rural-municipal wastewater. Sulfonamides constitute micropollutants with low

sorption potential to suspended solids and show good removal rates by constructed wetlands (Li et al., 2014b). They are mostly hydrophilic and were designed to be biologically resistant; they are therefore expected to mainly remain in the aqueous phase of the wastewater (Michael et al., 2013). Although little is known about the mechanisms that are responsible for these results, it has been proposed that plants are important for the bioremediation process (Xian et al., 2010) and that microorganisms could play an important role as well (Hijosa-Valsero et al., 2011). Of particular interest are the intermediates and metabolites that derive from these antibiotics, that could be more persistent and toxic that the original compounds (Michael et al., 2013). These by-products can be originated by the metabolism of living organisms (Gobel et al., 2007a) and it has been stated that photolysis could be also an important way of transforming certain sulfonamides, such as sulfamethoxazol, into another compounds (Trovó et al., 2009). In the case of HSSF-CW photolysis is not expected to play a role, as wastewater flows protected from sun light.

The removal of organic load can be followed by the biochemical oxygen demand (BOD) test. This test measures the biodegradable fraction of the wastewater by monitoring the assimilation of organic material by aerobic microorganisms over a set period of time under controlled conditions (Gray, 2004). Usually the test is carried out for in five days (BOD_5) at 20°C and if a nitrification inhibitor is added, BOD_5 is referred as C-BOD_5. Although aeration constitutes a common step in biological wastewater treatment due to a more efficient organic load removal, CW are not typically aerated. As oxygen is one of the main limiting factors in the removal of organic matter through HSSF-CW, aeration of these systems constitutes an improvement for wastewater treatment for the removal of carbonaceous and nitrogenous compounds (Nivala et al., 2013). On the other hand, many ARB of simultaneous clinical and environmental relevance are facultative or aerobic bacteria (Manaia et al., 2018). Therefore it is expected that aeration has an impact on ARB. A scheme of oxygen fluxes in CW wastewater treatment is shown in Figure 8.

The ability of CW to improve water quality in wastewater effluents depends largely on the bacterial communities present within them. Horizontal subsurface flow-CW are designed to simulate the processes that occur in natural wetlands but in a more controlled environment, and contaminants are removed by a wide range of mechanisms (García et al., 2010). Processes such as ammonia oxidation, nitrogen fixation and denitrification are mediated by different bacterial taxa (Berglund et al., 2014). Therefore, CW are viewed both as alternative wastewater treatment option for removal of ARB and ARG and as a near-natural habitat to investigate their environmental fate. A detailed literature evaluation on the fate of ARB and ARG in constructed wetlands reveals the multiplicity of

factors that could play a role in the fate of ARB and ARG. Some of the variables that have to be taken into account are: type of flow, operational parameters, if they are planted or unplanted, type of substrate and the type of influent including antibiotics concentrations. Furthermore, there is no standardized protocol to assess antibiotic resistance status (see Section 1.6) and what has been observed for one ARG might not be valid for another ARG, which makes the comparison between studies even more difficult. Another factor is that evapotranspiration and rain water are frequently not considered, which can affect the abundance of ARG and therefore the calculated removal efficiencies. Most of the studies are being done with small CW, which are easier in operation but differ from full scale WWTP and natural wetlands more than pilot-CW do. Additionally, most of the studies are carried out with samples from CW that have been operated for short periods of time, which can affect their efficiency (Samsó and García, 2013). It is also important to consider if the effluent from the primary, secondary or tertiary treatment is being analysed as it has been shown that removal of ARG is less efficient as the treatment proceeds (Nõlvak et al., 2013).

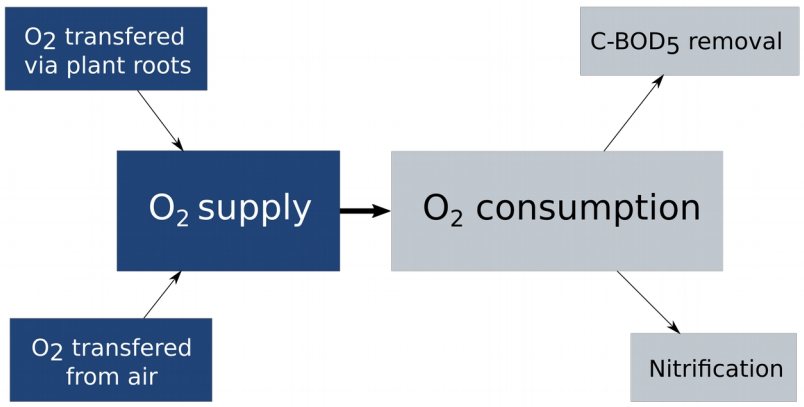


Figure 8| Scheme of oxygen fluxes in constructed wetlands wastewater treatment. Adapted from Ilyas and Masih, 2017.

Taking physicochemical parameters into account, Liu et al. (2014) observed that a higher pH level and dissolved oxygen (DO) showed higher abundances of ARG (*sul* and *tet* genes). Considering operational parameters, Chen et al. (2016a) found that the removal efficiency of ARG decreases with an increasing hydraulic loading rate (HLR) in mesocosm-scale CW. Regarding antibiotic concentrations, short-term treatment of CW with environmentally relevant concentrations (100–2000 ng/l) of different types of antibiotics did not significantly affect resistance gene abundances, suggesting that surface-flow constructed wetlands are well-suited for wastewater treatment purposes in terms of antibiotic removal (Berglund et al., 2014). On the other hand, Gao et al. (2012b) found a significant correlation between the total concentration of sulfonamides in WWTP and the quantity

of *sul* genes although this correlation was not observed in another study (Yi et al., 2017). The reason for the different results from theses studies could have been the variation in SMX concentrations which was between 845.7 and 2101.7 ng/L in the first study and between 62 and 438 ng/L in the second case.

In terms of HGT in CW, the conditions that are necessary for conjugation (see Section 1.1) can vary depending on the operational parameters: a high HRT increases the time when a HGT event can take place and a high organic loading rate (OLR) increases the connectivity. In addition, stress sources that can be present in wastewater such as heavy metals and sub-inhibitory concentrations of antimicrobials can increase HGT of ARG (Jutkina et al., 2018; Zhang et al., 2018). However, this data was obtained in-vitro with higher contaminants concentrations as found normally in wastewater. In the case of antimicrobials, the concentrations tested were in the range of 0.1-1.0 mg/ L and in the case of heavy metals 0.0001 to 100 mg/L. Oxygen levels can be seen as a operating parameter and a stress source at the same time (see Section 1.5). It has been seen that the abundance of *sul1* relative to 16S rRNA gene which is a hint of HGT (see Section 1.6.2), increased during wastewater treatment in CW. This effect was more pronounced in CW with higher oxidizing capacity (HSF compared to SF and VSF) (Liu et al., 2014; Yi et al., 2017).

1.5. Bacterial strategies to cope with oxidative stress can pose a risk for human health

Environmental changes in oxygen quantities constitute a source of stress. Molecular oxygen (O_2) is small and non-polar, and it diffuses across biological membranes as quickly as through water (Ligeza et al., 1998). Reactive oxygen species (ROS) are mainly formed in the cell as a by-product of aerobic metabolism and oxygenic photosynthesis (Figure 9). Flavoproteins, quinones, and iron-sulfur proteins found in virtually all cells can also catalyse the reduction of oxygen to superoxide. Thus, whether or not it can respire oxygen, a cell can be exposed to toxic oxygen species from time to time (Madigan, 2012).

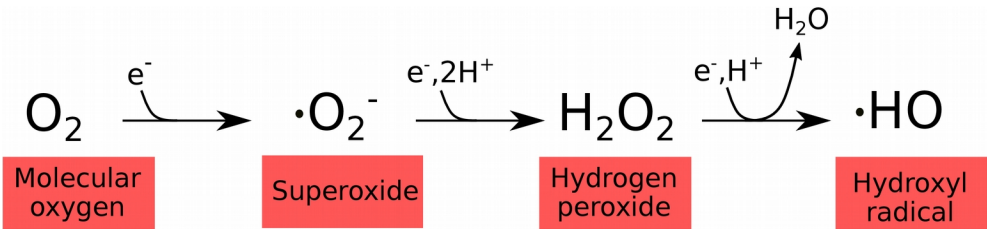


Figure 9| Reactive oxygen species formation. Reactive oxygen species (ROS) are mainly formed in the cell as a by-product of aerobic metabolism and oxygenic photosynthesis. Whether or not it can respire oxygen, a cell can be exposed to toxic oxygen species from time to time. Adapted from Imlay, 2013.

While some microorganisms circumvent oxidative stress by residing in anaerobic microhabitats, all others must contend with intracellular O_2. The ability to do so varies widely: obligate anaerobes cannot tolerate oxygen at all, microaerophiles require a low-micromolar O_2 concentration, and some aerobes can thrive in air-saturated fluids. The types of genotoxic stress induced by ROS include physical damage to the DNA base moiety and the sugar-phosphate backbone of incorporated or unincorporated ("free") nucleotides, as well as single- and double-stranded breaks within the double DNA helix; in addition, DNA can be damaged by by-products of lipid peroxidation. This also holds true for the facultative anaerobe *E. coli* (Imlay, 2013). Figure 10 shows H_2O_2 production in the presence of aeration for an *E. coli* strain deficient in catalase/peroxidase, which are enzymes that catalyse the conversion of ROS into innocuous products for the cell (Korshunov and Imlay, 2010).

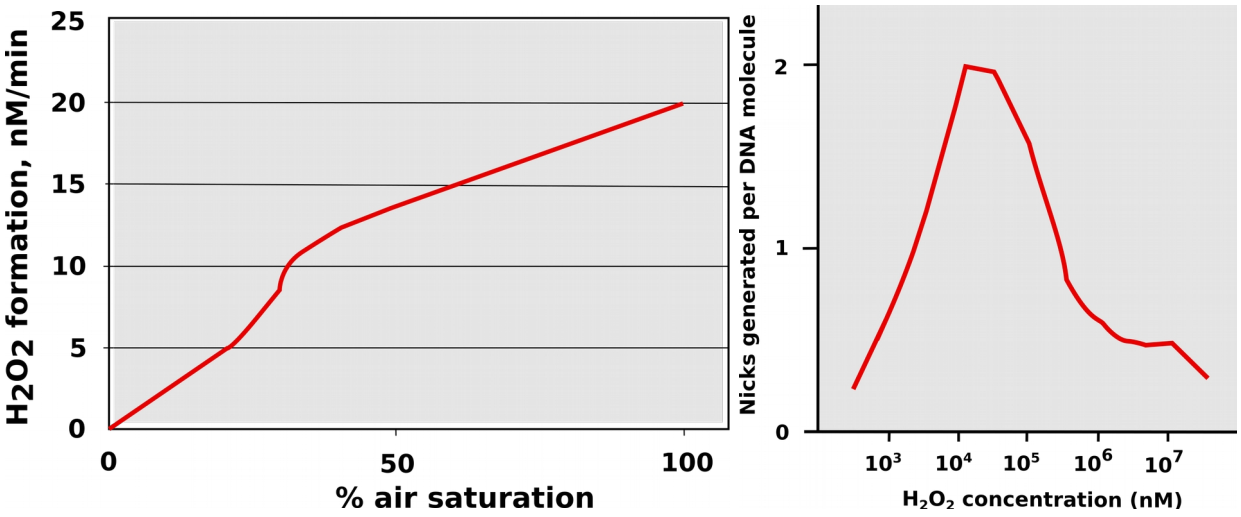


Figure 10| Dependence of H_2O_2 production on percentage of air saturation (left, adapted from Korshunov and Imlay, 2010) **and the number of DNA breaks as a function of H_2O_2 concentration** (right, adapted from Muiras et al., 1993)**.** The production of H_2O_2 as a function of oxygen concentration was studied for an *E. coli* strain, whereas DNA breaks were studied using plasmid DNA.

Figure 10 also shows the DNA damage as a function of H_2O_2 concentration. In this case, plasmid DNA was used (Muiras et al., 1993). It can be seen that the production of H_2O_2 is directly proportional to the dissolved oxygen present in the media. On the other hand, at low concentrations of H_2O_2, the amount of DNA breaks per plasmid is proportional to H_2O_2 concentration. The quantity of DNA breaks decreases after a certain concentration of H_2O_2 in the media. It was postulated that H_2O_2 has two modes of action: at low concentrations it acts by causing DNA damage and at high concentrations, by oxidizing other molecules in the cell (Uhl et al., 2015).

Cellular strategies have evolved to cope with ROS, and variations in external oxygen concentrations lead to physiological and molecular changes in order to maintain cellular homoeostasis (Imlay, 2013). Cuny et al. (2005) suggested that ROS play a role in the formation of dormant cells. Entering the dormant state may be a common adaptive mechanism of bacteria inhabiting different, stressful environments, instead of a specific mechanism limited to bacteria living in a particular niche (Pinto et al., 2015). Many bacteria, including a variety of important human pathogens, can enter into the dormant state in the presence of unfavourable environmental conditions. It has been suggested to be a survival strategy by which bacteria are able to withstand such conditions. While the ability to entering a dormant state is advantageous for the survival of bacteria, it poses a risk to public health. Dormant cells can pose a health risk because they aren't detected with standard cultivation tests but can resume growth under appropriate conditions. For some pathogens such as *E. coli* the resuscitation can be achieved by a increase in the temperature (Li et al., 2014a).

The mechanism of action of some antibiotics like quinolones involves ROS as a key factor for the damage of DNA and ultimately lethality (Luan et al., 2017). From an evolutionary perspective, acquiring defence mechanisms against ROS can protect bacteria against some antibiotics. It has been already shown that oxidative stress can lead to changes in antibiotic resistance by different means. On one hand, oxygen is thought to be an agent that in combination with environmental conditions can lead to a higher mutation rate and *de novo* acquisition of antibiotic resistance (Händel et al., 2016). On the other hand, damage of the DNA can activate the cellular SOS system, which has been linked to higher HGT rates of ARG (Beaber et al., 2004) .

1.6. Methodological approaches to study antibiotic resistance in the environment

Antibiotic resistance has been studied extensively in clinical isolates and the databases of ARB, their MGE and ARG are dominated by them. There are until now suggestions but no standardized procedure to monitor antibiotic resistance in wastewater treatment and to quantitatively assess the risk derived from their release into the environment (Berendonk et al., 2015; Exner and Schwartz, 2015). Although it is important to monitor ARB coming from WWTP as they represent a risk themselves, until now little is known about which ARG abundances are indicators of health risk. In order to determine the conditions at which resistance acquisition and selection is likely to occur in environmental compartments, combined molecular- and culture-based methods are necessary. Depending on the method that is being used, the antibiotic resistance load estimation may vary.

The main advantages of culture-dependent methods are that no sophisticated equipment is needed, an ARG can be linked to it's resistant phenotype and ARB can be identified. The major disadvantage of culture-dependent methods is that few species can be cultivated under standard conditions. Although the environmental prevalence of ARG primarily depends on the host and the type of MGE, the estimation of the levels of ARG has been proposed as a reliable and feasible method to monitor antibiotic resistance (Berendonk et al., 2015). Culture-independent methods like qPCR of ARG have the advantage of being able to quantify total ARG abundance, independently of whether their host are able to grow in the laboratory under selective conditions or not. This technique however doesn't allow to identify ARG hosts. Metagenomic studies also rely on culture independent methods which nowadays are largely based on next generation sequencing (NGS) technologies, like Illumina sequencing. These can reveal both bacterial taxonomy and the different ARG present in a sample but, although linking species with ARG is possible, it remains challenging (Burton et al., 2014).

In order to understand the mechanisms underlying the spread of ARG and ARB in the environment it is of interest to know the hosts outside the hospital walls. The standard procedure for the identification of ARB and/or ARG hosts is to cultivate bacteria in the presence of antibiotics and to identify the ones that can grow on the basis of their morphological characteristics or by sequencing of their 16S rRNA gene. As already mentioned, this approach has the drawback that just a little proportion of the environmental microbiota is culturable. The non-culturable part is made up of bacteria that don't grow in the laboratory under standard conditions and bacteria that are normally capable to grow under these conditions but are in a non-culturable state. A novel method called epicPCR (Spencer et al., 2015) allows to link phylogenetic information to predicted function without culturing and it has already been applied to ARG (Hultman et al., 2018). Another recently developed method called proximity-ligation Hi-C was also proposed to be appropriate for the same aim and was tested for wastewater (Stalder et al., 2019). Although these methods give a handful of useful information, the fact that a bacterium hosts an ARG doesn't necessarily mean that it is resistant towards a particular antibiotic. A metaproteomic approach would be more accurate (Provenzano et al., 2013) to predict antibiotic resistance but this approach doesn't allow to link function to taxonomy and just correlations can be made.

1.6.1. Antibiotic resistance: indicator bacteria and indicator genes

Although organisations at different levels have developed guidelines for action against antimicrobial resistance (Adler, N. et al., 2018; European Commission, 2017; World Health Organization, 2017), to date there no specific requirements concerning ARB and ARG. In Germany, emissions of ARB and ARG from WWTP have not been regulated, either in terms of regulatory law (Waste Water Ordinance) or tax law (Waste Water Charges Act). These is in part due to a lack of a unified protocol for assessing antibiotic resistance. In the attempt to look at the resistance status of a given environment and/or to evaluate wastewater treatment technologies it is necessary to find bacteria and genes that serve as indicators. Universal indicators or protocols haven't been established so far but there are a myriad of suggestions. Berendonk et al. (2015) and Exner and Schwartz (2015) have suggested members of the class Gammaproteobacteria or the phylum Firmicutes as indicator bacteria, as these are the most frequent carriers of acquired ARG. In particular, *Escherichia coli* and faecal enterococci have been recommended. They are already being used to control water quality and are well characterized with regards to antibiotic resistance. Other indicators were proposed for testing environmental samples in which faecal contamination is not expected: *Pseudomonas aeruginosa*, which is being used as water quality indicator and *Aeromonas* spp., which are commonly found in aquatic environments (Berendonk et al., 2015).

Koczura et al. (2012) found that class 1 integrons are often found not only in *E. coli* clinical isolates (56%) but also in *E. coli* isolated from environmental sources. Furthermore, in the same study it has been seen that WWTP effluent contributes to increased frequency of integron-carrying isolates in the river downstream the WWTP. In a study by Slekovec et al. (2012) it was concluded that, although WWTP can reduce *P. aeruginosa* counts in effluent, *P. aeruginosa* load in the river was higher downstream from the WWTP. Several ARG linked to class 1 integrons were found in environmental members of the genus *Pseudomonas* in contaminated waters (Adelowo et al., 2018). The *Aeromonas* spp. mobilome linked to ARG comprises several plasmids of broad host range, transposons, insertion sequences and class 1 integrons (Piotrowska and Popowska, 2015). Among multi-drug resistant *Aeromonas* spp. isolated by Harnisz and Korzeniewska (2018) from water samples taken upstream, within and downstream a WWTP, the majority (81.1%) were obtained downstream from the WWTP.

The absolute abundance of indicator genes is an important bit of information about the overall environmental antibiotic resistance state and for assessing risk associated with their presence. Indicator genes should be common in anthropogenic sources and scarce in non-contaminated

environments in order to allow spatial and temporal source tracking. Genes that are good candidates are linked to MGE and are persistent. Around 15 genes were proposed as indicators for assessing the resistance status in environmental settings and *sul1*, *sul2*, *dfrA1* and *intI1* were among them (Berendonk et al., 2015; Exner and Schwartz, 2015; Gillings et al., 2015). From all the genes studied in this work, *intI1* abundance is the one which can give more information about the resistance status, as it is linked to ARG and MGE. Furthermore, Gillings et al. (2015) designed primers that detect only the clinical allele of the class 1 integron, and thus the abundance obtained through the quantification with these primers is also an indicator of anthropogenic pollution.

1.6.2. Tracking horizontal gene transfer

Among all phenomena related to ARG hosts taking place in the environment, HGT of ARG is a great concern because it has the capacity to develop novel genetic arrangements leading to the emergence of multi-drug resistant bacteria. Also, environmental ARB could act as a reservoir of ARG, being able to transfer ARG to pathogens (Manaia et al., 2018). There is no way to study HGT in real time in the environment, and the only approach so far is to look at the outcomes after HGT had happened. The strongest data that indicates that a HGT event had taken place is a change in ARG host but as already mentioned, in order to study ARG hosts, culture-dependent methods are the most common approach and these reveal just a small percentage of the total bacterial population. Also, HGT is mostly likely to happen among bacteria of the same species (Thomas and Nielsen, 2005). If a change in bacterial host species is considered as a necessary condition for detecting HGT, these HGT events would remain unnoticed. The most common approach is to measure changes in the abundance of ARG relative to 16S rRNA gene abundance but this gives no information about the host. A multiphasic approach combining culture- dependent and independent methods is thus, the best way to address HGT in the environment (Figure 11).

The results obtained from culture- dependent and independent methods are often complementary, as they detect different subsets of the microbial community (McLain et al., 2016). Furthermore, a rise in ARG abundance detected by qPCR can not be observed by a PCR survey of isolates if this is produced by an intracellular increase of ARG copies (Nõlvak et al., 2013). Also, an increase in the relative abundance can indicate a selection (if the 16S rRNA abundance decreases to a higher extent than the ARG) or enrichment (if ARG abundance increases to a higher extent than 16S rRNA gene). If a selection of a particular ARG takes place, this could be due to co-selection, making it difficult to find out which environmental factors act as selective pressure. Co-selection can occur towards

genes that reside in the same bacterium or that are on the same genetic element, for example ARG that are part of class 1 integrons.

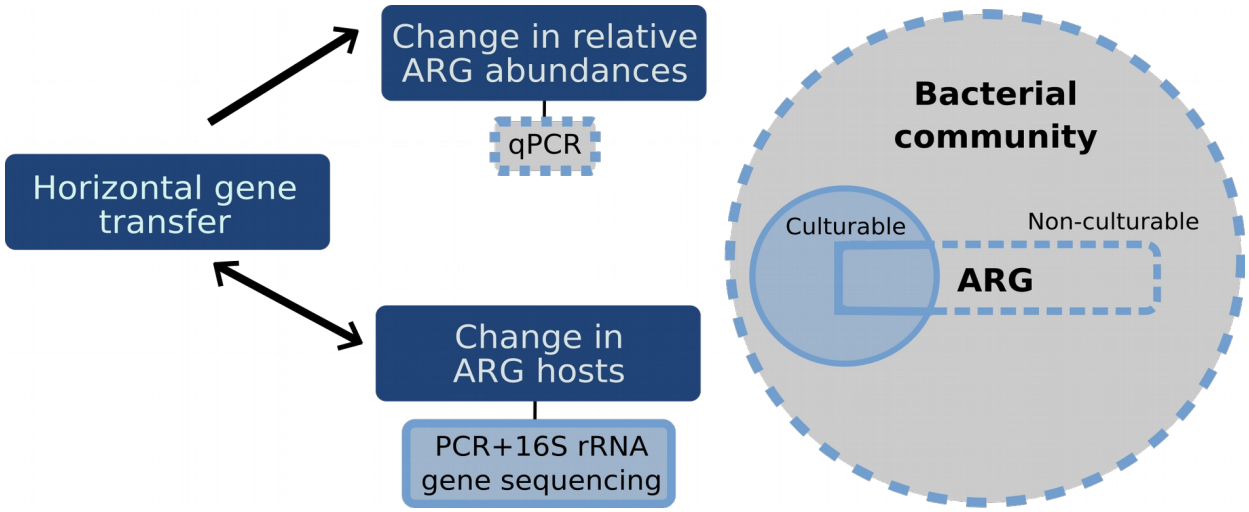


Figure 11| Multiphasic approach to study horizontal gene transfer in the environment. Culture-independent methods are shown with a dashed border and in filled border the culture-dependent method is depicted. Culture-dependent methods allow to identify ARG hosts. While a change in ARG abundance relative to 16S rRNA gene abundance can indicate HGT, a change in ARG hosts is sufficient to conclude that HGT has taken place.

2. Objectives

The WHO addresses antimicrobial resistance as a major health problem and it warns about a post-antibiotic era. Modern medicine relies on antibiotics and the consequences of an increased resistance are detrimental, both for human lives and for economical costs (OECD, 2018). While several strategies are taken in the clinical setting in several countries in order to prevent the development and the spreading of resistance, a lot remains to be done outside the hospitals walls. In a multivariate study (Collignon et al., 2018), poor sanitation was linked to increased antibiotic resistance.

Constructed wetlands constitute an alternative wastewater treatment that resemble natural wetlands and therefore serve as models for the processes taking place in natural aquatic environments (Hammer and Bastian, 1989). Aeration is beneficial for wastewater treatment by HSSF-CW in terms of final water quality, as low concentration subsurface oxygen is one of the main limiting factors in this kind of CW (Nivala et al., 2013). How aeration of CW affects the removal of ARB and ARG hasn't been addressed yet. The presence of reactive oxygen species coming from the incomplete reduction of oxygen causes DNA damage and this can enhance HGT of ARG through the activation of the SOS system. This work addresses the question whether aeration in CW causes an increased HGT of ARG. For the studying of HGT it is important to know ARG hosts, which hasn't been studied in CW before.

In this work, two CW treating municipal-rural wastewater were studied, one CW was mechanically aerated and the other was not. Comparing both CW helps to understand better the effects of aeration on antibiotic resistance during wastewater treatment and enables studying the physiochemical factors affecting it in the open environment. A common approach in wastewater treatment is to compare input and output in order to evaluate the systems. However, in a study on the same CW, Button et al. (2015) found that the microbial communities in the outflow and in internal samples differed significantly. That highlights the importance of obtaining internal samples to understand the processes involving bacteria during wastewater treatment. Therefore, water samples from the in- and outlet as well as from inside the CW were analysed.

With the aim of evaluating the performance of the aerated CW with regard to removal of ARB and ARG and to study HGT of ARG in the CW, the antimicrobials SMX and TMP and five genes were selected. The gene 16S rRNA is useful to quantify the total bacterial community, *sul1*, *sul2* and

dfrA1 are three ARG that give resistance to the aforementioned antibiotics; and *intI1* is related to ARG and MGE. A multiphasic approach was applied making use of culture- dependent and independent methods to analyse samples from in- and outflows as well as from internal points in both CW. The culture-independent method consisted in the quantification of the selected genes through qPCR. The culture-dependent approach consisted in the quantification of colony forming units on different solid culture media with and without the addition of the selected antibiotics. Over 1600 of these colonies were isolated and analysed through PCR for the selected genes. In addition, the 16S rRNA gene of over 250 isolates was sequenced in order to identify ARG hosts. The variable region of the class 1 integrons were studied through amplification by PCR and sequencing of the PCR product. As *E. coli* and *Aeromonas* spp. were common hosts of the studied genes, they were further quantified by culture dependent and independent methods. For *E. coli* the MPN method, CFU counting and qPCR of the *uidA* gene was used. In the case of *Aeromonas* spp., CFU counting and qPCR of the gene *gyrB* was applied. Standard wastewater parameters and antibiotic concentrations were also determined.

3. Materials and methods

3.1. Constructed wetlands at the ecotechnology in Langenreichenbach

The studied pilot-scale saturated HSSF-CW planted with common reed (*Phragmites australis*) are located in Saxony, Germany and receive the same wastewater from the surrounding rural-residential villages (~16000 inhabitants), after a pretreatment by a three-chamber septic tank (Nivala et al., 2013). They act then as secondary treatment and have the advantage that samples can be taken within the CW. Water samples were taken over a period of four years (May 2014 - October 2017) during periods were the CW were covered with fully grown plants (March-October). Three out of 22 sampling events took place outside this time period. Means of the values of this days were compared to the mean values of the vegetation period and the differences weren't statistically significant. Therefore, these data were included in the analysis. Operating details of both CW are provided in Table 1. H50p stands for **H**orizontal **50** cm depth and **p**lanted and HAp for **H**orizontal **A**erated and **p**lanted. Aeration in HAp takes place from the bottom of the CW, homogeneously and perpendicular to the direction of flow. For the calculation of HRT, the porosity of the media was taken into account to calculate the water volume in the CW (0.38).

Table 1| Design and operating details for the two sampled CW. HLR: hydraulic loading rate, HRT: hydraulic retention time. The HLR for HAp changed from 130 to 100 L/m^2.d in 10/2015.

System name	Length [m]	Width [m]	Depth [m]	HLR [L/m^2.d]	HRT [d]	Aeration [m^3 air/ m^2-h]
HAp	4.70	1.20	1.00	130/100	2.9/3.7	0.35
H50p	4.70	1.20	0.5	36	5.4	none

The two CW studied are stable wastewater treatment systems, as they have been run uninterruptedly for over 7 years with essentially constant performance levels regarding removal of C-BOD_5, inorganic nitrogen, and *E. coli* as microbial indicator for faecal contamination (Headley et al., 2013; Kahl et al., 2017). Because they are stable systems and they receive the same wastewater, the changes in wastewater that can be monitored in space are simultaneously changes in time, in contrast to systems like activated sludge which are mostly mixed. In this way, these CW offer a model to study flow- and therefore time-dependant changes of antibiotic resistance in a WWTP during aeration. In Table 2 the inflows, outflows and rain volume are shown for the months when sampling took place.

Table 2| Inflows, outflows and rain volume for the months in which sampling took place. "a" is the is the ratio between outflow and inflow. The inlet flow in HAp was modified by UBZ from 770 to 578 L/d on 10/2015.

Month	Rain (mm)	Q_{inlet} (L/d)		Q_{outlet} (L/d)		a (Q_{outlet}/Q_{inlet})	
		HAp	H50p	HAp	H50p	HAp	H50p
05/2014	95	770	195	689	141	0.9	0.7
06/2014	25	771	195	655	105	0.8	0.5
07/2014	73	770	194	615	116	0.8	0.6
08/2014	77	768	192	655	131	0.9	0.7
09/2014	35	769	192	701	168	0.9	0.9
10/2014	32	769	193	727	181	0.9	0.9
06/2015	60	770	197	669	121	0.9	0.6
07/2015	155	770	197	641	117	0.8	0.6
08/2015	113	770	197	734	164	1.0	0.8
09/2015	45	770	197	726	156	0.9	0.8
10/2015	73	770	197	749	176	1.0	0.9
Mean+SD		770±1		687±41		0.9±0.05	
11/2015	115	578	197	558	183	1.0	0.9
12/2015	46	578	197	571	191	1.0	1.0
07/2016	67	578	197	436	130	0.8	0.7
08/2016	40	578	197	436	143	0.8	0.7
09/2016	28	578	197	440	155	0.8	0.8
10/2016	89	577	197	533	186	0.9	0.9
11/2016	44	577	196	553	189	1.0	1.0
05/2017	37	578	197	516	159	0.9	0.8
06/2017	84	578	197	461	117	0.8	0.6
07/2017	109	578	197	469	130	0.8	0.7
08/2017	74	578	197	470	141	0.8	0.7
Mean+SD	68±33	578±0.5	196±20	495±50	150±27	0.9±0.1	0.8±0.1

In October 2015 the inflow changed from 770 L/d to 578 L/d for HAp for external reasons to this work. No changes in the values of the studied parameters were observed. It was already observed before (Kahl et al., 2017) that HAp it is a robust system. Robustness can be related to the extent to which the performance of a system is affected by operational conditions and the time needed for adaptation to changes.

It is worth noticing that due to a longer HRT and a higher surface/depth ratio, evapotranspiration is higher in H50p. On average, the proportion of inflow that leaves the CW was 90% in the case of HAp and 80% in the case of H50p. This calculations take evapotranspiration and rain into account. Whereas the correction due to these processes is of relevance for the evaluation of CW overall performance, the concentration of the substances within the CW is the one affecting microorganisms and their responses.

Areal removal rates were calculated using Equation 1. Calculating areal removal rate is of relevance in order to compare the treatment capacity (speed) of different CW.

$$areal\,removal\,rate = \frac{Q_{inlet} * [Abundance]_{inlet} - Q_{outlet} * [Abundance]_{outlet}}{A} \quad (1)$$

Where Q is flow in m^3/d, abundance is gene copies/m^3 or g/m^3 and area is the surface of the CW in m^2. An average of Q calculated with the values of the sampling months was used in order to simplify the analysis. Not all the parameters described were measured for every sampling day for logistical reasons. Water coming out from the septic tank entering the CW is called “inlet”.

3.2. Samples and standard wastewater parameters

An schematic representation of the sampling and pictures of the CW are shown in Figure 12. The sample collection procedure is described in detail by Nivala et al. (2013). Briefly, grab samples were taken at 0.12, 0.25, 0.50, 0.75 of the fractional length and 0.50 of the fractional depth of each CW, using a peristaltic pump. That means at a depth of 0.50 m in HAp and 0.25 m in H50p. Standard wastewater parameters (T, pH, redox potential and DO) were determined on site as described by Kahl et al. (2017). Water temperature and pH were measured with a SenTix 41 sensor, dissolved Oxygen (DO) with a ConOx sensor, and redox potential with a SenTix ORP sensor ($Pt/Ag^+/AgCl/Cl^-$). The sensors were part of a Multi 350i instrument (WTW, Weilheim, Germany). After arrival, part of the samples was immediately used for culturing on agar plates (Section 3.4) and some for DNA extraction (Section 3.7). The rest of the sample volume was transported in cold to the laboratory (around 2 h of transportation time). The systems were sampled at inlet/outlet simultaneously. This disregards transport delay within the treatment system, adding uncertainties whether the changes seen in water are a result of treatment or are affected by fluctuations in the incoming water. This is partly overcome by the stability of systems which allows repetition of the sampling and calculation of average values.

Removal efficiencies were calculated using Equation 2.

$$removal\,efficiency = \frac{[compound]_{out} - [compound]_{inlet}}{[compound]_{inlet}} * 100 \quad (2)$$

Variables in brackets represent concentration values. The ratio between in and outflows (called “a” in Table 2) was taken into account.

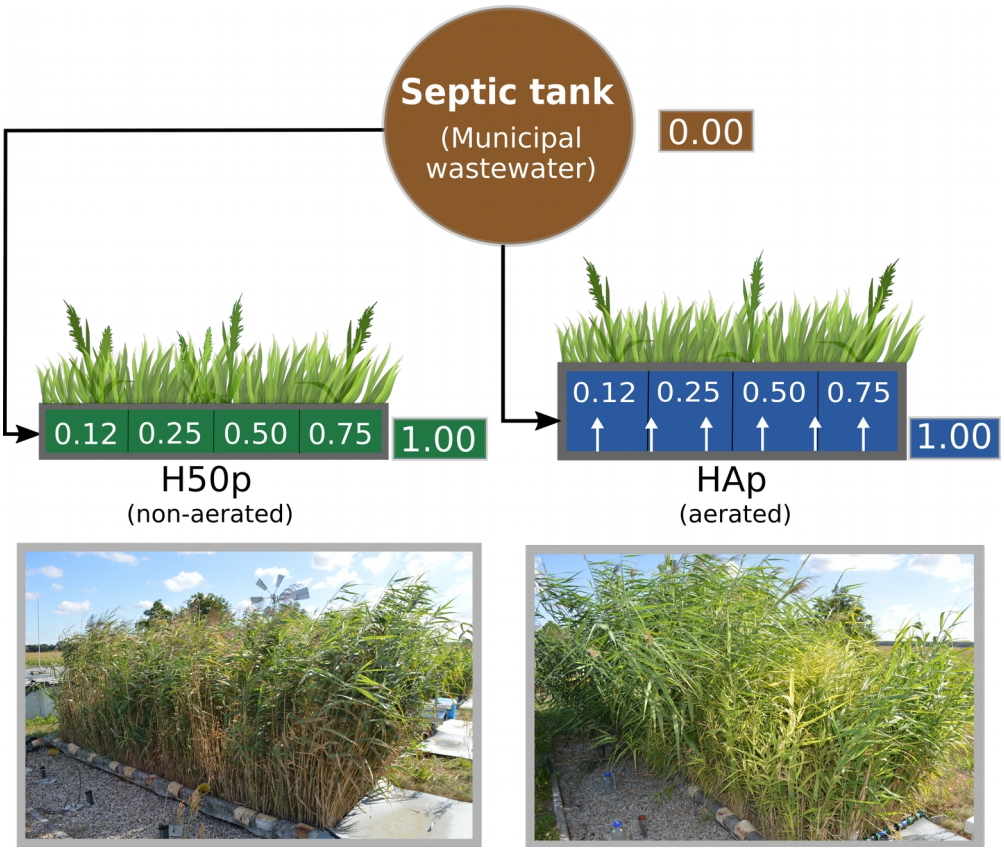


Figure 12| Sampling schema (top) and pictures (bottom) of the constructed wetlands. The numbers indicate the fractional length at which the samples were taken.

3.3. Chemical analysis of antibiotics

The compounds SMX, N^4-acetyl-sulfamethoxazole (N^4-Ac-SMX) and TMP were measured by liquid chromatography LC-MS in water and sediments, together with other two sulfonamides (sulfadiazin, sulfamethazin) which due to their low concentrations (<15 ng/L) were no longer considered. About 43-48% of SMX taken is excreted in the form of N^4-Ac-SMX by warm-blooded animals (Ven et al., 1995). This metabolite can be microbially transformed into its parental compound in the environment (Gobel et al., 2007) and has no anti-microbial activity.

Sample preparation

250 mL water samples were filtered (0.45 mesh, glass fibre filter, GE Healthcare, Buckinghamshire, UK) before solid phase extraction with Oasis HLB sorbent (200 mg, Waters, Milford, USA). The SPE sorbent was conditioned with methanol (Biosolve, Dieuze, France) and Milli-Q water before the sample was applied. After drying the sorbent under a gentle stream of inert gas for 30 min, the analytes were eluted with 10 mL methanol. The eluates were concentrated to 0.5 mL by evaporating the methanol (TurboVap II, Biotage, SWE). Adding 0.5 mL HPLC solvent A water, 5mM NH_4 acetate) gave the final sample volume of 1 mL from that 5 µL were injected for HPLC-MS-MS analysis.

HPLC-MS-MS analysis

High Performance Liquid Chromatography-tandem mass spectrometry (HPLC-MS-MS) analysis used an Agilent 1260 HPLC instrument (Agilent Technologies, Waldbronn, Germany) coupled to a triple stage quadrupol mass spectrometer ("QTrap 5500", SCIEX, Darmstadt, Germany). The chromatographic separation was performed with an "Ascentis Express C18" column (10 cm x 3 mm id and 2.7 µm particle size, Supelco, Seelze, Germany). Water with 0.1 % formic acid (solvent A) and methanol with 0.1 % formic acid (solvent B) were used to elute at the antibiotics at a flow rate of 300 µL min^{-1} and following linear gradient: to 1 min 95 % A, to 15 min 10 % A, from 20 – 25 min 95 % A. Column oven was set at 30 °C. Electrospray operated for ionization at positive mode with 5.5 kV spray voltage. Mass analysis at multiple reaction monitoring mode used the analyte-specific ion transitions listed in Table 3.

Table 3| Characteristics of the target antibiotics and the HPLC-MS-MS method.

	CAS-No./ logP*	Ion transitions (m/z)	HPLC-retention time (min)	SPE recovery (%), (n=4)	LOD_i (ng/L) ± SD (n=3)	LOD_m (ng/L) ± SD (n=3)
SMX	723-46-6 0.89	254.2 → 92.0 254.2 → 65.0	9.9	111	0.5 ± 0.02	1.50 ± 0.06
N_4-Ac-SMX	21312-10-7 0.86	295.9 → 134.1 295.9 → 65.0	6.5	89	1.0 ± 0.08	3.10 ± 0.30
TMP	738-70-5 0.91	290.9 → 230.1 290.9 → 261.0	8.5	109	0.5 ± 0.03	1.50 ± 0.08
Sulfadiazine	68-35-9 0.09	250.9 → 156.0 250.9 → 92.0	6.6	104	0.5 ± 0.03	1.50 ± 0.09
Sulfamethazine	57-68-1 0.89	279.4 → 186.1 279.4 → 92.0	9.0	111	1.0 ± 0.06	3.10 ± 0.20

CAS: chemical abstract service, SPE: solid-phase extraction, LOD_i: instrumental limit of detection. LOD_m: limit of detection of the method.*logP from PubChem open chemical database.

External calibration enabled quantification via calibration curves with 4-5 concentration points. Duplicates of each sample were measured randomly distributed in an analysis batch. Instrument controls were included multiple times within the series of analyses. Due to the good recoveries (89-111%) in solid-phase extraction (SPE) enrichment, concentration data were not corrected. Instrumental limits of detection and method limit of quantification (LOD_i, LOQ_m) as well as precision are included in Table 3.

3.4. Isolation of resistant bacteria

Different solid culture media were used in order to select common ARG hosts in wastewater: Mueller-Hinton agar, a rich media suitable for antibiotic resistance tests, Endoagar (EA), selective for *Enterobacteriaceae* spp. and differential for *E. coli*, Pseudomonas Isolation Agar (PIA), selective for *Pseudomonas* spp. and differential for *P. aeruginosa* and Aeromonas Isolation Agar (AIA), selective for *Aeromonas* spp. and differential for *A. hydrophila*. One set of petri dishes was plated without antibiotics, one was supplemented with SMX (64 mg/l) and another set with TMP (8 mg/l) (Czekalski et al., 2012). This type of selection was chosen and not a combination because in this way the dynamics of the ARB and ARG towards each antibiotic can be studied separately. This allowed a better comparison to qPCR data, where no selection or enrichment was carried out prior to analysis. Furthermore, using only SMX permits the selection of the total abundance of class 1 integrons with conserved 3' region and not only those which carry a *dfrA* allele in their variable region. Two serial dilutions differing in one order of magnitude were prepared per sampling point. The selected order of magnitude depended on the sampling point and the culture media. Before pipetting to plate, the containers were shaken in order to get a homogeneous sample and afterwards, 100 µL of each dilution were cultivated in duplicates. The incubation time and temperature were 16-43 h at 20 °C for all media when the differential property wasn't necessary. When also the differential property was needed, instructions were followed depending on the media (Appendix A). Bacterial cells were picked from the centre of well isolated colonies of each sample grown in the presence of antibiotics.

3.5. Characterization of SMX/TMP resistant isolates

Genomic DNA extraction from cultured SMX/TMP-resistant bacteria was performed by the microwave boiling method described by Orsini and Romano-Spica, 2001. Extracted DNA was stored at –20 °C until used.

As the abundances of the selected genes might vary in wastewater, an initial colony-PCR screening of different ARG was carried out. Fifty eight SMX resistant isolates were tested for *sul1*, *sul2* and *sul3* and fifty nine TMP resistant isolates were screened for the genes *dfrA1*, *dfrA5*, *dfrA7*, *dfrA12* and *dfrA17*. The frequency for *sul* genes was *sul1* > *sul2* >*sul3* and from the *dfrA* genes that were tested *dfrA1* was the most prevalent. Up to 17% of SMX resistant isolates carried *sul1* gene and 12% carried *sul2* gene. In the case of TMP resistant isolates, 9% carried *dfrA1*. The respective PCR amplicons were sequenced to confirm their identity.

As a consequence of the results of the first screening, *sul1*, *sul2* and *dfrA1* were the chosen ARG for further analysis. Resistant bacteria were PCR-tested for the presence of *sul1*, *sul2* and *dfrA1* as described before (Grape et al., 2007; Wang et al., 2014) using REDTaq® ReadyMix™ PCR Reaction Mix and the primers in Table 4. *intI1* (Gillings et al., 2015; Mazel et al., 2000) and gene cassettes (Machado et al., 2005) were also studied.

The primer pair developed by Mazel et al. detects environmental and clinical class 1 integrons, whereas primers designed by Gillings et al 2015 detect only 'clinical' alleles. 16S rRNA gene was amplified, in order to check the quality of the template and for sequencing purposes. Negative and positive controls were run in parallel to the samples. Negative control contained water instead of genomic DNA and the positive control consisted in genomic DNA from isolates which had been proven to be positive for the gene of interest.

Table 4| Description of primers used in this study. The prefix "q" is for primers used only for qPCR.

Gen	Primer	Sequence 5'-3'	Fragment size in bp	Reference
16S rRNA gene (PCR)	27F	AGAGTTTGATCMTGGCTCAG	1465	Wang and Qian, 2009
	1492R	CGGTTACCTTGTTACGACTT		
16S rRNA gene (qPCR)	519-F	CCAGCAGCCGCGGTAATAC	410	
	909-R	CCGTCAATTCCTTTRAGTTT		
uidA (*E. coli*)	Eco-F	CTGCTGCTGTCGGCTTTA	205	Kaushik and Balasubramanian, 2012
	Eco-R	CCTTGCGGACGGGTAT		
gyrB *(Aeromonas spp.)*	IA-F	CTGAACCAGAACAAGACCCCG	198	Khan et al., 2009
	IA-R	ATGTTGTTGGTGAAGCAGTA		

sul1	sul1-F	CGGCGTGGGCTACCTGAACG	433	Wang et al., 2014
	sul1-R	GCCGATCGCGTGAAGTTCCG		
	sul1-qF	TGTCGAACCTTCAAAAGCTG	113	
	sul1-qR	TGGACCCAGATCCTTTACAG		
*sul*2	sul2-F	GCGCTCAAGGCAGATGGCATT	293	Wang et. al. 2014
	sul2-R	GCGTTTGATACCGGCACCCGT		
	sul2-qF	ATCTGCCAAACTCGTCGTTA	89	
	sul2-qR	CAATGTGATCCATGATGTCG		
*dfr*A1	dfr1-F	TGGTAGCTATATCGAAGAATGGAGT	425	Grape et al., 2007
	dfr1-R	TATGTTAGAGGCGAAGTCTTGGGTA		
intI1 (all the alleles)	intI1-F	GGTCAAGGATCTGGATTTCG	483	Mazel et al., 2000
	intI1-R	ACATGCGTGTAAATCATCGTC		
intI1 (clinical allele)	intI1-F	CGAACGAGTGGCGGAGGGTG	311	Gillings et al., 2015
	intI1-R	TACCCGAGAGCTTGGCACCCA		
Gene cassettes	5’CS	GGCATCCAAGCAGCAAG	Variable	Levesque et al., 1995
	3’CS	AAGCAGACTTGCCTGA		

PCR products of 16S rRNA gene and gene cassettes were purified by using MinElute PCR purification Kit (Qiagen, Valencia, CA, USA) in order to be sequenced. Sequencing was carried out by the Sanger method (Macrogen, Netherlands). The sequence were then analysed using BLASTn. The number of isolates are shown in Table 5.

Table 5| Number of isolates analysed.

	SMX	TMP	total
screened	837	787	**1642**
sequenced	-	-	**256**
intI1(+), cassettes	294	207	**501**

3.6. Phylogenetic trees

Phylogenetic trees of the 16S rRNA gene were built in R using the package ‘ggtree’ (Yu et al., 2017). For alignment ‘aliview’ (Larsson, 2014) and ARB (Ludwig, 2004) was used, and ‘trex-online’ (Boc et al., 2012) for tree generation, using maximum likelihood method. Illumina sequencing of the 16 rRNA gene of the total community could not be finished within the scope of this research. These results coming from a culture-independent method would have provided data about the taxonomy and phylogeny of the total bacterial community.

3.7. Quantitative PCR analysis

In the case of DNA extraction, samples were centrifuged for 15 min at 13000 rpm and the pellets were the starting material for DNA extraction. The volume of sample that was centrifuged varied depending on the sample in order to get a visible pellet after centrifugation (1,5 to 60 mL). This was taken into account to correct the final concentration results. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), which is an appropriate Kit to extract total DNA from a myriad of samples, including water samples (Jeunen et al., 2019). DNA concentrations were quantified with a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE).

The abundances of the 16S rRNA gene, *sul1*, *sul2*, *dfrA1*, and *intl1* were determined by SYBR-green based real-time PCR with 3 technical replicates per sample. The assay was run on a StepOne Plus Cycler (Applied Biosystems) in a 12.5 µL reaction mixture per standard and sample. The PCR conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 20 s at the correspondent annealing temperature and 72 °C for 20 s. Dilutions of template DNA were used for each sample to compensate for the effect of PCR inhibitors in the samples. Standards were PCR-amplified fragments of the *uidA* gene of *E. coli* as well as the *sul* genes, *dfrA1* and *intI1* from bacteria isolated in this study. *gyrB* standard was obtained from an *A. aquatica* strain AE235 from the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

DNA concentrations of the standards were measured by Nanodrop spectrophotometry and the copy number (CN) per mL of sample was calculated using Equation 3.

$$CN = \frac{c * 6.022 * 10^{23}}{660 * N} \quad (3)$$

where *c* is the measured DNA concentration (µg/µL) and N is the DNA fragment length in bp. Average efficiency values were 90% for 16S rRNA gene, 84.5% for *sul1,* 94.1% for *sul2,* 91.3% for

intl1, 90% for *dfrA1*, >95% for *uidA* and 90% for *gyrB.* ARG removal efficiencies were calculated using Equation 4.

$$\text{ARG removal efficiency} = \frac{[\text{ARG}]_{\text{inlet}} - [\text{ARG}]_{\text{out}}}{[\text{ARG}]_{\text{inlet}}} * 100 \quad (4)$$

where [ARG] means gene abundance in copies/100 mL.

Fold change is defined as the change in abundance compared to the abundance in the inlet and was calculated using Equation 5.

$$fold\ change = \frac{[ARG]_{fractional\ length} - [ARG]_{inlet}}{[ARG]_{inlet}} \quad (5)$$

which further normalise the data for possible fluctuations in time in the incoming wastewater. Relative abundance of ARG was calculated with Equation 6.

$$ARG\ relative\ abundance = \frac{[ARG]}{[16S\ rRNA\ gene]} \quad (6)$$

Log reduction shows how many orders of magnitude the abundances changed after the treatment and was calculated with Equation 7.

$$\log reduction = \log_{10}[abundance]_{inlet} - \log_{10}[abundance]_{outlet} \quad (7)$$

In terms of antibiotic resistance, in order to compare absolute abundances from culture dependent and independent methods, the total ARG abundance from culture-dependent methods can be estimated using the Equation 8.

$$CFU^{R-ARG} * culturability\ factor \simeq [ARG] \quad (8)$$

where $CFU^{R\text{-}ARG}$ represents the frequency in which screened isolates were positive for ARG. The culturability factor considers that each cell has in average 4 copies of the 16S rRNA (Klappenbach, 2001) and it takes the data of the correspondent sampling point into account.

Which in the case of *sul1* is:

$$\left(CFU^{SMX-sul1} * CFU^{SMX}\right) * \frac{16S\ rRNA\ gene/4}{CFU} \simeq [sul1] \quad (9)$$

3.8. Quantification of specific bacteria

Quantification of Escherichia coli.

Culturable *E. coli* was measured by most probable number (MPN), adopting the Colilert18 Quanti-Tray method (IDEXX Laboratories, USA) and CFU counting on Endoagar. Abundance of total *E. coli* was carried out by measuring the *uidA* gene by qPCR. The selectivity of *uidA* primers as well as the differential property of the media towards *E. coli* was confirmed by sequencing the 16S rRNA gene.

Quantification of Aeromonas spp.

Culturable *Aeromonas* was quantified by CFU counting on Aeromonas isolation agar. Abundance of total *Aeromonas* spp was carried out by measuring the *gyrB* gene by qPCR. The selectivity of *gyrB* primers as well as the differential property of the media towards *Aeromonas* spp. was confirmed by PCR of CW isolates that had been identified as *Aeromonas* through 16S rRNA gene sequencing.

3.9. Statistics

No outliers treatment was carried out in order to include biological variability. Statistical significance was calculated with Wilcoxon test in R studio using the package ‘ggpubr’(Alboukadel, 2018).

3.10. Set up of emulsion, paired isolation and concatenation PCR (epicPCR)

The protocol was based on previous studies (Hultman et al., 2018; Spencer et al., 2015). Primers were designed with the online tool Primer-BLAST. A first set of three primers is necessary for one-step fusion PCR which amplifies part of a functional gene and part of the 16S rRNA gene. A second set of two primers is used to improve the specificity by the principle of nested-PCR and to introduce partial adaptor sequences for sequencing purposes. The primers giving the best results are shown in Tables 6 and 7. epicPCR products are being sequenced by the Illumina sequencing technic.

Polyacrylamide bead formation

Water samples were centrifuged at 8000 rpm for 15 in at room temperature. The volume centrifuged in order to get a visible pellet depended on the sample (between 2 and 180 mL depending on the sample, in order to get 1-2x10^7 cells). The pellets containing the cells were then resuspended in 100

µl of nuclease free water. 100 µl of 30% acrylamide/bis- acrylamide and 25 µl of ammonium persulfate (APS) were given to the cells and gently vortexed. After adding 600 µl of mineral oil the mixtures were vortexed at 3000 rmp for 30 s. After vortexing, 25 µl of tetramethylethylenediamine (TEMED) were added and vortexed again but this time at maximum speed (3000 rpm) for 30 s. The mixture was kept for 90 min at room temperature to allow polymerization.

For the extraction of the beads, 800 µl of diethyl ether was added and a precipitated formed. After adding 1 mL of nuclease free water, mixing and centrifuging for 30 s at 12000 g, three layers formed: bottom layer contained the beads, the middle layer contained a mixture of oil and water and the top layer was made of oil. The oil phase was discarded. This step was repeated until no top layer was visible. The water layer was discarded and 1 mL of TK-buffer (20 mM Tris-HCl, pH 7.5, 60 mM KCl) was added to the beads. The bead suspension was run through a 40 µm cell strainer and the flow through contained the beads.

Cell lysis

2 µl of lysozyme were added to 250 µl of the beads and incubated overnight at 37 °C. 2 aliquots were put together and centrifuged at 12000 g for 30 s. The supernatant was discarded and 400 µl of TK were used to resuspend the beads. 100 µl of proteinase K and 4 µl of triton X-100 were added to the samples and incubated 37 °C for 30 min and 95 °C for 10 min. Samples were aliquoted in 1 mL and centrifuged at 12000 g for 30 s. The beads were resuspended with enough TK buffer to reach 500 µl of total volume.

Fusion PCR

Four 2 mm glass beads and 900 µl ABIL emulsion oil were added to each tube. 110 µl of the flowing mix was added to 90 µl of the polyacrylamide beads:

- 40 µl of 5X Phusion High-Fidelity buffer (New England Biolabs Inc.)
- 5 µl of 10 mM dNTPs
- 20 µl of each each fusion primer (Forward and reverse: 10 µM, Fusion primer: 0,1 µM).
- 16 µl of Phusion Hot Start Flex (New England Biolabs Inc.)The mixture was emulsified by vortexing at 3000 rpm for 1 min and aliquoted in of 60 µl. The PCR program run was as follows: 98 °C 30 s, [98 °C 5 s, 55 °C 30 s, 72 °C 30 s]x33 cycles, 72 °C 5 min. After the purification of the products, 2 µl of 50 mM of ethylenediaminetetraacetic (EDTA) were added.

Table 6| Fusion PCR primers for epicPCR

Gene	Primer	Sequence	Size (bp)	Reference
sul1	sul1 qF	TGTCGAACCTTCAAAAGCTG	1180	Wang et al., 2014
	sul1 qR/519F'	GWATTACCGCGGCKGCTGTGGACCCAGATCCTTTACAG		
	pH'	AAGGAGGTGATCCAGCCGCA		Edwards et al., 1989
sul2	sul2 qF	ATCTGCCAAACTCGTCGTTA	1111	Wang et al., 2014
	sul2 qR/519F'	GWATTACCGCGGCKGCTGCAATGTGATCCATGATGTCG		
	pH'	AAGGAGGTGATCCAGCCGCA		Edwards et al., 1989
dfrA1	dfrA-F1 (2)	ATCGAAGAATGGAGTTATCG	1413	This work
	dfrA1-R1/519F'	GWATTACCGCGGCKGCTGTATGTTAGAGGCGAAGTCTTGGGTA		Grape et al. 2007
	pH'	AAGGAGGTGATCCAGCCGCA		Edwards et al., 1989
intI1	intI1-F1 (3)	CGAAGTCGAGGCATTTCTGTC	1241	Hultman et al., 2018
	intI1-R1/519F'	GWATTACCGCGGCKGCTGGCCTTCCAGAAAACCGAGGA		
	pH'	AAGGAGGTGATCCAGCCGCA		Edwards et al., 1989

Beads breaking and fusion-products purification

The samples were centrifuged at 13000 for 5 min at 22 °C and the upper phase was discarded. 1 mL of diethyl ether was added to each sample and vortexed gently. The samples were centrifuged for 1 min at 13000 g and the upper phase was disposed. This step was repeated. The same step was carried out using ethyl acetate instead of diethyl ether. Two more extraction were done with diethyl ether. After leaving the samples open in a laminar flow for 10 min, 100-150 µl of the bottom phase were taken. The kit NucleoSpin Gel and PCR clean-up was used to purify the fusion PCR products.

Nested/semi-nested PCR

Fusion-PCR products were purified and subjected to a new round of PCR, which had both the aims of increasing specificity and introducing partial adaptors for Illumina sequencing. In the case of *sul* genes a semi-nested approach was used and for the rest of the genes a nested approach was applied.

For the reaction different volumes of the samples (4-6 µl) were added to the following mix:

- 5 µl of 5X HF buffer
- 0.5 µl of 10 mM dNTP
- 1.5 µl of 10 µM of the primers
- 0.25 µl of 2 U/µl of Phusion Hot Start Flex
- 15.5 µl ddH_2O

Afterwards PCR was performed following the cycling program: 98 °C 30 s, [98 °C 10 s, 55 °C 30 s, 72 °C 30 s] x 40 cycles and a final elongation step of 5 min at 72 °C. The products were sent to be sequenced by the Illumina sequencing technique.

Table 7| Nested/semi-nested-Illumina PCR primers for epicPCR

Gene	Primer	Sequence	Size (bp)	Reference
sul1	sul1qF-TS	ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTCGAACCTTCAAAAGCTG	474	Wang et al., 2014
	785 R4 illumina	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTctgagtgGACTACHVGGGTATCTAATCC		Herlemann et al., 2011
sul2	sul2qF-TS	ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTGCCAAACTCGTCGTTA	449	Wang et al., 2014
	785 R43 illumina	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtctGACTACHVGGGTATCTAATCC		Herlemann et al., 2011
dfrA1	dfrA1-F1_TS	ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGAGCATTACCCAACCGAA	626	This work
	785 R4 illumina	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTctgagtgGACTACHVGGGTATCTAATCC		Herlemann et al., 2011
intI1	nested intI1-F_TS	ATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGGCATTTCTGTCCTGGCT	571	This work
	785 R3 illumina	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtctGACTACHVGGGTATCTAATCC		Herlemann et al., 2011

4. Research output

4.1. Fate of antibiotic resistance in CW: technology evaluation

Wastewater treatment plants weren't design with the aim of removing micropollutants and antibiotic resistance. In this respect it is important to mention that even in Germany, a leading country in wastewater treatment, the performance of WWTP is not normally evaluated in terms of microbiology. Only surface and drinking waters are evaluated in Germany for microbiological parameters.

Constructed wetlands have the potential to contribute to the removal of common pharmaceuticals that are usually present in urban wastewater. Their ability to remove a wide spectrum of pharmaceuticals can be ascribed to the coexistence of anoxic–aerobic–anaerobic microenvironments within the systems that favour the different mechanisms involved in their removal. The processes leading to removal are mainly biodegradation, sorption, plant uptake and, for surface systems, also photodegradation (Verlicchi and Zambello, 2014). Both SMX and TMP are hydrophilic (k_{ow} SMX= 0.89, k_{ow} TMP= 0.91), which means that they can be available for degradation and shouldn't accumulate in the substrate at a pH around neutral (Radke et al., 2009). Even though bacterial degradation of SMX has been the subject of several studies, the conditions affecting it and the metabolic pathways involved are still under investigation (Wang and Wang, 2018). Regarding DO, biodegradation of SMX occurs under both anaerobic conditions and aerobic conditions (Rodríguez-Escales and Sanchez-Vila, 2016). Adding readily biodegradable carbon sources has been shown to be beneficial for SMX degradation suggesting cometabolism (Müller et al., 2013; Yang et al., 2018). It was observed that the removal of TMP was poor in activated sludge (Li and Zhang, 2010), indicating a preference by the microorganisms for SMX.

The study of removal of antibiotic resistance is more difficult than studying the elimination of micropollutants for the reasons described in Section 1.6. Reductions of 16S rRNA gene and ARG abundances in conventional wastewater treatment facilities have been between two to three orders of magnitude (Auerbach et al., 2007; Gao et al., 2012a; Zhang et al., 2009). Gao et al. (2012b) quantified $1x10^8$ CFU^{SMX}/100 mL and detected a 2.5 log reduction in a three step WWTP. With respect to ARG, common abundances for *sul* genes and *intI1* in untreated wastewater are between $1x10^7$ and $1x10^9$ copies/100 mL (Chen et al., 2015a; Fang et al., 2017; Makowska et al., 2016). Activated sludge-based systems and membrane reactors show a reduction of around 2 orders of

magnitude in *sul1* abundances (Munir et al., 2011). Reductions of *sul1* and *sul2* in CW vary greatly from an increase in the abundance in a pilot-scale CW (Liu et al., 2014) to a reduction of 2 orders of magnitude in a WWTP integrating 5 sequential full-scale CW (Chen et al., 2015b). In the same study, the decrease in *intI1* abundance was of one order of magnitude. Abundance of *dfrA1* is not quantified as often as *sul* genes and *intI1* are, probably because it is in general less abundant. In a hybrid system including a full-scale CW, (Yi et al., 2017) found that *dfrA1* concentrations in raw landfill leachate were between $1x10^3$ and $1x10^5$ copies/100 mL and that it either remained the same or decreased one order of magnitude (wet and dry season respectively) .

Considering the treatment performance in the case of aerated CW, a recent review (Ilyas and Masih, 2017) showed that effluent DO was the most dominant factor for predicting organic matter and nitrogen removal. The organic loading rate and area could be stated as the second most influencing factors, whereas the wetland depth seemingly had the least influence on performance. The HLR, on the other hand, did not have a significant influence on the removal efficiencies of organic matter and nitrogen. This review didn't take antibiotic resistance into account and if these observations correlate with the behaviour of ARB and ARG remains unclear. Understanding which factors affect the removal mechanisms of ARB and ARG in CW is key for the design and operation of advanced treatment systems.

This chapter is a general analysis of the CW of LRB in terms of their potential to remove antibiotic resistance from wastewater. With this aim, only in- and outflow data and ARB isolated from Mueller-Hinton agar were included. Concentrations and areal removal rates were determined for C-BOD_5, DO, nitrogenous compounds and the antibiotics SMX and TMP. In the case of SMX, its most abundant metabolite N^4-Ac-SMX was also quantified. Log reductions and areal removal rate were calculated for *E. coli* (MPN), the aforementioned selected genes (qPCR) and CFU on Mueller-Hinton agar+SMX, Mueller-Hinton agar+TMP and on Mueller-Hinton agar without the addition of antibiotics. 328 resistant colonies were isolated and tested through PCR for the selected genes. 55 isolates were identified by sequencing of the 16S rRNA gene PCR product.

It should be noted that the resistance mechanism studied in the present work doesn't affect the concentrations of antibiotics, as it would be the case for inactivating enzymes that degrade antibiotics (See Figure 1). Identifying which bacteria carry out the degradation of SMX and TMP wasn't an objective of this work.

4.1.1. Effect of aeration on the reduction of antibiotic resistance in an HSSF-CW

4.1.1.1 Wastewater standard parameters and antibiotics concentration

Inlet and effluent concentrations

Common wastewater parameters are shown in Table 8 for inlet and the outlets of HAp and H50p. The total suspended solids (TSS) were more abundant in HAp than in H50p, even though the water in the outlet of HAp had a clearer appearance. This was already observed before for the same systems where TSS and turbidity were measured and turbidity was lower in HAp than in H50p (Button et al., 2015) although TSS was higher.

Table 8| Standard wastewater parameters of the in- and outflows of HAp and H50p (mean±SD)**.**
Data on T, pH, C-BOD_5, DO, redox potential and *Escherichia coli* data were recorded on the sampling dates (N=9). Redox potential was measured with a SenTix ORP sensor ($Pt/Ag^+/AgCl/Cl^-$, WTW, Weilheim, Germany). Data on nitrogen and TSS was recorded before by the UBZ (N=87). Total suspended solids were determined according to DIN EN 872. Ammonium, nitrate, and nitrite nitrogen were measured using a Gallery Plus photometric analyser.

Parameter	inlet	H50p out	HAp out
T (°C)	16.1 ±3.4	15.7 ± 4.4	15.8 ± 4.1
pH	7.2 ± 0.1	7.2 ± 0.1	7.5 ± 0.3
TSS (mg/L)	115.1±75.1	6.7±4.9	23.7±16.7
C-BOD_5 (mg/L)	314.6±66.8	37.6±7.1	2.1±1.7
NH_4^+-N (mg/L)	72.0±12.6	61.4±11.7	0.2±0.5
NO_3^--N (mg/L)	0.05±0.02	1.06±3.88	44.90±8.32
TN (mg/L)	87.9±10.0	68.7±8.7	47.6±9.3
DO (mg/l)	0.6±0.3	2.3±1.5	9.9±1.3
Redox potential (mV)	-263.4±25.8	-199.8±38.9	+217.7±14.8
***Escherichia coli* (MPN/100 mL)**	5.10^6±4.10^6	1.10^5±9.10^4	4.10^2±2.10^2

The most important effect that organic wastes can cause in receiving waters is a reduction in the DO concentration, which is normally due to the microbial breakdown of the organic matter present. The more biodegradable organic matter present in water, the higher BOD and the less DO if no external oxygen input is present. Some of the oxygen present can be used by bacteria for nitrification. N-nitrite was between below the level of detection (BLD), which was 0.04 mg/L, and 0.1 mg/L for the inlet as well as for the outlets.

While C-BOD_5 is related to oxygen as electron acceptor, the redox potential is also affected by other electron acceptors in the media, such as Fe^{3+} and NO_3^-. Measuring this parameters parallel to the study of ARB and ARG is useful to predict which conditions are more suitable for the treatment of ARB and ARG in wastewater by the means of CW. Comparing both CW, C-BOD_5, NH_4^+ and total nitrogen decreased in HAp to a larger extent than in H50p, while NO_3^- and DO increased in HAp more than in H50p. The German standards for WWTP of the size of the one operating in LRB (plant size 4) are: 20 mg/L for C-BOD_5 and 10 mg/L for NH_4^+-N. Whereas the values for C-BOD_5 and NH_4^+-N in the outflow of HAp were below the regulations standards, the treatment in H50p was insufficient. The values recorded from the WWTP in LRB, after a standard activated sludge treatment are: 10 mg/L of C-BOD_5 and 3 mg/l of NH_4^+ (values extracted from the WWTP in LRB: https://www.azv-heidelbach.de). The redox potential stayed below zero for H50p, whereas it increased to positive values in HAp.

Because there's still no consensus about how sub-inhibitory concentrations of antibiotics can affect antibiotic resistance in wastewater, antibiotics were quantified. Both SMX and TMP concentrations, as well as SMX's main metabolite N^4-Ac-SMX are shown in Table 9 for inlet and outflows. All concentrations were below 100 ng/L, which is several orders of magnitude lower than the MIC of SMX and TMP. Even in the inlet, SMX and TMP concentrations were at the lower limit of the range reported before in the literature for Saxony and other regions in Germany, which was about 100 ng/L (Gurke et al., 2015; Ternes et al., 2007). The value of 100 ng/L was proposed as an upper limit for SMX and TMP in groundwater by the German Federal Environment Agency (Hannappel et al., 2016). In terms of their reductions, the concentrations of all compounds decreased in both CW. Whereas for TMP and N^4-Ac-SMX the concentrations in the outflows were similar, SMX concentrations decreased in H50p to a larger extent than in HAp. However, the difference in concentrations in the outlets of the CW weren't statistically significant.

Table 9| Micropollutants median concentrations for the in in- and outflows for HAp and H50p. Minimal and maximal values are shown in brackets. N=6-10

Compound (ng/L)	inlet	H50p out	HAp out
SMX	96.9 (4.3-517.6)	19.8 (2.2-168.4)	59.9 (7.8-328.8)
TMP	8.2 (1.2-61.3)	0.8 (0.0-7.4)	0.8 (0.0-6.8)
***N*^{4}-Ac-SMX**	95.9 (18.3-766.3)	5.9 (0.5-20.7)	4.2 (0.0-58.9)

Removal rates

While the concentrations before and after the treatment indicate how water quality improves, areal removal rates (See Chapter 3) inform about how efficient the treatments are in cleaning pollutants from water. The capacity of the CW to remove organic matter, ammonium and nitrate is shown in Table 10. Areal removal rates of SMX, TMP and N^4-Ac-SMX are shown in Figure 13.

Table 10| Areal removal rates for HAp and H50p (means±SD). For *E. coli*, log reduction is also shown. N=9 and N=89 for nitrogen compounds data. Oxygen removal rate was calculated by Nivala et al., 2013.

Areal removal rate	H50p out	HAp out
C-BOD$_5$ (g/m^2-d)	9.9±2.2	38.6±8.1
NH$_4^+$-N (g/m^2-d)	0.8±0.4	8.6±2.5
NO$_3^-$-N (g/m^2-d)	1.9±0.0	-3.8±2.2
TN (g/m^2-d)	1.2±0.4	5.6±1.6
OCR (g/m^2-d)(minimum/medium/maximum)	1.3/7.6/12.9	15.4/46.0/87.5
***Escherichia coli* (MPN/m^2-d)**	1.9 x 10^9	5.7 x 10^9
***Escherichia coli* log reduction (MPN/100 mL)**	1.6±0.4	4.1±0.7

Although O_2 doesn't constitute a pollutant, it's areal removal rate can be estimated and is also shown. Oxygen consumption rate (OCR) was estimated as described by Headley (2013), where three equations can be used to calculate minimum (Equation 10), intermediate (Equation 11) and maximum (Equation 12) values. The carbonaceous component has different stoichiometric coefficients: 1.5 for the maximum case, 1.0 for the intermediate and zero for the minimum (where it is assumed that all the carbonaceous compounds are removed anaerobically).

$$OCR_{Maximum} = \frac{1.5(\Delta M_{CBOD_5}) + 4.6(\Delta M_{TKN})}{A} \quad (10)$$

$$OCR_{Intermediate} = \frac{1.0(\Delta M_{CBOD_5}) + 1.7(\Delta M_{TKN})}{A} \quad (11)$$

$$OCR_{Minimum} = \frac{1.7(\Delta M_{TKN})}{A} \quad (12)$$

where ΔM represents the mass removed per unit of time (g/d).

The maximum value for the stoichiometric coefficient (Equation 10) is calculated for the oxidation of the average molecular composition of organic molecules in wastewater ($C_{18}H_{19}O_9N$) (Wiesmann

et al., 2006). From equation 13 derives that ~1.5 g of oxygen are needed to oxidise 1 g of organic carbonaceous matter.

$$C_{18}H_{19}O_9N + 17.5O_2 + H^+ \rightarrow 18CO_2 + 8H_2O + NH_4^+ \quad (13)$$

The nitrogenous demand is calculated using Total Kjeldahl Nitrogen (TKN), which takes ammonium and organic nitrogen entering the system into account. The maximum case reflects conventional nitrification and intermediate and minimum consider alternative nitrogen pathways. Nitrification accounts for the use of ammonium by bacteria as electron donor for the reduction of oxygen with the concomitant production of energy. Conventional nitrification is represented in Equation 16, where the two biological steps shown in Equations 14 and 15 are combined. As a result, for every 14 g of nitrogen, 64 g of oxygen are needed giving a ratio of 4.6 g of oxygen for every gram of nitrogen.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O \quad (14)$$

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \quad (15)$$

$$NH_4^+ + 4O_2 \rightarrow 2NO_3^- + 4H^+ + 2H_2O \quad (16)$$

Under alternative nitrogen pathways anammox (**an**aerobic **amm**onium **ox**idation) is considered. The combination of the first step of nitrification (Equation 14) and anammox (Equation 17) results in equation 18. In turn, for every gram of nitrogen 1.7 gram of oxygen are needed. This considers that the nitrite necessary for Anammox is produced in the system and therefore still some oxygen is required. The processes leading to Equations 14 and 17 are compartmentalised in the system as anammox reaction can only take place in anoxic conditions.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (17)$$

$$4NH_4^+ + 3O_2 \rightarrow 2N_2^- + 4H^+ + 6H_2O \quad (18)$$

This method for estimating OCR doesn't take the oxygen that is used to oxidise sulfide into account, which is present in the incoming water at an average concentration of 8.6 mg/L (Saad, 2017). Such demand of oxygen was not accounted for in the determination of the C-BOD_5 which leads to an overestimation of the biodegradable carbonaceous compounds content in the wastewater.

Logarithmic areal removal of *Escherichia coli* (MPN/m^2-d) was calculated using the equation:

$$logarithmic\,areal\,removal\,rate = \log_{10}\frac{Q_{inlet} * [MPN]_{inlet} - Q_{outlet} * [MPN]_{oulet}}{A} \quad (19)$$

which in the case of H50p is:

$$areal\,removal\,rate_{H50p} = \frac{0.196\frac{m^3}{d} * [E.coli]_{inlet} - 0.150\frac{m^3}{d} * [E.coli]_{H50p\,outlet}}{5.64\,m^2} * 10000 \quad (20)$$

For HAp $Q_{inlet/outlet}$ represent different values depending if it was before or after 10/2015 (see Table 2). In the case of the faecal indicator *E. coli*, it's log reduction was also calculated using the equation 21

$$\log reduction\,of\,E.coli = \log_{10}[E.coli]_{H50p\,oulet} - \log_{10}[E.coli]_{inlet} \quad (21)$$

[*E. coli*] is given in MPN/100 mL for equations.

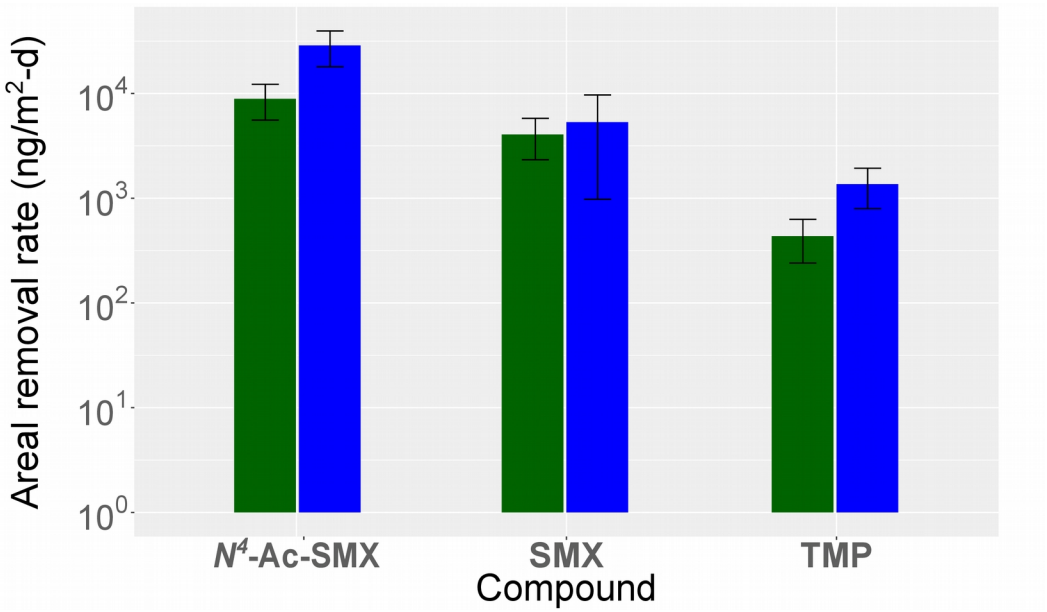


Figure 13| Mean areal removal rates for *N*^{4}-acetyl-sulfamethoxazole, sulfamethoxazole and trimethoprim for H50p (green) **and HAp** (blue).

The aerated CW showed a higher capacity than H50p to remove organic matter and ammonium, whereas it performed worse in terms of nitrate removal. This is a consequence of a much higher production of nitrate in HAp than in H50p. In H50p the amount of nitrate in the outflow is similar to the amount in the inflow. However, some TN is being removed in H50p and that is an indication that some denitrification is taking place, for which the formation of at least some nitrate is necessary within the system. *E. coli* MPN decreased in HAp about 4 orders of magnitude, whereas in H50p the reduction was less than 2 orders of magnitude. Areal removal rate of *E. coli* was around

3 times higher in HAp in comparison with H50p. The difference in areal removal rate wasn't as pronounced as the difference in log reduction because of the high removal of the *E. coli* MPN in both CW.

Considering the areal removal of SMX, TMP and N^4-Ac-SMX, HAp showed a greater capacity than H50p for all of them but the differences weren't statistically significant. The concentration values were between 100-1000 micrograms per day and square meter.

4.1.1.2 Reduction of antibiotic resistance genes and antibiotic resistant bacteria

Culture-dependent method

The selected ARG represent one of the many resistance mechanisms (See Figure 1) against SMX and TMP. To know to which extent this mechanism is responsible for the overall phenotypic resistance to these antibiotics in the system, culture dependent methods are necessary. Another reason for culturing is identifying resistant bacteria. This is important in order to detect resistant bacteria that are at the same time pathogens and therefore pose a health risk. Only results from Mueller-Hinton agar were taking into account for the analysis of the culture-dependent method in this chapter.

Colonies and isolates: sulfamethoxazole as selective pressure

The abundance of colony forming units (CFU) on Mueller-Hinton agar with the addition of SMX decreased in both CW and in HAp to a larger extent (Table 11). When comparing the CFU and CFU^{SMX} values between the CW's outlets, the differences weren't statistically significant. The same was true for the comparisons between CFU^{SMX}/CFU in the inlet and the outlet for a single CW. This last observation means that no significant selection of SMX resistant bacteria took place during the treatment, at least among bacteria that were culturable in the used conditions. To confirm whether this change is significant or not, more biological replicates should be carried out (in this case 3 biological replicates were analysed). Comparing the CFU that were counted on plates with SMX with those growing on plates without antibiotics, around 15% of bacteria were still resistant in the outlet of H50p and 8% in the outlet of HAp. Comparing the total number of isolates that were screened (isolates positive for ARG/screened isolates) the most common gene carried by the resistant isolates was *sul1* (46% of SMX-resistant isolates, inlet). That means that *sul1* accounts for the resistance of almost half of the sulfamethoxazole culturable resistant bacteria in the incoming

water. The frequency of all genes among the isolates decreased for both CW in comparison with the inlet and to a greater extent for HAp. Culture dependent methods are not useful to quantify the total abundance of ARG because they allow to know approximately ARG abundances only in culturable bacteria, which represent just a small proportion of the species in bacterial communities in the environment.

Table 11| Median total abundance and relative abundance of total colony forming units (CFU) on Mueller-Hinton agar, SMX resistant CFU (CFU^{SMX}) and the relative frequency with which they carried the studied genes (isolates positive for ARG/screened isolates). Maximum and minimal values are given in brackets. The number of isolates for each sampling point is also shown. Isolates from 3 different sampling days in 2014-2016.

	inlet	**H50p out**	**HAp out**
	Total abundance (CFU/100 mL)		
	$2.48x10^9$ ($9.50x10^8$-$4.08x10^9$)	$2.00x10^8$ ($1.78x10^8$-$2.45x10^8$)	$6.78x10^7$ ($6.08x10^7$-$1.11x10^8$)
	Total ARB abundance(CFU^{SMX}/100 mL)		
	$2.20x10^8$ ($2.3x10^8$-$7.9x10^8$)	$3.00x10^7$ ($1.9x10^7$-$1.3x10^8$)	$9.25x10^6$ ($3.0x10^6$-$2.0x10^7$)
	Relative abundance (CFU^{SMX}/CFU)		
	0.19 (0.09-0.21)	0.15 (0.11-0.54)	0.08 (0.08-0.30)
	Relative frequency (isolates positive for ARG/screened isolates)		
intI1	0.42	0.10	0.00
sul1	0.46	0.16	0.00
sul2	0.20	0.04	0.00
dfrA1	0.12	0.00	0.05
isolates	45	25	22

Colonies and isolates: trimethoprim as selective pressure

The absolute ARB abundance on TMP agar plates decreased for both CW by one order of magnitude and the relative abundance of CFU^{TMP} increased for both CW to a similar extent (Table 12). When comparing the CFU and CFU^{TMP} values between the CW's outlets, the differences weren't statistically significant. The same was true for the comparisons between CFU^{TMP}/CFU in the inlet and the outlet for a single CW. This last observation means that no significant selection of CFU^{TMP} took place during the treatment. The frequencies of isolates hosting the studied genes in the inlet and their tendencies were similar to what was observed for SMX: the highest frequency was for *sul1* in the inlet (50 %) and they decreased for both CW and to a greater extent for HAp.

Probably TMP indirectly selected for the different *dfrA* variants which are frequently found in the variable region of class 1 integrons. That would explain the high frequency of *sul1* positive isolates among TMP resistant bacteria. The frequencies in the outlets were higher for TMP than for SMX and for H50p higher than for HAp, with the exception of *dfrA1* that was higher on SMX for HAp (5%).

Table 12| Median total abundance and relative abundance of total colony forming units (CFU) on Mueller-Hinton agar, TMP resistant CFU (CFU^{TMP}) and relative frequency with which they carried the studied genes (isolates positive for ARG/screened isolates). Maximum and minimal values are given in brackets. The number of isolates for each sampling point is also shown. Isolates were from 3 different sampling days in 2014-2016.

	inlet	H50p out	HAp out
	Total abundance (CFU/100 mL)		
	$2.48x10^9$ ($9.50x10^8$-$4.08x10^9$)	$2.00x10^8$ ($1.78x10^8$-$2.45x10^8$)	$6.78x10^7$ ($6.08x10^7$-$1.11x10^8$)
	Total ARB abundance (CFU^{TMP}/100 mL)		
	$1.8x10^8$ ($4.0x10^7$-$2.0x10^8$)	$3.8x10^7$ ($3.8x10^7$-$7.9x10^7$)	$1.2x10^7$ ($1.1x10^7$-$2.4x10^7$)
	Relative abundance (CFU^{TMP}/CFU)		
	0.04 (0.04-0.08)	0.21 (0.00-0.32)	0.18 (0.17-0.22)
	Relative frequency (isolates positive for ARG/screened isolates)		
intI1	0.46	0.35	0.00
sul1	0.50	0.35	0.00
sul2	0.13	0.12	0.00
dfrA1	0.10	0.00	0.00
isolates	29	18	22

Reduction and removal of ARB

The differences observed between the CW in terms of CFU weren't statistically significant. The log reductions of culturable bacteria (Figure 14) ranged between 0.35 (TMP, H50p) and 1.80 (SMX, HAp) and were higher for HAp in all cases. In average, the abundance TMP resistant colonies decreased less than the control and that results in a higher relative TMP resistance abundance as it could be seen from Table 12.

Areal removal rates (Figure 15) were around 10^{10} and 10^{12} CFU/100 mL and were one order of magnitude higher for HAp than for H50p. This difference wasn't statically significant.

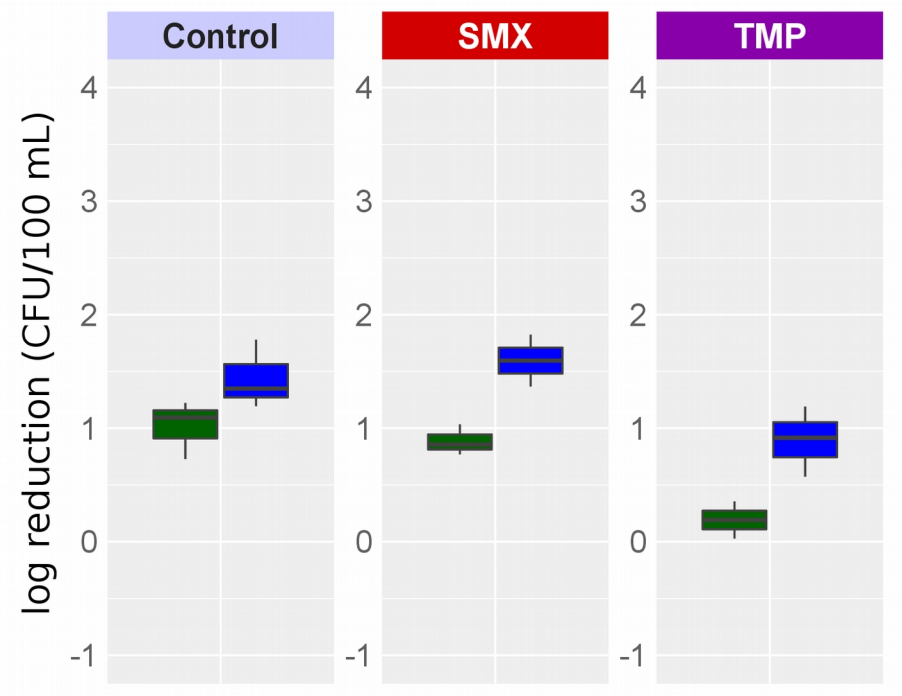


Figure 14| Median log reductions of colony forming units growing in the absence of antibiotics (Control)**, on sulfamethoxazole and on trimethoprim for HAp** (blue) **and H50p** (green).

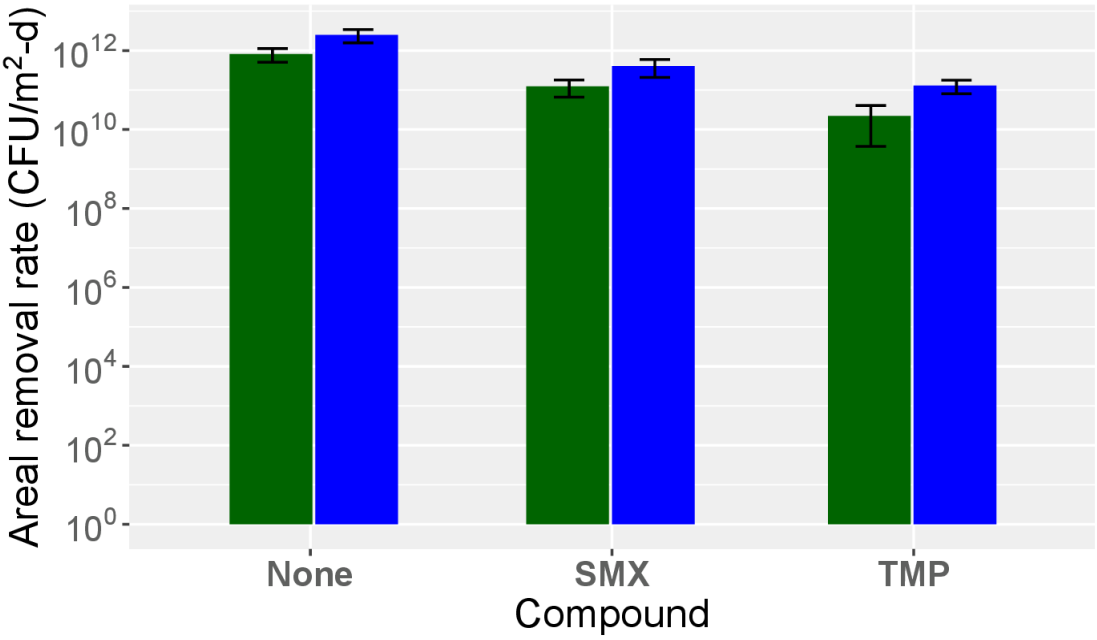


Figure 15| Mean areal removal rates of colony forming units on Mueller-Hinton agar without antibiotics, with sulfamethoxazole and with trimethoprim for H50p (green) **and HAp** (blue).

Taxonomy and phylogeny of ARB and ARG hosts

Sequencing of the 16S rRNA gene of 55 resistant isolates revealed no difference between CW and antibiotics in terms of taxonomy of ARG host (Figure 16 and 17). Most of the ARG hosts belonged to Gram-negative Gammaproteobacteria. More specifically, they classified as *Enterobacteriaceae, Pseudomonas* and *Aeromonas* in most cases. This method is limited to bacteria that can grow under the conditions used in this work, which represent a little proportion of the total bacterial diversity (for an overview of the different approaches to study antibiotic resistance see Section 1.6). Little can be said about the pathogenicity of the bacterial species found among the resistant isolates because the identification method doesn't allow to identify strains. However, in all of the groups detected there are members which can be pathogenic for humans. Some examples are several strains of *E. coli, Aeromonas hydrophyla, Pseudomonas aeruginosa* and *Enterococcus faecalis.*

Bacteria belonging to the genus *Pseudomonas* and to the Gram-positive phylum Firmicutes from the class *Bacilli*, like the families *Enterococcaceae* and *Carnobacteriaceae* rarely carried the studied genes. *Enterobacteriaceae* were more common in the inlet and in H50p, *Aeromonas* were evenly distributed and *Pseudomonas* and Gram-positive bacteria were more frequently found in the outlets than in the inlet. *Enterobacteriaceae, Aeromonas, Enterococcaceae* and *Carnobacteriaceae* are facultative anaerobes, whereas *Pseudomonas* are strict aerobes. A higher DO in the outflows might have favoured the growth of *Pseudomonas*.

Even though *Enterobacteriaceae* and *Aeromonas* were the most common ARG hosts, there were some members of these groups which didn't carry any of the genes but were resistant. This kind of isolates were found in all of the studied sample points but were more common in the outlets than in the inlet and for HAp more than for H50p. One explanation for this observation can be that the fitness of bacteria in the outlet was compromised. Plasmids can represent a burden to bacterial fitness (Smith and Bidochka, 1998). If the studied genes were on plasmids, the plasmids could have represented an extra burden for bacteria to resume growth on the plates.

Phylogenetic trees also show that bacteria carrying the same ARG weren't clones. Even though the studied genes weren't common in distantly related bacteria, their presence wasn't limited to one phylogenetic group. This confirms that HGT of the studied genes can take place among different bacterial families and genera. In the case of SMX resistant isolates, *Aeromonas* seemed to be the most common host on *intI1* and *sul1* whereas no pattern can be detected for *sul2* and *dfrA1*. The gene *dfrA1* was in little proportion of isolates and *sul2* was evenly distributed among all bacterial

groups. Only 3 of the 55 isolates carried *dfrA1* and *intI1* simultaneously. On the contrary, *intI1* and *sul1* were usually found together in isolates, even though there were some isolates that only had *intI1* or *sul1*. Some bacteria carried *sul1* and *sul2*. Although the biological explanation of this redundancy remains unknown, *sul1* and *sul2* were correlated in the environment (Chen et al., 2015b) and can be found on the same plasmid (del Castillo et al., 2013).

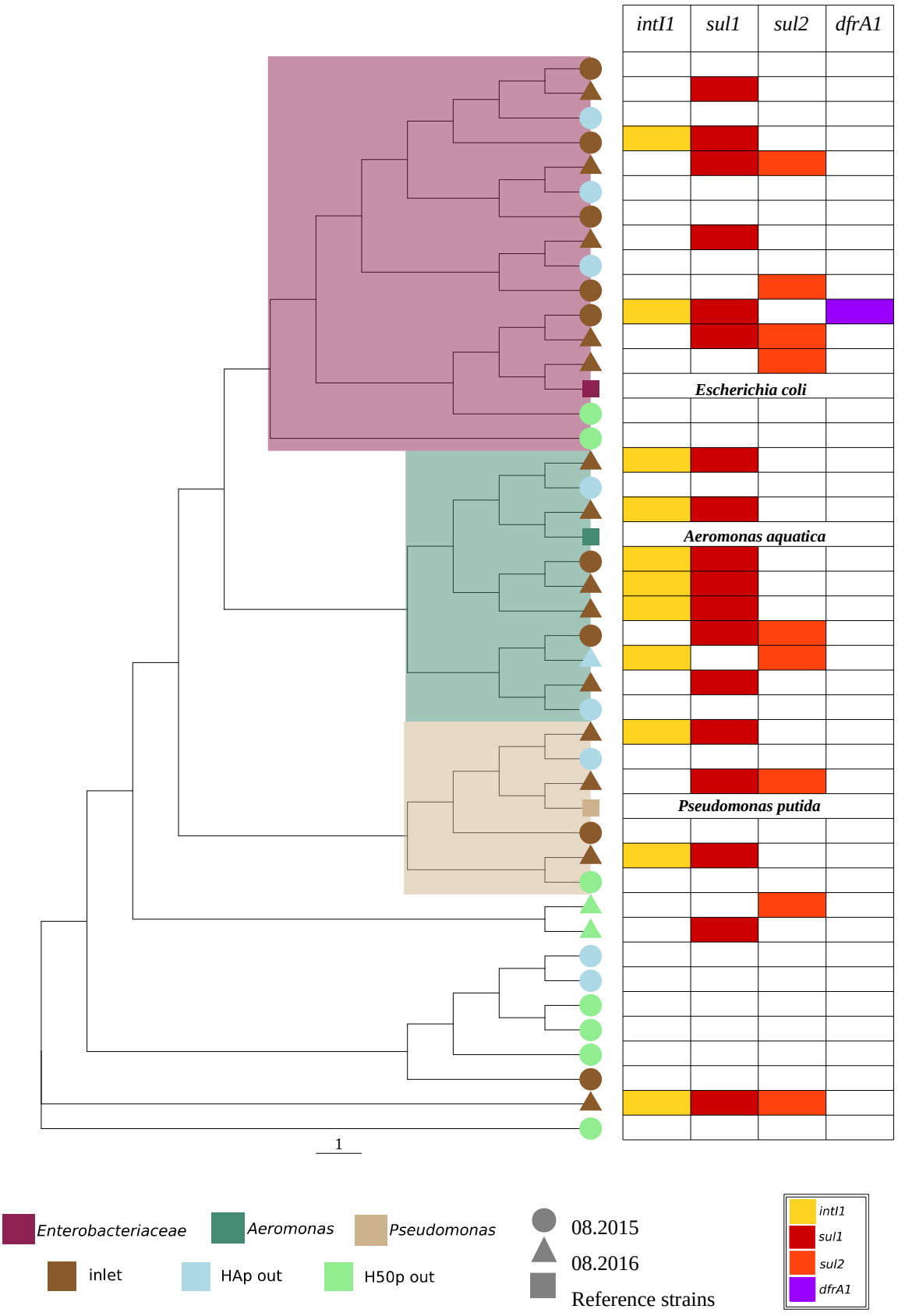


Figure 16| Phylogenetic tree of sequenced sulfamethoxazole resistant bacteria and reference strains. Carrying at least one of the studied genes was a eligibility criteria in 2015, whereas in 2016 the isolates were chosen randomly.

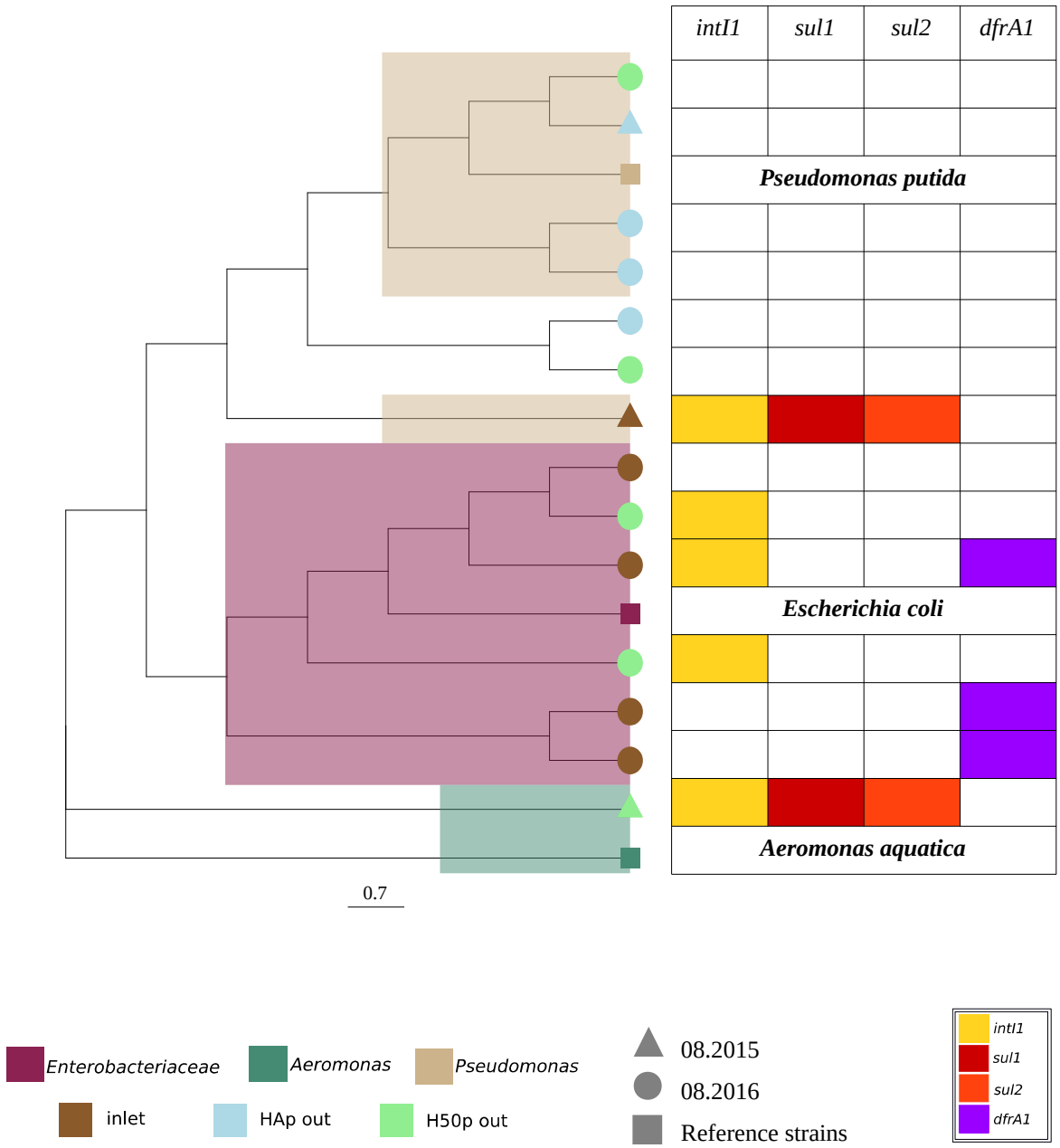


Figure 17| Phylogenetic tree of identified trimethoprim resistant bacteria and reference strains. Carrying at least one of the studied genes was a eligibility criteria in 2015, whereas in 2016 the isolates were chosen randomly.

Culture-independent method

Indicator genes abundances are useful to evaluate the wastewater treatment performance in terms of antibiotic resistance. Absolute gene abundances are shown in Table 13.

Table 13| Gene abundances. Median, minimal and maximal values for the inlet and outflows for HAp and H50p. N=10-11.

Gene	Gene abundance (copies/100 mL)		
	inlet	H50p outlet	HAp outlet
16S rRNA gene	$6.6x10^{10}$ ($1.6x10^{10}$-$2.1x10^{12}$)	$1.4x10^{9}$ ($4.5x10^{7}$-$2.2x10^{10}$)	$1.3x10^{8}$ ($5.2x10^{7}$-$2.3x10^{9}$)
intI1	$7.8x10^{7}$ ($7.1x10^{5}$-$3.3x10^{8}$)	$4.4x10^{6}$ ($2.5x10^{5}$-$4.6x10^{8}$)	$1.3x10^{5}$ ($7.4x10^{3}$-$1.6x10^{7}$)
sul1	$4.5x10^{8}$ ($4.7x10^{7}$-$1.4x10^{9}$)	$2.1x10^{7}$ ($3.6x10^{5}$-$9.5x10^{8}$)	$1.3x10^{6}$ ($2.6x10^{4}$-$4.4x10^{8}$)
sul2	$5.2x10^{7}$ ($3.6x10^{6}$-$3.9x10^{8}$)	$1.2x10^{6}$ ($5.9x10^{4}$-$8.1x10^{7}$)	$3.1x10^{5}$ ($5.3x10^{3}$-$1.4x10^{7}$)
dfrA1	$3.3x10^{6}$ ($1.6x10^{4}$-$2.4x10^{7}$)	$5.1x10^{4}$ ($8.3x10^{3}$-$1.9x10^{5}$)	$1.5x10^{4}$ ($3.2x10^{2}$-$1.5x10^{5}$)

The most abundant gene (excluding the 16S rRNA gene) was *sul1*. Both CW showed a decrease in ARG abundances (Figure 18), performing better than what was reported for HSSF-CW before (Chen et al., 2016a, 2016b). HAp performed better for all genes and this difference was significant for *intI1* (*p* value <0.01) and *sul1* (*p* value <0.05). The log reductions were between one (*intI1*, H50p) and three orders of magnitude (*intI1*, HAp). Comparing the reductions between genes within a particular CW (including the 16S rRNA gene), the differences weren't significant. That means that a selection of bacteria carrying the ARG or *intI1* during treatment could not be identified in neither of the CW.

Taking a look at the areal removal rate of ARG (Figure 19), the highest value was observed for *sul1* and the lowest for *dfrA1* for both CW. HAp performed better than H50p and in the case of 16S rRNA gene and *intI1*, those differences were significant.

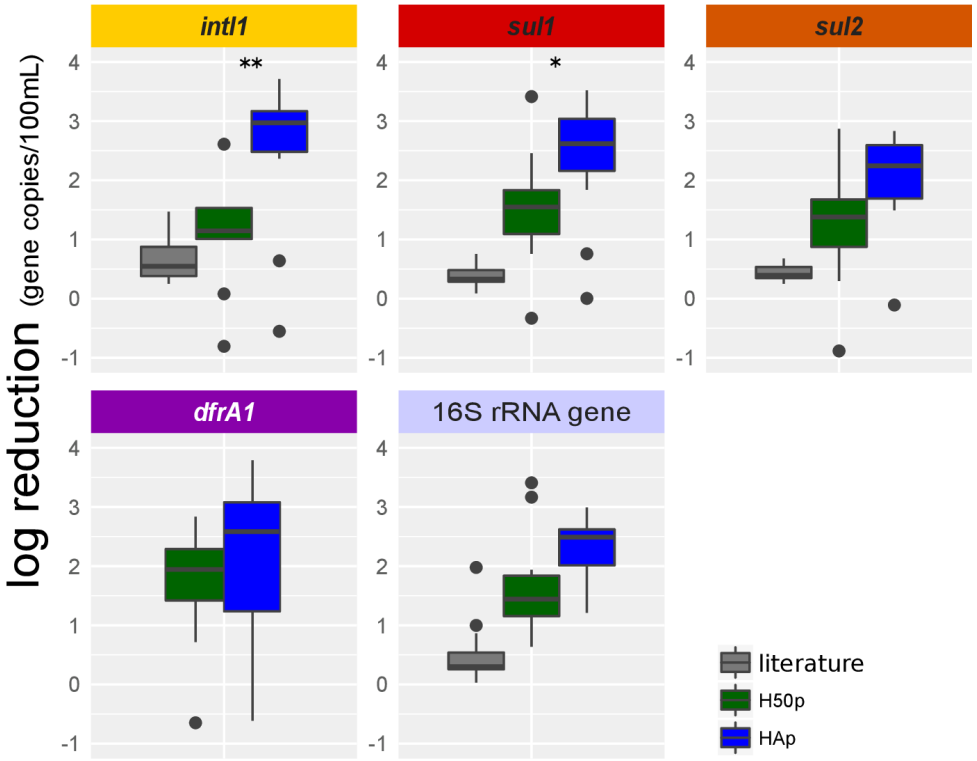


Figure 18| Median log reduction for *intI1*, *sul1*, *sul2*, *dfrA1* and 16S rRNA gene for the outlet of H50p (green) **and the outlet of HAp** (blue) **compared to the literature on HSSF-CW** (Chen et al., 2016a, 2016b). *=*p* value <0.05, **=*p* value <0.01

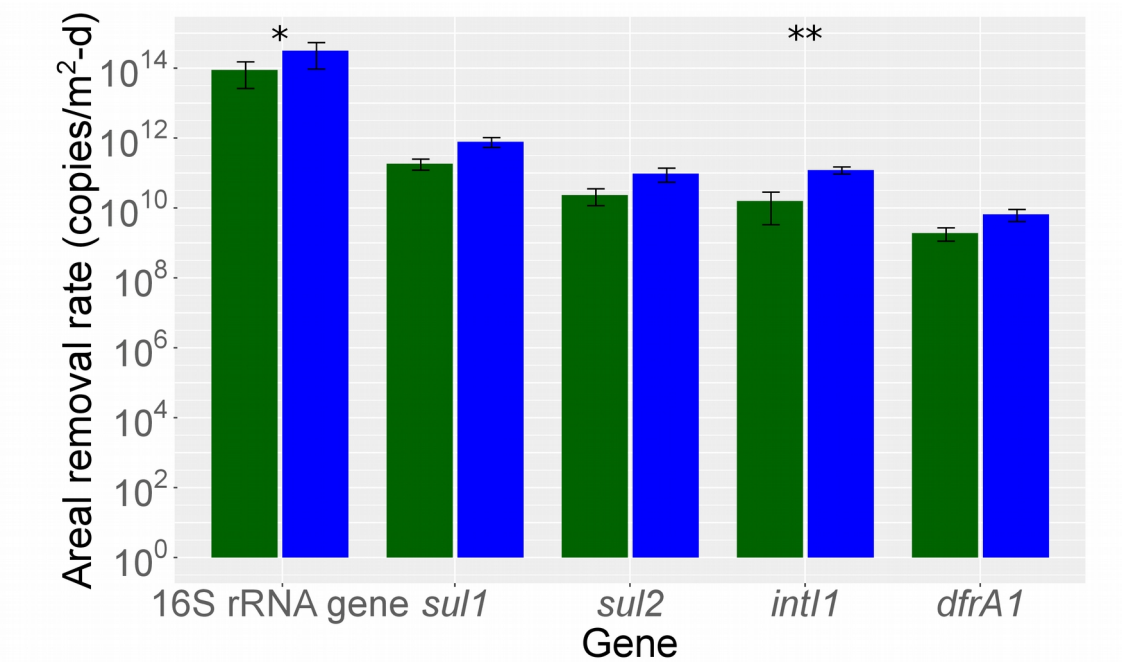


Figure 19| Mean log_{10} areal removal rate for *intI1*, *sul1*, *sul2*, *dfrA1* and 16S rRNA gene for H50p (green) **and HAp** (blue). *=*p* value <0.05, **=*p* value <0.01

Relative abundances

The abundances of the studied genes (ARG and *intI1*) relative to the total bacterial community (16S rRNA gene) (Figure 20) didn't show any statistically significant changes, with the exception of *intI1* which increased for H50p (p <0.05). A reduction of the relative abundance of studied genes in the CW wasn't observed, and this is consistent with what was reported for other wastewater technologies so far (Mao et al., 2015; Rodriguez-Mozaz et al., 2015). An increase in the relative abundance of genes linked to MGE, as in the case of *intI1* can indicate HGT. Therefore, the significant increase in the relative abundance of only the *intI1* gene was one of the reasons to look at the processes taking place inside the CW that could affect HGT of ARG.

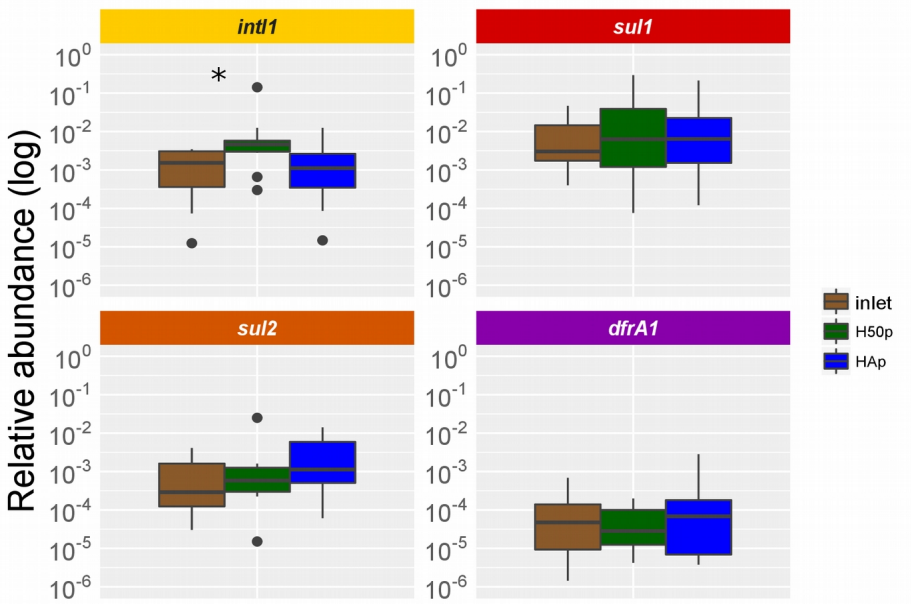


Figure 20| Median abundance of *intI1*, *sul1*, *sul2*, *dfrA1* relative to 16S rRNA gene (log10) for inlet (brown)**, H50p outlet** (green) **and HAp outlet** (blue). *=p value <0.05

4.1.2. Discussion

Standard wastewater parameters for H50p and HAp

The standard water parameters data recorded during this work showed the same treatment performance than what was reported in the literature for these systems (Headley et al., 2013; Nivala et al., 2013), indicating that the studied CW remained stable. Total suspended solids were higher for the outlet of HAp (in Table 8), probably because of the presence of elemental sulfur. The incoming sulfide oxidises to dispersed elemental sulfur at higher redox potential in HAp whereas in H50p it remains dissolved as sulfide as it was documented by Saad (2017). The average percentage reduction of C-BOD_5 in H50p was 88%, almost the same reported by Tanner et al. (1995) for a

HSSF-CW with a similar HRT (90%). However in our case, the C-BOD_5 in the outflow was double than what was stated in the literature. This can be caused by the high organic loading rate (OLR) which was around 11 g C-BOD_5 m^{-2} d^{-1}, almost double of the upper limit recommended by Kadlec and Wallace (2009) to achieve an effluent concentration of less than 30 mg/L. HAp showed a better treatment efficiency and fulfilled the minimum requirement in the outflow, even though the OLR was around 42 g C-BOD_5 m^{-2} d^{-1} before October 2015 and 32 g C-BOD_5 m^{-2} d^{-1} after that date. The redox potential in the outflow of H50p was around -200 mV and it was similar to the inlet. The negative values of redox potential in spite of the presence of 2.3 mg/l of O_2 can be caused by high amounts of sulfide in the media documented by Saad (2017). Differences between the system can be also observed in terms of nitrogenous compounds. N-ammonium was detected only in very low concentration in the outlet of HAp whereas nitrate concentration increased, indicating that most of the ammonium and organic-N was oxidised by bacteria (nitrification). The removal of total nitrogen was higher for HAp (~ 54%), indicating that denitrification is taking place in HAp, in spite of the presence of oxygen. In a review about the performance of aerated CW by Ilyas and Masih (2017), TN removal ranged between 10% and 86% but in the case of the highest value recorded the CW had limited aeration. Some TN was removed in H50p which is an indication that some denitrification is taking place, for which the formation of at least some nitrate is necessary inside the system. Various members of the genus *Pseudomonas* (a genus abundant among the culturable bacteria in the last fraction of HAp) are capable of this transformation under aerobic conditions (Ji et al., 2015). Furthermore, in spite of the overall oxygenic environment, there might be some micro-environments lacking DO. Examples of these are spaces in the interior of biofilms which are attached to the plant roots, the gravel and the basins walls.

Antibiotic concentrations for the inlet in this study (Table 9) were in the lowest limit of the range reported before in the literature for Saxony and other regions of Germany (146 ng/L-1100 ng/L) (Gurke et al., 2015; Ternes et al., 2007). The proportion SMX:TMP in the inlet was around 10:1 and it's not consistent with the ratio of Co-trimoxazole (5:1). This could be caused by a differential pharmacokinetics with which these substances are eliminated from the body. On the other hand, sulfonamides can be used in other combinations as anti-parasites in veterinary medicine which don't include TMP. This could be the case at the WWTP in Langenreichenbach where pig farming is taking place in the area. Although outlet SMX concentration was lower in H50p than in HAp, the latter showed higher areal removal rates for all studied compounds. The difference in removal rate of SMX between the CW wasn't as big as for TMP and N^4-Ac-SMX, probably because of the transformation of N_4-Ac-SMX back into its parental compound in HAp. Lower SMX concentration

in the outflow of H50p can be a consequence of a longer HRT and a higher C-BOD_5 throughout the CW than in HAp, which can enhance co-metabolism. The concentration-based removal efficiency for SMX was higher in H50p (80%) and lower in HAp (9%) than what was observed by Chen et al (2016) in mesocosm-scale HSSF-CW (46-69%). Higher areal removal rates for HAp can be caused by differences in water conditions such as redox potential (Rodriguez-Escales and Sanchez-Vila, 2016) and/or BOD_5 (Drillia et al., 2005). Sulfamethoxazol can be degraded both under aerobic and anaerobic conditions with different kinetics, the degradation being faster at higher redox potentials. The dependence on C-BOD_5 is valid for all the redox conditions and can be explained through co-metabolism, where the degradation of SMX is enhanced by the presence of external labile organic carbon. Furthermore, in contrast to what was observed for TMP before (Li and Zhang, 2010), TMP was highly degraded in the CW. Even though the concentrations of the selected antibiotics were very low, their presence in the incoming wastewater evidences their use and that selective pressure towards ARB and ARG could have taken place during medical treatment.

Regarding *E. coli*, HAp performed better than H50p and as it can be seen by looking at the load in the outlet and the log reduction although the areal removal rates were similar. The average culturable *E. coli* load (Q_{outlet}xMPN_{outlet}/L) coming from H50p was $2.4x10^8$ MPN/d and from HAp was $2.4x10^6$ MPN/d. With regard to *E. coli* reduction, HAp showed a reduction of 4 orders of magnitude and H50p 1.6 orders of magnitude. The reduction of *E. coli* in H50p was similar to the one observed by Decamp and Warren (2000) while treating secondary effluent with an HSSF-CW. The higher performance of the aerated CW is in accordance with Karimi et al. (2014), who observed a negative correlation between DO and *E. coli* abundance. A higher DO can indicate a lower BOD and therefore less organic matter available, leading to starvation. Another reason can be oxidative stress, as *E. coli* is a facultative anaerobe (see Section 1.5).

Characterization of antibiotic resistance for H50p and HAp

Considering the ARG abundances in the inlet and the outlets, HAp showed a similar reduction (~2 log reduction) as other treatment technologies such as activated sludge-based systems and membrane reactors (Munir et al., 2011). Compared to other studies of the same genes in CW (Chen et al., 2016b, 2016a; Yi et al., 2017) both studied CW showed a higher reduction. In those other studies the substrate material and hydraulic loading rate had an impact on ARG/ARB removal and they were laboratory scaled CW run for a shorter period of time. The time that the system has been running is important for stability and the latter for a higher C-BOD_5 removal (Samsó and García,

2013). It remains unknown if stability could be the reason why a high removal of ARB and ARG occurred in the studied systems.

In terms of bacterial reduction in absolute abundance, the comparison between culture- independent and dependent methods showed a similar reduction of CFU and 16S rRNA gene for H50p and in HAp a higher reduction of 16S rRNA gene than CFU. This could be caused by a change in bacterial species (which can be seen from the phylogenetical trees) as the 16S rRNA gene copy number is species-dependant (Klappenbach, 2001). In terms of antibiotic resistance, in order to compare absolute abundances, the number from culture-independent methods can be estimated using Equation 8.

For *sul1* (Equation 9) in the outlet of H50p this calculation results in $8x10^6$ which is of the order of magnitude as the mean value observed from qPCR. However, in the case of the outlet of HAp the result is zero although around $1x10^6$ copies could be still detected by qPCR. With these copy numbers the gene should be present at least with a frequency of 0.30 of the the resistant isolates. This incongruence could be due to a decrease in culturability of *sul1* hosts. A decrease in culturability could be caused by a higher stress pressure at the end of HAp, where oxygen is abundant and nutrients are scarce. The host lowered culturability could mean that they are dead or in a state in which they are no longer culturable by the used conditions but could grow again under appropriate conditions.

Another possible explanation for the difference in ARG between culture- dependent and independent methods is a change of hosts towards species that aren't culturable in the used conditions. Antibiotic resistant bacteria were identified as members of *Enterobacteriaceae*, *Pseudomonas, Aeromonas* and *Enterococcus.* While the hosts for the studied genes belonged to members of *Enterobacteriaceae* and *Aeromonas,* the other two groups rarely carried them. A shift in taxonomy of ARB inside the CW could be observed: Enterobacteriaceae and *Aeromonas* were more common among the culturable bacteria in the inlet whereas the other two groups were more common in the outlet of the CW. If this taxonomic change also encompassed a change in ARG hosts remains unanswered and the results of epicPCR will help to clarify this question.

The identified ARB belonging to *Enterobacteriaceae, Pseudomonas* and *Aeromonas* are members of the class Gammaproteobacteria and the phylum Proteobacteria. *Enterococcus* belong to the class Bacilli and the phylum Firmicutes. Proteobacteria, Firmicutes and Chloroflexi were shown to be the most predominant phyla in the total bacterial community in different CW types (Bouali et al., 2014;

He et al., 2016). In these studies different culture-independent methods involving 16S rRNA gene sequencing were applied. The bacterial diversity revealed in these studies was much higher than in the current work but the method used in this case targets a bacterial sub-community which has to be both culturable and resistant to SMX and/or TMP. In this sense, in the study from Bouali et al. (2014) it was concluded that only 20% of the 839 operational taxonomic units (OTU) were affiliated with cultured microorganisms in the microbial community from a HSF-CW planted with *Phragmites australis*. Chloroflexi wasn't detected in the current study but bacteria belonging to this phylum are anaerobic and the culturing conditions used in this study are aerobic. Data from Illumina sequencing of 16S rRNA gene of the total community has not been analysed within the scope of this work.

With both culture dependent- and culture independent methods, HAp showed better removal rates of ARG and ARB. Nevertheless, this difference wasn't evident when considering relative abundances of ARG, which remained the same with the exception of *intI1* for H50p. This can also be seen by the fact that log reductions of the studied genes aren't significantly different compared to the reduction of 16S rRNA gene in a particular CW. This means that no selection of the studied genes took place in the CW. Considering CFU^R/CFU, the median relative abundances increased for TMP but not for SMX. However, the maximum values detected were always higher for the outlet of both CW than for the inlet for both antibiotics. This can be a sign of selection or enrichment of ARB in the CW, which aren't hosts of the selected ARG. In the case of SMX resistance in the inlet, it can be seen that around 60% of the isolates carry *sul* genes and for TMP, *dfrA1* was in 10% of the isolates. For the outlets these frequencies were lower. Together, these results indicate that CW don't select ARG but select/enrich bacteria which are SMX/TMP resistant through other mechanisms. Bacteria like several members of *Pseudomonas* spp., which were more predominant in the outflows than in the inlet, are naturally resistant against TMP/SMX because of efflux pumps (Köhler et al., 1996).

4.1.3. Conclusion

In terms of physicochemical parameters (Tables 8 and 10) it was confirmed but it was already reported in the literature for both CW: they are stable and HAp performs better. Additionally, SMX and TMP were in very low concentrations (Table 9) and were partially removed in both CW presumably by biodegration, as photodegradation and sorption can be discarded. Photodegradation can be discarded because of the type of flow of the CW (subsurface) and sorption looking at the k_{ow}

of the studied compounds. In terms of antibiotic resistance, both CW showed higher reductions of ARB (Figure 14) and ARG (Figure 18) than what was observed for HSSF-CW before.

For the culture dependent method in this chapter, only results from Mueller-Hinton agar were taking into account for the analysis. An increase in CFU^{TMP}/CFU was seen in both CW (Table 12). In the case of CFU^{SMX}/CFU although the median values showed a reduction in CFU^{SMX}/CFU, single values showed an increase (Table 11). That means that there was a partial increase in the abundance of resistant bacteria relative to the total abundance among culturable bacteria. This difference wasn't statistically significant but this could be caused by a low number of biological replicates (N=3). In the inlet, *sul* genes accounted for ~60% of SMX resistance observed and *dfrA1* for ~10% of TMP resistance. In the outlets, these frequencies were lower and in HAp were lower than for H50p. ARB were identified as members of *Enterobacteriaceae*, *Pseudomonas*, *Aeromonas* and *Enterococcus.* While ARG hosts belonged to members of *Enterobacteriaceae* and *Aeromonas*, the other two groups rarely carried them. The decrease in the frequency of the isolates carrying the ARG at the same time that the ARB frequency increased, can be partly explained by the phylogenetical shift of the bacterial community into the CW. In the outflows, bacterial species that are resistant against TMP/SMX through other mechanisms, such as *Pseudomonas* spp. and *Enterococcus* spp. were more frequent. However, although a taxonomic change towards species that aren't normally host of the studied genes took place, the relative abundance of the studied genes didn't decrease during treatment (Figure 20).

Culture independent methods HAp performed better for all genes and this difference was significant for *intI1* and *sul1*. The log reductions were between one (*intI1*, H50p) and three orders of magnitude (*intI1*, HAp). Comparing the reductions between genes within a particular CW (including the 16S rRNA gene), the differences weren't significant. That means that a selection of bacteria carrying the ARG or *intI1* during treatment could not be identified in neither of the CW. The abundances of the studied genes (ARG and *intI1*) relative to the total bacterial community (16S rRNA gene) (Figure 20) didn't show any statistically significant changes, with the exception of *intI1* which increased for H50p.

Aeration seemed to be beneficial for the total reduction of ARB and ARG although a question arises from this evaluation: why can't ARG be detected in the cultivated isolates for the outlet of HAp although they can be detected in high abundances by the culture independent method? For this, at least two explanations seem plausible: the same bacterial species carrying the genes in the inlet became non-culturable during treatment and/or ARG are hosted by other bacterial species, which

are non-culturable under the conditions used in this work. This question and the fact that the relative abundance of *intI1* increased in the outlet of H50p led to a more detailed study inside the CW. This permitted to take a look at the parameters driving the changes observed between inflow and outflows and the differences between the CW.

4.2. Fate of antibiotic resistance in CW: internal behaviour

4.2.1. Effect of physicochemical parameters on antibiotic resistance

In order to improve the risk evaluation it is necessary to understand the biological processes linked to antibiotic resistance. In a recent study on antibiotic resistance in *E. coli* from a WWTP it was seen that, while the amount of bacteria released after treatment into the environment was reduced, the potential for antibiotic resistance of these bacteria wasn't (Mahfouz et al., 2018). However, this was limited to culturable bacteria belonging to one species. Taking a look inside the CW can help to understand which factors are responsible for the observed changes between the inlet and the outflows of the CW in terms of ARB taxonomy and ARG abundances. Assessing which environmental factors can increase the risk of the spreading of antibiotic resistance and the development of new antibiotic resistance bacteria remains a challenge due to several aspects. One major aspect is the lack of a standardised protocol for which choosing indicator bacteria and indicator ARG is crucial. Studies in wastewater treatment currently focus on antibiotics concentrations in water as a factor increasing the risk and to a minor extent on co-selection whereas more general conditions like physicochemical parameters are overlooked. However, physicochemical parameters have a big influence on the environmental microbiome. Small changes in temperature, BOD or DO can have a large impact on the microbial community.

Aeration is a common procedure in wastewater treatment as it increases the removal efficiency of organic compounds, which if were released into the environment would cause pollution. Also oxygen can indirectly inhibit bacterial growth through the effects that ROS have on biomolecules like DNA. Many bacteria suffer oxidative stress under a higher DO than what is optimal for their growth. It has been determined that DNA damage can activate the SOS response and the latter can affect HGT of ARG linked to integrons. How aeration affects wastewater treatment, specially in terms of ARB and ARG, and if the additional stress caused by oxygen could increase HGT of ARG is the main research question of this chapter (Figure 21).

With this aim and in order to address the questions that arose from Chapter 4.1, we analysed internal samples of the CW and the same procedures that were carried out in Chapter 4.1 was repeated with the internal samples of the CW. Taking the results coming from Mueller-Hinton media into account, three selective and differential agar media for the most common ARB, *Enterobacteriaceae*, *Pseudomonas* and *Aeromonas*, were chosen. This was a way to zoom into the

bacterial groups to look for patterns in antibiotic resistance within and between the phylogenetical groups. Among the three major groups studied *E. coli* and *Aeromonas* spp. were common hosts of the studied genes and at the same time were proposed as good indicators for antibiotic resistance. They were quantified through culture- dependent and independent methods, in order to assess their abundance and their physiological status. Additionally, the variable regions of class 1 integron in SMX resistant isolates were sequenced. At last, the method emulsion paired isolation and concatenation concatenation PCR (epicPCR) was set up and applied to prepare samples for Illumina sequencing. The sequencing of the epicPCR products has not been finished within the scope of this research.

Figure 21| Main research question. The blue boxes show what has been demonstrated before and the grey boxes, the research question addressed in this chapter.

4.2.2. The role of aeration in horizontal gene transfer of ARG

4.2.2.1 Standard wastewater parameters within H50p and HAp

Changes in C-BOD_5 content and DO along the flow path of H50p and HAp are depicted in Figure 22. These values as well as other raw data are shown in Appendix B. DO value increased sharply after 0.25 of the fractional length, reaching oxygen saturation in the outlet of HAp. For H50p, DO remained low (~1 mg/L) throughout the watercourse, with the exception of H50p out. Together, C-BOD_5, DO, redox potential and nitrogenous compounds concentrations give information of the predominant overall microbial metabolisms taking place in the CW.

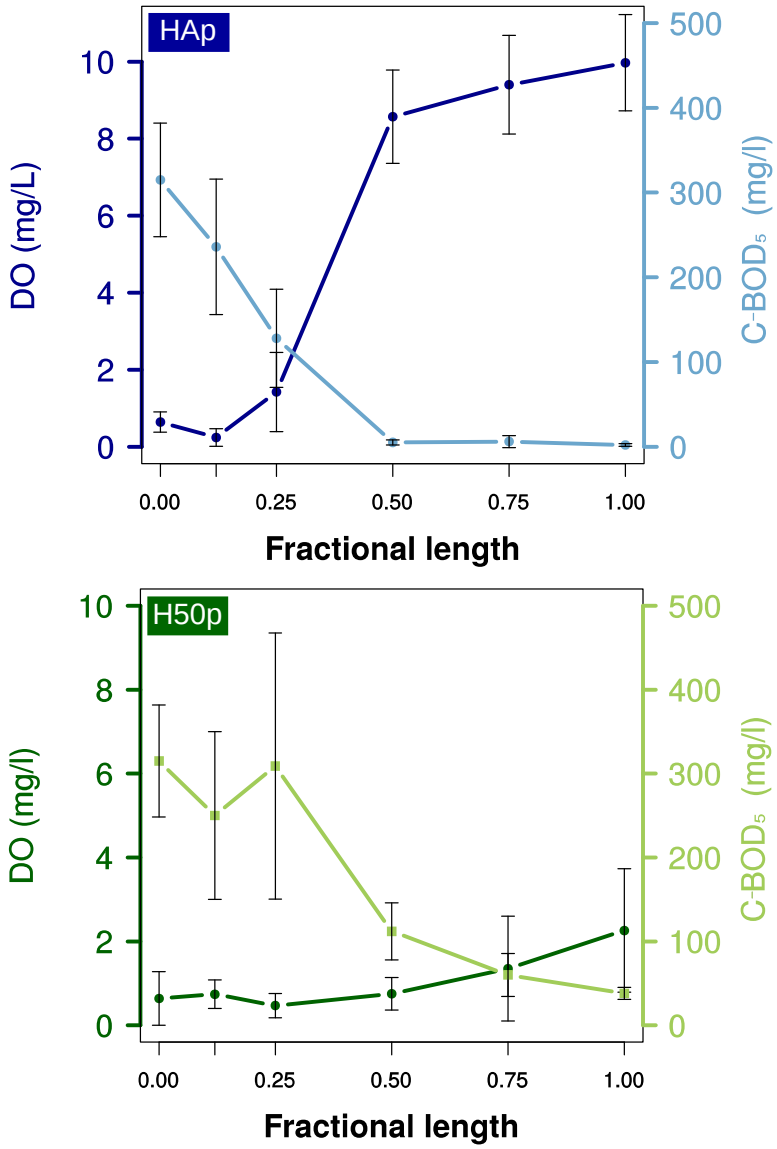


Figure 22| Mean dissolved oxygen and carbonaceous oxygen demand along the flow path. DO (dark colours) and C-BOD_5 (light colours) values along the flow path for HAp (blue) and H50p (green), N=9.

Figure 22 also shows that dissolved oxygen is absent for the first fraction of HAp although aeration is taking place uniformly along the flow path path. Figure 23 shows how ammonia was transformed to nitrate, which indicates that oxygen is being introduced to the system although it was not measurable. The internal behaviour of nitrogenous compounds wasn't measured for H50p as the concentration of nitrogenous compounds didn't change as much as in HAp (Table 8). Nitrate-N concentration in the inlet was always below the level of detection and in 0.12 of the fractional length of HAp the value rose to 0.1 mg/L. Nitrite-N concentration was mostly zero, although for same samples in HAp 0.25 and 0.50 it had values of 0.1-0.8 mg/l.

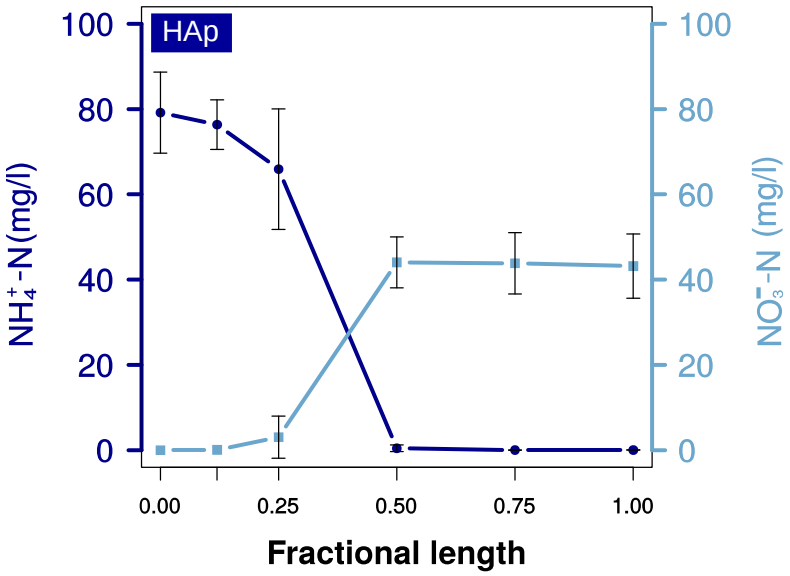


Figure 23| Mean nitrogenous compounds along the flow path for HAp. NH_4^+-N (dark blue) and NO_3^--N (light blue), N=8.

Antibiotics concentrations.

The concentrations of SMX, N^4-Ac-SMX and TMP along the flow path are shown in Figure 24 and 25. Other antibiotics belonging to the sulfonamide family; sulfadiazine and sulfamethazine were detected in even lower concentrations in all the samples (<2 ng/L). The concentration of SMX and TMP in sediments were normally below the level of detection (except for one sample where the concentration was 2 ng/g of sediment). This confirmed what is reported about SMX and TMP in the literature, that these antibiotics don't accumulate in the sediment and discarded the possibility of a higher selection pressure in the sediments than in water. The difference in concentrations between the CW weren't statistically significant for any of the sampling points inside the CW. The average concentrations of SMX and TMP in the inlet were at least 7 and 8 orders of magnitude lower than

their Minimal Inhibitory Concentrations (MIC), respectively (Czekalski et al., 2012). Trimethoprim concentrations were one order of magnitude lower than SMX concentrations.

For both wetlands, a sustained decrease in N^4-Ac-SMX was seen. For SMX, H50p showed a decrease all along the flow path, whereas in HAp it remained around constant. For TMP the concentration decreased until 0.5 of the fractional length for both CW and to a larger extent for HAp. For 0.75 and 1.00 of HAp the concentration remained almost constant. In the case of H50p, the concentration continued decreasing till 0.75 of the fractional length and remained constant towards the outlet. Nevertheless, it is worth noticing that in this case the values are close to the limit of detection of the method (1.5 ng/mL).

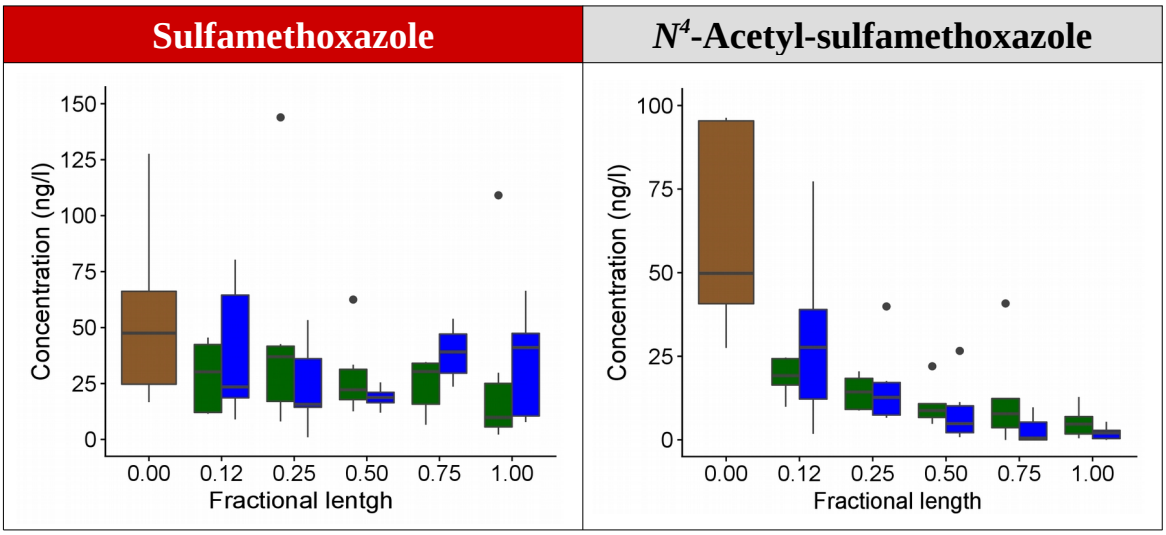


Figure 24| Sulfamethoxazole (left) **and N^4-acetylsulfamethoxazole concentrations** (right) **for the inlet** (brown), **HAp** (blue) **and H50p** (green). N=6

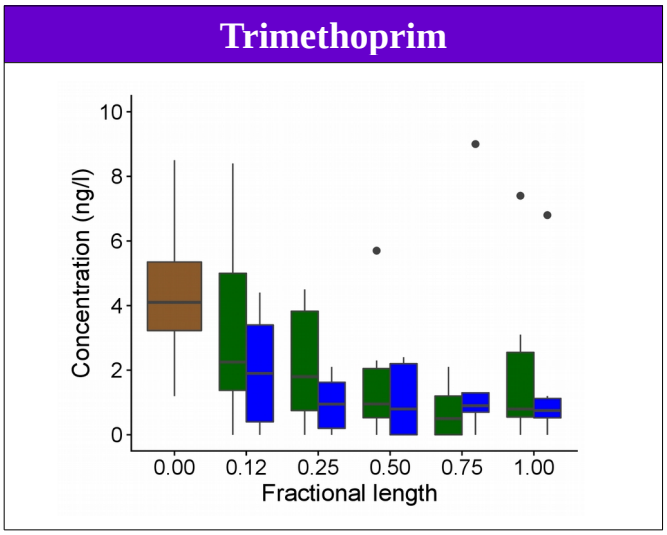


Figure 25| Trimethoprim concentration for the inlet (brown), **HAp** (blue) **and H50p** (green). N=6

4.2.2.2 Abundance of 16S rRNA gene and ARG

The abundance of the 16S rRNA gene showed a sustained decrease in both CW (Figure 26 A) with no statistically significant difference between the CW. Fold change (calculated by Equation 5) normalises the data for possible fluctuations in time in the incoming wastewater (Figure 26 B). The fold change was significantly different between the CW for 0.12, 0.25 and 1.00.

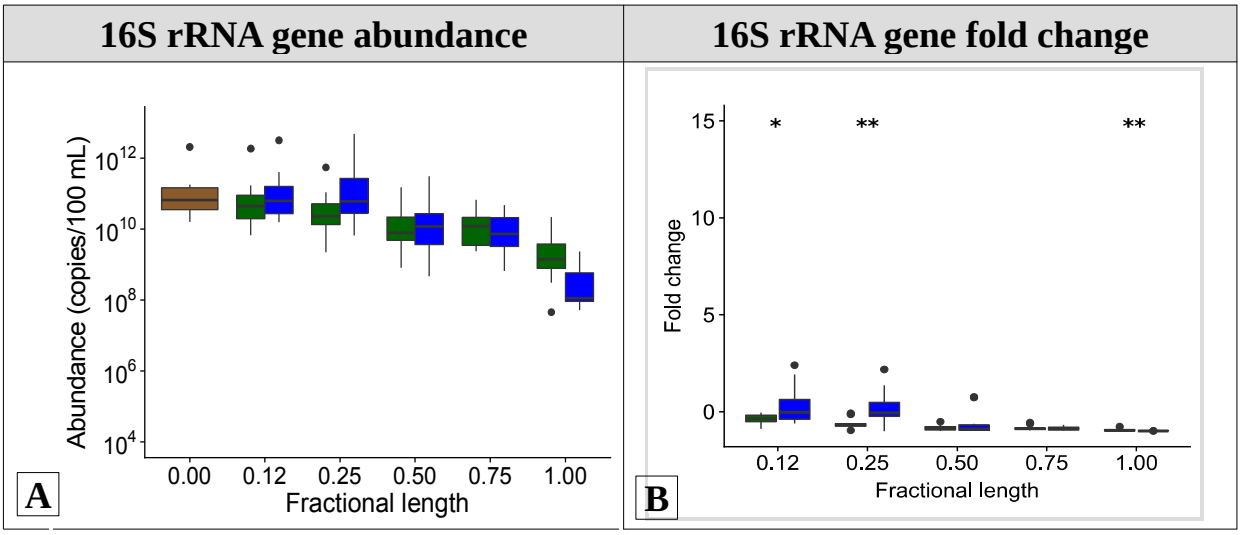


Figure 26| 16S rRNA gene abundances measured by qPCR along the flow path. Inlet in shown in brown, H50p in green and HAp in blue. **A|** Absolute abundance **B|** Fold change. N=11. *=*p* value <0.05, **=*p* value <0.01

The changes in the abundances of some of the ARG are shown in Figures 27 and 28. The change differed between genes and between CW along the flow path path: for *sul* genes and *intI1*, the abundance increased in the first half of HAp and the difference in fold change between the CW was statistically significant. For *dfrA1*, the values were similar for both CW, decreasing all along the flow path until reaching a minimum in the outlet and the fold change didn't show statistically significant differences. The abundances of all studied genes started decreasing from 0.50 of the fractional length. For *intI1*, an additional qPCR was carried out for some of the samples (randomly picked) with primers that specifically target the clinical allele of *intI1* (Figure 30). Being able to differentiate between clinical alleles and environmental alleles was of relevance in order to be able to predict if the phenomena observed were allele-dependant. The clinical allele was the most abundant and most probably the one responsible for the overall behaviour. The tendency along the flow path was similar as for *intI1* total abundance, and in this case, the difference between CW was statistically significant for 0.12, 0.25 and 1.00 of the fractional length.

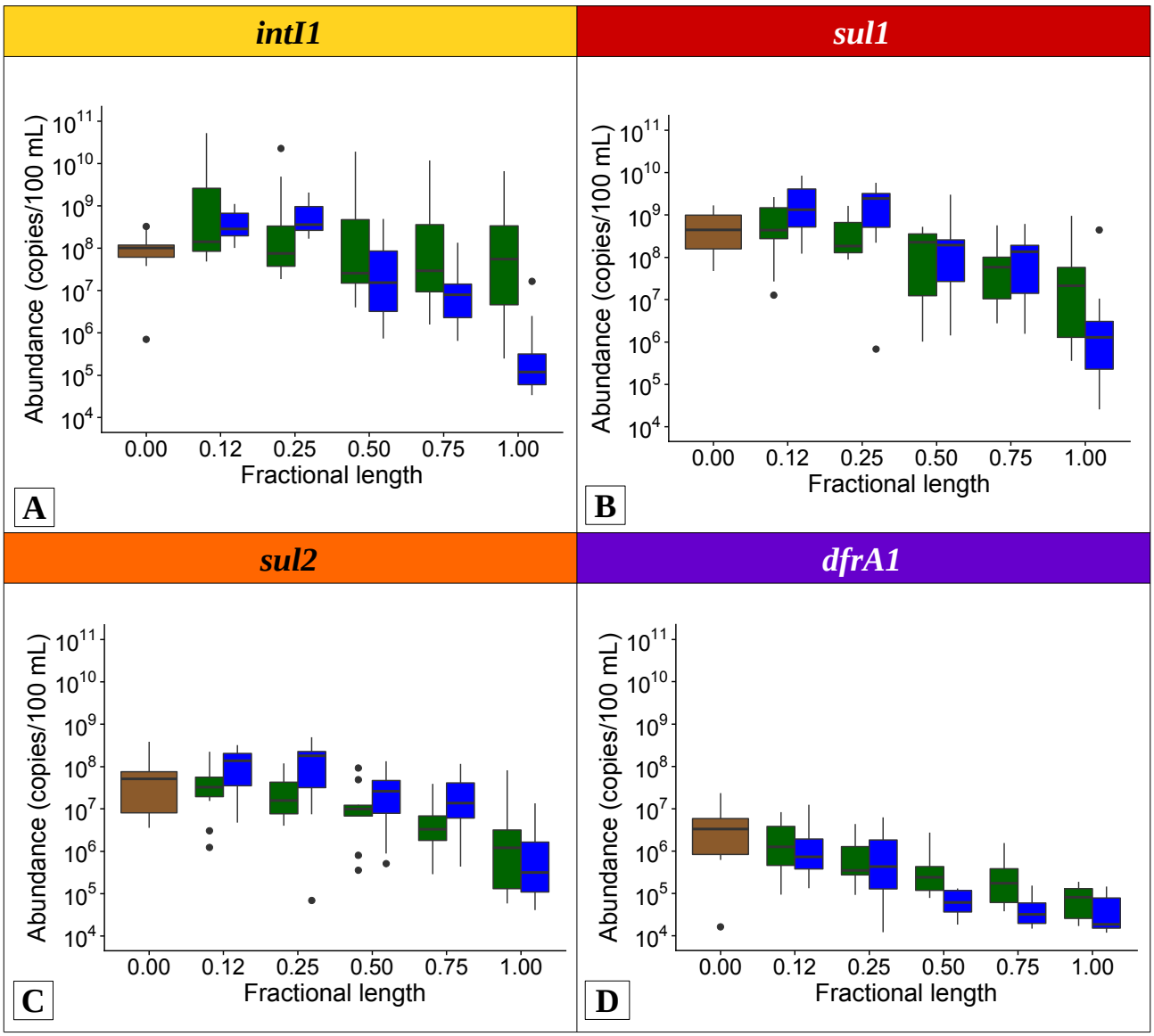


Figure 27 | Absolute gene abundances measured by qPCR along the flow path. Inlet in shown in brown, H50p in green and HAp in blue. **A|** *intI1* **B|** *sul1 C|* *sul2* **D|** *dfrA1***.** N=11

The genes *intI1* and *sul1* are frequently genetically linked in the class 1 integron structure and thus their similar behaviour indicates that the increase in the first sampling points of HAp are due to this type of integron. Furthermore, an increase in *sul2* abundance was also seen although this gene hasn't been detected previously in class 1 integrons. A correlation in the abundances of *intI1* and *sul2* has already been observed by Chen et al. (2015). In this study the concentrations of *intI1* and several resistance genes were measured by qPCR in samples from a river. A possible explanation is that the integron class 1 and *sul2* can be on the same plasmid, as in the case of plasmid p148 (del Castillo et al., 2013). This plasmid belongs to the incA/C group, which are conjugative and broad host range.

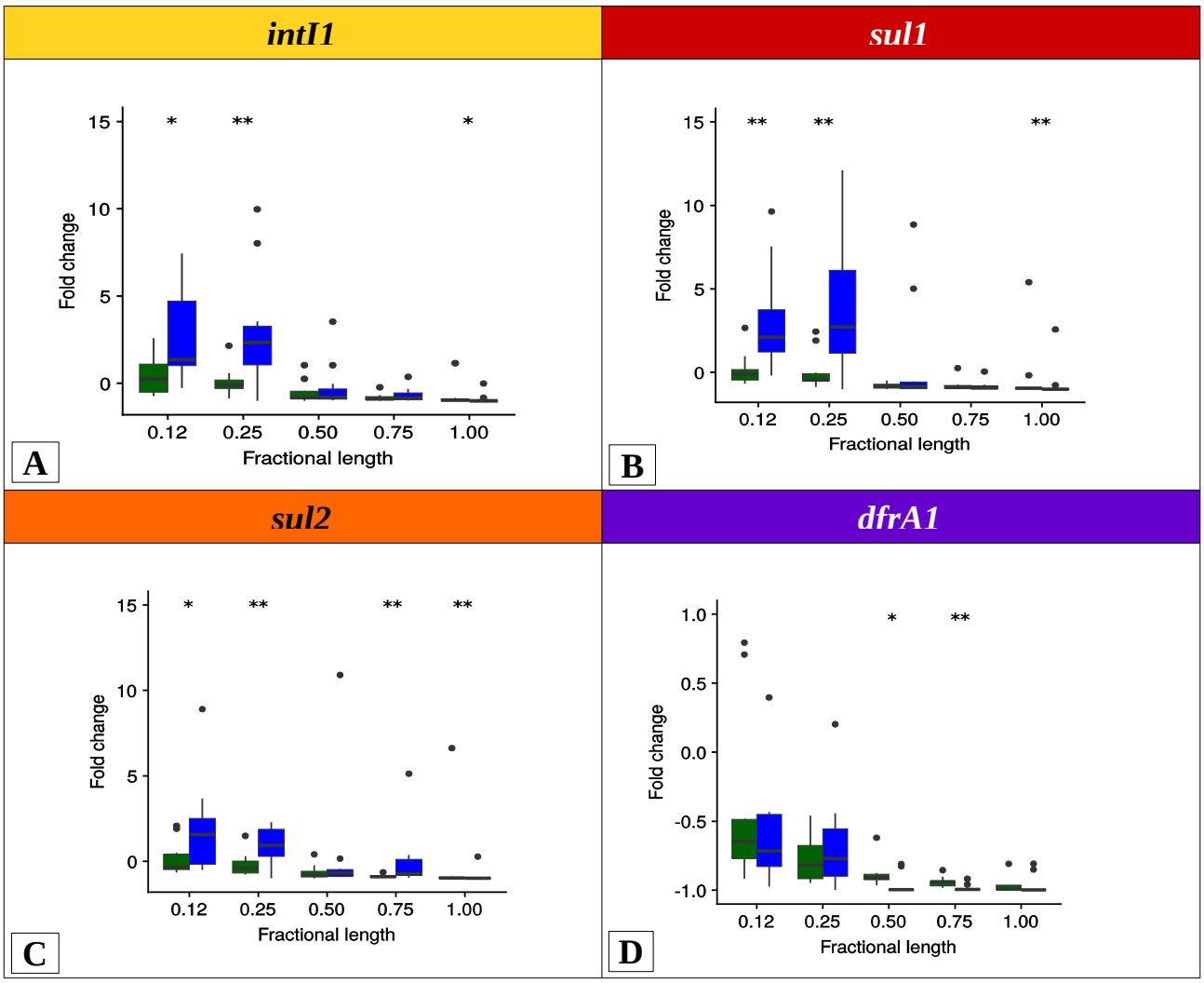


Figure 28 | Fold change of gene abundances along the flow path. H50p is shown in green and HAp in blue. **A|** *intI1* **B|** *sul1* **C|** *sul2* **D|** *dfrA1*. N=11. *=*p* value <0.05, **=*p* value <0.01

Although *dfrA1* can be found as gene cassette in integrons, in a study in aquaculture farms (Muziasari et al., 2014), *intI1* and *dfrA1* abundances weren't correlated, suggesting that other genetic context are prevalent for *dfrA1*. The trend seen for 16S rRNA gene was similar to the one observed for *dfrA1*, indicating that the increase in *sul* genes and *intI1* is specific and it's not a result of clonal amplification. In Figure 29, the abundance of *intI1* relative 16S rRNA gene is shown against 16S rRNA gene abundance. That the relative abundance is not correlated with 16S rRNA gene abundance further confirms that the increase in *intI1* isn't a result of clonal amplification and that is not a consequence of a higher bacterial density (higher connectivity). That this increase took place only in the first fraction of HAp and was specific for *intI1* led to the hypothesis that oxidative stress was the cause for this observation. Oxidative stress can damage the DNA and DNA damage has already been linked to a higher integrase activity (Guerin et al., 2009) and to an increased HGT of *int* and ARG (Beaber et al., 2004).

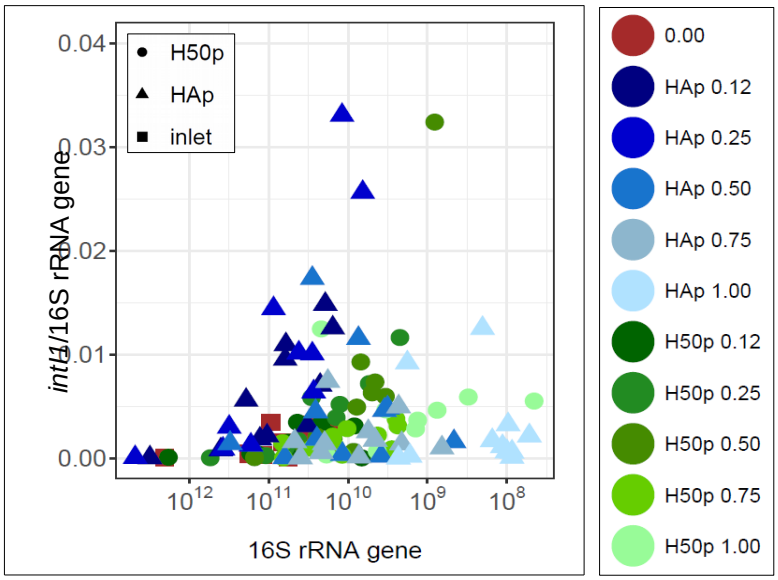


Figure 29| *intI1* abundance relative to 16S rRNA gene vs. 16S rRNA gene abundance. x-axis decreases to the right in order to reflect the flow path in the CW, where the 16S rRNA gene abundance decreases towards the outflow. N=11

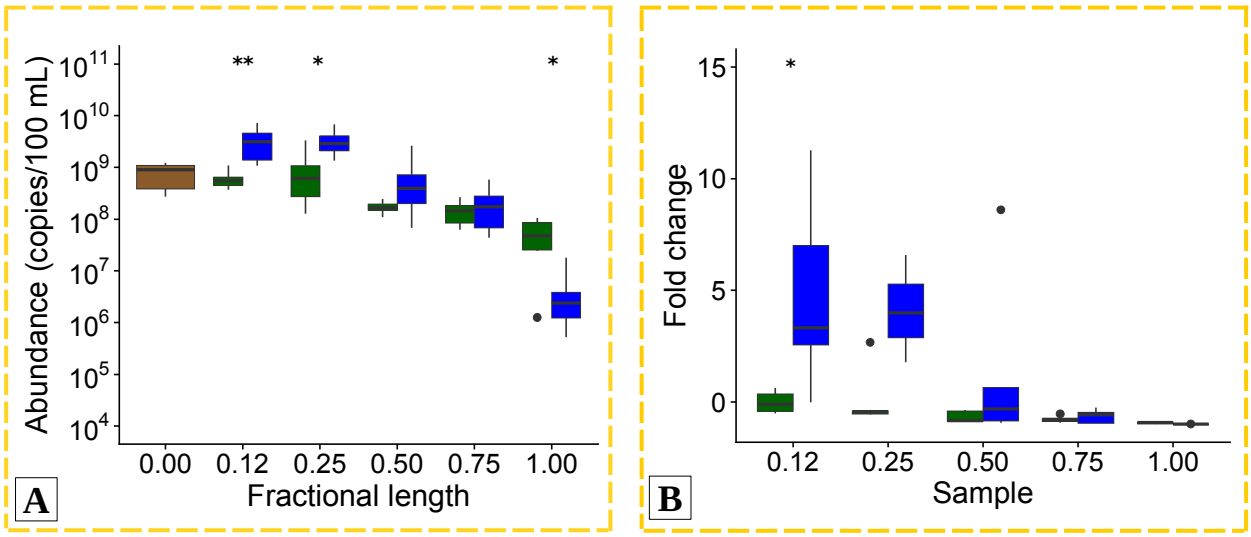


Figure 30| Abundance of *intI1* measured by qPCR along the flow path. Inlet is shown in brown, H50p in green and HAp in blue. **A|** Absolute abundance **B|** Fold change. N=6. *=*p* value <0.05, **=*p* value<0.01

Antibiotic resistant bacteria quantification and characterization.

Culturing and sequencing of the 16S rRNA gene of 256 SMX or TMP resistant isolates further confirmed that the culturable hosts of the studied genes in our systems belonged to the genera *Aeromonas* and *Pseudomonas* and to the family *Enterobacteriaceae*. In Figure 31 the 256 isolates that were sequenced are shown without distinguishing between agar media, antibiotic used or CW from which they originated.

The taxonomic distribution of *sul1* hosts was similar to the taxonomic distribution of *intI1* hosts and *Aeromonas* spp. was the most abundant group. The taxonomic distribution of *sul2* hosts was similar to the taxonomic distribution of *dfrA1* hosts and in this case *Enterobacteriaceae* spp. was the most abundant group. The results for *intI1* and *sul1* were not identical in terms of their host. This further confirms the observations from qPCR results, where the results for *sul1* were similar yet not identical to those for *intI1*. Although *sul1* is part of the 3' conserved region of the class 1 integron, some variants lacking this region have been already detected before (Chah et al., 2010).

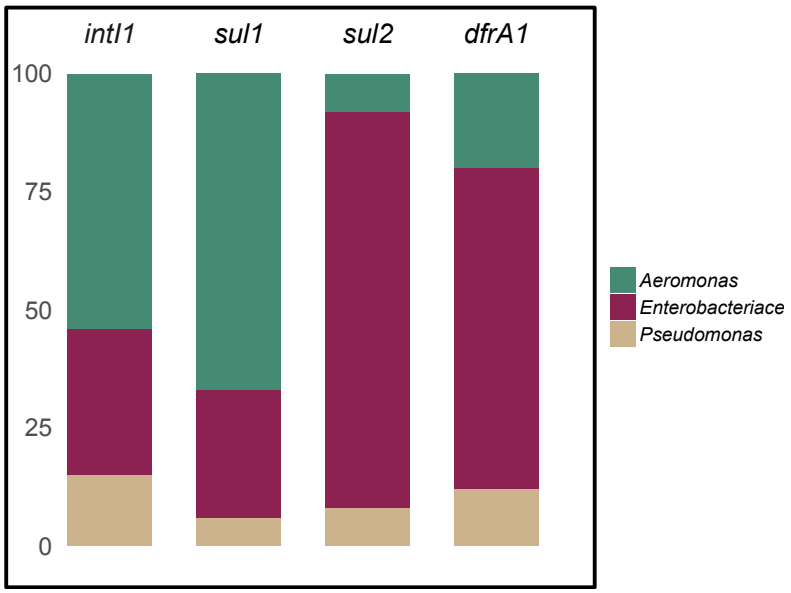


Figure 31| Proportion of the gene hosts grouped in the main three hosts types detected by sequencing of the resistant (SMX and TMP) isolates in percentage. The hosts for each gene are shown without distinguishing between sampling points, constructed wetland variety or isolation media. N= 256.

The selectivity property of the agar media isn't 100% effective. These agar media are useful in clinical diagnostic in order to be able to differentiate among some pathogens. The results obtained with environmental samples can vary due to the great diversity of the bacterial community. Endoagar permitted the growth of *Aeromonas* and *Pseudomonas*, and *Enterobacteriaceae* and *Aeromonas* were able to grow on Pseudomonas isolation agar.

Sulfamethoxazole-resistant isolates

The results of CFU counting along the flow path are shown in Figures 32-34 (A-B). For Mueller-Hinton agar, the tendency was similar without or with SMX. For EA and PIA, CFU numbers were similar in HAp and H50p on the plates without SMX. On the plates with SMX, the counting was higher for HAp in the first two sampling points and for PIA it was higher than in the inlet, even

though those differences weren't statistically significant. In order to find out the genotype responsible for the resistance, the resistant isolates were PCR-screened for the *sul1* and *sul2*. *intI1* was also studied (32-34, C-D panels). The same incubation conditions were used for all the media in order to be able to compare the results between the media.

Mueller-Hinton agar (MH agar):

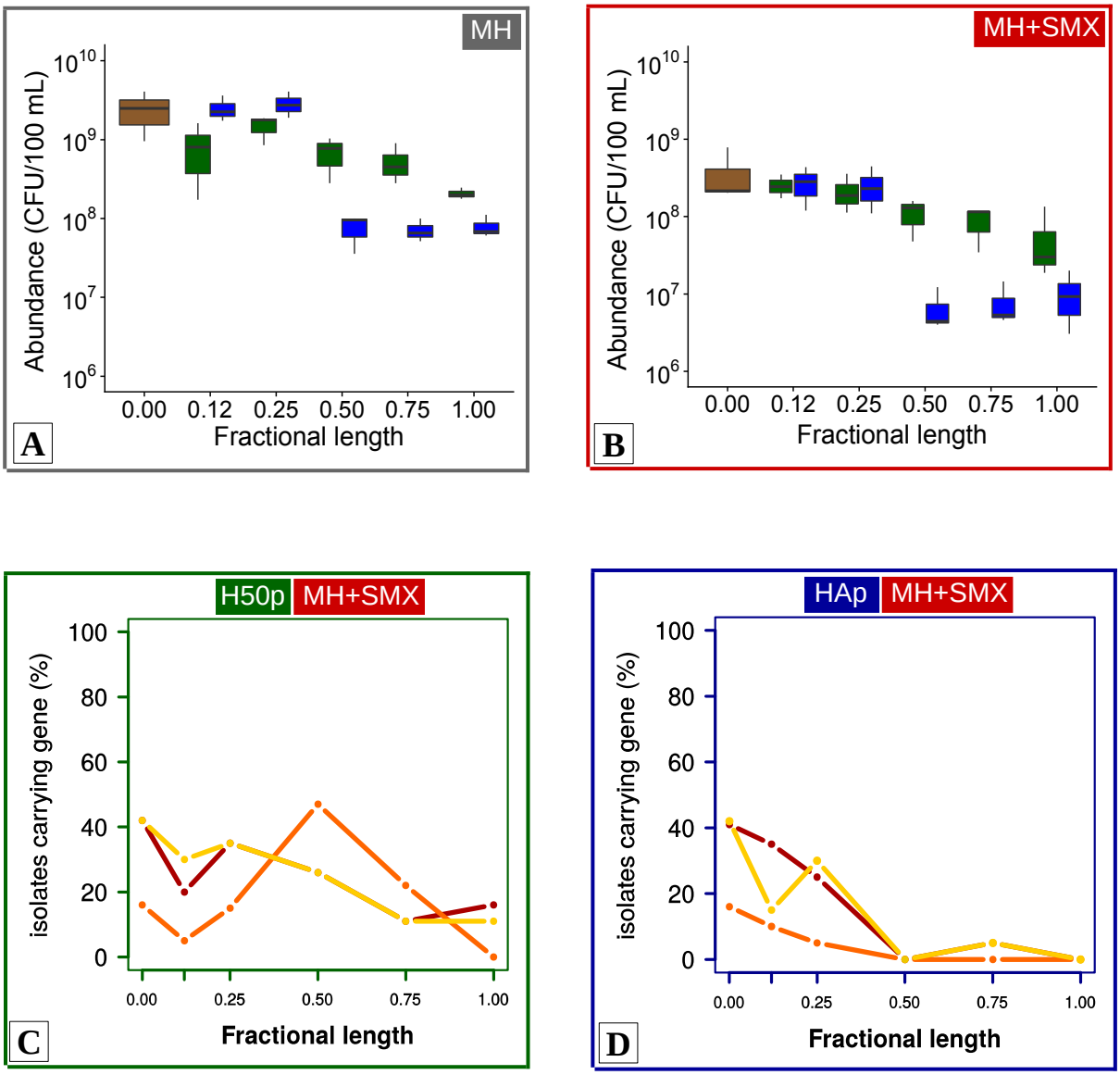


Figure 32| Mueller-Hinton agar (MH agar) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU without antibiotics are shown with grey border and CFU on MH agar+SMX with red border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from MH agar+SMX carrying *intI1* (—), *sul1* (—) and *sul2* (—) for H50p and HAp. N=20 isolates per sampling site on one sampling day.

The CFU on PIA for HAp out was zero at the time of counting but a longer incubation time at higher temperature allowed the isolation of colonies. Although the number of resistant CFU for the inlet was similar on Mueller-Hinton agar as on PIA, the percentage of isolates carrying the studied genes was lower on Mueller-Hinton agar. On one hand, Mueller-Hinton agar allows the growth of Gram-positive bacteria like *Enterococcus*, which are resistant towards SMX without carrying *sul* genes whereas PIA doesn't. On the other hand, PIA+SMX showed an increased selectivity for *Aeromonas* and *Enterobacteriaceae* carrying *sul* genes than the other media. This might be also the reason why CFU counting on PIA+SMX showed similar tendencies to the qPCR results.

Endoagar (EA):

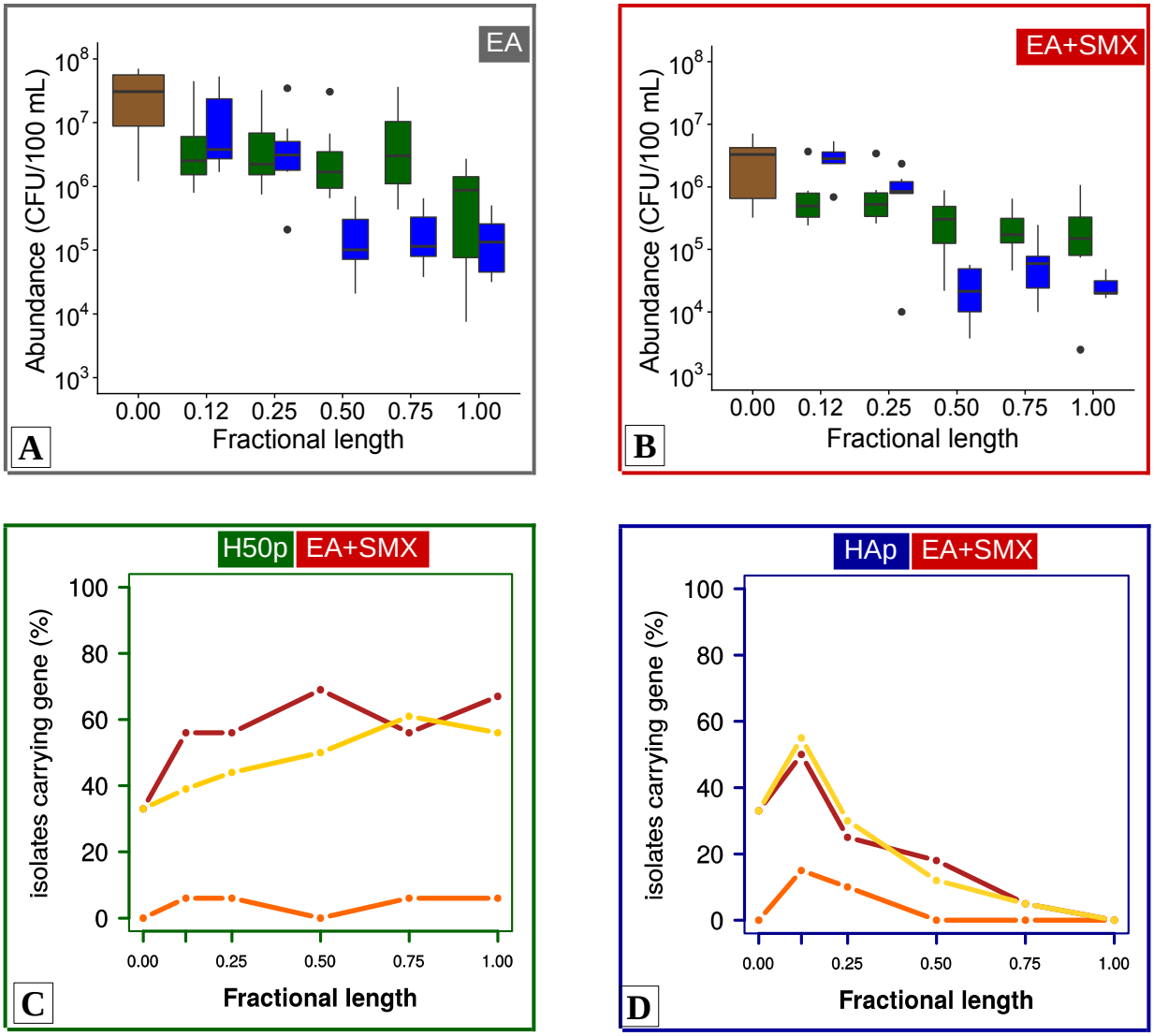


Figure 33| Endoagar (EA) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU on EA without antibiotics is shown with grey border and CFU on EA+SMX with red border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from EA+SMX carrying *intI1* (—), *sul1* (—) and *sul2* (—) for H50p and HAp. N=20 isolates per sampling site on one sampling day.

Furthermore, *sul1* abundance and CFU on PIA+SMX had the same order of magnitude, whereas for the other media the CFU counting was an order of magnitude higher than *sul1* abundance. For the inlet, CFU without antibiotics on PIA had the same order of magnitude as on the plates with SMX, whereas CFU on the other media with antibiotics showed one order of magnitude less than the corresponding plates without SMX. 20 isolates from PIA plates without antibiotics were screened and the colonies didn't carry *sul* genes, with no difference in taxonomy.

Pseudomonas isolation agar (PIA):

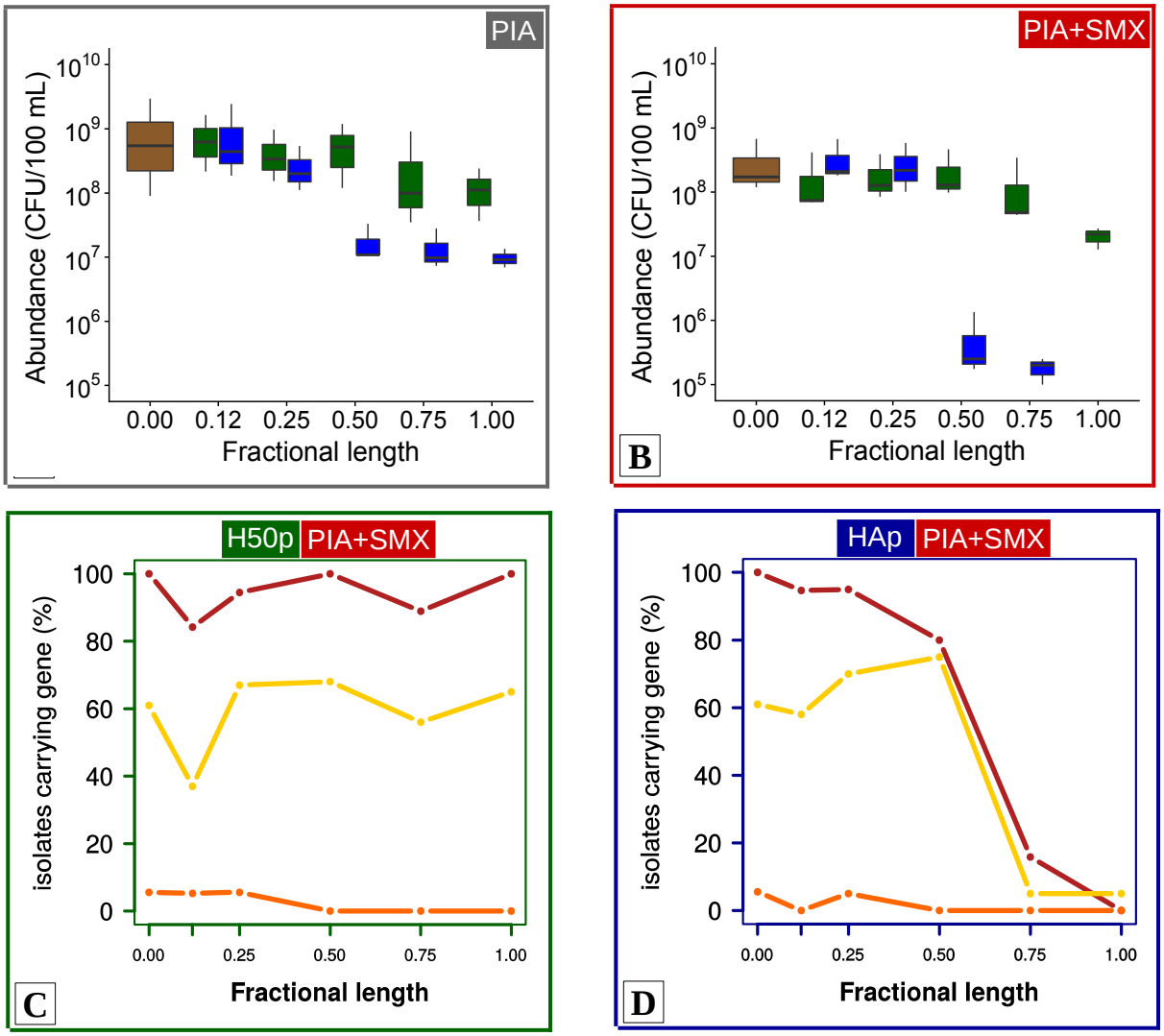


Figure 34| Pseudomonas isolation agar (PIA) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU on PIA without antibiotics is shown with grey border and on PIA+SMX with red border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from PIA+SMX carrying *intI1* (—), *sul1* (—) and *sul2* (—) for H50p and HAp N=20 isolates per sampling site on one sampling day.

In Figure 35, the proportion out of 825 SMX resistant isolates carrying *sul* genes and *intI1* are shown along the flow path for both CW. In the inlet, most of the resistant isolates tested carried at least one of the genes. This remained constant for H50p but decreased to almost zero in HAp. The change in HAp is likely partly due to a shift in the bacterial community along the flow path in HAp to species that are naturally resistant to SMX, like *Pseudomonas* spp or *Enterococcus* spp (Figures 16-17, 35-38). However both *intI1* and *sul* genes were still abundant in the outlet of HAp but were in hosts that weren't culturable in the used conditions. Either hosts culturability had been compromised through environmental stress (starvation and ROS) or a change in hosts had taken place towards species that can't be cultured. Sulfamethoxazole resistant isolates from all media that were *intI1* positive with Mazel's primers (environmental and clinical) were also tested with Gillings primers (clinical). Of all bacteria carrying integrons, 13% of the isolates hosted an environmental variant.

The phylogenetic trees of bacteria isolated from different agar media showed similar results as what was seen in chapter 4.1 for Mueller-Hinton agar. In this case, isolates coming from 0.12 and 0.25 of the fractional length in HAp were included. Only one of the isolates identified as *Pseudomonas* this time were host of the studied genes. Finding *Pseudomonas* which carry the studied genes depends on the selection criteria. In the case of the isolates in the phylogenetic trees in this chapter, the resistant isolates were randomly picked. If only those resistant isolates carrying one of the studied genes were picked to be sequenced, some *Pseudomonas* could be found as it can be seen from Figure 31. In the case of Mueller-Hinton agar, resistant *Enterobacteriaceae* outweighed resistant *Aeromonas* and *Pseudomonas*. Gram-positive bacteria can grow on Mueller-Hinton agar and can be recognised from the tree because they have no background colour. In the case of EA and PIA, *Aeromonas* outweighed *Enterobacteriaceae* and no resistant Gram-positive bacteria were detected. In the case of PIA, all the resistant isolates that weren't identified as *Pseudomonas* carried at least one of the *sul* genes. One isolate belonging to the Nitrobacterium family carried both *sul1* and *sul2* genes. The results from this media showed that the hosts of class 1 integrons in the inlet belonged to Enterobacteriaceae, while for the rest of the sampling points they were identified as *Aeromonas*. This wasn't the case for EA, where some of the class 1 integron hosts were identified as *Aeromonas* both in the inlet and in the rest of the sampling points. These results confirm *Aeromonas* as good indicator bacteria for antibiotic resistance monitoring. They also suggest the importance of *Aeromonas* as ARG reservoirs in aquatic environments.

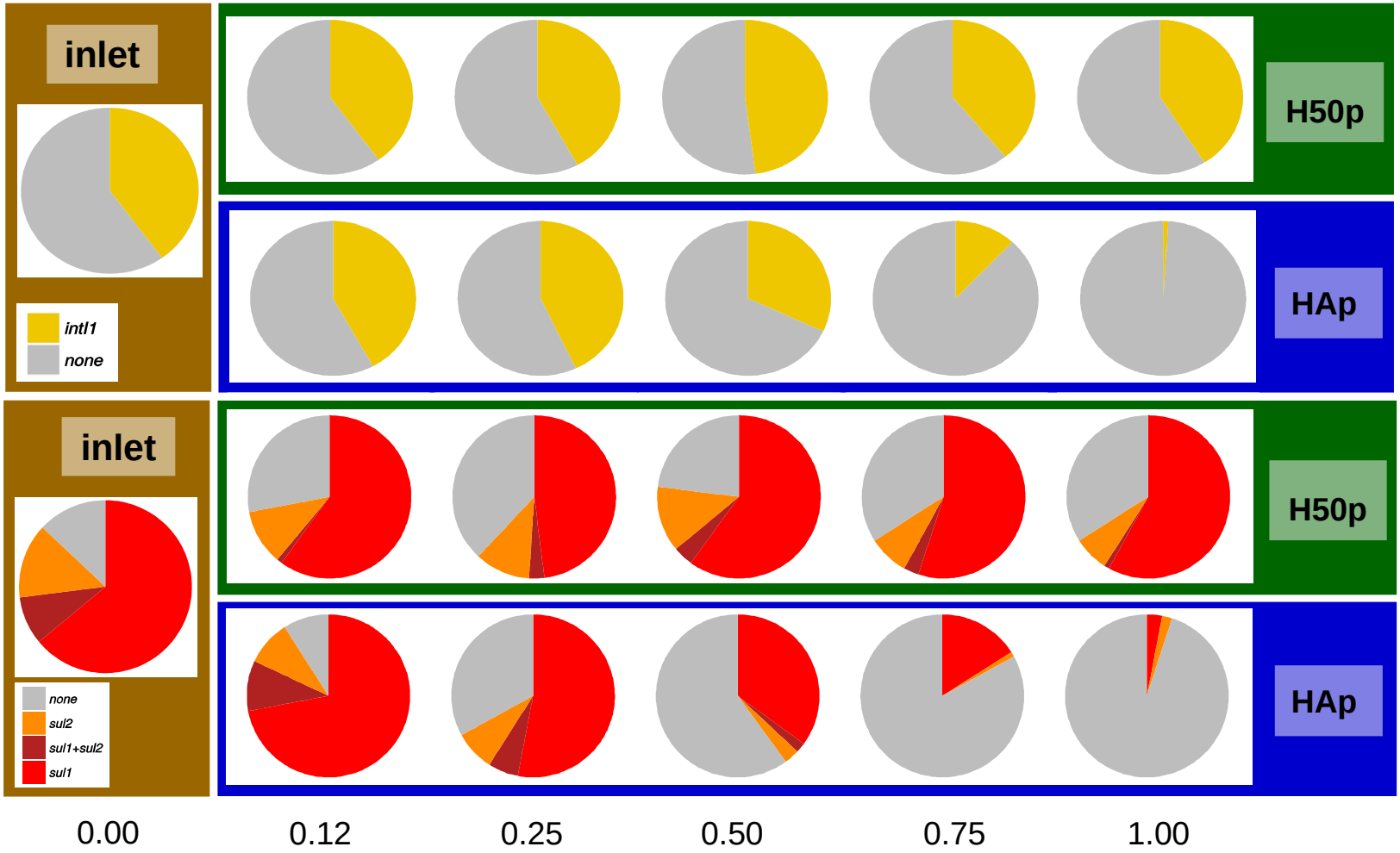


Figure 35| Genotypes of sulfamethoxazole resistant bacteria isolated from the same sampling day on Mueller-Hinton agar, Pseudomonas isolation agar and Endoagar along the flow path. Percentage of isolates carrying *intI1* in yellow. Percentage of isolates carrying *sul1* in light red, *sul2* in orange and *sul1+sul2* in dark red. The inlet is shown on the left, H50p on top and HAp at the bottom, n: 53-60 isolates per sampling point.

Mueller-Hinton agar

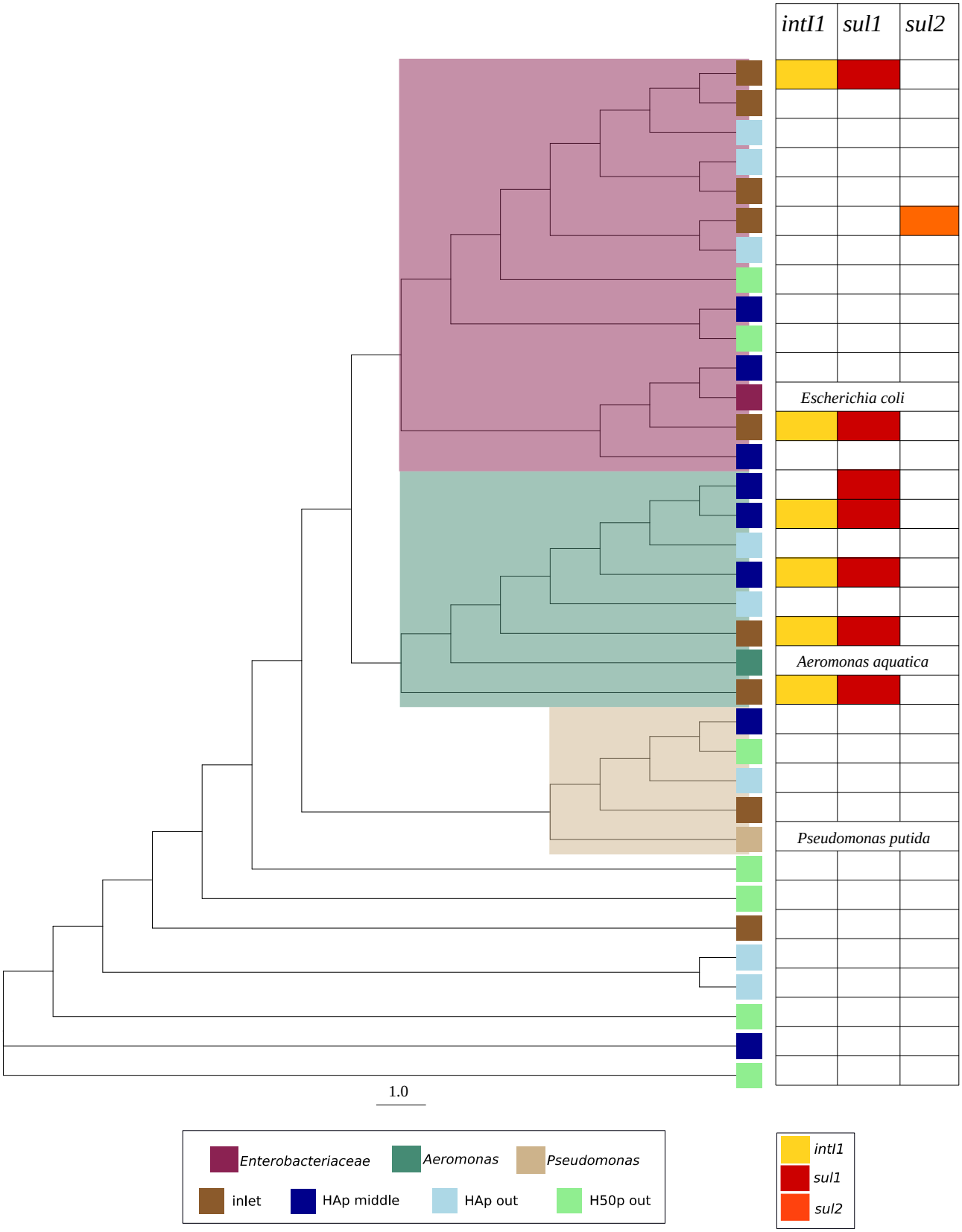


Figure 36| Phylogenetic tree of sequenced sulfamethoxazole resistant bacteria from Mueller-Hinton media and reference strains. N=7 per sample point.

Endoagar

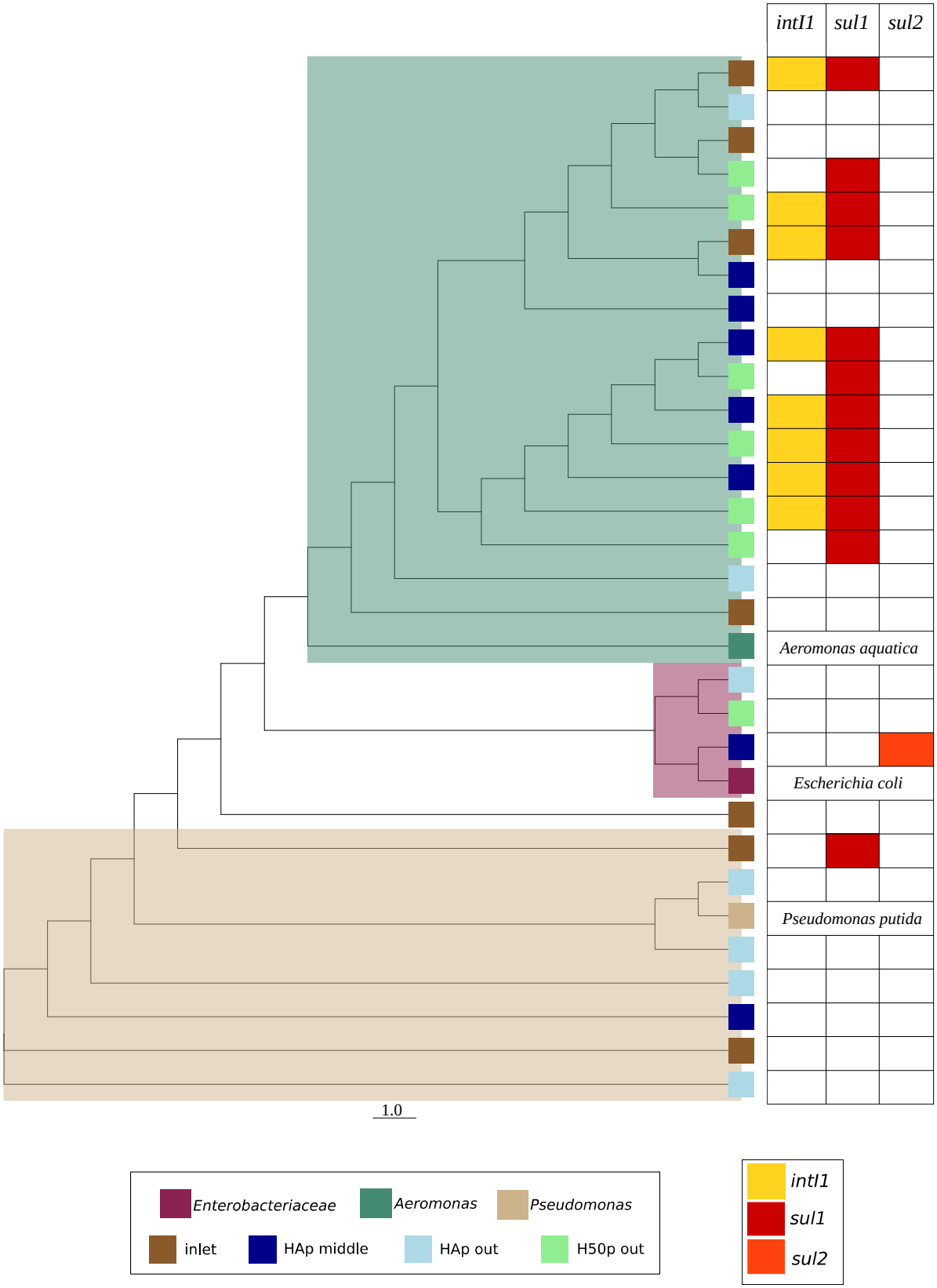


Figure 37| Phylogenetic tree of sequenced sulfamethoxazole resistant bacteria from endoagar media and reference strains. N=7 per sample point.

Pseudomonas isolation agar

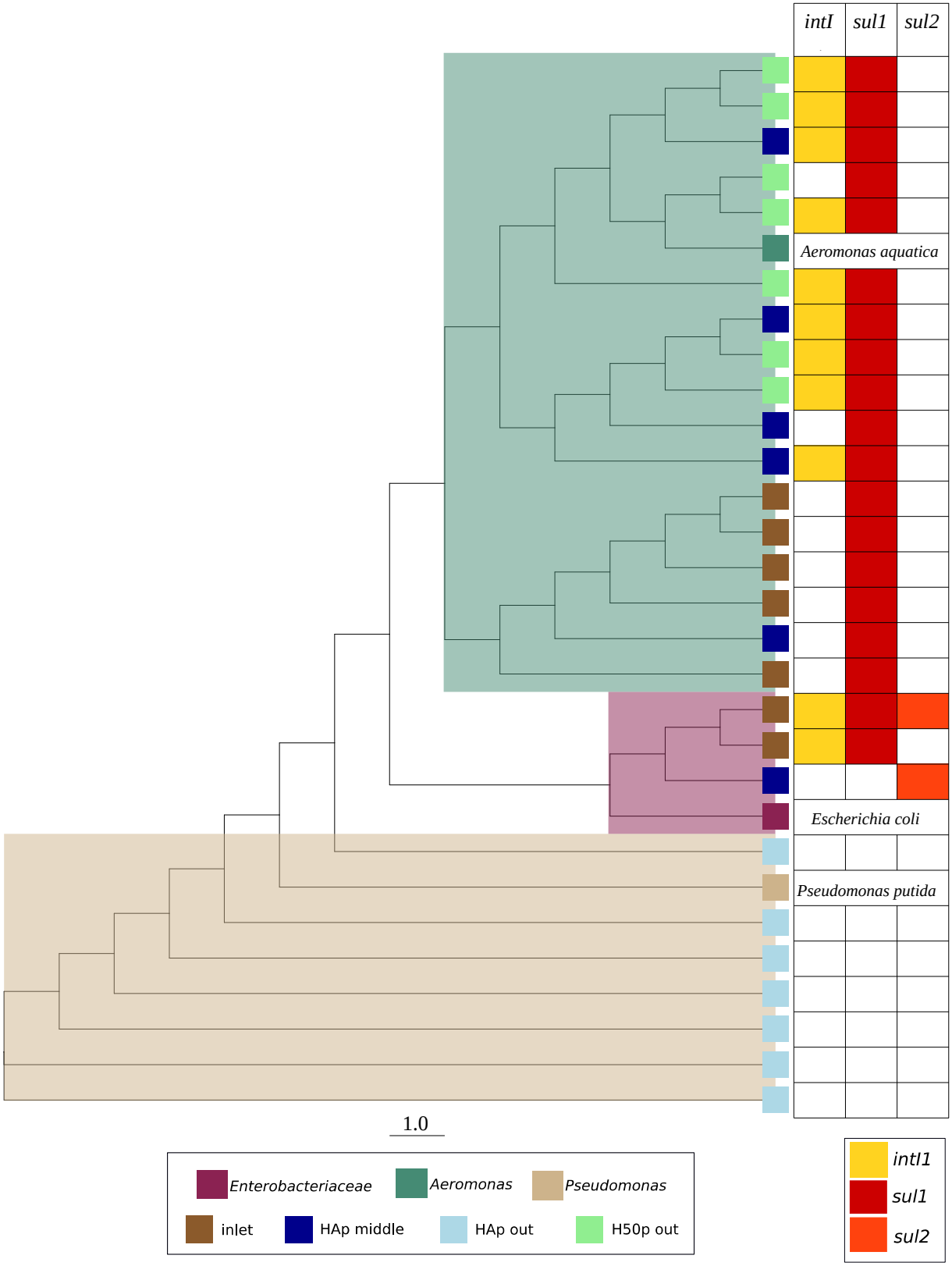


Figure 38| Phylogenetic tree of sequenced sulfamethoxazole resistant bacteria from Pseudomonas isolation agar media and reference strains. N=7 per sample point.

Trimethoprim resistant isolates

The results of CFU counting along the flow path are shown in Figures 39-41, A-B panels, and the proportion of resistant isolates carrying *dfrA1* or *intI1* is shown in Figures 39-41, C-D panels.

Mueller-Hinton agar (MH agar)

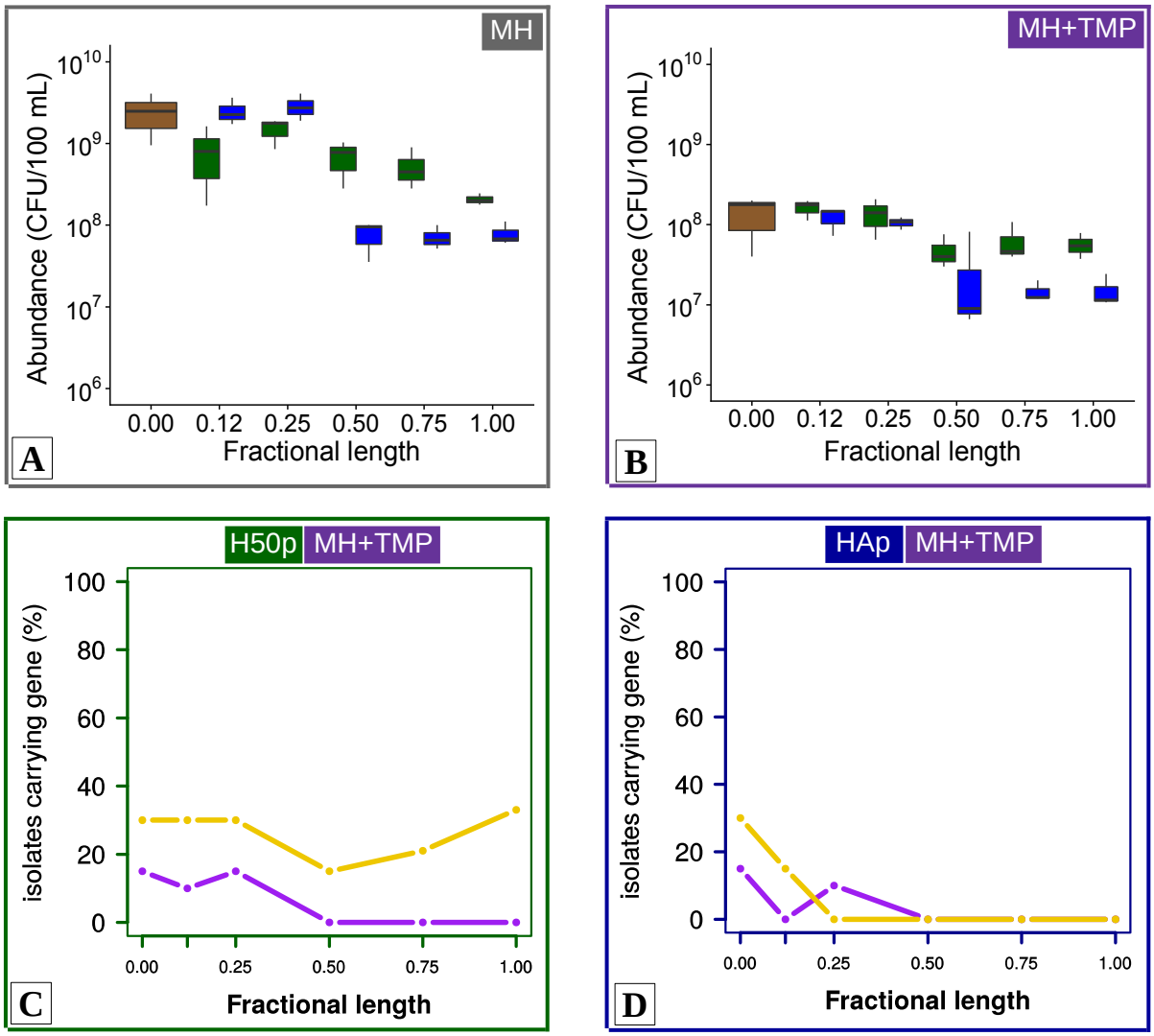


Figure 39| Mueller-Hinton agar (MH agar) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU without antibiotics are shown with grey border and CFU on MH agar+TMP with violet border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from MH agar+TMP carrying *intI1* (—) and *dfrA1* (—) for H50p and HAp. N=20 isolates per sampling site on one sampling day.

CFU counting of TMP resistant bacteria had the same orders of magnitude for Mueller-Hinton agar and EA than for SMX (Figures 32-34, A-B) but the abundance of *dfrA1* (Figure 26, E) was three orders of magnitude lower than *sul1* (Figure 26, C). CFU on PIA+TMP (Figure 41, B) for the inlet was one order of magnitude lower than for SMX (Figure 34, B).

Endoagar (EA)

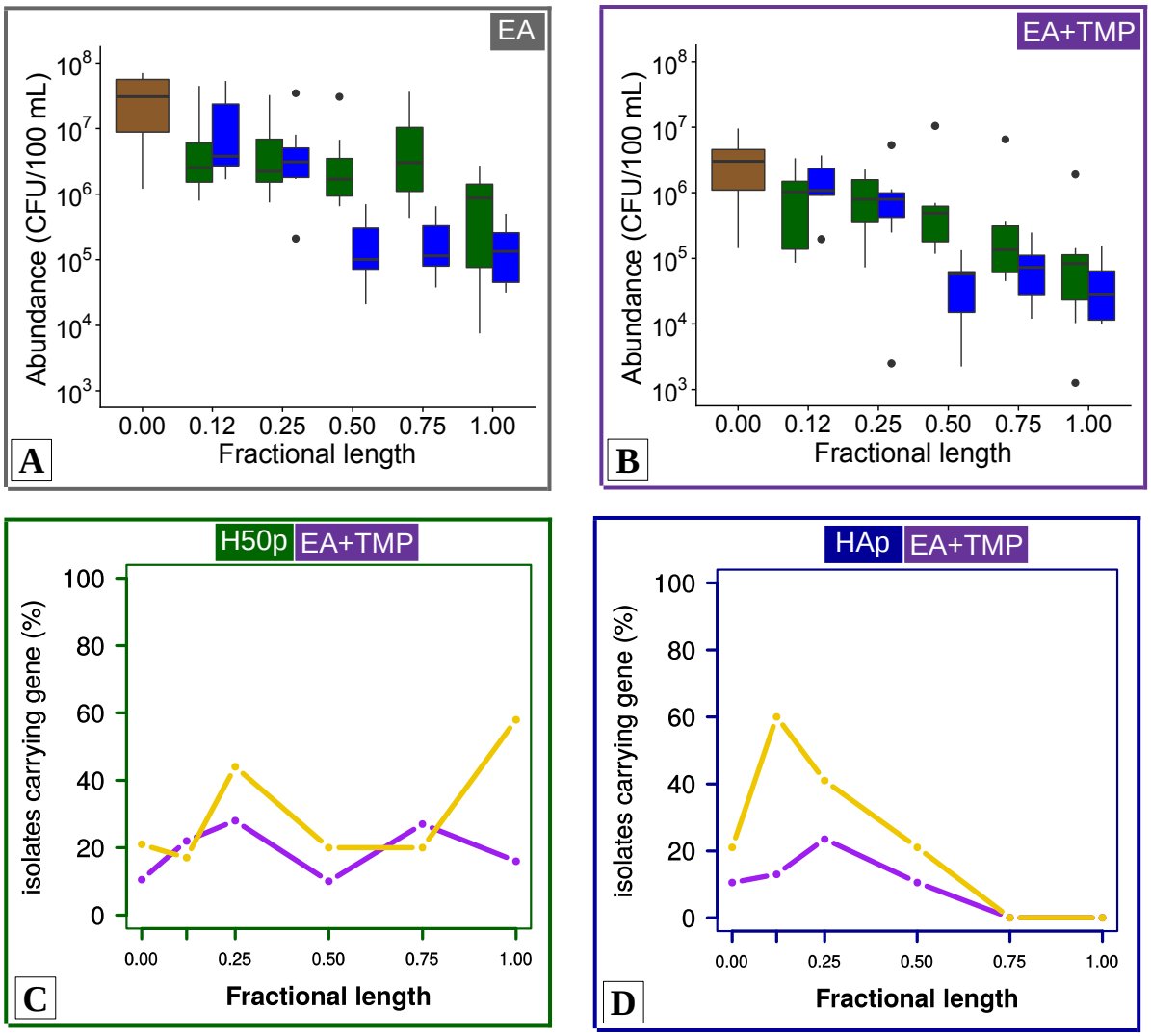


Figure 40| Endoagar (EA) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU without antibiotics are shown with grey border and CFU on EA+TMP with violet border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from EA agar+TMP carrying *intI1* (—) and *dfrA1* (—) for H50p and HAp. N=20 isolates per sampling site on one sampling day.

The proportion of isolates on TMP carrying the *intI1* and *dfrA1* genes showed similarities with those from SMX (Figure 32-34, C-D) in that the percentage was the lowest for Mueller-Hinton agar, intermediate for EA and highest for PIA. In contrast to the observed trend for SMX and *sul1* (Figures 32-34, C-D), the proportion of ARB carrying *intI1* often outweighed the proportion of ARB carrying *dfrA1*. Furthermore, about 75% of the isolates that were *dfrA1* positive, didn't carry this gene in the genetic context of class 1 integrons. Most of the isolates for which this observation was true, were identified as *Enterobacteriaceae* (Figures 43-45). The presence of *dfrA1* in a genetic context different than class 1 integrons was detected more frequently on TMP than on SMX. SMX

indirectly selects for *intI1* because *sul1* is often in the conserved regions of these integrons. When comparing the results for the inlet in the case of TMP, CFU^{TMP} were also higher than the abundance of *dfrA1* measured by qPCR (Figure 26, E). In this case, the difference in orders of magnitude between the qPCR results and the CFU were higher than for SMX.

Pseudomonas isolation agar (PIA)

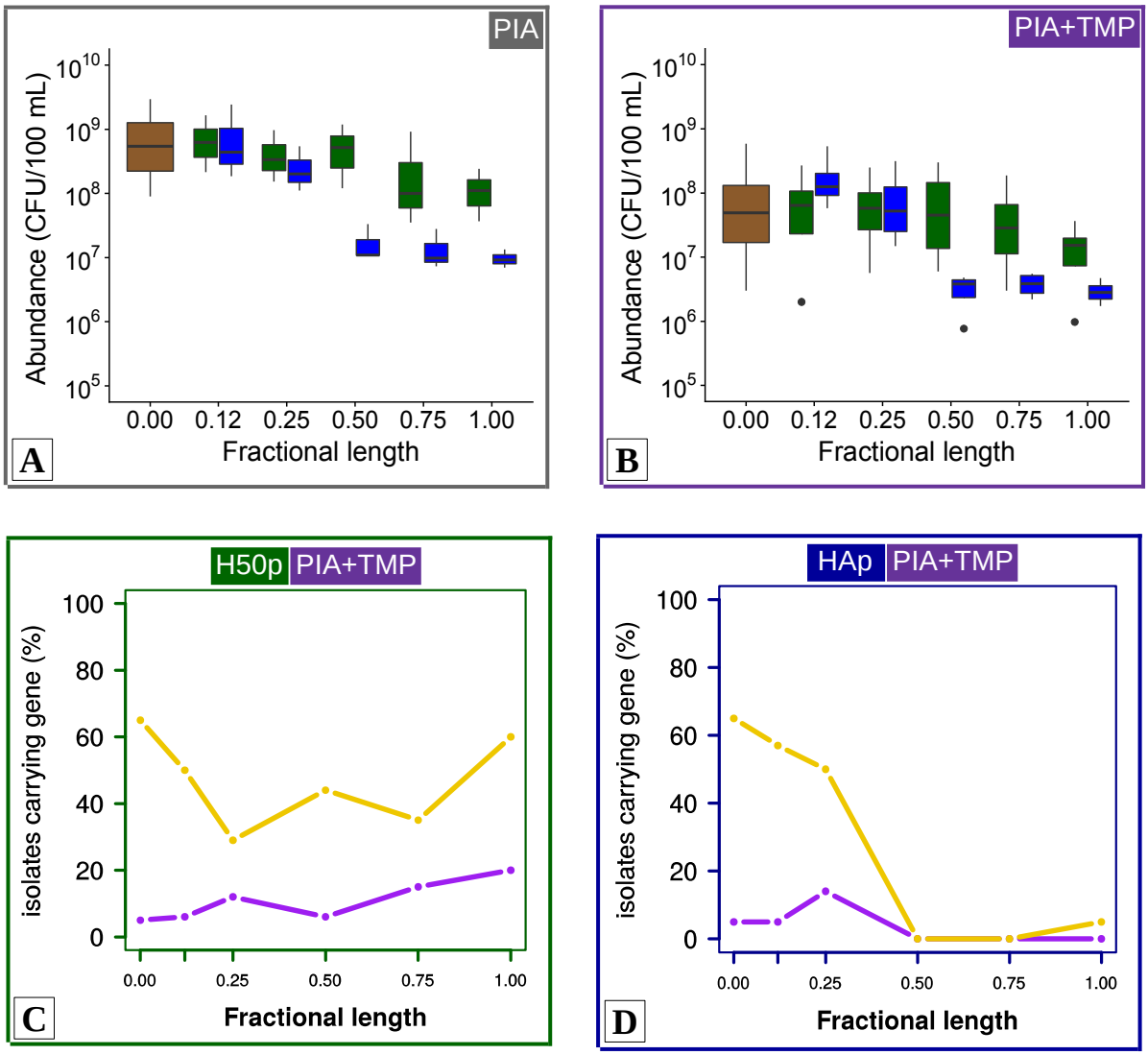


Figure 41| Pseudomonas isolation agar (PIA) colony forming units (CFU) counting and colony-PCR survey along the flow path. A-B| Inlet in shown in brown, H50p in green and HAp in blue. CFU without antibiotics are shown with grey border and CFU on PIA+TMP with violet border. N=3 sampling days. **C-D|** Percentage of bacteria isolated from PIA agar+TMP carrying *intI1* (—) and *dfrA1* (—) for H50p and HAp. N=20 isolates per sampling site on one sampling day.

In Figure 42, the proportion out of 787 TMP resistant isolates carrying *dfrA1* genes and *intI1* are shown along the flow path for both CW. Comparing the proportion of isolates that carry *dfrA1* with the abundance of this gene from qPCR data (Figure 26, E), it can be seen that for HAp the reduction

is reflected whereas for H50p is not: while the abundance of the gene decreased for both CW, the proportion of isolates remained the same for H50p and decreased for HAp.

The percentage of isolates carrying *intI1* were comparable for both antibiotics on PIA, and that is the reason why the results of CFU^{TMP} were also similar to qPCR results, as it was seen for SMX. An increase of CFU^{TMP} for the first sampling points of HAp could be observed, although *dfrA1* abundance decreased. Another difference between PIA+SMX and PIA+TMP was that on the latter there were still 10^6 CFU/100 mL in the outlet for HAp, where for SMX there was none. This can be a consequence of the growth of Gram-positive bacteria, like *Enterococcus* spp. (Figures 43-45). Figures 43-45 also show that TMP resistant bacteria weren't different to SMX resistant bacteria in terms of their taxonomy. As in the case of PIA+SMX, isolates from PIA+TMP carried the studied genes more frequently than isolates on the other two agar media. On Mueller-Hinton agar no TMP resistant *Aeromonas* were detected but these could be observed in the other two agar media with a similar frequency to *Enterobacteriaceae*.

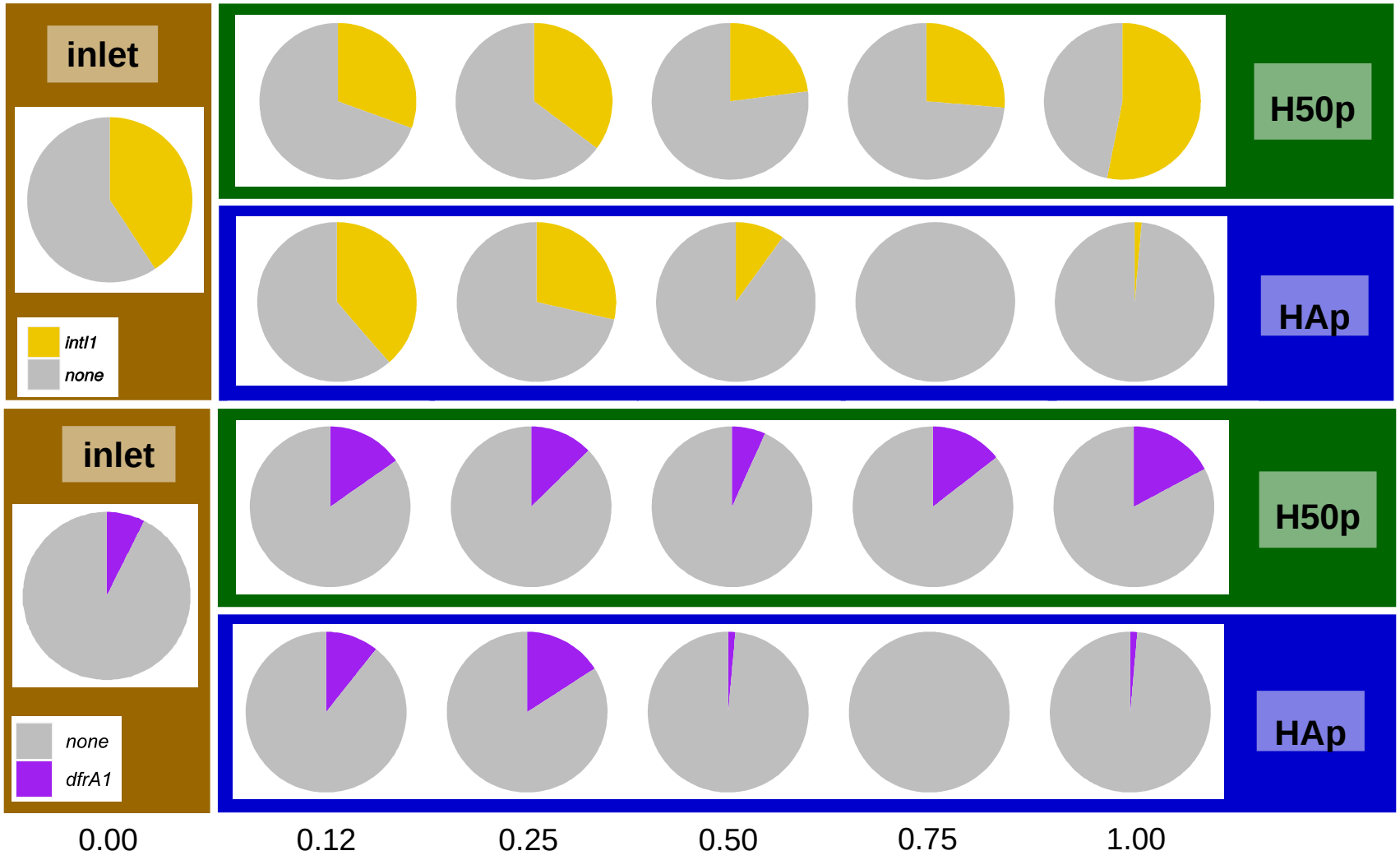


Figure 42| Genotypes of trimethoprim bacteria isolated from the same sampling day on Mueller-Hinton agar, Pseudomonas isolation agar and Endoagar along the flow path. Percentage of isolates carrying *intI1* in yellow, percentage of isolates carrying *dfrA1* in purple. The inlet is shown on the left, H50p on top and HAp at the bottom, N=52-59 per sampling point.

Mueller-Hinton

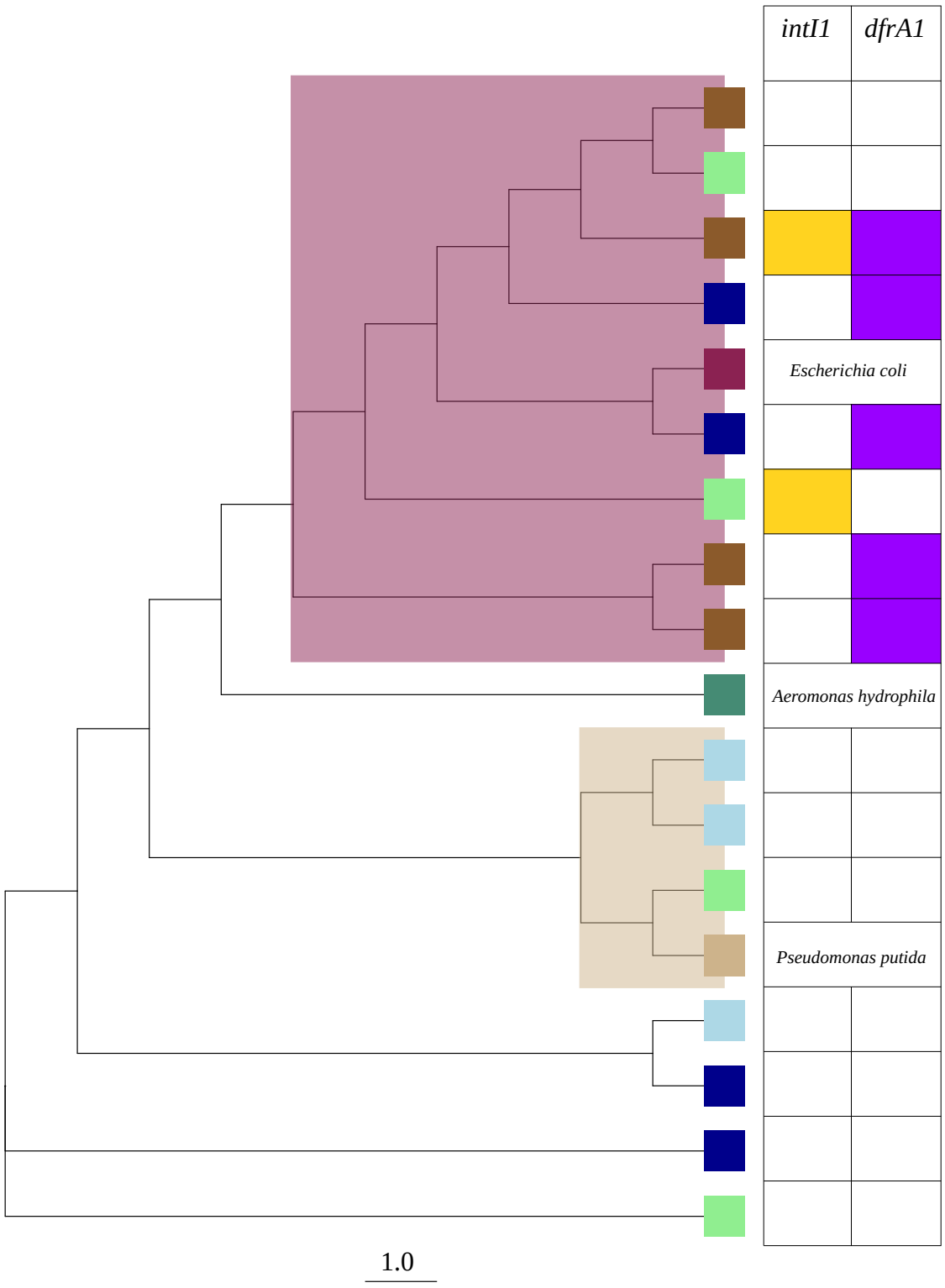


Figure 43| Phylogenetic tree of sequenced trimethoprim resistant bacteria from Mueller-Hinton media and reference strains. N=4 per sampling point.

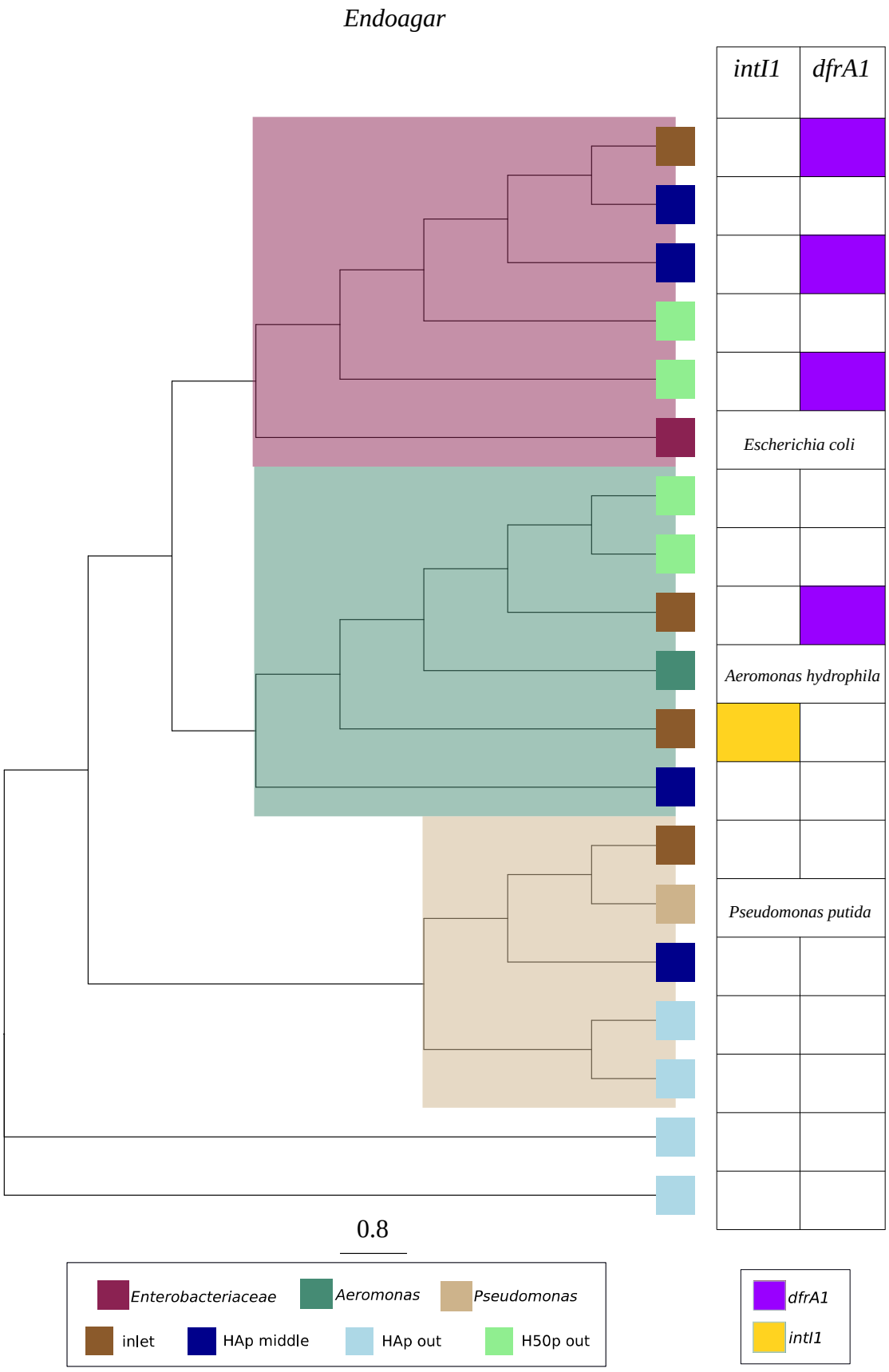


Figure 44| Phylogenetic tree of sequenced trimethoprim resistant bacteria from endoagar media and reference strains. N=4 per sampling point.

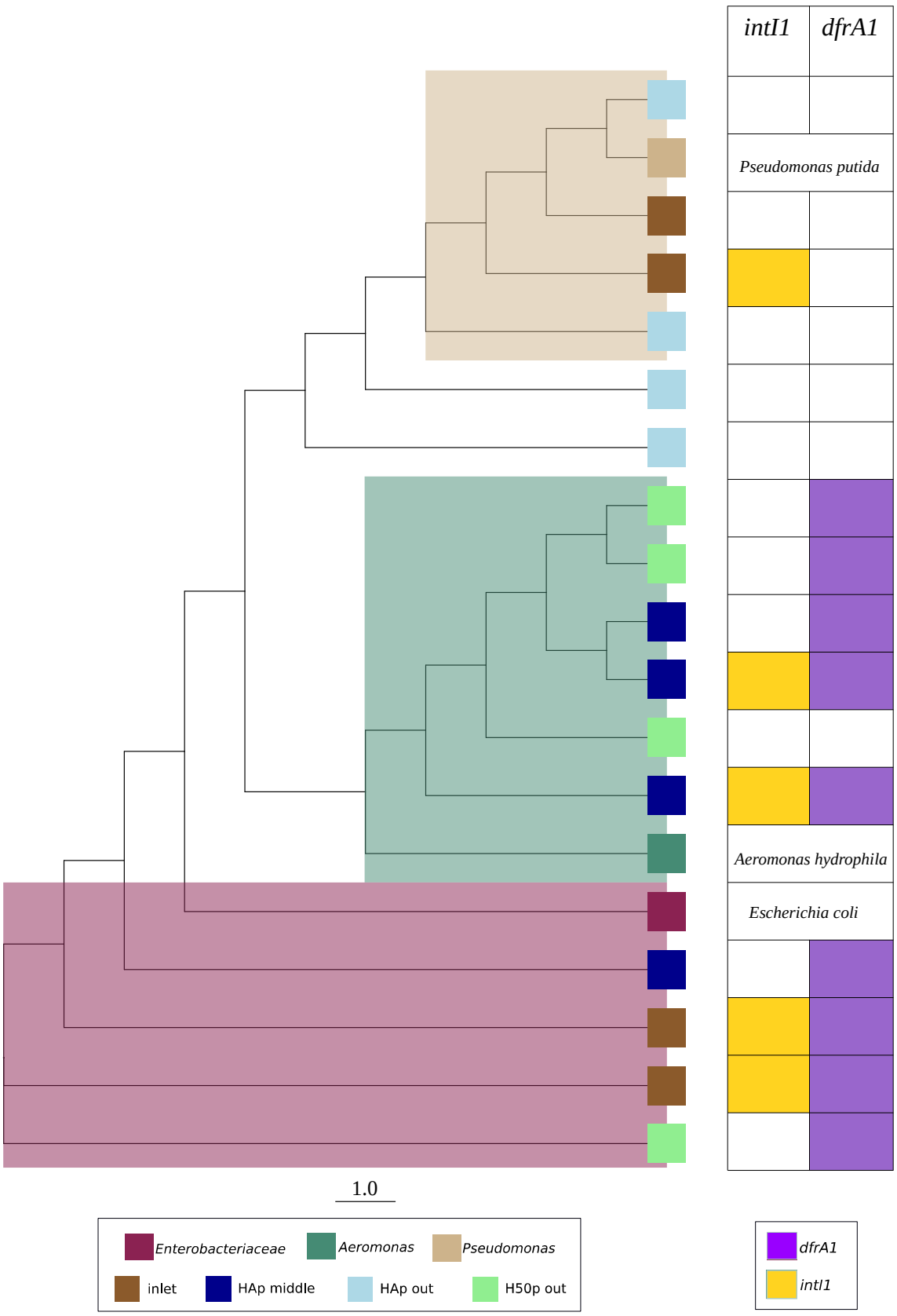


Figure 45| Phylogenetic tree of sequenced trimethoprim resistant bacteria from Pseudomonas isolation agar media and reference strains. N=4 per sampling point.

Gene cassettes

The enzyme IntI1 mediates the recombination of antibiotic-resistant gene cassettes between different integrons in the same cell, facilitating the persistence and dissemination of these genes (Shearer and Summers, 2009). If a cell carries only one copy of class 1 integron, the PCR product run in an agarose gel will show only one band. If the cell carries more than one integron class 1 with different variable regions, the PCR product shows more than one band in an agarose gel (polycarriers). In Figure 46, those isolates showing more than one band as a result of the amplification of the variable region of class 1 integron were taken into account. The number of combinations of different types in a cell are shown in the y axis for the different sampling sites. HAp showed a higher integron diversity than H50p, being highest in isolates from the first half of the bed and thus mirroring the increase in *intl1* abundance. The antibiotic resistance genes found in the variable region were: *dfrA1*, *aadA1*, *aadA2 and catB3* in different combinations (Table 14). Data on which inserts combinations were found and their frequency are shown in Table 15.

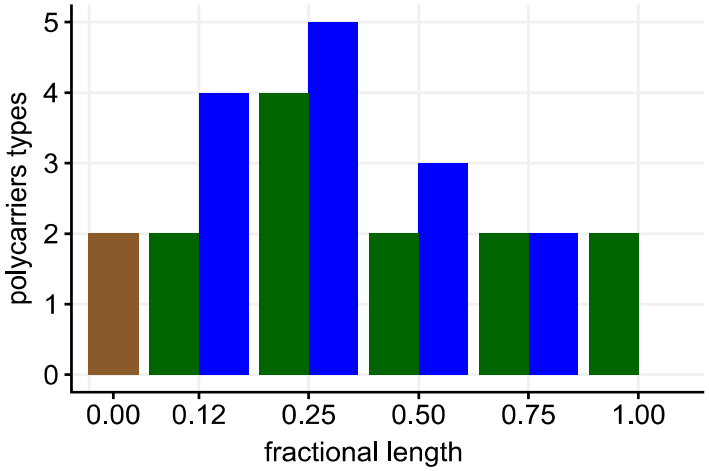


Figure 46| Class 1 integrons diversity of polycarriers isolates along the flow path for inlet (brown), **HAp** (blue) **and H50p** (green)**.** Polycarriers host more than class 1 integron type per cell. Type is assigned depending on the size of the variable region.

Table 14| Gene cassettes types inserted in class 1 integrons in the studied isolates. The type was determined according to their sizes (in brackets). Cassettes sequences are listed in Appendix C

Cassette type	Gene cassettes	hosts taxonomy
A (150 bp)	-	*Aeromonas*
B (600-850 bp)	*aadA1*	*Enterobacteriaceae*
C (1000 bp)	*aadA2|catB3*	*Enterobacteriaceae*
D (2200 bp)	*dfrA1|catB3|aadA4*	*Aeromonas/Enterobacteriaceae*
E (2700 bp)	*aadA|pkg|ereA|Orf*	*Enterobacteriaceae*

Table 15| Gene cassettes types inserted in class 1 integrons for the different sampling points. The type was determined according to their sizes. In red appear the number of isolates carrying more than one integron type per cell.

Cassettes combinations	**0.00**	**0.12**		**0.25**		**0.50**		**0.75**		**1.00**	
	inlet	HAp	H50p	HAp	H50p	HAp	H50p	HAp	H50p	HAp	H50p
A “empty”	(1)	(1)	(2)	(2)	(3)	(3)	(1)		(3)		(2)
B	1	1	1		3	2	2		1		1
C	2	2	8	2	3		3		4		1
D	7	4	1	9	4	5	1	2	3		4
E	2	1	2		2		5				1
A-B	**7**	**9**	**1**	**4**	**1**	**3**	**4**		**5**		**11**
A-D	**1**		**1**								
A-E		**2**		**1**							
B-D		**1**		**1**	**1**	**3**	**1**		**1**		**1**
B-E		**1**									
C-D					**1**			**1**			
D-E				**1**							
A-B-D				**1**	**1**						
A-B-E						**1**		**1**			
Polycarriers type	**2**	**4**	**2**	**5**	**4**	**3**	**2**	**2**	**2**	**0**	**2**

The genes *aadA* confer resistance against aminoglycosides (30S inhibitors) and *catB3* against chloramphenicol (50S inhibitors). The gene *pkg* codifies for a protein kinase. Even though the search for the sequence identified as *pkg* in the different databases didn't match to any known ARG, kinases have been related to resistance to several antibiotics (Burk and Berghuis, 2002).

4.2.2.3 Quantification of specific bacteria

Escherichia coli

In addition to the MPN method, *E. coli* CFU were counted, another culture dependent method that in contrast to MPN, has the advantage of allowing the study of resistance. In this work, *E. coli* abundance was additionally measured by qPCR targeting the *uidA* gene as culture independent method. The comparison of the quantifications made by different methods is shown in Figure 47.

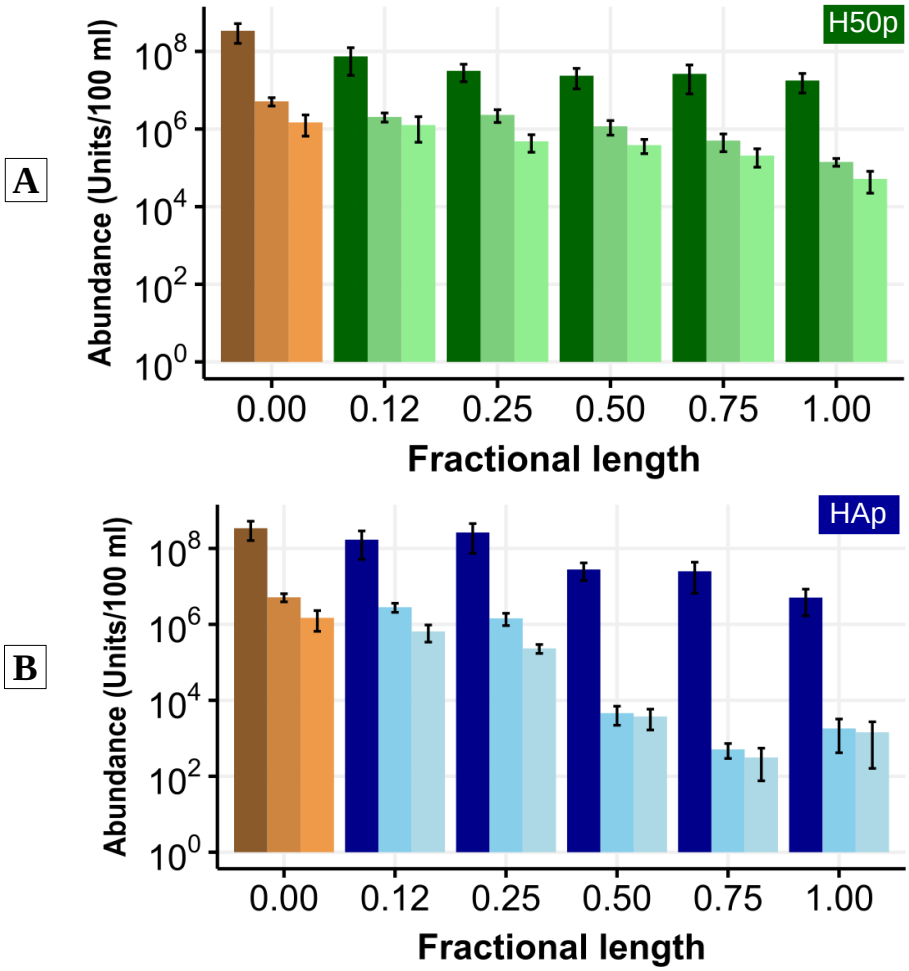


Figure 47| *Escherichia coli* abundance along the flow path, measured by qPCR (dark tones)**, most probable number** (middle tones) **and colony forming units** (light tones) **in 100 mL.** Abundance in the inlet is shown in brown, abundance in H50p in green and in HAp in blue. **A|** Inlet and H50p **B|** Inlet and HAp. N_{qPCR}=12, N_{MPN}= 9, N_{CFU}=4.

The *uidA* gene codifies for the enzyme ß-glucuronidase, which catalyses the breakdown of complex carbohydrates (Kaushik and Balasubramanian, 2012). The specificity of these primers was confirmed by colony-PCR of the *uidA* gene and sequencing of the 16S rRNA gene. Sequencing of the 16S rRNA gene of 10 *uidA* positive isolates was consistent and no false positive were detected.

The comparison of the results of the different methods shows that in H50p the difference between culture-dependent and culture-independent methods remained constant (being the culturable *E. coli* two orders of magnitude lower). That wasn't the case for HAp, where the abundance measured by culture-dependent method dropped by two orders of magnitude starting from 0.50 of the fractional length while the reduction measured by the culture-independent method didn't. For the outlet of HAp, viable *E. coli* (culture-dependent method) was four orders of magnitude lower than total *E. coli* (culture-independent method).

Taking a look at resistant *E. coli*, it can be seen that for the inlet the abundance of *E.coli* resistant to SMX and TMP is similar, so are the tendencies along the flow path (Figure 48).

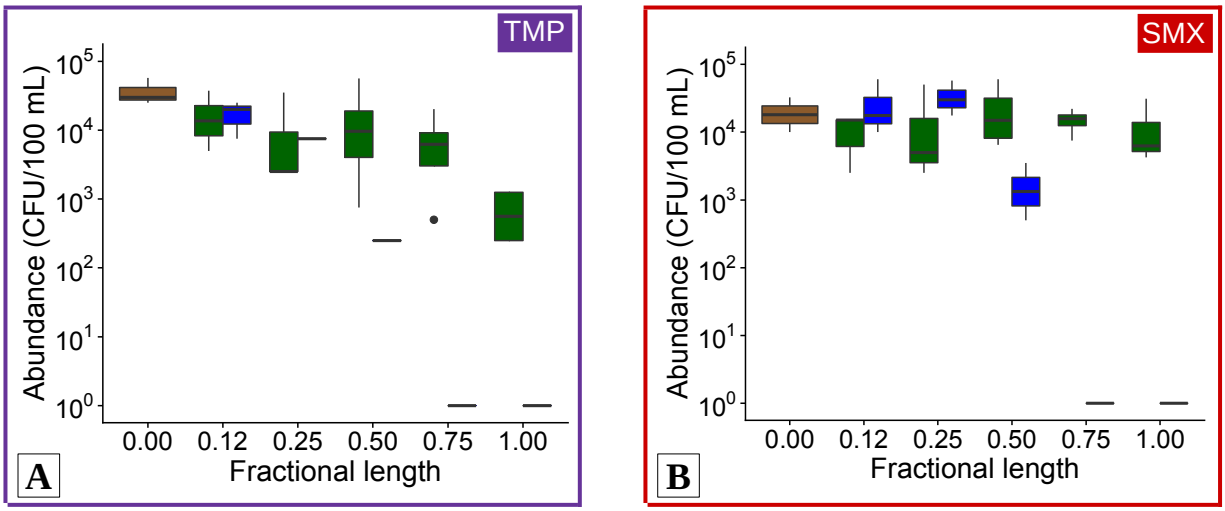


Figure 48| Resistant *E. coli* quantified by colony forming units on endoagar with the addition of antibiotics along the flow path. Inlet is shown in brown, H50p in green and HAp in blue. **A|** EA+trimethoprim (TMP) **B|** EA+sulfamethoxazole (SMX). N=3

In the case of SMX, a higher number of CFU^{R} was observed for HAp than for H50p in the first sampling points while the CFU^{TMP} were similar for the same samples. For the last two sampling points for HAp no resistant *E. coli* was detected. In average for all sampling points 10% of *E. coli* colonies were SMX resistant and 4% were TMP resistant and thus around 10^2 CFU^{SMX} and 10 CFU^{TMP} should have been detected. In the case of CFU^{TMP} it could be to a sampling artefact as 100 µL are plated on the petri dishes and that would mean in average one CFU. In the case of CFU^{SMX} 10

CFU should be present in average in 100 µL but no *E. coli* CFU was observed. However, as it can be seen from the difference between molecular and culture dependent methods, this doesn't necessarily mean that SMX resistant *E. coli* was absent in the effluent of HAp, it means that it couldn't be cultured. *E. coli* isolates were also tested for the studied genes, their 16S rRNA gene was sequenced and the data was included in Figure 31.

Aeromonas spp

A similar procedure to what was done for *E. coli* was carried out for *Aeromonas* spp. (Figure 49), as they were also common hosts of the studied genes (Figure 31). As a culture-independent method the gene *gyrB* was quantified by qPCR using published primers (Khan et al., 2009). This gene codifies for a DNA gyrase, which is an enzyme that relieves molecular tension in DNA supercoiling. As a culture dependent method, CFU on AIA were counted. *Aeromonas* spp. appear on this medium as dark green colonies .

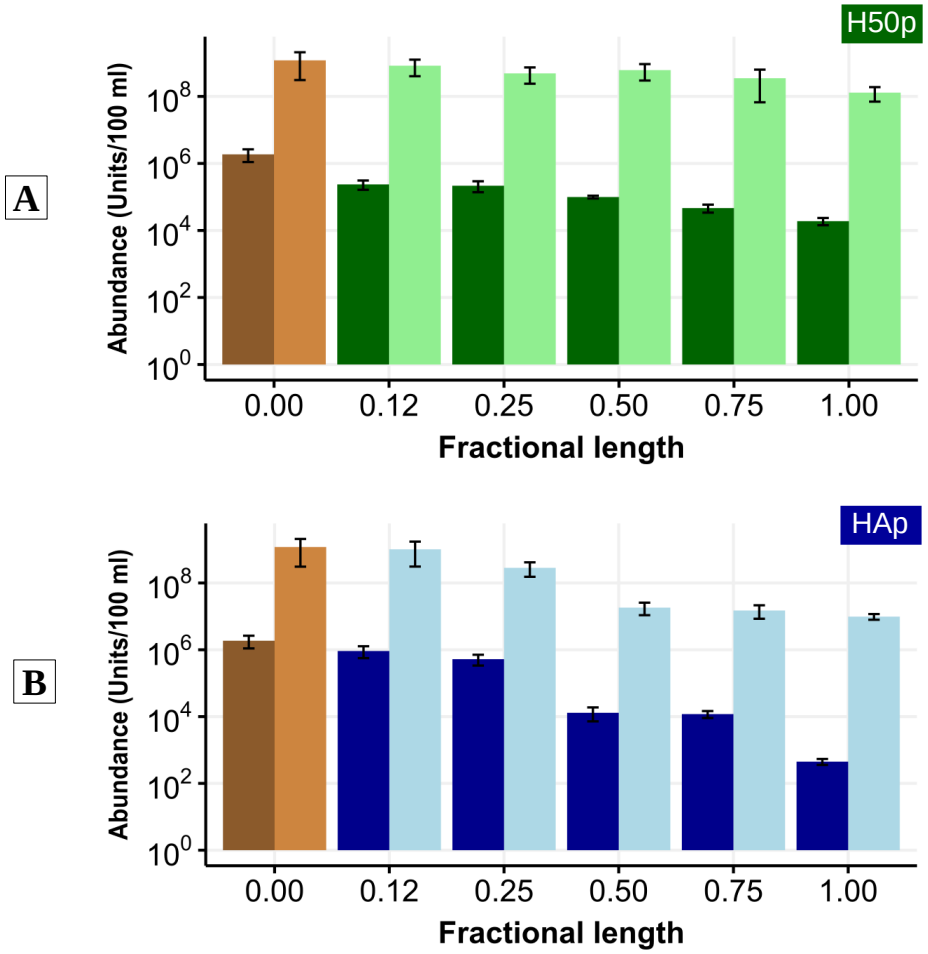


Figure 49| *Aeromonas* spp abundance along the flow path, measured by qPCR (dark tones) **and colony forming units** (light tones) **in 100 mL**. Abundance in the inlet is shown in brown, abundance in H50p in green and in HAp in blue. **A|** Inlet and H50p **B|** Inlet and HAp. N=3.

In this case and other than expected, the abundance detected by the molecular method was around 2 orders of magnitude lower than the culture-dependent method. 17 putative *Aeromonas* colonies from AIA were tested by PCR with *gyrB* primers and just 5 (30%) were PCR-positive. That means that either the primers were too specific and didn't detect all the *Aeromonas* spp. in the samples or the medium is not selective enough and some dark green colonies didn't belong to this genus. The sequencing of colonies that were *gyrB* negative were identified as *Pseudomonas* spp. by Sanger sequencing of their 16S rRNA gene.

Otherwise, the spatio-temporal changes towards the outlets of the CW were comparable with the results obtained for *E. coli* (Figure 50). For H50p, neither the abundance nor the difference between methods changed along the flow path. For HAp there was a decrease that could be seen by both methods starting from 0.50 of the fractional length (one order of magnitude in culture-dependent method and three orders of magnitude in culture-independent method). This change correlates with multiple changes in HAp in terms of physicochemical parameters like DO, C-BOD_5 and nitrogen species. It also correlated with changes regarding the taxonomy of cultured resistant bacteria and the abundance of ARB and ARG.

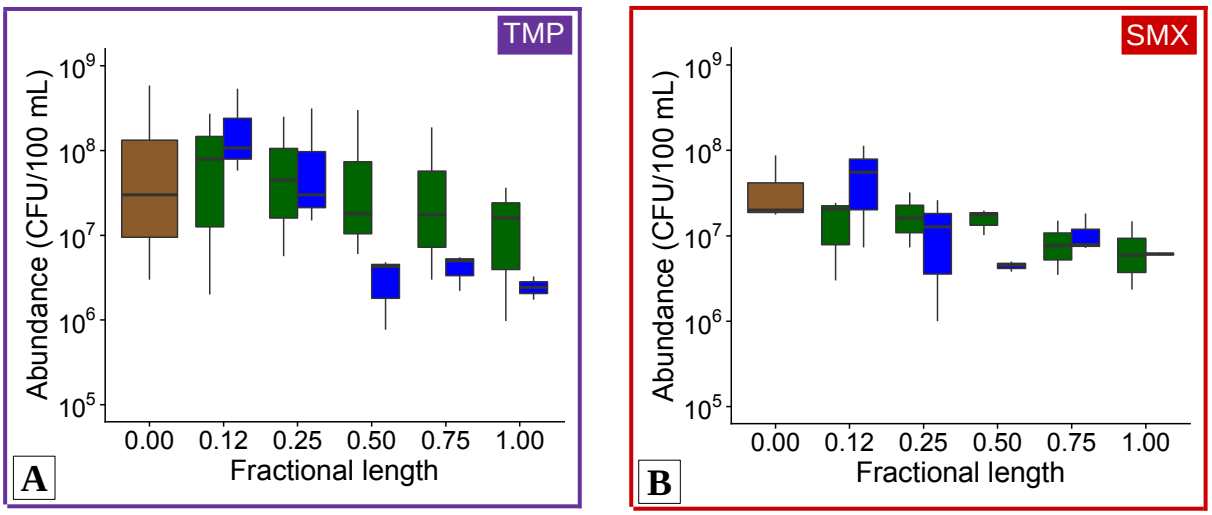


Figure 50| Resistant *Aeromonas* spp. quantified by colony forming units on Aeromonas isolation agar with the addition of antibiotics along the flow path. Inlet is shown in brown, H50p in green and HAp in blue. **A|** AIA+trimethoprim (TMP) **B|** AIA+sulfamethoxazole (SMX). N=3.

The total abundance was higher than for *E. coli* partly because it is a whole genus which is being detected and not only one species. There were still 10^7 CFU^{SMX}/100 mL in the outflow of HAp (Figure 50), comparable to what has been observed on Mueller-Hinton agar (a non-selective medium), which further suggests that there was an overestimation of *Aeromonas* spp. CFU on AIA. While the total abundance might be overestimated, there was also a slight increase in CFU^R in the

first sampling point of HAp for both antibiotics, although these difference weren't significant. The difference wasn't statistically significant but the data is coming from 3 biological replicates. In order to confirm or discard the significance of this difference more biological replicates should be considered.

That in this case this increase can also be observed for TMP resistant isolates in HAp might be a consequence of the selective conditions in Aeromonas isolation agar which contains ampicillin (see Appendix A). The gene *pkg* could account for ampicillin resistance (Li et al., 2016) and it was often found in the variable region of the integrons in the CW (see Table 14). Even though an increase could be seen also for H50p in the first sampling point, the increase is higher for HAp.

4.2.2.4 Emulsion paired isolation and concatenation PCR (epicPCR)

As already mentioned throughout this work, linking phylogenetic information with function by culture independent methods in environmental samples represents a big challenge. Some methods have been recently developed and are currently being validated and applied. Among them, epicPCR allows to link taxonomic and antibiotic resistance genetic information without culturing by physically linking genes and sequencing of the fused PCR product. This is achieved by the formation of polyacrylamide beads that trap single cells. Afterwards, a fusion PCR is carried out which links part of the 16S rRNA gene with part of the ARG. The products are then purified and a second PCR is carried out which increases the specificity and prepares the products to be sequenced.

The protocol was first set up for pure cultures. Genomic DNA of isolates that were hosts of the studied genes and whose 16S rRNA gene had been sequenced were used for the set up of the PCR. Several primers for the first and the second PCR steps and variation of the protocols were tested. Once that specific amplification and that the products showed the predicted size in agarose gels, the final products were sequenced by Sanger sequencing and the specificity was confirmed.

In Table 16 agarose gel pictures of the different epicPCR products are shown. This products are obtained in the second PCR step (nested or semi-nested PCR of fusion products obtained from first step PCR) and included:

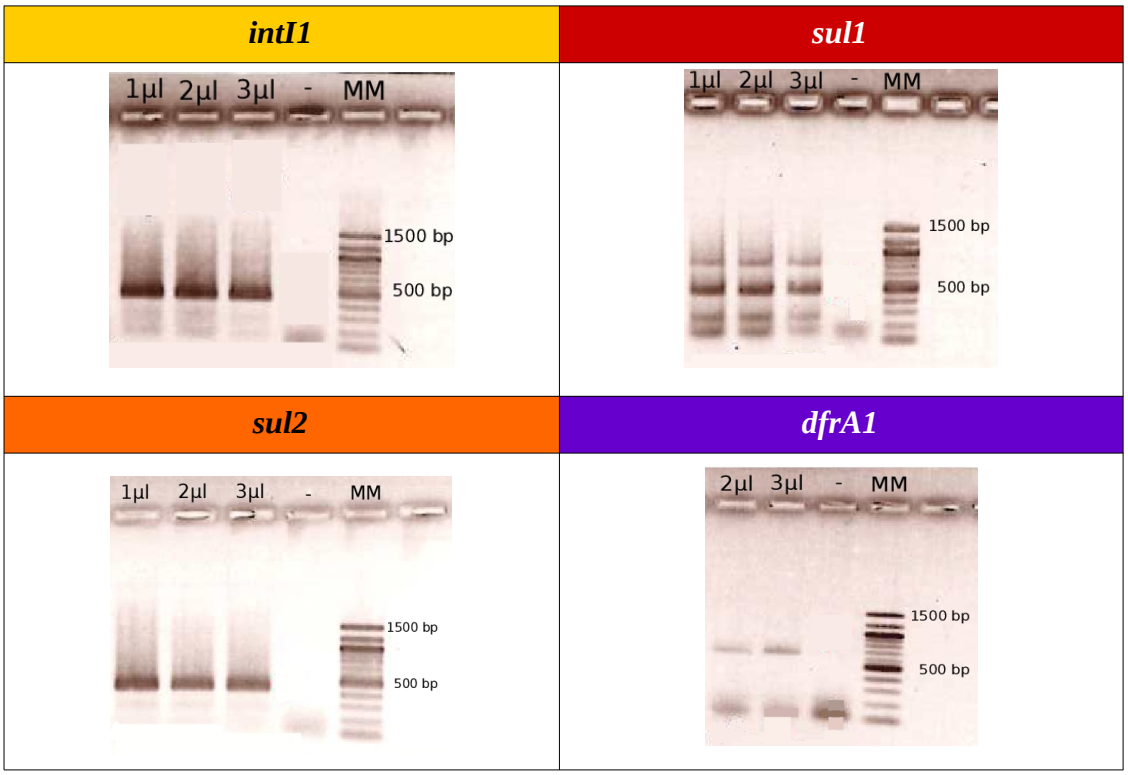
1) part of the ARG or *intI1* gene

2) part of 16S rRNA gene and

3) partial sequences of Illumina adaptors necessary for subsequent sequencing.

For the polyacrylamide bead formation, these isolates were cultured in liquid media in order to trap single cells. About 3 cells in 100 of the beads contained a single cell (Figure 51), which was comparable with what already reported by Spencer et al. (2015) where 1 of 100 beads contained a single cell.

Table 16| Agarose pictures of the PCR products of the last step of epicPCR before Illumina sequencing. Different volumes refer to the amount of the fusion product used as a template for nested/semi-nested PCR.



MM: molecular marker, bp: base pairs.

After setting up the method for pure cultures, the same procedure was carried out for samples coming from the studied CW. Illumina sequencing of epicPCR products coming from the different sampling points of both CW as well as total 16S rRNA gene amplicon is undergoing. This will hopefully help to answer one of the open question that remain to be answered: if there are ARG hosts species which couldn't be cultured.

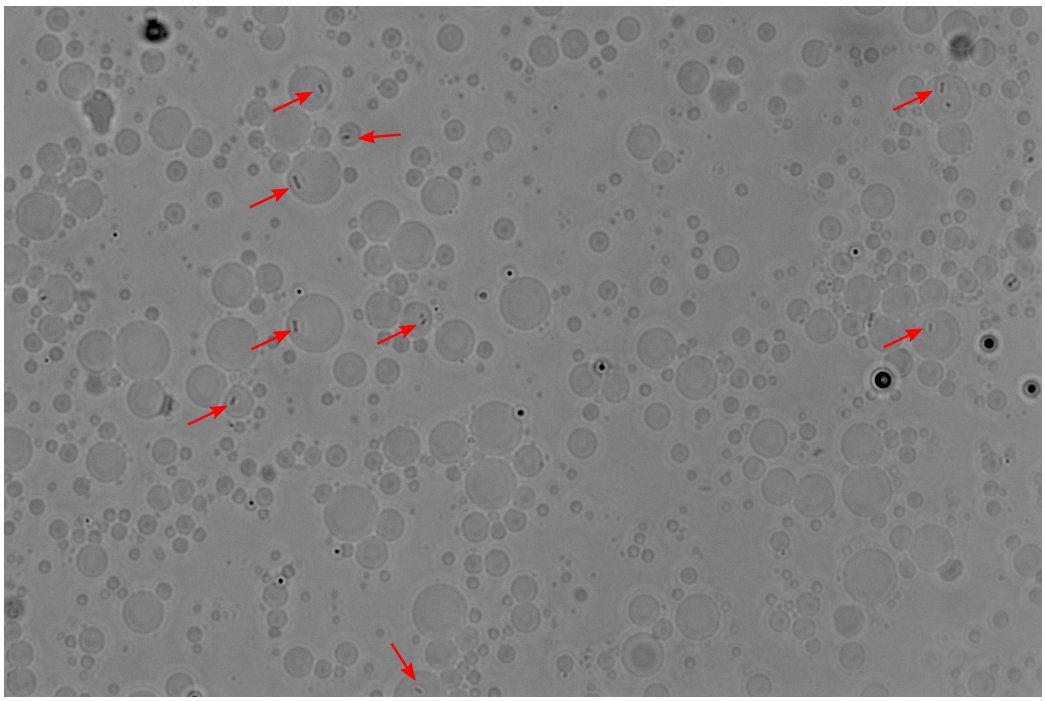


Figure 51| Polyacrilamyde beads from a pure culture of an isolate obtained from the studied constructed wetlands. Red arrows point towards bacterial cells trapped in polycrilamyde beads. 3 % of the beads contained a single cell, which is in the range of what has been reported by the developers of the method (Spencer et al., 2015).

4.2.3. Discussion

Physicochemical conditions for H50p and HAp

Antibiotics in wastewater have been thought to be the main driving force for the development of antibiotic resistance in wastewater and the environment. Therefore, several studies tried to figure out whether there was a correlation pattern between antibiotic concentrations and antibiotic resistance. Physicochemical parameters in this context are often overlooked although they vastly affect the bacterial community, indirectly affecting ARB and ARG hosts. Specifically oxygen content together with the nutritional status can lead to oxidative stress and this could directly affect antibiotic resistance. In H50p changes in standard wastewater parameters showed the same continuous tendency all along the flow path, whereas in HAp showed a shift after 0.50 of the fractional length. Whereas in H50p a sustained decrease in C-BOD_5 was seen, in HAp the organic biodegradable matter was totally consumed before reaching half of the CW. This acute change reflected in the DO concentration, which increased after 0.50, and in the concentration of nitrogenous compounds. Ammonium-N decreased till 0.50 of HAp where it reached a value below the detection limit. At the same time, NO_3^--N reached a constant value of around 45 mg/L. The

absence of DO in the first fraction of HAp in spite of active aeration together with the transformation of NH_4^+ into NO_3^- and NO_2^- indicates high aerobic metabolism. Although ROS weren't measured, they are an unavoidable intracellular by-product of aerobic metabolism. In that sense, ROS are being constantly produced *in situ* in the first fraction of HAp. The amount of ROS produced and the effect on the cell is difficult to predict, as there is no simple correlation between DO and oxidative stress since ROS production is species dependent (Korshunov and Imlay, 2006). The study of the oxidative damage of a particular bacterial species is difficult in the systems investigated, due to the diversity of the microbial community and the risks of introducing labelled bacteria into the open environment. One possibility for measuring the impact of ROS in the cells is by the quantification of carbonylated proteins through mass spectrometry. If the aim was to quantify how a particular species is affected by this modifications in a bacterial community in the investigated CW. One possibility of labelling is introducing a gene which codifies for a fluorescent protein, which allows to select the cells by flow cytometry. It would be necessary to introduce genetically modified cells of this species in the open environment which is not allowed in Germany.

Antibiotics median concentrations in the inlet were 96.9 ng/L for SMX and 8.2 ng/L for TMP, 8 orders of magnitude lower than their Minimal Inhibitory Concentrations (MIC) (Czekalski et al., 2012). The concentrations inside the CW were always lower than in the inlet. Because their concentrations were very low and because the differences between the CW weren't statistically significant, the presence of SMX/TMP in the media was unlikely the cause for the difference in resistance observed between the CW. All in all, both CW are run in parallel and receive the same municipal-rural wastewater. Besides from aeration and the physicochemical parameters related to DO, both CW are the same.

Characterization of antibiotic resistance in H50p and HAp

In the chapter 4.1 it was observed that the relative abundance of *intI1* for H50p was higher than in the inlet. In chapter 4.2 a transient increase of *sul* genes and *intI1* (Figure 27) abundance in the first half of HAp correlated with the highest oxygen turnover (Figures 22 and 23). This increase wasn't seen for the first fraction of H50p. One possibility is that at the end of H50p, with a higher oxygen availability and lower C-BOD_5, the same phenomena that take place at he beginning of HAp take place at the end of H50p. For *dfrA1*, the values were similar for both CW, decreasing all along the flow path until reaching a minimum in the outlet (Figure 27). Furthermore, qPCR of the total abundance of *intI1* and it's the clinical allele had the same tendency, showing an increase in the first portion of HAp (Figure 30). The clinical nature of most of the class 1 integrons was further

confirmed by the fact that just 13% of the SMX resistant isolates hosted an environmental allele. Dissolved oxygen concentration has been positively correlated with *intI1* copy number measured by qPCR in a river samples with different grade of anthropogenic pollution (Koczura et al., 2016). This is anyway a study carried out in the environment were no controlled aeration conditions are possible and where therefore no evidence for the amount of aerobic metabolism were provided. Furthermore this is not what we observed in our study, as with higher measured DO intI1 abundance was lower. In another study carried out by Sulfikar et al. (2018), it was observed that resistance against amoxicillin or norfloxacin was more likely to increase in the aeration tank than in the primary sedimentation tank or final sedimentation tank. This study was nevertheless limited to the determination of the MIC of *E. coli* and the time of residence in the aeration tank was longer than in the other tanks.

Looking at the isolates from the studied CW, when comparing resistance patterns of isolates from the various agar media, it is better to look at the inlet samples in order to reduce the number of variables. That means to compare isolates before the treatment in order to be able to elucidate which differences observed in the isolates from internal samples are caused by treatment in the CW and which by the selection on the different agar media. The CFU^{R} in the inlet were the same on PIA as on Mueller-Hinton agar (Figures 32, 34, 39 and 41, B). Isolates carrying the studied genes in the inlet were more frequent on PIA than on Mueller-Hinton agar. This can be attributed partly to the selectivity of PIA towards Gram-negative bacteria (32, 34, 39 and 41, C and D). Gram-positive bacteria rarely carry these genes, as they often have efflux pumps that make them resistant. The increased selectivity on PIA is due to its triclosan content, a wide spectrum biocide. The observations that the percentage of isolates carrying *intI1* on PIA (Figures 34 and 41, C and D) is the same for both antibiotics and higher than for the other media could be explained through co-selection. The *qacΔE* gene on the integron could serve as an efflux pump for triclosan but this hypothesis warrants further investigation. Pseudomonas isolation agar in combination with SMX represents a good methodological approach to monitor and study antibiotic resistance in culturable indicator bacteria.

The number of CFU^{SMX} in the outlet of HAp varied between the agar media to a greater extent than for the outlet of H50p (Figures 32-34 and 39-42, B). This can be explained by the difference in bacterial communities and in bacterial viability between the CW. Endoagar and, to a broader extent, PIA exert an additional constraint on the growth of resistant bacteria in comparison to Mueller-Hinton. In the case of the outlet of HAp, bacterial viability is compromised to a greater extent than

in the case of H50p at least for *E. coli* and *Aeromonas* spp (Figures 47 and 49). As mentioned before, if bacteria aren't culturable but still detected by culture-independent methods there's the risk that they are activated under appropriate conditions.

The analysis of the ARB selected on TMP is not as straight forward as with SMX selection because *dfrA1* is less frequently the cause of TMP resistance than *sul* genes is of SMX resistance (compare Figures 35 and 42). Alternative possibilities for TMP resistance are other mechanisms or different *dfrA* alleles. Although there are less frequent as the first screening showed (Chapter 3.5), there are more than 20 *dfrA* alleles. Different *dfrA* alleles can be part of the variable region of class 1 and class 2 integrons (Labar et al., 2012). This can be further confirmed by the web-search tool INTEGRALL: http://integrall.bio.ua.pt/. By setting the search parameters integrase "1" and cassette "*dfrA"*, the several alleles for the gene can be seen in different arrangements with no apparent preference. As a consequence, the proportion carrying *intI1* was higher than the *dfrA1* frequency in TMP resistant isolates (Figures 39-41, C and D), in contrast to what was observed with *sul1* in SMX resistant bacteria (Figures 32-34, C and D). The data from this study suggested that whether *dfrA1* is part of the variable region of class 1 integron is species-dependent. Bacteria carrying *dfrA1* in a different genetic context than in class 1 integron belonged to the family *Enterobacteriaceae*. This can explain why even though there was an increase in *intI1* and *sul* genes for the first half of HAp, *dfrA1* decreased. The possible increase in *dfrA1* as an indirect consequence of the increase in class 1 integron abundance was smaller than the decrease in bacteria carrying *dfrA1* in other genetic contexts and an overall reduction in *dfrA1* abundance took place.

Colony forming units on TMP (Figures 39-41, B) for the inlet had the same order of magnitude than on SMX for all media except for PIA+TMP (Figures 32-34, B), where it was one order of magnitude lower. In this case co-selection (TMP+triclosan) could also be a possible explanation for this observation, although this hypothesis needs further investigation. A gene codifying for resistance against SMX (*sul1*) is in a conserved region of class 1 but resistance against TMP is in a variable region. In that way, the probability of co-selection is lower. An increase of CFU^{R} for the first sampling points of HAp can be seen on PIA+TMP (Figure 41, B), although *dfrA1* abundance decreases (Figure 27, D). These could be caused by the selection of those bacteria carrying class 1 integrons with different *dfrA* alleles in their variable region or due to a change in the position of *dfrA1* in the integron, which can result in a more resistant phenotype.

Viability of *Escherichia coli* and *Aeromonas* spp in H50p and HAp with treatment

Comparing qPCR results from *E. coli* (Figure 47) and *sul1* abundance (Figure 27) it can be seen that they have the same orders of magnitude. This is consistent with what was observed for *E. coli* isolates, which was proven to be a common host of the studied genes. This further confirms *E. coli* as good indicator for antibiotic resistance. Studying the physiological status of bacteria in the environment is complex from a methodological point of view. By quantifying the same bacteria by both culturable dependent and independent method we were able to observe not only that bacteria are under stress in the last portion of HAp but also that this stress is species dependant. The comparison of the values obtained through culture- dependent and independent method can indicate the viability of a particular organism or group of organisms. In the case of *E. coli*, an increased difference between the culture- dependent and independent methods could be seen for HAp, whereas this was not observed for H50p (Figure 47). For the case of *Aeromonas* spp. an absolute difference between culture- dependent and independent method that accounted for viability wasn't observed but a relative increase in the difference between culture- dependent and independent methods in HAp could be seen (Figure 47). This data was also important in order to discard that the increase in *sul1-intI1* in the first sample points of HAp was due to a clonal amplification of *Aeromonas*, which are the most frequent host of class 1 integrons.

The conditions compromising the viability can be a combination of oxidative stress and starvation. It is not clear if this difference between culture- dependent and independent method is due to dead bacteria or bacteria that are a non-culturable state but still active/viable. Furthermore, if they were no longer viable the question remains whether the ARG that they carry could be still be transferred. By qPCR 5.10^4 *dfrA1* copies could be detected (Table 9) and this corresponds with 20% of isolates that carry the gene in the outflow of H50p (Figure 42). A similar copy number of *sul2* in the outflow of HAp (Table 9) reflects in just 2% of the isolates carrying the gene (Figure 35). This is another proof on how much ARG detection is affected by culturing bias when bacterial viability is compromised. This is of relevance as *E. coli* is a common host of the studied ARG: about 44% of the isolated Enterobacteriaceae or 21% of all isolates that were host of the indicator genes were identified as *E. coli*.

Horizontal gene transfer

In Figure 52, the molecular mechanism linking O_2 to an increased activity of the integrase and it's consequence observed in this study are shown. This observation has to be done at the isolate level

(culture-dependent method). In this case, cells hosting more than one type of class 1 integron were considered.

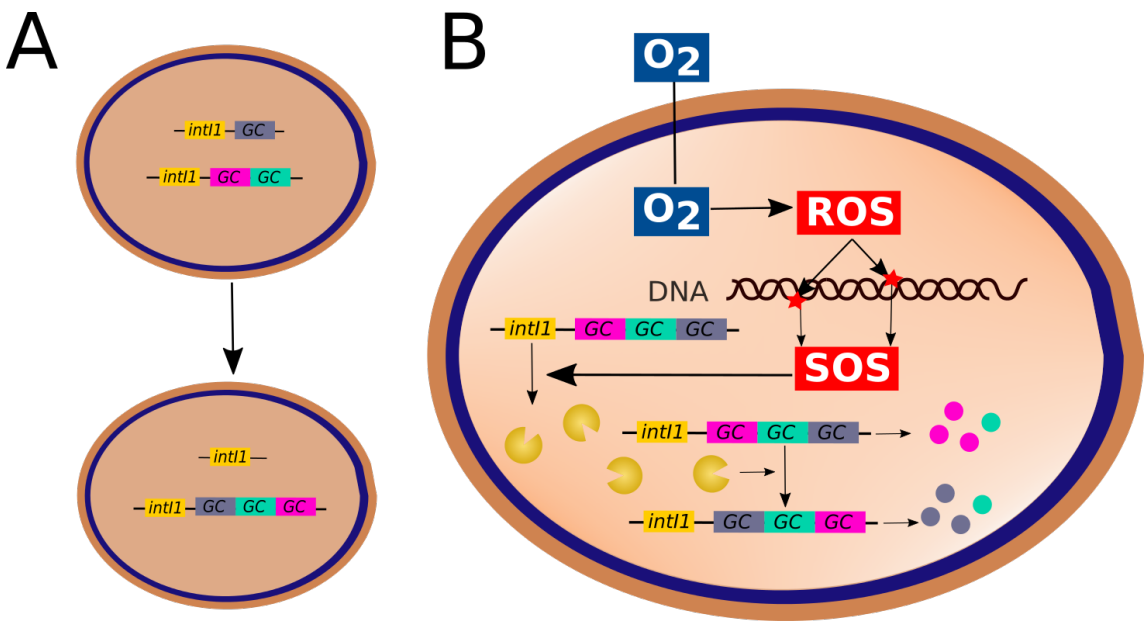


Figure 52| Molecular mechanisms linking oxidative stress with an increased diversity in class 1 integrons and the observable consequence of it. Genes are shown in rectangles and proteins in circles in the correspondent colour. **A|** The gene cassettes in cells hosting more than one class 1 integron can be recombined leading to a higher diversity of gene cassettes arrangements. Not all genes of integron type E are shown for simplicity purposes. **B|** Molecular oxygen diffuses through the cell wall and cell membrane and once inside the cell, ROS are produced. ROS in turn, cause DNA nicks which activate the SOS system. As a result, more intI1 protein copies are produced which are responsible for an increased gene cassette recombination. The order of the cassettes in the variable region are differentially transcribed and more protein are produced from the gene cassette nearer to the *intI1* gene. ROS: reactive oxygen species, GC: gene cassette. Molecular mechanisms from Guerin et al., 2009.

In the outlet for HAp, the abundance of *sul1* and *sul2* measured by qPCR (culture-independent method) were still high (Figure 27) but these genes were found seldom in resistant isolates by colony-PCR (culture-dependent method) in this study (Figure 35). This difference between the culture- dependent and independent method can result from the fact that bacteria carrying the ARG are non-culturable by the methods we used and/or by an increase in the intracellular number of ARG. A bacterium can contain multiple compatible plasmids or multiple copies of the same plasmid. The high proportion of non-culturable *E. coli* in HAp supports the first statement and it is an indicator of bacterial stress. The main stress sources in HAp are ROS and/or starvation. These conditions were already related to a higher integrase activity through the activation of the SOS response (Baharoglu and Mazel, 2011; Guerin et al., 2009). This was observed for a pure culture of *E. coli* but it's relevance in the environment is still unknown. Independently from the presence of antibiotics in the environment, once ARB are released some natural environmental conditions like a

change in DO can trigger cellular responses that lead to health risk related to antibiotic resistance. A greater integrase activity can lead to novel genetic arrangements of ARG and a higher HGT. This two phenomena combined can enhance the probability of the development of multi-drug resistant bacteria. Furthermore environmental bacteria could act as resistance reservoirs and with an enhanced HGT could transfer the ARG to pathogenic bacteria. The broader cassette diversity in *intI1* polycarriers in HAp is an evidence for an increased integrase activity which can render novel genetic arrays of ARG. Although we have recorded a considerable amount of data supporting this hypothesis, it is difficult to predict the actual risk that this posses.

A higher HGT rate was related to the activation of SOS system for an ICE called SXT (Beaber et al., 2004), which shares with class 1 integron the characteristics of codifying for a integrase and giving resistance against TMP and SMX. Although SXT is not expected to be common in the studied systems, the molecular mechanism could be similar. In Figure 53, the molecular mechanisms linking O_2 with an increased horizontal gene transfer and its effect observed in this study are shown. This was to be observed at the community level (culture-independent method). A greater cassette variety in polycarriers can be a result from a combination of the molecular mechanisms shown in Figure 52 and 53.

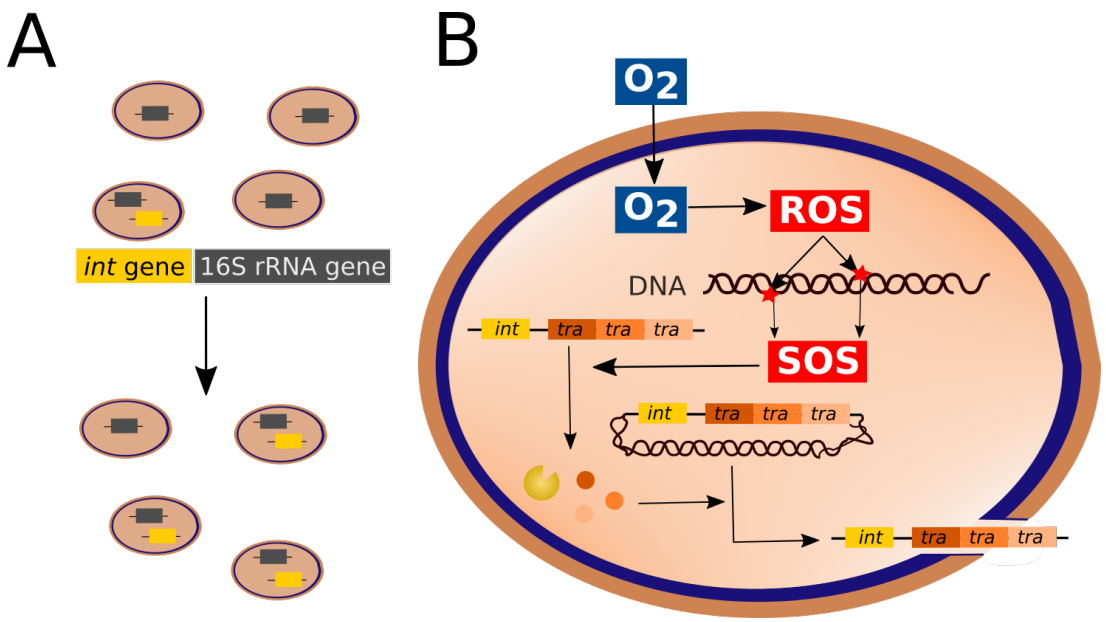


Figure 53| Molecular mechanisms linking oxidative stress with an increased horizontal gene transfer of an *int* gene on the ICE SXT and the observable consequence of it. Genes are shown in rectangles and proteins in circles. **A|** *int* copies in the community increase without an increase of the 16S rRNA gene. **B|** Molecular oxygen diffuses through the cell wall and cell membrane and once inside the cell, ROS are produced. ROS in turn, cause DNA nicks which activate the SOS system. As a consequence, the horizontal gene transfer of an *int* gene is increased by the generation of *tra* proteins. Molecular mechanisms from Beaber et al., 2004.

Furthermore, an increase in *sul2* abundance was also seen although this gene hasn't been detected in class 1 integrons. A possible explanation is that the integron class 1 and *sul2* can be on the same plasmid, as in the case of plasmid p148 (del Castillo et al., 2013). This plasmid belongs to the incA/C group, which are conjugative and broad host range. In an in vivo study by Matsushita et al. (2018) it was seen that this kind of plasmids can be transferred between *E. coli* and *A. caviae*. Furthermore, this plasmid group was identified as the closest relatives of ICE (Fricke et al., 2009).

4.2.4. Conclusion

Constructed wetlands in this study showed different internal behaviour with regard to antibiotic resistance. In H50p, a continuous decrease in ARB and ARG abundances was observed whereas the tendencies in HAp were different depending on the gene and on the fractional length. The progress of waste water treatment in HAp can be divided in two sections: from inlet to 0.50 of the fractional length and from there to the outlet. In the first fraction of HAp, substantial aerobic metabolism took place and an increase in *intI1* and *sul* genes abundances was seen. The abundance of the 16S rRNA gene remained almost constant and *dfrA1* decreased, indicating that the increase in *intI1* and *sul* abundances was specific. Furthermore, *Aeromonas* which were the most common hosts of class 1 integrons based on culture-dependent method, didn't show an increase in this fraction of the CW. Cells carrying more than one class 1 integron copy showed more diversity than in the inlet and the rest of HAp. The rest of the HAp showed a decrease in genes abundances higher than for the total length of H50p. The main stress sources in HAp are probably ROS and/or starvation and whereas oxidative stress is present for the totality of HAp, after 0.50 of its fractional length starvation might play a major role. Because of their very low concentrations and the absence of statistically significant differences between the CW, the presence of SMX/TMP in the media was unlikely the cause for the difference in resistance observed between the CW, even though a synergistic effect can't be discarded.

Oxidative stress is proposed as an important factor increasing the HGT of ARG in some wastewater treatment plants. In order to do so, it would be important to answer the following questions: are bacteria that are not detectable by culture dependent methods still viable? If they aren't viable, can their ARG still be transferred by transformation? Would epicPCR reveal a change of host during the treatment? Another question is if there is an optimal C-BOD_5/bacterial concentration/DO ratio which can minimize HGT? And if a choice had to be made on one criteria for wastewater treatment in terms of antibiotic resistance, would quantity (total abundance of ARG) matter more than the

quality (the risks associated with HGT)? It would be also important to find out the mobile genetic elements that carry integrons in the studied systems. For that a novel method called inverse PCR could could be useful (Pärnänen et al., 2016).

5. General discussion and outlook

Both HAp and H50p were effective in the reduction of ARG/ARB acting as a second step in municipal wastewater treatment. Cultured ARB were identified as members of *Enterobacteriaceae*, *Pseudomonas, Aeromonas, Enterococcaceae and Carnobacteriaceae*. In the outflows, bacterial species that are resistant against TMP/SMX through mechanisms different from the one studied in this work were more frequent than in the inlet and this was more pronounced for HAp.

Whereas aeration was beneficial for the total reduction of ARB and ARG, the first portion of HAp showed a rise in the abundance of some ARG which could be a hint of HGT of ARG. The same factors can represent stress sources or cause death, depending on a the combination with other environmental factors. The main stress sources in HAp are thought to be ROS and/or starvation. One of the resources that bacteria have in order to deal with stress is an increase conjugation to transfer genes that might be improve community's chances of survival. The observations in this study suggest that while oxidative stress at first enhanced HGT, at the end of HAp and in combination with starvation, it led to the death of some ARG hosts or at least their inactivation.

While some questions remain open, antibiotic resistance poses a present burden and a big risk not only for human health but also in economical terms. As bacteria and ARG represent a special type of contaminants that are capable of amplification and susceptible to several environmental conditions to make use of the precautionary principle, preventing ARB and ARG to end up in the environment in the first place is highly recommended. For that lowering the use of antibiotics is essential. Measures have to be implemented worldwide, and which are priority depends on the socio-economical situation of each country. Investing in hygiene, sanitation and wastewater treatment is necessary specially in the Global South (Collignon et al., 2018), and reducing the amount of antimicrobials used for the production of meat and dairy products, considering the One Health approach is also a needed in the Global North (Van Boeckel et al., 2017).

In October 2018 the European Parliament approved restrictions on the use of antimicrobials in healthy livestock which are planned to become law in 2022. The new legislation bans the use of antibiotics for animals that are important for human medicine and prohibits the use of any antimicrobials in livestock without a prescription from a veterinary. This is a big step in the right direction and others steps worldwide should follow.

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Appendices

Appendix A: Culture media composition

Mueller-Hinton

Mueller Hinton Agar is a medium very rich in nutrients that is used primarily for sensitivity testing of microorganisms.

Component	Quantity (g/L)
Beef Infusion	2.0
Corn starch	1.5
Acid Casein Peptone	17.5
Agar	17.0
pH 7.4±0.2	

Endo Agar

Endo Agar is recommended for the detection of coliform and other enteric organisms. It is used for the microbiological examination of potable water, waste water, dairy products and food.

Component	Quantity (g/L)
Peptic Digest of Animal Tissue	10.0
Lactose	10.0
Dipotassium Phosphate	3.5
Sodium Sulphite	2.5
Basic Fuchsin	0.5
Agar	15.0
pH 7.5±0.2	

Sodium sulphite and basic fuchsin have an inhibitory effect on Gram-positive microorganisms (selective). Lactose fermenting coliforms produce aldehyde and acid. The aldehyde in turn liberates fuchsin from the fuchsin-sulphite complex giving rise to red colonies. With *Escherichia coli*, this reaction is very pronounced as the fuchsin crystallizes (differential). The differential characteristic develops after 18-24 hours at 35-37 °C.

Pseudomonas isolation agar.

Component	Quantity (g/L)
Peptic digest of animal tissue	20.0
Magnesium chloride	1.4
Potassium sulfate	10.0
Triclosan (Irgasan)	0.025
Agar	13.6
pH 7.0±0.2	

Peptic digest of animal tissue provides nitrogenous compounds. Glycerol serves as carbohydrate source and promotes the phycocyanin (blue-green pigment) production (differential for *Pseudomonas Aeruginosa*). Magnesium is a cofactor for many metabolic reactions and together potassium sulfate stimulate phyocyanin production as well. Irgasan is an antibiotic and selectively inhibits Gram-positive and Gram-negative bacteria other than Pseudomonas spp (selective). Cultural characteristics are evident after 24-48 hours at 35 °C.

Aeromonas isolation agar

Aeromonas media are used for the detection of *Aeromonas* in tap water, bottled water and foods including meat, poultry, fish and seafood

Component	Quantity (g/L)
Peptone, special	5.0
Yeast extract	3.0
L-Lysine hydrochloride	3.5
L-Arginine hydrochloride	2.0
Inositol	2.5
Lactose	1.5
Sorbose	3.0
Xylose	3.8
Bile salts	3.0
Sodium thiosulfate	10.7
Sodium chloride	5.0
Ferric ammonium citrate	0.8

Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	12.5
Ampicilin	$1.125x10^{-3}$
Final pH 7.0±0.2	

Peptone special, amino acid and yeast extract provide nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex, which are essential for the growth of *Aeromonas.* Inositol, Lactose, Sorbose and Xylose are the carbohydrate substrates and sodium thiosulfate is important for the neutralisation of chlorinated water. Complex sodium thiosulfate-ferric ammonium citrate produces black -centered colonies when ferric ammonium citrate precipitates (H_2S production) (differential). The mixed indicator bromothymol blue and thymol blue changes its colour to yellow, when acid is formed. Sodium chloride is for the osmotic balance and bile salts inhibits growth of Gram-positive bacteria (selective).

Appendix B: Raw data

Physicochemical parameters

C-BOD$_5$	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	456	383	513	152	87.4	53.1	299	203	7.9	25	1.4
30/08/2016	332	400	597	115	113	43.5	135	84.4	5.1	3.6	2
08/11/2016	245	366	462	174	50.7	30	132	66.2	2.8	0.3	0
22/11/2016	298	225	237	67.6	64.4	37.3	134	45.8	3.4	1.4	1.7
09/05/2017	222	242	192	90	38	29.4	197	208	0.6	no data	no data
22/05/2017	281	143	228	86	47.5	34.5	315	189	3.1	2	no data
20/06/2017	369	203	158	100	46.9	32.8	281	127	5.9	4.5	4.2
04/07/2017	282	141	248	83.1	50.9	36.7	304	90	5.1	3.6	0.6
01/08/2017	346	149	146	141	39	41.3	324	135	9.5	6.7	5.1
Mean	**314.6**	**250.2**	**309.0**	**112.1**	**59.8**	**37.6**	**235.7**	**127.6**	**4.8**	**5.9**	**2.1**

DO (mg/L)	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	0.43	0.44	0.08	0.96	1.33	2.56	0.07	1.85	7.4	8.51	9.22
30/08/2016	0.16	0.18	0.09	0.25	0.25	0.36	0.06	0.22	7.66	7.96	9.34
08/11/2016	0.73	0.37	0.27	0.57	4.72	4.42	0.22	3.14	9.63	10.59	11.16

22/11/2016	1.12	0.88	0.43	0.49	0.62	0.72	0.22	0.49	9.79	10.33	11.84
09/05/2017	0.55	0.82	0.52	0.65	1.15	1.14	0.35	0.17	9.37	10.5	11.15
22/05/2017	0.61	1.41	0.99	1.65	1.64	1	0.85	1.16	10.54	11.63	11.12
20/06/2017	0.7	0.86	0.77	0.74	0.79	4.06	0.19	0.9	7.32	8.36	8.68
04/07/2017	0.55	0.78	0.43	0.96	0.89	2.25	0.11	2.64	8.32	8.66	8.75
01/08/2017	0.9	0.9	0.68	0.45	0.79	3.86	0.09	2.21	7.1	8.08	8.47
Mean	**0.64**	**0.74**	**0.47**	**0.75**	**0.93**	**2.26**	**0.24**	**1.42**	**8.57**	**9.40**	**9.97**

HAp												
	NH_4^+ (mg/L)						**NO_3^- (mg/L)**					
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
04/09/2014	84.33	71.04	41.49	0.04	0.04	0.05	<LOD	0.07	12.22	34.38	31.99	29.98
09/09/2014	71.10	68.70	48.26	0.06	0.05	0.05	<LOD	<LOD	11.00	38.61	35.94	35.49
07/10/2014	100.24	81.54	no data	0.02	0.07	0.05	<LOD	<LOD	no data	42.58	48.66	41.63
19/05/2015	77.17	83.02	79.02	2.43	0.07	<LOD	<LOD	0.09	0.32	52.07	51.57	50.17
26/05/2015	81.41	80.46	79.43	0.71	<LOD	0.04	<LOD	<LOD	0.16	50.26	50.40	50.62
02/06/2015	77.30	79.08	74.17	0.08	<LOD	<LOD	<LOD	0.07	0.32	52.07	no data	53.00
09/06/2015	75.55	79.47	74.06	0.18	0.05	0.03	<LOD	0.07	0.29	48.68	51.30	49.52
23/06/2015	66.40	67.45	64.98	0.03	<LOD	0.05	<LOD	<LOD	0.11	42.38	38.79	38.31
30/06/2015	74.95	72.34	67.72	<LOD	<LOD	<LOD	<LOD	<LOD	0.07	41.32	41.93	39.85
Mean	78.72	75.90	66.14	0.45	0.05	0.04	<LOD	0.07	3.06	44.70	43.82	43.17

SMX (ng/L)	inlet	H50p					HAp				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	16	12	8	25	34	4	9	1	463	23	47
14/08/2016	159	172	143	62	6	8	212	216	174	223	264
07/11/2016	47	45	38	33	33	29	18	15	17	39	66
22/05/2017	24	11	11	12	11	109	23	14	25	53	7
20/06/2017	66	30	35	17	34	2	64	36	12	29	10
01/08/2017	127	42	42	19	27	10	80	53	19	47	41
Median	56.5	36	36.5	22	30	9	43.5	25.5	22	43	44

N^4-Ac-SMX	inlet	H50p					HAp				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	27.5	9.7	6.7	11.3	<LOD	<LOD	16.4	14.3	22.0	11.9	12.8
14/08/2016	657.1	77.3	39.9	26.6	6.7	5.4	187.7	343.4	130.9	40.8	7.3
07/11/2016	40.7	1.8	6.6	1.9	9.7	1.5	24.6	8.8	10.8	3.6	5.9
22/05/2017	49.8	19.7	9.6	6.8	1.1	2.9	9.9	9.1	8.79	3.7	0.5
20/06/2017	96.3	40.1	17.6	2.9	<LOD	2.6	24.3	20.5	4.8	BLD	1.2
01/08/2017	95.4	35.6	15.8	0.8	<LOD	<LOD	19.2	18.4	6.7	12.5	3.5
Median	**72.6**	**27.6**	**12.7**	**4.8**	**6.7**	**2.8**	**21.8**	**16.3**	**9.8**	**11.9**	**4.7**

< LOD: limit of detection

TMP (ng/L)	inlet	H50p					HAp				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	8.5	4.4	2.1	2.2	1.3	0.9	8.4	4.5	5.7	153.7	3.1
14/08/2016	20.2	3.8	1.8	12.1	31.4	6.8	5.8	4.3	2.3	2.1	7.4
07/11/2016	61.3	<LOD	0.8	<LOD	9.0	0.6	2.6	2.4	0.5	<LOD	0.7
22/05/2017	4.3	1.6	<LOD	2.4	0.9	1.2	1.2	0.6	0.61	0.5	0.5
20/06/2017	1.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
01/08/2017	3.9	2.2	1.1	0.8	0.7	0.5	1.9	1.2	1.3	1.2	0.9
Median	6.4	3.0	1.5	2.3	1.3	0.9	2.6	2.4	1.3	1.7	0.9

Gene abundances

***intI1* total (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
05/2014	7.19E+07	1.42E+08	3.97E+07	no data	1.68E+07	4.60E+08	2.39E+08	9.43E+08	4.33E+08	7.57E+07	1.65E+07
06/2014	7.06E+05	7.81E+07	7.20E+07	2.41E+07	2.91E+07	1.56E+07	1.58E+08	1.67E+08	8.54E+07	1.34E+08	2.52E+06
07/2014	4.95E+07	5.97E+07	2.66E+07	2.58E+07	9.42E+06	4.86E+06	1.96E+08	3.93E+08	4.88E+08	1.14E+07	1.11E+05
09/2014	1.01E+08	1.06E+08	3.21E+07	3.76E+07	2.69E+07	2.48E+05	1.08E+09	no data	4.56E+07	2.77E+07	2.56E+05
10/2014	7.83E+07	2.87E+08	3.94E+07	2.61E+07	9.87E+07	5.60E+06	2.25E+08	2.13E+08	4.32E+06	1.19E+07	3.36E+05
06/2015	1.52E+08	4.88E+07	1.86E+07	4.02E+06	1.57E+06	no data	1.23E+08	2.99E+08	2.61E+06	1.05E+06	3.41E+04
07/2015	1.18E+08	1.15E+08	7.99E+07	1.38E+07	7.34E+06	3.47E+06	5.92E+08	4.24E+08	1.60E+07	2.94E+06	1.26E+05
08/2015	1.04E+08	8.51E+07	1.04E+08	1.33E+07	9.37E+06	3.97E+06	2.88E+08	1.73E+08	1.53E+07	7.90E+06	5.04E+04

12/2015	3.26E+08	2.54E+08	1.72E+08	1.53E+07	3.28E+07	2.71E+08	6.67E+08	1.26E+09	1.15E+08	1.42E+07	3.10E+05
10/05/2017	6.12E+07	2.88E+07	2.10E+08	1.06E+07	2.50E+06	1.79E+06	1.01E+08	2.84E+08	7.37E+05	6.45E+05	6.07E+04
22/05/2017	3.79E+07	1.23E+07	1.10E+08	6.20E+06	3.30E+06	2.83E+06	3.24E+08	3.31E+08	7.74E+05	9.59E+05	7.39E+03
Median	**7.83E+07**	**8.51E+0**	**7.20E+07**	**1.45E+07**	**9.42E+06**	**4.42E+06**	**2.39E+08**	**3.15E+08**	**1.60E+07**	**1.14E+07**	**1.26E+05**

***intI1* clinical (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
06/2014	1.09E+09	6.54E+08	5.04E+08	1.09E+08	2.64E+08	8.77E+07	1.08E+09	no data	7.61E+08	5.78E+08	1.79E+07
07/2014	2.72E+08	3.67E+08	1.28E+08	1.60E+08	6.26E+07	2.44E+07	1.17E+09	1.36E+09	2.61E+09	1.15E+08	5.23E+05
09/2014	3.82E+08	6.24E+08	2.20E+08	2.44E+08	1.79E+08	1.25E+06	4.69E+09	no data	6.30E+08	2.86E+08	1.79E+06
07/2015	1.23E+09	1.10E+09	7.30E+08	1.70E+08	7.43E+07	2.83E+07	4.37E+09	3.42E+09	1.87E+08	5.72E+07	4.11E+06
08/2015	no data	4.91E+08	1.23E+09	2.05E+08	1.82E+08	7.86E+07	2.26E+09	2.42E+09	2.42E+08	2.58E+08	3.16E+06
05/2017	8.99E+08	4.33E+08	3.30E+09	1.45E+08	1.18E+08	1.05E+08	7.18E+09	6.81E+09	6.83E+07	4.37E+07	1.08E+06
Median	**8.99E+08**	**5.57E+08**	**6.17E+08**	**1.65E+08**	**1.48E+08**	**5.35E+07**	**3.31E+09**	**2.92E+09**	**4.36E+08**	**1.86E+08**	**2.48E+06**

***sul1* (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
05/2014	4.45E+08	1.30E+09	5.23E+08	1.02E+06	8.11E+07	9.54E+08	1.13E+09	4.88E+09	2.01E+09	6.10E+08	4.41E+08
06/2014	1.05E+09	5.36E+08	8.33E+08	5.25E+08	1.03E+08	2.13E+07	1.33E+09	2.79E+09	2.30E+08	6.03E+08	2.35E+06
07/2014	1.84E+08	3.68E+08	1.83E+08	3.74E+08	5.84E+07	3.21E+07	1.62E+09	3.65E+09	3.01E+09	7.37E+07	1.77E+06
09/2014	1.99E+08	4.37E+08	9.51E+07	2.52E+08	5.74E+07	6.93E+05	3.84E+09	6.81E+05	1.95E+08	1.35E+08	5.77E+05

10/2014	7.33E+08	2.64E+09	4.66E+08	4.27E+08	5.66E+08	6.97E+07	4.36E+09	2.44E+09	8.98E+07	1.96E+08	1.06E+07
06/2015	9.29E+08	3.66E+08	1.31E+08	4.14E+07	7.61E+06	3.57E+05	6.77E+08	1.98E+09	1.42E+07	9.42E+06	2.81E+05
07/2015	1.67E+09	1.90E+09	1.32E+09	3.40E+08	9.86E+07	4.72E+07	8.42E+09	5.76E+09	2.61E+08	1.64E+08	4.03E+06
08/2015	1.36E+09	1.69E+09	1.64E+09	2.26E+08	1.73E+08	9.32E+07	8.06E+09	2.70E+09	2.59E+08	1.86E+08	1.28E+06
12/2015	1.36E+08	2.04E+08	1.24E+08	1.49E+07	1.45E+07	1.66E+06	2.77E+08	6.19E+08	5.03E+07	2.09E+07	1.21E+05
10/05/2017	5.57E+07	2.68E+07	8.87E+07	1.03E+07	2.76E+06	9.84E+05	1.21E+08	2.21E+08	1.42E+06	1.57E+06	1.84E+05
22/05/2017	4.72E+07	1.27E+07	1.49E+08	6.66E+06	4.00E+06	1.81E+06	3.99E+08	4.26E+08	2.92E+06	1.69E+06	2.56E+04
Median	**4.45E+08**	**4.37E+08**	**1.83E+08**	**2.26E+08**	**5.84E+07**	**2.13E+07**	**4.37E+08**	**1.83E+08**	**2.26E+08**	**5.84E+07**	**2.13E+07**

***sul2* (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
05/2014	1.06E+07	3.28E+07	1.38E+07	0.00E+00	2.23E+06	8.12E+07	2.88E+07	1.79E+08	1.27E+08	6.52E+07	1.36E+07
06/2014	8.37E+07	2.99E+07	5.01E+07	4.88E+07	8.65E+06	1.96E+06	4.27E+07	2.43E+08	4.64E+07	1.16E+08	2.22E+06
07/2014	5.31E+06	1.54E+07	6.51E+06	7.46E+06	1.45E+06	5.98E+05	5.25E+07	2.00E+08	1.33E+08	7.28E+06	4.92E+04
09/2014	1.64E+07	2.47E+07	5.42E+06	1.25E+07	5.82E+06	5.89E+04	2.87E+08	6.89E+04	1.88E+07	1.41E+07	no data
10/2014	5.15E+07	6.77E+07	1.58E+07	1.16E+07	6.75E+06	5.20E+06	1.78E+08	9.95E+07	5.88E+06	1.37E+07	1.66E+06
06/2015	1.33E+08	7.33E+07	3.09E+07	6.62E+06	3.31E+06	1.80E+05	1.36E+08	2.70E+08	1.04E+07	5.07E+06	3.04E+05
07/2015	6.36E+07	4.64E+07	3.62E+07	9.35E+06	2.59E+06	1.21E+06	2.24E+08	2.10E+08	2.62E+07	1.38E+07	3.16E+05
08/2015	6.87E+07	4.52E+07	5.44E+07	1.05E+07	6.91E+06	1.71E+06	3.21E+08	1.28E+08	2.60E+07	2.63E+07	1.09E+05
12/2015	3.86E+08	2.24E+08	1.19E+08	9.22E+07	3.92E+07	1.61E+07	1.88E+08	4.94E+08	4.79E+07	6.47E+07	1.36E+06
10/05/2017	6.23E+06	3.04E+06	4.02E+06	7.99E+05	3.25E+05	9.57E+04	4.78E+06	7.44E+06	5.10E+05	4.38E+05	4.06E+04

22/05/2017	3.58E+06	1.23E+06	8.94E+06	3.58E+05	2.85E+05	6.47E+04	8.71E+06	1.03E+07	8.87E+05	6.21E+05	5.27E+03
Median	**5.15E+07**	**3.28E+07**	**1.58E+07**	**9.35E+06**	**3.31E+06**	**1.21E+06**	**1.36E+08**	**1.79E+08**	**2.60E+07**	**1.38E+07**	**3.10E+05**

***dfrA1* (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
05/2014	6.24E+05	1.12E+06	2.61E+05	7.78E+04	9.10E+04	1.19E+05	3.54E+05	2.78E+05	1.18E+05	5.20E+04	9.32E+04
06/2014	1.63E+04	6.64E+05	3.57E+05	1.04E+05	3.88E+05	7.22E+04	4.58E+05	8.74E+04	3.59E+04	3.29E+04	6.65E+04
07/2014	7.58E+05	3.89E+05	2.46E+05	2.89E+05	4.71E+04	2.90E+04	1.67E+05	9.86E+04	1.32E+05	3.16E+04	1.46E+05
09/2014	6.13E+06	1.41E+06	3.23E+05	4.75E+05	5.98E+05	8.94E+03	2.10E+06	1.22E+04	3.74E+04	1.47E+04	1.37E+04
10/2014	5.10E+06	1.68E+06	3.41E+05	1.78E+05	3.36E+05	1.89E+05	1.33E+05	4.98E+05	1.84E+04	2.05E+04	1.69E+04
06/2015	2.97E+06	5.08E+06	1.61E+06	3.24E+05	6.07E+04	8.27E+03	1.25E+07	3.58E+06	0.00E+00	1.71E+04	9.05E+03
07/2015	1.17E+07	5.62E+06	2.13E+06	1.05E+06	3.77E+05	9.27E+04	6.32E+06	2.66E+06	6.12E+04	8.96E+04	1.19E+04
08/2015	2.35E+07	8.30E+06	4.37E+06	2.73E+06	1.54E+06	1.70E+05	6.28E+05	6.28E+06	1.15E+05	1.54E+05	1.86E+04
10/05/2017	3.71E+06	4.07E+05	6.59E+05	2.00E+05	6.26E+04	1.70E+04	8.47E+05	3.77E+05	3.09E+03	5.29E+03	6.03E+02
22/05/2017	1.11E+06	9.35E+04	9.20E+04	9.19E+04	3.81E+04	1.80E+04	1.55E+06	6.17E+05	2.15E+03	2.40E+03	3.24E+02
Median	**3.34E+06**	**1.26E+06**	**3.49E+05**	**2.45E+05**	**2.13E+05**	**5.06E+04**	**7.38E+05**	**4.38E+05**	**3.67E+04**	**2.60E+04**	**1.53E+04**

Escherichia coli

***uidA* (gene copies/100 mL)**	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
05/2014	2.29E+08	1.36E+07	9.90E+06	3.92E+06	6.20E+06	7.62E+06	1.73E+07	3.33E+07	3.26E+07	6.52E+06	6.48E+06
06/2014	2.14E+09	1.55E+07	1.97E+07	1.25E+07	5.11E+06	8.35E+07	9.72E+06	1.01E+07	1.50E+06	1.07E+06	6.03E+05
07/2014	2.06E+08	1.75E+07	1.66E+07	1.82E+07	6.65E+06	2.53E+07	1.99E+007	5.15E+007	3.91E+007	9.20E+006	5.30E+006
09/2014	3.20E+08	1.55E+08	4.37E+07	5.69E+07	7.26E+07	8.05E+06	1.41E+009	6.51E+008	1.07E+008	5.51E+007	5.23E+006
10/2014	9.17E+08	6.11E+08	1.75E+08	1.56E+08	2.18E+08	8.60E+07	4.55E+08	2.06E+09	1.22E+08	2.02E+08	3.81E+07
07/2015	1.99E+07	2.07E+07	7.28E+06	3.38E+06	1.02E+06	2.85E+05	1.58E+07	5.06E+06	0.00E+00	0.00E+00	0.00E+00
08/2015	1.65E+07	6.97E+06	4.55E+06	9.89E+05	4.29E+05	1.86E+04	1.25E+06	6.36E+06	no data	no data	no data
12/2015	2.10E+07	1.68E+07	no data	8.18E+06	1.46E+05	5.56E+05	8.73E+06	1.08E+07	3.89E+06	0.00E+00	0.00E+00
10/05/2017	1.07E+08	1.81E+07	4.57E+07	7.82E+06	1.94E+06	7.22E+05	3.60E+07	2.22E+07	8.38E+04	1.43E+05	2.62E+04
22/05/2017	6.24E+07	4.92E+06	1.60E+07	9.44E+06	3.20E+06	6.77E+05	4.56E+07	3.23E+07	1.75E+05	1.51E+05	7.45E+03
20/6/2017	3.60E+07	4.77E+06	6.03E+06	2.59E+06	8.46E+05	2.02E+05	1.27E+07	8.23E+06	1.63E+05	2.85E+05	1.67E+04
04/07/2017	1.08E+07	7.53E+06	2.93E+06	4.01E+06	7.32E+05	3.87E+05	8.46E+06	6.39E+06	1.64E+05	1.91E+05	1.58E+04
Median	**8.46E+07**	**1.61E+07**	**1.60E+07**	**8.00E+06**	**2.57E+06**	**7.00E+05**	**1.66E+07**	**2.22E+07**	**1.50E+06**	**2.85E+05**	**2.62E+04**

MPN (MPN/100 mL)	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
09/08/2016	6.49E+06	3.26E+06	3.87E+06	2.10E+06	2.42E+06	1.42E+05	6.13E+02	1.67E+02	3.55E+01	4.88E+01	3.65E+02

30/08/2016	1.20E+07	5.17E+06	8.16E+06	4.61E+06	5.17E+05	3.08E+05	6.87E+06	4.61E+06	2.25E+04	1.94E+03	4.88E+02
08/11/2016	2.28E+06	1.26E+06	1.16E+06	4.65E+05	1.72E+05	2.48E+05	2.14E+06	5.04E+05	2.01E+03	3.09E+02	1.44E+02
22/11/2016	2.06E+06	1.21E+06	1.21E+06	4.48E+05	1.85E+05	1.41E+05	1.42E+06	4.37E+05	2.21E+03	4.71E+02	5.56E+02
09/05/2017	7.70E+06	3.45E+06	3.26E+06	1.50E+06	3.87E+05	2.10E+05	4.88E+06	2.91E+06	1.97E+03	3.01E+02	5.65E+02
22/05/2017	5.17E+06	8.62E+05	9.09E+05	4.39E+05	1.94E+05	5.91E+04	4.11E+06	2.28E+06	1.89E+03	1.00E+01	5.83E+02
20/06/2017	8.16E+06	2.25E+06	1.31E+06	5.81E+05	3.26E+05	3.99E+04	4.11E+06	1.39E+06	8.66E+03	6.13E+02	4.95E+02
04/07/2017	9.59E+05	4.10E+05	4.22E+05	2.75E+05	1.50E+05	8.36E+04	6.63E+05	2.59E+05	1.66E+03	3.87E+02	1.46E+02
01/08/2017	1.66E+06	5.73E+05	4.87E+05	1.60E+05	1.79E+05	4.52E+04	1.31E+06	5.94E+05	4.20E+02	no data	no data
Median	**5.17E+06**	**1.26E+06**	**1.21E+06**	**4.65E+05**	**1.94E+05**	**1.41E+05**	**2.14E+06**	**5.94E+05**	**1.97E+03**	**3.48E+02**	**4.92E+02**

CFU coli (CFU/100 mL)	**inlet**	**H50p**					**HAp**				
Date	**0.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**	**0.12**	**0.25**	**0.50**	**0.75**	**1.00**
22/05/2017	3.73E+06	3.60E+06	1.17E+06	7.28E+05	5.05E+05	1.33E+05	3.00E+05	2.80E+05	7.50E+03	0.00E+00	5.00E+02
20/06/2017	4.00E+05	5.25E+04	2.65E+05	9.25E+04	5.00E+04	7.50E+03	3.00E+05	3.35E+05	2.50E+02	2.50E+02	5.25E+03
04/07/2017	1.70E+06	1.13E+06	3.13E+05	5.60E+05	1.78E+05	7.50E+03	1.58E+06	2.63E+05	7.25E+03	1.00E+03	0.00E+00
01/08/2017	1.00E+05	2.88E+05	1.83E+05	1.63E+05	9.50E+04	6.00E+04	4.25E+05	5.50E+04	0.00E+00	0.00E+00	0.00E+00
Median	**1.05E+06**	**7.08E+05**	**2.89E+05**	**3.61E+05**	**1.36E+05**	**3.38E+04**	**3.63E+05**	**2.71E+05**	**3.75E+03**	**1.25E+02**	**2.50E+02**

Appendix C: Cassettes sequences

Cassette A:

>MH_SMX_108
GTtanGgagCagCACGATGTTACGCAGCAGGgCagTCGCCCTAAAACAAAGTTAGATGCACTAAGCACATAAT
TGCTCACaGCCAAACTATCAGGTCAAGT

Cassette B:

>S11
CGCGTCATATGTGCTTAGTGCATCTACGCTTGAGTTAAGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATT
GTTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAACTGATCTG
CGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAAGTATGACGGGCTGATACTG
GGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTAC
CAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTCGGGCGGCGAGTTCCATAGCGTTA
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CAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGATCAATGTCGATCGTGGCTGGCTCGAAG
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GATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCGGCGATCACCGCTTCCCTCATGAT
GTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACATCGACCC
ACGGCGTACGCTGGCCCCTGGCGGGAACACAAACAAAAAAAAGGCGGGGGCTTGTTG

Cassette C:

>S21

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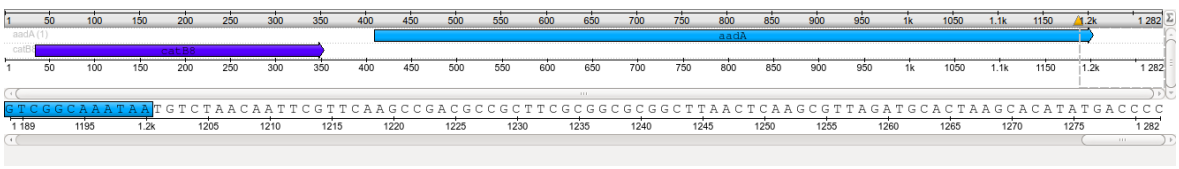

Cassette D:

>MH SMX 124 (2273 bp)

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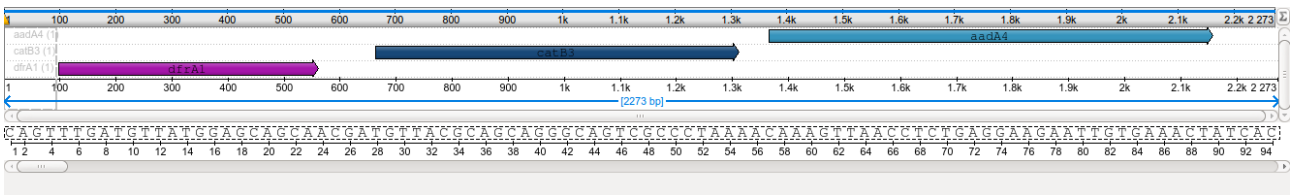

Cassette E:

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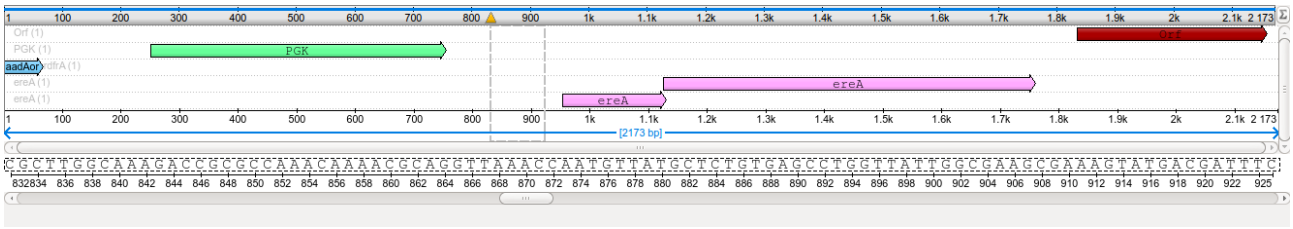

Curriculum vitae

Oral presentations

C. Knecht, M. Krüger, H. Dambeck, J. Nickolaus, A. Ahmed, S. Kellmann, I. Mäusezahl, M. Möder, M. Kästner, O. O. Adelowo, J. Nivala, J. A. Müller. Fate of antibiotic resistant bacteria in two different constructed wetland types. VAAM Annual Meeting • 69th Annual Meeting of the DGHM Wurzburg, Germany from March the 5th to 8th, 2017.

Poster presentations

Knecht C., Dambeck, H., Krüger, M., Nikolaus, J., Ahmed, A., Kellman, S., Mäusezahl, I., Kästner, M., Adelowo, O.O., Nivala, J., Müller, J. A. Fate of antibiotic resistant bacteria in two different constructed wetland types. VAAM Annual Meeting, Jena, Germany from March the 13th to 16th, 2016.

C. Knecht, M. Krüger, A. Buchholz, H. Dambeck, J. Nickolaus, A. Ahmed, S., Kellmann, I. Mäusezahl, M. Möder, M. Kästner, O. O. Adelowo, J.Nivala, J. A. Müller. Fate of antibiotic resistant bacteria in two different constructed wetland types, 17th International Symposium on Microbial Ecology, Leipzig, Germany from August the 12th to 17th, 2018.

Camila Knecht, Heinz Köser, Jochen A. Müller. Bioremediation von Antibiotika-resistenten Bakterien und Antibiotika-Resistenzgenen in Pflanzenkläranlagen, Spurenstoffe und Krankheitserreger im Wasserkreislauf, Frankfurt, Deutschland, von dem 23. zu dem 24. Oktober, 2018.

Camila Knecht, Markus Krüger, Arif Ahmed, Simon Kellmann, Ines Mäusezahl, Monika Möder, Heinz Köser, Olawale. O. Adelowo, Jaime Nivala, Jochen A. Müller. Enhanced horizontal transfer of antibiotic resistance genes in an aerated constructed wetland. 8th International Symposium on Wetland Pollutant Dynamics, Aarhus, Denmark from June the 17th to 21st, 2018.

Distinctions

17th International Symposium on Microbial Ecology, Poster award.

Scholarship from the Heinrich Böll Foundation (2015-2018)

Published manuscripts

Camila Knecht, Cecilia L. Balaban, Joaquín V. Rodríguez, Eduardo A. Ceccarelli, Edgardo E. Guibert, Germán L. Rosano (2018). Proteome variation of the rat liver after simple cold storage assayed in an ex vivo model, Cryobiology 85, 47-55.

Adelowo, O.O., Helbig, T., Knecht, C., Reincke, F., Mäusezahl, I., Müller J. A. (2018). High abundances of class 1 integrase and sulfonamide resistance genes, and characterisation of class 1 integron gene cassettes in four urban wetlands in Nigeria. PLoS ONE 13.

Manuscript in preparation

Camila Knecht, Markus Krüger, Simon Kellmann, Arif Ahmed, Ines Mäusezahl, Monika Möder, Olawale O. Adelowo, Heinz Köser, Jaime Nivala, Jochen A. Müller. Oxidative stress enhances horizontal transfer of antibiotic resistance genes during wastewater treatment.

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10/2015 - today	**PhD** at the Otto-von-Guericke University, Magdeburg, Germany. Faculty of Process & Systems Engineering Field: Waste water treatment
10/2015 – today	**Member** of the Helmholtz Interdisciplinary GRADuate School for Environmental Research (HIGRADE)
03/2006 - 06/2014	**Master in biotechnology** at the National University of Rosario, Argentina Faculty of Biochemical and Pharmaceutical sciences. Master thesis: “Proteomic analysis of livers subjected to injuries due to hypoxia/reperfusion.”

Languages

Spanish	Mother tongue
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