UNIVERSITY OF INSUBRIA

Could *Aeromonas* spp. be considered a vector of resistance genes from the environment to human intestinal flora?

Ph.D. School:
Biological and Medical Sciences

Ph.D. Program in:
"ANALYSIS, PROTECTION AND MANAGEMENT OF BIODIVERSITY"
-XXVI Course-

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December 2013

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Antibiotic resistant bacteria and antibiotic resistance genes cause increasing problems in clinical setting and are considered emerging environmental contaminants. In the environment, antibiotic resistant bacteria may be inactivated but their resistance determinants may become part of the environmental gene pool and may move back to human and animal intestinal flora via food and drinking water.

In order to assess the role of *Aeromonas* spp. in the flow of resistant determinants, we analyzed by resistance profiles, plasmidic and integrons content and characterization, 231 *Aeromonas* spp. and 250 Fecal Coliforms isolated from different aquatic environments. Resistance patterns could be related to both the presence of multiple plasmids and to the origin of the strains. The 31.7% of the plasmids carried by the *Aeromonas* strains were mobilizable as assess by dot-blot of MOB-subfamily covering the BHR (Broad Host Range) incompatibility groups. The majority (75%) of the Class 1 integrons were chromosomally locate and were found in the 27.7% of the *Aeromonas* spp. tested.

The number and type of resistance genes identified so far on *Aeromonas* spp. support the hypothesis of considering these bacteria as a "reservoir" of resistance genes.

The capability of some *Aeromonas* strains to transfer their resistance to Fecal Coliform has been demonstrated, thus allowing to assert its role as a “vector” of resistance genes to members of the human intestinal flora.

Keywords: Aquatic environments; bacteria; *Aeromonas* spp.; Fecal Coliforms; plasmids; antibiotic resistance genes; reservoir; conjugation; vector.

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1 INTRODUCTION

1.1 Aquatic Environments – antibiotics and reservoirs of resistance determinants

Freshwater environment includes groundwater and surface freshwater. Groundwater holds low amount of nutrients but is rich in mineral content due to rocks erosion, while surface water covering lakes, rivers and canal water, carries a diverse range of flora and fauna and manifold physical and chemical conditions (**Holmes et al., 1996**). In Europe the fresh waters are subject of a framework for the protection and the improvement of the ecological status by the European Water Framework Directive 2000/60/CE. The directive focuses on the evaluation of the ecological effects, biological communities and multiple anthropogenic stressors (**European Community, 2000**).

Aquatic ecosystems are exposed to a broad range of natural stressor elements, such as temperature and oxygen depletion acting on individuals, populations and communities, as well as to a wide spectrum of different product categories (micro-pollutants) released in the environment. The micro-pollutants could include plant protection products, biocides, drugs and components of consumers goods (e.g. body creams, detergents) (**UFAM, 2009**) originating from municipal and industrial wastewater as well as from urban and agricultural areas (**Connon et al., 2012**).

In recent years, the pharmacological treatment of human infections caused by pathogenic bacteria has become difficult due to their increasing resistance, giving rise to a further increase in the use of antibiotics and in the bacterial resistance. Nowadays, among the micro-pollutants of the aquatic environment there are also antibiotics used in human and veterinary medicine and multiresistant pathogenic bacteria. The contamination by human and veterinary antibiotics was found in studies conducted either on wastewater treatment plants than on watersheds; generally the concentration was less than 1μg/L. Veterinary antibiotics are introduced in the environment because they are used as fertilizer or for veterinary purpose. Antibiotics are found in soils and can reach basins and rivers by the rainfalls (**Kim et al., 2010; Campagnolo et al., 2002**).

The antibiotic concentrations tend to increase along the rivers due to the polluted incoming water. Even when antibiotic concentrations are sub-therapeutic, there is a high probability of resistant bacteria development. (**Zuccato et al., 2010**).

The presence of pollutants, antimicrobial resistant bacteria and resistant determinants in the aquatic systems enables and facilitates the horizontal gene transfer (HGT) between bacteria. The interactions among non-pathogenic environmental and pathogenic human/animal bacteria can lead to the establishment of resistance in clinical relevant isolates (Figure 1.1.1) (**Taylor et al., 2011**).

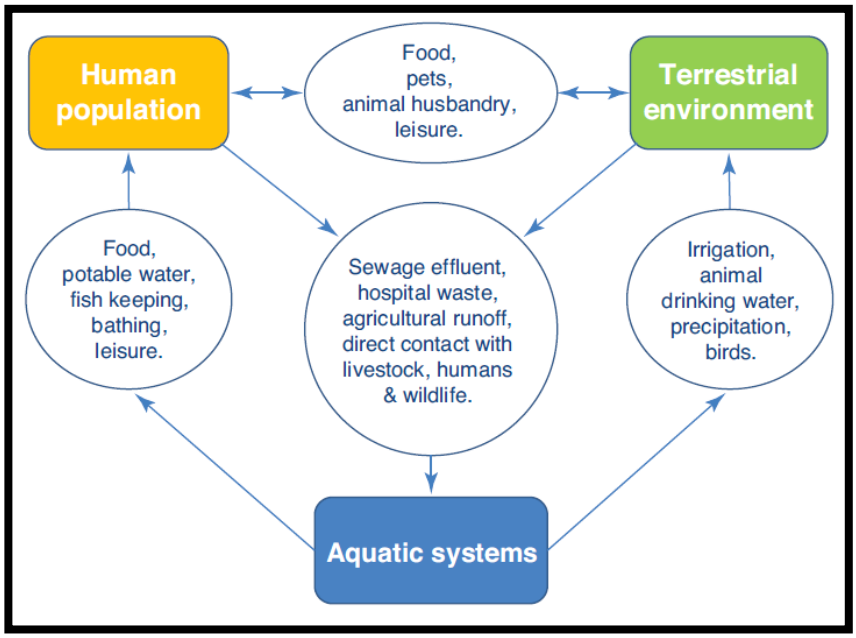


Figure 1.1.1: **Contact flow between antimicrobial-resistant bacteria and resistant genes through terrestrial environment, aquatic system and humans. (From Taylor et al., 2011)**

Bacteria have different methods to adapt to stressful conditions in their environments, either in nature or in human beings. The mechanisms allowing a survival increasing are molecular adaptations and acquisition of exogenous DNA by horizontal gene transfer, thus leading to the bacterial evolution (**Moura et al., 2010**).

Bacteria have spontaneously developed five methods to bypass the impact of small bio-molecules or bioactive compounds (Figure 1.1.2): (i) exclusion by the membrane; (ii) intracellular modification/deactivation of the antimicrobial compound; (iii) extrusion; (iv) reduction of the target sensitivity; and (v) intracellular sequestration. These survival mechanisms can be acquired by different pattern: (a) transduction via the action of viruses; (b)

transformation by the uptake of foreign DNA; (c) conjugation by the interaction of two bacteria (**Taylor et al., 2011**).

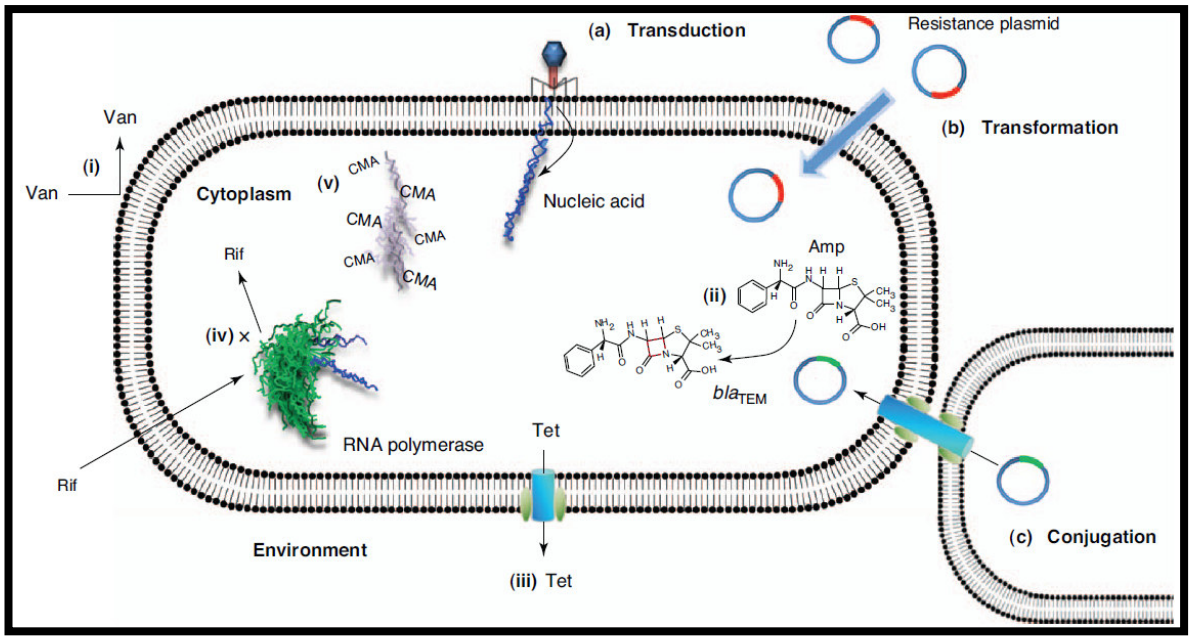


Figure 1.1.2: Horizontal Gene Transfer (HGT) and Antimicrobial Resistance in bacteria (AMR): (i) exclusion by the membrane; (ii) intracellular modification and/or deactivation of the antimicrobial; (iii) extrusion; (iv) reduction in sensitivity of the cellular target; and (v) intracellular sequestration. Exogenous DNA can be obtain by (a) viruses –Transduction-, (b) environment –Transformation-, (c) other bacteria –Conjugation-. Antibiotics: Amp, ampicillin; CMA, coumermycin; Rif, rifampicin; Tet, tetracycline; Van, vancomycin. (**From Taylor et al., 2011**)

The dissemination of antimicrobial resistance genes is supported by many bacteria which encode resistant and other accessory genes on broad-host range (BHR) plasmids. Plasmids holding clinical relevant resistant genes were isolated from effluents of wastewater treatment plants, thus promoting the environmental spread of resistant determinants (**Rahube et al., 2010**). The process of conjugation is believed to be the main pathway for the horizontal gene transfer among bacteria as conjugative plasmids can be transferred between different genera or domains. In addition to the resistance plasmids, there are other genetic structures involved in the acquisition and/or expression of resistant genes such as the integrons, which can be mobilized, and the transposons (**Moura et al., 2012 –a–**).

The resistance gene flow between the natural environment and the human microbial flora, has to be considered bidirectional. Therefore, any selective pressure acting in the environment can increase the danger to the human health through the enhancement of the resistant determinant transfer among bacteria (**Cabello et al., 2013**).

Two environmental bacteria groups with mobile genetic elements are aeromonads and enterobacteria, respectively found in aquatic environment and common residents of polluted

water (**Moura et al., 2012 –b–**). Aeromonads and enterobacteria can carry plasmids harboring multiple antibiotic resistant determinants of clinical and environmental concern.

1.2 *Aeromonas* spp.

1.2.1 Identification and taxonomy

Aeromonas belong to the bacteria domain, proteobacteria kingdom, phylum gamma-proteobacteria, class *Aeromonadales*, and genus *Aeromonas*. Aeromonads are gram-negative bacilli, oxidase-positive, facultative anaerobic, glucose fermenting bacteria, autochthonous of the aquatic environments.

Aeromonads are natural inhabitants of water environments and have been isolated from brackish, fresh, estuarine, marine, chlorinated and un-chlorinated water worldwide; their density is higher in the warmer months. Strains were isolated also from cold and warm-blood animals, in which they can cause diseases. The first isolate from a human date from the early 1950s. The pathologies caused by aeromonads affect both compromised and immunocompromised human hosts. Aeromonads can be classified in two major groups based on their growth characteristics and biochemical features: the mesophilic group, including *Aeromonas* species able to grow well at 35 to 37°C, motile, which can be associated to human pathologies, and the psychrophilic, composed by strains showing an optimal growth temperatures of 22 to 25°C, non-motile and associated to fish diseases (**Carnahan et al., 1996**).

Studies based on DNA-DNA hybridization reveled that in each group are present multiple hybridization groups (HGs) (**Janda et al., 2010**) that are hardly distinguishable with biochemical methods (**Abbott et al., 2003**). Many molecular methods have been applied to *Aeromonas* to unravel their classification: the 16S rRNA gene sequencing, the *rpoB* (β-subunit of the multi-subunit enzyme DNA-dependent RNA polymerase), the *gyrB* (B-subunit of DNA gyrase), the *rpoD*, and the MLSA (Multilocus Sequence Analysis) sequencing methods. This last method, which combines the information derived from the sequences of seven housekeeping genes (*gyrB*, *rpoD*, *recA*, *dnaJ*, *gyrA*, *dnaX*, and *atpD*) has been recently proposed during the 10th International Symposium on *Aeromonas* and *Plesiomonas,* whereas a MLSA based on *gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA* can be used to identify unequivocally all the species of the genus (http://pubmlst.org/aeromonas) (**Küpfer et al., 2006; Beaz-Hidalgo et al., 2012**).

The number of validated *Aeromonas* species had increase rapidly in the last 20 years; currently the genus comprises the following species: *A. hydrophila* (subspecies: *hydrophila* and *ranae*), *A. bestiarum*, *A. salmonicida* (subspecies: *salmonicida*, *masoucida*, *smithia*, *achromogenes* and *pectinolytica*), *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (subspecies *veronii* and *sobria)*, *A. encheleia*, *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. popoffii, A. simiae, A. molluscorum, A. bivalvium, A. tecta, A. aquariorum, A. piscicola, A. fluvialis, A. taiwanensis, A. sanarellii, A. diversa* and *A. rivuli* (**Beaz-Hidalgo et al., 2012**).

1.2.2 *Aeromonas* and environment

As ubiquitous bacteria in the biosphere, aeromonads can be virtually isolated from every bacterial ecosystem such as aquatic environments, fishes, domestic pets, farm animals, birds, invertebrate species, insects, foods and natural soils. Their constant presence lead to the continuous exposure and interaction between *Aeromonas* and human beings. Table 1.2.2.1 resumes the distribution of *Aeromonas* spp. in diverse niches (**Janda et al., 2010**).

Table 1.2.2.1: Distribution of Aeromonas spp. [a] 0, not reported; **±**, rare; **+**, uncommon; **++** common; **+++**, predominant species. Data were selected in different previous study: population, identification, method of analysis and other factor. [b] Includes bivalves and snail; [c] Insects and arachnids; [d] Includes leeches; [e] Estuaries; [f] Excludes fish shellfish and crustaceans (**From Janda et al., 2010**).

Species	Presence of species[a]							
	Vertebrates		Invertebrates			Water		
	Primates	Others	Molluscs[b]	Arthropods[c]	Others[d]	Fresh	Saline[e]	Foods[f]
A. allosaccharophila	±	±	0	0	0	0	0	0
A. aquariorum	0	±	0	0	0	±	0	0
A. bestiarum	+	±	±	0	0	++	0	0
A. bivalvium	0	0	±	0	0	0	0	0
A. caviae	+++	+++	++	++	0	++	+++	+++
A. encheleia	0	++	±	0	0	+	0	0
A. eucrenophila	±	+	0	0	0	+	0	0
A. hydrophila	+++	+++	±	±	0	+++	++	++
A. jandaei	+	++	±	0	+	±	0	0
A. media	+	0	0	0	0	+	0	0
A. molluscorum	0	0	±	0	0	0	0	0
A. popoffii	±	0	0	0	0	+	0	0
A. salmonicida	+	+++	0	0	0	++	0	0
A. schubertii	+	±	0	0	0	0	0	±
A. simiae	±	0	0	0	0	0	0	0
A. sobria	0	++	0	0	0	±	0	0
A. tecta	±	±	0	0	0	0	0	0
A. trota	+	0	0	0	0	0	0	±
A. veronii	+++	++	0	±	++	±	++	++

Members of the genus *Aeromonas* are a proper example of bacteria able to colonize the aquatic environments in the urban water cycle (Figure 1.2.2.1), which is composed by

drinking water, household tap water, municipal wastewater and river; these environments are also potential spots for antimicrobial resistance dissemination (**Figueira et al., 2011**).

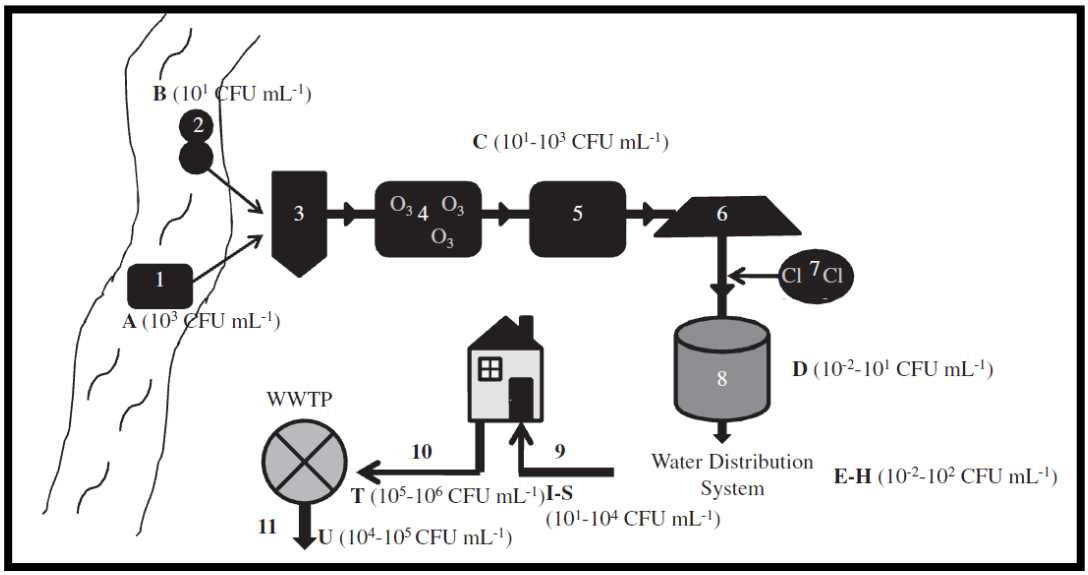


Figure 1.2.2.1: Schematic representation of urban aquatic cycle. 1, raw surface water; 2, ground water; 3, filtration; 4, ozonation; 5, coagulation/flocculation; 6, flotation and filtration; 7, chlorination; 8, treated water reservoir; 9, drinking water; 10, row waste water; 11, treated waste water. A-U, drinking and waste water treatment plant and water treatment stages. CFUmL^{-1}, culturable count of *Aeromonas*. (**From Figueira et al., 2011**)

Invertebrates and insects such as leeches and mosquitoes can be carriers of *Aeromonas* strains. In the leeches, *Aeromonas* act as symbionts, helping to digest the blood. *A. culicicola* was isolated from the midgut of *Culex quinquefasciatus* and *Aedes aegypty*, where the normal bacterial flora inhibit the development of pathogens within the mosquitoes (**Pidiyar et al., 2002**).

1.2.3 Human pathologies

There is a strong correlation between *Aeromonas* and aquatic environment, in fact many microbiologists consider the word "*Aeromonas*" to be a synonymous for "water". However, the association between human infection/colonization and aeromonads is not always obviously connected to the water. Indeed, the pathway of the human infection/colonization by aeromonads (Figure 1.2.3.1) may include: ingestion of contaminated drinking water or food (e.g. meat and dairy products contaminated during the "farm-to-table" operations or bivalves as mussels and oysters directly in contact with these bacteria by filtering the water), direct contamination of major or unapparent traumas during boating, fishing or diving activities, and

zoonotic origin such as the reptile and vertebrate bites or contact with pets and other animals (**Janda et al., 2010**).

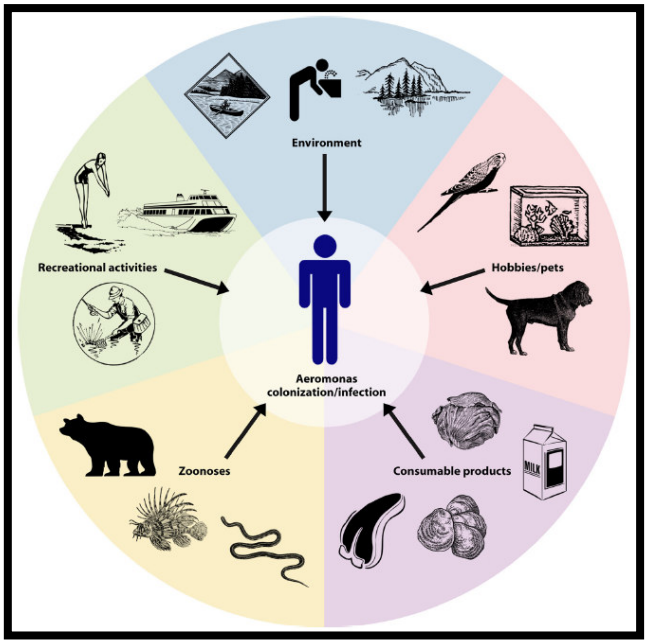


Figure 1.2.3.1: Possible environmental routes to infection or contamination of human beings with *Aeromonas*. The black arrows indicated the interaction between the environments and humans. (**From Janda et al., 2010**)

Among the recognized twenty-five *Aeromonas spp*., three species (*A. hydrophila*, *A. caviae*, and *A. veronii bv. sobria*) are responsible for the majority (over 85%) of human infections. Other species, for example *A. aquariorum*, *A. tecta*, *A. schubertii*, can occasionally be identified in intestinal and extra-intestinal infections (septicemia, peritonitis, osteomyelitis and soft-tissue infections) (**Puah et al., 2013; Puthucheary et al., 2012; Janda et al., 2010**). The pathologies related to *Aeromonas* infections affect different tissues and apparatus. These bacteria have been recognized as the etiological agent in: (i) gastroenteritis; (ii) blood-borne infections; (iii) skin and soft tissue infections (SSTIs); (iv) intra-abdominal infections; (v) respiratory tract infections; (vi) urological tract infections and (vii) eye infections (**Janda et al., 2010**). *Aeromonas* strains causing enteritis are not limited to a single genomospecies or a peculiar biotype/genotype (**Albert et al., 2000**). The illness seems closely correlate with water and seafood as well as fishes consumption. The symptoms includes fever, diarrhea and vomiting, and may occur in five clinical forms: mild enteritis, severe form accompanied by bloody stools, sub-acute or chronic intestinal syndrome, rare cholera-like disease and episodic traveler's diarrhea. *A. veronii* biotype *sobria* and *A. caviae* are the most prevalent species identified in traveler's diarrhea from Asia, Africa and Latin America (**Demarta et al., 2000; Vila et al., 2003**). Some cases of *Aeromonas* colitis may evolve in long term in a chronic condition such as ulcerative colitis or pan-colitis. The severe gastroenteritis with bloody stools can lead to the death of the patients (**Janda et al., 2010**). The main species involved in

the blood-borne infections are *A. hydrophila*, *A. caviae* and *A. veronii* bv. *sobria*. Sepsis can be caused, although less frequently, also by *A. jandaei*, *A. veronii* bv. *veronii* and *A. schubertii*. Skin and soft tissue infections can appear as a mild external problem such as pustule lesions until to serious or deadly infections. Intra-abdominal infections caused by *Aeromonas* include pancreatitis, acute cholangitis and peritonitis. *A. hydrophila* is the most common species implicated in peritonitis in Southeast Asia, although *A. veronii* bv. *sobria* may also be the causative agent (**Janda et al., 2010**). Respiratory tract infections include asymptomatic colonizations, but also pneumonia that may be caused by a near-drowning event (**Bossi-Küpfer et al., 2007**) or non obvious event that lead to a respiratory sufferance with fever, productive cough, vomiting and/or respiratory failure (**Rodriguez et al., 2005**). *A. hydrophila* and *A. veronii* bv *sobria* have caused spontaneous bacterial empyema in males with cirrhosis in Southeast Asia (**Chen et al., 2006; Wang et al., 2000**). *Aeromonas* occasionally cause infection of the urological tract; cases involving *A. veronii* bv *sobria*, *A. popoffii* and *A. caviae* were reported (**Hua et al., 2004**). *A. caviae* may be involved in dysuria, hematuria and cystitis (**Al-Benwan et al., 2007**). Finally, *Aeromonas* can cause ocular diseases ranging from endophtalmitis to keratitis and corneal ulcerations that are not related to a previous ocular trauma or environmental contamination. **Pinna et al., (2004)** described a case of *A. caviae* keratitis associated with contact lens wear.

1.2.4 Fish diseases

The mesophilic and psychrophilic species of aeromonads, such as *A. hydrophila*, *A. veronii* and *A. salmonicida*, are implicated in fish diseases.

The role of aeromonads as etiological agent of fish diseases has been known for several years. The mesophilic *Aeromonas hydrophila* and *A. veronii*, can cause septicemias (hemorrhagic septicemias) in many species of fishes including carps, tilapias, perches, catfishes and salmons. The red sore and ulcerative infections in basses, carps, catfishes, cods, gobies are other diseases related to *Aeromonas*. *A. hydrophila* is considered the major cause of fish death around the Earth (**Janda et al., 2010**).

The furunculosis (Figure 1.2.4.1), caused by the psychrophilic species *A. salmonicida*, is one of the main diseases affecting fish industry with a high rate of mortality and economic losses in the european salmonid aquacultures. Although vaccination programs are established, the aquacultures are regularly exposed to furunculosis outbreaks requiring antibiotic treatments (**Holten-Andersen et al., 2012**). The capacity of *A. salmonicida* to cause infection is due to its intrinsic virulence, as for the strain 01-B526 (**Charette et al., 2012**), and its ability to

evade the host immune system (**Kilpi et al., 2013**). The furunculosis presents a wide range of serious manifestations such as acute septicemia with hemorrhages at the fin base, inappetence, and melanosis, but it can also assume a subacute or even chronic form in older fish which manifest lethargy, slight exophthalmia and bleeding in muscle and internal organs (**Janda et al., 2010**). The risk of mortality increases after the tenth day of infection (**Holten-Andersen et al., 2012**).

Figure 1.2.4.1: Furunculosis in Atlantic salmon. *From AGDAFF (2008) Aquatic Animal Diseases Significant to Australia: Identification Field Guide. Australian Government Department of Agriculture, Fisheries and Forestry, 2008. Canberra. Diseases — Furunculosis (infection with Aeromonas salmonicida subsp. salmonicida).*

1.3 Fecal Coliforms

The term of fecal coliforms is referred to bacteria that are gram-negative non-sporulating bacilli, aerobic or facultative anaerobic, oxidase-negative, able to growth in presence of bile salts, and able to ferment lactose with acid and gas production at 44±0.5°C in 48 hours (**Doyle et al., 2006**). This non taxonomic group includes genera of bacteria that originate in feces (e.g. *Escherichia*) as well as genera not of fecal origin (e.g. *Enterobacter, Klebsiella, Citrobacter*). *E. coli* is therefore the most specific indicator of fecal contamination, not only in water but also in foods (shellfishes, oysters, fresh vegetables and beverages or tap water).

The fecal contamination of water is detected by evaluating the presence of the total coliforms, fecal coliforms and *Escherichia coli*. This analysis is carried out to prevent human exposure to microbial pathogens (**Girones et al., 2010; Kishinhi et al., 2013, Ballestè et al., 2010**).

1.3.1 Aquatic environment

Water is the major resource for all form of life, it is required to sustain health, for sanitation needs, as solvent and for industrial applications. Inadequate treatments of this resource have implications on the public health as it can contain pathogens (**Akturk et al., 2012**); in fact, the World Health Organization (WHO) estimates that 3.4 million persons, in large part children, die every year from water-related diseases.

The fecal contamination is an important way of spreading pathogenic bacteria, virus and protozoa in public water supplies. The potential sources of the fecal contamination in aquatic

environments are the domestic, the wild animals and the birds defecations, septic tanks or wastewater treatment plant malfunctions, agriculture run-off, and municipal and industrial effluents (**Kishinhi et al., 2013**).

Although the sewage treatment plants remove most of the microbial pathogens, many of these are discharged through the effluent water and enter in the environment as a point source of contamination (**Girones et al., 2010**).

Usually, the risk for humans to contract a pathogen from domestic or agricultural animal feces is lower than from human feces, in part because of the high microorganisms host specificity. This is not true for skin infections, which are more frequent in beaches with contaminated water from animal wastes. Moreover, the fecal contamination by wild animals seems to have increased the number of infections due to waterborne zoonotic pathogens (**Field et al., 2007**).

Infections by fecal pathogens may be acquired by direct contact with a skin lesion, air (aerosol) through the respiratory system, or by ingestion of contaminated water. As a matter of fact, during a swimming session of about eighty minutes a child, a woman and a man can absorbs 0.63 mL/min, 0.34 mL/min and 0.50 mL/min, respectively (**Thorolfsdottir et al., 2013**).

1.3.2 *Escherichia coli*

Escherichia coli is a common member of the intestinal flora in humans and animals. *E. coli* is a gram-negative bacteria, facultative anaerobic whose cells are typically rod-shaped. This enteric species comprises both commensally and pathogenic strains. *E. coli* is also used as indicator organism of fecal contamination, because *E. coli* cells are always present in the feces of warm-blooded animals (**Janezic et al., 2013**).

In the environment, *E. coli* is able to survive for extended periods of time in various habitats such as soil, sediments, sand of the beaches and aquatic vegetation. The ability of these intestinal bacteria to survive in soil or in surface waters seems to be related to a high concentration in nutrients which may originate from runoff of residential areas, from animal feeding operations and from other urban sources (**Goto et al., 2011Ibekwe et al., 2011**). Beside the presence of nutrients, other environmental factors affect the survival of these bacteria such as UV light, temperature and predation.

The *Escherichia coli* populations are genetically diverse, and organized in distinct clones **(Topp et al., 2003; Ibekwe et al., 2011).** The genetic diversity is also accompanied by the gain/loss of genes that are useful only in the current/preferred habitat. However, there are some highly conserved genes maintained in all *E. coli* strains , such as stress-associate genes

(i.e. oxidative, acidic, osmotic condition, heat/cold shock, toxic compound, DNA protection/repair, etc.), whereas others, such as *evgAS*, *umuDC*, *nhaR* and *betBT* probably conferring an advantage for the colonization of the human gastrointestinal tract, those implicate in adherence (hemolysin E, *hlyE*; fimbrial-like adhesin, *sfmH*) and nutrient uptake, can be lost by environmental strains (**Oh et al., 2012**).

The extreme plasticity of the *E. coli* strains assumes an important role in the emergence of new virulent strains that are of concern to public health (**Middleton et al., 2013**). The environments contaminated by these *E. coli* strains can serve as reservoirs for the dissemination of the existing and/or the new variant strains. The issues for the public health may arise from their metabolic richness, virulence factors and antibiotic resistances (**Janezic et al., 2013**).

The genome plasticity and the evolutionary behavior of the *E. coli* strains are due to the horizontal gene transfer. During an outbreak reported in Germany in 2011, the genomes of two *Escherichia coli* O104:H4 were sequenced. This demonstrated that the strains in charge of the outbreak had acquired several mobile elements (plasmids, transposons, and pathogenicity islands) encoding for tellurite and mercury resistance, type IV pilus system, colicin, hemolysin E, and F fimbriae (involved in high frequency of recombination) as well as mobilization and transfer genes (**Brzuszkiewicz et al., 2011**).

1.4 Plasmids

Plasmids are circular double-stranded deoxyribonucleic acid molecules. The main feature of these accessory DNA molecules are genes allowing it to replicate autonomously in the host cell (initiation and control of replication). Some plasmids have genes that ensure their stable inheritance, such as equipartitioning during bacterial division or conjugal transfer, and many contain useful genes to themselves and to the bacterial hosts. The length can vary from few to several hundred kilobase (kb) pairs (**Couturier et al., 1988**).

Historically, bacterial plasmids are divided in two main groups: the R (resistance) plasmids and the F (fertility) plasmids. The R plasmids possess two central characteristics, namely the ability to render the bacteria resistant to antibiotics and eventually enable them to transmit resistances to other bacteria. Plasmids named F (for fertility factor or “sex factor”) are those able to be transmitted and/or to promote integration into the chromosome (**Meynell et al., 1968**).

When released in the environment, after bacterial lysis, the plasmids can be found in three different topological conformation (Figure 1.4.1): (i) supercoiled or covalently close circular,

(ii) relaxed circular or (iii) linear (**Demanèche et al., 2002**). In the first topological conformation the DNA is uncut and presents an integral twist, which confers a compact form. The second topoform shows a fully intact DNA enzymatically relaxed, while the third is a linearized plasmid due to cutting of the DNA. In addition, it is possible to find a fourth topological form of plasmids in which the DNA has only one strand cut, the nicked open-circular.

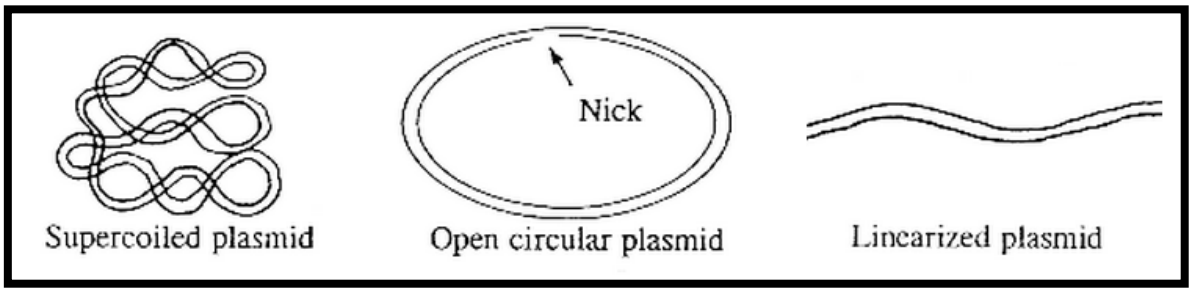
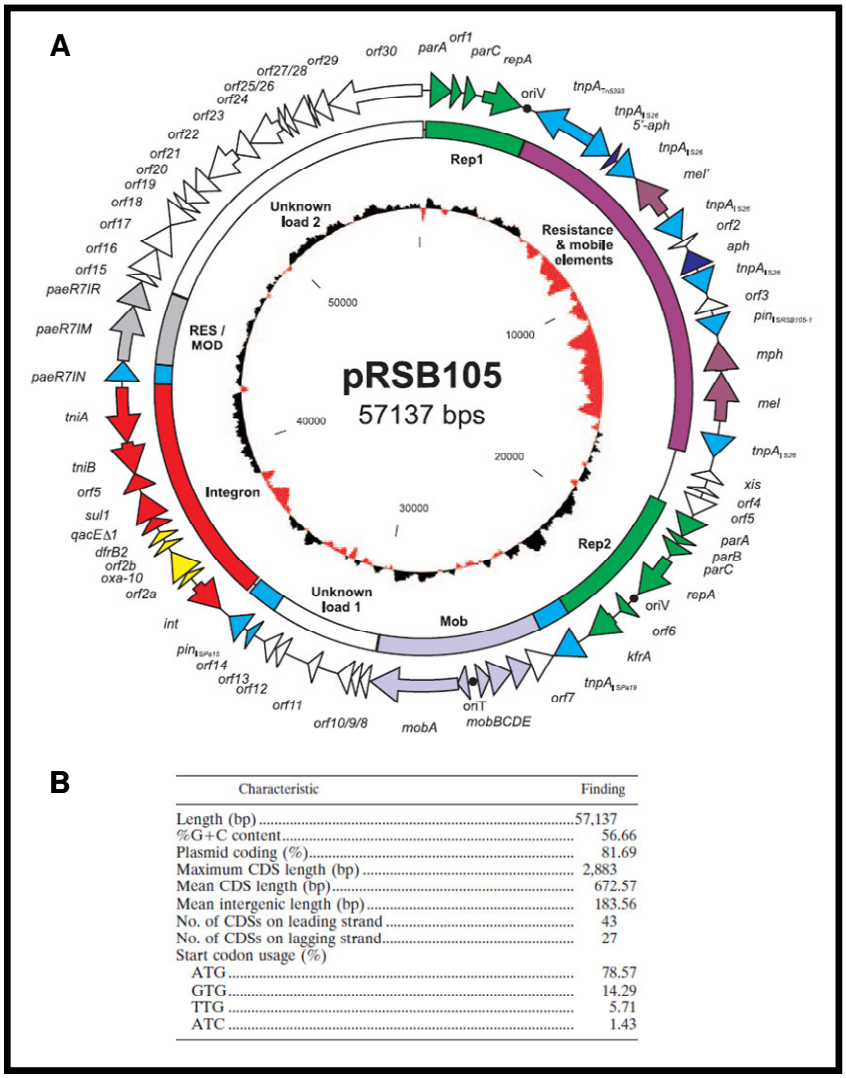


Figure 1.4.1: Topological conformations of plasmids. (Modified images from Google)

Plasmids do not harbor functional genes needed for the bacterial growth and/or multiplication, but they may have other sets of genes conferring advantages to survive particular environmental situations, such as polluted aquatic environments or in the presence of antibiotics. In fact, these accessory genetic materials may carry a variety of genes including antibiotic resistance determinants and/or genes conferring resistance to toxic heavy metals such as mercury (Hg), cadmium (Cd), silver (Ag), genes that code for enzymes that increase bacterial nutritional versatility, virulence determinants for invasion and survival in animal hosts, and genes which enhance the DNA repair (**Bennett, 2008**). These genes are generally inserted in transposons (mobile genetic elements) allowing the formation of highly variable and flexible plasmids (**Couturier et al., 1988**).

The complexity of a plasmid depends on the number and type of genes contained. The coding sequences (CDSs) can be categorized as follows: replication (Rep genes), stable inheritance and portioning (Par genes), mobilization (Mob genes), transposition and recombination (transposons), resistance, restriction and modification, and unknown function (Figure 1.4.2).



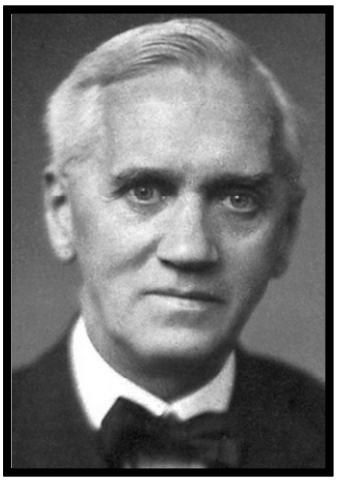
Characteristic	Finding
Length (bp)	57,137
%G+C content	56.66
Plasmid coding (%)	81.69
Maximum CDS length (bp)	2,883
Mean CDS length (bp)	672.57
Mean intergenic length (bp)	183.56
No. of CDSs on leading strand	43
No. of CDSs on lagging strand	27
Start codon usage (%)	
ATG	78.57
GTG	14.29
TTG	5.71
ATC	1.43

Figure 1.4.2: Example of the genetic complexity within a plasmid.
A: Genetic map of a *Ralstonia eutropha* resistance plasmid. Arrows indicated the orientation of the coding region. The modular structures is shown in the median circle: plasmid replicons, Rep1 e Rep2 (green); mobilization region, Mob (light blue); first resistance region includes macrolide and truncated aminoglycoside-phosphotransferase (purple); integron contain Tn402-like element(red); modification/restriction region (gray); unknown functions (white); insertion sequence, IS, and transposon, Tn (blue); genes within integron gene cassettes (yellow); putative genes (white arrows). OriV, origin of vegetative and OriT, origin of transfer replication (black dots).
B: General features of the plasmid.
(**From Schülter, 2007**)

The mobilization modules may belong to different MOB families (e.g. MOBp) or subfamilies that include different mobilization genes such as *mobA*, *mobB*, *mobC*, *mobD* and *mobE*. Their product recognizes and binds to the origin of transfer (*ori* genes). Therefore, the presence of those particular genes increases and optimizes the replication and the dissemination of plasmids in different host bacteria. The resistance genes can be included in integrons (as gene cassettes), in transposons or spread along the plasmid (e.g. *mel* gene, for the ABC-type efflux pump) (**Schülter et al., 2007**).

1.5 Antibiotics and resistance genes

The discovery of antibiotics in the first half of 1900 has reduced the mortality of humans and increased life expectancy. In the 1928, Sir Alexander Fleming (born: 6 August 1881, Lochfield, Scotland; died: 11 March 1955, London, United Kingdom), during an experiment with staphylococci, noticed that a plate was contaminated by a fungus that repressed bacterial growth in the surrounding area. This fungal contamination (*Penicillium rubens*) was able to produce a substance inhibiting numbers of pathogenic bacteria. The substance found by Fleming was named penicillin, the first antimicrobial compound (β-lactam antibiotic) discovered. Nowadays the number of used antibiotics is grown, but, on the other hand, bacteria have acquired the ability to resist to these compounds.



Sir Alexander Fleming

Antibiotic resistance may be due to different mechanisms that are: permeability changes, active efflux pumps, enzymatic modifications and/or degradations of the antibiotics, acquisition of alternative metabolic pathways, modification of the targets, and overproduction of the targets (**van Hoek et al., 2011**).

1.5.1 Aminoglycosides

Aminoglycosides act as inhibitors of the protein synthesis through the ribosome binding. The resistance to these antimicrobial agents (Figure 1.5.1.1) consists in the production of modified enzymes (that catalyze the covalent modification of specific amino or hydroxyl functions of the drugs) reducing the binding of the antibiotics to the ribosomes. On the other hand, the inactivation of the antibiotics are caused by three enzymes: (i) phosphotransferases (e.g. resistance to gentamicin); (ii) nucleotidyl-transferases or adenyl-transferases; (iii) acetyl-transferases (e.g. resistance to kanamycin) enzymes (**Taylor et al., 2004**).

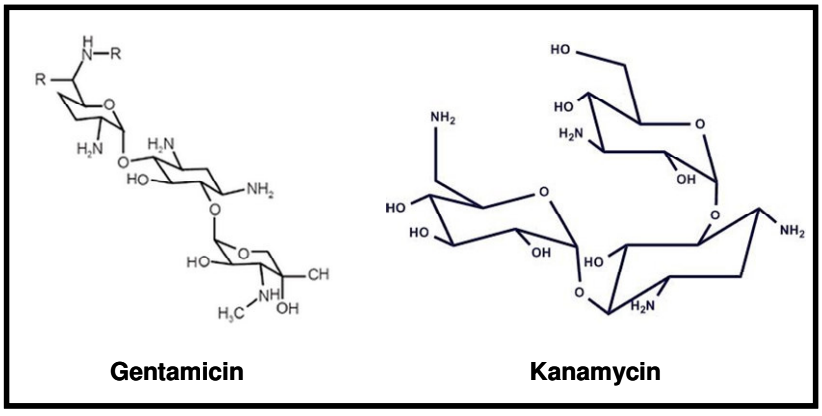


Figure 1.5.1.1: Examples of aminoglycosides antibiotics

1.5.2 β-lactams

The β-lactams are a wide family of antibiotics which includes penicillins and the derivatives, cephalosporins, carbapenemes, monobactams. The starting point to design these compounds is the core of the penicillin (6-APA, 6-aminopenicillinic acid) (Figure 1.5.2.1).

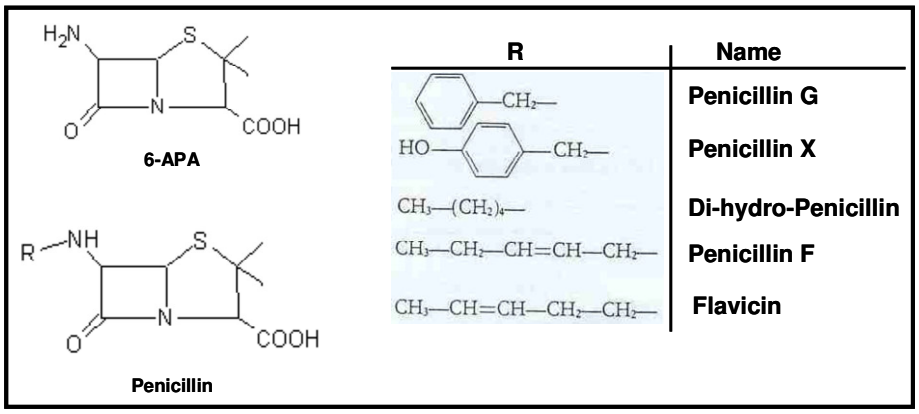


Figure 1.5.2.1: Penicillin: core and derivatives. The 6-APA is the base structure for the drug design of derivatives of the penicillin compound. In the Table are indicate the possible lateral chains (R) of the basic structure of the Ampicillin.

The mechanism of action of this family of antibiotics is the inhibition of the cell wall synthesis starting by the binding of the compound to the penicillin-binding proteins (PBPs) in bacteria, hence interfering with the cross-linking of the peptidoglycan (**van Hoek et al., 2011**). Resistance to the β-lactams is due often to the production of β-lactamases enzymes, which are able to hydrolyze of β-lactam ring therefore inhibiting the activity of the drugs (**Taylor et al., 2004**).

The β-lactamases families were clustered either based on their functionality (Bush-Jacoby-Medeiros groups) or on their molecular characteristic (Amber classes). The Bush classification, is based on biochemical parameters (i.e. enzymatic activity). This classification form four major groups: groups 1, 2 and 4 are serine-β-lactamases, while the group 3 includes the metallo-β-lactamases. The Amber classes take into consideration the molecular characteristics of the molecules (i.e. amino acid homology) but is lacking of the enzymatic activity information. Here too, the Amber classification allows to separate the enzymes in four classes: A,C and D include the serine-β-lactamases, while the class C includes the metallo-β-lactamases that require Zinc for their activity (**van Hoek et al., 2011**).

Extended spectrum β-lactamases (ESBLs) are enzymes that hydrolyze extended-spectrum cephalosporins such as cefotaxime, ceftriaxone, and ceftazidime, as well as aztreonam (Figure 1.5.2.2). The ESBLs are frequently plasmid encoded and arise from amino acid substitutions in the sequence of TEM-1, TEM-2 (TEM, from Temoniera patient's name) and SHV-1 (SHV, from Sulfhydryl reagent Variable) enzymes (**Shaheen et al., 2011; van Hoek et al., 2011**). Carbapenems are the treatment of choice for serious infections due to ESBL-producing

organisms, yet carbapenem-resistant isolates have recently been reported (**Nordmann et al., 2011**).

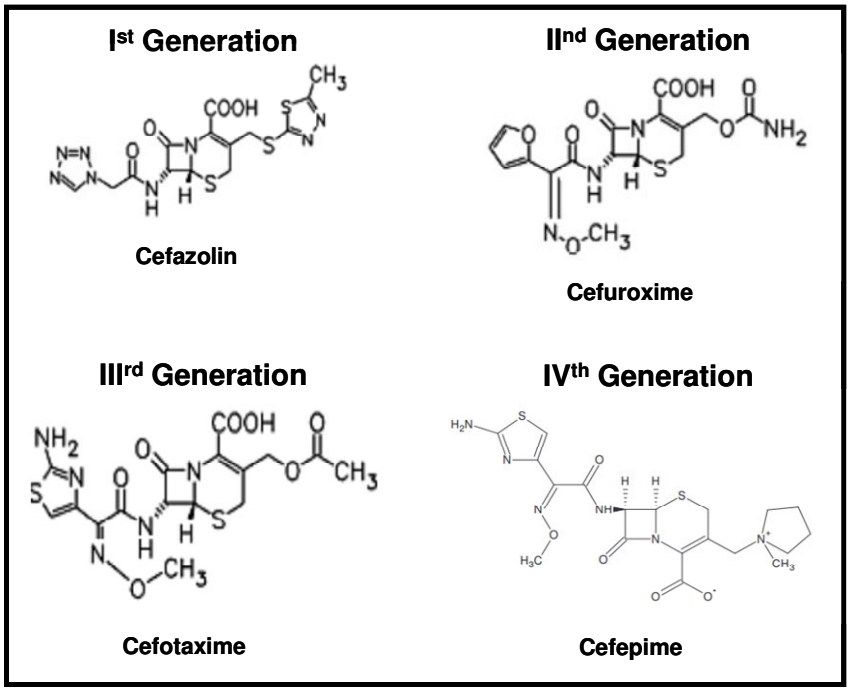


Figure 1.5.2.2: Example of cephalosporins from the first to the fourth generation. The ESBLs genes are able to confer the resistances to the first three generations of antibiotics (bold font).

The mutations in the bla_{TEM} gene allow the enzyme to hydrolyze the cephalosporins. The first variant of this gene is the enzyme TEM-2, where the lysine in 39-position is replaced by a glutamine. Other mutations in the bla_{TEM} gene led to the formation of more than ninety TEM derivatives ESBLs enzymes with specific resistance patterns for a wide range of β-lactam compounds. Mutations of the bla_{SHV-1} gene has in parallel expanded the ability to resist to the extended-spectrum cephalosporins and monobactams (**Rupp et al., 2003**).

Within the clinical setting, the variants ESBL TEM-3 and SHV-12 are the most frequent encountered in enterobacteria (**Poirel et al., 2012 -a-**).

ESBLs are not only composed by the TEM-1 and TEM-2 derivative variants, they also include the OXA-type and the CTX-M-type β-lactamases, commonly found within the *Enterobacteriaceae* and *Pseudomonas aeruginosa* (**Rupp et al., 2003**).

1.5.3 Chloramphenicol

The genes named *cat* (chloramphenicol acetyltransferase) catalyze the transfer of an acetate group from the Acetyl-coA (acetyl-coenzyme A) to the carbon-3 of the chloramphenicol (Figure 1.5.3.1). These genes are present in strains of gram-positive and gram-negative bacteria and are associated with plasmids. In the bi-acetylated form, the antibiotic is inactive, because unable to bind to the prokaryotic ribosomes (**Taylor et al., 2004**)

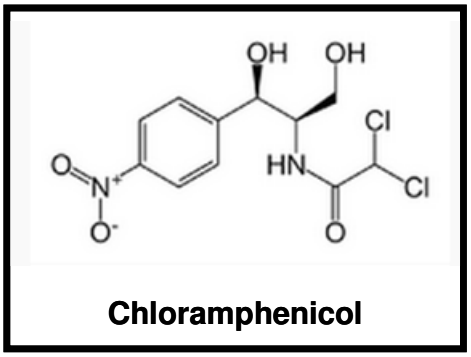


Figure 1.5.3.1: Molecular structure of chloramphenicol.

1.5.4 Glycopeptides

The antimicrobial activity of the first glycopeptide isolated (vancomycin, Figure 1.5.4.1) was found in a fermentation product from *Streptomyces orientalis*. The glycopeptides bind to the target, the D-alanyl-D-alanine (D-Ala-D-Ala), thereby blocking the transglycosylase, interfering with the polymerization of the peptidoglycan. The resistance to vancomycin is due to the modification of the peptidoglycan precursor causing a low binding affinity for the glycopeptide. The *vanA* and *vanB* genes are plasmid and/or chromosomal located, while *vanC1*, *vanC2/3*, *vanD*, *vanE*, and *vanG* have exclusively been found in the chromosome (**van Hoek et al., 2011**).

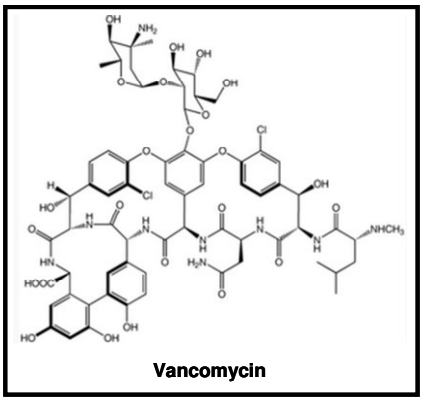


Figure 1.5.4.1: Molecular structure of the vancomycin.

1.5.5 Quinolones

The quinolones were discovered by chance in 1962, when nalidixic acid was discovered as by-product of the chloroquine production, an antimalarial compound. Since the frequence of resistance to the quinolones increased just after their introduction as clinical treatments, the fluoroquinolones were developed. These antibiotics (ciprofloxacin (Figure 1.5.5.1), norfloxacin, perfloxacin) originate through the substitution of a fluorine in 6-position (**Kim et al., 2011**).

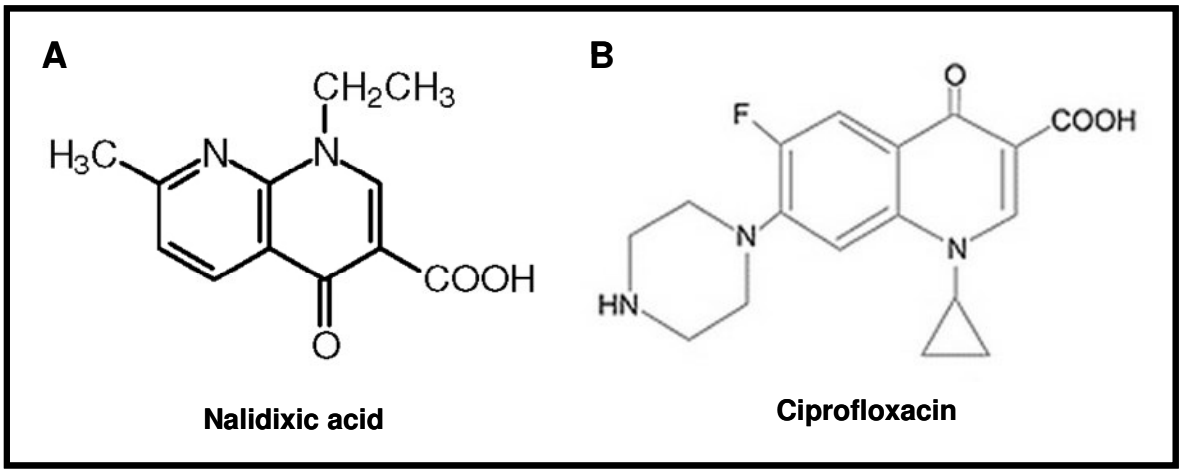


Figure 1.5.5.1: Examples of quinolones compounds. **A**, proto-type of quinolone; **B**, fluoroquinolone: fluorine modified quinolone in 6-position.

Quinolones are broad-spectrum antibiotics used in both human and animal treatments, so they are easily found in the environment at low concentrations. The resistance to quinolones (*Qnr*) depends on DNA gyrase A and topoisomerase IV chromosomal mutations, or on the overexpression of efflux pumps (*Qep*). Resistance can be also plasmid-mediated, therefore transferable (PMQR, plasmid-mediate transferable quinolone resistance); in this case, resistance is conferred by: (i) the Qnr-type pentapeptide proteins (QnrA, QnrB, QnrC and QnrS) that are able to protect the target gene, DNA gyrase; (ii) the AAC(6')-Ib-cr, modified aminoglycoside acetyltransferase in two specific amino acid that acetylates both ciprofloxacin and norfloxacin; (iii) and QepA proteins, an efflux pump. Findings suggest that the *qnrS* and the *aac(6')-ib-cr* may spread silently among bacteria, without expressing the phenotypic resistance (**Picão et al., 2008**). The mutations causing resistance are in the N-terminus of the GyrA protein, named "quinolone resistance determinant region" (QRDR), between the amino acids Ala67 and Gln106. A double mutation, near the active site of the enzyme, the amino acids Ser83→Ile and Asp87→Asn, increase the resistances to the fluoroquinolones (**Goñi-Urriza et al., 2002; Poirel et al., 2012 -b-**). The QnrA inhibits the activity of quinolones protecting the DNA gyrase and the topoisomerase IV, increasing the resistance to the fluoroquinolones from four- to eightfold. The efflux pump coded by the *qepA* gene confers resistance to the hydrophilic quinolones such as ciprofloxacin, norfloxacin, and enrofloxacin (**Crémet et al., 2011**).

1.5.6 Tetracyclines

Tetracyclines were characterized in 1948 from *Streptomyces aureofaciens*. These antibiotics are largest used throughout the world. They can be separated in two groups (Figure 1.5.6.1):

typical (i.e. chlortetracycline, doxycycline, etc.) and atypical (e.g. anhydrotetracycline and 6-thiatetracycline) tetracyclines. The mechanisms of resistance can be divided into three categories: energy-efflux pumps, ribosomal protection proteins (RPPs), and enzymatic inactivation. Genes involved in tetracycline resistances are *tet* and *tcr* (tetracycline resistance), and *otr* (oxytetracycline resistance). Of these genes, 25 *tet*, 2 *otr* and 1 *tcr* code for efflux pumps, 10 *tet* and 1 *otr* code for RPPs, while the *tet* (U) gene code for an unknown mechanism. *tet* (M) had the broadest host range of all tetracycline resistance gene. Three other *tet* genes confer resistance through an enzymatic inactivation. In gram negative bacteria the most common gene conferring resistance to tetracyclines is *tet* (B) (**van Hoek, 2011**).

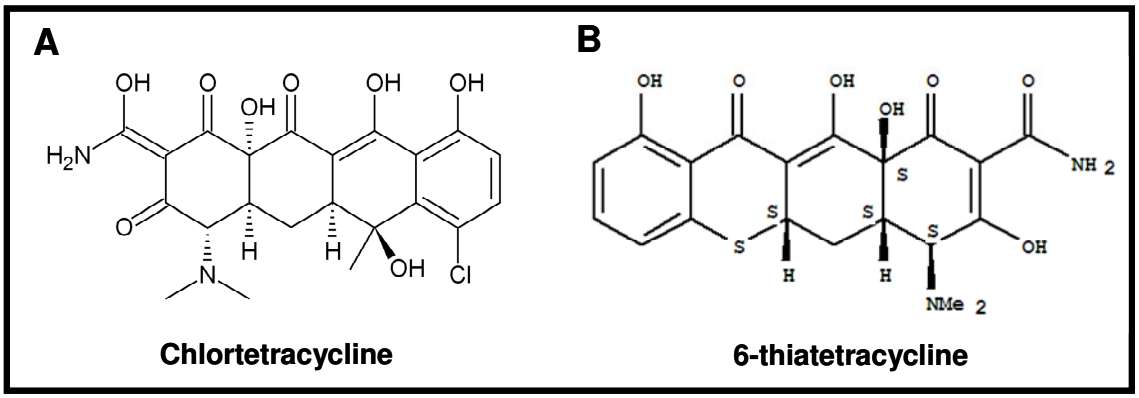


Figure 1.5.6.1: Examples of the structure of tetracyclines. The two molecules are part of the tetracycline typical (**A**) and atypical (**B**) groups.

1.5.7 Trimethoprim

Trimethoprim (Figure 1.5.7.1) is completely synthetic and belong to the diaminopyrimidine group of molecules (**van Hoek et al., 2011**). The activity of this synthetic antibiotic is the competition with the dihydrofolate reductase (DHFR), inhibiting the synthesis of the folic acid necessary to the replication of the genetic material. The resistance to trimethoprim is due to the specific genes *dfr* and is achieved by a bypass mechanism in which a non-allelic and drug-insusceptible variant of a chromosomal DHFR is produced. These acquired DHFRs genes are divided in distinct groups: the *dfrA* (474 nucleotides) family is composed of more than 30 genes, in the *dfrB* (237 nucleotides) family there are at least 8 different genes while the *dfrK* family includes only few genes (**van Hoek et al., 2011**). The *dfr1* seems

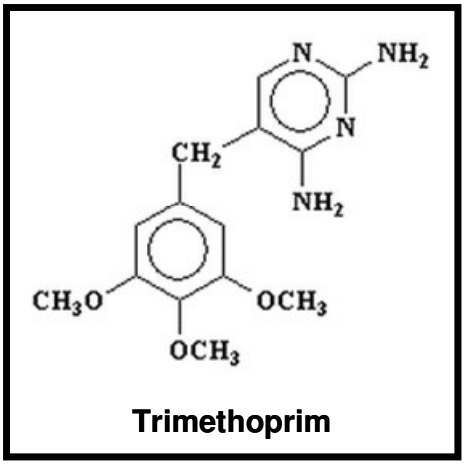


Figure 1.5.7.1: Molecular structure of trimethoprim

to be the most common trimethoprim resistance gene in gram-negative bacteria: it is usually carried on the plasmid-located transposons Tn7 (**Taylor et al., 2004**).

1.6 Integrons

Integrons are mobile elements able to capture gene cassettes by site-specific recombination mechanisms. They are a mean to spread genes, particularly in gram-negative bacteria. Given the high divergence of the genes that encode their integrase, eight classes of integrons were discovered and characterized (**Rowe-Magnus and Mazel, 2001; Zhao and Hu, 2011**).

The class 1 integrons are the most important in the acquisition, expression and spread of antibiotic resistance determinants, though little is known about the flux of genes in this class within and between bacterial communities (**Nardelli et al., 2012**).

The structure of a class 1 integron (Figure 1.6.1) is based on three regions: a 5' and a 3' conserved segments (5'CS and 3'CS) separated by a variable region. The 5'CS region of the class 1 integron includes: the integrase-encoding gene (*intI1*), the insertion site for the gene cassette (*attI*) and a promoter region (Pc) for the transcription of the gene cassettes. The 3'CS includes a partial deleted *qac* gene (*qacE*Δ1) fused to a *sul1* gene, that confer resistance to the quaternary ammonium compounds (antiseptics) and to sulphonamides (**Zhao and Hu, 2011**).

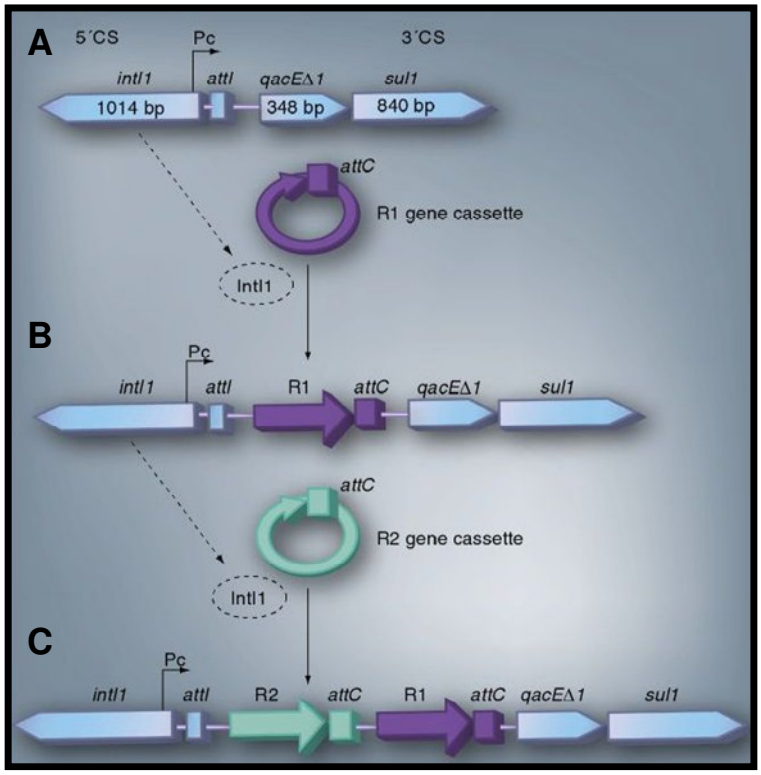


Figure 1.6.1: Class 1 integron and acquisition of gene cassettes.
A, basic structure of the class 1 integrons. The 5'CS region includes the *intI1*, the *attI* and the promoter region (Pc), while the 3'CS includes the *qacE*Δ1 (quaternary ammonium resistance) and the *sul1* (sulphonamide resistance) genes.
B-C, A class 1 integron after insertion of resistance gene cassettes (R1 and R2). The gene cassettes carry the resistance genes (R1 and R2) and the recognition site for the integrase (*attC*, also called 59 base element).
(**From Zhao and Hu, 2011**)

The cassettes are mobile genetic elements holding a gene (in class 1 integrons commonly an antibiotic resistance gene) encoding a protein able to increase bacterial adaptation to environmental stressful conditions, and the *attC* (or 59 base element, 59be), an integrase-specific recombination site (**Rowe-Magnus and Mazel, 2001; Hall and Collis, 1995**).

Most of the *attC* sites are unique and their lengths and sequences can vary significantly (57-141bp) apart at the ends, which correspond to the inverse core site (ICS) and to the core site (CS), whose sequences are RYYAAC and GTTRRRY (R, purine; Y, pyrimidine) respectively (**Rowe-Magnus and Mazel, 2001**).

The gene cassettes are always inserted into the integron with the same orientation to allow gene expression; the insertion is however reversible and the cassettes may also be lost (**Hall and Collis, 1998**). Their transcription is not uniform along the variable zone of the integron: expression of distal genes may be reduced due to an early termination of the transcripts at or near the end of the preceding cassette (**Hall and Collis, 1995**).

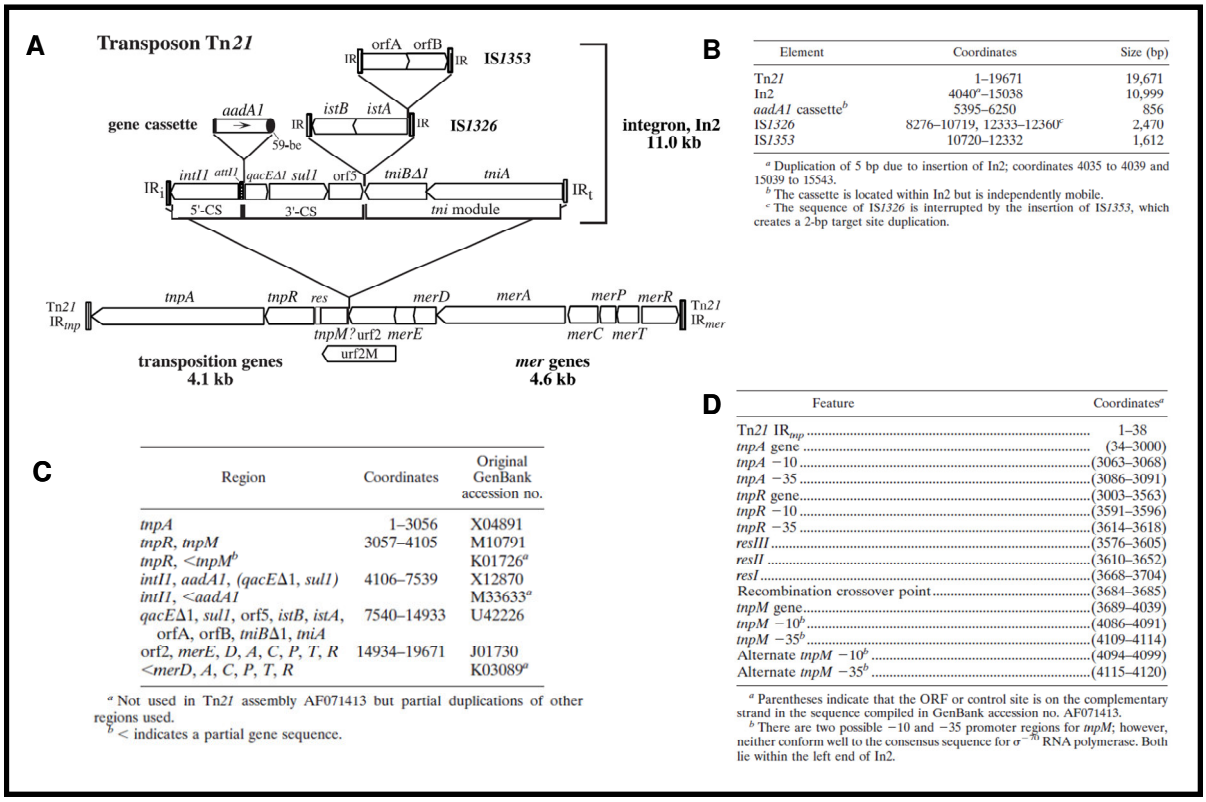
The frequency of integron-carrying bacterial strains is influenced by the surrounding environment and the lack of gene cassettes in integrons does not seem to be related with the absence of antibiotic contaminations. In most cases, the same strain can carry both an empty integron and one carrying resistance genes (**Mokracka et al., 2012**).

The high number of studies and information on these mobile genetic elements have permitted to create a database called Integrall (http://integrall.bio.ua.pt) that collect, organize and search the integrons and gene cassettes. The current version (1.2) of this database includes: (i) background information on integrons; (ii) list tool; (iii) search tool and BLAST interface; (iv) guideline for nomenclature of the gene cassettes; (v) glossary of integron-associated genes; (vi) forum discussion on mobile genetic elements (**Moura et al., 2009**). To now, the database includes 6575 entries, 1507 integrase genes, 8549 gene cassettes, 116 genera, and 242 bacterial species.

Class 1 integrons can be found in more complex structures, such as the Tn 21 transposons (Fig. 19). In gram-negative bacteria, and in particular in the *Enterobacteriaceae*, the transposons that encode multiple antibiotic resistances belong to the Tn 21 class, subgroup of the Tn3 family. The integrons within the transposons are potentially independent mobile DNA fragments. In fact, the integrons possess a site-specific integration system, encoded by RecA-indipendent, for gene cassettes acquisition, while the Tn21 transposon carries genes for the transposition (*tnp*) and a mercury resistance (*mer*) operon (**Liebert et al., 1999**).

Transposons Tn21 may be associated to many resistant gene cassettes in the class 1 integrons, such as *dfrA1*, *dfrA12*, *dfrA17* and *dfrA7*. On the other hand, class 1 integrons can be found in

other transposons such as Tn5086 and Tn5090, and includes other gene cassettes such as *dfrIIc* (Tn5086) and *dfrVII* (Tn5090) (**Kiiru et al., 2013; Radström et al., 1994**).



Element	Coordinates	Size (bp)
Tn*21*	1–19671	19,671
In2	4040[a]–15038	10,999
aadA1 cassette[b]	5395–6250	856
IS*1326*	8276–10719, 12333–12360[c]	2,470
IS*1353*	10720–12332	1,612

[a] Duplication of 5 bp due to insertion of In2; coordinates 4035 to 4039 and 15039 to 15543.
[b] The cassette is located within In2 but is independently mobile.
[c] The sequence of IS*1326* is interrupted by the insertion of IS*1353*, which creates a 2-bp target site duplication.

Region	Coordinates	Original GenBank accession no.
tnpA	1–3056	X04891
tnpR, *tnpM*	3057–4105	M10791
tnpR, <*tnpM*[b]		K01726[a]
intI1, *aadA1*, (*qacE*Δ1, *sul1*)	4106–7539	X12870
intI1, <*aadA1*		M33633[a]
*qacE*Δ1, *sul1*, orf5, *istB*, *istA*, orfA, orfB, *tniB*Δ1, *tniA*	7540–14933	U42226
orf2, *merE*, *D*, *A*, *C*, *P*, *T*, *R*	14934–19671	J01730
<*merD*, *A*, *C*, *P*, *T*, *R*		K03089[a]

[a] Not used in Tn*21* assembly AF071413 but partial duplications of other regions used.
[b] < indicates a partial gene sequence.

Feature	Coordinates[a]
Tn*21* IR_{tnp}	1–38
tnpA gene	(34–3000)
tnpA −10	(3063–3068)
tnpA −35	(3086–3091)
tnpR gene	(3003–3563)
tnpR −10	(3591–3596)
tnpR −35	(3614–3618)
resIII	(3576–3605)
resII	(3610–3652)
resI	(3668–3704)
Recombination crossover point	(3684–3685)
tnpM gene	(3689–4039)
tnpM −10[b]	(4086–4091)
tnpM −35[b]	(4109–4114)
Alternate *tnpM* −10[b]	(4094–4099)
Alternate *tnpM* −35[b]	(4115–4120)

[a] Parentheses indicate that the ORF or control site is on the complementary strand in the sequence compiled in GenBank accession no. AF071413.
[b] There are two possible −10 and −35 promoter regions for *tnpM*; however, neither conform well to the consensus sequence for σ^{70} RNA polymerase. Both lie within the left end of In2.

Figure 1.6.2: Structure and characteristic of the Tn21 transposon. **A**, structure of the Tn21 transposon: the genes for the transposition (*tnpA*, *tnpR*) and the mercury resistance operon (*merD*, *merA*, *merC*, *merP*, *merT* and *merR)* are shown. The transposon includes the In2, the class 1 integron (*intI1*, *attI1*, *qacEΔ1*, *sul1*, *orf5*), and the *tni* module (*tniBΔ1* and *tniA*). The *aadA1* gene cassette and the insertion sequence IS1326 followed by the IS1353, are located inside and outside the class 1 integron, respectively. Both the transposon, integron and insertion sequences are flanked by inverted repeat sequences (IR). **B**, coordinates and size of the Tn21 and the included mobile elements. **C**, sequenced regions and GeneBank accession numbers. **D**, features of the transposition region of the Tn21 transposon. (**From Liebert et al., 1999**)

1.7 Mobilization and incompatibility groups

Plasmids are characterized by a backbone of core genes, highly conserved during a long evolutionary time, and by genetic material which is integrated or lost in the plasmid structure but that does not change its characteristics. Therefore, the identification and characterization of a plasmid allow to obtain information on its physiology and mode of transmission. The relaxase gene is the most informative unit of the plasmid backbone (**Garcillàn-Barcia et al., 2011**). The catalytic domain of the relaxases is located at the N-terminus of the protein (300 amino acids), and its sequence has permitted the classification of plasmids in different families.

The main super-families of small mobilizable plasmids are: the MOB_Q, the ColE1, the pMV158, and the CloDF3. The MOB_Q and a part of the ColE1 overlapping with the MOB_P

family, are the only families that are mobilizable and transferable, while plasmids of the other families require a second plasmid for their transfer (**Francia et al., 2004**). The same method of classification was applied to the large conjugative plasmids. The conjugative plasmids are for the most part included in the MOB_F, the MOB_H, the MOB_C, the MOB_V and in two families which grouped also the small mobilizable plasmids, MOB_Q and MOB_P (**Garcillàn-Barcia et al., 2009**).

During the mobilization and conjugation processes (Figure 1.7.1), the relaxase is the key enzyme that recognizes the origin of transfer (*oriT*), catalyzes the initial and the final steps in the conjugation cleaving the *oriT* in the donor and catalyzes also the re-ligation of the plasmid transferred in the recipient bacterium.

The mobilization/transfer process require the presence of two other enzymatic complex: the type IV coupling protein (T4CP) and the type IV secretion system (T4SS). Mobilizable plasmids may carry the T4CP, while transferable plasmids require both enzymatic complexes (**Smillie et al., 2010**).

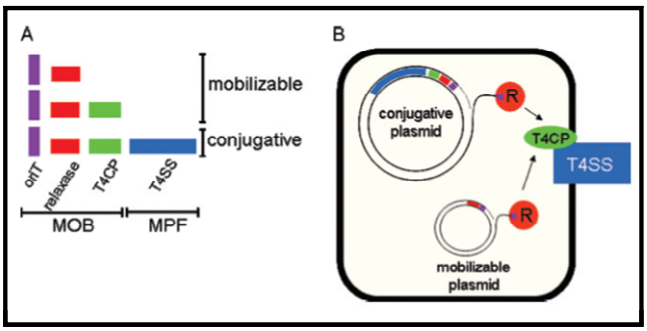


Figure 1.7.1: Schematic view of mobilizable and transmissible plasmids. A, necessary genes in the mobilizable or conjugative plasmids. **B**, schematic interaction among the relaxase and the secretion complex. *oriT*, origin of transfer (violet); R, relaxase (red); T4CP, type IV coupling protein (green); T4SS, type IV secretion system (blue); MPF, mating pair formation. (**From Smillie et al., 2010**).

The analysis of the relaxases and the replicons has highlighted a high correlation between the MOB families and the incompatibility groups (Inc groups): in a single MOB subfamily can be grouped different Inc plasmids (Figure 1.7.2) (**Alvarado et al., 2012**).

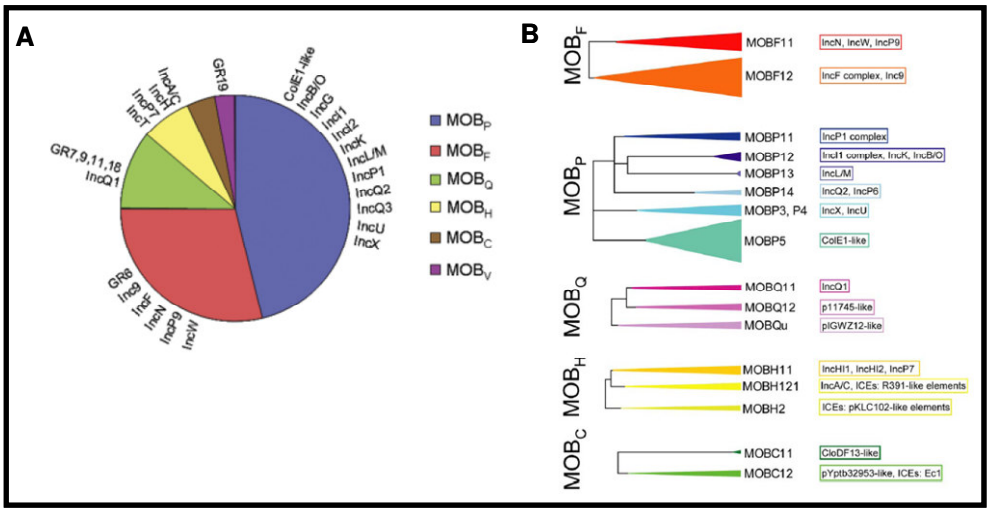


Figure 1.7.2: Relation between MOB and incompatibility groups. **A**: distribution of the inc/rep groups in the MOB families (**From Garcillàn-Barcia, 2009**). **B**: representation of the abundance of the MOB subfamilies, rendered by the width and depth of the colored triangles, and relative distribution of the incompatibility groups (color boxes) (**From Alvarado et al., 2012**).

The incompatibility was defined as the inability of two coresident plasmids to be permanently inherited in absence of external selection. More generally, the introduction of a second plasmid destabilize the inheritance of the first (**Novick, 1987**).

The autonomous replication of a plasmid is under the control of genes that maintain the average number of copy per cell in a given bacterium and growth condition. The replicons consist in: the origin of replication (*cop*), the control region of initiation of the replication (*inc*) and the replication and its control (*rep*) region. Based on these plasmidic regions, it was possible to formulate a schema for the classification of the incompatibility groups of plasmids. The incompatibility groups include: FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P,W, T, A/C, K, B/O, X, Y, F, and FIIA replicons (**Couturier et al., 1988**). Among these incompatibility groups, the most important are represented by the broad-host range (BHR) groups that confer the ability to the plasmids to be spread and maintained in a wide range of bacteria. The BHR plasmids belong to the incompatibility groups *IncN, IncP, IncW, Inc L/M, IncK* and *IncX* (**Götz et al., 1996**), *IncA/C* (**McIntosh et al., 2008**), and *IncU* (**Cattoir et al., 2008**). The incompatibility group Q (*IncQ*) is considered a BHR due to the high degree of mobilization and not to its ability to transfer (**Loftie-Eaton and Rawling, 2012**).

Recent studies have shown that mobilizable plasmids are generally of small size: they present an average size around 5 kb though some can reach 50 to 300 kb. The (self-transmissible) conjugative plasmids are generally larger with an average size of 100 kb. The majority of the plasmids are non transmissible and have variable sizes but can be grouped in sizes around 5, 35 and 400 kb (**Smillie et al., 2010; Garcillàn-Barcia et al., 2011**). The distribution of the mobilizable, conjugative, and non-transmissible plasmids is reported in Figure 1.7.3.

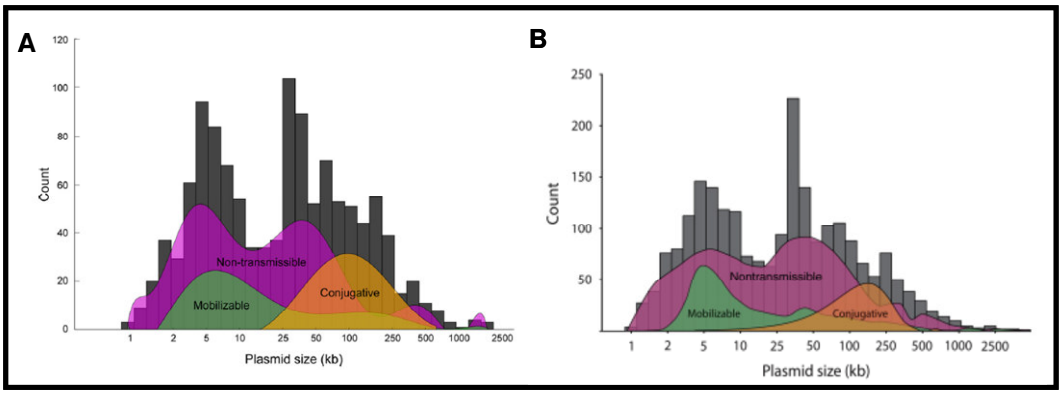


Figure 1.7.3: Comparison of the size of plasmids. Distribution of plasmids according to the size of the mobilizable plasmids (green curves), conjugative plasmids (yellow curve) and non-transferable plasmids (violet curve). The bars indicate the number of plasmids with the same size. **A:** Size and distribution of 1730 plasmids **(From Smillie et al., 2010). B:** Update of the database used from Smillie et al. (2010) **(From Garcillàn-Barcia et al., 2011).**

1.8 Plasmid Curing

The "plasmid curing" is a method resulting in the chemical elimination of the accessory DNA plasmids from bacteria. The lost of plasmids can be spontaneous or forced by different chemical compounds. Historically, the chemical compounds used to eliminate resistance (R) or sex (F) factors carried by plasmids were cobalt, nickel ions and acridine dyes. The effect of these treatments can vary depending on bacteria species; as example, the acridine dyes have a lower efficacy in *E. coli* than in *Shigella* where the effect can reach the 100%. The treatment with sodium dodecyl sulfate 10% (SDS), a reagent routinely used in laboratory for the bacterial lysis, permitted to increase the number of cured bacteria following the incubation time (**Tomoeda et al., 1968**). SDS treatment provide a slow partial lysis of the bacteria that have reach the stationary growth phase, thus facilitating the loss of the plasmids. Bacterial cultures remain viable up to 72 hours after the treatment (**Inuzuka et al., 1969**).

Plasmid curing can also be obtained through the repetitive culture transfer of the bacteria at sub-optimal temperature in absence of chemical compounds. In any case, curing methods have to be adapted to both the plasmids and the bacteria (**Ghosh et al., 2000**).

1.9 Horizontal gene transfer

The genetic material can be moved within a bacterium (intracellular mobility) or between bacteria (intercellular mobility). The sequences of DNA, that encode enzymes or other proteins, which may be mobilized, are defined mobile genetic elements (MGEs). The intercellular exchange of these MGEs can take place in three ways: conjugation, transformation, and transduction (**Frost et al., 2005**).

1.9.1 Conjugation

The conjugative transfer of MGEs needs the cell-to-cell junction and a pore formation allowing the DNA to be transferred from a donor to a recipient bacteria, as well as a type IV secretion apparatus (Type IV Secretion System, T4SS) to produce a pilus. The interaction between the donor and the recipient bacteria is due to the combination of the mating-pair apparatus with the lipopolysaccharides and outer-membrane proteins of the recipient cell surface (**Thomas and Nielsen, 2005**). The conjugative transfer is commonly associated with plasmids which are transferred to recipient bacteria (**Thomas and Nielsen, 2005**). The high horizontal transfer frequency of plasmids may allow them to be maintained as molecular parasites, whilst other mobile elements are transmitted vertically (**Madsen et al., 2012**). Different kind of plasmids provide the cell to form flexible or rigid pili. Strains harboring IncIα, IncI2, and IncK plasmids show thin and flexible pili able to conjugate in aquatic environments, while strains containing IncM, IncN, IncP, IncW plasmids, produce rigid pili able to conjugate prevalently on a solid substrate (**Bradley et al., 1980**).

The conjugation is an important way to spread resistance genes, heavy metal, and virulence factor between pathogenic resistant strains to other bacteria belonging to the endogenous human flora (**Anjum et al., 2011; van den Bogaard and Stobberingh, 2000**).

1.9.2 Transformation

In the environment, the natural transformation of bacteria is the mechanism of acquisition of foreign DNA. This DNA can be integrated in the genome and can supply the bacterium with new functions. To allow the transformation mechanism, bacteria need to be in a peculiar physiological state that involves approximately 20 to 50 regulatory proteins: this state is defined competence. In most of the cases the competence state is time-limited, and it is activated in response to particular condition such as altered growth conditions or nutrient

accessibility, cell density or starvation, that can vary among bacterial species or even strains. In the environment only 1% of bacteria is considered naturally competent while in clinical settings, different human pathogenic bacteria are naturally transformable: *Campylobacter, Haemophilus, Helicobacter, Neisseria, Pseudomonas, Staphylococcus*, and *Streptococcus*. The mechanisms favoring the DNA uptake varies among bacteria, in general, the foreign double-stranded DNA is converted to a single-strand during the translocation across the membrane. The majority of competent bacteria are able to translocate, both plasmids and chromosomal DNA (**Thomas and Nielsen, 2005**).

1.9.3 Transduction

Transduction is the mechanism of horizontal gene transfer that exploit viruses, in particular bacteriophages, carrying bacterial genes in their own genome. Transduction can be generalized, when any bacterial gene can be transferred, or specialized, when a restricted set of bacterial genes can be transferred. Since bacteriophages are one of the most abundant biological particles in the world, it seems likely that they can play a significant role in the horizontal gene transfer between bacteria **(Muniesa et al., 2013).**

2 PURPOSE

The aim of the study is to evaluate the role of *Aeromonas* spp. in the environmental spread of genes conferring resistance to antibiotics. Since *Aeromonas* are natural inhabitants of water environments and some species are pathogenic for both humans and animals, it can be assumed that they play a crucial role in the dissemination of resistant genetic determinants. Through the observation of the biodiversity of *Aeromonas* strains regarding their resistance against antibiotics, we wanted to verify if these bacteria are reservoirs of resistance genes or can be considered as vectors of the same.

In the last decades, bacterial resistance to antibiotics has assumed an increasing importance with regard to its impact on public health, making difficult the treatment of some life-threatening infections. However antibiotic resistance is not only found in pathogenic bacteria but also in environmental organisms inhabiting terrestrial and aquatic habitats. In fact, the extraordinary capacity of bacteria to adapt to their environments soon allows these organisms to develop mechanisms of resistance enabling them to overcome the toxic effects of antibiotics (**Stalder et al., 2012**).

In the environment, the higher numbers of resistant bacteria is found in polluted habitats, indicating that humans contribute substantially to their increase proportion. Possible mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria include the discharge of antibiotics and of resistant bacteria into the sewage. The environmental contamination with antibiotics, resistant bacteria and resistance genes affects the biodiversity of natural ecosystems by the suppression of susceptible organisms and the selection of the resistant ones. Moreover, in these environments, bacteria can play a role as reservoir and/or vector for the spread of antibiotic resistance genes. The main risk for public health is that resistance genes pass from environmental bacteria to human pathogens by genetic transfer such as conjugation, transformation and transduction. Humans can become contaminated by the contact with or by ingestion of contaminated surface or drinking water (**Baquero et al., 2008**).

3 MATERIAL AND METHODS

3.1 Water Sampling

Water sampling were taken from: the Ticino river (before and after the wastewater treatment plant), the Bellinzona hospital wastewater, the active sludge of the Giubiasco wastewater treatment plant and from the alpine lake Cadagno (Figure 3.1.1).

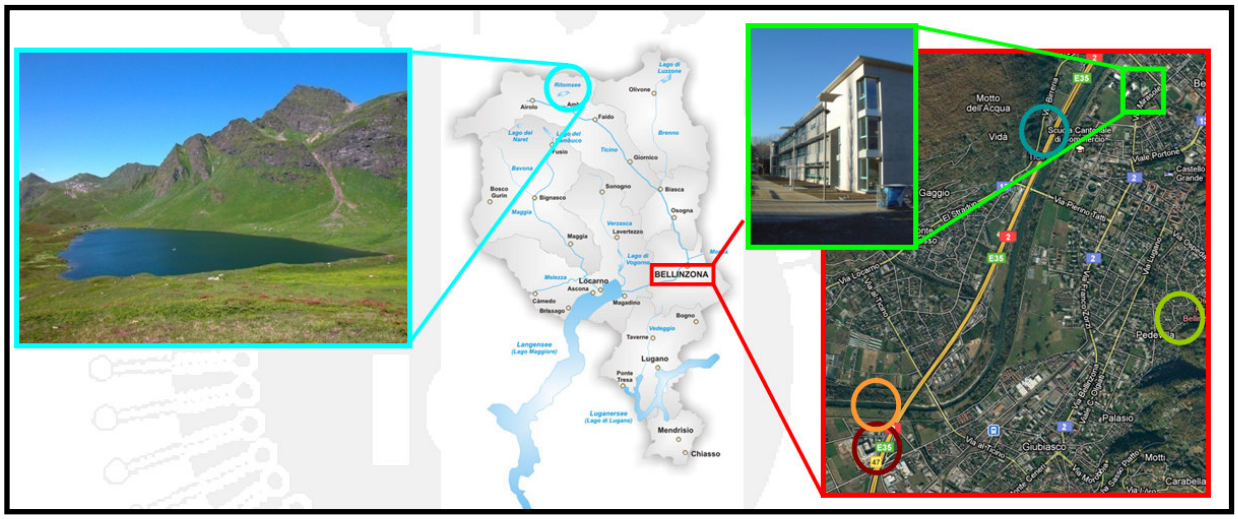


Figure 3.1.1: Map of sampling locations in Canton Ticino. Lake Cadagno (in light blue) and Bellinzona (in red) with the four sampling locations: Ticino river before wastewater treatment plant (blue), Bellinzona hospital (dark green), wastewater treatment plant (brown) and Ticino river after wastewater treatment plant (orange). In light green area is indicated the Laboratory of Applied Microbiology in Bellinzona.

The wastewater treatment plant serves nine municipalities of the Bellinzona district and it is dimensioned to treat 66000 equivalent inhabitant/24 h. Table 1 resumes sample collection temperature, dates and hour.

Table 3.1.1: Temperatures and time of sample collection

Areas	Abbreviations	Temperature	Data	Hour
Ticino River before Wastewater Treatment Plant	TR-b-WWTP	3 °C	18.01.2011	9.30 am
Bellinzona Hospital wastewater	HWW	16°C	26.04.2011	8.45 am
Giubiasco Wastewater Treatment Plant	WWTP	17°C	08.08.2011	8.00 am
Ticino River after Wastewater Treatment Plant	TR-a-WWTP	13°C	08.08.2011	12.00 am
Cadagno Lake	CL	10°C	13.09.2011	10.00 am

At each sampling site, 3 liters of water were collected in sterile/autoclaved glass bottles. Samples were then transported in a refrigerate bag at 4°C and processed within 2 hours of collection.

3.2 Bacterial isolation

Logarithmic dilutions (from 10^{2}ml to 10^{-2}ml) of the water samples were prepared in 0.1% peptone (enzymatic digest of animal proteins) water and filtered through 0.45 µm-pore size sterile filter (Millipore) of 47 mm diameter. Filters were placed on m-*Aeromonas* selective agar base (Biolife) supplemented with Ampicillin and on C-EC Mug agar to isolate *Aeromonas spp.* and Fecal Coliforms, respectively. *Aeromonas* spp. selective plates were incubated at 30°C for 24h, while fecal coliforms C-EC Mug agar plates were incubated at 44°C for 24 hours. Fifty colonies of *Aeromonas spp.* (yellow colonies) and fecal coliforms (blue colonies) were randomly selected and plated onto COL-S 5% SB agar (Columbia agar 5% Sheep Blood, BD BBL™ Stacker™ Plates). After 24 hours of incubation at 30°C or 37°C, the strains were inoculated into Skim-Milk (Difco, BD) and frozen at -20°C for 2 hour. These cultures were then stored at -80°C.

3.3 Identification of the bacterial species

Identification of *Aeromonas spp.* and Fecal Coliforms to the species level was achieved using MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization – Time Of Flight, Mass Spectrometry). Pure cultures were preliminarily screened by the oxidase test revealing the presence of the Cytochrome C enzyme in aerobic and facultative anaerobic bacteria: *Aeromonas spp*. are always positive whereas Fecal Coliforms lack this enzyme.

Biomarkers (the majority represented by ribosomal proteins) with a specific mass signal (2 - 20kDa) can be detected by MALDI-TOF MS. Mass spectra are generated as positive lines by scanning the samples with a laser beam (Figure 3.3.1). After the signal acquisition, the spectra are automatically processed removing the baseline. The useful picks identified are compared with each reference spectra present in the database (SARAMIS – Spectral Archive And Microbial Identification System) for the identification of microorganisms and the results are expressed in percentage of confidence relating to the reference spectra (**Benagli et al., 2012**).

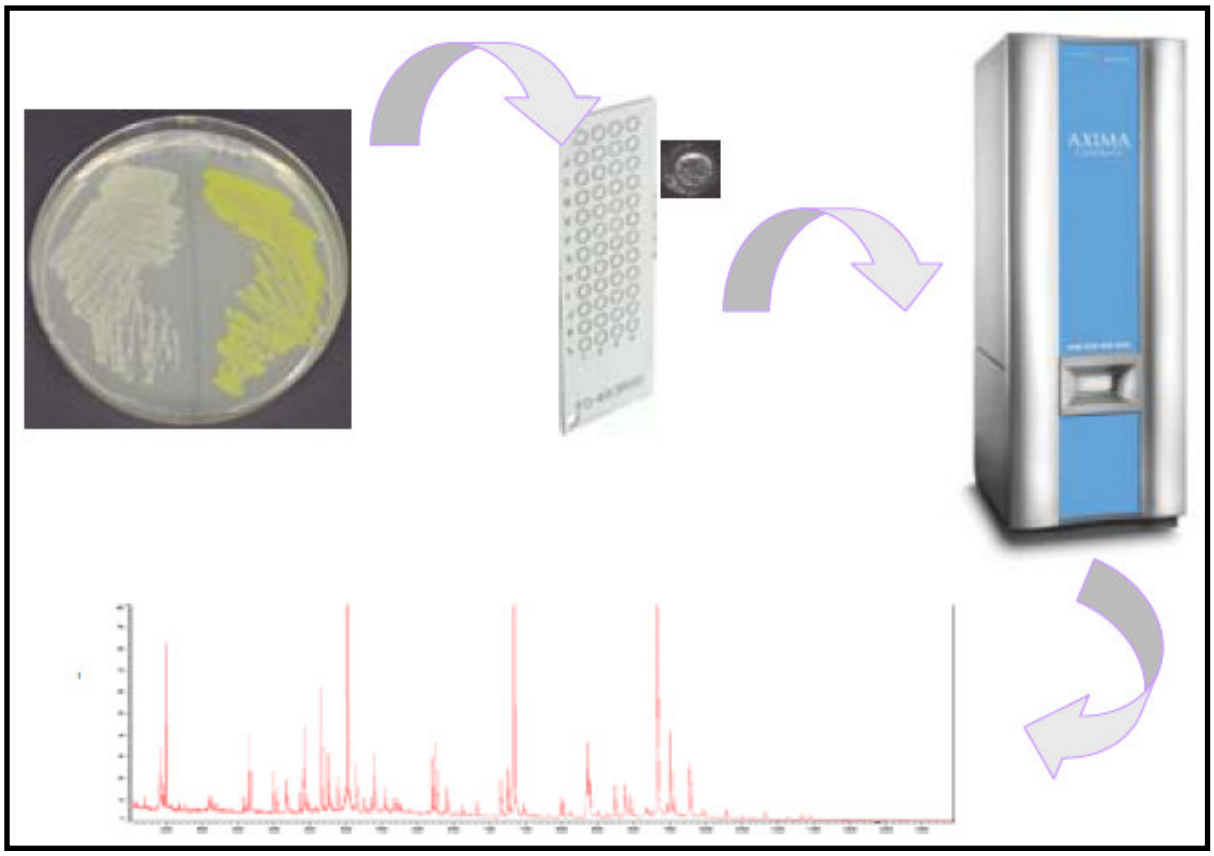


Figure 3.3.1: **Schematic representation of the identification process by MALDI-TOF MS**. From poster: *Development and validation of a MALDI-TOF MS microbial database for rapid identification of public-, animal-, and plant-health relevant environmental bacteria.* **Tonolla M., Vogel G., Duffy B., Rezzonico F. KTI Medtech Event 2010.**

A bacterial colony was spotted twice on the wells of the MALDI plate; after the addition of 1 µL of α-cyano matrix (composition and volumes are listed in Table 3.18.1), the colony is processed by the MALDI-TOF. Bacteria are shown as a list of proteins mass spectra and the presence of specific picks allows genus and species identification.

3.4 Antibiotic susceptibility test

Antibiotic susceptibility was determined by the disc diffusion method (Kirby-Bauer) on Mueller Hinton agar (composition and volumes are listed in Table 3.18.1), according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2011) guidelines. Three colonies of an overnight culture were inoculated in 2 mL of TSB (Trypticase Soy Broth, Oxoid) for 3-5 hours. The bacterial suspension was diluted to a 0.5 McFarland turbidity, corresponding approximately to $1.8 \cdot 10^8$ CFU/mL, in 5 mL of saline solution (NaCl 0.9%) and inoculated on the Mueller Hinton Petri dish using a cotton swab. The antibiotics

(listed in Table 3.4.1) were chosen among different antibiotic groups covering different resistance mechanisms. They were applied on the plate within 15 minutes after inoculation of the bacterial strain. After 24 hours of incubation at 30°C or 37°C, for *Aeromonas spp*. and for Fecal Coliforms respectively, the strains were classified as resistant, intermediate or sensitive, accordingly to the EUCAST guidelines.

Table 3.4.1: Antibiotics used to determine the phenotypic profiles of resistance in bacteria. The resistance phenotypes were determined by comparing the diameters of inhibition. Bacteria were considered resistant or sensitive when the diameters were less than equal or equal than higher of those indicated.

			Diameters (mm)		
Antibiotics	**Abbreviation**	**Concentration (µg)**	**Resistant**	**Intermediate**	**Sensitive**
Cefazolin	CZ	30	19	20-22	23
Cefuroxime	CXM	30	18	---	18
Ceftriaxone	CRO	30	20	21-22	23
Cefoxitin	FOX	30	19	---	19
Gentamicin	GM	10	14	15-16	17
Bactrim	SXT	23.75/1.25	13	14-15	16
Ciprofloxacin	CIP	5	19	20-21	22
Meropenem	MEM	10	16	17-21	22
Polymyxin	PB	300 unit	11	---	12
Chloramphenicol	C	30	17	---	17
Nalidixic Acid	NA	30	13	14-18	19
Amikacin	AN	30	13	14-15	16
Ampicillin	AM	10	14	---	14
Tetracycline	Te	30	11	12-14	15
Streptomicyn	S	10	11	12-14	15
Trimetoprim	TMP	5	15	16-17	18
Sulfamethoxazole	RL	100	18	19-21	22
Aztreonam	ATM	30	24	25-26	27
Tobramicyn	NN	10	13	14-15	16
Kanamicyn	K	1000	10	---	10
Netilmicin	NET	10	12	13-14	15

3.5 Detection of Extended Spectrum β-Lactamases (ESBLs)

The presence of ESBLs was tested by the disk diffusion method described above (Table 3.5.1) only for those strains showing resistances to the first, second and third generation of cephalosporins but susceptibility to Cefoxitin. ESBL strains show a synergy between Ceftriaxone/Augmentin and Cefotaxine/Augmentin (Figure 3.5.1) causing an increase of the inhibition diameter due to the inhibitory effect of the clavulanic acid, contained in Augmentin (Amoxicillin and Clavulanic Acid 2:1) on the ESBL enzymes. In absence of synergy but with the resistance profiles described above, the ESBL phenotype was confirmed by E-Test® (Biomèrieux) with CT/CTL (Cefotaxime/ Cefotaxime-Clavulanic Acid) (Figure 3.5.2)

Figure 3.5.1: Example of synergy between CTX/AMC. The synergy of Cefotaxine (CTX) and Augmentin (AMC) is characterized by the presence of an increasing inhibition zone between the two antibiotics (black arrow). **Modified image from Calvo J. et al 2011.**

Table 3.5.1: Antibiotics used to determine the phenotypic ESBL profile in bacteria. The concentration 20/10 μg of Augmentin is referred to Amoxicillin and Clavulanic Acid.

			Diameters (mm)		
Antibiotics	Abbreviation	Concentration (μg)	Resistant	Intermediate	Sensitive
Ceftriaxone	CRO	30	20	21-22	23
Cefoxitin	FOX	30	19	---	19
Ampicillin	AM	10	14	---	14
Cefuroxime	CX	30	19	---	19
Cefpodoxime	CPD	10	21	---	21
Augmentin	AMC	20/10	17	---	17
Aztreonam	ATM	30	24	25-26	27
Cefepime	FEP	30	21	22-23	24
Cefotaxine	CTX	5	18	19-20	21

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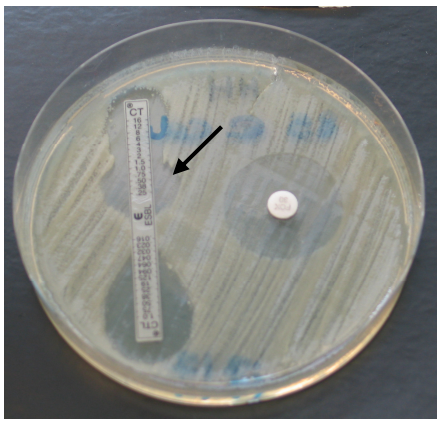


Figure 3.5.2: E-Test® CT/CTL on a *E.coli* strain. The presence of the Clavulanic Acid in the CTL reduce the resistance to the Cefotaxime content in the upper part of the strip visible as a "rounded" phantom inhibition zone (black arrow).

3.6 Plasmid extractions

Plasmid extractions were carried out by a modified alkaline/SDS lysis procedure (buffer, composition and volumes are listed in Table 3.6.1). In order to have a large amount of total plasmid extracts, plasmid extraction was performed following a "midi-prep" procedure (Plasmid DNA Purification, NucleoBond® PC100, Machery-Nagel). When little amounts were needed, plasmid extractions were executed following a "mini-prep" protocol (Plasmid DNA Purification NucleoBond® PC20, Machery-Nagel) (Table 3.6.2). Both protocols were suitable for the extraction of a wide range of plasmids, cosmids and large construct (BACs, PACs), ranging from 3 to 300 Kbp of size. An overnight bacterial culture, obtained inoculating an appropriate volume of LB medium (depending to the employed procedure), was centrifuged at 4°C, 6000 x g for 15 minutes, the supernatant was discharged and the pellet subsequently resuspended in Buffer S1+RNase A. The bacterial alkaline lysis was performed adding Buffer S2, mixing by inverting 6-8 times and incubating for 2-3 minutes at room temperature, to denature the total DNA (chromosomal and plasmidic DNA). The addition of Buffer S3 (Potassium acetate buffer) and the subsequent ice incubation for 5 minutes caused the formation of a precipitate containing the chromosomal DNA and other cellular compound (debris). The potassium acetate buffer neutralized the lysate and revert the plasmidic DNA to its negative supercoiled structure that remained in solution. The anion-exchange resins of the NucleoBond® Column were equilibrated with buffer N2. To remove the "debris", the lysates were centrifuge at 12000 x g for 10 minutes at 4°C (for "mini-prep") or filtered by NucleoBond® Folded filter (for "midi-prep"). The cleared lysates were loaded on the NucleoBond® Columns where plasmidic DNA bound to the resin; after the washing step with Buffer N3, the plasmids could be eluted with Buffer N5. Plasmidic DNA was afterwards purified by alcoholic precipitation adding 750 µL or 3.5 mL of 2-propanol and centrifuging at 15800 x g for 30 minutes at 4°C. Pellets were washed with 500 µL or 2 mL of ethanol 70% and centrifuged at 16100 x g for 10 minutes at room temperature, and dried for 10-20 minutes at room temperature. Finally, plasmid extracts were resuspended in TE buffer or PCR grade water.

Table 3.6.1: Kit contents, buffer composition and reaction volumes.

Activity	Buffer	Composition	Volumes (mL)	
			Mini	Midi
Resuspension	S1	50 mM Tris-HCl 10 mM EDTA 100 µg/mL RNase A pH 8.0	0.4	4
Lysis	S2	200 mM NaOH 1% SDS	0.4	4
Neutralization	S3	2.8 M Kac pH 5.1	0.4	4
Column Equilibration	N2	100 mM Tris 15% ethanol 900 mM KCl 0.15% Triton X-100 pH 6.3 (adjusted by H_3PO_4)	1	2.5
Wash	N3	100 mM Tris 15% ethanol 1.15 M KCl pH 6.3 (adjusted by H_3PO_4)	1 (3x)	10
Elution	N5	100 mM Tris 15% ethanol 1 M KCl pH 8.5 (adjusted by H_3PO_4)	1	5

Table 3.6.2: Correlation between bacterial culture volume to expected plasmid yield.

Copy plasmids	LB culture volume	NucleoBond® Column	Average yield
High copy	1 - 5 mL	PC20 (mini)	3 - 20 µg
	5 - 30 mL	PC100 (midi)	20 - 100 µg
Low copy	3 - 10 mL	PC20 (mini)	3 - 20 µg
	10 - 100 mL	PC100 (midi)	20 - 100 µg

3.7 PCR detection of BHR Incompatibility Groups

Plasmids of *Aeromonas spp*. and Fecal Coliforms were screened in order to detect the Broad Host Range (BHR) incompatibility groups by PCR, using the primers and the PCR conditions listed in Table 6. The PCR mix consisted in 2.5 U/reaction of Hot Start Taq (Qiagen), 2.5 µL Buffer 10X (Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7), 0.5 µL dNTPs 10 mM, 0.5 µL of each primer Forward and Reverse 10 µM, $MgCl_2$ (see Table 3.7.1), 1-5 µL of sample and water to a final volume of 25 µL.

Table 3.7.1: List of primers and PCR condition for BHR incompatibility groups. The initial denaturation and the final elongation were set at 95°C for 15 minutes and 72°C for 10 minutes for all PCR programs.

Primer Name	Primer Sequence (5'-3')	Final $MgCl_2$ concentration (mM)	PCR condition	Amplicon size (bp)	Reference
Inc A/C For Inc A/C Rev	GAGAACCAAAGACAAAGACCTGGA ACGACAAACCTGAATTGCCTCCTT	1.5	30 cycles, 94°C for 30'', 60°C for 1', 72°C for 1'	465	*Carattoli A. 2005*
Inc P For Inc P Rev	CTATGGCCCTGCAAACGCGCCAGAAA TCACGCGCCAGGGCGCAGCC	1.5	30 cycles, 94°C for 30'', 60°C for 1.5', 72°C for 1.5'	534	*Carattoli A. 2005*
Inc W For Inc W Rev	CCTAAGAACAACAAAGCCCCCG GGTGCGCGGCATAGAACCGT	1.5	30 cycles, 94°C for 30'', 60°C for 1.5', 72°C for 1.5'	242	*Carattoli A. 2005*
Inc N For Inc N Rev	GTCTAACGAGCTTACCGAAG GTTTCAACTCTGCCAAGTTC	1.5	30 cycles, 94°C for 30'', 60°C for 1.5', 72°C for 1.5'	559	*Carattoli A. 2005*
Inc U-For Inc U-Rev	CTGGCTGAAATGCTGTTGCC GCTTCATAGGCTTCACGCTC	3.75	35 cy1cles, 94°C for 1', 57°C for 1', 72°C for 1'	590	*Cattoir V. 2008*
Inc Q repB1 Inc Q repB2	TCGTGGTCGCGTTCAAGGTACG CTGTAAGTCGATGATCTGGGCGTT	3.75	35 cy1cles, 94°C for 1', 62°C for 1', 72°C for 1'	1160	*Götz A. 1996*
Inc Q oriT 1 Inc Q oriT 2	TTCGCGCTCGTTGTTCTTCGAGC GCCGTTAGGCCAGTTTCTCG	4.75	35 cy1cles, 94°C for 1', 57°C for 1', 72°C for 1'	191	*Götz A. 1996*

3.8 Bacterial conjugations

Bacterial conjugations were performed using *Aeromonas spp*. as donor and fecal coliforms or *Aeromonas spp*. as recipient. The choice of bacterial strains was made considering the presence of plasmids (at least one plasmid for the donors and no plasmids for the recipients) and the resistance phenotype of the strains. The concentration of antibiotics used for the selection of transconjugants was established according to the minimum inhibitory concentration (MIC) reported in the EUCAST and CLSI (Clinical and Laboratory Standard Institute) tables. Solid and liquid conjugations were performed in three steps: **I**, donors and recipients were grown in 2 mL of TSB for 3 hours at 30°C (*Aeromonas* spp.) and 37°C (Fecal coliforms) and spread on single, double selective and non-selective LB agar plate to check the sensitivity to the selections; **II**, donor and recipient were inoculated for 3 hours in 2 mL of TSB at the optimal temperature condition; after having reached a 0.5 McFarland concentration in 5 mL saline solution, they were spread on the non selective plate in ratio of 1:2 (100 µL and 200 µL) and 1:4 (100 µL and 400 µL) and incubated for 24 hours at 30°C. The liquid conjugations were performed inoculating the same ratio volumes in 5 mL of LB broth; **III**, after conjugation on solid media, the colonies were collected in 5 mL of saline solution up to a concentration of 3 McFarland from which 100 µL were spread on selective, double selective or non-selective LB agar plate subsequently incubated at 37°C or 30°C, if the recipient are Fecal Coliforms or *Aeromonas* spp. respectively, for 24 hours. The same amount of the liquid conjugation was spread on the selective or non selective agar plates. Transconjugated bacteria (Fecal Coliforms or *Aeromonas*) were plated for other 24 hours at

37°C or 30°C on a double selective LB agar. Antibiograms and plasmidic extractions were carried out to confirm the transfer of plasmids from the donors to the recipients.

3.9 Total DNA extraction

Total DNA was extracted from *Aeromonas spp*. and Fecal Coliforms by a boiling method in presence of matrix beads (InstaGene Matrix, Biorad). Briefly, one bacterial colony was resuspended in 200 µL of InstaGene matrix, incubated at 56°C for 30 minutes, vortexed at high speed for 10 seconds then placed in a 100°C heated block for 8 minutes and vortexed at high speed for 10 seconds. The bacterial lysates were spun at 12000 rpm for 3 minutes. Total DNA is contained in the supernatant, while the debris are bound to the matrix in the pellet.

3.10 Chemically competent bacteria ($CaCl_2$) and bacterial transformation

To increase the ability of bacteria to acquire exogenous DNA by transformation, strains were treated with $CaCl_2$. Two *E.coli* strains (DH5α, BL21) were inoculated in 3 mL of LB broth at 37°C for 24 hours. This suspension was diluted in 300 mL of LB broth and grown up to an absorbance of 0.6 (OD_{600}) for 3-5 hours at 37°C prior to incubate on ice for 10 minutes. Portions of 50 mL were centrifuged for 10 minutes at 3000 rpm at 4°C and supernatants were removed. The pellets were resuspended in 0.5 mL of LB broth to which were added 20 mL of 100 mM $CaCl_2$ and then incubated again on ice for 20 minutes. Once more, the bacterial suspensions were centrifuged for 10 minutes at 3000 rpm at 4°C and the supernatants were discarded. Pellets were resuspended in 0.5 mL, 4 mL of 100 mM $CaCl_2$ were added and incubated at room temperature (RT) for 20 minutes. The bacterial suspensions were centrifuged for 10 minutes at 3000 rpm at RT and supernatants were removed. Finally, bacteria were resuspended one last time in 0.5 mL of 100 mM $CaCl_2$ and 0.12 mL of glycerol 50%. Portions of 50 µL were frozen in liquid Nitrogen for 10 minutes and stored at -80°C.

For transformations, the competent bacteria were exposed to plasmids of environmental strains. The portions of 50 µL of chemically competent bacteria were thawed on ice. 1 to 5 µL of plasmid DNA (1 µg) was added and mixed gently. As positive control, 1µL of the plasmid pUC19 (1 µg/µL) was added to a separate vial. Vials were heated at 42°C for 30 seconds, and incubated for 2 minutes on ice. The bacteria were placed at 37°C for 1 hour after addition of 250 µL of LB broth to increase the number of transformed cells. 100 µL of the bacteria and 1:10, 1:100 dilutions were spread on a selective LB agar plate containing antibiotics and incubated for 24 hours at 37°C.

The transformation efficiencies (expressed as **Transformants/µg DNA**) were calculated by the following formula:

$$\textbf{n° colonies/pg DNA} \cdot 10^6 \textbf{pg/µg} \cdot \textbf{300 µL/ X µL plated} \cdot \textbf{dilution factor}$$

3.11 PCR amplification of Gyrase A (*GyrA*) gene

The Gyrase A gene (*GyrA*) was amplified by PCR using the total DNA extract from *Aeromonas spp*. and Fecal Coliforms. PCR were carried out using 2.5 U/reaction of Hot Start Taq (Qiagen), 2.5 µL Buffer 10X (Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7), 0.5 µL dNTPs 10 mM, 0.5 µL of each primer Forward and Reverse 10 µM, $MgCl_2$ (Table 3.11.1), 1-5 µL of DNA extract and water to a final volume of 25 µL. Primer sequences and PCR conditions are listed in Table 3.11.1.

Table 3.11.1: List of primers and condition of the PCR Gyrase A gene. The initial denaturation and the final elongation were of 95°C for 15 minutes and 72°C for 10 minutes for all PCR programs.

Primer Name	Primer Sequence (5'-3')	Final $MgCl_2$ concentration (mM)	PCR condition	Amplicon size (bp)	Reference
GyrA For GyrA Rev	TTCTATCTTGATTACGCCATG CATGCCCACCGCRATACC	3	35 cycles, 95°C for 1', 55°C for 1', 72°C for 1'	482	Goni-Urizza, 2002
GyrA Fw entero GyrA Rv entero	TCCTATCTGGAYTATGCGATG CWTMCCKACSGCGATACC	3	35 cycles, 95°C for 1', 55°C for 1', 72°C for 1'	482	This study

3.12 PCR amplification of Integrase I gene (*Int I*), 5'-3' CS and Pant-QacEΔ1

The Integrase I gene (*Int I*), the 5'-3' CS and the Pant-QacEΔ1 regions were amplified by PCR in the total plasmidic extracts from *Aeromonas spp*. and Fecal Coliforms. Primer sequences and PCR conditions are listed in Table 3.12.1. The PCR mix consisted in a 12.5 µL Taq PCR Master Mix (2.5 U/reaction Taq DNA polymerase, Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7; 200 µM of each dNTPs. Qiagen), 0.75 µL of each primer, 1-5 µL of samples and water to a final volume of 25 µL.

Table 3.12.1: Primers and PCR conditions to amplify Integrase I gene, variable region (5'-3'CS) and gene cassettes (PantF-qacEΔ1).

Primer Name	Primer Sequence (5'-3')	Final $MgCl_2$ concentration (mM)	PCR condition	Amplicon size (bp)	Reference
Int I-L Int I-R	CTGCGTTCGGTCAAGGTTCT GGAATGGCCGAGCAGATCCT	1.5	94°C for 3'; 35 cycles, 94°C for 1', 68°C for 1', 72°C for 1'; 72°C for 7'	882	Lanz, 2003
5'CS-R 3'CS-L	GATGCCCGAGGCATAGACT TGCTCACAGCCAAACTATCA	1.5	95°C for 15'; 30 cycles, 95°C for 1', 55°C for 30'', 72°C for 1.5'; 72°C for 7'	Variable	This study
Pant F qacEΔ1	GTCGAAACGGATTAAGGCACG CAAGTCTTTGCCCATGAAGC	1.5	94°C for 3'; 31 cycles, 95°C for 45'', 60°C for 45'', 72°C for 6'; 72°C for 7'	Variable	Sandvang, 1999

3.13 PCR amplification of ESBL genes

Total plasmidic extracts were amplified with specific primers to identify ESBL genes. The conditions and the primer sequences are listed in Table 3.13.1. The Hot Start Taq (Qiagen) and the Taq PCR Master Mix (Qiagen) were used to amplify the following genes: bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{OXA}.

Table 3.13.1: Primers and PCR conditions for OXA, SHV, TEM and CTX-M genes.

Primer Name	Primer Sequence (5'-3')	Final $MgCl_2$ concentration (mM)	PCR condition	Amplicon size (bp)	Reference
OXA-F OXA-R	ATATCTCTACTGTTGCATCTCC AAACCCTTCAAACCATCC	1.5	95°C for 5'; 32 cycles, 94°C for 30'', 54°C for 30'', 72°C for 1'; 72°C for 10'	619	Colom, 2003
SHV-A SHV-B	ATGCGTTATWTTCGCCTGTGT TTAGCGTTGCCAGTGCTCG	1.5	95°C for 3'; 30 cycles, 95°C for 30'', 55°C for 30'', 72°C for 1'; 72°C for 10'	821	Mammeri, 2005
PRE-TEM-1 PRE-TEM-2	GTATCCGCTCATGAGACAATA TCTAAAGTATATATGAGTAAACTTGGTCTG	1.5	95°C for 3'; 30 cycles, 95°C for 30'', 55°C for 30'', 72°C for 1'; 72°C for 10'	1000	Mammeri, 2005
CTX-M A1 CTX-M A2	SCSATGTGCAGYACCAGTAA CCGCRATATGRTTGGTGGTG	1.5	95°C for 3'; 30 cycles, 95°C for 30'', 55°C for 30'', 72°C for 1'; 72°C for 10'	504	Lartigue, 2007

3.14 PCR clean-up and gel extraction

Amplicons were purified before sequencing using the PCR Clean-up (Macherey-Nagel) kit, while DNA was extracted from gels with the NucleoSpin® Gel (Macherey-Nagel) following the manufacturer's instructions. The PCR products were purified as reported in the user manual. Due the excessive size of the plasmids, the extracts were purified increasing the amount of samples loaded in the column.

200 µL of buffer NT1 were added to 100 µL of PCR product or 100 mg of agarose gel. For the gel extraction the samples were incubate for 10 minutes at 50°C. Samples are then loaded into the NucleoSpin® columns and centrifuged for 30 seconds at 11000 x g. The flow-through is discarded. The silica membranes are washed twice with 700 µL of buffer NT3, centrifuged

for 30 seconds at 11000 x g, discharging the flow-through, and dried for 1 minute at 11000 x g to remove the buffer. The DNA was eluted by addition of 15-30 µL of buffer NE (5 mM Tris/HCl, pH 8.5), followed by an incubation at room temperature for 1 minute (70° for large fragments) and centrifuge for 1 minute at 11000 x g.

3.14.1 Sequencing

Sequencing was carried out using BigDye Terminator® v3.1 (Applied Biosystems). The reactions were carried out with 1µL of BigDye Terminator®, 1.5 µL of 5X BigDye Buffer, DNA as listed in Table 3.14.1.1, 2 µL of primer forward or reverse 1 µM and water to a final volume of 10 µL. The thermal cycling was set with a denaturation step at 96°C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

Table 3.14.1.1: Amount of PCR product to use in a sequencing reaction based on the size of the amplicon.

Template	Quantity
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	20–50 ng

Products were purified by Illustra™ Sephadex™ G50 Fine DNA Grade (GE Healthcare) and were sequenced with the addition of 5 µL HiDi™ Formamide (Applied Biosystems) on a genetic Analyzer ABI/Hitachi 3500 (Applied Biosystems) (figure 3.14.1.1).

Figure 3.14.1.1: Analyzer ABI/Hitachi 3500. Image from http://www.chikyu.ac.jp/laboratories/public_E/instruments_E/DNAseq_E.html.

Data were analyzed by the 3500 Data Collection Software v.1.00 (Applied Biosystems) and multiple alignments were performed using MEGA version 4 (Molecular Evolutionary Genetics Analysis) **(Tamura et al., 2007)**.

3.15 Plasmid curing

Plasmid curing was performed on *Aeromonas* spp. following five methods: plate curing, 10% SDS curing, Minimal medium curing, water curing and acridine orange curing. The first method consisted in plating repeatedly and growing at 30°C on COL-S 5% SB agar a single bacterial colony. This treatment was repeated every 48 hours for eight weeks. The second method was performed incubating a single bacterial colony in 5 mL of MH broth with 10% SDS (modified method of **Molina-Aja et al., 2002**) for 24 hours at 30°C and plating 100 µL of this suspension on COL-S 5% SB agar for other 24 hours at 30°C. The third method was carried out growing the bacteria in 5 mL of minimal medium (Table 15) for one week at 15°C. The fourth method consisted in the inoculation of one colony in filtered 0.45µm (0.45µm-pore size filter, Millipore) water lake and grown for one week at 15°C. The last method (modified from **Molina-Aja et al., 2002**) was executed by inoculation of the bacteria in LB broth containing different concentration of acridine orange (50, 75, 100 and 125 µg/mL) and growing for 24 hours at 30°C. After each treatment, bacteria were submitted to an antibiotic susceptibility test, and in case of resistance variations, the plasmids were extracted and visualized through electrophoresis in agarose gel at 0.7%.

3.16 PCR amplification of Mobilizable Groups (MOBs)

Total plasmid extracts were amplified using the specific MOB subfamily primers and the conditions listed in Table 3.16.1. The polymerase chain reactions were carried out using Taq PCR Master Mix (Qiagen) in a final volume of 25 µL.

Table 3.16.1: Primer sequences and MOB PCR condition. All the primers shown degenerate bases in 3' terminal region to include most subfamily members.

Primer Name	Primer Sequence (5'-3')	PCR condition	Amplicon size (bp)	Reference
H121-f H121-r	GCCAGCTTCCGAATCACAYCAYCAYCG GTCGCTTGTCGCGCCACCGDATRAARTA	94°C for 4'; 25 cycles, 94°C for 30'', 59°C for 30'', 72°C for 30''; 72°C for 10'	313	Alvarado, 2012
F12-f F1-r	AGCGACGGCAATTATTACACCGACAAGGAYAAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	94°C for 4'; 25 cycles, 94°C for 30'', 55°C for 30'', 72°C for 30''; 72°C for 10'	234	Alvarado, 2012
P12-f P1-r	GCACACTATGCAAAAGATGATACTGAYCCYGTTTT ACGGATGTGGATGTGAAGGTTRTCNGTRTC	94°C for 4'; 30 cycles, 94°C for 30'', 53.8°C for 30'', 72°C for 30''; 72°C for 10'	189	Alvarado, 2012
P131-f P1-r	AACCCACGCTGCAARGAYCCVGT ACGGATGTGGATGTGAAGGTTRTCNGTRTC	94°C for 4'; 30 cycles, 94°C for 30'', 59°C for 30'', 72°C for 15''; 72°C for 10'	180	Alvarado, 2012
P14-f P1-r	CGCAGCAAGGACACCATCAAYCAYTAYRT ACGGATGTGGATGTGAAGGTTRTCNGTRTC	94°C for 4'; 25 cycles, 94°C for 30'', 50°C for 30'', 72°C for 30''; 72°C for 10'	174	Alvarado, 2012
H11-f H11-r	CCGGCGTCGGAGAAYCAYCAYCA AAGGTCGTATACCTTYCCKGCRTCRTG	94°C for 4'; 11 cycles, 94°C for 30'', start at 65°C ΔTa=-1 for 30'', 72°C for 30''; 15 cycles, 94°C for 30'', 55°C for 30'', 72°C for 30''; 72°C for 10'	207	Alvarado, 2012
F11-f F1-r	GCAGCGTATTACTTCTCTGCTGCCGAYGAYTAYTA ACTTTTGGGCGCGGARAABTGSAGRTC	94°C for 4'; 25 cycles, 94°C for 30'', 53°C for 30'', 72°C for 30''; 72°C for 10'	234	Alvarado, 2012

3.17 Dot blotting

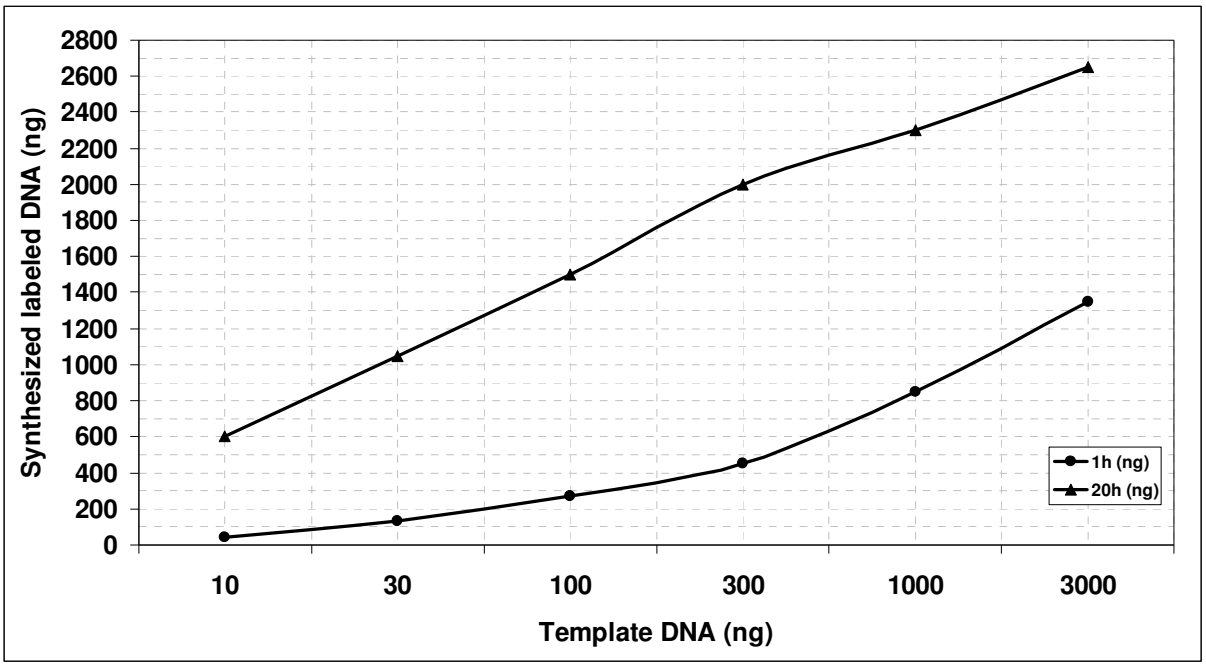
Dot-Blot experiments were performed using the DIG High Prime DNA Labeling and Detection Starter Kit I and DIG Wash and Block Buffer Set (Roche Applied Science). Buffers and contents are listed in Table 3.17.1. Purified DNA probes were labeled with Digoxigenin and used to identify by hybridization the target genes either on plasmids and on chromosomal DNA, which were spotted on a nylon membrane.

Table 3.17.1: Kit contents and buffers composition.

Reagents/Buffers	Composition
DIG-High Prime (5x)	Random primers Nucleotides DIG-dUTP (alkali-labile)
DIG-labeled Control DNA	pBR328 (linearized with BamHI) 5 µg/mL
DNA dilution buffer	Fish sperm DNA 50 µg/mL Tris-HCl 10 mM EDTA 1 mM pH 8.0
Anti-Digoxigenin-AP conjugate	Fab-fragment (sheep) 750 U/mL
NBT/BCIP (50x)	nitroblue tetrazolium chloride 18.75 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate 9.4 mg/mL DMSO 67% (v/v)
DIG Easy Hyb granules	not specified
Washing buffer	Maleic Acid 0.1 M NaCl 0.15 M pH 7.5 Tween-20 0.3% (v/v)
Maleic Acid buffer	Maleic Acid 0.1 M NaCl 0.15 M pH 7.5 (adjusted with NaOH solid)
Detection buffer	Tris-HCl 0.1 M NaCl 0.1 M pH 9.5

3.17.1 Digoxigenin-DNA labeling

DNA was labeled with Digoxigenin-11-dUTP by DIG-High Prime. From 10 ng to 3 µg of amplicon was added to double distilled water to a final volume of 16 µL. The DNA was denatured by heating in a boiling bath for 10 minutes and quickly chilled on ice/water bath. 4 µL of DIG-High Prime were added to the samples and incubated for 20 hours at 37°C in a thermocycler (Verity, Applied Biosystems). Reactions were blocked adding 2 µL of EDTA 0.2 M (pH 8.00) and incubated for 10 minutes at 65°C. The theoretical amount of probes was extrapolated by means of the graphic 3.17.1.1.



Graphic 3.17.1.1: Theoretical correlation between the amount of template DNA and synthesized labeled probe. The two curves represent the theoretical increase of the amount (in ng) of probe from 1 hour (dots) to 20 hours (triangles) of synthesis.

3.17.2 Determination of labeling efficiency

The determination of the labeling efficiency is an important step to perform reproducible results. A too high or too low probe concentrations could cause respectively background or weak signals. Labeled probes and positive control (pBR328 linearized with *BamHI*) were diluted up to 1 ng/µL, according to the theoretical probes concentration, and used as start samples for the subsequent dilutions (Table 3.17.2.1).

Table 3.17.2.1: Volumes and final concentrations of labeled probes and positive control dilutions.

Tube	DNA (µL)	From Tube	DNA Dilution Buffer (µL)	Dilution	Final Concentration
1		Diluited original	-	-	1 ng/uL
2	2	1	198	1:100	10 pg/uL
3	15	2	35	1:333	3 pg/uL
4	5	2	45	1:1000	1 pg/uL
5	5	3	45	1:3333	0.3 pg/uL
6	5	4	45	1:10000	0.1 pg/uL
7	5	5	45	1:33333	0.03 pg/uL
8	5	6	45	1:100000	0.01 pg/uL
9	-	-	50	-	0

One micro-liter (1 µL) of dilutions 2 to 9 (see Table 3.17.2.1) of the labeled probes and of the positive control were applied to the nylon membrane (Roche). Nucleic acids were fixed for 1 hour at 100°C in an air incubator. Then, the membrane was transferred in a plastic container with 20 mL of Maleic Acid buffer, incubated under shaking for 2 minutes at room temperature and subsequently treated with 10 mL of Blocking buffer for 30 minutes. The immunological detection was carried out adding 10 mL of Antibody solution (1:5000) for 30 minutes. The membrane was washed twice with 10 mL of Washing buffer for 10 minutes. To visualize the spot, the membrane was treated with 2 mL of Color substrate solution freshly prepared. The reaction was stopped after 2 or 2.5 hours with 50 mL of PCR grade water. If 0.1 pg dilution spots of the probe and the positive control were visible, then the labeled probes had the same concentration as this calculated theoretically.

3.17.3 DNA transfer, fixation and hybridization

Total plasmid extracts or chromosomal DNAs were boiled for 10 minutes, chilled in ice/water bath, and spotted (1 or 2 µL respectively) on a nylon membrane placed on a soaked 3MM paper with 10x SSC. After the fixation of the samples in an air incubator for 1 hour at 100°C, the membrane was washed briefly in water and dried at room temperature. The appropriate hybridization temperatures (Table 3.17.3.1) were calculated according to the CG content and the homology of the probes to their target according to the following equations:

$$\mathbf{T_m = 49.82 + 0.41\ (\%\ C + G) - (600/l)}$$

$$\mathbf{T_{opt} = Tm - 20\ to\ 25°C}$$

where T_m is the melting temperature of the probe, l is the length of the hybrid in base pair and T_{opt} is the optimum temperature used for the hybridization (high or low stringency).

Table 3.17.3.1: Probes and optimal temperatures to hybridize. The stringency used in the hybridization experiments is indicated.

Probes	%G+C	length (bp)	Tm (°C)	Topt (°C)	Stringency
MOB H121	**57**	**326**	**71**	**51**	**High**
MOB F12	**59**	**234**	**71**	**49**	**Middle**
MOB P12	**48**	**189**	**66**	**44**	**Middle**
MOB P13	**47**	**180**	**66**	**43**	**Middle**
MOB P14	**55**	**174**	**69**	**46**	**Middle**
MOB H11	**45**	**207**	**65**	**43**	**Middle**
IncU	**57**	**590**	**72**	**50**	**Middle**
Integrase I	**61**	**882**	**74**	**49**	**Low**

The DIG Easy Hyb buffer was pre-heat to the appropriate hybridization temperature (10mL/100cm^2 filter) and used to prehybridize the nylon membrane for 30 minutes with gentle agitation. The DIG-labeled probes were boiled for 5 minutes at 100°C in a water bath and rapidly cooled in ice/water. Denatured probes were added to the pre-heated DIG Easy Hyb buffer (3.5 mL/100 cm^2 filter) to a final concentration of 25 ng/mL. The prehybridization solution was poured off and replaced by the probe/hybridization mixture. Hybridization was carried out overnight with gentle agitation.

3.17.4 Immunological detection

The hybridized membrane was washed twice with 25 mL of 2x SSC, 0.1% SDS at room temperature for 5 minutes to remove the unbound probes, then twice with high stringency washing made of 25 mL 0.5x SSC, 0.1% SDS at 68°C for 15 minutes to remove the weakly bound probes. The incubation steps for the immunological detection took place at room temperature with agitation. After the high stringency washings, the membranes were rinsed in Washing buffer for 5 minutes and subsequently incubated with 100 mL of Blocking solution for 30 minutes. Afterwards membranes were incubated with 20 mL of Antibody solution for 30 minutes and washed twice with 100 mL of Washing buffer for 15 minutes.

The detection of positive samples were performed incubating the membranes in 10 mL of freshly prepared Color substrate solution in the dark, and the reactions were stopped after 2.5 hours washing the membranes for 5 minutes in 50 mL of PCR grade water. Results were recorded in a tif format picture.

3.18 Culture media and solution.

Table 3.18.1: Solution, medium and broth.

Solution/Medium/Broth	Reagents	Amount	Sterilization	Cooling
m-Aeromonas selective agar base	Tryptose	5 g		
	Yeast Extract	2 g		
	Dextrin	11.4 g		
	Sodium Chloride	3 g		
	Potassium Chloride	2 g		
	Magnesium Sulphate	0.1 g		
	Ferric Chloride	0.06 g		
	Sodium Desoxycholate	0.1 g		
	Bromothymol Blue	0.08 g		
	Agar	13 g		
	H_2O	up to 1L		
	---		121°C for 15'	40-45°C
	Ampicillin	10 µg/mL		
C-EC MUG Agar	Peptone	5 g	121°C for 15'	40-45°C
	Tryptophan	1 g		
	Tryptose	10 g		
	Yeast Extract	3 g		
	Bile Salts n.3	1.5 g		
	Sodium Chloride	5 g		
	IPTG	0.1 g		
	X-GAL	0.08 g		
	MUG	0.05 g		
	Agar	13 g		
	H_2O	up to 1L		
	pH	7.4 ± 0.1 (25°C)		
Peptone water 0.1%	Bacteriological Peptone	1 g	121°C for 15'	
	Distilled water	up to 1L		
Skim-Milk	Skim-Milk	10 g		
	H_2O	70 mL		
	---		121°C for 15'	
	Calf serum	10 mL		
	Glycerin	20 mL		
TSB	Trypticase Soy Broth	30 g	121°C for 15'	
	H_2O	up to 1L		
Saline solution	NaCl	9 g	121°C for 15'	
	H_2O	up to 1L		

Continued…

Solution/Medium/Broth	Reagents	Amount	Sterilization	Cooling
Mueller Hinton agar	**Mueller Hinton Agar**	**38 g**	**121°C for 15'**	
	H_2O	**up to 1L**		
Mueller Hinton broth-10% SDS	**Mueller Hinton pounder**	**38 g**		
	H_2O	**up to 1L**		
	---		**121°C for 15'**	**20°C**
	SDS	**100 g**		
α-cyano	**α-cyano-4-hydroxy-cinnamic acid, 97%**	**40 mg**		
	acetonitrile	**330 µL**		
	Ethanol	**330 µL**		
	H_2O	**330 µL**		
	Trifluoroacetic Acid	**30 µL**		
Minimal medium	**TSB**	**100 mL**		
	NaCl solution	**900 mL**		
EDTA 0.2 M	**EDTA**	**37.224 g**	**121°C for 15'**	
	H_2O	**500 mL**		
	pH	**8.00**		
TE	**Tris HCl 1 M**	**10 mL**	**121°C for 15'**	
	EDTA 0.5 M	**2 mL**		
	H_2O	**up to 1 L**		
20x SSC	**Sodium Citrate**	**88.2 g**	**121°C for 15'**	
	NaCl	**175.3 g**		
	H_2O	**up to 1 L**		
	pH	**7.00**		
2x SSC, 0.1% SDS	**20x SSC**	**5 mL**		
	SDS	**50 mg**		
	H_2O	**45 mL**		
0.5x SSC, 0.1% SDS	**20x SSC**	**1.25 mL**		
	SDS	**50 mg**		
	H_2O	**48.75 mL**		

4 RESULTS

4.1 Sampling

The result of the quantification of *Aeromonas* and Fecal Coliforms, expressed in CFU/100 mL, in the various samples is summarized in Table 4.1.1.

The *Aeromonas* strains were recovered from the five sampling areas with concentration ranging from $2 \cdot 10^3$ (Ticino river before the wastewater treatment plant) to $3 \cdot 10^6$ (activated sludge of the wastewater treatment plant in Giubiasco) CFU in 100 mL. The Fecal Coliforms concentration reached $2.07 \cdot 10^6$ CFU/100 mL in the Bellinzona Hospital wastewaters, whereas they were 3 CFU/100 mL in the Lake Cadagno. The ratio between the two bacterial groups was higher in the Lake Cadagno (about 1283), while it was under the unit (0.7005) in the wastewaters of Bellinzona Hospital where the Fecal Coliforms are predominant.

We isolated a total of 231 Aeromonas strains and 250 Fecal Coliforms which were identified by MALDI-TOF MS. The most prevalent *Aeromonas* species were *A. hydrophila*, *A. media*, *A. caviae*, and *A. salmonicida* (Table 4.1.2). In the Bellinzona Hospital sampling, *A. hydrophila* represented the 84.78% of the identified species, while in the Ticino River before and after the wastewater treatment plant the species *A. media* counted for the 57.14% and 45.65%, respectively; this species was predominant also in the Giubiasco waste water treatment plant representing the 51.22% of the *Aeromonas* species. On the other hand, the principal specie collected in Lake Cadagno was *A. salmonicida* (87.76%).

The most representative species belonging to the Fecal Coliforms group was *Escherichia coli*, which was highly present at each sampling site (Table 4.1.3). The recovery of *Klebsiella pneumoniae* and *Enterobacter cloacae* was also significant in the waste water treatment plant (34% and 20% respectively). *K. pneumoniae* represented the 22% of the species isolated from the Ticino river after the treatment plant of Giubiasco.

Table 4.1.1: Concentrations of *Aeromonas* spp. and Fecal Coliforms, expressed in CFU/100 mL (Colony Forming Units in 100 mL), and their relative ratios in the five sampling areas. The colored squares refer to the sampling areas indicated on the map shown in "Material and Methods". WW.T.P, wastewater treatment plant.

	CFU/100mL		
	Aeromonas	Fecal Coliforms	Ratio (Aer/FC)
TICINO RIVER BEFORE THE WW.T.P	2*10^3	1.65*10^2	12.1212
BELLINZONA HOSPITAL	1.45*10^6	2.07*10^6	0.7005
WW.T.P. GIUBIASCO	3*10^6	7.05*10^5	4.2553
TICINO RIVER AFTER WW.T.P.	3.7*10^4	1.63*10^4	2.2699
LAKE CADAGNO	3.85*10^3	3	1283.34

Table 4.1.2: *Aeromonas* spp. identified in the five sampling areas.

	AEROMONAS				
	TICINO RIVER before WW.T.P.	BELLINZONA HWW	WW.T.P GIUBIASCO	TICINO RIVER after WW.T.P.	CADAGNO LAKE
SPECIES	Percentage	Percentage	Percentage	Percentage	Percentage
A.bestiarum	10.20	0.00	0.00	2.17	0.00
A.caviae	12.24	2.17	17.07	32.61	0.00
A.hydrophila	6.12	84.78	29.27	17.39	0.00
A.media	57.14	4.35	51.22	45.65	4.08
A.salmonicida	4.08	6.52	2.44	0.00	87.76
A.sobria	10.20	0.00	0.00	0.00	0.00
A.veronii	0.00	2.17	0.00	2.17	0.00
A.eucrenophila	0.00	0.00	0.00	0.00	6.12
A.popoffii	0.00	0.00	0.00	0.00	2.04

Table 4.1.3: Fecal Coliforms identified in the five sampling areas.

	FECAL COLIFORMS				
	TICINO RIVER before WW.T.P.	BELLINZONA HWW	WW.T.P GIUBIASCO	TICINO RIVER after WW.T.P.	CADAGNO LAKE
SPECIES	Percentage	Percentage	Percentage	Percentage	Percentage
E.coli	80.00	90.00	34.00	64.00	90.00
E.cloacae	2.00	2.00	20.00	2.00	4.00
C.freundii	4.00	2.00	8.00	12.00	2.00
E.hermannii	2.00	0.00	0.00	0.00	0.00
K.pneumoniae	10.00	6.00	34.00	22.00	0.00
K.oxytoca	2.00	0.00	4.00	0.00	0.00
P.shigelloides	0.00	0.00	0.00	0.00	4.00

4.2 Plasmid profiles

Plasmids were extracted from 231 *Aeromonas* spp. and 250 Fecal Coliforms. The plasmid profiles were compared using the BioNumerics software (Applied Maths, NV), that allowed to identify six major groups based on their complexity (Figure 4.2.1). The blue group clusters the plasmid profiles showing the higher number of bands (1 to 300 kbp), whereas bacteria without plasmids belong to the light blue group (61 strains). The others groups shown different intermediate range of complexity as plasmids with predominantly high molecular weight (over 23 kbp, red group), plasmids with molecular weight ranging from 1 to 170 kbp (yellow group), one or two plasmids with an intermediate molecular weight (approx. 23 kbp, purple group), and plasmids with predominantly medium/low molecular weight (1 to 23 kbp, green group).

Some bacteria, belonging to different genera and species, shared similar or identical profiles (see e.g. Figure 4.2.2). The distribution (in percentages) of the *Aeromonas* and Fecal Coliforms strains in the different plasmid complexity groups is reported in Table 4.2.1. The origin of the strains was related to the complexity of the plasmid profiles. *Aeromonas* strains isolated from the Ticino River before the treatment plant outlet belonged mostly to the blue and green groups (30.61% and 26.53%, respectively), whereas those isolated from the same river but after the treatment plant belonged to the blue group (58,70%). Most of the *Aeromonas* spp. isolated from the Bellinzona Hospital wastewater clustered in the yellow group (63.04%); the majority of those from the wastewater treatment plant ended in the blue and green groups (39.02% and 26.83%); *Aeromonas* strains from the Lake Cadagno belonged to the purple group(73.47%). The Fecal Coliforms of the two river samplings were distributed in the groups blue (26,00% and 30,00%) and green (28.00% of the strains isolated before the WWTP); in the yellow group were clustered 26.00% of the Fecal Coliforms isolated from the river before the WWTP but only the 12,00% of those isolated after the WWTP. The Fecal Coliforms of the Bellinzona Hospital wastewater were mainly present in the yellow group (28.00%). The Fecal Coliforms isolated from the wastewater treatment plant clustered in the blue (38.00%) and those of the Lake Cadagno in the green (56.00%) groups.

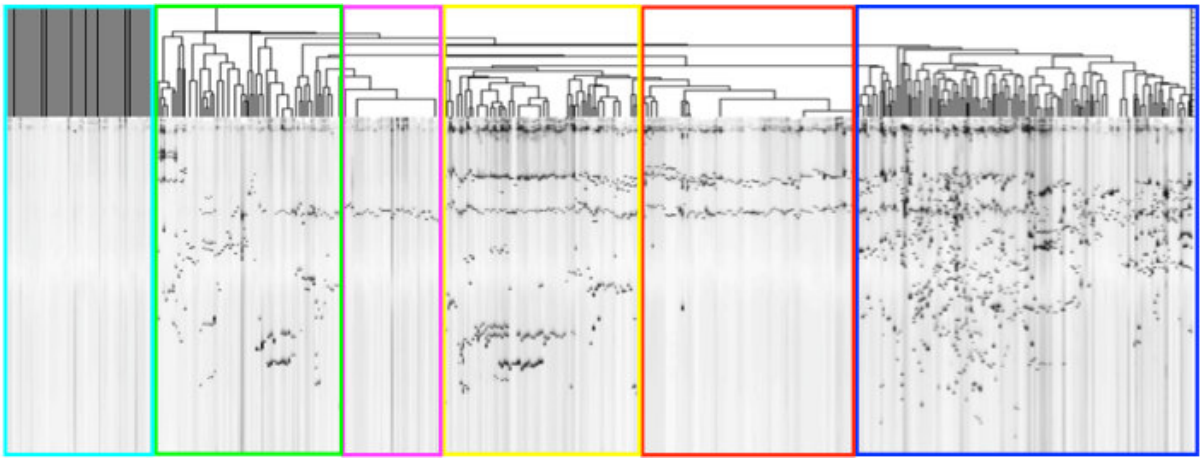


Figure 4.2.1: *Aeromonas* and Fecal Coliforms plasmid profiles. The Blue group includes the most complex plasmid profiles; the Yellow and Green groups comprise intermediate complex profiles; the Purple and Red groups include strains with only one or two bands, respectively; the Light blue group comprises the strains without visible bands on gel.

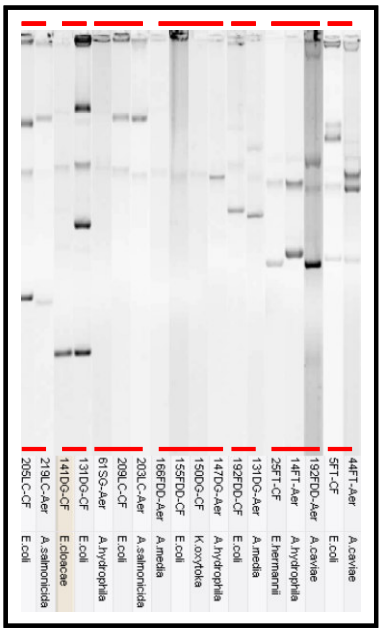


Figure 4.2.2: example of similar or identical plasmidic profiles. Red lines indicated common profiles

Table 4.2.1: Distribution of *Aeromonas* spp. and Fecal Coliforms in the six groups of plasmid complexity. In the Table are indicated the percentage of the strains per group, the total number of bacteria per group, and the six colored squares, representing the groups of plasmidic complexity.

		TICINO RIVER BEFORE WW.T.P.		BELLINZONA HWW		WW.T.P. GIUBIASCO		TICINO RIVER AFTER WW.T.P.		LAKE CADAGNO	
		Aer	F.C.	Aer	F.C.	Aer	F.C.	Aer	F.C.	Aer	F.C.
	Strains	49	50	46	50	41	50	46	50	49	50
		Percentage (%)		Percentage (%)		Percentage (%)		Percentage (%)		Percentage (%)	
	120	30.61	26.00	4.35	18.00	39.02	38.00	58.70	30.00	0.00	8.00
	40	10.20	8.00	2.17	12.00	4.88	14.00	4.35	14.00	4.08	8.00
	89	12.24	26.00	63.04	28.00	12.20	8.00	2.17	12.00	2.04	20.00
	61	2.04	6.00	2.17	12.00	4.88	10.00	2.17	12.00	73.47	0.00
	110	26.53	28.00	13.04	12.00	26.83	14.00	21.74	12.00	18.37	56.00
	61	18.37	6.00	15.22	18.00	12.20	16.00	10.87	20.00	2.04	8.00

4.3 Resistance phenotypes

The resistance phenotypes were established by the Kirby-Bauer method for all the *Aeromonas* spp. and Fecal Coliforms. The antibiograms conducted on the 481 strains were based on 20 antibiotics (Table 4.3.1). The majority of the resistances observed were Cefazolin (66.11%), Streptomycin (31.81%), Nalidixic Acid (30.98%), Sulfamethoxazole (25.57%), and Cefoxitin (21.41%). As genus *Aeromonas* are constitutively resistant to Ampicillin, this antibiotic was tested only on the Fecal Coliforms: the 16.22% (78 out of 481) of the strains were resistant. However, all the *Aeromonas* strains were sensitive to the Amikacin while the Fecal Coliforms were not tested for this antibiotic.

The resistant phenotypes of the strains according to the six groups of plasmid complexity, are listed in the Table 4.3.2.

Of the 481 strains tested, 198 (namely the 41.16%) presented three or more antibiotic resistances; 189 (namely the 39.29%) had one or two resistances, and 94 (19.54%) were sensitive to all the antibiotics tested. Most of the strains with three or more resistances were included in the blue group (58.33%), while the majority of the strains with one or two resistances were comprised in the purple group (67.21%). Strains without any antibiotic resistances were spread along the six groups with different percentages: from the 25.00% in the red group to the 13.11% in the purple one. The distribution of the strains according to the number of antibiotic resistances is shown in Table 4.3.3.

In Table 4.3.4 are reported the percentages of *Aeromonas* spp. and Fecal Coliforms carrying plasmids. In the five sampling areas the percentages of *Aeromonas* spp. carrying plasmids were comprised between the 81.63% of the Ticino River before the wastewater treatment plant and the 97.96% of the Cadagno Lake. For the Fecal Coliforms, the strains with plasmids ranged from 80.00% to 96.00% of the river after and before the treatment plant. In the Lake Cadagno the 92.00% of the strains carried plasmids.

Only a portion of the strains with plasmids shown three or more antibiotic resistances, as reported in Table 4.3.5. The strains carrying plasmids and showing three or more antibiotic resistances were particularly abundant in the wastewater treatment plant in Giubiasco (87.80% and 40.00% for *Aeromonas* spp. and Fecal Coliforms, respectively), while they were rarely isolated in the Lake Cadagno (2.04% and 18.00% for *Aeromonas* spp. and Fecal Coliforms, respectively).

Table 4.3.1: Number of resistant strains to each antibiotic, and relative percentages.. CZ, Cefazolin; CXM, Cefuroxime; CRO, Ceftriaxone; FOX, Cefoxitin; GM, Gentamycin; SXT, Bactrim; CIP, Ciprofloxacin; MEM, Meropenem; PB, Polymyxin B; C, Chloramphenicol; NA, Nalidixic Acid; AN, Amikacin; AMP, Ampicillin; RL, Sulfamethoxazole; Te, Tetracycline; S, Streptomycin; TMP, Trimethoprim; ATM, Aztreonam; NN, Tobramycin; K, Kanamycin; NET, Netilmicin.

	CZ	CXM	CRO	FOX	GM	SXT	CIP	MEM	PB	C	NA
Strains	318	23	21	103	7	24	46	0	2	12	149
Percentages	66,11	4,78	4,37	21,41	1,46	4,99	9,56	0,00	0,42	2,49	30,98

	AN	AMP	RL	Te	S	TMP	ATM	NN	K	NET
Strains	0	78	123	32	153	29	11	17	1	5
Percentages	0,00	16,22	25,57	6,65	31,81	6,03	2,29	3,53	0,21	1,04

Table 4.3.2: *Aeromonas* spp. and Fecal Coliforms resistant phenotypes. The names, the sampling areas, and the specie of the *Aeromonas* spp. and Fecal Coliforms are listed and divided in the six group of plasmid complexity (colored ribbons). The resistances to the antibiotics are indicated by the black squares. The gray squares represent the antibiotics not tested.

Groups	Key	Origins	Species	Resistances: CZ	CXM	CRO	FOX	GM	SXT	CIP	MEM	PB	C	NA	AN	AMP	RL	Te	S	TMP	ATM	NN	K	NET
	190FDD-CF	TR-a-WWTP	*K.pneumoniae*																					
	24FT-CF	TR-b-WWTP	*K.pneumoniae*				■									■	■		■					
	129DG-CF	WWTP	*K.pneumoniae*																					
	104DG-CF	WWTP	*K.pneumoniae*													■								
	130DG-CF	WWTP	*E.coli*																					
	199FDD-CF	TR-a-WWTP	*E.coli*																■					
	53SG-CF	HWW	*K.pneumoniae*													■								
	169FDD-CF	TR-a-WWTP	*K.pneumoniae*													■			■					
	182FDD-CF	TR-a-WWTP	*K.pneumoniae*													■	■		■					
	233LC-CF	CL	*E.coli*	■															■					
	105DG-CF	WWTP	*K.pneumoniae*														■							
	154FDD-CF	TR-a-WWTP	*K.pneumoniae*													■								
	163FDD-CF	TR-a-WWTP	*E.coli*																					
	111DG-CF	WWTP	*E.coli*																					
	134DG-CF	WWTP	*K.pneumoniae*																■					
	165FDD-CF	TR-a-WWTP	*E.coli*																					
	132DG-CF	WWTP	*K.pneumoniae*													■	■		■					
	148DG-CF	WWTP	*K.pneumoniae*																					
	146DG-CF	WWTP	*K.pneumoniae*																					
	171FDD-CF	TR-a-WWTP	*E.coli*																					
	128DG-CF	WWTP	*E.coli*	■						■				■		■	■		■					
	22FT-CF	TR-b-WWTP	*E.coli*	■					■							■	■			■				
	73SG-CF	HWW	*E.coli*	■												■								
	80SG-CF	HWW	*E.coli*	■	■		■	■	■	■			■	■		■	■	■	■	■		■		■
	45FT-CF	TR-b-WWTP	*E.coli*	■												■	■							
	88SG-CF	HWW	*E.coli*																					
	138DG-CF	WWTP	*K.pneumoniae*	■													■							
	139DG-CF	WWTP	*K.pneumoniae*																					
	16FT-Aer	TR-b-WWTP	*A.media*	■										■			■		■					
	109DG-Aer	WWTP	*A.eucrenophila*	■										■										
	77SG-Aer	HWW	*A.hydrophila*	■	■	■		■		■				■			■		■		■	■		■

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85SG-CF	HWW	*E.coli*	
179FDD-Aer	TR-a-WWTP	*A.caviae*	
23FT-CF	TR-b-WWTP	*E.coli*	
11FT-CF	TR-b-WWTP	*K.pneumoniae*	
195FDD-CF	TR-a-WWTP	*K.pneumoniae*	
137DG-CF	WWTP	*K.pneumoniae*	
120DG-CF	WWTP	*E.cloacae*	
161FDD-CF	TR-a-WWTP	*E.coli*	
32FT-CF	TR-b-WWTP	*E.coli*	
101DG-CF	WWTP	*K.pneumoniae*	
32FT-Aer	TR-b-WWTP	*A.bestiarum*	
8FT-Aer	TR-b-WWTP	*A.media*	
7FT-Aer	TR-b-WWTP	*A.sobria*	
86SG-CF	HWW	*E.coli*	
105DG-Aer	WWTP	*A.caviae*	
39FT-Aer	TR-b-WWTP	*A.media*	
153FDD-Aer	TR-a-WWTP	*A.media*	
155FDD-Aer	TR-a-WWTP	*A.media*	
195FDD-Aer	TR-a-WWTP	*A.veronii*	
231LC-CF	CL	*E.coli*	
180FDD-Aer	TR-a-WWTP	*A.hydrophila*	
44FT-Aer	TR-b-WWTP	*A.caviae*	
5FT-CF	TR-b-WWTP	*E.coli*	
42FT-Aer	TR-b-WWTP	*A.media*	
1FT-Aer	TR-b-WWTP	*A.media*	
6FT-Aer	TR-b-WWTP	*A.media*	
181FDD-Aer	TR-a-WWTP	*A.media*	
117DG-Aer	WWTP	*A.media*	
101DG-Aer	WWTP	*A.caviae*	
133DG-Aer	WWTP	*A.hydrophila*	
5FT-Aer	TR-b-WWTP	*A.hydrophila*	
97SG-Aer	HWW	*A.media*	
151FDD-CF	TR-a-WWTP	*E.coli*	
218LC-CF	CL	*E.coli*	
151FDD-Aer	TR-a-WWTP	*A.caviae*	
180FDD-CF	TR-a-WWTP	*K.pneumoniae*	
199FDD-Aer	TR-a-WWTP	*A.hydrophila*	
123DG-CF	WWTP	*E.cloacae*	
102DG-CF	WWTP	*K.pneumoniae*	
14FT-Aer	TR-b-WWTP	*A.hydrophila*	
192FDD-Aer	TR-a-WWTP	*A.caviae*	
25FT-CF	TR-b-WWTP	*E.hermannii*	
102DG-Aer	WWTP	*A.media*	
171FDD-Aer	TR-a-WWTP	*A.media*	
135DG-Aer	WWTP	*A.media*	
50FT-CF	TR-b-WWTP	*E.coli*	
139DG-Aer	WWTP	*A.hydrophila*	
143DG-Aer	WWTP	*A.hydrophila*	
3FT-CF	TR-b-WWTP	*K.pneumoniae*	
43FT-CF	TR-b-WWTP	*E.coli*	
38FT-Aer	TR-b-WWTP	*A.media*	
131DG-Aer	WWTP	*A.media*	
192FDD-CF	TR-a-WWTP	*E.coli*	
28FT-Aer	TR-b-WWTP	*A.media*	
12FT-Aer	TR-b-WWTP	*A.media*	
59SG-CF	HWW	*E.coli*	
16FT-CF	TR-b-WWTP	*K.pneumoniae*	
198FDD-Aer	TR-a-WWTP	*A.media*	
10FT-Aer	TR-b-WWTP	*A.media*	
150DG-Aer	WWTP	*A.caviae*	

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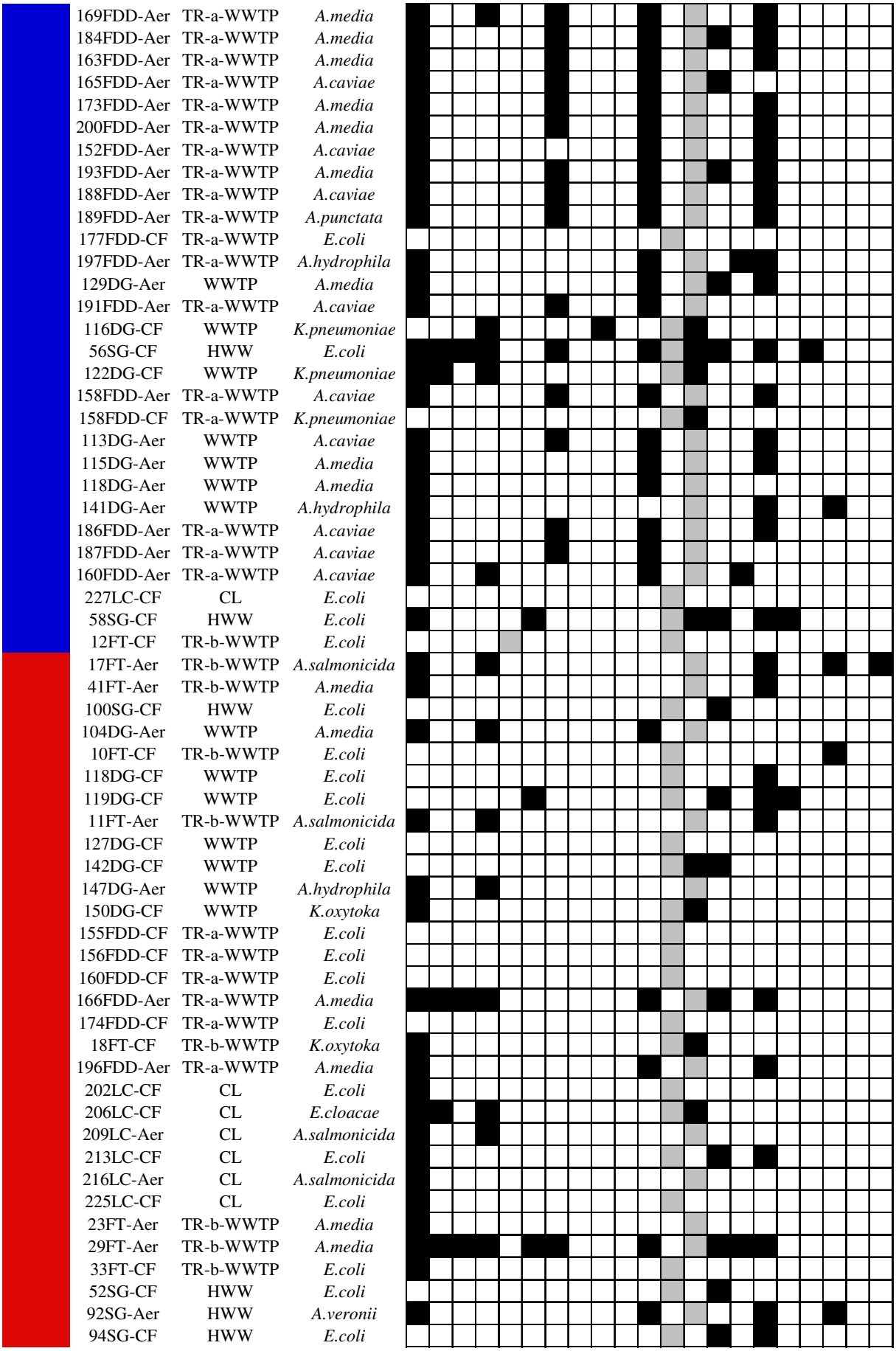

169FDD-Aer	TR-a-WWTP	*A.media*
184FDD-Aer	TR-a-WWTP	*A.media*
163FDD-Aer	TR-a-WWTP	*A.media*
165FDD-Aer	TR-a-WWTP	*A.caviae*
173FDD-Aer	TR-a-WWTP	*A.media*
200FDD-Aer	TR-a-WWTP	*A.media*
152FDD-Aer	TR-a-WWTP	*A.caviae*
193FDD-Aer	TR-a-WWTP	*A.media*
188FDD-Aer	TR-a-WWTP	*A.caviae*
189FDD-Aer	TR-a-WWTP	*A.punctata*
177FDD-CF	TR-a-WWTP	*E.coli*
197FDD-Aer	TR-a-WWTP	*A.hydrophila*
129DG-Aer	WWTP	*A.media*
191FDD-Aer	TR-a-WWTP	*A.caviae*
116DG-CF	WWTP	*K.pneumoniae*
56SG-CF	HWW	*E.coli*
122DG-CF	WWTP	*K.pneumoniae*
158FDD-Aer	TR-a-WWTP	*A.caviae*
158FDD-CF	TR-a-WWTP	*K.pneumoniae*
113DG-Aer	WWTP	*A.caviae*
115DG-Aer	WWTP	*A.media*
118DG-Aer	WWTP	*A.media*
141DG-Aer	WWTP	*A.hydrophila*
186FDD-Aer	TR-a-WWTP	*A.caviae*
187FDD-Aer	TR-a-WWTP	*A.caviae*
160FDD-Aer	TR-a-WWTP	*A.caviae*
227LC-CF	CL	*E.coli*
58SG-CF	HWW	*E.coli*
12FT-CF	TR-b-WWTP	*E.coli*
17FT-Aer	TR-b-WWTP	*A.salmonicida*
41FT-Aer	TR-b-WWTP	*A.media*
100SG-CF	HWW	*E.coli*
104DG-Aer	WWTP	*A.media*
10FT-CF	TR-b-WWTP	*E.coli*
118DG-CF	WWTP	*E.coli*
119DG-CF	WWTP	*E.coli*
11FT-Aer	TR-b-WWTP	*A.salmonicida*
127DG-CF	WWTP	*E.coli*
142DG-CF	WWTP	*E.coli*
147DG-Aer	WWTP	*A.hydrophila*
150DG-CF	WWTP	*K.oxytoka*
155FDD-CF	TR-a-WWTP	*E.coli*
156FDD-CF	TR-a-WWTP	*E.coli*
160FDD-CF	TR-a-WWTP	*E.coli*
166FDD-Aer	TR-a-WWTP	*A.media*
174FDD-CF	TR-a-WWTP	*E.coli*
18FT-CF	TR-b-WWTP	*K.oxytoka*
196FDD-Aer	TR-a-WWTP	*A.media*
202LC-CF	CL	*E.coli*
206LC-CF	CL	*E.cloacae*
209LC-Aer	CL	*A.salmonicida*
213LC-CF	CL	*E.coli*
216LC-Aer	CL	*A.salmonicida*
225LC-CF	CL	*E.coli*
23FT-Aer	TR-b-WWTP	*A.media*
29FT-Aer	TR-b-WWTP	*A.media*
33FT-CF	TR-b-WWTP	*E.coli*
52SG-CF	HWW	*E.coli*
92SG-Aer	HWW	*A.veronii*
94SG-CF	HWW	*E.coli*

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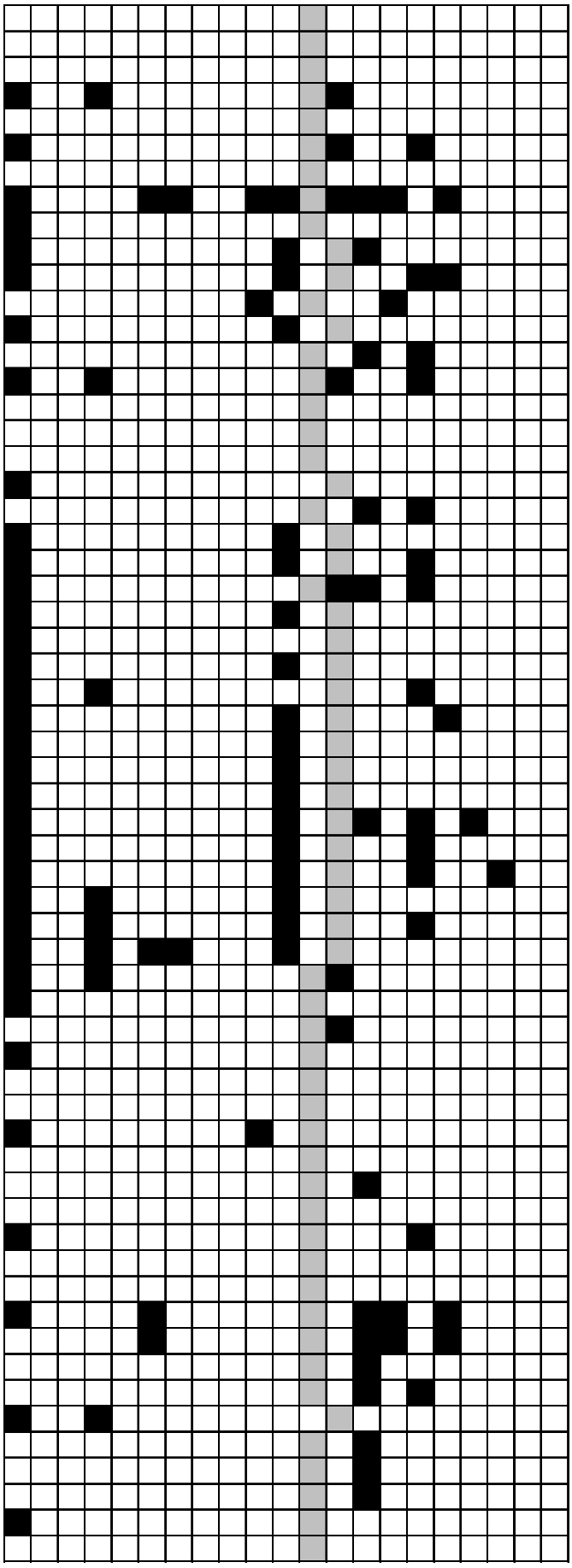
97SG-CF	HWW	*E.coli*	
98SG-CF	HWW	*E.coli*	
99SG-CF	HWW	*E.coli*	
125DG-CF	WWTP	*E.cloacae*	
191FDD-CF	TR-a-WWTP	*E.coli*	
136DG-CF	WWTP	*K.oxytoka*	
187FDD-CF	TR-a-WWTP	*E.coli*	
189FDD-CF	TR-a-WWTP	*E.coli*	
26FT-CF	TR-b-WWTP	*E.coli*	
78SG-Aer	HWW	*A.hydrophila*	
80SG-Aer	HWW	*A.hydrophila*	
133DG-CF	WWTP	*E.coli*	
98SG-Aer	HWW	*A.hydrophila*	
135DG-CF	WWTP	*E.coli*	
143DG-CF	WWTP	*E.cloacae*	
166FDD-CF	TR-a-WWTP	*E.coli*	
168FDD-CF	TR-a-WWTP	*E.coli*	
173FDD-CF	TR-a-WWTP	*E.coli*	
203LC-Aer	CL	*A.salmonicida*	
209LC-CF	CL	*E.coli*	
60SG-Aer	HWW	*A.hydrophila*	
61SG-Aer	HWW	*A.hydrophila*	
70SG-CF	HWW	*E.coli*	
72SG-Aer	HWW	*A.hydrophila*	
73SG-Aer	HWW	*A.salmonicida*	
79SG-Aer	HWW	*A.hydrophila*	
81SG-Aer	HWW	*A.salmonicida*	
86SG-Aer	HWW	*A.hydrophila*	
88SG-Aer	HWW	*A.hydrophila*	
94SG-Aer	HWW	*A.hydrophila*	
95SG-Aer	HWW	*A.hydrophila*	
96SG-Aer	HWW	*A.hydrophila*	
106DG-Aer	WWTP	*A.media*	
110DG-Aer	WWTP	*A.hydrophila*	
112DG-Aer	WWTP	*A.caviae*	
119DG-Aer	WWTP	*A.hydrophila*	
137DG-Aer	WWTP	*A.hydrophila*	
157FDD-CF	TR-a-WWTP	*E.cloacae*	
178FDD-CF	TR-a-WWTP	*E.coli*	
184FDD-CF	TR-a-WWTP	*K.pneumoniae*	
203LC-CF	CL	*E.coli*	
204LC-CF	CL	*E.coli*	
210LC-CF	CL	*E.coli*	
212LC-CF	CL	*E.coli*	
217LC-CF	CL	*E.coli*	
222LC-CF	CL	*E.coli*	
226LC-CF	CL	*E.coli*	
232LC-CF	CL	*E.coli*	
234LC-CF	CL	*E.coli*	
28FT-CF	TR-b-WWTP	*E.coli*	
30FT-CF	TR-b-WWTP	*E.coli*	
35FT-CF	TR-b-WWTP	*E.coli*	
38FT-CF	TR-b-WWTP	*E.coli*	
40FT-CF	TR-b-WWTP	*E.coli*	
46FT-Aer	TR-b-WWTP	*A.bestiarum*	
51SG-CF	HWW	*E.coli*	
57SG-CF	HWW	*E.coli*	
61SG-CF	HWW	*E.coli*	
63SG-CF	HWW	*E.coli*	
64SG-CF	HWW	*E.coli*	



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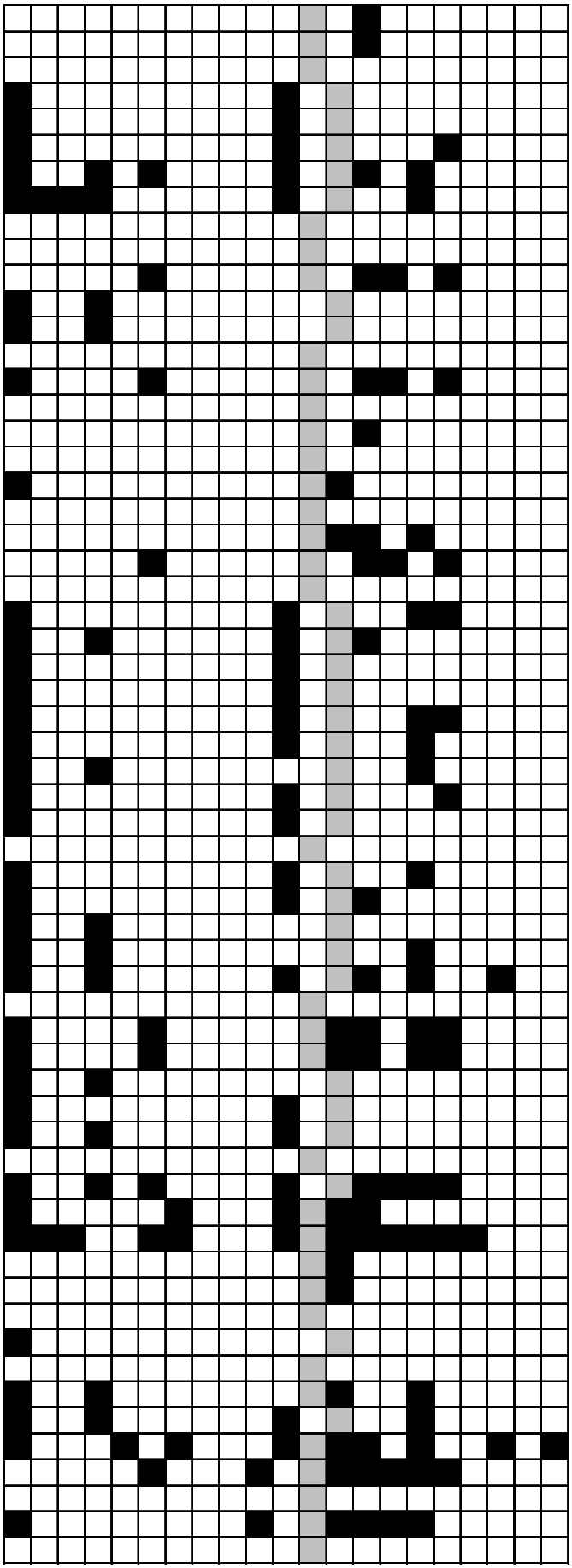
	69SG-CF	HWW	*E.coli*
	71SG-CF	HWW	*E.coli*
	74SG-CF	HWW	*E.coli*
	83SG-Aer	HWW	*A.hydrophila*
	84SG-Aer	HWW	*A.hydrophila*
	85SG-Aer	HWW	*A.hydrophila*
	161FDD-Aer	TR-a-WWTP	*A.caviae*
	19FT-Aer	TR-b-WWTP	*A.caviae*
	19FT-CF	TR-b-WWTP	*E.coli*
	20FT-CF	TR-b-WWTP	*E.coli*
	21FT-CF	TR-b-WWTP	*E.coli*
	24FT-Aer	TR-b-WWTP	*A.caviae*
	26FT-Aer	TR-b-WWTP	*A.caviae*
	42FT-CF	TR-b-WWTP	*E.coli*
	6FT-CF	TR-b-WWTP	*E.coli*
	75SG-CF	HWW	*E.coli*
	76SG-CF	HWW	*E.coli*
	93SG-CF	HWW	*E.coli*
	49FT-CF	TR-b-WWTP	*E.coli*
	79SG-CF	HWW	*E.coli*
	48FT-CF	TR-b-WWTP	*K.pneumoniae*
	34FT-CF	TR-b-WWTP	*E.coli*
	82SG-CF	HWW	*E.coli*
	51SG-Aer	HWW	*A.hydrophila*
	52SG-Aer	HWW	*A.hydrophila*
	54SG-Aer	HWW	*A.hydrophila*
	55SG-Aer	HWW	*A.hydrophila*
	62SG-Aer	HWW	*A.hydrophila*
	63SG-Aer	HWW	*A.hydrophila*
	64SG-Aer	HWW	*A.salmonicida*
	65SG-Aer	HWW	*A.hydrophila*
	69SG-Aer	HWW	*A.hydrophila*
	145DG-CF	WWTP	*E.coli*
	59SG-Aer	HWW	*A.hydrophila*
	67SG-Aer	HWW	*A.hydrophila*
	13FT-Aer	TR-b-WWTP	*A.bestiarum*
	30FT-Aer	TR-b-WWTP	*A.bestiarum*
	57SG-Aer	HWW	*A.hydrophila*
	14FT-CF	TR-b-WWTP	*E.coli*
	96SG-CF	HWW	*E.coli*
	65SG-CF	HWW	*E.coli*
	123DG-Aer	WWTP	*A.media*
	71SG-Aer	HWW	*A.hydrophila*
	154FDD-Aer	TR-a-WWTP	*A.media*
	68SG-CF	HWW	*E.coli*
	130DG-Aer	WWTP	*A.media*
	170FDD-CF	TR-a-WWTP	*E.coli*
	172FDD-CF	TR-a-WWTP	*E.coli*
	107DG-CF	WWTP	*K.pneumoniae*
	167FDD-CF	TR-a-WWTP	*K.pneumoniae*
	144DG-CF	WWTP	*E.coli*
	229LC-Aer	CL	*A.salmonicida*
	131DG-CF	WWTP	*E.coli*
	141DG-CF	WWTP	*E.cloacae*
	34FT-Aer	TR-b-WWTP	*A.media*
	140DG-CF	WWTP	*E.coli*
	81SG-CF	HWW	*E.coli*
	164FDD-CF	TR-a-WWTP	*E.coli*
	72SG-CF	HWW	*E.coli*
	27FT-CF	TR-b-WWTP	*E.coli*



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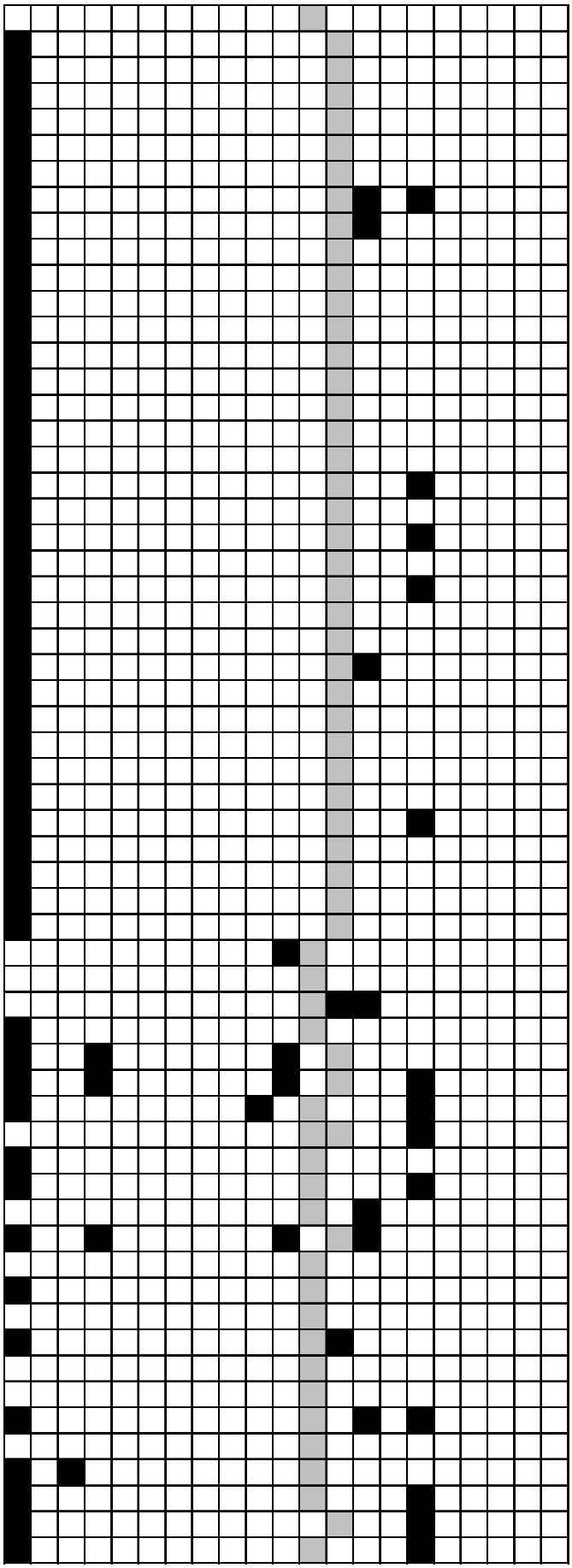
	31FT-CF	TR-b-WWTP	*E.coli*
	238LC-Aer	CL	*A.salmonicida*
	215LC-Aer	CL	*A.salmonicida*
	210LC-Aer	CL	*A.salmonicida*
	214LC-Aer	CL	*A.salmonicida*
	220LC-Aer	CL	*A.salmonicida*
	227LC-Aer	CL	*A.salmonicida*
	235LC-Aer	CL	*A.salmonicida*
	236LC-Aer	CL	*A.salmonicida*
	237LC-Aer	CL	*A.salmonicida*
	240LC-Aer	CL	*A.eucrenophila*
	211LC-Aer	CL	*A.salmonicida*
	247LC-Aer	CL	*A.salmonicida*
	208LC-Aer	CL	*A.salmonicida*
	217LC-Aer	CL	*A.salmonicida*
	218LC-Aer	CL	*A.salmonicida*
	224LC-Aer	CL	*A.salmonicida*
	226LC-Aer	CL	*A.salmonicida*
	228LC-Aer	CL	*A.salmonicida*
	239LC-Aer	CL	*A.salmonicida*
	245LC-Aer	CL	*A.salmonicida*
	249LC-Aer	CL	*A.salmonicida*
	231LC-Aer	CL	*A.salmonicida*
	233LC-Aer	CL	*A.salmonicida*
	243LC-Aer	CL	*A.salmonicida*
	222LC-Aer	CL	*A.salmonicida*
	225LC-Aer	CL	*A.salmonicida*
	244LC-Aer	CL	*A.eucrenophila*
	213LC-Aer	CL	*A.salmonicida*
	230LC-Aer	CL	*A.salmonicida*
	223LC-Aer	CL	*A.salmonicida*
	242LC-Aer	CL	*A.salmonicida*
	202LC-Aer	CL	*A.salmonicida*
	207LC-Aer	CL	*A.salmonicida*
	212LC-Aer	CL	*A.salmonicida*
	221LC-Aer	CL	*A.salmonicida*
	159FDD-CF	TR-a-WWTP	*E.coli*
	179FDD-CF	TR-a-WWTP	*E.coli*
	95SG-CF	HWW	*K.pneumoniae*
	228LC-CF	CL	*E.coli*
	99SG-Aer	HWW	*A.hydrophila*
	58SG-Aer	HWW	*A.hydrophila*
	221LC-CF	CL	*E.coli*
	47FT-CF	TR-b-WWTP	*E.coli*
	201LC-CF	CL	*E.coli*
	216LC-CF	CL	*E.coli*
	114DG-CF	WWTP	*E.coli*
	136DG-Aer	WWTP	*A.media*
	13FT-CF	TR-b-WWTP	*E.coli*
	2FT-CF	TR-b-WWTP	*E.coli*
	41FT-CF	TR-b-WWTP	*E.coli*
	46FT-CF	TR-b-WWTP	*E.coli*
	4FT-CF	TR-b-WWTP	*E.coli*
	9FT-CF	TR-b-WWTP	*E.coli*
	230LC-CF	CL	*E.coli*
	152FDD-CF	TR-a-WWTP	*E.coli*
	236LC-CF	CL	*E.coli*
	205LC-CF	CL	*E.coli*
	219LC-Aer	CL	*A.salmonicida*
	220LC-CF	CL	*E.coli*



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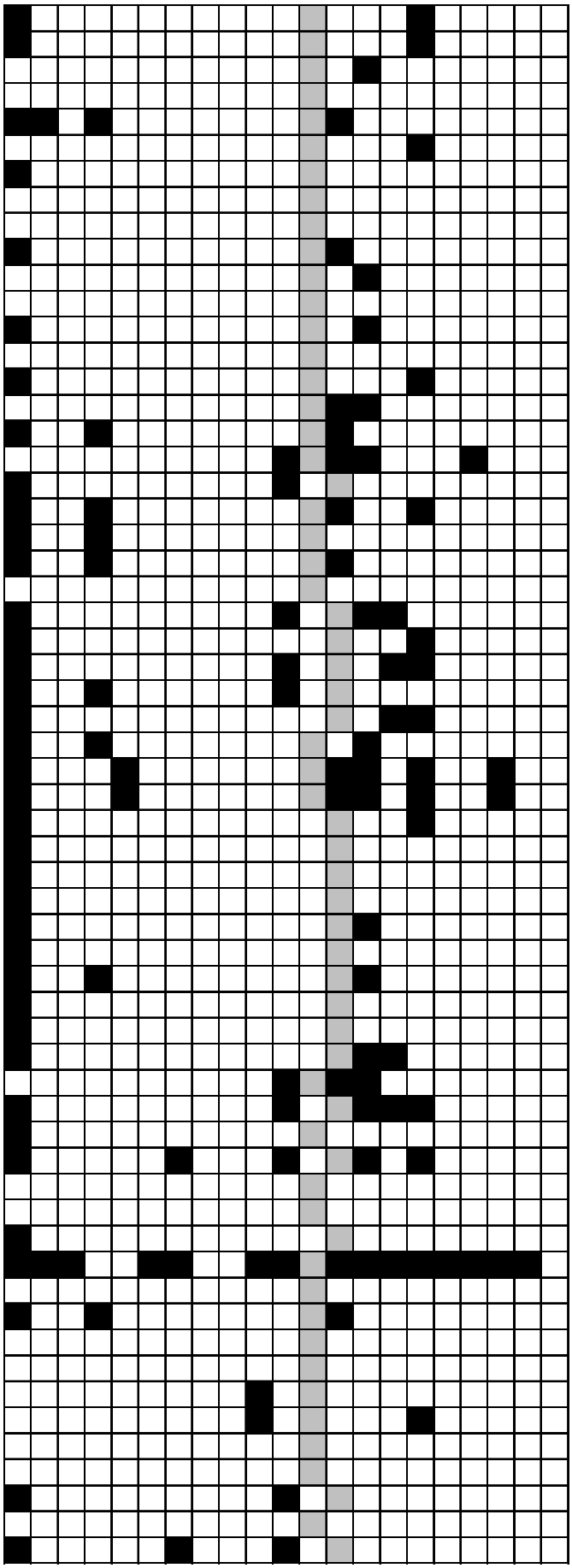
223LC-CF	CL	*E.coli*	
208LC-CF	CL	*E.coli*	
17FT-CF	TR-b-WWTP	*E.coli*	
29FT-CF	TR-b-WWTP	*E.coli*	
67SG-CF	HWW	*E.cloacae*	
78SG-CF	HWW	*E.coli*	
211LC-CF	CL	*E.coli*	
219LC-CF	CL	*E.coli*	
60SG-CF	HWW	*E.coli*	
175FDD-CF	TR-a-WWTP	*E.coli*	
37FT-CF	TR-b-WWTP	*E.coli*	
39FT-CF	TR-b-WWTP	*E.coli*	
44FT-CF	TR-b-WWTP	*E.coli*	
7FT-CF	TR-b-WWTP	*E.coli*	
229LC-CF	CL	*E.coli*	
181FDD-CF	TR-a-WWTP	*K.pneumoniae*	
185FDD-CF	TR-a-WWTP	*C.freundii*	
121DG-CF	WWTP	*E.coli*	
145DG-Aer	WWTP	*A.hydrophila*	
117DG-CF	WWTP	*E.cloacae*	
149DG-CF	WWTP	*E.cloacae*	
124DG-CF	WWTP	*E.cloacae*	
176FDD-CF	TR-a-WWTP	*E.coli*	
3FT-Aer	TR-b-WWTP	*A.caviae*	
9FT-Aer	TR-b-WWTP	*A.media*	
148DG-Aer	WWTP	*A.media*	
40FT-Aer	TR-b-WWTP	*A.media*	
120DG-Aer	WWTP	*A.bestiarum*	
15FT-CF	TR-b-WWTP	*E.cloacae*	
214LC-CF	CL	*P.shigelloides*	
215LC-CF	CL	*P.shigelloides*	
204LC-Aer	CL	*A.media*	
206LC-Aer	CL	*A.media*	
234LC-Aer	CL	*A.salmonicida*	
246LC-Aer	CL	*A.salmonicida*	
201LC-Aer	CL	*A.popoffii*	
205LC-Aer	CL	*A.salmonicida*	
175FDD-Aer	TR-a-WWTP	*A.media*	
232LC-Aer	CL	*A.salmonicida*	
250LC-Aer	CL	*A.salmonicida*	
68SG-Aer	HWW	*A.hydrophila*	
54SG-CF	HWW	*K.pneumoniae*	
91SG-Aer	HWW	*A.media*	
188FDD-CF	TR-a-WWTP	*E.coli*	
167FDD-Aer	TR-a-WWTP	*A.media*	
244LC-CF	CL	*E.coli*	
247LC-CF	CL	*E.coli*	
164FDD-Aer	TR-a-WWTP	*A.hydrophila*	
55SG-CF	HWW	*E.coli*	
239LC-CF	CL	*E.coli*	
248LC-CF	CL	*E.cloacae*	
241LC-CF	CL	*E.coli*	
243LC-CF	CL	*E.coli*	
245LC-CF	CL	*E.coli*	
240LC-CF	CL	*E.coli*	
238LC-CF	CL	*E.coli*	
246LC-CF	CL	*E.coli*	
172FDD-Aer	TR-a-WWTP	*A.caviae*	
237LC-CF	CL	*E.coli*	
127DG-Aer	WWTP	*A.media*	



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27FT-Aer	TR-b-WWTP	*A.sobria*	
128DG-Aer-A	WWTP	*A.media*	
92SG-CF	HWW	*E.coli*	
144DG-Aer	WWTP	*A.media*	
25FT-Aer	TR-b-WWTP	*A.media*	
4FT-Aer	TR-b-WWTP	*A.media*	
2FT-Aer	TR-b-WWTP	*A.media*	
156FDD-Aer	TR-a-WWTP	*A.hydrophila*	
183FDD-Aer	TR-a-WWTP	*A.media*	
235LC-CF	CL	*E.coli*	
43FT-Aer	TR-b-WWTP	*A.media*	
114DG-Aer	WWTP	*A.hydrophila*	
140DG-Aer	WWTP	*A.media*	
66SG-Aer	HWW	*A.hydrophila*	
90SG-Aer	HWW	*A.hydrophila*	
112DG-CF	WWTP	*K.pneumoniae*	
224LC-CF	CL	*E.coli*	
157FDD-Aer	TR-a-WWTP	*A.hydrophila*	
47FT-Aer	TR-b-WWTP	*A.media*	
45FT-Aer	TR-b-WWTP	*A.media*	
50FT-Aer	TR-b-WWTP	*A.media*	
48FT-Aer	TR-b-WWTP	*A.media*	
36FT-Aer	TR-b-WWTP	*A.media*	
110DG-CF	WWTP	*E.coli*	
128DG-Aer-B	WWTP	*A.caviae*	
159FDD-Aer	TR-a-WWTP	*A.hydrophila*	
121DG-Aer	WWTP	*A.media*	
176FDD-Aer	TR-a-WWTP	*A.media*	
178FDD-Aer	TR-a-WWTP	*A.caviae*	
103DG-CF	WWTP	*K.pneumoniae*	
106DG-CF	WWTP	*C.freundii*	
107DG-Aer	WWTP	*A.media*	
108DG-CF	WWTP	*C.freundii*	
109DG-CF	WWTP	*E.cloacae*	
111DG-Aer	WWTP	*A.media*	
113DG-CF	WWTP	*E.coli*	
115DG-CF	WWTP	*E.cloacae*	
116DG-Aer	WWTP	*A.media*	
122DG-Aer	WWTP	*A.hydrophila*	
126DG-Aer	WWTP	*A.salmonicida*	
126DG-CF	WWTP	*C.freundii*	
147DG-CF	WWTP	*C.freundii*	
153FDD-CF	TR-a-WWTP	*E.coli*	
15FT-Aer	TR-b-WWTP	*A.sobria*	
162FDD-CF	TR-a-WWTP	*C.freundii*	
168FDD-Aer	TR-a-WWTP	*A.hydrophila*	
174FDD-Aer	TR-a-WWTP	*A.media*	
177FDD-Aer	TR-a-WWTP	*A.bestiarum*	
182FDD-Aer	TR-a-WWTP	*A.media*	
183FDD-CF	TR-a-WWTP	*C.freundii*	
186FDD-CF	TR-a-WWTP	*E.coli*	
18FT-Aer	TR-b-WWTP	*A.caviae*	
190FDD-Aer	TR-a-WWTP	*A.media*	
193FDD-CF	TR-a-WWTP	*E.coli*	
194FDD-CF	TR-a-WWTP	*K.pneumoniae*	
196FDD-CF	TR-a-WWTP	*C.freundii*	
197FDD-CF	TR-a-WWTP	*C.freundii*	
198FDD-CF	TR-a-WWTP	*C.freundii*	
1FT-CF	TR-b-WWTP	*C.freundii*	
200FDD-CF	TR-a-WWTP	*E.coli*	

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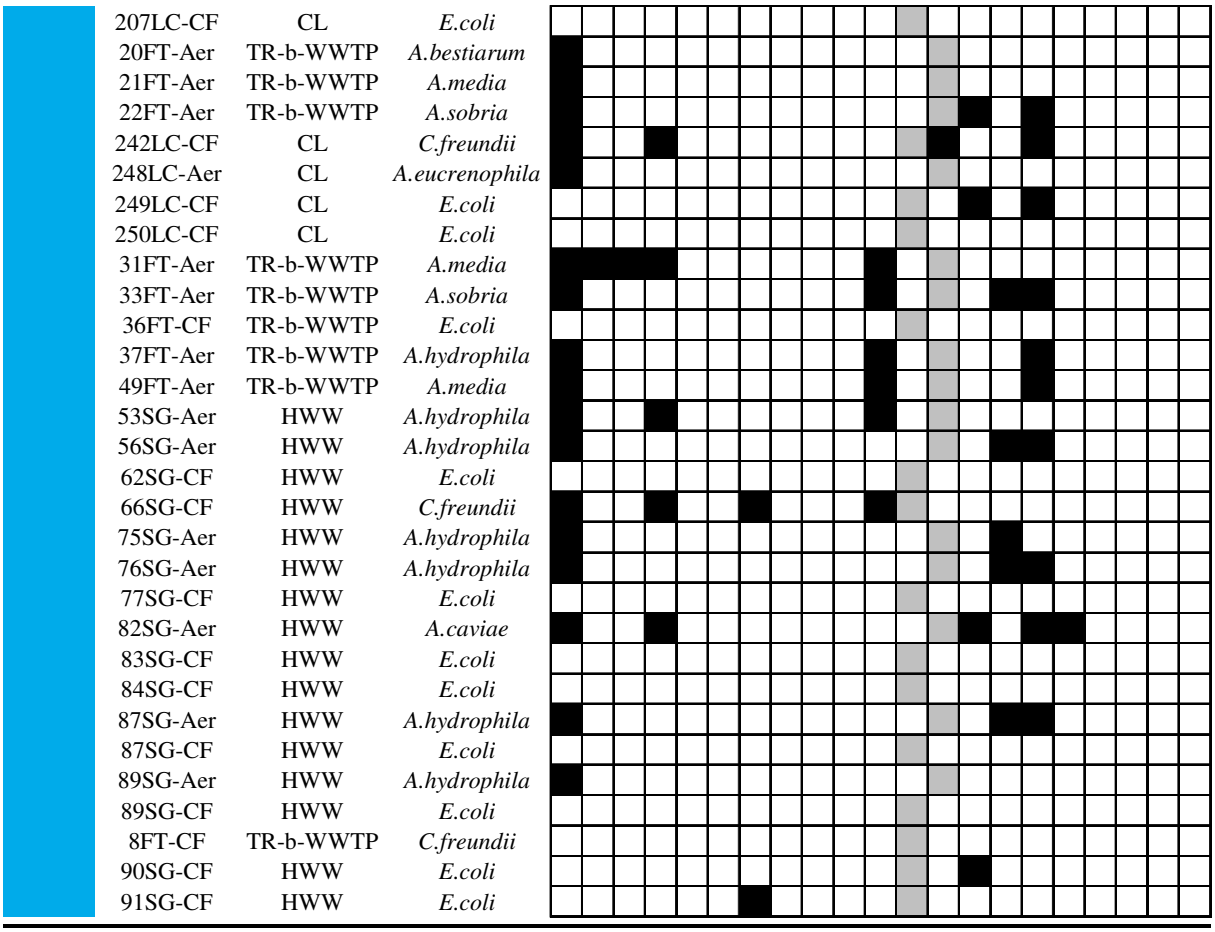

Table 4.3.3: Distribution of the resistances within the six groups of plasmid complexity. The number and the percentage of the strains with three or more resistances, one or two resistances and no antibiotic resistances in the groups are reported.

	Strains with resistaces			Percentage with resistaces (%)		
	> 3	2 - 1	0	> 3	2 - 1	0
	70	28	22	58,33	23,33	18,33
	13	17	10	32,50	42,50	25,00
	33	37	19	37,08	41,57	21,35
	12	41	8	19,67	67,21	13,11
	43	47	20	39,09	42,73	18,18
	27	19	15	44,26	31,15	24,59
	Total			Total		
	198	189	94	41,16	39,29	19,54
		481				

Table 4.3.4: Percentages of *Aeromonas* spp. and Fecal Coliforms carrying plasmids.

	Percentage of strains carrying plasmids	
	Aeromonas spp.	Fecal Coliforms
TICINO RIVER BEFORE WW.T.P.	81.63%	96.00%
BELLINZONA HWW	82.61%	82.00%
WW.T.P. GIUBIASCO	90.24%	84.00%
TICINO RIVER AFTER WW.T.P.	91.30%	80.00%
LAKE CADAGNO	97.96%	92.00%

Table 4.3.5: Percentages of *Aeromonas* spp. and Fecal Coliforms carrying plasmids with three or more antibiotic resistances.

	Percentage of strains with 3 or more resistances	
	Aeromonas spp	Fecal Coliforms
TICINO RIVER BEFORE WW.T.P.	69.39%	22.00%
BELLINZONA HOSPITAL	60.87%	28.00%
WW.T.P. GIUBIASCO	87.80%	40.00%
TICINO RIVER AFTER WW.T.P.	73.91%	22.00%
LAKE CADAGNO	2.04%	18.00%

4.4 Extended spectrum β-lactamases

The research of the extended spectrum β-lactamases production was carried out on presumptive positive strains.

Of the 231 *Aeromonas* spp., 13 strains were resistant to the Cephalosporins (first to third generation) and were further investigated for the synergy of PM/PML (Cefepime/Cefepime + Clavulanic Acid). The strain 77 SG-Aer, isolated from the Bellinzona Hospital wastewater, showed the rounded "phantom" in the PM/PML E-Test®.

36 over 250 Fecal Coliforms, resistant to the Cephalosporins (first to third generation) and growing on the chromogenic selective plates for ESBL producers (chromID ESBL, BioMérieux), were further investigated for the detection of the ESBL phenotype. Strains 55 SG-CF (HWW) and 172 FDD-CF (TR-a-WWTP) presented the typical rounded "phantom", due to the synergy between the two antibiotics CTX and AMC. The CT/CTL E-Test® confirmed the ESBL phenotype for the strains 56 SG-CF (HWW) and 85 SG-CF (HWW) (Table 4.4.1).

The five phenotypical ESBL strains were screened by PCR for the plasmidic *bla* genes; sequencing of the amplicons showed the presence of the genes $bla_{SHV\text{-}12}$, $bla_{OXA\text{-}1}$ in the

Aeromonas 77 SG-Aer strain as well as in the Fecal Coliforms 56 SG-CF and 85 SG-CF, bla_{SHV-12} in the strain 55 SG-CF, and $bla_{CTX-M27}$ in the strain 172 FDD-CF.

Table 4.4.1: Fecal Coliforms tested for the ESBL phenotypes. The colored rows indicated the ESBL phenotype of the four strains. The gray rows indicated the strains with a clear synergy (rounded "phantom") between CTX and AMC. The orange rows indicated the CT/CTL E-Test® positive strains. (---) sensitive strains to the antibiotics tested.

Source	Species	Strains	Resistace phenotypes	Source	Species	Strains	Resistace phenotypes
HWW	*E.coli*	55SG-CF	AM,CPD,CRO,AMC,CTX,ATM,FEP(I)	WWTP	*C.freundii*	126 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM
HWW	*E.coli*	56SG-CF	AM, FOX,CXM,CPD,CTX,ATM,FEP(I)	WWTP	*E.coli*	130 DG-CF	---
HWW	*E.coli*	58SG-CF	AMC	WWTP	*E.coli*	133 DG-CF	---
HWW	*C.freundii*	66SG-CF	AM,CXM,CPD,CRO,AMC,CTX,ATM	WWTP	*E.coli*	135 DG-CF	---
HWW	*E.cloacae*	67SG-CF	CPD,CRO,AMC,CTX,ATM,	WWTP	*K.oxytoca*	136 DG-CF	---
HWW	*E.coli*	72SG-CF	---	WWTP	*E.cloacae*	141 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM
HWW	*E.coli*	80SG-CF	CPD,AMC,CTX,ATM,FOX	WWTP	*E.cloacae*	143 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM
HWW	*E.coli*	85SG-CF	CPD,CTX,ATM	WWTP	*C.freundii*	147 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM
WWTP	*C.freundii*	106 DG-CF	AM,CXM,CPD,CRO,AMC,CTX,ATM	WWTP	*K.oxytoca*	150 DG-CF	---
WWTP	*C.freundii*	108 DG-CF	AM,CXM,CPD,CRO,AMC,CTX,ATM	TR-a-WWTP	*E.cloacae*	157 FDD-CF	CXM,CPD,CRO,AMC,CTX,ATM
WWTP	*E.cloacae*	109 DG-CF	CXM,CPD,AMC,CTX,ATM	TR-a-WWTP	*C.freundii*	162 FDD-CF	CXM,CPD,CRO,AMC,CTX,ATM,FEP(i)
WWTP	*E.coli*	110 DG-CF	---	TR-a-WWTP	*E.coli*	172 FDD-CF	AM,CXM,CPD,CRO,AMC,CTX,ATM,FEP
WWTP	*E.cloacae*	115 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM	TR-a-WWTP	*C.freundii*	183 FDD-CF	CXM,CPD,CRO,AMC,CTX,ATM
WWTP	*E.cloacae*	117 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM	TR-a-WWTP	*C.freundii*	185 FDD-CF	CXM,CPD,CRO,AMC,CTX,ATM
WWTP	*E.cloacae*	120 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM	TR-a-WWTP	*E.coli*	189 FDD-CF	---
WWTP	*E.cloacae*	123 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM,FEP(i)	TR-a-WWTP	*C.freundii*	197 FDD-CF	CXM,CPD,CRO,AMC,CTX,ATM
WWTP	*E.cloacae*	124 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM	CL	*E.coli*	213 LC-CF	---
WWTP	*E.cloacae*	125 DG-CF	CXM,CPD,CRO,AMC,CTX,ATM	CL	*E.cloacae*	248 LC-CF	CXM,CPD,CRO,AMC,CTX,ATM

4.5 Nalidixic acid and Ciprofloxacin resistances

29/231 *Aeromonas* spp. and 10/250 Fecal Coliforms carrying the phenotypical resistance to Ciprofloxacin, as well as 6 *Aeromonas* spp. resistant to Nalidixic Acid were screened by PCR in order to investigate the specific Gyrase A gene (*GyrA*) resistance mutation. Results showed the existence of mutations localized near the active site of the enzyme in all the *Aeromonas* spp. tested. In particular, mutations were found in the amino acid Ser83→Ile or Ser83→Arg of the GyrA enzyme. The mutation Ser83→Phe was found in one *Aeromonas caviae* strain resistant only to Nalidixic acid. In addition, three *Aeromonas media* showed a second mutation Asp87→Asn, and one *A. caviae* in Asp87→Ala. On the other hand, the Fecal Coliform 85 SG-CF (HWW) was found wild-type, showing the sequence expected for the two controls sensitive to Ciprofloxacin (*Aeromonas* 195 FDD-Aer and Fecal coliform 235 LC-CF (CL)). The other nine strains presented the mutation in the position Ser83→Val. 7 of the 9 resistant Fecal Coliform showed also a second mutation in Asp87→Asn.

The single and/or double mutation, and the wild type strains are reported in Figure 4.5.1.

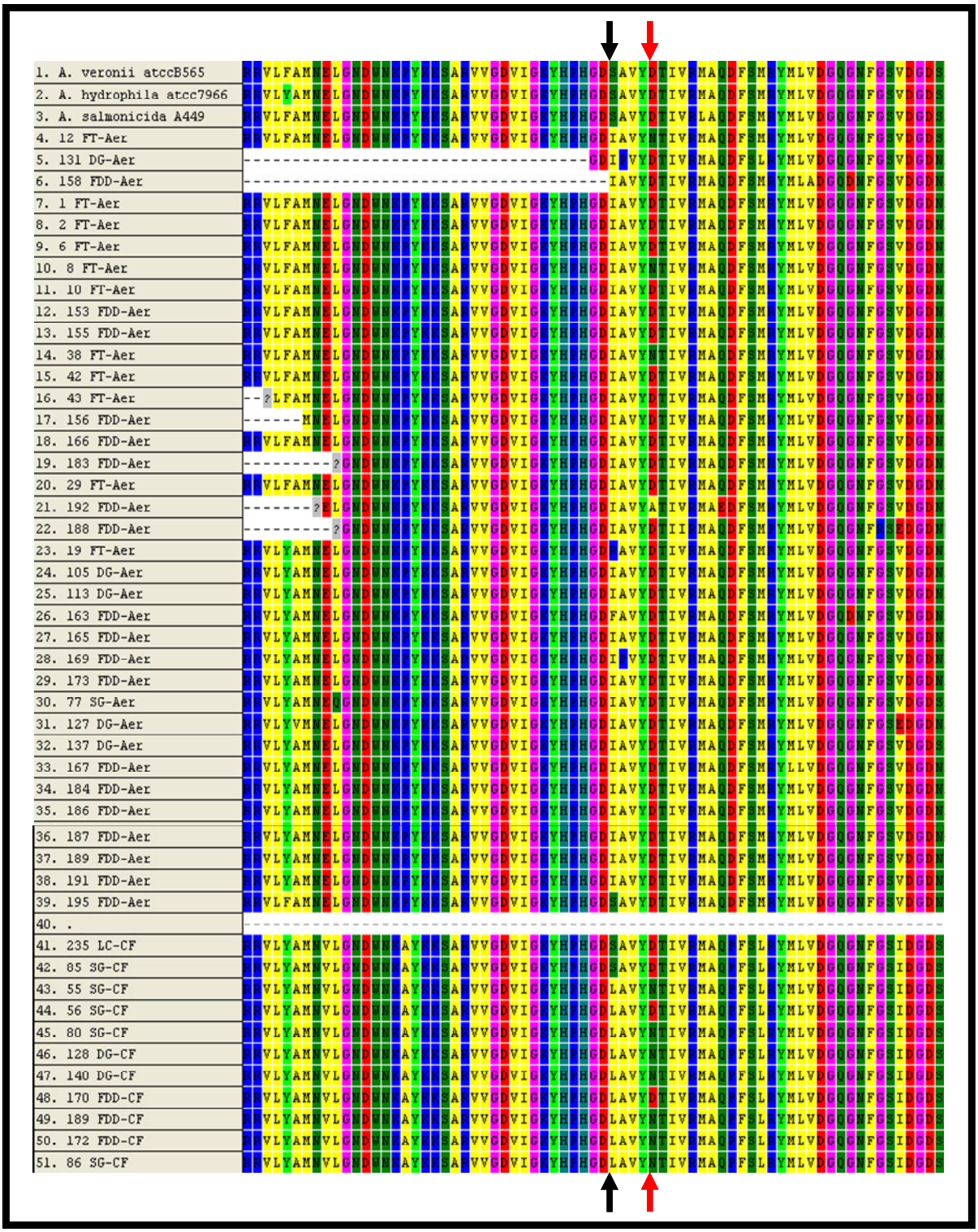

Figure 4.5.1: Mutations in the Gyrase A enzyme. The figure resumes the *Aeromonas* and Fecal Coliforms strains carrying the mutation in the amino acid 83 (the Serine can be substituted with Isoleucine, Arginine, Phenylalanine or Leucine) or in the amino acid 87 (Aspartic acid substituted with Asparagine). The amino-acid 83 is indicated with the black harrow, while the amino acid 87 with red harrow. The sequences of the reference strains (ATCC) were retrieved from NCBI. S, Serine; I, Isoleucine; R, Arginine; A, Alanine; D, Aspartic acid; N, Asparagine; L, Leucine.

4.6 Mobilization and incompatibility groups

The MOB sub-families screening carried out by Dot-blot of the *Aeromonas* spp. plasmids (205 strains) has shown that the 31.7% of the *Aeromonas* strains had mobilizable and potentially transferable plasmids belonging to the MOB_{P13}, and MOB_{P14} subfamilies. Approximately 69% of the Fecal Coliforms (149 out of 216 strains) had mobilizable and potentially transferable plasmids belonging to the MOB_{F12}, MOB_{P12}, and MOB_{H11} (Table 4.6.1). The MOB_{P13}, one of the largest subfamily in *Aeromonas* plasmids, was observed in the 2.08% of the Lake Cadagno isolates and the 17.07% of the strains isolated from the Giubiasco wastewater treatment plant. Plasmids of the MOB_{P14} were distributed in the 2.17% of the Bellinzona Hospital strains and in the 46.34% of the wastewater treatment plant isolates. The *Aeromonas* plasmids from Lake Cadagno were hardly characterizable by the MOB screening. The MOB sub-families identified in the Fecal Coliforms were: the MOB_{F12}, detected in 16.00% of strains from wastewater treatment plant and in 72.00% of those from Lake Cadagno; the MOB_{P12}, identified in 12.50% and 28.00% of the strains from the Ticino River after and before the wastewater treatment plant, respectively. Finally, the MOB_{H11} was distributed in the 8.00% of the Ticino River after the treatment plant and Bellinzona Hospital strains, and in the 14.00% of those isolated from the Giubiasco wastewater treatment plant.

The MOB sub-families identified in the plasmid extracts of the *Aeromonas* spp. and Fecal Coliforms strains are listed in Table 4.6.2.

Table 4.6.1 reports the distribution of the MOB sub-families as well as the Dot-Blot based screening results for the incompatibility group U (Inc U) performed on the plasmid extracts. All the other BHR groups, namely Inc A/C, Inc N, Inc P, and Inc W, which were screened by PCR, resulted negative. In *Aeromonas* spp., four strains (139 DG-Aer, 145 DG-Aer, 114 DG-Aer and160 FDD-Aer isolated from WWTP and after the WWTP in the river Ticino) were positives for the presence of plasmids belonging to the incompatibility group U, whereas in Fecal Coliforms, only one strain (133 DG-CF isolated from WWTP) was found positive.

The majority of the mobilizable plasmids identified belonged to the MOB_{P13} (17 strains), and MOB_{P14} (37 strains), for the *Aeromonas* spp., and to the MOB_{F12} (79 strains), MOB_{P12} (42 strains), and MOB_{H11} (21 strains), for the Fecal Coliforms.

Table 4.6.1: Mobilizable and transferable plasmids in *Aeromonas* spp. and Fecal Coliforms strains.

		MOB subfamilies						Incompatibility group
		H121	F12	P12	P13	P14	H11	IncU
Aeromonas spp.	TR-b-WWTP	-	-	-	4.08%	8.16%	-	-
	HWW	2.17%	2.17%	2.17%	2.17%	2.17%	-	-
	WWTP	2.44%	-	4.88%	17.07%	46.34%	7.32%	7.32%
	TR-a-WWTP	-	-	4.65%	13.95%	34.88%	-	2.33%
	CL	-	-	-	2.08%	-	-	-
Fecal Coliforms	TR-b-WWTP	4.00%	26.00%	28.00%	-	-	12.00%	-
	HWW	6.00%	26.00%	14.00%	-	-	8.00%	-
	WWTP	-	16.00%	16.00%	2.00%	2.00%	14.00%	2.00%
	TR-a-WWTP	-	18.00%	12.50%	-	-	8.00%	-
	CL	-	72.00%	16.00%	-	-	-	-

Table 4.6.2: MOB sub-families in *Aeromonas* spp. and Fecal Coliforms. 231 Aeromonas spp. and 250 Fecal Coliforms are listed and divided by sampling areas. The black squares indicate the positive plasmids for the MOB sub-families; colored squares indicated the groups of plasmidic complexity.

Aeromonas spp.

Groups	Key	Origine	Specie	MOB Subfamilies H11	H121	F12	P12	P13	P14
	1FT-Aer	TR-b-WWTP	*A.media*						
	2FT-Aer	TR-b-WWTP	*A.media*						
	3FT-Aer	TR-b-WWTP	*A.caviae*					■	
	4FT-Aer	TR-b-WWTP	*A.media*						
	5FT-Aer	TR-b-WWTP	*A.hydrophila*						
	6FT-Aer	TR-b-WWTP	*A.media*						
	7FT-Aer	TR-b-WWTP	*A.sobria*						
	8FT-Aer	TR-b-WWTP	*A.media*						
	9FT-Aer	TR-b-WWTP	*A.media*						
	10FT-Aer	TR-b-WWTP	*A.media*						
	11FT-Aer	TR-b-WWTP	*A.salmonicida*						
	12FT-Aer	TR-b-WWTP	*A.media*						
	13FT-Aer	TR-b-WWTP	*A.bestiarum*						
	14FT-Aer	TR-b-WWTP	*A.hydrophila*						
	15FT-Aer	TR-b-WWTP	*A.sobria*						
	16FT-Aer	TR-b-WWTP	*A.media*					■	■
	17FT-Aer	TR-b-WWTP	*A.salmonicida*						
	18FT-Aer	TR-b-WWTP	*A.caviae*						
	19FT-Aer	TR-b-WWTP	*A.caviae*						■
	20FT-Aer	TR-b-WWTP	*A.bestiarum*						
	21FT-Aer	TR-b-WWTP	*A.media*						
	22FT-Aer	TR-b-WWTP	*A.sobria*						
	23FT-Aer	TR-b-WWTP	*A.media*						
	24FT-Aer	TR-b-WWTP	*A.caviae*						
	25FT-Aer	TR-b-WWTP	*A.media*						
	26FT-Aer	TR-b-WWTP	*A.caviae*						
	27FT-Aer	TR-b-WWTP	*A.sobria*						
	28FT-Aer	TR-b-WWTP	*A.media*						■
	29FT-Aer	TR-b-WWTP	*A.media*						
	30FT-Aer	TR-b-WWTP	*A.bestiarum*						
	31FT-Aer	TR-b-WWTP	*A.media*						
	32FT-Aer	TR-b-WWTP	*A.bestiarum*						
	33FT-Aer	TR-b-WWTP	*A.sobria*						
	34FT-Aer	TR-b-WWTP	*A.media*						
	36FT-Aer	TR-b-WWTP	*A.media*						

Fecal Coliforms

Groups	Key	Origine	Specie	MOB Subfamilies H11	H121	F12	P12	P13	P14
	1FT-CF	TR-b-WWTP	*C.freundii*						
	2FT-CF	TR-b-WWTP	*E.coli*						
	3FT-CF	TR-b-WWTP	*K.pneumoniae*						
	4FT-CF	TR-b-WWTP	*E.coli*						
	5FT-CF	TR-b-WWTP	*E.coli*				■		
	6FT-CF	TR-b-WWTP	*E.coli*			■	■		■
	7FT-CF	TR-b-WWTP	*E.coli*						
	8FT-CF	TR-b-WWTP	*C.freundii*						
	9FT-CF	TR-b-WWTP	*E.coli*	■			■		
	10FT-CF	TR-b-WWTP	*E.coli*						
	11FT-CF	TR-b-WWTP	*K.pneumoniae*	■			■		
	12FT-CF	TR-b-WWTP	*E.coli*						
	13FT-CF	TR-b-WWTP	*E.coli*						
	14FT-CF	TR-b-WWTP	*E.coli*			■			
	15FT-CF	TR-b-WWTP	*E.cloacae*						
	16FT-CF	TR-b-WWTP	*K.pneumoniae*						
	17FT-CF	TR-b-WWTP	*E.coli*						
	18FT-CF	TR-b-WWTP	*K.oxytoka*						
	19FT-CF	TR-b-WWTP	*E.coli*						
	20FT-CF	TR-b-WWTP	*E.coli*			■			
	21FT-CF	TR-b-WWTP	*E.coli*			■	■		
	22FT-CF	TR-b-WWTP	*E.coli*			■			
	23FT-CF	TR-b-WWTP	*E.coli*			■			
	24FT-CF	TR-b-WWTP	*K.pneumoniae*						
	25FT-CF	TR-b-WWTP	*E.hermannii*						
	26FT-CF	TR-b-WWTP	*E.coli*						
	27FT-CF	TR-b-WWTP	*E.coli*						
	28FT-CF	TR-b-WWTP	*E.coli*				■		
	29FT-CF	TR-b-WWTP	*E.coli*	■					
	30FT-CF	TR-b-WWTP	*E.coli*			■	■		
	31FT-CF	TR-b-WWTP	*E.coli*	■					
	32FT-CF	TR-b-WWTP	*E.coli*				■		
	33FT-CF	TR-b-WWTP	*E.coli*						
	34FT-CF	TR-b-WWTP	*E.coli*			■	■		
	35FT-CF	TR-b-WWTP	*E.coli*			■	■		

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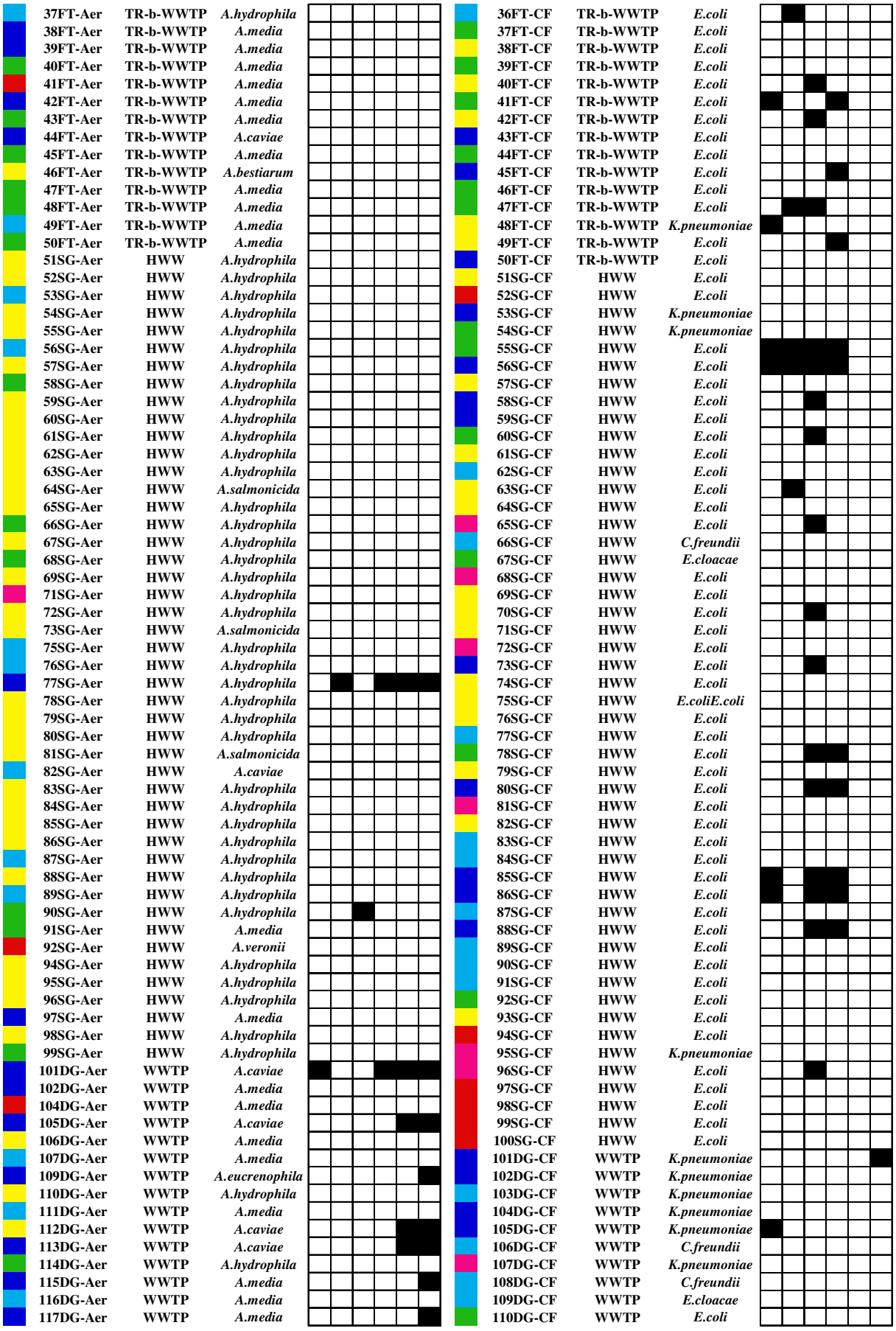
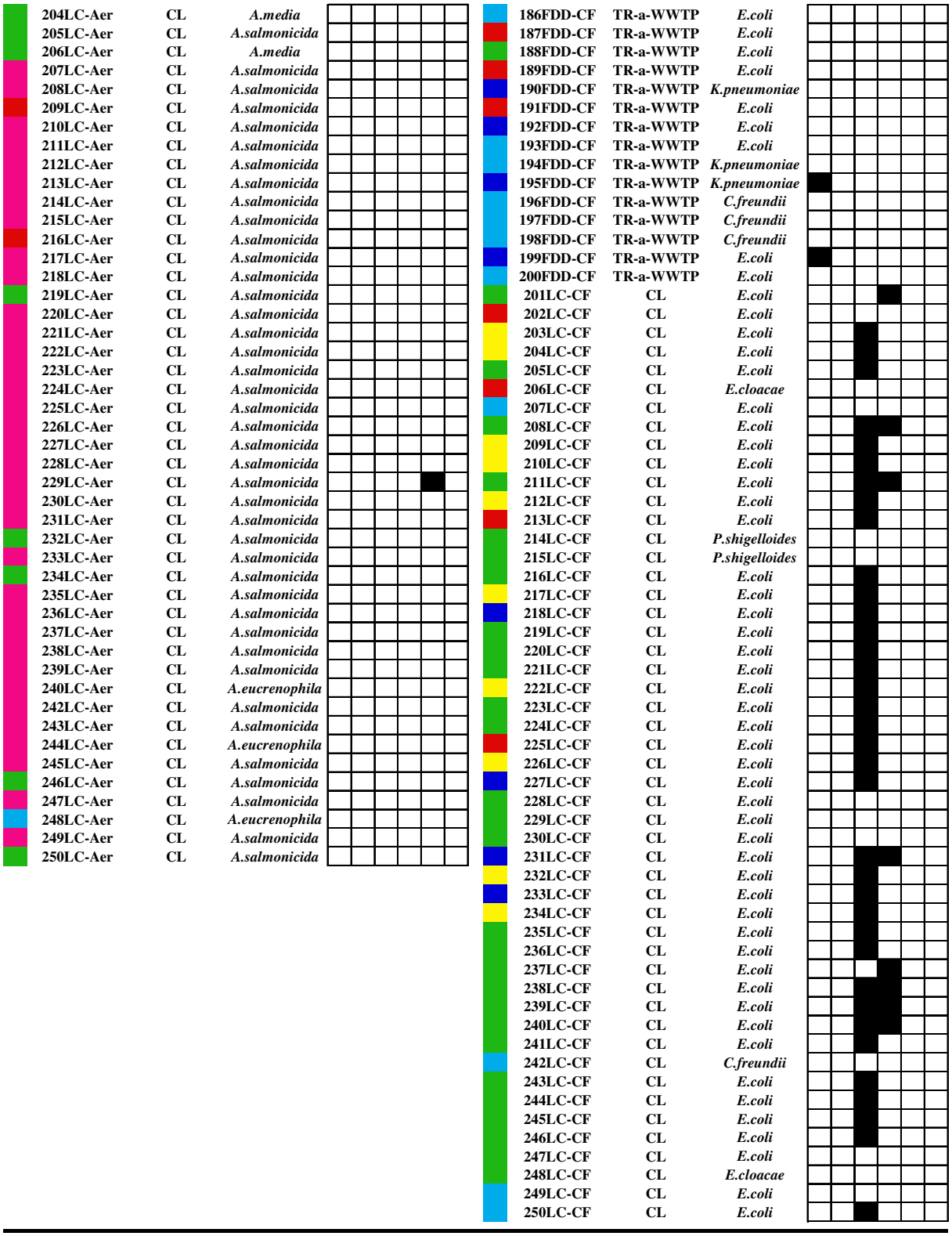

37FT-Aer TR-b-WWTP A.hydrophila
38FT-Aer TR-b-WWTP A.media
39FT-Aer TR-b-WWTP A.media
40FT-Aer TR-b-WWTP A.media
41FT-Aer TR-b-WWTP A.media
42FT-Aer TR-b-WWTP A.media
43FT-Aer TR-b-WWTP A.media
44FT-Aer TR-b-WWTP A.caviae
45FT-Aer TR-b-WWTP A.media
46FT-Aer TR-b-WWTP A.bestiarum
47FT-Aer TR-b-WWTP A.media
48FT-Aer TR-b-WWTP A.media
49FT-Aer TR-b-WWTP A.media
50FT-Aer TR-b-WWTP A.media
51SG-Aer HWW A.hydrophila
52SG-Aer HWW A.hydrophila
53SG-Aer HWW A.hydrophila
54SG-Aer HWW A.hydrophila
55SG-Aer HWW A.hydrophila
56SG-Aer HWW A.hydrophila
57SG-Aer HWW A.hydrophila
58SG-Aer HWW A.hydrophila
59SG-Aer HWW A.hydrophila
60SG-Aer HWW A.hydrophila
61SG-Aer HWW A.hydrophila
62SG-Aer HWW A.hydrophila
63SG-Aer HWW A.hydrophila
64SG-Aer HWW A.salmonicida
65SG-Aer HWW A.hydrophila
66SG-Aer HWW A.hydrophila
67SG-Aer HWW A.hydrophila
68SG-Aer HWW A.hydrophila
69SG-Aer HWW A.hydrophila
71SG-Aer HWW A.hydrophila
72SG-Aer HWW A.hydrophila
73SG-Aer HWW A.salmonicida
75SG-Aer HWW A.hydrophila
76SG-Aer HWW A.hydrophila
77SG-Aer HWW A.hydrophila
78SG-Aer HWW A.hydrophila
79SG-Aer HWW A.hydrophila
80SG-Aer HWW A.hydrophila
81SG-Aer HWW A.salmonicida
82SG-Aer HWW A.caviae
83SG-Aer HWW A.hydrophila
84SG-Aer HWW A.hydrophila
85SG-Aer HWW A.hydrophila
86SG-Aer HWW A.hydrophila
87SG-Aer HWW A.hydrophila
88SG-Aer HWW A.hydrophila
89SG-Aer HWW A.hydrophila
90SG-Aer HWW A.hydrophila
91SG-Aer HWW A.media
92SG-Aer HWW A.veronii
94SG-Aer HWW A.hydrophila
95SG-Aer HWW A.hydrophila
96SG-Aer HWW A.hydrophila
97SG-Aer HWW A.media
98SG-Aer HWW A.hydrophila
99SG-Aer HWW A.hydrophila
101DG-Aer WWTP A.caviae
102DG-Aer WWTP A.media
104DG-Aer WWTP A.media
105DG-Aer WWTP A.caviae
106DG-Aer WWTP A.media
107DG-Aer WWTP A.media
109DG-Aer WWTP A.eucrenophila
110DG-Aer WWTP A.hydrophila
111DG-Aer WWTP A.media
112DG-Aer WWTP A.caviae
113DG-Aer WWTP A.caviae
114DG-Aer WWTP A.hydrophila
115DG-Aer WWTP A.media
116DG-Aer WWTP A.media
117DG-Aer WWTP A.media
36FT-CF TR-b-WWTP E.coli
37FT-CF TR-b-WWTP E.coli
38FT-CF TR-b-WWTP E.coli
39FT-CF TR-b-WWTP E.coli
40FT-CF TR-b-WWTP E.coli
41FT-CF TR-b-WWTP E.coli
42FT-CF TR-b-WWTP E.coli
43FT-CF TR-b-WWTP E.coli
44FT-CF TR-b-WWTP E.coli
45FT-CF TR-b-WWTP E.coli
46FT-CF TR-b-WWTP E.coli
47FT-CF TR-b-WWTP E.coli
48FT-CF TR-b-WWTP K.pneumoniae
49FT-CF TR-b-WWTP E.coli
50FT-CF TR-b-WWTP E.coli
51SG-CF HWW E.coli
52SG-CF HWW E.coli
53SG-CF HWW K.pneumoniae
54SG-CF HWW K.pneumoniae
55SG-CF HWW E.coli
56SG-CF HWW E.coli
57SG-CF HWW E.coli
58SG-CF HWW E.coli
59SG-CF HWW E.coli
60SG-CF HWW E.coli
61SG-CF HWW E.coli
62SG-CF HWW E.coli
63SG-CF HWW E.coli
64SG-CF HWW E.coli
65SG-CF HWW E.coli
66SG-CF HWW C.freundii
67SG-CF HWW E.cloacae
68SG-CF HWW E.coli
69SG-CF HWW E.coli
70SG-CF HWW E.coli
71SG-CF HWW E.coli
72SG-CF HWW E.coli
73SG-CF HWW E.coli
74SG-CF HWW E.coli
75SG-CF HWW E.coliE.coli
76SG-CF HWW E.coli
77SG-CF HWW E.coli
78SG-CF HWW E.coli
79SG-CF HWW E.coli
80SG-CF HWW E.coli
81SG-CF HWW E.coli
82SG-CF HWW E.coli
83SG-CF HWW E.coli
84SG-CF HWW E.coli
85SG-CF HWW E.coli
86SG-CF HWW E.coli
87SG-CF HWW E.coli
88SG-CF HWW E.coli
89SG-CF HWW E.coli
90SG-CF HWW E.coli
91SG-CF HWW E.coli
92SG-CF HWW E.coli
93SG-CF HWW E.coli
94SG-CF HWW E.coli
95SG-CF HWW K.pneumoniae
96SG-CF HWW E.coli
97SG-CF HWW E.coli
98SG-CF HWW E.coli
99SG-CF HWW E.coli
100SG-CF HWW E.coli
101DG-CF WWTP K.pneumoniae
102DG-CF WWTP K.pneumoniae
103DG-CF WWTP K.pneumoniae
104DG-CF WWTP K.pneumoniae
105DG-CF WWTP K.pneumoniae
106DG-CF WWTP C.freundii
107DG-CF WWTP K.pneumoniae
108DG-CF WWTP C.freundii
109DG-CF WWTP E.cloacae
110DG-CF WWTP E.coli

continued…

	118DG-Aer	WWTP	*A.media*								111DG-CF	WWTP	*E.coli*						
	119DG-Aer	WWTP	*A.hydrophila*								112DG-CF	WWTP	*K.pneumoniae*						
	120DG-Aer	WWTP	*A.bestiarum*								113DG-CF	WWTP	*E.coli*						
	121DG-Aer	WWTP	*A.media*								114DG-CF	WWTP	*E.coli*						
	122DG-Aer	WWTP	*A.hydrophila*								115DG-CF	WWTP	*E.cloacae*						
	123DG-Aer	WWTP	*A.media*								116DG-CF	WWTP	*K.pneumoniae*						
	126DG-Aer	WWTP	*A.salmonicida*								117DG-CF	WWTP	*E.cloacae*						
	127DG-Aer	WWTP	*A.media*								118DG-CF	WWTP	*E.coli*						
	128DG-Aer-A	WWTP	*A.media*								119DG-CF	WWTP	*E.coli*						
	128DG-Aer-B	WWTP	*A.caviae*								120DG-CF	WWTP	*E.cloacae*						
	129DG-Aer	WWTP	*A.media*								121DG-CF	WWTP	*E.coli*						
	130DG-Aer	WWTP	*A.media*								122DG-CF	WWTP	*K.pneumoniae*						
	131DG-Aer	WWTP	*A.media*								123DG-CF	WWTP	*E.cloacae*						
	133DG-Aer	WWTP	*A.hydrophila*								124DG-CF	WWTP	*E.cloacae*						
	135DG-Aer	WWTP	*A.media*								125DG-CF	WWTP	*E.cloacae*						
	136DG-Aer	WWTP	*A.media*								126DG-CF	WWTP	*C.freundii*						
	137DG-Aer	WWTP	*A.hydrophila*								127DG-CF	WWTP	*E.coli*						
	139DG-Aer	WWTP	*A.hydrophila*								128DG-CF	WWTP	*E.coli*						
	140DG-Aer	WWTP	*A.media*								129DG-CF	WWTP	*K.pneumoniae*						
	141DG-Aer	WWTP	*A.hydrophila*								130DG-CF	WWTP	*E.coli*						
	143DG-Aer	WWTP	*A.hydrophila*								131DG-CF	WWTP	*E.coli*						
	144DG-Aer	WWTP	*A.media*								132DG-CF	WWTP	*K.pneumoniae*						
	145DG-Aer	WWTP	*A.hydrophila*								133DG-CF	WWTP	*E.coli*						
	147DG-Aer	WWTP	*A.hydrophila*								134DG-CF	WWTP	*K.pneumoniae*						
	148DG-Aer	WWTP	*A.media*								135DG-CF	WWTP	*E.coli*						
	150DG-Aer	WWTP	*A.caviae*								136DG-CF	WWTP	*K.oxytoka*						
	151FDD-Aer	TR-a-WWTP	*A.caviae*								137DG-CF	WWTP	*K.pneumoniae*						
	152FDD-Aer	TR-a-WWTP	*A.caviae*								138DG-CF	WWTP	*K.pneumoniae*						
	153FDD-Aer	TR-a-WWTP	*A.media*								139DG-CF	WWTP	*K.pneumoniae*						
	154FDD-Aer	TR-a-WWTP	*A.media*								140DG-CF	WWTP	*E.coli*						
	155FDD-Aer	TR-a-WWTP	*A.media*								141DG-CF	WWTP	*E.cloacae*						
	156FDD-Aer	TR-a-WWTP	*A.hydrophila*								142DG-CF	WWTP	*E.coli*						
	157FDD-Aer	TR-a-WWTP	*A.hydrophila*								143DG-CF	WWTP	*E.cloacae*						
	158FDD-Aer	TR-a-WWTP	*A.caviae*								144DG-CF	WWTP	*E.coli*						
	159FDD-Aer	TR-a-WWTP	*A.hydrophila*								145DG-CF	WWTP	*E.coli*						
	160FDD-Aer	TR-a-WWTP	*A.caviae*								146DG-CF	WWTP	*K.pneumoniae*						
	161FDD-Aer	TR-a-WWTP	*A.caviae*								147DG-CF	WWTP	*C.freundii*						
	163FDD-Aer	TR-a-WWTP	*A.media*								148DG-CF	WWTP	*K.pneumoniae*						
	164FDD-Aer	TR-a-WWTP	*A.hydrophila*								149DG-CF	WWTP	*E.cloacae*						
	165FDD-Aer	TR-a-WWTP	*A.caviae*								150DG-CF	WWTP	*K.oxytoka*						
	166FDD-Aer	TR-a-WWTP	*A.media*								151FDD-CF	TR-a-WWTP	*E.coli*						
	167FDD-Aer	TR-a-WWTP	*A.media*								152FDD-CF	TR-a-WWTP	*E.coli*						
	168FDD-Aer	TR-a-WWTP	*A.hydrophila*								153FDD-CF	TR-a-WWTP	*E.coli*						
	169FDD-Aer	TR-a-WWTP	*A.media*								154FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	171FDD-Aer	TR-a-WWTP	*A.media*								155FDD-CF	TR-a-WWTP	*E.coli*						
	172FDD-Aer	TR-a-WWTP	*A.caviae*								156FDD-CF	TR-a-WWTP	*E.coli*						
	173FDD-Aer	TR-a-WWTP	*A.media*								157FDD-CF	TR-a-WWTP	*E.cloacae*						
	174FDD-Aer	TR-a-WWTP	*A.media*								158FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	175FDD-Aer	TR-a-WWTP	*A.media*								159FDD-CF	TR-a-WWTP	*E.coli*						
	176FDD-Aer	TR-a-WWTP	*A.media*								160FDD-CF	TR-a-WWTP	*E.coli*						
	177FDD-Aer	TR-a-WWTP	*A.bestiarum*								161FDD-CF	TR-a-WWTP	*E.coli*						
	178FDD-Aer	TR-a-WWTP	*A.caviae*								162FDD-CF	TR-a-WWTP	*C.freundii*						
	179FDD-Aer	TR-a-WWTP	*A.caviae*								163FDD-CF	TR-a-WWTP	*E.coli*						
	180FDD-Aer	TR-a-WWTP	*A.hydrophila*								164FDD-CF	TR-a-WWTP	*E.coli*						
	181FDD-Aer	TR-a-WWTP	*A.media*								165FDD-CF	TR-a-WWTP	*E.coli*						
	182FDD-Aer	TR-a-WWTP	*A.media*								166FDD-CF	TR-a-WWTP	*E.coli*						
	183FDD-Aer	TR-a-WWTP	*A.media*								167FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	184FDD-Aer	TR-a-WWTP	*A.media*								168FDD-CF	TR-a-WWTP	*E.coli*						
	186FDD-Aer	TR-a-WWTP	*A.caviae*								169FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	187FDD-Aer	TR-a-WWTP	*A.caviae*								170FDD-CF	TR-a-WWTP	*E.coli*						
	188FDD-Aer	TR-a-WWTP	*A.caviae*								171FDD-CF	TR-a-WWTP	*E.coli*						
	189FDD-Aer	TR-a-WWTP	*A.punctata*								172FDD-CF	TR-a-WWTP	*E.coli*						
	190FDD-Aer	TR-a-WWTP	*A.media*								173FDD-CF	TR-a-WWTP	*E.coli*						
	191FDD-Aer	TR-a-WWTP	*A.caviae*								174FDD-CF	TR-a-WWTP	*E.coli*						
	192FDD-Aer	TR-a-WWTP	*A.caviae*								175FDD-CF	TR-a-WWTP	*E.coli*						
	193FDD-Aer	TR-a-WWTP	*A.media*								176FDD-CF	TR-a-WWTP	*E.coli*						
	195FDD-Aer	TR-a-WWTP	*A.veronii*								177FDD-CF	TR-a-WWTP	*E.coli*						
	196FDD-Aer	TR-a-WWTP	*A.media*								178FDD-CF	TR-a-WWTP	*E.coli*						
	197FDD-Aer	TR-a-WWTP	*A.hydrophila*								179FDD-CF	TR-a-WWTP	*E.coli*						
	198FDD-Aer	TR-a-WWTP	*A.media*								180FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	199FDD-Aer	TR-a-WWTP	*A.hydrophila*								181FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	200FDD-Aer	TR-a-WWTP	*A.media*								182FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	201LC-Aer	CL	*A.popoffii*								183FDD-CF	TR-a-WWTP	*C.freundii*						
	202LC-Aer	CL	*A.salmonicida*								184FDD-CF	TR-a-WWTP	*K.pneumoniae*						
	203LC-Aer	CL	*A.salmonicida*								185FDD-CF	TR-a-WWTP	*C.freundii*						

continued…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205LC-Aer CL A.salmonicida
206LC-Aer CL A.media
207LC-Aer CL A.salmonicida
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209LC-Aer CL A.salmonicida
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211LC-Aer CL A.salmonicida
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216LC-Aer CL A.salmonicida
217LC-Aer CL A.salmonicida
218LC-Aer CL A.salmonicida
219LC-Aer CL A.salmonicida
220LC-Aer CL A.salmonicida
221LC-Aer CL A.salmonicida
222LC-Aer CL A.salmonicida
223LC-Aer CL A.salmonicida
224LC-Aer CL A.salmonicida
225LC-Aer CL A.salmonicida
226LC-Aer CL A.salmonicida
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231LC-Aer CL A.salmonicida
232LC-Aer CL A.salmonicida
233LC-Aer CL A.salmonicida
234LC-Aer CL A.salmonicida
235LC-Aer CL A.salmonicida
236LC-Aer CL A.salmonicida
237LC-Aer CL A.salmonicida
238LC-Aer CL A.salmonicida
239LC-Aer CL A.salmonicida
240LC-Aer CL A.eucrenophila
242LC-Aer CL A.salmonicida
243LC-Aer CL A.salmonicida
244LC-Aer CL A.eucrenophila
245LC-Aer CL A.salmonicida
246LC-Aer CL A.salmonicida
247LC-Aer CL A.salmonicida
248LC-Aer CL A.eucrenophila
249LC-Aer CL A.salmonicida
250LC-Aer CL A.salmonicida
186FDD-CF TR-a-WWTP E.coli
187FDD-CF TR-a-WWTP E.coli
188FDD-CF TR-a-WWTP E.coli
189FDD-CF TR-a-WWTP E.coli
190FDD-CF TR-a-WWTP K.pneumoniae
191FDD-CF TR-a-WWTP E.coli
192FDD-CF TR-a-WWTP E.coli
193FDD-CF TR-a-WWTP E.coli
194FDD-CF TR-a-WWTP K.pneumoniae
195FDD-CF TR-a-WWTP K.pneumoniae
196FDD-CF TR-a-WWTP C.freundii
197FDD-CF TR-a-WWTP C.freundii
198FDD-CF TR-a-WWTP C.freundii
199FDD-CF TR-a-WWTP E.coli
200FDD-CF TR-a-WWTP E.coli
201LC-CF CL E.coli
202LC-CF CL E.coli
203LC-CF CL E.coli
204LC-CF CL E.coli
205LC-CF CL E.coli
206LC-CF CL E.cloacae
207LC-CF CL E.coli
208LC-CF CL E.coli
209LC-CF CL E.coli
210LC-CF CL E.coli
211LC-CF CL E.coli
212LC-CF CL E.coli
213LC-CF CL E.coli
214LC-CF CL P.shigelloides
215LC-CF CL P.shigelloides
216LC-CF CL E.coli
217LC-CF CL E.coli
218LC-CF CL E.coli
219LC-CF CL E.coli
220LC-CF CL E.coli
221LC-CF CL E.coli
222LC-CF CL E.coli
223LC-CF CL E.coli
224LC-CF CL E.coli
225LC-CF CL E.coli
226LC-CF CL E.coli
227LC-CF CL E.coli
228LC-CF CL E.coli
229LC-CF CL E.coli
230LC-CF CL E.coli
231LC-CF CL E.coli
232LC-CF CL E.coli
233LC-CF CL E.coli
234LC-CF CL E.coli
235LC-CF CL E.coli
236LC-CF CL E.coli
237LC-CF CL E.coli
238LC-CF CL E.coli
239LC-CF CL E.coli
240LC-CF CL E.coli
241LC-CF CL E.coli
242LC-CF CL C.freundii
243LC-CF CL E.coli
244LC-CF CL E.coli
245LC-CF CL E.coli
246LC-CF CL E.coli
247LC-CF CL E.coli
248LC-CF CL E.cloacae
249LC-CF CL E.coli
250LC-CF CL E.coli

4.7 Class 1 integrons

The Integrase I (*IntI1*) gene has been investigated by Dot-blot on the total DNA extracts of 231 *Aeromonas* spp. and 250 Fecal Coliforms (Table 4.7.1). The *IntI1* gene was detected in the 60.98% of the *Aeromonas* strains collected from the wastewater treatment plant, and in the 55.81% of those sampled in the Ticino river after the treatment plant. In the Bellinzona Hospital wastewater only the 8.70% of the strains resulted positive for the *IntI1* gene, while in the river before the wastewater treatment plant the frequency was 22.45%. The strains of the Lake Cadagno did not have any Integrase 1 gene. The *IntI1* gene was present in the 2.00% of the Fecal Coliforms isolated from the wastewater treatment plant and from the Lake Cadagno, and in the 18.00% of those isolated from the Hospital wastewater.

All the strains which tested positive for the genomic presence of the *IntI1* gene were further investigated through the amplification and sequencing of the variable regions of the class 1 integrons performed on the plasmid extracts. Class 1 integrons had a plasmid localization in all the Fecal Coliforms harboring the *IntI1* gene except for the strains from the Ticino river after the wastewater treatment plant, in which integrons were located on plasmids in only the 50% of the strains. In *Aeromonas* spp., class 1 integrons were plasmid located in the 45.45% and 12.50% of the strains isolated from the Ticino river before and after the wastewater treatment plant, respectively, in the 16.00% of those from the Giubiasco wastewater treatment plant, and in all strains originated from the Bellinzona Hospital wastewaters.

The most common gene cassettes (Table 4.7.2) within the class 1 integrons found in the *Aeromonas* strains were *aadA1* (62.5%), *CatB8* (37.5%), and *CatB3* (25%); in the Fecal Coliforms the most common gene cassettes were *aadA1* (63.6%), *CatB2* (36.4%) and *dfrA14* (22.7%).

Table 4.7.1: Percentage of Integrase 1 gene positive strains from the total DNA extracts.

	Integrase I			
	***Aeromonas* spp.**		**Fecal Coliforms**	
	Strains	**Percentages**	**Strains**	**Percentages**
TR-b-WWTP	**49**	**22.45%**	**50**	**16.00%**
HWW	**46**	**8.70%**	**50**	**18.00%**
WWTP	**41**	**60.98%**	**50**	**2.00%**
TR-a-WWTP	**43**	**55.81%**	**50**	**12.00%**
CL	**48**	**-**	**50**	**2.00%**

Table 4.7.2: Plasmidic class 1 Integrons in *Aeromonas* spp. and Fecal Coliforms. The variable regions of the class 1 Integrons were amplified between the *PantF* and the *qacEΔ1* genes. The gene cassettes colored in red showed incomplete sequences or portions of hypothetical proteins.

Plasmidic Class I Integron							
Aeromonas spp				Fecal Coliforms			
Source	Species	Strains	PantF-qacEΔ1	Source	Species	Strains	PantF-qacEΔ1
TR-b-WWTP	*A.sobria*	7FT-Aer	dfrB1-aadA1b-CatB2	TR-b-WWTP	*E.coli*	6FT-CF	dfrA14-aadA1-CatB2
	A.media	16FT-Aer	OXA10-aadA1		*E.coli*	21FT-CF	dfrA14-aadA1-CatB2
	A.sobria	27FT-Aer	Empty		*E.coli*	22FT-CF	dfrA17-aadA5
	A.media	39FT-Aer	dfr22		*K.pneumoniae*	24FT-CF	aadA4a-hypotetical protein
	A.media	42FT-Aer	CatB3- aadA1		*E.coli*	30FT-CF	dfrA14-aadA1-catB2
					E.coli	34FT-CF	dfrA14-aadA1-CatB2
HWW	*A.hydrophila*	52SG-Aer	CatB8-aadA1		*E.coli*	35FT-CF	dfrA14-aadA1-CatB2
	A.hydrophila	57SG-Aer	CatB8-aadA1		*K.pneumoniae*	48FT-CF	aadA2
	A.hydrophila	77SG-Aer	aacA4cr-OXA1-CatB3-aar3				
	A.hydrophila	96SG-Aer	CatB8-aadA1	HWW	*E.coli*	55SG-CF	aacA4-aadA1-CatB2
					E.coli	56SG-CF	aacA4-aadA1-CatB2
WWTP	*A.caviae*	105 DG-Aer	CatB8-Transposase		*E.coli*	58SG-CF	dhfrA1-aadA1
	A.media	130 DG-Aer	dfr?-aadA1		*E.coli*	65SG-CF	dfrA1-aadA1
	A.media	135 DG-Aer	aadA2		*E.coli*	72SG-CF	EstX putative esterase/hydrolase
	A.hydrophila	137 DG-Aer	CatB3-aadA1		*E.coli*	80SG-CF	dfrA17-aadA5
					E.coli	81SG-CF	dfrA12-OrfF-?
TR-a-WWTP	*A.caviae*	161 FDD-Aer	CatB8-aadA1		*E.coli*	85SG-CF	aacA4-aadA1-CatB2
	A.media	167 FDD-Aer	CatB8-aadA1		*E.coli*	96SG-CF	dfrA1-aadA1
	A.media	171 FDD-Aer	aacA3-OXA21-CatB3-aadA16				
				WWTP	*E.coli*	110 DG-CF	dfrA1-aadA1
				TR-a-WWTP	*K.pneumoniae*	169 FDD-CF	Empty
					E.coli	172 FDD-CF	dfrA17-aadA5
					E.coli	189 FDD-CF	dfrB4
				CL	*E.coli*	235 LC-CF	aadA1

4.8 Plasmid curing

205 of 231 *Aeromonas* spp. strains harbored plasmids. All the strains with these accessory DNA molecules were grown in presence of SDS 10%, but most of them (62.44%) were not able to replicate in these conditions. Of the remaining strains (Table 4.8.1), only two strains (5 FT-Aer and 91 SG-Aer) showed the loss of plasmids. The antibiotic resistance phenotype (resistance to Nalidixic Acid) was lost only for the strain 91 SG-Aer (Table 4.8.2).

In 13.17% of the strains no variations of the resistance phenotype were observed, whereas the chemical curing caused some other changes in a number of *Aeromonas* strains such as a decrease of resistance (0.98%), an increase of resistance in approx. 21% of the strains, and a mixed effect of increased resistance for some antibiotics and decreased for others (1.46%) (Table 4.8.1).

The antibiotic resistance phenotype before and after SDS 10% curing of six strains, used as example to summarize the possible resistance and plasmidic variations of the *Aeromonas* spp. strains, are shown in Table 4.8.2.

The strains unable to grow in presence of SDS 10% and the strain 91 SG-Aer were submitted to the plate curing by the culture transfer on solid agar medium. Plasmids were again cured only from the strain 91 SG-Aer (Figure 4.8.1). All the other attempts of curing plasmids (culture transfer in liquid minimal medium, culture transfer in water or curing using acridine

orange) failed. No strain tested showed loss of plasmids, but some developed an increased resistance to one of the fallowing antibacterial compounds: Cefoxitin, Gentamicin, Bactrim and Ciprofloxacin (data not shown).

Table 4.8.1: Percentages of each variation in *Aeromonas* spp. strains following the curing with SDS 10%.

Aeromonas spp. (205)	
Lost of plasmids	**0.98%**
Decrease of resistances	**0.98%**
Increase of resistances	**20.98%**
Any variations	**1.46%**
No variations	**13.17%**
No growth	**62.44%**

Table 4.8.2: Summary of the changes observed after the chemical curing (SDS 10%). The red rectangles indicate the phenotype "antibiotic resistant"; the green rectangles indicate the phenotype "antibiotic susceptible". The numbers in the color rectangles indicate the diameters of inhibition. In the bottom of the Table are indicated the types of variations.

Antibiotics		91 SG-Aer		5 FT-Aer		119 DG-Aer		60 SG-Aer		19 FT-Aer		123 DG-Aer	
		wild type	Curing	wild type	Curing	wild type	Curing	wild type	Curing	wild type	Curing	wild type	Curing
Cefazolin	CZ	11	16	8	8	7	6	9	6	6	6	6	6
Cefuroxime	CXM									6	31		
Ceftriaxone	CRO									11	36		
Cefoxitin	FOX			17	18	18	20	20	15	6	16	6	6
Ciprofloxacin	CIP							25	19				
Polymyxin	PB									17	9		
Nalidixic Acid	NA	6	23	8	6	6	6	6	6	11	6		
		Lost of plasmids Decrease of resistances		Lost of plasmids No variations		Decrease of resistances		Increase of resistances		Any variations		No variations	

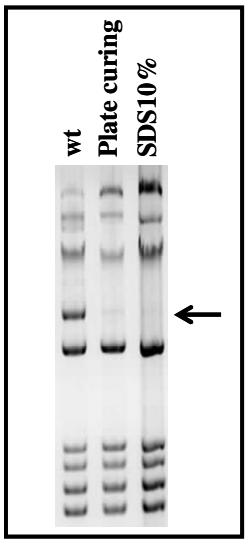

Figure 4.8.1: Agarose gel of the total plasmid extracts of the strain 91 SG-Aer before and after the SDS 10% and plate curing. The black harrow indicates the loss of one plasmid.

4.9 Bacterial conjugations

The results of conjugations on solid supports or in liquid media performed between *Aeromonas* spp. and Fecal Coliforms are listed in Table 4.9.1. Strains of both groups were used alternatively as donors and recipients. On the 55 solid and 33 liquid conjugation, only the 7.27% of the first group gave a positive conjugation. A positive transfer of plasmids, and of the related antibiotic resistance phenotype, was obtained with four *Aeromonas* strains used as donors in conjugations performed on solid support. In liquid media the same strains were not able to transfer their plasmids by conjugation.

The resistance profiles of the transconjugants obtained by conjugation between the four *Aeromonas* spp. strains (2 FT-Aer, 19 FT-Aer, 156 FDD-Aer, and 183 FDD-Aer) used as donors, and the Fecal Coliforms (66 SG-CF) used as recipient, are listed in Table 4.9.2. The double selection was performed using Ciprofloxacin (chromosomal resistance) and Ceftriaxone, antibiotics for whom the donor and the recipient were sensitive, respectively. The resistance phenotype of the transconjugants confirms the transfer of the resistance to Ceftriaxone.

The plasmid profile of the donors and the transconjugants were visualized by electrophoresis on an agarose gel. The gel reported in Figure 4.9.1 shows the transfer of one plasmid from the donors to the recipient bacteria.

Table 4.9.1: Conjugations experiments on solid supports or in liquid media.

	Donor				Recipient					
Conjugation type	Strain	Species	Source	Resistances	Strain	Species	Source	Resistances	Double Selection	Concentration (µg/mL)
Solid	121 DG-Aer	*A.media*	WWTP	CZ, FOX, C, RL	126 DG-Aer	*A.salmonicida*	WWTP	CZ, NA, S(I), RL(I)	C, NA	16, 32
					190 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA		
					66SG-CF	*C.freundii*	HWW	CZ, FOX, NA, AM, CXM, CPD, CRO, AMC, CTX, ATM		
Solid	138 DG-Aer	*Alcaligenes faecalis*	WWTP	CZ, GM	126 DG-Aer	*A.salmonicida*	WWTP	CZ, NA, S(I), RL(I)	GM, NA	32, 32
					190 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA		
					66SG-CF	*C.freundii*	HWW	CZ, FOX, NA, AM, CXM, CPD, CRO, AMC, CTX, ATM		
Solid	2FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S, TMP	66SG-CF	*C.freundii*	HWW	CZ, FOX, NA, AM, CXM, CPD, CRO, AMC, CTX, ATM	CRO, CIP	4, 4
	19FT-Aer	*A. caviae*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S						
	156 FDD-Aer	*A.hydrophila*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA						
	183 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA(I)						
Solid	119 DG-CF	E.coli	WWTP	SXT, S, TMP, RL(I)	J53	*E.coli*	Ref. strain	C, NA, Rif, NaN3	SXT, C	4, 8
	29FT-Aer	A.media	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I),S, RL, CRO(I),SXT(I),CIP(I)						
Solid, Liquid	173 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), S(I)	J53	*E.coli*	Ref. strain	C, NA, Rif, NaN3	CIP, C	1, 8
	184 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), S(I), RL(I)	29FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I), S, RL, CRO(I) ,SXT(I) ,CIP(I)	CIP, SXT	1, 4
	186 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	187 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA						
	188 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	189 FDD-Aer	*A.punctata*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	191 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, NA, CIP(I), RL(I)						
	192 FDD-Aer	*A.caviae*	TR-a-WWTP	CIP, NA, CZ(I)						
	193 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I), RL(I)						
	200 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I)						

continued…

Solid, Liquid	1FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, CIP(I)	J53	*E.coli*	Ref. strain	C, NA, Rif, NaN3	CIP, C	1, 8
	6FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, CIP(I)	29FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I), S, RL, CRO(I) ,SXT(I) ,CIP(I)	CIP, SXT	1, 4
	8FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, CIP, NA, S(I),RL(I)						
	10FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, CIP(I), RL(I)						
	12FT-Aer	*A.media*	TR-b-WWTP	CZ, FOX,CIP,NA, S(I)						
	43FT-Aer	*A.media*	TR-b-WWTP	CZ, FOX, NA, NN, CRO(I), CIP(I), S(I)						
	77SG-Aer	*A.hydrophila*	HWW	CZ, CXM, CRO, GM, CIP, NAS, RL, ATM, NN, NET						
	105 DG-Aer	*A.caviae*	WWTP	NA, S, RL, CIP(I)						
	113 DG-Aer	*A.caviae*	WWTP	CZ, CIP, NA, S(I)						
	127 DG-Aer	*A.media*	WWTP	CZ, NA, CIP(I)						
	131 DG-Aer	*A.media*	WWTP	CZ, CXM, CRO, FOX, NA, S(I), CIP(I)						
	137 DG-Aer	*A.hydrophila*	WWTP	CZ, (FOX), CIP, NA, SXT(I)						
	151 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	153 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)						
	155 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)						
	158 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	163 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I)						
	165 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, NA, CIP(I), RL(I)						
	167 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), RL(I), S(I)						
	169 FDD-Aer	*A.media*	TR-a-WWTP	CZ,FOX,CIP,NA, S(I)						
Solid, Liquid	42FT-Aer	*A.media*	TR-b-WWTP	CZ, FOX, CIP, C, NA, S(I), RL, NN	29FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I),S, RL, CRO(I) ,SXT(I), CIP(I)	CIP, SXT	1, 4
	127 DG-Aer	*A.media*	WWTP	CZ, NA, CIP(I)						
	167 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), S(I), RL						
Solid	1FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, CIP(I)	J53	*E.coli*	Ref. strain	C, NA, Rif, NaN3	CIP, NaN3	1, 100
	12FT-Aer	*A.media*	TR-b-WWTP	CZ,FOX,CIP,NA, S(I)						
	105 DG-Aer	*A.caviae*	WWTP	NA, S, RL, CIP(I)						
	127 DG-Aer	*A.media*	WWTP	CZ,, NA, CIP(I)						
	137 DG-Aer	*A.hydrophila*	WWTP	CZ, (FOX), CIP, NA, SXT(I)						
	153 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)						
	155 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)						
	167 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), RL(I), S(I)						
Solid	2FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S, TMP	J53	*E.coli*	Ref. strain	C, NA, Rif, NaN3	CRO, NaN3	2, 100
	19FT-Aer	*A. caviae*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S						
	38FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, SXT, NA, CRO(I), S(I)						
	156 FDD-Aer	*A.hydrophila*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA						
	166 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA, S(I), RL(I)						
	183 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA(I)						

Table 4.9.2: Resistant phenotypes of Donors, Recipient and Transconjugants strains. The red rectangles indicate the phenotype "antibiotic resistant"; the green rectangles indicate the phenotype "antibiotic susceptible". The numbers indicate the diameters of inhibition.

	Strains			Strains		
	2 FT-Aer	66 SG-CF	66/2	19 FT-Aer	66 SG-CF	66/19
Antibiotics						
CZ	6	14	6	6	14	6
CXM	6	23	6	6	23	6
CRO	6	28	11	11	28	9
FOX	6	6	6	6	6	6
GM	21	19	20	22	19	20
SXT	24	26	25	29	26	25
CIP	22	17	15	35	17	14
MEM	32	32	30	32	32	30
PB	16	17	17	17	17	17
C	28	22	16	33	22	17
NA	6	6	6	11	6	6
AM		16	6		16	6

	Strains			Strains		
	156 FDD-Aer	66 SG-CF	66/156	183 FDD-Aer	66 SG-CF	66/183
Antibiotics						
CZ	6	14	6	6	14	6
CXM	6	23	6	6	23	6
CRO	14	28	9	16	28	10
FOX	6	6	6	6	6	6
GM	23	19	20	26	19	20
SXT	29	26	24	31	26	24
CIP	29	17	16	31	17	16
MEM	35	32	30	38	32	30
PB	17	17	16	19	17	16
C	35	22	16	37	22	16
NA	13	6	6	15	6	6
AM		16	6		16	6

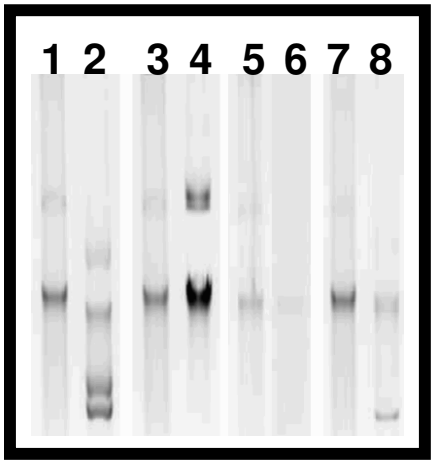

Figure 4.9.1: Plasmid profiles of Transconjugants (T) **and Donor (D) strains.** The recipient strain (66SG-CG) was not included in the picture because it didn't have any plasmid. Lane 1, 66/2 (T); lane 2, 2FT-Aer (D); lane 3, 66/19 (T); Lane 4, 19FT-Aer (D); lane 5, 66/156 (T); lane 6,

4.10 Bacterial transformations

Plasmids from *Aeromonas* spp. and Fecal Coliforms (listed in Table 4.10.1) were used in the transformation experiments. In the 36 transformations performed with the competent bacteria *E. coli* DH5α, and in the 5 with the *E. coli* BL21, the uptake of the exogenous DNA was not successful, and no colonies grew after 24-48 hours of incubation in selective agar culture media.

Table 4.10.1: Transformation of competent bacteria with plasmids extracted from *Aeromonas* spp. and Fecal Coliforms.

Competent Bacteria	Plasmids				Concentration (ng/transformation)	Antibiotics	
	Stranis	Species	Source	Resistances		Selection	Concentration (µg/mL)
DH5α	6FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, CIP(I)	100	CIP, CRO	1, 4
	8FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, CIP, NA, S(I),RL(I)			
	10FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, CIP(I), RL(I)			
	29FT-Aer	A.media	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I),S, RL, CRO(I),SXT(I),CIP(I)			
	43FT-Aer	*A.media*	TR-b-WWTP	CZ, FOX, NA, NN, CRO(I), CIP(I), S(I)			
	77SG-Aer	*A.hydrophila*	HWW	CZ, CXM, CRO, GM, CIP, NAS, RL, ATM, NN, NET			
	131 DG-Aer	*A.media*	WWTP	CZ, CXM, CRO, FOX, NA, S(I), CIP(I)			
DH5α	1FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, CIP(I)	100	CIP	1
	12FT-Aer	*A.media*	TR-b-WWTP	CZ, FOX,CIP,NA, S(I)			
	105 DG-Aer	*A.caviae*	WWTP	NA, S, RL, CIP(I)			
	113 DG-Aer	*A.caviae*	WWTP	CZ, CIP, NA, S(I)			
	151 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	153 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)			
	155 FDD-Aer	*A.media*	TR-a-WWTP	CZ, FOX, NA, CIP(I)			
	158 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	163 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	165 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, NA, CIP(I), RL(I)			
	167 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), RL(I), S(I)			
	169 FDD-Aer	*A.media*	TR-a-WWTP	CZ,FOX,CIP,NA, S(I)			
	173 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), S(I)			
	184 FDD-Aer	*A.media*	TR-a-WWTP	CZ, NA, CIP(I), S(I), RL(I)			
	186 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	187 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA			
	188 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	189 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, CIP, NA, S(I)			
	191 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, NA, CIP(I), RL(I)			
	192 FDD-Aer	*A.caviae*	TR-a-WWTP	CIP, NA, CZ(I)			
	200 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I)			
DH5α	2FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S, TMP	100	CRO	4
	19FT-Aer	*A. caviae*	TR-b-WWTP	CZ, CXM, CRO, FOX, NA, S			
	166 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CXM, CRO, FOX, NA, S(I), RL(I)			
DH5α	55SG-CF	*E.coli*	HWW	CZ, CXM, CRO, SXT, CIP, C, NA, AM, Te, S, TMP, RL, ATM, NN(I), K	100	CIP, CRO	1, 4
	56SG-CF	*E.coli*	HWW	CZ, CXM, CRO, FOX, CIP, NA, AM, S(I), RL, ATM			
	85SG-CF	*E.coli*	HWW	CZ, CXM, CRO, AM, CIP(I), NA(I), S(I), RL, ATM			
	172 FDD-CF	*E.coli*	TR-a-WWTP	CZ, CXM, CRO, SXT, CIP, NA, AM, Te, S, TMP, RL, ATM			
DH5α	161 FDD-Aer	*A.caviae*	TR-a-WWTP	CZ, FOX, NA, SXT(I), S(I), RL	100	SXT	4
BL21	29FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, NA, Te(I),S, RL, CRO(I),SXT(I),CIP(I)	100	SXT+C	4, 8
	38FT-Aer	*A.media*	TR-b-WWTP	CZ, CXM, FOX, SXT, NA, CRO(I), S(I)			
	137 DG-Aer	*A.hydrophila*	WWTP	CZ, (FOX), CIP, NA, SXT(I)			
BL21	127 DG-Aer	*A.media*	WWTP	CZ, NA, CIP(I)	100	CIP+C	1, 8
	193 FDD-Aer	*A.media*	TR-a-WWTP	CZ, CIP, NA, S(I), RL(I)			

5 DISCUSSION

The concentrations of *Aeromonas* in the surface water, the wastewater and the water before and after the wastewater treatment plant ranged from $2 \cdot 10^3$ CFU/100 mL into the Ticino river before the treatment plant to $3 \cdot 10^6$ CFU/100 mL in the Giubiasco wastewater treatment plant, and were similar to those reported by **Figueira et al. (2011).**

The concentrations of Fecal Coliforms in the sampling areas followed the trend expected. In fact in presence of high amount of organic matter of fecal origin, the number of these bacteria was particularly high, such as in the Bellinzona Hospital wastewater ($2.07 \cdot 10^6$ CFU/100 mL) and in the wastewater treatment plant ($7.05 \cdot 10^5$ CFU/100 mL). In the Ticino river the sampling collected in the vicinity of the exhaust duct of the sewage plant showed a concentration of $1.63 \cdot 10^4$ CFU/100 mL, which was higher than the one from the same river but before the outlet ($1.65 \cdot 10^2$ CFU/100 mL). Therefore it clearly appears that the wastewater treatment plant has an impact on the microbiology of the river even if the wastewater treatment plants are generally able to reduce about the 99% of the bacteria present in the input waters. On the other hand, the presence of Fecal Coliforms at low concentrations (3 CFU/100 mL) in the alpine lake may be due to the leaching from the pastures that surround it.

The identification of the bacterial species was performed by MALDI-TOF MS, which is a useful and very rapid method able to identify both *Aeromonas* and Fecal Coliforms (**Benagli, et al., 2012; Thevenon et al., 2012**). The most common mesophilic and psychrophilic species of *Aeromonas* were: *A. hydrophila* (up to 84.78%), *A. caviae* (up to 36.61%), *A. media* (up to 57.14%) and *A. salmonicida* (up to 87.76%). The distribution of the species reflected what expected to find in different types of surface water (**Janda et al., 2010**) that had, in addition, different temperatures from each other. It is not surprising that the major part of the *A. salmonicida*, the mesophilic species of the genus, were found in the Lake Cadagno (1921 m.a.s.l.).

Among the Fecal Coliforms the species *Escherichia coli* was the most frequently identified (representing up to the 90% of the strains). In the sample of the wastewater treatment plant, *E. cloacae* (20%) and *K. pneumoniae* (34%) were also present in large amounts. It is known that the presence and concentration of Fecal Coliforms in environmental waters are not closely related to the kind of water, the temperature or the season (**Byappanahalli et al., 2006**), but rather to the fecal contamination of the environment.

The complexity of the extrachromosomal genomes of the *Aeromonas* spp. and Fecal Coliforms isolates was visualized by total plasmid extracts electrophoresis (plasmid profile).
We decided to make a comparison of the plasmid profiles as such even if the bands on the gels may be due to different topological forms of the same plasmid. In fact, the common topological forms of plasmids comprises the covalently close circular (CCC), the open circular (OC), the open linear (OL), and the positive and negative supercoiled forms (**Barth et al., 2009**). All the profiles shared a band of approx. 23kbp. In other works (**Kado and Liu, 1981; Anderson and McKay, 1983; Takahashi and Nagano, 1984; Pedraza and Ricci, 2002**), the same band was described to be composed by chromosomal DNA. The restriction analysis (using *EcoRI*, *SmaI*, and *SalI*) of this single band taken from the gels of some *Aeromonas* spp., Fecal Coliforms, and *Flavobacterium psychrophilum* (a fish pathogen) plasmid extracts, has shown that the band corresponded to a combination of the plasmids contained in each single bacterial strain (data not shown).

The plasmid profiles presented from few to many bands. The comparison of the plasmid profiles obtained from 231 *Aeromonas* strains and 250 Fecal Coliforms, has permitted to create six groups of profiles based on their complexity: 120 plasmidic profiles were clustered in the group of higher complexity (blue), 40 in the red group (low complexity with 2 or 3 bands per profile), 89 in the yellow group (moderately complex profiles with bands from 1 to 170 kbp), 61 plasmid profiles were placed in the purple group (with only one band of 23 kbp), and 110 in the green group (moderately complex profiles with bands from 1 to 23 kbp). The last group (light blue) includes 61 strains (27 *Aeromonas* spp. and 34 Fecal Coliforms) with no visible plasmids.

About 60% of the profiles of the *Aeromonas* strains isolated from the river before the wastewater treatment plant clustered in the blue and green groups. In these groups clustered also approx. the 80% of the profiles of the *Aeromonas* isolated from the river after the plant. Therefore, there was a substantial difference between the two sampling areas, which may be due to the input in the river of the strains coming from the wastewater treatment plant. In fact, 65% of the strains isolated in the plant presented plasmid profiles belonging to the blue and green groups.
The 63% of the *Aeromonas* plasmid profiles isolated from the Bellinzona hospital wastewater were assigned to the yellow group, while the 73% of those isolated from the Lake Cadagno were included in the purple group. As this last complexity group clustered profiles with only

one band, we may thus speculate on the reduced need to have accessory genetic material for the strains living in this low polluted environment.

The biggest part (80%) of the Fecal Coliforms plasmid profiles isolated from the Ticino river before the treatment plant were distributed in the blue, yellow and green groups, i.e. the groups showing an elevated complexity. This may be due to the presence of pollutants and/or of peculiar strains in the water collected, probably linked in part to the rain fall in the period in which the sampling was made. In fact, it has already been reported (**Kim et al., 2010**) that antibiotics and resistant bacteria can be transported into surface waters from soils by surface runoff or leaching after rainfall events. The Fecal Coliforms isolated from the hospital wastewater showed plasmid profiles clustered mainly in the yellow group (28%), while there has been a shift toward the blue group (38%) for those isolated from the wastewater treatment plant. These findings could be explained by differences of the environmental conditions resulting in different selective pressures. In the wastewater treatment plant the distribution of the plasmid profiles into the groups ranged between the 8% in the yellow group and the 38% in the blue group. Similar results were obtained in the river after the treatment plant, probably because of the partial mixing of the water leaving the plant with that of the river. Even if the changes observed in the plasmid profiles of the Fecal Coliforms were not as evident as those observed for *Aeromonas*, we can conclude that the outlet of the wastewater treatment plant affects the microbial flora of the receiving waters at least regarding the gene pool.

More than the fifty percent of the stains isolated from the Lake Cadagno were included in the green group (moderately complex profiles). The presence of Fecal Coliforms with an elevated diversity of extrachromosomal elements may indicate the need for these bacteria to adapt to an hostile environment (oligotrophic waters and low temperature). Moreover, the selection of antibiotic resistant strains can not be excluded, given the presence on the neighboring land of a large herd of cows.

The 481 strains showed resistance to Cefazolin (66.11%), Streptomycin (31.81%), Nalidixic acid (30.98%), Sulfamethoxazole (25.57%), Cefoxitin (21.41%), Ampicillin (16.22% of the Fecal coliforms), and Ciprofloxacin (9.56%) among the 20 antibiotics tested. Previous works carried out on *Aeromonas* strains isolated from two wastewater treatment plants (**Igbinosa and Okoh, 2012**), and from fishes, shellfishes and waters (**Borrego et al., 1991**) showed *Aeromonas* to be resistant to Ampicillin, Chloramphenicol, Gentamicin, Kanamycin, Nalidixic acid, Streptomycin, Tetracycline, Tobramycin, and Sulfamethoxazole. These resistances were related to the presence of small to large plasmids. Similar results have been

reported for Fecal Coliforms isolated in four hospitals (**Gu et al., 200**8) and in a slaughterhouse wastewater treatment plant (**Moura et al., 2007**). These antibiotic resistances seem therefore to be commonly present in various environments and bacteria.

The 80.5% of the 481 *Aeromonas* spp. and Fecal Coliforms analyzed was resistant to at least one antibiotic. Only 9.5% of the strains with one or more resistances belonged to the group without plasmids. On the contrary, more than the 50% of the strains resistant to one or more antibiotics, were included in the groups with the higher plasmid complexity, namely the blue (20.37%), the yellow (14.55%) and the green (18.71%) groups.

Among the *Aeromonas* strains carrying plasmids isolated from the Ticino river before the wastewater treatment plant, approx. the 69% had three or more resistances. Among those isolated from the Bellinzona hospital wastewater, the strains resistant to three or more antibiotics represented the 60.87%, from the Giubiasco wastewater treatment plant the percentage was 87.80%, whereas from the Ticino river after the treatment plant, it was 73.91%. The 97.96% of the *Aeromonas* strains isolated from the Lake Cadagno had plasmids but only 2% were resistant to three or more antibiotics.

Fecal Coliforms harbouring plasmids and being resistant to at least three antibiotics, represented the 22% of the strains isolated from the river before and after the treatment plant, the 28% of the strains from the hospital wastewater, the 40% of the strains isolated from the wastewater treatment plant, and the 18% of the alpine Lake Cadagno strains. These results indicate a relation between the plasmid carriage and the resistance phenotype. In the environments where selective pressure of any type is expected, both the plasmid complexity and the number of resistances increased.

During the characterization of the extended spectrum β-lactamases associated with plasmids, one *A. hydrophila* (77 SG-Aer, bla_{SHV-12}, bla_{OXA-1}) and four *E. coli* (55 SG-CF, bla_{SHV-12}; 56 SG-CF, bla_{SHV-12}, bla_{OXA-1}; 85 SG-CF, bla_{SHV-12}, bla_{OXA-1}; 172 FDD-CF, $bla_{CTX-M27}$) were identified. Four of these strains (one *Aeromonas* and three *E. coli*) were isolated from the Bellinzona hospital wastewater, while one *E. coli* from the Ticino river after the treatment plant. The strains carried on their plasmids the ESBL genes bla_{OXA-1}, $bla_{CTX-M27}$, and bla_{SHV-12} which are resistant determinants often encountered in clinical isolates all over the world (**Yano et al., 2013; Carattoli A., 2009**). Furthermore, the bla_{OXA-1} has been found also in animals, in addition to human samples (**Shaheen et al., 2011**). The finding of ESBL strains in the wastewater of a hospital is therefore not unexpected. On the contrary, it may be of concern the isolation of such strains from the river after the outlet of the wastewater treatment plant. In

fact, this result demonstrate the possibility for this worrisome resistance to disseminate into the natural environment through environmental, aquatic bacteria such as *Aeromonas* strains.

About 12.6% of the *Aeromonas* spp. and 4% of the Fecal Coliforms were Ciprofloxacin resistant. Usually, the resistance to this antibiotic is due to the presence of genes, such as those of the *qnr* family, located on the chromosome or on plasmids. The resistance gene *qnrS2* has been reported in an *A. hydrophila* isolated from an infected fish (**Majumdar et al., 2011**), a *qnrS1* gene in an *E.coli* isolated from cattle (**Kirchner et al., 2011**), and *qnrA1*, *qnrS1*, and *qnrB19* genes were found in *E.coli* and *K. pneumoniae*, in a collection of 232 Ciprofloxacin resistant or ESBL-producing isolates (**Richter et al., 2010**). Another study conducted on *Aeromonas* strains recovered from a Swiss lake identified the presence of the *qnrS2* gene and of a class 1 integron, containing the *acc(6')-Ib-cr* cassette, in an *A. allosaccharophila* (**Picão et al., 2008**).

On the contrary, the resistance to Ciprofloxacin found in our strains was due to mutations in the Gyrase A protein (encoded by *GyrA*), the target of the quinolones and fluoroquinolones antibiotics. Among the 29 *Aeromonas* spp. resistant to Ciprofloxacin and the six resistant to Nalidixic Acid, 29 strains (seven from the Ticino river before and 16 after the WW.T.P outlet) were resistant due to a single mutation in position 83 of the *gyrA* gene, leading to the substitution Ser83→Ile in the protein, and one strain (*A. caviae*) to the mutation Ser83→Arg. These results were in agreement with those reported by other authors (**Goñi-Urriza et al., 2002**), who found that the substitution of the Serine 83 in Isoleucine was more frequent than that in Arginine in *A. caviae*, *A. hydrophila* and *A. sobria* isolates from European rivers. One of our strains, *A. media* isolated from the river before the treatment plant, showed a different mutation Ser83→Phe. To our knowledge, this mutation was never reported in *Aeromonas* spp. In fact, this substitution has been described previously only in *E. coli* isolated from outpatients (**Minarini et al., 2008**), and in *S. typhimurium* isolated from clinical specimens (**Reyna et al., 1995**). This unusual mutation seems related to the Nalidixic acid and Ciprofloxacin resistances but not to other fluoroquinolones, since these last showed milder increases of the MIC (Minimal Inhibitory Concentration) (**Reyna et al., 1995**). Another substitution that confers resistance to quinolones, already described in *Aeromonas* isolates from feces (**Alcaide et al., 2010**) but not present in our strains, was the Ser83→Val. In three *A. media* and one *A. caviae* the resistance was a result of a double substitution. In two *A. media* resistant to Ciprofloxacin and in the one resistant to Nalidixic acid, we showed the substitutions Ser83→Ile and Asp87→Asn. The *A. caviae*, which was resistant to Nalidixic

acid and Ciprofloxacin, had the double substitution Ser83→Ile and Asp87→Ala. These double mutations are not described in the scientific literature, and do not seem to be directly related to the phenotype of resistance to fluoroquinolones and quinolones. Actually, other strains presented the same resistance profile but harbored only the single mutation Ser83→Ile. However, we would have to check any change of the MIC regarding these antibiotics.
The single substitution Ser83→Leu was found in two *E.coli*, isolated from the wastewater of the Bellinzona Hospital and from the river after the treatment plant in Giubiasco. These mutations were already reported in *E.coli* and *K. pneumoniae* (**Fu et al., 2013**). The double mutation Ser83→Leu and Asp87→Asn was detected in seven *E. coli* isolated from the same sampling areas (three from the hospital wastewater and two strains from the river after the WWTP) and in two strains isolated from the wastewater treatment plant in Giubiasco. The *E. coli* 85 SG-CF, isolated from the hospital wastewater, showed a wild-type sequence of the *GyrA* gene. Its resistance might therefore be due to the presence of a resistance determinant, such as the *qnr* or the *aac(6')-Ib-cr* genes, conferring resistance to quinolones. These genes may be carried on plasmids, rare in *E. coli* (**Richter et al., 2010**), or on the chromosome (**Ruiz et al. 2012**).

In order to verify the presence of a relationship between the phenotypic resistances and the genotype of our strains, we investigated the distribution of Class 1 integrons and of their associated gene cassettes. The integrase I gene (*IntI1*) was detected in the 18.5% of the strains analyzed; in details, about the 28% (64 out of 231) of the *Aeromonas* spp. and the 10% (25 out of 250) of the Fecal Coliforms had this gene. In a study carried out in a slaughterhouse wastewater treatment plant the *IntI1*-carrying bacteria (*Aeromonas* spp. and *Enterobacteriaceae*) were the 30.7% (**Moura et al., 2007**). Our findings were in agreement with those reported by these authors since we found that 28.5% of the strains isolated from the wastewater treatment plant of Giubiasco were *IntI1* positive. The rate of *Aeromonas* strains carrying Class I integrons was found to be 65% in a set of 40 *A. salmonicida* isolated during a clinical outbreak of furunculosis (**Schmidt et al., 2001**), and 10% of the 133 *Aeromonas* strains arising from samples suspected of a foodborne outbreak (**Chang et al., 2007**). 221 out of 1832 (12.1%) *Enterobacteriaceae* isolated from a wastewater treatment plant in a municipal area in Poland were *IntI1* positive (**Mockraka et al., 2012**). Considering only the species *E. coli*, *K. pneumoniae*, *K. oxytoca*, *C. freundii*, and *E. cloacae*, i.e. those we also found in the sampling done in the wastewater treatment plant of Giubiasco, the percentage of positive *IntI1* isolates drops to 7.5%, a rate very close to our percentage. In

another study conducted in Kenya during a 19-year period, the authors analyzed *E. coli* strains isolated from hospitalized and non-hospitalized patients, and found that the 35% (464 out of 1327) of the strains were positive for the Class 1 integrons (**Kiiru et al., 2013**). In our study the percentage of *IntI1* positive *E. coli* strains, considering only those isolated from the Bellinzona hospital wastewater, was of 20% (9 out of 45). These different rates, are probably due to their different origin and degree of pathogenicity.

The 25% (16 out of 64) of the *Aeromonas* strains and the 88% (22 out of 25) of the Fecal Coliform carried the *IntI1* gene on plasmids. The sequencing of the gene cassettes within the Class 1 integrons was performed exclusively in the total plasmid extracts of the positive *IntI1* strains, in order to investigate only the resistance determinants associated to plasmids.

The *IntI1* gene located on plasmids was detected in the 43.75% of the *A. media*, in the 31.25% of the *A. hydrophila*, and in the 12.5% of the *A. caviae* and *A. sobria*. In the Fecal Coliforms the percentage of the plasmidic *IntI1* genes was 88% (22 out of 25); the majority of strains, about the 86% (19 out of 22) were *E. coli*.

In a work conducted on 267 *Aeromonas* strains from human origin isolated in Taiwan, the *IntI1* gene was present in the 13.8% of the isolates, but only nine of them carried plasmids; therefore, merely the 0.75% of the strains carried *IntI1* on plasmids (**Lee et al., 2008**). Similar results were obtained in strains of *E. coli* isolated from animal samples: the *IntI1* gene was present in the 27% of the bacteria but only in about the 3.7% of the strains the gene was plasmid located (**Shaheen et al., 2010**). Our results differ from those reported above since the values we found are substantially higher, but it should be considered that the strains had a different origin. It is likely that our set of environmental strains was submitted to a significant selective pressure in the water ecosystems we studied, that favored the selection of more versatile strains, possessing an elevated ability to survive in these hostile environments thanks to a large availability of extrachromosomal material. The presence of Class 1 integrons indicates that our environmental strains are able to store, to use and to eventually mobilize some of their genes when needed.

In the Class 1 integrons of our *Aeromonas* spp. and Fecal Coliforms, we identified genes that confers resistance to aminoglycosides (*aadA*- and *aacA*-like gene), carbapenems (*blaOXA*-like gene), trimethoprim (*dfrA-, dfrB-, dfr*- like gene), and chloramphenicol (*catB*-like gene). In *Aeromonas,* the most common plasmid integrons contained the gene cassettes *catB8-aadA1*. *aadA1* was included in the 62.5% of the integrons, *CatB8* in the 37.5%, *CatB3* in the 25%, and *dfr*- in the 18.75%. These rates are in agreement with the results reported by **Chang**

and colleagues (**2007**). Only one strain shown an empty Class 1 integron. Usually, the first cassette resistance gene in the variable part of the Class 1 integrons confers a phenotypic resistance, while the other genes positioned from the second position onwards confers low resistances or do not affects the phenotype. In the 50% of the integrons detected in the *Aeromonas* strains, the first cassette, represented by the genes *CatB8* or *CatB3*, did not confer the resistance to Chloramphenicol. A similar observation was reported by **Moura and colleagues** (**2007**) regarding the *aadA1* gene cassette. This is probably due to the presence of weak promoters that cause the absence or the low expression of the gene cassette. In some cases, as in the strain 42 FT-Aer (*A. media*) showing the *CatB3-aadA1* gene cassettes, the resistances to Chloramphenicol, Streptomycin, and Tobramycin were phenotypically detectable. In Fecal Coliforms the most common gene cassettes were *dfrA14-aadA1-catB2*, *aacA4-aadA1-catB2*, and *dfrA1-aadA1*. *aadA1* was detected in 59.1% of the integrons, *CatB2* in the 36.4%, and *DfrA14* in the 22.7%. Consequently, the resistances to the aminoglycosides and to the trimethoprim were the most common resistances found in the Class 1 integron as reported by other studies (**Shaheen et al., 2010; Kiiru et al., 2013**). As found in *Aeromonas*, the presence of the gene cassette *CatB2* was not related to the Chloramphenicol resistance, but it has to be noted that the gene was always present in the third position. The only exception was represented by the strain 55 SG-CF (*E. coli*) that was Chloramphenicol resistant despite the position of *CatB2*. In few strains, we noted the presence of genes with no clear functions in resistance. In one *E. coli* the *ExtX* (putative Esterase/Hydrolase) sequence was integrated in the variable part of the integron. This genetic construct was also reported by **Mokracka and colleagues** (**2012**), but its function remains unexplained. We were not able to complete the sequence of the integron gene cassettes *DfrA12-OrfF-?* of an *E. coli* strain despite many attempts. **Gestal and colleagues** (**2005**) reported a variable region of a Class 1 integron composed by the genes *DfrA12-OrfF-aadA8b* that conferred resistance to Streptomycin/Spectinomycin; the gene *aadA8b* is probably an hybrid cassette that results from the recombination of the *aadA2* and the *aadA1* genes. Our strain was phenotypically resistant to Trimethoprim/Sulfamethoxazole, Trimethoprim, and Streptomycin, making plausible the presence of the *aadA8b* cassette.

All the *Aeromonas* spp. and the Fecal Coliforms that possessed integrons were resistant to Sulfamethoxazole; this resistance is normally due to the presence of the *sul* gene in all the Class 1 integron. As already stated by **Moura and colleagues** (**2007**), the other antibiotic resistances detected by phenotypical methods in our strains reflected the presence of genes or

mechanisms of resistance located on the chromosome or on plasmids, but not associated with integrons.

Approximately the 32% of the *Aeromonas* spp. and the 69% of the Fecal Coliforms had mobilizable plasmids belonging to the subfamilies MOB_{H121}, MOB_{H11}, MOB_{F12}, MOB_{P12}, MOB_{P13}, and MOB_{P14}. The MOB_P subfamily was the most frequent in *Aeromonas* spp. (25.54%), while in the Fecal Coliforms the 31.6% of the strains harbored plasmids belonging to the MOB_F. Plasmids characterized by a MOB family were more frequent in the strains with complex plasmid profiles, i.e. belonging to the Blue, the Yellow and the Green groups.
According to a previous study carried out on *Gammaproteobacteria* (**Garcillàn-Barcia et al., 2011**), the MOB_P, MOB_H. and MOB_F families are characteristic of plasmids of large size. Contrary to the finding reported, the MOB_F were found solely in *Aeromonas* showing plasmids with medium/low size. The MOB_P was the family most frequently found in environmental bacteria (**Smillie et al., 2010**) as in the *Aeromonas* spp. we analyzed.

Plasmid extracts were investigated in order to identify the presence of sequences permitting the transfer of plasmids by conjugation. The search for the incompatibility groups Inc A/C, Inc P, Inc W, Inc N, or Inc Q by PCR was, in our experience, unsuccessful: whereas this failure may be explained for the *Aeromonas* extracts by the use of primers designed on the *Enterobacteriaceae* (**Carattoli et al., 2005; Cattoir et al., 2008; Götz et al., 1996**), other reasons have to be at the base of this negative result in the Fecal Coliforms.
Dot-Blot was performed only for the screening of the incompatibility group U allowing the detection of some positive plasmid extracts. Plasmids belonging to the IncU group and carrying resistance determinants have already been described in *Aeromonas* strains (**Picão et al., 2008; Rhodes et al., 2004; Sørum et al., 2003**). This incompatibility group was identified in the plasmid extract of *Aeromonas* spp. isolated from the wastewater treatment plant of Giubiasco (three strains) and from the river after the treatment plant (one strain), and in a Fecal Coliforms isolated from the wastewater treatment plant. Since the annealing of the Dot-Blot probes are less specific than those of the primers, the finding of some positive extracts only by Dot-Blot is conceivable. The incompatibility groups Inc N, Inc P, Inc W, and Inc U, were found in 29 of 32 plasmids tested by **Coutourier and colleagues** (**1988**). The percentage of positivity was therefore about 91%, and for the Inc U alone the rate was 25%. This result was in contrast with the present study where only few representatives of the Inc U group were found at the very low rate of 1.19%.

Treating *E. coli* K-12 strains with 10% SDS, **Inuzuka and colleagues (1969)** obtained the loss of plasmids without reducing the growth. In our case, the use of the same protocol has led the inability to survive in approx. 63% of the *Aeromonas* strains. The loss of plasmids was obtained in two strains, and only in one (91 SG-Aer), it was coupled to the loss of the resistance to Nalidixic Acid. Other attempts of plasmid curing based on minimal medium, water and plate curing or using the DNA-intercalating acridine orange were carried out. In particular, the DNA-intercalating acridine orange proved to be effective in *Pseudomonas aeruginosa* in which the 66% of the isolate lost concomitantly plasmids and resistances such as those for Amikacin, Tetracycline, and Chloramphenicol (**Shahid et al., 2003**). Since none of the curing procedures tested was effective, we concluded that plasmids are stably maintained in *Aeromonas*. On the other hand, it has to be noted that the stress induced to the *Aeromonas* strains has led to the development of adaptations, which were expressed in the 23% of the strains through changes in the profiles of resistance. These variations in the resistance profiles may be a signal of changes in pathways used by *Aeromonas* to survive in polluted aquatic environments.

To investigate the ability of the *Aeromonas* spp. to horizontally transfer the resistance genes, some strains were used as donors in conjugation experiments, while Fecal Coliforms were employed as the recipients. No conjugations were successful when performed in liquid media, whereas it was possible to obtain transconjugants when the conjugation was performed in a solid medium. The efficiency of conjugation in liquid or solid media is linked to the morphology of the sexual pili (**Bradley et al., 1980**): with rigid pili, as those expressed by the IncM, IncN, IncP and IncW plasmids, the efficiency is higher in solid media, whereas the flexible pili expressed by the incompatibility groups IncH1, IncH2 or IncT, are more efficient in conjugation experiments carried out in liquid media. *A. media*, *A. caviae*, and *A. hydrophila* were able to transfer one of their plasmids to a *Citrobacter freundii*, isolated from the wastewater of the Bellinzona Hospital that became resistant to Ceftriaxone, the antibiotic used as selection for the donors. It was therefore possible to transfer horizontally a conjugative plasmid to an environmental bacterium. This result reinforces the role *Aeromonas* can play in the diffusion of resistances in the aquatic environment. It has already been demonstrated that *Aeromonas* are able to transfer their resistances to other bacteria, but the recipients were generally laboratory strains such as *E. coli* J53, *E. coli* K12 14R525, and *P. putida* KT2442-GFP (**Moura et al., 2012a; Cattoir et al., 2008; Lee et al., 2008; Schmidt et al., 2001;**

Mokracka et al., 2012). It is therefore not surprising that the efficiency of the conjugations found in those experiments was higher compared to our experiments.
We were unable to determine the MOB subfamilies of the plasmids present in the strains used in the conjugation experiments, except for the plasmid extract of the strain 19 FT-Aer, that was positive for the MOBP14. This MOB subfamily is related to the incompatibility groups IncQ2 and IncP6. Given that this strain was able to conjugate on a solid support, we can thus assume that the strain 19 FT-Aer harbors and is able to transfer a plasmid belonging to the incompatibility group P. Furthermore, it is possible that all the strains that conjugated possessed rigid sexual pili because they were able to transfer their resistance plasmids exclusively on solid supports.

The few attempts of transformation we carried out gave negative results, perhaps because the excessive size or number of the plasmids used. Generally, the bacterial transformation is performed through electroporation to facilitate the entry of the plasmids into the recipient strain, using naturally competent bacteria, and plasmids of limited size, i.e. max. 13-14 kbp (**Sota et al., 2010; Demanèche et al., 2002**). In order to confirm the ability of *Aeromonas* strains to acquire exogenous DNA from the aquatic environment further experiments of transformation should be carried out using: plasmids in the CCC form (Covalently Closed Circular) to eliminate any topological obstacle, electroporation, and/or naturally competent bacteria.

6 CONCLUSIONS

The hypothesis of this PhD thesis was that bacteria of the genus *Aeromonas* could play a substantial role in the diffusion of resistant genes in aquatic environments. Since these bacteria are natural inhabitants of hydric ecosystems but can also be find as pathogens in humans, we assumed that they could be considered not only a "reservoir" of antibiotic resistance genes but possibly also a "vector" in the aquatic environment for the spread of these genes to bacteria of clinical relevance, such as the Fecal Coliforms.

The experimental planning has permitted to isolate and to identify *Aeromonas* spp. and Fecal Coliforms from five sampling areas, representing five different aquatic ecosystems, and to characterize their plasmid content and their antibiotic resistances.

The rationale behind this study is the growing awareness of the existence of a pool of resistance genes, which are selected by the presence in the environment of increasing amounts of micro-pollutants, such as antibiotics (**Campagnolo et al., 2002**). These resistance genes can be transferred horizontally to pathogenic bacteria, thereby increasing the risk of infections caused by resistant germs.

Several studies (**Shaheen et al., 2010; van Hoek et al., 2011**) have shown that the use of antimicrobial compounds in aquaculture, in veterinary and in clinical settings, can lead to the selection of resistant bacteria, and, even more important, to the selection of mobile genetic elements containing resistance determinants that can be transmitted among bacteria (**Nardelli et al., 2012; Shaheen et al., 2011**).

Pollutants and resistant and/or virulent bacteria can reach the water environment through the sewage waters but also through the rainfall or watering of the fields which drain the soils (**Cabello et al., 2013; Kim et al, 2010**). On the other hand, the waterborne infections are well known pathologies not only in developing countries. (**Puthucheary et al., 2012; Majumdar et al., 2011; Cremonesini and Thomson, 2008**).

In this study we demonstrated that several antibiotic resistances found in the analyzed *Aeromonas* were located on Class 1 integrons associated to plasmids. Other resistance genes were located on plasmids outside integrons or in the chromosomal DNA, both in Class 1 integrons and in other regions. As found in a study looking for presence of ESBL resistance genes on plasmids in the Seine river (**Girlich et al., 2011**), we were able to isolate from the hospital wastewater an *Aeromonas* strain producing extended spectrum beta-lactamases thank to genes located on a plasmid. These findings confer to these bacteria a more important

contribution for the spread of resistance determinants in the aquatic environment. It should be emphasized that *Aeromonas* are aquatic bacteria that can reach significant high concentrations; considering the proportion that we obtained in our analysis of one ESBL producing *Aeromonas* each 250 strains, the number of *Aeromonas* ESBL positive that could be found in the analyzed waters could range from $2*10^{12}$ to $2*10^{15}$ CFU/day.
The number and type of resistance genes identified so far on *Aeromonas* spp. plasmids allows to support the hypothesis of considering these bacteria an environmental "reservoir" of plasmid resistance genes.

In presence of variable stress conditions, the totality of the *Aeromonas* strains was able to adapt and grow, highlighting the capacity of this bacterial genus to persist under adverse conditions. The strains maintained their plasmids even under extreme stress conditions, such as growing in 10% SDS. We observed some phenotypic changes regarding the antibiotic resistance, which might be due to nonspecific mechanisms of resistance as those induced by chemical compounds or heavy metals (**Yilmaz et al., 2013; Aktan et al., 2013**). Furthermore, the stability of the plasmids contained in *Aeromonas* promotes their role as environmental "reservoirs" of antibiotic resistance genes.

The presence in *Aeromonas* strains of mobilizable (MOB_P) and potentially transferable plasmids endorses the assumptions of a plausible role of these bacteria in the horizontal gene transfer mediated by plasmids in the aquatic environment. We verified in a preliminary study that *Aeromonas* were able to transfer their resistant plasmids to other *Aeromonas* strains, as also reported by **Bello-Lòpez and colleagues** (**2011**). In our study, four strains of *Aeromonas* transferred one or more plasmids and the respective resistances to a *C. freundii* strain. The horizontal transfer of resistance genes from *Aeromonas* spp. strains to a strain of fecal origin, allowed to demonstrate the ability of *Aeromonas* spp. to relocate the resistance plasmids to other bacterial genera, thus leading to the formation of transconjugants potentially resistant to a given pharmacological treatment. In the aquatic environment that we studied here, these exchanges of genetic material through conjugation might involve a huge number of *Aeromonas* strains, in the order of $4.5*10^{12}$ to 10^{14} CFU/day. The ability of *Aeromonas* strains to transfer their resistance plasmids at inter-species and inter-genera levels, confirms also its role as a "vector" of genetic resistance determinants possibly to the human intestinal flora or to pathogenic bacteria.

Given the significant number of resistant and conjugative *Aeromonas* strains in the various aquatic environments, the role that *Aeromonas* hold in the spread of resistant genes seems to be very important. However, further analysis will be needed in order to characterize unknown MOB or BHR groups of plasmids and therefore to better understand the horizontal transfer of resistance determinants among *Aeromonas* strains and other potentially pathogenic bacteria.

7 REFERENCES

Abbott S.L, Cheung W.K.W., Janda J.M. The Genus *Aeromonas*: Biochemical characteristic, atypical reaction, and phenotypic identification schemes. *Journal of Clinical Microbiology, 41 (6), 2348-2357, 2003*.

Aktan Y., Tan S., Icgen B. Characterization of lead-resistant river isolate *Enterococcus faecalis* and assessment of its multiple metal and antibiotic resistance. *Environmental Monitoring and Assessment, 185 (6), 5285-5293, 2013*.

Akturk S., Dincer S., Toroglu S. Determination of microbial quality and plasmid-mediated multidrug resistant bacteria in fountain drinking water source in Turkey. *Journal of Environmental Biology, 33 (6), 1127-1136, 2012*.

Al-Benwan K., Abbott S., Janda J.M., Huys G., Albert M.J. Cystitis caused by *Aeromonas caviae. Journal of Clinical Microbiology, 45 (7), 2348-2350, 2007*.

Albert M.J., Ansaruzzaman M., Talukder K.A., Chopra A.K., Kuhn I., Rahman M., Faruque A.S.G., Sirajul Islam M., Bradley Sack R., Mollby R. Prevalence of enterotoxin genes in *Aeromonas* spp. isolated from children with diarrhea, healthy controls, and the environment. *Journal of Clinical Microbiology, 38 (10), 3785-3790, 2000*.

Alcaide E., Blasco M.-D., Esteve C. Mechanisms of quinolone resistance in *Aeromonas* species isolated from humans, water and eels. *Research in Microbiology, 161 (1), 40-45, 2010*.

Alvarado A., Garcillàn-Barcia M.P., de la Cruz F. A Degenerate Primer MOB Typing (DPMT) method to classify gamma-proteobacterial plasmids in clinical and environmental settings. *Plos One, 7 (7), 1-15, 2012*.

Anderson D.G., McKay L.L. Simple and rapid method for isolating large plasmid DNA from Lactic Streptococci. *Applied and Environmental Microbiology, 46 (3), 549-552, 1983*.

Anjum R., Grohmann E., Malik A. Molecular characterization of conjugative plasmids in pesticide tolerant and multi-resistant bacterial isolates from contaminated alluvial soil. *Chemosphere, 84 (1), 175-181, 2011*.

Ballestè E., Bonjoch X., Belanche L.A., Blanch A.R. Molecular indicators used in the development of predictive models for microbial source tracking. *Applied and Environmental Microbiology, 76 (6), 1789-1795, 2010*.

Baquero F., Luis Martınez J. and Canton R. Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology, 19 (3), 260-265, 2008*.

Barth M.C., Dederich D.A., Dedon P.C. An improved method for large-scale preparation of negatively and positively supercoiled plasmid DNA. *Biotechniques, 47 (1), 633-635, 2009*.

Beaz-Hidalgo R., Figueras M.J. Molecular detection and characterization of Furunculosis and other *Aeromonas* fish infection. *In: Health and Environment Aquaculture, Edited by Carvalho E., (In Tech). 97-132, 2012*.
(Available from: http://www.intechopen.com/books/health-and-environment-in-aquaculture/molecular-detection-and-characterization-of-furunculosis-and-other-aeromonas-fish-infections)

Bello-Lòpez J.M., Vàzquez-Ocampo N.J., Fernàndez-Redòn E., Curiel-Quesada E. Inability of some *Aeromonas hydrophila* strains to act as recipients of plasmid pRAS1 in conjugal transfer experiments. *Current Microbiology, 64 (4), 332-337, 2011*.

Benagli C., Demarta A., Caminada AP., Ziegler D., Petrini O., Tonolla M. A rapid MALDI-TOF MS identification database at genospecies level for clinical and environmental *Aeromonas* strains. *Plos one, 7 (10), 1-6, 2012*.

Bennett P.M. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology, 153 (S1), S347-S357, 2008*.

Borrego J.J., Morinigo M.A., Martinez-Manzanares E., Bosca M., Castro D., Barja J.L. Toranzo A.E. Plasmid associated virulence properties of environmental isolates of *Aeromonas hydrophila*. *Journal of Medical Microbiology, 35 (5), 264-269, 1991*.

Bossi-Küpfer M., Genini A., Peduzzi R., Demarta A. Tracheobronchitis caused by *Aeromonas veronii biovar sobria* after near-drowning. *Journal of Medical Microbiology, 56 (Pt11), 1563-1564, 2007***.**

Bradley D.E., Taylor D.E., Cohen D.R. Specification of Surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *Journal of Bacteriology, 143 (3), 1466-1470, 1980*.

Brzuszkiewicz E., Thürmer A., Schuldes J., Leimbach A., Leisegang H., Meyer F.-D., Boalter J., Petersen H., Gottschalk G., Daniel R. Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic Escherichia coli. *Archives of Microbiology, 193 (12), 883-891, 2011*.

Byappanahalli M.N., Whitman R.L., Shively D.A., Sadowsky M.J., Ishii S. Population structure, persistence, and seasonally of autochthonous *Escherichia coli* in temperate, coastal forest soil from a Great Lakes watershed. *Environmental Microbiology, 8 (3), 504-513, 2006*.

Cabello F.C., Godfrey H.P., Tomova A., Ivanova L., Dölz H., Millanao A., Bushmann A.H. Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. *Environmental Microbiology, 15 (7), 1917-1942, 2013*.

Calvo J., Cantòn R., Cuenca F.F., Mirelis B., Navarro F. Detección fenotípica de mecanismos de resistencia en gramnegativos. *Procedimientos en Microbiología Clínica, Ed. Emilia Cercenado y Rafael Cantòn, 2011*.

Campagnolo E.R., Johnson K.R., Karpati A., Rubin C.S., Kolpin D.W., Mayer M.T., Esteban J.E., Currier R.W., Smith K., Thu K.M., McGeehin M. Antimicrobial residues in animal waste and resources proximal to large-scale swine and poultry feeding operations. *The Science of the Total Environment, 299(1-3), 89-95, 2002*.

Carattoli A. Resistance plasmid families in *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy, 53 (6), 2227-2238, 2009*.

Carattoli A., Bertini A., Villa L., Falbo V., Hopkins K.L., Threlfall E.J. Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods, 63 (3), 219-228, 2005*.

Carnahan A.M., Altwegg M. Taxonomy. *In: The genus Aeromonas, Edited by Austin B., et al. (John Wiley & Sons Ltd, Chichester). 1-38, 1996.*

Cattoir V., Poirel L., Aubert C., Soussy C-J and Nordmann P. Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas spp. Emerging infectious diseases, 14 (2), 231-237, 2008*.

Chang Y.-C., Shih D.Y.-C., Wang J.-Y., Yang S.-S. Molecular characterization of class 1 integrons and antimicrobial resistance in *Aeromonas* strains from foodborne outbreak-suspect samples and environmental sources in Taiwan. *Diagnostic Microbiology and Infectious Disease, 59 (2), 191-197, 2007*.

Charette S.J., Brochu F., Boyle B., Filion G., Tanaka K.H., Derome N. Draft genome sequence of the virulent strain 01-B526 of the fish pathogen *Aeromonas salmonicida*. *Journal of Bacteriology, 194 (3), 722-723, 2012*.

Chen W.-C., Huang J.-W., Chen K.-Y., Hsueh P.-R. Yang P.-C. Spontaneous bilateral bacteria empyema in a patient with nephritic syndrome. *Journal of Infection, 53 (3), e131-134, 2006*.

Colom K., Pérez J., Alonso R., Fernandez-Aranguiz A., Larino Eva., Cisterna R. Simple and reliable multiplex PCR assay for detection of bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes in *Enterobacteriaceae. FEMS Microbiology letters 223 (2), 147-151, 2003*.

Connon R.E., Geist J., Werner I. Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. *Sensor (Basel), 12 (9), 12741-12771, 2012*.

Couturier M., Bex F., Bergquist P.L., Maas W.K. Identification and classification of bacterial plasmids. *Microbiological Reviews, 52 (3), 375-395, 1988.*

Crémet L., Caroff N., Dauvergne S., Reynaud A., Lepelletier D., Corvec S. Prevalence of plasmid-mediated quinolone resistance determinants in ESBL *enterobacteriaceae* clinical isolates over a 1-year period in a French hospital. *Pathologie Biologie, 59 (3), 151-156, 2011.*

Cremonesini D., Thomson A. Lung colonization with *Aeromonas hydrophila* in cystic fibrosis believed to have come from a tropical fish tank. *Journal of the Royal Society of Medicine, 101 (S1), S44-S45, 2008* .

Demanèche S., Jocteur Monrozier L., Chapel J.P., Simonet P. Influence of plasmid conformation and inserted sequence homology on natural transformation of *Acinetobacter* sp. *Annals of Microbiology, 52, 61-69, 2002.*

Demarta A., Tonolla M., Caminada AP., Beretta M., Peduzzi R. Epidemiological relationships between *Aeromonas* strains isolate from symptomatic children and household environments as determined by ribotyping. *European Journal of Epidemiology, 16 (5), 447-453, 2000.*

Doyle M.P., Erickson M.C. Closing the door on the fecal coliform assay. *Microbe, 1, 162-163, 2006.*

European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 1.3, valid from 05-01-2011.

European Community. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000, establishing a framework for Community action in the field of water policy. *Official Journal of the European Communities, L 327-22.12.2000: 72; 2000.*

Field K.G., Samadpour M. Fecal source tracking, the indicator paradigm, and managing water quality. *Water Research, 41 (16), 3517-3538, 2007.*

Figueira V., Vaz-Moreira I., Silva M., Manaia C.M. Diversity and antibiotic resistance of *Aeromonas* in drinking and waste water treatment plants. *Water Research, 45 (17), 5599-5611, 2011*.

Francia M.V., Varsaki A., Garcillàn-Barcia M.P., Latorre A., Drainas C., de la Cruz F. A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiology Review, 28 (1), 79-100, 2004*.

Frost L.S., Leplae R., Summers A.O., Toussaint A. Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology, 3 (9), 722-732, 2005*.

Fu Y., Zhang W., Wang H., Zhao S., Chen Y., Meng F., Zhang Y., Xu H., Chen X., Zhang F. Specific patterns of *gyrA* mutations determine the resistance difference to ciprofloxacin and levofloxacin in *Klebsiella pneumoniae* and *Escherichia coli*. *BMC Infectious diseases, 13 (8), 1-6, 2013*.

Garcillàn-Barcia M.P., Alvarado A., de la Cruz F. Identification of bacterial plasmids based on mobility and plasmid population biology. *FEMS Microbiology Reviews, 35 (5), 936-956, 2011*.

Garcillàn-Barcia M.P., Francia M.V., de la Cruz F. The diversity of conjugative relaxase and its application in plasmid classification. *FEMS Microbiology Review, 33 (3), 657-687, 2009*.

Gestal A.M., Stokes H.W., Partridge S.R., Hall R.M. Recombination between the *dfrA12-orfF-aadA2* cassette array and an *aadA1* gene cassette creates a hybrid cassette, *aadA8b*. *Antimicrobial Agents and Chemotherapy, 44 (11), 4771-4774, 2005*.

Ghosh S., Mahapatra N.R., Ramamurthy T., Benerjee P.C. Plasmidic curing from an acidophilic bacterium of the genus *Acidocella*. *FEMS Microbiology Letters, 183 (2), 271-274, 2000*.

Girlich D., Poirel L., Nordmann P. Diversity of Clavulanic acid-inhibited Extended-Spectrum β-Lactamases in *Aeromonas* spp. from the Seine river, Paris, France. *Antimicrobial Agents and Chemotherapy, 55 (3), 1256-1261, 2011*.

Girones R., Ferrùs M.A., Alonso J.L., Rodriguez-Manzano J., Calgua B., de Abreu Correa A., Hundesa A., Carratala A., Bofill-Mas S. Molecular detection of pathogens in water – The pros and cons of molecular techniques. *Water Research, 44 (15), 4325-4339, 2010*.

Goni-Urriza M., Arpin C., Capdepuy M., Dubois V., Caumette P., Quentin C. Type II Topoisomerase quinolone resistance-determining region of *Aeromonas caviae*, *A. hydrophila* and *A. sobria* complex and mutations associated with quinolone resistance. *Antimicrobial Agents and Chemotherapy, 46 (2), 350-359, 2002.*

Goto D.K., Yan T. Genotypic diversity of *Escherichia coli* in water and soil of tropical watersheds in Hawaii. *Applied and Environmental Microbiology, 77 (11), 3988-3997, 2011*.

Götz A., Pukall R., Smit E., Tietze E., Prager R., Tschäpe H., van Elsas J.D., Smalla K. Detection and characterization of Broad-Host-Range plasmids in Environmental Bacteria by PCR. *Applied and Environmental Microbiology, 62 (7), 2621-2628, 1996*.

Gu B., Pan S., Wang T., Zhao W., Mei Y., Huang P., Tong M. Novel cassette arrays of integrons in clinical strains of Enterobacteriaceae in China. *International Journal of Antimicrobial Agents, 32 (6), 529-533, 2008*.

Hall R.M., Collis M. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resistance Updates, 1 (2), 109-119, 1998*.

Hall R.M., Collis M. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular Microbiology, 15 (4), 593-600, 1995*.

Holmes P., Niccolls L.M., Sartory D.P. The ecology of mesophilic *Aeromonas* in the aquatic environment. *In: The genus Aeromonas, Edited by Austin B., et al. (John Wiley & Sons Ltd, Chichester). 127-150, 1996.*

Holten-Andersen L., Dalsgaard I., Buchmann K. Baltic Salmon, *Salmo salar*, from Swedish river Lule Älv is more resistant to furunculosis compared to rainbow trout. *Plos One, 7 (1), Pg. 1-5, 2012*.

Hua H.T., Bollet C., Tercian S., Drancourt M., Raoult D. *Aeromonas popoffii* urinary tract infection. *Journal of Clinical Microbiology, 42 (11), 5427-5428, 2004*.

Ibekwe A.M., Murinda S.E., Graves A.K. Genetic diversity and antimicrobial resistance of *Escherichia coli* from human and animal sources uncovers multiple resistances from human sources. *Plos One, 6 (6), 1-11, 2011*.

Igbinosa I.H., Okoh I. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. *The Scientific World Journal, v2012, 1-6, 2012*.

Inuzuka N., Nakamura S., Inuzuka M., Tomoeda M. Specific action of sodium dodecyl sulfate on the sex factor of *Escherichia coli* K-12 Hfr strains. *Journal of Bacteriology, 100 (2), 827-835, 1969*.

Janda J.M, Abbot S.L. The Genus *Aeromonas*: pathogenicity, and infection. *Clinical Microbiology Reviews, 23 (1), 35-73, 2010*.

Janezic K.J., Ferry B., Hendricks E.W., Janiga B.A., Johnson T., Murphy S., Roberts M.E., Scott S.M., Theisen A.N., Hung K.F., Daniel S.L. Phenotypic and genotypic characterization of *Escherichia coli* isolated from untreated surface waters. *The Open Microbiology Journal, 7, 9-19, 2013*.

Kado C.I., Liu S.-T. Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology, 145 (3), 1365-1373, 1981*.

Kiiru J., Butaye P., Goddeeris B.M., Kariuki S. Analysis for prevalence and physical linkages amongst integrons, ISEcp1, ISCR1, Tn21 and Tn7 encountered in *Escherichia coli* strains from hospitalized and non-hospitalized patients in Kenya during a 19-year period (1992-2011). *BMC Microbiology, 13, 1-14, 2013*.

Kilpi M., Nikoskelainen S., Grannas S., Nuutila J., Järvisalo O., Kause A., Lilius E.-M. Resistance to bacterial infection diseases in rainbow trout (Oncorhynchus mykiss). *Veterinary Immunology and Immunophathology, 153 (3-4), 267-278, 2013*.

Kim K-.Y., Park J-.H., Kwak H-.S., Woo G-.J. Characterization of the quinolone resistance mechanism in foodborne *Salmonella* isolates with high nalidixic acid resistance. *International Journal of Food Microbiology, 146 (1), 52-56, 2011*.

Kim S.C., Davis J.G., Truman C.C. Ascough II J.C., Carlson K. Simulated rainfall study for transport of veterinary antibiotics – mass balance analysis. *Journal of Hazardous Materials, 175 (1-3), 836-843, 2010.*

Kirchner M., Wearing H., Teale C. Plasmid-mediated quinolone resistance gene detected in *Escherichia coli* from cattle. *Veterinary Microbiology, 148, Pg. 434-435, 2011*.

Kishinhi S.S., Paul B., Farah T., Farah I.O. Molecular approach to microbiological examination of water quality in the Grand Bay National Estuarine Research Reserve (NERR) in Mississippi, USA. *Environmental Health Insights, 7, 33-41, 2013*.

Küpfer M., Kuhnert P., Korczak B.M., Peduzzi R., Demarta A. Genetic relationships of *Aeromonas* strains from 16S rRNA, *gyrB* and *rpoB* gene sequences. *International Journal of Systemic and Evolutionary Microbiology, 56 (Pt12), 2743-2751, 2006*.

Lanz R., Kuhnert P., Boerlin P. Antimicrobial resistance and resistance gene determinants in clinical *Escherichia coli* from different animal species in Switzerland. *Veterinary Microbiology, 91 (1), 73-84, 2003.*

Lartigue M-F., Zinsius C., Wenger A., Bille J., Poirel L., Nordmann P. Extended-Spectrum β-Lactamase of the CTX-M type now in Switzerland. *Antimicrobial Agents and Chemotherapy, 51 (8), 2855-2860, 2007*.

Lee M.-F., Peng C.-F., Lin Y.-H., Lin S.-R., Chen Y.-H. Molecular diversity of class 1 integron in human isolates of *Aeromonas* spp. from Southern Taiwan. *Japanese Journal of Infectious Diseases, 61 (5), 343-349, 2008*.

Liebert C.A., Hall R.M., Summers A.O. Transposon Tn21, flagship of floating genome. *Microbiology and Molecular Biology Reviews, 63 (3), 507-522, 1999*.

Loftie-Eaton W., Rawling D.E. Diversity, biology and evolution of IncQ-family plasmids. *Plasmid, 67 (1), 15-34, 2012*.

Madsen J.S., Burmolle M., Hansen L.H., Sorensen S.J. The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology and Medical Microbiology, 65 (2), 183-195, 2012*.

Majumdar T., Das B., Bhadra R.K., Dam B., Mazumder S. Complete nucleotide sequence of a quinolone resistance gene (*qnrS2*) carrying plasmid of *Aeromonas hydrophila* isolated from fish. *Plasmid, 66 (2), 79-84, 2011*.

Mammeri H., Van deLoo M., Poirel L., Martinez-Martinez L., Nordmann P. Emergence of Plasmid-Mediated Quinolone resistance in *Escherichia coli* in Europe. *Antimicrobial Agents and Chemotherapy, 49 (1), 71-76, 2005.*

McIntosh D., Cunningham M., Ji B., Fekete F.A., Parry E.M., Clark S.E., Zalinger Z.B., Gilg I.C., Danner G.R., Johnson K.A., Beattie M., Ritchie R. Transferable, multiple antibiotic and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. *Journal of Antimicrobial Chemotherapy, 61 (6), 1221-1228, 2008*.

Meynell E., Meynell G.G., Datta N. Phylogenetic relationship of drug-resistance factors and other transmissible bacterial plasmids. *Bacteriological Reviews, 32 (1), 55-83, 1968*.

Middleton J.H., Salierno J.D. Antibiotic resistance in triclosan tolerant fecal coliforms isolated from surface waters near wastewater treatment plant outflows. *Ecotoxicology and Environmental Safety, 88, 79-88, 2013*.

Minarini L.A.R., Poirel L., Cattoir V., Darini A.L.C., Nordmann P. Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *Journal of Antimicrobial Chemotherapy, 62 (3), 474-478, 2008*.

Mokracka J., Koczura R., Kaznowski A. Multiresistant *Enterobacteriaceae* with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water Research, 46 (10), 3353-3363, 2012*.

Molina-Aja A., Garcìa-Gasca A., Abreu-Grobois A., Bolàn-Mejìa C., Roque A., Gomez-Gil B. Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured penaeid shrimp. *FEMS Microbiology letters, 213 (1), 7-12, 2002*.

Moura A., Oliveira C., Henriques I., Smalla K., Correia A. Broad diversity of conjugative plasmids in integron-carrying bacteria from wastewater environments. *FEMS Microbiology Letters, 330 (2), 157-164, 2012 -a-*.

Moura A., Pereira C., Henriques I., Correia A. Novel gene cassettes and integrons in antibiotic-resistant bacteria isolated from urban wastewater. *Research in Microbiology, 163 (2), 92-100, 2012 -b-*.

Moura A., Henriques I., Smalla K., Correia A. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. *Research in Microbiology, 161 (1), 58-66, 2010*.

Moura A., Soares M., Pereira C., Leitão N., Henriques I., Correia A. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics Application Note, 25 (8), 1096-1098, 2009*.

Moura A., Henriques I., Ribeiro R., Correia A. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. *Journal of Antimicrobial Chemotherapy, 60 (6), 1243-1250, 2007*.

Muniesa M., Imamovic L., Jofre J. Bacteriophages and genetic mobilization in sewage and faecally polluted environments. *Microbial Biotechnology , 4 (6), 725-734, 2013.*

Nardelli M., Scalzo P.M., Ramìrez M.S., Quiroga M.P., Cassini M.H., Centròn D. Class 1 integrons in environments with different degrees of urbanization. *Plos One, 7 (6), 1-13, 2012*.

Nordmann P., Naas T., Poirel L. Global spread of Carbapenemase-producing *Enterobacteriaceae*. *Emerging Infectious Diseases, 17 (10), 1791–1798, 2011.*

Novick, R.P. Plasmid incompatibility. *Microbiological Reviews, 51 (4), 381-395, 1987.*

Oh S., Buddenborg S., Yoder-Himes D.R., Tiedje J.M., Konstantinidis K.T. Genomic diversity of *Escherichia coli* isolated from diverse habitats. *Plos One, 7 (10), 1-9, 2012.*

Pedraza R.O., Ricci D. In-well cell lysis technique reveals two new megaplasmids of 103.0 and 212.6 MDa in the multiple plasmid-containing strain V517 of *Escherichia coli*. *Letters in Applied Microbiology, 34 (2), 130-133, 2002.*

Picão R.C., Poirel L., Demarta A., Ferreira Silva C.S., Corvaglia A.R., Petrini O., Nordmann P. Plasmid-mediate quinolone resistance in *Aeromonas allosaccharophila* recovered from a Swiss lake. *Journal of Antimicrobial Chemotherapy, 62 (5), 948-950, 2008.*

Pidiyar V., Kaznowski A., Narayan N.B., Patole M., Shouche Y. *Aeromonas culicicola* sp. Nov., from the midgut of *Culex quinquefasciatus*. *International Journal of Systemic and Evolutionary Microbiology, 52 (Pt5), 1723-1728, 2002.*

Pinna A., Secchi L.A., Zanetti S., Usai D., Carta F. *Aeromonas caviae* keratitis associated with contact lens wear. *Ophthalmology, 111 (2), 348-351, 2004.*

Poirel L., Cattoir V., Nordmann P. Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies. *Frontiers in Microbiology, 3:24, 1-7, 2012 -a-.*

Poirel L, Bonnin A., Nordmann P. Genetic support and diversity of acquire extended spectrum β-lactamases in Gram-negative rods. *Infection, Genetics and Evolution, 12 (5), 883-893, 2012 -b-.*

Puah S.M., Puthucheary S.D., Liew F.Y., Chua K.H. *Aeromonas aquariorum* clinical isolates: antimicrobial profiles, plasmids and genetic determinants. *International Journal of Antimicrobial Agents, 41 (3), 281-284, 2013.*

Puthucheary S.D., Puah S.M., Chua K.H. Molecular characterization of clinical isolates of *Aeromonas* species from Malaysia. *Plos One, 7 (2), 1-7, 2012*.

Radström P., Sköld O., Swedberg G., Flensburg J., Roy P.H., Sundström L. Transposon Tn5090 of plasmid R751, which carries an integron, is related to Tn7, Mu, and retroelements. *Journal of Bacteriology, 176 (11), 3257-3268, 1994*.

Rahube T.O., Yost C.K. Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. *African Journal of Biotechnology, 9, Pg. 9183-9190, 2010*.

Reyna F., Huesca M., Gonzàlez V., Fuchs Y. *Salmonella typhimurium gyrA* mutations associated with fluoroquinolone resistance. *Antimicrobial Agents and Chemotherapy, 39 (7), 1621-1623, 1995*.

Rhodes G., Parkhill J., Bird C., Ambrose K., Jones M.C., Huys G., Swings J., Pickup R.W. Complete Nucleotide sequence of the conjugative Tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. *Applied and Environmental Microbiology, 70 (12), 7497-7510, 2004*.

Richter S.N., Frasson I., Bergo C., Manganelli R., Cavallaro A., Palù G. Characterization of qnr plasmid-mediated quinolone resistance in Enterobacteriaceae from Italy: association of the qnrB19 allele with the integron element ISCR1 in *Escherichia coli*. *International Journal of Antimicrobial Agents, 35 (6), 578-583, 2010*.

Rodriguez C.N., Campos R., Pastran B., Jiminez I., Garcia A., Meijomil P., Rodriguez-Morales A.J. Sepsis due to extended-spectrum β-lactamase-producing *Aeromonas hydrophila* in a pediatric patient with diarrhea and pneumonia. *Clinical Infectious Diseases, 41 (3), 421-422, 2005*.

Rowe-Magnus D.A., Mazel D. Integrons: natural tools for bacterial genome evolution. *Current Opinion in Microbiology, 4 (5), 565-569, 2001*.

Ruiz E., Sàenz Y., Zarazaga M., Rocha-Gracia R., Martìnez-Martìnez L., Arlet G., Torres C. *qnr, aac(6')-Ib-cr* and qepA genes in *Escherichia coli* and *Klebsiella* spp.: genetic environments and plasmid and chromosomal location. *Journal of Antimicrobial and Chemotherapy, 67 (4), 886-897, 2012*.

Rupp M.E., Fey P.D. Extended spectrum β-lactamase (ESBL)-producing *enterobacteriaceae*; considerations for diagnosis, prevention and drug treatment. *Drug, 63 (4), 353-365, 2003*.

Sandvang D. Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 integron isolated from *Escherichia coli*. *Antimicrobial Agents and Chemotherapy, 43 (12), 3036-3038, 1999*.

Schmidt A.S., Bruun M.S., Larsen J.L., Dalsgaard I. Characterization of class 1 integrons associated with R-plasmids in clinical *Aeromonas salmonicida* isolates from various geographical areas. *Journal of Antimicrobial Chemotherapy, 47 (6), 735-743, 2001*.

Schülter A., Szczepanowski R., Kurz N., Schneiker S., Krahn I., Pühler A. Erythromycin resistance-conferring plasmid pRSB105, isolated from a sewage treatment plant, harbors a new Macrolide resistance determinant, an integron-containing Tn402-like element, and a large region of unknown function. *Applied and Environmental Microbiology, 1952-1960, 2007*.

Shaheen B.W., Nayak R., Foley S.L., Kweon O., Deck J., Park M., Rafii F., Boothe D.M. Molecular characterization of resistance to Extended-spectrum Cephalosporins in clinical *Escherichia coli* isolated from companion animals in the United States. *Antimicrobial Agents and Chemotherapy, 55 (12), 5666-5675, 2011*.

Shaheen B.W., Oyarzabal O.A., Boothe D.M. The role of the class 1 and 2 integrons in mediating antimicrobial resistance among canine and feline clinical *E. coli* isolates from the US. *Veterinary Microbiology, 144 (3-4), 363-370, 2010*.

Shahid M., Malik A., Sheeba. Multidrug-resistant *Pseudomonas aeruginosa* strains harbouring R-plasmids and AmpC β-lactamases isolated from hospitalized burn patients in a tertiary care hospital of North India. *FEMS Microbiology Letters, 228 (2), 181-186, 2003*.

Smillie C., Garcillàn-Barcia M.P., Francia M.V., Rocha E.P.C., de la Cruz F. Mobility plasmids. *Microbiology and Molecular Biology Reviews, 74 (3), 434-452, 2010*.

Sørum H., L'Abée-Lund T.M., Solberg A., Wold A. Integron-containing Inc U R plasmids pRAS1 and pAr-32 from the fish pathogen *Aeromonas salmonicida*. *Antimicrobial Agents and Chemotherapy, 47 (4), 1285-1290, 2003*.

Sota M., Hirokazu Y., Hughes J., Daughdrill G.W., Abdo1 Z., Forney L.J., Top E.M. Shifts in host range of a promiscuous plasmid through its parallel evolution of replication initiation protein. *N.I.H., 4 (12), 1568-1580, 2010*.

Stalder T., Barraud O., Casellas M., Dagot C., Ploy M.C. Integron involvement in environmental spread of antibiotic resistance. *Frontiers in Microbiology, 3:119, 1-14, 2012*.

Takahashi S., Nagano Y. Rapid procedure for isolation of plasmid DNA and application to epidemiological analysis. *Journal of Clinical Microbiology, 20 (4), 608-613, 1984*.

Tamura K., Dudley J., Nei M., Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24 (8), 1596-1599, 2007*.

Taylor D.E., Gibreel A., Lawley T.D., Tracz D.M. Antibiotic resistance plasmids. *In: Plasmid Biology, Edited by Funnell B.E., and Philips G.J. (ASM Press, Washington, D.C.), 473-491, 2004*.

Taylor N.G.H., Verner-Jeffreys D.W., Baker-Austin C. Aquatic systems: maintaining, mixing and mobilizing antimicrobial resistance? *Trends in Ecology and Evolution, 26 (6), 278-284, 2011*.

Thevenon F., Regier N., Benagli C., Tonolla M., Adatte T., Wildi W., Poté J. Characterization of fecal indicator bacteria in sediments cores from the largest freshwater lake of Western Europe (Lake Geneva, Switzerland). *Ecotoxicology and Environmental Safety, 78 (50-6), 50-56, 2012.*

Thomas C.M., Nielsen K.M. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology, 3 (9), 711-721, 2005*.

Thorolfsdottir B.O.T., Marteinsonn V.T. Microbiological Analysis in three diverse natural geothermal bathing pools in Iceland. *International Journal of Environmental Research and Public Health, 10 (3), 1085-1089, 2013*.

Tomoeda M., Inuzuka M., Kubo N., Nakamura S. Effective elimination of drug resistance and sex factor in *Escherichia coli* by sodium dodecyl sulfate. *Journal of Bacteriology, 95 (3), 1078-1089, 1968*.

Topp E., Welsh M., Tien Y.-C., Dang A. Lazarovits G., Conn K., Zhu H. Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology, 44 (3), 303-308, 2003*.

UFAM (Ufficio Federale dell'Ambiente), Berna. Microinquinanti nelle acque. Valutazione e riduzione dei carichi inquinanti provenienti dallo smaltimento delle acque urbane (Riassunto). *Ed. UFAM, 1-9, 2009.*

van den Bogaard A.E., Stobberingh E.E. Epidemiology of resistance to antibiotics links between animals and humans. *International Journal of Antimicrobial Agents, 14 (4), 327-335, 2000*.

van Hoek A.H.A.M., Mevius D., Guerra B., Mullany P., Roberts A.P., Aarts H.J.M. Acquired antibiotic resistance genes: an overview. *Frontiers in Microbiology, Antimicrobial, Resistance and Chemotherapy, 2:203, 1-27, 2011*.

Vila J., Ruiz J., Gallardo F., Vargas M., Soler L., Figueras M.J., Gascon J. *Aeromonas* spp. and traveler's diarrhea: clinical features and antimicrobial resistance. *Emerging Infectious Disease, 9 (5), 552-555, 2003*.

Wang J.T., Fang C.-T., Hsueh P.-R., Chang S.-C., Luh K.-T. Spontaneous bacteria empyema caused by *Aeromonas veronii* biotype sobria. *Diagnostic Microbiology and Infectious Disease, 37 (4), 271-273, 2000*.

Yano H., Uemura M., Endo S., Kanamori H., Inomata S., Kakuta R., Ichimura S., Ogawa M., Shimojima M., Ishibashi N., Aoyagi T., Hatta M., Gu Y., Yamada M., Tokuda K., Kunishima H., Kitagawa M., Hirakata Y., Kaku M. Molecular characteristics of Extended-Spectrum β-Lactamases in clinical isolates from *Escherichia coli* at a japanese tertiary Hospital. *Plos One, 8 (5), 1-6, 2013*.

Yilmaz F., Orman N., Serim G., Kochan C., Ergene A., Icgen B. Surface water-borne multidrug and heavy metal-resistant *Staphylococcus* isolates characterized by 16S rDNA sequencing. *Bulletin of Environmental Contamination and Toxicology, 10.1007/s00128-013-1112-6, 2013*.

Zhao W-.H., Hu Z-.Q. Epidemiology and genetics of VIM-type metallo-β-lactamases in gram-negative bacilli. *Future Microbiology, 6 (3), 317-333, 2011*.

Zuccato E., Castiglioni S., Bagnati R., Melis M., Fanelli R. Source, occurrence and fate of antibiotics in the Italian aquatic environment. *Journal of Hazardous Materials, 179 (1-3), 1042-1048, 2010*.