Evaluation of the impact on the environment of antimicrobials used in livestock mass medication

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Ai miei genitori e a nonno Armando

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Summary

In intensive animal farming, mass medication involves the administration of large quantities of antimicrobial drugs that may be unmetabolized by the animal and eliminated as such, or metabolized and excreted as active or inactive metabolites. Since the manure/slurry from these farms is commonly used for the fertilization of agricultural soil, these treatments may lead to an environmental drug contamination. Subsequently, the residues of active ingredients can be transferred from the soil to surface water through seepage and/or runoff. The environmental consequences of soil fertilization with drug-contaminated manure are a topic of great interest. Indeed, the persistence of antimicrobials in the environment can: 1.represent an ecotoxicological risk for populations of *non-target* organisms and 2.lead to the emergence and spread of bacterial resistance to antimicrobials (AMR).

For the evaluation of the possible effects in non-target organisms, fluoroquinolones (FQs) were selected among the classes of emerging contaminants; in particular, two drugs used in veterinary medicine (enrofloxacin and flumequine) and one used in human medicine (levofloxacin), with which root-elongation inhibition tests were run on three terrestrial plants (*Lycopersicon esculentum, Lactuca sativa* and *Daucus carota*). All these dicotyledons showed limited sensitivity to the three FQs; for this reason, subsequent studies with the same antibiotics were instead carried out on the freshwater model-organism *Daphnia magna*. Innovative tests were developed and fine-tuned to highlight delayed toxic effects after embryonic or neonatal exposure. The toxicological interactions of the three drugs, in binary and ternary mixtures, were also verified. The obtained results showed that the official tests have some limitations, since they lead to an overestimation of the EC_{50}, and that the mixtures of these compounds show a degree of toxicity that can be explained by additive interactions. Another test, again involving FQs and *Daphnia magna*, was a minor contribution to a project of other researchers studying the biocompatibility and therapeutic efficacy of magnetic nanoparticles engineered with flumequine. This nano-immobilized antibiotic, which is recoverable by the application of a magnetic field, represents a promising tool for “green aquaculture”. The core-shell nanocarrier was tested on a *D. magna* culture naturally infected with bacteria known to be sensitive to the drug: it showed to be effective and relatively non-toxic. Lastly, other research activities concerning FQs represented the launch of a new research project aimed at evaluating the possible epigenetic effects of flumequine (transgenerational toxicity) on *D. magna*. Preliminary results showed, in addition to the already known toxic effects caused by the drug to the first generation (dwarfism, mortality), phenomena of lethargy and less responsiveness to light sources, occurring in the next two generations non-exposed to the drug. The molecular analysis, currently undertaken by other researchers participating to the project, aims to highlight any alterations of the genome that may justify the various effects observed at the phenotypic level.

Regarding the potential spread of antimicrobial resistance following the use of antimicrobials in the livestock sector, animal manure, and agricultural soil before and after fertilization, were investigated. The aim was to evaluate the changes in the composition of the microbial communities in the aforementioned types of

samples using Next Generation Sequencing (NGS) analysis and assess the presence and abundance of antimicrobial resistance genes (ARGs) against four antimicrobials classes (*i.e.*, fluoroquinolones, macrolides, polymyxins and β-lactams) by employing gene specific SYBRGreen Real-Time PCR assays paired with melting curves analysis. To this end, previously published assays were utilized. However, when not available, new assays were designed and developed (*i.e.,* assays for the detection and quantification of colistin resistance genes, *mcr-4* and *mcr-5*) or optimized and validated with the SYBRGreen chemistry (*i.e*., assays for the detection and quantification of *ermB, bla*$_{CTXM1\text{-}like}$*, bla*$_{CMY\text{-}2}$*, qnrA* and *qnrS* genes).

This doctoral thesis is divided into four sections: 1. General Introduction; 2. Studies on Ecotoxicology; 3. Studies on Antimicrobial Resistance; 4. General Conclusions.

Riassunto

Negli allevamenti intensivi, i trattamenti di massa implicano la somministrazione di grandi quantità di antimicrobici che possono essere non metabolizzati dall'animale ed eliminati tal quali, o essere metabolizzati ed escreti come metaboliti attivi o inattivi. Poichè le deiezioni (letami/liquami) provenienti da tali allevamenti vengono comunemente usate per la fertilizzazione dei terreni agricoli, può derivarne una contaminazione ambientale da farmaci. I residui di principi attivi possono poi essere trasferiti dal suolo alle acque superficiali tramite dilavamento o infiltrazione. Le conseguenze ambientali derivanti dalla concimazione del suolo con letame contaminato da farmaci costituiscono un argomento di grande interesse, poiché la persistenza di antimicrobici nell'ambiente può da un lato rappresentare un rischio ecotossicologico per popolazioni di organismi *non-target,* e dall'altro portare alla comparsa e alla diffusione di resistenza agli antimicrobici (AMR).

Per una prima valutazione dei possibili effetti in organismi *non-target*, sono stati selezionati, tra le classi di contaminanti emergenti, i fluorochinoloni (FQs). In particolare, due farmaci ad uso veterinario (enrofloxacina e flumechina) ed uno ad uso umano (levofloxacina), con i quali sono stati effettuati dei test di inibizione dell'allungamento radicale su tre piante terrestri (*Lycopersicon esculentum, Lactuca sativa* e *Daucus carota*); queste dicotiledoni hanno mostrato una limitata sensibilità ai tre FQs. Per tal motivo, successivi studi sono stati invece effettuati, utilizzando gli stessi antibiotici, sull'organismo modello di acqua dolce *Daphnia magna*. Sono stati sviluppati e messi a punto dei test innovativi in grado di evidenziare effetti tossici ritardati in seguito ad esposizione embrionale o neonatale. Si è verificata, inoltre, la modalità di interazione tossicologica dei tre composti in miscele binarie e ternarie. I risultati ottenuti hanno mostrato che i test ufficiali presentano dei limiti, in quanto portano a una sovrastima dell'EC_{50}, e che le miscele di questi composti mostrano un grado di tossicità che si giustifica con interazioni di tipo additivo. Parallelamente, disponendo di nanoparticelle magnetiche ingegnerizzate con flumechina che potrebbero trovare futura applicazione in acquacoltura, è stata saggiata anche la biocompatibilità e l'efficacia terapeutica di questo core-shell nanocarrier, su una coltura di *D. magna* naturalmente infettatasi con batteri sensibili alla flumechina. Altre attività di ricerca concernenti i FQs hanno, infine, rappresentato l'avvio di un progetto di ricerca volto a valutare i possibili effetti epigenetici di flumechina (tossicità transgenerazionale) su *D. magna*. Dai primi risultati sono emersi, oltre ai già noti effetti tossici causati dal farmaco in prima generazione (nanismo armonico, mortalità), dei fenomeni di letargia e di minore responsività alle fonti luminose che si manifestano nelle due generazioni successive non esposte al farmaco. Le analisi biomolecolari, attualmente in corso di svolgimento da parte di altri ricercatori che partecipano al progetto, mirano ad evidenziare eventuali alterazioni del genoma che giustifichino i diversi effetti finora osservati a livello fenotipico.

L'altra parte del progetto di tesi ha investigato il fenomeno della diffusione dell'antimicrobico resistenza conseguente ai trattamenti di massa in ambito zootecnico, prendendo in esame le deiezioni degli animali ed i terreni agricoli prima e dopo la fertilizzazione con letami/liquami. Lo scopo di tale progetto è stato quello di

valutare le modificazioni nella composizione delle comunità microbiche nelle diverse tipologie di campione mediante analisi di Next Generation Sequencing (NGS), nonché la presenza e l'abbondanza, in tali campioni, di geni di antimicrobico resistenza nei confronti di 4 classi di antimicrobici (fluorochinoloni, macrolidi, polimixine e β-lattamici), utilizzando metodi biomolecolari (Real-Time PCR con SYBRGreen e le curve di *melting*). A tal fine sono stati impiegati saggi pubblicati in letteratura, ma è anche stato necessario sviluppare dei nuovi saggi in Real-Time PCR per l'identificazione e la quantificazione di geni di resistenza alla colistina (*mcr-4* e *mcr-5*) ed ottimizzare e validare 5 saggi di qPCR, quali *ermB, bla*$_{CTXM1\text{-}like}$*, bla*$_{CMY\text{-}2}$*, qnrA* e *qnrS.*

La seguente tesi di dottorato è suddivisa in quattro sezioni: 1. Introduzione generale; 2. Studi di ecotossicologia; 3. Studi di antimicrobico resistenza; 4. Conclusioni generali.

SECTION 1: General Introduction

Use of antimicrobials in farm animals

Antimicrobials play an essential role in the treatment and prevention of diseases in human and veterinary medicine. Over the past decades, the global consumption of these compounds has increased dramatically (Klein *et al.,* 2018) and, consequently, there is a growing concern about the potential impact on the biota, given their continuous release into the environment (Kummerer, 2009; Tijani *et al.,* 2013).

In intensive animal farming, antimicrobials are used not only for the individual treatment of sick animals, but also for mass medication (administration of drugs through feed or drinking water to a large number of animals). In particular, three different types of mass medication are typically carried out: auxinic, prophylactic and metaphylactic. Auxinic treatments (Gaskins *et al.,* 2002) involve the daily administration of sub-therapeutic doses of antimicrobials during most of the production cycle, with the aim of increasing productivity. It is believed that these growth-promoting treatments can contribute to the spread of bacterial drug resistance. For this reason, they were banned in EU countries as early as 2006 (Sarmah *et al.,* 2006); however, in other countries such as China and India they have been allowed until very recently (Laxminarayan *et al.,* 2015). On the other hand, prophylactic treatments are carried out under particular circumstances of the production cycle, such as weaning, transportation or arrival of new animals to the farm, in order to prevent the onset of diseases that are typically related to stressful events. Even these treatments, up to now tolerated in the EU, are strongly discouraged by the health authorities and will indeed be prohibited with the entry into force of the new European legislation on veterinary medicines, starting from January 2022 (EU Regulation 2019/6). Once again, the reason behind the ban refers to the problem of the spread of bacterial resistance, with the aim of limiting the use of antibiotics as much as possible. Lastly, metaphylactic treatments are not planned, but take place at the first signs of an infectious disease, with the aim of preventing the transmission of the infection within the farm. In the same way as the prophylactic use, this type of treatment, involving the administration of the antimicrobials at full dosage for at least 5-7 days, will in turn be strictly limited with the advent of the EU Regulation 2019/6. It is therefore desirable that the contribution of livestock activities to the spread of bacterial resistance will decrease significantly in the coming years; this will be possible if all the major producing countries will harmonize their regulations. Indeed, the lack of homogeneity of regulations in the various continents may undermine the efforts that many countries are making to limit the use of antimicrobials, in order to protect human and animal health.

To date, the use of mass medication in livestock has generated a non-negligible environmental load of either parent drugs or active metabolites. Indeed, after treatment, these active compounds can be excreted in the urine or faeces (Wei *et al.,* 2011; Xia *et al.,* 2019) and accumulate in manure and slurry, which are used for the fertilization of agricultural soil (Boxall *et al.,* 2003). Then, the contamination can be transferred from the manured soil to surface water through ground seepage and/or runoff (Tarazona *et al.,* 2010). Moreover, a direct transfer of antimicrobials to the aquatic compartment may occur after mass medication in aquaculture facilities.

Of course, the use of antimicrobials in humans also contributes to the environmental impact. For this reason, in recent years, guidelines for a prudent use of these drugs have been published for human medicine (WHO, 2019). In developed countries, urban wastewater may contain considerable residues of drugs that are not always efficiently removed, despite the processing at the Wastewater Treatment Plants (WTP), and may end-up in effluents and be eventually transferred to waterways. Besides, even the sludge obtained through WTP systems may contain traces of pharmaceuticals and it is often used for the fertilization of agricultural soils (Van Doorslaer *et al*., 2014).

Once in the environment, antimicrobials or their active metabolites, not only continue to exert a selective pressure on bacterial populations, but also may have an impact on *non-target* organisms. Indeed, these molecules, designed to interfere with the biological activity, are often not sufficiently selective and, therefore, can also harm eukaryotic organisms.

Data on the use of antimicrobials in human and veterinary medicine

Excessive consumption of antimicrobials is a public health concern in Europe and in other parts of the world, representing a social and economic problem and a threat to animal and human health. To obtain a comprehensive picture of the actual use of antimicrobial drugs in the human and veterinary fields, it is important to refer to the report produced by the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA). The third report, requested by the European Committee, reports the results of an analysis of both the consumption of antimicrobial agents and the presence of resistant bacteria in humans and animals (Report JIACRA III 2016-2018, 2021). The results and conclusions of this report are mainly based on the data collected in the three-year period 2016-2018. For the comparison between the consumption of antimicrobials in food-producing animals (FPA) and in humans, data from 2017 were analysed, while data from 2014 and 2015 were included in the trend analyses. To facilitate the comparison between Antimicrobial Medicines Consumption (AMC) in humans and food-producing animals, data were converted into mg of the active antimicrobial per kg of estimated biomass.

In 2017, weighted average AMC in the population for the European Union (EU) and the European Economic Area (EEA) in 29 EU/EEA countries was 130.0 mg kg^{-1} in humans (range 52.8- 212.6) and 108.3 mg kg^{-1} (range 3.1–423.1) in FPA. However, there is a certain variability when considering both the different countries and the various classes of antimicrobials.

In 2017, out of the 29 EU/EEA countries, 20 countries reported higher AMC in humans than in FPA, 8 countries lower AMC in humans than in FPA, and 1 country a balanced consumption. These results differ from those of the previous JIACRA report, in which the overall AMC in FPA was higher than in humans. The shift is explained by a significant decrease in the weighted average consumption of antimicrobials in FPA between 2014 and 2018, while the trend in human medicine remained substantially stable (Figure 1). It took place thanks to the

implementation by veterinarians of the so-called antimicrobial stewardship actions, *i.e*. coordinated measures to promote the correct use of antimicrobials (Report JIACRA III 2016-2018, 2021).

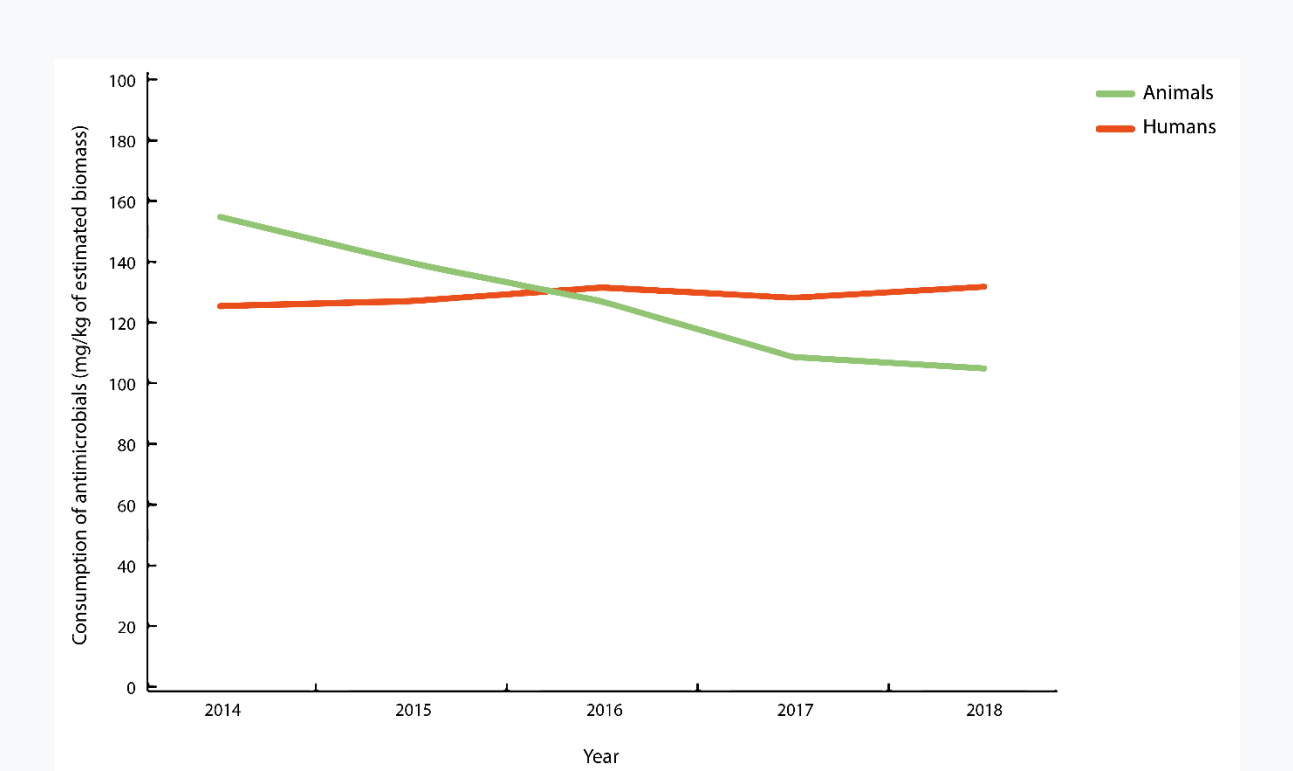

Figure 1. Population weighted-mean of the total consumption of antimicrobials in humans and food-producing animals in 27 EU/EEA countries(*) (from JIACRA report, 2021. Modified).

(*)AT, BE, BG, CY, DE, DK, EE, ES, FI, FR, HR, HU, IE, IS, IT, LT, LU, LV, NL, NO, PL, PT, RO, SE, SI, SK, UK.

In Figure 2, the different ratio between the AMC of humans and FPA is depicted for 29 countries, with reference to the year 2017. Italy ranks second in terms of antimicrobial consumption in intensive animal farming, while the Scandinavian countries (Norway, Sweden and Finland) are among the most virtuous, since they have significantly decreased the use of antimicrobials in FPA.

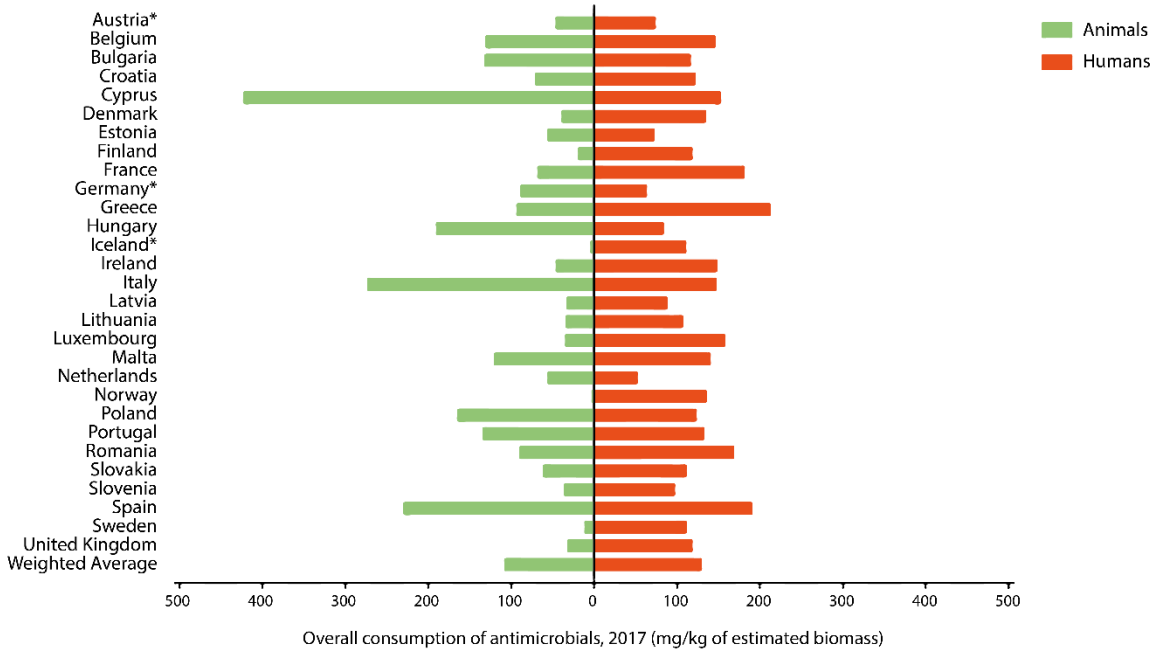

Figure 2. Comparison of biomass-corrected consumption of antimicrobials (mg/kg of estimated biomass) in humans and food-producing animals in 29 EU/EEA countries, 2017 (from JIACRA report, 2021. Modified).

Asterisks (*) indicates that only community consumption was provided for human medicine.

Antimicrobials used in food producing-animals

Antimicrobials are classified based on their chemical structure, spectrum of action, type and mechanism of action. There are different classes of substances such as β-lactams, fluoroquinolones, macrolides, tetracyclines, polymyxins, sulfonamides, which in turn are classified into subgroups. Given the importance of these molecules for the treatment of various diseases in the veterinary and human fields, their correct and responsible use is essential.

Considering the critical value of some classes of drugs, not only in veterinary medicine, but also in humans, the results of the monitoring of the antimicrobial consumption in humans and FPA animals are of interest (Table 1; Figure 3).

Antimicrobial Class	**Antimicrobial Consuption (mg kg^{-1} estimated biomass)**						**Correlation Coefficient[(b)] (p-value)**
	Humans			Food-producing animals			
	Range	Median	Mean[(a)]	Range	Median	Mean	
3th and 4th-generation cephalosporins	0,1-11-4	2,8	4,0	<0,01-0,8	0,2	0,2	0,32 (0,087)
Fluoroquinolones and other quinolones	2,2-24,0	6,4	7,7	<0,01-15,3	1,1	2,8	0,72 (<0,001)
Polymyxins	0-0,2	0,03	0,06	0-14,9	1,7	3,7	0,36 (0,056)
Aminopenicillins	7,3-128,8	50,0	66,3	-0,1-78,3	11,2	26,1	0,54 (0,003)
Macrolides	1,2-18,0	6,4	7,9	0-22,0	5,7	8,0	0,46 (0,013)
Tetracyclines	0,2-11,7	1,4	3,1	0,05-173,5	22,3	33,0	-0,32 (0,095)
Total consumption	**52,8-212,6**	**122,8**	**130**	**3,1-423,1**	**61,9**	**108,3**	**0,33 (0,082)**

Table 1. Range, median and population weighted mean consumption of antimicrobials overall and for the classes selected for analysis in humans and food- producing animals, and correlation analysis of antimicrobials consumption in humans and food-producing animals, 29 EU/EEA countries[(*)] for which data were available both for humans and food-producing animals, 2017 (data from JIACRA report, 2021).

[(*)]AT, BE, BG, CY, DE, DK, EE, EL, ES, FI, FR, HR, HU, IE, IS, IT, LT, LU, LV, MT, NL, NO, PL, PT, RO, SE, SI, SK, UK.
[(a)]Population weighted mean.
[(b)]Spearman's rank correlation coefficient (rho) for consumption in humans and consumption in food-producing animals.

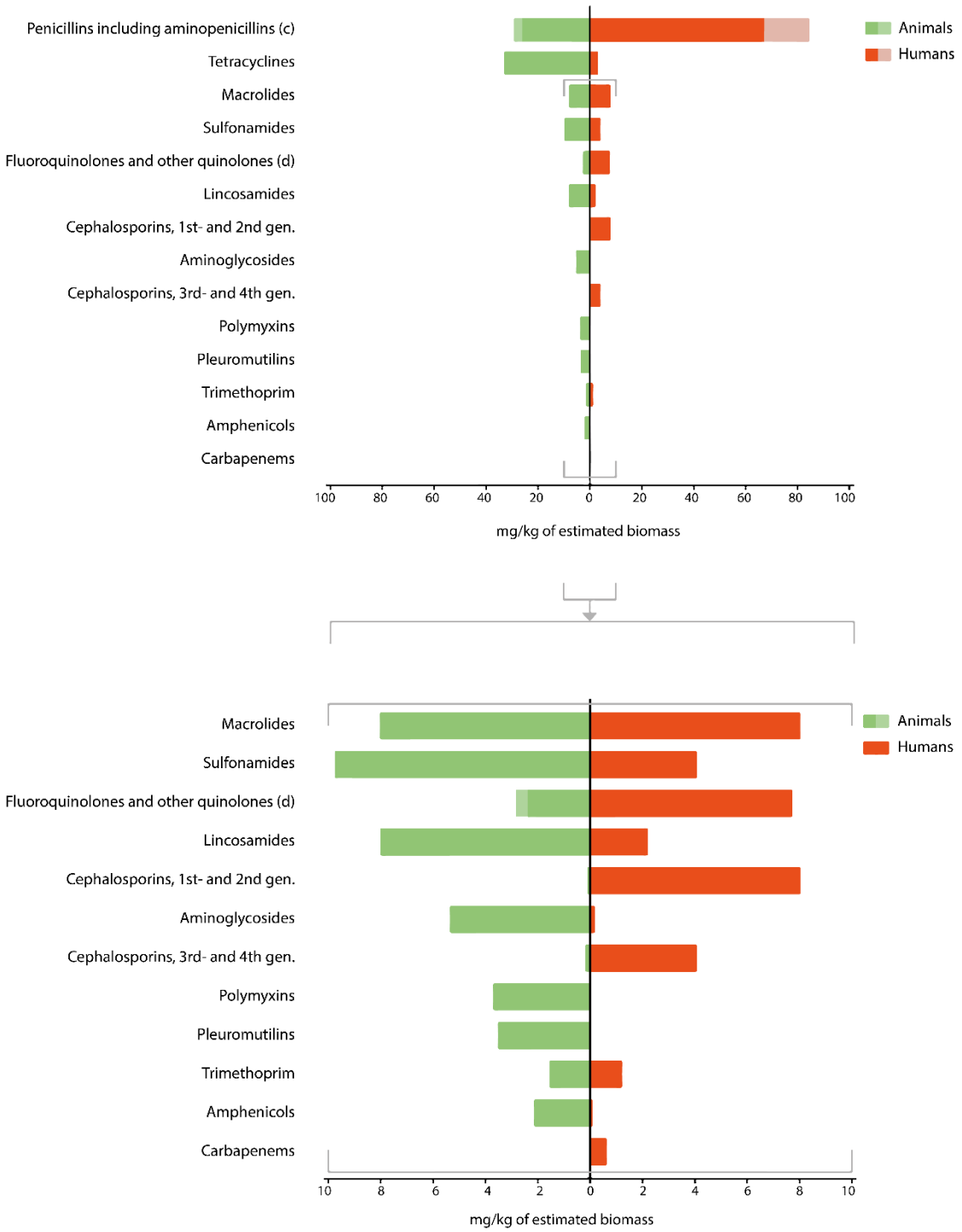

Figure 3. Comparison of consumption of antimicrobial classes in humans[(a)] and food-producing animals[(b)], in 29 EU/EEA countries for which data were available, both for humans and food-producing animals, 2017 (from JIACRA report, 2021. Modified).

[(c)]Aminopenicillins are shown in dark colour and all other penicillins in light colour.
[(d)]Fluoroquinolones and other quinolones are shown in dark and light colour, respectively.
Notes: 1) The x-axis scale differs between graphs A and B.
2) The estimates presented are crude and must be interpreted with caution.

In this PhD project, four classes of antimicrobials commonly used in farm animals have been considered: β-lactams, fluoroquinolones, macrolides and polymyxins.

β-lactams → this class of antibiotics gains the name from the characteristic four-membered ring structure. They act by inhibiting the synthesis of the bacterial cell wall, and are particularly effective against Gram-positive bacteria. They are divided into groups based on their chemical structure; the most important β-lactams are penicillins, cephalosporins and carbapenems.

Penicillins, discovered by Alexander Fleming in 1928, remain among the most widely used antibiotics in the world, *e.g.,* for the treatment of mastitis in cow, gastrointestinal, urinary and respiratory infections. According to the indications of the Antimicrobial Advice *ad hoc* Expert Group (AMEG), carbapenems are included in "Category A", or "Avoid" (EMA, 2019); therefore, their use in veterinary medicine is not allowed. They can be used, and only as an exception, for pets.

Other important β-lactams are cephalosporins, among which those belonging to the 3rd and 4th generation are considered of great value. Indeed, these drugs are essential for the treatment of serious infections, such as meningitis and diseases related to *Salmonella* spp. in particular categories of patients (*e.g.*, children). They are often one of the few alternatives for the treatment of sepsis and respiratory tract infections in various animal species, in cases where resistance to drugs considered less problematic by the AMEG has already been confirmed. In human medicine, this group of β-lactams is considered by the WHO as Highest Priority Critically Important Antimicrobials (HPCIA) (WHO, 2019) and has been listed by the World Organization for Animal Health (OIE) among the Veterinary Critically Important Antimicrobial Agents (VCIA) (OIE, 2019). Following the AMEG, 3rd and 4th generation cephalosporins belong to "Category B", with a "Restrict" use in the veterinary field (EMA, 2019). In this case, it is intended that the use in veterinary medicine is allowed only if drugs belonging to lower categories (C or D, "Caution" or "Prudence") are ineffective. Lastly, 5th generation cephalosporins cannot be used for animals and belong to “Category A” (“Avoid”).

Fluoroquinolones → these antimicrobials have a fluorine atom on the central ring, usually in position 6; they are also called 2nd generation quinolones. Some are administered to FPA, such as cattle, pigs, poultry and fish, for the treatment of infections by Gram-positive and Gram-negative bacteria, like *Streptococcus pneumoniae*, *Escherichia coli, Salmonella* spp. and *Campylobacter* spp. Fluoroquinolones appear to be one of the few alternatives for *e.g.* the treatment of diarrhoea in piglets (caused by *E. coli*) or for sepsis in various animal species (caused by Enterobacterales). These antimicrobials, likewise 3rd and 4th generation cephalosporins, have been defined by the OIE as Veterinary Critically Important Antimicrobial Agents (VCIA). According to AMEG, fluoroquinolones fall into "Category B" (EMA, 2019), *i.e.* drugs whose use must be limited (“Restrict”) only to situations in which other, less essential antimicrobials (“Categories C” and “D”) are ineffective.

Macrolides → this is a class of drugs used in human medicine for the treatment of *Legionella* spp., *Campylobacter* spp*.,* multidrug-resistant *Salmonella* spp. and *Shigella* spp. In veterinary medicine, this group

of antimicrobials appears to be one of the few solutions for the treatment of ileitis in pigs (caused by *Lawsonia intracellularis*) and mycoplasma infections in pigs and poultry. Recently, WHO classified macrolides as a category of drugs that must be monitored (WHO, 2019) and they are considered Veterinary Critically Important Antimicrobial Agents (VCIA) (OIE, 2019). Based on the AMEG categorization, macrolides belong to "Category C" ("Caution") (EMA, 2019) and can be used in the veterinary field when less dangerous drugs ("Category D") are not available.

Polymyxins → the most widely used drug belonging to this class is colistin. Polymyxins are used particularly in human medicine, in hospitals, as a last resort treatment for infections caused by multidrug-resistant Gram-negative bacteria that are not sensitive to the action of carbapenems. For this reason, they are defined by the WHO as Highest Priority Critically Important Antimicrobials (HPCIA) (WHO, 2019). In the veterinary field, polymyxins are defined as VHIA (OIE, 2019) and they have been included by AMEG in "Category B" ("Restrict") (EMA, 2019). The administration of these drugs to animals should be limited to particular conditions in which antimicrobials of "Categories C" and "D" have not led to desired outcomes.

Aim of the thesis

Mass medication in intensive animal farming may pose a threat to the health of the environment. Indeed, the subsequent use of manure/slurry as fertilizer can cause a significant environmental load of active ingredients, which contaminate the soil and might be transferred to the water compartment through seepage and/or runoff (Tarazona *et al.,* 2010).

These active substances, once in the environment, may be responsible for:

1. Toxicological effects in *non-target* organisms;
2. Spread of antimicrobial resistance genes (ARGs).

The evaluation of these two issues is the aim of this thesis. In detail, the toxic effects that fluoroquinolones can cause in three terrestrial plants (*Lycopersicon esculentum, Dacutus carota* and *Lactuca sativa*) and in the aquatic model organisms *Daphnia magna,* are presented and discussed in Section 2; the presence and dissemination of antimicrobial resistance genes in the environment due to the fertilization of soil with manure/slurry are addressed and discussed in Section 3.

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SECTION 2: Studies on Ecotoxicology

Chapter 2.1

Introduction

The present section of this PhD thesis concerns fluoroquinolones (FQs). This class of antimicrobials was selected for an ecotoxicological evaluation, taking into account previous data on its toxicity towards *non-target* organism and its widespread contamination of the aquatic environment. FQs belong to a broader class of synthetic antibiotics, called quinolones, that have been marketed since the 1960s. Over time, due to their narrow antimicrobial spectrum (limited to Gram-negative bacteria, with the exception of *Pseudomonas aeruginosa* and *Serratia* spp.), the high MIC (Minimum Inhibitory Concentration) values and the rapid onset of antimicrobial resistance phenomena, the resort to the therapeutic use of quinolones has strongly decreased in favour of FQs.

In the human and veterinary field, the use of FQs offers several advantages: the possibility of oral administration, the wide tissue distribution, and the strong activity against Gram-negative aerobes combined with a certain activity towards Gram-positive aerobes. These drugs are very effective both in the treatment of urinary infections, because of their renal excretion in active form, and of severe septicaemia and respiratory infections. Thanks to their ability to penetrate phagocytic cells, FQs are also useful in the therapy of infections caused by *mycoplasma, mycobacteria, brucellae, chlamydiae, ehrlichiae*, etc.

The mechanism of action, responsible for a rapid and dose-dependent bactericidal activity, involves the interaction with two enzymes: topoisomerase II (DNA gyrase) and topoisomerase IV. The first enzyme has the function of introducing a negative supercoiling into the DNA while the second one is involved in the unwinding of the ATP-dependent DNA. FQs act specifically on the subunits that make up the aforementioned enzymes, preventing DNA replication and transcription, with consequent chromosome fragmentation and cell death. These enzymes were thought to be present only in bacterial cells, thereby guaranteeing a highly selective activity (Blondeau, 2004); however, other studies had shown that the DNA gyrase enzyme is present both in algae (Thompson and Mosig, 1985) and in higher plants (Wall *et al.*, 2004). Over the years, numerous *in vitro* studies have highlighted not only the ability of FQs to interact with the genetic material present in eukaryotic cells but also their toxicity towards terrestrial and aquatic organisms, thus indicating that these drugs can have a non-negligible impact on the environment (Khadra *et al.*, 2012; Thomé *et al.*, 2012).

Meanwhile, for many of these compounds, widespread use in humans had led to pharmacovigilance warnings concerning side effects of a certain severity. As a result, some of these compounds have been withdrawn from the market, and for those still on the market the FDA and the EMA have given indication of limiting their prescription to circumstances of strict necessity (Report JIACRA, 2021).

FQs are considered emerging contaminants for various reasons: widespread use in both human and veterinary medicine; toxicity towards a wide range of *non-target* organisms; poor biodegradability in the environment. In particular, these antibiotics have a tendency to adsorb to the soil and sediments where, protected from light, they can progressively accumulate. Indeed, the maximum levels of contamination found in these matrices are strikingly high (mg kg^{-1}) (Van Doorslaer *et al.,* 2014). On the other hand, very high concentrations (mg L^{-1}) have also occasionally been found in the water column, in relation to uncontrolled

discharges from pharmaceutical industries (Larsson *et al.,* 2007) or bad practices in the aquaculture sector (Le and Munekage, 2004). Indirectly, the soil pollution generated by the use of manure and slurry from animal farms can also contribute, through progressive desorption and subsequent seepage and/or runoff phenomena, to the contamination of the aquatic environment (Picó and Andreu, 2007). On this regard, a research recently carried out in Northern Italy (Lombardia and Veneto) has shown that, among the various classes of antibacterials used in animal husbandry, the FQs reach the highest concentrations in agricultural soil, both before and after fertilization (Laconi *et al.,* 2021).

Taking all of this into account, it was decided to focus on this class of compounds the research activities concerning the ecotoxicological features of antibacterials. In particular, two drugs typically used in veterinary mass-medication (enrofloxacin and flumequine), and one drug largely employed in human medicine (levofloxacin) were investigated.

First of all, considering the presence of FQs residues in the agricultural soil of Northern Italy, an investigation on the toxicity of these molecules towards crop plants was carried out. On this regard, indeed, data available in the literature are very scarce. Being this an exploratory investigation, simple root elongation inhibition tests were run on three very common vegetables (tomato, lettuce and carrot) by exposing their seeds to FQs solutions. The experimentation is presented in chapter 2.2. The three plants showed poor sensitivity to the three fluoroquinolones studied and, as it will be seen, provided contradictory and not strictly dose-related responses. For all these reasons, the topic was not explored further.

Subsequently, considering previous studies that had highlighted the toxicity of FQs to the microcrustacean *Daphnia magna* (Dalla Bona *et al.,* 2016; De Liguoro *et al.,* 2019), it was deemed important to deepen, in the same model-organism, aspects related to the delayed toxicity of these antibacterials following pre-natal and post-natal exposure, and their possible interactions (additive or non-additive) within binary and ternary mixtures. These investigations were the topic of the scientific article available in chapter 2.3 (Tolosi and De Liguoro, 2021).

The appearance of a spontaneous infection during the maintenance of microcrustacean cultures represented a starting point for some bacteriological investigations and, above all, for a subsequent collaboration with a research group that was studying the possible application in aquaculture of flumequine immobilized on iron-oxide nanoparticles. The contribution offered to their research project consisted in a preliminary evaluation of the therapeutic effects and biocompatibility of nano-immobilized flumequine on individuals of *D. magna* affected by spontaneous infection with *Aeromonas* spp. and *Pseudomonas* spp. The abstract and conclusions of the original scientific publication (Bortoletti *et al.,* 2020), together with an extract from the materials and methods and the results related to this contribution, are presented in chapter 2.4.

The last research activity in the ecotoxicological field is still in progress. The aim, in this case, is to study the transgenerational effects of flumequine in *Daphnia magna* at the biomolecular level; more specifically, to identify any transcriptional and epigenetic modifications that can be related to the already observed

phenotypic effects. Previous research (De Liguoro *et al.,* 2019) has indeed highlighted the appearance of effects such as developmental inhibition, mortality and reproduction inhibition. An introduction, the materials and methods and the preliminary results of this experiment, related only to the effects on the phenotype, are presented in chapter 2.5.

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Chapter 2.2

Evaluation of the phytotoxicity of three fluoroquinolones to tomato, lettuce and carrot, using the Root Elongation Assay

Data not published

Abstract

Fluoroquinolones (FQs) are a class of antibiotics largely used both in human and veterinary medicine. Consequently, their environmental load is profuse and, as they can persist and accumulate in soil and sediments, may represent a threat to the ecosystem. However, only few studies have evaluated the toxicity of these compounds to terrestrial plants. In this experimentation the toxicity of three FQS towards three common crops was evaluated. Enrofloxacin (ENR) and flumequine (FLU), typically used in intensive animal farming, and levofloxacin (LEV), one of the FQs most used in human medicine, were assayed on tomato (*Lycopersicon esculentum*), lettuce (*Lactuca sativa*) and carrot (*Daucus carota*) by means of the Root Elongation Assay (ASTM, 2014). On the basis of preliminary tests, six sequential concentrations of each drug, with five replicates, in geometric series and with a separation factor of four, were assayed (100, 25, 6.25, 1.56, 0.4, 0.1 mg L^{-1}). Notwithstanding the wide range of concentrations tested, the dose-response correlation was generally limited and even at the highest exposure level (100 mg L^{-1}) the Root Elongation inhibition was always ≤70%. However, it was possible to obtain dose-response curves with a tolerable R^2 coefficient, by simulating hypothetical 100% responses under extremely high concentrations. The calculated EC_{50s}, for ENR, FLU and LEV, respectively, were the following: 1.01, 6.44, 18.73 (*L. esculentum*); 33.55, 12.01, 61.15 (*L. sativa*); 50.32, 21.98, n.d. (*D. carota)*. Overall, results indicated that among the three crops *L. esculentum* is the most sensitive to FQs and that the two veterinary FQS are more toxic then LEV to the three terrestrial plants. It was concluded that more information regarding the water/soil partition coefficient (K_d) of FQs are necessary, in order to determine if the concentration actually detected in soil (up to few mg kg^{-1}) might represent a threat to terrestrial plants.

Introduction

Antimicrobials for veterinary use play a fundamental role in the treatment of various diseases, not only for pets but also in animal husbandry. In recent years, the study of the environmental impact of antimicrobials used in food-producing animals has been the subject of growing interest by researchers, given the substantial amount of active ingredients applied to “mass medication”, their ability to interfere with biological processes, and their possible resistance to degradation.

Antimicrobials, as parent compounds or as active metabolites, can be eliminated with animal excrements and urine and reach the environment directly (grazing animals, aquaculture) or indirectly (manure/slurry applied to agricultural soil). Indeed, traces of a large number of these substances have been found in soil, surface water and sediments (Tong *et al.*, 2009; Tamtam *et al.,* 2011).

Fluoroquinolones (FQs) are a class of antibacterials that have raised concern regarding possible effects on *non-target* organisms. In fact, these molecules have been overused in recent years both in human and veterinary medicine, are resistant to degradation and able to interact with the genetic material of animal and plant cells, causing toxic effects even under relatively low levels of exposure (Hillis *et al.,* 2011; Pan and Chu,

2016). Compared to other classes of antibacterials, the concentrations found in soil and sediments are remarkably high, and concentrations in the order of mg L^{-1} have also been occasionally found in water (Van Doorslaer *et al.,* 2014).

In a recent study, conducted in northern Italy and concerning the contamination of agricultural soil as a consequence of the use of different classes of antibacterials in animal husbandry, these data were essentially confirmed: flumequine (FLU) and enrofloxacin (ENR), two fluoroquinolones used in animal farming, were the mostly detected molecules, with concentrations up to 437.38 and 217.8 µg Kg^{-1}, respectively (Laconi *et al.,* 2021).

Whilst there is already a certain amount of data concerning the toxicity of FQs towards aquatic organisms (Janecko *et al.,* 2016), the same is untrue for soil organisms. For this reason, experimentations were carried out to assess the possible impact of these emerging contaminants on terrestrial plants. Being essentially an exploratory assessment, a relatively simple test was used for the purpose, namely the Root Elongation Assay (ASTM, 2014). This assay, over the years, has been exploited by various authors to evaluate the ecotoxicity of pesticides (Santelman, 1972; Horowitz, 1976; Gorsuch *et al.,* 1990, Bettiol, 2016), heavy metals (Imai and Siegel, 1973; Walley *et al.,* 1974; Di Salvatore, 2008; Guzman-Rangel *et al.,* 2017) and other potentially toxic xenobiotics (Rubinstein *et al.,* 1975; Hikino, 1978; Pan and Chu, 2016; Yang, 2021).

The present assay involved, in addition to FLU and ENR, also levofloxacin (LEV), an antibiotic widely used in human medicine; this in order to make a comparison between the toxicity to terrestrial plants of veterinary and human FQs.

Plants are crucial components of the terrestrial ecosystem as they produce substances that are essential to other living beings and improve the quality of soil and sediments (Wang and Freemark, 1995). Some xenobiotics are able to interfere with the processes of germination, development and growth of plants, thus causing damage to biodiversity and a major impact on crops (ASTM, 2014; Bártíková *et al.,* 2016; Wang and Freemark, 1995). The assay used in this research does not provide results that are readably spendable for Risk Assessment, as they expose the plants not to contaminated soil but to contaminated water. Indeed, for any risk assessment related to the presence of FQs in the soil, the measured EC_{50s} should be critically evaluated, taking into account the water/soil partion of the molecules under study. In the case of FQs, this would be unrealistic, because their partition coefficients have not been sufficiently defined and are subjected to wide variability according to the type of soil (Van Doorslaer *et al.,* 2014). However, as already mentioned, this study had only an exploratory aim, and its main purpose was to provide an idea of the sensitivity of terrestrial plants to FQs and to understand whether the subject is worth investigating. Concerning the effects of FQs on plants, the few data available in literature are quite contradictory, as in some cases they point to EC_{50}> 100 mg L^{-1} (Jin *et al.,* 2009; Timmerer *et al.,* 2020), and therefore of very little or no ecotoxicological interest, but in other cases, relevant effects even under concentrations <1 mg L^{-1} are claimed (Hillis *et al.,* 2011; Pan and Chu, 2016). After some preliminary tests conducted in pure medium, in which the germination

capacity of the seeds of a dozen crops of agricultural interest was evaluated under the conditions provided for by the ASTM assay, three very common crops were selected for the tests, namely tomato *(Lycopersicon esculentum*), lettuce (*Lactuca sativa*), and carrot (*Daucus carota*).

Materials and methods

1.Reagents

Enrofloxacin (ENR), flumequine (FLU) and levofloxacin (LEV) were provided by Sigma-Aldrich (Milan, Italy). Their purity was ≥ 98%. A stock solution of 100 mg L^{-1} in Rocchetta© mineral water (pH 7.6, dry residue 181.6 mg L^{-1}) was prepared for each antibiotic, and stored at 4°C in the dark. The solubilisation for each compound was obtained by gentle stirring overnight at 37°C.

2.Plants (seeds)

After performing a preliminary germination test, in pure medium and under the conditions foreseen by the official test (ASTM, 2014), with the seeds of five species commonly used for phytotoxicity tests (*Cucumis sativus, Avena sativa, Lactuca sativa, Lycopersicon esculentum* and *Daucus carota*), three species were selected for the ecotoxicity tests with FQs: *Lycopersicon esculentum*, *Lactuca sativa* and *Daucus carota*. Their seeds were supplied by a specialized dealer and kept at room temperature (21 ± 2°C), in the dark, pending the test. Any seed with anomalous shape, size or colour was not used in the assays.

3.Root elongation test

Starting for each drug from the stock solution of 100 mg L^{-1}, six sequential concentrations in geometric series were prepared in Rocchetta© water, with a separation factor of 4 (100, 25, 6.25, 1.56, 0.4 , 0.1 mg L^{-1}). A filter paper disc (Whatman, 9mm) was placed in each Petri dish (9.2 x 1.6 cm), and soaked in 3 mL of drug solution or pure medium (negative control). Ten seeds of a single species, uniform in size and colour, were then placed in each Petri dish and uniformly spaced. For each concentration level (including negative control), 5 replicates were set up (ASTM, 2014). Therefore, for assaying each drug in a given species, (1 + 6) x 5 Petri dishes were used, amounting to a total of 35. The dishes were closed with the lid and kept in the incubator, in the dark, at 24±2°C, for 120 hours. To limit, as much as possible, evaporation from the filters soaked with the drug solutions, sheets of moistened paper were placed between the various layers of Petri dishes. At the end of the experiment, the percentage of germination was determined first; indeed, the test is considered valid if there is at least 80% germination of the seeds in the negative controls. The length of the roots was then measured using a calliper. Measurements were made from the transition point between the hypocotyl and the root to the end of the root tip (Figure 1). Based on drug concentrations and root length, the dose-response curves were plotted.

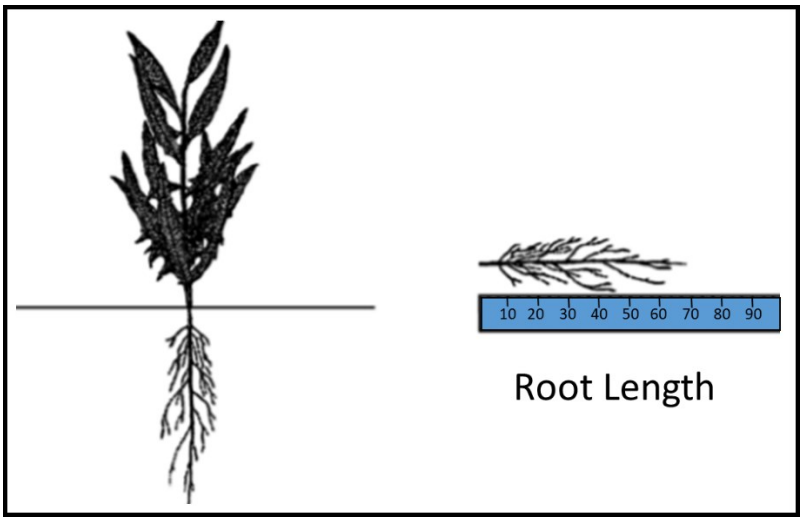

Figure 1. Illustration of Root Length Measurement (from ASTM, 2014. Modified)

4.Statistical analysis

The results of the radical elongation assay were processed with Prism© (8.4) in order to obtain the best dose-response curve (least squares fit) based on the variable slope model with four parameters.

Results

1.Preliminary assessment of germination capacity

The germination percentages calculated in the preliminary test for the selected plants were the following: *Lycopersicon esculentum* 81%; *Lactuca sativa*, 87%; *Daucus carota* 80%.

2.Root Elongation Test: Levofloxacin

Results obtained with LEV in *Daucus carota* are not presented, since, despite the favourable indication of the preliminary test, the germination percentage of the control group (pure medium) was lower than that required by the test protocol (80%). Therefore, only *Lycopersicon esculentum* and *Lactuca sativa* are considered here. The results of the root elongation assays show that, of the two species, *Lycopersicon esculentum* is the more sensitive, with an EC_{50} of 18.73 mg L^{-1} (CI 0.383-51.06) (Table 1, Fig. 2,A). A favourable effect on root elongation at the lowest concentration (0.1 mg L^{-1}) is also evident for this species. The dose-response curve of *Lactuca sativa* (Fig. 2,B) shows that at 100 mg L^{-1} the effect was only slightly greater than 50%; however, the calculated EC_{50} was 61.15 mg L^{-1} (CI 37.95-111.3) since at 25 mg L^{-1} the effect was still slightly lower than 50%.

3. Root Elongation Test: Enrofloxacin

In this test, all the three species showed a sufficient percentage of germination in the control group. As can be seen in Table 1, *Lycopersicon esculentum* was the most sensitive species also to ENR, with an EC_{50} equal

to 1.014 mg L^{-1} (CI 0.58-1.75), followed by *Lactuca sativa* with an EC_{50} of 33.55 mg L^{-1} (CI 20.50-60.08). The carrot, on the other hand, displayed a lower sensitivity, with an EC_{50} of 50.32 mg L^{-1} (CI 15.03-396.2). In Figure 3, the dose-response curves of the three assays are depicted.

4. Root Elongation Test: Flumequine

Also in this assay with FLU, the three species showed a sufficient percentage of germination in the control group. The results in Table 1 show that *Lycopersicon esculentum* was the most sensitive species, with an EC_{50} of 6.44 mg L^{-1} (CI 4.28-9.70), followed by *Lactuca sativa*, with an EC_{50} of 12.01 mg L^{-1} (CI 7.30-20.83), and *Daucus carota*, with EC_{50} of 50.32 mg L^{-1} (CI 15.03-396.20), thus reproducing the sensitivity ranking already observed with ENR.

When comparing the R^2 coefficents for each seed exposed to FLU (Table 1), it is evident that *Daucus carota*, as already observed with ENR, has a remarkably lower R^2. This can be noticed also in Figure 4 C, where the points of the dose-response curve of *Daucus carota* show a greater dispersion.

In Figure 4 the dose-response curves of tomato, lettuce and carrot assays are depicted.

DRUG	SPECIES	EC_{50} (CI 95%)	R^2
Levofloxacin	*Lycopersicon esculentum*	18,73 (0,38-51,06)	0,7061
	Lactuca sativa	61,15 (37,95-111,30)	0,8447
Enrofloxacin	*Lycopersicon esculentum*	1,01 (0,58-1,75)	0,8972
	Lactuca sativa	33,55 (20,50-60,08)	0,7706
	Daucus carota	50,32 (15,03-396,20)	0,4507
Flumequine	*Lycopersicon esculentum*	6,44 (4,28-9,70)	0,7696
	Lactuca sativa	12,01 (7,30-20,83)	0,8740
	Daucus carota	21,98 (9,49-65,11)	0,4389

Table 1. EC_{50} (CI 95%) and R^2 of levofloxacin, enrofloxacin and flumequine for three terrestrial plants.

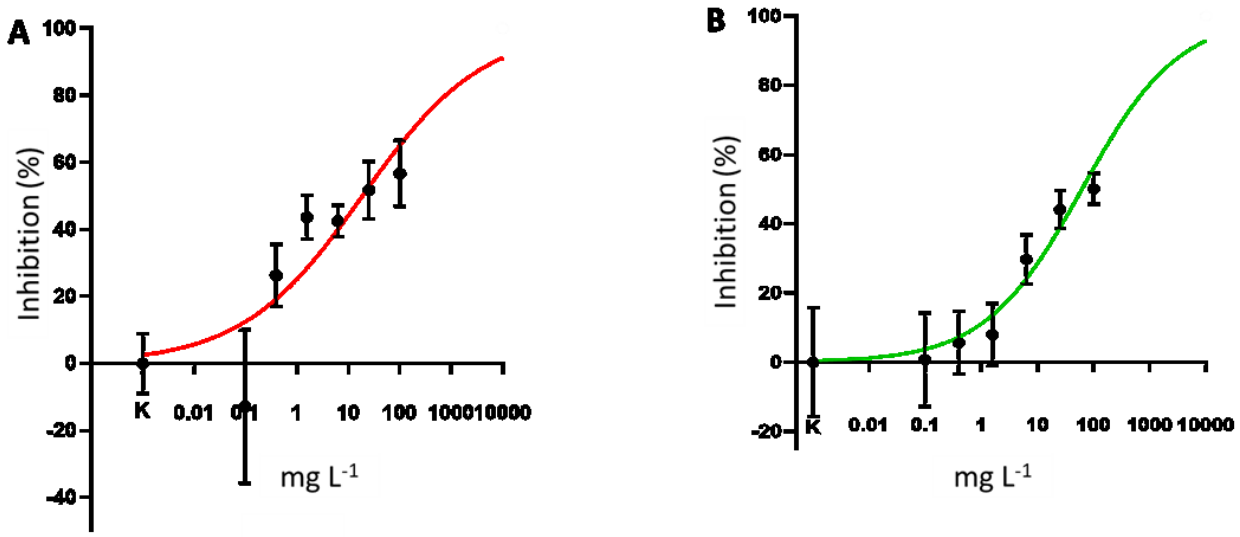

Figure 2. Dose-response curves of tomato (A) and lettuce (B), exposed to levofloxacin.

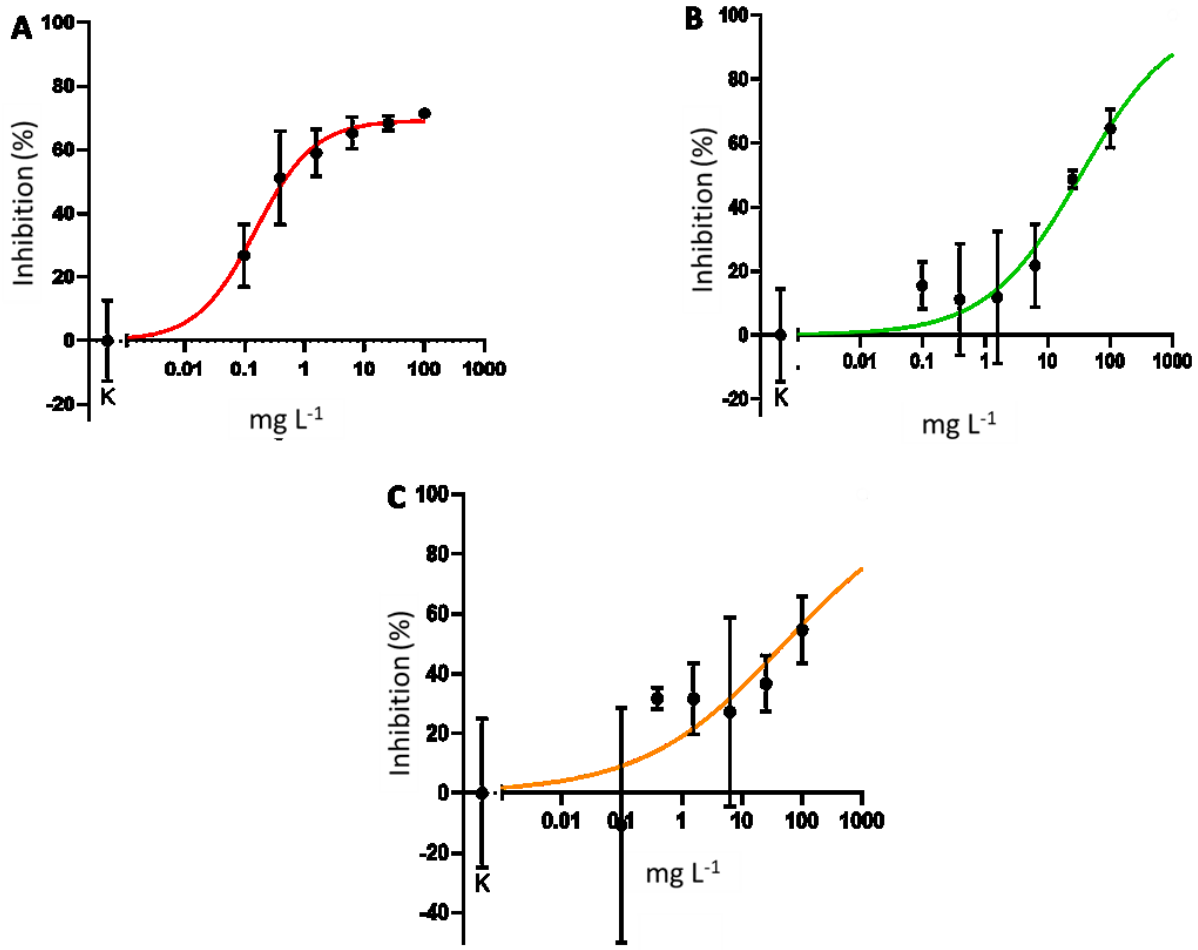

Figure 3. Dose-response curves of tomato (A), lettuce (B) and carrot (C), exposed to enrofloxacin.

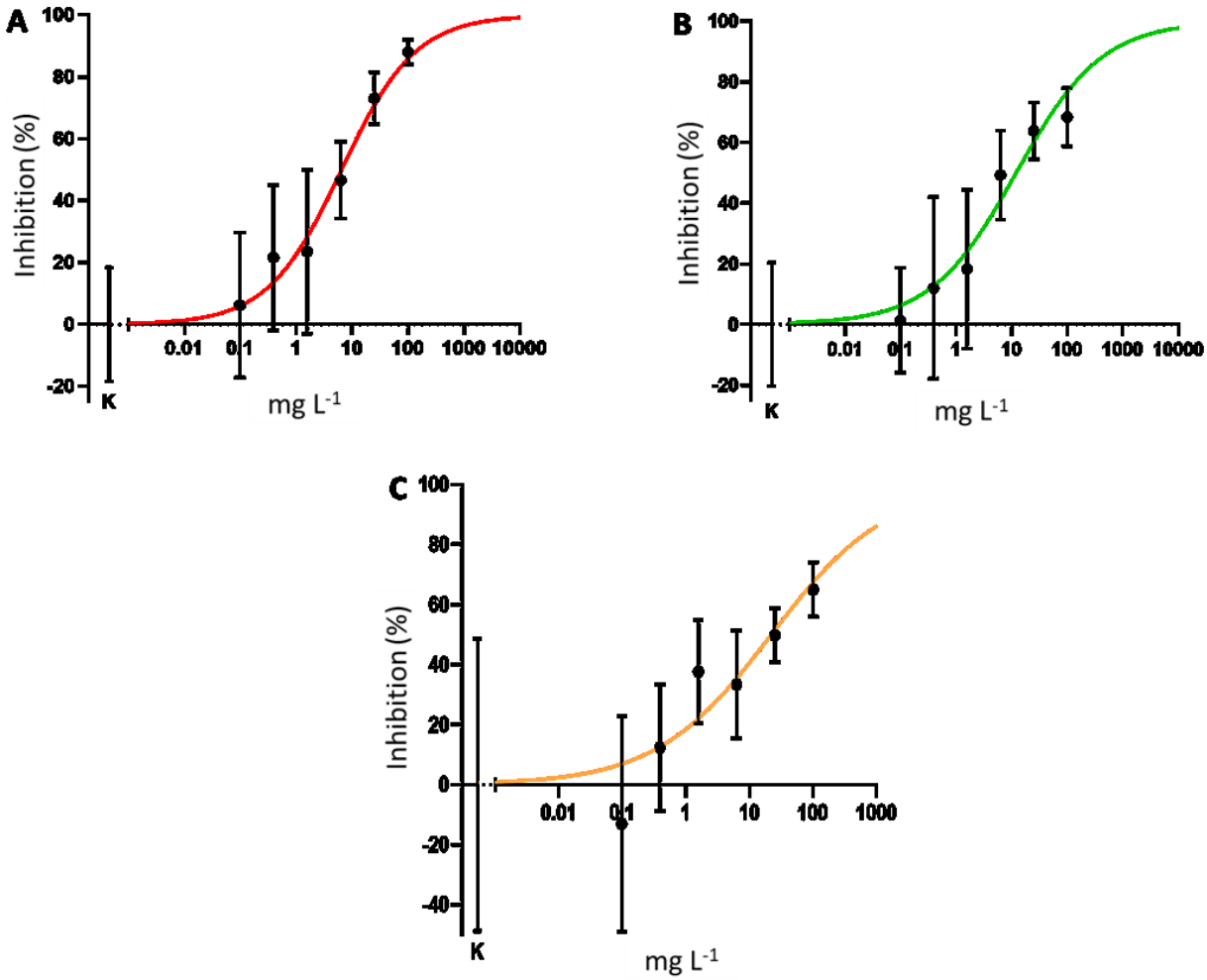

Figure 4. Dose-response curves of tomato (A), lettuce (B) and carrot (C), exposed to flumequine.

Discussion

Drug degradation was not evaluated during the tests since it can be considered negligible as already observed in our laboratory by Dalla Bona *et al.,* in 2015 and De Liguoro *et al.,* in 2019 for ENR and FLU, respectively, and by Czyrski *et al*., in 2019 for LEV. Indeed, FQs are photosensitive but poorly biodegradable and are not subjected to spontaneous hydrolysis reactions. Given their considerable economic importance and their widespread consumption all over the world, *Lycopersicon esculentum, Lactuca sativa* and *Daucus carota* are among those species identified in the regulatory documents and standard procedures of a number of tests, not only by the American Society for Testing and Materials (ASTM) but also by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Toxic Substance Control Act (TSCA), Federal Drug Administration (FDA) and the Organization for Economic Cooperation and Development (OECD). After selecting the three species used in the tests, we performed some preliminary assays with them (data not shown) to identify the most appropriate concentration range to perform the assays. In these pilot tests we applied a concentrations separation factor of 2, which is generally the maximum recommended for ecotoxicological tests. However,

this separation factor did not allow to obtain a sufficiently wide range of concentrations to generate dose-response curves, since an identical response was often observed under two subsequent concentrations. For this reason, a factor of 4 was applied in the final tests, which seemed the minimum necessary to define a range of concentrations suitable for obtaining a dose-response curve. Besides, in a similar study conducted with different drugs, Pan and Chu (2016) applied a separation factor of 10. This suggests that compared to other organisms used in toxicity tests (*e.g.*, the aquatic plant *Lemna minor*), these terrestrial dicotyledonous plants are able to activate compensation mechanisms that can somehow balance, up to a certain level of exposure, the effects of the drug. More generally, the responses observed in these ecotoxicity assays with FQs were not always closely related to the drug concentration levels. In particular, at the lowest dose, both with LEV in tomato and, to a lesser extent, with FLU in carrot, a stimulation of root elongation was detected. This type of result is described by many authors as the phenomenon of hormesis, according to which "Within the hormetic zone, the biological response to low exposures to toxins and other stressors generally is favourable" (Calabrese *et al.,* 2007; Mattson, 2008). In our case, it is possible to hypothesize that the hormetic effect may be linked to the toxic action of the drug that, at low doses, would be able to limit the competition of bacteria against the seed in the course of germination, without causing harm to the plant itself. It is worth noting that an "hormetic" was also observed by Migliore *et al.* (2003) when exposing various plant species to 0.05-5 mg L^{-1} ENR.

In general, the obtained results allow to assert that of the three assayed species, *Lycopersicon esculentum* is the most sensitive to FQs. The EC_{50} values calculated for this species, with the three FQs under study, are similar to those obtained in the prolonged acute immobilization test already carried out on the microcrustacean *Daphnia magna* (Tolosi and De Liguoro, 2021). *Daucus carota*, on the other hand, was the less sensitive species, with EC_{50} levels up to 50 times higher, in the case of ENR, when compared to *Lycopersicon esculentum*. Even the EC_{50} levels of *Lactuca sativa*, although lower than those of *Daucus carota*, seem too high to be of concern in the real environment. Indeed, these results, with reference to the soil, must be framed in the dynamics of drug partitioning between soil and water which, in the case of FQs, indicate a strong tendency of adsorption to soil particles, with very high K_d values that are explained by electrostatic interactions (Van Doorslaer *et al.,* 2014). It is therefore to be assumed that if the same experiments had been carried out by placing the seeds in contaminated soil, the toxic effects would have been lessened considerably. Other features that limit the validity of the results obtained in these assays are the poor correlation between exposure levels and observed doses, despite the application of a high separation factor, and the wide variability of the replicates. Furthermore, the limited sensitivity of the plants has meant that at the highest concentration tested, a 100% effect was generally missed. This would instead be recommended in a toxicity test, in order to obtain a good fit of the dose response curve. Indeed, to obtain curves with a tolerable R^2, a hypothetical 100% response under a very high dose was simulated. In the case

of the carrot, however, this was still not sufficient: the R^2 was ≤0.45 in both tests, with extremely wide confidence intervals of the EC_{50}.

Lastly, it is interesting to note that the FQs for veterinary use (ENR and FLU) are more toxic, based on the calculated EC_{50s}, when compared to the FQ for human use (LEV). This data is in good agreement with what was subsequently observed in *Daphnia magna* (Tolosi and De Liguoro, 2021).

Conclusions

The toxic effects of three FQs in three common crop species were evaluated in the present study. Although preliminary tests had been carried out for the optimization of the assays, the results are somewhat unsatisfactory and seem to indicate, generally, a low response of terrestrial plants to the toxicity of FQs, even if the sensitivity of *Lycopersicon esculentum* appears to be non-negligible.

In any case, before investigating the possible toxic effects of FQs on *Lycopersicon esculentum*, and on some other species that could be equally or even more sensitive, it will be necessary to gather further data on the partition of these drugs between soil and infiltration water. Then, assays should be carried out using selected types of contaminated soil. Being this one an exploratory study, it was limited to the exposure of plant seeds to the water solutions of FQs. The few indications that have been obtained from this study, and that should be considered for future research projects, are the following:

1) A selection of seeds should be made on the basis of their germination capability;

2) The range of assayed concentrations should be as large as reasonable;

3) *Lycopersicon esculentum* is an advisable species to evaluate the toxicity of FQs;

4) The veterinary FQs (ENR and FLU), display a higher environmental impact than LEV.

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Chapter 2.3

Delayed toxicity of three fluoroquinolones and their mixtures after neonatal or embryonic exposure, in Daphnia magna.

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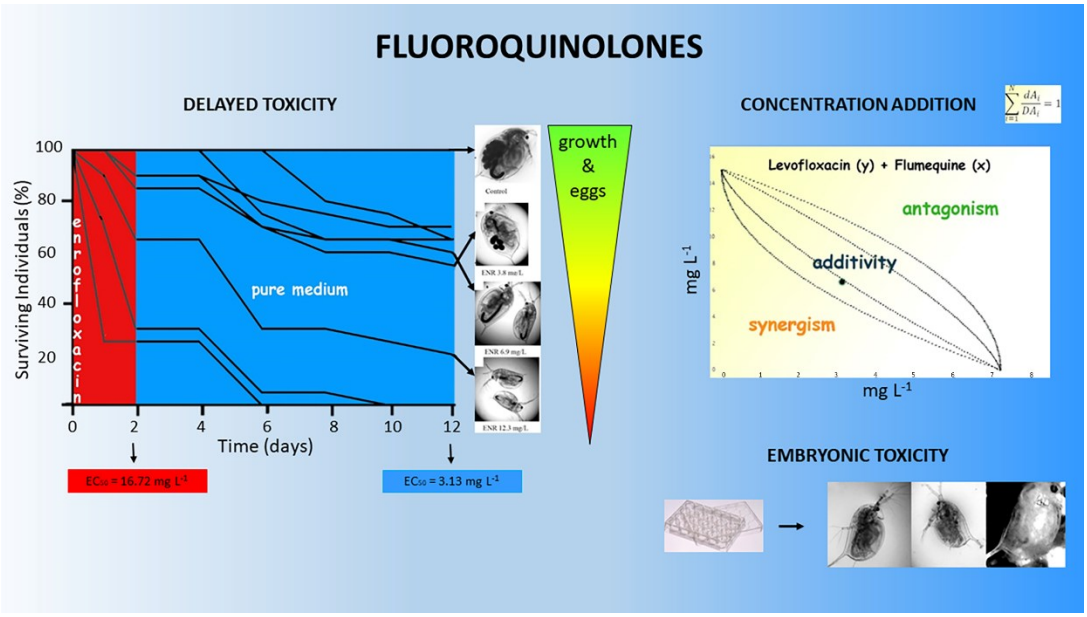
Graphical Abstract



Keywords: *Daphnia magna*, fluoroquinolones, delayed toxicity, mixtures, embryonic test.

Highlights

- Fluoroquinolones cause delayed toxic effects (lethality/growth inhibition) in *D.magna*
- The acute immobilization test can overestimate EC_{50}
- The toxicity of fluoroquinolone mixtures tends to follow the CA principle
- Levofloxacin is less toxic to *D. magna* than enrofloxacin and flumequine

Abstract

Fluoroquinolones (FQs) are antibacterial drugs, used both in human and veterinary medicine, that are currently considered as emerging micropollutants. This study investigated the delayed toxic effects of enrofloxacin (ENR), flumequine (FLU), levofloxacin (LEV) and their binary mixtures in *D. magna*. For this purpose, a 10-day follow-up in pure medium was added to the standard *D. magna* immobilization test. During this follow-up, phenotypic alterations were evidenced, which were related to scarce or zeroed egg production and early mortality. Consequently, the EC_{50s} recalculated at the end of the follow-up were always remarkably lower than those obtained after the 48h immobilization test: ENR 3.13 vs. 16.72 mg L^{-1}; FLU 7.18 vs. 25.35 mg L^{-1}; LEV 15.11 vs. >40 mg L^{-1}. To analyse the possible interactions within the binary mixtures, the method of nonlinear additive isoboles was applied. The three compounds showed invariably to follow the principle of concentration addition. Furthermore, as previous experiments showed toxicity of FLU and ENR after embryonic exposure of *D. magna* at a concentration of 2 mg L^{-1}, an additional two embryonic tests were conducted with identical design: one with 2 mg L^{-1} LEV and the other with a ternary mixture containing 0.66 mg L^{-1} of each of the three FQs. The embryos were exposed for three days *in vitro* to the drug solutions and were then reconducted to pure medium for 21 days observation. Both the tests ended-up with only non-significant effects on growth and reproduction, confirming the lower toxicity of LEV, when compared to ENR and FLU, and the absence of any evident synergistic interaction among the three FQs. Overall, these studies have shown two relevant features related to the toxicity of the three FQs: (1) they give rise to delayed toxic effects in *D. magna* that are undetectable by the standard immobilization test; (2) their interaction in mixtures follow the principle of Concentration Addition. Both these indications concern the Environmental Risk Assessment of FQs and may be of interest to regulatory authorities.

1.Introduction

Antibacterial drugs play a crucial role in the treatment and prevention of diseases in human and veterinary medicine. In recent decades, global consumption of these compounds has increased drastically (Klein *et al*., 2018) and there is growing concern about the potential impact on biota given their continuous release into the environment (Kümmerer, 2009; Tijani *et al*., 2013).

The environmental prevalence of these agents has been increasingly evident thanks to the progress of analytical chemistry, and their possible harmful effects on ecosystems made clear by the development of refined ecotoxicity tests. It is now straightforward for toxicologists to evidence the sublethal effects that may be critical for the dynamics of wild populations (Beiras, 2018).

In intensive animal farming, antibacterials are used mainly for mass prophylactic/metaphylactic treatment. Their use as growth promoters was banned in the EU in 2006 (Sarmah *et al.,* 2006), but has continued until recently in many countries outside the EU, China and India included (Laxminarayan *et al*., 2015). Use of any of these agents generates a non-negligible environmental load of either parent compounds or active

metabolites because slurry and manure are used for soil fertilization (Boxall *et al.,* 2003a). Antibacterials can be transferred from contaminated soils to surface water through ground seepage and/or runoff (Tarazona *et al.,* 2010). Another significant, more direct impact of antibacterials on surface water, relates to fish farming. In addition, the use of antibacterials in humans results in the contamination of municipal sewage, from both households and hospitals. This can be transferred to surface water through deliberate or accidental sewer overflow during periods of high rainfall (Laville, 2021) or inefficient wastewater treatment (Vieno *et al*., 2007). Another possible source of surface water contamination is discharge from pharmaceutical manufacturing facilities, particularly in countries with less stringent controls (Scott *et al*., 2018).

Fluoroquinolones (FQs) have been widely used both in human (Hamad, 2010), and veterinary medicine (EMA, 2011). They act through the inhibition of bacterial DNA-gyrase and topoisomerase IV, with consequent bactericidal action (Martinez *et al.,* 2006). In human medicine, they are mainly used in the treatment of urinary and respiratory infections, whilst their primary indication in farm animals is the control of diseases such as colibacillosis, salmonellosis, mycoplasmosis and pasteurellosis.

Significant factors that contribute to the potential environmental impact of FQs are the persistence of the parent compounds and the toxicity of their metabolites. Indeed, both can persist and accumulate in soil and sediments (Sukul and Spiteller, 2007; Gao *et al*., 2012). Furthermore, when FQs are degraded by sunlight, their photolysis products may also be active, and can be even more toxic than the parent compounds in some cases (Li *et al.,* 2011).

Given their environmental prevalence, FQs have been detected repeatedly in surface water (Gunnarsson *et al*., 2009; Santos *et al.,* 2010) and the most troubling agents have been given high priority for environmental risk assessment (Boxall *et al*., 2003b; Kim *et al.,* 2008). Whilst actual concentrations in watercourses and standing water are usually very low (ng to µg per liter), there are notable exceptions where remarkably high levels of contamination occur. Concentrations as high as mg L^{-1} have been evidenced in relation to manufacturing facilities (Larsson *et al.,* 2007; Gothwal and Thatikonda, 2017) or fish farming (Le and Munekage, 2004). Moreover, given their tendency to adsorb to solid matrices, higher concentrations of FQs are generally detected in freshwater sediments, from 210 ng kg^{-1} up to 20 mg kg^{-1}, with an average of 760 µg kg^{-1} (Van Doorslaer *et al*., 2014).

The potential environmental impact of three different FQs, Flumequine (FLU), Enrofloxacin (ENR) and Levofloxacin (LEV) was considered in this study. FLU and ENR are largely used in farm animals, whilst LEV, together with Ciprofloxacin (a metabolite of ENR), are amongst the most widely-used FQs in human medicine. The cladoceran crustacean *Daphnia magna* was the model organism selected for testing the ecotoxicity of the three FQs, given its well documented sensitivity to this class of pharmaceuticals (Isidori *et al*., 2005; Yang *et al., 2013;* Dalla Bona *et al.,* 2016; De Liguoro *et al*., 2019).

Preliminary, acute immobilisation tests (OECD, 2004) run in our laboratory showed lethargic behavior of *D. magna* after 48h exposure to FLU or ENR. The same observation was made by Robinson and coll. (2005) for

both FLU and clinafloxacin. In order to investigate any possible delayed toxic effects of ENR, FLU and LEV, in this study our experimental design was based on a standard 48h exposure with a follow-up of ten days in pure medium. Moreover, this experimental design involved exposure of the crustacean to binary mixtures of the three FQs and thus, any possible interactions between these compounds was assessed. This type of evaluation is worthwhile because various FQs can be present simultaneously in contaminated waters (Gao *et* al., 2012; Rutgersson *et al*., 2014). Whether any such interactions are less than additive, additive or synergistic is of considerable interest in environmental risk assessment (Cedergreen, 2014).

Finally, as previous experiments have shown toxicity of FLU and ENR after embryonic exposure of *D. magna*, an additional two embryonic experiments were conducted with identical design; one with LEV and the other with a ternary mixture of the three FQs.

The standard *D. magna* immobilization test has a pivotal role in Environmental Risk Assessment, however, neither does it consider delayed toxicity, nor is there a single agreed protocol to evaluate mixtures of environmental micropollutants (Cedergreen, 2014).

Given the significance of this emerging class of environmental micropollutants (Van Doorslaer *et al*., 2014), it is hoped that data generated in this study may contribute to the further evolution of the methodologies used in Environmental Risk Assessment.

2. Materials and methods

2.1Chemicals

Enrofloxacin (ENR), flumequine (FLU) and levofloxacin (LEV) were supplied by Sigma-Aldrich (Milan, Italy). Their purity was ≥98%.

For each compound, a 100 mg L^{-1} stock solution in Rocchetta© still mineral water (pH 7.6, dry residue 181.6 mg L^{-1}) was prepared before each test and stored in the dark at 4°C. Solubilization of the test compounds was obtained by gentle stirring overnight at 37 °C, and pH was measured using a BASIC20 pH-meter (CRISON, Carpi, Italy).

2.2 Test organism and culture conditions

Ephippia of *D. magna* were originally provided by ECOTOX (Milan, Italy) and a single cloned population was cultured in our lab. During culturing, the medium was Rocchetta© water. A temperature of 20°C +/- 1°C temperature was maintained by a thermo-refrigerated incubator. A photoperiod of 16h light (100 lx): 8h dark was selected. The sensitivity of the *D. magna* clone was checked every 4 months by exposure to potassium dichromate (ISO, 1996). The high quality health status of the culture was evidenced over time by the low mortality rate (≤ 2% per week), the high reproduction rate (about 10 neonates per day per individual), and the absence of *ephippia* and/or males. Daphnids were fed three times per week with *Scenedesmus dimorphus*

(8×10^5 cells mL^{-1}). Details of the algal culturing method have previously been reported (De Liguoro *et al*., 2012).

2.3. Acute immobilization test with 10-day follow-up

The acute immobilization test itself was performed in accordance with the Guideline 202 "*Daphnia sp.,* Acute immobilization Test" (OECD, 2004). However, in order to evidence any delayed toxicity, a follow-up of 10 days in pure medium was added to the standard incubation time (48h).

The three FQs were assayed individually (ENR; FLU; LEV) or in binary combinations (ENR+FLU; ENR+LEV; FLU+LEV). In each test, we used a negative control (pure medium) and eight sequential concentrations in a geometric series, with a separation factor of 1.8. The individual tests were run at the following concentrations: 0.7, 1.2, 2.1, 3.8, 6.9, 12.3, 22.2, and 40 mg L^{-1}. The concentrations of compounds within the binary mixtures followed an equi-toxicity ratio design that was based on the EC_{50} calculated at the end of the three individual tests (Figure 1).

Before the beginning of each test, in order to evaluate growth inhibition, 30 offspring not intended for the tests were isolated, fixed in 70% ethanol and photographed. Their length was measured, using Photoshop© software, as the distance between the eye and the base of the tail spine. The same procedure was repeated with all surviving individuals at the end of the assay, and hence their daily growth calculated.

For the test, only daphnids younger than 24h and obtained from the third to fifth brood were used. They were fed 1h before the beginning of the experiment with spirulina powder (15 mg in 100 mL of Rocchetta© water) and then incubated in 4 groups of 5, in 10 mL pure medium (control) or drug solutions, under the same light and temperature conditions used for culturing. Pre-feeding of the organisms is not deemed necessary by the test guideline, however in our experience is beneficial and therefore advisable (as it helps to sustain 100% survival in the control groups). After 48h of incubation, the number of immobilized daphnids was recorded and the EC_{50} calculated. Then, the follow-up in pure medium was carried out: surviving individuals were transferred to 36 individual beakers (preserving the group/exposure identity). Each beaker contained 50 mL of Rocchetta© water and the daphnids fed in every other day by adding *S. dimorphus* to a concentration of 8×10^5 cells mL^{-1}. Follow-up lasted 10 days during which time *D. magna* ability to survive, grow and produce eggs was evaluated. At day 12, when many daphnids had already produced their first clutch, all surviving adults were collected, fixed and measured. The endpoints considered were mortality and growth inhibition.

2.4. Embryonic test with 21-day follow-up

As previous experiment had shown toxicity of FLU and ENR after embryonic exposure of *D. magna* at a concentration of 2 mg L^{-1} (Dalla Bona *et al*., 2016; De Liguoro *et al*., 2019), an analogous test with 2 mg L^{-1} LEV was performed. Then, in order to understand whether one of the three different toxicity patterns

displayed by the individual compounds would prevail on exposure to their mixture, another test was run with a combination containing 0.66 mg L^{-1} of each one. In both tests the following procedure was used. Gravid daphnids were collected from cultures and examined microscopically for the level of embryo development in the brood chamber. To obtain sufficient embryos, approximately thirty specimens, carrying embryos in early development (stage1) (LeBlanc *et al*., 2000), were selected. Embryos were extracted by immobilizing the head of the adult with a dissecting probe whilst a second probe was used to free the embryos gently by separating the carapace (LeBlanc *et al*., 2000). The collected embryos were taken at random and transferred individually to each well of 24-well Suspension Culture Plate (CELLSTAR, Greiner bio-one) containing either 1 mL of the drug solution (*n*=72) or 1 mL of Rocchetta© (*n*=72). They were incubated for 3 days under the conditions (light and temperature) normally used for *D. magna* culture (see above). After incubation, the number of embryos hatched was recorded and 10 apparently-healthy neonates from the population of exposed embryos were randomly selected and assigned to group 1 (prenatal exposure), whilst 10 neonates randomly selected from the control group were assigned to group 2 (controls). Daphnids from each group were individually allocated to 100 mL beakers containing 50 mL of pure Rocchetta© water and incubated for 21 days under the culture conditions described earlier. Every other day the medium was replaced, the neonates removed and counted, and feed (*S. dimorphus*, $8x10^5$ cells mL^{-1}) supplied. At the end of the test, the growth rate, reproductive activity and mortality rate of the two groups were measured and compared (OECD, 2012).

2.5. Data analysis

The EC_x values with confidence limits were calculated using "Probit Analysis" software (USEPA, 2012). The applied Concentration Addition (CA) model for binary mixture toxicity prediction was calculated as:

$$\sum_{i=1}^{N} \frac{dA_i}{DA_i} = 1$$

where dAi is the dose/concentration of Ai in a mixture that produces a specified effect, and DAi is the dose/concentration of the single agent which on its own elicits the same effect as the mixture (Kortenkamp and Altenburger, 1998). To strictly evaluate any possible deviation from the CA model, a curvilinear isobologram analysis at EC_{50} was performed, with confidence limits based on Hill coefficient variability (De Liguoro *et al*., 2018). According to Tallarida (2006), for compounds with a variable potency ratio, synergy (or antagonism) is detected only if the EC_{50} of the mixture lies below (or above) the region of the plane bounded by the two curves of additivity for a 50% effect.

To infer differences in growth and reproduction between the control and the exposed group in the immobilization and embryo tests, the Student T-test was used. P values <0.05 were considered significant.

3.Results

Considering the exposure conditions adopted in the various tests (incubation 48-72 h, temperature 20±1° C, 100 lux), any degradation of the three FQs under study can be considered negligible. Indeed, previous studies have already indicated that ENR (Dalla Bona *et al.*, 2015) and FLU (De Liguoro *et al*., 2019) are largely stable under these conditions, while LEV was found to be stable in three different saline solutions, even after a 4-week exposure to daylight (Czyrski *et al*., 2019). Based on the Criteria for Reporting and Evaluation of ecotoxicity Data (CRED; Moermond *et al.,* 2016), in acute toxicity tests with stable substances, nominal concentrations without further measurements are acceptable. Accordingly, in the present study the use of HPLC analysis was deemed redundant and consequent undesirable excess use of solvents was avoided, with test results being based on nominal concentrations. In the immobilization tests, validity criteria (OECD, 2004) were fulfilled as control survival (mobility) was ≥ 90%, and the recorded values of water quality parameters were always within the following ranges: pH 7.5–7.7, dissolved oxygen 7.9–8.4 mg L^{-1}.

Data from acute immobilization tests with follow-up are shown in Figure 1. Invariably, a decline in survival was recorded during the 10-day follow-up. As a result, the calculated 48h EC_{50s} were considerably higher than those calculated at the end of the test (12 days). In particular, for the individual tests: ENR 16.72 vs. 3.13 mg L^{-1}; FLU 25.35 vs. 7.18 mg L^{-1}; LEV >40 vs. 15.11 mg L^{-1}. And for the binary tests: ENR+FLU 9.98+22.89 vs 1.44+3.30 mg L^{-1}; ENR+LEV 9.98+47.85 vs 1.86+8.92 mg L^{-1}; LEV+FLU >47.85+22.89 vs 6.58+3.14 mg L^{-1}. Thanks to the follow-up, effects on development were evidenced in some individuals. These were testified by the scarcity or absence of eggs/embryos in the brood chamber. In Figure 2, a control individual (a) is compared to exposed daphnids displaying proportionate dwarfism, which apparently was dose-correlated (b-f), and to surviving individuals from a single group exposed to 22.2 mg L^{-1} LEV (g), ranging from perfectly healthy to seriously altered. Reproductive potential was inevitably compromised by these phenotypic alterations, which were generally also the precursor to premature death. Significant effects on daily growth were detected mainly at higher concentrations, in some cases with large within-group variability. These effects were more evident with ENR in the individual tests and with ENR+FLU in the binary tests (Figure 3).

In Figure 4, the predicted dose-response curves based on the CA principle were matched to the curves obtained by testing the binary mixtures. In all cases the two curves were closely similar to each other, thereby indicating no relevant deviation from the CA principle. This tendency was confirmed by the curvilinear isobolograms (Tallarida, 2006), where the EC_{50s} of the three mixtures invariably lay within the plane bounded by the two curves of additivity and their confidence limits (Figure 5).

The exposure of *D. magna* embryos to 2 mg L^{-1} LEV did not affect their survival (Table 1); however, two newborns (out of 70) showed an incorrect arrangement of their antennae (Figure 6.a,b) and were unable to swim properly. Healthy newborns transferred to pure medium and followed-up for 21 days showed no phenotypic alterations, and their reproduction and growth rates were not significantly lower than those of the control group (Table 1). Similarly, the exposure of *D. magna* embryos to the mixture composed by 0.66

mg L^{-1} of each compound caused only a non-significant reproduction inhibition (Table 1). 10 % mortality during the 21-day follow-up should be considered casual from a statistical point of view; however, the only dead individual (out of ten) showed (Figure 6.c) the typical phenotypic alteration (discolouration, poor growth) known to be brought about by FQs (De Liguoro *et al*., 2019).

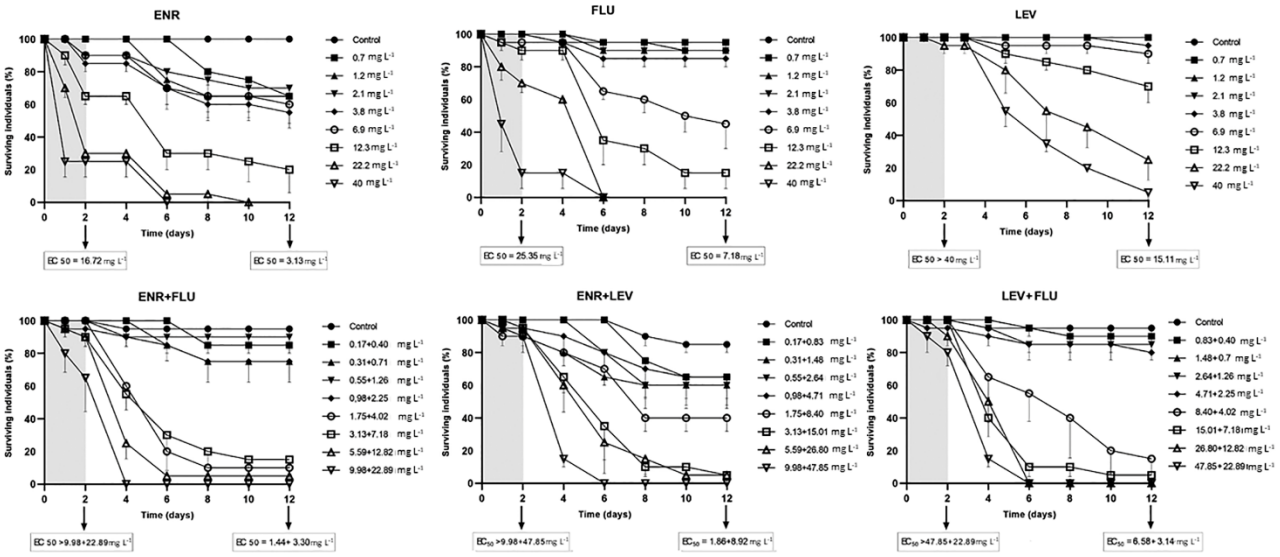


Figure 1. Survival curves of *D. magna* over 48 hours exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. Error bars show standard error (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

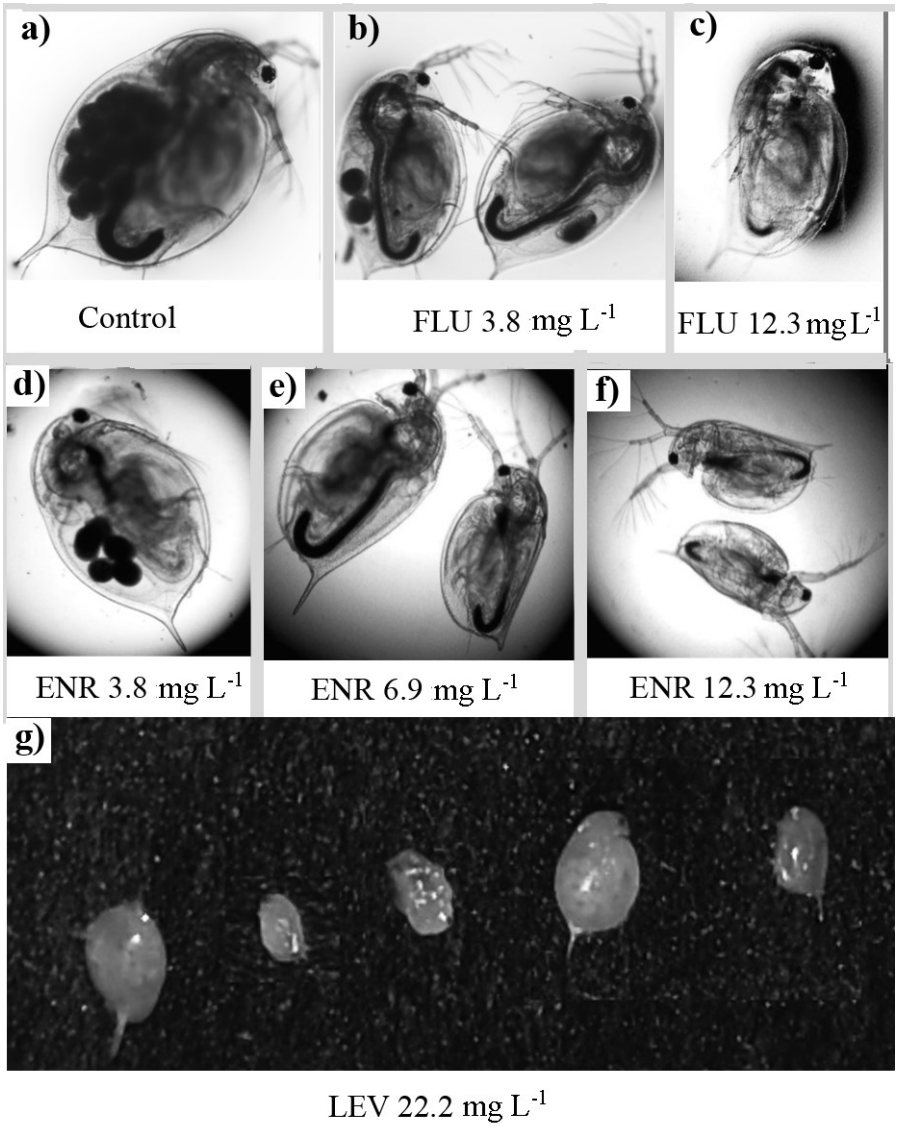


Figure 2. Phenotypic and functional alterations in *D. magna* after 48 hours exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. a) Control; b-f) individuals exposed to different concentrations of FLU and ENR; g) surviving individuals from a single group, exposed to LEV (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

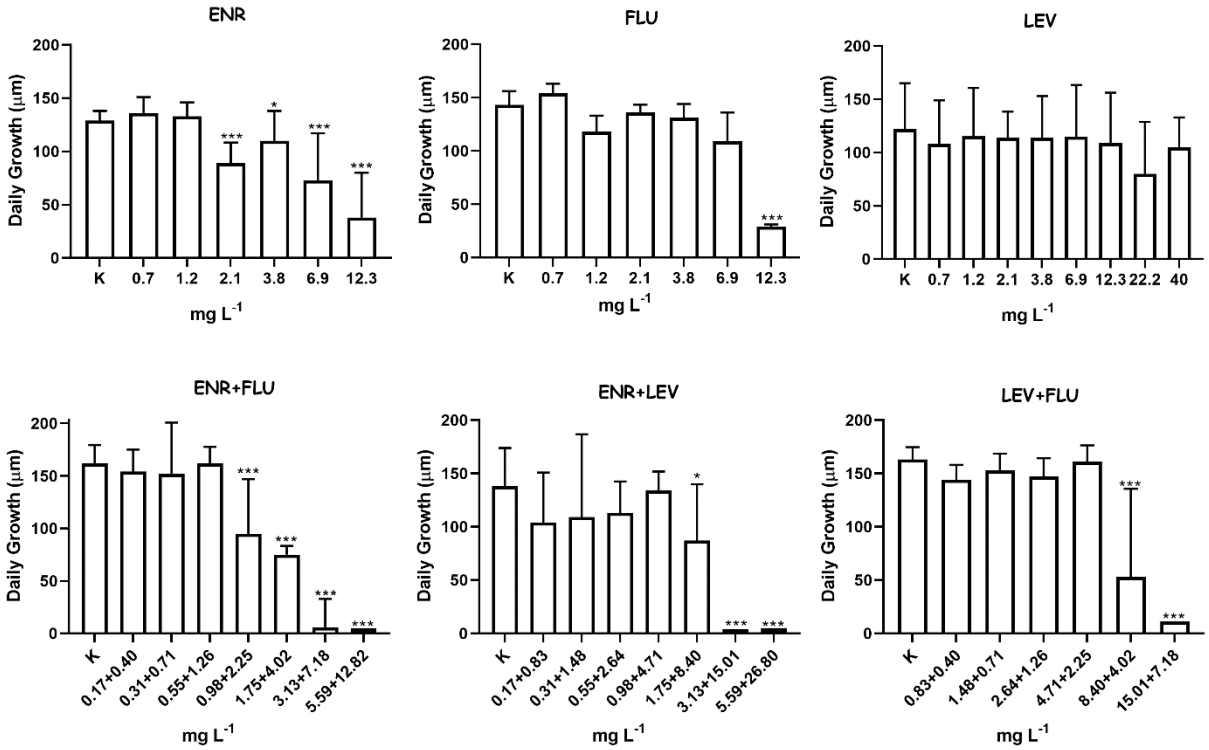


Figure 3. Daily growth of *D. magna* after 48 hours exposure to fluoroquinolones, individually or in binary combinations, with 10 days follow-up in pure medium. Error bars show standard deviation (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

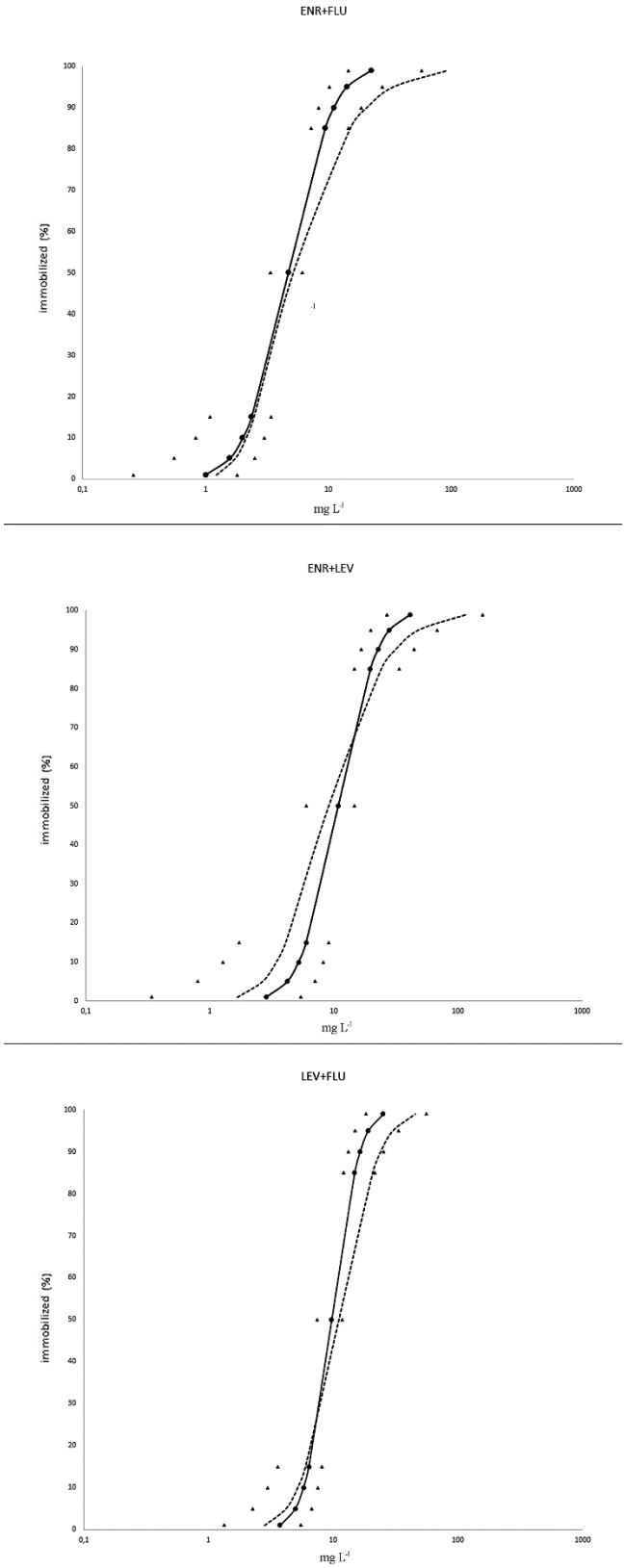


Figure 4. Concentration-effect curves of fluoroquinolone binary mixtures assayed on *D. magna*, obtained by connecting the EC_x values generated by Probit analysis of the experimental data. Triangles indicate the 95% confidence limits. Dashed lines are the predicted concentration-effect curves according to the Concentration Addition principle (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

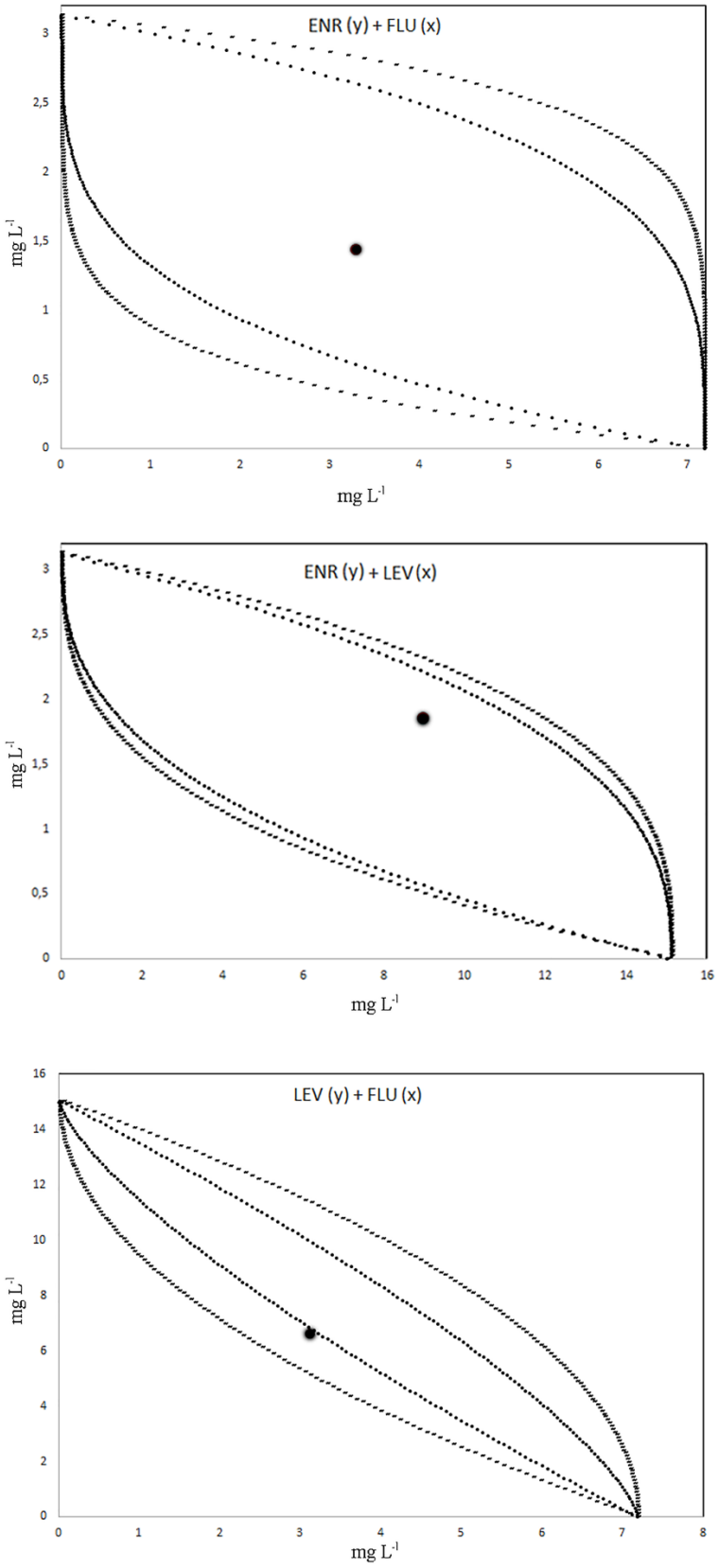


Figure 5. Isobolograms of fluoroquinolone binary mixtures assayed on *D. magna*. Dotted lines represent curves of additivity. Dashed lines are their confidence limits based on Hill coefficient variability (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin).

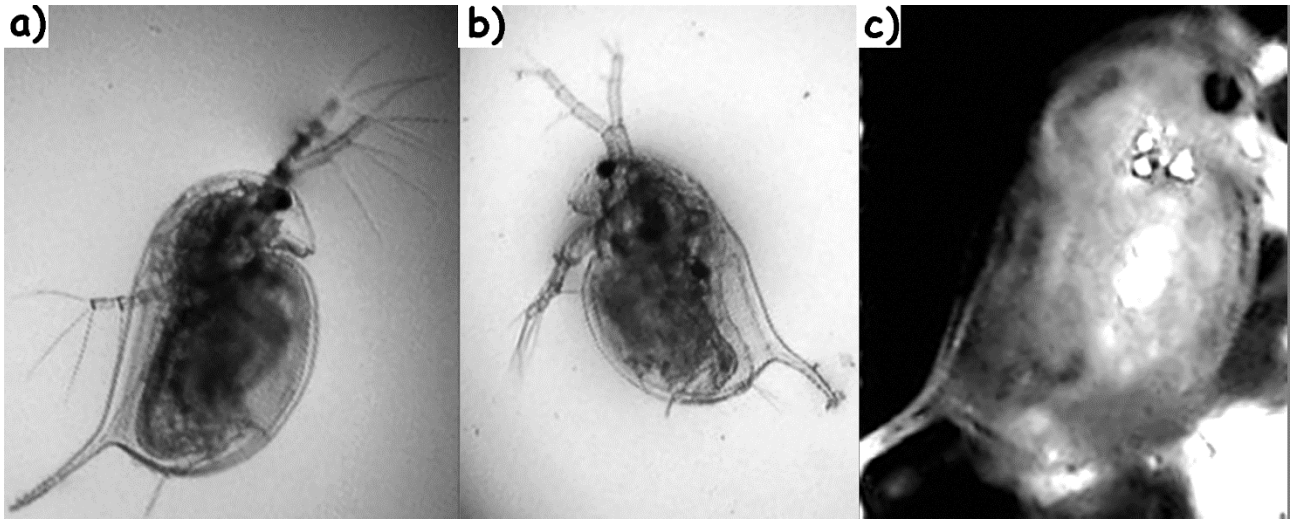


Figure 6. Phenotypic and functional alterations in *D. magna* after 3 days embryo exposure to LEV individually or to ternary mixture, with 21 days follow-up in pure medium. a-b) Individuals exposed to 2 mg L^{-1} levofloxacin (LEV); c) individual exposed to the ternary mixture of fluoroquinolones.

a)

Group	Healthy newborns	Early developmental arrest	Late developmental arrest	Stillbirth	Birth defects (antennae or tail spine)	References
Control (n:35)	97%	1	0	0	0	Dalla Bona *et al*., 2016
ENR	69%	9	2	0	0	
Control (n:72)	93%	3	1	1	0	De Liguoro *et al*., 2019
FLU	78%	6	1	3	6	
Control (n:72)	92%	4	1	1	0	This work
LEV	93%	1	1	1	2	
Control (n:72)	92%	6	0	0	0	This work
ENR+FLU+LEV	94%	4	0	0	0	

b)

Group	Mortality rate (%) (*n*=10)	Time to production of first brood (days)	Neonates per parent animals at the start of the test	Neonates per surviving parent animals	Average daily growth (length, µm)	Daily growth inhibition (%)	References
Control	0	n.d.	122 ± 27	122 ± 27	183 ± 30		Dalla Bona *et al*., 2016
ENR	0	n.d.	119 ± 46	119 ± 46	177 ± 30	3	
Control	10	10	73 ± 30	79 ± 26	152±31		De Liguoro *et al*., 2019
FLU	70	10	17 ± 30***	59 ± 27	152±24	0	
Control	20	10	74 ± 9	73 ± 8	160± 30		This work
LEV	20	10	60 ± 26	64 ± 15	148± 35	8	
Control	0	10	107±18	107±18	141		This work
ENR+FLU+LEV	10	10	84±33	93±15	144	-2	

***p<0.001

Table 1. Embryonic test with 21-day follow-up. **a)** Results of the 3-day *in vitro* test on *D. magna* embryos exposed to 2 mg L^{-1} of ENR, FLU or LEV, or to a ternary mixture (ENR+FLU+LEV) containing 0.66 mg L^{-1} of each compound (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin). **b)** Mortality, reproduction performance and daily growth in the 21-day follow-up of apparently healthy individuals hatched after 3-day embryo exposure to ENR, FLU or LEV, or to a ternary mixture (ENR+FLU+LEV) (ENR, enrofloxacin; FLU, flumequine; LEV, levofloxacin)

4. DISCUSSION

As already observed in a previous, multigenerational experiment (De Liguoro *et al*., 2019), the toxic effects of FQs on the cladoceran crustacean *D. magna* occur stochastically in a certain percentage of individuals. They are characterized by phenotypic alterations of varying severity, accompanied by functional effects, ranging from limitation of reproduction rate to early mortality. The exposure to eight concentrations in a geometric series permitted the observation that both the percentage of harmed individuals (Figure 1) and the severity of damage (Figure 2.b-f) increase with increasing concentrations of FQs. Based on the measured EC_{50s}, the following toxicity ranking was inferred: ENR> FLU> LEV. This ranking was apparent also when taking into account the growth inhibition endpoint (Figure 3). Considering that, at neutral pH, ENR has a lower aqueous solubility and a higher membrane permeability than LEV (Blokhina *et al*., 2016), a possible explanation for its higher toxicity could be the easier absorption by the cells of *D. magna*. However, the toxic effects of the three drugs appeared to be qualitatively alike. For example, the alterations observed after acute exposure to LEV (Figure 2.g) are strikingly similar to those previously found after chronic exposure to FLU (De Liguoro *et al*., 2019).

In general, inhibition of daily growth was poorly correlated to the exposure level since the measurement of this endpoint was limited to individuals alive at the end of the test. Indeed, randomly harmed individuals within groups either survived till the end of the test or died prematurely, thereby affecting in one sense or another the average daily growth of their group. Moreover, the occasional presence of healthy and harmed individuals in the same group, explains the large standard deviation of the dataset seen in some cases (Figure 3).

The follow-up to the acute immobilization test evidenced, for all the compounds tested and their binary mixtures, delayed toxic effects that may have crucial consequences for *D. magna* populations. The EC_{50} measured at 48h is clearly overstated when compared to that recalculated after 10 days of maintenance in pure medium. Considering that the EC_{50} of *Daphnia* immobilization test is, within the Environmental Risk Assessment of pharmaceuticals (EMEA, 2005), one of the primary parameters used for the calculation of the PNEC (Predicted No Effect Concentration), its overstatement implies an underestimation of the Risk Quotient (PEC/PNEC) and potentially inadequate protection of the environment. Furthermore, the same test is required under EU regulations concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (EC, 2006). For these reasons, we believe that a follow-up period post acute immobilisation test should always be considered in those cases where, after 48h exposure, the surviving daphnids display signs of lethargy, discolouration, or any other evident harm.

The aim of testing binary mixtures was to verify if the three FQs could interact synergistically, and thereby jointly exert a larger effect than predicted by the CA principle. This information is crucial because, for those regulations where mixtures are considered, CA is proposed as the default model (Backhaus *et al*., 2010). Data generated thus far on the toxicity of chemical mixtures, justify this proposition, as in 95% of cases no

interaction (simple additivity) or antagonism (less than additivity) have been detected (Cedergreen, 2014). The compounds investigated in this experiment were no exception to the rule; invariably, they acted in combination by following the principle of CA. Therefore, at least for these three FQs, the simple additivity concept seems adequate for evaluating the ecotoxicity of their mixtures using *D. magna* populations. However, the assayed binary mixtures were all composed of equi-toxic concentrations of the three pharmaceuticals and synergic or antagonistic interactions at other toxic ratios cannot be excluded. Indeed, the mixture-ratio dependence of drug interactions is not an unusual phenomenon and has been observed by various authors (Berenbaum, 1989; Kortenkamp and Altenburger, 1998; De Liguoro *et al*., 2009). Moreover, any generalization of the obtained results to the entire class of FQs should be avoided: the fact that FQs share the same mechanism of action does not necessarily imply that any or all of their possible combinations should follow the CA principle. Indeed, in previous tests on *D. magna* with sulphonamides, another class of antibacterials, after testing 15 different binary combinations, a tendency towards a range of possible interactions (antagonistic, additive, synergistic) was observed (De Liguoro *et al*., 2018).

The embryonic test with 2 mg L^{-1} LEV confirmed its lower toxicity to *D. magna* when compared to the other two FQs. Taking into account previously published data from analogous tests conducted with ENR and FLU (Dalla Bona *et al.* 2016; De Liguoro *et al.* 2019), the three FQs showed different impact patterns after prenatal exposure of the crustacean (Table 1): ENR featured an "all or nothing" toxicity pattern, causing the death of a substantial percentage of embryos without any delayed toxicity over postnatal development and reproduction; FLU caused a slight mortality of embryos and some birth defects, but had also delayed, relevant effects on survival and reproduction; LEV had no apparent effect, with the exception of two cases of phenotypic alterations in *D. magna* newborns. The two individuals (Figure 6.a,b) bore an altered conformation of the antennae and, consequently, they were unable to swim properly, which would have destined them to a very short life in a natural environment, given the crucial role of swimming in finding the optimum position relative to food availability and predator pressure (Christensen *et al*., 2005).

The results of the test with the ternary mixture (ENR+FLU+LEV) indicate a pattern very similar to the one elicited by LEV alone. Considering that LEV was the least toxic of the three compounds composing the mixture, any synergistic interaction can be excluded for the assayed ternary combination.

Conclusions

A 10-day follow-up in pure medium added to the standard (48h) immobilization test on *D. magna*, led to lower values of acute EC_{50} with each of three FQs (ENR, FLU and LEV). At the end of the standard immobilization test, in the case of some survived daphnids displaying signs of illness, this addition of a 10-day follow-up in pure medium can be recommended for all the survived individuals. Indeed, in that it can evidence delayed mortality, it permits a more accurate environmental risk-assessment.

Tests using mixtures of the three compounds have indicated that their toxicity to the crustacean tends to obey the reference model of Concentration Addition. Whilst this limited observation cannot be generalized to the entire class of FQs, it strengthens the concept that for drugs sharing the same mechanism of action, such as the FQs, the Concentration Addition model (simple additivity) is probably appropriate.

The exposure of *D.magna* embryos to 2 mg L^{-1} LEV did not cause any mortality and had only non-significant effects on subsequent daily growth and reproduction activity. This confirmed the lower toxicity of LEV when compared to ENR and FLU, already observed with the extended immobilization test. Overall, the three compounds can harm this crustacean at concentrations of few mg L^{-1}. Whilst levels of FQs in the freshwater environment are generally very low (ng to µg L^{-1}), far higher concentrations (mg L^{-1}) have been occasionally reported either in the water column or sediment. Consequently, possible harm to crustacean populations in heavily contaminated watersheds cannot be ruled out.

Given the mechanism of action of FQs, any future research aimed at investigating possible alteration or regulation of *D. magna* genetic material would contribute to a better understanding of the delayed toxicity evidenced in this study and the previously reported transgenerational toxicity.

Acknowledgements

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Chapter 2.4

Nano-immobilized flumequine with preserved antibacterial efficacy

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I have contributed only to a minor part of the following paper; for this reason, I will present in this thesis only the abstract and conclusions of the study, whilst limiting the other sections to the paragraphs that pertain to my personal collaboration.

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Abstract

Flumequine was nano-immobilized by self-assembly on iron oxide nanoparticles, called surface active maghemite nanoparticles (SAMNs). The binding process was studied and the resulting core-shell nanocarrier (SAMN@ FLU) was structurally characterized evidencing a firmly immobilized organic canopy on which the fluorine atom of the antibiotic was exposed to the solvent. The antibiotic efficacy of the SAMN@FLU nanocarrier was tested on a fish pathogenic bacterium (*Aeromonas veronii*), a flumequine sensitive strain, in comparison to soluble flumequine and the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were assessed. Noteworthy, the MIC and MBC of soluble and nanoparticle bound drug were superimposable. Moreover, the interactions between SAMN@FLU nanocarrrier and microorganism were studied by transmission electron microscopy evidencing the ability of the complex to disrupt the bacterial wall. Finally, a preliminary *in vivo* test was provided using *Daphnia magna* as animal model. SAMN@FLU was able to protect the crustacean from the fatal consequences of a bacterial infection and showed no sign of toxicity. Thus, in contrast with the strength of the interaction, nano-immobilized FLU displayed a fully preserved antimicrobial activity suggesting the crucial role of fluorine in the drug mechanism of action. Besides the importance for potential applications in aquaculture, the present study contributes to the nascent field of nanoantibiotics.

2. Materials and Methods

2.1. Chemicals

Iron(III) chloride hexahydrate (97 %), sodium borohydride (NaBH4), ammonium hydroxide solution (35 % in water) and flumequine (≥ 97 % HPLC) were purchased from Aldrich (Sigma-Aldrich, Italy) at the highest commercially available purity and were used without further treatment. All solutions were prepared using Milli-Q (Merck KGaA, Germany) grade water. The synthesis of SAMNs was already described (Magro *et al.,* 2012), and was briefly summarized in Supplementary Information.

2.3. Evaluation of the therapeutic effect of nano-immobilized FLU on infected Daphnia magna

An acute toxicity test with SAMN@FLU was performed on *Daphnia magna* individuals affected by a spontaneous bacterial infection (*Aeromonas* spp. and *Pseudomonas* spp. assessed by polymerase chain reaction, PCR, 16S rRNA gene amplicons). The test was carried out in accordance with the OECD Guideline n. 202 "*Daphnia* spp., Acute Immobilization Test". However, after the canonic 48 h incubation, a follow-up of another 8 days in pure medium was envisaged. Rocchetta medium (De Liguoro *et al.,* 2019) was used for control and compared with 20 mg L^{-1} SAMN@FLU. A positive control with daphnids exposed to 24 mg L^{-1} soluble FLU (48 h EC_{50},) (Tolosi *et al.,* 2019) was also settled up. Daphnids (< 24 h old) were fed for about 1 h with dried Spirulina powder (15 mg in 100 mL Rocchetta medium) just before the starting of the experiment, and then each group (4 replicates, n = 5) was incubated in 10 mL of the test solution under the same

conditions (photoperiod 16 h light, 2.6 µE m^{-2} s^{-1}, 8 h darkness, 20 ± 1 °C temperature) used for culturing. Agitation was provided by gentle shaking at 100 rpm. Shaking, which is not envisaged by the OECD protocol, was chosen to minimize nanoparticle sedimentation and was considered tolerable as it reproduces random flows experienced by the animals in nature (Magro *et al.,* 2018). The number of immobile daphnids was recorded after 48 h incubation. Then, in order to evidence delayed toxicity (Tolosi *et al.,* 2019), all groups (treatments and controls) were transferred into pure medium, maintained under normal culturing conditions and followed-up for another 8 days during which, every other day, they were fed with *Scenedesmus dimorphus* (8×10^5 cells mL^{-1}) and any immobilized individual was removed and counted. The protocol for FLU immobilization, the detailed description of the electron microscopy analysis (TEM), as well as the instrumentation used in the present study were reported in Supplementary Materials.

3. Results and Discussion

3.5. Evaluation of the therapeutic effect of nano-immobilized FLU on infected Daphnia magna

In order to provide a proof of concept of the applicability of the SAMN@FLU hybrid in a real scenario, its biocompatibility as well as effectiveness were assessed on a *Daphnia magna* clone naturally infected with FLU-sensitive bacteria (*Pseudomonas* spp. and *Aeromonas* spp.) (Moir *et al.,* 2007; Le Coadic *et al.,* 2012). On this regard, it is important to mention that an alarming chronic toxicity of FLU (2 mg L^{-1}) was already reported on *D. magna*, causing transgenerational effects, such as phenotypic alterations, reduced survival and impaired reproductive capacity (De Liguoro *et al.,* 2019). Moreover, a delayed toxicity has recently been observed after a 48 h immobilization test, resetting the acute EC_{50} value of flumequine at a lower concentration, from 24 down to 7 mg L^{-1} (Tolosi *et al.,* 2019). Herein, individuals of the infected clone of *D. magna* were exposed for 48 h to SAMN@FLU at a concentration corresponding to the in vitro measured MIC (20 mg L^{-1}) and their survival was compared to that of controls and groups exposed to 24 mg L^{-1} soluble FLU (48 h, EC_{50}). After 48 h incubation, no immobilization was recorded in unexposed and SAMN@FLU-exposed individuals, while 8 individuals out of 20 were immobilized in the FLU exposed groups. At the end of the test (tenth day), all individuals exposed to FLU and 9 out of 20 of the unexposed group were immobilized, while all members of the group exposed to SAMN@FLU were alive and apparently healthy (Fig. S3). These latter were observed by light microscope and showed the appearance of nanomaterial aggregates on the carapace surface. Likely, the crustacean surface acted as a nucleation seed for SAMN@FLU aggregation, and the phenomenon took place as a response to the destabilization of the nanomaterial colloid induced by the salinity of the medium. Interestingly, the binding of SAMN@FLU on the crustacean surface seemed to stimulate the molting, as witnessed by the presence a multitude of released carapaces bearing agglomerated nanoparticles (Fig. 6). The aggregation of nanoparticles to the *D. magna* carapace has already been documented for TiO_2 (Dabrunz *et al.,* 2011), CeO_2 (Gaiser *et al.,* 2012), and pristine SAMNs (Magro *et al.,* 2018). Indeed, the treatment of *D. magna* with SAMN@FLU revealed no sign of toxicity. Instead, it

significantly improved the survival of individuals infected by *Pseudomonas* spp. and *Aeromonas* spp., suggesting the feasibility as nanoantibiotic in aquaculture of this functional coreshell nanomaterial. Nevertheless, this aspect deserves a deeper investigation and will be object of further studies.

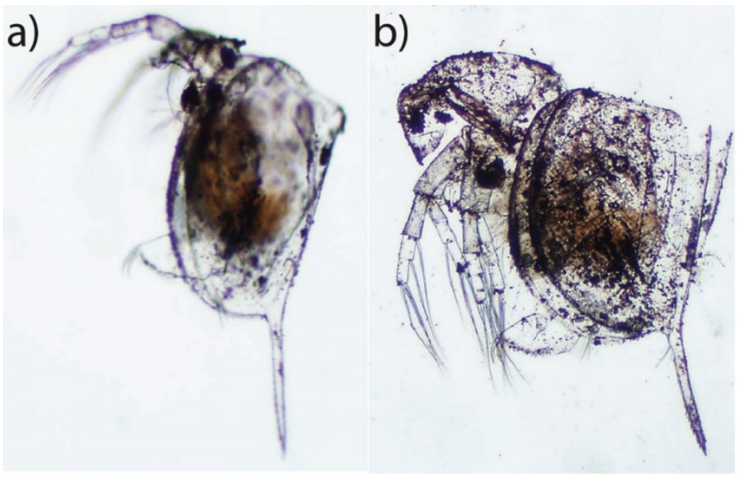

Figure 6. Effect of SAMN@FLU on *D. magna*. (a–b) Aggregates of SAMN@FLU on *D. magna* carapace after exposure to 20 mg L^{-1} SAMN@FLU (4× magnification).

4. Conclusions

Flumequine was nano-immobilized on iron oxide nanoparticles and a core-shell antimicrobial nanocarrier (SAMN@FLU) was developed and fully characterized. The SAMN@FLU hybrid showed interesting features, such as good colloidal stability, recoverability by the application of a magnetic field and bacteriostatic activity on *A. veronii* suggesting the possible application of SAMN@FLU in aquaculture. Moreover, a further added value is represented by the first evidences of the therapeutic effect of SAMN@FLU on a model aquatic organism, *D. magna*, combined to a high tolerability. Importantly, the action of the nano-immobilized drug appears correlated with the solvent exposed fluorine atom. This represents an informative insight in the view of a deeper comprehension of the mechanism of action of fluoroquinolones. Overall, the present contribution helps in promoting the ongoing evolution of drug delivery aimed at overcoming the drawbacks of the widespread use of antibiotics by developing, novel, multifunctional nanoantibiotics.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2020.111019.

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Supplementary Material

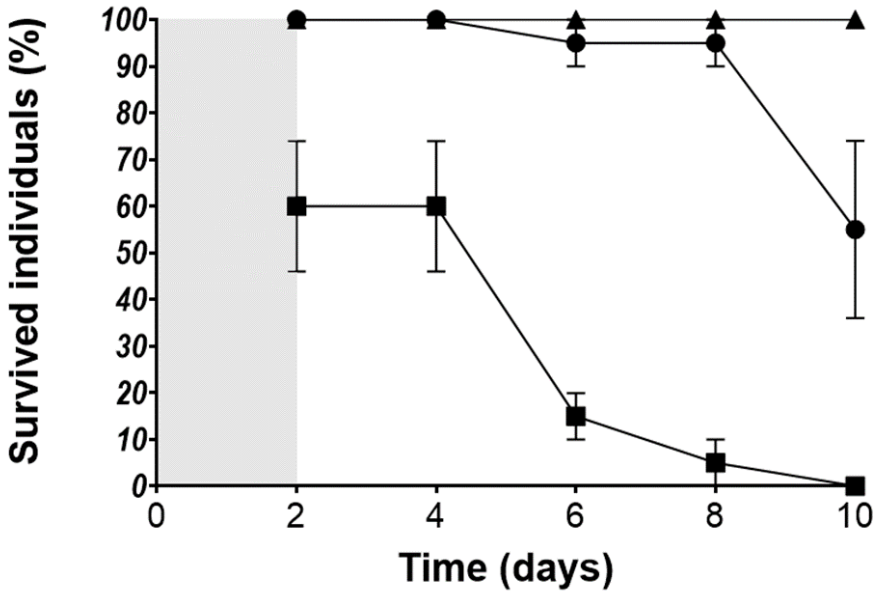

Figure S3. Fraction of survived individuals of a *Daphnia magna* clone naturally infected with *Pseudomonas spp. and Aeromonas spp*., after 48 h exposure to either pure medium (●), 24 mg/L flumequine (■) or 20 mg/L SAMN@FLU (▲); vertical error bars show standard deviation (4 vessels, each with 5 daphnids).

Chapter 2.5

In vivo experimentation related to a project aimed at studying, at the biomolecular level, the multigenerational/transgenerational toxicity of flumequine, in Daphnia magna

Data not published

Introduction

Fluoroquinolones (FQs) are antibacterials used in both human and veterinary medicine, by virtue of their efficacy and broad spectrum of action. Over the years, many studies have highlighted the ability of FQs to interact with the genetic material of eukaryotic organisms as well. For example: genotoxicity of ofloxacin (Isidori *et al.,* 2005); single-strand DNA breakdown induced by ciprofloxacin and norfloxacin (Itoh *et al.,* 2006); genotoxicity of flumequine (FLU) and its contribution to the genotoxicity of contaminated waterways and groundwaters (Zounkovà *et al.,* 2011; Ma *et al.,* 2012). More recently, Bhattacharya *et al.* (2020) showed that different FQs exert cytotoxic effects due to their ability to intercalate in DNA and to cause oxidative stress. *In vivo*, FLU has been found to act as an initiator of liver tumours in mice, due to its ability to induce DNA strand breaks (Kashida *et al.;* 2002). Furthermore, Goto *et al.* (2008) showed a significant alteration of gene expression profiles in the joint cartilages of rats treated with ofloxacin, whilst Thomé *et al.* (2012) highlighted the recombinant and mutagenic activity of ciprofloxacin and enrofloxacin (ENR) in *Drosophila melanogaster*. Lastly, with regard to plant organisms, Khadra *et al.* (2012) found genotoxic effects of ciprofloxacin and ENR on *Vicia faba*. Meanwhile, there has been a growing evidence of FQs side effects in humans, with severe and disabling consequences (Tennyson and Averch, 2017); some of these effects (renal toxicity, tendinopathies) would be explained by epigenetic alterations induced by FQs in mammalian cells (Badal *et al.,* 2015). For these antibacterials, which are widely used, persistent in the environment (Hektoen *et al.,* 1995) and non-selective, present and future concerns must also consider the environmental impact as a possible consequence of their toxicity to non-target organisms.

Tests performed a few years ago in our laboratory on the crustacean *Daphnia magna*, evidenced that both ENR and FLU show a toxicity that increases over generations, and is characterized by phenotypic alterations, reproduction inhibition and mortality. Interestingly, these effects, in the case of FLU, were also present after three generations non-exposed to the drug, thereby indicating a possible transgenerational toxicity (De Liguoro *et al.,* 2019). The persistence of phenotypic alterations evidenced stochastically in clones of *D. magna*, pointed to an epigenetic inheritance (Jablonka and Raz, 2009). *D. magna* is a model organism that shows a series of advantages for this type of study since, in controlled environmental conditions, it has a parthenogenetic reproduction cycle that allows to obtain genetically homogeneous clones, in which epigenetic effects can be studied without any confounding factor linked to genetic differences. In this species, sex determination and occasional sexual reproduction are epigenetically determined, as are many other phenotypic characters that arise in response to environmental stressors (Harris *et al.,* 2012). Epigenetic patterns in *D. magna* are influenced by environmental stressors and can be passed on to subsequent generations non-directly exposed to the substance (Jeremias *et al.,* 2018; Hearn *et al.,* 2019). Furthermore, a vast literature is available on this subject, recently supplemented by the genome sequence of this species (Lee *et al.,* 2019). Moreover, details of the genome sequence of *Daphnia pulex* revealed a higher number of genes shared with humans when compared to that of any other arthropod until then sequenced (Ebert,

2011). This genetic overlap means that, in addition to its relevance for ecotoxicological tests, daphnids could be useful model organisms for predicting the possible toxic effects in humans.

Whole genome sequencing approaches can be extremely useful in determining the potential transgenerational inheritance of epigenetic modifications. However, in an ecotoxicological context, only a very limited number of studies have evaluated DNA methylation patterns. Studies reporting contaminant-induced transgenerational epigenetic inheritance remain mainly limited to mammalian models (Mirbahai and Chipman, 2014; Shaw *et al.,* 2017); in invertebrates, a study reporting the transgenerational inheritance of DNA methylation concerns *Daphnia* exposed to gamma radiation (Trijau *et al.,* 2018).

At present, only the first part of the research project on "the transgenerational toxicity of flumequine, in *D. magna*" has been developed, namely: exposure of the first generation to two different concentrations of FLU and follow-up in pure medium of the three following generations; detection of phenotypic alterations in the various generations; collection of samples for biomolecular analyses. These analyses, which are currently being performed by other members of the research team, aim to identify the epigenetic effects and consequent modulation of gene expression induced by FLU in the freshwater model-organism *Daphnia magna*.

Materials and methods

1. Reagents

Flumequine (FLU) was supplied by Sigma-Aldrich (Milan, Italy) (CAS number 42835-25-6). A 100 mg L^{-1} stock solution was prepared in the culture medium (Rocchetta© water, dry residue 174.1 mg L^{-1}, pH 7.61) which was stored in the dark, at 4° C. Solubilization was obtained by gentle stirring, overnight, at 37° C. The two flumequine solutions (0.2 and 2 mg L^{-1}) used in the experiments were prepared by further dilution of the 100 mg L^{-1} stock solution, in the culture medium.

2. Culture conditions

The original *Daphnia magna* specimens were courteously supplied by an aquaculture farmer. From a single mother, a clone was generated which was then kept in culture for two months, until experimentation. The excellent state of health of the organisms was constantly evidenced by the low mortality (≤2% per week), the high reproduction rate (about 15 newborns per clutch) and the absence of winter eggs (*ephippia*) and/or males. The crustaceans were kept in Rocchetta© water at 20 ± 1°C; a photoperiod of 16 h light (100 lx): 8 h dark was selected. They were fed three times per week (when the medium was renewed) with the green alga *Scenedesmus dimorphus* (stock of $4x10^8$ cells mL^{-1}). The amount of feed was calibrated according to the age of the daphnids:

- Up to 4 days of age → 1.5 mL of the algal stock (100 individuals in 750 mL of medium);
- Up to the production of eggs → 2 mL of the algal stock (50 individuals in 750 mL of medium);

- After the production of eggs → 2.5 mL of the algal stock (50 individuals in 750 mL of medium).

3. Experimental design

The test was carried out on four consecutive generations of *D. magna*, named F0, F1, F2 and F3. Starting from the maintenance cultures, 300 daphnids less than 24 h old (the mothers had been isolated the day before) were collected and randomly assigned to three groups of 100 individuals. The first group (negative control) was kept in pure medium, the second group was exposed to 2 mg L^{-1} FLU and the third to 0.2 mg L^{-1} FLU. Under these conditions; in accordance to the standard Reproduction Test (OECD, 2012), all the specimens were followed for 21 days by regularly applying the conditions of number of specimens/volume of medium, amount of feed/renewal of the solutions, already used for the maintenance of cultures (see above). When the solutions were renewed, all the newborns were collected, counted and excluded from the test. However, from the newborns produced on the 21st day, 300 individuals were randomly collected, in order to set up the three groups of the next generation. Starting from F1, drug exposure was discontinued, and therefore not only the control group, but also the second (ex-FLU 2 mg L^{-1}) and the third group (ex-FLU 0.2 mg L^{-1}) were reconducted to pure medium. With the same criteria (newborns collection from the last clutch of the previous generation and maintenance for 21 days in pure medium), the experimentation continued over the F2 and F3 generations. At the end of each generation, up to 20 mothers, among those who survived, were randomly collected, fixed in 70% ethanol, photographed next to a calliper, and subsequently measured (from the edge of the eye to the base of the tail spine) using Adobe Photoshop©. A scheme of the experimental design is shown in Figure 1.

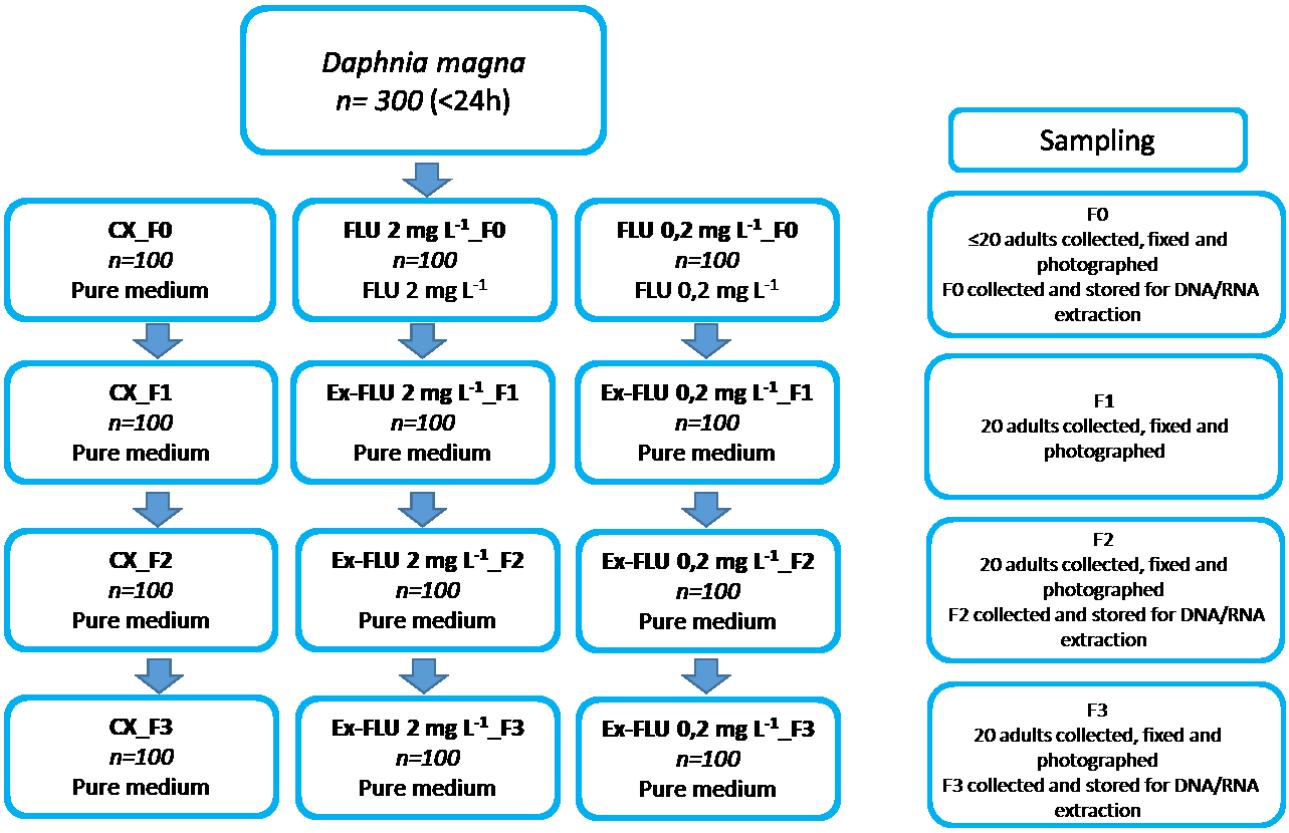

Figure 1. Experimental design (Study of multigenerational/transgenerational toxicity of flumequine, in *Daphnia magna*). CX, negative control; FLU, flumequine.

4.Statistical analysis

The average daily growth was calculated by subtracting from the mean length of each experimental group, the average length of a newborn less than 24 hours old (1 mm), and dividing the result by 21 (days of the test).

Data on daily growth and the number of newborns produced in the control group and the FLU/ex-FLU groups were analysed using the Student T-test. A P-value <0.05 was considered significant. To confirm results obtained with the Student T-test, data on reproduction were also analysed with the Kruskal–Wallis test followed by the Dunn test.

5.Preparation of samples for molecular analysis

For all groups of generations F0, F2 and F3, no feed was administered at the time of the renewal of the medium/solution on the 20th day; this was done in order to limit, as far as possible, interference due to the undigested feed in those individuals that would have been collected the following day to be used for biomolecular analysis. Once collected, these individuals were immediately placed under the stereomicroscope and, with two dissecting needles, their carapace was opened, in order to deprive them of the eggs. They were then frozen in liquid nitrogen and stored at -80° C, pending extraction of RNA and DNA.

Results

Mortality rate in the different groups (Table 1) over the various generations was always low (≤ 9%), except for the group of the F0 generation exposed to FLU 2 mg L^{-1}; in this group, indeed, the mortality rate was 46%. As a proof of the excellent state of health of the organisms enrolled in the experimentation, control groups showed a very low mortality rate (3%, on average) over the 4 generations, keeping well under the validity treshold (20%) set by the standard Reproduction Test (OECD, 2012).

Generation	Group	Mortality rate (%) *n* =100	Neonates per parent animal at the start of the test (mean)	Reproduction Inhibition (%)	Average Daily Growth (length, µm)	Daily growth inhibition (%)
F0	CX	6	42		130 ±13	
	FLU 2 mg L^{-1}	46	22	47.62*	119 ±8	0.85
	FLU 0.2 mg L^{-1}	9	41	0.23	129 ± 7	0.08
F1	CX	1	53		115 ± 10	
	Ex-FLU 2 mg L^{-1}	4	42	16.98	111 ± 7	0.35
	Ex-FLU 0.2 mg L^{-1}	2	53	0	121 ± 8	-0.90
F2	CX	2	56		116 ±7	
	Ex-FLU 2 mg L^{-1}	3	42	25.00	110± 10	0.52
	Ex-FLU 0.2 mg L^{-1}	3	47	16.07	116± 10	0
F3	CX	3	49		115 ± 11	
	Ex-FLU 2 mg L^{-1}	3	41	16.32	114 ± 10	0.09
	Ex-FLU 0.2 mg L^{-1}	2	48	0.20	116 ± 80.85	-0.09

Table 1. Mortality rate (%), mean of neonates per parent animal at the start of the test, reproduction inhibition (%), average daily growth (µm) and daily growth inhibition (%) over four *Daphnia magna* generations.

The particular set-up of the experiment, with groups of 50 individuals kept in the same vessel, did not allow to calculate the number of newborns produced by each surviving mother. Indeed, it was not possible to distinguish the newborns generated by mothers who died through the test, from those produced by mothers who survived until the end of the test. This would have been possible only by housing the 100 *Daphnia* individually, which would have implied a large waste of time, space and drug solutions. In any case, since the

mortality of the groups FLU/ex-FLU and the relative controls was very limited (≤ 9%), it can be assumed that even having the number of newborns produced only by surviving mothers, the statistical analysis would not have found any significant differences. In F0, many of the daphnia exposed to 2 mg L^{-1} of FLU showed poor development and lethargic behavior, with consequent piling-up of algae on the vessel bottom, due to a reduced feed consumption. These individuals died and therefore, their poor development, despite being evident during the test, was not detected by the measurements made at the end of the test. In all other cases, instead, the minimal percentage of mortality appeared to be completely random and unattributable to evident alterations of the phenotype, the daily growth being almost identical in all groups (Table 1).

It should be emphasized, however, that the lethargic behaviour continued to occur along the group ex-FLU 2 mg L^{-1} up to the F2 generation, though with less intensity and frequency. Another effect observed inconstantly in these groups was the poor responsiveness to light. To facilitate the collection of newborns when they were counted, a light source was used to attract them to a specific area of the vessel, exploiting the phenomenon of phototaxis (Dojmi Di Delupis, 1997). It was therefore possible to observe, occasionally, their scarce tendency to be attracted to light, compared to the newborns of the control group.

Discussion

The results reported in Table 1 show that only the FLU 2 mg L^{-1} group of the F0 generation has a statistically significant difference in reproductive capacity compared to the control. In subsequent generations this difference is no longer significant and tends to flatten over the four generations. In fact, in F3, the reproduction rate is almost identical in the various groups. This result is in disagreement with previously published data (De Liguoro *et al.,* 2019), in which a significant inhibition of reproduction (30%) and a non-random mortality (30%) was detected even in the third non-exposed generation. On the other hand, if we compare the mortality rate in F0 generations, the situation is reversed, with a 46% in the current test, compared to a 20% reported in the previous study. This more drastic selection taking place in F0, may have made it possible to obtain stronger populations, in the subsequent non-exposed generations, able to better tackle the toxic effects of the drug.

The lethargic behaviour, which was particularly observed in the ex-FLU 2 mg L^{-1} groups, could be linked to the ability of FQs to interact with receptors of the nervous system, thereby altering their functionality (Ayad, 2014). This phenomenon, for F1 generation, could be attributed to the presence of FLU in the medium of F0, leading to an inevitable perinatal exposure of the newborns; whilst, for F2 generation, the only exposure that can be considered concerns the F1 germ cells, with toxic effects that would be, also in this case, intergenerational. However, a transgenerational toxicity cannot be excluded, and this would only be confirmed by the possible finding of epigenetic alteration in F3.

Regarding the phototaxis inhibitory effects observed in F1 and F2, it is important to consider that other authors reported visual impairment in cats following the intake of FQs. The damage was due to the

accumulation, in the ocular tissue, of these photoreactive antibiotics. Indeed, FQs are able to produce reactive oxygen species (ROS) that can seriously harm the retina (Ramirez *et al.,* 2011). Similar effects were observed in the rabbit (Kumbahar *et al.,* 2014; Rampal *et al.*, 2008) and in humans (Etminan *et al.*, 2012), albeit with a lower incidence. It is therefore conceivable that this damage was also done, with a similar mechanism, to the retinula of the daphnids. For individuals belonging to F1, this could be a consequence of direct exposure to FLU during the perinatal life; this circumstance is not plausible for the F2 generation, and therefore the observed phenomenon could be a consequence of epigenetic inheritance.

Conclusions

Standard acute and chronic ecotoxicity tests on *D. magna* (OECD, 2004; OECD, 2012) have shown to have crucial limitations when used to assay drugs such as FQs, capable of expressing delayed acute toxicity (Tolosi and De Liguoro, 2021) or multigenerational/transgenerational toxicity (Dalla Bona *et al.,* 2016; De Liguoro *et al.,* 2019). In both standard tests, toxicity parameters of the FQs (EC_{50} and NOEC, respectively) are overestimated, thereby leading to an underestimation of the environmental risk. Molecular mechanisms underlying the aforementioned forms of toxicity expressed by this class of compounds may depend on their intrinsic ability to interact with enzymes that regulate DNA.

The particular design of this experiment, which used a high number of daphnids, made it possible to collect at the end of the generations F0, F2 and F3, a sufficient amount of tissues to be submitted for the extraction of DNA and RNA and subsequent biomolecular analyses. The excellent performance of the control groups (growth, survival, reproductive capacity) over the 4 generations under study, testified the particular adequacy of the culture conditions and increased the statistical power of the assay.

The results obtained so far, with the ex-FLU groups of the F3 generation responding like the control group, seem to deny the hypothesis of transgenerational toxicity. In any case, this does not avoid the possibility that genetic, epigenetic or gene expression alterations may emerge from the molecular analyses still in progress. These alterations whilst not causing damage at the phenotypic level, may still indicate the ability of FLU to interfere with the homeostasis of the nucleic acids in the eukaryotic organism *D. magna*. On this regard, it should not be overlooked that in F1 and F2, alterations of the phenotype (decreased phototaxis and lethargic behavior) were recorded in groups previously exposed to FLU. This could simply be due to perinatal exposure, of the newborns generated by F0, and of their germ cells, for F1 and F2 respectively. However, it cannot be excluded that the observed toxicity is instead a hereditary transgenerational toxicity. On this issue, the biomolecular analyses shall have the last word.

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SECTION 3: Studies on Antimicrobial Resistance

Chapter 3.1

Introduction

1. Antimicrobial resistance

Since penicillin was discovered in 1928, antibiotics changed our society and economy; in fact, previous fatal diseases became treatable diseases. Nowadays, the effectiveness of these drugs is at risk due to their excessive or inappropriate use, exerting a selective pressure and contributing to the emergence and dissemination of resistant microorganisms (Tang *et al.,* 2017). Resistant bacteria have the ability to grow after the exposure to concentrations of antimicrobials that would otherwise inhibit or kill them (Schwarz *et al.,* 2017). Without an effective action to reverse this trend, we could face a return to the pre-antibiotic era, where simple infections or routine medical procedures might pose a risk for human and animal health.

However, the phenomenon of AMR exists also in nature, as clearly shown by the presence of resistant microorganism in permafrost samples (D'Costa *et al.,* 2011) but the discovery of AMR dates 1940, when Abraham and Chain identified an enzyme capable of destroying penicillin (Abraham and Chain, 1940).

Later in 1945, Fleming stated: "The reckless person who plays with the penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism. I hope that this evil can be averted" (Fleming's speeches, 1945), highlighting immediately what the consequences of this phenomenon could have been.

In this section of the thesis, the possible contribution to the spread of AMR linked to *e.g.* the use of animal manure from intensive farms for the fertilization of agricultural soil was investigated. This agricultural practice improves soil fertility and productivity and mitigates soil degradation (Das *et al.,* 2017).

However, since for many decades antimicrobials have been widely used in livestock production, soil fertilization with manure from treated animals, can cause the spread of antimicrobial residues to the surrounding environment, contributing to the emergence of AMR and the dissemination of antimicrobial resistance genes (ARG) (Munk *et al.,* 2018; Rovira *et al.,* 2019; Xia *et al.,* 2019).

These genes can be integrated into mobile gene elements and transmitted through horizontal gene transfer (HGT), making thus bacteria that initially did not have this characteristic potentially resistant (Qiang *et al.,* 2006).

In a study reported in one of the following chapters (Chapter 3.3), we studied the impact of the application of manure from dairy cattle, chicken, and pig farms located in Northern Italy, by analysing the composition of the microbial community, the antimicrobial concentrations, and the ARGs abundance in animal waste and agricultural soil before and after fertilization (Laconi *et al.,* 2021).

2. Mechanisms of resistance

In this section, we will discuss the mechanisms of resistance against four classes of antimicrobials: fluoroquinolones, macrolides, β-lactams and polymixins.

Common mechanisms of resistance to all these classes are limiting uptake of the drug, modification of the target of the drug, inactivation of the drug, and active efflux of the drug. These mechanisms may be native to microorganisms or acquired through horizontal gene transfer. Improving the understanding of these mechanisms and the ability to detect of ARGs is likely to reduce the emergence and dissemination of AMR and increase treatment options for infectious diseases.

2.1 Resistance to fluoroquinolones (FQs)

In 1998, plasmid-mediated resistance to quinolones in clinical isolates was reported for the first time in a *Klebsiella pneumoniae* strain (Martinez-Martinez *et al.,* 1998). Mechanisms of resistance to this class of drugs, transmitted horizontally or vertically, include alteration of the target site, increased expression of efflux pumps, protection of the target site, and modification of the drug (Hooper and Jacoby, 2016). The most common mutation mechanisms that cause high resistance levels to these antimicrobials are related to the mutation in one or more chromosomal genes that encode topoisomerase II and are located in the quinolone resistance-determining region (QRDR) (Redgrave *et al.*, 2014; Rodriguez-Martinez *et al.,* 2016). The main families of genes involved in this mechanism are *gyrA, gyrB, and parC* that are transmitted vertically.

However, resistance can also be caused by plasmid-mediated quinolone resistance (PMQR) genes. The first gene characterized has been *qnr.* Nowadays, several PMQR gene families have been identified (*e.g., qnrA, qnrB, qnrC, qnrD, qnrS,* and *qnrVC* (Redgrave *et al.,* 2014; Rodriguez-Martinez *et al.*, 2016). The mechanism of action of PMQR seems to rely on the binding of qnr proteins to topoisomerases, which does not allow the binding of the fluoroquinolones to their target enzyme. Plasmids carrying the *qnrA* and *qnrB* genes are large and conjugative, while those with *qnrS* are often small and non conjugative (Tomova *et al.,* 2018).

Another PMQR gene is *aac (6 ')-Ib-cr,* which encodes a variant of aminoglycoside acetyl-transferase.

Another group of PMQR genes includes *qepA, oqxAB, and quauBIII.* These genes encode efflux systems (Redgrave *et al.*, 2014; Rodriguez-Martinez *et al.,* 2016; Yanat *et al.,* 2017; Tomova *et al.,* 2018) and appear to confer moderate resistance to fluoroquinolones.

PMQR have been detected in food, animal, human and environment samples (Zhao *et al.,* 2010; Veldam *et al.,* 2011; Yassine *et al.,* 2019; Nndadozie and Odume, 2019).

The genes conferring resistance to fluoroquinolones investigated in the following chapters are: *qnrA, qnrB, qnrS, oqxA,* and *oqxB*.

2.2 Resistance to macrolides

Mechanisms of resistance to this class of antimicrobials include ribosomal modifications, increased efflux, and inactivation of the antimicrobial (Leclercq and Courvalin, 2002).

Mechanisms of ribosomal modifications that confer resistance to macrolides can be transmitted either horizontally or vertically. Genes of the *erm* family (*erythromycin ribosomal methyltransferase*) are primarily responsible for resistance to this class of antimicrobials and are subject to horizontal transfer. These genes encode homonymous enzymes erm methyltransferases that add metal groups and prevent binding with the drug. There are more than 30 genes belonging to this family, including, in descending order, *ermA, ermB, ermC, ermF, ermX,* and *ermV* (Fyfe *et al.,* 2016).

Resistance to macrolides can also be due to membrane pumps mef and msr efflux pumps of the membrane pumps (Dinos, 2017) or to inactivation of antimicrobial caused by esterases ereA and ereB esterases.

Macrolide resistance genes that can be transmitted horizontally, especially *erm* genes, are commonly detected in fields fertilized with animal waste, wastewater, food products, and multidrug-resistant human isolates (Alexander *et al.,* 2011; Flórez *et al.,* 2014; Fyfe *et al.,* 2016; Li *et al.,* 2020; Lim *et al.,* 2012; Lima *et al.,* 2020; Tan *et al.,* 2021).

Therefore, the ARGs conferring resistance to macrolide investigated in this thesis project belong to the *erm* family (*i.e., ermA* and *ermB*).

2.3 Resistance to β-lactams

β-lactams are bactericidal agents that inhibit bacterial wall synthesis by binding to penicillin-binding proteins (PBPs), *i.e.* enzymes that lead to the formation of the peptidoglycan layer in both Gram-positive and Gram-negative bacteria (Bush and Bradford, 2016). Resistance to this class occurs through inactivation of the drugs by specific enzymes (β-lactamases), decreased binding to PBPs, reduced permeability of the bacterial cell and increased expression of efflux pumps (Van Duijkeren *et al.,* 2018). The β-lactamases constitute the most important mechanism of resistance to these drugs, especially against clinically relevant Gram-negatives (Bush and Jacoby, 2010).

These enzymes are also commonly known by their enzyme family; *e.g,* the TEM (named after the first patient) family, the SHV (sulphydryl variable) family, and the CTX (preferentially hydrolyze cefotaxime) family.

Based on their spectrum of action and sensitivity to some drugs, these enzymes can be divided into several groups:

Group I → cephalosporinases. The genes encoding these proteins are located mainly in the bacterial chromosome, even if they have also been also found at plasmidic level (*e.g., bla*$_{CMY}$) (Van Duijkeren *et al.,* 2018).

Group II → contains the highest number of β-lactamases. Genes belonging to this group investigated in the present thesis are: bla_{TEM-1}, bla_{SHV}, and bla_{CTX-M}. $bla_{OXA\text{-}type}$ genes that encode the oxacillinases protein and belong to a distinct subgroup of group 2 β-lactamases.

Group III → metallo-enzymes that hydrolyse carbapenems and other β-lactams, except monobactams. These enzymes are frequently found in *P. aeruginosa*, *Bacteroides fragilis,* and *Stenotrophomonas maltophilia.*

Group IV → enzymes that inactivate carbapenems encoded by *e.g.* bla_{VIM} and bla_{NDM} (Bush and Jacoby, 2010; Ghafourian *et al.,* 2014; Van Duijkeren *et al.,* 2018). The spread of Gram-negative bacteria producing carbapenemase and oxacillinase-48 has led to the establishment of multidrug-resistant infections that are often not treatable (Ferri *et al.,* 2017).

Another mechanism of resistance to β-lactams is conferred by the presence of altered PBPs that result in a decreased ability of the drug to bind to the bacterial cell.

The resistance genes investigated for this class of antimicrobials are: bla_{TEM-1}, bla_{SHV}, bla_{CTX-M}, bla_{CMY-2}, bla_{OXA-1}, bla_{OXA-48}, bla_{VIM-2} and bla_{NDM}.

2.4 Resistance to colistin

Colistin, or polymyxin E, is an antibiotic that binds to lipopolysaccharides (LPS), increasing the permeability of the bacterial cell and causing the lysis (Rhouma *et al.,* 2016; Van Duijkeren *et al.,* 2018).

Mechanisms of acquired resistance to colistin include modification or complete absence of LPS, overexpression of efflux pumps, overexpression of components of the polysaccharide capsule, and the overexpression of protein oprh that prevents the binding of the drug to LPS (Bialvaei and Kafil, 2015 ; Van Duijkeren *et al.,* 2018).

Until the last decade, it was believed that resistance to colistin was due only to chromosomal mutations (McEwen and Collignon, 2018); however, in 2015 a plasmid-mediated resistance gene to colistin (*mcr-1*) was discovered. Interestingly, evidence suggests possible spread from animals (Liu *et al.,* 2016).

Since then, several *mcr* genes have been identified and characterized; *mcr-2* (Xavier *et al.,* 2016), *mcr-3* (Yin *et al*., 2017), *mcr-4* (Carattoli *et al.*, 2017), *mcr -5* (Borowiak *et al.,* 2017), *mcr-6* (AbuOun *et al.,* 2017), *mcr-7* (Yang *et al.,* 2018), *mcr-8* (Wang *et al.,* 2018), *mcr-9* (Carroll *et al*., 2019) and *mcr-10* (Wang *et al.,* 2020).

In the present PhD thesis, genes from *mcr-1* to *mcr-5* have been analysed; to this end, two novel Real-Time PCR assays based on SYBR Green chemistry were developed.

In the following chapters, the presence and abundance of the aforementioned resistance genes were investigated in manure and soil samples (before and after fertilization with manure) (chapter 3.3, Laconi *et al.,* 2021). These analyses were carried out by means of Real-Time PCR assays.

Chapter 3.2 presents the work on the development of two novel Real-Time PCR assays based on SYBR Green paired with melting curves analysis for the detection and quantification of resistance genes *mcr-4* and *mcr-5.* In chapter 3.4 the optimization and validation of five Real-Time PCR SYBR green assays, from previously published end-point or probe-based PCRs, is reported (Tolosi *et al.,* 2020; Tolosi *et al.,* 2021).

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Chapter 3.2

Rapid detection and quantification of plasmid-mediated colistin resistance genes (mcr-1 to mcr-5) by real-time PCR in bacterial and environmental samples

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Abstract

Aim: The aim of the study was to validate a rapid method to detect and quantify colistin resistance genes (*mcr-1* to *mcr-5*) by real-time polymerase chain reaction (RT-PCR) in diverse matrices.

Methods and results: The detection limit of two newly designed SYBR Green real-time PCR assays for *mcr-4* and *mcr-5* and of previously published protocols for *mcr-1* to *mcr-3* was assessed using serial dilutions of reference strains. The assays could detect all five *mcr* genes with the lower limit of 10^2 copy numbers. *Escherichia coli* isolates (*n*=1062) and environmental samples (*n*=93) were tested for the presence of *mcr* genes. The assays enabled the detection of colistin resistance genes both in bacterial isolates and in complex environmental samples.

Conclusions: This method represents a set of sensitive, rapid and effective assays for the screening of colistin resistance directly from the environment.

Significance and impact of the study: Colistin is an antimicrobial commonly used in animals and has recently emerged as a last-resort treatment in humans. Plasmid-mediated *mcr* genes confer resistance to colistin and represent a major threat for public health since they can be easily disseminated through horizontal gene transfer. The rapid and sensitive detection of *mcr* genes is of utmost necessity.

Introduction

Colistin is a critically important antimicrobial and a last-resort treatment against human infections caused by multi-drug resistant Gram-negative bacteria (WHO CIA List, 2017). Currently, the clinical utility of colistin is facing a serious threat due to the emergence and dissemination of plasmid-mediated colistin resistance. The first report of a mobile colistin resistance gene was dated November 2015, and the discovered gene, identified in an *Escherichia coli* strain isolated from a pig, was named *mcr-1* (Liu *et al.*, 2016). Since then, novel variants of plasmid-mediated colistin resistance genes have been described, *mcr-2* (Xavier *et al.*, 2016), *mcr-3* (Yin *et al.*, 2017), *mcr-4* (Carattoli *et al.*, 2017), *mcr-5* (Borowiak *et al.*, 2017), *mcr-6* (AbuOun *et al.*, 2018), *mcr-7* (Yang *et al.*, 2018), *mcr-8* (Wang *et al.*, 2018), *mcr-9* (Carroll *et al.*, 2019) and *mcr-10* (Wang *et al.*, 2020). These additional *mcr* genes have been reported in different bacterial species isolated from multiple sources (*i.e*. human and livestock), raising concerns related to the emergence and spread of mobile colistin resistance. Moreover, *mcr* genes are harboured on conjugative plasmids belonging to various incompatibility groups, including IncI2 , IncX4, IncFIA, IncFII and IncHI2 (Xavier *et al.*, 2016; Yin *et al.*, 2017; Wang *et al.*, 2018; Yang *et al.*, 2018; Wang *et al.*, 2020). Although *mcr* genes have been discovered only recently, retrospective studies testing isolates dating back to 1980s identified bacteria harbouring *mcr* genes (Shen *et al.*, 2016). Of notice, most *mcr*-positives were detected in samples collected from 2009 onwards (Shen *et al.*, 2016). In the past few years, *mcr* genes have been identified in 47 different countries across six continents in human and animal associated bacteria (*i.e. Salmonella*, *E. coli*, *Pseudomonas*, etc.) (Nang *et al.*, 2019). Livestock is considered the main reservoir for *mcr* genes due to the use of colistin for prophylaxis, metaphylaxis and

therapeutic purposes (Kempf *et al.*, 2016; Liu *et al.*, 2016). More worryingly, *mcr* genes have been also identified in water sources (Petrillo *et al.*, 2016; Zurfuh *et al.*, 2016) and soil (Xia *et al.*, 2019) suggesting that these resistance determinants might be transferred from the environment to humans via food of both vegetal and animal origin (Salisbury *et al.*, 2002; Hao *et al.*, 2014).

Due to the emergence of multiple mobile colistin resistance genes in human, animal and environmental samples (Osei Sekyere, 2019), an increased demand of rapid and reliable methods for their detection is warranted. Routine susceptibility testing is considered unreliable since colistin is a large cationic molecule, diffuses poorly into media and adheres to common plastic labware (Kempf *et al.*, 2016), making the agreement of results between replicates and between laboratories difficult (Poirel *et al.*, 2017). A previous study proposed a molecular method for detection of *mcr-1* to *mcr-5* (Rebelo *et al.*, 2018); however, this approach does not allow detection and quantification in complex environmental matrices and, as a conventional method, it does not offer results in real-time. Recently, a real-time polymerase chain reaction (RT-PCR) method for *mcr-1, mcr-2* and *mcr-3* detection has been proposed (Li *et al.*, 2017). In the present study we aimed to implement this method towards the detection and quantification of *mcr-4* and *mcr-5* by using newly designed *mcr* specific RT-PCR primers. Furthermore, we optimised the *mcr-1* to *mcr-5* detection protocols and validated their application in both bacterial isolates and complex environmental samples.

Materials and methods

Reference strains

The reference strains for *mcr-1* (*E. coli* 412016126), *mcr-2* (*E. coli* KP37), *mcr-3* (*E. coli* 2013-SQ352), *mcr-4* (*E. coli* DH5a) and *mcr-5* (*Salmonella* Paratyphi B 13-SA01718) (Table 1) were kindly provided by the EU Reference Laboratory for Antimicrobial Resistance (DTU, Denmark) and were used for the optimization, the assessment of efficiency, sensitivity and specificity of the assays and as positive control when testing bacterial isolates and environmental samples. Briefly, reference strains were streaked onto nutrient agar (Microbiol, Uta, Italy) and incubated at 37±0.5°C for 20±2h. Bacterial genomic DNA (gDNA) was extracted with the Invisorb Spin Tissue Mini Kit (Invitek Molecular, Berlin, Germany) according to the manufacturer's instructions. Quality and quantity of positive controls' DNA was assessed with NanoDrop spectrophotometer (ThermoScientific, Massachusetts, United States). Based on the assessment of the limit of detection of the assay, we used 0.4 ng μl^{-1} as cut-off value to select the sample to be tested.

mcr-4 and mcr-5 RT-PCR primers design and confirmation by end-point PCR

Primer BLAST software (Jian *et al.*, 2012) was used to design the specific RT-PCR primers for *mcr-4* and *mcr-5* genes and the specificity was checked *in silico* against the NCBI database (Table 1) (Johnson *et al.*, 2008). Primer specificity was confirmed by end-point PCRs, using reference strains DH5a and 13-SA01718 harbouring *mcr-4* and *mcr-5*, respectively. To avoid false positive results, together with the *in silico*

evaluation, the assays for the detection of *mcr-4* and *mcr-5* developed in the present study were tested against DNA extracted from bacteria not harbouring the respective *mcr* target gene, including the positive reference strains for *mcr-1* (412016126), *mcr-2* (KP37) and *mcr-3* (B 13-SA01718) genes. Following this experimental setting, we also evaluated the absence of cross-reactivity among the five assays. Both end-point PCRs were performed in a final volume of 25 µL amplification mix containing 12.5 µL of DreamTaq PCR Master Mix 2X (ThermoScientific), 0.5 µL of 10 pmol mL^{-1} of each primer (Table 1), and 5 ng of gDNA as template. The thermal profile for the end-point PCR was initialised by holding 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 20 s, and 72 °C for 20 s, and a single final elongation at 72 °C for 7 min using an Applied Biosystems 2720 Thermal Cycler (ThermoScientific). The expected amplicon lengths (Table 1) were evaluated by agarose gel electrophoresis (2% agarose, 100 V, 60 min).

Optimization of RT-PCR conditions for mcr-1 to mcr-5 assays

The optimisation steps were conducted for both novel (*mcr-4*, *mcr-5*) and previously published (*mcr-1* to *mcr-3*) primers (Li *et al.*, 2017) in order to acquire a unique protocol for detection and quantification of genes. RT-PCR amplification was performed in a LightCycler®480 Roche (Basel, Switzerland), using a final volume of 10 µL. Two different reactions were carried out using either 2.5 µL of DNA template at different concentrations (2.0-0.2-0.02-0.002 ng μL^{-1}) or 2.5 µL of previous end-point PCR amplicons diluted at 0.0001 ng μL^{-1}, to assess respectively the optimal DNA concentration to analyse bacterial and environmental samples and the highest dilution to create a standard curve.

The PowerUp™ SYBR® Green Master Mix (ThermoScientific) was used together with different concentrations of each primer (*i.e.* 300/300 pmol mL^{-1}, 300/600 pmol mL^{-1}, 600/300 pmol mL^{-1}, 600/600 pmol mL^{-1}, 600/900 pmol mL^{-1}, 900/600 pmol mL^{-1} and 900/900 pmol mL^{-1} for forward and reverse primer, respectively). The cycling conditions were as follows: initial incubation at 50 °C for 2 min, followed by 2 min at 95 °C, and 45 cycles at 95 °C for 10 s and 60 °C for 40 sec. A melting curve between 40 and 95 °C was determined by adding a dissociation step after the last amplification cycle at a temperature transition rate of 4.4 °C s^{-1}. RT-PCR data analysis was performed using LightCycler®480 software version 1.5 (Roche). For each sample, the crossing point (Cp) was used to determine the amount of target gene. Specificity of RT-PCR products was determined by the analysis of amplification profiles and melting curves. Furthermore, positive RT-PCR products were purified using the SPRIselect purification kit (Beckman Coulter, Pasadena, United States) and templates were sent to Macrogen Inc. (Madrid, Spain) for direct Sanger sequencing. The visualization, analysis and editing of chromatograms were performed with FINCHTV 1.4.0 software. Standard BLAST search against the CARD (Comprehensive Antibiotic Resistance Database) (Jia *et al.*, 2017) reference sequences was performed. All RT-PCR reactions were performed in duplicate.

Efficiency, sensitivity and specificity of mcr-1 to mcr-5 RT-PCR assays

To evaluate the efficiency and the dynamic range of each primer pair, serial dilutions (1:4 and 1:5) were prepared for each DNA template. Standard curves were constructed to evaluate the efficiency and the sensitivity of *mcr* primer sets using serial dilutions of gDNA. The assays' sensitivity was further tested by pooling five *E. coli* DNA samples with different concentrations and combinations of positive and negative samples (median concentration: 11.9 ng μL^{-1}, min: 0.4 ng μL^{-1}, max: 23.4 ng μL^{-1}). Melting profile analysis was used to assess the specificity of the amplification plots.

Validation of mcr-1 to mcr-5 RT-PCR assays

To validate the assays, DNA was extracted from *E. coli* isolates and environmental samples. Samples were stored at -80°C up to DNA isolation, while extracted DNAs were stored at -20°C up to processing. A total of 1,062 *E. coli* isolated from various stages of the broiler production pyramid (*i.e.* breeders, broilers and carcasses) and assessed for the presence of extended-spectrum β-lactamase (ESBL)- and plasmid-mediated AmpC β-lactamase (pAmpC) genes (n=587 positive) (Apostolakos *et al.*, 2019) were tested. gDNA was extracted by suspending 1-2 bacterial colonies in a solution of 5% Chelex (BioRad, Hercules, United States) and boiling for 10 min. Debris in the Chelex suspension was removed by centrifugation at 12000 ×g for 5 min. Bacterial isolates were analysed by pooling five DNA samples; when a pool tested positive, each sample was analysed individually to detect the positive sample(s) within the pool. No 16S amplification of *E. coli* isolates was performed because the aim of the study was to assess the diagnostic performance of the assays, that is, their ability to identify rather than quantify *mcr* genes, and to optimize the assays with pooled isolates. DNA was extracted also from 93 environmental samples, namely agricultural soil (62 samples) and manure from dairy (11 samples), swine (10 samples) and chickens farms (10 samples), using the PowerSoil® DNA Isolation kit (Qiagen, Hilden, Germany). In each RT-PCR round, a positive (*mcr*+) and negative (*mcr*-) control for each gene was used. When testing the environmental samples, the 16S rRNA gene was used (Nadkarni *et al.*, 2002) as internal process control and to normalize the *mcr-1* to *mcr-5* gene copy numbers.

Results

Analytical performances of mcr-1 to mcr-5 RT-PCR assays

We established optimal amplification conditions for all *mcr* genes with 600 pmol mL^{-1} concentration of each primer. Assays' efficiencies ranged from 90.7% to 92.9%, and all R^2 values were >0.98 (Fig. 1). The limit of quantification copies was 13.7, 31.8, 82.4, 60.5 and 149.1 for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* assays, respectively. No primer-dimers or non-specific peaks were observed in any of the assays. In detail, no increase in fluorescence associated with a sigmoidal amplification curve was observed for none of the template controls or when *mcr* primer pairs were used against non-target *mcr* genes. Pooling of bacterial

DNA enabled fast screening of multiple samples in a single assay, since detection of positive controls was possible in all tested concentrations and combinations.

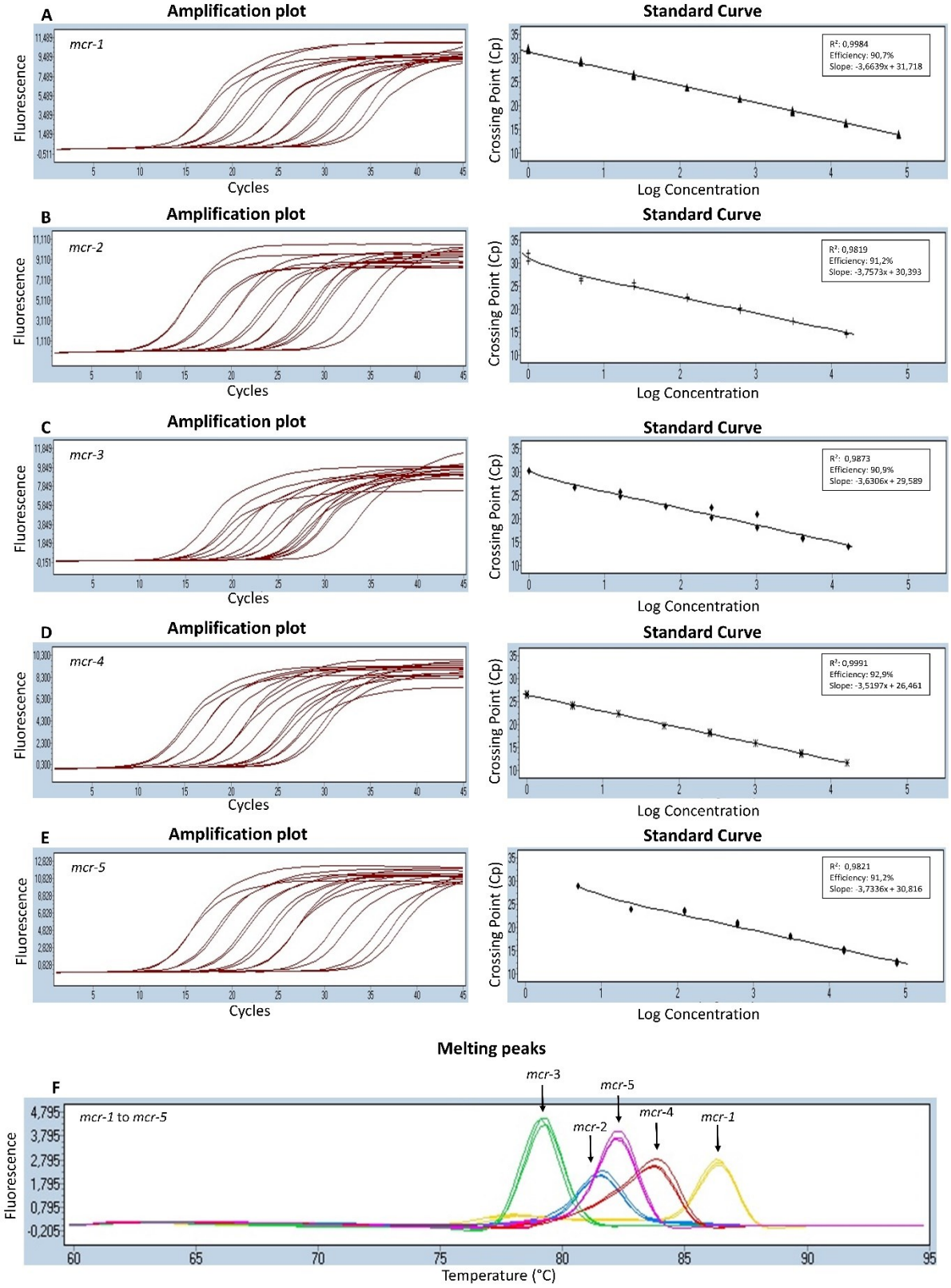

Figure 1. Real-time PCR amplification curves, standard curves and melting curves. (A-E) show real-time PCR amplification and standard curves for *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5. For each standard curve R^2, efficiency and slope are reported. (F) shows melting curves for each *mcr* gene.

SYBR Green RT-PCR followed by melting curve analysis enables the detection of *mcr-1* to *mcr-5* from bacterial isolates and environmental samples.

Two different matrices were screened in this study: bacterial (*i.e*. *E. coli* cultures) and complex environmental (*i.e.* manure and soil) samples. Starting from 1062 pure cultures, seven samples were found positive for *mcr* genes. In particular, two samples were positive for *mcr-3* (one breeder, one broiler), one for *mcr-4* (breeder) and four for *mcr-5* (broilers). Three of the *mcr*-carrying isolates were also harbouring ESBL/pAmpC genes. Out of 93 environmental samples, 43 (46.2%) were positive for *mcr* genes; 20 (eight manure, twelve soil) for *mcr-1*, three (manure) for *mcr-2*, eleven (seven manure, four soil) for *mcr-3*, four (three manure, one soil) for *mcr-4* and five (two manure, three soil) for *mcr-5* (Fig. 2). The RT-PCR assays showed that copies number in the environmental samples for *mcr-1*, *mcr-3* and *mcr-5* ranged from below the limit of quantification to 21.0, 2072.68 and 30.6 copies, respectively. The copies number for *mcr-2* and *mcr-4* was below the limit of quantification in all positive samples.

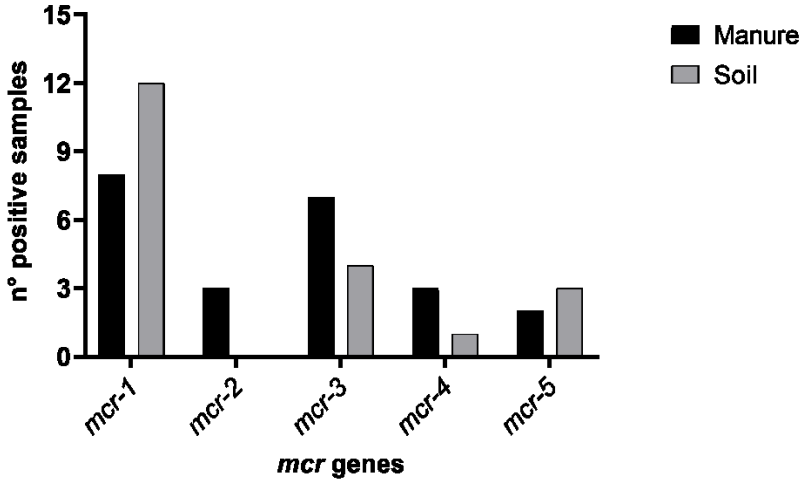

Figure 2. Detection of *mcr* genes in soil and manure samples.

Discussion

The aim of this study was to develop a fast, sensitive and reliable method for detection and quantification of colistin mobile resistance genes (*mcr-1* to *mcr-5*). We extended and optimized the method of Li *et al.* (2017) by constructing two novel assays for *mcr-4* and *mcr-5*. The method, based on a SYBR Green RT-PCR paired with melting curve analysis, enables the rapid and confident detection and quantification of *mcr-1* to *mcr-5* genes in bacterial isolates and environmental samples.

In recent years, end-point PCR, real-time PCR, loop-mediated isothermal amplification and microrray-based methods have been developed to detect colistin resistance genes. Although these methods proved to be sensitive and effective, they present some limitations, such as detection of only one to three *mcr*-genes (Chabou *et al.*, 2016; Irrgang *et al.*, 2016; Xavier *et al.*, 2016; Bernasconi *et al.*, 2017; Donà *et al.*, 2017; Li *et al.*, 2017) and/or requirement of specific and expensive equipment (Bernasconi *et al.*, 2017; Zhong *et al.*,

2019). A multiplex PCR for the detection of *mcr-1* to *mcr-5* has been also recently developed (Rebelo *et al.*, 2018); however, this method is a standard end-point PCR, hence it is time consuming as it has to rely on gel electrophoresis to visualize the results of the amplification and more prone to contamination. By comparison, SYBR Green-based assays are faster and less expensive, making our set of assays an ideal tool for *mcr-1 to mcr-5* genes detection, in particular when testing large number of samples including complex matrices such as those from the environment. The set of assays described here supports samples pooling, allowing the screening of five individual samples in a single reaction tube, further decreasing time and cost of the analyses. Due to its cost-effectiveness, our method might also have wide employment in developing countries, where financial limitations represent a huge issue toward antimicrobial resistance gene surveillance studies. The method proposed here is not itself deprived of limitations, as it cannot detect all five *mcr* genes in a single reaction, unlike the multiplex PCR developed by Rebelo et al. (Rebelo *et al.*, 2018), nor emerging *mcr* genes. However, SYBR Green RT-PCRs are relatively easy to implement, and the detection of emerging *mcr* genes might be easily achieved via designing new specific primer pairs.

The detection and quantification of colistin resistance genes are crucial because they are considered an emerging risk for public health (Poirel *et al.*, 2017), since colistin is employed as a last-resort antimicrobial for the treatment of multidrug-resistance Gram-negative bacterial infection in humans. Non-prudent use of colistin in human medicine but also in farming settings contributed to the spread and persistence of resistance genes in the natural environment. The simultaneous presence of *mcr* and ESBL/pAmpC genes in three *E. coli* isolates is notable given the importance of third generation cephalosporins and colistin in human health.

The novel set of RT-PCR primers and the optimised *mcr-1* to *mcr-5* assays developed in the present study represent a useful method for future surveillance studies for determination of *mcr* genes prevalence in human, animal and environmental samples in a fast, sensitive and reproducible way.

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Conflict of interest

None to declare.

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Table 1. Primers and positive controls used for the detection of *mcr-1* to *mcr-5* and 16S genes in RT-PCR assays.

Primer name	Target gene	Sequence (5' - 3 ')	Product length (bp)	Reference	Positive control strains	
					Strain	Origin
mcr1-qf	*mcr-1*	AAAGACGCGGTACAAGCAAC	213	(Li *et al.*, 2017)	*E. coli* 412016126	Poultry
mcr1-qr		GCTGAACATACACGGCACAG				
mcr2-qf	*mcr-2*	CGACCAAGCCGAGTCTAAGG	92	(Li *et al.*, 2017)	*E. coli* KP37	Swine faeces
mcr2-qr		CAACTGCGACCAACACACTT				
mcr3-qf	*mcr-3*	ACCTCCAGCGTGAGATTGTTCCA	169	(Li *et al.*, 2017)	*E. coli* 2013-SQ352	Sewage
mcr3-qr		GCGGTTTCACCAACGACCAGAA				
mcr4-qf	*mcr-4*	AGAATGCCAGTCGTAACCCG	230	This study	*E. coli* DH5a	Swine faeces
mcr4-qr		GCGAGGATCATAGTCTGCCC				
mcr 5-qf	*mcr-5*	CTGTGGCCAGTCATGGATGT	98	This study	*Salmonella* Paratyphi B 13-SA01718	Poultry
mcr 5-qr		CGAATGCCCGAGATGACGTA				
16S-qf	16S	TCCTACGGGAGGCAGCAGT	467	(Nadkarni *et al.*, 2002)		
16S-qr		GGACTACCAGGGTATCTAATCCTGTT				

Chapter 3.3

Microbial community composition and antimicrobial resistance in agricultural soils fertilized with livestock manure from conventional farming in Northern Italy

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Graphical Abstract

Keywords: Antimicrobials, Antimicrobial resistance, Microbiome, Manure application, Agricultural soil, Conventional animal farming

Highlights

- Manure application does not strongly influence soil microbiome.
- Fertilization enriches the abundance of some ARGs (i.e. *ermA, ermB,* bla_{OXA-1} and *oqxA*) harboured in agricultural soil.
- Manure-derived ARGs in soil display different dissipation patterns.
- Flumequine residues correlate with *oqxA* and *qnrS* abundances.
- Higher ARG abundances are present in swine vs. dairy and poultry manure.

Abstract

Antimicrobials are commonly used in conventional livestock production and manure is widely applied to agricultural lands as fertilizer. This practice raises questions regarding the effects of fertilization on (i) soil microbiota composition and (ii) spread of antimicrobials and antimicrobial resistance (AMR) in the environment. This study was conducted in a high-density farming area of Northern Italy and aimed at assessing the impact of (dairy cattle, chickens and swine) manure application on soil microbiome, antimicrobial concentrations and antimicrobial resistance gene (ARG) abundance. We found the microbial community composition in manure to be different and less diverse than in soil, with manure application altering only marginally the soil microbiome. Exceptions were the phyla Firmicutes, Tenericutes and Cloacimonetes, which significantly enriched in fertilized soil. Of the antimicrobials investigated, only flumequine concentrations increased after manure application, albeit non-significantly. ARGs were more abundant in manure, with *ermA*, *ermB,* bla_{OXA-1} and *oqxA* being significantly enriched in fertilized soil. Positive correlations between *oqxA* and *qnrS* abundances and flumequine concentrations were observed, together with the co-occurrence of some ARGs and microbial taxa (*e.g*. *oqxA* correlated with Acidobacteria and Gemmatimonadetes). This study showed that manure application has little effect on soil microbiome but may contribute to the dissemination of specific ARGs into the environment. Moreover, flumequine residues seem to enhance the emergence of *oqxA* and *qnrS* in soil.

1. Introduction

Manure derived from conventional livestock production systems is commonly used in agriculture to improve soil quality and, as organic fertilizer, to provide nitrogen enrichment and increase organic matter (Das *et al.,* 2017). However, microbial communities in manure may influence soil microbiome, either directly through competition or indirectly by spreading antimicrobial resistance (AMR). The extent to which the manure microbiome influences the soil microbial community remains unclear. Although some studies have found that organic manure application significantly alters the soil microbiome (Stocker *et al.,* 2015; Zhang *et al.,* 2020), other studies have reported changes limited to a few taxa, whereas the main microbial composition of the soil remains unmodified (Lopatto *et al.*, 2019). Antimicrobial drugs have been widely used for several decades in conventional livestock production. While some of these antimicrobials are scarcely metabolized in the animal body and eliminated as such, others are metabolized and excreted as active or inactive metabolites in urine and faeces (Wei *et al.,* 2011; Xia *et al.,* 2019a). After soil fertilization, antimicrobial residues may spread into the surrounding environment, potentially inducing the emergence of resistant bacteria and antimicrobial resistance genes (ARGs) (Hou *et al.,* 2015; Munk *et al.,* 2018; Qiao *et al.,* 2018; Rovira *et al.*, 2019; Xia *et al.,* 2019a). The dissemination of ARGs in the environment represents a great concern for public health, since they can be integrated into mobilizable genetic elements, such as plasmids or transposons, and propagated via horizontal gene transfer (HGT) among bacteria, including pathogenic and

non-pathogenic ones (Qiang *et al.,* 2006). Indeed, ARGs disseminated into the environment have the potential to be transferred to humans, via dispersion into waterways, through runoff and drainage from the soil, or by entering the food chain (Berendonk *et al.,* 2015; Hruby *et al.*, 2016; Marti *et al.*, 2013; Pruden *et al.,* 2012).

In the present study, we investigated for the first time the impact of the application of manure from three different livestock sectors, namely dairy cattle, chickens and swine, located in a high-density farming area of Northernern Italy on the microbial community composition, antimicrobial concentrations and ARG abundances in agricultural soil. The main purpose of the study was then to improve our understanding of the impact of fertilization with manure from conventional livestock farms on soil microbiome and AMR spread into the environment, as well as the correlation between antimicrobial concentrations and ARG abundance. Furthermore, we aimed to assess whether the different livestock sectors differed in their microbial communities, in the concentration of antimicrobial residues and in ARG abundance.

2. Materials and Methods

2.1 Sampling procedure

Samples were collected from 31 conventional farms (10 chicken farms, 10 swine farms, and 11 dairy cattle farms) located in two regions in Northern Italy from October 2017 to March 2019. Farms were chosen because of their location in a high-density farming area and because of the application of an integrated agricultural system, meaning that the manure produced by each farm was used to fertilize the surrounding farmland. In each farm, three samples were collected from: 1) manure or slurry (one sample), 2) soil (one sample before fertilization with manure/slurry and one sample one month after fertilization with manure/slurry), accounting for a total of 93 samples (*i.e*. 3 samples x 31 farms). Details of the sampled farms are summarized in Supplementary material 1.

Manure/slurry samples were collected according to Kumari *et al.* (2015). Briefly, manure samples were collected by taking 1 kg of 10 manure cores (1.5 cm in diameter and 12 cm deep) at randomly selected locations and then pooled; slurry samples were collected by taking 1 L of slurry from five different points at 1 m depth from the surface of the storage tank. Soil samples were collected according to Dong *et al.* (2014) by sampling in the low layer (0-20 cm) and using an auger with a 5 cm internal diameter at five randomly selected locations and then pooled.

2.2 Quantification of antimicrobials

The analytical method was set up to detect and quantify 14 different antimicrobials: amoxicillin, ampicillin, cefquinome, ceftiofur, ciprofloxacin, danofloxacin, enrofloxacin, flumequine, marbofloxacin, erythromycin, spiramycin, tilmicosin, tylosin and colistin. The 14 antimicrobials were chosen as representing some of the most commonly used antimicrobials in animal farming and/or because listed as critically important

antimicrobials by the World Health Organisation (2019). Soil and manure (dairy cattle and chickens) or slurry (swine) sample purification was performed as previously described (Chiesa *et al.,* 2018), with some modifications. An aliquot (1 g weight) of mixed soil or manure/slurry from dairy cattle, chickens or swine, spiked with IS (enrofloxacin d5) at a final 50 ng/g, 100 µl of 20% TCA for protein precipitation and 10 ml McIlvaine buffer (pH 4.0), were combined. Samples were vortexed and sonicated for 10 minutes (min). After centrifugation at 2500g, 4°C for 5 min, the supernatant was transferred to a clean falcon tube and defatted with 10 ml n-hexane, then vortexed and centrifuged (at the previously reported conditions) to discard the n-hexane layer. Solid phase extraction Oasis HLB cartridges was used to purify the obtained extracts; then the eluate was dried and reconstituted as reported by Chiesa *et al.* (2018). Chromatographic separation was obtained with the same gradient elution for all compounds except for colistin, as in Chiesa *et al.* (2018). For colistin, the elution started with 90% A (aqueous formic acid 0.1%; B methanol), maintained for 5 min, followed by a decrease to 10% A at 6 min, and maintained till 11 min. Subsequently, the mobile phase was gradually increased back to 90% A at 12 min and then held constantly until 17 min.

Mass spectrometric (MS) analysis was performed on a triple-quadrupole TSQ Quantum MS (Thermo Fisher Scientific, Massachusetts, U.S.A.) equipped with an electrospray interface (ESI) set in the positive (ESI+) electrospray ionization mode for all analytes (Chiesa *et al,* 2018). The selected diagnostic ions, one of which was chosen for the quantitation, and the collision energies are reported in supplementary material 2. Acquisition data were recorded and elaborated using Xcalibur software from Thermo Fisher Scientific.

After the identification of samples in which the absence of antimicrobials was detected, through a preliminary screening of soil or manure and slurry samples, the method was validated according to the Commission Decision 2002/657/EC criteria (European commission, 2002). For the validation procedure on manure, samples of manure/slurry from dairy cattle, swine and chickens were mixed to obtain pooled manure samples.

For each analyte, the method performance was evaluated by the determination of retention time (RT), transition ion ratios, recovery, accuracy (trueness), precision (expressed as the intra- and inter-day repeatability), linearity, as well as the decision limit (CCα) and detection capability (CCβ), which were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008). The detailed procedure is reported in Chiesa *et al.* (2018). All the results of the method validation for all the compounds are reported in supplementary material 3.

2.3 DNA extraction

Twenty-five grams (manure or soil) or 25 ml (slurry) of sample were placed in a sterile Filtra-bag (280 µm pore size), added with 25 ml of Phosphate Buffered Saline (PBS) and mixed by hands for 1 min. The filtered liquid was centrifuged at 4000 rpm for 10 min at 4°C; DNA was extracted from 250 mg of the resulting pellet using DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA quality

and quantity were assessed using a UV-Vis spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, United States).

2.4 16S rRNA gene amplification, sequencing, and data analysis

To evaluate differences in bacterial communities among manure/slurry, fertilized and unfertilized soils, the V3-V4 regions of the 16SrRNA gene were amplified with primers 341F/R806 (Takahashi *et al.,* 2014) modified with overhangs to add index adapters using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). PCRs were carried out in a 2720 thermal cycler (Applied Biosystems, Waltham, MA) with 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds, followed by a final extension for 7 minutes at 72°C. PCR products were purified using the SPRIselect purification kit (Beckman Coulter, Brea, CA), and barcodes introduced via a second PCR using platform-specific barcode-bearing primers (Milan *et al.,* 2018). Following a second purification, libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen, Life Technologies, Monza, Italy) and pooled. Pooled DNA concentration and integrity were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), and then sequenced using the Illumina MiSeq sequencing platform (San Diego, California, USA) with a 2×300 bp paired-end approach.

DADA2 package within the Quantitative Insights into Microbial Ecology 2 (QIIME2 version 2019.4) software was used for 16S rRNA data analysis (Bolyen *et al.,* 2019; Callahan *et al.*, 2016). To assign taxonomy categories, a Naive Bayes classifier and the q2-feature-classifier plugin were used. Taxa assignment was carried out using SILVA- Naive Bayes sklearn trained database (Yilmaz *et al.,* 2014). The raw sequence reads have been deposited in the NCBI Short Read Archive under the accession number PRJNA600160. The on-line based software Calypso (http://cgenome.net/wiki/index.php/Calypso) was used for microbial community characterization and α- and β-diversity statistics (Zakrzewski *et al.*, 2017). Default parameters were employed for data filtering. Total sum normalization (TSS) and SquareRoot data transformation were used. The microbial community composition was visualized using heatmap and network analyses. To quantify the microbiome diversity within each sample group, Shannon index and Chao1 methods were employed. To assess the overall differences in microbial community composition among type of sample and livestock sectors, principal coordinate analysis (PCoA) plots and non-metric multidimensional scaling (NMDS) were used for visualization, and permutational multivariable analysis of variance (PERMANOVA) based on the Bray-Curtis dissimilar measure for significance testing using the Adonis function.

2.5 Quantitative PCR (qPCR) analysis of antimicrobial resistance genes (ARGs)

The detection of ARGs to antimicrobials commonly used in conventional farming was carried out by quantitative polymerase chain reaction (qPCR). Gene-specific qPCRs paired with melting curve analysis were employed for detecting the following ARGs: *ermA, ermB, oqxA, oqxB, qnrS, qnrA, qnrB, mcr-1, mcr-2, mcr-3, mcr-4, mcr-5,* bla_{TEM-1}*,* bla_{SHV}*,* $bla_{CTX-M-1like}$*,* bla_{CMY-2}*,* bla_{OXA-1}*,* bla_{OXA-48}*,* bla_{VIM-2} *and* bla_{NDM}. All qPCRs were

performed in a final volume of 10 µL using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) with optimal concentration of each primer (600/600nM) in a LightCycler®480 Roche (Roche, Basel, Switzerland) real-time platform. ARG-specific primers and reference strains used as positive control are summarized in supplementary material 4. Cycling conditions were as follows: initial incubation at 50 °C for 2 min, followed by 2 min at 95°C, and 45 cycles at 95°C for 10 sec and 50-60°C for 40 sec. Melting curves between 40 and 95°C were determined by adding a dissociation step after the last amplification cycle at a temperature transition rate of 4.4°C/sec. The absolute abundance of ARGs in samples was calculated based on standard curves obtained using serial dilutions of previous end-point PCR amplicons of target DNA isolated from the reference strains. However, the absolute abundance of ARGs in a given sample is not a significant value, as it is proportional to the total DNA present in the sample; hence, ARG relative abundance was calculated, by normalizing the ARG copy number to 16S rRNA gene copy, and used in the statistical analysis.

2.6 Statistical analysis

Differences in ARG occurrence (presence/absence) over types of samples (manure/slurry, soil before fertilization, or soil 1 month after fertilization) and livestock farms (*i.e.* dairy cattle, swine or chickens) were tested using generalized linear models (GLMs) with a logit link function, binomial error distribution and a cluster-robust sandwich variance estimator to account for clustering of samples collected from the same farms, while differences in ARG abundance were compared using Kruskal-Wallis test with Dunn's test for multiple comparisons. Differences in antimicrobial residues over livestock farms and types of samples were tested using Tobit regression on log-transformed residue concentrations with the left-censoring limit set at the minimum detection threshold. To assess the association between antimicrobial residues concentration and ARG relative abundance over sample type, Spearman rank correlation analysis was performed. To assess the association between the relative abundance of microbial taxa at phylum and family levels with the ARGs, multivariate regression analysis with several dependent variables (*i.e*. log-transformed relative abundances of the microbial taxa) was used to jointly regress on the same independent variables (*i.e.* presence/absence of the different ARGs), while adjusting for livestock farm and type of sample, and accounting for clustering of observations at the farm level (cluster variable) using bias-corrected and accelerated cluster-bootstrapped standard errors (1000 replications). Since manure/slurry were considered as a unique statistical unit, hereafter we will use the term manure to refer to manure/slurry samples. Statistical analysis and data visualization were carried out in R (version 3.6.3) (https://www.r-project.org/).

3. Results

3.1 Prevalence and quantification of antimicrobials

Out of 93 samples, only 39 were found positive to at least one antimicrobial. Flumequine was found in 38 samples, although in eight it was detected as traces (<CCα). Of the 14 antimicrobials investigated, six were

detected in at least one sample; flumequine was the most detected (38/93; 40.86% of the total samples), followed by enrofloxacin (7/93; 7.53%), tylosin (3/93; 3.22%), and marbofloxacin, ampicillin and ciprofloxacin (2/93; 2.15%) at a much lower prevalence. Flumequine and enrofloxacin were the only compounds identified also in soil, while the remainders were detected only in manure. The prevalence of antimicrobials in swine and chicken farms was similar (22 *vs*. 16 samples, respectively). However, flumequine was more prevalent in swine samples (73.33%) than in chicken samples (53.33%), although the difference was not significant ($p>0.05$). Marbofloxacin was detected only in swine, ciprofloxacin only in chickens and swine, and ampicillin in one sample of chicken and dairy cattle farms, respectively. Antimicrobial concentrations ranged from below the decision limit (CCα) to 437.38 ng/g (flumequine). The average concentration of flumequine in manure was 24.79 ng/g, while in soil before and after fertilization was 77.77 ng/g and 109.36 ng/g, respectively (Fig. 1A). However, the difference between soil and manure samples and between fertilized and unfertilized soil was not significant ($p>0.05$). Considering the livestock sector, flumequine concentrations were not different in swine and chicken samples, but these were significantly higher ($p < 0.001$) in comparison to samples from dairy cattle, in which it was never detected (Fig. 1B).

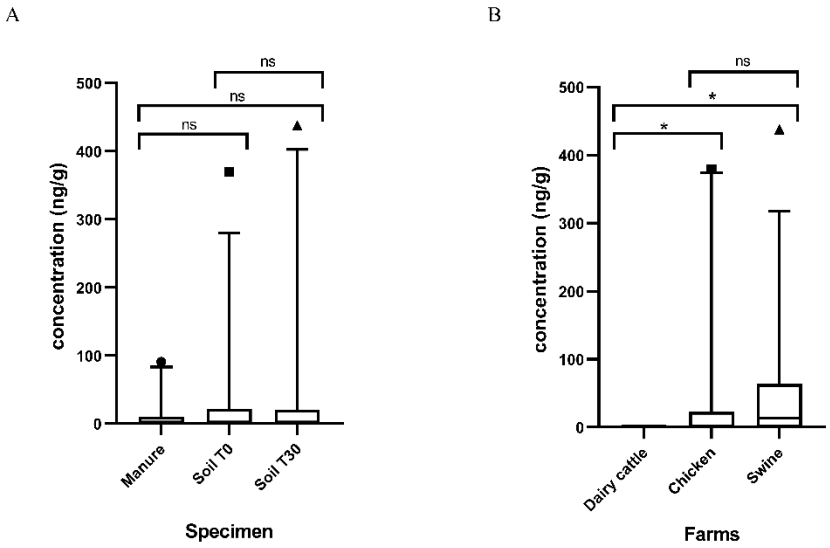

Figure 1. Concentration of flumequine in A) manure and soil samples and in B) dairy cattle, chicken and swine samples. $p < 0.05$ shows as *. Non-significant differences are indicated by ns.

3.2 General description of DNA sequences

After the quality-filter step, removal of chimeric fragments and reads merging, a total of 2,851,500 reads were obtained with 35,921 different features, with an average of 30,661 sequences per individual sample. Filtering by quality, three samples were excluded and 90 were considered in the downstream analyses. The

rarefaction curves for samples plateaued, indicating that the obtained sequencing depth was good (supplementary material 5).

3.3 Composition of bacterial communities

Using 16S rRNA gene sequencing, the microbial community structure of samples was characterized. At phylum level, members of the Firmicutes and Bacteroidetes dominated the community composition of manure samples. While Bacteroidetes were dominant also in soil samples, Firmicutes were significantly ($p<0.05$) less abundant. On the contrary, Acidobacteria and Proteobacteria were significantly more abundant ($p < 0.05$) in soil than in manure and, as a result, the heatmap at phylum level shows two main clusters, one grouping manure samples and one grouping soil samples (Fig. 2). Network analysis at operational taxonomy unit (OTU) level based on the 3000 more abundant OTUs corroborated this finding, showing a marked separation between manure and soil samples (Fig. 3). Despite this clear separation when considering the whole bacterial community, significant ($p < 0.05$) changes in specific phyla (*i.e.* Cloacimonetes, Firmicutes and Tenericutes) after fertilization were observed. Within manure samples, while Firmicutes were highly abundant in all farm types, Proteobacteria and Actinobacteria phyla were more abundant in chicken manure samples, while a higher percentage of Bacteroidetes was identified in dairy cattle and swine. As a result, two distinct clusters within manure samples were observed, one containing chicken manure samples and one containing dairy cattle and swine samples (Fig. 2).

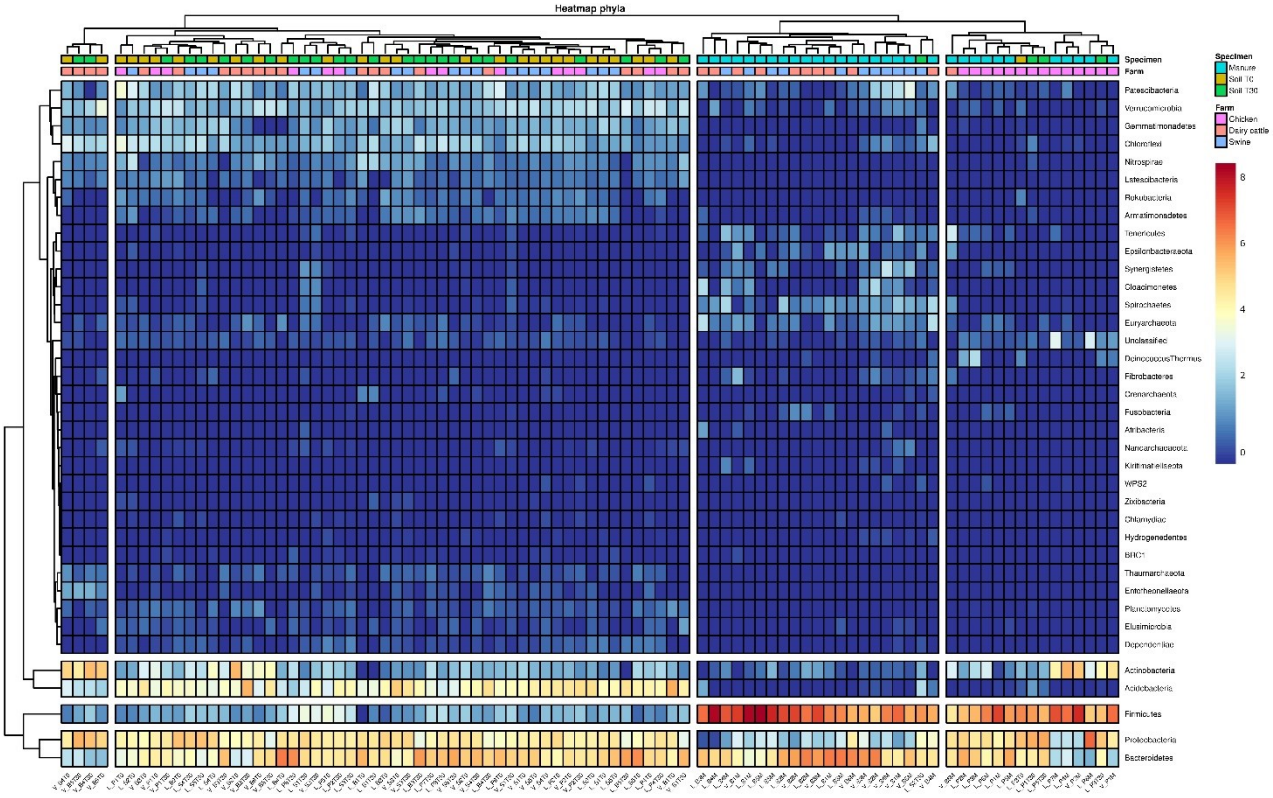

Figure 2. Heatmap representing the microbial community composition of manure and soil samples at phylum level.

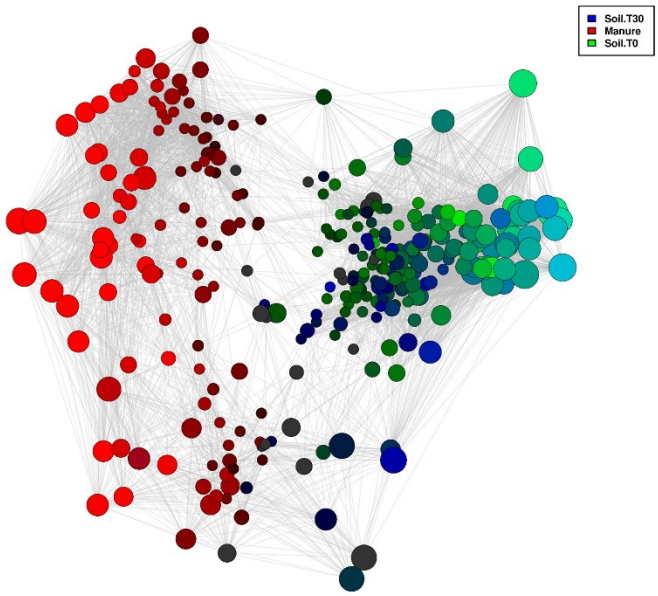

Figure 3. Network analysis representing the microbial composition of manure and soil samples at OTU level. The 3000 most abundant OTUs were considered to generate the network.

3.4 Bacterial community α-diversity

The α-diversity, *i.e.* the diversity within each sample type, was evaluated at OTU level using two methods, the Shannon index and Chao1 (Fig. 4). Both measurements showed a significantly ($p < 0.05$) lower α-diversity in manure samples in comparison to soil samples, both pre- and post-fertilization. On the contrary, the α-diversity was comparable between soil samples before and after manure application. When considering the diversity within samples belonging to the same livestock sector, dairy cattle farms were characterized by the lowest α-diversity, while swine showed the highest α-diversity. The difference in α-diversity between farm type samples was highly significant ($p < 0.001$).

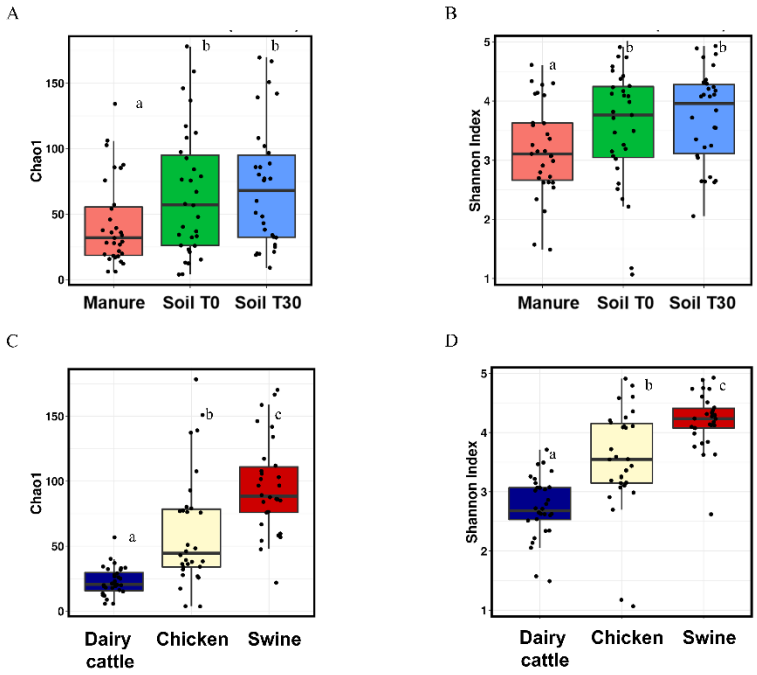

Figure 4. α-diversity within manure and soil samples (A and B) and within dairy cattle, chicken and swine sectors (C and D). Boxplots represent 25th to 75th percentiles and whiskers showing a maximum of 1.5x the interquartile range (IQR), and different letters indicate significant differences within the α-diversity indexes ($P < 0.05$).

3.5 Comparison among bacterial communities (β-diversity)

To compare the bacterial communities between sample types (β-diversity) at OTU level, PCoA and NMDS ordination together with PERMANOVA were used. This analysis showed that the microbial community in manure was significantly different ($p < 0.001$) from that of soil before and after fertilization, whereas there was no significant difference between fertilized and unfertilized soil. This finding was supported by PCoA and NMDS graphs (Fig. 5A, B), showing a marked difference in bacterial communities composition between manure and soil and the clustering of soil samples before and after fertilization. Pairwise PERMANOVA also showed that differences in microbial community composition between farm types were highly significant ($p<0.001$), and PCoA and NMDS graphs confirmed these observations (Fig. 5C, D).

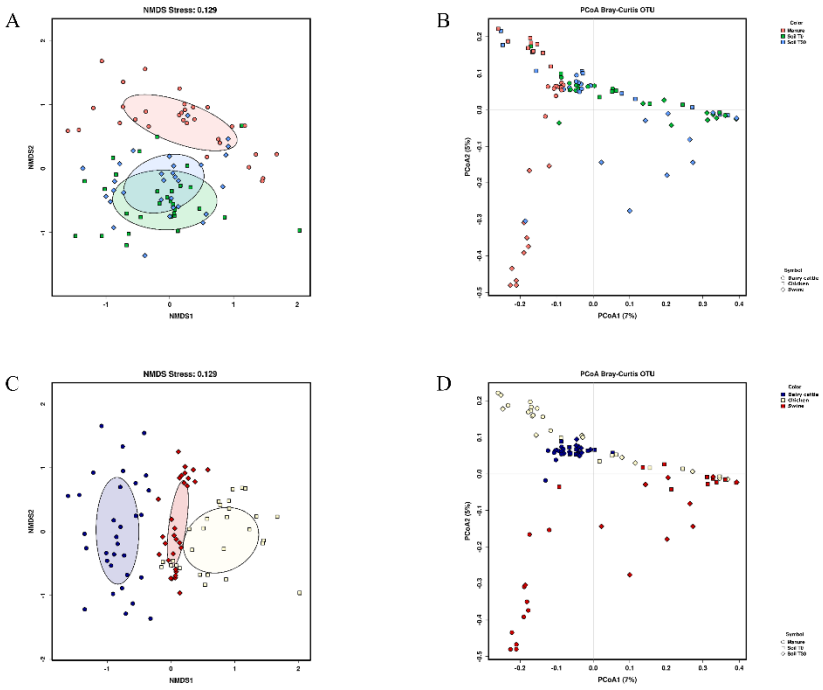

Figure 5. β-diversity between manure and soil samples (A and B) and between dairy cattle, chicken and swine samples (C and D). In both NDMS and PCoA analysis, samples are clustered according to Bray-Curtis distances.

3.6 Prevalence and relative abundance of ARGs

Besides microbial community characterization, the prevalence and abundance of ARGs in manure and soil samples was investigated to assess whether manure application enriched ARG abundance and diversity in the soil. Of the 20 ARGs investigated, all but *qnrA* and bla_{NDM} genes, were detected in at least one sample. Bla_{TEM-1} (89.25%) and *ermB* (81.72%) were the most prevalent ARGs, followed by *ermA* (65.59%), bla_{CMY-2} (58.06%) and bla_{OXA-1} (45.16%), while the prevalence of the other genes ranged from 30.11% ($bla_{CTX-M-1LIKE}$) to 3.23% (*mcr-2*) (Supplementary material 6). *Mcr-2* was detected only in manure samples, while bla_{VIM-2} was present only in soil. No target genes emerged in soil after manure application (Supplementary material 6). A significant increase in the number of ARG positive soil samples after fertilization was observed for *ermA*, *ermB*, *qnrS*, bla_{CMY-2} and bla_{OXA-1} (Fig. 6A). When considering the different livestock sectors, only swine samples were positive for *mcr-2*, whereas no bla_{OXA-48} and bla_{SHV} were detected in samples from this sector. The total abundance of ARGs normalized to 16S rRNA for each sample ranged from 1.24×10^{-14} to 0.06 ARG copy per million copy of 16S rRNA. Soil samples before manure application showed lower total ARG abundance in comparison to soil after fertilization, possibly reflecting the legacy of manure application, as manure samples had the highest total abundance of the target genes (Fig. 6B). Target genes did not show the same abundance patterns, and the relative abundance of some genes varied between sample categories; *ermA*, *ermB,* bla_{OXA-1}, and *oqxA* had similar dynamics, being significantly enriched ($p < 0.05$) in soil after manure application (Fig. 6C, D, E and F), while $bla_{CTX-M-1LIKE}$, bla_{SHV}, and bla_{TEM-1} were significantly more

abundant in manure than soil ($p < 0.05$), but their relative abundance decreased after fertilization (Fig. 6G, H, and J). *QnrS* was significantly more abundant in manure than in untreated soil ($p < 0.001$), with trends towards enrichment after fertilization ($p = 0.048$) (Fig. 6I). The remaining target genes showed a low relative abundance and no differences between sample categories were observed. Comparisons between livestock sectors showed higher numbers of target gene copies in swine samples, whereas dairy cattle samples had the lowest abundance (Fig. 6B). Specifically, bla_{OXA-1}, *ermB*, *mcr-1* and *qnrS* were significantly ($p < 0.05$) more abundant in swine samples than the other two sectors (Fig. 7).

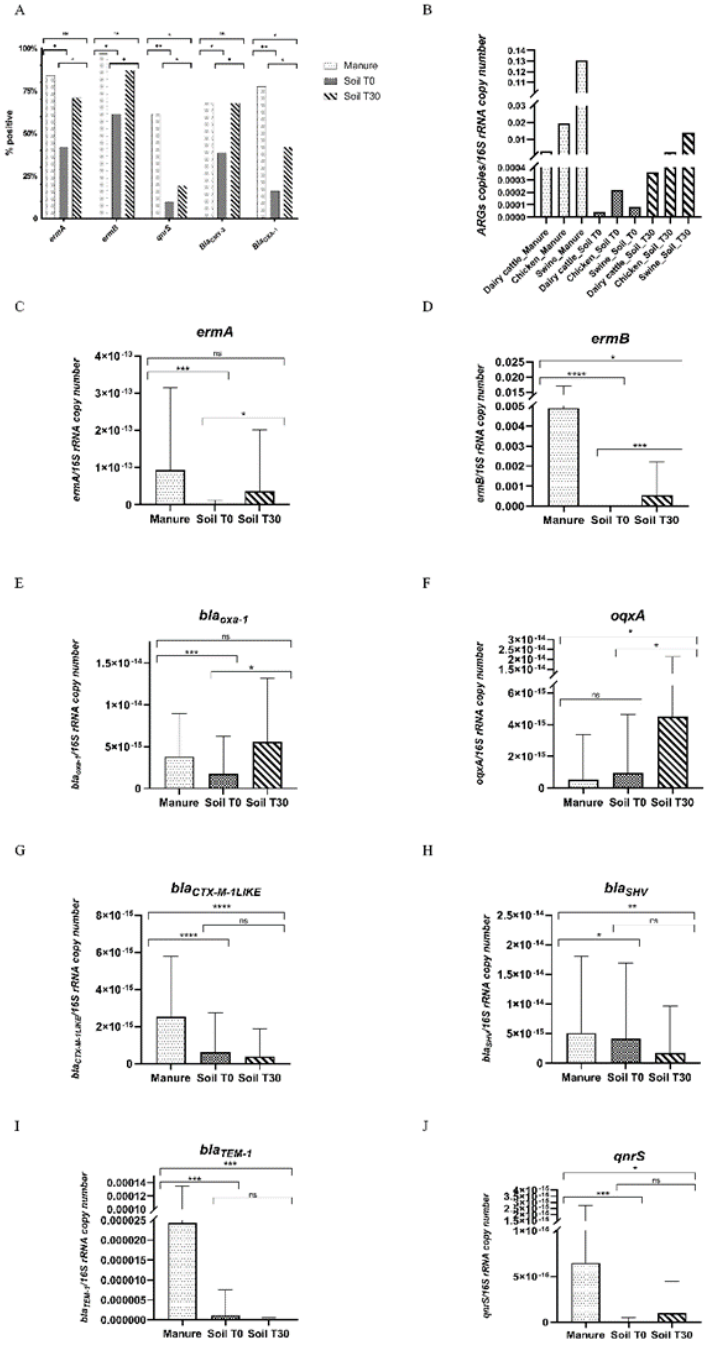

Figure 6. Prevalence and relative abundance of target genes to 16S rRNA copy number in manure and soil samples. $p<0.05$ shown as *, $p < 0.01$ as **, $p < 0.001$ as *** and $p < 0.0001$ as ****. Non-significant differences are indicated by ns. For easiness of representation, only ARGs showing significant differences among sample types are reported.

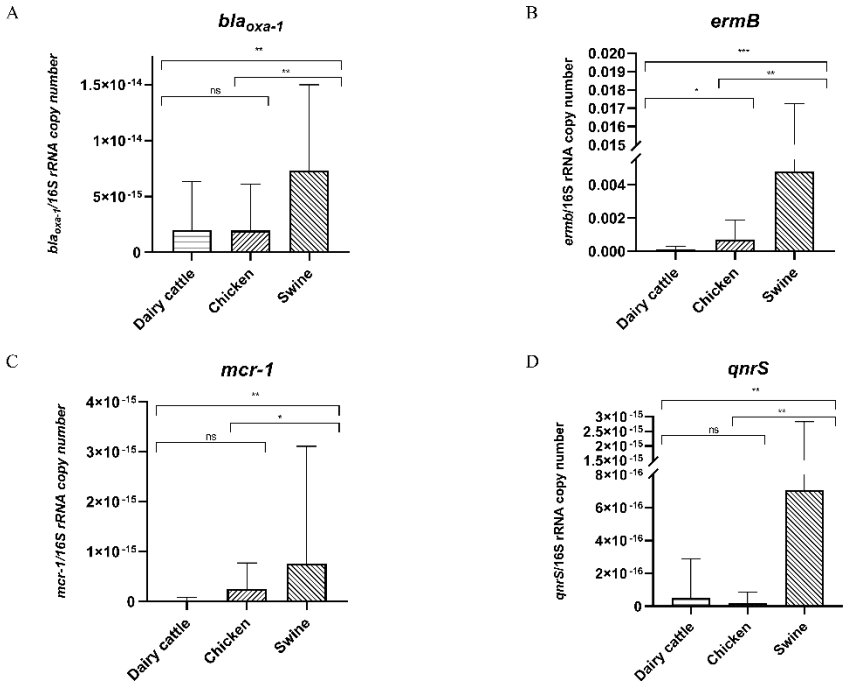

Figure 7. Relative abundance of target genes to 16S rRNA copy number in dairy cattle, chicken and swine samples. For easiness of representation, only ARGs showing significant differences among farm types are reported. $p < 0.05$ shown as *, $p < 0.01$ as ** and $p < 0.001$ as ***. Non-significant differences are indicated by ns.

3.7 Co-occurrence between ARGs and bacterial taxa

Co-occurrence patterns between the ARGs and the main microbial taxa were explored at the phylum level by using multivariate regression analysis. A positive correlation was observed between *ermA* and two phyla, Bacteroidetes (β-coefficient = 0.173, $p < 0.01$) and Hydrogenedentes (β-coefficient = 0.059, $p = 0.04$), suggesting that these phyla may harbour *ermA*. Acidobacteria (β-coefficient = 0.144, $p = 0.046$) and Gemmatimonadetes (β-coefficient = 0.138, $p = 0.044$) co-occurred with *oqxA*, while Spirochaetes may be potential hosts for bla_{OXA-1} (β-coefficient = 0.169, $p = 0.05$) and *mcr-1* (β-coefficient = 0.347, $p < 0.001$) genes.

3.8 Correlation analysis between antimicrobials and ARGs

The correlation analysis between antimicrobial concentration and the relative abundance of the 18 ARGs detected using Spearman's bivariate correlation analysis was performed considering only flumequine, since the low number of observations for the remaining antimicrobials made the analysis not possible. The analysis showed positive correlations between flumequine concentration and the abundance of *oqxA* (Spearman $r = 0.2836$, $p = 0.006$) and *qnrS* (Spearman $r = 0.2715$, $p = 0.008$), while no significant correlation was observed for the other genes conferring resistance to (fluoro)quinolones, nor any other ARGs.

4. Discussion

4.1 Antimicrobials prevalence in soil and manure

A low percentage of positive samples (42%) was found in the study, and only six out of the 14 antimicrobials screened were detected. Although flumequine is an old-generation (fluoro)quinolone relatively used in chickens and swine due to its low costs and good tolerability mainly, it was the most detected compound (97.4%) in these livestock and the only antimicrobial detected in soil. This is in agreement with its known high persistence in manure, where it can remain after one year, thus its environmental presence was quite expected (Berendsen *et al.*, 2018).

4.2 Impact of manure application on soil microbiome

The microbial composition for both manure and soil samples is in agreement with previous studies; the most abundant phyla identified are those typical of manure and soil microbiomes and the manure microbiome is confirmed to be less diverse than that of soil (Chen *et al.,* 2015; Fierer, 2017; Hamm *et al.,* 2016; Looft *et al.,* 2012). In accordance with previous observations (Riber *et al.,* 2014; Xie *et al.,* 2018), manure application showed limited influence on soil microbial community and did not increase soil diversity, but rather caused significant changes only in a few phyla. Firmicutes, representing the most abundant phylum in manure, was enriched in soil after fertilization as previously reported (Rieke *et al.,* 2018). However, several studies have shown an increase of soil microbial diversity after manure application and significant changes in the whole microbial community composition (Chen *et al.,* 2015, 2019; Zhen *et al.,* 2014), indicating that in some cases fertilization may have a strong impact on soil. These contrasting results might be due to the inability of most manure-associated bacteria to survive for long periods in soil, making the time of sampling a key factor and suggesting that manure microbiome might influence only temporarily the soil microbial community (Leclercq *et al.,* 2016; Rieke *et al.,* 2018). Furthermore, factors other than manure application (*i.e.* temperature, moisture, pH, seasonality) are known to influence soil microbial composition over time, and might be accountable for such contrasting results (Classen *et al.,* 2015; Fierer, 2017; Lopatto *et al.,* 2019). Here, the sampling was carried out over 18 months (but mostly during winter); however, pH, humidity and temperature were comparable among most of the farms (Supplementary material 1). Overall, data gathered in this study indicate that manure application has only a limited effect on soil microbiome at day 30 post-fertilization.

4.3 Abundance of ARGs and their fate in soil after fertilization

Aiming to understand whether ARGs disseminate from manure to soil, the prevalence and relative abundance of selected genes were assessed in manure and soil before and after fertilization. The highest number of total ARG copies was detected in manure and a clear increase in ARG abundance was observed in soil after fertilization. According to previous studies, this finding indicates that manure increased ARG abundance in soil (Marti *et al.,* 2014; Xia *et al.*, 2019a; Xiong *et al.*, 2015; Zhao *et al.,* 2017). None of the selected ARGs emerged in soil after manure application, suggesting that such practice might effectively

enrich but not introduce any of the screened genes. The ARGs investigated in our study did not show the same abundance pattern, and only four genes (*i.e*. *ermA*, *ermB*, bla_{oxa-1} and *oqxA*) were significantly enriched in manure-amended soil. Both *ermA* and *ermB* have been demonstrated to be able to spread and persist in soil after fertilization with swine manure (Lopatto *et al.,* 2019; Marti *et al.,* 2013; Zhang *et al.,* 2017), while an increase in bla_{oxa-1} abundance in farmland soil has been previously associated with irrigation with wastewater from swine farms showing high abundance of this gene (Yang *et al.,* 2019). Xiong *et al.* (2015) reported higher *oqxA* abundance in fertilized soil in comparison with the untreated control at one month after manure application; however, at day 60, the relative abundance of the gene was lower than in the control group, potentially indicating dissipation of *oqxA* in the soil. A similar dynamic was also observed for other plasmid-mediated quinolone resistance (PMQR) genes, which disappeared within two months after manure application (Xiong *et al.,* 2015). Lopatto *et al.* (2019) traced *ermB* and *ermC* abundance in treated soil over a period of six months, while the former was more abundant in the fertilized soil up to the last time point, the latter decreased after three months, suggesting that each ARG possessed a different dissipation dynamic. Bla_{TEM-1} and the extended-spectrum β-lactamase (ESBL)-encoding genes, $bla_{CTX-M-1LIKE}$ and bla_{SHV}, known to be widespread in the environment (Graham *et al.,* 2016), were significantly more abundant in manure than in soil and showed a decrease after manure application, pointing at a dilution effect in the soil after fertilization (Yang *et al.,* 2019). Here, ARG abundance was investigated only at day 30 post-fertilization, hampering to assess the impact of manure-derived ARGs on the soil at multiple time points and to establish the dissipation dynamic of the genes in agricultural soil, and future studies should aim at this. Together with the dissemination of ARGs from manure to the environment, we investigated the differences in ARG abundance in dairy cattle, chicken and swine farms. Not surprisingly, swine farms showed the highest total ARG abundance, and bla_{oxa-1}, *ermB*, *mcr-1* and *qnrS* were significantly more abundant in this sector in comparison to the others (Chen *et al.*, 2019; Marti *et al.,* 2014; Xia *et al.,* 2019a). Differences in ARG levels between livestock sectors might rely on differences in physiologies of animals, but also on differences in manure treatment and storage (Chen *et al.*, 2007; Sandberg and LaPara, 2016). Most of the sampled farms employed the same manure storage strategy (*i.e*. open-air pit), while the duration of the storage before manure application ranged from no longer than 120 days for dairy cattle farms, to 360 days for swine farms, pointing out that a longer storage might contribute to a high ARG abundance in manure. Despite the low prevalence and not being enriched by fertilization, the detection of the carbapenemase-gene bla_{oxa-48} both in manure and soil raises public health concerns due to the clinical importance of these drugs as last resort treatment in human medicine (Nordmann *et al.,* 2011). Interestingly, the carbapenemase-gene bla_{VIM-2} was detected merely in soil, suggesting that this gene of human/animal origin can be maintained in the environment (Scotta *et al.,* 2011). Manure application has been historically recognized as the main source of dissemination in the environment of *mcr* genes, conferring resistance to colistin, another critically important antimicrobial and last resort drug against human infections caused by multidrug resistant Gram-negative

bacteria (Kempf *et al.*, 2016; Liu *et al.,* 2016; Xia *et al.,* 2019b). *Mcr-1* to *mcr-5* genes were detected with a prevalence as high as 25% in manure; however, since none of the genes was enriched in soil after fertilization, and *mcr-2* and *mcr-4* were not detected in fertilized soil, manure application may not be a driver of the spread of these genes in the environment. As a whole, the ARGs investigated did not display uniform dynamics in soil after manure application.

4.4 Correlation analysis between flumequine concentration, taxa and ARGs abundances

As previously mentioned, due to the low positivity for most of the antimicrobials investigated in this study, the possible correlation between antimicrobial concentration and ARG abundance was explored only for flumequine. The increase in flumequine concentration in soil after manure application was correlated to an increase in relative abundance of two genes conferring resistance to (fluoro)quinolones (*i.e. oqxA* and *qnrS*), suggesting that flumequine might enhance the accumulation of these PMQR genes in manure-amended soil or slow down their dissipation, as previously reported for other (fluoro)quinolones (Xiong *et al.,* 2015). *OqxA* and *qnrS* accumulation in soil under the selective pressure of flumequine might be the result of different processes; indeed, the increase of ARGs in soil after fertilization might be due to the direct addition of bacteria originating from the manure, from proliferation of bacteria present in the soil or indirectly via gene spreading mediated by HGT (Ahmed *et al.,* 2018; Heuer *et al.*, 2011; Marti *et al.,* 2014; Rieke *et al.,* 2018). *OqxA* co-occurrence with Acidobacteria and Gemmatimonadetes, which were more abundant in soil than in manure, suggests that the application of manure carrying flumequine may promote the proliferation of bacteria harbouring *oqxA* already present in the environment. On the contrary, *qnrS* did not correlate to any specific phyla and consequently its spread in soil might be due to HGT. Independently from the process by which the accumulation of these two genes occurred, this finding represents a concern for public health and should lead to reconsider the use of flumequine in the veterinary field. Indeed, both *oqxA* and *qnrS* confer resistance not only to flumequine, which is a first generation (fluoro)quinolone with a limited spectrum of activity (Daly and Silverstein, 2009), but also to other (fluoro)quinolones (*e.g.* ciprofloxacin, levofloxacin, norfloxacin, and nalidixic acid) widely used to treat human bacterial infections (Jacoby *et al.,* 2014; Kim *et al.,* 2009). *ErmA* co-occurred with two phyla (*i.e.* Bacteroidetes and Hydrogenedentes) showing similar abundance in manure and soil; hence the increase in *ermA* abundance in soil after fertilization might be due to HGT (Murphy, 1985). Spirochaetes, a phylum more abundant in manure, showed a positive correlation with bla_{oxa-1} and *mcr-1*, two genes characterized by a different dynamic in amended soil; while bla_{oxa-1} abundance significantly increased after manure application, *mcr-1* seems to follow the fate of Spirochaetes, not being enriched in the soil (Gao *et al.,* 2019). Bla_{oxa-1} enrichment in soil might be due to HGT to other bacteria present before manure application, since this gene is commonly found in plasmids and integrons in several Gram-negative bacteria (Poirel *et al.,* 2010).

5. Conclusion

In the present study we analysed manure and soil samples from integrated farms located in Northern Italy; by combining LC-MS/MS, qPCR and 16S rRNA gene sequencing, we demonstrated that fertilization may affect the abundance of specific ARGs in soil. The main conclusions of our study are:

- Manure-derived bacteria does not survive in soil, and manure application do not drastically affect the soil microbiome, since at 30 days after fertilization only three phyla were significantly enriched.
- ARGs showed different dynamic patterns in soil; while *ermA*, *ermB,* bla_{OXA-1}, *oqxA* and *qnrS* enriched, $bla_{CTX-M-1LIKE}$, bla_{SHV}, and bla_{TEM-1} disappeared after manure application, suggesting that different manure-derived genes experience different fates in soil.
- Flumequine may exert a selective pressure for the accumulation of *oqxA* and *qnrS* in fertilized soil; hence, the use of flumequine in the veterinary field should be reconsidered.
- The different dairy cattle, chicken and swine farms displayed different microbial communities, and the latter was characterized by the highest abundance of ARGs, of which bla_{OXA-1}, *ermB*, *mcr-1* and *qnrS* were significantly more abundant than in the other two livestock sectors.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Andrea Laconi: Formal analysis; Conceptualization; Data Curation; Visualization; Writing - Original Draft; Writing - Review & Editing. **Lapo Mughini-Gras:** Formal analysis; Writing - Original Draft; Writing - Review & Editing. **Roberta Tolosi:** Investigation; Data Curation; Validation. **Guido Grilli:** Conceptualization; Investigation; Writing - Review & Editing. **Angela Trocino:** Conceptualization; Investigation; Writing - Review & Editing. **Lisa Carraro:** Investigation; Data Curation. **Federica Di Cesare**: Investigation; LC-MS/MS analysis and data analysis (oppure Formal analysis o molecules quantification, data analysis) **Petra Cagnardi:**

Conceptualization; Supervision; Project administration; Funding acquisition; Investigation; Validation; Writing - Original Draft; Writing - Review & Editing. **Alessandra Piccirillo:** Conceptualization; Supervision; Project administration; Funding acquisition; Writing - Original Draft; Writing - Review & Editing.

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Supplementary material

				pH		Treatment																													Manure			Enviromental conditions		
																																				Storage					
Unit	label	Farm	Specimen	field	lab	Aminosidine	amoxicillin	Ampicillin	cefalexin	Cefagrin	cefquinome	ceftiofur	Chlortetracyc	cloxacillin	colistin	Dexamethas	Doxycycline	enrofloxacin	Erythromycii	Fenbendazo	fluoroquinol	vermectin	Ketoprofen	Lincomycin	mamycin	Marbofloxac	meloxicam	micospector	Monensin	Neomycin	Oxytetracycl	Penethamat	penicillin	penicillin g b	Rifaximin	Spectinomy	Sulfadimeth	Sulfamonom	tetracycline	Tiamulin	Tildipirosin	Trimethopri	Tulathromy	Tylosin	Harvesting	Type	Duration	Pretreatment before fertilization	Temperature (°C)	Humidity (%)	Rain (mean mm / month)
1	B1_B1M	Dairy cattle	Manure	7,8	MD													x									x																MD	open-air slurry pit	120 days	MD	4,8	84	MD
1	B1_B1T0	Dairy cattle	Soil T0	MD	MD																																												
1	B1_B1T30	Dairy cattle	Soil T30	7,9	7,46																																												
1	B1_B2M	Dairy cattle	Manure	7,05	7,47						x																																MD	open-air slurry pit	90 days	MD	4,1	88	MD
1	B2_B2T0	Dairy cattle	Soil T0	7,5	7,45																																											
1	B2_B2T30	Dairy cattle	Soil T30	7,31	7,31																																											
2	D1_B3M	Dairy cattle	Manure	7,81	MD				x																																		MD	open-air slurry pit	120 days	biogas	4,6	81	MD
2	D1_B3T0	Dairy cattle	Soil T0	7,25	7,41																																											
2	D1_B3T30	Dairy cattle	Soil T30	7,9	7,57																																											
2	L1_B4M	Dairy cattle	Manure	7,7	7,92				x									x									x																MD	open-air slurry pit	MD	MD	4,2	83	MD
2	L1_B4T0	Dairy cattle	Soil T0	7,34	7,54																																											
2	L1_B4T30	Dairy cattle	Soil T30	7,45	7,74																																											
2	Z1_B5M	Dairy cattle	Manure	7,61	7,94			x	x																	x																	MD	open-air slurry pit	120 days	MD	4	83	MD
2	Z1_B5T0	Dairy cattle	Soil T0	7,46	7,45																																											
2	Z1_B5T30	Dairy cattle	Soil T30	7,48	8,01																																											
1	1_P1M	Chicken	Manure	7,58	6,67												x																										MD	NA	MD	MD	18	81	MD
1	1_P1T0	Chicken	Soil T0	7,5	6,9																																											
1	1_P1T30	Chicken	Soil T30	8,04	7,45																																											
2	1_P2M	Chicken	Manure	8,18	8,12												x																									x	mechanical	enclosed manure pit	MD	MD	4,3	82	MD
2	1_P2T0	Chicken	Soil T0	7,7	7,1																																											
2	1_P2T30	Chicken	Soil T30	8,44	7,95																																											
3	1_P3M	Chicken	Manure	8,48	8,07		x																																				MD	open-air manure pit	MD	MD	4,2	83	MD
3	1_P3T0	Chicken	Soil T0	7,54	6,71																																											
3	1_P3T30	Chicken	Soil T30	7,81	7,11																																											
4	1_P4M	Chicken	Manure	7,88	7,42										x						x																				x	MD	open-air manure pit	MD	MD	4,9	83	MD
4	1_P4T0	Chicken	Soil T0	7,63	6,99																																											
4	1_P4T30	Chicken	Soil T30	7,68	6,8																																											
5	1_P5M	Chicken	Manure	7,33	7,04												x																									x	MD	open-air manure pit	MD	biogas	4,5	84	MD
5	1_P5T0	Chicken	Soil T0	7,5	7,24																																											
5	1_P5T30	Chicken	Soil T30	7,83	6,98																																											
29	L_P6M	Chicken	Manure	7,71	7,52																																						mechanical	open-air manure pit	90-120 days	MD	10	51	MD
29	L_P6T0	Chicken	Soil T0	7,43	7,25																																											
29	L_P6T30	Chicken	Soil T30	7,61	7,27																																											
30	L_P7M	Chicken	Manure	8,15	7,65																																							mechanical	enclosed manure pit	120 days	MD	3	79	MD
30	L_P7T0	Chicken	Soil T0	7,43	7,6		x								x				x																														
30	L_P7T30	Chicken	Soil T30	7,7	7,3																																											
31	L_P8M	Chicken	Manure	6,9	7,2		x								x				x																									mechanical	enclosed manure pit	120 days	MD	5	75	MD
31	L_P8T0	Chicken	Soil T0	7,3	7,2																																											
31	L_P8T30	Chicken	Soil T30	7,8	7,4																																											
6	L_S1M	Swine	Manure	7,75	8,39		x																																				MD	open-air slurry pit	90 days	MD	4,2	87	MD
6	L_S1T0	Swine	Soil T0	7,6	7,13																																											
6	L_S1T30	Swine	Soil T30	7,7	6,98																																											
7	L_S2M	Swine	Manure	7,12	7,91		x																																				MD	open-air slurry pit	90-120 days	MD	3,2	91	MD
7	L_S2T0	Swine	Soil T0	7,54	6,97																																											
7	L_S2T30	Swine	Soil T30	7,08	4,69																																											
8	L_S3M	Swine	Manure	6,95	7,14		x																x																				MD	open-air slurry pit	90 days	no	3,5	87	MD
8	L_S3T0	Swine	Soil T0	7,58	6,92																																											
8	L_S3T30	Swine	Soil T30	7,62	7,21																																											
9	L_S4M	Swine	Manure	7,63	8,36		x																x																				MD	open-air slurry pit	90 days	no	4,2	88	MD
9	L_S4T0	Swine	Soil T0	7,34	5,88																																											
9	L_S4T30	Swine	Soil T30	7,47	6,86																																											
10	L_S5M	Swine	Manure	6,2	5,7		x																x																				MD	open-air slurry pit	90-120 days	no	3,8	89	MD
10	L_S5T0	Swine	Soil T0	7,11	7,16																																											
10	L_S5T30	Swine	Soil T30	6,92	5,91																																											
23	V_B1M	Dairy cattle	Manure	8,25	8,5						x	x		x									x					x	x	x	x		x										mechanical	open-air manure pit	180 days	no	7,86	75,76	4,11
23	V_B1T0	Dairy cattle	Soil T0	6,68	7,44																																											
23	V_B1T30	Dairy cattle	Soil T30	7,71	7,37																																											
24	V_B2M	Dairy cattle	Manure	6,72	6,96			x								x			x				x						x									x					mechanical	open-air slurry pit	60/90 days	no	7,3	75,13	4,11
24	V_B2T0	Dairy cattle	Soil T0	5,68	6,51																																											
24	V_B2T30	Dairy cattle	Soil T30	7,04	6,07																			x	x		x				x	x			x												
25	V_B3M	Dairy cattle	Manure	6,72	7,59																																							mechanical	open-air slurry pit	90 days	no	5,79	76	3,54
25	V_B3T0	Dairy cattle	Soil T0	8,39	6,74																																											
25	V_B3T30	Dairy cattle	Soil T30	7,42	6,91																																											
26	V_B4M	Dairy cattle	Manure	8,58	8,29					x													x	x					x	x	x		x		x					x			mechanical	open-air slurry pit	90/120 days	no	5,6	76	3,4
26	V_B4T0	Dairy cattle	Soil T0	6,99	7,47																																											
26	V_B4T30	Dairy cattle	Soil T30	7,23	7,26																																											
27	V_B5M	Dairy cattle	Manure	8,63	8,36						x					x	x						x						x		x	x			x		x	x			x		manual	open-air manure pit	60/90 days	litter added with phosphate	4,4	78	1,45
27	V_B5T0	Dairy cattle	Soil T0	7,75	7,18																																											
27	V_B5T30	Dairy cattle	Soil T30	7,76	7,47																																											
28	V_B6M	Dairy cattle	Manure	7,44	7,88																																							mechanical	open-air manure pit	180 days	no	4,17	73	2,97
28	V_B6T0	Dairy cattle	Soil T0	6,06	6,95																																											
28	V_B6T30	Dairy cattle	Soil T30	7,15	6,44																																											
11	V_P1M	Chicken	Manure	7,3	7,61												x																											manual	open-air manure pit	360 days	no	5,88	80	2,22
11	V_P1T0	Chicken	Soil T0	7,44	7,55																																											
11	V_P1T30	Chicken	Soil T30	7,46	7,66																																											
12	V_P2M	Chicken	Manure	6,08	5,66		x												x																									manual	enclosed manure pit	>180 days	no	MD	MD	MD
12	V_P2T0	Chicken	Soil T0	7,54	7,46																																											
12	V_P2T30	Chicken	Soil T30	6,75	7,44																																											
13	V_S1M	Swine	Manure	7,85	7,92		x						x							x																								mechanical	two open-air slurry pit, one enclosed slurry pit	180 days	no	4,24	69	3
13	V_S1T0	Swine	Soil T0	7,25	7,71																																											
13	V_S1T30	Swine	Soil T30	7,73	7,63																																											
14	V_S2M	Swine	Manure	7,66	7,84		x						x								x	x				x																	mechanical	open-air slurry pit	180 days	no	5,65	73,9	3,95
14	V_S2T0	Swine	Soil T0	7,87	7,98																																											
14	V_S2T30	Swine	Soil T30	6,79	6,1																																											
15	V_S3M	Swine	Manure	7,15	7,25	x	x						x				x				x					x																	mechanical	enclosed slurry pit	180 days	no	MD	MD	MD
15	V_S3T0	Swine	Soil T0	8,01	8,2																																											
15	V_S3T30	Swine	Soil T30	6,66	6,07																																											
16	V_S4M	Swine	Manure	7,36	7,92														x				x			x	x																mechanical	open-air slurry pit	150 days	no	MD	MD	MD
16	V_S4T0	Swine	Soil T0	7,92	7,14																																											
16	V_S4T30	Swine	Soil T30	6,84	6,3																																											
17	V_S5M	Swine	Manure	7,83	7,74		x									x							x	x			x																mechanical	open-air slurry pit	180 days	no	8,41	74,71	5,53
17	V_S5T0	Swine	Soil T0	6,89	7,71																																											
17	V_S5T30	Swine	Soil T30	7,44	7,34																																											

Supplementary material 1. Information regarding the pH of samples, treatments employed in each farm, details regarding the management of manure/slurry in each farm and the environmental conditions at the time of sampling.

	Parent (m/z)	**Product ions (m/z)**			**ESI**
Amoxicillin	366	114	134	349	(+)
Ampicillin	350	106	114	160	(+)
Cefquinome	529	134	324	396	(+)
Ceftiofur	524	210	241	285	(+)
Ciprofloxacin	332	268	288	314	(+)
Danofloxacin	358	82	314	340	(+)
Enrofloxacin	360	245	316	342	(+)
Flumequine	262	174	202	244	(+)
Marbofloxacin	363	72	320	345	(+)
Erytromycin	735	116	158	576	(+)
Spiramycin	844	142	174	540	(+)
Tilmicosin	870	132	156	174	(+)
Tylosin	917	156	174	772	(+)
Colistin A*	391	101	379	385	(+)
Colistin B*	386	101	374	380	(+)
* The $[M+3H]^{3+}$ ions were considered					

Supplementary material 2. List of ions selected for the quantification and their collision energies.

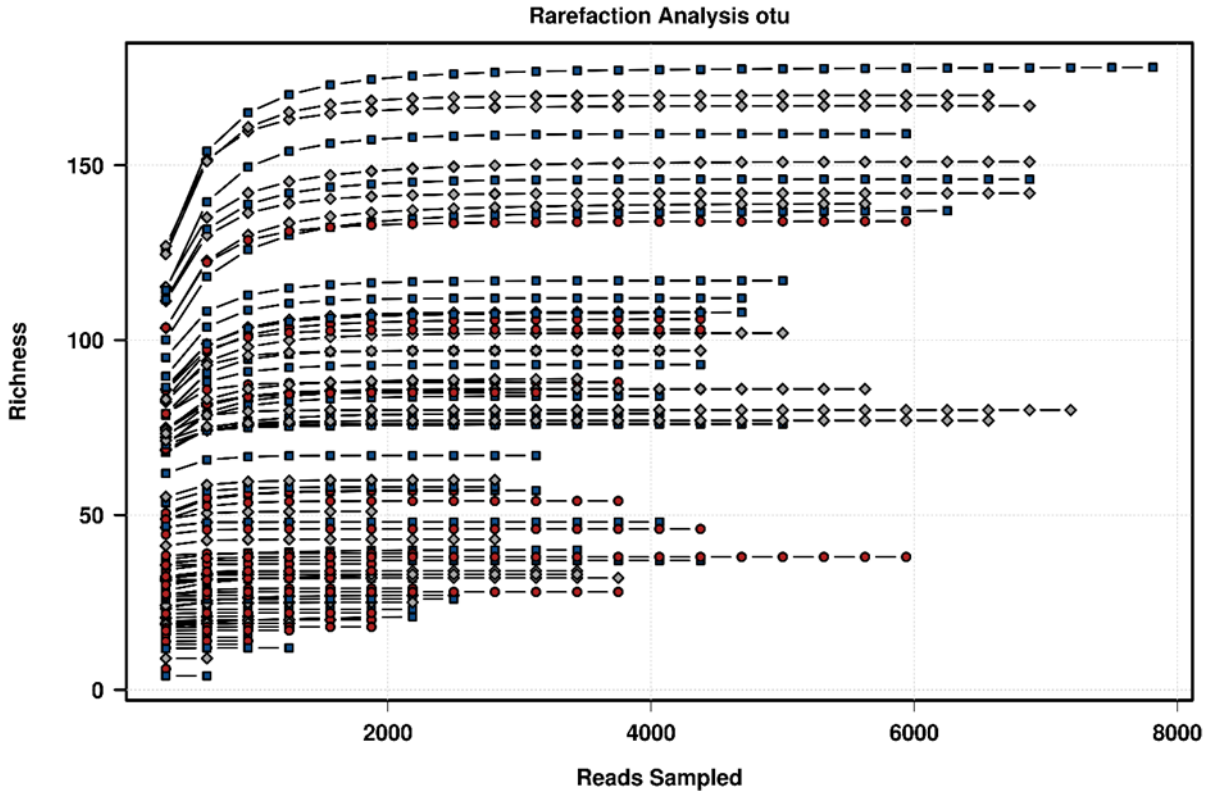
POOLED MANURE							
Analyte	**Validation levels C0, 2C0, 3C0 (ng/g)**	**CCα (ng/g)**	**CCβ (ng/g)**	**Recovery %**	**Trueness %**	**Intra-day Precision CV%**	**Inter-day Precision CV%**
Amoxicillin	20.00, 40.00, 60.00	20.04	20.78	94	106	14	17
Ampicillin	0.50,1.00,1.50	0.51	0.72	93	97	11	15
Cefquinome	1.00, 2.00, 3.00	1.13	1.32	91	97	11	16
Ceftiofur	0.50,1.00,1.50	0.53	0.70	95	101	13	16
Ciprofloxacin	1.00, 2.00, 3.00	1.03	1.25	89	97	14	18
Danofloxacin	1.00, 2.00, 3.00	1.09	1.31	87	102	16	19
Enrofloxacin	1.00, 2.00, 3.00	1.12	1.34	90	92	17	20
Flumequine	0.50,1.00,1.50	0.52	0.74	98	95	10	14
Marbofloxacin	1.00, 2.00, 3.00	1.10	1.32	93	94	15	18
Erytromycin	1.00, 2.00, 3.00	1.11	1.3	97	98	11	15
Spiramycin	0.50,1.00,1.50	0.50	0.71	97	94	8	13
Tilmicosin	0.50,1.00,1.50	0.51	0.75	96	92	9	13
Tylosin	0.50,1.00,1.50	0.55	0.78	94	107	12	15
Colistin	100.00, 200.00, 300.00	100.07	100.94	89	101	15	18

SOIL							
Analyte	**Validation levels C0, 2C0, 3C0 (ng/g)**	**CCα (ng/g)**	**CCβ (ng/g)**	**Recovery %**	**Trueness %**	**Intra-day Precision CV%**	**Inter-day Precision CV%**
Amoxicillin	20.00, 40.00, 60.00	20.53	21.05	92	93	16	20
Ampicillin	5.00, 10.00, 15.00	5.11	5.51	95	95	12	17
Cefquinome	5.00, 10.00, 15.00	5.15	5.49	96	99	17	20
Ceftiofur	5.00, 10.00, 15.00	5.21	5.60	93	100	12	16
Ciprofloxacin	10.0, 20.00, 30.00	10.28	10.61	87	93	17	20
Danofloxacin	10.0, 20.00, 30.00	10.06	10.68	84	100	13	18
Enrofloxacin	10.0, 20.00, 30.00	10.32	10.83	89	94	18	20
Flumequine	5.00, 10.00, 15.00	5.04	5.42	96	94	12	18
Marbofloxacin	10.0, 20.00, 30.00	10.37	10.98	87	96	17	20
Erytromycin	5.00, 10.00, 15.00	5.07	5.47	98	104	12	16
Spiramycin	5.00, 10.00, 15.00	5.02	5.43	99	103	11	15
Tilmicosin	5.00, 10.00, 15.00	5.12	5.61	97	101	14	17
Tylosin	5.00, 10.00, 15.00	5.16	5.69	95	98	15	20
Colistin	100.00, 200.00, 300.00	100.73	101.87	87	95	18	20

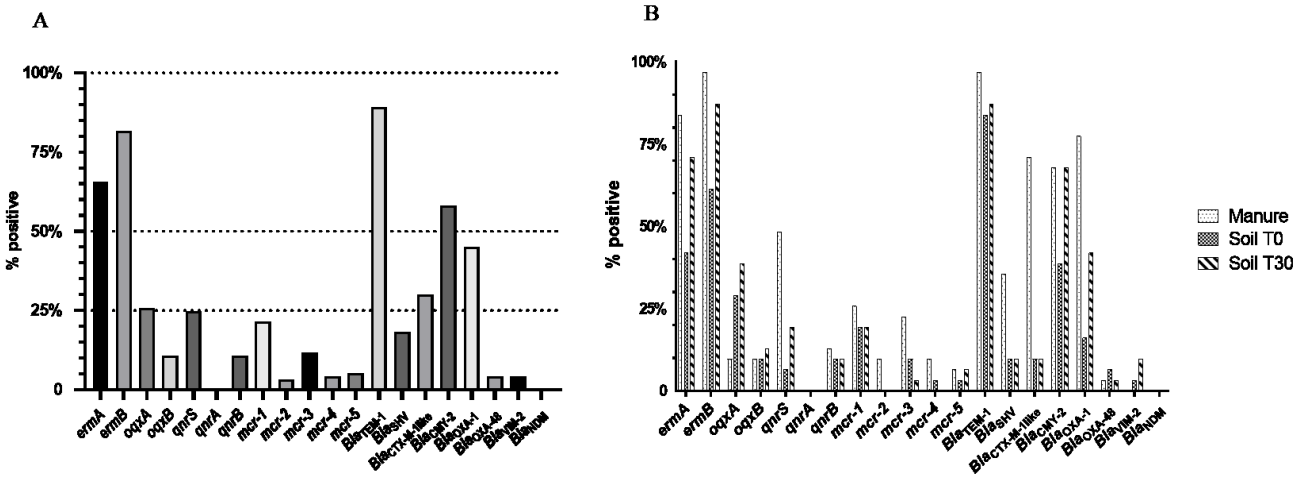
Supplementary material 3. Results of the method validation for all the compounds investigated in the study.

Name	Sequence (5'-->3')	Annealing temperature	Target gene	Reference	Refernce strain
mcr1-qf	AAAGACGCGGTACAAGCAAC	60°C	*mcr-1*	(Li et al., 2017)	*E. coli* 412016126
mcr1-qr	GCTGAACATACACGGCACAG				
mcr2-qf	CGACCAAGCCGAGTCTAAGG	60°C	*mcr-2*	(Li et al., 2017)	*E. coli* KP37
mcr2-qr	CAACTGCGACCAACACACTT				
mcr3-qf	ACCTCCAGCGTGAGATTGTTCCA	60°C	*mcr-3*	(Li et al., 2017)	*E. coli* 2013-SQ352
mcr3-qr	GCGGTTTCACCAACGACCAGAA				
mcr4-qf	AGAATGCCAGTCGTAACCCG	60°C	*mcr-4*	(Tolosi et al., 2020)	*E. coli* DH5a
mcr4-qr	GCGAGGATCATAGTCTGCCC				
mcr5-qf	CTGTGGCCAGTCATGGATGT	60°C	*mcr-5*	(Tolosi et al., 2020)	*Salmonella* Paratyphi B 13-SA01718
mcr5-qr	CGAATGCCCGAGATGACGTA				
ermAF	CCTTCTCAACGATAAGATAGC	56°C	*ermA*	(Beukers et al., 2018)	*S .aureus* 1206::Tn554 ermA
ermAR	ATGGAGGCTTATGTCAAGTG				
ermB1	CCGAACACTAGGGTTGCTC	56°C	*ermB*	(Di Cesare et al., 2013)	*E. faecalis* JH2-2::Tn1545
ermB2	ATCTGGAACATCTGTGGTATG				
blaTEM1F	CATTTTCGTGTCGCCCTTAT	56°C	*bla TEM-1*	(Wang et al., 2015)	*E.coli* 31 Holland
blaTEM1R	GGGCGAAAACTCTCAAGGAT				
blaSHVF	CGCTTTCCCATGATGAGCACCTTT	60°C	*bla SHV*	(Beukers et al., 2018)	*S. Keurmassar* DAK 2
blaSHVR	TCCTGCTGGCGATAGTGGATCTTT				
RTCTX-M-F	CTATGGCACCACCAACGATA	58°C	*bla CTX-M-1*	(Marti et al., 2013)	*E. coli* O 149 77-30108-11
RTCTX-M-R	ACGGCTTTCTGCCTTAGGTT				
FW3_CMY-2	AGACGTTTAACGGCGTGTTG	58°C	*bla CMY-2*	(Schmidt et al., 2015)	*S. Heidelberg* 5-12893-1
RV4_CMY-2_	TAAGTGCAGCAGGCGGATAC				
blaOXA1F	TATCTACAGCAGCGCCAGTG	56°C	*bla OXA-1*	(Wang et al., 2015)	*E. coli* 53/RGN238
blaOXA1R	CGCATCAAATGCCATAAGTG				
OXA-rtF	AGGCACGTATGAGCAAGATG	56°C	*bla OXA-48*	(Subirats et al., 2017)	*E. coli* MSC234 (pMSC122)
OXA-rtR	TGGCTTGTTTGACAATACGC				
VIM-F	GTTTGGTCGCATATCGCAAC	58°C	*bla VIM-2*	(Poirel et al., 2011)	*E. coli MSC23* 1 (pMSC119)
VIM-R	AATGCGCAGCACCAGGATAG				
Ndm-rtF	GATTGCGACTTATGCCAATG	56°C	*bla NDM*	(Subirats et al., 2017)	*E. coli* MSC229 (pMSC116)
Ndm-rtR	TCGATCCCAACGGTGATATT				
qnrAm-F	AGAGGATTTCTCACGCCAGG	56°C	*qnrA*	(Cattoir et al., 2007)	*E. cloacae* 03-577
qnrAm-R	TGCCAGGCACAGATCTTGAC				
qnrBF	GATCGTGAAAGCCAGAAAGG	56°C	*qnrB*	(Liu et al., 2012)	*K. pneumoniae* Kp15
qnrBF	ACGATGCCTGGTAGTTGTCC				
qnrSm-F	GCAAGTTCATTGAACAGGGT	60°C	*qnrS*	(Cattoir et al., 2007)	*E. coli* pHC19
qnrSm-F	TCTAAACCGTCGAGTTCGGCG				
oqxAF	CTCGGCGCGATGATGCT	60°C	*oqxA*	(Kim et al., 2009)	*E. coli* 396 pOLA52
oqxAR	CCACTCTTCACGGGAGACGA				
oqxBKF	TCCTGATCTCCATTAACGCCCA	60°C	*oqxB*	(Kim et al., 2009)	*E. coli* 396 pOLA52
oqxBKR	ACCGGAACCCATCTCGATGC				
16S-qF	TCCTACGGGAGGCAGCAGT	60°C	*16S*	(Nadkarni et al., 2002)	NA
16S-qR	GGACTACCAGGGTATCTAATCCTGTT				

Supplementary material 4. List of primers used in qPCR assays for the detection of the selected ARGs.



Supplementary material 5. Rarefaction curves per sample type.



Supplementary material 6. A) Prevalence of ARGs. B) Prevalence of ARGs per sample type

Chapter 3.4

Optimization of five qPCR protocols toward the detection and the quantification of antimicrobial resistance genes in environmental samples.

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ABSTRACT

Here, we describe the optimization and validation of five quantitative PCR (qPCR) assays by employing the SYBRGreen chemistry paired with melting curve analysis to detect and quantify clinically relevant antimicrobial resistance genes (ARGs) (*i.e. ermB,* $bla_{CTXM1\text{-}like}$*,* $bla_{CMY\text{-}2}$*, qnrA* and *qnrS*) from environmental samples (*i.e*. soil and manure). These five protocols accurately detected and quantified the aforementioned ARGs in complex environmental matrices and represent useful tools for both diagnostic and monitoring activities of resistant bacteria and ARGs into the environment.

SPECIFICATIONS TABLE

Subject Area	Immunology and Microbiology
More specific subject area	Antimicrobial resistance genes detection and quantification
Protocol name	SYBRgreen qPCRs paired with melting curve analysis for the detection and quantification of *ermB,* $bla_{CTXM1\text{-}like}$*,* $bla_{CMY\text{-}2}$*, qnrA* and *qnrS* genes in environmental samples.
Reagents/tools	**Reagents** PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA) Primers (Macrogen, The Netherlands) ermB1 = 5'-CCGAACACTAGGGTTGCTC-3' ermB2 = 5'-ATCTGGAACATCTGTGGTATG-3' RTCTX-M-F = 5'-CTATGGCACCACCAACGATA-3' RTCTX-M-R = 5'-ACGGCTTTCTGCCTTAGGTT-3' FW3_CMY-2_Lahey = 5'-AGACGTTTAACGGCGTGTTG-3' RV4_CMY-2_Lahey = 5'-TAAGTGCAGCAGGCGGATAC-3' qnrAm-F = 5'-AGAGGATTTCTCACGCCAGG-3' qnrAm-R = 5'-TGCCAGGCACAGATCTTGAC-3' qnrSm-F = 5'-GCAAGTTCATTGAACAGGGT-3' qnrSm-F = 5'-TCTAAACCGTCGAGTTCGGCG-3'
Experimental design	This work represents the optimization of previously published qPCR assays for the detection of five antimicrobial resistance genes (ARGs) by using the SYBRGreen chemistry paired with melting curve analysis. The primer pairs were tested firstly against DNA extracted from reference strains harbouring the target genes and then against total DNA extracted from complex environmental samples (*i.e.* soil and manure). These methods enable the detection and quantification of *ermB,* $bla_{CTXM1\text{-}like}$*,* $bla_{CMY\text{-}2}$*, qnrA* and *qnrS* genes.
Trial registration	*NA*
Ethics	*NA*
Value of the Protocol	Rapid and reliable methods for the detection and quantification of clinically relevant antimicrobial resistance genes (ARGs) from complex matrices.

	Useful for research and monitoring of macrolide, (fluoro)quinolone and β-lactam resistance. Assays cheap and easy to adapt to the emergence of new ARG variants, being based on SYBRGreen chemistry.

Description of protocol:

The emergence of bacteria resistant to macrolides, (fluoro)quinolones and β-lactams represents a threat for human health, since antimicrobial drugs (AMDs) belonging to these classes are listed among the critically important antimicrobials by the World Health Organization (WHO, 2019). Of great concern is the dissemination in the environment of antimicrobial resistance genes (ARGs) conferring resistance to these AMDs, since they can be transferred to humans through different routes, including the food chain (Hruby *et al*., 2016; Laconi *et al.,* 2020). Here, we report the optimization of previously published assays, four end-point PCRs and one probe-based qPCR (Cattoir *et al.,* 2007; Di Cesare *et al.* 2013; Marti *et al.,* 2013; Schmidt *et al*., 2015), for the detection and quantification of five ARGs (*i.e. ermB*, $bla_{CTXM1\text{-}like}$, $bla_{CMY\text{-}2}$, *qnrA* and, *qnrS*) conferring resistance to the aforementioned AMDs classes. The SYBRGreen chemistry paired with the melting curve analysis was chosen being specific, cost-effective and easy to adapt to the emergence of new ARG variants. The optimization and then the validation of the assays were carried out by using DNA from both bacterial isolates and complex environmental samples (*i.e.* soil and manure). The analytical performances of the assays, *i.e*. specificity, dynamic range, limit of detection (LoD), limit of quantification (LoQ) and efficiency, were determined, and the amplicons from environmental samples were also analyzed by Sanger sequencing to further confirm the specificity of the assays.

Major equipment and supplies for DNA extraction and quality/quantity assessment

Sterile 1.5 ml Eppendorf style microcentrifuge tubes (Sarstedt, Germany)

Adjustable micropipettes (0.5–1000 ml) (Gilson, USA)

Aerosol resistant micropipette tips (0.5–1000 ml) (Sarstedt, Germany)

Vortex Mixer (Velp, Italy)

Benchtop microcentrifuge (Eppendorf, Germany)

UV-Vis spectrophotometer NanoDrop ND-1000 (Nanodrop Technologies, USA).

Qubit 2.0 Fluorometer™ (Thermo Fisher Scientific, USA)

Reagents for DNA extraction and quality/quantity assessment

Invisorb Spin Tissue Mini Kit (Invitek Molecular, Germany)

DNeasy PowerSoil kit (Qiagen, Germany)

Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA)

Major equipment and supplies for qPCR and amplicon purification

LightCycler®480 Roche (Roche, Switzerland)

LightCycler®480 software version 1.5 (Roche, Switzerland)

Sterile 0.5-1.5-2.0 ml Eppendorf style microcentrifuge tubes (Sarstedt, Germany)

Adjustable micropipettes (0.1–1000 ml) (Gilson, USA)

Aerosol resistant micropipette tips (0.1–1000 ml) (Sarstedt, Germany)

Vortex Mixer (Velp, Italy)

Benchtop microcentrifuge 5424 (Eppendorf, Germany)

Plates centrifuge 5810R (Eppendorf, Germany)

Optically clear plates and foils for qPCR (Euroclone, Italy)

Reagents for qPCR

PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA)

UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, USA)

Primer HPSF purified (Macrogen, The Netherlands). Primers stock concentration 100 µM.

Procedures

DNA extraction

DNA extraction was performed under a sterile microbiological laminal flow cabinet to avoid contaminations. Micropipettes were used with aerosol resistant filter tips.

Genomic DNA was extracted from the reference strains (Table 1), kindly provided by the EU Reference Laboratory for Antimicrobial Resistance (DTU, Denmark), using the Invisorb Spin Tissue Mini Kit (Invitek Molecular, Germany) according to the manufacturer's instruction. DNeasy PowerSoil kit (Qiagen, Germany) was used to extract DNA from 93 environmental samples (*i.e.* soil and manure) following manufacturer's instructions. Both reference strains and environmental samples were stored at -80 °C and thawed in ice.

DNA quantity was assessed by using the NanoDrop spectrophotometer (ThermoScientific, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA), following the manufacturer's recommendations. A260/280 values equal or higher than 1.6 were considered acceptable. The extracted DNA was stored at -20 °C until qPCR analysis.

qPCR assays

The amplification mix was prepared under a sterile microbiological laminal flow cabinet to avoid contamination and all qPCR reagents were thawed in ice. Micropipettes were used with aerosol resistant filter tips. The cabinet and the pipettes were not the same used in DNA extraction steps. The qPCR assays

were performed in a final volume of 10 µl reaction mixtures, containing 2.5 µl of DNA template, 1.3 µl of UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, USA), 5 µl of 2X PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA) and 0.6 µl of each primer (Table 1). The composition of the reagents in the qPCR mix is reported in the Table 2. qPCR mixes were mixed using the vortex and aliquot in optically clear plates kept in ice until being placed in the real-time machine. All qPCRs were performed in a LightCycler®480 Roche (Roche, Switzerland) real-time platform. All qPCR reactions were performed in triplicate.

Primer name	Sequence (5'–3')	Annealing temperature	Amplicon size (bp)	Reference	Reference strains
ermB1	CCGAACACTAGGGTTGCTC	56°C	139	Di Cesare *et al.* 2013	*E. faecalis* JH2-2::Tn1545
ermB2	ATCTGGAACATCTGTGGTATG				
RTCTX-M-F	CTATGGCACCACCAACGATA	58°C	103	Marti *et al.,* 2013	*E. coli* O 149 77-30108-11
RTCTX-M-R	ACGGCTTTCTGCCTTAGGTT				
FW3_CMY-2_Lahey	AGACGTTTAACGGCGTGTTG	58°C	128	Schmidt *et al*., 2015	*S. Heidelberg* 5-12893-1
RV4_CMY-2_Lahey	TAAGTGCAGCAGGCGGATAC				
qnrAm-F	AGAGGATTTCTCACGCCAGG	56°C	580	Cattoir *et al.,* 2007	*E. cloacae* 03-577
qnrAm-R	TGCCAGGCACAGATCTTGAC				
qnrSm-F	GCAAGTTCATTGAACAGGGT	60°C	428	Cattoir *et al.,* 2007	*E. coli* pHC19
qnrSm-F	TCTAAACCGTCGAGTTCGGCG				

Table 1. List of primers and reference strains.

Reagents	Final concentration	µl per reaction
PowerUp™ SYBR® Green Master Mix 2X	1X	5
Primer forward 10 µM	600 nM	0.6
Primer reverse 10 µM	600 nM	0.6
UltraPure™ DNase/RNase-Free Distilled Water	-	1.3
DNA template	-	2.5

Table 2. qPCR reaction mix.

The following PCR thermal profile was used: initial incubation at 50°C for 2 minutes (min), followed by 2 min at 95°C, and 45 cycles at 95°C for 10 seconds (s) and 56–60°C for 40 s. The exact annealing temperatures of the assays are depicted in table 1.

Melting curves were determined by adding a dissociation step after the last amplification cycle with a temperature transition rate of 4.4 °C/s between 40 and 95°C.

For the analysis of the environmental samples, in each plate a "positive control" (*i.e.* DNA obtained from the strain harbouring the ARG under analysis) was included, together with a "No Template Controls" (NTC) (*i.e.* a sample containing all qPCR reagents with the exception of the DNA template).

Optimization of qPCR conditions

Assay optimization is crucial to ensuring the best qPCR performances. We optimized the annealing temperatures used in the previously published studies by comparing the amplification plots and dissociation curves. Furthermore, we tested different concentrations of each primer (300/300 nM, 300/600 nM, 600/300 nM, 600/600 nM, 600/900 nM, 900/600 nM and 900/900 nM for forward and reverse primer respectively). The combination of concentrations yielding the lowest Cp, the best efficiency of amplification as well as negative NTC was chosen for the validation steps.

Interpretation of qPCR amplification plots

For each run the baseline and the Crossing Point (Cp) of the amplification curves were calculated using the LightCycler®480 software version 1.5 (Roche). The specificity of the amplification was assessed by melting curve analysis. The melting temperature for *ermB*, $bla_{CTXM1\text{-}like}$, $bla_{CMY\text{-}2}$, *qnrA* and *qnrS* were 80.5 ±0.2 °C, 84.4 ± 0.2 °C, 86.9 ± 0.2 °C, 72.5 ± 0.2 °C and 82.5 ± 0.2 °C, respectively. As shown in Figure 1, each of the optimized assays was able to identify correctly its target ARG based on the melting curve analysis. No amplification was observed for any NTCs. To confirm the specificity of the amplifications, PCR products of each assay were run on 1.5% gel; all amplicons showed the expected size (bp) and no non-specific bands were observed (Figure 2).

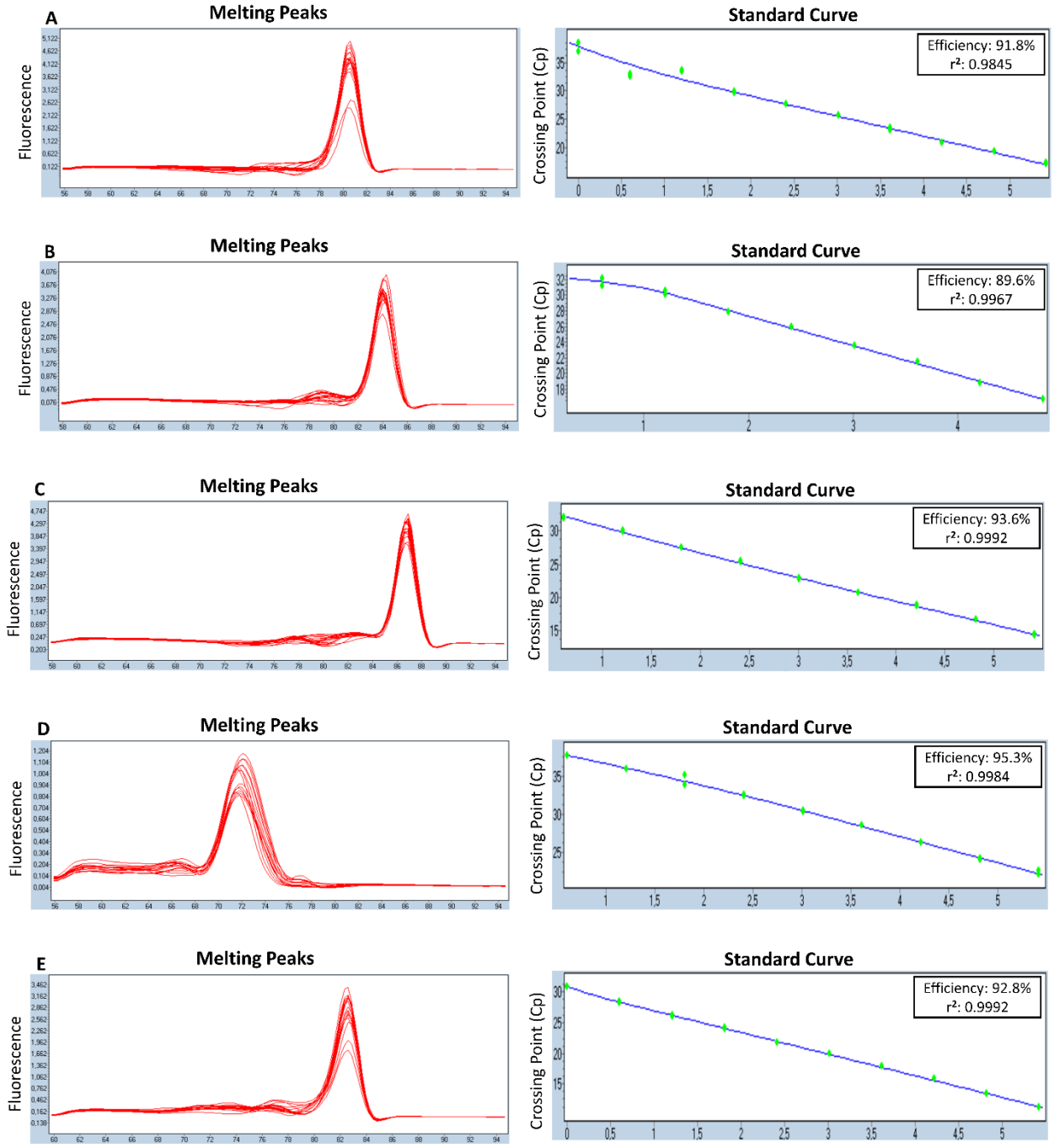

Figure 1. qPCR standard curves and melting curves for *ermB* (A), *bla*$_{CTXM1\text{-}like}$ (B), *bla*$_{CMY\text{-}2}$ (C), *qnrA* (D) and, *qnrS* (E). For each standard curve, efficiency, slope, intercept, and r^2 are reported.

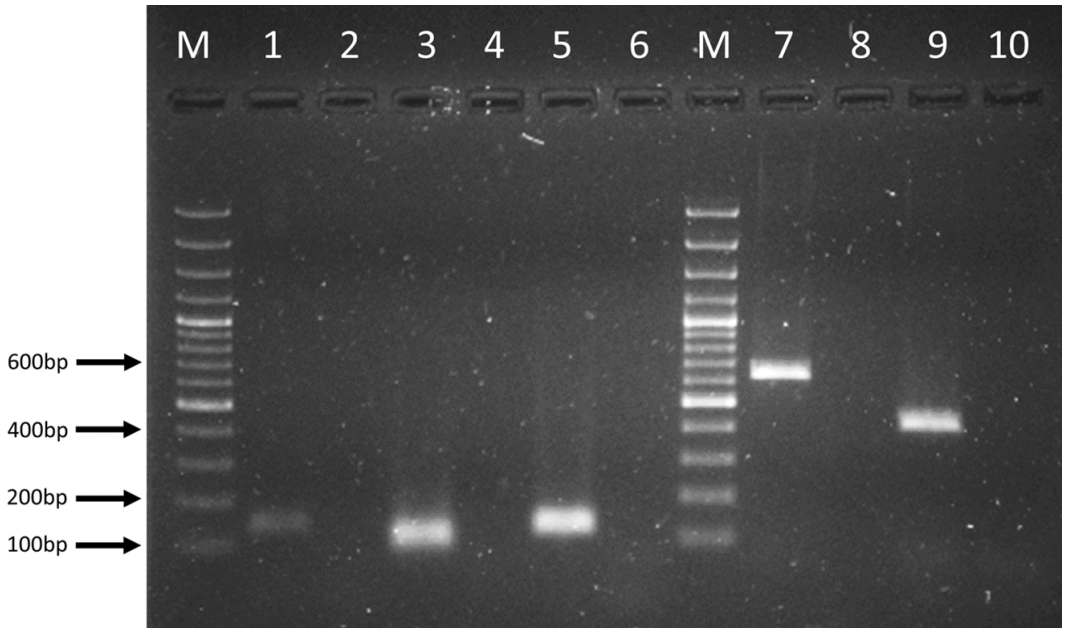

Figure 2. Agarose gel electrophoresis of amplicons obtained for each assay. Lane M = 100bp DNA ladder, lane 1 = *E. faecalis* JH2 2::Tn1545 (*ermB*), lane 2 = negative control *ermB*, lane 3 = *E. coli* O 149 77-30108-11 ($bla_{CTXM1\text{-}like}$), lane 4 = negative control $bla_{CTXM1\text{-}like}$, lane 5 = *S. Heidelberg* 5-12893-1 ($bla_{CMY\text{-}2}$), lane 6 = negative control $bla_{CMY\text{-}2}$, lane M = 100bp ladder, lane 7 = *E. cloacae* 03-577 (*qnrA*), lane 8 = negative control *qnrA*, lane 9 = *E. coli* pHC19 (*qnrS*), lane 10 = negative control *qnrS*.

Analytical specificity

To avoid false positive results, each assay was tested against DNAs extracted from bacteria not harbouring its target gene. This experimental setting enabled to evaluate the cross-reactivity among the assays; no increase in fluorescence associated with a sigmoidal amplification curve was observed for any assays when tested against no-target genes (Supplementary material 1).

Efficiency, analytical sensitivity and intra-assay variability

Positive control DNAs (previously amplified by end-point PCR) of known concentration (ng/µl) were serially diluted (1 : 4 or 1 : 5) and used to construct the standard curves. The Cp values of these standards were plotted against the logarithm of their concentration. The technical assay dynamic range along with the limit of detection (LoD), the limit of quantification (LoQ) and the efficiency of the assays was determined. The correlation coefficient (r^2), which provides an estimate of the goodness of fit of the data points to the linear trend-line, was also calculated. As depicted in Table 3, all the assays show good efficiency (from 89.6% to 95.3%), linearity ($r^2 > 0.98$), LoQ (from 8.13 to 320.80 gene copies number) and LoD (from 1.50 to 35.94 gene copies number). Furthermore, all assays showed good intra-assay repeatability, and mean Ct (three replicates), standard deviation (SD), and coefficient of variation (CV) for low (*i.e*. LoD), medium and high concentrations are reported in table 4.

Target gene	Efficiency	r^2	Dynamic Range (Cp values)	LoD (copies number)	LoQ (copies number)
ermB	91.8%	0.9845	17.18-37.69	5.01	320.80
$bla_{CTXM1\text{-}like}$	89.3%	0.9966	16.72-31.62	35.94	57.50
$bla_{CMY\text{-}2}$	93.6%	0.9992	14.45-31.94	28.92	46.27
qnrA	95.3%	0.9984	22.32 - 37.77	1.50	96.10
qnrS	92,8%	0.9992	11.25-30.84	2.03	8.13

Table 3. Efficiency, r^2, dynamic range, LoD and LoQ of the five assays.

Target gene	Concentration ng/µl	Mean	SD	CV
ermB	4.25E-05	19.26	±0.09	0.48
	6.60E-07	25.67	±0.03	0.11
	6.48E-10	37.69	±1.12	2.96
$bla_{CTXM1\text{-}like}$	4.25E-05	16.72	±0.06	0.34
	6.60E-07	23.55	±0.07	0.30
	2.59E-09	31.62	±0.66	2.10
$bla_{CMY\text{-}2}$	4.25E-05	16.67	±0.01	0.04
	6.64E-07	22.85	±0.07	0.31
	2.59E-09	31.93	±0.19	0.60
qnrA	4.25E-05	24.08	±0.20	0.82
	6.64E-07	30.44	±0.16	0.51
	2.59E-09	37.77	±0.85	2.25
qnrS	4.25E-05	13.37	±0.01	0.05
	6.64E-07	19.94	±0.02	0.11
	6.48E-10	30.84	±0.03	0.09

Table 4. Repeatability of the qPCR assays. Mean Ct (three replicates), standard deviation (SD) and coefficient of variation (CV) are reported for each ARG according to target gene DNA concentration.

Prevalence and absolute abundance of target ARGs in environmental samples

The assays were tested against 93 environmental samples (*i.e.* soil and manure); *ermB* was the gene showing the highest prevalence (81.72%), followed by $bla_{CMY\text{-}2}$ (58.06%), $bla_{CTXM1\text{-}like}$ (30.11%) and *qnrS* (24.73%), while *qnrA* was not detected in any sample. To further confirm the specificity of the assays, the amplicons obtained from the environmental samples were Sanger sequenced and BLAST searched against the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca) using the FASTA sequences; 100% agreement between the results yielded by the qPCR assays and the sequences was observed. Kappa (ƙ) values were calculated as a measure of overall agreement between each qPCR assay and the sequencing results, which proved to be perfect (ƙ = 1). Statistical analysis was performed using in GraphPad Prism version 9.1.1 (https://www.graphpad.com). The absolute abundance of each ARG in the environmental samples was calculated based on the respective standard curve and it ranges from below the LoQ to 206.28, 242.38, 229.09 and 134.659 copies number for *ermB,* $bla_{CTXM1\text{-}like}$*,* $bla_{CMY\text{-}2}$ and *qnrS*, respectively. However, the absolute abundance of ARGs in a given sample is not a significant value, as it is proportional to the total DNA

present in the sample; therefore, 16S rRNA gene copy number should be obtained (*e.g.* by analysing the samples with the qPCR assay developed by Nadkarni *et al.* (2002) and ARGs relative abundance should be calculated by normalizing the ARG copy number to 16S rRNA gene copies.

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None

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101488.

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SECTION 4: General Conclusions

General Conclusions

The ecotoxicological section of this thesis focused on three FQs, for veterinary (ENR, FLU) and human (LEV) use, that are currently considered as emerging contaminants. The results of the first experimentation indicated that these pharmaceuticals have moderate toxicity towards tomato, lettuce and carrot, especially if we consider that the applied assay, in which the seeds are exposed to aqueous solutions of the active ingredients, does not take into account the water/soil partition of the three antimicrobials. Indeed, they have a significant tendency to adsorb to soil particles; therefore, in the natural environment, their availability to be absorbed by roots would be presumably very low. Everything considered, it was decided to shift focus on the toxicity of the three antimicrobials towards the freshwater model organism *Daphnia magna*. The particular design of the tests carried out on embryos and newborns of the crustacean, with follow-up in pure medium, allowed to highlight a delayed toxicity that the standard tests (acute and chronic) are not able to detect. Indeed, the official tests overestimate the ecotoxicity parameters (EC_{50}, NOEC), leading to an inevitable underestimation of the environmental risk. The risk assessment should also take into account the possible coexistence of the different contaminants in the environmental matrices. For this reason, the type of interactions that the three FQs carry out in the context of binary or ternary mixtures was also investigated. The results indicated their tendency to additive interactions. This represents another information that may be useful to regulatory authorities. Moreover, since the standard chronic test is carried out on a single generation of daphnids, it is not able to highlight any phenomenon of multigenerational/transgenerational toxicity. However, previous research had highlighted the ability of flumequine to cause severe effects on the phenotype of daphnids, even in the generation that followed three non-exposed generations. It was therefore considered of interest to investigate also this phenomenon. This was done by setting up a particular test, over four generations, that would provide sufficient sample size for subsequent biomolecular analysis (DNA, RNA), aimed at highlighting epigenetic and gene expression effects. This test evidenced new sublethal effects on the phenotype (lethargy, reduced sensitivity to light) and provided samples for the genome analysis, which is currently ongoing. The final results of this study could be of particular interest because the genome sequence of *Daphnia* has a high number of genes shared with that of the human species. This genetic overlap means that, in addition to its relevance for ecotoxicological tests, *Daphnia magna* can represent a useful model to explain the dangerous side effects observed in human patients treated with FQs, which are the subject of particular interest by pharmacovigilance authorities.

The section related to antimicrobial resistance investigated the effects of animal manure fertilization on the spread of antimicrobial resistance genes (ARGs) and antimicrobial residues in the enviroment, and on the composition of the soil. The results of this study showed that ARGs were more abundant in manure/slurry than in soil, and that, after fertilization, some ARGs (*i.e. ermA, ermB,* bla_{OXA-1} and *oqxA*) were transferred to the soil. Furthermore, the analysis revealed that the composition of the microbial community in the manure/slurry is different and less rich than that in the soil. After fertilization, the soil microbiome is only

partially altered, with the exception of a few phyla (Firmicutes, Tenericutes, and Cloacimonetes). Therefore, the data gathered seem to indicate that the use of manure/slurry in agricultural soil fertilization has a limited impact on soil microbial communities, but can contribute to the spread of specific ARGs in the environment. The residues of flumequine and enrofloxacin were the compounds detected most in all samples, not only in manure but also in soil. The remainder antimicrobials were detected only in manure.

Real-Time PCR is the method used in our studies for the detection and quantification of resistance genes. The SYBRGreen chemistry paired with the melting curve analysis has proven to be a specific and sensitive method, cost-effective and reproducible, and therefore represents a useful tool for studies on surveillance of AMR dissemination, in particular when testing a large number of samples in very complex matrices, such as environmental samples. This tecnique can also be easily implemented by designing new specific primer pairs for the detection and the quantification of new emerging ARGs. For the Real-Time PCR analyses, two novel assays have been developed for the detection and quantification of *mcr-4* and *mcr-5* and five previously published assays *(i.e.* four end-point PCR and one probe-based qPCR) have been optimized, increasing the assays available for the screening of ARGs by Real-Time PCR.

List of Publications

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