Environment International 115 (2018) 312–324

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Review article

# Antibiotic resistance in wastewater treatment plants: Tackling the black box

Célia M. Manaia[a,*], Jaqueline Rocha[a], Nazareno Scaccia[a], Roberto Marano[b,c], Elena Radu[d,e], Francesco Biancullo[f,g], Francisco Cerqueira[h], Gianuário Fortunato[a], Iakovos C. Iakovides[i], Ian Zammit[j], Ioannis Kampouris[k], Ivone Vaz-Moreira[a,l,*], Olga C. Nunes[l,**]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal
[b] Department of Agroecology and Plant Health, Robert H. Smith Faculty of Agriculture, Food, and Environment, The Hebrew University of Jerusalem, Rehovot, Israel
[c] Institute of Soil, Water, and Environmental Sciences, Agricultural Research Organization, Volcani Center, Rishon Lezion, Israel
[d] University of Technology Vienna, Institute for Water Quality and Resources Management, Karlsplatz 13/226, A-1040 Vienna, Austria
[e] AGES - Austrian Agency for Health and Food Safety, Spargelfeldstraße 191, A-1220 Vienna, Austria
[f] Laboratory of Separation and Reaction Engineering - Laboratory of Catalysis and Materials (LSRE-LCM), Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal
[g] Adventech-Advanced Environmental Technologies, Centro Empresarial e Tecnológico, Rua de Fundões 151, 3700-121 São João da Madeira, Portugal
[h] Department of Environmental Chemistry, IDAEA-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain
[i] NIREAS-International Water Research Center and Department of Civil and Environmental Engineering, University of Cyprus, P.O. Box 20537, 1678 Nicosia, Cyprus
[j] Department of Civil Engineering, University of Salerno, SP24a, 84084 Fisciano, SA, Italy
[k] Institute for Hydrobiology, Technische Universität Dresden, 01217 Dresden, Germany
[l] LEPABE, Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

ARTICLE INFO

*Keywords:*
Antibiotic resistance monitoring
SWOT analysis
Wastewater treatment optimization

ABSTRACT

Wastewater is among the most important reservoirs of antibiotic resistance in urban environments. The abundance of carbon sources and other nutrients, a variety of possible electron acceptors such as oxygen or nitrate, the presence of particles onto which bacteria can adsorb, or a fairly stable pH and temperature are examples of conditions favouring the remarkable diversity of microorganisms in this peculiar habitat. The wastewater microbiome brings together bacteria of environmental, human and animal origins, many harbouring antibiotic resistance genes (ARGs). Although numerous factors contribute, mostly in a complex interplay, for shaping this microbiome, the effect of specific potential selective pressures such as antimicrobial residues or metals, is supposedly determinant to dictate the fate of antibiotic resistant bacteria (ARB) and ARGs during wastewater treatment. This paper aims to enrich the discussion on the ecology of ARB&ARGs in urban wastewater treatment plants (UWTPs), intending to serve as a guide for wastewater engineers or other professionals, who may be interested in studying or optimizing the wastewater treatment for the removal of ARB&ARGs. Fitting this aim, the paper overviews and discusses: i) aspects of the complexity of the wastewater system and/or treatment that may affect the fate of ARB&ARGs; ii) methods that can be used to explore the resistome, meaning the whole ARB &ARGs, in wastewater habitats; and iii) some frequently asked questions for which are proposed addressing modes. The paper aims at contributing to explore how ARB&ARGs behave in UWTPs having in mind that each plant is a unique system that will probably need a specific procedure to maximize ARB&ARGs removal.

## 1. Introduction

Urban wastewater treatment plants (UWTPs) have a pivotal role in the protection of the environment, in particular, the natural water bodies. The removal of organic matter, chemical pollutants and undesirable microorganisms from sewage, using combinations of physico-chemical and biological treatments, was a major technological achievement of the last century, allowing the return to the environment

*Abbreviations:* ARB, Antibiotic Resistant Bacteria; ARGs, Antibiotic Resistance Genes; BOD, Biological Oxygen Demand; COD, Chemical Oxygen Demand; epicPCR, Emulsion, paired isolation and concatenation Polymerase Chain Reaction; FISH, Fluorescence in situ hybridization; HGT, Horizontal gene transfer; PCR, Polymerase Chain Reaction; qPCR, Quantitative PCR; SWOT, Strengths, Weaknesses, Opportunities and Threats; UWTPs, Urban wastewater treatment plants

* Corresponding authors at: Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal.
** Corresponding author at: LEPABE, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal.
*E-mail addresses:* cmanaia@porto.ucp.pt (C.M. Manaia), ivmoreira@porto.ucp.pt (I. Vaz-Moreira), opnunes@fe.up.pt (O.C. Nunes).

https://doi.org/10.1016/j.envint.2018.03.044
Received 20 December 2017; Received in revised form 5 March 2018; Accepted 28 March 2018

of water with good quality. However, the final UWTPs effluents are far from being sterile and, hence, release to the environment high amounts of bacteria, many of which are of animal (e.g. pets or small husbandry or animal farms) or human origin (Berendonk et al., 2015; Manaia, 2017; Rizzo et al., 2013). Many of these bacteria harbour acquired antibiotic resistance genes (ARGs) and are potential carriers for the dissemination of these genes in the environmental microbiome (Berendonk et al., 2015; Manaia, 2017; Pruden, 2014). As such, these bacteria are considered a potential threat to humans and/or animals health since they may lead to more cases of difficult-to-treat infections. Moreover, although only part of the ARB released from UWTP will be able to cause disease in humans or animals, the risk of enriching the environmental resistome either through selection or horizontal gene transfer (HGT), and therefore contribute to the emergence of resistance in pathogenic bacteria cannot be neglected (Manaia, 2017). UWTPs bring together antibiotic resistant bacteria (ARB), antibiotic residues and other potential selectors that favour the selection towards these bacteria and, simultaneously, offer a rich supply of nutrients and close cell-to-cell interaction, capable of facilitating the horizontal transfer of ARGs. These arguments make the UWTPs environment one of the most exciting niches to unveil the fate of ARB&ARGs. This paper is the result of a think tank of Early Stage Researchers summer school organized by the Marie Skłodowska-Curie Innovative Training Networks, project ANSWER (http://www.answer-itn.eu/), and discusses the tools and the environmental conditions that may rule the fate of ARB&ARGs throughout the wastewater treatment.

The paper is divided into four major sections: 1) one dissecting the UWTPs compartments where analyses of ARB&ARGs may be relevant given the potential constraints that are imposed to the microbiota, as well as 2) the bio-physico-chemical conditions that may shape the dynamics of populations and genes within the bacterial communities; 3) another revising the pros and cons of the most commonly used methods to analyse antibiotic resistance in environmental samples; and 4) a final section where the previous three are combined to give an integrated overview of the major information on ARB&ARGs ecology, exemplified through the answers to some frequently asked questions. Above all, this work intends to serve as a guide for wastewater professionals who aim at optimizing wastewater treatment for the removal of ARB&ARGs.

## 2. Urban wastewater treatment plant, the big black box

UWTPs were first developed to assure the removal of debris, high organic loads and pathogens from sewage before discharging into environmental receptors (water streams/rivers, lakes, sea). Benefits of their worldwide implementation include avoidance of eutrophication and the spread of potentially harmful microorganisms (Henze et al., 2008). However, socio-economic evolution and increasing human population density created new challenges for an efficient wastewater treatment, with the consensual recognition that improvements are required in order to produce final effluents that effectively will protect the environment and humans.

Nowadays, a wide variety of UWTPs designs are available. Nonetheless, all of them assemble at least 3 sequential steps: the preliminary (pre)-, the primary-, and the secondary-treatment (Grady et al., 2011; Henze et al., 2008). Pre-treatment aims at removing from the raw wastewater all the materials that can damage the downstream equipment, including bulky solids and sand which are mechanically removed or settled. In addition, in some UWTPs this step includes an equalization tank, not only to avoid flow peaks but also to homogenise the raw wastewater composition, avoiding the sporadic income of high loads of chemicals, which could inhibit the following secondary treatment. The removal of the floating fat and grease is also undertaken in some large UWTPs. The remaining sedimentable solids are removed in the primary settling tanks, and channelled into the sludge treatment facilities, whereas the effluent of this primary treatment enters the secondary treatment. A wide variety of processes are nowadays available for secondary treatment, but all of them aim at removing the biodegradable compounds from wastewater (Henze et al., 2008). Suspended and/or dissolved compounds are mainly those resultant from human excreta, food waste, and detergents, but a wide variety of inorganic (e.g. heavy metals) and organic compounds (e.g. pharmaceutical residues, pesticides) is also present (Henze and Comeau, 2008; Köck-Schulmeyer et al., 2013; Rizzo et al., 2013; Tchobanoglous et al., 2003). Hence, wastewater does not only contain microorganisms and readily biodegradable compounds but also recalcitrant substances, some of which may be potentially toxic to at least a fraction of the cells entering and/or inhabiting the reactor(s), i.e., substances capable of generating selective pressure. Nevertheless, the high organic load of the wastewater supports the growth of the microbiota able to cope with the prevailing conditions, which consequently can reach high densities. The resultant excess of biomass must be removed, although its release to the environmental receptors should be avoided. This is possible thanks to the prevailing conditions in the secondary treatment that favour the floc/biofilm forming organisms. The extracellular polymer substances (EPS) produced by these cells act as adsorbents not only of microorganisms unable to produce EPS but also of organic and/or inorganic chemical compounds, the so-called activated sludge, which is settled in the secondary sedimentation tanks. Hence, the microbial load of the secondary effluent is 1 to 2 log-units lower than raw wastewater (EPA, 1986), and the spent biomass is channelled to the sludge treatment line. Indeed, microbes that enter, survive or even proliferate during the wastewater treatment can be pollutants themselves if released in the environment, in the sense that they will occur in an environment to which they do not belong, and where they can cause directly or indirectly any kind of damage.

At least in some countries, conventional wastewater treatment relies mostly upon activated sludge tanks to reduce the organic load of the primary effluent to values compatible with its discharge in the environment (EEA, 2017; Grady et al., 2011). However, upgraded UWTPs include re-circulation of the mixed liquor between aerobic, anoxic, or even anaerobic tanks to ameliorate the removal of inorganic N and P, respectively, from the secondary effluents (EEA, 2017; Grady et al., 2011). The tertiary treatment has been increasingly regarded as a measure to obtain a final treated wastewater of high quality, i.e., not only without readily organic metabolizable compounds but also free of nutrients (N/P) and recalcitrant chemical micropollutants as well as with very low microbiological loads (Henze et al., 2008). Given the high costs involved in the removal of the chemical micro-pollutants, most of the currently operating UWTPs if include any additional step, this is the disinfection of the secondary effluent, before discharge in the environment (EEA, 2017). Chlorination, UV radiation, and ozonation are the most common disinfection technologies currently applied in WWTPs (e.g., EPA-Victoria, 2002; EPA, 1986). Fig. 1 summarizes the main steps of the majority of the UWTPs operating nowadays worldwide.

### 2.1. Wastewater treatment events affecting ARB and ARGs

Looking into the process from a microbiological point of view, sharp variations occur in each wastewater treatment step. Sewage microbiota is mainly composed of human commensal bacteria, which is mixed with bacteria from distinct origins that may be entering and colonizing the sewage system (e.g., Cai et al., 2014; Shanks et al., 2013; Shchegolkova et al., 2016; Wang et al., 2014). In this environment, the fraction of ARB may reach more than 50% at least within a given group (e.g., enterobacteria or enterococci) (e.g., Manaia et al., 2016; Rizzo et al., 2013). A high fraction of the organisms thriving in sewage adheres to organic and/or inorganic particles, which in first instance can be removed from wastewater if retained in the primary sedimentation tank. Nonetheless, those in suspension or forming less dense flocs end up in the biological treatment tank(s). The secondary treatment is thus where the fraction of ARB&ARGs not removed in the primary treatment gets in

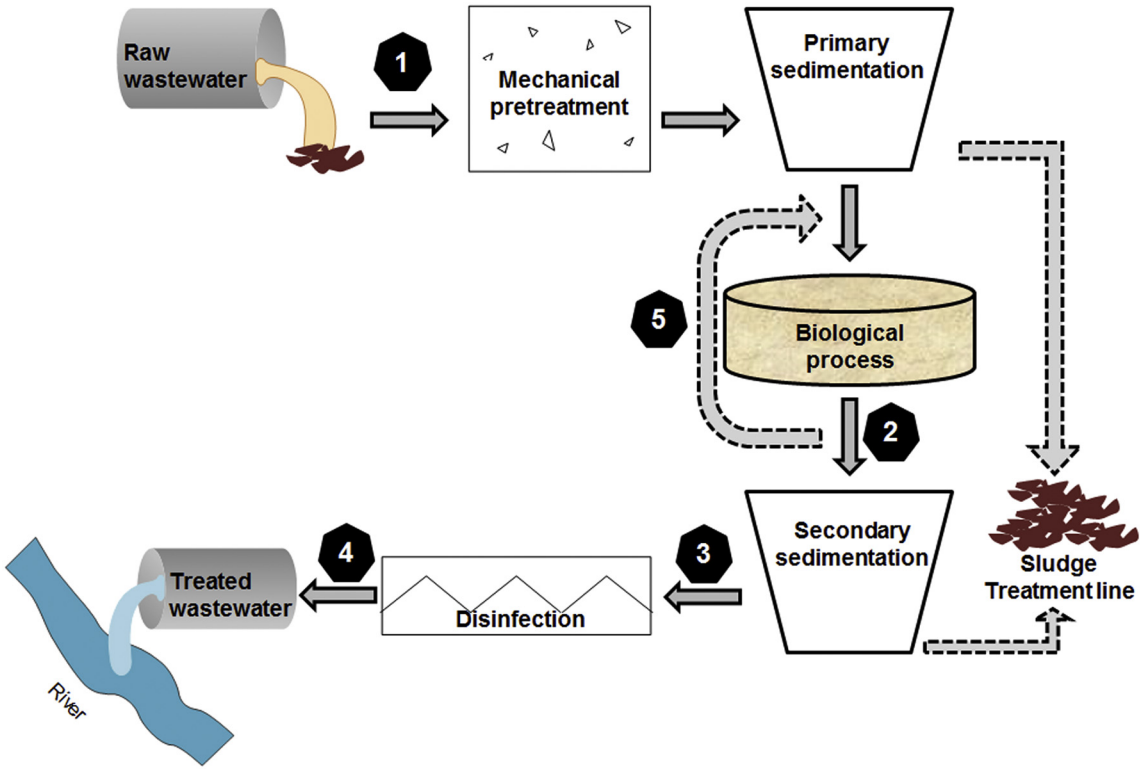


**Fig. 1.** Schematic of the UWTP with sampling sites 1–5 labelled: 1) pre-treated raw wastewater; 2) biological treatment; 3) secondary effluent; 4) final effluent; 5) recirculating activated sludge. (Recommended: 24 h composite water samples or multiple sludge sub-samples; 1–2 L volume; three days in a row each trimester; for DNA samples, preferentially three replicates at each sampling time.)

contact with sludge bacteria, and with potential selective pressures present in the raw inflow. To potentiate the degradation process, previously formed biomass (activated sludge), settled in secondary sedimentation tanks, returns to the biological reactor(s) where it is mixed with the arriving primary effluent. This process ensures enough cells to reduce the organic load of the wastewater in the shortest time possible, i.e. achieve a low hydraulic retention time. Hence, the arriving microbiota, including ARB, is stimulated to compete with sludge bacteria for the available organic matter. The intense metabolic activity that takes place during the biological treatment leads to important bacterial community dynamics. The shifts occurring in the bacterial community can be dramatic at this stage and the fitness of ARB and the success with which their ARGs are spread to other bacteria, through HGT (Marano and Cytryn, 2017), will be crucial to dictate the effectiveness of treatment on resistance removal.

Besides the readily metabolised organic matter, wastewater contains substances that may exert an array of effects on bacteria, being sometimes designated as stressors, of which are examples heavy metals, and recalcitrant natural or synthetic compounds, including antibiotic residues and metabolites thereof. These stressors may also shape the surviving community because different organisms or groups of related organisms have different degrees of tolerance or defensive responses against their adverse effects (Berendonk et al., 2015; Manaia et al., 2016; Seiler and Berendonk, 2012). Moreover, the stressors, together with the high nutrient load, stable pH and temperature, and the close proximity of cells in the flocs, may facilitate HGT of genetic elements encoding for resistance, including against antibiotics (Di Cesare et al., 2016; Dröge et al., 2000; Kim et al., 2014; Marano and Cytryn, 2017). Altogether, the conditions prevailing inside the biological treatment tanks may lead to the numerical or proportional increase of the cells capable of tolerating adverse conditions, including antibiotics. Eventually, the majority of these cells settle in the secondary sedimentary tank, and only a minority will leave the conventional UWTPs without further treatments, suspended in the secondary effluent. However, as described above, part of these cells return to the biological tank, in a cycle that allows the enrichment of sludge with cells highly adapted to the conditions prevailing in the UWTPs, including a wide array of stressors. Hence, when finally released, the secondary effluent may represent a discharge of up to $10^{12}$ ARB/day or $10^{18}$ ARGs/day (Manaia et al., 2016; Vaz-Moreira et al., 2014). In this context, the time ARB stay in the reactor may be a critical determinant of the likelihood of their proliferation or of being involved in HGT events. In this aspect, it is arguable that the re-recirculation of biomass between different tanks can promote the enrichment of the sludge in ARB and ARGs, clearly an undesirable consequence of upgrading UWTPs with improved nutrient removal (EEA, 2017). Table 1 reviews the parameters that may influence the fate of ARB&ARGs in each treatment stage, and how their variations and potential impact can be assessed throughout the treatment process.

In UWTPs with a disinfection step, the cells face again the stressful conditions imposed by the disinfection process before discharge into the environment. From the microbiological and genetic points of view, there is no reason to expect that ARB can survive better to disinfection than their susceptible counterparts. Hence, disinfection, given its capacity to reduce the microbial loads, will contribute to the removal of ARB&ARGs (Manaia et al., 2016). However, attention must be given to the disturbance of microbial communities and reactivation of ARB&ARGs during treated wastewater storage. It has been demonstrated that when disinfection is applied, a great fraction of cells will die, including ARB, while others will enter a state of dormancy due to the stress, recovering when the stressors are released (Becerra-Castro et al., 2016; Moreira et al., 2018; Sousa et al., 2017), which in nature is what happens, for example, due to the dilution of the final effluent in the receiving water body or water storage. Disinfection and the fate of ARB &ARGs is an issue far from being fully understood. It is known that the effectiveness of processes such as chlorination, UV radiation, and

**Table 1**
Sampling sites and type of microbiological and potentially related parameters to analyse and expected outcomes regarding antibiotic resistance removal (according to Fig. 1).

| Site (No. refers to Fig. 1) | Parameters | Expected outcomes |
|---|---|---|
| Raw wastewater (1) | Total microbial load | Indication of the "contamination index" of the inflow and the required treatment effort |
| Biological treatment (2) and secondary effluent (3) | Quantification of ARB&ARGs (abundance or prevalence)[b] | Measure of the increase/decrease of the total microbial and/or ARB&ARGs loads<br>Inference about selection and/or HGT due to selective pressures (antibiotics, heavy metals) or treatment |
| | Concentration/dose of other contaminants (e.g. heavy metals, biocides, antibiotic residues) | The difference between 2 and 3 will give an insight of the fraction of ARB&ARGs that was removed (meaning incorporated in the sludge, loss of viability or outcompeted)<br>The difference between 1 and 3 will give an insight of the fraction of total microbial load, ARB&ARGs that was removed<br>Evaluation of possible improvements of biological/secondary treatment regarding ARB/ARGs removal |
| Final effluent[a] (4) | | The difference between 3 and 4 will give an insight of the disinfection/tertiary treatment efficiency<br>The overall efficiency of wastewater treatment<br>Potential impact of the discharge of treated wastewater on the surrounding/receptor environment |
| Recirculating activated sludge (5) | Quantification of ARB&ARGs (abundance or prevalence)[b] | Compared with 2 and 3 gives an indication if sludge recirculation may contribute to ARB&ARGs enrichment |
| | Concentration/dose of other contaminants (e.g. heavy metals, biocides, antibiotic residues) | May contribute to estimate the critical sludge age (e.g. when desorption of contaminants or ARB&ARGs starts)<br>Inference about co-selection of ARB/ARGs due to sorption of chemicals (e.g. antibiotics) on particles |

ARB, antibiotic resistant bacteria; ARGs, antibiotic resistance genes; HGT, horizontal gene transfer.
[a] Can be secondary effluent, disinfected secondary effluent, or tertiary effluent, depending on the UWTP configuration.
[b] Abundance, is the number of cells or gene copy number *per* mass or volume of sample; prevalence or relative abundance, is the number of cells or gene copy number *per* total number of cells or gene copies of a housekeeping gene (e.g. 16S rRNA gene).

ozonation are not only dose-dependent but also influenced by several physico-chemical factors (Alexander et al., 2016; Di Cesare et al., 2016; McKinney and Pruden, 2012). While high doses of disinfectant may impose severe microbial community rearrangements, it is also observed that higher doses may be needed to inactivate ARGs than bacteria (McKinney and Pruden, 2012; Moreira et al., 2018). In addition, some disinfectants, as peracetic acid or chlorine were even observed to have a selective effect on ARGs (Di Cesare et al., 2016). This selective effect means that even if a decrease in abundance (gene copies *per* mL of sample) is observed, the relative abundance or prevalence of the gene (gene copies *per* total bacteria) may increase. However, considering that disinfection can lead to reductions of 2–4 log-units in the bacterial loads, even in the presence of increases of ARB&ARGs prevalence after disinfection, it may be a preferable alternative, in particular for critical effluents as those discharged by hospitals (Manaia, 2017). Nevertheless, the recommended disinfection approach in face of type, volume and final destiny of the effluent, as well as, the balance environmental benefits versus associated costs is an issue still under debate and for that reason, no specific disinfection process was considered in Table 1 or Fig. 1.

## 3. Major factors affecting the fate of ARB and ARGs

In spite of the expected influence that abiotic factors (e.g. temperature, pH, electric conductivity, among others) may have on the bacterial community dynamics and, hence, on the fitness of ARB during wastewater treatment, no evidence has been gathered so far in a way that the measurement of a specific bio-physico-chemical factor or condition could be used as unequivocal predictor of the efficiency of ARB removal during secondary treatment. While it is not expected that any of these conditions will have a selective effect on ARB in relation to antibiotic susceptible bacteria, they will certainly have the potential to influence the development of specific physiological and biochemical groups of bacteria. In this sense, these factors may influence the survival of ARB. Many ARB of simultaneous clinical and environmental relevance are strictly chemoorganoheterotrophic, mesophilic, neutrophilic and facultative or aerobic bacteria, whose survival and proliferation will be conditioned by the adequate balance of all these variables. Some studies explored the effect of temperature and pH on the removal of ARGs during sludge treatments to conclude that high temperatures or high pH were more effective than other conventional or low temperature treatments in the removal of ARGs or class 1 integrons (Diehl and Lapara, 2010; Gao et al., 2012; Munir et al., 2011; Zhang et al., 2015). Arguably, temperature or pH values deviating from the mesophilic (temperature) and neutral (pH) range where most ARB thrive have the potential to contribute to ARB&ARGs removal. This rationale may explain the observations of those authors that studied the effects of these parameters on ARGs removal from sludge. However, it is not possible to extrapolate such effects for wastewater treatment, where pH and temperature are maintained in the mesophilic and neutral range. For wastewater treatment, it is probably the combination of all the factors listed in Table 2, and eventually many others, instead of a single factor per se, that rules the fate of ARB&ARGs during wastewater treatment.

Despite these considerations, different studies aimed at finding relevant associations between some of the listed bio-physico-chemical factors or conditions and the variation of antibiotic resistance during wastewater treatment. For instance, Novo et al. (2013) observed a positive correlation between temperature and loads of sulfonamide resistant bacteria in treated wastewater, although no significant correlation between ARB and the chemical oxygen demand (COD), biological oxygen demand (BOD), water flow or temperature in raw wastewater. Also, Di Cesare et al. (2016) observed a relationship between the prevalence of the genes *tetA*, *ermB*, *qnrS* and the different biotic and abiotic factors (total organic carbon, the overall prokaryotic cell number, and the number of bacterial aggregates). Kim et al. (2007) tried to understand the fate of tetracycline resistant bacteria as a function of activated sludge organic loading rate and growth rate, using lab-scale sequencing batch reactors (SBRs). These authors concluded that the organic load (achieved by altering the inflow wastewater flux) and growth rate (achieved by altering sludge retention time, SRT) resulted in an increase of tetracycline resistance.

The measurement of antibiotic residues in wastewater samples and the attempt to correlate with the occurrence of antibiotic resistance

**Table 2**
Bio-physico-chemical factors and conditions which measurement is recommended and the type of information that can provide (according to Fig. 1).

| Bio-physico-chemical factors and conditions | Reasons to study this factor | Site (Fig. 1) |
|---|---|---|
| Temperature; pH; EC; DO | These factors may affect the microbial community composition and structure because they may impose stress conditions capable of inducing the survival/overgrowth of specific populations; | 1–5 |
| COD; BOD; Total N, C, P; TSS | The removal of total organic load (biodegradable and non-biodegradable) indicates treatment efficiency;<br>The organic load and other nutrients (P and N) affect bacterial growth/survival;<br>TSS reflects the bacterial load and the cell aggregation capacity (likelihood of HGT and bulking risk); | 1–4 |
| HRT, SRT, wastewater flow and expected dilution factor in the receptor environment | Retention times are important as an estimate of the potential opportunities for bacterial selection and/or genetic recombination (HGT);<br>Wastewater flow is useful to assess the impact of the wastewater discharge in the surrounding/receptor environment regarding ARB&ARG dissemination; | 1–5 |
| Antibiotic residues, metals (e.g. As, Pb, Hg, Cd, Cu, Ni, Zn, Cr, etc) and other contaminants | Potential impact on the wastewater and sludge microbial communities as selective pressures;<br>Impact of the wastewater discharge in the surrounding/receptor environment regarding micropollutants contamination; | 1–5 |
| Bacterial community composition and ARB&ARG quantification | Variations in the bacterial community composition and structure in each step of the treatment;<br>Potential correlations between specific populations and some ARB&ARG;<br>Combined with wastewater flow and expected dilution factor in the receptor environment allows the assessment of the impact of wastewater regarding ARB&ARG dissemination; | 1–5 |

EC, electric conductivity; DO, dissolved oxygen; COD, chemical oxygen demand; BOD, biological oxygen demand; TSS, total suspended solids; HRT, hydraulic retention time; SRT, sludge retention time.

may be challenging. First, because of the lifetime of antibiotics, which can be biodegraded, transformed or adsorbed to sediments, is too short when compared with that of ARB that can proliferate and transfer ARGs. Second, because the concentrations measured in bulk samples hardly reflect what can be found in a bacterial microenvironment. However, although not detected in the commonly used sampling and analytical protocols, these potential selectors may have exerted their effect, affecting the bacterial community, and possibly giving an advantage to some bacteria, including ARB. This is a hypothesis that should be considered, even if difficult to prove in field conditions. In spite of these constraints, some putative indications of selective pressures have been reported. Novo et al. (2013) found a positive correlation between tetracyclines concentration in raw wastewater and the loads of resistant bacteria present in the final effluent. In a study with municipal and hospital effluents, Varela et al. (2014) found a significant positive correlation between ciprofloxacin and arsenic concentration and the loads of quinolone resistant bacteria, while Narciso-da-Rocha et al. (2014) also observed significant positive correlations between the concentration of sulfonamides and tetracycline and the prevalence of the genes *intI1*, $bla_{TEM}$, and *vanA*. In none of these studies, it was possible to conclude about a cause-effect relationship, although it is possible to witness the association of resistance removal or enhancement with a specific environmental variable. Yet, it is worth mentioning that such associations may result simply from the simultaneous occurrence of two events. For example, high concentrations of antibiotics co-occurring with a high density of ARB or removal of organic matter co-occurring with starvation of bacteria and hence the decrease of bacterial loads. In theory, selective pressures can either exert their effect due to the favouring of a subset of individuals in a community and/or by promoting HGT. Indeed, estimates of the predicted antibiotics concentration that might contribute to select for ARB has been proposed by different authors (Bengtsson-Palme and Larsson, 2016; Gullberg et al., 2011, 2014; Tello et al., 2012). According to these studies, concentrations able to select for ARB or to promote HGT can be very low, below the inhibitory concentrations (sub-inhibitory concentrations). Kim et al. (2014) observed that the pB10 plasmid transfer rate to the activated sludge microbiota could be significantly increased in the presence of 10 to 100 ppb of tetracycline or sulfamethoxazole. Similar results were observed by Jutkina and colleagues (Jutkina et al., 2016, 2018) for tetracycline (10 μg/L), chlorhexidine (24.4 μg/L), triclosan (100 μg/L), gentamicin (100 μg/L) and sulfamethoxazole (1000 μg/L). While these concentrations do not inhibit antibiotic susceptible bacteria, it is known that they are normally found in wastewater environments (Kulkarni et al., 2017; Kümmerer, 2009; Michael et al., 2013). In spite of these evidences, it is consensual that, in practice, in extremely complex microbial communities subjected to variable environmental conditions and in the presence of a myriad of chemical (organic, inorganic, polymeric) substances with variable life-times, it is difficult to predict the selective effect taking place in a UWTP in a given moment.

Other ecological drivers are also supposed to affect the ecology of ARB&ARGs in the biological reactors and sedimentation tanks. For example, the selective and unselective predation or the viral lysis may have an impact on ARB&ARGs fate, and that deserves further investigation. For instance, the interactions of ARB with predatory bacteria (e.g. *Bdellovibrio*), protozoa or virus (Miki and Jacquet, 2008) may be worth of studying. Indeed, heterotrophic nanoflagellates (HNF), together with ciliates, are recognized as a major source of bacterial predation/mortality in soil, marine, and freshwater environments (Miki and Jacquet, 2008). They may affect the bacterial community through the excretion of growth stimulating compounds, enhancing the bacterial activity, or by contrast, the selective predation of some bacteria (Faust and Raes, 2012; Miki and Jacquet, 2008). In addition, these eukaryotes can also work as a vehicle for some bacteria that can survive, and even replicate, within predatory protozoa. Also, virus may impact the bacterial community composition through selective mortality through bacterial lysis (Bouvier and Del Giorgio, 2007; Winter et al., 2004). The role of these agents as biotechnological tools for ARB control should not be overlooked.

### 3.1. Where to sample and what for?

To estimate the contribution of UWTPs on the fate of ARB&ARGs, the sites where samples are collected and the parameters analysed must be selected based on specific questions. Because all the steps of the treatment are interconnected, when following the fate of ARB&ARGs in a UWTP, a sampling schedule should be carefully planned. It may be important to sample each of the described major steps of the treatment (Fig. 1, Table 1). It is also important to evaluate the influence of possible selective pressures through the analysis of contaminants such as heavy metals, biocides or antibiotic residues, as well as other physico-chemical parameters currently analysed in UWTPs (Table 2). A sufficient number of representative samples should be collected over time, allowing statistical analyses in order to assess whether a given event is consistently observed or is simply a random occurrence (Novo et al., 2013; Novo and Manaia, 2010; Varela et al., 2014). Yet, even if it is a random event, it may be relevant to infer about the implications it might have on the fate of ARB&ARGs.

## 4. How to track ARB and ARGs in the UWTP?

Different methods are available to study either the diversity of ARB &ARGs or to measure the abundance (*per* mass or volume of sample) or prevalence (*per* total bacteria) in a given environment (Supplementary Table S1). These methods are often designated as culture-dependent and culture-independent, depending on the use of cultivation methods or direct analyses of nucleic acids (DNA or RNA). In addition, methods can be classified as targeted and non-targeted, according to the capability of searching for a specific bacterial group or gene or of surveying the community as a whole, respectively. Culture-dependent methods can survey microorganisms that are viable at a given moment and for which the growth conditions are known. In spite of skipping non-culturable microorganisms, culture-dependent methods have the important advantage of permitting the phenotypic characterization of isolates, relevant to assess for instance their profile of multidrug resistance, the minimum inhibitory concentration of different antibiotics or the capability to take part in HGT processes. However, depending on the type of environment, less than 1–10% of bacteria can be culturable (Amann et al., 1995; Vaz-Moreira et al., 2013), with the largest majority of environmental bacteria falling, permanently or transiently, within the wide category of non-culturable microorganisms. Viable but non-culturable bacteria (VBNC) have been extensively studied, being recognized that in some situations, the physiological state of non-culturability, does not preclude viability, capacity to infect and activation of virulence mechanisms (Pommepuy et al., 1996; Rozen and Belkin, 2005). These arguments underlie the need to explore the potential occurrence of ARB among the non-culturable bacteria. In these situations, the direct analyses of DNA to survey or quantify genes or of RNA to assess gene expression are important approaches, used alone or in combination with culture-dependent methods, to unveil the fate of ARB &ARGs in the environment. In addition, the use of DNA-based analyses to characterize ARB, which bring important epidemiological insights about ARB and ARGs became more and more popular over the last years.

The selection of the best method to analyse ARB and ARGs must be made based on the questions the researchers seek to answer. If all resources are available, the best approach will comprise a combination of different methods, but sometimes one or two approaches can give reliable answers. The next sections make an overview of three major approaches used, two targeted (culture-based and quantitative PCR) and one non-targeted (metagenomics) and discuss the associated strengths, weaknesses, opportunities, and threats (SWOT) (Table 3). While most of these methods may not be available in wastewater treatment companies, the highly desirable and increasing collaboration between the research institutions, services and companies may make possible and fruitful the efforts to combat antibiotic resistance.

### 4.1. Culture-based methods

One of the main advantages of cultivation is the possibility of determining phenotypic traits, many of which are crucial for understanding the ecology of a given bacterial group. In the case of antibiotic resistance, this knowledge is the basis to assess the propagation or gene transfer potential of specific ARB under environmental conditions. In this aspect, relevant phenotypic traits that can be examined in bacterial isolates include metabolism (e.g. carbon source utilization, identification of auxotrophies), required physico-chemical conditions for growth (e.g. pH or temperature range, salinity), biofilm formation capacity, sporulation, motility, tolerance against stressful conditions, among others (McLain et al., 2016). In addition, culture-based methods allow the enumeration of viable cells, and the possibility for assessing antibiotic resistance profiles (i.e. minimal inhibitory concentration and antibiotic resistance spectrum) of isolates (Buthelezi et al., 2010; Garcha et al., 2016; Li et al., 2009), being the most commonly used method to determine multidrug resistance phenotypes. Other methods can be used to assess resistance phenotypes but may imply sophisticated equipment and know-how, e.g. flow cytometry combined with live/dead assays after exposure of the sample/bacteria to multiple drugs (Berney et al., 2007).

In addition, the analysis of ARB isolates facilitate the analysis of the harboured ARGs, as well as the mobile genetic elements to which they are associated, and above all supports the identification of the species or clones that may be of major relevance for the spread of a given ARG (Guardabassi et al., 2002; Hembach et al., 2017; Kaplan et al., 2015; Varela et al., 2015; Vaz-Moreira et al., 2014). Last, but not least, standardized procedures based on cultivation methods, currently used for water quality monitoring worldwide, may be adapted to develop guidelines for ARB surveillance in aquatic environments, for example, based on the epidemiological cut-off values (ECOFFs) (Kronvall, 2010).

With the advent of the culture-independent methods, with increasing effulgence over the last decades, culture-based methods became unpopular. Besides the limited capacity to survey microbial communities, the fact that these methods can be time-consuming, requiring experienced operators, and involving high costs for proper post-isolation analyses (e.g. in molecular epidemiology), often resulted in the preference for direct DNA analyses. Also important is the fact that working with ARB isolates requires adequate safety equipment and procedures due to the potential biohazard contamination of exposed operators and environment. This can be a limitation in some laboratories. However, the usefulness of cultivation is increasingly becoming evident, mainly because it is now supported by information provided by culture-independent methods. Indeed, when information available in databases refers essentially to DNA analyses, in which gene functions are mainly inferred based on in silico analyses, cultivation, i.e. phenotype, is again regarded as a crucial need to advance the microbiology knowledge. The designated *culturomics* efforts, involving high throughput culture-based methods are now considered a priority (Greub, 2012; Lagier et al., 2012, 2015). In summary, the combination of culture-based methods with culture-independent approaches may be the ideal way to explore the environmental resistome (Batt et al., 2007; Li et al., 2015; Port et al., 2014; Yang et al., 2014).

### 4.2. Culture-independent methods

Culture-independent approaches rely, primarily, on the extraction of genetic material, most of the times DNA, and not so often RNA, from a sample. As happens with the choice for the cultivation conditions, also the nucleic acids extraction may introduce biases in these analyses. For example, the DNA or RNA extraction efficiency may vary with the protocol used as well as with the matrix to analyse; it may favour the extraction of nucleic acid from specific bacterial groups (e.g. Gram-negative in comparison to Gram-positive) or it may be more or less effective on the removal of inhibitors (Knauth et al., 2013; Li et al., 2018; McCarthy et al., 2015; Terrat et al., 2012; Thomas et al., 2012). For these reasons, it is clear that the choice of the nucleic acids extraction method is relevant and it should be maintained in any comparative analyses as, for example, of raw and treated wastewater. The direct analyses of DNA may bring an additional limitation that is the incapability to distinguish living from dead cells. Some studies have shown methods useful to overcome such limitation, for example, the use of propidium monoazide (PMA) to distinguish membrane injured-cells from intact cells (Cangelosi and Meschke, 2014; Nocker et al., 2007). However, it is important to consider that other mechanisms of cell inactivation, e.g. those caused by UV radiation, may be not detected when using PMA. While DNA-based analyses are the best choice for gene survey, RNA-based analyses will be the option when gene expression, rather than gene presence, is to be investigated. ARGs expression analyses in environmental samples are not common, in contrast with the surveying of ARGs based on DNA analyses, which became highly popular. Two major approaches, the quantitative PCR (qPCR) and the metagenomics, have been used to assess the wastewater

**Table 3**
SWOT analysis of the most commonly used methods to analyse antibiotic resistance in urban wastewater treatment plants (UWTP).

| | |
|---|---|
| **Culture-based** | |
| Strengths | • Permits enumeration of viable cells<br>• Allows purification and further phenotype characterization, including determination of minimal inhibitory concentrations<br>• Permits the identification and monitoring of clinically relevant species or strains<br>• It makes the link with worldwide implemented methods to assess the microbiological water quality<br>• Enable to link phenotype of resistance to a given genetic and/or physiological mechanism (e.g. virulence mechanisms; tolerance to oxidative stress) |
| Weaknesses | • Laborious, time consuming and expensive if post-isolation processing is aimed at (e.g. molecular epidemiology)<br>• Culture are not representative of the whole bacterial community diversity<br>• Under non-selective condition the limits of quantification may be very high |
| Opportunities | • May lead to the isolation of new antibiotic resistant bacteria, for example harbouring emerging antibiotic resistance genes<br>• Permits the establishment of reproducible/standardized procedures<br>• Supports the characterization of multidrug-resistance profiles<br>• Supports further assessments of potential horizontal gene transfer (transformation, transduction, conjugation)<br>• Supports gene-function and/or regulation studies<br>• Permits the establishment of guidelines for environmental ARB (ECOFF)<br>• Detection limits can be lowered to enable the targeting of rare antibiotic resistance phenotypes |
| Threats | • Potential for biohazards contamination – requires that good environmental and personal safety practices are adopted<br>• Requires practical professional microbiology skills<br>• Biases, leading to the over- or sub- quantification of antibiotic resistant bacteria |
| **Quantitative PCR** | |
| Strengths | • It is a targeted analysis that allows the search for a specific gene or gene mutation<br>• Allows the quantification of abundance (*per* volume of sample) or prevalence (*per* total bacteria) of specific antibiotic resistance genes (ARG) in a given sample<br>• Permits the simultaneous quantitative analysis of multiple ARG as well as of housekeeping genes<br>• Methods (primers, references and conditions) are consistently described in the literature and it is possible to reproduce in different labs |
| Weaknesses | • The limit of quantification may be considered too high compared with the risk associated with some ARGs<br>• DNA extraction may represent an important bias on the genes quantification<br>• The presence of PCR inhibitors in complex matrices may jeopardize the accuracy of ARGs quantification<br>• The protocols available are limited to ARG that were previously characterized<br>• Complex matrices may lead to incorrect amplifications (e.g. incorrect melting temperature, shoulders, double peaks) |
| Opportunities | • Possibility to infer about ARG selection or horizontal gene transfer in a trans-sectional analysis (time or space)<br>• Possibility to settle universal guidelines to survey ARG in environmental samples, e.g. in a water treatment plant<br>• When based on live cells (e.g. PMA) or in total cDNA can give an overview of active/expressed ARGs<br>• It is possible to analyse extracellular DNA |
| Threats | • Due to the high specificity or poor sensitivity, slight variations in ARG abundance or rare ARGs may be neglected or sub-estimated<br>• The analysis is independent from the phenotype or ARB host<br>• Even using standardized methods, the results may be affected by factors such as the batch of reagents, the equipment used or even the nature of the sample matrix<br>• The protocols are settled according with the materials and machines, making standards and methods specific from the laboratory that settled it<br>• Difficulty of design primers that are completely universal or sufficiently specific |
| **Metagenomics** | |
| Strengths | • Based on high throughput methods, it is capable of providing wide sets of genomic information<br>• Gives an overview of the majority of organisms and/or genes and/or putative functions present in a sample<br>• Biases due to primer use or cultivation methods can be avoided |
| Weaknesses | • Requires sophisticated equipment and expertise in bioinformatics<br>• The amount of sample that can be analysed and DNA extraction efficiency can limit the analyses outcome<br>• Sequencing depth may be a shortcoming when rare genes are searched<br>• High variation between replicas may be observed, meaning that higher numbers of replicates are required to detect statistically significant differences<br>• Read length may be too short to perform accurate sequences assembly<br>• Does not allow inferences about the antibiotic resistance phenotypes and, hence, may have a limited value on the assessment of risks<br>• It does not provide info about the host that carry a plasmid or an ARG<br>• The lack of standard methods for data analysis reduces the reproducibility and comparison between similar projects |
| Opportunities | • Analyses can be performed as an external service<br>• Used mainly as a non-targeted method that may support the finding of novel ARGs<br>• It permits the continuous enrichment of public databases, where genes information of different geographic origins can be compiled over time and used for future studies dealing with historic presence of newly discovered genes<br>• The costs of high throughput sequencing methods decreased considerably over the last years<br>• Metatranscriptomics allow inferences about the expressed/active genes or populations<br>• The read length is now higher and tends to increase, overcoming gene identification and assemblage limitations<br>• Using adequate informatics tools and methods may facilitate the inference about the ARGs associations or putative hosts |
| Threats | |

(*continued on next page*)

**Table 3** (*continued*)

- Technologies evolve fast and data provided by currently used sequencing platforms can be obsolete in few years
- Combined skills on bioinformatics and microbiology are essential to correctly analyse metagenomics data, under both data analyses and biological meaning perspectives
- The analyses and hence the results can be biased by the database used
- It may be difficult to detect rare/specific genes in metagenomics data pools, giving the wrong perspective of gene absence (risk absence)
- It is not known if the method is sensitive enough to infer about a gene fate in a UWTP
- Non-redundancy (if the coverage is not adjusted to the sample diversity)

PMA, propidium monoazide.

resistome and will be the focus of the next paragraphs.

The qPCR is designed to follow in real-time the amplification of a specific gene fragment, through the use of specific primers and the development of fluorescence, which emission is proportional to the PCR amplicon produced, due to the use of a fluorescent dye or a probe (Stratagene®, 2005). The continuous improvements of the method, in terms of chemistry (dye and probes) and machines, have conducted to improve the sensitivity and specificity of the qPCR process. SYBR Green, a double-stranded intercalating agent, and TaqMan, based on a dual labelled oligonucleotide and exonuclease activity of Taq polymerase, are the most popular fluorescent dye and probe used, being the first preferable due to its lower costs, in spite of the lower sensitivity. TaqMan systems may be preferred for gene mutation detection or multiplex gene quantification (Fyfe et al., 2007). When applied to ARGs analyses, qPCR can be adapted to assess prevalence values, through the ratio ARGs/housekeeping gene, normally the 16S rRNA gene, or when using a housekeeping gene that can be considered the signature for a species (e.g. *uidA* for *E. coli*) it may allow inferences on ARGs loss or acquisition (Stefani et al., 2015). Due to its potential for quantitative and highly specific analyses, the qPCR has been increasingly used, with an ever-increasing number of recommended primers and reference conditions, consistently described in the literature (Czekalski et al., 2015; Rocha et al., 2018). The use of qPCR array is, in this respect, an important advance, allowing the simultaneous analyses of a large number of genes (Karkman et al., 2016).

Nevertheless, qPCR has still some limitations that should be taken into account when data is to be analysed and interpreted. An important limitation refers to the impossibility to design primers for new/unknown genes, since the primers design is based on reference sequences that are already described and deposited in the databases. For both universal and specific primers, primer design may be a difficult task. A universal primer, working for all the taxa with the same efficiency, may be difficult to achieve (Wintzingerode et al., 1997). Conversely, when ARGs are the targets, a primer set that is too specific may fail to detect variants of the gene. On the contrary, since primers only require homology with a small fragment of DNA, annealing may occur with non-target genes with regions of high identity with the primers. However, these false positive reactions can be easily identified based on melting temperature of the amplicon (Rocha et al., 2018). Other troubleshooting of qPCR analyses of environmental samples, refers to different types of often unknown or unexpected interferences or inhibitor agents (e.g. humic acids), which can reduce the accuracy of the process (Sidstedt et al., 2015; Smith and Osborn, 2009). In addition, the limits of quantification of the method may be too high compared with the risk associated with the ARGs, being important a critical analysis of the results (Christou et al., 2017).

One of the limitations that may be pointed out to qPCR is the fact that it represents a targeted analysis, meaning that we only find what we are looking for. Metagenomics is a suitable approach to overcome such a limitation since it circumvents possible biases due to primer use through the sequencing of whole metagenome present in the sample (Schmieder and Edwards, 2012). An important asset of metagenomics analyses is that it has the potential to provide not only an overview of the already known ARGs but also of their variants or possible new ARGs that may exist in a given environment (Oulas et al., 2015). As for qPCR primer design, in metagenomics analyses the availability of representative databases, from which it is possible to extract reliable information, may also constitute a bottleneck, due to the limited size and phylogenetic and geographic coverage of the databases (Arango-Argoty et al., 2017; Bengtsson-Palme et al., 2017; Carr and Borenstein, 2014). Compared with the qPCR, which permits the expression of the results as gene copy number *per* volume of wastewater sample, the metagenomics allows the estimation of relative abundance values, either calculated based on specific versus total reads number, or versus the number of reads of a housekeeping gene, most commonly the 16S rRNA gene (Bengtsson-Palme et al., 2017; Parsley et al., 2010; Schmieder and Edwards, 2012; Thomas et al., 2012; Tian et al., 2016; Zhang et al., 2012). Metagenomics has benefited from the technical and scientific development of high throughput sequencing methods, combined with a progressive reduction of costs (Thomas et al., 2012), a trend that is now even more encouraged by the increasing read length offered by techniques such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (Nanopore) (Escalona et al., 2016; Goodwin et al., 2016).

The usefulness of metagenomics to study ARGs and bacterial diversity in several environments, including wastewater, has been widely demonstrated (Li et al., 2015; Munck et al., 2015; Tang et al., 2016). However, it is arguable if this approach is sensitive enough to infer about the fate of ARGs in a UWTP. For instance, metagenomics may have a limited capacity to explore i) if the abundance of a given ARG decreases with the treatment, ii) an extremely rare ARG in a given sample, or iii) different ARGs variants (polymorphisms) with distinct clinical relevance in the same sample. In some of these cases, the sequencing depth may have to be extremely increased to support a reliable assessment. In alternative to metagenomics, a targeted analysis based on the PCR amplification of a specific genetic element, i.e., metagenetics, may be preferable (Handelsman, 2009). This approach may circumvent the problems noted for metagenomics in what concerns rare or diverse genetic elements, allowing the study of antibiotic resistance gene cassettes known for their variability (Gatica et al., 2016). As for qPCR, neither metagenetics nor metagenomics allows the identification of the hosts of a specific ARG (Luby et al., 2016). In addition, the lack of standard methods or universal tools to be used in metagenomics analyses, may represent a constraint for the analyses of reproducibility and comparability of data obtained in different occasions or places (Escobar-Zepeda et al., 2015). In summary, ARGs metagenomics represented a tremendous advancement in the exploitation of the diversity and biogeography of the environmental antibiotic resistomes. Besides showing the distribution of the contaminant resistome, metagenomics has also the potential to unveil the occurrence of other genes, some potentially emerging ARGs in the future. The possibility of metagenomics data archiving and sharing in public databases is an invaluable contribute to writing the history of antibiotic resistance evolution.

## 5. Frequently asked questions (FAQs)

The recognition that UWTPs may represent important ARB&ARGs reservoirs, raises concerns related to the capability of treatment processes to maximize the removal of these contaminants. In this section, we address five frequently asked questions regarding the role of UWTPs

**Table 4**
Frequently asked questions (FAQs) on the suitable approaches to explore the AR throughout the treatment process.

| FAQs | How |
|---|---|
| Is a given wastewater treatment process contributing to increase or to remove the ARB and/or ARGs? | Quantify and compare the loads of ARB or ARG over the different UWTP stages<br><br>Culture-dependent methods: culture<br>Estimate ARB abundance: population resistant to an antibiotic vs the volume of sample<br><br>Culture-independent methods: qPCR; metagenomics<br>Estimate ARG abundance: specific gene *per* mass of sample |
| Are there any possible positive or negative selective pressures or conditions contributing to increase the ARB or ARGs in this UWTP? | Assess possible associations between physico-chemical and microbiological data:<br><br>Physico-chemical analysis: mass spectrometry, chromatographic methods, etc. (e.g., temperature, HRT, SRT, metals, antibiotics and other micropollutants quantification)<br><br>Culture-dependent methods: culture<br>Estimate ARB prevalence: population resistant to an antibiotic vs total population<br>Analysis of bacterial population changes<br><br>Culture-independent methods: qPCR; metagenomics; metatranscriptomics<br>Estimate ARG prevalence: specific gene *per* 16S rRNA gene copies or reads of a gene per reads of a housekeeping gene<br>Analysis of bacterial population changes<br>Quantification of ARGs<br>Detection or quantification of expressed genes |
| In the complex wastewater microbiome, which bacterial groups may facilitate or represent an obstacle to the proliferation of invasive ARB or ARGs? | Culture-independent methods: Microbiome analysis (NGS); qPCR (with PMA or cDNA based, if only live bacteria are targeted) over the different treatment stages<br>Targeted analysis of total or live bacterial populations<br>Culture-dependent methods: culture<br>Targeted analysis of bacterial populations recognized as relevant carriers of ARGs (e.g. faecal coliforms; enterococci) using selective culture media |
| Where is a given ARG or ARGs set? Looking for the bacterial host or genetic environment | Culture-dependent methods: culture; PCR; Whole genome sequencing<br>Targeted analysis of ARB populations followed by detection of the ARG by PCR and identification by sequencing, or by sequencing of the whole bacterial genome<br><br>Culture-independent methods: epicPCR; Whole genome sequencing<br>Amplification of the ARB by PCR and sequencing of the final product or by culture-independent whole genome sequence analyses |
| Does the final effluent of this UWTP contain new, emerging or extremely rare ARGs?<br><br>Examples of these ARB/ARG: carbapenemase producing *Klebsiella pneumoniae* and *Enterobacteriaceae*; methicillin-resistant *Staphylococcus aureus*; and *vanA*, *mecA*, $bla_{CTX\text{-}M}$, $bla_{NDM\text{-}1}$, $bla_{KPC}$, $bla_{IMP}$, $bla_{VIM}$, and *mcr1* | Culture-dependent methods: culture; PCR<br>Targeted analysis of ARB culturable populations recovered under selective pressure, followed by detection of the ARG by PCR and identification by nucleotide sequencing<br><br>Culture-independent methods: PCR; qPCR; FISH<br>Detection of targeted ARB or ARG by PCR<br>Detection of ARB with FISH<br>Quantification of ARB or ARG by qPCR<br>Functional metagenomics<br>Sequencing of mobile genetic elements using high-throughput sequencing methods (e.g. PacBio) |

NGS, next generation sequencing; ARB, antibiotic resistant bacteria; ARG, antibiotic resistance genes; PMA, propidium monoazide; FISH, fluorescence in situ hybridization.
Underlined are the different types of methods that can be used to answer the questions.

in the dissemination of ARB&ARGs (Table 4). We suggest that through an adequate experimental design, combining the proper selection of sampling sites and analytical parameters, it will be possible to enlarge the body of information that may contribute to a better understanding of the fate of ARB&ARGs over wastewater treatment. In the next paragraphs the five FAQs proposed are listed.

### *5.1. Is a given wastewater treatment process contributing to increase or to remove the ARB and/or ARGs?*

To address this question it is required the measurement of the abundance *per* unit of sample (volume if a liquid sample or mass if a solid sample) of the specific ARB or ARG, over the different steps of the wastewater treatment process. The simplest approach is the comparison of ARB and/or ARGs abundance in the raw inflow and in the final effluent.

### *5.2. Are there any possible positive or negative selective pressures or conditions contributing to increase the ARB or ARGs in this UWTP?*

This question may refer to variations in the prevalence of ARB or ARGs, meaning if their abundance varied (increased or decreased) in relation to the total bacteria. Using culture-dependent methods it can be assessed based on the ratio between ARB and total bacteria, measured by cultivation on antibiotic-supplemented culture medium or cultivated on antibiotic-free culture medium, respectively. Using qPCR or metagenomics it will be the ratio between the ARGs and a housekeeping gene (most commonly the 16S rRNA gene), measured in gene copy numbers or reads number, respectively. However, it is not only the observation of possible variations on ARB or ARGs that are of interest in this case. It is also intended the inference of possible associations between the increase or decrease of the aforementioned ratios and the occurrence of some conditions, such as higher or lower concentrations of antibiotic residues or metals in the inflow, the occurrence of specific

ARB populations in the raw inflow, or the observation of an extreme rain event (in *lato sensu* designated as stressor conditions). This kind of studies may also benefit from gene expression insights, based on a RNA-based analysis. This can involve the measurement of a specific gene based on qPCR or a metatranscriptomics analysis. The reliability of all these analyses and possible associations between stressors, ARGs or gene expression patterns, is strongly dependent on a robust sampling scheme and statistical analyses. It should be noted however, that variations on ARB or ARGs ratios, even if statistically associated with putative selective pressure factors, may be due to shifts in the microbial community and not necessarily resultant from a direct effect of a given selective pressure factor on a specific ARB or ARG. Hence, additional studies may be required to study the effect of the putative selective pressure factor or the behaviour of a specific target ARB or ARG. In this case, the study must be focused on the specific target in order to unveil the physiological or genetic mechanisms involved.

### *5.3. In the complex wastewater microbiome, which bacterial groups may facilitate or represent an obstacle to the proliferation of invasive ARB or ARGs?*

To assess this aspect, the first step will be the characterization of the bacterial community, in the raw inflow, sludge, and final effluent, and how it changes during treatment. This involves a nowadays routine approach (normally 16S rRNA gene metagenetics analysis), based on the analysis of the microbiome using high-throughput sequencing technologies (e.g. Illumina) to compare the total bacterial community over the UWTP. Culture-dependent methods may be used as a complement, in an attempt to assess which bacterial populations or metabolic groups might be favoured/disfavoured during wastewater treatment.

### *5.4. Where is a given ARG or ARGs set? Looking for the bacterial host or genetic environment*

The identification of the ARB that may play a pivotal role in the dissemination of a given ARG, may be determinant to control its occurrence over the wastewater treatment and, hence, for reducing its presence in the final effluent. Emulsion, Paired Isolation and Concatenation PCR (epicPCR) (Spencer et al., 2016) and inverse PCR (Pärnänen et al., 2016) are two approaches of choice to meet this objective. It relies on PCR and DNA sequencing of the final products and thus may be affected by the limitations already described above for the methodologies that require the use of DNA amplification. EpicPCR is specifically designed to link the presence of a target gene (e.g. an ARG) to the phylogeny of the bacterial host for that gene, whereas inverse PCR identifies the association of a target ARG with any type of mobile genetic element that is associated within a bulk DNA extract (Pärnänen et al., 2016; Spencer et al., 2016). Both have the specificity and sensitivity advantages of PCR-based procedures, target one ARG at the time and have the potential to associate an ARG to a host or mobile genetic element. In addition to these methods, whole genome sequencing approaches are promising tools to unveil the preferential hosts of relevant ARGs (Fournier et al., 2013).

### *5.5. Does the final effluent of this UWTP contain new, emerging or extremely rare ARGs?*

This must be one of the most important questions for wastewater treatment professionals, mainly because emerging ARGs and some of the most hazardous ARB are at extremely low abundance and prevalence in environmental samples, being easily ignored in spite of the high risk that they represent. Examples of these ARB are the carbapenemase-producing *Klebsiella pneumoniae* or other *Enterobacteriaceae* and methicillin-resistant *Staphylococcus aureus*. Examples of ARGs of concern are, for example, the *vanA*, *mecA*, $bla_{CTX\text{-}M}$, $bla_{NDM\text{-}1}$, $bla_{KPC}$, $bla_{IMP}$, $bla_{VIM}$, and *mcr-1*, that in spite of the low levels at which occur in wastewater may spread rapidly and widely, representing a higher risk than other environmental bacteria considered as antibiotic resistance reservoirs (Manaia, 2017). For the detection and measurement of these genes, it is recommended the use of targeted methods, either qPCR or cultivation under selective pressure conditions, meaning in the presence of the antibiotic to which confer resistance. However, for the detection of unknown ARGs the abovementioned approaches may not work. In such situations, the use of functional metagenomics analysis or the targeted analyses of integrons or plasmids, where the probability of finding ARGs may be higher, may be the best option (Gonzalez-Plaza et al., 2017; Lau et al., 2017; Razavi et al., 2017).

## 6. Conclusions

The study of the fate of ARB&ARGs during wastewater treatment is complex, influenced by a myriad of external factors, difficult to control and monitor in real-world systems. This is probably the reason why we can find contradictory findings in the literature and many unanswered questions, albeit the important efforts researchers around the world have been making. Although researchers did not find a magic formula to study ARB&ARGs in UWTPs, it is now possible to settle some recommendations, which are unanimous among researchers, as it is consensual the evidence that we need a larger body of information to be able to open this black box and maximize the removal of ARB&ARGs during wastewater treatment. Irrespective of the type of methods to use and the plethora of information that can be collected, the establishment of solid hypotheses as a basis for experimental design and the incisive critical thinking on data analyses are essential to advance even more our current knowledge in the field.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.03.044.

## Acknowledgments

This work is part of a project that has received funding from the European Union's Horizon 2020, under the Innovative Training Networks (ITN-ETN) programme Marie Skłodowska-Curie grant (ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions) agreement No 675530.

This work was financially supported by Projects UID/Multi/50016/2013-CBQF and POCI-01–0145-FEDER-006939 (LEPABE – UID/EQU/00511/2013) funded by European Regional Development Fund (ERDF) through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), and by national funds through FCT – Fundação para a Ciência e a Tecnologia; project “LEPABE-2-ECO-INNOVATION” – NORTE-01-0145-FEDER-000005, funded by Norte Portugal Regional Operational Programme (NORTE 2020), under PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).VM and JR were supported by the FCT grant SFRH/BPD/87360/2012 and the International PhD Programme in Biotechnology – BIOTECH.DOC, NORTE-08-5369-FSE-000007, respectively.**Disclaimer**

The content of this publication reflects only the authors' views and the Research Executive Agency is not responsible for any use that may be made of the information it contains.

## References

Alexander, J., Knopp, G., Dotsch, A., Wieland, A., Schwartz, T., 2016. Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. Sci. Total Environ. 559, 103–112. http://dx.doi.org/10.1016/j.scitotenv.2016.03.154.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Arango-Argoty, G.A., Garner, E., Pruden, A., Heath, L.S., Vikesland, P., Zhang, L., 2017. DeepARG: a deep learning approach for predicting antibiotic resistance genes from metagenomic data. In: bioRxiv, http://dx.doi.org/10.1101/149328.

Batt, A.L., Kim, S., Aga, D.S., 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere 68, 428–435. http://dx.doi.org/10.1016/j.chemosphere.2007.01.008.

Becerra-Castro, C., Macedo, G., Silva, A.M.T., Manaia, C.M., Nunes, O.C., 2016. Proteobacteria become predominant during regrowth after water disinfection. Sci. Total Environ. 573, 313–323.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ. Int. 86, 140–149. http://dx.doi.org/10.1016/j.envint.2015.10.015.

Bengtsson-Palme, J., Larsson, D.G.J., Kristiansson, E., 2017. Using metagenomics to investigate human and environmental resistomes. J. Antimicrob. Chemother. 72, 2690–2703. http://dx.doi.org/10.1093/jac/dkx199.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. http://dx.doi.org/10.1038/nrmicro3439.

Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.U., Egli, T., 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. Appl. Environ. Microbiol. 73, 3283–3290. http://dx.doi.org/10.1128/AEM.02750-06.

Bouvier, T., Del Giorgio, P.A., 2007. Key role of selective viral-induced mortality in determining marine bacterial community composition. Environ. Microbiol. 9, 287–297. http://dx.doi.org/10.1111/j.1462-2920.2006.01137.x.

Buthelezi, S.P., Olaniran, A.O., Pillay, B., 2010. Production and characterization of bioflocculants from bacteria isolated from wastewater treatment plant in South Africa. Biotechnol. Bioprocess Eng. 15, 874–881. http://dx.doi.org/10.1007/s12257-009-3002-7.

Cai, L., Ju, F., Zhang, T., 2014. Tracking human sewage microbiome in a municipal wastewater treatment plant. Appl. Microbiol. Biotechnol. 98, 3317–3326. http://dx.doi.org/10.1007/s00253-013-5402-z.

Cangelosi, G.a., Meschke, J.S., 2014. Dead or alive: molecular assessment of microbial viability. Appl. Environ. Microbiol. 80, 5884–5891. http://dx.doi.org/10.1128/AEM.01763-14.

Carr, R., Borenstein, E., 2014. Comparative analysis of functional metagenomic annotation and the mappability of short reads. PLoS One 9, e105776. http://dx.doi.org/10.1371/journal.pone.0105776.

Christou, A., Agüera, A., Bayona, J.M., Cytryn, E., Fotopoulos, V., Lambropoulou, D., Manaia, C.M., Michael, C., Revitt, M., Schröder, P., Fatta-Kassinos, D., 2017. The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – a review. Water Res. http://dx.doi.org/10.1016/j.watres.2017.07.004.

Czekalski, N., Sigdel, R., Birtel, J., Matthews, B., Bürgmann, H., 2015. Does human activity impact the natural antibiotic resistance background? Abundance of antibiotic resistance genes in 21 Swiss lakes. Environ. Int. 81, 45–55. http://dx.doi.org/10.1016/j.envint.2015.04.005.

Di Cesare, A., Fontaneto, D., Doppelbauer, J., Corno, G., 2016. Fitness and recovery of bacterial communities and antibiotic resistance genes in urban wastewaters exposed to classical disinfection treatments. Environ. Sci. Technol. 50, 10153–10161. http://dx.doi.org/10.1021/acs.est.6b02268.

Diehl, D.L., Lapara, T.M., 2010. Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. Environ. Sci. Technol. 44, 9128–9133. http://dx.doi.org/10.1021/es102765a.

Dröge, M., Pühler, A., Selbitschka, W., 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol Gen Genet 263, 471–482. http://dx.doi.org/10.1007/s004380051191.

EEA, 2017. Urban waste water treatment map. (WWW Document).

EPA, 1986. Ambient water quality criteria for bacteria. In: Regulations and Standards Criteria and Standards Division Office of Water, Washington DC, USA.

EPA-Victoria, 2002. Disinfection of treated wastewater. Guidel. Environ. Manag. 30.

Escalona, M., Rocha, S., Posada, D., 2016. A comparison of tools for the simulation of genomic next-generation sequencing data. Nat. Rev. Genet. 17, 459–469. http://dx.doi.org/10.1038/nrg.2016.57.

Escobar-Zepeda, A., De León, A.V.P., Sanchez-Flores, A., 2015. The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. Front. Genet. 6. http://dx.doi.org/10.3389/fgene.2015.00348.

Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nat. Rev. Microbiol. 10, 538–550. http://dx.doi.org/10.1038/nrmicro2832.

Fournier, P.-E., Drancourt, M., Colson, P., Rolain, J.-M., La Scola, B., Raoult, D., 2013. Modern clinical microbiology: new challenges and solutions. Nat. Rev. Microbiol. 11, 574–585. http://dx.doi.org/10.1038/nrmicro3068.

Fyfe, J.a.M., Lavender, C.J., Johnson, P.D.R., Globan, M., Sievers, A., Azuolas, J., Stinear, T.P., 2007. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. Appl. Environ. Microbiol. 73, 4733–4740. http://dx.doi.org/10.1128/AEM.02971-06.

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421–422, 173–183. http://dx.doi.org/10.1016/j.scitotenv.2012.01.061.

Garcha, S., Verma, N., Brar, S.K., 2016. Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability. Water Resour. Ind. 16, 19–28. http://dx.doi.org/10.1016/j.wri.2016.10.002.

Gatica, J., Tripathi, V., Green, S., Manaia, C.M., Berendonk, T., Cacace, D., Merlin, C., Kreuzinger, N., Schwartz, T., Fatta-Kassinos, D., Rizzo, L., Schwermer, C.U., Garelick, H., Jurkevitch, E., Cytryn, E., 2016. High throughput analysis of integron gene cassettes in wastewater environments. Environ. Sci. Technol. 50, 11825–11836. http://dx.doi.org/10.1021/acs.est.6b03188.

Gonzalez-Plaza, J.J., Šimatović, A., Milaković, M., Bielen, A., Wichmann, F., Udikovic-Kolic, N., 2017. Functional repertoire of antibiotic resistance genes in antibiotic manufacturing effluents and receiving freshwater sediments. Front. Microbiol. 8, 2675.

Goodwin, S., McPherson, J.D., McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nat. Rev. Genet. 17, 333–351. http://dx.doi.org/10.1038/nrg.2016.49.

Grady, C.P.L., Daigger, G.T., Love, N., Filipe, C.D.M., 2011. Biological Wastewater Treatment. CRC press, NW.

Greub, G., 2012. Culturomics: a new approach to study the human microbiome. Clin. Microbiol. Infect. 18, 1157–1159. http://dx.doi.org/10.1111/1469-0691.12032.

Guardabassi, L., Lo Fo Wong, D.M.A., Dalsgaard, A., 2002. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res. 36, 1955–1964. http://dx.doi.org/10.1016/S0043-1354(01)00429-8.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158. http://dx.doi.org/10.1371/journal.ppat.1002158.

Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. MBio 5. http://dx.doi.org/10.1128/mBio.01918-14.

Handelsman, J., 2009. Metagenetics: spending our inheritance on the future. Microb. Biotechnol. http://dx.doi.org/10.1111/j.1751-7915.2009.00090_8.x.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the *mcr-1* colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8. http://dx.doi.org/10.3389/fmicb.2017.01282.

Henze, M., Comeau, Y., 2008. Wastewater characterization. Biol. Wastewater Treat. Princ. Model. Des. 33–52. http://dx.doi.org/10.2166/wst.2009.242.

Henze, M., Van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D., 2008. Biological wastewater treatment: principles, modelling and design. In: Proceedings of the National Academy of Sciences of the United States of America, pp. 511.

Jutkina, J., Rutgersson, C., Flach, C.F., Joakim Larsson, D.G., 2016. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. Sci. Total Environ. 548–549, 131–138. http://dx.doi.org/10.1016/j.scitotenv.2016.01.044.

Jutkina, J., Marathe, N.P., Flach, C.F., Larsson, D.G.J., 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. Sci. Total Environ. 616–617, 172–178. http://dx.doi.org/10.1016/j.scitotenv.2017.10.312.

Kaplan, E., Sela, N., Doron-Faigenboim, A., Navon-Venezia, S., Jurkevitch, E., Cytryn, E., 2015. Genomic and functional characterization of qnr-encoding plasmids from municipal wastewater biosolid *Klebsiella pneumoniae* isolates. Front. Microbiol. 6. http://dx.doi.org/10.3389/fmicb.2015.01354.

Karkman, A., Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. FEMS Microbiol. Ecol. 92. http://dx.doi.org/10.1093/femsec/fiw014.

Kim, S., Jensen, J.N., Aga, D.S., Weber, A.S., 2007. Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere 66, 1643–1651. http://dx.doi.org/10.1016/j.chemosphere.2006.07.066.

Kim, S., Yun, Z., Ha, U.H., Lee, S., Park, H., Kwon, E.E., Cho, Y., Choung, S., Oh, J., Medriano, C.A., Chandran, K., 2014. Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative micro-contaminant concentrations. Sci. Total Environ. 468–469, 813–820. http://dx.doi.org/10.1016/j.scitotenv.2013.08.100.

Knauth, S., Schmidt, H., Tippkötter, R., 2013. Comparison of commercial kits for the extraction of DNA from paddy soils. Lett. Appl. Microbiol. 56, 222–228. http://dx.doi.org/10.1111/lam.12038.

Köck-Schulmeyer, M., Villagrasa, M., López de Alda, M., Céspedes-Sánchez, R., Ventura, F., Barceló, D., 2013. Occurrence and behavior of pesticides in wastewater treatment plants and their environmental impact. Sci. Total Environ. 458–460, 466–476. http://dx.doi.org/10.1016/j.scitotenv.2013.04.010.

Kronvall, G., 2010. Normalized resistance interpretation as a tool for establishing epidemiological MIC susceptibility breakpoints. J. Clin. Microbiol. 48, 4445–4452. http://dx.doi.org/10.1128/JCM.01101-10.

Kulkarni, P., Olson, N.D., Raspanti, G.A., Goldstein, R.E.R., Gibbs, S.G., Sapkota, A., Sapkota, A.R., 2017. Antibiotic concentrations decrease during wastewater treatment but persist at low levels in reclaimed water. Int. J. Environ. Res. Public Health 14. http://dx.doi.org/10.3390/ijerph14060668.

Kümmerer, K., 2009. Antibiotics in the aquatic environment—a review—part I. Chemosphere 75, 417–434(https://doi.org/S0045-6535(08)01510-5 [pii]https://doi.org/10.1016/j.chemosphere.2008.11.086).

Lagier, J.C., Armougom, F., Million, M., Hugon, P., Pagnier, I., Robert, C., Bittar, F., Fournous, G., Gimenez, G., Maraninchi, M., Trape, J.F., Koonin, E.V., La Scola, B., Raoult, D., 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin. Microbiol. Infect. 18, 1185–1193. http://dx.doi.org/10.1111/1469-0691.12023.

Lagier, J.C., Hugon, P., Khelaifia, S., Fournier, P.E., La Scola, B., Raoult, D., 2015. The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. Clin. Microbiol. Rev. 28, 237–264. http://dx.doi.org/10.1128/CMR.00014-14.

Lau, C.H.F., van Engelen, K., Gordon, S., Renaud, J., Topp, E., 2017. Novel antibiotic resistance determinants from agricultural soil exposed to antibiotics widely used in human medicine and animal farming. Appl. Environ. Microbiol. 83. http://dx.doi.org/10.1128/AEM.00989-17.

Li, D., Yang, M., Hu, J., Zhang, J., Liu, R., Gu, X., Zhang, Y., Wang, Z., 2009. Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. Environ. Microbiol. 11, 1506–1517. http://dx.doi.org/10.1111/j.1462-2920.2009.01878.x.

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 9, 2490–2502. http://dx.doi.org/10.1038/ismej.2015.59.

Li, A.-D., Metch, J.W., Wang, Y., Garner, E., Zhang, A.N., Riquelme, M.V., Vikesland, P.J., Pruden, A., Zhang, T., 2018. Effects of sample preservation and DNA extraction on enumeration of antibiotic resistance genes in wastewater. FEMS Microbiol. Ecol. http://dx.doi.org/10.1093/femsec/fix189.

Luby, E., Ibekwe, A.M., Zilles, J., Pruden, A., 2016. Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: prospects and challenges. J. Environ. Qual. 45, 441–453. http://dx.doi.org/10.2134/jeq2015.07.0367.

Manaia, C.M., 2017. Assessing the risk of antibiotic resistance transmission from the environment to humans: non-direct proportionality between abundance and risk. Trends Microbiol. http://dx.doi.org/10.1016/j.tim.2016.11.014.

Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. http://dx.doi.org/10.1007/s00253-015-7202-0.

Marano, R., Cytryn, E., 2017. The mobile resistome in wastewater treatment facilities and downstream environments. In: Antimicrobial Resistance in Wastewater Treatment Processes, pp. 129.

McCarthy, A., Chiang, E., Schmidt, M.L., Denef, V.J., 2015. RNA preservation agents and nucleic acid extraction method bias perceived bacterial community composition. PLoS One 10. http://dx.doi.org/10.1371/journal.pone.0121659.

McKinney, C.W., Pruden, A., 2012. Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater. Environ. Sci. Technol. 46, 13393–13400. http://dx.doi.org/10.1021/es303652q.

McLain, J.E., Cytryn, E., Durso, L.M., Young, S., 2016. Culture-based methods for detection of antibiotic resistance in agroecosystems: advantages, challenges, and gaps in knowledge. J. Environ. Qual. 45, 432. http://dx.doi.org/10.2134/jeq2015.06.0317.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. Water Res. 47, 957–995. http://dx.doi.org/10.1016/j.watres.2012.11.027.

Miki, T., Jacquet, S., 2008. Complex interactions in the microbial world: underexplored key links between viruses, bacteria and protozoan grazers in aquatic environments. Aquat. Microb. Ecol. http://dx.doi.org/10.3354/ame01190.

Moreira, N.F.F., Narciso-da-Rocha, C., Polo-López, M.I., Pastrana-Martínez, L.M., Faria, J.L., Manaia, C.M., Fernández-Ibáñez, P., Nunes, O.C., Silva, A.M.T., 2018. Solar treatment (H 2 O 2, TiO 2-P25 and GO-TiO 2 photocatalysis, photo-Fenton) of organic micropollutants, human pathogen indicators, antibiotic resistant bacteria and related genes in urban wastewater. Water Res. 15, 195–206. http://dx.doi.org/10.1016/j.watres.2018.01.064.

Munck, C., Albertsen, M., Telke, A., Ellabaan, M., Nielsen, P.H., Sommer, M.O. a, 2015. Limited dissemination of the wastewater treatment plant core resistome. Nat. Commun. 6, 8452. http://dx.doi.org/10.1038/ncomms9452.

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693. http://dx.doi.org/10.1016/j.watres.2010.08.033.

Narciso-Da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. BlaTEM and vanA as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. J. Glob. Antimicrob. Resist. 2, 309–315. http://dx.doi.org/10.1016/j.jgar.2014.10.001.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K., 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol. 73, 5111–5117. http://dx.doi.org/10.1128/AEM.02987-06.

Novo, A., Manaia, C.M., 2010. Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. Appl. Microbiol. Biotechnol. 87, 1157–1166. http://dx.doi.org/10.1007/s00253-010-2583-6.

Novo, A., André, S., Viana, P., Nunes, O.C., Manaia, C.M., 2013. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. 47, 1875–1887. http://dx.doi.org/10.1016/j.watres.2013.01.010.

Oulas, A., Pavloudi, C., Polymenakou, P., Pavlopoulos, G.A., Papanikolaou, N., Kotoulas, G., Arvanitidis, C., Iliopoulos, I., 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinf. Biol. Insights 9, 75–88. http://dx.doi.org/10.4137/BBI.S12462.

Pärnänen, K., Karkman, A., Tamminen, M., Lyra, C., Hultman, J., Paulin, L., Virta, M., 2016. Evaluating the mobility potential of antibiotic resistance genes in environmental resistomes without metagenomics. Sci. Rep. 6, 1–9. http://dx.doi.org/10.1038/srep35790.

Parsley, L.C., Consuegra, E.J., Kakirde, K.S., Land, A.M., Harper, W.F., Liles, M.R., 2010. Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. Appl. Environ. Microbiol. 76, 3753–3757. http://dx.doi.org/10.1128/AEM.03080-09.

Pommepuy, M., Butin, M., Derrien, A., Gourmelon, M., Colwell, R.R., Cormier, M., 1996. Retention of enteropathogenicity by viable but nonculturable Escherichia coli exposed to seawater and sunlight. Appl. Environ. Microbiol. 62, 4621–4626.

Port, J.A., Cullen, A.C., Wallace, J.C., Smith, M.N., Faustman, E.M., 2014. Metagenomic frameworks for monitoring antibiotic resistance in aquatic environments. Environ. Health Perspect. 122, 222–228. http://dx.doi.org/10.1289/ehp.1307009.

Pruden, A., 2014. Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. Environ. Sci. Technol. 48, 5–14. http://dx.doi.org/10.1021/es403883p.

Razavi, M., Marathe, N.P., Gillings, M.R., Flach, C.-F., Kristiansson, E., Joakim Larsson, D.G., 2017. Discovery of the fourth mobile sulfonamide resistance gene. Microbiome 5, 160. http://dx.doi.org/10.1186/s40168-017-0379-y.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Env. 447, 345–360. http://dx.doi.org/10.1016/j.scitotenv.2013.01.032S0048-9697(13)00042-9.

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., 2018. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. http://dx.doi.org/10.1016/j.jece.2018.02.022. (in press).

Rozen, Y., Belkin, S., 2005. Survival of enteric bacteria in seawater: molecular aspects. In: Oceans and Health: Pathogens in the Marine Environment, pp. 93–107. http://dx.doi.org/10.1007/0-387-23709-7_4.

Schmieder, R., Edwards, R., 2012. Insights into antibiotic resistance through metagenomic approaches. Future Microbiol 7, 73–89. http://dx.doi.org/10.2217/fmb.11.135.

Seiler, C., Berendonk, T.U., 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. Front. Microbiol. 3, 399. http://dx.doi.org/10.3389/fmicb.2012.00399.

Shanks, O.C., Newton, R.J., Kelty, C.A., Huse, S.M., Sogin, M.L., McLellan, S.L., 2013. Comparison of the microbial community structures of untreated wastewaters from different geographic locales. Appl. Environ. Microbiol. 79, 2906–2913. http://dx.doi.org/10.1128/AEM.03448-12.

Shchegolkova, N.M., Krasnov, G.S., Belova, A.A., Dmitriev, A.A., Kharitonov, S.L., Klimina, K.M., Melnikova, N.V., Kudryavtseva, A.V., 2016. Microbial community structure of activated sludge in treatment plants with different wastewater compositions. Front. Microbiol. 7. http://dx.doi.org/10.3389/fmicb.2016.00090.

Sidstedt, M., Jansson, L., Nilsson, E., Noppa, L., Forsman, M., Rådström, P., Hedman, J., 2015. Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. Anal. Biochem. 487, 30–37. http://dx.doi.org/10.1016/j.ab.2015.07.002.

Smith, C.J., Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67, 6–20. http://dx.doi.org/10.1111/j.1574-6941.2008.00629.x.

Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C., Manaia, C.M., 2017. Ozonation and UV254nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. J. Hazard. Mater. 323, 434–441. http://dx.doi.org/10.1016/j.jhazmat.2016.03.096.

Spencer, S.J., Tamminen, M.V., Preheim, S.P., Guo, M.T., Briggs, A.W., Brito, I.L., A Weitz, D., Pitkänen, L.K., Vigneault, F., Virta, M.P., Alm, E.J., 2016. Massively parallel sequencing of single cells by epicPCR links functional genes with phylogenetic markers. ISME J. 10, 427–436. http://dx.doi.org/10.1038/ismej.2015.124.

Stefani, F.O.P., Bell, T.H., Marchand, C., De La Providencia, I.E., El Yassimi, A., St-Arnaud, M., Hijri, M., 2015. Culture-dependent and -independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. PLoS One 10, e0128272. http://dx.doi.org/10.1371/journal.pone.0128272.

Stratagene®, 2005. Introduction to quantitative PCR. In: Methods and Application Guide, (IN #70200–01/Revision #105002 48 pages).

Tang, J., Bu, Y., Zhang, X.-X., Huang, K., He, X., Ye, L., Shan, Z., Ren, H., 2016. Metagenomic analysis of bacterial community composition and antibiotic resistance genes in a wastewater treatment plant and its receiving surface water. Ecotoxicol. Environ. Saf. 132, 260–269. http://dx.doi.org/10.1016/j.ecoenv.2016.06.016.

Tchobanoglous, G., Stensel, H.D., Burton, F.L., Eddy, M., 2003. Wastewater engineering: treatment and reuse. McGraw Hill.

Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environ. Health Perspect. 120, 1100–1106. http://dx.doi.org/10.1289/ehp.1104650.

Terrat, S., Christen, R., Dequiedt, S., Lelièvre, M., Nowak, V., Regnier, T., Bachar, D., Plassart, P., Wincker, P., Jolivet, C., Bispo, A., Lemanceau, P., Maron, P.A., Mougel, C., Ranjard, L., 2012. Molecular biomass and MetaTaxogenomic assessment of soil microbial communities as influenced by soil DNA extraction procedure. Microb. Biotechnol. 5, 135–141. http://dx.doi.org/10.1111/j.1751-7915.2011.00307.x.

Thomas, T., Gilbert, J., Meyer, F., 2012. Metagenomics - a guide from sampling to data analysis. Microb. Inform. Exp. 2, 3. http://dx.doi.org/10.1186/2042-5783-2-3.

Tian, Z., Zhang, Y., Yu, B., Yang, M., 2016. Changes of resistome, mobilome and potential hosts of antibiotic resistance genes during the transformation of anaerobic digestion from mesophilic to thermophilic. Water Res. 98, 261–269. http://dx.doi.org/10.1016/j.watres.2016.04.031.

Varela, A.R., Andre, S., Nunes, O.C., Manaia, C.M., 2014. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. Water Res. 54, 327–336. http://dx.doi.org/10.1016/j.watres.2014.02.003.

Varela, A.R., Manageiro, V., Ferreira, E., Guimarães, M.A., Da Costa, P.M., Caniça, M., Manaia, C.M., 2015. Molecular evidence of the close relatedness of clinical, gull and wastewater isolates of quinolone-resistant Escherichia coli. J. Glob. Antimicrob. Resist. 3, 286–289. http://dx.doi.org/10.1016/j.jgar.2015.07.008.

Vaz-Moreira, I., Egas, C., Nunes, O.C., Manaia, C.M., 2013. Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. FEMS Microbiol. Ecol. 83, 361–374. http://dx.doi.org/10.1111/1574-6941.12002.

Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS Microbiol. Rev. 38, 761–778. http://dx.doi.org/10.1111/1574-6976.12062.

Wang, Z.H., Yang, J.Q., Zhang, D.J., Zhou, J., Zhang, C.D., Su, X.R., Li, T.W., 2014. Composition and structure of microbial communities associated with different domestic sewage outfalls. Genet. Mol. Res. 13, 7542–7552. http://dx.doi.org/10.4238/2014.September.12.21.

Winter, C., Smit, A., Herndl, G.J., Weinbauer, M.G., 2004. Impact of Virioplankton on archaeal and bacterial community richness as assessed in seawater batch cultures. Appl. Environ. Microbiol. 70, 804–813. http://dx.doi.org/10.1128/AEM.70.2.804-813.2004.

Wintzingerode, F.V., Göbel, U.B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229. http://dx.doi.org/10.1111/j.1574-6976.1997.tb00351.x.

Yang, Y., Li, B., Zou, S., Fang, H.H.P., Zhang, T., 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res. 62, 97–106. http://dx.doi.org/10.1016/j.watres.2014.05.019.

Zhang, T., Shao, M.-F., Ye, L., 2012. 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J. 6, 1137–1147. http://dx.doi.org/10.1038/ismej.2011.188.

Zhang, T., Yang, Y., Pruden, A., 2015. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. Appl. Microbiol. Biotechnol. 99, 7771–7779. http://dx.doi.org/10.1007/s00253-015-6688-9.