Antibiotics, Antibiotic Resistant Bacteria (ARB), and Antibiotic Resistance Genes (ARGs): Increasing removal with wetlands and reducing environmental impacts

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Running Title: A Review on emerging contaminants removal using wetlands

ABSTRACT

There is a growing concern of the presence of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in treated wastewaters. In this article we review the occurrence and impact of antibiotics, other pharmaceutical and personal care products (PPCPs), ARB and ARGs in the environment specially addressing the potential of wetlands to their removal. The capacity of different types of constructed wetlands (CWs) including surface flow, and vertical and horizontal subsurface flow is analized. Also the efficiency of small onsite systems (septic tank and infiltration system) is evaluated, as well as wetlands receiving secondary treated effluents (natural assimilation wetlands). CWs have proven effective in removing many antibiotics, other PPCPs, ARB and ARGs. Small onsite systems are not effective for removal of such contaminants and can be an important source. Tertiary wetland-based systems are very appropriate for removing these contaminants. In the paper we suggest future research needs in this topic.

Keywords: constructed wetlands, assimilation wetlands, antibiotic resistance, green treatments, pharmaceuticals, emerging organic contaminants.

INTRODUCTION

In recent years there has been growing concern about the release of organic compounds of anthropogenic origin, known as emerging organic contaminants (EOCs), to the environment. These EOCs include a diverse group of thousands of chemical compounds, such as pharmaceuticals and personal care products (PPCPs), pesticides, hormones, surfactants, flame retardants, plasticizers and industrial additives, among others. Metabolites and intermediate degradation products of parent compounds are also included (Farré et al., 2008). The ubiquity of EOCs in the environment poses a threat to many non-target living organisms since they are designed to remain biologically active for long periods.

Antibiotics are a type of PPCPs and their presence in the environment is of special concern due to the development of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). These substances are extensively used in both human and veterinary medicine against microbial infections and are excreted from the body of the treated organisms, together with their metabolites, within a few days of consumption. It has been widely demonstrated that conventional sewage treatment plants (STPs) are inefficient in the removal of many antibiotics, ARBs and ARGs, thus contaminanting the receiving ecosystems with a complex mixture of bioactive agents and bacteria (Cacace et al., 2019; Corno et al., 2019; Manaia et al., 2018, 2016).

Once in the environment, antibiotics can lead to the continuous selection for ARB that contain ARGs (Choo, 1994; Costanzo et al., 2005; White et al., 2006: Ávila and García, 2015; Sui et al., 2015; Shiffett and Schubauer-Berigan, 2019). Although ARB are a threat to public health, ARGs are the underlying mechanism of an increasing antibiotic tolerant microbial consortia. In recent years, medical professionals and scientists globally have become concerned over the prevalence of ARGs and ARB that have appeared as the result of over prescription/production of antibiotics (Zhang et al., 2009). Overuse of antibiotics can range from doctors prescribing them ineffectively to patients for viral infections (González et al., 1997), patients using other people's antibiotics or old antibiotics and the use of antibiotics as growth promotors and feed additives in livestock and poultry (Kim and Aga, 2007). In 2019, antibiotic-resistant bacteria and fungus caused more than 2.8 million infections and 35,000 deaths in the United States alone (CDC, 2019). Currently, there are approximately 260 different antibiotics in about 20 different families or classes (Everage et al., 2014).

In this paper, we review the occurrence and impact of antibiotics, ARB and ARGs in the environment and address the potential of wetlands to remove these compounds from wastewaters. We focus on antibiotics among other types of PPCPs because they are the main cause of ARB and ARGs. However, because the review explores the potential of wetlands as bioremediation alternative for EOCs, in the Sections dealing with wetlands we also include descriptions on their potential for other PPCPs different than antibiotics. There are already several reviews on the occurrence of PPCPs in the environment and removal by constructed wetlands (Matamoros et al., 2006; García et al., 2010; Velicchi et al., 2014; Zhang et al., 2014; Vymazal et al., 2017), but few specifically focused on antibiotics, ARB and ARGs (Liu et al., 2019). Also the new feature of the present review is that there are specific Sections dealing on small-scale domestic (septic) treatment systems and natural assimilation wetlands, which are natural wetlands which receive secondary treated effluents.

1. Sources of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs)

After intake, antibiotics rarely become fully metabolized in the body and thus are partially excreted in their original form together with their metabolites through urine and feces within a few days of consumption (Zhang et al., 2009). In rural areas, direct excretion from medicated cattle in animal husbandry facilities is the main entrance route into the environment (Tasho and Cho, 2016; Wei et al., 2011), together with manure application as fertilization amendments (considering also biosolids from STPs), farm runoff (Bird et al., 2019; Chee-Sanford et al., 2009; Dolliver and Gupta, 2008; Sabourin et al., 2009) and fish farming (Sapkota et al., 2008). In urban areas, the regular discharges of STP effluents into aquatic bodies (e.g. rivers, lakes), including hospital wastewater effluents, is the main entrance pathway of these substances into the environment (Hocquet et al., 2016; Rodríguez-Mozaz et al., 2015; Michael et al., 2013). It is estimated that 30-60% of all prescribed antibiotics can end up in STPs, which act as primary reactors creating ARB and ARGs (Costanzo et al., 2005). Recent studies show that incomplete metabolism in humans and improper disposal of antibiotics to sewage treatment plants (STPs) has been a main source of antibiotic release into the environment (Everage et al., 2014; Naquin et al., 2015; Boopathy, 2017; Grabert et al., 2018).

There are many commonly prescribed antibiotics that are found in ng to μg L^{-1} concentration levels in sewage wastewater and treated effluents (Grabert et al., 2018; Bird et al., 2019). These antibiotics exert constant selective pressure on microbial populations in developing antibiotic resistance. Conventional STPs, based on activated sludge (CAS) biological treatment, are an ideal place for the development of ARB and ARGs as the sewage is concentrated in the activated sludge process and the microbes are actively multiplying with constant exposure to low concentrations of a multitude of antibiotic drug types. This mixture of differentially acting drugs and high microbial activity becomes an ideal environment for adaptive evolution of antibiotic resistance in microbes. There are various reports on the occurrence of ARGs in treated municipal wastewater effluents worldwide. Tables 1 and 2 summarize a representative selection of the genes reported, but there are numerous other studies not included here because of the presence of the same genes in different geographical locations.

2. Antibiotic resistance acquisition

It is important to understand that antibiotic resistance can be present in all bacteria, not solely pathogenic bacteria (Hawkey, 1998). Bacteria often have two distinct types of resistance to antibiotics, intrinsic and acquired resistance. Intrinsic resistance is a naturally occurring trait in the organism, while acquired resistance is the evolution of sensitive bacteria to resistant bacteria (Hawkey, 1998). Organisms most often develop resistance to antibiotics because of spontaneous mutations in their DNA structure, regardless of the amount of antibiotics actually present.

Bacteria with resistance to antibiotics started to appear soon after antibiotic use became widespread (Bronzwaer et al., 2002). Although scientists noted the phenomenon of resistance as early as the mid-twentieth century, it is now understood that antibiotic resistance has likely existed for as long as microorganisms have been able to produce antibiotics naturally. Because antibiotics remove all the susceptible bacteria from the treated individuals, the only bacteria left are those resistant to the treatment, which may flourish and spread resistance throughout the environment. Multi-drug resistant bacteria have been cultured from habitats isolated from anthropogenic disturbances for hundreds of years (Bhullar et al., 2012). It is expected that in the near future many bacteria will evolve to become completely resistant to most or all antibiotics used today that will increase risks to the environment and to humans.

Bacteria are able to acquire ARGs through mutations and through gene transfer. The main method of antibiotic gene transfer is through horizontal gene transfer (Salyers et al., 2004). In this method, bacteria acquire the genes from other bacteria within and across different species. Every time bacteria multiply and divide, the ARGs spread vertically in an exponential scale, enabling this rapid spread also in the aquatic environment. Depending on the antibiotic, many different genes may exist that allow the bacteria to survive to antibiotic exposure. For example, 38 different tetracycline resistance genes have been found and described in bacteria (Roberts, 2005; Grabert et al., 2018).

3. Antibiotics, ARB and ARGs in the environment and their impacts

The continued use of antibiotics is likely to increase the frequency of antibiotic resistance in the environment (Gillings and Stokes 2012). For instance, soil samples in the Netherlands were shown to contain up to 15 times more genes-encoding resistance in 2008 when compared to soil samples from 1970 (Knapp et al., 2010). Furthermore, antibiotics can survive for extended periods of time in the environment and free DNA carrying ARGs can last up to two years in the soil.

Urban water bodies may become reservoirs of ARGs, particularly where wastewater is improperly managed or treated. A clear example of this is demonstrated in wetlands associated with the Tijuana River that discharges to the Pacific Ocean near the US-Mexico border (Cummings et al. 2017). This river receives drainage from the Tijuana metropolitan area including inadequately treated sewage and landfill runoff and stormwater. Cummings et al. (2017) analyzed surface sediments from the river estuary and a nearby urban wetland, Famosa Slough, that is not directly affected by sewage. They found plasmid-mediated quinolone resistance genes *qnrA, qnrB, qnrS, qepA, and aac(6')-Ib-cr* in the Tijuana River Estuary, and only *qnrB, qnrS, and qepA* at Famosa Slough.

Belding and Boopathy (2018) examined natural surface waters near Chauvin and Port Fourchon, Louisiana, in the lower Mississippi River Delta, and found significant numbers of ARB and ARGs, indicating their widespread presence in coastal waters used for recreation and fishing. Bird et al. (2019) also identified ARB and ARGs in Bayou Lafourche, an important waterway in southeastern Louisiana. In this case, the primary cause for this contamination was related to livestock. Although the antibiotics were found in small concentrations, antibiotics at sublethal concentrations provided the selection pressure needed to promote genetic exchange leading to ARB and ARGs without killing the entire microbial pool (Conkle et al., 2012).

Individual septic tanks contribute significantly to the increase and spread of ARGs due to the low level of treatment and lack of any significant disinfection. ARB and ARGs have been reported in the Mississippi River which receives treated wastewater and agricultural runoff from a large expanse of the central United States (Bird et al. 2019).

4. Impacts of conventional wastewater treatment on antibiotics, other PPCPs, ARB and ARGs

Secondary municipal wastewater treatment using CAS does not substantially remove antibiotics and other PPCPs, and removal rates are highly compound specific (Conkle et al., 2008; Matamoros et al., 2006; Ávila and García, 2015; García et al., 2010; Velicchi et al., 2014; Zhang et al., 2014; Vymazal et al., 2017). Removal mechanisms of such compounds during CAS treatment include microbial degradation and sorption to particulate matter (Conkle et al, 2010). However, sludge can retain significant concentrations of compounds that can be released back into the environment after biosolids application onto land. Hydraulic retention time (HRT) in CAS treatment is short, often less than 12 hours, and is not generally adequate for significant removal of most of PPCPs compounds, including antibiotics.

In addition, STPs can increase ARB and ARGs in wastewater during the treatment process. In a municipal treatment system in south Louisiana, Boopathy (2017) reported that raw sewage as well as secondary effluent from a treatment plant tested positive for Methicillin-Resistant *Staphylococcus aureus* (MRSA), and free DNA of *mecA* gene. The antibiotic resistance was significantly higher in treated sewage than in raw sewage, indicating that bacteria were acquiring ARGs during the treatment process.

There are a number of advanced tertiary treatment technologies that can effectively remove antibiotics and other PPCPs such as ozonation, advanced chemical oxidation or ultraviolet (UV) radiation, but these approaches are generally very expensive, especially for small municipalities (White et al., 2006). For these reasons, in the context of small municipalities, Ávila and García (2015) suggested the use of decentralized, low-cost technologies including constructed and natural wetland treatments.

5. Removal of antibiotics, other PPCPs, ARB and ARGs in constructed wetlands

CWs for wastewater treatment are nowadays a state of the art technology with thousands of full-scale applications at a global scale. CWs are being increasingly used for decentralized wastewater treatment due to their simple design and because operation and maintenance costs are typically much lower than conventional systems.. CWs can tolerate fluctuations in daily flow rate and load, a signature of wastewater production (Zurita et al., 2012). Wetlands do not require any chemical addition and their sludge production is negligible (Álvarez et al. 2017; Paing et al. 2015). However, constructed wetlands (CWs) are more land intensive and require from 2 to 8 m^2/PE (person equivalent) compared to 0.06 m^2/PE for conventional treatment. Natural assimilation wetlands used for tertiary treatment need about 30 m^2/PE (Figure 1) (White et al., 2006). Efficiency of CWs for the improvement of conventional water quality parameters has been widely evaluated and demonstrated over many decades, and very positive results have also been obtained regarding EOCs removal (Ávila et al. 2016; Ávila and García 2015; Conkle et al. 2008; Verlicchi and Zambello 2014). A number of studies have shown that wetlands can remove many antibiotics and other PPCPs (White et al., 2006; Conkle et al., 2008; Chen et al., 2016; Fang et al., 2017; Dires et al., 2018; Hayward et al., 2018; Santos et al., 2019; Yi et al., 2019).

CWs are classified based on how water flows through the wetland (i.e., surface flow and subsurface flow). In general, subsurface flow constructed wetlands (SSF-CWs) are used in the framework of decentralized sanitation, while surface flow systems are usually larger in scale than SSF-CWs, and are frequently applied as tertiary treatment. Recent studies on CWs efficiency have shown the capacity of these systems to remove not only antibiotics and other PPCPs, but also ARB and ARGs from wastewaters (Chen et al., 2019; Liu et al., 2019). The direct discharge of STP effluent to natural assimilation wetlands also results in significant removal of these substances (White et al. 2006: Conkle et al. 2008).

The main removal mechanisms in CWs include biodegradation, substrate adsorption, precipitation, plant uptake, photolysis and hydrolysis (García et al., 2003; Uggetti et al., 2016; Ávila et al., 2017; Álvarez et al., 2017; Pelissari et al., 2018). However, there is a lack of knowledge on the relative importance of these mechanisms and on the factors influencing the prevalence of one or/and another. Most of the CWs studied achieved desirable antibiotic and other PPCPs, and ARGs removals, and outperformed conventional wastewater treatment systems (Chen and Zhang, 2013; Chen et al., 2016; Hijosa-Valsero et al., 2010; Xu et al., 2015; Zhang et al., 2014). For instance, detectable concentrations of ARGs (*tetA, tetM,* and *ampC*) in municipal wastewater were removed in a horizontal subsurface flow constructed wetland (HSSF-CW) mesocosm experiment after 150 days of treatment (Nõlvak et al., 2013). These authors observed a clear correlation between the high removal of NH_4–N and organic matter, and the reduction of ARGs in the CW effluent.

Matamoros and Bayona (2006) evaluated the removal of 11 PPCPs in a decentralized sanitation plant in Spain, consisting of 2 parallel HSSF-CWs with different water depths, both planted with *Phragmites australis*. Removal was generally greater in the shallower bed due to a higher passive aeration and a less negative redox potential that enhanced biodegradation of the compounds. The most hydrophobic compounds (with higher K_d), such as the musks tonalide and galaxolide, had 80% removal from the aqueous fraction and were retained in the gravel of the filter bed. Only the non-steroideal anti-inflammatories diclofenac and ketoprofen were not efficiently removed in both systems, due to their high hydrophobicity and low biodegradation potential. The recalcitrance of these two compounds has been widely demonstrated in conventional STPs (Gros et al., 2010, 2007; Mamo et al., 2018). In another work by the same authors, the removal of 13

PPCPs was studied in a variety of field scale, onsite confined domestic wastewater treatment systems in Denmark, including biological sand filters, compact biofilters, vertical subsurface flow constructed wetlands (VSSF-CWs) and HSSF-CWs serving from 2 to 280 PE (Matamoros et al., 2009). The removal rate for PPCPs was generally >80% with the exception again of diclofenac and ketoprofen, and the antiepileptic drug carbamazepine. Carbamazepine was also poorly removed in a surface flow wetland system in Lousiana (Conkle et al., 2008). Matamoros et al. (2009) reported that the VSSF-CWs, with greater oxygenation in the unsaturated gravel filter, yielded better removals. White et al. (2006) reported the complete removal of five out of nine PPCPs occurring in a sewage effluent pumped to a large treatment wetland in Orlando, FL, but again carbamazepine was not efficielty removed.

Liu et al. (2019) reviewed the capacity of 106 different CW treatment systems to eliminate 39 antibiotics, including sulfonamides, quinolones, tetracyclines, macrolactones, chloramphenicol, polyethers and beta-lactams. CW configurations included surface flow constructed wetlands (SF-CWs), HSSF-CWs, VSSF-CWs and hybrid flow constructed wetlands, all at microcosm-scale or mesocosm-scale. VSSF-CWs were the most effective in eliminating antibiotics (>70% for most of the antibiotics), and these results were significantly different ($p < 0.05$) from the other types of CWs (Figure 2). It should be noted that the systems compared in this study operated under very different conditions (solar radiation, temperature, size, influent quality, etc.). Nevertheless, this review confirmed the crucial role of the wetland configuration in the overall removal efficiency of the system, as confirmed by a number of other studies (Chen et al., 2016; Hijosa-Valsero et al., 2011; Huang et al., 2017, 2015).

VSSF-CWs are generally more efficient than HSSF-CWs in the removal of organic matter, ammonia and also EOCs. Intermittent discharge in VSSF-CWs creates unsaturated conditions between pulses, promoting aeration/oxygenation of the filter medium during those intervals (Kahl et al., 2017; Nivala et al., 2019). Nitrification capacity is greatly enhanced by these intermittent, unsaturated conditions (García et al., 2010), which can also enhance the degradation of different antibiotics by co-metabolism (He et al., 2018; Kassotaki et al., 2016; Müller et al., 2013). In contrast, in HSSF-CWs the granular medium remains continously saturated and still, positive results have been obtained for these systems. Nõlvak et al. (2013) demonstrated that detectable concentrations of ARGs (*tetA, tetM* and *ampC*) in municipal wastewater influents were removed in a HSSF-CWs mesocosm experiment.

A relatively new configuration of VSSF-CW, in which the top part of the filter medium is only partially saturated, enhances a variety of redox gradients through the filter bed and also diversifies the bacterial metabolism developed in the system, improving nitrogen transformation pathways and achieving complete nitrification-denitrification (Ávila et al., 2017; Dong and Sun, 2007; Pelissari et al., 2018, 2017). This partial saturation has already achieved high removal rates of different EOCs including antibiotics such as trimethoprim and sulfamethoxazole (Sgroi et al., 2018). All in all, SSF-CWs are more efficient in activating and removing ARGs than SF-CWs (Chen et al., 2016b; Decamp and Warren, 2001). Inside SSF-CWs, VSSF-CWs have a higher efficiency than HSSF-CWs. These findings are in agreement with previous research on organic matter and nutrient removal in CWs (Carvalho et al., 2014; Vymazal, 2011). Greater general efficiency of SSF-CWs than SF-CWs may be due to a greater exposure to particles of the granular medium resulting in higher sorption capacity and more complex biological processes occurring in the filter media with close proximity of aerobic and anaerobic zones. However, a clear correlation has not been established yet between redox mediated biological processes and ARGs removal, and this is a topic that deserves intensive research. The reader has to be aware that SSF-CWs and SF-CWs are not straightforward comparable since they are used for different applications.

The removal efficiency of different antibiotic families in CWs varies with the physical characteristics of the compounds (e.g., water solubility, octanol-water distribution coefficient (K_{ow})). Those compounds with the best removal rates (e.g., quinolones and tetracyclines at 70-90%) generally have a low solubility, a high tendency to adsorb tofilter medium of SSF-CWs, and are prone to photodegradation in SF-CWs (Jia et al. 2012). For instance, high K_{ow} values and low water solubilities, as in the case of macrolides, lead to high adsorption by plants and the filter medium (Liu et al. 2019). In this study average removal efficiencies of macrolides were high in VSSF-CWs (81.73%). In contrast, sulfonamides, generally highly hydrophilic, are removed mainly by microbial degradation due to their higher solubility and bioavailability. Average eliminations for sulfonamides have therefore wide ranges of removals from 13% to 99.9%. Among the different antibiotics studied, sulfamethoxazole and erythromycin have raised scientific concern due to their high concentrations and frequencies of detection in feed waters to CWs, and the low reduction rates obtained (Liu et al. 2018; Yang et al. 2018). Conkle et al., (2008) demonstrated the strong sorption to soil particles of three fluoroquinolone antiobiotics individually. However, when mixtures of the 3 antibiotics were applied, there was substantially less sorption of two of the compounds, clearly indicating a competitive sorption among compounds that sorb well alone. Considering how many different antibiotics can be in a particular waste stream, sorption behavior in mixtures needs to be further evaluated.

On the other hand, different studies have reported negative removals of antibiotics in CWs, meaning that higher concentrations were detected in the effluents of the systems than in the corresponding influents (Fang et al., 2017; Conkle et al, 2010). These results are not infrequent either in conventional STPs, and are usually related to the presence of second-phase metabolites in influent wastewaters, such as acetylates or glucuronides. These metabolites, which are not considered or measured in most of the studies, can deconjugate and transform back into the parent compound (García-Galán et al., 2012; Kasprzyk-Hordern et al., 2009), thus accounting for the higher concentrations observed in the CWs effluents than in the influent to the systems. For more hydrophobic antibiotics, which are adsorbed and retained in the filter medium of the CWs, their eventual release has also been confirmed, contributing also to these negative removal rates (Li et al. 2014). Indeed, solid particles tend to act as a reservoir for ARB and ARGs in CWs, thus a pulsing regime that allows the systems to "rest" (such as in VSSF-CWs) will favor continued reduction in antibiotics, PPCPs, ARB and ARGs. Fang et al., (2017) observed that the concentration of ARGs increased in the soil of a CW consisting of 3 SF-CWs connected in series and planted with different macrophytes. This facility has been operated for 10 years, receiving domestic sewage from a population of roughly 4000 people in Nanchang, (Jiangxi province, China). The authors evaluated ARGs and ARB removal efficiency, and observed that most of the ARGs detected in the aqueous phase were eliminated, but their levels increased in the solid phase. As a result, this phase could become an important source of aqueous ARGs through the desorption and release of microbes from soil to the water phase.

It should also be considered that hydraulic loading rate (HLR) and hydraulic retention time (HRT) are also key parameters in the CWs removal efficiencies of ARGs (Anderson et al., 2013; Maal-Bared et al., 2013; Miller et al., 2014). Longer HRT in CWs (in contrast to CAS) may lead to ARGs accumulation in the soil or the medium which eventually can be discharged in the effluent. Seasonal variability in CWs retention efficiencies were also observed in the study by Fang et al., (2017) on SF-CWs. Absolute and relative abundances of ARGs in the CWs soil were higher during summer than winter, due mainly to warmer temperatures promoting the survival of bacterial communities in the soil and also larger communities of ARGs carriers. Higher HLR (and therefore shorter HRT) during summer may have also enhanced the transport and exchange of these ARGs carriers between the soil and water phase, due to a higher mechanical turbulence of the water phase. In VSSF-CWs higher HLRs and infiltration rates, compared to those in HSSF-CWs, would filter out bacteria (size exclusion) and bind extracellular DNA onto soil particles (Cardinal et al., 2014).

The type of filter medium has also been studied and considered as a relevant determinant feature of CWs removal efficiency. For instance, different studies have demonstrated that antibiotics and ARGs removal in zeolite were higher than those of volcanic rock in VSSF-CWs (Liu et al., 2013) and also higher than those obtained in oyster shell, medical stone and ceramic media in HSSF-CWs (Chen et al., 2016). Higher relative surface area, micropores and Si-OH structures for chemical sorption and microbial attachment improve the removal of ARGs (Hu et al., 2012). Dires et al. (2018, 2019) evaluated the effectiveness of broken brick as filtering material in 8 HSSF-CWs (mesocosms scale) to remove nutrients and ARB from hospital wastewater in Ethiopia. The wetlands achieved 7.1 log10 and 5.1 log10 removal of total and fecal coliforms, respectively. Absolute abundances of ARB were reduced from the influent to the effluent by up to 93.2%.

Dires et al. (2018, 2019) also highlighted the role of vegetation in CWs, as they observed that ARB removals were higher in planted CWs (81% - 93%) than in unplanted wetlands (42% - 74%). Plants do not seem to be directly involved in the removal efficiency of ARGs, but they are involved indirectly. They are crucial in filtering solid particles and delivering small amounts of oxygen to microbial communities, which in turn create unfavorauble conditions for ARGs (Anderson et al., 2013; Chen and Zhang, 2013; Fang et al., 2017). They also provide the surface for biofilm development, which in turn enhances the microbial removal ability, and their subsequent harvesting in surface systems can reduce ARGs accumulation (Huang et al., 2015). Common reed (*Phragmites australis*) is widely used in CWs systems, and its presence has proved quite efficient in reducing ARGs and 16sRNA levels compared to other plants (Fang et al., 2017; Yi et al., 2017). Other aquatic plants such as *Pontederia cordata*, *Myriophyllum verticillatum* and *Cyperus alternifolius* can also contribute to removal of ARGs (Chen et al., 2015). Chen et al., (2016b) observed significant removal of antibiotics and ARGs in 6 different mesocosm-scale CWs, all of them with different flow configurations and plant species. As previously reported, SSF-CWs yielded better aqueous removal rates than SF-CWs, and the authors also observed that biodegradation was the main removal pathway, in terms of mass removal, compared to substrate adsorption or plant uptake. By contrast, Helt et al., (2012) found that adding ciprofloxacin to mesocosm-scale wetlands planted with *P. australis* led to an increase in resistance, not only to ciprofloxacin, but to other classes of antibiotics as well (cephalosporins, penicillins, tetracyclines, and sulfonamides), highlighting the potential for a single antibiotic exposure event to increase the antibiotic resistance profile to different antibiotics.

In another study by Chen and Zhang (2013), the fate of ARGs of *tetM*, *tetO*, *tetQ*, *tetW* (from tetracycline) *sulI*, *sulII* (sulfadiazine) *intI1*, and 16S rDNA genes was evaluated in a HSSF-CW, resulting in a reduction of 1–3 orders of magnitude of the ARGs initial concentrations. The authors concluded that the HSSF-CW could perform better than advanced treatments such as UV disinfection or biological aerated filters.

Solar radiation is an important factor in SF-CWs as UV light alters DNA structures and can be lethal for bacteria. In the study by Fang et al., (2017), the removal of 14 ARGs was evaluated in 3 field-scale SF-CWs in series operating since 2005. The authors measured ARGs removal of 78% and 60% in winter and summer, respectively, demonstrating the influence of season on the performance of CWs, as higher temperatures may favor the activity of microbial communities and ARGs-containing microorganisms. The concentration of ARGs in the effluent was still 1 or 2 orders of magnitude higher than the levels in the efffluent stream. Indeed, concentrations of five ARG (*sul1, tetA, tetC, tetE, and qnrS)* in winter and of six ARGs *(sul1, sul3, tetA, tetC, tetE, and qnrS*) in summer increased throughout the treatment process. In contrast, Yi et al., (2017) measured high removal rates for several ARGs (*sulI*, *sulII*, *intI1*, qnrA) and 16 sRNA during a summer study in a 5.1 ha HSSF-CW and reported that the effluent from the CW had ARGs concentrations comparable to those of the receiving surface waters.

6. Antibiotics, PPCPs, ARB and ARGs in small-scale domestic (septic) treatment systems.

In many areas with a low-density population, such as suburban and semi-rural situations, sewage treatment for a significant proportion of the population is provided by small-scale (septic tanks) and package plant treatment systems. For example, over 85% of the population in some areas of the United States use septic systems (Godfrey et al. 2007) mainly because conventional centralized systems for such populations is very expensive (Matamoros et al. 2009). Coastal Louisiana is an example of an area with large numbers of septic systems. The southeastern part of the state is the most populous area in Louisiana, but only in the metropolitan areas of New Orleans and Baton Rouge are most of the population served by conventional STPs. In these two areas, a large proportion of treated municipal effluent is discharged to the Mississippi River. In the rest of that area, and in much of Louisiana, most effluent flows, directly or indirectly, into open water systems. Approximately 1.3 million residents in Louisiana treat and dispose of sewage on-site (not connected to municipal treatment) and an estimated 50% of these systems may be failing or malfunctioning (Figure 3) (LDEQ 2018; Martínez et al. 2019). This is similar in other areas in the US.

Septic systems and other on-site treatment systems such as package plants are also important sources of antibiotics and other PPCPs (Sui et al., 2015). Most studies of small-scale onsite wastewater treatment systems include limited information on antibiotics and other PPCPs in septic tanks and/or soil zones (Hinkle et al., 2005; Carrara et al., 2008; Huntsman et al., 2006). Swartz et al. (2006) monitored the concentrations of caffeine and its metabolite paraxanthine, as well as other micropollutants, in a residential septic system and downgradient groundwater. Concentrations of caffeine and paraxanthine were extremely high (caffeine ranged from 17,000 to 23,000 ng/L and paraxanthine from 55,000 to 65,000 ng/L) in the septic tank discharge, resulting in high concentrations of the compounds in the nearest well (>1700 ng/L) that declined with distance and depth. A recent study concerning the occurrence and fate of different micropollutants in groundwater networks affected by septic systems showed that concentrations of several PPCPs such as carisoprodol (a muscle relaxant) and lidocaine (a typical anesthetic) could be greater than 0.1 µg/L in groundwater below and downgradient of the leaching bed (Phillips et al., 2006).

Considering that individual septic systems do a very poor job in removing EOCs from wastewater (Phillips et al., 2015; Swartz et al., 2006), the high number of septic systems and other decentralized treatment systems in the US pose a serious threat to the aquatic ecosystems health. For instance, only in Louisiana there are about 90,000 septic systems discharging to natural waters that drain to Lakes Pontchartrain and Maurepas (Figure 3). Thus, antibiotics, other PPCPs, ARB and ARGs may be a significant problem in the Lake Pontchartrain Basin due to the large number of individual septic systems. Decentralized treatment systems where septic tanks are connected to CWs, natural wetlands or drainage fields offer an economical and effective way of reducing the concentrations of antibiotics, other PPCPs, ARB and ARGs from septic systems.

Decentralized treatment approaches for septic systems and package plants offer an economic way of achieving better treatment. In this approach, liquid waste is collected in a small diameter (2-3 in) flexible piping ditch-witched into the ground. This flow can then be further treated in a conventional STP, CW or a natural wetland.

7. Reduction of antibiotics, other PPCPs, ARB and ARGs in natural assimilation wetlands

Natural wetlands can also reduce antibiotics, other PPCPs, as well as ARB and ARGs, in treated sewage effluents. Direct discharge to natural wetlands after treatment in a centralized STP can often result in significant removal of these compounds in a relatively small area. Hayward et al. (2018) reported that ARGs generally decreased in tundra wetland ecosystems receiving domestic wastewater, and that removal rates were directly correlated with HRT. In this study, short HRTs (2 days) produced the highest ARGs absolute abundance concentrations in the effluents. The authors indicated that soils may act as a reservoir for ARB, potentially enriching overlying waters by desorption. Similarly to the study by Fang et al. (2017) in CWs, seasonal variability in the removal efficiency of the tundra wetlands was also observed, with lower removal rates during summer.

Conkle et al. (2008) measured the uptake of various pharmaceutically active compounds (PhACs) in the Mandeville, Louisiana wastewater treatment system (Table 3), which included aerated ponds, a constructed nitrification-denitrification wetland, and the Bayou Chinchuba natural forested wetland system that discharges to Lake Pontchartrain. Thirteen of 15 PhACs studied were detected in the wastewater inflow to the treatment plant. Nine of the 13 compounds were above detection limits in the effluent of the treatment plant but concentrations of most compounds were reduced > 90% within the plant, while carbamazepine and sotalol were only reduced by 51% and 82%, respectively. The removal rates observed in the Mandeville system including the forested wetland (Tables 3 and 4) were greater than those reported for conventional STPs, probably due to the longer total HRT (>30 days). Most of the target PhACs were reduced to very low levels or below detection limits before discharge into the Lake Pontchartrain estuary, and their total annual loading was reduced from greater than 200 kg to less than 1 kg (Conkle et al. 2008). These results can be used to estimate the area of wetland required to reduce PhACs to near background or non-detect levels. The dry weather discharge from the plant is about 6500 m^3/day. The area of wetlands required to achieve high reductions in PhACs, considering the results by Conkle et al. (2008) in the overall treatment stream (STP + wetlands) would be about 23 ha. The area of the Bayou Chinchuba wetland is about 40 ha and the population of Mandeville is about 13,000 people and, thus, the surface per person equivalent (PE) to achieve the reduction rates reported by Conkle et al. (2008) is about 30 m^2. This constrasts with the 2 to 8 m^2/PE for constructed wetlands and 0.06 m^2 for conventional STPs (Ávila and García 2015) (Figure 1). Additional reduction can be achieved with larger wetland areas. Because all existing assimilation wetlands in Louisiana have areas that are in excess of what is required to reduce nutrients to background levels, it is likely that most if not all PPCPs are reduced to background or non-detectable levels in these more extensive systems.

Nutrient and sediment reduction in secondarily treated municipal effluents using natural assimilation wetlands has a long history in coastal Louisiana (Hunter et al., 2018; Day et al., 2019). There are 11 assimilation wetlands, five of which have operated for 27-70 years, with more than a combined 250 system-years of operation. There has been extensive studies of the hydrology, biogeochemistry, vegetation productivity, decomposition rates, faunal communities and PPCPs dynamics in these assimilation wetlands in coastal Louisiana. These studies have demonstrated that, generally, nutrients are reduced to background levels before flowing into open water bodies, vegetation productivity and accretion are enhanced, and decomposition rates are not increased compared to reference wetlands not receiving input of treated effluent. The information included in this review suggests that an optimally managed assimilation wetland could serve to reduce antibiotics, other PPCPs, ARB and ARGs.

8. Conclusions and future reseach needs

Paired investigations have shown that constructed wetlands outperformed conventional wastewater treatment systems for the removal of antibiotics, other PPCPs, ARG and ARBs. There is not a clear explanation reason, but much shorter hydraulic retention times could be a strong driver for lower efficiency in conventional systems.

Constructed wetlands with subsurface flow have higher removals than surface flow systems, and this seems to be linked to the presence of granular medium. Inside subsurface flow, vertical systems ourperform horizontal systems because higher oxygenation (alternance of saturated and unsaturated conditions) and resting periods which provide "time" for sorption. Pilot studies on testing the effects of wetland configuration and design and operation factors such as type and size of granular medium and hydraulic retention time using the same wastewater source are crucial to understand the effect on antibiotics, other PPCPs, ARB and ARGs removal. This type of studies will help to get insight on the relevance of the mechanisms involved in removal. Nowadays there is a clear lack of knowledge on the relative relevance of the different mechanisms. Sorption seems to be quite important because it is generally assumed that is fast in comparison with other processes; however, sorption is not in fact a removal mechanism, just a "change in phase" phenomenon.

There is a clear consensus in different studies that solid substrate (granular medium in subsurface wetlands and soil in surface wetlands, including natural assimilation wetlands) can become a source for is a reservoir for ARB and ARGs. Studies have shown lower ARGs removal in summer than in winter suggesting that in warmer periods there is higher survival and activity which favours transport from solid phase to bulk water. This is important in constructed wetlands, but also of high relevance for natural assimilation wetlands. Studies on survival rates on full-scale systems linked to risk assessment are rapidly needed to give light to these issues.

Information on removal capacity of antibiotics, other PPCPs, ARB and ARGs in small onsite systems is very scarce. In general, it seems that systems with septic tanks and somekind of infiltration technique have low removals. Connecting septic tanks to new small constructed wetlands will clearly increase efficiency. Retrofitting these systems will be easy and will require low investment from owners. Demostration projects are needed to encourage this type of development.

Natural assimilation wetlands and tertiary surface flow constructed wetlands have a great potential for secondary effluent microbial quality improvement and natural disinfection. Studies at field-scale facilities are needed to study distribution of antibiotics, ARB and ARGs in water an soil. Seasonal variations are also of great interest as well as influence on changes in hydraulic conditions.

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Table 1 ARGs for sulfonamide and tetracycline reported in the sewage treatment plant effluents

Reported Gene	Sewage Plant Location	Reference
Sulfonamide Resistance Genes		
Sul-1	Michigan, USA	Munir et al., 2011
Sul-1	Lausanne, Switzerland	Czekalski et al., 2012
Sul-II	Lausanne, Switzerland	Czekalski et al., 2012
Sul-1	Minnesota, USA	Burch et al., 2013
Sul-1	Shafdan, Israel	Negreanu et al., 2012
Sul-1I	Shafdan, Israel	Negreanu et al., 2012
Sul-1	Hangzhou, China	Chen & Zhang, 2013
Sul-1I	Hangzhou, China	Chen & Zhang, 2013
Sul-I	Louisiana, USA	Naquin et al., 2015
Tetracycline Resistance Genes		
tetW	Michigan, USA	Munir et al., 2011
tetO	Michigan, USA	Munir et al., 2011
TetQ	Wisconsin, USA	Auerbach et al., 2007
tetG	Wisconsin, USA	Auerbach et al., 2007
tetC	Hong Kong, China	Zhang et al., 2009
tetA	Nanjing, China	Zhang et al., 2009
tetA	Minnesota, USA	Burch et al., 2013
tetW	Minnesota, USA	Burch et al., 2013
tetX	Minnesota, USA	Burch et al., 2013
tetM	Hangzhou, China	Chen & Zhang, 2013
tetX	Louisiana, USA	Naquin et al., 2015
tetA	Louisiana, USA	Bird et al. 2018
tetX	Louisiana, USA	Belding&Boopathy, 2018.

Table 2 ARGs for Beta-Lactam and Macrolide sulfonamide and tetracycline reported in sewage treatment plant effluents

Reported Gene	**Sewage Plant Location**	**Reference**
Beta-Lactam		
Bla$_{TEM\text{-}uni}$	Massachusetts, USA	Lachmayr et al., 2009
blaM-1	South Carolina, USA	Uyaguari et al., 2011
Bla$_{\text{-}vim}$	Germany	Schwartz et al., 2003
amp-C	Germany	Schwartz et al., 2003
amp-C	Spain, Italy, Belgium	Bockelmann et al., 2009
Blas$_{hv\text{-}5}$	Spain, Italy, Belgium	Bockelmann et al., 2009
mecA	Gothenburg, Sweden	Borjesson et al., 2009
mecA	Louisiana, USA	Boopathy, 2017
mecA	Louisiana, USA	Naquin et al., 2015
Macrolide Resistance Genes		
ermB	Minnesota, USA	Burch et al., 2013
ermF	Shafdan, Israel	Negreanu et al., 2012
ermB	Shafdan, Israel	Negreanu et al., 2012
ermB	Spain, Italy, Belgium	Bockelmann et al., 2009
Others		
VanA	Spain, Italy, Belgium	Bockelmann et al., 2009
VanA	Germany	Schwartz et al., 2003

Table 3. Percent removal of pharmaceutially active compounds from the wastewater treatment plant at Mandeville Louisiana (USA), from the receiving forested wetland and from the total system . Adapted from Conkle et al. 2008 STP= sewage treatment plant, ND = non-detected.

Class	Compound	STP (%)	Wetland Discharge	Total % Removal
Alkaloids (estimulants)	Cotinine	>99	-	>99
	Caffeine	>99	-	>99
Psychiatric drugs	Carbamazepine	-53	105	51
	Fluoxetine	ND	ND	ND
β-blockers	Atenolol	>99	6	>99
	Nadolol	77	23	>99
	Propranolol	ND	ND	ND
	Metoprolol	92	8	>99
	Sotalol	30	52	>99
Antibiotics (sulfonamides)	Sulfapyridine	76	24	>99
	Sulfamethoxazole	100	-	>99
Anti-inflammatories	Acetaminophen	100	-	>99
	Naproxen	99	1	>99
	Ibuprofen	>99	-	>99
Lipid regulator	Gemfibrozil	64	31	95

Table 4. Percent removal and concentrations in treated wastewater for target pharmaceutical compounds in conventional wastewater treatment systems compared to constructed/natural wetland systems. Adapted from Conkle et al. (2008). STP= sewage treatment plant, CBZ = carbamazepine, SMX = sulfamethoxazole

Compound	% Reduction in Wetlands	% Reduction in STPs	STPs effluent concentration (µg/L)
Caffeine	>99	94-99	0.18-0.22
Carbamazepine	51	7-30	1.18-2.10
Gemfibrozil	91	69-75	0.18-0.40
Ibuprofen	99	90-96	0.15-0.37
Metoprolol	>99	30-65	0.19
Naproxen	99	66-93	0.25-0.30
Sotalol	82	25	0.25
Sulfamethoxazole	92	24	0.62

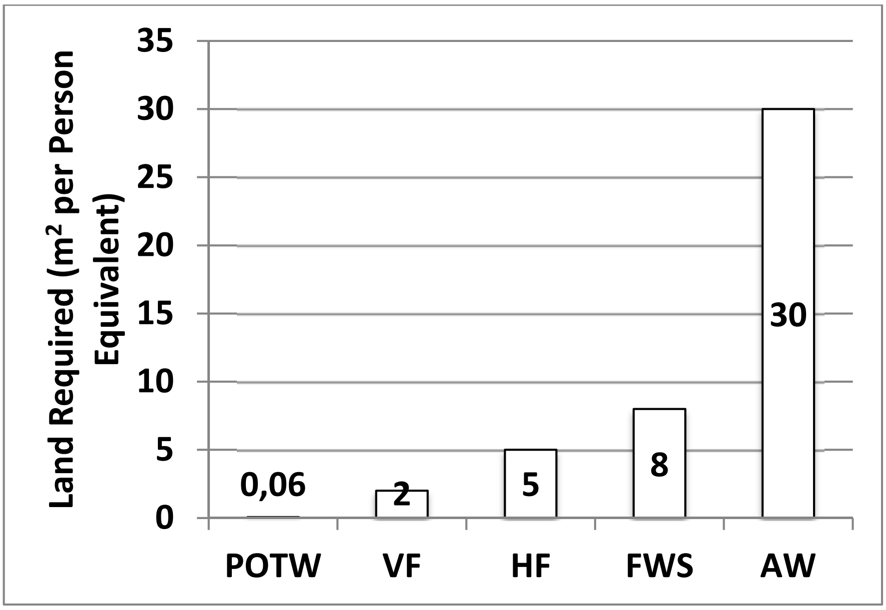

Figure 1. Land requirements (m^2 per person equivalent per 6500 m^3/day) for publicly-owned treatment works (POTW), vertical flow constructed wetlands (HF), horizontal flow constructed wetlands (HF), free water surface wetlands (FWS), and assimilation wetlands (AW). Values fro POTW, VF, HF, and FWS from Ávila and García (2015). Value for AW calculated from Conkle et al. (2008).

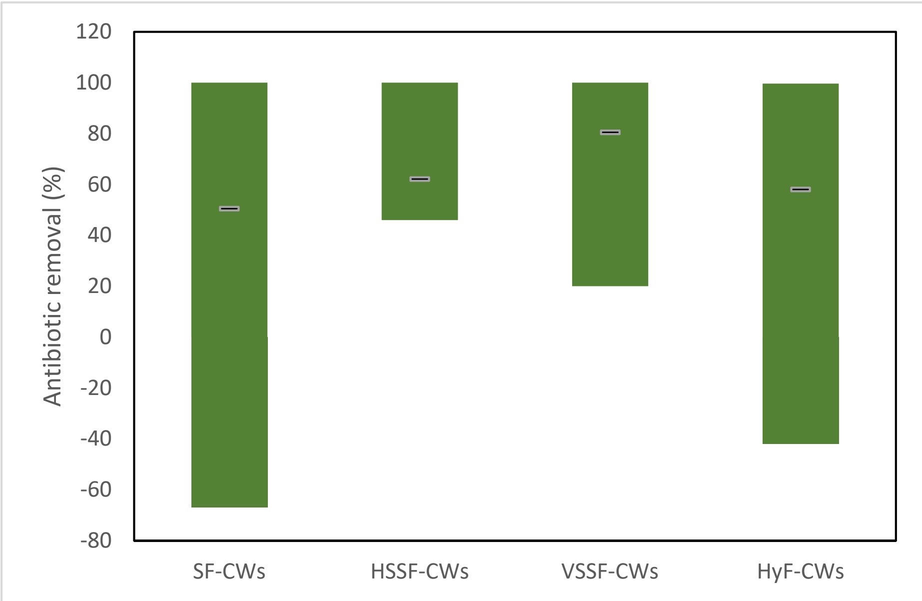

Figure 2. Antibiotic removal efficiencies of different constructed wetlands (average values). SF-CWs: surface flow constructed wetlands (n=31); horizontal subsurface flow constructed wetland (HSSF-CWs) (n=24); vertical subsurface flow constructed wetlands (VSSF-CWs) (n=31); hybrid flow constructed wetlands (HyF-CWs) (n=20). The different studies were carried out at a microcosm-scale or mesocosm-scale (Liu et al., 2019).

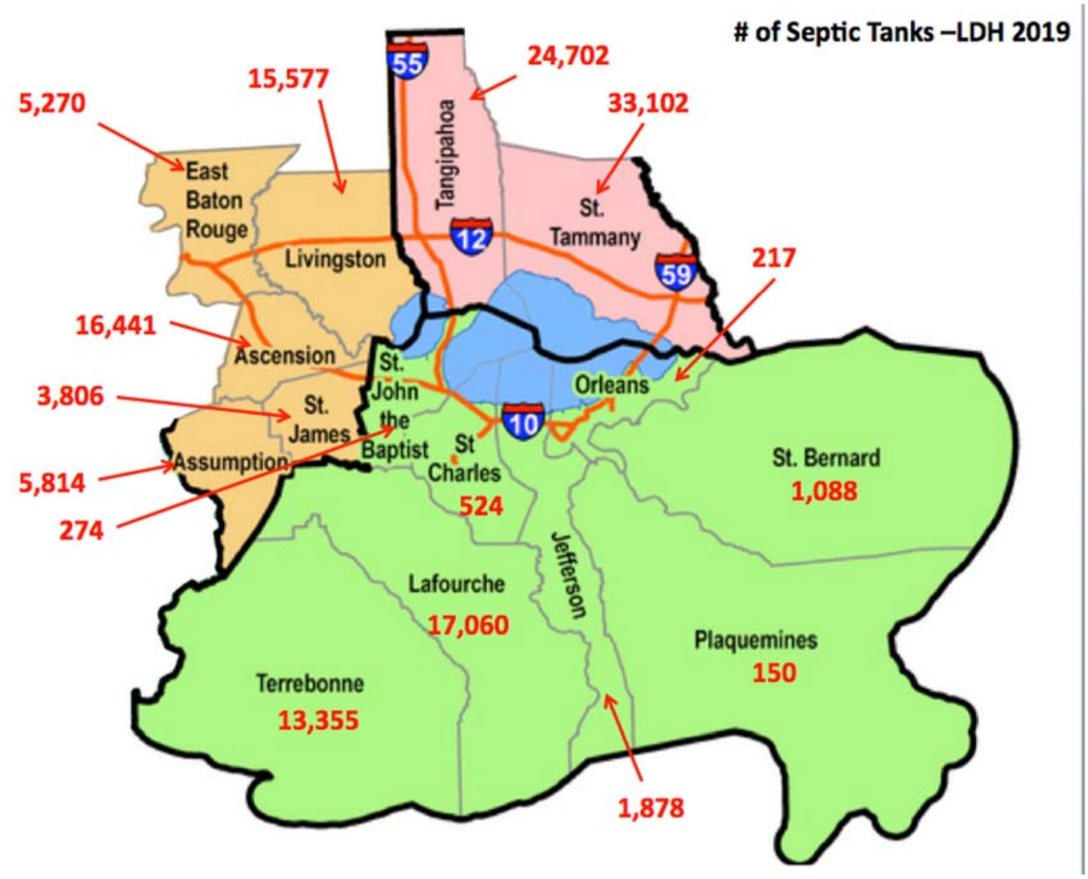

Figure 3. Number of individual septic systems by Parish in southeast Louisiana. Lakes Pontchazrtrain and Maurepas are shown in the middle of the figure. (Source LDH 2019).