Environmental occurrence of Oxytetracycline and the potential selection of antibiotic resistance in bacteria

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Oxytetracycline (OTC) is one of the most commonly administered antibiotics in livestock and has been categorized by the World Health Organization (WHO) as “critically important”. The misuse, overuse and partial metabolism of OTC in humans and the animal-producing industry over the years, has been accompanied by unintentional environmental antibiotic contamination. This work presents an inventory of OTC concentrations reported in different environmental media across the world, including aqueous and solid media. OTC concentrations are compared to threshold limits that delineate selective windows of resistance to assess the selective potential for *Salmonella typhimurium* in aqueous media and *Salmonella sp.* and *E. coli* in soil.

Introduction

Antibiotics in the environment can alter the composition and diversity of indigenous soil microbial communities, which can lead to inhibited decomposition of organic matter, altered nutrient cycling and changes in energy flow (Kennedy, Smith 1995, Baquero, Martínez & Cantón 2008, Kong et al. 2012). Additional concerns for antibiotic presence in the environment are the effects from long-term environmental exposure to low concentrations of antibiotic, in the range of ng/L to µg/L.

OTC, the subject antibiotic of this work, is commonly used to treat bacterial infections in human and is widely administered to farm animals to prevent (prophylactically) and treat (therapeutic) diseases, as well as increase productivity (growth promotion) due to its low toxicity profile, broad spectrum of activity and low cost (Chopra, Roberts 2001, Miranda, Zemelman 2002, Watanabe et al. 2010, Kong et al. 2012). In 2009, WHO categorized OTC as a critically important antibiotic because it is used as an alternative treatment of serious infections in humans and to treat diseases caused by bacteria that may be transmitted to humans from non-human sources. In the US, OTC has been used as a feed additive since 1953 (Chopra, Roberts 2001) for cattle, swine, poultry, turkeys, sheep, honey bees, pacific salmon, salmonids, cat fish, rainbow trout and lobster (CFR 2017). OTC is also one of the most common used antibiotics on plants but in a more modest degree relative to human and veterinary uses (McManus et al. 2002). OTC is partially absorbed in the digestive track, about 40-80% of the administered dose (Hirsch et al. 1999, Merck and Co. 2002, Liguoro et al. 2003, Sarmah, Meyer & Boxall 2006). With the increased incidence of antibiotic resistance detected and reported, increased attention has been paid to the environmental fate of OTC (Chopra, Roberts 2001).

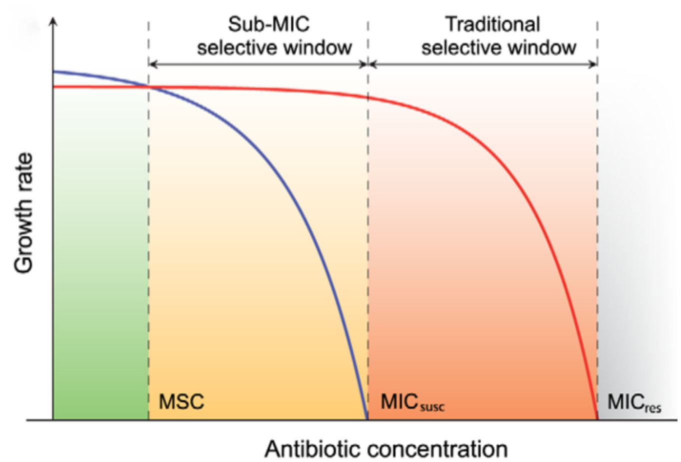

Figure 1 Schematic diagram of growth rates as a function of antibiotic concentration. The green region indicates the interval were sensitive strains (blue line) outcompete resistant strains (red line). The orange (sub-MIC selective window) and red (traditional selective window) regions indicate concentration intervals were resistant strains outcompete sensitive strains. MSC = minimal selective concentration; MIC_{susc} = minimal inhibitory concentration for the sensitive strain; MIC_{res} = minimal inhibitory concentration for the resistant strain. Figure adapted from Gullberg et al. (2011)

Even at low concentration levels, OTC and its transformation products have been shown to facilitate the development of antibiotic-resistant bacteria (ARB) and accelerate the evolution, mobilization and transfer of antibiotic resistant genes (ARG) to pathogens (Brown et al. 2006, Huang et al. 2012, Bengtsson-Palme, Larsson 2015, Lundström et al. 2016). Several authors have highlighted water and soil environments as recipients, reservoirs and sources of ARGs to pathogens (Pruden et al. 2006, Martínez 2008, Zhang et al. 2013). However, the scale at which the environmental presence of OTC contributes to the enrichment and prevalence of ARBs and ARGs is still unclear.

A general assumption in pharmacodynamics models is that selection of ARBs occurs on the range of a minimal inhibitory concentration (MIC) of the susceptible type (MIC_{susc}) and the MIC of resistant bacteria (MIC_{res}) as depicted in the orange shaded section in Figure 1 (Drlica 2003, Drlica, Zhao 2007, Gullberg et al. 2011). However, studies have shown that low concentration levels are relevant for the enhancement and prevalence of selected new mutants as well as pre-existing resistant mutants depicted by the yellow shaded section of Figure 1 (Gullberg et al. 2011, Liu et al. 2011, Quinlan et al. 2011, Lundström et al. 2016). This limit value is referred to as the minimal selective concentration (MSC), which can also be defined as the minimum concentration at which the resistant mutants can outgrow sensitive wild-type strains (Gullberg et al. 2011).

In the following review, we seek to provide a through inventory of OTC concentrations reported in the many environmental compartments and an assessment for the potential development of ARBs. Understanding the occurrence of OTC in the different environmental compartments (surface water, runoff, sediment and soil) is being used to identify potential hot-spots for antibiotics resistance. Comparison of environmental concentrations of a specific media with MSCs (thresholds for ARB development) is a potential means of identifying the selective pressure risk (Ashbolt et al. 2013). Such data, could aid in developing guidelines and/or regulations for safe limits that take into account the risk of antibiotic resistance development (Lundström et al. 2016).

Methodology

OTC has been detected in several different media around the world by numerous investigations (45 journal papers). The data reported for OTC concentrations were initially categorized as either aqueous or solid. The aqueous media data were further delineated based on origin, either agricultural (Ag), urban (Urb) or Industrial (Ind). The data from each of these sources (Ag, Urb or Ind) were then sorted based on sample origin: surface water, groundwater, runoff, WWTP influent and effluent, WW lagoon and anaerobic digester. OTC concentrations reported for solid media were also sorted based on sample origin: sediments (Ag, Urb, Ind or Fish farm), sludge (Urb, Ind, Cattle or Swine), fresh or aged manure (Cattle, Swine or Poultry), and soil.

Agricultural sediment is defined as those river sediments over areas influenced by agricultural activity, near manure composting facilities and concentrated animal feeding operations. Urban and industrial sediments are defined as river sediments over areas influenced by urban (domestic) and industrial (OTC production facilities) discharges, respectively. Fish farm sediment include river and marine sediment at or near fish farms lagoons. Urban and industrial sludge are defined as activated sludge samples from urban and OTC production WWTPs, respectively. Animal sludge is defined as samples taken from WW lagoons which collect surface runoff, wash water and manure generated at the agricultural facility. Fresh manure is defined as those sample taken directly in the house or barn, or from the influent of a waste treatment operation. Aged manure samples originated from some type of treatment such as: compost, storage tanks, digesters, manure heaps adjacent to the field.

To represent the OTC data by these categories, boxplots of aqueous and solid media were generated and are presented in Figure 2 and Figure 3, respectively. The number of observations is also identified on the figures. A total of 103 observations were reported for aqueous media and 429 were reported for solid media.

To assess if OTC presence in aqueous and soil media promotes development of resistance, concentrations were compared with threshold limits that delineate the sub-MIC selective window of resistance (Figure 1). For aqueous media, effective concentration of 10% activity inhibition (EC_{10}) and MIC on a pure sensitive strain (*E. coli* ATCC 25922) were assumed as MSC and MIC_{susc}, respectively (Peng et al. 2014). For soil media, 10% and 100% of decline in colony forming units (CFU) of *E. coli* ATCC 25922 in a silt loam soil were assumed as MSC and MIC_{susc}, respectively (Peng et al. 2014). EC_{10} and EC_{50} effect on substrate-induced respiration (SIR) from indigenous microbial population on a sandy loam soil were assumed as MSC and MIC_{susc}, respectively (Thiele-Bruhn, Beck 2005). Values are presented in Table 1.

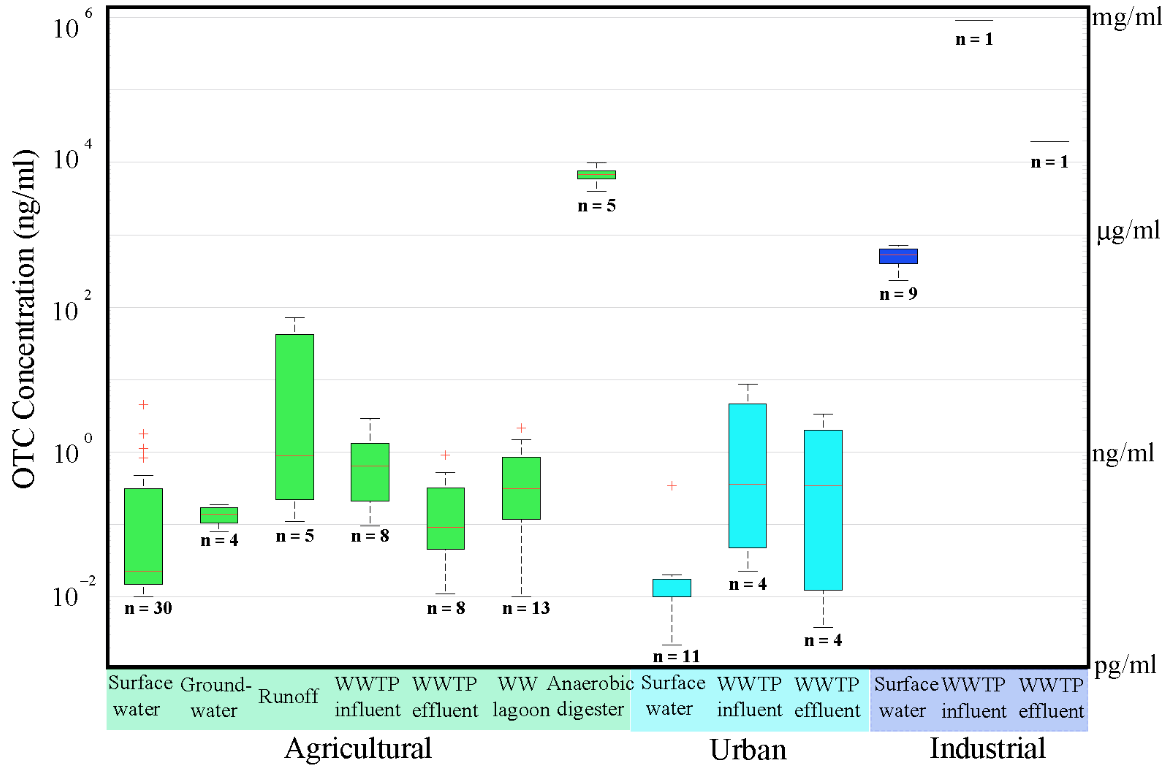

Figure 2 OTC Concentrations in aqueous media

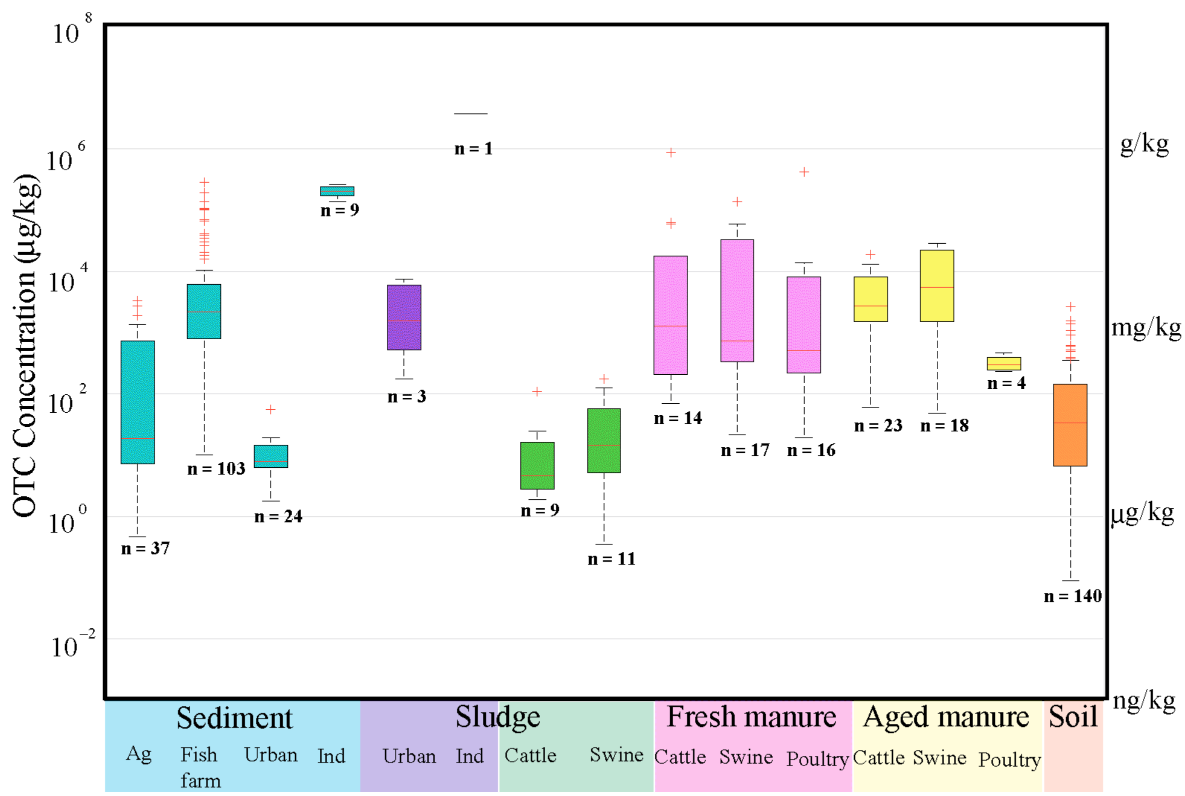

Figure 3 OTC concentrations in solid media

Results

Occurrence in aqueous media

A total of 73, 19 and 11 observations were reported for agricultural, urban and industrial areas, respectively, for aqueous media. Surface water presented the highest frequency of detection for the three sources. The rest of the categories reported a low detection frequency, below 10 observations, except for Ag WW lagoon with 13 (Figure 2).

OTC aqueous concentrations from agricultural areas range from 10^{-2} to 1 ng/ml, except for runoff and anaerobic digester (AD). Runoff concentrations range from approximately 10^{-1} to 10^{2} ng/ml. In this extensive review, only three authors reported OTC presence in runoff (Kay, Blackwell & Boxall 2005, Blackwell, Kay & Boxall 2007, Popova et al. 2013) from overland flow experiments due to land application of manure contaminated with OTC. (Kay, Blackwell & Boxall 2005) and (Blackwell, Kay & Boxall 2007) report that the slurry application rates were characteristic of standard agricultural practices in the UK (45,000 and 33,000 L/ha, respectively). In both cases, OTC was spiked into the slurry at concentrations of 18.85 and 26.2 mg/L, respectively. (Popova et al. 2013) used a different approach in which the OTC contaminated manure (1 kg) was applied as a strip or pat across the packed soil box. Manure was treated with OTC to achieve a concentration of 200 µg/kg.

Kay, Blackwell & Boxall (2005) identified peak concentration of OTC on the first rainfall event (24 hr after manure application) with 71.7 ng/ml and 32 ng/ml for tilled and no tilled plot soils, respectively. Blackwell, Kay & Boxall (2007) detected OTC only on the composite sample (0.9 ng/ml) collected 6 days after treatment. Popova et al. (2013) collected composite samples weekly over a 3 week period. An average total mass of 4.1 and 1.7 µg of OTC were detected over the three weeks from two silt loam soils representative of Northern California, USA. Reported mass losses in surface water runoff of initially applied OTC were estimated to be below 0.1% for the Kay, Blackwell & Boxall (2005) study and 2.5% for Popova et al. (2013) study. These observations indicate that even compounds with high sorption coefficients for soil, such as OTC, are transported via surface runoff.

OTC concentrations in AD were in the range of 10^{3} ng/ml and are approximately three to four orders of magnitude higher (10^{3} to 10^{4} ng/ml) than the highest OTC concentration reported for Ag WWTP effluent and influent, respectively.

Reported OTC concentrations from urban sources ranged from 10^{-3} to 1 ng/ml while industrial concentrations range from 10^{2} to 10^{5} ng/ml. For both groups, surface water concentration are lower than WWTP effluent (dilution effect), and WWTP effluent are lower than WWTP influent. Only one study was found to report OTC concentrations related to OTC production facilities (Li et al. 2008).

Occurrence in solid media

OTC presence in sediments and soils were the most frequently reported detections with 173 and 140 observations, respectively. Sludge, fresh and aged manure present 24, 47 and 45 observations, respectively. OTC concentrations in urban sediment range from 1 to 10^1 µg/kg. Agricultural and fish farm sediment range from 10^{-1} to 10^3 µg/kg and 10^1 to 10^5 µg/kg, respectively, as depicted in Figure 3. Industrial sediment range at 10^5 µg/kg and are much higher in comparison with Ag, Urb and fish farm sediment.

OTC is commonly used in the aquaculture industry which is reflected in a high detection frequency (103 observations). All urban sediment samples are from a study by Kim, Carlson (2007) along the Cache La Poudre River in northern Colorado. These watershed is a relatively complex system as it includes several municipal WWTP effluents, numerous tributaries and withdrawals along the river. Ag sediment observations include studies from Simon (2005), Boxall et al. (2006a), Kim, Carlson (2007) and Awad et al. (2014).

OTC was detected in sludge from urban, industrial and agricultural sources. OTC concentrations in urban and industrial activated sludge range from 10^2 to 10^3 µg/kg and at 10^6 µg/kg, respectively. OTC sludge concentrations from treatment lagoons for animal operations (cattle and swine) range from 10^{-1} to approximately 10^2 µg/kg. Treatment lagoons and storage ponds or pits are common structure used for management of waste in cattle and swine animal feeding operations to collect surface runoff, wash water and manure generated (Mackie et al. 2006, Watanabe et al. 2010, Zhang et al. 2013, Zhou et al. 2013). OTC concentration in fresh and aged manure (cattle, swine and poultry) range from 10^1 to approximately 10^5 µg/kg and 10^1 to 10^4 µg/kg, respectively.

OTC concentrations in soil include measurements from eight countries (China, Germany, Korea, Italy, Spain, UK, Turkey and USA) and range from approximately 10^{-1} µg/kg to 10^3 µg/kg. Soil samples include measurements at various depths, times after treatment (time after land application of manure or slurry), and random sampling from agricultural areas (animal husbandry and crop fields).

Discussion

Aqueous media

Aqueous phase OTC was detected in several wastewater treatment facilities, groundwater and surface water sources. Antibiotic removal by conventional wastewater treatment has been demonstrated to be incomplete (Kim et al. 2005, Jia et al. 2009, Shao et al. 2009). Traditional wastewater treatment processes are ineffective in completely eliminating aqueous phase OTC, as indicated by the presence of OTC in the effluent of urban and industrial discharges.

Despite OTCs high water solubility and low *n*-octanol/water partition potential based on its partition coefficient, OTC is known to be highly sorbed onto solids (manure, sediment and soil) (Tolls 2001, Sassman, Lee 2005, Kim et al. 2005, Figueroa 2012, Huang et al. 2012). Consequently, sorption can be a significant removal mechanism for OTC from

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WWTPs (Kim et al. 2005, Huang et al. 2012), as OTC sorbs to biomass in the activated sludge (Kim et al. 2005). However, great attention has to be given when sludge is recycled for agricultural purposes (de Cazes et al. 2014). Ozonation has been reported as effective in reducing OTC concentrations levels from industrial WWTP facilities (Zheng et al. 2010).

OTC occurrence in shallow groundwater has been associated with seepage from treatment lagoons (Mackie et al. 2006). OTC occurrence in surface water influenced by agricultural activities suggests that its sorption to solid medium is not an irreversible process and that certain conditions could facilitate their mobility in the environment (Kim et al. 2005, Watkinson, Murby & Costanzo 2007).

Solid media

OTC is commonly used in the fish farming industry to protect against bacterial diseases which are a frequent problem. The occurrence and persistence of OTC in sediments from fish farm lagoons has been investigated by several authors (Jacobsen, Berglind 1988, Björklund, Bondestam & Bylund 1990, Samuelsen, Torsvik & Ervik 1992, Capone et al. 1996, Kerry et al. 1996) and include measurements from Finland, Ireland, Norway and the US. OTC persistence in sediment may be significantly affected by the physical and chemical characteristics of sediment, temperature, pH (Björklund, Bondestam & Bylund 1990). Half-life values for marine sediments originating from fish farming operations range from 32 to 419 days (Samuelsen 1989, Björklund, Bondestam & Bylund 1990).

Even substances with high sorptive capacity are not necessarily immobile. The presence of OTC-contaminated river sediment in areas influenced by agricultural activities could be explained by transport from overland flow. Overland flow from manure treated farmlands has been previously identified by Kay, Blackwell & Boxall (2004) as a pathway by which OTC enters surface waters and can be transported either solubilized or associated with suspended sediments.

OTC occurrence in soil has been extensively documented in crop fields and animal production farms (De Liguoro et al. 2003, Kay, Blackwell & Boxall 2004, Aga et al. 2005, Brambilla et al. 2007, Andreu et al. 2009, Karcı, Balcıoğlu 2009, Zhou et al. 2013, Huang et al. 2013). Its presence in soil has usually been associated with land application of OTC-contaminated manure. To a lesser extent relative to veterinary and human use, OTC is used on several fruit crops (pears, peaches, nectarines and apples) to control bacterial diseases (McManus et al. 2002).

ARB selective pressure assessment

Potential selection of resistant bacteria in aqueous media was assessed by comparing aqueous OTC concentrations with MSC and MIC_{susc} values for *E. coli* (Table 1). As depicted in Figure 4, all aqueous sources were found to be below the sub-MIC selective window except samples taken from industrial sources and Ag anaerobic digester. This comparison illustrates that OTC surface water receiving WWTP discharges from OTC production facilities have the potential to select for resistant strains of *E. coli ATCC 25922* under the sub-MIC selective window concept.

Table 1 Assumed values for sub-MIC selective window assessment

Bacterial strain	Reported response	Assumed response	Media	Values		Reference
E. coli ATCC 25922	EC_{10} MIC	MSC MIC_{susc}	Aqueous	78.5 1000	ng/ml ng/ml	Peng et al. (2014)
E. coli ATCC 25922	10% decline CFU 100% decline CFU	MSC MIC_{susc}	Silt loam	4643 200000	µg/kg µg/kg	Peng et al. (2014)
Indigenous microbial population	EC_{10} EC_{50}	MSC MIC_{susc}	Sandy loam	500 11800	µg/kg µg/kg	Thiele-Bruhn, Beck (2005)

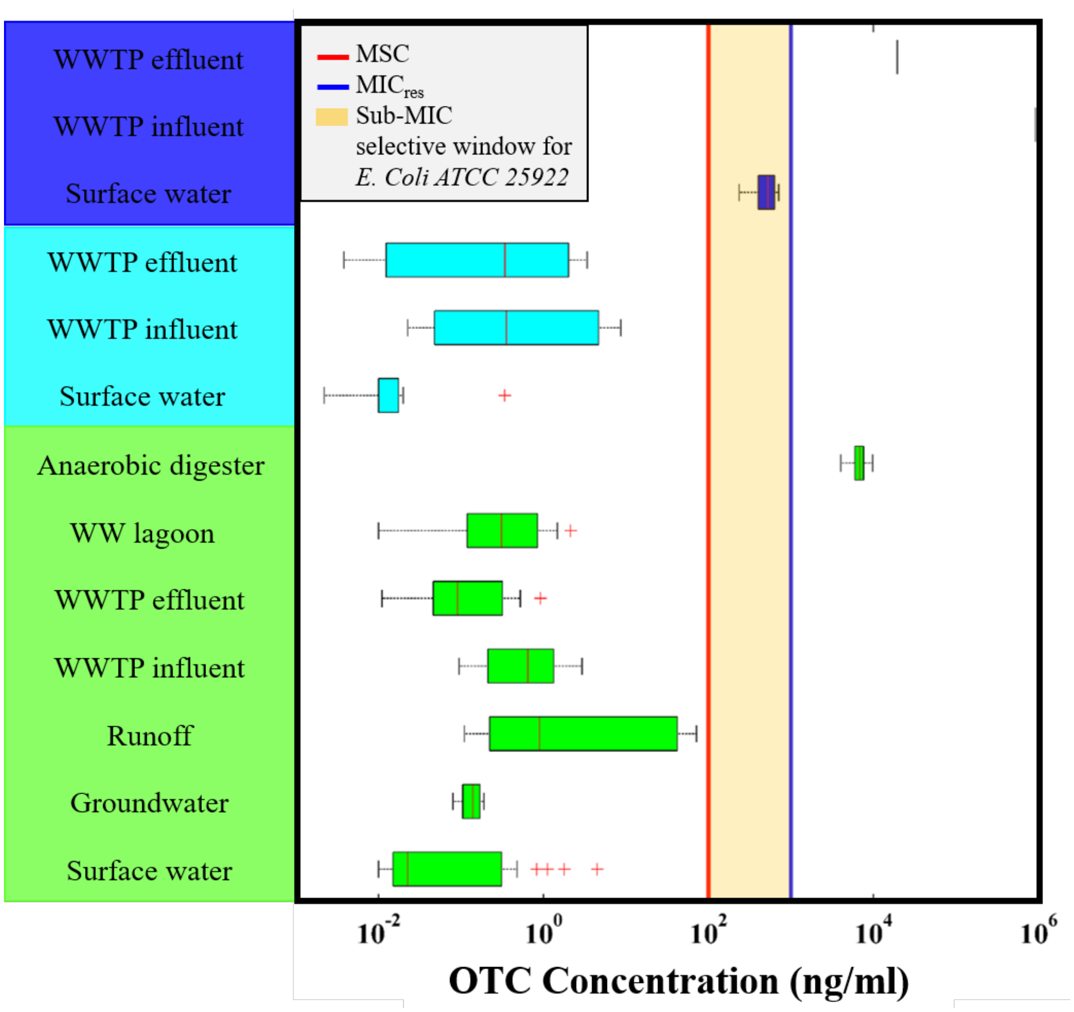

Figure 4 Comparison of OTC concentrations in aqueous media with sub-MIC selective window for E. coli ATCC 25922

Potential selection of resistant bacteria in soil was assessed by comparing soil OTC concentrations with MSC and MIC_{susc} for *E. coli* and indigenous microbial population in loamy sand and sandy loam, respectively. As depicted in Figure 5, no OTC concentration measurements are inside the sub-MIC selective window for an *E. coli* sensitive strain (Figure 5a), while several OTC concentration are within the sub-MIC selective window for the indigenous microbial population in a sandy loam soil sample (Figure 5b). These comparisons suggest that residual OTC concentrations in soil induce a selective pressure for resistance in soil microorganisms, however, the real extent of the sub-MIC selective window is unknown since EC_{50} is being used as the upper end of the resistance selective range. Results from Figure 5 illustrate that the potential selective pressure of a particular bacterial strain, cannot be extrapolated to bacterial populations in soil where bacterial diversity can harbor a variety of antibiotic resistant genes that can be exchanged potentially.

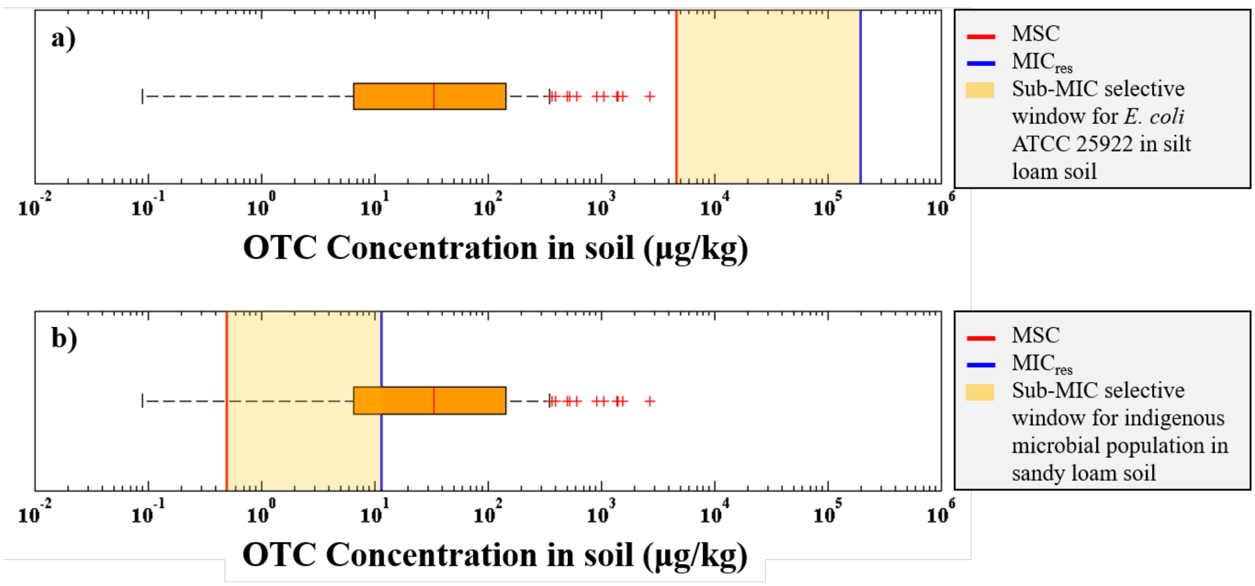

Figure 5 Comparison of OTC concentrations in soil with sub-MIC selective window for a) E. coli ATCC 25922; b) indigenous microbial population

Conclusion

The inventory of OTC presence in aqueous and solid media revealed multiple sources and a range of concentrations. Surface water receiving industrial WWTP effluent was identified as a potential source of OTC concentrations that might select for OTC resistance of *E. coli* in aqueous media. Strategies aimed at reducing WWTP effluent concentrations of OTC should be implemented to minimize the potential selection of resistant bacteria once discharged.

Soil media was identified as a reservoir for OTC concentrations with the potential to select for resistance among *indigenous soil microorganism*. Comparisons demonstrate that the presence of OTC can result in the selection of resistant bacterial strains in soil. However, is important to highlight that MICs of a given antibiotic differ from organism to organism and within species as depicted in Figure 5a and 5b.

Several MIC assays of tetracycline have been reported for single bacterial strains and complex bacterial communities in aquatic environments. However, no reported MSC and MIC_{susc} range was found for OTC. Further studies are required to identify OTC concentration ranges for resistance selection in aqueous and solid media considering the biodiversity of indigenous microbial populations. Such data can provide a basis to develop guidelines and/or regulations for usage of antibiotics and their subsequent release to the environment.

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