Demonstration of antibiotic-induced tolerance development in tropical agroecosystems through physiological profiling of sediment microbial communities

Agricultural use of antibiotics differs quantitatively and qualitatively in tropical and temperate countries. To gain insight into the nature and magnitude of physiological adaptations prompted by these drugs in microbial communities from tropical agroecosystems, we compared community-level physiological profiles of sediment bacteria from a protected wetland (PV), a pig farm (RD), treated (TIL1) and untreated effluents (TIL2) from a tilapia farm, an estuary close to shrimp farms (CA), and an irrigation channel adjacent to a rice plantation (AZ) exposed to a range of oxytetracycline (OTC) concentrations in Ecoplates (Biolog®). In addition, we used LC/MS/MS and plate counts to determine the concentration of OTC and the number of OTC-resistant bacteria in the samples, respectively. Water samples collected at RD contained maximum amounts of OTC (640 ng L^{-1}), followed by TIL2 (249 ng L^{-1}), TIL1 (72 ng L^{-1}), and CA (85 ng L^{-1}). In average, the microbial community of RD was more tolerant to OTC (EC_{50}: 14.30 ± 3.12 mg L^{-1}) than bacteria from CA (8.83 ± 1.85 mg Ll^{-1}), TIL2 (EC_{50}: 4.97 ± 1.43 mg L^{-1}), TIL1 (4.25 ± 0.60 mg L^{-1}), AZ (3.66 ± 0.97 mg L^{-1}) and PV (3.77 ± 0.62 mg L^{-1}). Congruently, PV, AZ, TIL1, CA, TIL2, and RD appeared in that order in a cumulative distribution of individual EC_{50} values and higher plate counts of bacteria resistant to 10 µg mL^{-1} ($5.0x10^5$- $1.5x10^7$) and 100 µg mL^{-1} of OTC ($1.5x10^4$-$8.4x10^5$) were obtained for RD than for the other sites (10 µg ml^{-1}: $4.8x10^4$-$3.3x10^5$ and 100 µg mL^{-1}: $1.0x10^2$-$4.4x10^3$). These results are compatible with a scenario in which the basal level of tolerance to OTC that characterizes pristine environments (PV) is amplified in proportion to the intensity of antibiotic exposure (agriculture<aquaculture<swine farming).

PeerJ PrePrints | http://dx.doi.org/10.7287/peerj.preprints.228v1 | CC-BY 4.0 Open Access | received: 31 Jan 2014, published: 31 Jan 2014

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Introduction

Antibiotics from diverse families have been recently found in sewage treatment plants, sewage sludge, surface waters, sediments, and groundwater all over the world (Heberer 2002; Hernando et al. 2006). Many of these compounds should be regarded as high-priority emerging pollutants on account of their synthetic nature, abnormal environmental levels (Kümmerer 2009), long persistence, and lack of toxicological and ecotoxicological information or published health and environmental standards.

As a result of their historical efficacy in human medicine, low cost, and extensive commercialization, different tetracyclines have been used for decades in the control of bacterial diseases of fruits, vegetables and animals (Kümmerer 2009), for growth promotion of poultry and livestock (Pijpers et al. 1991), and as infection control agents in aquaculture (Avisar et al. 2009). These substances tend to be poorly absorbed by animals (Lunestad and Goksayr 1990; Seyfried et al. 2010); hence they are administered in high concentrations and after excretion they accumulate in agricultural soils (Piotrowska-Seget et al. 2008) and fishpond sediments (Lalumera et al. 2004; Maki et al. 2006), where they can be mobilized outside the farms or persist for up to 5 months (Carson et al. 2002; Rubert and Pedersen 2006).

Our study focuses on the environmental effects elicited by oxytetracycline (OTC) for several reasons. First of all, OTC is widely used in tropical agriculture (Rodríguez 2008). Second, studies of acute and chronic toxicity of OTC in microorganisms, invertebrates, and fish urge closer monitoring and further toxicological research on this substance (Park and Choi 2008). Third, OTC was heavily consumed and linked to very high hazard quotients in a recent risk assessment performed in the area studied here (de la Cruz et al. 2014). Finally, there are reports of the finding of OTC-resistant bacteria and *tet* genes in crops (Rodríguez et al. 2006) and farm soil from Costa Rica (Rodríguez et al. 2007), of the detection of mg kg^{-1} of OTC and other tetracyclines in locally-produced animal feed (Gutiérrez et al. 2010; Granados et al. 2012), and of ng L^{-1} of this substance in surface waters across this tropical country (Spongberg et al. 2011).

Molecular methods have proved that the structure of microbial communities changes in response to antibiotic exposure and that this type of disturbance increases the abundance of resistant bacteria (Liu et al. 2012). Nonetheless, a retrospective demonstration of a cause-and-effect relationship between antibiotic usage and tolerance development has never been tested under field conditions in the tropics, where the ecotoxicology of antibiotics may be unique due to biotic and abiotic factors and highly valued ecosystems, such as wetlands, rain forests, coral reefs, and

rivers, are exposed to these compounds due to their proximity to agroecosystems. With this in mind, and aiming to assess the nature and magnitude of physiological adaptations that OTC exposure could have caused in tropical agroecosystems, we determined the *in vitro* level of tolerance to OTC of sediment bacteria from 5 locations and a reference wetland located in Northwestern Costa Rica.

Materials and methods

Description of the sites

We studied sediment samples collected in February, May, August, and November 2009 at the drainage of the last of a series of three oxidation ponds collecting wastewater from a pig farm with 8000 animals (RD), a channel receiving effluents from numerous ponds in a tilapia farm of 210 ha (TIL2), the drainage of an artificial wetland used to treat wastewater in the same tilapia farm (TIL1), an estuary receiving wastewater from various shrimp farms (CA), an irrigation channel next to a rice plantation of approximately 300 ha (AZ), and a protected wetland in the Palo Verde National Park (PV). These sites are located in a tropical dry region in Northwestern Costa Rica that is irrigated with water from the Lake Arenal through a system of channels covering ca. 28 000 Ha (Arenal Tempisque Irrigation District; ATID). de la Cruz and collaborators (2014) recently estimated that 0.014-0.340 Kg/Ha/year, 0-1.93 Kg/Ha/year, and 0.82-107.3 Kg/Ha/year of OTC are consumed in agriculture, aquaculture, and swine production activities in the ATID, respectively.

Sediment collection and analysis

Three samples of approximately 6 L of sediment were collected with a shovel from the upper 30 cm of the horizon. These materials were transported to the laboratory on ice in plastic jars filled to their maximum capacity and covered with overlaying water. Once in the laboratory, samples were maintained at 4°C for a maximum of 24 h before analysis. The dissolved oxygen, pH value, temperature, and conductivity of overlaying water associated with the sediments were measured at the field with a portable multimeter (HQ11d, Hach, Loveland, CO, USA). This data, together with the organic carbon content (LOI 550ºC) and the texture of the sediments, appears in Table 1.

Tetracycline screening by LC/MS/MS in water and sediment samples

OTC, chlortetracycline (CTC), and tetracycline (TC) were determined in surface water samples with the protocol of Christian et al. (2003). Briefly, 500 ml of water samples cleared through glass fibre filters (GC/C, Whatman, ø 47 mm, 1.2 mm) and whose pH was adjusted to 4.0 with 2 M H_2SO_4 were mixed with 1 mL of EDTA 200 mg mL^{-1} to prevent bonding of the analytes to glass. Thereafter, they were passed with a flow rate <10 mL min^{-1} through SPE OASIS HLB cartridges (Waters 200 mg/6 mL) that were conditioned with 6 mL of methanol and 6 mL of ultrapure water (18.2 MΩ cm^{-1}). The SPE cartridges were dried by centrifugation for 2 min at

5000 rpm and then by vacuum during 10 min, and the analytes were eluted with 5 mL of methanol. The extracts were concentrated to approximately 0.05 mL in a water bath at 35 °C under a gentle nitrogen flow and redissolved in 0.8 mL of a mixture of water:acetonitrile (9:1, V/V) applying ultrasonic bath for 1 min. Extracts were filtered through a 0.45 µm membrane filter and transferred to polypropylene injection vials. Blank samples of ultrapure water (18.2 MΩ cm^{-1}) were run to control for possible contamination of the analytical procedure. Sediment samples were processed by liquid extraction with a method described by Hamscher et al. (2002). In this procedure, sediments were homogenized using a 1 mm sieve, their excess water was drained, and tubes and glassware were washed with water and methanol, heated at 400 °C for 1 h, and rinsed with a saturated methanolic solution of EDTA prior to extraction to avoid losses due to association of the analytes with organic matter and divalent cations. One g of each sediment was homogenized with 1 mL of 1 M citrate buffer solution (pH 4.7) using a vortex. Thereafter, 6 ml of ethyl acetate were added to the suspensions and they were shaken for 15 minutes prior to centrifugation for 10 minutes at 1000 x g. The organic phase was recovered and the extraction procedure was repeated once. Pooled organic fractions were concentrated under a gentle flow of nitrogen and the residue was resolved in 1 mL of water/acetonitrile (9:1 V/V). These concentrated extracts were filtered through 0.45 µm membranes and transferred to polypropylene injection vials. Every sediment sample was extracted in duplicate. For both water and sediment extracts, analyte separation and detection was achieved by LC-MS/MS using a triple quadrupole mass analyser (4000 QTrap, Applied Biosystems/MDS SCIEX) with electrospray ionization (ESI) connected to a Shimadzu HPLC system and operated in the positive ion mode. The samples were injected on a ACE column (5 µm, C18, 150 x 3 mm; Advanced Chromatography Technologies, Aberdeen, UK) at 30 °C. As mobile phase we used (A) 1 mM ammonium acetate buffer in sub-boiled water and methanol (ULC/MS grade Biosolve) (95/5 V/V) and (B) 1 mM ammonium acetate buffer in sub-boiled water and methanol (5/95 V/V), both containing 0.1% formic acid (V/V). The total flow rate of eluent A and B was 0.4 mL min^{-1}. The gradient program was: 80% A (2 min), 70% A (9 min), 10% A (13-19 min) and finally 80% A. The total run time was 21 min.

Determination of CLPP and calculation of OTC-induced community tolerance

Bacteria were extracted from the sediments with the methods of Burke et al. (2002) and Schmitt et al. (2004). Briefly, suspensions prepared with 10 g of solid matter from the sediments and 40 mL of 0.1% sodium pyrophosphate were shaken for 2 min by hand and homogenized 5 times by means of sonication for 10 seconds at 47 kHz. Thereafter, soil particles were separated from bacteria by centrifugation at 500 x *g* for 15 min and the supernatants were immediately frozen in

liquid N_2. Each extraction was performed by triplicate and the extracts were stored at -70°C for not more than 6 days. Extracted bacteria were exposed to 0.1, 1, 3, 6, 10, 25, 50, and 100 mg L^{-1} of OTC HCl (Sigma) in EcoPlates® (Biolog®) that were incubated for 6 days at 25°C and 85% relative humidity in the dark. These plates contain 31 carbon sources representing amines, aminoacids, carbohydrates, carboxylic acids, phenolic compounds, and polymers. The Ecoplates® were incubated for a long period of time to appraise the contribution of slow growing bacteria. The utility of the range of OTC concentrations assayed to reveal effects of low, medium, and strong magnitude was verified in preliminary experiments. Moreover, we followed the dilution-based method recommended of Schmitt et al. (2004) to ensure that equal amounts of bacteria were added to the plates. Daily absorbance measurements of plate wells at 595 nm were transformed into WCD (standardized well color development) or AWCD (average well color development) values and thereafter into AUC values (area under the curve). This data treatment was favored because AUC, unlike AWCD and WCD, contemplate irregularities in color formation across time and provide a more comprehensive view of respiration kinetics. To express OTC effects in a relative scale, normalized AUC (nAUC) were calculated by dividing the AUC of plates containing OTC by the AUC of control plates without OTC. Finally, nAUC were exploited to calculate logistic dose–response curves from which the concentration of OTC needed to reduce color formation in 50% (Effect Concentration 50%; EC_{50}) was estimated with the formula: Y=Bottom + (Top-Bottom)/(1+10^((X-logEC_{50}))) (Hill slope=-1.0). Curve fitting was considered appropriate if a non-lineal regression exhibited a $r^2 \geq 0.3$ and the logarithm of the standard error of the EC_{50} was <1 (Schmitt et al. 2004; Schmitt et al. 2005; Kamitani et al. 2006).

Plate counts of OTC-resistant bacteria

Serial dilutions of sediment suspensions in 0.8% saline solution were inoculated by triplicate onto trypticase soy agar plates (TSA, Oxoid) supplemented with 1, 10, or 100 µg mL^{-1} of OTC. All plates included 50 µg mL^{-1} of cycloheximide to inhibit the growth of mycelial fungi and 2% of agar to limit bacterial swarming. Plate counts were recorded after incubation for 120 h at 30ºC under aerobiosis.

Statistical analyses

nAUC and EC_{50} values, as well as plate counts, were compared by means of analyses of variance (ANOVA) or appropriate non-parametrical tests at a 0.05 level of significance. All differences were corroborated with Post-Hoc tests. Linear models were calculated to determine the influence

of several physicochemical characteristics of the sediments (PCS) on the development of microbial tolerance to OTC. When required, PCS were log transformed to obtain normal distributions. Plots depicting cumulative distributions of individual EC_{50} values were also prepared to assess OTC tolerance across the range of concentrations and carbon sources tested.

Results

LC/MS/MS screening of tetracyclines

OTC was the only tetracycline detected in our survey of environmental samples (Table 2). More water than sediment samples rendered positive results and, without exception, concentrations were in the order of ng L^{-1} and ng g^{-1}, respectively (Table 2). OTC was detected in all samples collected at the fish farm (TIL1 and TIL2). However, higher amounts were found in the pig farm (RD). While rather low concentrations were detected in samples from CA, no tetracyclines were detected in AZ or in PV.

Carbon source respiration

Sigmoid curves typified the respiration of the carbon sources analyzed. Variations in the initial response times, curve slopes, and the time elapsed until respiration ceased, were observed across the samples. In general, nAUC diminished in proportion to the concentration of OTC added to the plates. However, a few communities showed increased catabolic activities in presence of 0.1 mg L^{-1} of OTC or an OTC-dependent stimulation of respiration in some substrates (Fig. S1). When nAUC from all substrates and OTC concentrations were averaged, the microbial community from RD showed higher values (5.27 ± 0.11) than bacteria from TIL1 (3.59 ± 0.08), TIL2 (2.76 ± 0.07), CA (2.73 ± 0.08), AZ (2.87 ± 0.10), and PV (2.85 ± 0.07) (Fig. 1; $p< 0.05$). A two-way ANOVA evidenced highly significant differences in bacterial respiration across OTC concentrations ($F=265.0$, $p<0.0001$). Moreover, a subsequent ANOVA test at fixed OTC concentrations confirmed that RD had higher respiration values than all other sites at OTC concentrations above 1 mg L^{-1}. This predominance of RD can also be graphically seen in Figure S2.

OTC tolerance

A total of 369 dose-response curves showing inhibition were considered in the calculation of EC_{50}. Most of these inhibitory curves were documented in wells containing carbohydrates

(*n*=130), aminoacids (*n*=82), or carboxylic acids (*n*=76). RD gave rise to the highest average OTC tolerance values for 4 of the 6 types of carbon sources assayed (Table 3). While bacteria from AZ and CA had comparable or higher EC_{50} for phenolic compounds and polymers than bacteria from RD, all other sites showed lower individual and average EC_{50} than RD (Table 3). The average OTC tolerance of RD (14.30 ± 3.12 mg L^{-1}) was significantly higher than that of TIL1 (4.25 ± 0.60 mg L^{-1}), TIL2 (4.97 ± 1.43 mg L^{-1}), CA (8.83 ± 1.85 mg L^{-1}), AZ (3.66 ± 0.97 mg L^{-1}), and PV (3.77 ± 0.62 mg L^{-1}) (p=0.0001). However, a cumulative distribution of individual EC_{50} revealed a right-skewed behavior of tolerance, with reference wetland PV at the far left, AZ and TIL1 followed by CA and TIL2 in the middle, and RD in the far right (Fig. 2). This trend was more apparent at higher OTC concentrations.

Linear models calculated with the physicochemical parameters included in Table 1 revealed that Clay (F=7.19, *P*=0.01; Pearson correlation=-0.49) and to a lesser extent log transformed Sand (F=4.42, *P*=0.047, Pearson correlation=0.409) explained the OTC tolerance and elevated EC_{50} recorded for RD. Furthermore, a linear regression model of EC_{50} created by a forward stepwise regression assigned the highest predictive values to suspended solids, silt and clay in combination (data not shown).

Plate counts of OTC-resistant bacteria

Culturable bacteria resistant to 1, 10, or 100 µg mL^{-1} of OTC were found in all sites. Counts of bacteria resistant to 1 µg mL^{-1} of OTC were comparable across the sites. By contrast, bacteria from PV, AZ, CA, TIL1 and TIL2 were more severely inhibited by 10 µg L^{-1} and 100 µg L^{-1} of OTC than bacteria from RD (Fig. 3). In detail, the abundance of OTC-resistant bacteria in RD (10 µg mL^{-1}: $5.00x10^5$-$1.50x10^7$; 100 µg mL^{-1}: $1.50x10^4$-$8.40x10^5$) was one or two orders of magnitude higher than that recorded for all other sites (10 µg mL^{-1}: $4.83x10^4$-$3.33x10^5$; 100 µg mL^{-1}: $1.00x10^2$-$4.37x10^3$).

Discussion

The pollution-induced community tolerance (PICT) concept postulates that exposition of a community to a contaminant will result in increased tolerance of its members against the xenobiotic compound. This concept is applicable to microorganisms (van Beelen et al. 2001; van Beelen 2003) and allows for evaluation of toxic effects in a short period of time without ignoring the role of biological interactions (Segner 2007). Others have used PICT to study the impact of

metals such as Zn, Cu, Pb and Cd (Pennanen et al. 1996; Díaz-Raviña and Bååth 1996; Díaz-Raviña 1994; Bååth 2005) and antibiotics such as sulfonamide (Schmitt et al. 2004) on the functioning of communities of environmental bacteria. We exploited it to assess the nature and magnitude of the physiological adaptations triggered by a widely used antibiotic in tropical sediments from diverse locations.

The respiration of most carbon sources and the concentration of OTC added to the wells of the Ecoplates® were inversely related. This dose-dependent inhibition was consistent across the samplings and sustained notwithstanding that the time elapsed between the last *in situ* exposure of the sediments to antibiotics to OTC and their *de novo* exposition to this compound in the laboratory is unknown. We therefore conclude that the adaptations developed by the bacterial communities assayed are stable.

The stimulation of the respiration of certain substrates in presence of 0.1 mg l^{-1} of OTC is an example of hormesis; a phenomenon that has previously been reported for tobramycin, tetracycline, and norfloxacin (Linares et al. 2006) and for OTC in a tropical soil (Solís et al. 2011). The interpretation of responses to such low concentrations of toxicants requires further research; however, recent investigations demonstrate that natural concentrations of antibiotics influence gene expression and intercellular communication at the community level (Yim et al. 2007; Fajardo and Martinez 2008). On the other hand, as pollutants may serve as carbon source (Dantas et al. 2018) or stimulate nutrient release by pollutant degraders (Cycoń et al. 2006), it is plausible that OTC-degraders contributed to the development of OTC tolerance. In this regard, Liu et al. (2012) recently noted that chlortetracycline and sulfamethoxazole may serve as nutrients for soil bacteria.

The sediment from RD and one of the sediments from the fish farm (TIL1) showed top respiration rates. The clearest differentiation between sites was obtained with OTC concentrations between 3 and 6 mg l^{-1}, presumably because concentrations > 6 mg l^{-1} give rise to attenuated profiles that sacrifice relevant information and exposition to < 3 mg l^{-1} does not elicit detectable phenotypic responses. On the other hand, the key contribution of the carbohydrates and the aminoacids in the differentiation of bacterial communities seems to be related to more favorable conditions for the growth of r-strategists over K-strategists under high-nutrient conditions (Preston-Mafhamet al. 2002; Stefanowicz 2006). We recommend considering these concentrations and carbon sources in the design of bioassays dealing with the ecotoxicology of tetracyclines.

The graph with cumulative EC_{50} showed that the community extracted from the protected area (PV) was, in comparison to the other communities studied, more sensitive to OTC. The communities of AZ, CA and TIL1 were characterized by intermediate EC_{50} values, whereas bacteria from one of the sites in the fish farms where OTC was commonly detected (TIL2), and from the swine farm illustrating higher antibiotic usage and containing greater amounts of OTC residues, were more tolerant to OTC. Thus, our data indicates a scenario where a basal level of natural tolerance to OTC becomes amplified in line with the intensity of antibiotic usage in agriculture. This interpretation is supported by the OTC consumption figures presented in the Materials & Methods section.

The increased tolerance of the microbial community of RD was corroborated by the fact that it retained most catabolic functions at high OTC concentrations. Therefore, it is likely that OTC tolerance was developed in diverse bacterial groups. In future studies, microscopic and molecular analyses could be considered to identify key players and to estimate their individual contribution to the community phenotype.

In agreement with the notion that antibiotic resistance is a natural phenomenon (Martinez 2009), we found large numbers of OTC-resistant bacteria in pristine locations. However; their abundance and level of susceptibility was much lower than those of bacteria from human-impacted sediments in farms. The growing antibiotic resistance of bacterial pathogens, along with the contamination of the environment and of foodstuff with antibiotics, antibiotic-resistant bacteria, and antibiotic-resistance genes, is a global concern from sanitary, economic, and ecological perspectives. In Costa Rica and in many other developing countries, pig manure is exploited as soil fertilizer or in cow nutrition and fishpond sediments are used to fertilize sugarcane plantations. Therefore, the OTC-resistant bacteria reported here can find a way out of the farms. This situation is particularly worrisome because resistant bacteria can persist in natural reservoirs in absence of obvious selective pressures (Miranda and Zemelman 2002) and also because biocides and other substances commonly used to disinfect farm facilities may co-select resistant bacteria (Sheldon 2005). This could explain why degraders of phenolic compounds and polymers from AZ and CA and from RD exhibited similar OTC tolerances.

It is known that abiotic factors can shape tolerance through interactions with pollutants. For example, Boivin et al (2005) reported an effect of temperature on the magnitude of cooper-induced tolerance in aquatic microbial communities, probably due to exposure enhancement. Among other factors, the concentration of organic matter (Doi and Stoskof 2000), clay (Chang et al. 2009), oxygen, divalent metals (MacKay and Canterbury 2005), and degrading bacteria in the

matrix, has been shown to influence the fate and activity of OTC in the environment and thereby the exposure that microbial communities may encounter. In our study, the low percentage of clay in the sediment from RD is a likely explanation for the elevated OTC tolerance of its bacterial community. An additional non-excluding reason for the high tolerance recorded for RD is the massive and intensive use of β-lactams, sulfonamides, and tetracyclines in pig farming (Sarmah et al. 2006); a situation that was also confirmed in RD (de la Cruz et al. 2014).

Overall, our PICT findings provide more clear-cut indications than studies addressing the relationship between antibiotic consumption and resistance development. For instance, the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme -a surveillance and research programme for antibiotic consumption and resistance in bacteria from animals, food and humans- concluded that the occurrence of tetracycline resistance in Danish pig production rises steadily even though tetracycline use has decreased over the last two years, and that resistance to vancomycin and quinupristin/dalfopristin persists at low levels among *Enterococcus faecium* isolates from pigs despite of the ban of avoparcin and virginiamycin called more than ten years ago (DANMAP 2010). Given that tolerance development to antibiotics reflects real selection pressures rather than multidrug resistance patterns and that PICT experiments in controlled microcosms and ecotoxicological test systems of equivalent complexity deliver comparable results (Schmitt et al. 2009), PICT investigations may find an application in environmental impact assessments on the field. In this respect, our data strongly postulate intermediate concentrations of OTC as valuable markers of antibiotic exposure.

Conclusions

Our study shows a causal relationship between antibiotic exposure and OTC tolerance development in tropical sediments. To the best of our knowledge, this is the first report of an antibiotic PICT experiment in tropical and/or subtropical areas, where most studies in aquaculture farms and aquaculture-impacted environments have aimed to inventory antibiotic resistant bacteria and antibiotic resistance genes (Su et al. 2011; Thuy et al. 2011) in full disregard of the contribution of environmental variables to the results. Our results were interpreted in function of the physicochemical characteristics of the sediments analyzed and, in opposition to most investigations for Europe and USA (Schmitt et al 2004; Brandt et al. 2009; Demoling et al. 2009), tolerance profiles were obtained for microbial ensembles subjected to different exposure regimes at the field.

Developing countries like Costa Rica have consistently and traditionally showed difficulties in the regulation of pesticides and the implementation of good agricultural practices (Castillo et al. 2006), residual water treatment (OPS 2003), and according to our experience and appreciation, in the overall management of antibiotics in medicine and farming (Gutiérrez et al 2010; Granados et al. 2012). Our results support the latter observation and nourish the limited knowledge on the ecotoxicology of antibiotics in aquatic ecosystems (Ding and He 2010). Furthermore, since diversity losses lead to higher ecosystem vulnerability (Girvan et al. 2005; Szabó et al. 2007) and macromolecular carbon degraders are critical to ecosystem stability (Waldrop and Firestone 2004), our results justify the design and execution of monitoring programs of antibiotics and antibiotic resistance and of robust risk assessments to increase the awareness of farmers and consumers on the public and environmental health implications of antibiotic use in the tropics.

Acknowledgements The authors thank Heike Schmitt for her help with the setup and interpretation of the PICT experiments and Frans van der Wielen (University of Amsterdam) for his support with the LC/MS/MS analyses.

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Table 1(on next page)

Table 1. Site and sediment description.

	Site[a]					
Characteristic	PV	AZ	CA	TIL2	TIL1	RD
Site Vegetation	Dry tropical forest, covered with aquatic plants	Open	Mangrove Forest	Open	Open	Open
			Overlaying Water			
pH	7.0±0.3	6.7±0.4	7.1±0.05	6.2±0.5	5.8±0.3	7.3±0.9
Conductivity ($\mu S\ cm^{-1}$)	1743±748	587±261	18233±6529	115±4	110±7	5933±332
Dissolved oxygen ($mg\ L^{-1}$)	3.9±0.7	6.2±0.7	5.3±0.5	6.4±0.6	3.5±0.9	3.7±0.5
Total soluble solids ($mg\ L^{-1}$)	244±206	517±408	360±180	35±18	35±18	253±11
			Sediment (% dry weight)			
Organic Matter	8.8 ± 0.8	6.5 ± 1.8	10.4 ± 0.5	8.3 ± 0.3	9.2 ± 0.4	12.1 ± 1.3
Sand	32.8 ± 4.3	50 ± 5.8	18.5 ± 7.2	28.0 ± 4.6	20.0 ± 4.8	66.3 ± 11.6
Silt	23.8 ± 1.1	18.8 ± 2.2	32.3 ± 3.1	20.8 ± 1.6	20.0 ± 1.9	22.3 ± 10.5
Clay	44.0 ± 3.8	31.3 ± 3.8	49.3 ± 9.9	51.3 ± 4.5	60.0 ± 6.3	11.8 ± 1.3

[a]PV=Palo Verde (reference wetland); CA= estuary close to shrimp farms; AZ= irrigation channel adjacent to a rice plantation; TIL2= untreated effluent inside tilapia farm; TIL1= treated effluent from tilapia farm; RD= oxidation lagoon in swine farm. Values represent mean±SD.

Table 2(on next page)

Table 2. Results of a LC-MS/MS residue analysis of tetracyclines in water and sediment samples collected at 4 agroecosystems and a reference wetland.

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	Site[a]					
	PV	AZ	CA	TIL2	TIL1	RD
Sampling period	Water samples (ng L^{-1})[b]					
February	n.d.	n.d.	n.d	OTC (*t*)	OTC (12)	n.d
May	n.d.	n.d.	n.d.	OTC (249)	OTC (43)	n.d
August	n.d.	n.d.	n.d.	OTC (89)	OTC (33)	OTC (462)
November	n.d.	n.d.	OTC (26)	OTC (26)	OTC (72)	OTC (640)
	Sediment samples (ng g^{-1})[b]					
February	n.d.	n.d.	OTC (*t*)	n.d.	n.d.	n.d
May	n.d.	n.d.	n.d.	n.d.	n.d.	n.d
August	n.d.	n.d.	n.d.	n.d.	n.d.	n.d
November	n.d.	n.d.	n.d.	OTC (*t*)	OTC (*t*)	n.d

[a]PV=Palo Verde (reference wetland); CA= estuary close to shrimp farms; AZ= irrigation channel adjacent to a rice plantation; TIL2= untreated effluent inside tilapia farm; TIL1= treated effluent from tilapia farm; RD= oxidation lagoon in swine farm

[b]OTC= oxytetracycline; *t*= traces; n.d. = non detected

Table 3(on next page)

Table 3. Tolerance to OTC of sediment bacterial communities from 4 agroecosystems and a reference wetland.

Site[a]	OTC tolerance (EC_{50}; mg L^{-1})						Average EC_{50} (mg L^{-1})
	Type of carbon source assayed[b,c]						
	A	AA	C	CA	P	PC	
PV	2.38	1.49	5.69	4.01	3.27	1.67	3.77 ± 0.62
AZ	0.13	3.67	1.92	4.30	10.61	7.27	3.66 ± 0.97
CA	14.18	5.51	7.11	5.31	**21.35**	ND	8.83 ± 1.85
TIL2	1.76	8.93	4.90	4.37	2.32	1.12	4.97 ± 1.43
TIL1	1.45	3.87	6.49	3.06	2.56	2.69	4.25 ± 0.60
RD	**16.92**	**14.66**	**20.53**	**9.53**	9.70	**7.60**	14.30 ± 3.12

[a]PV=Palo Verde (reference wetland); CA= estuary close to shrimp farms; AZ= irrigation channel adjacent to a rice plantation; TIL2= untreated effluent inside tilapia farm; TIL1= treated effluent from tilapia farm; RD= oxidation lagoon in swine farm

[b]A: amines; AA: aminoacids; C: carbohydrates; CA: carboxylic acids; P: polymers; PC: phenolic compounds

[c]The highest EC_{50} for each type of carbon source assayed appears in bold

Figure 1

Figure 1.

Average catabolic activity of sediment bacterial communities from agroecosystems and a protected wetland upon exposure to a range of OTC concentrations.

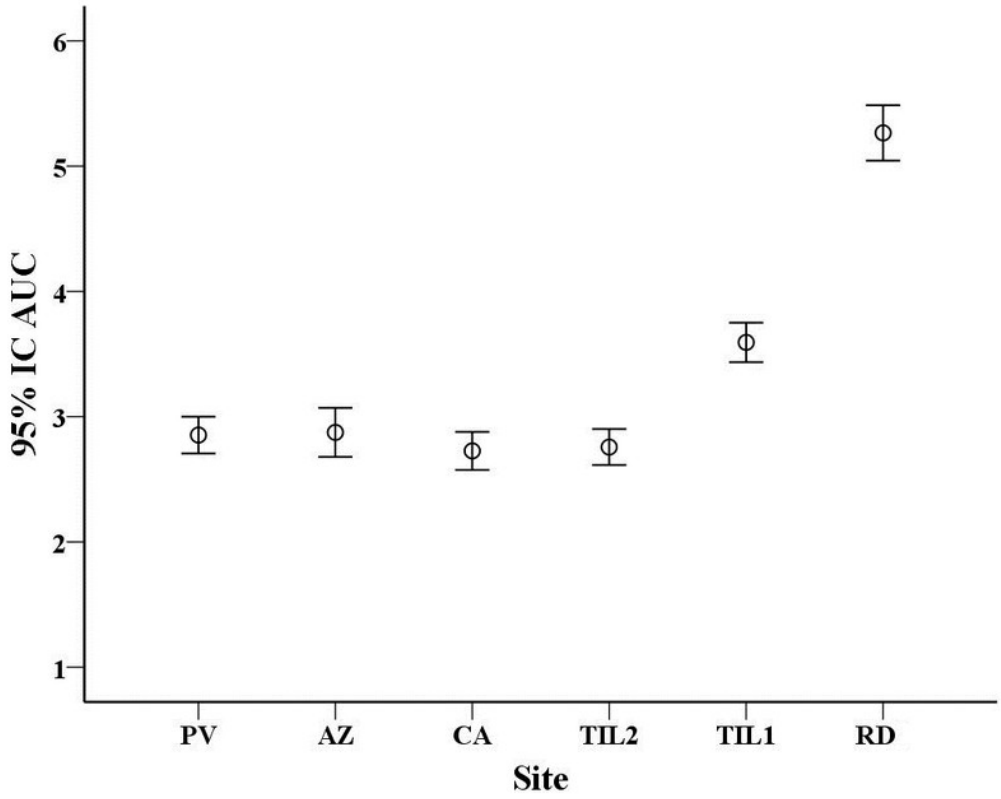

Figure 2

Figure 2.

Cumulative distribution of EC_{50} of OTC for sediment bacterial communities from agroecosystems and a protected wetland.

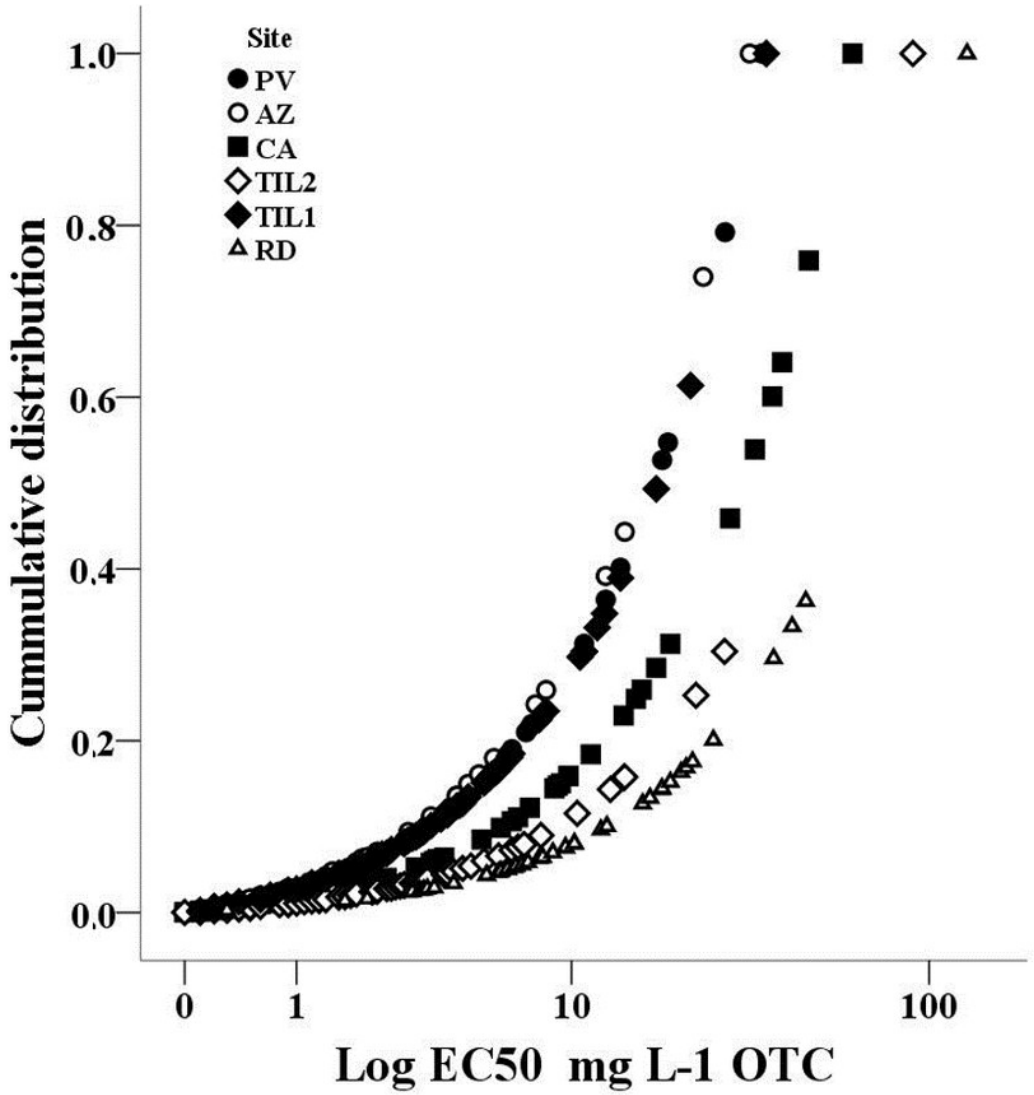

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Figure 3

Figure 3.

Plate counts of culturable OTC-resistant bacteria of sediment bacterial communities from agroecosystems and a protected wetland. PV=Palo Verde (wetland), AZ= Rice farm drainage, TIL2= Effluent into tilapia farm, TIL1= Drainage of treated tilapia farm effluent, RD2= Swine farm oxidation lagoon