Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

JEB
Journal of Environmental Biology

ISSN: 0254-8704
CODEN: JEBIDP

Ecotoxicological evaluation of aquaculture and agriculture sediments with biochemical biomarkers and bioassays: antimicrobial potential exposure

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Publication Info

Paper received:
29 March 2013

Revised received:
10 June 2013

Accepted:
05 September 2013

Abstract

Inappropriate practices and lack of regulations regarding antimicrobial use in agricultural production of developing countries increase the risk of exposure to aquatic ecosystems. Sediments may act as sink of antimicrobial compounds and can provide a historical record of pollution. In the present study, toxic potential of sediments receiving effluents from a fish farm (TIL_1), rice farm (AZ) and swine farm (RD_2) and from a reference natural wetland (PV) in a tropical dry region was evaluated. According to local surveys of antimicrobials and national product registries, sites were classified from highest to lowest potential exposure as following: RD_2>TIL_1>AZ>PV. Both, whole sediment and interstitial water tests, showed a high toxicity of pig farm sediments to the behavior of *Anodontites luteola* and the survival of *Daphnia magna* (EC_{50} -48hrs: 2.4 -11.8 %) (ANOVA, $p<0.05$). Integrated responses from Cholinesterase activity (ChE), Gluthathion-S-transferase (GST) and Lipoperoxidation (LPO) measured in *A. luteola* tissue pointed at the pig and rice farms as sites influenced by activities with an intensive use of xenobiotic substances. The assessment of toxicity pointed at the need of more research on sub-lethal effects of antimicrobials on aquatic invertebrates. With this purpose, we analyzed biomarker response of *A. luteola* to oxytetracycline *in vitro* and found a decrease of ChE and GST in concentrations of 100 µg l^{-1}.

Key words

Antimicrobial, Bioassay, Biomarker, Sediment

Introduction

Sediments receiving effluents from human activities accumulate metals and organic pollutants that may be later mobilized to aquatic ecosystems. Agriculture effluents contain pesticides, antimicrobials and heavy metals that are known to accumulate or adsorb into sediments (Warren *et al*., 2003; Díaz-Cruz and Barceló, 2004; Green-Ruiz and Páez-Osuna, 2003; Zhang and Shan, 2009). Since sediments play a major role in the storage, transport, bioavailability, and toxicity of contaminants, it is an important matrix to consider when analyzing toxicological risks from agriculture to aquatic ecosystems (Birch *et al*., 2001).

Little is known about the contribution of agriculture antimicrobials to ecotoxicity risks in the neotropics. Agriculture has been identified as a major source of antibiotics in the sediments as compared to residential and industrial areas of other regions (Hu *et al*. 2012). With the use of interviews to local producers, de la Cruz *et al*. (2010) analyzed the antibiotic use pattern in the Arenal-Tempisque Irrigation District (ATID) located north dry region of Costa Rica during 2008-2009, and estimated maximal amounts applied in swine production, agriculture and aquaculture at intervals of 821-107310, 14,8-340 and 0-1925 g ha^{-1} $year^{-1}$ respectively for each crop . The experience of analyzing chemical import information and its relation to health bio indicators has been used for pesticides and human health hazards in Central America (Bravo *et.al*., 2011).

Information regarding broad numbers of registered chemical products for swine, rice and aquaculture production in

Costa Rica is provided in Table 1. It also includes numbers of products containing antimicrobials, and results of analytical measurements of antibiotics, in samples from sites in the ATID that were sampled in the present study. In addition, Table 2 presents the different subgroups of antimicrobials in those products and their ecotoxicological profiles. For specific detail, supplementary Tables 1 and 2 provide the specific substances that include these groups for each activity.

The main objective of the present study was to evaluate the toxicity of sediments receiving effluents from agriculture and aquaculture sites in the ATID, which are known to have different patterns of antimicrobial use, based on product registration data. An assessment of sediment toxicity was made with the sediment dwelling freshwater clam *Anodontites luteola* (whole sediment test) and the zooplankton species *Daphnia magna* (interstitial water test).

The toxicity of sediments towards bivalves by changes in behavior and physiological responses were analyzed. Sediment avoidance in clams represents a serious threat to these animals in their natural habitat as they normally burrow in sediment for feeding and protection from predators. Variations in burrowing behavior are considered an easy to record and relevant marker in bivalves (Byrne and O'Halloran, 2001; Shin *et al*., 2002). Biochemical responses are being proposed as sensitive tools to assess (sub-) lethal effects of antimicrobials on aquatic biota after potential exposure episodes (Tu *et al*., 2010; Bineli *et al*., 2009). In the present study, toxicity of sediment interstitial water to aquatic invertebrates were evaluated by means of well-known acute toxicity tests with *D. magna*.

Additionally, we assessed the biochemical response of clams exposed *in vitro* to oxytetracycline (OTC), one of the few antibiotics that is used likewise in agriculture, aquaculture and swine production in the ATID (de la Cruz *et al*., 2010). This substance is considered to have an important ecotoxicological potential (Park and Choi, 2008) and limited information exists on the effects of this and other antibiotics on physiological bio markers of oxidative stress and tissue damage.

Material and Methods

Site description and sediment collection : Sediment samples were collected during four different sampling times (February, May, August and November, 2009) at four sites: the drainage of the last of a series of 3 oxidation ponds that collects wastewater from a swine farm with 8000 heads (RD_2); the drainage of an artificial wetland used to treat wastewater from a 210 ha tilapia farm (TIL_1); an irrigation channel next to a rice plantation of

Table 1 : Chemical and antimicrobial use profile according to National Databases and monitoring of antibiotic concentrations in environmental samples.

Activity/Site in the ATID	Maximun concentrations[a]				N°Chemical products registered[b]	N°Antibiotic/Antimicrobial in products registered[b]
	SMTw ng l^{-1}	OTCw ng kg^{-1}	SMTs	OTCs		
Fish aquaculture/TIL_1	ND	82	T	T	17	6
Protected wetland/PV	ND	ND	ND	ND	0	0
Rice culture/AZ	ND	ND	ND	ND	800	3
Swine farm/RD_2	98000	640	645	ND	1700	74

[a]Antibiotic residue analysis of tetracyclines and sulfonamides in 6 samples per site by LC-MS/MS in the period of 2008-2009 by Ruepert C. (manuscript in prep.); [b]Information of products from the MediVet database of the National Service of Animal Health, SENASA (http://www.senasa.go.cr/medivet/) categories: "aquaculture fish", "swine" and from the Insumosys database of the National Phytosanitary Service, SFE (http://www.sfe.go.cr/insumosys/) categories of "rice"; SMT= sulfametazine, OTC= oxytetracycline. The letter s and w indicate the sample analyzed was sediment and overlying water respectively; PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1=Fish farm drainage, RD_2= swine farm lagoon effluent.

Table 2 : Number of antimicrobial ingredients per species or crop in products registered and approved for use in the period between 2008-2009 and toxic profile of substances.

Species or crop	Antimicrobial type	N° of substances identified[a]	Toxic profile of substances[b]
Swine	Antibiotics	52	(10 Moderate, 16 Low toxicity)
	Antiparasitics	12	(5 Extreme, 1 High, 1 Moderate, 1 Low)
	Antifungals	4	(1 Extreme, 2 High)
	Other antimicrobials	6	(2 Low)
Aquaculture Fish	Antibiotics	6	(3 Moderate, 2 Low)
Rice	Antibiotics	3	(1 Moderate, 1 Low)

[a]Information from the MediVet database of the National Service of Animal Health, SENASA (http://www.senasa.go.cr/medivet/) (products with active registries between 2008-8009) and the Insumosys database of the National Phytosanitary Service, SFE (http://www.sfe.go.cr/insumosys/); [b]Information of acute toxicity to cladocerans based on ecotoxicology databases and published information. For some substances information was not available.

approximately 300 Ha (AZ), and a protected wetland (reference site) located inside the Palo Verde National Park (PV). 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 280 km^2 (Arenal Tempisque Irrigation District; ATID).

The dissolved oxygen, pH, temperature, and conductivity of overlaying water associated with the sediments were measured at the field with a portable meter (HQ40d, Hach). The above mentioned information with organic content, texture of the sediment and the measurements of some metals are presented in Table 3. Considering: the number of chemical products registered for use nationwide, the available information on residues in environmental samples (both in Table 1) and the maximum amounts (g/Ha/year) used according to local survey by de la Cruz *et al*., the sites were ranked from higher to lower toxic potential in the following way: RD_2>TIL_1>AZ>PV, with the latter considered as reference area with non-intentional exposure.

Samples of approximately 6 l of sediment were collected from the first 30 cm of the horizon using a shovel. These materials were homogenized in the field, 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 kept at 4°C for a maximum of 24 hrs until the start of the toxicity experiments.

96 hour avoidance assay with *A. luteola* : The clams were bred and bought at the Costa Rican Institute of Fishery (INCOPESCA) and kept for a month in filtered (MILLIPORE) and UV-treated (PURA) water in the laboratory before tests. After sieving in a 2.5 cm mesh, subsamples of sediments (400 ml) from PV, RD, AZ, and TIL_1 were distributed by triplicate in 1 l plastic recipients, and 500 ml of filtered (MILLIPORE) and UV-treated (PURA) water was added. Three clams were weighed and placed on the surface of the sediment in each recipient (15 organisms per sample). The organisms were 7.0 ± 0.7 cm length and 2.7 ± 0.3 cm width (mean ± SD). Exposure lasted for 96 hrs at 20 ± 2 °C with constant aeration. Every 24 hrs, physical chemical parameters (dissolved oxygen, temperature and conductivity) were recorded. After 96 hrs the survival and avoidance to the sediment (valve closure and not buried) of each clam was recorded and classified as: clam not buried =100% avoidance, partially buried = 50% avoidance or completely buried = 0% avoidance. At the end of the test, organisms were retrieved, dried with paper cloth, each replicate re-weighed and the weight difference between the start and the end of the experiment was calculated. The avoidance percentages recorded for the clams, as well as the differences in weight loss after 96 hrs, were compared between sites by analysis of variance (ANOVA) using the SPSS software (SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc).

Biochemical analysis of *A. luteola*´s tissue : Clams were dissected immediately after the 96 hours exposure described and individual samples of hepatopancreas and foot muscle samples were collected, placed in a microtube and stored at –20°C until further analysis (Monteiro *et al*., 2007). Sample homogenization, protein quantification and biomarker analyses were carried out as described in Mena *et al*., (2012). Briefly, Cholinesterase (ChE) activity was measured according to methods described by Ellman *et al*., (1961), Gluthathion-S-transferase (GST) followed methods by Habig *et al*., (1974) and Booth *et al*., (2000), and Lipoperoxidation (LPO) was analyzed according to Torres *et al*., (2002). Biochemical determinations (enzyme activities and TBARS abundance) were normalized to protein content in the samples, according to the Bradford method (Bradford, 1976). All reagents were purchased from Sigma Chemical Company (USA) and Sigma-Aldrich Chemie Gmbh (Germany). Results from biomarker activities were only analyzed for the May and November samples, and are expressed as means ± SE; values were tested for normality (Kolmogorov-Smirnov) and sites were compared (p <0.05) using one-way ANOVA and the Tukey test using the SPSS software. Data was transformed to generate an integrated biomarker response (IBR) according to Beliaeff and Burgeot (2002). High LPO or GST, or low ChE were considered positive responses. Linear models were calculated using the R software (*Rcmdr* package) to determine the influence of several physical chemical characteristics of the sediments and overlying waters in the individual biochemical responses and the score of the IBR.

Acute test with *D. magna* and sediment interstitial water : *D. magna* culture media consisted of Reconstituted Hard Water (RHW) supplemented with 0.5ml l^{-1} YFC, 2µgl^{-1} B12, 2µg^{-1} Se^{+2} and 30ml l^{-1} green algae 3.5x106 cel ml^{-1} (*Selenastrum capricornutum* and *Chlorella* sp). Medium was renewed three times per week and cultures were maintained at 20±2ºC under 16-hrs light: 8-hrs dark photoperiod. *D. magna* adults were discarded after 3 weeks. The *D. magna* stock complied with quality parameters such as no ephippia, ≤ 10% mortality, time to first brood ≤ 10 days, average number per brood ≥ 10. The EC_{50}-48hrs for Cr^{+6} was 0.10 µg ml^{-1}, and compatible with quality criteria of this type of test.

In the laboratory overlaying water was removed from sediments and samples were homogenized by mixing, distributed in 250 ml centrifuge bottles and centrifuged at 3000 rpm for 20min (Beckman, TJ-6) to obtain interstitial water from each sample. The acute 48-hrs static test was based on standard procedures (Dutka, 1989) and was conducted with *D. magna* aged less than 24-hrs at the beginning of the assay. Treatments consisted of 100% interstitial water and four dilutions 1:2 of each sample (i.e., 50, 25, 12.5, 6.25%), done in 3 plastic cups (30-ml) and containing 10 organisms and 25 ml of test solution. The temperature of the experiment was kept at 20±2 °C. The negative control consisted of 3 test units containing 25 ml of reconstituted hard water and 10 *D. magna* individuals. Immobility, defined as lack of movement after gentle prodding, was recorded at 24-h and

48-h. Oxygen, temperature, pH and conductivity of the interstitial water were measured at the beginning of the tests. EC50 and its 95% confidence limits were calculated by Probit analysis method using the SPSS software. Linear models were calculated using the R software (*Rcmdr* package) to determine the influence of several physic chemical characteristics of the sediments and overlying waters in the toxicity to *D. magna*.

***In vitro* toxicity of *A. luteola* to OTC :** Oxitetracycline dihydrate (cod 04636 Sigma ultra) was purchased from Sigma Chemical Company, St. Louis MO, USA. A 5mg ml^{-1} stock solution of OTC was prepared with 70% ethanol in a volumetric flask and with the aid of an analytical balance for mass determination. The solution was filtrated by a 0.22 µm filter (Millipore) and kept at -20°C in eppendorf tubes. It was used in another experiment to elaborate dilutions of 0.1, 1, 3, 6, 10, 25, 50 and 100 mgl^{-1} of OTC in MilliQ water (Millipore), that were quantified by LC/MS/MS. This parallel experiment revealed that the dilutions contained between 81 to 117% of the nominal concentrations. Clams were exposed in a static test for 96 hrs to nominal concentrations of OTC 0, 0.1 and 100 μgl^{-1} using UV treated water as dilution media and control, and kept at 20°C during exposure. The dilutions were prepared in volumetric flasks. Each treatment consisted of 15 clams. At the end of the exposure, clams were dissected and samples were taken for biomarker analysis as described above. Analysis if variances (ANOVA, Dunnett) were used to compare OTC treatments with control for each biomarker.

Results and Discussion

Acute toxicity to *A. luteola* and *D magna* : Complete avoidance to the sediment (100%) was only observed in clams exposed to sediment from RD2 in all sampling periods, and mortality was seen in the exposure to this sediment in one sampling period (Supp Table 1). Oxygen (DO) level in overlaying waters were similar between samples during the whole exposure (6.1 ± 0.5 mg l^{-1}). Lower conductivities (ANOVA, $p<0.05$) were found in AZ and TIL1 (275 ± 40 and 209 ± 49 µS cm^{-1}) in comparison to PV, and RD2 (936±221 and 1080±212 µS cm^{-1}).

Opposite to TIL_1, PV and AZ, the net weight change in clams exposed to RD_2 sediment was always negative, due to clam avoidance behavior that prevented from active filtering (Fig. 1). Overall, these results differentiated the pig farm from the rest of the sites in all sampling periods but November ($p<0.05$, ANOVA, Tukey).

The interstitial water of sediments from the natural wetland (PV), the fish farm (TIL_1) and the rice plantation (AZ) were not toxic to *D. magna*, opposite to those from the pig farm (RD2) according to the results shown in Table 3. No effect was observed in the Hard Reconstituted Water control. Physical Chemical characteristics of the interstitial water such as DO, pH and conductivity were measured at the beginning of the test. The average DO was similar among samples with values of 5.5±0.9, 5.0±1.8, 7.1±1.7 and 6.1±2.4 mg l^{-1} for PV, AZ, TIl^{-1} and RD_2 respectively. The same was found for pH with values of 8.0±0.5, 7.1±0.5, 7.2±0.2 and 8.3±0.3 (same order as above). The conductivity in RD_2 and PV (3534.7±1739.9 and 2703.3±749.7 µScm^{-1}) was higher (ANOVA, post hoc Tukey, $p<0.05$) than in TIL1 (367.7±82.3 µScm^{-1}), and the conductivity of AZ (667.3±169.3 µScm^{-1}) was lower than in RD2.

Linear models were calculated with the physical chemical characteristics in Table 3, with antibiotic concentrations in sediments and overlying waters (Table 1) and with the number of chemical and antimicrobials registered for each activity, to explain

Table 3 : Description of the sediment samples and overlying waters at the sites.

Site	Sediments						
	Organic matter	Sand	Lime	Clay	Cd	Zn	Cu
	(%. dry weight)				(mg kg^{-1})		
RD2	12.1±1.3	66.3±11.6	22.3±10.5	11.8±1.3	6.7±2.0	52.3±16.3	10.0±12.7
TIL_1	9.2±0.4	20.0±4.8	20.0±1.9	60.0±6.3	6.3±0.9	50.0±7.8	48.0±3.6
AZ	6.5±1.8	50±5.8	18.8±2.2	31.3±3.8	4.4±1.0	40.3±8.1	26.3±7.2
PV	8.8 ±0.8	32.8±4.3	23.8±1.1	44.0±3.8	4.3±8.3	46.3±.3	40.0±18.1

Site	Overlaying water				
	Dissolved oxygen (mg l^{-1})	pH	Conductivity (µS cm^{-1})	Total soluble solids (mg l^{-1})	Nitrates (mg l^{-1})
RD2	3.7±0.5	7.3±0.9	5933±332	253±11	5,62
TIL_1	3.5±0.9	5.8±0.3	110±7	35±18	2,8
AZ	6.2±0.7	6.7±0.4	587±261	517±408	1,24
PV	3.9±0.7	7.0±0.3	1743±748	244±206	0,52

PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1=Fish farm drainage, RD_2= swine farm lagoon effluent; Values indicate mean of four replicates ±SD.

acute effects. The metals measured did not explain the acute effects on *D. magna* and the same was obtained with oxygen and pH, organic matter, texture and nitrates. On the other hand, antibiotic (sulfonamides and oxytetracycline) maximum residue concentrations, conductivity, total solids in overlying waters and total number of registered antimicrobials explained acute mortality of daphnids ($p<0.05$).

The sediment with higher potential exposure to antimicrobials and chemicals (the swine farm) caused acute effects in aquatic invertebrates. The effluent from the pig farm can contain a hundred times more concentration of tetracyclines and sulfonamides than fish farm effluent (Table 1), but the concentrations of these substances considered toxic to aquatic invertebrates are in the range of mgl^{-1} (Park and Choi, 2008). Swine sewage could be source of other highly toxic pollutants, but also of nutrients like ammonia that can cause acute toxicity in these kinds of effluents (De la Torre *et al*., 2000). Nevertheless, considering that at least one substance was found in the range of $ug\ l^{-1}$ in RD_2 (Table 1), and the vast diversity of substances used in this activity (Supp Table 1), acute toxicity to aquatic invertebrates can´t be ruled out from other substances in those concentrations, such as ivermectin (Garric *et al*., 2007). It is important to consider that substances such as tetracyclines and fluroquinolones in the concentrations found at these sites may be more toxic to other groups of organisms such as cyanobacteria, aquatic plants and green algae (Robinson *et al*., 2005; Ebert *et al*., 2011).

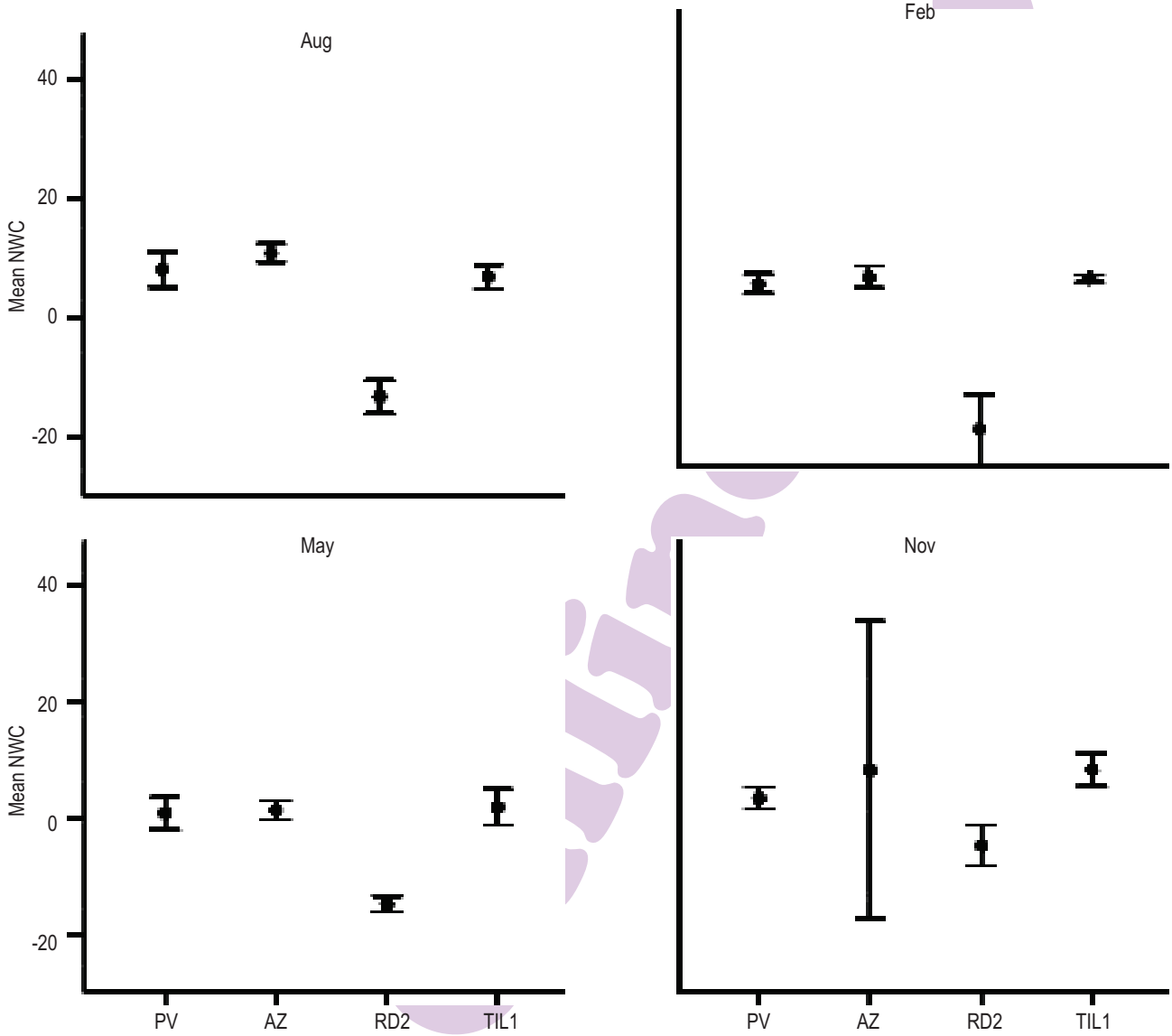

Fig. 1 : Net weight change (NWC) in clams exposed to the sediments in the four sampling periods. Error bars indicate 95% confidence interval. Differences were found between RD_2 and the rest of the sites in all samplings but November (ANOVA, $p<0.05$); PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1 = Fish farm drainage, RD_2 = swine farm lagoon effluent.

According to national databases and local surveys of the activities analyzed in the present study, antibiotics and antiparasitics with high or extreme toxicity are only used in swine production (Table 2). These are included in the pig feed daily and are regularly being excreted in feces and urine almost unaltered (Boxall, 2010). Oxidation lagoons for waste management as used in the pig farm are not designed for chemical degradation, and thus their effluents are a recognized source of pollution to aquatic ecosystems (Kolz *et al*., 2005; Luo *et al*., 2011). The pig farm sediments showed the lowest clay content from all sites (Table 1). It has been reported that an increase in clay content favors adsorption of some veterinary pharmaceuticals (Xu *et al*., 2009), which may lower substance toxicity due to less availability.

Biochemical response of *A. luteola* : During May exposure, both ChE and GST activity of clams exposed to sediment from pig farm (RD2) were higher than in clams exposed to rest of the sites (AZ, PV and TIL_1) (ANOVA, $p<0.05$; Fig 2. A and B). Meanwhile, organisms exposed to samples collected in November did not show significant difference in ChE or GST activity between sites. Overall, ChE activity and LPO in clams exposed in May was higher than during November, except for LPO in TIL_1 which was similar at both times. On the other hand, none of the sites showed significant GST activity variation between both periods, except for pig farm (RD_2), where GST varied between extremes with the highest GST activity in organisms exposed during May, and the lowest activity in organisms exposed during November (Fig 2).

High GST activity in bivalves has been related to exposure to organophosphates, polycyclic aromatic hydrocarbons and metals (Damásio *et al*., 2010). Depletion of glutathione (which is substrate for GST activity) has been observed in mussels exposed to pyretroids (Simsek-Koprucu *et al*., 2008). In contrast, inhibition of GST activity, as observed in clams exposed to November RD_2 sample, have been associated with exposure to organochlorines, aromatic compounds and acidic herbicides (Damásio *et al*., 2010). Effect of antibiotics on biomarkers of oxidative stress or tissue damage are less known, but it has been reported that OTC can induce increase in lipid peroxidation and gluthatione-S-transferase in rainbow trout (Yonar, 2012).

Regarding variation in ChE activity observed in clams exposed to May-RD_2 sample, it has been stated that bivalve ChE is not a sensitive biomarker for some pollutants such as pesticides and that its activity shows seasonal variation, as observed in the present study (Damásio *et al*., 2010). Additionally, other authors have documented that clam sediment avoidance containing cholinesterase inhibitors results does not result in observable effects when measuring ChE activity (Cooper and Bidwell, 2006). Meanwhile Tu *et al.* (2009) suggested that changes in ChE activity can indicate exposure to antibiotics like enrofloxacin and furazolidone in black tiger shrimp, revealing health impairment.

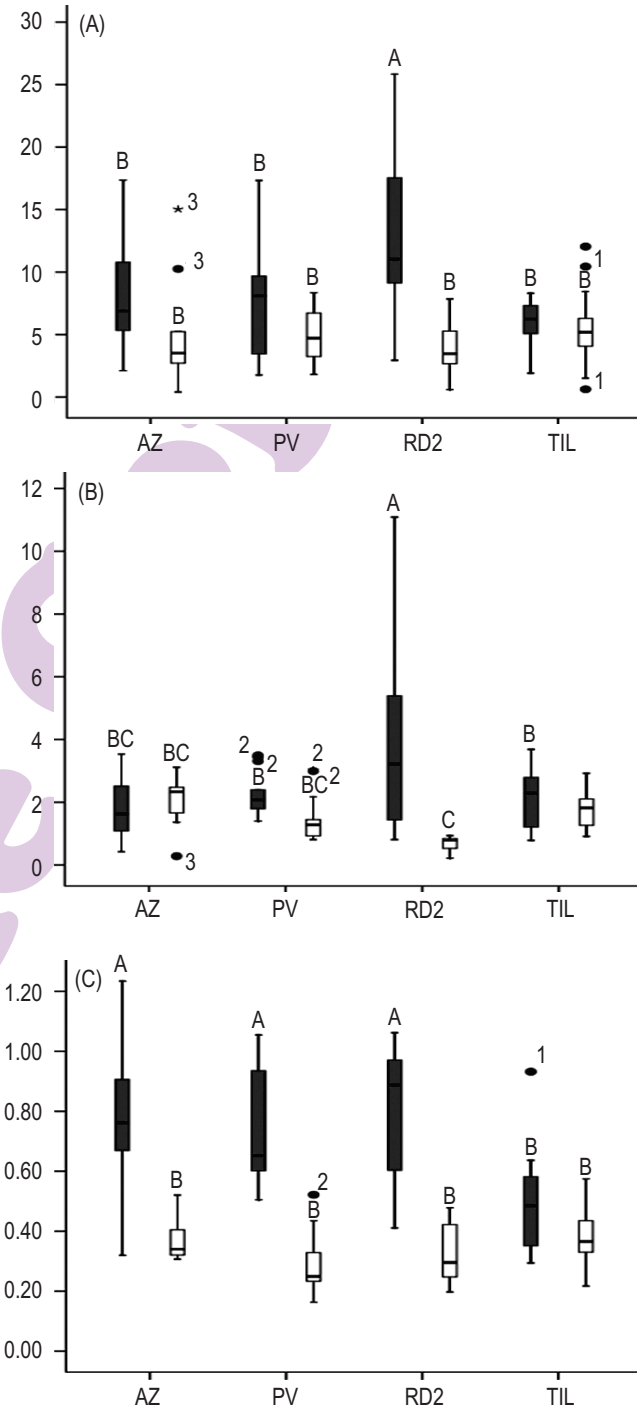

Fig. 2 : Biomarkers measured in *A. luteola* after 96-h exposure to sediment from the four sampled locations: (A) feet muscle ChE activity in U mg^{-1} ptotein; (B) hepatopancreas GST activity in U mg^{-1} ptotein; (C) hepatopancreas LPO in nmol TBARS mg^{-1} protein. Gray boxes series represent assays carried out with samples collected in May-2009; white boxes series represent represent assays carried out with samples collected in November 2009. Significant differenses bettwen samples are represented with different letters. The lines in the boxplot indicate 95% CI of the mean. PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1=Fish farm drainage, RD_2= swine farm lagoon effluent

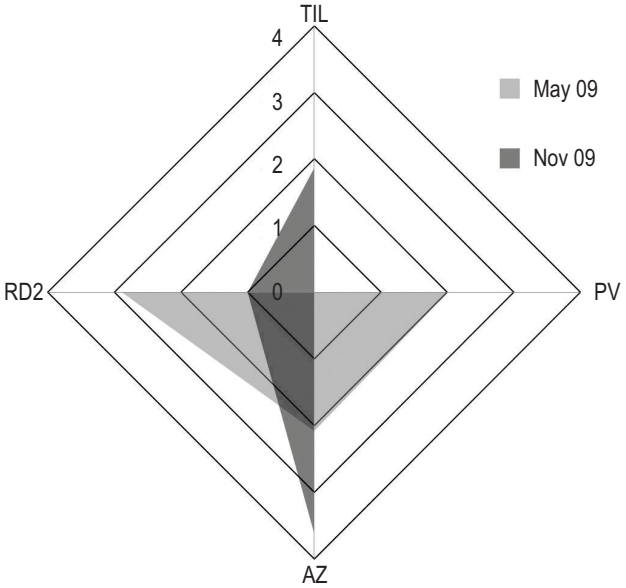

Fig. 3 : Integrated Biomarker Response (IBR), calculated with ChE, GST and LPO data measured in *A. luteola* exposed to sediment from four locations influenced by different economic activities; in two sampling campains (May and November 2009). PV=Palo Verde (reference wetland), AZ= Rice farm drainage; TIL_1=Fish farm drainage; RD_2= swine farm lagoon effluent

When integrating the results from three biomarkers, the IBR values in May indicated different degrees of effects at AZ, PV and RD_2 sites, later being most impacted (TIL_1 site was least affected) (Fig 3). Meanwhile, in November, moderate signs of response were observed towards TIL_1 and RD_2 sites, while AZ site was clearly differentiated as the most impacted. Furthermore, AZ was also the site spotted with the major overlapping between May and November assays (Fig. 3).

Integration of biomarkers in this study supports the results observed in acute effects elicited by the pig farm sediment. On the other hand, indication that AZ is more impacted than TIL_1 does not relate to the proposed classification of sites by their exposure to antimicrobials. A refinement on our exposure assessment requires improvement of information related to environmental residues and concentrations applied at the sites, as important discordances between labeling and measured concentrations for antibiotics have been detected in animal foods in Costa Rica (Granados *et al*., 2012; Gutiérrez *et al*., 2010).

In the linear models calculated, conductivity and nitrates in overlying waters explained the integrated biomarker response (IBR) of clams (p<0.05). This was not obtained with other physical chemical characteristics, maximum antibiotic residue concentrations, and total number of registered chemicals and antimicrobials.

***In vitro* toxicity of oxytetracycline to *A. luteola* :** Although not statistically significant, a decrease in ChE activity was observed

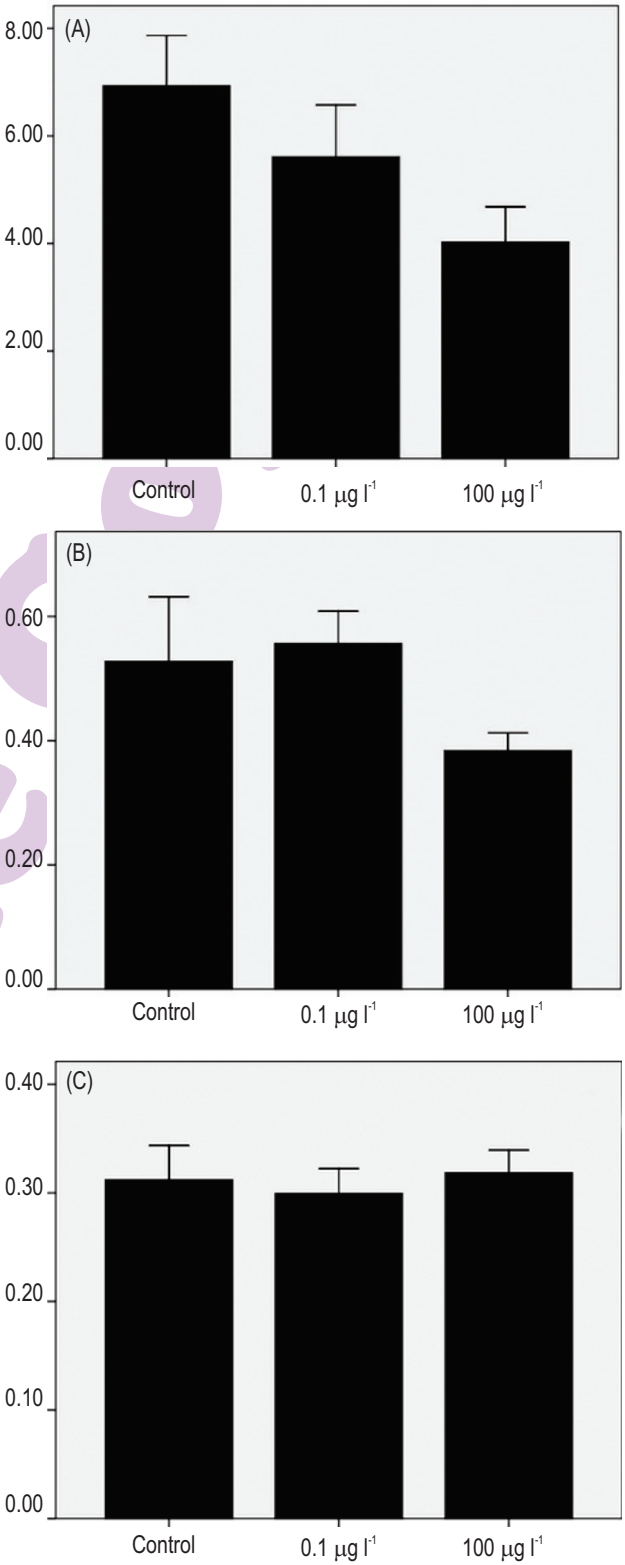

Fig. 4 : Biomarkers measured in A. luteola after 96-hr exposure to Oxytetracycline: (A) feet muscle ChE activity in U mg^{-1} protein; (B) hepatopancreas GST activity in U mg^{-1} ptotein; (C) hepatopancreas LPO in nmol TBARS mg^{-1} protein. Data expressed as mean + SE

Table 4 : Acute (48 hr) toxicity of sediment interstitial water to *Daphnia magna*

Site	Sampling period			
	February	**May**	**August**	**November**
AZ	7% inmobility in 100% concentration	ND	ND	ND
PV	ND	ND	ND	ND
RD_2	EC50: 11.76% (10.23-13.32)	EC50: 8.97% (7.84-10.24)	EC50: 2.42% (1.03-3.37)	EC50: 8.97% (7.84-10.24)
TIL_1	ND	ND	ND	ND

Values represent dilution % of sediment interstitial water and numbers in parenthesis indicate 95% confidence interval of the EC50; ND: no acute effect could be detected; PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1=Fish farm drainage, RD_2= swine farm lagoon effluent

in clams exposed to oxytetracycline (Fig. 4A). Regarding GST activity, a decrease was only observed in organisms exposed to 100 µg l^{-1} of antibiotic (Fig 4B). This is a high concentration compared to the ng l^{-1} found at the sites. However, no effect was observed at LPO level (Fig 4C).

Biomarker analysis of organisms interacting with the sediments is designed to detect physiological changes prior or even in the absence of more evident responses, such as mortality, immobility or behavioral changes. Response of anti-oxidative and biotransformation defenses in organisms is a complex process which depends on intensity and duration of exposure (Livingstone, 2001). The use of a multiplex (biochemical, physiological and molecular) biomarker approach has been indicated as an accurate and sensitive method to assess the past or present exposure of shrimp to drugs and chemicals used in tropical aquaculture (Tu *et al*., 2009, 2010). Effects of antimicrobials used in aquaculture in environmentally relevant conditions have also been found in fish (Yonar, 2012) and other mollusks like mussels (Bineli *et al*., 2009). However, information on sub-lethal effects of antimicrobials on different biological groups is still scarce.

In Costa Rica, products approved in swine species operations have extreme to low toxicity profiles to aquatic invertebrates. Hence, actions should be taken in order to address the high toxic profile observed in swine farm effluents, considering nutrients and solids, but also chemical characteristics such as organic pollutants and metals. Antimicrobial concentrations in animal foods and improvement in waste management need to be considered in the risks associated with swine operations. The analysis of chemical registration databases is an important first approach to address the toxicity hazards of antimicrobial use in those activities. However, efforts should be made to increase residue monitoring in effluents and gather on-farm use information in the neotropics in order to improve the exposure characterization.

A deeper knowledge of the responses of biomarkers towards antimicrobials in aquatic invertebrates may improve our understanding of results with environmental samples as in our study, and will influence future biomarker and species choice for antimicrobial risk assessment. The knowledge on antimicrobial effects on aquatic invertebrates would also benefit from the use of chronic-response assessments.

Acknowledgments

This work was supported by the National Council of Rectors (CONARE). Job Sloot and the IBED-University of Amsterdam for the determination of OTC by LC/MSMS in parallel experiment.

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Supplementary Table 1 : Antimicrobials included in products registered in MediVet during 2008-2009 for swine production

Antimicrobial type	Subgroup			
Antibiotics	Aminoglycoside	B Lactams	Macrolides	Sulfonamides
	Apramycin	Amoxicillin	Erythromycin	Sulfadiazine
	Spectinomycin	Ampicillin	Esperamicin	Sulfadimethoxine
	Streptomycin	Cefalexin	Josamycin	Sulfadimidine
	Gentamicin	Cefotaxime	Kitasamycin	Sulfadoxine
	Kanamycin	Cefquinome	Tilmicosin	Sulfaguanidine
	Kasugamycin	Ceftiofur	Tylosin	Sulfamerazine
	Neomycin	Ceftriaxone	Tulathromycin	Sulfamerazine
		Penicillins	Virginiamycin	Sulfamethoxazole
				Sulfamethoxypyridazine
	Orthosomycins	Pleuromutilins	Polimixins	Sulfathiazole
	Avilamicine	Tiamulin	Colistin	
	Quinolones	Polipeptides	Tetracyclines	Amphenicols
	Ciprofloxacin	Bacitracin	Chlortetracycline	Florfenicol
	Danofloxacin	Enramycin	Doxycycline	Thianfenicol
	Difloxacin		Oxytetracycline	
	Enrofloxacin	Lincosamide	Tetracycline	Glycopeptides
	Flumequine	Lincomycin	Flavofosfolipol	
	Norfloxacin			
Antiparasitics	Anticoccidials	Anthelmintics	Avermectins	Antiprotozoals
	Amprolium	Abamectin	Doramectin	Pyrimethamine
	Narasin	Fenbendazole	Ivermectin	
	Toltrazuril	Flubendazole		
		Levamisole		
		Mebendazole		
		Oxibendazole		
Antifungals	Clotrimazole			
	Enilconazole			
	Diiodohydroxyquinoline			
	ketoconazole			
Others	Phosphonates			
	Olaquindox			
	Trimethoprim			
	Salinomycin			
	Sulfaclozina			
	Sulfaquinoxaline			

Supplementary Table 2 : Antimicrobials included in products registered in MediVet during 2008-2009 for "aquaculture fish" and in Insumosys for "Rice"

Species or crop/ Antimicrobial type	Antibiotics			
Aquaculture Fish				
Subgroup	Sulfonamides	Amphenicols	Tetracyclines	Others
Substance	Chloramine t	Florfenicol	Doxycycline	Trimethoprim
	Sulfamethoxazole		Oxytetracycline	
Rice				
Antimicrobial type	Antibiotics			
Subgroup	Aminoglycosides	Tetracyclines		
Substance	Streptomycin		Oxytetracycline	
	Kasugamycin			

Supplementary Table 3 : Number of clams with 0, 50 and 100% avoidance to the sediment after 96 hours of exposure (n=15 exposed / site / period)

Site	Sampling period											
	February			May			August			November		
	0%	50%	100%	0%	50%	100%	0%	50%	100%	0%	50%	100%
PV	15	0	0	14	0	1	15	0	0	15	0	0
AZ	15	0	0	15	0	0	15	0	0	14	0	1
RD2	0	0	13[a]	0	0	15	0	0	15	0	0	15
TIL_1	15	0	0	13	1	1	15	0	0	15	0	0

[a] Two clams were found dead at the end of the exposure; PV=Palo Verde (reference wetland), AZ= Rice farm drainage, TIL_1=Fish farm drainage, RD_2= swine farm lagoon effluent.