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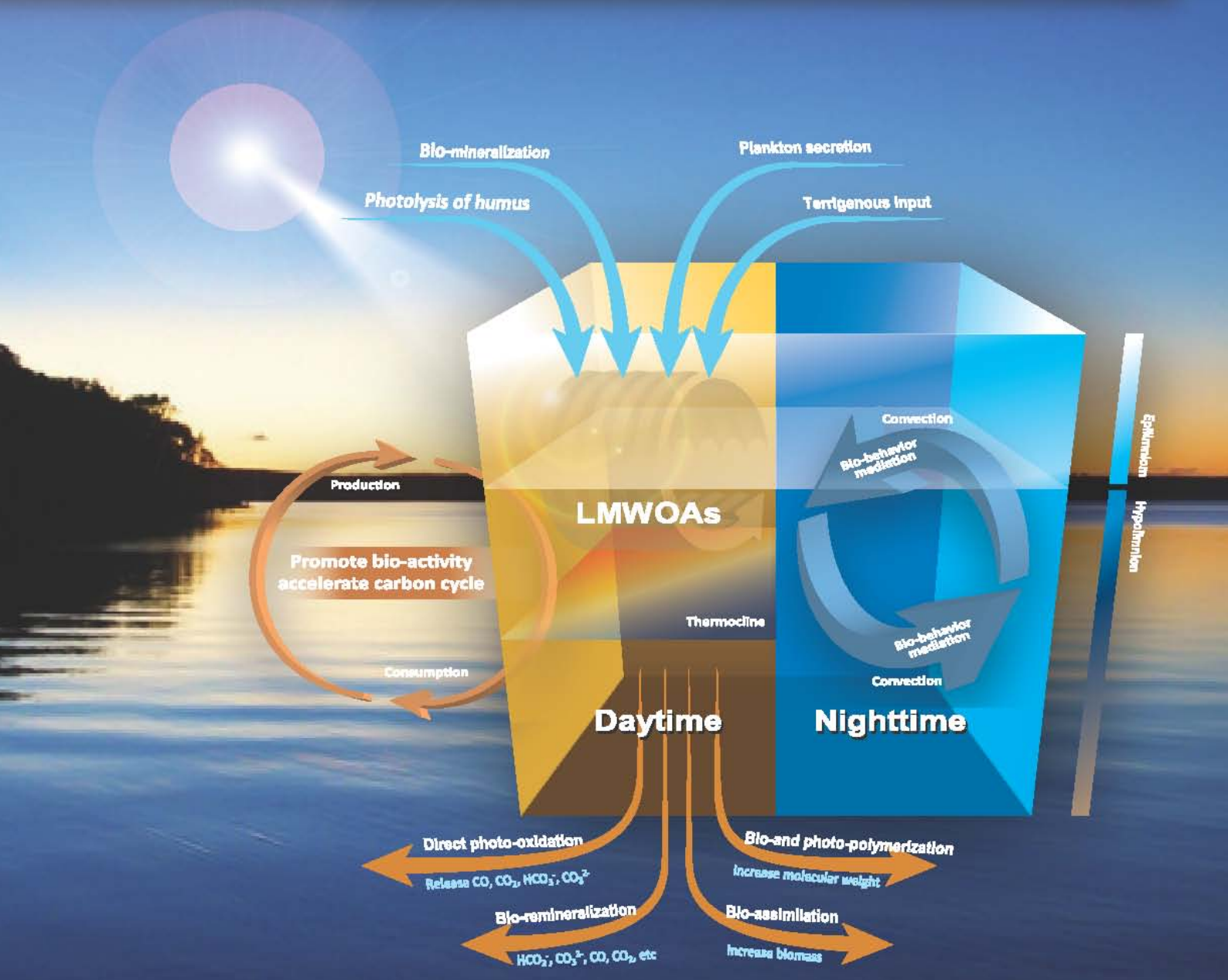

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Preliminary studies on occurrence of monensin antibiotic in Bosque River Watershed

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Abstract

Water quality impact due to excessive nutrients has been extensively studied. In recent years, however, micro-pollutants such as pharmaceuticals and hormonal products used in animal agriculture have added an additional impact to overall water quality. Pharmaceuticals used in the poultry, swine, beef, and dairy industries have been detected in various environmental matrices such as, soil, groundwater and surface water. In this study, 26 surface water samples were collected throughout the Bosque River Watershed (BRW) with samples representing a range of land use conditions and locations of major dairy operations. Samples were analyzed using commercially available Enzyme-Linked Immunosorbent Assay test. Of the 26 samples, three samples consistently tested positive for monensin antibiotic with concentration ranging from 0.30 to 3.41 μg/L. These three samples were collected from sites that received varying amount of agriculture wastes (11.7% to 31.3%) and located downstream from sites associated with moderate levels of animal agriculture. The preliminary results suggest that there is a potential for monensin occurrence in the BRW, although initial findings indicate only very low levels.

Key words: ELISA; ionophore; monensin antibiotic; dairy waste; emerging pollutants; pharmaceuticals

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Introduction

In recent years, occurrence of antibiotics (ABs) in environmental matrices such as soil, groundwater and surface water has been reported in scientific journals, periodicals and national newspapers with increasing frequency. Although, potential human health risks due to occurrence of ABs in the environment are not yet fully known; the presence of ABs in the environment may pose human health and ecological risks (Boxall et al., 2003). Sources through which ABs enter the environment include human therapeutic usages and residual concentrations of ABs resulting from their use in intensive animal agriculture. Sources such as direct discharge of animal waste containing partially metabolized ABs, surface run-off from land application of manure containing trace ABs and direct grazing of ABs fed animals all may contribute to the occurrence of ABs in the environment. **Figure 1** shows the schematic representation of sources and pathways of monensin antibiotic in the environment.

Some of the major classes of ABs used in animal agriculture are tetracyclines, sulfonamides, aminoglycosides, β-lactums, macrolides, lincosamides and ionophores (Thiele-Bruhn, 2003; McGuffey et al., 2001). Monensin is the single most widely used antibiotic in ruminant animals to improve feed efficiency and overall production (Watanabe et al., 2008). It alone accounts for approximately 13% of total sub-therapeutic livestock antibiotic usages in the United States (Dolliver et al., 2008). Antibiotics are commonly administered to milk replacer for calves, as a feed additive to accelerate the growth of heifers and for treatment during lactation (Brown et al., 2006). The USDA (2008) reported that 17.5% of dairy operations used antibiotics for sub-therapeutic purposes as a feed additive

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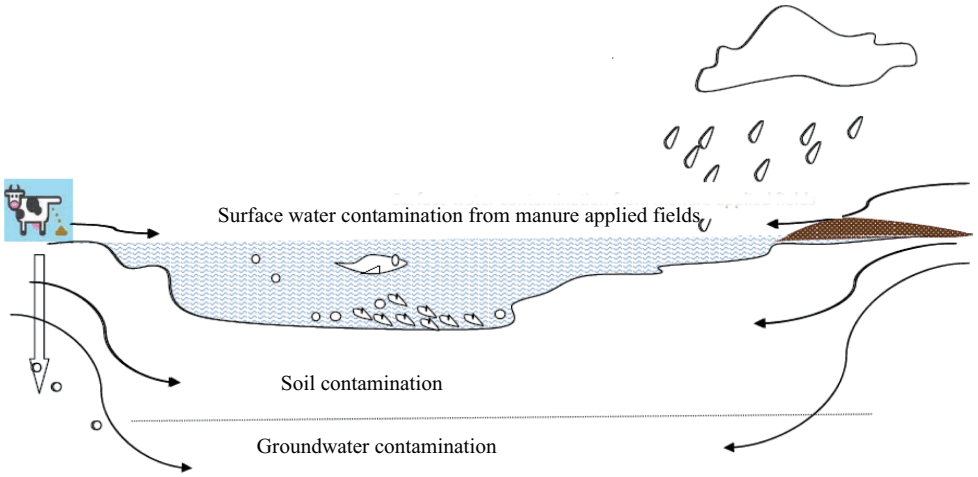

Fig. 1 Schematic representation environmental pathways of monensin antibiotic from animal agriculture operations.

and monensin often comes pre-mixed in feed. It is reported that 94% of dairy operators administer antibiotic injections to at least 1% of their herd and nearly 43% dairy operators inject antibiotics to nearly 10% of their herd (Brown et al., 2006). Depending on the type and size of the animal and the class of antibiotic used, the dose can vary anywhere between 1 to 100 g/Mg of feed (Kumar et al., 2004).

Due to the routine use of ionophores including monensin as feed additive, ionophores have been termed as an emerging environmental contaminant in diverse environmental matrices, such as soil, groundwater and surface water (Hansen et al., 2009). Research by Watanabe et al. (2008) reported partially metabolized monensin in groundwater samples on dairy farms and in wells located downstream from the dairy wastewater lagoons. Environmental occurrence of monensin due to intensive bovine production system in Argentina is confirmed by Yoshida et al. (2007). Kim and Carlson (2006) confirms the presence of monensin in river water and aquatic sediments in Colorado and attributes this detection of monensin to feedlots in the watershed. In Southern Ontario, Lissmore et al. (2006) reported detecting monensin in nearly 75% of stream samples with concentration ranging from 0.006 to 1.2 μg/L. Alternatively Kemper et al. (2008), reported that manure and leachate samples collected from dairy farm waste did not indicate the presence of antibiotics. They attributed the lack of antibiotics in manure and in leachate samples to the relatively low administration of antibiotics in dairy farming compared to swine and poultry operations, the potential degradation of antibiotics due to storage, and the sorption of antibiotics to soil material.

The Bosque River Watershed (BRW) region approximately covers 4300 km^2 (1660 $mile^2$) in north central Texas and is ranked as the number one milk producing region in the state (USDA-AMS, 2010; Cabrera, 2005). To date, there are no studies in this region regarding occurrence of ABs in the environment. This research presents preliminary data from surface water samples collected from 26 sites within the BRW. Samples were collected in March and May 2010, focusing on sites in the upper third of the watershed where a large number of dairy operations are located. Enzyme Linked Immunosorbent Assay (ELISA) was conducted to determine the presence and distribution of the monensin antibiotic in the BRW region.

1 Experimental

1.1 Chemicals

ELISA kits were purchased from Immuno-Diagnostic Reagents (Vista, CA). Kits consist of pre-coated 96 well microtiter plates and standards of 0, 1, 2.5, 5, 10 and 25 μg/L. Monensin sodium salt (CAS# 22373-78-0; Assay 90%–95%) was purchased from Sigma Aldrich, St. Louis, MO, USA. Because monensin is slightly soluble in water but very soluble in organic solvents (Merck Index 2001), a stock solution of monensin was prepared in methanol and standard solutions of monensin were prepared in nanopure water (18.2 MΩ·cm at 25°C) by serially diluting the stock solution. Molecular structure and a standard curve for monensin are shown in **Fig. 2**.

1.2 Surface water sample collection

Surface water samples were collected on March 2–3 and May 10–12, 2010, from throughout the BRW with samples close to and far from animal agricultural activities in the region. The locations of the sampling sites within the BRW are shown in **Fig. 3**. Routine grab samples were generally taken at a depth of about 0.08 to 0.15 meters (0.25 to 0.5 ft.) below the surface, as recommended in Texas Commission on Environmental Quality (TCEQ) surface water quality monitoring procedures (TCEQ, 2003). The sampling sites are represented by alphanumeric codes with first two

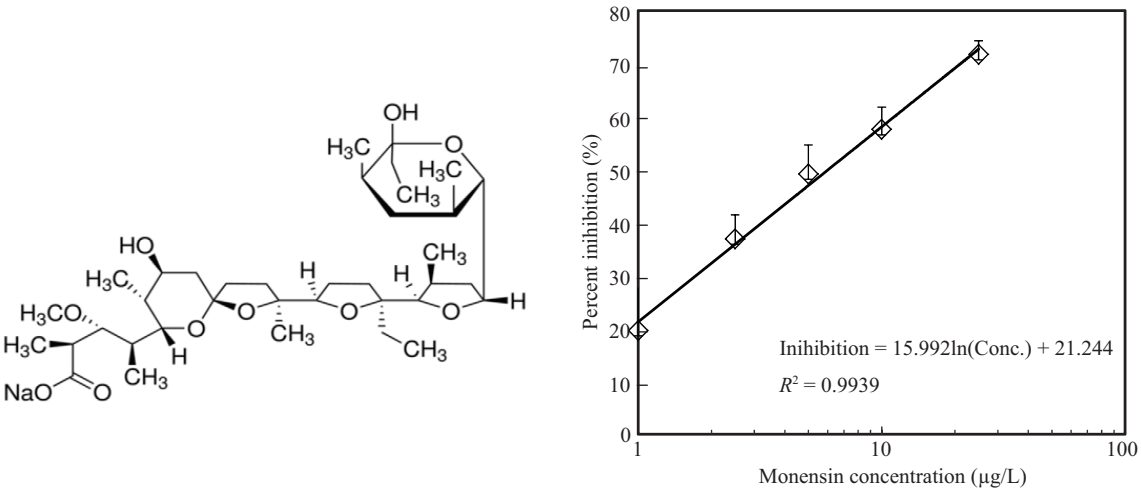

Fig. 2 Molecular structure (source: Sigma Aldrich) and standard curve for monensin ELISA.

letters specifying the tributary or river (BO for North Bosque River) on which the site is located, while the last three digits indicating the relative location of the site. Sites near to the headwaters are represented by lower numeric values, whereas, sites further downstream are represented by higher numeric values.

Sampling sites were categorized based on drainage area or location within the watershed. Category 1 sites are on the micro-watersheds, Category 2 sites are on major tributaries to the North Bosque River, Category 3 sites are on the North Bosque River and Category 4 sites are on the other rivers and major tributaries to Lake Waco. These sites were selected with regard to general land-use descriptions based on classification of satellite imagery collected from 2001 through 2003 and processed by the Spatial Sciences Laboratory of the Texas Agricultural Experiment Station (Narasimhan et al., 2005). Manure or waste application sites were compiled from the database maintained by the TCEQ and were used to supplement the satellite imagery classification.

1.3 Experimental method

Assay samples were prepared for ELISA by centrifugation at 3000 r/min for 15 min followed by filtration through 0.45 μm Whatman syringe filters (part# 6869-2504, Fisher Scientific) and pH adjustments of sample aliquots (pH 7.0 ± 1.0). A 25-μL aliquot of standard or sample was added to each well followed by addition of 100 μL of monensin alkaline phosphate conjugate. The plate was gently tapped to ensure even distribution of sample and enzyme conjugate and incubated at room temperature for 40 min, after which the well contents were removed by inverting and shaking. Plates were washed three times with wash buffer to remove the non-bound conjugate and 100 μL of p-nitrophenyl phosphate substrate was added to each well. Plates were incubated for another 20 min followed by addition of 50 μL of stop solution to the wells. Optical density was measured at 405 nm using a Thermo Scientific Multiskan EX microplate reader. Monensin quantification is dependent on percent inhibition relative to the blank sample. Percent inhibition (I, %) was calculated from the optical density using the formula:

$$I = \left(1 - \frac{\mathrm{OD_s}}{\mathrm{OD_0}}\right) \times 100\%$$

where, $\mathrm{OD_s}$ is average absorbance of each standard or sample; $\mathrm{OD_0}$ is average absorbance of 0 μg/L standard.

A five point (0, 1, 2.5, 5, 10 and 25 μg/L) standard curve was developed for quantification of monensin in surface water samples (**Fig. 2**). The kit did not include the 2.5 and 10 μg/L standards; these were prepared by diluting 5 and 25 μg/L standards respectively. Sensitivity of the monensin ELISA kit was established through the analysis of serially diluted laboratory standards of pure monensin at various concentrations. All the standards and samples were run in duplicate.

2 Results and discussion

The enzyme immunoassay for monensin is based on the competition between the monensin to be assayed and the monensin-alkaline phosphate conjugate for binding to the antibodies coated on the microwells. When the sample containing monensin, and the monensin-alkaline phosphate conjugate added to the microtiter wells, they compete for binding on to the limited number of antibodies coated on to the microwells. Incubation followed by rinsing facilitates removal of non-bound components. The bound enzymatic activity is then measured by adding the substrate that forms chromogen. It should be noted that the intensity of color formed due to addition of such substrate is inversely proportional to the concentration of monensin in the sample. Monensin concentration in the surface water samples was quantified using the standard curve established by analyzing different concentrations of monensin (**Fig. 2**).

Fig. 3 Surface water sampling locations in the BRW region.

At least four samples showed positive detection for the monensin antibiotic for more than one sample preparation method at different date (**Table 1**). Three of these samples were consistently positive and were from sites (AL020, LD040 and IC020) associated with moderate levels of animal agriculture. The fourth sample was from an eco-region reference site (NC060) with limited agricultural activity. Samples from three sites were the only ones that consistently showed positive inhibition and detection of monensin, although other samples also represented sites in the vicinity of animal agriculture. All three samples AL020, LD040 and IC020 have 11.7%, 31.3% and 19.3% of the area used for animal waste application and approximately 45%, 26.9% and 35.1% of the land area in pasture. This land use pattern is consistent with the high frequency of detection in these sampling sites. Although sites GB020 and NF020 have large animal waste application fields, the overall extent of land application is small compared to other sites. Both GB020 and NF020 have total areas of 440 and 800 ha respectively which are significantly smaller than the site that has tested positive concentration of monensin, an observation indicating that extent of land application of animal waste may correlate with the frequency of detection of monensin. The samples AL020, LD040 and IC020 have consistently shown to be positive for monensin antibiotics under different sample

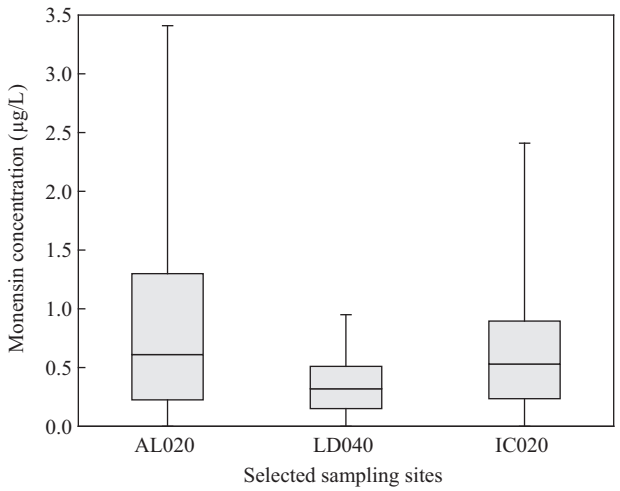

Fig. 4 Surface water samples showing positive detection of monensin in three sites due to land application of animal agriculture waste.

preparation techniques (**Fig. 4**). Although other samples were ELISA positive for monensin, inconsistencies were observed in the results, which may be due to several factors such as seasonal variation, river flow stages, and potential matrix effect and cross reactivity of ELISA with other potentially present ionophores.

Monensin is a polyether ionophore and is practically insoluble in water. As such, it might sorb on to the soil and

Table 1 Concentration of monensin in various surface water samples collected on different dates (unit: μg/L)

Sample name	Total area (ha)	Area waste application (%)	Centrifuged-Unfiltered			Centrifuged and Filtered			
			20-Mar	30-Mar		22-May	30-Mar		24-May
			pH adjusted	pH adjusted	Non-pH adjusted	Non-pH adjusted	pH adjusted	Non-pH adjusted	Non-pH adjusted
Category 1: Sites on micro-watersheds									
AL020	4720	11.7	0.61	3.41	0.85	ND	1.75	0.45	ND
DB035	2130	14.3	ND	ND	ND	ND	ND	ND	ND
GC045	11900	7.8	ND	ND	ND	ND	ND	ND	ND
GM060	4410	5.8	ND	ND	ND	ND	ND	ND	ND
IC020	17402	19.3	0.68	2.41	0.8	ND	0.47	0.53	ND
LD040	2960	31.3	0.32	0.3	0.68	ND	0.6	0.42	ND
LG060	4260	10.3	ND	ND	ND	ND	ND	ND	ND
NF009	520	13.5	ND	ND	ND	ND	ND	0.53	ND
NF020	800	41.3	ND	ND	ND	ND	ND	ND	ND
SP020	1560	0	ND	ND	ND	ND	ND	ND	1.83
TC020	2990	0	ND	ND	ND	ND	ND	ND	ND
WC020	950	0	ND	ND	ND	ND	ND	ND	ND
Category 2: Sites on micro-watersheds									
GC100	25200	7.9	ND	ND	ND	ND	ND	ND	ND
NC060	35200	0	ND	0.9	0.48	ND	ND	ND	ND
NF050	8370	17.7	ND	ND	ND	ND	ND	ND	ND
SF085	12900	16.7	ND	ND	ND	ND	ND	ND	ND
Category 3: Sites on micro-watersheds									
BO020	21700	16.9	ND	1.12	ND	ND	ND	ND	ND
BO040	25700	15.5	ND	ND	ND	ND	ND	ND	ND
BO070	93100	8.9	ND	ND	ND	ND	ND	ND	ND
BO083	178000	6.3	ND	ND	ND	ND	ND	ND	ND
BO090	253000	4.4	ND	5.13	ND	ND	ND	ND	ND
BO095	267000	3.8	ND	ND	ND	ND	ND	ND	ND
17500	0	0	ND	ND	ND	ND	ND	ND	1.07
Category 4: Sites on micro-watersheds									
HC060	20200	0	ND	ND	ND	ND	ND	ND	ND
MB063	46900	0.1	ND	ND	ND	ND	ND	ND	ND
SB065	22200	0	ND	ND	ND	ND	ND	ND	ND

ND: non-detect.

sediments and persist in aquatic environment. Results of ELISA assays performed on surface water samples collected from 26 sites throughout the BRW indicate that some headwater creeks may contain monensin antibiotic. While BRW had positive detection of monensin, the measured concentrations were quite low and below current levels of concern for environmental impact (McGregor et al., 2007; Lissmore et al., 2006). Although ELISA is accurate, matrix interference might produce inaccurate results as is evidenced from a relatively higher concentration of monensin in unfiltered samples compared to the filtered samples (**Table 1**). It should be noted that antibody used in these monensin ELISA kits has potential cross-reactivity issues for other similar types of ionophoric antibiotic such as salinomycin (Dolliver et al., 2008). Further research is warranted to establish any potential cross-reactivity issues and use of other ionophores in the BRW.

3 Conclusions

The use of antibiotics in animal agriculture has substantially increased the efficiency of production of meat, milk, and fiber for consumers. In the United States, low levels of various veterinary pharmaceuticals (including antibiotics) have been detected in soil, groundwater, and surface water, and as such potential impact on human health and the environment is naturally a concern. Because the occurrence of pharmaceuticals in the environment is often from non-point (diffused) sources, it is difficult to identify the source and quantity the amount of such compounds introduced in the environment.

Although monensin ELISA gives reasonably consistent results, further research is warranted regarding the potential matrix interference and cross reactivity of other antibiotics. In order to conclusively demonstrate and identify the source of monensin, a comprehensive study would need to be performed. In summary, the results suggest that there is a potential for monensin detection in the BRW, although initial findings indicate only very low levels. Further studies are required to fully understand the influence of land application of animal waste and occurrence of monensin antibiotic in surface water samples.

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