# Reducing nutrients, organic micropollutants, antibiotic resistance, and toxicity in rural wastewater effluent with subsurface filtration treatment technology

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**Abstract**

The ability of a sub-surface treatment filtration system to remove nutrients, thirty-nine organic contaminants, metals, and antibiotic resistant gene (ARG)-bearing organisms, and to attenuate acute toxicity of wastewater lagoon effluents, was assessed. Significant removal was observed for nutrients between the conventional primary and secondary sewage lagoons, with further average attenuation of 59% and 50% of ammonia and total phosphorus (TP), respectively, within the filter. Effluent concentrations of ammonia ranged from 0.4 to 2.6 mg/L and concentrations of TP from 1 to 4.1 mg/L, with decreasing acute toxicity from primary to secondary lagoons, and no toxicity observed in the filtration system based on Microtox® assays. Most organic micropollutants were also efficiently removed between the primary and secondary lagoons (e.g., up to 98% for atenolol). However, in general, little attenuation occurred within the filter for estrogenic compounds (e.g., 17 $\alpha$ -ethinylestradiol); $\beta$ -blockers (e.g., metoprolol); antidepressants (e.g., fluoxetine--Prozac); antibacterial agents (e.g., triclosan), non-steroidal anti-inflammatory drugs (e.g., diclofenac); lipid regulators (e.g., clofibric acid); and macrolide (e.g., clarithromycin) and sulfonamide (e.g., sulfamethazine) antibiotics; or metals (Cr, Cu, Fe, Mn, Ni, and Zn). This lack of removal was likely due to a minimal hydraulic residence time within the filter (~6 h) under current operating conditions. The lagoon treatment system effectively removed ~99% of sulfonamide resistant bacteria, but the filter both reduced tetracycline-resistant bacteria (~58%) in wastewater and harbored them in the biofilms, as relative abundances of *sul* and *tet* genes were greatest there. The filter also harbored nitrifying and denitrifying bacteria, respectively, contributing to N removal. These results

suggest that the constructed sub-surface treatment filtration system can provide a low-cost, low-maintenance, and effective means to reduce nutrient loading and improve microbial community structure and function.

**Keywords:** Wastewater lagoons; Subsurface Filtration; Pharmaceuticals; Antibiotic resistance genes (ARGs)

## 1. Introduction

With increased pressure on global water resources, concerns over wastewater contaminants and their effects on water quality continue to grow. Nutrient enrichment and subsequent eutrophication continue to threaten water quality in freshwater systems downstream of areas of agricultural intensification and urbanization (Smith, 2003). In addition, the ubiquitous presence of organic contaminants, including human- and veterinary- use pharmaceuticals, has been well-established to pose a hazard to aquatic organisms in receiving waters, and a challenge for wastewater treatment (Fent et al., 2006; Kolpin et al., 2002). Also of concern for wastewater treatment systems are organisms bearing antibiotic resistance genes (ARGs), which could promote future outbreaks by antibiotic-resistant pathogens (Rowan et al., 2010; WHO, 2000). To address the risks posed by these chemical and biological wastewater contaminants, effective treatment systems are required, along with an improved understanding of the mechanisms by which these contaminants can be removed prior to their entry into vulnerable ecosystems.

Wastewater lagoons are a common technology for sewage treatment in rural communities around North America (US EPA, 2002), including the province of Manitoba,

46 Canada (Federation of Canadian Municipalities, 2004). In many communities, decisions
47 around design, implementation, and management of lagoon systems were made before
48 water quality impairment, such as eutrophication, was a widespread environmental concern
49 resulting in a more stringent regulatory environment around releases. In addition,
50 wastewater guidelines are very new for other ubiquitous emerging contaminants, such as
51 chemical micropollutants and organisms bearing ARGs (Kolpin et al., 2002; Pruden, 2014),
52 if guidelines exist at all. One example, intended to regulate release of synthetic estrogens
53 (e.g., 17 $\alpha$ -ethinylestradiol in birth control pills) in the UK, may cost billions of dollars to
54 achieve compliance (Owen and Jobling, 2012). In Canada, regulations are becoming stricter
55 for phosphorus (P), total suspended solids (TSS), and biochemical oxygen demand (BOD)
56 (Government of Canada, 2012). Performing upgrades to existing lagoons to improve
57 nutrient and emerging contaminant removal, the latter of which lagoons are not inherently
58 designed to mitigate (Fent et al., 2006), can be costly. As a result, research to develop
59 effective, low-cost, and low-maintenance polishing systems is vital for rural municipalities
60 seeking to meet regulatory expectations within financial constraints.
61 Free-flow surface wetlands are a popular tool to polish wastewaters of small
62 communities (Kadlec and Wallace, 2008), but these have drawbacks, especially in
63 climatically challenged regions, i.e., harsh winters, or drought conditions. While relatively
64 easy to construct, their contribution to removing wastewater contaminants beyond nutrients
65 and suspended solids can be limited. For example, lack of maintenance of the natural plant
66 assemblages and water flow in a surface wetland can restrict overall removal efficiency of
67 pharmaceutical contaminants (Anderson et al., 2013). While some select emerging

68 contaminants are removed in free-flow wetlands (Breitholtz et al., 2012; Dordio et al.,
69 2011), others such as ARGs may not be, possibly due to a lack of significant biomass
70 separation from the waste stream (Anderson et al., 2013). The limited research to date
71 suggests that aerobic environments promote growth of microbial consortia involved in
72 nutrient and micropollutant elimination in surface (Dordio et al., 2011) and sub-surface
73 flow wetlands (Avila et al., 2013).

74 A novel passive sub-surface filtration system was developed that can promote a
75 more efficient aerobic state for removing wastewater contaminants. A pilot-scale facility
76 was installed in 2009 for the Village of Dunnottar, Manitoba, Canada, near the shores of
77 Lake Winnipeg. This system was designed to polish lagoon wastewater effluent by
78 removing traditional wastewater contaminants (e.g., nutrients, coliforms), and serves as a
79 model for a planned full-scale system. One outstanding question of interest was whether
80 emerging wastewater contaminants common in sewage (e.g., ARGs and organic
81 micropollutants, such as pharmaceuticals and personal care products, and pesticides) could
82 be removed by the filters in conjunction with a traditional lagoon system, despite it (and
83 many other wastewater treatment systems) not being expressly designed to do so.
84 Furthermore, the potential, and extent of, reduction in observed toxicity by removing these
85 emerging contaminants needed to be assessed. To this end, water was collected regularly
86 throughout the treatment and discharge season (May-September) with the aim of
87 determining: 1) removal efficiency of the current lagoon system; 2) efficiency of each filter
88 configuration; and 3) possible toxicological impacts on receiving waters for traditional and
89 emerging wastewater contaminants.
90

## 2. Materials and Methods

### *2.1 Study location*

The wastewater facility used for this study is comprised of a primary and secondary lagoon system (Fig. 1) that provides treatment services for the Village of Dunnottar, a community in rural Manitoba. While the Village has fewer than 1000 permanent residents, summer use from cottagers, tourists, and other vacationers increases the population significantly relative to the winter season by up to several-fold. Municipal sewage from septic tanks at homes and cottages is transported by septic trucks to the primary lagoon during the active treatment season (~May until September). All valves are open between the primary and secondary lagoons, except for about three weeks before release when access to the secondary lagoon is closed and its water tested for regulatory compliance purposes.

An array (Fig. 2) containing four pilot-scale filter cells, each lined with an impermeable synthetic liner, was installed at the facility in 2009. Two of the filter cells (each 10 m long × 3.6 m wide × 1.2 m deep with a capacity of 44 $m^3$) were used in the current study to test their efficiency in removal of nutrients, organic contaminants, and organisms imparting antibiotic resistance from municipal wastewater. The filter beds are lined with PVC and clay, have natural local meadow plants on the surface growing within an organic soil layer (0.4 m depth), and an unsaturated sub-surface filter comprised of a combination of natural substrates (e.g. soils, gravel, rocks) and artificial matrices (i.e. proprietary materials from Dillon Consulting Ltd., who designed and constructed the filter). Water is pumped from the secondary lagoon through a transfer pipe, which splits into the

two filter systems ("north" and "south"). This water is added to the filter surface through a transverse perforated distribution pipe, and allowed to percolate through the solid substrates to the bottom of the filter into a collection pipe. Treated wastewater is collected at the end of the filter, where water from both filters is then directed back into a single outflow point, which flows into a shallow creek. Testing was performed at a relatively high flow vertical rate of ca. 0.5 m/d, resulting in an overall water residence time of 6 h within the filter. No other energy or chemical inputs are performed during treatment.

### *2.2 Sample collection*

The *in situ* conditions (e.g., temperature, pH, dissolved oxygen, redox, nutrients, BOD, TSS) in the secondary lagoon and filters were assessed by Dillon, as part of their routine monitoring, by established methods (APHA, 2005). For other analyses, water was sampled from seven locations around the study site: ~15 m away from the sewage delivery location in the primary lagoon ("primary lagoon"), entry point into the filters from the secondary lagoon ("secondary lagoon"), at the outflow from the filters ("north filter" and "south filter"), at the point where the treated water from the filters joined ("outflow"), 20 m downstream of the outflow ("creek"), further downstream in the creek towards the highway ("highway") (Fig. 1).

Sampling was conducted over the course of the licensed discharge season in 2013 on June 4 and 18, July 2, 16, and 30, Aug. 13 and 27, and Sept. 10 and 24. Grab samples for measurement of organic compounds, metals, and toxicity (as indicated by Microtox®) were collected as single samples at each time and location, except for a rotating triplicate (i.e. one location had triplicates each sampling day). Water for organics was sampled in 1 L

136 pre-ashed glass amber bottles, and for Microtox® and metals, in 50 mL sterile Falcon tubes
137 (pre-washed with 50% nitric acid for metals). Bottles were rinsed 3 times with sample
138 water before being filled to the top with no headspace, except for Microtox® where
139 headspace was left to allow for freezing at -20°C upon return to the laboratory. Both field
140 blanks and laboratory blanks were employed to ensure quality of the analyses for organic
141 compounds, metals, and Microtox® measurements.
142
143 ***2.3 Biofilm and water sample collection for ARGs***
144 For establishment of biofilms, samplers comprised of 600 grit sandpaper squares
145 (3.8 cm length) were tied to weighted fishing line and deployed at the lagoon bottom at
146 three locations: the secondary lagoon, the north filter, and the south filter, which were the
147 same locations where water samples were taken. The sandpaper was sterilized with ethanol
148 prior to deployment. Samplers were deployed on June 18 and sampled every 2 weeks either
149 one at a time or in triplicate. A second round of samplers was also deployed in the
150 secondary lagoon on July 16 and sampled on the same schedule as the first round.
151 Personnel wore gloves disinfected with 70% isopropanol while handling both ARGs
152 and biofilm samplers. Collected biofilms were placed in 15 mL sterile falcon tubes. Grab
153 samples of water for analysis of ARGs were collected in autoclaved 500 mL polyethylene
154 bottles on all sampling days from all sampling locations, with rotating triplicate sampling.
155 Bottles were rinsed 3 times with sample water before being filled to the top with no
156 headspace. Samples were kept on ice for transport to the laboratory, and then they were
157 filtered in a sterile environment. Filters and biofilm tubes were kept at -20°C until shipment
158 to the University of Strathclyde, Glasgow, UK, for analysis.

159

160 ***2.4 Determination of nutrient, pharmaceutical, and metal concentrations***

161 Following previously described methods (Carlson et al., 2013), grab samples for
162 pharmaceutical analyses were processed by solid phase extraction using Oasis HLB
163 (Waters, Milford MA). Ultra-high performance liquid chromatography-tandem mass
164 spectrometry (UHPLC/MS/MS) with isotope dilution was used to quantify chemicals of
165 interest in water samples, as described in previously published work (e.g., Anderson et al.,
166 2013; Cardinal et al., 2013; Carlson et al., 2013). These compounds included a suite of
167 thirty-nine commonly used pesticides and human or veterinary pharmaceuticals that are
168 commonly found in wastewaters (MacLeod and Wong, 2010; Anderson et al., 2013;
169 Carlson et al., 2013), including: estrogenic compounds (e.g., 17 $\alpha$ -ethinylestradiol); $\beta$ -
170 blockers (e.g., metoprolol); antidepressants (e.g., fluoxetine--Prozac); antibacterial agents
171 (e.g., triclosan), non-steroidal anti-inflammatory drugs (e.g., diclofenac); lipid regulators
172 (e.g., clofibric acid); and macrolide (e.g., clarithromycin) and sulfonamide (e.g.,
173 sulfamethazine) antibiotics.

174 Concentrations of nutrients were determined by ALS Environmental Laboratory
175 (Winnipeg, MB) using standard methods (APHA, 2005). Analysis of total dissolved metals
176 was performed using flame atomic absorption spectroscopy (flame AAS) for Fe, Mn, and
177 Zn with detection limits from 0.05-0.29 mg/L, or graphite furnace atomic absorption
178 spectroscopy (GFAAS) for Ni, Cr, and Cu (APHA, 2005) with detection limits from 0.05-
179 0.4 μg/L.

180

181 ***2.5 Quantifying abundances of bacterial genes***

182 Abundances of the ARGs were quantified in water and biofilm samples according to
183 methods described in detail by Cardinal et al. (2013) and based upon previously established
184 protocols (Knapp et al., 2010). The genes of interest were *sul*1, *sul*2, and *sul*3 for
185 sulfonamide resistance (Pei et al., 2006), and *tet*1, *tet*2, *tet*3, and *tet*4 for tetracycline
186 resistance (Ng et al., 2001). Additionally, genes related to nitrogen transformation were
187 quantified: *nir*K (Henry et al., 2004) and *nir*S (Throbäck et al., 2004) for denitrifying
188 bacteria and *amo*A for ammonia oxidation. 16S rRNA genes were quantified as a measure
189 of 'total bacteria'. DNA was extracted using MoBio PowerDNA extraction kits (Cambio,
190 Cambridge, UK) according to the manufacturer's instructions. Reaction efficiencies were
191 determined to be most efficient (83-107%, depending on assays) at 1:100 dilutions with
192 DNase-free water (Knapp et al., 2010), and all extracts were diluted accordingly.
193 Quantitative PCR was run on a BioRad iQ cycler (BioRad, Hercules, CA). Standards and
194 post-analytical melting curves were generated (Smith et al., 2004) to verify PCR reaction
195 efficiencies, quantify results, and check for the presence of PCR artifacts.
196
197 ***2.6 Toxicity assessment***

198 Sample toxicity was assessed using the Microtox® assay, which measures relative
199 bioluminescence of the marine bacterium, *Vibrio fischeri*, following exposure to test
200 mixtures. Samples collected for Microtox® analysis were analyzed according to adapted
201 standard protocols with recommended QA/QC on a Microbics M500 Analyzer
202 (Environment Canada, 1992). In brief, individual frozen samples (-20°C) were thawed at

203 4°C and the change in *Vibrio fischeri* bioluminescence was measured in triplicate at 100%
204 sample strength. This deviation from the standard protocol, which analyzes a serial dilution
205 of the test mixture and results in a generated $IC_{50}$ (Azur Environmental, 1995), was utilized
206 to allow for a time- and cost-effective screening of the large sample set under investigation.
207 All samples were pre-adjusted to optimal salinity for the microorganism and the response
208 was compared to control after 15 minutes of exposure as the mean percent of control
209 performance.
210
211 ***2.7 Statistical analyses***
212 Concentrations of nutrients and organic compounds, as well as abundance of ARGs,
213 were assessed using analysis of variance (ANOVA) followed by Tukey's test where log,
214 square root, or reciprocal-transformed data met the assumptions of normality and equal
215 variance. Normality and equality of variance were assessed by Shapiro-Wilk and Levene's
216 median tests, respectively, and non-normal data were analyzed by Kruskal-Wallis rank
217 tests. Data were analyzed using SigmaPlot 11.0 (San Jose, CA) and are presented as mean ±
218 standard deviation (SD) unless otherwise indicated. Differences were considered significant
219 at $p<0.05$.
220
221 **3. Results**
222 ***3.1 Water quality and nutrients***
223 Nutrients and selected water quality parameters (Table 1) were monitored on six
224 occasions in the secondary lagoon and at the confluence point of the outflow from the two
225 filters (Dillon Consulting Limited, 2014). Average influent pH was 8.8 and average effluent

226 pH was 7.8. Nitrate + nitrite was not detected in grab samples at any time (< 0.35 mg/L).
227 Post-filtration concentrations of ammonia ranged from 0.4 to 2.6 mg/L and concentrations
228 of TP ranged from 1 to 4.1 mg/L, representing mean respective reductions of 59% and 50%
229 compared to the secondary lagoon, except for the increase observed on July 16, 2013 for
230 ammonia. Total Kjeldahl nitrogen (TKN) was also reduced by 47% with passage through
231 the filter. Other improvements in water quality with passive filtration included reductions in
232 biochemical oxygen demand (BOD) (>25% mean reduction), chemical oxygen demand
233 (59%), total dissolved solids (TDS) (4%), total suspended solids (TSS) (62%), and fecal
234 coliforms (92%). There was no observed reduction in total coliforms from the secondary
235 lagoon to post-filtration between mid-June and mid-July (Table 1). However, after the end
236 of July, coliform counts were reduced by filtration by an average of 91% over the
237 remaining study period.
238
239 ***3.2 Pharmaceutical concentrations***
240 Nearly all of the thirty-nine target organic compounds were detected at least once in
241 the system, with measured concentrations in the ng/L range (Table S1). Atenolol,
242 diclofenac, ibuprofen, naproxen, and sulfamethazine were only detected in the primary
243 lagoon, while propranolol, metoprolol, triclosan, and trimethoprim were also occasionally
244 detected in the secondary lagoon. Most other compounds were detected sporadically with
245 no obvious temporal or spatial trends (Table S1). None of the target compounds were
246 consistently removed by passage through either of the filters.
247 Concentrations of atrazine, a corn herbicide, decreased significantly over time at all
248 sites except the primary lagoon and highway (Fig. 3A, $p<0.05$). Concentrations of

249 carbamazepine, an anticonvulsant, were relatively consistent across all sites, with no
250 significant changes over time at any site (Fig. 3B, $p>0.05$). The antibiotic clarithromycin
251 was detected in the two filters and outflow site, as well as inconsistently in the primary
252 lagoon, but there was no obvious trend in concentration over time or location (Fig. 3C,
253 $p>0.05$). In the case of gemfibrozil, a lipid-regulator, significant removal was observed
254 between the primary and secondary lagoons (Fig. 3D, $p<0.05$). In addition, a significant
255 increase in concentration was observed over time in the primary lagoon ($p<0.05$),
256 suggesting increased inputs over the season. For the antibiotic sulfamethoxazole, the
257 greatest reduction in concentration occurred between the primary and secondary lagoons
258 (Fig. 3E, $p<0.01$). While there was some evidence of removal by the filters, changes in
259 concentrations of sulfamethoxazole were not significant between the secondary lagoon and
260 the filters. Finally, sulfapyridine was detected in the primary lagoon at every sampling time
261 but concentrations were significantly lower in the secondary lagoon (Fig. 3F, $p<0.05$) and
262 other sites (when detections occurred).
263
264 ***3.3 Metal concentrations***
265 All six of the metals detected in an initial screening of the primary lagoon (Cr, Cu,
266 Fe, Mn, Ni, and Zn) were also detected in at least one sample from each of the other
267 sampling locations (Fig. 4). Concentration ranges were as follows: Cr – 0.18 to 2.1 μg/L;
268 Cu – 0.05 to 3.9 μg/L; Fe – 0.3 to 1.6 mg/L; Mn – 0.05 to 1.0 mg/L; Ni – 2.3 to 3.8 μg/L;
269 and Zn – 0.08 to 0.3 mg/L. There was no evidence for targeted removal of metals by the

filters, and the small number of samples (n=1-3) collected during each sampling event precluded statistical comparisons over time at individual sites.

### *3.4 Abundances of ARGs*

Measured abundances of 16S rRNA genes, representing "total" bacterial populations, in water samples were greatest in the primary lagoon ($10^{7.3}$ gene copies/mL). Bacterial gene abundance was reduced by 80% in the secondary lagoon (to $10^{6.9}$ copies/mL) and by 89% when compared to the outfall (Table 2). Concentrations in the filtration units were slightly lower on average than the outflow, but differences were not statistically significant ($p>0.05$).

Individual genes, or clusters of genes, were analyzed and the results were summed (Table S2) according to resistance types (i.e., sulfonamide or tetracycline) to facilitate assessment of resistance patterns. Of the ARGs harvested from the water samples, the greatest abundances of $tet^R$ (sum of tetracycline resistance genes) were found in the secondary lagoon. These abundances were nearly 50% higher than in samples from the primary lagoon and significantly greater than in samples from downstream "natural" areas (i.e., "creek" and "highway" locations) ($p<0.05$). However, concentrations were reduced by 58% by the outfall from the secondary lagoon. Abundances of $sul^R$ (sum of sulfonamide resistance genes) were greatest in the primary lagoon ($p<0.001$). These genes immediately declined in abundance (by 99%) in the secondary lagoon effluent, and levels remained constant through the remainder of the treatment process ($p>0.05$). Among the three sulfonamide gene determinants measured, *sul*2 was most prevalent. Tetracycline gene clusters tended to be more evenly distributed among the different gene determinants.

293 To facilitate further analysis and account for differences in prevalence of bacteria
294 throughout the treatment process, abundances of genes were divided by the abundance of
295 16S rRNA genes to represent relative gene abundances. Greater proportions of resistant
296 bacteria were found in the filtration units, although the primary lagoon also had elevated
297 $sul^R$ (0.8%). In addition, the filter units had more than twice higher relative abundances of
298 $sul^R$/16S (0.22-0.24%) than the outflow (0.10%). $Tet^R$/16S values averaged 0.28% and
299 0.42% in north and south filters, respectively, while all other treatments had relative gene
300 abundances of $tet^R$ less than 0.12%. These findings suggest a greater potential for ARG-
301 bearing bacteria to exist in the primary lagoon and within the filters.
302 Biofilms were also sampled in the secondary lagoon and the two filter units.
303 Abundances of 16S rRNA genes (i.e., total bacteria) averaged between $10^{6.8}$ and $10^{7.3}$
304 gene/$cm^2$, with no significant differences among sites ($p>0.05$) (Table 2, Table S2). Similar
305 abundances of ARGs were found in biofilms collected from the secondary lagoon and north
306 filter unit ($tet^R$/16S rRNA genes ranged from 0.3-0.8%, and $sul^R$/16S rRNA genes
307 represented 0.26-0.45%), with the south filter having significantly fewer resistant genes for
308 both ARG types (approximately 0.01% of 16S rRNA genes; $p<0.01$).
309
310 ***3.5 Abundances of denitrification and nitrification genes***
311 In addition to ARGs, three genes related to nitrogen cycling processes in wastewater
312 treatment were also quantified: *nirK*, *nirS,* and *amoA* (Table 2). The *nir* genes encode for
313 nitrite-reductases, enzymes responsible for the conversion of nitrite to nitric oxide within
314 the denitrification pathway. The enzyme *nirS* is a non-haeme iron-containing enzyme, and

315 *nirK* contains copper. A subunit of ammonia monooxyenase (*amoA*), which is required for
316 the first step in nitrification, is found in lithoautotrophic ammonia oxidizers.
317 Relative abundances of nitrite reductase genes (both *nirS* and *nirK*) ranged from ~1
318 to 22% in the water, and ~4 to 31% in the biofilms. Abundances of *nirS* were often 1-3
319 orders of magnitude greater than *nirK*; as such, it represents the dominant denitrifying gene
320 in the community. Relative abundances of denitrifying populations were generally greater
321 in the filter units for both the biofilm (log-transformed ANOVA, $p<0.05$) and the water
322 ($p<0.001$). Relative abundances of ammonia oxidizing bacteria were also greater in close
323 proximity to the filters (~3-6% of "total bacteria", versus <1% elsewhere). The values were
324 significantly higher for the community in the water ($p<0.05$), but not quite significant for
325 biomass ($p=0.127$).
326
327 ***3.6 Toxicity of wastewater towards bacteria***
328 With the exception of the primary lagoon and creek samples, the average
329 bioluminescence of *Vibrio fischeri*, represented as percent of control, was greater than 90%
330 (Table 3, Table S3). In the primary lagoon, *V. fischeri* bioluminescence was generally
331 about 50% of the control response. After water had been treated in the secondary lagoon
332 and moved into the north and south filters, responses were ≥90% of control, indicating
333 recovery and conditions suitable to the promotion of bacterial growth. The notable
334 exception to this trend was the creek sample which elicited *V. fischeri* bioluminescence that
335 was ≈42% of control. As a point of reference, water from Lake Winnipeg ("lake blank"
336 sample, Table S3) elicited a response that was ≈95% of controls.
337

## 4. Discussion

### *4.1 Water quality and nutrients*

Overall, the passive filtration system achieved some degree of nutrient removal and improved the water quality of effluent from the lagoon system. Removal efficiencies in 2013 were on par with that observed in prior years (e.g., at least 50-75% above existing lagoon treatment) for BOD, nitrogen, phosphorus, and TSS (Village of Dunnottar, 2012). Concentrations of ammonia and TP in the final effluent (Table 1) were generally within discharge water quality guidelines, as were pH and TSS (6.5 to 9.0 and 25 mg/L, respectively) (CCME, 2011). It should be noted, however, that samples from the secondary lagoon typically met or exceeded the available guidelines already for Water Quality for the Protection of Aquatic Life (CCME, 2011), as could be expected from an operational wastewater lagoon (Federation of Canadian Municipalities, 2004). Therefore, the system was providing sufficient nutrient removal without the additional filter, but use of filtration further improves the effluent quality entering the environment.

In terms of coliforms, while fecal coliforms were consistently removed by filtration, there was a trend of increased total coliforms with filtration during the first half of the season and decreased total coliforms during the second part of the season. In the secondary lagoon, there was a considerable spike in total coliforms in July and August (counts of 15,000, 110,000, and 9,300 per L vs. 210-750 per L earlier in the season, Table 1). These counts were reduced to 430-2,300 per L with filtration, while the increases with filtration earlier in the season were to 930-4,300 per L, so final effluents were generally fairly consistent in their total coliform contents across the sampling season. The guidelines for fecal and total coliforms outlined on Manitoba Conservation's wastewater license for the

361 facility were set at 200 and 1,500 per 100 mL of sample, respectively. Fecal and total
362 coliform counts in effluent from the filtration system were below these guideline values, as
363 were nearly all counts in the secondary lagoon, which would be expected for a well-
364 operated lagoon system (Federation of Canadian Municipalities, 2004; US EPA, 2002).
365
366 ***4.2 Pharmaceutical detection in, and removal from, wastewater***
367 The concentrations of pharmaceuticals measured in grab samples of receiving
368 waters from the Dunnottar system were generally consistent with those from other
369 wastewater systems in Manitoba (Table 4) (Anderson et al., 2013; Carlson et al., 2013) and
370 elsewhere (Conkle et al., 2008; Kolpin et al., 2002; MacLeod and Wong, 2010). Many
371 detectable compounds had highest concentrations in the primary lagoon and were not
372 detected in the creek or highway sites. Exceptions were atrazine, carbamazepine,
373 gemfibrozil, and sulfamethoxazole, which tended to persist throughout the treatment
374 process and were released in the effluent, though concentrations had been reduced from
375 those measured in the primary lagoon.
376 Based on hazard quotients (HQs) calculated in previous studies (Anderson et al.,
377 2013; Carlson et al., 2013), none of the compounds detected in the outfall or downstream of
378 the effluent discharge point would pose a significant hazard for macrophytes, aquatic
379 invertebrates, or fish. Calculated HQs ranged from 0.01 to 2.4 in the worst-case scenario of
380 the primary lagoon, with both sulfamethoxazole and gemfibrozil exceeding the threshold of
381 1 (HQs of 2.4 and 1.2, respectively). However, the greatest concentrations of
382 sulfamethoxazole and gemfibrozil measured in the outflow, creek, or highway sites,
383 calculated with the toxicity value of the most sensitive aquatic species yielded HQs of 0.78

384 and 0.12, respectively. This observation suggests that concentrations of these
385 pharmaceuticals are sufficiently low enough in effluent from the wastewater system that
386 they would not be expected to pose a hazard to aquatic life in receiving waters. It should be
387 noted that current HQs are based primarily on acute toxicity endpoints, so it is unknown if
388 concentrations observed in this study play a role for subchronic endpoints e.g., disruption
389 of Na/K-ATPase activity, as observed in fish with ng/g levels of fluoxetine (Lajeunesse et
390 al., 2011).

391 The widespread detection of atrazine across sites at the low levels quantified was
392 consistent with its use in the region and perhaps disposal into collected wastewater. This
393 trend was also observed for atrazine in the Dead Horse Creek system (Carlson et al., 2013),
394 which receives treated wastewater from several rural communities and ultimately flows to
395 Lake Winnipeg. The observed persistence of carbamazepine over time is consistent with
396 steady use patterns and a relatively recalcitrant compound in the environment (Conkle et
397 al., 2008; Hai et al., 2011). A decline in the concentration of carbamazepine in the primary
398 lagoon was reported at the end of the study, likely a result of reduced inputs as cottages
399 were closed down and temporary residents were no longer contributing to the sewage
400 lagoon. In contrast, there was an increase in the concentration of gemfibrozil in the primary
401 lagoon over time. However, there was also a distinct decline at the very end of the study,
402 which may again be due to a declining population of cottagers at the end of the season.
403 Much of the gemfibrozil present in the primary lagoon dissipated before water entered the
404 secondary lagoon, which is consistent with previously observed dissipation in aeration
405 basins (Conkle et al., 2008).

406 Concentrations of the sulfonamide antibiotics sulfamethoxazole and sulfapyridine
407 declined in the primary lagoon over time, which may be due to increased photodegradation
408 (Ryan et al., 2011) as light intensity and duration of daylight in the summer months.
409 Similar reductions in concentrations of these antibiotics have been reported in primary
410 aeration basins (Conkle et al., 2008) and a model surface constructed wetland (Anderson et
411 al., 2013).
412 Because of the large and variable transient cottager population, whose wastewater
413 inputs to the facility are ill-defined, it is difficult to determine if treated wastewater
414 concentrations correlated to per-capita use and loading of organic micropollutants, as
415 shown at other sewage lagoons in Canada (MacLeod and Wong, 2010). Further
416 complicating any such correlation is the fact that unlike lagoon systems receiving inputs by
417 municipal sewage collection pipes (MacLeod and Wong, 2010; Carlson et al., 2013), most
418 wastewater inputs to the Dunnottar system come from septic systems, in which residence
419 time of wastewaters and degradation of micropollutants is unknown and likely quite
420 variable (Anderson et al., 2013).
421
422 ***4.3 Metals***
423 Iron and zinc were present within the system at concentrations surpassing their
424 respective guidelines (0.3 and 0.03 mg/L, respectively) for the protection of aquatic life
425 (CCME, 2011), while Cu and Cr may have exceeded guideline values depending on their
426 speciation (2 μg/L for Cu, depending on hardness, and 8.9 μg/L for Cr). Concentrations of
427 Ni were below guideline values (minimum value 25 μg/L depending on hardness) and there

428 is not currently a water quality guideline for Mn. Concentrations of metals tended to be
429 quite variable, both over time and between sampling locations within the system. The filters
430 did not significantly affect metals, but this trend cannot be further explained without
431 additional knowledge of the proprietary materials within the filters themselves.
432 There are no heavy industries and no indication of man-made pollution in the area to
433 contribute to the load of metals in the water treatment system. The concentrations of metals
434 found are likely consistent with natural levels in this part of Manitoba.
435
436 ***4.4 Removal of ARGs***
437 Abundances of sulfonamide and tetracycline resistance genes in the Dunnottar
438 lagoon system were consistent with those measured in a nearby lagoon and constructed
439 wetland wastewater treatment system located in Grand Marais, Manitoba (Anderson et al.,
440 2013). In our study system, there was an overall reduction of ARG-harbouring bacteria (in
441 terms of absolute abundances) for downstream areas, especially in terms of *sul*-resistance,
442 which declined by two-orders of magnitude. Removal of total bacteria by wastewater
443 lagoons under summer operating conditions has been demonstrated in other systems (e.g.,
444 Mezrioui and Baleux, 1994), including one serving Grand Forks, North Dakota (Walter and
445 Vennes, 1985), which ultimately feeds into Lake Winnipeg.
446 Comparing conditions between the outflow and secondary lagoon, there was a 75%
447 reduction of total bacteria, as measured by 16S-rRNA gene abundances, in water passing
448 through the subsurface filters; however, there were variable effects on abundances of
449 antimicrobial resistant organisms. While total $tet^R$ declined ($T_{10} = 4.08$, $p < 0.01$), total $sul^R$
450 remained similar ($T_{10} = 0.30$, $p = 0.77$). Following the 99% reduction between the two

451 lagoons, $sul^R$ concentrations through the subsurface filters likely represent background
452 abundances, with further removal being unlikely. Unfortunately, wastewater systems have a
453 highly variable ability to reduce antimicrobial resistance (e.g., Mezrioui and Baleaux,
454 1994). For example, Christgen et al. (2015) inversely found high rates of $tet^R$ decline, but
455 minimal $sul^R$, in anaerobic-aerobic sequencing reactors. .Generally, resistant bacteria
456 numbers decline in wastewater treatment as bacteria are removed; but patterns require
457 further investigations, as it remains a function of bacterial community, operating conditions
458 and bioreactor design (e.g, Christgen et al. 2015).

459 Baquero and Canto (2008) refer to wastewater and its biological components as one
460 of four genetic reactors in the development of antibiotic resistance. Wastewater treatment
461 plants stabilize waste materials and reduce overall bacterial load discharged to receiving
462 waters, but evidence suggests that resistance rates (ratio of resistant bacteria to total
463 bacteria) may be amplified in effluent (Czekalslo et al., 2012; Lachmayr et al., 2009;
464 Martinez and Baquero, 2000). While fewer bacteria were entering the environment at the
465 outflow of our study system, a greater proportion was found to carry genes for tetracycline
466 or sulfonamide (or both), which corroborates concerns from many other wastewater
467 treatment systems (Czekalslo et al., 2012; Lachmayr et al., 2009; Martinez and Baquero,
468 2000).

469 In removing bacteria from this system, there was an accumulation of genes in the
470 filter systems and formation of biofilms, especially in the north filter. Wastewater
471 treatments provide optimal conditions for development and dissemination of ARGs via
472 horizontal genetic processes in dense microbial communities (Schlüter et al., 2007) and
473 continuous exposure to chemical stressors (e.g., pharmaceutics, metals, and detergents).

474 Harbouring of resistant bacteria into peripheral biofilms has been observed previously
475 (Engemann et al., 2008; Zhang et al. 2009). The cause for gene-density differences between
476 filters remains unknown, but could be attributable to conditions such as biofilm age and
477 bacterial composition (Patel, 2005). However, the removal and disposal of accumulated
478 biomass material could help alleviate the risk of downstream movement of ARGs (Pruden
479 et al., 2013). As such, the technology has some promise of reducing loading of ARGs to the
480 environment with proper operational management.
481
482 ***4.5 Maintenance of nitrogen-transforming bacteria***
483 In the current study, substrates for harvesting biofilm samples were inserted at the
484 start of the filtration operations, and the first samples were collected two weeks later. While
485 it requires time for the biofilm communities to establish, the population of microorganisms
486 (based on gene abundances) appeared to have stabilized by July 20 (Fig. S1). Relative
487 abundances of *nirS*, *nirK*, and *amoA* genes were consistent with other studies involving
488 aerobic wastewater treatment systems (You, 2005; Limpiyakorn et al., 2011; Chom et al.,
489 2011).
490 Many wastewater treatment processes rely on the retention of high densities of
491 bacteria in biofilms to reduce the concentrations of dissolved organic matter and nutrients.
492 Further, floc- or biofilm-attached growth micoorganisms allow slow-growth populations to
493 be retained in the system and avoid wash-out conditions, especially under low HRT such as
494 the subsurface treatment system (HRT = 6 hr). This is often the case for the ammonia
495 oxidizing bacteria, which commonly occur floc- or biofilm-attached in freshwater and
496 wastewater systems (generation time ~17 hrs; Koops et al. 2006). Further, biofilms create

497 micro-environmental gradients, such as dissolved oxygen, which may enhance the
498 performance of bacteria. Diffusional limitations of dissolved oxygen often exist within the
499 biofilms (e.g., Costerton et al., 1994). Communities of ammonia oxidizing bacteria, which
500 produce nitrite as a metabolic by-product, locate themselves in aerobic zones (near root
501 zones). In areas of reduced oxygen, either within biofilms (Münch et al., 1996) or within
502 the soil matrix (Brix, 1987), the oxidized nitrogen by-products (nitrate and nitrite) can be
503 reduced by denitrifying bacteria to $N_2$. However, limited nitrite and nitrate concentrations
504 in the effluent suggest poor nitrification, and the presence of genes does not guarantee
505 biochemical activity, but does suggest a developing readiness for the system. Whether
506 caused by simultaneous nitrification-denitrification process (e.g., Yoo et al., 1999),
507 adsorption of ammonia to particles (e.g., Brix, 1987), or the assimilatory nitrogen reactions,
508 ammonia levels are effectively reduced with minimal nitrite and nitrate accumulation.
509
510 ***4.6 Toxicity of wastewater towards bacteria***
511 Represented as *V. fischeri* bioluminescence in test samples relative to controls, the
512 input water in the primary lagoon elicited the greatest toxic response with an average of
513 ~50% bioluminscence (Table 3, Table S3). Inhibition of bacterial luminescence using the
514 Microtox® assay has been reported at levels between 15 and 100% in raw wastewaters
515 entering wastewater treatment facilities (Katsoyiannis and Samara, 2007 and references
516 therein). Therefore, the inhibition observed in the primary lagoon of the Dunnottar system
517 is expected and is moderate. All other sample sites, with the exception of the creek, elicited
518 >90% bioluminescence from the exposed bacteria, indicating effective water treatment.
519 Attenuation of toxicity within the secondary lagoon is also consistent with trends observed

in the secondary sedimentation stage of a sewage treatment plant in Greece (Katsoyiannis and Samara, 2007). The elevated toxicity in the creek sample (average bioluminescence of 42% of control) was an unexpected result given the greater levels of luminescence observed in the secondary lagoon, north and south filter, outflow, and highway samples, in addition to the fact that chemical analyses of this sample did not indicate elevated levels of any of the target compounds relative to the remainder of the sample set. As such, the observed toxicity in the creek sample is not likely due to inefficient treatment by the Dunnottar facility, but warrants further investigation.

## 5. Conclusions

The subsurface filters were effective at removing nutrients, but residence time under the current operational conditions was likely insufficient to provide effective removal of pharmaceuticals. The majority of removal of pharmaceuticals from the wastewater typically occurred in the primary lagoon, so the standard lagoon features without the additional filters do have the ability to remove chemical micropollutants to some degree. As well, the presence of the filters did not have a detrimental effect on concentrations of pharmaceuticals. In general, the Dunnottar wastewater treatment lagoon system removed bacteria well, in addition to reducing acute toxicity as characterized via the Microtox® assay. The filters promoted growth of desirable bacteria (i.e., denitrifying and nitrifying bacteria) and significantly reduced the abundances of antibiotic resistances genes. However, in removing the ARGs from wastewater, the filters do harbor these genes, which will affect the way in which filters must be cleaned and ultimately disposed of once they

542 reach their life expectancy. Overall, the filters were effective at removing nutrients and
543 certain ARGs from rural wastewater and are worth exploring further. Additional
544 optimization of operating conditions may result in improved removal of pharmaceutical
545 compounds as well and will be investigated as part of a full-scale installation in the near
546 future.
547
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**Figure captions**

Fig. 1: Map of study site and its relative position within the province of Manitoba, Canada. Sampling was performed at the primary lagoon, secondary lagoon, north filter, south filter, outflow, creek, and highway (main road to the north of the site). North and south filter sampling sites are located on east side of filters (see Fig. 2), but are depicted here for clarity on west side of filter.

Fig. 2: Schematic of pilot-scale filter (not to scale). Wastewater flow paths indicated by grey arrows.

Fig. 3: Concentrations of (A) atrazine, (B) carbamazepine, (C) clarithromycin, (D) gemfibrozil, (E) sulfamethoxazole, and (F) sulfapyridine at sampling sites in the lagoons, filter, and discharge stream over summer and fall 2013. Wastewater in the secondary lagoon, filter, and creek were not available on September 24, 2013.

Fig. 4: Box plot of metal concentrations in the primary and secondary lagoons. Centerline is median concentrations, top and bottom of boxes are 25th and 75th percentiles respectively, and top and bottom whiskers are 5th and 95th percentiles respectively.

**Figure 1**
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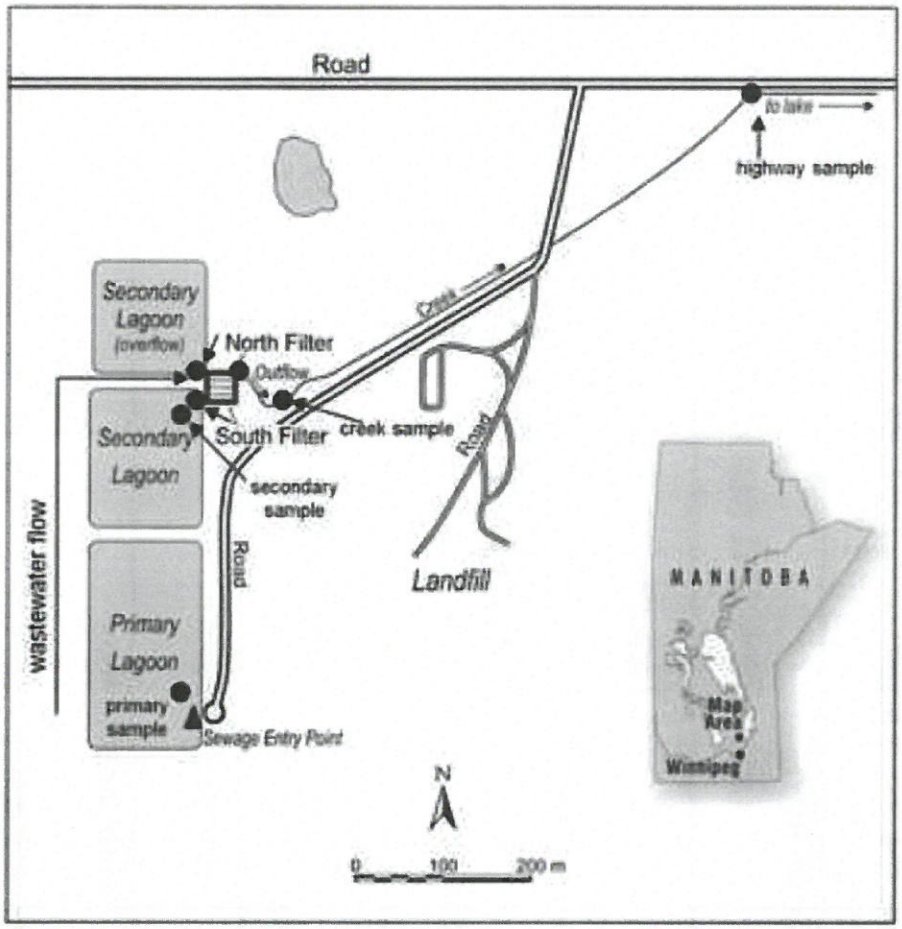

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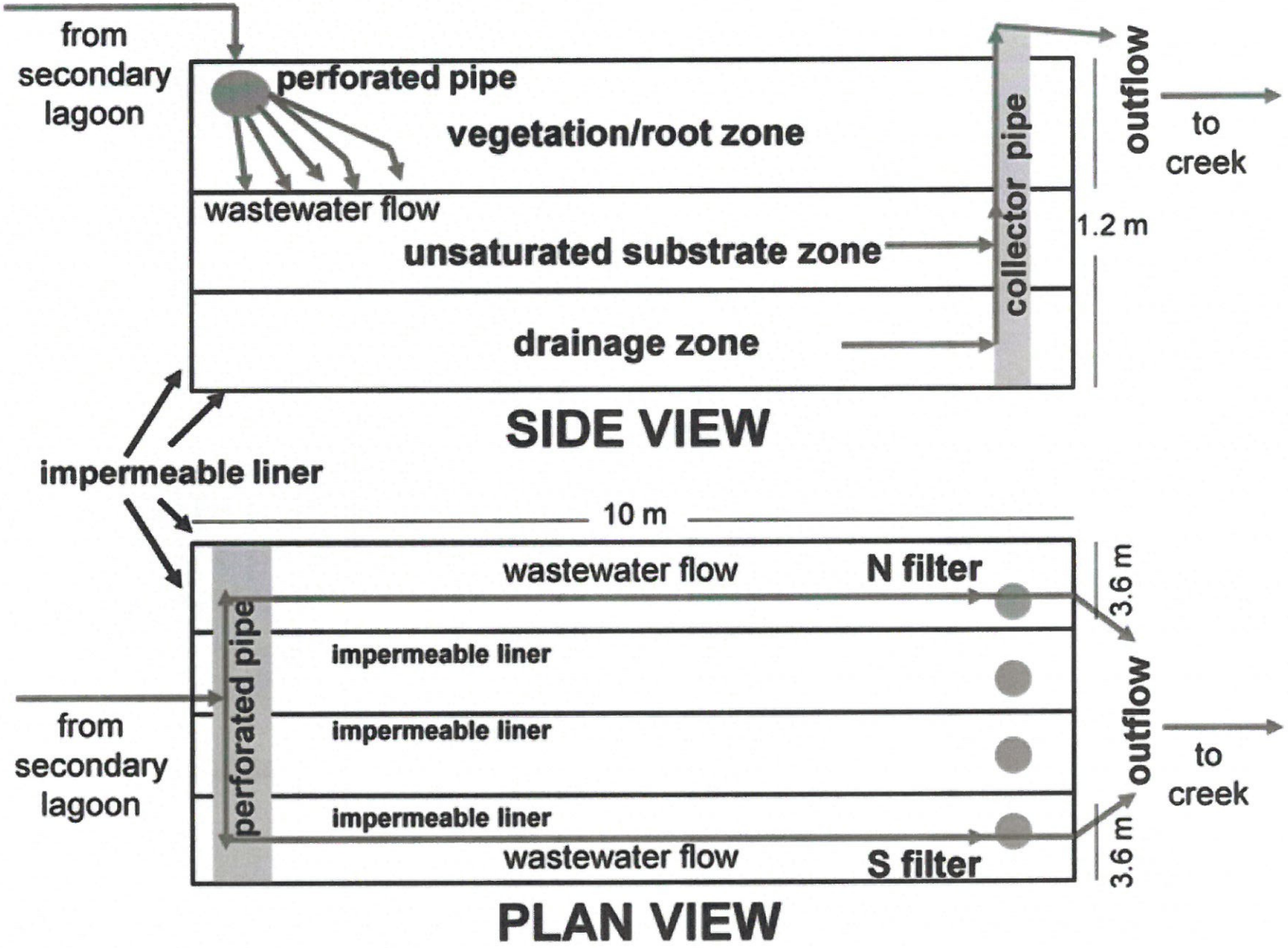

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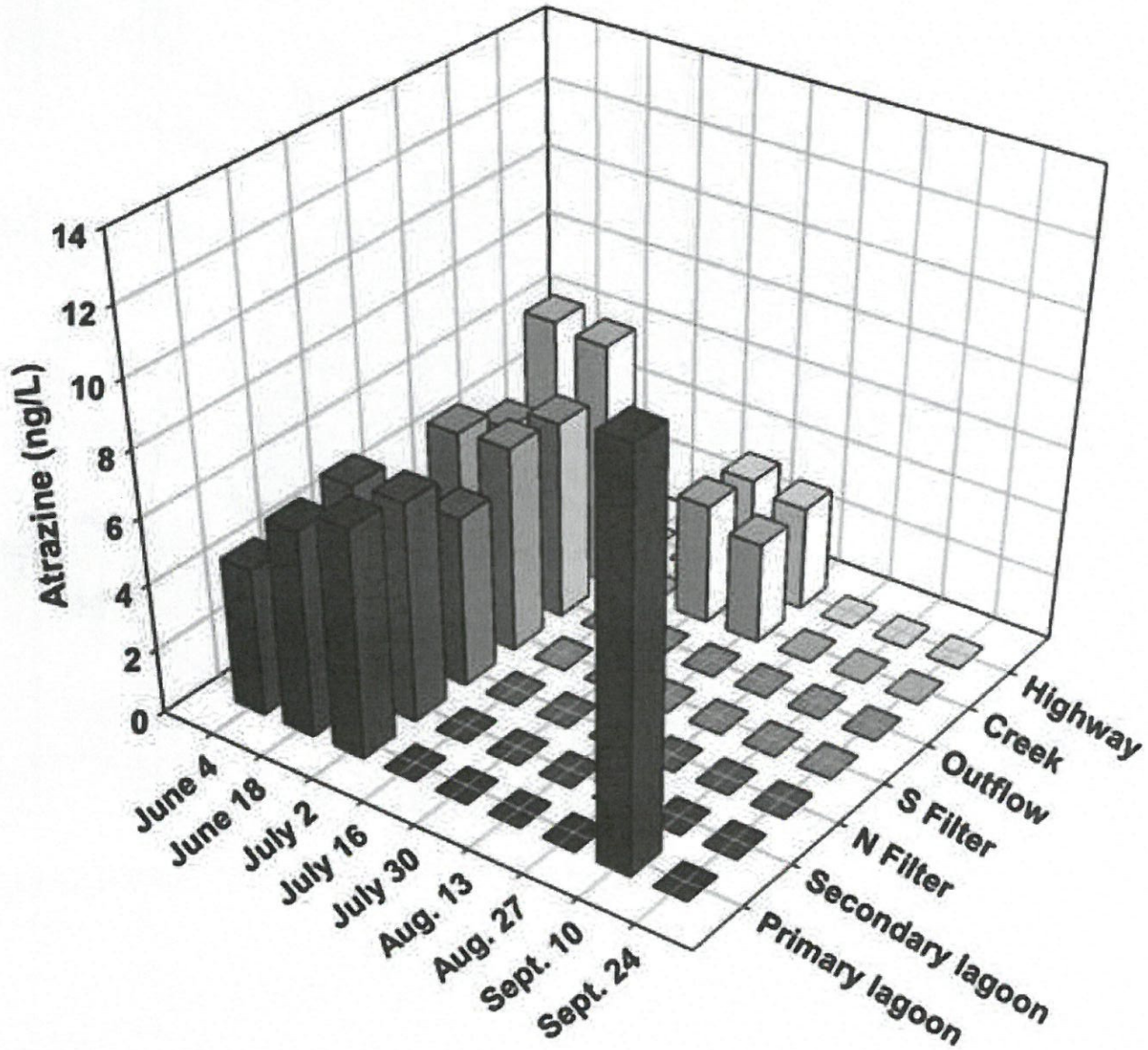

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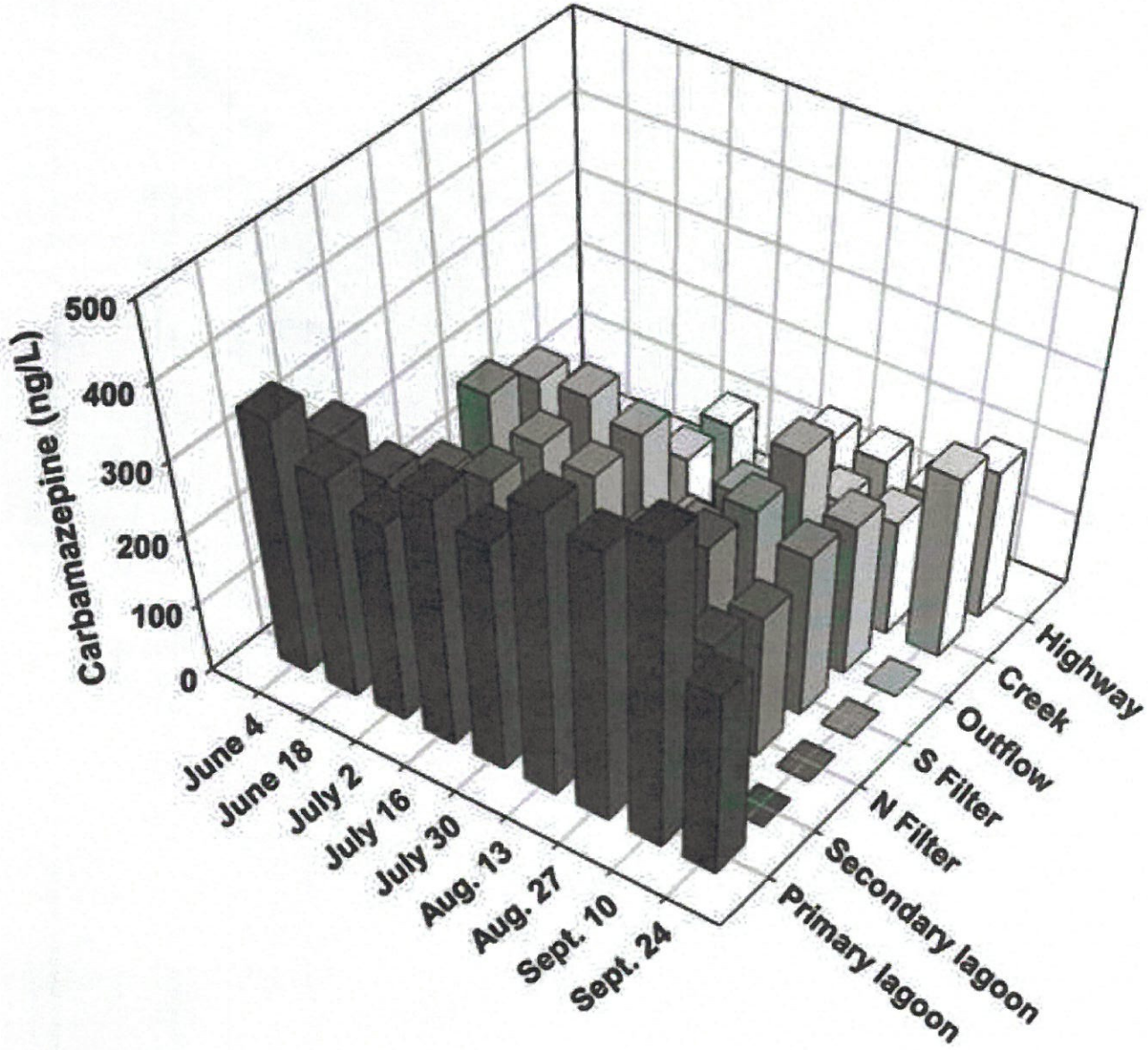

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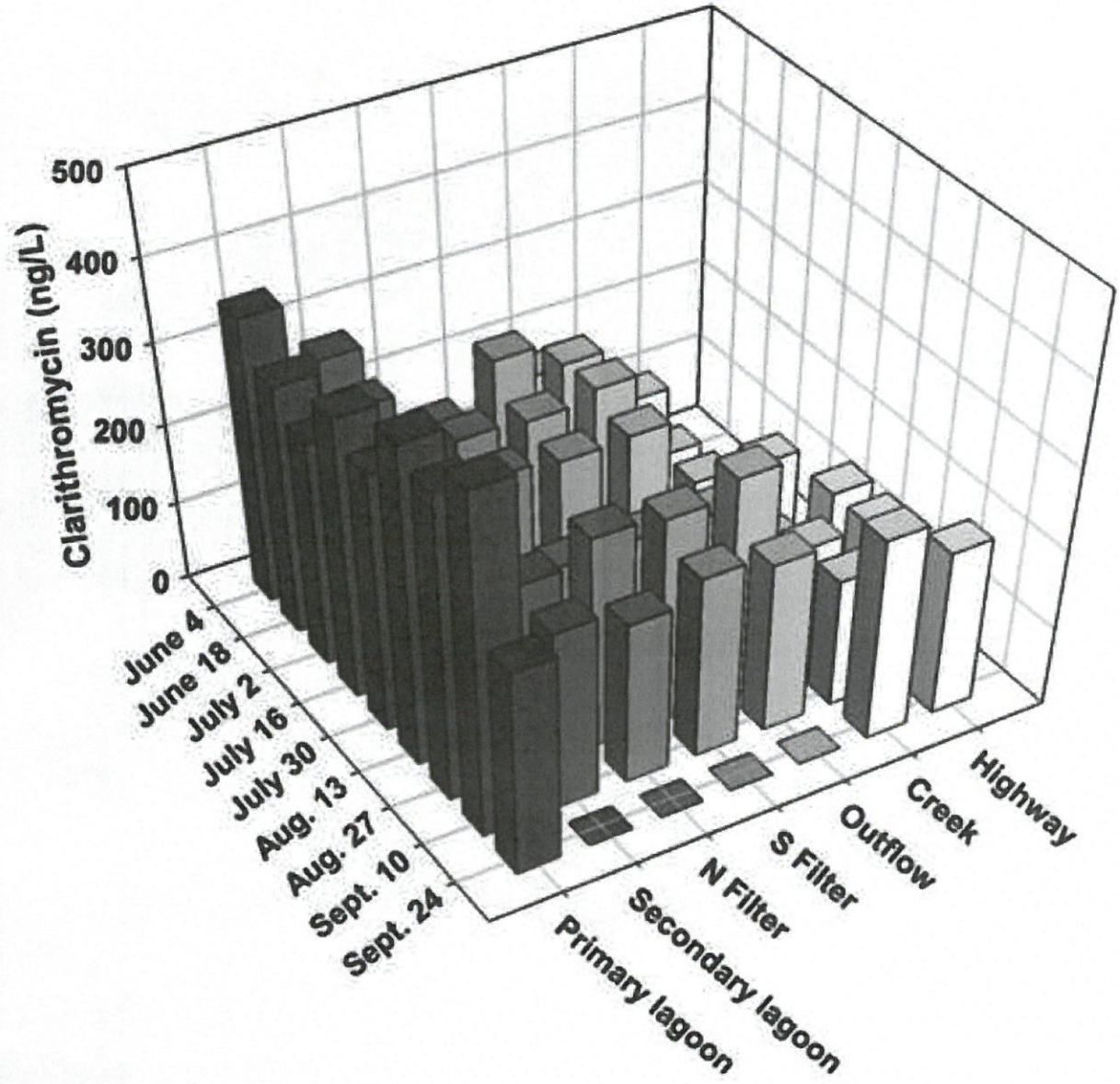

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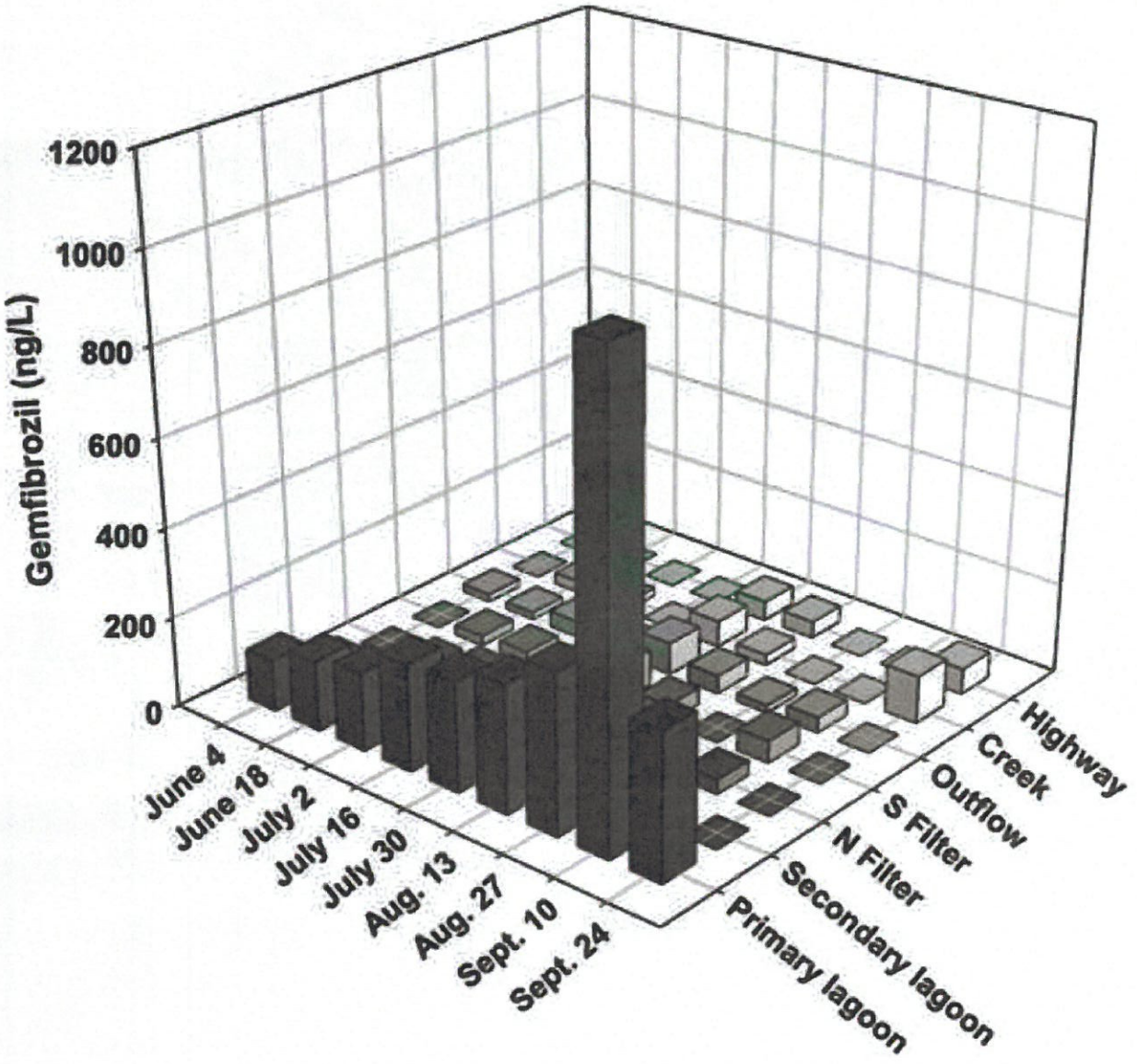

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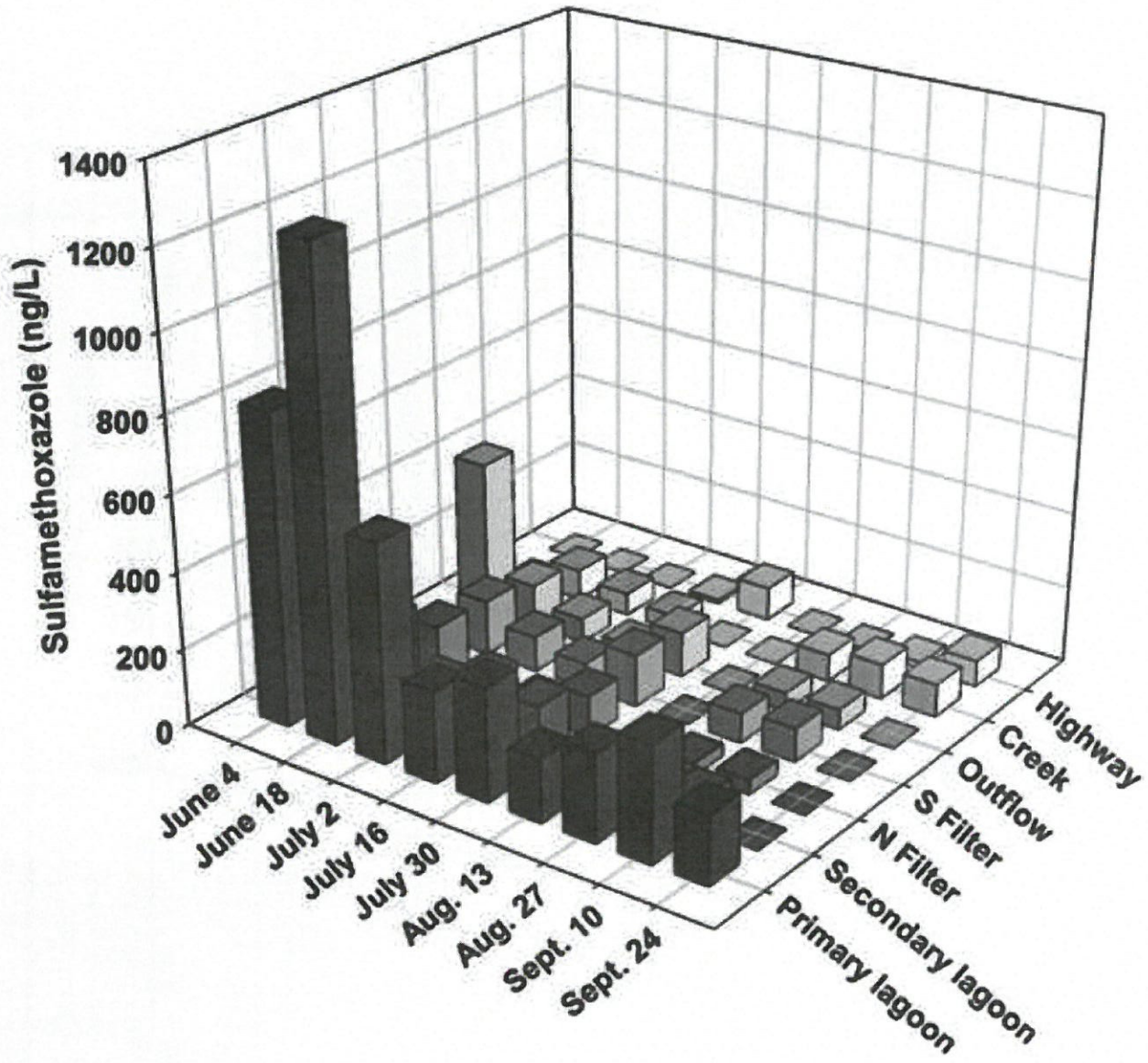

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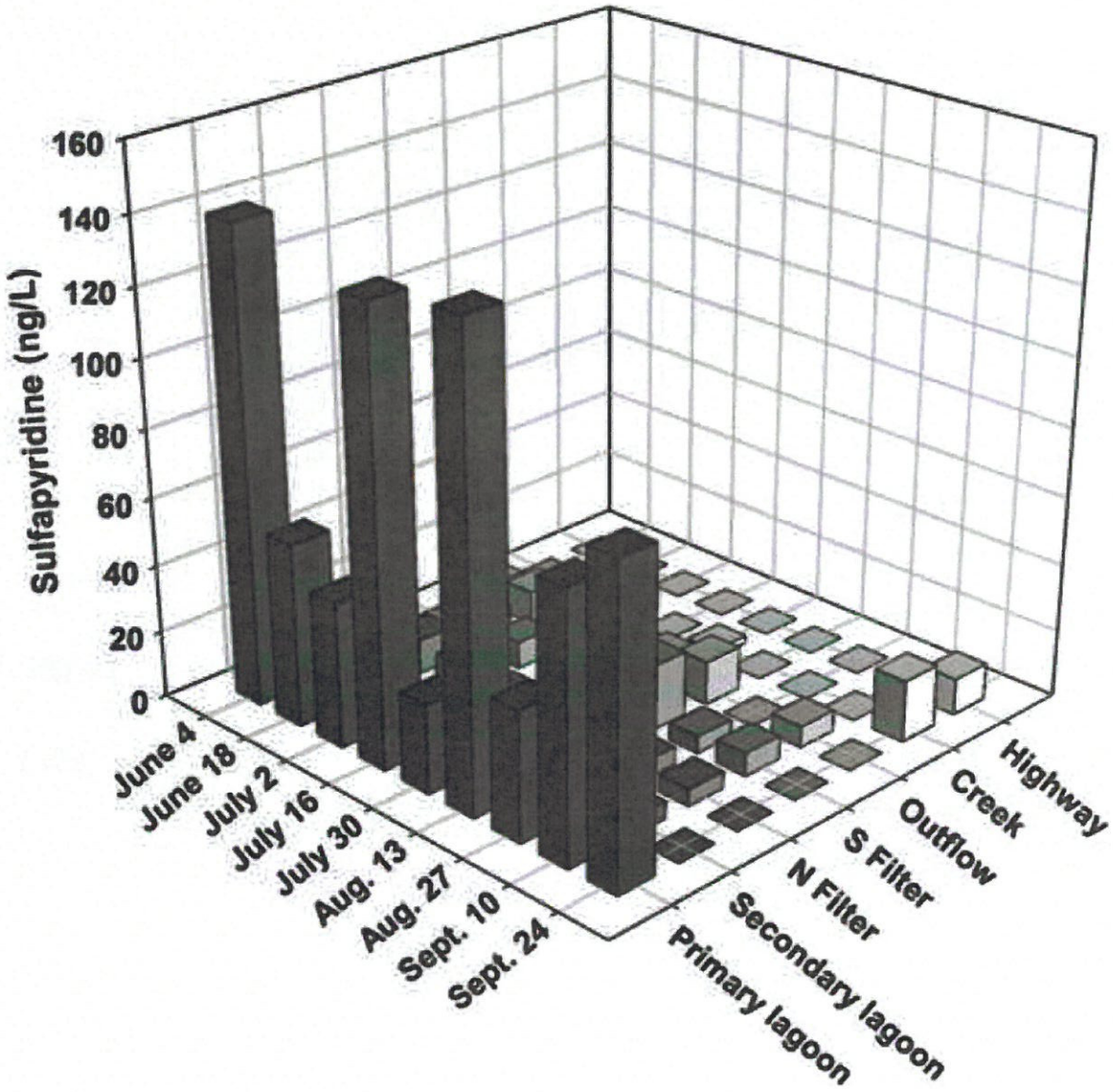

**Figure 4**
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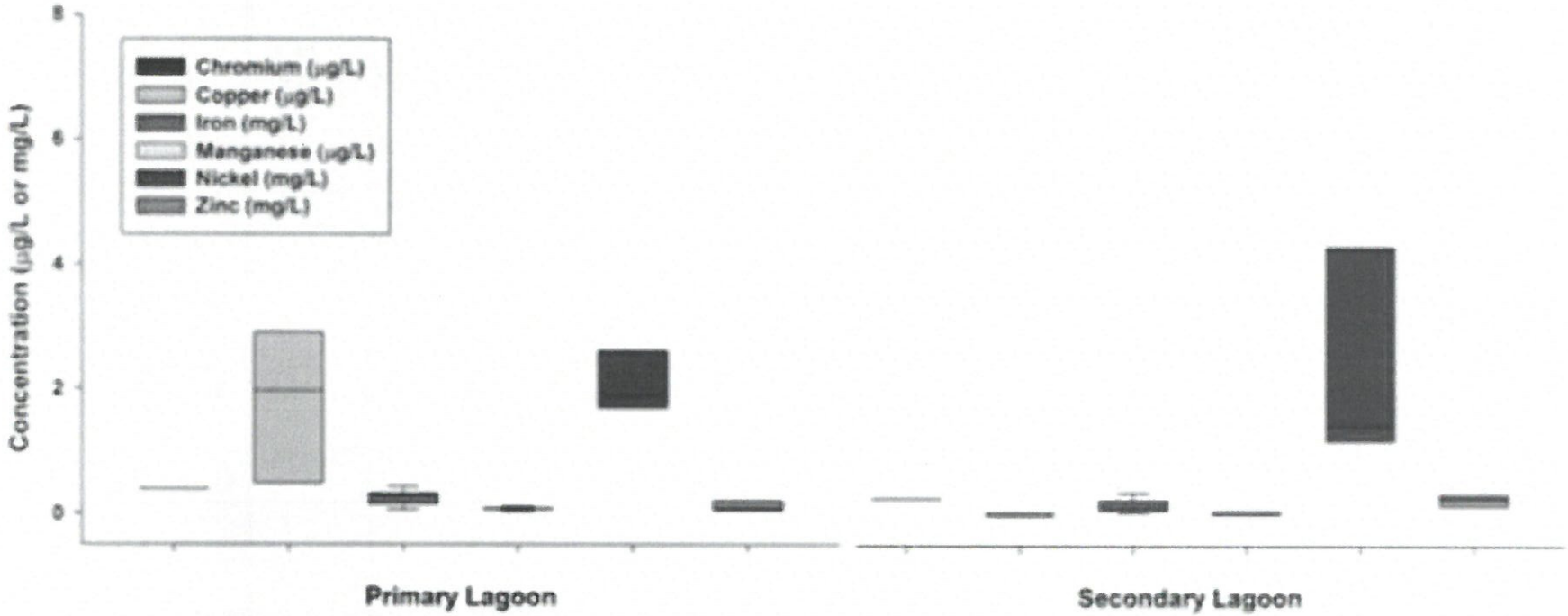

**Table 1**

Table 1. Levels of nutrients and other traditional wastewater contaminants in the secondary lagoon ("pre-filter" input water) and outflow of both subsurface filters ("post-filter" output water) of the passive filter at Dunnottar, Manitoba. BOD = biochemical oxygen demand, COD = chemical oxygen demand, TDS = Total dissolved solids, TKN = total Kjeldahl nitrogen; TSS = total suspended solids. Units are mg/L for all except coliforms (counts/L). Data from Dillon Consulting Ltd. (2014).

| **Parameters** | **June 18, 2013** | | **July 2, 2013** | | **July 16, 2013** | | **July 30, 2013** | | **August 13, 2013** | | **August 27, 2013** | | **% reduction pre- to post-filter** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **[mg/L] or counts/L** | Pre-filter | Post-filter | Pre-filter | Post-filter | Pre-filter | Post-filter | Pre-filter | Post-filter | Pre-filter | Post-filter | Pre-filter | Post-filter | range | average |
| $NO_2 + NO_3$ | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | <0.35 | N/A | N/A |
| Ammonia | 2.54 | 2.36 | 4.43 | 2.62 | 0.171 | 1.04 | 12.0 | 0.489 | 14.3 | 0.420 | 2.23 | 1.05 | -510-97 | 58 |
| BOD | <6.0 | <6.0 | 6.6 | <6.0 | <6.0 | <6.0 | 12.9 | <6.0 | 24.5 | <6 | 7.0 | <6.0 | 0-76 | >25 |
| COD | 121 | 46 | 113 | 44 | 135 | 52 | 88 | 49 | 163 | 52 | 115 | 50 | 44-68 | 59 |
| Total P | 3.59 | 1.91 | 5.30 | 4.05 | 2.25 | 1.0 | 5.45 | 1.43 | 5.30 | 1.30 | 1.90 | 1.44 | 24-75 | 50 |
| TDS | 956 | 981 | 1070 | 1030 | 1040 | 1080 | 1130 | 984 | 1140 | 988 | 1110 | 1110 | -3-13 | 4 |
| TKN | 5.82 | 4.72 | 8.20 | 5.04 | 3.03 | 2.93 | 16.6 | 2.27 | 21.7 | 2.40 | 5.52 | 2.97 | 3-89 | 47 |
| TSS | 8.0 | 6.0 | 25 | 6.0 | 18 | 12 | 22 | <5.0 | 65 | 5.0 | 17 | <5.0 | 25-92 | 62 |
| pH | 8.44 | 7.40 | 8.30 | 7.44 | 9.52 | 7.34 | 8.37 | 8.20 | 8.73 | 8.68 | 9.04 | 7.61 | 0.6-23 | 11 |
| Fecal Coliform | 150 | 9 | 9 | <3 | 230 | 23 | 430 | 4 | 4300 | 4 | 9300 | 43 | 67-99.9 | 92 |
| Total Coliform | 210 | 930 | 210 | 1500 | 750 | 4300 | 15000 | 430 | 110000 | 430 | 9300 | 2300 | -614-99.6 | |

**Table 2**

Table 2: Mean (±SE) abundances of antibiotic resistance genes (ARGs), nitrification, and denitrification genes within water and biofilm samples collected from the Dunnottar wastewater treatment and downstream areas in 2013.

| Water [a] | 16S-rRNA | Total *tet*$^R$ | Total *sul*$^R$ | *nir*S+K | *amoA* |
|---|---|---|---|---|---|
| **Primary lagoon** | 19000 (±4400) | 4.1 (±1.0) | 151 (±58) | 422 (±96) | 15 (±3) |
| **Secondary lagoon** | 8540 (±4050) | 6.4 (±2.2) | 2.0 (±0.4) | 97 (±18) | 30 (±12) |
| **North filter** | 1070 (±210) | 3.0 (±0.9) | 2.3 (±0.8) | 233 (±35) | 62 (±16) |
| **South filter** | 1190 (±580) | 4.9 (±1.6) | 2.8 (±1.3) | 119 (±22) | 38 (±12) |
| **Outflow** | 2130 (±1410) | 2.6 (±0.6) | 2.1 (±0.7) | 87 (±19) | 31 (±10) |
| **Creek** | 1370 (±430) | 0.9 (±0.2) | 5.6 (±3.2) | 86 (±23) | 13 (±2) |
| **Highway** | 1020 (±300) | 1.0 (±0.2) | 4.6 (±3.6) | 87 (±17) | 19 (±8) |

| Biofilm [b] | 16S-rRNA | Total *tet*$^R$ | Total *sul*$^R$ | *nir*S+K | *amoA* |
|---|---|---|---|---|---|
| **Secondary lagoon** | 6950 (±3070) | 51 (±13) | 31 (±11) | 2150 (±370) | 31 (±11) |
| **North filter** | 20500 (±14900) | 68 (±23) | 54 (±22) | 779 (±266) | 46 (±17) |
| **South filter** | 80800 (±49300) | 0.8 (±0.4) | 0.5 (±0.4) | 1820 (±850) | 11 (±4) |

[a] $10^3$ genes per mL

[b] $10^3$ genes per $cm^2$

**Table 3**

Table 3: *Vibrio fischeri* (Microtox® assay) bioluminescence presented as percent of control (±SD) after 15 minutes exposure to test water samples. *V. fischeri* bioluminescence values less than 75% of control are highlighted; values with ' * ' are the averaged bioluminescence values from the triplicate samples collected on that day as part of the rotating sampling schedule. While the pH of the samples ranged from 6-9, there was no observable impact on *V. fischeri* bioluminescence (data not shown). "-" indicates sample was lost due to breakage.

| **Water Sample Locations** | 04-Jun-13 | 18-Jun-13 | 02-Jul-13 | 16-Jul-13 | 30-Jul-13 | 13-Aug-13 | 27-Aug-13 | 10-Sep-13 | 24-Sep-13 | Average |
|---|---|---|---|---|---|---|---|---|---|---|
| **Primary lagoon** | 31 (±1) | 72 (±2) | 43 (±1) | 45 (±1) | 48 (±3) | 50 (±1) | 52 (±2)* | 48 (±1) | 62 (±1) | 50 |
| **Secondary lagoon** | 57 (±1) | 101(±13)* | 115 (±2) | - | 114 (±4) | 69 (±3) | 95 (±2) | 97 (±3) | - | 94 |
| **North filter** | - | 106 (±1) | 108 (±6)* | 128 (±4) | 126 (±4) | 113 (±4) | 102 (±7) | 112 (±1) | - | 114 |
| **South filter** | - | 94 (±1) | 101 (±2) | 101 (±3)* | 119 (±2) | 100 (±1) | 102 (±1) | 81 ± (6) | - | 100 |
| **Outflow** | 59 (±1) | 103 (±2) | 90 (±2) | 135 (±7) | 120 (±2)* | 106 (±2) | 120 (±4) | 113 (±1) | - | 106 |
| **Creek** | 44 (±1) | 51 (±1) | 62 (±1) | 39 (±2) | 54 (±5) | 14 (±4)* | 9 (±1) | 43 (±2) | 63 (±3) | 42 |
| **Highway** | - | - | - | 105 (±4) | 116 (±5) | 80 (±1) | 110 (±4) | 75 (±6)* | 114 (±3) | 100 |
| **Lake Blank** | 110 (±2) | 85 (±2) | 72 (±1) | 89 (±2) | 105 (±2) | 106 (±4) | 91 (±3) | 87 (±2) | 123 (±4) | 97 |
| **Field Blank (milli-q)** | - | 103 (±1) | 114 (±1) | 105 (±5) | 120 (±4) | 111 (±1) | 114 (±2) | 124 (±2) | 108 (±6) | 112 |

**Table 4**

Table 4: Comparison of concentrations of target pharmaceutical compounds in grab water samples from receiving waters of different Manitoban wastewater systems.

| Compound | Dunnottar | Grand Marais[1] | Winkler/Morden[2] |
|---|---|---|---|
| **Carbamazepine** | 44-256 ng/L | 85-500 ng/L | 1-85 ng/L |
| **Gemfibrozil** | ND-107 ng/L | ND-15 ng/L | ND |
| **Metoprolol** | ND-26.7 ng/L | ND | ND-19 ng/L |
| **Sulfamethoxazole** | ND-403 ng/L | ND-21 ng/L | ND-70 ng/L |

ND = not detected

[1]Anderson et al. (2013); [2]Carlson et al. (2013)

**Supplementary Material**

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