Effects of erythromycin, trimethoprim and clindamycin on attached microbial communities from an effluent dominated prairie stream

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A B S T R A C T

In this study, differing metrics were utilized to measure effects of erythromycin (ER), trimethoprim (TR) and clindamycin (CL) on the structure and function of attached Wascana Creek, SK microbial communities. All three test antibiotics, especially ER, affected community structure and function of biofilms grown in rotating annular reactors. Biofilm thickness, bacterial biomass, and lectin binding biovolume (exopolymeric substances) were consistently less in ER treated biofilms when compared to the control. As well negative effects on protozoan numbers, and carbon utilization were detected. Finally, PCA analyses of DGGE results indicated that bacterial community diversity in ER exposed biofilms was always different from the control. ER exhibited toxic effects even at lower concentrations. Observations on TR and CL exposed biofilms indicated that bacterial biomass, lectin binding biovolume and carbon utilization were negatively affected as well. In terms of bacterial community diversity, however, CL exposed biofilms tended to group with the control while TR grouped with nutrient additions suggesting both nutritive and toxic effects. This study results represent an important step in understanding antibiotic effects, especially ER, on aquatic microbial communities. And because ER is so ubiquitous in receiving water bodies worldwide, the Wascana study results suggest the possibility of ecosystem disturbance elsewhere.

Capsule abstract: Erythromycin (ER) is ubiquitous in waterbodies receiving sewage effluent. Structure and function of microbial communities from an effluent dominated stream were negatively affected by ER, at realistic concentrations.

1. Introduction

In Canada, systemic anti-infectives are among the top prescribed medications (Morgan et al., 2005) while in Europe, 13,500 tons of antibiotics are used each year with 65% for humans and the remaining 35% for animals (Christensen et al., 2006). Despite variation in excretion rates and types of metabolites, some antibiotics are excreted from humans and animals relatively unchanged. Further, many human antibiotics are not fully removed during sewage treatment. Consequently, surface waters worldwide receiving treated sewage effluent and animal waste contain antibiotics in the ng/L to μg/L range. Of these antibiotics, erythromycin (ER) and trimethoprim (TR), appear to be ubiquitous in receiving water bodies. A recent British investigation, for example, revealed average ER and TR concentrations of 159 ng/l and 12 ng/l, respectively, 1 km downstream of five sewage treatment plants (STPs) (Ashton et al., 2004). Maximum erythromycin-H₂O concentration (metabolite) in a United States survey of 139 streams was 1.7 μg/l (Kolpin et al., 2002) while in Italy, median ER concentrations in the Lambro and Po Rivers were 4.5 and 3.2 ng/l, respectively (Zuccato et al., 2006). Because they are so commonly found in receiving water bodies, many scientific reviews rank ciprofloxacin, TR, ER and sulfamethazine of particular concern (Johnson et al., 2015).

The presence of antibiotics in aquatic ecosystems is of concern for a number of reasons. Most antibiotics interact with a biological target (membranes, enzymes) shared by humans, animals, plants (e.g. plastids) and bacteria (Brain et al., 2008). They act at relatively low concentrations, need time to achieve effects and resist biodegradation (Jones et al., 2004). Proliferation of antibiotic resistance has been linked to chronic bacterial exposure (Eggen et al., 2004; Fent et al., 2006; Gullberg et al., 2011) and high nutrient conditions typifying water bodies receiving treated effluent may exacerbate such proliferation (Castiglioni et al., 2008). Low antibiotic concentrations may also stimulate or depress bacterial gene expression at the transcriptional level at concentrations significantly lower than the minimum inhibitory concentration (MIC).
(Goh et al., 2002). Finally, their constant addition to aquatic ecosystems at rates exceeding transformation means not only that they are considered pseudo-persistent (Daughton and Ternes, 1999) but that aquatic organisms are chronically exposed.

Not a great deal, however, is known regarding such chronic effects as most research has been directed towards single species toxicity with acute exposures. Although these controlled studies have figured largely in risk assessment, their results lack environmental realism and extrapolation to communities at the ecosystem level is difficult. Community-based effects assessments, however, offer the potential of evaluating ecosystem level effects at relevant contaminant concentrations. Ideal candidates for community level effects studies are microorganisms. Within rivers, streams and other aquatic habitats they not only serve as an important food source for benthic invertebrates and protozoans, they are also major players in biogeochemical nutrient cycling and organic matter biodegradation. Due to their short life cycles, toxic induced community succession (TIS) can be observed in the laboratory over relatively brief time scales (Backhaus et al., 2011). Microbial communities, therefore, are potentially excellent indicators of changes in ecosystem health.

The purpose of this study was to investigate effects of three antibiotics, ER, TR and clindamycin (CL) on the structure, indicators of changes in ecosystem health. Microbial communities, therefore, are potentially excellent indicators of changes in ecosystem health.

The purpose of this study was to investigate effects of three antibiotics, ER, TR and clindamycin (CL) on the structure, development, functioning and biodiversity of biofilm communities from an effluent dominated creek (Wascana Creek) in southern Saskatchewan, Canada using a laboratory approach. Previous seasonal monitoring studies here indicated that ER, CL, and TR were all present in ng/L and sometimes μg/L concentrations. With regard to their toxicity, single species research has revealed that a dosage of 1 mg/L ER inhibits Synechocystis sp. and Lemma minor growth by 70% and 20% (Pomati et al., 2004). Other studies indicated that the median inhibition concentration (IC50) of TR to Pseudokirchneriella subcapitata was 1000 μg/L (Yang et al., 2008). According to hazard quotients (HQ) generated from earlier Wascana Creek research, ER and TR were present at concentrations which could present a risk to aquatic organisms (Waiser et al., 2011b). Although CL did not generate a hazard quotient indicative of risk, it was added to this study due to its presence on all sampling dates and its persistence downstream of the STP. It was hoped that such community based research would play an important role not only in establishing effects of these antibiotics but in structuring future environmental risk assessments for Wascana Creek and perhaps other similarly affected aquatic ecosystems (c.f. Backhaus et al., 2011).

2. Materials and methods

2.1. Study area

Wascana Creek receives tertiary treated sewage effluent from the city of Regina, SK sewage treatment plant. From late October through to March most flow in the creek is treated sewage effluent. Wascana Creek is considered hypereutrophic with average seasonal total phosphorus (TP) concentration 0.24 mg/L, soluble reactive phosphorus (SRP) 0.09 mg/L, ammonia-N (NH3) 0.04 mg/L and total dissolved nitrogen (TDN) 0.79 mg/L (Waiser et al., 2011a).

2.1.1. Attached microbial communities; rotating annular reactor experiments

Biofilms recruited from Wascana Creek (algae, bacteria and exopolysaccharide) were grown for 8 weeks under controlled conditions in rotating annular biofilm reactors as described by Lawrence et al. (2004). Wascana creek water was collected in August 2006 and November 2006 at a site approximately 30 km upstream of the sewage treatment plant. Previous monitoring studies indicated that none of the study antibiotics were present here (Waiser et al., 2011b). Creek waster served as the microbial inocula as well as a source of carbon (C), nitrogen (N) and phosphorus (P).

2.2. Experimental design

The August biofilm experiment (BEXP1) tested the effects of the three antibiotics, each at 4 μg/L (nominal concentrations), on creek biofilms. Control reactors with no additions were run at the same time. The November experiment (BEXP2) repeated these three treatments but also added 1, 2, 3 and 4 μg/L of ER (nominal concentrations) to the investigation. Each treatment and control had three identical replicate reactors randomly assigned to it in a block. Antibiotics were continuously added to the reactors using peristaltic pumps. Nutrient controls were also run where the molar equivalent of the antibiotic treatment was added as carbon (glucose) and nitrogen (ammonium chloride) (cf Lawrence et al., 2012). These treatments are designed to test whether the impact of the contaminant is more similar to degradation and utilization or toxicity. These nutrient controls are designated as CL control, ER control and TR control respectively.

The central rotating shaft of each biofilm reactor contained 12 identical polycarbonate slides on which the biofilms grew. At the end of the experiment, slides were randomly selected, removed and in some cases small subsections cut so that biofilms on these sections (coupons ~ 1 cm²) could be subjected to microscopic examination and other assays.

2.3. Algal biomass (Chl a)

Biomass on each 1 × 10 cm coupon (one from each replicate) was scraped into a 50–ml centrifuge tube containing 10 ml of 90% ethanol. Chl a was extracted coupons in 90% boiling ethanol and analysed fluorometrically using a Turner Designs Model 10-AU digital fluorometer (Turner Designs, Sunnyvale, CA) (Waiser and Robarts, 1997).

2.4. Bacterial production (BP)

One coupon from each replicate was used to estimate BP. Each coupon was placed in a plastic Petri dish containing 10 ml of 0.2 μm filter sterilized creek water. Approximately 30 nM of 3H labeled thymidine (Tdr – Perkin Elmer, Waltham, MA) was added to each dish and dishes swirled. One coupon from each treatment was placed into a corresponding ‘killed’ control dish to which 500 μl of formalin had been previously added. Samples were incubated for thirty minutes, at the end of which time live samples were killed with 500 μl of formalin. Bacterial DNA was extracted and bacterial production was estimated using [methyl-3H] thymidine (Tdr) incorporation into bacterial DNA according to established methods (Robarts and Wicks, 1989).

2.5. Confocal laser scanning microscopy and image analysis

Coupons with biofilms attached were mounted in small petri dishes using Dow Corning #3140 acid-free silicone coating (WPI, Inc., Sarasota, Fla.). Bacteria were then stained with a fluorescent nucleic acid stain (SYTO 9) (excitation wavelength, 488 nm [ex 488]; emission wavelength, 522–532 nm [em 522/32]). Biofilms were observed using a Bio-Rad MRC 1024 confocal laser scanning microscope (Zeiss, Jena, Germany) attached to a Microphot SA microscope (Nikon, Tokyo, Japan). Two water submersible lenses, a 63 × 0.9 numerical aperture (Zeiss, Jena, Germany) and a 40 × 0.55 numerical aperture (Nikon) were used for biofilm examination. Laser scanning microscopy imaging was done at five positions on five randomly chosen transects traversing each biofilm coupon.
These scans were subsequently utilized to estimate biofilm thickness and biomass. For biomass estimates, cyanobacteria and algae were differentiated based on wavelengths of their autofluorescent pigments and bacteria on the basis of the SYTO 9 emission wavelength (three channel procedure - Neu et al., 2004; Lawrence et al., 2004, 2005). Digital image analysis of resulting optical thin biofilm sections in each channel was then performed (Neu et al., 2001).

2.6. Exopolymer analyses

Specific sugar residues in biofilm exopoly saccharide were detected using fluor-conjugated lectins (fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate (TRITC) - Sigma, St. Louis, Mo.) alone or in combination. Cy5 labeling was done using a commercial kit according to instructions provided (Research Organics, Cleveland, Ohio). Specific lectins utilized included, Arachis hypogaea (galactose, N-acetyl galactosamine), Canavalia ensiformis (mannotose, glucose), Glycine max (N-acetyl galactosamine), Triticum estivum (N-acetyl glucosamine residues and oligomers), and Ulex europaeus (fucose). Image analyses and lectin binding volume calculations were carried out using equations of Neu et al. (2001).

2.7. Carbon use spectra

Carbon-use spectra were determined using commercial EcoPlates (Biolog, Hayward, CA, USA) as described in Lawrence et al. (2004). Biofilm coupons (1 x 10 cm) were scraped with a sterile silicon rubber spatula to remove biofilm and sonicated for 5 min in a Branson 5120 water bath sonifier (Branson Ultrasonics, Danbury, Conn.) to disperse cells. Each of the 96 wells of the Biolog microtiter plates was subsequently inoculated with 150 μl of the biofilm slurry and plates subsequently incubated at 23 °C. Absorbance (590 nm) was measured using a standard microtiter plate reader at 24-h intervals for 7 d.

2.8. Protozoan and micrometazoan enumeration

Biofilm samples were removed from reactors weekly and protozoan and micrometazoans counted manually on replicate 2 cm² coupons (3 subsamples from each treatment replicate therefore n=9 for each treatment) using phase contrast microscopy according to Packroff et al. (2002).

2.9. Molecular analyses

Bacterial cells in frozen biofilm samples were removed from the polycarbonate strip with a sterile metal scraper and total DNA extracted using the Powersoil DNA isolation kit (MO Bio Laboratories, Mississauga, ON) consisted of an initial denaturation step (94 °C for 5 min), followed by 10 cycles of denaturation (94 °C for 1 min), annealing at 66 °C (decreasing in each cycle by 1 °C) for 1 min and an elongation step (72 °C for 1 min). Then another 20 cycles of 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min with a final elongation step of 72 °C for 7 min. PCR product was confirmed by electrophoresis on a 1.5% w/v agarose gel in 1.0 × Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) for 1.0 h at 100 V. Gels were stained using ethidium bromide and documented using the Alphalmager 3300 gel documentation and image analysis system (Alpha Innotec Corporation, San Leandro, CA.).

2.11. Denaturing gradient gel electrophoresis analysis (DGGE)

After checking specificity and size of amplified products on agarose gels, the PCR product was separated by DGGE using an Ingeny phorU2 system (Ingeny, Leiden, the Netherlands). All lots of PCR product (45 μl) were mixed with 5 μL of loading dye buffer and resolved on a 6% (w/v) polyacrylamide gel in 1.0 × TAE buffer, using denaturing gradients from 45% to 65% for Bacteria (100% denaturant contains 7 M urea and 40% deionised formamide). Electrophoresis was carried out at 60 V for 30 min, then 100 V for 18 h at 60 °C. After electrophoresis, the gel was stained with SYBR green I (110,000 dilution; Molecular Probes, Eugene, OR) for 15 min with gentle agitation and photographed using the Alphalmager 3300 gel documentation and image analysis system (Alpha Innotec Corporation, San Leandro, CA, USA).

2.12. Statistical analysis

Each antibiotic treatment had three identical reactors randomly assigned to it on the reactor bench (replications). Furthermore, each analysis was done on subsamples of randomly selected biofilm coupons from among the 12 replicate coupons in each replicate reactor. In the biofilm experiments, a one way ANOVA followed by a post-hoc Tukey’s test was used to detect significant differences among sample means for all investigated variables at p < 0.05 when the data were normally distributed (MiniTab, State College, PA, USA).

For DGGE gels, band detecting, matching and processing were completed with the GelCompare II software 4.6 (Applied Maths, Kotriek, Belgium). Fingerprint data were processed by generating a band matching table (Boon et al., 2002). The binary data were exported and compared by principal component analysis (PCA) with PRIMER v6 software (PRIMER-E Ltd Lutton, UK). Statistical analyses of PCA scores generated from the first two axes were run using an analysis of similarity (ANOSIM) with PRIMER v6 software (Clarke, 1993). The inclusion of DGGE ladders allowed

<p>| Table 1. Oligonucleotide primers used for PCR amplification of bacterial genes. |</p>
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer consensus sequencea Position (length)</th>
<th>Reference organism</th>
<th>PCR fragment size (bp)</th>
<th>Target bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA Forward</td>
<td>5′– CTC ACG GGA GGC AGC AG–3′</td>
<td>Escherichia coli</td>
<td>586</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Position</td>
<td>341–357 (17 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′– CCG TCA ATT CMT TTG AGT TT–3′</td>
<td></td>
<td>907–927 (20 nt)</td>
<td></td>
</tr>
</tbody>
</table>

| Footnotes |
| Forward and Reverse indicate the orientation of the primers in relation to the rRNA sequence. |
| Preceded by a GC clamp on 5′ end for DGGE (not for sequencing) = CGC CCG CCG CCC CCC GCC CCG CCC CCG CCC C CGG (40 nt) Muyzer et al., 1993. |
| Muyzer et al. (1993). |
| E. coli numbering according to Brosius et al. (1981). |
| Casamayor et al. (2000). |

GelCompareII to normalize the position of bands in all of the lanes under examination.

3. Results

3.1. Biofilm thickness

Biofilm thickness in the TR and CL treatments in BEXP1 was greater than the control, while the ER treatment was significantly (p < 0.05) less (Fig. 1(A)). There was no significant difference between the antibiotic nutrient controls and the control treatment.

In BEXP2 biofilm thickness in all ER treatments was less than CL, TR, the antibiotic nutrient control and control (Fig. 1(B)), similar to results from BEXP1. Within ER treatments, thickness in the 4 μg/L treatment was less than any of the other three concentrations. Thickness in the CL treatment was less than the control, while no difference was noted between the control and TR. TR was significantly greater than the comparative nutrient treatment.

3.2. Biomass and production

In BEXP1, although no difference was noted in algal biomass (Chl a) when all antibiotic treatments were compared to the control, bacterial biomass in the same treatments was significantly less than the control (p < 0.05; Fig. 2(A)). A within antibiotic treatment comparison indicated that bacterial biomass was least in the ER treatment, as was bacterial production (data not shown) while biomass in the CL treatment was greater than in the other two (p < 0.05). Bacterial production in the TR and CL treatment was not significantly different from the control (data not shown). The only treatment where there was an effect on cyanobacteria was seen in the TR treatment (p < 0.05; Fig. 2(A)). In all treatments except ER, bacterial biomass was greater than that of algae indicating heterotrophy in these biofilms. The nutrient treatments had no significant effects relative to control and antibiotic exposures.

In contrast to BEXP1, significant depression of algal biomass (from confocal laser analysis) was noted in TR and all ER treatments in BEXP2 when compared to the control (p < 0.05; Fig. 2(B)). Within ER treatments, algal biomass in the 3 and 4 μg/L treatments was less than in 1 and 2 μg/L while biomass in 2 μg/L was less than in 1 μg/L (p < 0.05). Bacterial biomass was also affected being far less in the TR and all ER treatments when compared to either CL or the control. Within ER treatments, bacterial biomass in the 1 and 4 μg/L treatments were greater than the remaining two, while biomass in the 4 μg/L was less than that in the 1 μg/L (p < 0.05). The 4 μg/L nutrient control resulted in increased bacterial biomass relative to CL, while cyanobacteria and bacterial biomass increased relative to TR. With regard to ER 1 μg/L bacterial biomass increased relative to the nutrient addition, while for the 2, 3, and 4 μg/L treatments algal biomass was significantly reduced relative to the nutrient addition (Fig. 2(B)). In contrast to BEXP1, in all treatments, algal biomass was greater than that of bacteria, indicating autotrophy. All antibiotic treated biofilms, except TR had cyanobacterial biomass which was less than the control (Fig. 2(B)). No effect of any antibiotic on bacterial production was noted (data not shown).

Fig. 1. Influence of antibiotics on biofilm thickness in BEXP1 (A) and BEXP2 (B) as assessed at the microscale using confocal laser microscope analysis. Treatments indicated by different letters are significantly different from the control (p < 0.05; n=3 ± SD).

Fig. 2. Results of image analyses of confocal laser micrographs illustrating the effect of antibiotics on the relative abundance of algae, cyanobacteria, and bacteria in Wascana Creek biofilms in BEXP1(A) and BEXP2 (B) Treatments indicated by different letters are significantly different from the control (p < 0.05; n=3 ± SD).
3.3. Lectin binding volume

In BEXP1, the least lectin binding volume was observed in the ER treatment, while the most was in the TR treated biofilms (Fig. 3(A)). In all cases the respective nutrient controls differed significantly from the control. CL and TR treatments were also significantly different from their nutrient controls. In the case of ER, however, lectin binding volumes were not significantly different between antibiotic and nutrient treatments (Fig. 3(A)). In BEXP2 slightly different results were noted in that biofilms from all drug treatments had less lectin binding volume than the control (Fig. 3(B)). Similar to BEXP1, however, the least lectin binding volume was observed in the ER treatments. Additionally, no significant difference was observed between the four ER treatments.

3.4. Carbon utilization spectra

In BEXP1, significant differences were noted when antibiotic nutrient control and antibiotic treatments were compared to the controls in all carbon categories except phosphorylated compounds. In most cases, antibiotics significantly decreased utilization of the tested carbon substrates (Fig. 4(A),(B),C). Of the number of significant effects noted, decreased utilization ranged from 64% in CL treatments to 100% for both ER and TR. Interestingly, the addition of the antibiotic nutrient equivalents similarly resulted in a general trend of decreased carbon substrate utilization. Due to technical difficulties (spectrophotometer not zeroing correctly) the carbon utilization data from BEXP2 could not be utilized.

3.5. Protozoan and micrometazoan enumeration

By the end of BEXP1 and BEXP2, protozoan and rotifer numbers were higher in the TR and lower in ER treatment when compared to the control (Fig. 5(A)). In contrast, although protozoan numbers in the CL treatment in BEXP1 were less than the control, rotifer numbers were greater on the final day of the experiment. Results for the CL treatment in BEXP2 were somewhat different with protozoan and rotifer numbers less than the control by experiment end (Fig. 5(B)).

Within the ER dose response treatments, numbers of protozoans and rotifers were slightly higher in the 1 µg/L treatment, protozoans were similar to the control in the 3 µg/L treatment, while protozoans and rotifers in the remaining treatments were less than the control (Fig. 5(B)). Oddly, the ER nutrient addition of 4 µg/L resulted in the least numbers of both protozoans and
microinvertebrates by experiment end (Fig. 5(B)).

3.6. Molecular analyses - PCA and Anosim results

3.6.1. BEXP1

Principal component analyses of the DGGE results indicated that each antibiotic treatment resulted in significantly different communities. The control and CL treatments grouped together, while the ER and TR treatments were different from each other as well as from the CL/control grouping (Fig. 6(A)). Further the TR treatment grouped not only with its nutrient control but the other nutrient controls as well. ANOSIM pair-wise analysis showed significant differences ($R = 0.799$, $p < 0.001$) in the structure of microbial communities between antibiotic treatments. The MSDS below 0.2 indicated that the graph was highly interpretable and the model statistically significant. According to this analysis, CL was the only treatment not significantly different from the control (Fig. 6(A)). Bacterial diversity in the remaining treatments (TR and ER) was significantly different from the control. The greatest effect was observed in the ER treatment with diversity here grouping away from both control and all other treatments (Fig. 6(A)). The apparent similarity between ER and CL nutrient control was likely due to the outlier and does not necessarily reflect a true similarity.

3.6.2. BEXP2

DGGE principle components analysis indicated as in BEXP1 that
4. Discussion

It has been stated that the ability of biofilms to detect toxic effects of anthropogenic stressors both in the short (changes in physiological function) and long term (changes in community structure) make them ideal early indicators of stress in aquatic ecosystems (Sabater et al., 2007; Sandin et al., 2009; Lawrence et al., 2015). It is evident from the results of the Wascana experiments presented here just how ideal biofilms can be as such indicators. These results clearly show that the test antibiotics (ER, TR and CL) changed the amount of bacterial and algal biomass produced, carbon utilization patterns, microbial community composition and structure, as well as the amount and composition of exopolysaccharide created in stream biofilms.

One of the first observations was the significant depression of algal biomass in all ER treatments in the second experiment (BEXP2). This observation contrasted with results of the first experiment (BEXP1) where no depression was noted. There are a number of explanations for this disparity. It may be that different algal communities, with differing sensitivities to antibiotics, are recruited to biofilms during warmer August as opposed to cooler November temperatures. In Wascana Creek, water temperatures vary from about 21 °C in July to 5 °C in November (Waiser et al., 2011a). Research into diclofenac effects on South Saskatchewan River biofilms, has noted similar effects with spring and summer biofilms differing in their microbial communities and response (Lawrence et al., 2007). According to the authors, seasonal factors like flow rate, water temperature and levels as well as amount of photosynthetically active radiation likely influenced microorganisms available for recruitment to the biofilm (Lawrence et al., 2007; Besemer et al., 2007).

There are, however, other explanations for the declines in biofilm algal and bacterial communities. Although depression of algal biomass in the ER treatment only occurred in BEXP2, bacterial biomass was always significantly less than the control no matter what the season, or ER concentration. Erythromycin and other macrolide antibiotics inhibit bacterial protein synthesis by binding to the 23S rRNA molecule (in the 50S subunit) of the bacterial ribosome blocking exit of the growing peptide. Declines in bacterial biomass may therefore have been the result of the direct action of ER on bacteria protein production. Depression of algal biomass in BEXP2 may also have been due to the coherence of green algae phylogeny with plastid genes containing consensus prokaryotic promoters and bacterial-like sigma factors (Brain et al., 2008). Because of the existence of these transcription/translation homologues, macrolide antibiotics, like ER, may disturb the transcription/translation process in the photosynthetic chloroplast (Brain et al., 2008) thereby lowering algal biomass.

But a depression in algal biomass was also noted in the TR treatment of BEXP2. The basis of TR toxicity appears to be its ability to bind and reversibly inhibit dihydrofolate reductase, an enzyme involved in folate synthesis (Crane et al., 2006). Essential for one-carbon transfer reactions in plants, folates are also needed for photosynthesis and production of nucleic acids, amino acids and lignins (Brain et al., 2008). Because the folate synthetic pathway in plants and bacteria is essentially the same, TR could potentially elicit a physiological response in phytoplankton thereby providing a possible explanation for the depressed algal biomass noted here.

Additionally, the decline in bacterial biomass may also be related to the depression in algal biomass. Within biofilms, algae have been referred to as ‘ecosystem engineers’, physically modifying the microenvironment and forming a structural template on which bacteria can grow (Besemer et al., 2007). They also excrete labile organic carbon that can be readily used by bacteria for growth. As such they play an important role in biofilm structure and function. In BEXP2, the depression in algal biomass may have been accompanied by a decline in excreted labile carbon. This decline in turn may have been linked to the concomitant changes noted in the bacterial communities. Within aquatic ecosystems the bacterial constituents are also important in conditioning detritus, making it more palatable for grazers (Maul et al., 2006). Consequently if there are less ‘conditioning’ bacteria, there might be a spill down effect on those grazers relying on biofilm bacteria to perform this function.

Within aquatic ecosystems, bacteria and algae both play key roles in the biogeochemical cycling of carbon, nitrogen and phosphorus. Disruption of their communities by exposure to antibiotics may affect these important cycles (Rosi-Marshall and Royer, 2012). In the Wascana studies presented here biofilm carbon utilization patterns were affected with significantly decreased use of some carbon substrates observed in all antibiotic treatments. This observation suggests that some disruption of bacterial carbon cycling may be occurring as a result of antibiotic exposure, a similar result to those noted in other studies. Using Biolog Eco plates, stream microbial communities exhibited declines in carbon utilization patterns after exposure to 100 μg/L ciprofloxacin (Maul
et al., 2006), while Lawrence et al. (2009) found that exposure to 10 μg/L of triclocarban and tricosan also resulted in depressed carbon utilization.

There are a number of possible explanations for the observation of depressed carbon utilization. Results of the PCA analysis on the DGGE gels clearly showed that the microbial communities in TR and ER exposed communities were different from the control. The extent to which these changes affected carbon cycling is unknown, but remains a possibility. Additionally, some studies have demonstrated that antibiotics act as metabolic signaling molecules with bacteria responding to exposure by up or down regulation of genes (Goh et al., 2002; Fajardo and Martínez, 2008). Disruption of carbon cycling or other cellular functions may occur as a result. At exposures of 50 and 500 μg/mL of ER, for example, activation or repression of a significant proportion of genes (~5%) involved in transport, virulence and DNA repair were noted in Escherichia coli and Salmonella typhimurium (Goh et al., 2002). With respect to Wascana Creek, a recent metagenomics and meta-transcriptomic study (using the same reactor approach and water source as in this study) investigated the effects of ER (at 1 μg/L) on Wascana Creek biofilms. Here, active community composition at the RNA level (taxonomic affiliation of mRNA sequencing) in Cyanobacteria and Proteobacteria was strongly affected by ER exposure (Yergeau et al., 2012). Effects on photosynthesis-related genes were also noted (Yergeau et al., 2012). When compared to the control, ER exposure caused expression of several unique genes which resulted in large changes in metabolic networks specifically those related to C, N and P cycling. Such observed genetic regulatory changes may explain the decreased carbon utilization noted in the present study.

A number of studies have indicated that decreased carbon substrate utilization influences biofilm exopolysaccharide (EPS) composition (Mohamed et al., 1998; Lawrence et al., 2007, 2015). In the Wascana experiments presented here, definite changes in EPS composition were observed in antibiotic exposed biofilms. Such changes are of concern because the EPS matrix is responsible for maintaining cell spatial organization and promoting cross-linking or scaffolding within the biofilm, thereby fostering growth of bacterial microcolonies. As well, some EPS’s make biofilms more viscous thereby favouring biofilm spreading (Chew et al., 2014). Decreased carbon utilization may also affect the amount of EPS produced (Lawrence et al., 2007, 2015).

Results from the Wascana experiments clearly indicated that antibiotic exposure, especially to ER also resulted in thinner biofilms and this observation is of concern. According to some authors, physiological changes, including declines in biofilm thickness, are indicative of an initial stress response and may be an early sign of direct ecosystem damage (Bonnineau et al., 2010). In stream and river ecosystems biofilms integrate nutrients and organic matter into a structure subsequently utilized as a food source to support higher trophic levels (invertebrate grazers). Biofilm ingestion is also thought to provide endosymbionts which excrete enzymes (apparently lacking in some invertebrate grazers) capable of breaking down cellulose and other refractory leaf constituents (Maul et al., 2006; Sinsabaugh et al., 1985). Less biofilm of differing composition (as noted in the present study) and perhaps of less nutritional value may have negative consequences for invertebrate grazers. If the negative effect is great enough or occurs often enough it may resonate to higher trophic levels. In the Wascana experiments there was an indication that such resonance might be occurring as protozoan and metazoan numbers in most of the ER treatments and rotifers in the higher ER treatments were significantly lower than the control by experiment end.

Although decreased carbon utilization may affect the amount of EPS produced, there may be other factors which influence this process. Recent research has suggested that antibiotic exposure may affect cell surfaces, making bacterial adhesion more difficult. ER at concentrations of 0.5 and 50 μg/L, for example, significantly inhibited adhesion of Escherichia coli, Bacillus subtilis and Aquabacterium commune, to uncoated polystyrene beads (Schreiber et al., 2008).

One of the interesting and perplexing observations from BEXP1 was the significant difference not only in biomass amount but EPS composition in both the CI and ER nutrient controls when compared to the creek water only control. One reasonable explanation would be that nutrient addition in these two treatments may have caused an initial rapid growth in both biofilm bacteria and algae. This rapid growth may have been followed by an increase in protozoan numbers and subsequent grazing pressure. The grazing effort in turn may have been enough to contribute to the differences in biofilm biomass and EPS composition noted in BEXP1. There is some support for this idea in that at experiment end, highest protozoan numbers were noted in the ER and CI nutrient controls. Research has shown that the structure and production of periphyton (biofilms) communities can be regulated by such interplay between consumers and nutrients (Liess and Hillebrand, 2006). Unfortunately we do not have a time series of biomass or EPS composition to indicate the initial response of biofilms to the nutrient treatment. Other biofilm experiments, however, have shown that phosphorus additions were responsible for increased biofilm bacterial growth and concomitant increase in protists numbers. Protist grazing in turn rapidly reduced the amount of extracellular polymeric substances accumulated by biofilm bacteria (Mohamed et al., 1998).

The same explanation, however, does not hold for BEXP2. Here although the biomass in the nutrient treatment was significantly less than the control, protozoan and metazoan grazer numbers were the lowest of all treatments. Research has shown that community composition within biofilms changes seasonally. In 2005, for example, a study of the seasonal response of biofilms to dissolved organic matter and nutrient enrichments in the Mahoning River, NE Ohio revealed that the relative abundance of differing bacteria phylogenetic groups varied seasonally and with nutrient treatment. Specifically, bacterial community structure of biofilms grown in the fall and exposed to phosphate enrichment was significantly different from the control (Olapade and Leff, 2005). The second biofilm experiment was conducted with water collected from Wascana Creek in November. Results from the BEXP2 DGGE PCA analyses clearly indicate that the bacterial community grown in the nutrient amendment was significantly different from the control as well as the antibiotic treatments. The dissimilarity in the bacterial community noted in addition to the time of year in which water was collected, may have contributed to the differences noted between the nutrient control and the control.

5. Conclusion

Taken together, results of this study represent an important step in our understanding of the effects of antibiotics (especially ER) on attached stream microbial communities. They provide strong evidence that chronic exposure to antibiotics at environmentally relevant concentrations can cause changes not only in the amount of EPS produced and its composition but the amount of algal and bacterial biomass produced. Changes in microbial community composition as well as some disruption in carbon utilization as also occurred as a result of antibiotic exposure. Knowledge gained from this research is also important because ER is ubiquitous—found worldwide in almost every studied waterbody receiving treated sewage effluent. The Wascana Creek research presented here suggests that ER may be responsible, at least in part, for observed ecosystem disturbance in
other waterbodies receiving treated sewage effluent.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.05.026.

References


