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Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge☆

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HIGHLIGHTS

- Fish and mussels were caged up- and down-stream of wastewater treatment discharge.
- The aim was to determine the best biomarkers for contaminants of emerging concern.
- Several emerging contaminants were elevated downstream of wastewater discharge.
- Biological effects were measured downstream of wastewater discharge.
- Biomarkers of emerging contaminants were elevated in fish exposed to effluent.

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ABSTRACT

Contaminants of emerging concern (CECs), including pharmaceuticals, personal care products and estrogens, are detected in wastewater treatment plant (WWTP) discharges. However, analytical monitoring of wastewater and surface water does not indicate whether CECs are affecting the organisms downstream. In this study, fathead minnows (*Pimephales promelas*) and freshwater mussels *Pyganodon grandis* Say, 1829 (synonym: *Anodonta grandis* Say, 1829) were caged for 4 weeks in the North Saskatchewan River, upstream and downstream of the discharge from the WWTP that serves the Edmonton, AB, Canada. Passive samplers deployed indicated that concentrations of pharmaceuticals, personal care products, an estrogen (estrone) and an androgen (androstenedione) were elevated at sites downstream of the WWTP discharge. Several biomarkers of exposure were significantly altered in the tissues of caged fathead minnows and freshwater mussels relative to the upstream

Abbreviations: AHTN, Tonalide® synthetic musk; ALP, alkali labile phosphate; AND, androstenedione; BCA, bicinchoninic acid; BFC, benzoxy-4-trifluoromethyl-coumarin; BSA, bovine serum albumin; CAT, catalase; CBZ, carbamazepine; CEC, contaminants of emerging concern; CFU, colony forming units; CYP1A1, cytochrome P450 1A1; CYP3A, cytochrome P450 3A; DO, dissolved oxygen; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid) or Ellman's reagent; EC, electrical conductivity; EDCs, endocrine disrupting compounds; EDTA, ethylenediaminetetraacetic acid; EPCOR, Edmonton Power Corporation; EROD, ethoxyresorufin-O-deethylase; EST, Estrone; GC, gas chromatography; GMZ, gemfibrozil; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; HFC, 7-hydroxy-4-trifluoromethylcoumarin; HHCB, Galaxolide® synthetic musk; IBU, ibuprofen; KPB, potassium phosphate buffer; LC, liquid chromatography; LOD, below detection limits; LOQ, below limits of quantitation; LPO, lipid peroxidation; MDA, malonaldehyde; MOPS, 3-(N-morpholino) propanesulfonic acid; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; NSR, North Saskatchewan River; POCIS, polar organic contaminants integrated sampler; PPCPs, pharmaceuticals and personal care products; SDS, sodium dodecyl sulfate; SI, supplementary information; SMX, sulfamethoxazole; SOD, superoxide dismutase; SPMD, semi-permeable membrane device; TBARS, THIOBARBITURIC acid reactive substances; TCS, triclosan; Temp, water temperature; TGSH, TOTAL glutathione; TPM, trimethoprim; Vtg, vitellogenin; WWTP, waste water treatment plant.

☆ Roles of authors: EJJ conducted the field research and drew all figures except for the multivariate analyses; GGG was responsible for field research, logistics, site selection, tissue sample collection and initial processing; AASM did the multivariate analyses and corresponding figures; PG, AASM and MG performed analysis of oxidative stress for mussel tissues; GGG analyzed VTG expression, TWM and AM measured glutathione and oxidative stress activity and mRNA, MG and JW performed BFC and EROD analysis in fathead minnows. CDM and TS performed chemical analyses of pharmaceuticals in water. CDM was lead PI on the project. All authors played a role in writing and editing of the MS.

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Endocrine disrupting compounds
Oxidative stress
Fathead minnows
Freshwater mussels
Passive samplers

reference sites. Biomarkers altered in fish included induction of CYP3A metabolism, an increase in vitellogenin (Vtg) gene expression in male minnows, elevated ratios of oxidized to total glutathione (i.e. GSSG/TGSH), and an increase in the activity of antioxidant enzymes (i.e. glutathione reductase, glutathione-*S*-transferase). In mussels, there were no significant changes in biomarkers of oxidative stress and the levels of Vtg-like proteins were reduced, not elevated, indicating a generalized stress response. Immune function was altered in mussels, as indicated by elevated lysosomal activity per hemocyte in *P. grandis* caged closest to the wastewater discharge. This immune response may be due to exposure to bacterial pathogens in the wastewater. Multivariate analysis indicated a response to the CECs Carbamazepine (CBZ) and Trimethoprim (TPM). Overall, these data indicate that there is a 1 km zone of impact for aquatic organisms downstream of WWTP discharge. However, multiple stressors in municipal wastewater make measurement and interpretation of impact of CECs difficult since water temperature, conductivity and bacteria are also inducing biomarker responses in both fish and mussels.

1. Introduction

Contaminants of emerging concern (CECs) have been detected in discharges from municipal wastewater treatment plants (WWTPs) and these include pharmaceuticals, personal care products and estrogens (Anderson et al., 2003; Metcalfe et al., 2003; Carballa et al., 2004; Servos et al., 2005; Lishman et al., 2006; Chen et al., 2006; Brun et al., 2006; Kerr et al., 2008; Yargeau et al., 2008; Metcalfe et al., 2010). Direct water analysis monitoring of CECs in receiving waters is expensive and does not provide direct information on the biological impacts of exposure to these contaminants (Hecker and Hollett, 2009). Monitoring biomarker expression in aquatic organisms can be an effective way to evaluate whether CECs in wastewater effluents affect the receiving environment. Biomonitoring has many advantages: it integrates the response temporally, accounts for bioavailability and more directly assesses exposure under ambient conditions (Schmitt and Dethloff, 2000). In rivers, caging appropriate organisms provides consistent and reliable sample numbers with a defined exposure.

Widespread sexual disruption and reduced fertility in wild fish was studied and documented downstream of wastewater effluents (Jobling et al., 1998, 2002). More recent studies of fish endocrine responses to wastewater include intersex (occurrence of ova-testes) (Bahamonde et al., 2013), altered gene expression and physiology (altered steroid production) (Bahamonde et al., 2014; Tanna et al., 2013; Tetreault et al., 2011). These responses, including induction of vitellogenin (Vtg) are consistent with the exposure of fish to an environmental estrogen (17α-ethinylestradiol) that led to a population collapse of fathead minnows (*Pimephales promelas*) during a whole lake experiment (Kidd et al., 2007, 2014). Larsson et al. (1999) found distinct induction of Vtg in caged juvenile rainbow trout (*Oncorhynchus mykiss*) downstream of Swedish sewage treatment works. Harries et al. (1997) found estrogenic activity in caged rainbow trout downstream of sewage treatment works in the UK. Ings et al. (2011, 2012) observed changes in gene expression and stress responses in juvenile rainbow trout exposed to a tertiary treated wastewater plant in Ontario. Cazenave et al. (2014) observed activation of antioxidant enzymes and lipid oxidative damage, among other biomarkers of fish health in a neotropical fish species caged downstream of wastewater discharges in Argentina.

In mussels, likewise, biomarker responses to CECs have been observed. Gagné et al. (2004) observed several biomarker responses in freshwater mussels (*Elliptio complanata*) caged for a year downstream of a municipal WWTP that indicated exposure to both estrogenic and serotonergic compounds. In marine mussels (*Mytilus edulis*) collected from intertidal regions impacted by municipal wastewater, biomarker responses included reduced levels of Vtg-like proteins in females and elevated lipid content in males (Hellou et al., 2003). Gillis et al. (2014a) observed induction of oxidative stress biomarkers and modulation of immune function in freshwater mussels (*Lasmigona costata*) caged in a river influenced by wastewater. Bianchi et al. (2014) found that in the freshwater mussel, *Diplodon chilensis*, gGST and gCAT were suitable biomarkers for high fecal bacteria pollution.

In a survey of pharmaceuticals and endocrine disrupting compounds (EDCs) in Alberta, Canada, measurable concentrations of a number of EDCs were identified in the treated effluents from the Gold Bar WWTP (serves the City of Edmonton). These EDCs included estrone, bisphenol A and nonyphenol (Sosiak and Hebben, 2005). Therefore, aquatic organisms in the North Saskatchewan River (NSR) downstream of this WWTP discharge could be impacted by exposure to EDCs and other contaminants released in the treated effluent. However, sampling of fish and mussels at this site would be very difficult because of the nature of the plume and river volume and velocity. Caging studies are particularly useful because natural exposure gradients can be studied by placing cages at varying distances downstream from the source of contamination and upstream sites can be used for reference locations. This strategy has been used in variety of studies to examine the effects environmental contamination and responses in organisms to exposure to municipal wastewaters (e.g. Stien et al., 1998) seldom, however, with both fish and bivalves exposed at the same time.

In this study, we used an essentially sedentary invertebrate species (freshwater mussel) and a highly mobile vertebrate (fathead minnow) to increase the likelihood of identifying sensitive and robust biomarkers of CECs downstream of the discharge from the Gold Bar WWTP. Fish and mussels were caged for 4 weeks at sites over an exposure gradient downstream of the WWTP discharge, as well as at two upstream reference sites. Biological effects in caged fish and mussels were assessed using biomarkers of oxidative stress, immunomodulation and endocrine disruption, as well as induction of the microsomal Phase I detoxification enzymes, CYP3A and CYP1A1, two enzyme activities involved in metabolism of pharmaceuticals, polycyclic aromatic hydrocarbons and coplanar polychlorinated biphenyls. In addition, passive samplers were deployed in the river at the fish and mussel caging sites to assess the distributions of selected CECs in the receiving waters.

2. Materials and methods

2.1. Study sites

The study sites were located upstream and downstream of the effluent discharge from the Edmonton Power Corporation (EPCOR) Gold Bar WWTP that serves the city of Edmonton. The WWTP serves approximately 730,000 people and has an average daily flow of 255 ML/day. It has tertiary treatment facilities for biological nutrient removal and UV disinfection. Full operating conditions can be found at: http://corp.epcor.com/watersolutions/operations/edmonton/goldbar/pages/goldbar-wastewater-treatment-plant.aspx.

The satellite imagery of the study area obtained using Google Earth (taken in 14 Sep 2008) showed a dark effluent plume on the south side of the river that extended at least 10 km downstream of the WWTP discharge. Two reference sites, NSR1 and NSR2, were established on the south side of the river, 1.25 and 1.10 km, respectively upstream of the WWTP discharge. There are no WWTPs upstream of the reference sites, only stormwater inputs. Downstream of the WWTP discharge

there were also intermittent stormwater discharges, however these were typically dry. Four downstream sites were established at 0.15 km (NSR3), 1 km (NSR4), 2.5 km (NSR5) and 9.9 km (NSR6) downstream of the effluent discharge (Fig. 1). At each of the six sites, fathead minnows (*P. promelas*) and giant floater freshwater mussels (*Pyganodon grandis*) Say, 1829 (synonym: *Anodonta grandis* Say, 1829) (Turgeon et al. 1998) were caged for four weeks from September 13 to October 13, 2011. Both of these aquatic species are native to the NSR.

2.2. Water quality

Conductivity, temperature and dissolved oxygen were monitored at each of the 6 sites (immediately upstream of cages), at least twice per week, over the four week duration of the study, starting on September 13, 2011. On 6 October, 2011 pH was also measured at each site. Water samples for analysis of total coliforms and total heterotrophic counts were collected from all sites on October 12, 2011 and sent for analyses to PBR Laboratories Inc., Edmonton, AB, for testing using standard plate count methods (http://www.epa.gov/ogwdw/methods/pdfs/methods/methods_tcr.pdf).

2.3. Passive samplers

Passive samplers were deployed alongside the mussel cages over the 4-week study period: Polar Organic Contaminants Integrated Sampler (POCIS) for polar contaminants and the Semi-permeable Membrane

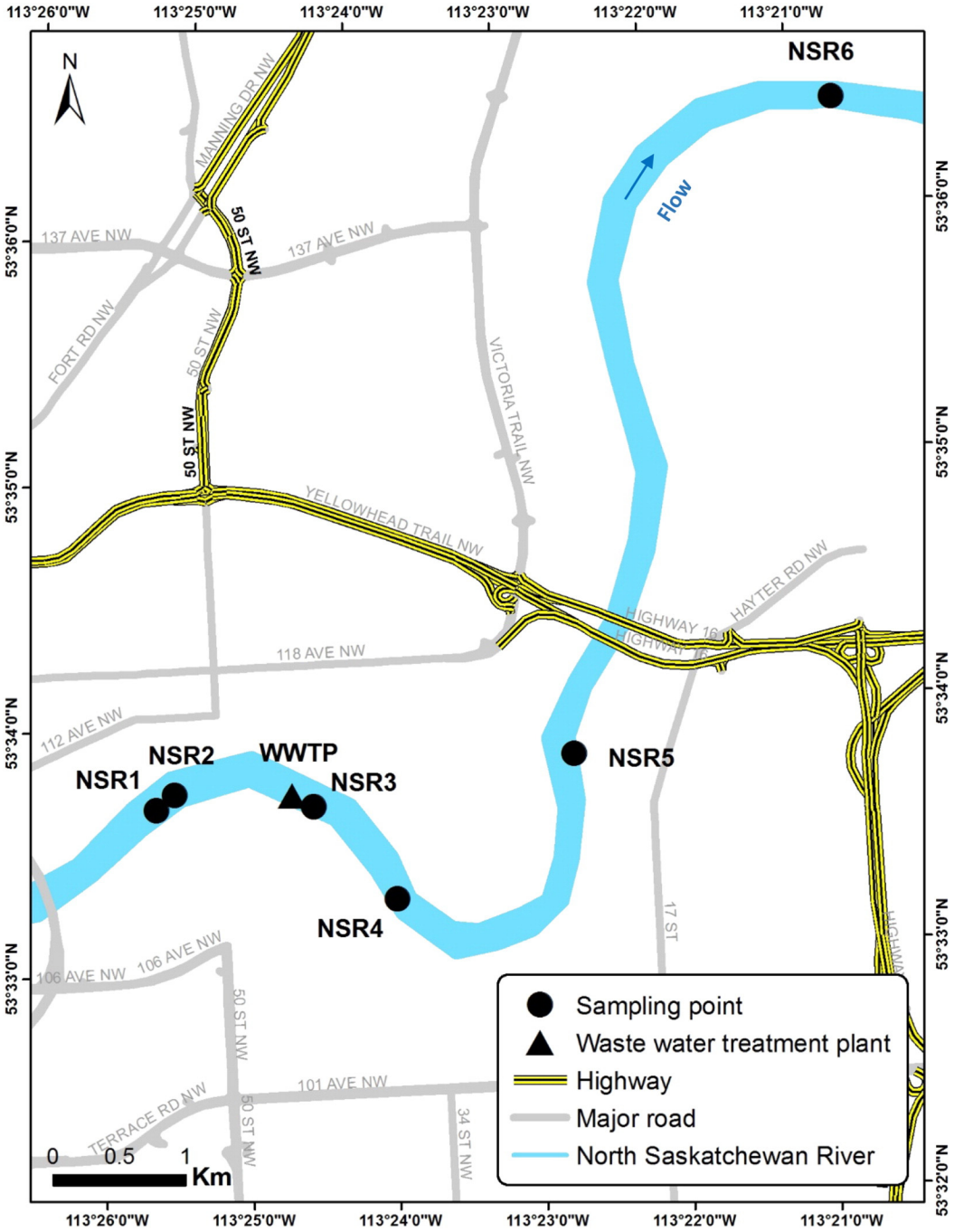

Fig. 1. Location of study sites and the effluent outfall from the Gold Bar Wastewater Treatment Plant (WWTP) on the North Saskatchewan River (NSR), Edmonton, AB, Canada. Control sites are on the south bank, 1.25 km (NSR1) and 1.10 km (NSR2) upstream of the WWTP effluent outfall. Treatment sites are downstream of the outfall at 0.15 km (NSR3), 1 km (NSR4), 2.5 km (NSR5) and 9.9 km (NSR6).

Device (SPMD). At each site, the SPMDs (n = 3) and POCIS (n = 3) were mounted in perforated stainless steel cages, as described previously (Helm et al., 2012; Gillis et al., 2014a) and anchored to the iron rods that were used to hold the mussel cages in place in the substrate of the river.

Non-polar contaminants were extracted and analyzed from the SPMDs using methods described by Helm et al. (2012) and polar contaminants were extracted and analyzed from the POCIS using methods described by Li et al. (2010). Data on the amounts (ng) of these compounds accumulated in the passive samplers over the deployment period were used to estimate the cumulative average concentrations of the target analytes in water (ng/L). These concentrations were estimated using sampling rates (L/d) at 15 °C that were previously determined for the target compounds in POCIS (Li et al., 2010) or in SPMDs (Helm et al., 2012).

2.4. Fathead minnows

2.4.1. Caging and sample collection

Wild, fathead minnows (female: mean weight 1.9 g, range 1.0–3.2 g; mean length: 55 mm, range 40–77 mm; male: mean weight 2.4 g, range 0.8–6.3 g; mean length: 58 mm, range 40–75 mm) were collected from an unnamed pond in Sherwood Park, AB within the NSR watershed according to an approved Alberta Sustainable Resource Development fish research license. All experiments were conducted according to approved University of Alberta Animal Use Committee Protocol #753. The fish were held at the University of Alberta aquatic facility at 12 °C for two weeks prior to deployment. During this acclimation period, the fish were fed 1 mm sinking trout pellets ad libitum every 3 days. A total of 100 fathead minnows were caged at each study site. Approximately the same number of males and females were deployed at each site. The sex of the fish was assessed using secondary sex characteristics, such as coloration, head shape and the presence of nuptial tubercles. The 100 minnows at each site were split into four groups of 25 fish each. Each group was placed in Frabill™ bait-buckets (~6 L) and these, in turn, were placed in larger perforated containers (Rubbermaid™), with two bait-buckets per container. To reduce contamination from the holding conditions, containers were first prepared by removing labels and metal and then pre-soaked in a pond for one month at the Meanook Biological Field station prior to use. The containers were anchored with iron rods at each of the six sites in the river at depths of 40–70 cm. During the study, the fish were fed weekly, ad libitum, by injecting 1 mm sinking trout pellets through tubing placed into each bait bucket.

On October 13, 2011 the bait buckets were removed from the river and transported in river water to the laboratory (<15 km), where the fathead minnows were euthanized in buffered tricaine methane sulfonate (50 mg/L; Syndel). The carcasses were blotted dry and measured for length (mm) and mass (g). Once measured, the fish used for biomarker analysis were dissected and definitively sexed. Liver and gill samples were removed from each fish, placed in 1.5 mL polypropylene microcentrifuge tubes and flash frozen in liquid nitrogen. All frozen tissues were stored at −80 °C.

2.4.2. Biomarkers

The biochemical indicators of contaminant-induced effects selected for fish were conducted using liver and/or gill tissues (Table 1). These biomarkers included induction of two hepatic microsomal enzymes, ethoxyresorufin-O-deethylase (EROD) as an indicator of cytochrome P4501A activity, and benzoxy-4-trifluoromethyl-coumarin (BFC) as an indicator of cytochrome P4503A activity. Biomarkers also included various indicators of oxidative stress including the levels of total glutathione (TGSH), reduced (GSH) and oxidized (GSSG) glutathione, as well as activities of the antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), catalase (CAT), and superoxide dismutase (SOD). Liver of male fish was analyzed for expression of genes coding for CYP1A1 and vitellogenin (Vtg) protein normalized to 18S RNA (Table 1).

Frozen liver microsomes were analyzed for total protein content, and for activity of EROD and BFC. All tissues and homogenates were held on ice during tissue preparation. Liver microsomes were prepared as described by Smith and Wilson (2010), except that 16 mL of homogenization buffer was used per gram of sample, and the final pellet was re-suspended in 5 mL TEDG buffer (0.1 M Tris, pH 7.6, with 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) per gram of original liver sample. Microsomal protein concentrations were determined using the bicinchoninic acid (BCA) assay with a Pierce™ BCA kit (Thermo-Fisher, Toronto, ON, Canada), using bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON, Canada) as a standard. The protein concentration was determined to normalize enzyme activities. For EROD and BFC assays, the microsomes were diluted 5× before reading the fluorescence. For all analyses, the means of replicates (n = 3) were determined, and the absorbance of the blank was subtracted from the mean absorbance of each sample. Concentrations were adjusted for dilution.

EROD activity was determined as described by Smith and Wilson (2010), but miniaturized for a 384-well plate. The mean rates for fluorescence generation per minute were normalized for microsome protein concentrations and converted into pmol product/min/mg protein. BFC activity was determined as described by Smith and Wilson (2010), but modified so that 30 μL of a BSA stock solution was added to each well prior to the addition of substrate to decrease substrate binding to the well. The mean replicate blank-corrected rates for fluorescence generation per minute were normalized for microsome protein concentrations and converted into reaction rates in pmol/min/mg protein using the slope of the standard curve for the 7-hydroxy-4-trifluoromethylcoumarin (HFC; BD Biosciences, San Jose, CA, USA). Glutathione and antioxidant enzyme assays levels in liver and gill tissues were measured as previously described in Massarsky et al. (2013). The activity of SOD (EC 1.15.1.1) was measured using a SOD Assay Kit (Sigma-Aldrich), which uses an indirect assay method based on xanthine oxidase and a color reagent, which absorbs at 450 nm. All enzyme activities were based on protein contents of homogenates assayed using the BCA method (see above) and assessed at 25 °C.

For detecting the expression of vitellogenin (Vtg) and cytochrome P450 1A1 (CYP1A1) in male fish, primer sequences (Table 2) were designed using Primer Express (Applied Biosystems Inc, Burlington, ON, Canada) based on GenBank sequences for the genes of interest. Amplification efficiencies were determined using a standard curve prepared from a serially diluted pool of cDNA and were between 90 and 110%. The resulting primer efficiencies and y-intercepts were used to interpolate the relative abundance of mRNA transcripts in the experimental samples. For quantification of relative abundance of mRNA transcripts, total RNA was extracted from liver tissue using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol (Life Technologies, Burlington, ON, Canada), and RNA quantified using a Nanodrop8000 (Thermo Scientific, Waltham, MA).

One microgram of total RNA was treated with DNase 1 (Sigma-Aldrich) to remove trace genomic DNA contamination and samples were reverse transcribed using a Moloney's-Murine Leukemia Virus reverse transcriptase kit (Life Technologies) according to the manufacturers' protocols. Resulting cDNA was diluted for interpolation from the respective standard curve and genes of interest were amplified using a final concentration of 200 nM of each sense and antisense primer and 7.5 μL PerfeCTa SYBR green fast mix (Quanta Biosciences; Gaithersburg, MD, USA) in a total reaction volume of 15 μL. Samples were cycled 40 times at 95 °C for 15 s + 60 °C for 1 min. All results were normalized to the transcript abundance of 18S RNA using the Ct method according to the manufacturer's directions (Applied Biosystems, Foster City, CA). Normalized data were expressed as fold change relative to the control site NSR1.

Table 1
Biomarkers used to assess biological responses in fathead minnows and freshwater mussels caged at 6 sites in the NSR, including the tissues analyzed and the units of measurement.

Biomarker	Tissue	Units
Fathead minnows		
Microsomal CYP enzymes (EROD, BFC)	Liver (males + females)	pmol product/min/mg protein
Glutathione (TGSH, GSSG, GSH)	Liver, gill (males + females)	nmol/g tissue
Antioxidant enzymes (GST, GR, GPx, SOD, CAT)	Liver, gill (males + females)	μmol or nmol/min/mg protein
Expression of Vtg gene	Liver (males only)	Fold difference from control[a]
Expression of CYP1A1 gene	Liver (males only)	Fold difference from control[a]
Freshwater mussels		
Lipid peroxidation (TBARS)	Digestive gland	μmol/MDA per mg protein
Antioxidant enzyme (GST)	Digestive gland	Absorbance change/min/mg protein
Vtg-like protein	Gonad	μg ALP/mg protein
Hemocyte density	Hemolymph	Number hemocytes/mL hemolymph
Lysozyme activity	Hemolymph	U/mg protein[b]

[a] Normalized to expression 18S RNA.
[b] One unit (U) of lysozymal activity is equal to the quantity of enzyme that causes a decrease in absorbance of 0.001 per minute at pH 6.2 and 25 °C.

2.5. Mussels

2.5.1. Caging and sample collection

Freshwater mussels (*P. grandis*- 12–26 g — whole animal wet weight) were collected from Long Lake in the NSR watershed approximately 1 week before deployment and kept together with the fathead minnows at 12 °C in the University of Alberta aquatic facility. The mussels were placed in cages in the NSR at the 6 study sites noted above on September 13, 2011. The design and construction of the mussel cages was as described by Gillis et al. (2014a). The cages were anchored to iron rods and submerged to a depth of 40–70 cm. Each cage contained 25 mussels and two cages were placed at each site (50 mussels per site).

Mussels were removed from the cages on October 13, 2011 and transported in river water to the laboratory for processing. The total depuration time in river water was approximately 2 h. Whole animal wet weight was determined and digital calipers were used to measure the external parameters of shell length, height and width. The tissues were collected as described previously by Gillis et al. (2014a). Hemolymph was collected from the sinus of the posterior abductor muscle and immediately distributed into either 96-well micro-plates for hemocyte density and protein assessment, or centrifuged to separate plasma (supernatant) from the cell pellet. Plasma was preserved at −80 °C for lysozyme activity assessment. Mussels were dissected to obtain digestive gland and gonad tissues for biomarker analysis. Gravidity (i.e. presence of brooding glochidia) of the mussels was noted upon dissection. Tissue samples were stored at −80 °C until analyzed for biomarkers.

2.5.2. Biomarkers

Lipid peroxidation (LPO) and glutathione-*S*-transferase (GST) activities were examined in digestive gland and vitellogenin (Vtg)-like proteins in gonadal tissue were selected as biochemical indictors of contaminant-induced biological effects (Table 1). All biomarker measurements were normalized against total protein content in the tissue homogenate using a Bradford (1976) assay as noted below.

For GST analysis, digestive gland tissues were dissected on ice, and homogenized with 10 mM Hepes-NaOH buffer (pH 7.4), containing 125 mM NaCl, 0.1 mM EDTA and 0.1 mM dithiothreitol at a 1:5 (w/v) ratio. The homogenate was centrifuged, and the supernatant was separated from the pellet and utilized for determination of GST activity. GST was measured following the method developed by Boryslawskyj et al. (1988). Briefly, 50 μL supernatant was added to 200 μL of 1 mM GSH and 1 mM CDNB in 10 mM Hepes-NaOH buffer (pH 6.5) containing 125 mM NaCl. Absorbance (340 nm) was measured at 0, 5, 10, 20 and 30 min to determine the appearance of the glutathione conjugate. Results were expressed as absorbance change per min per mg protein.

LPO in digestive gland was determined by the thiobarbituric acid reactive substances (TBARS) method as described by Oakes and Van Der Kraak (2003). Fluorescence was measured at 520 nm excitation and 590 nm emission with a Chameleon® fluorescence microplate reader (Bioscan, Washington, DC, USA). The results were expressed as μmol MDA produced per mg of total protein.

The relative levels of Vtg-like proteins were determined using the alkali labile phosphate assay performed on high molecular weight proteins (Bouchard et al., 2009). Gonad tissues for Vtg analysis were homogenized at 4 °C in 25 mM Hepes buffer at pH 7.5, containing 125 mM NaCl, 1 mM dithiothreitol and 1 mM EDTA. The homogenate was then centrifuged and the supernatant was removed. The poorly soluble high-molecular-weight proteins in this supernatant were precipitated with 35% acetone. The pellet was resuspended in the homogenization buffer and mixed with one volume of 0.8 M sucrose containing 10 mM MOPS buffer, pH 7.0, 1% SDS and 0.001% bromophenol blue (gel tracking) The levels of Vtg-like proteins were determined by high resolution denaturing gel electrophoresis (Invitrogen Inc., USA) and silver staining. A standard solution of BSA was used for inter-gel calibration and Vtg standards from rainbow trout were used. Band intensity was analyzed from pictures of stained gels using Un-scann-it gel software (Silk Scientific Inc. Orem, Utah, USA).

2.5.3. Immune function

Biomarkers of immune function (Table 1) were determined using hemolymph (~1 mL). Lysozyme activity was measured in mussel plasma according to Lee and Yang (2002). Lysozyme activity was normalized to plasma protein concentration following the protein–dye binding method (Bradford, 1976) with BSA as a standard.

Hemocyte density was determined by flow cytometry using a Guava easyCyte HT instrument (Millipore, Hayward, CA, USA). A 200 μL sample of fresh hemolymph was preserved with an equal volume of 0.1% formalin and held at 4 °C pending analysis. A 100 μL aliquot of preserved hemolymph was then diluted (1:1) with ViaCount® reagent (EMD Millipore, Billerica, MA, USA). Hemocyte density was quantified using a Guava® ViaCount® assay and expressed as the number of cells per mL (± SE) of hemolymph. Immune activity in the mussels was expressed as the ratio of lysozyme activity per hemocyte.

Table 2
Primer sequences used for analysis of mRNA abundance in male fathead minnows caged at 6 sites in the NSR.

Accession number	Primer name	Sequence (5′-3′)	Amplicon size (bp)
AF130354	VTG-sense	GGG AGA GGC TGC CAA TAA TGT	109
	VTG-antisense	TCA CTG TTC GTC GCT CTT TCA A	
AJ277866.1	CYP1a1-sense	TGC AGC CCG TAG AAG AGG AT	83
	CYP1a1-antisense	CGT TGT TTC TCC GCT GAT GTT	

2.6. Statistical analysis

Data are presented as means with standard error of the mean (±SE). One-way ANOVA was used to determine statistical differences in biomarker responses; control sites were not pooled. For fathead minnows, log transformed data were used for data on gene expression and Student–Newman–Keuls test was chosen as the *post-hoc* test whenever differences occurred. For mussels, the ANOVA was followed by Tukey's Test. Where data that did not meet the normality and/or equal variance assumptions, differences were identified with Kruskal–Wallis ANOVA on Ranks followed by Dunn's Test. In all cases $p < 0.05$ was considered significant. Each individual (fish or mussel) at a particular site was considered a replicate.

Multivariate analyses were carried out using Principal Component Analysis (PCA) and Correspondence Analysis. The average values of biomarker and contaminants per site were compiled in contingency tables. To achieve all statistical pre-requisites for correspondent analysis, each variable was z-normalized plus the corresponding lowest z-score. These data were used for correspondence analysis performed with the library 'ca' (Nenadic and Greenacre, 2007) available on R software (R Core Team, 2013). Correspondence analysis was used to examine the association of contaminants of emerging concerns and (1) immune function (mussels), (2) biochemical biomarkers (mussels), biochemical and genetic biomarkers in (3) liver (fathead minnows) and (4) gills (fathead minnows).

PCA of variability in biomarkers of fathead minnows in relation to sex and in mussels in relation to gravidity was performed with the library vegan (Oksanen et al., 2015). For the fathead minnows, homoscedasticity and homogeneity were not guaranteed since both liver and gills were analyzed together.

3. Results and discussion

3.1. Water quality

Water temperatures in the river fluctuated during the experiment, both over time and with distance from the effluent plume [data presented in Supplementary Information (SI)]. Temperatures at all sites demonstrated a cooling trend over time, consistent with seasonal cooling over September to October. Water temperatures at the NSR1 and NSR2 reference sites were consistently cooler than the impacted downstream sites; the reference site temperatures ranged from 14.0 °C on September 26 to 8.1 °C on October 14 (SI Fig. 1). At the NSR3 site, located 0.15 km downstream of the effluent discharge, water temperatures varied between 15.4 °C and 18.6 °C, or up to 7 °C higher than temperatures at the reference sites. The water temperature at NSR4 (i.e. 1 km downstream from effluent discharge) was typically mid-way between the reference sites and the NSR3 site. At the NSR5 (i.e. 2.5 km downstream) and NSR6 (i.e. 9.9 km downstream) sites, the temperatures were still up to 1 °C warmer than at the reference sites.

Electrical conductivity of river water (SI Fig. 2) was consistently lowest at both reference sites, ranging from 329 μS/cm to 342 μS/cm. The conductivity at NSR3 at 0.15 km downstream of the discharge (580 μS/cm to 838 μS/cm) was typically double the conductivity at the reference sites. The electrical conductivity at the site 1 km downstream (NSR4) was clearly elevated, at >500 μS/cm. At the sites 2.5 km and 9.9 km downstream (i.e. NSR5 and NSR6), electrical conductivity was still slightly elevated by approximately 20 μS/cm, relative to the reference sites.

All sites were well oxygenated, although the NSR3 site had the lowest concentrations of Dissolved Oxygen (DO), ranging from 9.5 to 10.5 mg/L. The remainder of the sites generally had DO concentrations between 11 and 12 mg/L. Since temperature has an impact on oxygen saturation, the sites were compared using %DO saturation (SI Fig. 3). All sites except NSR3 showed >100% saturation at all times. Overall, site NSR3 had the lowest %DO saturation, but on only one occasion (i.e. between October 9 to14) was measured at <100% saturation (approximately 97% saturation). Measurements of pH also showed differences between upstream and downstream sites. The pH at the reference sites was pH 8.5, while the pH at NSR3 and NSR4 was 7.5 and at NSR6 the pH was 8.3.

Heterotrophic bacteria were detected at all sites (SI Fig. 4a) with plate counts of colony forming units (CFU — an estimate of viable bacteria) of approximately 300 CFU/mL at each reference site (one CFU count per site). The highest counts were at the NSR3 site, with counts of 2,200 CFU/mL, and at all the other downstream sites (i.e. NSR4 to NSR6) the heterotrophic plate counts were >1500 CFU/mL. The trends in total coliform bacteria were similar, with the highest counts occurring at the four downstream sites (SI Fig. 4b). Curiously, total coliform counts were highest at the NSR5 site (i.e. 38,000 CFU/100 mL), while at the NSR3 site located 0.150 km downstream of the treated wastewater discharge, the total coliform counts were 5000 CFU/100 mL. Coliform bacteria were detected at the reference sites at counts between 800 to 900 CFU/100 mL each (SI Fig. 4b).

The field measurements of conditions in the river (see SI figures) confirmed that the treated wastewater definitely impacted the water quality at the four downstream study sites, with temperature, conductivity and total heterotrophic bacteria counts that were consistently elevated compared to the upstream control sites. The NSR at Edmonton is a large hydrological system that can have discharges >4000 m^3/s at peak flow. However, during the study period, the discharges ranged from 96 to 200 m^3/s, which are near the historical lows for flows in the river at this time and location, according to records published online by Water Survey of Canada (i.e. hydrological station 05DF001). Therefore, the conditions during the study period are likely to represent a scenario of near maximum exposure of aquatic organisms to the wastewater from the Gold Bar WWTP.

3.2. Passive samplers

The POCIS and SPMD passive samplers deployed in the NSR at sites downstream of the discharge from the WWTP accumulated all of the target analytes in amounts that were detectable by LC–MS/MS or GC–MS. The estimated time weighted average concentrations in water in ng/L (±SE) are summarized in Table 3. At the two reference sites, the target analytes were present in amounts close to or below the limits of detection. Without exception, all target analytes were present at the greatest estimated concentrations at the NSR3 site, just downstream of the WWTP effluent discharge. Notably, estrone was detected only at this site at a time weighted average concentration of 0.22 ± 0.01 ng/L. The estimated time weighted average concentrations of the remaining target analytes decreased from NSR3 to NSR6. With the exception of estrone, all target analytes were still detectable at NSR6, located 9.9 km downstream from the WWTP effluent discharge. Although androstenedione was detected at NSR6, the estimated concentration was below the limit of quantitation (<LOQ). The synthetic musk, HHCB (Galaxolide®), was present at by far the highest concentrations among the target analytes, with an estimated time weighted average concentration of approximately 382 ng/L at the NSR3 site (Table 3). While HHCB does not induce vitellogenin induction in carp (Smeets et al., 1999) it is known to inhibit multixenobiotic resistance transporters in mussel gills (Luckenbach et al., 2004) and this indicates that oxide may act by reducing the clearance of other organic anions. However, values found in this study are <10% of the probable no effect level (4.34 ug/L) as outlined in the JRC EU Risk Assessment report (2008).

The target analytes selected for analysis comprise pharmaceuticals and personal care products (PPCPs) and two natural steroid hormones commonly detected in municipal wastewater and in surface waters downstream of WWTP discharges. Several of these indicator compounds were also selected by Dickenson et al. (2011) for monitoring a small number of indicator PPCPs in wastewater treatment facilities in the USA.

Table 3
Estimated time weighted average (TWA) concentrations (ng/L ± SE) of contaminants of emerging concern accumulated in POCIS and SPMD passive samplers at sites in the NSR located upstream (i.e. NSR1 and NSR2) and downstream (i.e. NSR3 to NSR6) of the discharge from the Gold Bar WWTP. For codes used in the table see Abbreviations section.

Site	POCIS (n = 3 per station)							SPMDs (n = 3 per station)		
	CBZ	IBU	TPM	SMX	GMZ	EST	AND	TCS	HHCB	AHTN
NSR1	0.14 ± 0.0	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	0.39 ± 0.18	0.11 ± 0.01
NSR2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
NSR3	22.27 ± 3.18	60.47 ± 1.95	17.93 ± 4.30	5.34 ± 0.63	0.90 ± 0.06	0.22 ± 0.01	0.37 ± 0.10	8.85 ± 0.78	381.65 ± 19.64	21.64 ± 1.00
NSR4	21.63 ± 0.24	48.37 ± 4.01	14.94 ± 2.79	3.87 ± 0.40	0.78 ± 0.05	<LOD	0.32 ± 0.07	5.46 ± 018	169.78 ± 45.36	7.27 ± 1.16
NSR5	13.60 ± 1.75	24.15 ± 4.68	14.60 ± 4.67	1.85 ± 0.18	0.37 ± 0.06	<LOD	0.20 ± 0.04	1.25 ± 0.08	122.64 ± 21.35	5.46 ± 0.42
NSR6	10.96 ± 1.57	14.46 ± 0.67	6.95 ± 2.76	1.41 ± 0.23	0.24 ± 0.04	<LOD	<LOQ	0.53 ± 0.09	102.09 ± 12.15	3.93 ± 0.97

<LOD = Below detection limits; <LOQ = Below limits of quantitation.

The data show that the concentrations of all analytes increased at the first deployment location immediately downstream of the WWTP discharge (i.e. NSR3) and then declined with distance downstream. The declines in estimated concentrations with distance downstream of the plant discharge are in agreement with the attenuation of conductivity measurements at these sampling sites in the river. The time weighted average concentrations of the indicator compounds estimated from the passive sampler data are consistent with the concentrations of these compounds that have been measured in surface waters and in WWTP effluents at other locations in Canada (Li et al., 2010; Lishman et al., 2006; Yang and Metcalfe, 2006; Chen et al., 2006; Brun et al., 2006; Sosiak and Hebben, 2005; Servos et al., 2005; Metcalfe et al., 2003). Overall, these data indicate that the fathead minnows and freshwater mussels caged downstream of the WWTP were exposed to CECs at ng/L concentrations over the 4 week deployment period.

3.3. Fathead minnows

All fathead minnows, survived the caging experiment.

3.3.1. Biomarkers

In the fathead minnows, there were no statistically significant differences in mean EROD activities between fish caged at the 6 sites, although there was a non-significant trend of higher activities in the livers of fish caged at sites NSR3 and NSR4 (Fig. 2A). The lack of significance was due to the high variability in responses among fish caged at each site. For hepatic BFC activities in fathead minnows, there was also a trend of higher mean activity in fish caged at the NSR3 and NSR4 sites relative to the fish caged at upstream and downstream sites (Fig. 2B). However, only in fish from the NSR4 site was the mean BFC activity significantly elevated relative to fish from the reference sites. There was also considerable variability in BFC activity among the fish caged at each site, although this variability was not as extreme as for EROD. Note that the mean BFC activity in fish from NSR1 was calculated after removing data for a single fish judged to be an outlier (Fig. 2B).

Principal Component Analysis (presented in the final section) indicated that there was similar variability in biomarker responses in male and female fathead minnows (SI Fig. 7). This variability in microsomal activity may have been due to site-specific temperature and conductivity fluctuations.

Juvenile rainbow trout exposed to pharmaceutical industry effluents also showed elevated EROD (Gunnarsson et al., 2009). In mammals, isoforms of the CYP3A subfamily are involved in the metabolism of many pharmaceuticals (Hemeryck and Belpaire, 2002). Importantly, CYP3A4 has been identified from rainbow trout microsomes with similar substrate specificity and catalytic activity as the human CYP3A isoform (Buhler and Wang-Buhler, 1998). Therefore, it is possible that exposure to pharmaceuticals in the wastewater plume was responsible for induction of these microsomal enzymes. On the other hand, exposure of fish to some pharmaceuticals has been shown to inhibit CYP-mediated metabolism, including EROD and BFC activity (Smith et al., 2012), suggesting that the obtained results may reflect the complex synergistic/antagonistic dynamics of CECs and other constituents present in the effluent.

Levels of TGSH and GSH in the liver of fathead minnows caged at the first upstream reference site (i.e. NSR1) were significantly higher than in minnows caged at the rest of the sites, including the second upstream reference site (Table 4). At the rest of the sites, there were no significant differences in the mean levels of TGSH, GSSG and GSH in liver (Table 4). There were no significant differences in the mean levels of any of the glutathione parameters in the gill tissues of fathead minnows caged at the various locations (Table 4). The hepatic GSH levels (1226–2436 nmol/g) reported in this study were slightly higher than those previously reported by Wallace (1989) for fathead minnows (i.e. 0.9 ± 0.6 μmol/g). The GSSG levels were higher and the TSGH levels were lower in gill compared to the liver. There were no obvious spatial trends in the data for glutathione parameters in gill tissue.

In healthy tissues, more than 90% of the total glutathione pool is in the reduced form (GSH) and less than 10% exists in the oxidized form

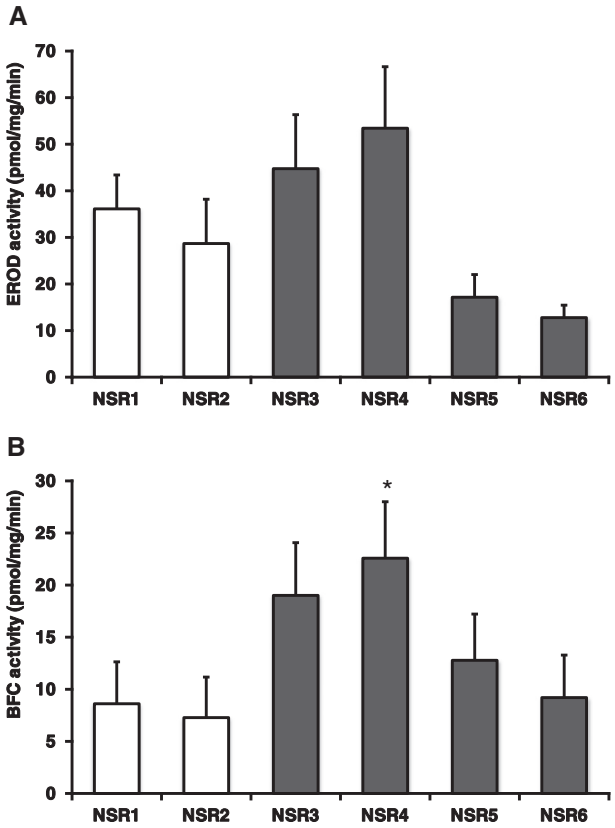

Fig. 2. Activity of EROD (A) and BFC (B) in the livers of fathead minnows (n = 10 for all except for NSR1, BFC where n = 9) caged at 6 sites in the NSR. The bar (mean ± SE) identified with an asterisk (*) was significantly ($p < 0.05$) different from the reference sites. Open bars represent control sites. Note that one outlier data point was removed from the BFC data for the NSR1 site.

Table 4
Mean levels (±SE) of the various forms of glutathione (nmol/g tissue) in the liver and gills of fathead minnows caged in the NSR at 6 sites (n = 10 per site).

	NSR1	NSR2	NSR3	NSR4	NSR5	NSR6
Liver						
TGSH	2688 ± 245[a]	1934 ± 203	1993 ± 200	1543 ± 213	1593 ± 105	1527 ± 111
GSH	2436 ± 192[a]	1671 ± 254	1605 ± 215	1226 ± 229	1363 ± 96	1281 ± 62
GSSG	126 ± 29	132 ± 29	194 ± 20	158 ± 22	115 ± 10	123 ± 26
Gill						
TGSH	1554 ± 66	1547 ± 15	1705 ± 140	1784 ± 51	1474 ± 145	1684 ± 68
GSH	1025 ± 46	1087 ± 20	1131 ± 143	1313 ± 54	922 ± 164	1222 ± 74
GSSG	264 ± 16	230 ± 9	287 ± 6	236 ± 6	276 ± 16	231 ± 12

[a] Significantly different from all other sites.

(GSSG). An increased GSSG-to-TGSH ratio is considered indicative of oxidative stress; it is the result of the conversion of glutathione from reduced to oxidized form as radicals with oxidative potential are neutralized (Kelly et al., 1998). The mean GSSG to TGSH ratios in the livers of the fish caged at the most impacted sites (i.e. NSR3, NSR4) were significantly elevated relative to the reference sites (Fig. 3). Therefore, these data for glutathione levels in liver show evidence of oxidative stress in fathead minnows exposed to the wastewater plume. There were no similar trends in the GSSG to TGSH ratios for glutathione parameters in gill tissue. In contrast, Gunnarsson et al. (2009) did not observe any changes in TGSH, GSSG or the ratios of these proteins in the liver of juvenile rainbow trout exposed to diluted effluents from the pharmaceutical industry; however these differences could be attributed to the different exposure regimens and fish species used.

The data for GR activity in the liver demonstrated differences for fish caged at the NSR3 and NSR4 sites, where levels were significantly elevated relative to fish caged at the other sites (Fig. 4). For GST activity in liver, the fish caged at the NSR3 site also had significantly elevated levels relative to fish caged at both upstream and downstream sites (Fig. 4). Hepatic SOD activities were significantly lower in fish caged at the NSR3 site relative to most other locations, except that SOD activity was also depressed in fish caged at the NSR6 site (Fig. 4). The data for the activities of the remaining antioxidant enzymes in liver showed no obvious trends that would reflect exposure to wastewater. The activities of the antioxidant enzymes in the gill were generally lower than those in the liver, and there were no obvious exposure-related trends, except for a slight but significant elevation of gill GR and GST activities in fish caged at the NSR3 and NSR4 sites (Fig. 4).

The activities of the hepatic antioxidant enzymes reported in the present study are comparable to those reported previously for fathead minnows following metal exposure (Lapointe et al., 2009). The antioxidant enzyme activities reported previously for gill tissue of rainbow trout exposed to triazole fungicide were similar to the enzyme activities in the gills of fathead minnows reported in the present study (Li et al., 2011).

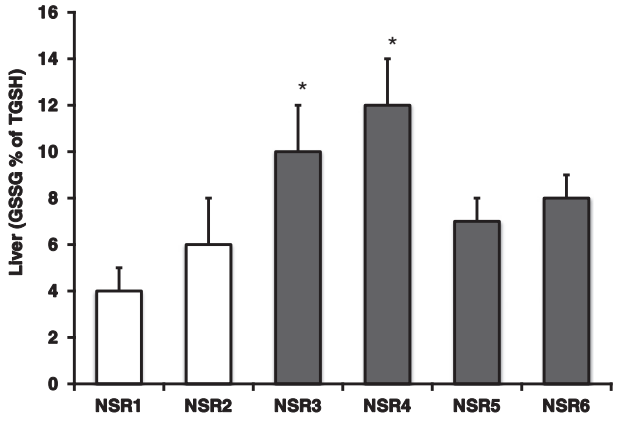

Fig. 3. Oxidized glutathione (GSSG) as a percentage of total glutathione (TGSH) in the livers of fathead minnows (n = 10) caged at 6 sites in the NSR. Bars (mean ± SE) identified with an asterisk (*) were significantly ($p < 0.05$) different from the reference site NSR1. Open bars represent control sites.

Significantly elevated GR and GST activities were measured in the livers and gills of fish caged at the NSR3 and/or NSR4 sites. However, the hepatic SOD activity was reduced at the NSR3 site, as well as at the NSR6 site. The increase in the activities of some of the antioxidant enzymes (i.e. GR and GST) in the livers and gills of fish caged at the wastewater-impacted sites suggests that there was oxidative stress in these fish. Sturve et al. (2008) reported that GR and CAT were elevated in the livers of rainbow trout exposed to municipal wastewater. Cazenave et al. (2014) observed elevated GST and GR in the livers of fish caged in a river 0.2 km downstream of a wastewater discharge, but fish caged 2 km downstream did not show elevated antioxidant enzyme activities. It has been shown previously that some pollutants, such as cadmium, are able to inhibit catalase and SOD activities in fish (Roméo et al., 2000; Asagba et al., 2008; Ling et al., 2011), so the decline in hepatic SOD activity in fish from the NSR3 site may indicate that metal contaminants not assessed here were present downstream of the WWTP. It should be noted that increased GST activity may not solely reflect oxidative stress, but also elevated xenobiotic metabolism, since this enzyme conjugates GSH with not only harmful products of lipid peroxidation, but also with various xenobiotics (Lushchak et al., 2001).

Gene expression analysis revealed further impacts of effluent exposure. CYP1A1 mRNA abundance in liver tissue from male fathead minnows was significantly increased in fish caged at NSR3, NSR4 and NSR5 relative to fish caged at the upstream reference site, NSR1 (Fig. 5A). These data lend support to the EROD data showing a trend of induction of the activity of a microsomal enzyme from the CYP1A class, even though this trend was not statistically significant (Fig. 2A). The selection of only male fathead minnows for the analysis of gene expression may be the reason why there was much reduced variability in CYP1A expression among fish caged at each site relative to the EROD data (combined male and female data). Miller et al. (2003) observed a strong correlation between EROD activity and levels of CYP1A protein in English sole (*Pleuronectes vetulus*) collected from contaminated sites in Puget Sound, USA. Gunnarsson et al. (2009) detected elevated expression of CYP1A in the livers of juvenile rainbow trout exposed to effluents from the pharmaceutical industry. Therefore, it is possible that this biomarker may be useful for detection of effect of WWTP effluents as it showed strong correlation with distance from the WWTP. However, as our PCA analysis now demonstrates, the potential impacts of other similar abiotic parameters that co-vary with distance (e.g. temp,) make exact associations of cause and effect difficult.

Vtg mRNA abundance was also elevated in the livers of male fish caged at sites NSR3 and NSR4 relative to the upstream reference sites (Fig. 5B). No significant differences in abundance were found for female fish from any of the exposure sites (data not shown). Increased levels of the Vtg egg yolk precursor protein in male fish are used as a general biomarker of exposure to contaminants with estrogenic activity (Mylchreest et al., 2003; Kavanagh et al., 2004). Recently, molecular techniques to quantify abundance of the Vtg gene have provided another biomarker for exposure to environmental estrogens (Silva de Assis

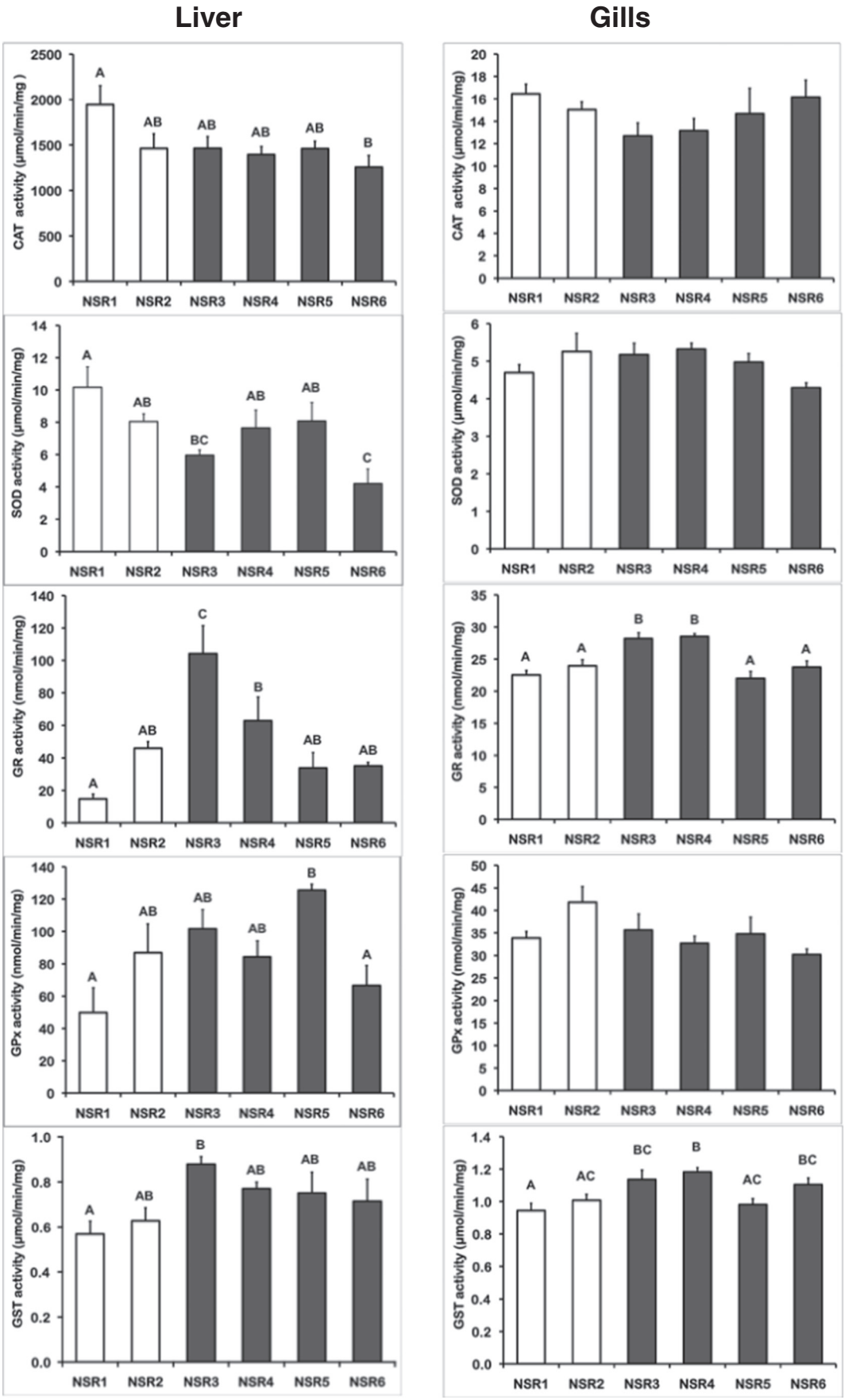

Fig. 4. Activity of antioxidant enzyme catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione-*S*-transferase (GST) in liver and gill tissues of fathead minnows (n = 5) caged at 6 sites in the NSR. Bars identified with different letters are significantly ($p < 0.05$) different. Open bars represent control sites.

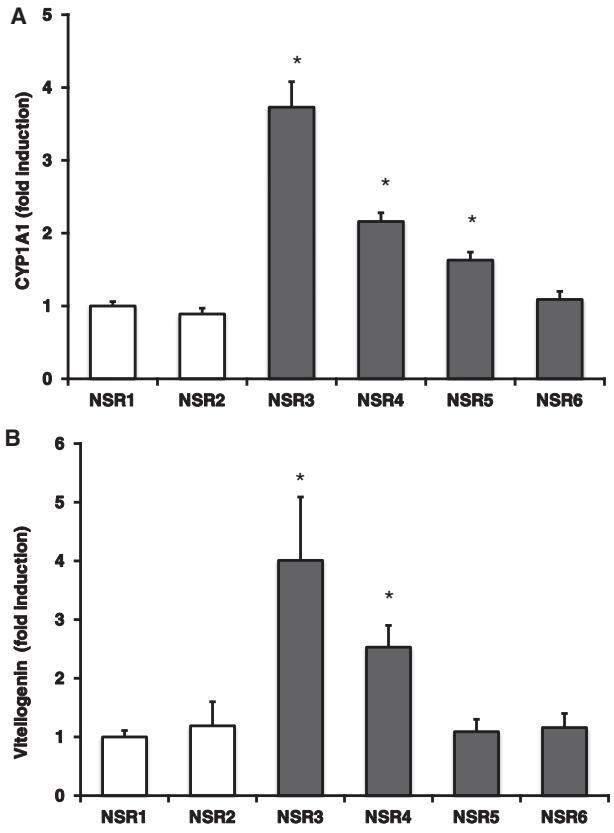

Fig. 5. mRNA abundance of (A) CYP1A1 (n = 12) and (B) vitellogenin (n = 6) normalized to 18S RNA and presented as fold-induction relative to abundance at NSR1 in the livers of male fathead minnows caged at 6 sites in the NSR. Bars (mean ± SE) identified with an asterisk (*) were significantly ($p < 0.05$) different from the reference sites. Open bars represent control sites.

et al., 2013). Notably, NSR3 was the only site with measurable amounts of estrone (Table 3) with the concentrations at the remainder of the sites falling below the limits of detection. It is likely that other co-occurring estrogens were also present that may account for the measured response.

3.4. Freshwater mussels

All mussels survived the caging experiment.

3.4.1. Biomarkers

On retrieval from the caging exposures, many of the mussels were gravid i.e. brooding glochidia in the gill. The percentage of gravid mussels in the cages was >50% at all stations, except at NSR4, where the percentage of gravid mussels was reduced to approximately 20% (SI Fig. 5). Perhaps elevated water temperature (SI Fig. 1) but somewhat diluted effluent induced the mussels to release their glochidia. *P. grandis* typically brood glochidia over winter and release them the following spring (Watters et al., 2009) when water temperature increases. However, freshwater mussels have also been shown to prematurely release glochidia when exposed to pharmaceuticals such as selective serotonin reuptake inhibitors (Cunha and Machado, 2001; Bringolf et al., 2010). Nevertheless, multivariate analysis (presented at the end of the paper) demonstrated that gravidity had no effect on biomarker expression (SI Fig. 8).

Two biomarkers of oxidative stress were examined in the digestive gland of the caged mussels. Lipid peroxidation (LPO) was analyzed using the TBARS assay. Although significant differences ($p < 0.05$) existed between sites in the mean LPO values for mussel digestive gland, the responses did not reflect spatial variations in the degree of exposure to wastewater in the NSR (Fig. 6). However, Gagné et al. (2011), Gillis et al. (2014b), and Machado et al. (2014) observed elevated LPO in other species of wild freshwater mussels collected from locations downstream of municipal wastewater effluent outfalls, and Gillis et al. (2014a) reported a significant increase in LPO in freshwater mussels caged downstream of a WWTP. Data on the mean levels of GST in mussels caged in the NSR showed no significant differences between sites (SI Fig. 6). In contrast, previous studies have shown significant elevation of GST in both caged (Farcy et al., 2011; Gillis et al., 2014a), and wild (Machado et al., 2014) freshwater mussels exposed to municipal wastewater effluents, reflecting perhaps a species- and/or exposure-specific difference.

The mean levels of Vtg-like protein in the gonad of mussels caged in the NSR varied between sites (Fig. 7), with significantly lower levels of Vtg-like protein in mussels caged at NSR3, NSR4 and NSR6. Gillis et al. (2014a) observed slightly elevated levels of Vtg-like proteins in mussels (*L. costata*) caged in a plume of municipal wastewater, but only when data for females were excluded. In contrast, Hellou et al. (2003) observed reduced levels of Vtg-like proteins in the gonads of female marine mussels (*M. edulis*) collected from intertidal regions impacted by municipal wastewater, a response similar to the one seen here in the gravid mussels caged at NSR3 and NSR4.

The species used in the present study (*P. grandis* Say, 1829; synonym: *A. grandis* Say, 1829) is known to exhibit hermaphroditism: specimen containing both sperm and eggs have been described histologically (van der Schalie and Locke, 1941). A well-studied population in Narrow Lake (near source of mussels for this study) of this species was assumed to be hermaphroditic by Jansen and Hanson (1991) based on the proportions of gravid specimens in various months and years in the population. A larger sample size of non-gravid mussels would have been preferred in the present study to confirm whether the changes observed in the levels of Vtg-like protein reflect exposure to CECs with estrogenic activity in wastewater.

3.4.2. Immune function

To investigate the impact of the exposure to wastewater on immune function in mussels, hemocyte concentrations and plasma lysozyme activities were determined in the hemolymph. As illustrated in Fig. 8, the mussels deployed directly in the effluent plume at NSR3 had significantly fewer hemocytes compared to the sites upstream of the WWTP (NRS1 and NSR2) and sites further downstream (NSR5 and NSR6). A significant increase in lysosomal activity per hemocyte was identified in caged mussels from the NSR3 site compared to upstream sites (NSR1 and NSR2). The significant increase in lysosomal activity per hemocyte in the mussels caged in the effluent plume probably results from exposure to bacteria of wastewater origin, which is consistent with the elevated counts of indicator bacteria detected in the river at these downstream sites. Lysozymes are a key component in the immune response of bivalves to waterborne pathogenic bacteria. The decline in hemocytes also observed at these impacted sites is consistent with prolonged exposure to bacteria. Gillis et al. (2014a) observed a decline in hemocytes and an increase in lysozymal activities in freshwater mussels caged at a site downstream of a wastewater discharge. Gagné et al. (2008) observed similar immunological effects in freshwater mussels exposed to wastewater. However, Canesi et al. (2007) reported that exposure of marine mussels to estrogenic compounds decreased hemocyte viability by increasing lysosomal membrane permeability. In our study, correlation analysis between lysozyme activities and the presence of heterotrophic bacteria were significant. However, it could not be ruled out that here estrogenic chemicals contributed to the immunological effects observed in the present study.

3.5. Multivariate analysis

Correspondence analysis for association of contaminants of emerging concerns and biomarker responses in fathead minnows and mussels

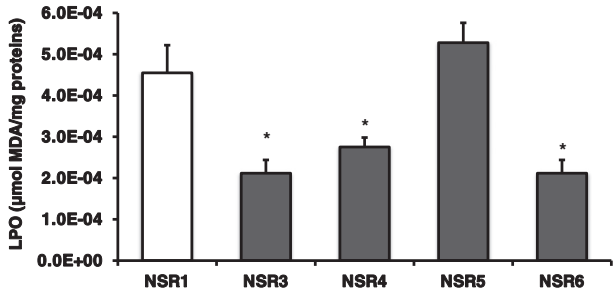

Fig. 6. Lipid peroxidation (LPO) in the digestive gland of mussels caged at 5 sites (only one reference site used for this component) within the NSR ($n_{NSR1} = 10$; $n_{NSR3} = 10$; $n_{NSR4} = 10$; $n_{NSR5} = 10$; $n_{NSR6} = 10$). Bars (mean ± SE) identified with an asterisk (*) are significantly ($p < 0.05$) different from control. Open bar represents control site.

identified the wastewater effluent plume as the primary cause for the ordering of the caging sites along the correspondence dimension 1 axis (Fig. 10A, B, C and D).

The negative association between hemocyte numbers and lysosome activity supports the trends illustrated in Figs. 8 and 9, as is the association of high lysosome activity with the most impacted sites (NSR3 and NSR4) (Fig. 10A). The emergent contaminants, Carbamazepine (CBZ) and Trimethoprim (TPM) an anti-epileptic and an antibiotic respectively, show a close association with lysosome activity while temperature (temp) and conductivity (EC) appear to be less important (Fig. 10A). CBZ and TPM were below detection at the control sites and had highest concentrations at NSR3 and NSR4 (Table 3). Mussel biochemical biomarkers did not show much association with any plume-parameter (Fig. 10B).

In fathead minnow livers, GST activity and GSSG/TGSH was most related to CBZ and TPM rather than to the measured environmental plume parameters (Fig. 10C). In the gills of fathead minnows, GR appeared to be the best biomarker, having a significant response at NSR3 and NSR4 (Fig. 4) and the Correspondence Analysis showing that water temperature, pH and electrical conductivity had little influence on fish GR expression (Fig. 10D).

Principal component analysis of variability in biomarkers of fathead minnows in relation to sex illustrated that there was similar variability in male and female biomarker responses (SI Fig. 7). This supports the pooling of both sexes for fish biomarker analyses. Likewise, for mussels, the PCA of variability in immunobiomarkers in relation to gravidity showed similar variability of responses in gravid and non-gravid mussels (SI Fig. 8).

4. Conclusions

Biomarkers altered in fathead minnows caged in the wastewater plume included induction of BFC metabolism, an enzyme activity

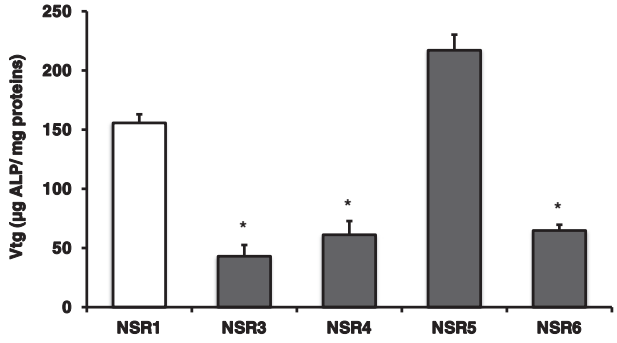

Fig. 7. Vitellogenin abundance per mg in the gonads of mussels caged at 5 sites (only one reference site is used for this component) within the NSR ($n_{NSR1} = 10$; $n_{NSR3} = 10$; $n_{NSR4} = 8$; $n_{NSR5} = 10$; $n_{NSR6} = 10$). Bars (mean ± SE) identified with an asterisk (*) are significantly ($p < 0.05$) different from control. Open bar represents control site.

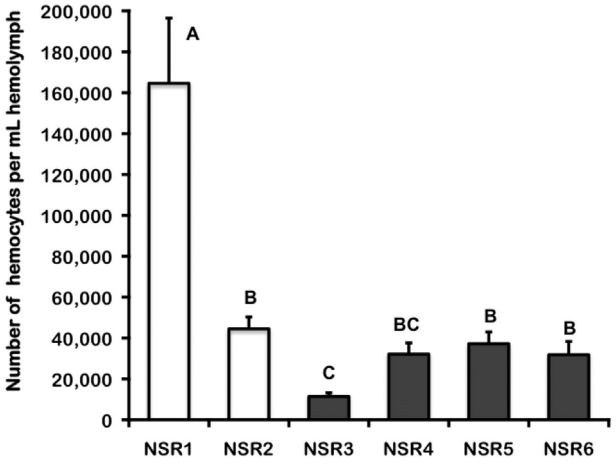

Fig. 8. Numbers of hemocytes per mL hemolymph in mussels caged at 6 sites within the NSR ($n_{NSR1} = 13$; $n_{NSR2} = 16$; $n_{NSR3} = 17$; $n_{NSR4} = 12$; $n_{NSR5} = 16$; $n_{NSR6} = 12$). Bars (mean ± SE) identified with different letters are significantly ($p < 0.05$) different. Open bars represent control sites.

associated with microsomal CYP3A enzyme, elevated mRNA abundance of CYP1A1 and Vtg in male minnows, elevated ratios of GSSG/TGSH, and an increase in the activity of the antioxidant enzymes GR and GST. In mussels, there were no significant changes in biomarkers of oxidative stress and the levels of Vtg-like proteins were reduced, not elevated, in mussels caged at sites closest to the wastewater discharge. Immune function was altered in mussels, as indicated by elevated lysosomal activity per hemocyte. It is possible this immune response is due to exposure to bacterial pathogens in the wastewater, and not to exposure to CECs, although immunomodulation from exposure to estrogens cannot be ruled out.

Wastewater effluent streams present a rather complex and variable environment with differences in temperature, salinity, oxygen levels, bacterial load and even ammonia (not measured in this study) all combining with the presence of CECs and other chemical to effect potential changes in resident aquatic biota. Given this complexity, is difficult to attribute these biomarker responses directly to exposure to CECs within the effluent stream. Moreover, for many CECs, there are no specific biomarkers that can be measured to accurately define effect. Regardless, induction of BFC metabolism and elevated expression of CYP1A may be of use to indicate exposure to pharmaceuticals and personal care products. However, these biomarkers may alternatively be due to exposure to chemicals from industrial effluents or surface drainage/stormwater

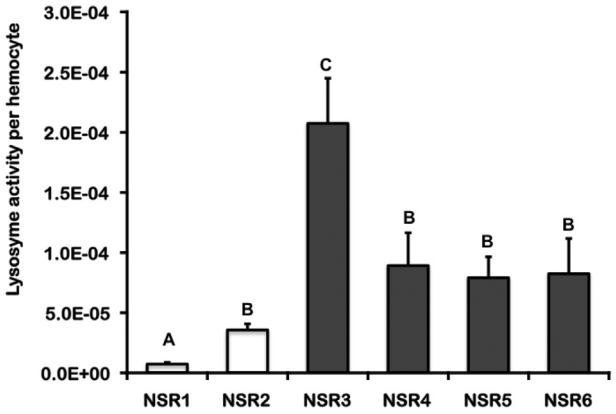

Fig. 9. Lysozyme activity per hemocyte in mussels caged at 6 sites in the NSR ($n_{NSR1} = 12$; $n_{NSR2} = 15$; $n_{NSR3} = 15$; $n_{NSR4} = 12$; $n_{NSR5} = 16$; $n_{NSR6} = 11$). Bars (mean ± SE) identified with different letters are significantly ($p < 0.05$) different. Open bars represent control sites.

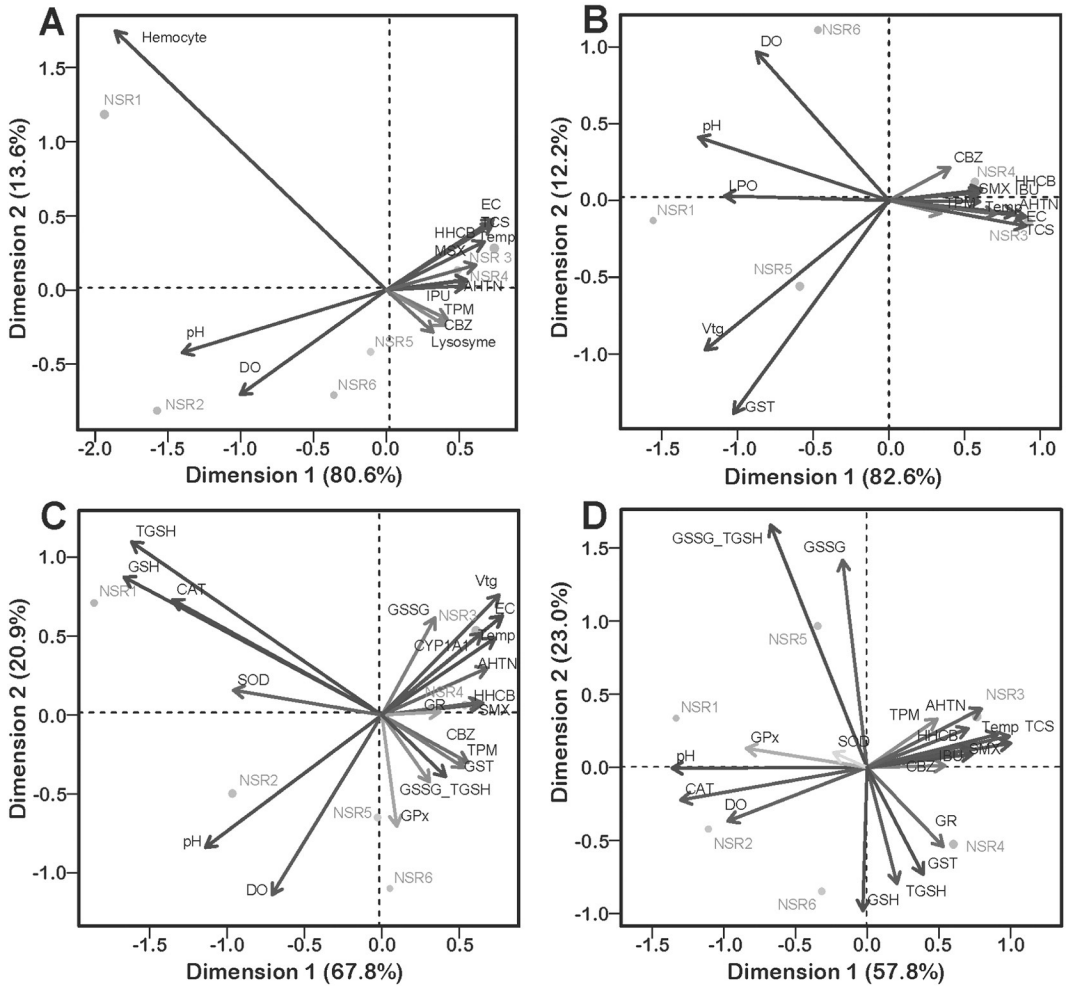

Fig. 10. Correspondence analysis for association of contaminants of emerging concerns and, (A) immune function in mussels, (B) biochemical biomarkers in mussels, (C) biochemical and genetic biomarkers in liver and (D) gills of fathead minnows caged at 6 sites in NSR. Sites are represented as solid circles. Measured parameters are shown as arrows. The darkness of arrows is directly proportional to the strength of correspondence. Codes used in the figure are defined in the Abbreviations section.

entering the WWTP. Induction of Vtg in male fish is a strong indicator of exposure to estrogenic chemicals and this is seen in our study. However, the multivariate analyses indicated (particularly for gills of fathead minnows) that biomarkers of oxidative stress, GR and GST, did not appear to be primarily influenced by water temperature, DO, pH or conductivity but they were significantly elevated at the impacted sites, this could be due either to bacteria in the waste water or CECs. More work is needed to elucidate whether CECs typically found in wastewater can cause oxidative stress in aquatic organisms before a definitive link can be made between exposure to CECs in wastewater and biomarkers of oxidative stress. Overall, several of the parameters show promise as biomarkers of exposure to CECs in wastewater, including induction of CYP450 enzymes, induction of Vtg in male organisms and possibly changes in ratios of oxidized and reduced forms of glutathione. However, interpretation of data is hampered by the biological variability observed in biomarker responses, even with organisms caged directly in the effluent plume.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.05.080.

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