RESEARCH REPORT

The effects of cyanobacterial blooms on the immune system of *Elliptio complanata* in urban and agricultural areas in the Yamaska River watershed

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Abstract

The cumulative effects of cyanobacterial blooms at sites impacted by urban or agricultural activity, could be detrimental to local freshwater mussels. The purpose of this study was to examine and compare the resulting toxicity using an upstream–downstream approach at four sites in the Yamaska River. Mussels were caged and placed at a site where cyanobacterial blooms were present, a site that receives municipal effluent from a small city of 75,000 inhabitants, and a site that drains a large agricultural area—plus a reference site in Lake Saint-Pierre (Quebec, Canada) for four months spanning the summer and fall (June to October). Effects on immunocompetence were monitored by testing for hemocyte counts, viability, phagocytosis, production of nitric oxide (NO), lysozyme (LY), reactive oxygen species (ROS), thiol contents, and inflammation induced by cyclooxygenase (COX) activity and glutathione S-transferase (GST) activity. Cyanobacterial blooms occurred at the target site only, reaching levels of 2.1 million cyanobacteria/L and 3 μg/L of microcystin-LR in surface waters. Although most of the immune parameters were affected at the urban, agricultural and cyanobacteria sites, ROS and LY were the most responsive to cyanobacterial bloom, with a significantly greater response than the agriculture, urban and reference sites. The results also suggest that the effects of cyanobacterial blooms are spatially localized; they are not found at the downstream urban and agriculture sites in the Yamaska River.

Key Words: cyanobacteria, microcystins, hemocytes, immunocompetence, lysozymes, oxidative stress

Introduction

The Yamaska River is located in southeastern Quebec (Canada). It is 177 km long, covers a watershed of 4,784 km^2 and originates from Lake Brome. The river ends in Lake Saint-Pierre, which is part of the St. Lawrence River. The watershed consists mainly of small townships (<75000 inhabitants) and supports intense agricultural activity. Urban and agricultural pollutants including nutrients (total phosphorus and nitrogen) are released into the river, degrading its water quality. Eutrophic conditions in aquatic environments can lead to blooms of pelagic and benthic cyanobacteria. For example, blooms of unicellular planktonic *Microcystis aeruginosa* or filamentous *Anabaena*, *Aphanizomenon* or *Planktothrix* can develop in lakes enriched with high nutrient loads.

M. aeruginosa produce toxic secondary metabolites called microcystins (MYCST). These toxins are potent inhibitors of protein phosphatase 1 and 2A and cause liver damage and death to animals (Jochimsen *et al.*, 1998). The production of toxins is also associated with other unfavourable environmental conditions (e.g., hypoxia and high concentrations of suspended solids, organic matter and other associated pollutants), and the combined or cumulative effects can have profound effects on aquatic organisms.

In freshwater environments, harmful algal blooms often occur in locales where bivalve molluscs are found. As sedentary filter-feeder organisms, mussels accumulate (cyano)bacteria, fungi, algae and suspended matter as sources of nourishment, thereby ingesting an important variety of environmental contaminants. Thus, they may filter and accumulate large quantities of possibly toxic cyanobacteria. In a previous study, freshwater mussels (*Elliptio complanata*) fed with *Anabaena flos-aqua* in the laboratory for five days showed toxic effects, although the cyanobacteria were not

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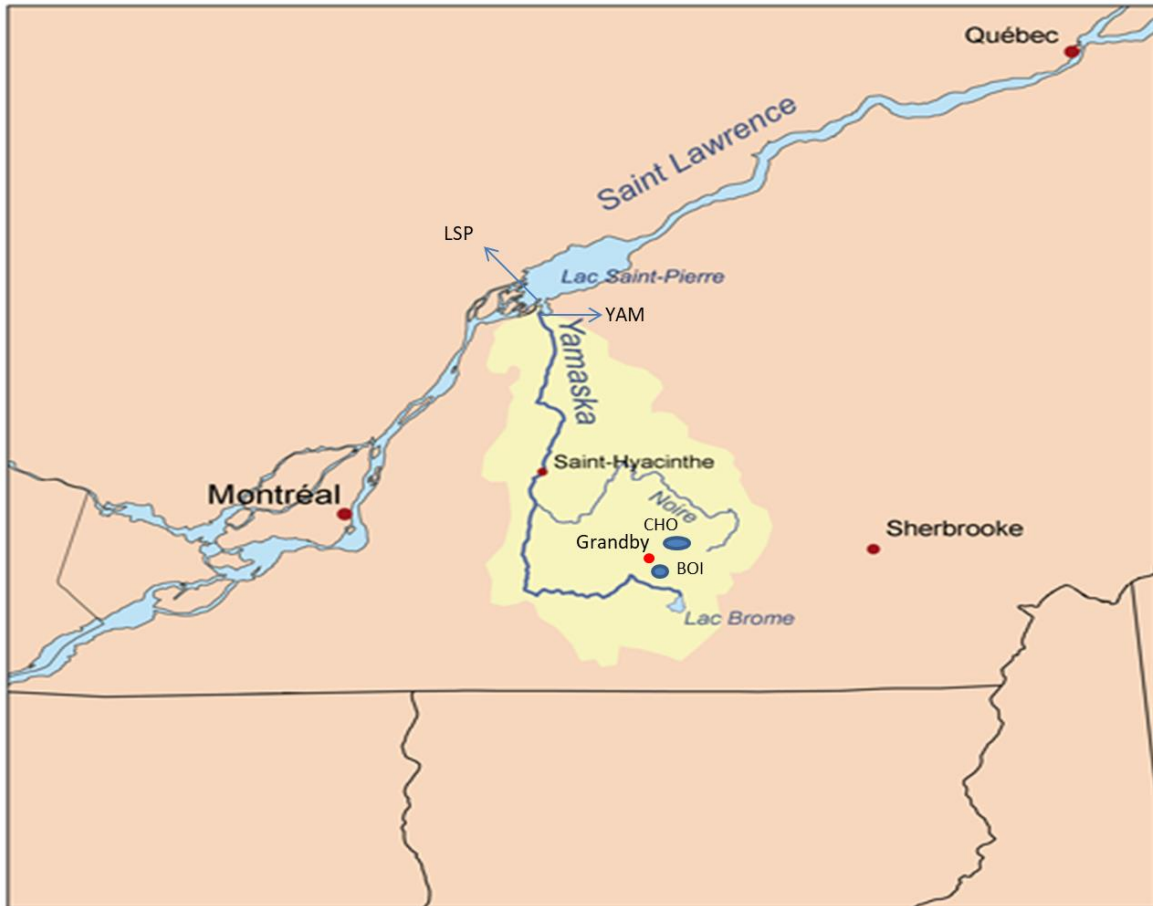E-mail: francois.gagne@canada.ca

Fig. 1 Site location. The Lake Saint-Pierre (LSP) site is part of the St. Lawrence River and is located downstream from the other sites. The YAM site is located 0.5 km from the junction of the Yamaska River and Lake Saint-Pierre. Lake Boivin (BOI) is within the municipality of Granby. The Choinière (CHO) basin is northwest of Granby.

actively producing microcystins (Gélinas *et al.*, 2013). Indeed, acetylcholinesterase activity and lipid peroxidation were higher in mussels fed with the cyanobacteria. Although cyanobacteria are considered Gram-negative, they contain unusual surface mucopeptides thick enough to react weakly with the Gram stain (Hoiczyk and Hansel 2000). In addition, exposure of fish for 90 days to *M. aeruginosa* stimulated the immune system, including lysozyme activity, which is involved in the degradation of cell walls of Gram-positive bacteria (Das *et al.*, 2013). In clams exposed to 100 µg/L microcystin-LR for 10 days, an increase in enzymes involved in oxidative stress, such as superoxide dismutase and catalase, was observed (Pham *et al.*, 2016). These responses were observed at tissue microcystin levels of 12.7 ± 2.4 µg/g dry weight, indicating that *Corbicula leana* clams are relatively tolerant to these toxins. Decreased glutathione S-transferase (GST) activity was observed in *Dreissena polymorpha* exposed to 10 µg/L and 50 µg/L microcystin-LR for 24 h and 7 days (Burmester *et al.*, 2012). The long-term and cumulative impacts of urban, agricultural and cyanobacteria-related pollution on organisms exposed to multiple contaminants are currently not well understood. For

example, decreased GST activity in organisms exposed to cyanobacterial blooms could limit biotransformation and elimination of other organic pollutants to which they are exposed concurrently.

Increased levels of cyanobacteria in mussel digestive glands could have major impacts on the immune system. As a mussel filters its food, these harmful algae interact directly with the gills, digestive gland and other tissues during ingestion, allowing diffusion to the hemolymph throughout the bivalve's open circulatory system. The internal defences against invading foreign bodies are mainly based upon non-specific innate immune system responses, such as phagocytosis and the production of humoral factors such as cytokines (Auffret, 2005). Hemocytes are responsible for phagocytosis and the production of humoral factors (Cheng *et al.*, 1996). These cells are not only found in the circulatory system, but also nest in interstitial tissues waiting to return to circulation following a stress signal. The release of hemocytes following an invasion by foreign bodies is a first response; phagocytosis and lysozyme (LY) production usually follow. Hemocyte viability could be also affected following exposure to cyanobacteria extracts in *Crassostrea gasar* oysters (Queiroga *et al.*, 2017). LY

is an enzyme involved in the degradation of cell walls of most Gram-positive bacteria and cyanobacteria (which are Gram-indeterminate), leading to lysis. This process is supported by phagocytocis, a further attempt to eradicate the pathogens/toxins in which the ingested bodies in phagosomes are destroyed by oxidative burst involving the production of nitric oxide (NO) and hydrogen peroxide (peroxynitrite) as the main form of reactive oxygen species (ROS). Increased cyclooxygenase (COX) activity is associated with the production of pro-inflammatory precursors to assist the immune response (Ottaviani 2004; Gagné et al., 2005). Recently, ROS formation and antioxidant enzyme responses have been reported in organisms exposed to purified microcystins or Microcystis extracts (Wiegand and Pflugmacher 2005; Pietro et al., 2006; Amado et al., 2010). ROS, when improperly inactivated, can inflict damage on a wide range of cellular components (DNA, proteins and lipids), and it is linked with the development of many pathological states. The observed resistance of mussels against cyanotoxins could also be attributed to increased biotransformation through GST activity and GSH, a main source of reduced thiols in cells (Fernandes et al., 2009). The increase in GST activity coincided with peak levels of microcystin-LR in tissues, followed by a phase of decreasing activity after 8-day and 12-day depuration periods in toxin-free water.

Given that toxic marine algae could impair the immune system, we investigated whether blooms of M. aeruginosa could affect the immune system of a freshwater mussel, E. complanata, already multiply stressed by agricultural and urban activities in the Yamaska River. This mussel is typical of the native fauna of the Yamaska watershed and is representative of a sessile species that would endure blooms of M. aeruginosa in addition to other pollution inputs from agricultural and urban activities. The aim of the study was therefore to examine the effects of M. aeruginosa blooms on the immune system in caged mussels during the summer/fall months. We included sites contaminated by urban activities (municipal discharges) and agriculture during the same period to assess the contribution of other stressors to the health status of caged mussels.

Materials and Methods

Study area

Sites were selected using an upstream-downstream approach: the sites polluted by urban activity and algal blooms are located upstream in the Yamaska River which discharges on the south shore of Lake Saint-Pierre (LSP), which is part of the St. Lawrence River (Figure 1). Three sites were located in the Yamaska watershed, which supports agricultural activity and an urban area (Granby; population circa 75,000). Two sites were located upstream from Granby: Lake Boivin (BOI) and the Choinière Reservoir (CHO). CHO, the site located farthest upstream, is a 4.7 km² reservoir built in 1972 that is prone to frequent cyanobacterial blooms (Rolland et al., 2005). The BOI site, which is a little farther downstream, was also formed by dams in the early 19th century. It receives the municipal effluents from the city of Granby (aeration lagoons) and is also subject to cyanobacterial blooms. However, in this study, a cyanobacterial bloom occurred only at the CHO site. The third site (YAM) was in the Yamaska River 3 km upstream from where it flows into Lake Saint-Pierre, which drains more than 50 km of intensely farmed land. The fourth site was the reference site, LSP, located in Lake Saint-Pierre near Île Plate which does not receive input from the Yamaska River.

Mussel collection and caging exposure

Wild freshwater Elliptio complanata mussels were collected in the first week of June 2011 in Lake Achigan (Quebec, Canada), which is not subjected to any direct sources of pollution. The animals were maintained in an aquarium at 15 °C under constant aeration, with a 16h-light/8h-dark photoperiod. They were fed daily with concentrates of phytoplankton (Phytoplex®) and laboratory-cultured Pseudokirchneriella subcapitata algae. Mussels were then placed in two cylindrical nets (65×30 cm), with 30 mussels in each net (which includes extra mussels in case of unexpected mortality), and immersed at the four sites (LSP, YAM, BOI and CHO) on June 20 and 21. The cages were secured with a cable and 10-kg cinderblocks. Mussels were collected (10 randomly collected mussels per time) for analysis after one month (on July 30) and three months (September 30), placed in coolers at 4 ℃ and brought back to the laboratory for immediate immunocompetence assessments, as described below.

Water sampling and analysis

Water sampling was carried out monthly (in July, August, September and October). Temperature, oxygen concentration, conductivity and pH were measured at 1 m depth in the water column with a YSI multiprobe. Water samples were transferred into two 1 L polypropylene containers: a dark one for chlorophylla a (Chl a) analysis and a clear one for the measurement of total phosphorus (TP), total nitrogen (TN), dissolved organic carbon (DOC), ammonia (NH_3) and nitrite-nitrate (NO_2-NO_3) (Environment Canada 2005). In the laboratory, Chla water samples were filtered on GF/F Whatman filters (0.7 µm pore size) that were kept frozen until analysis. Chla pigments were extracted in cold 95% ethanol for 24 h and measured before and after acidification at 665 nm and 750 nm (Spectronic Genesys 5 spectrophotometer) (Rice et al., 2012). TP and total dissolved phosphorus (TDP) were determined by acid digestion followed by colorimetry with ammonium molybdate (Rice et al., 2012). TN was analyzed with a LACHAT Continuous Flow Quick-Chem 8000. NO_2-NO_3 was measured by reducing nitrate to nitrite in a cadmium column prior to colorimetry; NH_3 was analyzed by colorimetry after the addition of sodium nitroprusside and phenate sodium. DOC was oxidized to carbon dioxide by the addition of persulfate prior to infra-red detection (Shimadzu TCO-5000) (Rice et al., 2012). The levels of MC-LR toxins in the water were determined using a commercial competitive immunoassay kit (Enzo Life Sciences, USA).

Hemolymph collection

Mussel hemolymph of 10 individuals per site and time of collection (July and end of September) was drawn (between 750 μL and 1,000 μL) from the posterior adductor muscle with a 3 mL syringe and a 23G needle. A portion of the hemolymph was set aside to measure hemocyte concentration and viability, reactive oxygen species (ROS), nitric oxide production (NO) and reduced thiols by flow cytometry. About 800 μL was kept to measure lysozyme (LY), cyclooxygenase (COX) activities and nitric oxide (NO) concentration. For LY, the hemolymph was centrifuged at 1,000 × g at 4 °C for 10 min, 100 μl of supernatant (plasma) was withdrawn for activity measurements, and the remaining pellet was re-suspended in 150 μL of phosphate buffered saline (PBS; pre-diluted 1/3 in water) for COX activity. Total proteins in the cell-free hemolymph and hemocytes were determined by the protein-dye binding principle (Bradford, 1976).

Flow cytometric analysis

Hemocyte counts and viability were evaluated by flow cytometry using a 3-colour Guava EasyCyte Plus cytometer with a laser emitting at 488 nm, using the supplied Viacount kit (Guava Technologies, Hayward, CA, USA). An aliquot of 20 μL of hemolymph was mixed with 80 μL of Viacount solution after 10 min, in accordance with the supplier's recommended procedure. The flow rate was set at 0.6 μL/sec, and 5,000 events were counted. The data were expressed as the number of cells/mL and percentage of live cells for hemocyte concentration and viability respectively.

For phagocytosis activity, hemocytes were incubated with fluorescently labelled beads based on the protocol of Brousseau *et al.*, 2000. Fluorescent latex beads (Polysciences, PA, USA) were added to the cell suspensions at a 30:1 (beads:cell) ratio and incubated for 18 h at 15 °C in a humidified incubator. After the incubation period, the hemocyte suspension was re-suspended in pre-diluted PBS and layered over 4 mL of RPMI cell culture media (diluted 1/4 in water) supplemented with 3% bovine serum albumin (BSA) (Sigma, Ontario, Canada). Elimination of free or loosely bound beads was followed by centrifugation at 150 × g for 8 min at 4 °C. The cell pellet was then suspended in 0.5 mL of 0.5% formaldehyde and 0.2% sodium azide (Sigma Chemicals, Canada) diluted in PBS (Becton Dickinson, CA, USA). Cells were analyzed using flow cytometry, and at least 10,000 events were recorded for analysis. Hemocyte populations were defined on the basis of their forward and right-angle scatter properties (FSC and SSC, respectively). Results were analyzed with the Cell Quest Pro software (Becton Dickinson). The percentage of cells (hemocytes) that engulfed at least one bead and at least three beads was measured in order to assess phagocytosis activity and efficiency, respectively (Farcy *et al.*, 2011).

Intracellular ROS and reduced thiol were measured as previously described (Brousseau *et al.*, 2000). The probe dihydrochlorofluorescein diacetate (H$_2$DCFDA; CAS 4091-99-0) was used to measure ROS levels in hemocytes (Collén and Davison 1997). A 2 μM final concentration of

H$_2$DCFDA was prepared and mixed with hemocytes and allowed to stand for 45 min in the dark. The presence of intracellular ROS was determined by oxidation of H$_2$DCFDA dye and the dye is retained in viable cells by non-specific esterases (deacetylation of the diacetates into carboxylates). Fluorescence was measured for 3,000 events using flow cytometry. For reduced thiol, the 5-chloromethylfluorescein diacetate probe (CMFDA) was used. As the first step, non-specific thiol fluorescence background was blocked using N-ethylmaleimide (NEM) as a negative control: hemocytes were treated with 100 μM NEM for 10 min and washed in pre-diluted PBS. Afterward, all hemocytes were stained with 5-μM CMFDA and a total of 2,500 events were acquired by flow cytometry.

Immunological biomarkers

The production of NO was estimated by measuring the levels of nitrite concentration in the plasma (Verdon *et al.*, 1995). Because NO reacts readily with oxygen to produce nitrites and nitrates, nitrate reductase was added to convert nitrates into nitrites. Following a 30 min pre-incubation step of 50 μL of hemolymph with 25 μL of nitrate reductase (80 units/L) and 25 μL of NADPH (3 mM), the concentration of NO was measured by adding 100 μL of Griess reagent and reading the absorbance at 450 nm after 30 min. Results are expressed as μmol of NO/mg proteins. The activity of COX in hemocytes was measured by oxidation of the 2,7-dichlorofluorescein in the presence of arachidonic acid (Gagné, 2014). A volume of 50 μL of the suspension (hemocytes and 1/4 PBS) was mixed with 150 μl of Tris-HCL buffer 50 mM, pH 8.0, containing 0.05% Tween 20, 0.1 μg/mL horseradish peroxidase, 25 μM arachidonic acid and 2 μM 2,7-dichlorofluorescein. Fluorescence readings were taken at 485 nm for excitation and 520 nm for emission every 10 min for 30 min at 30 °C. A standard solution of fluorescein in PBS at 1 μM was used for instrument calibration. COX activity was expressed as fluorescein units/min/mg proteins. Total protein concentration was determined using bovine serum albumin as standard, via the protein-dye binding principle (Bradford, 1976), with absorption at 595 nm measured by a microplate reader (PowerWave, BioTek).

Statistical analysis

The biomarker responses were determined in n=10 mussels for each site and time of collection (season). The biomarkers were normalized to the LSP reference site for each season. The data was tested for normal distribution and homogeneity of variance using the Shapiro–Wilk and Bartlett tests respectively. Two-way factorial analysis of variance (ANOVA) was used to test for the effects of sites (BOI, CHOI, LSP, YAM), seasons (summer and fall) and their interaction. Intersite and between time comparisons were done using the Least Square Difference Test as the post-hoc test. Correlation was examined using the Pearson-moment procedure. Significance was set at p<0.05. All statistical analyses were performed using Statistica version 8.

Table 1 Change in physico-chemical characteristics of surface waters

Parameter	Turbidity	Oxygen (mg/L)	Chl a (mg/mL)	Blue-green algae (cells/mL)	Total N/NH3/NO2 (mg/L)	DOC[1] (mg/L)	Conductivity/pH μS.cm-1	Dissolved and total phosphates (mg/L)
LSP								
J	4.4	8.9	0.62	ND	0.545/0.04/0.18	3.5	260/8.5	0.18/0.01
A	3.3	8.7	2.7	ND	0.475/0.04/0.17	3.5	276/8.6	0.18/0.01
S	2.5	9.0	1.9	ND	0.54/0.05/0.19	3.4	272/8.3	0.02/0.01
O	6.1	10.3	2.8	ND	0.58/0.04/0.22	3.4	271/8.2	0.03/0.01
YAM								
J	15.8	10.1	1.13	ND	0.48/0.02/0.05	3.2	280/8.3	0.03/0.008
A	7.8	9.2	7,7	ND	0.69/0.02/0.03	3.3	286/8.7	0.03/0.01
S	59.1	8.5	6.3	ND	0.54/0.05/0.2	7.5	289/8.1	0.19/0.07
O	27.7	9.7	4.3	ND	1.9/0.05/1.3	7.5	332/8.3	0.14/0.06
CHO								
J	10.2	13.1	9.9	ND	0.06/0.001/0.02	5.2	129/9.2	0.02/0.01
A	8.2	9.6	**15.3**	613	0.68/0.02/0.02	5.4	129/8.6	0.03/0.01
S	18.8	8.6	**17.6**	2424	0.75/0.08/1.2	6.8	122/7.5	0.04/0.01
O	**261**	7.8	**18**	20900	0.75/0.01/0.02	6.8	130/6.9	0.04/0.01
BOI								
J	3.7	6.0	48	60	0.1/0.02/0.02	9.5	156/7.4	0.22/0.1
A	4.2	6.3	29.7	ND	1.1/0.03/0.02	9.5	161/7.8	0.17/0.1
S	3.3	6.8	8.6	ND	0.84/0.02/0.02	11	180/7.2	0.11/0.09
O	1.5	9.0	9	ND	0.84/0.02/0.03	11	218/8.0	0.11/0.09

1. Dissolved organic carbon.

Results

Surface water chemistry

During the first four weeks of the summer period, water temperature was stable at around 24 ℃. During the fall period, the mean water temperature was 17 °C and varied between 20 °C and 14 ℃. Chl a measurement was higher at the BOI site in the first summer month (July) but did not contain blue-green algae. Chl a was higher at the CHO site in fall, during the *M. aeruginosa* bloom (Table 1). The number of blue-green algae increased considerably during the fall at CHO site exclusively (Table 1). Cyanobacteria toxins were measured on every sampling date at all sites. No toxins were detected at any site in the summer, but during just one month in September at CHO site. Microcystin-LR equivalents were detected in filtered water (0.45 µm) taken from the water column (not the algae) at concentrations between 1.5 µg/L and 3 µg/L.

Immunocompetence responses

For hemocyte density, two-way factorial ANOVA revealed a positive interaction (F=32; p<0.001) between site and season (Figure 2A). Hemocyte density was significantly higher in the summer than in the fall: the mean cell density at the reference site, LSP, was 960 000 and 580 000 cells/mL in summer and fall respectively. In the summer, hemocyte density did not change significantly between sites. In the fall, hemocyte counts were significantly higher at the CHO (5-fold)

and BOI (3.5-fold) sites than at the LSP and YAM sites. The CHO and BOI levels were also significantly different from each other. Hemocyte density was significantly correlated with ammonia (r=0.52; p<0.05), nitrates (r=-0.53; p<0.05) and pH (r=0.54; p<0.05). In respect to hemocyte metabolic activity (viability), based on general esterase activity, two-way factorial ANOVA confirmed a significant interaction (F=15.7, p<0.001) between site and season (Figure 2B). The initial metabolic activity (viability) index was 82% and 50% at the LSP site in summer and fall respectively. In the summer, hemocyte metabolic activity was significantly lower at the CHO site (0.9-fold) compared to the LSP site. In the fall, hemocyte activity was significantly higher at the YAM (1.2-fold), BOI (1.5-fold) and CHO (1.45-fold) sites relative to the LSP site. Hemocyte density was not significantly different between the CHO and BOI sites. Hemocyte activity was significantly correlated with hemocyte density (r=0.39, p<0.05). Phagocytosis activity and efficiency were also examined in mussels (Figure 3). Two-way factorial ANOVA revealed a significant interaction (F=18.3, p<0.001) with site and season for phagocytosis activity. Phagocytosis activity was significantly higher in the summer than in the fall (Figure 3A). The initial activity was 41% and 14% at the LSP site for summer and fall respectively. In the summer, phagocytosis activity was significantly higher at the BOI site (1.4-fold) compared to the LSP site. In the fall, phagocytosis activity at CHO (2.3-fold), BOI (2.1-fold) and YAM (0.6-fold) were significantly
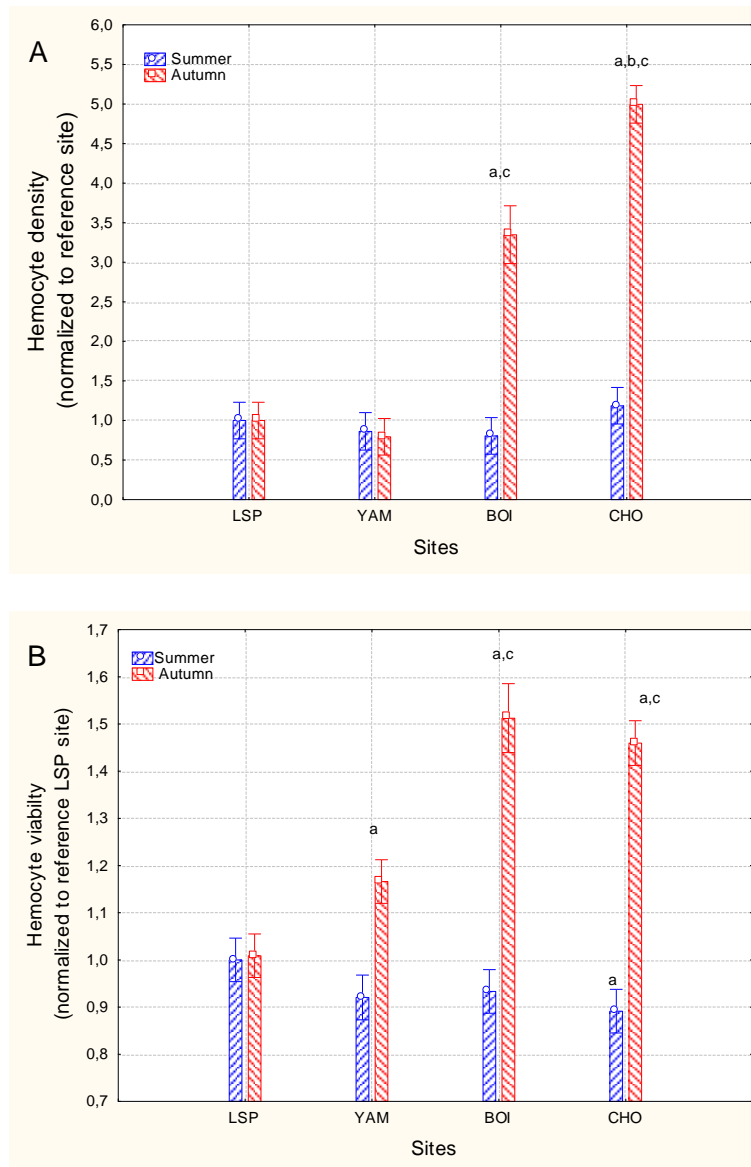
Fig. 2 Change in hemocyte density and esterase activity. Mussels were caged at the sites in June and removed at the end of July and September. The letter a indicates a significant difference between the site and LSP (the reference site). The letter b indicates a significant difference from BOI (impacted by urbanization but no blue-green algae). The letter c indicates a significant difference from YAM.

different than at LSP. Phagocytosis activity was significantly correlated with hemocyte density ($r=0.55$; $p<0.001$), viability ($r=0.43$; $p<0.001$), nitrates ($r=-0.67$; $p<0.01$), Chl a ($r=0.61$; $p=0.01$) and water conductivity ($r=-0.53$; $p<0.01$). Phagocytosis efficiency responded similarly to phagocytosis activity with a significant interaction ($F=22.8$, $p<0.001$) between site and time (Figure 3B). The efficiency values were 27% and 5% at the LSP site in summer and fall respectively. In the case of LY activity, two-way factorial ANOVA revealed a significant interaction ($F=7.0$, $p<0.001$) between site and season (Figure 3C). LY activity was at 3.9 and

1.5 absorbance change/min/mg proteins at the LSP site in the summer and fall respectively. In the summer season, LY activity remained unchanged between sites. However, a significant increase in LY activity was observed at BOI (6-fold) and CHO (10-fold) compared to the LSP. There was also a significant difference in LY activity between the CHO site (rural cyanobacterial bloom) and the BOI (urban) site. Correlation analysis revealed that LY activity was significantly correlated with blue-green cyanobacteria counts ($r=0.50$; $p<0.05$), ammonia ($r=-0.52$; $p<0.05$) and conductivity ($r=-0.60$; $p=0.01$). Multiple regression analysis revealed a correlation
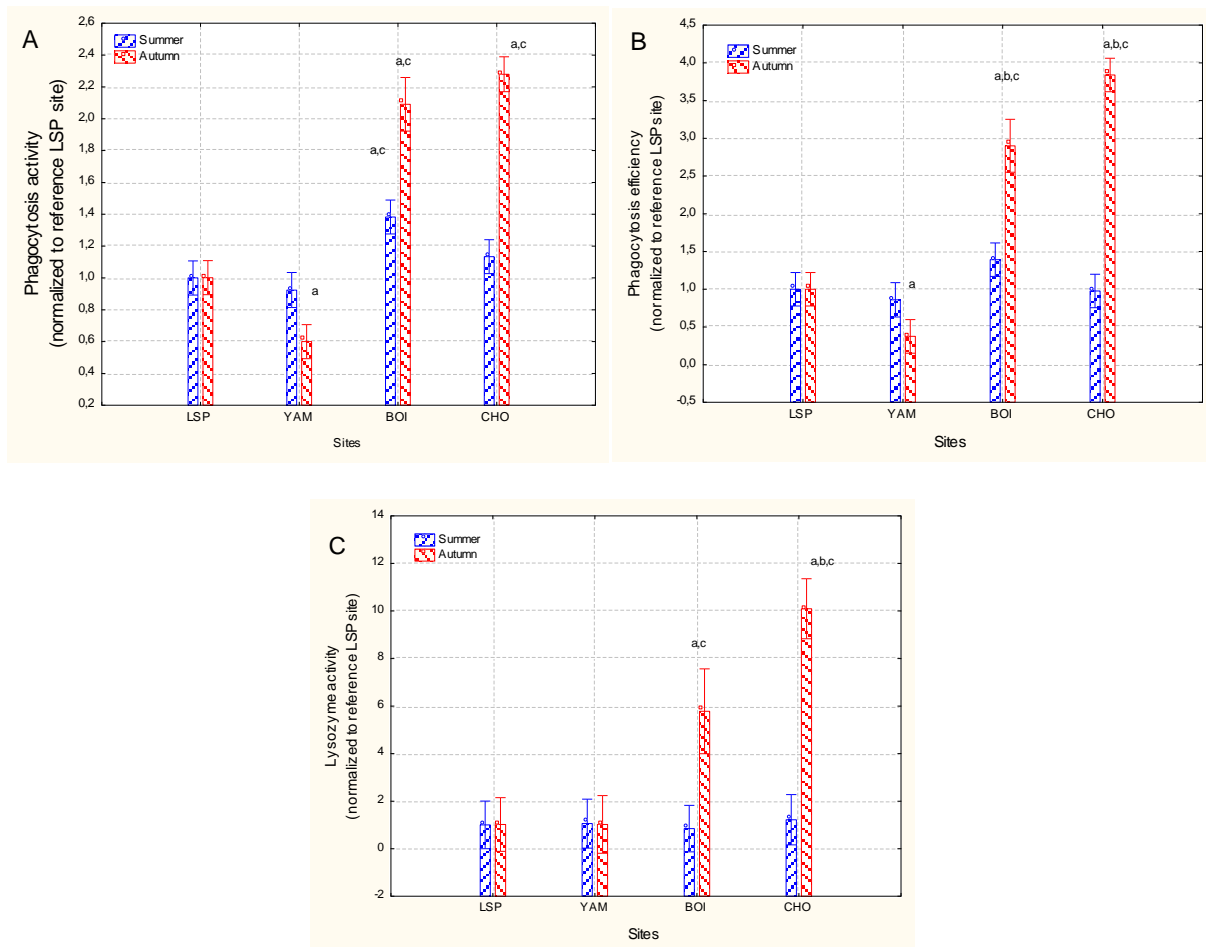
Fig. 3 Change in phagocytosis and lysozyme activity in mussels exposed to multiple sources of pollution. Mussels were caged at four sites from June to October. The letter a indicates a significant difference between the site and LSP (the reference site). The letter b indicates a significant difference from BOI (impacted by urbanization but no blue-green algae). The letter c indicates a significant difference from YAM.

between lysozyme activity (dependent variable) and blue-green cell counts (β=0.50) with conductivity (β=-0.44) (r=0.80; p=0.002), suggesting that hemolymph LY activity increases with blue-green cell levels in water that has low conductivity.

ROS production following phagocytosis was measured, as well as the production of total nitrites in hemocytes (Figure 4). For ROS production, two-way factorial ANOVA showed a significant interaction (F=12.6, p<0.001) between season and site (Figure 4A). Mean ROS levels were 2,941 and 36 fluorescence units at the LSP site during the summer and fall seasons, respectively. In the summer, ROS production did not differ significantly between sites. In the fall, ROS production was significantly higher at the CHO site only (20-fold) than at the YAM and LSP sites. Although the ROS levels were high (5-fold) at BOI, the difference was not significant owing to the variability. ROS levels between CHO and BOI were significantly different. Correlation analysis revealed that ROS production was significantly correlated with hemocyte density

(r=0.32; p=0.001), viability (r=0.42; p<0.001) and dissolved P/total P ratio (r=-0.68; p<0.01). For NO production, two-way factorial ANOVA revealed a significant interaction (F=4.6, p<0.01) between season and site (Figure 4B). The mean NO levels were 3.3 µg/mg and 0.1 µg/mg protein at the LSP site in the summer and fall respectively. In the summer, NO production showed no significant difference between sites. In the fall, NO production was significantly higher at CHO (8-fold) compared to the LSP site. Correlation analysis revealed that NO level was significantly correlated with hemocyte density (r=0.37; p<0.001), phagocytosis efficiency (r=0.25; p=0.05) and dissolved P/total P ratio (r=-0.49; p=0.05). COX activity was measured in hemocytes to assess inflammation in freshwater mussels (Figure 4C). Two-way factorial ANOVA revealed a significant interaction (F=4.5, p<0.01) between site and season. COX activity was at 8,700 and 7,300 fluorescein units/min/mg proteins at the LSP site in the summer and fall respectively. In the summer, COX activity did not differ significantly by
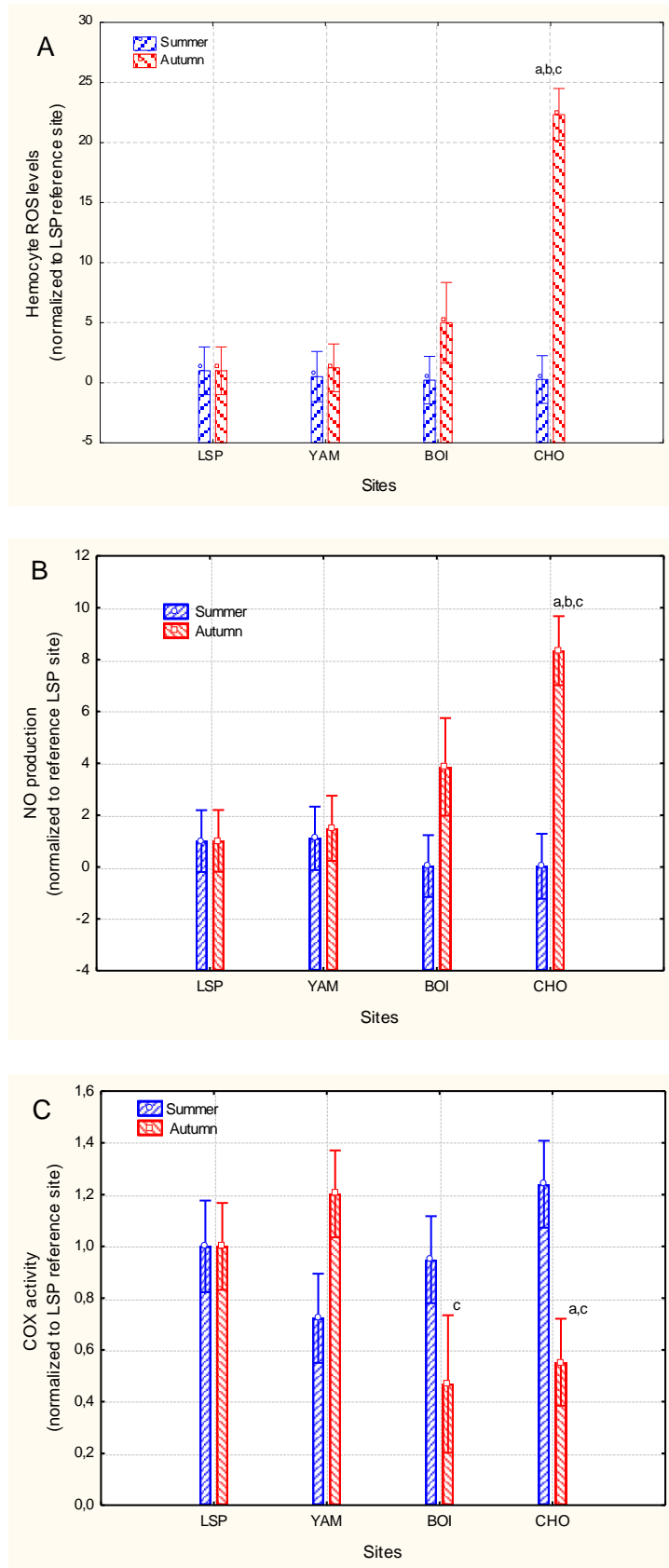
Fig. 4 Oxidative burst and inflammation in freshwater mussel hemolymph. Mussels were caged at four sites during the summer and fall. The letter a indicates a significant difference between the site and LSP (the reference site). The letter b indicates a significant difference from BOI (impacted by urbanization but no blue-green algae). The letter c indicates a significant difference from YAM.

site. In the fall, COX activity was significantly lower at CHO (0.6-fold) and BOI (0.5-fold) compared to the reference site, LSP. Correlation analysis revealed that COX activity was significantly correlated with hemocyte density (r=0.56; p<0.01), GST (r=0.26; p<0.01), thiol content (r=0.83; p<0.001) and lysozyme activity (r=-0.67; p<0.01).

Biotransformation activity was followed by reduced thiol content (for metals and ROS inactivation) and GST (for organic pollutants) activity. For hemocyte thiol content, two-way factorial ANOVA revealed a significant interaction (F=10.9, p<0.001) between site and season. The

mean thiol content was 109 and 121 fluorescence units at the LSP site in the summer and fall respectively. In the summer, thiol content was significantly higher at CHO (1.8 fold) and lower at YAM (0.3-fold) compared to LSP (Figure 5A). Thiol content was significantly higher at the CHO site than at the BOI site. In the fall, thiol content was significantly lower at CHO (0.2-fold) and BOI (0.18-fold) compared to YAM and LSP. There was no significant difference between the BOI and CHO sites. Thiol content was significantly correlated with pH (r=0.61; p=0.01) and oxygen content (r=0.51; p<0.05).
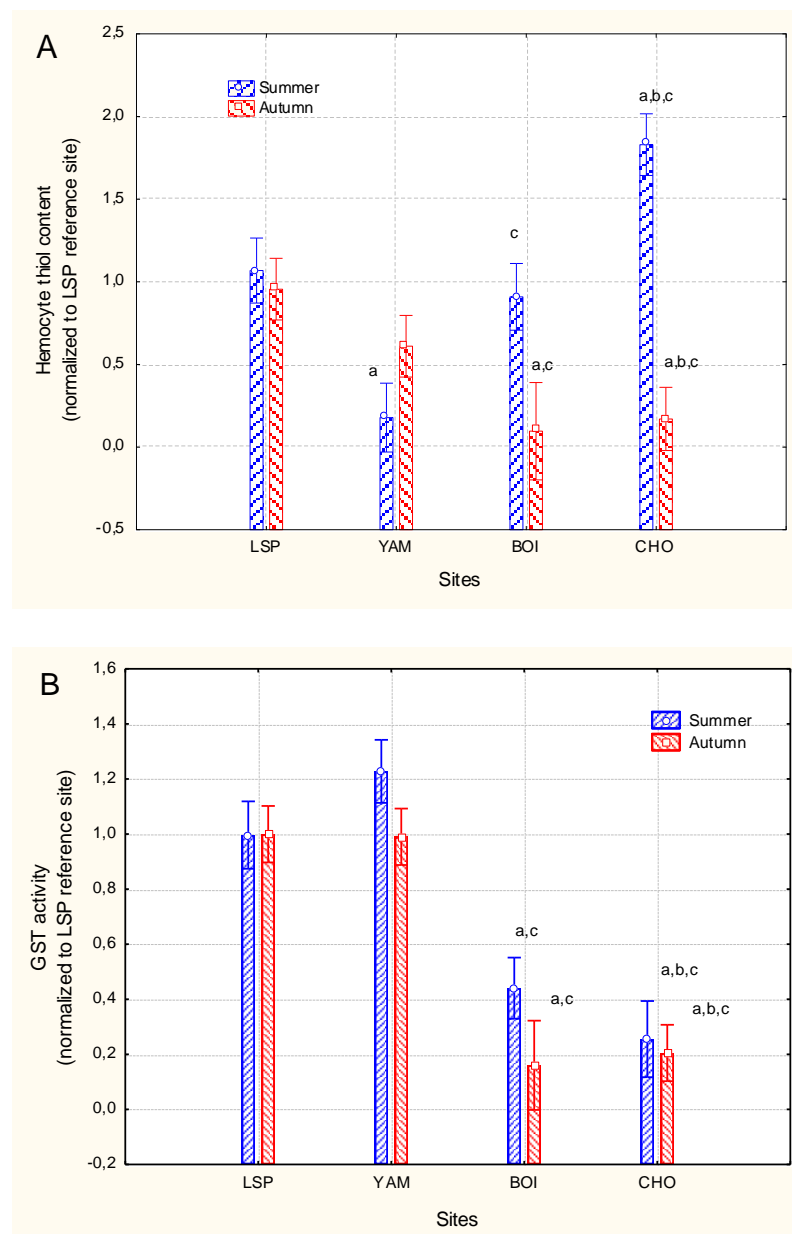

Fig. 5 Biotransformation activity in hemocytes. Mussels were caged at four sites in the Yamaska River during the summer and fall. Biotransformation activity was determined by total thiol content and GST activity. The letter a indicates a significant difference between the site and LSP (the reference site). The letter b indicates a significant difference from BOI (impacted by urbanization but no blue-green algae). The letter c indicates a significant difference from YAM.

For GST activity, two-way factorial ANOVA revealed a significant difference for sites only (F=29.5, p<0.001) i.e. no seasonal effects and no interaction between season and sites. GST activity was globally similar in the summer and in the fall (Figure 5B). The mean GST activity was 0.11 and 0.06 absorbance change/min/mg proteins at the LSP site in the summer and the fall. In the summer, GST activity significantly decreased at BOI (0.45-fold) and CHO (0.25-fold) compared to LSP. In the fall, the same pattern was observed but more intensely than in the summer, with 0.18-fold and 0.2-fold decreases at BOI and CHO respectively. There was no significant difference between the BOI and CHO sites by season. Correlation analysis revealed that GST activity was significantly correlated with hemocyte density (r=0.23; p<0.05), phagocytosis efficiency (r=0.23; p<0.05), NO production (r=0.42; p<0.001), DOC (r=-0.66; p<0.01), conductivity (r=0.61; p=0.01)

and dissolved P/total P ratio (r=-0.56; p<0.05).

We examined the immunocompetence data to attempt to determine whether cyanobacterial blooms could produce specific, discernible effects in mussels. In respect to cyanobacterial blooms that occurred at the CHO site only in the fall, we sought biomarkers that responded to the CHO site and differed significantly from the urban pollution (BOI), agriculture (YAM) and reference (LSP) sites. We found that hemocyte density, PHAG efficiency, ROS, NO and LY activity satisfied the above criteria based on two-way factorial ANOVA (Table 2). Moreover, LY activity was significantly correlated (r=0.67; p<0.01) with the levels of blue-green algae (cyanobacteria) in surface waters. Multiple regression revealed that lysozyme activity was predicted (R=0.85) by blue-green algae (β =0.54; p<0.05), NO_3/total N (β=0.32; p<0.05) and conductivity (β=-0.42; p<0.05).

Table 2 Identification of effects in caged freshwater mussels associated with cyanobacterial blooms

Biomarker	Difference before and after blooms at CHO site	Difference from reference site LSP	Difference from urban pollution (BOI)	Difference from agricultural pollution	Relationship with water parameters
Hemocyte density	yes (+)	yes(+)	yes(+)	yes(+)	pH (r=0.5)
Hemocyte viability	yes(+)	yes(+)	no	yes(+)	nil
Phagocytosis activity	yes (-)	yes (+)	no	yes (+)	turbidity (r=-0.51) Chl *a* (r=0.61) Cond (r=-0.59)
Phagocytosis efficiency	yes (+)	yes (+)	yes(+)	yes (+)	Chl *a* (r=0.61) Cond (r=-0.53)
Lysozyme	yes (+)	yes(+)	yes(+)	yes(+)	cyanobacteria (r=0.66) cond (r=-0.63) pH (r=-0.60)
ROS	yes(+)	yes(+)	yes(+)	yes(+)	Pdissol/total P (r=-0.68)
NO	yes(+)	yes(+)	yes(+)	yes(+)	COD (r=-0.58) Pdissol/total P (r=-0.57)
Thiol content	yes (-)	yes (-)	no	yes (-)	O_2 (r=0.50) pH (r=0.61)
GST activity	no	yes (-)	no	yes (-)	COD (r=-0.66) Cond (r=0.61) pH (r=0.5) Pdissol/total P (r=-0.56)
COX activity	yes (-)	yes (-)	no	yes (-)	pH (r=0.67)

The shaded rows indicate biomarkers that responded more strongly to cyanobacterial blooms than to urban and agricultural pollution.

Discussion

In nutrient (N and P)-rich environments, exponential growth (blooms) of cyanobacteria develop annually during summer and fall in lakes, reservoirs and slow-flowing rivers in temperate latitudes. However, it was found that total P levels alone were not always able to predict the presence of algal blooms (Shimoda *et al.*, 2016). Multiple regression analysis showed that the occurrence of blue-green algae was significantly correlated with acidification of surface waters (pH, r=-0.52; p<0.05) but not with total phosphate and nitrogen levels. Conditions in the Yamaska River watershed are known to favour cyanobacterial blooms, which usually occur in late summer and fall (MDDEFP: http://www.mddelcc.gouv.qc.ca/eau/inter.htm). Our two upstream sites, CHO and BOI, were located within the urban and agricultural area and one of them, CHO, exhibited cyanobacterial bloom during the fall period, as confirmed by the presence of blue-green algae and microcystin-LR in surface waters and visual inspection (a thick green mat covered the water). Given that mussel populations also thrive in the Yamaska watershed, which drains both urban and agricultural areas, freshwater mussels are exposed to the cumulative input of urban and agricultural pollution and cyanobacterial blooms. The health consequences of this exposure are currently not well understood.

GST activity in hemocytes was significantly depressed in both summer and fall at both the CHO (algal blooms) and BOI (municipal effluent) sites relative to LSP (the reference site), suggesting that not only cyanobacterial blooms but also other factors were at play in decreasing the activity. The decrease in GST activity could reflect a depletion of GSH stores from oxidative stress and biotransformation in mussels exposed long-term to pollution. This was corroborated by reduced thiol content in hemocytes in the fall (Figure 3). Interestingly, thiol content levels were significantly higher in the summer, which suggests that thiol stores were not fully depleted at this time. In a study with the freshwater clam *Diplodon chilensis patagonicus*, clams were fed with a toxic strain of *M. aeruginosa* for 6 weeks, then oxidative stress and biotransformation activity were determined (Sabati *et al.*, 2011). Antioxidant enzymes (superoxide dismutase and catalase), reduced GSH and GST activity were all significantly upregulated after 6 weeks. The reverse was found with decreased thiol content and GST in mussels caged at the CHO site in the fall during cyanobacterial blooms. Moreover, the ROS increase was much greater than the increase in NO production and was weakly correlated (r=0.19; p<0.05) with NO, suggesting that oxidative stress involved other mechanisms than oxidative burst following phagocytosis. Shrimps exposed for 70 days to cyanobacterial blooms had decreased hemocyte density and increased phagocytosis activity (Gao *et al.*, 2017). Phagocytosis activity was increased at both the urban (BOI) and cyanobacterial bloom (CHO) sites, and the increase was stronger in the fall. However, both CHO (cyanobacterial blooms) and BOI (urban) showed increased phagocytosis activity and

efficiency, although phagocytosis efficiency differed significantly between the CHO and BOI sites during the cyanobacterial bloom in the fall. Nevertheless, the increase in phagocytosis activity and hemocyte density in mussels exposed to municipal effluents was reported elsewhere (Farcy *et al.*, 2011). Interestingly, LY activity was not significantly increased by the effluents, indicating less sensitivity to human-generated sources of bacteria than to cyanobacterial blooms.

Microcystins from algal blooms could be toxic to clams (Pham *et al.*, 2016). Exposure to crude extracts of cyanobacteria containing 400 µg/L of microcystin-L in clams for 10 days resulted in increased GST, superoxide dismutase and catalase in the gills and mantle, and inhibition of catalase and GST activities in the foot and other tissues. GST activity was also decreased at CHO site, which contained cyanobacteria and microcystins at 3 µg/L but exposure was for much longer times than 10 days. GST activity was also reduced at BOI, which receives urban discharges from the city of Granby. No signs of cyanobacterial blooms were found there, indicating that other contaminants could also reduce the activity. In another study, freshwater clams (*D. chilensis*) were fed a toxic strain of *M. aeruginosa* for 6 weeks (Sabatini *et al.*, 2011). Although neither protein nor lipid damage were observed, significant increases in GSH (thiol), superoxide dismutase, catalase and GST were observed, suggesting that antioxidant enzymes and cofactors were able to contain oxidative stress. This is in keeping with decreased inflammation (COX activity) in mussels caged at BOI and CHO. However, total thiol content was decreased at CHO and BOI indicating that other factors could have contributed to reduced thiol levels in the hemolymph. Another possible explanation for the observed decreased in GST activity and the tolerance of bivalves to toxins is compensation mechanisms involving a multi-xenobiotic resistant P-glycoprotein (P-gp) pump (Contardo-Jara *et al.*, 2008). The activity of the P-gp pump showed a significant increase up to 72 h in clams exposed to 100 µg/L microcystin-LR, indicating enhanced excretion of the toxin. This was accompanied by decreased GST and catalase activities after 72 h exposure times. Hemocyte metabolic activity was greater when hemolymph was exposed *in vitro* to *M. aeruginosa* than when exposed to *Lyngbia wollei* extracts (Gélinas *et al.*, 2014). This is consistent with increased hemocyte metabolic activity and density observed at the CHO site, which was undergoing cyanobacterial blooms. However, hemocytes exposed to *M. aeruginosa* extracts had elevated ROS levels and COX activity. A possible explanation is that protein thiol content was depleted by sustained exposure to contaminants including cyanotoxins and oxidative stress in mussels after four months. Lower water temperatures in the fall might also have reduced the turnover rate of thiols and proteins in the hemolymph.

NO production, LY activity and ROS levels appeared to be the most responsive and selective endpoints to cyanobacterial blooms when compared to urban and agriculture sites. Indeed, LY activity

was maximal at the cyanobacterial bloom (CHO) site, differing significantly from the urban (BOI) and agriculture (YAM) sites (Table 2). LY activity was elevated at BOI, but significantly less than at CHO, compared to the reference LSP site, which makes sense given the occurrence of microorganisms in municipal effluents. Increased hemolymph LY activity was also observed in *Hyriopsis cumingii* mussels exposed to *M. aeruginosa* (Hu *et al.*, 2015). Moreover, hypoxia was found to dampen the increase in LY activity. Although oxygen content at CHO (7.8 mg/L) was lower than at BOI (9 mg/L), there was no significant correlation between LY activity and O_2 levels in surface waters, within the range of O_2 measured in the present study (6 mg/L to 13.1 mg/L).

In conclusion, exposure to cyanobacterial blooms could contribute to contamination from urban and agricultural pollution in caged freshwater mussels. ROS production and LY activity were the most responsive biomarkers for cyanobacterial blooms at the CHO site and responded more strongly there than at the BOI site, which receives municipal effluents from the city of Granby. ROS levels were not correlated with phagocytosis activity and were correlated only weakly with NO levels, which suggests that there are other sources of ROS from the immune response (oxidative burst), perhaps from mitochondria activity and biotransformation. The results support the hypothesis that cyanobacterial blooms exert additional stress on freshwater mussels by contributing to oxidative stress and to enhanced immunocompetence, as with other contaminants.

Acknowledgments

References

Amado LL, Monserrat JM. Oxidative stress generation by microcystins in aquatic animals: Why and how. Environ. Internat. 36: 226-235, 2010.

Auffret M. Bivalves as models for marine immunotoxicology. In: Tryphonas H, Fournier M, Blakley BR, Smits JEG., Brousseau P. (eds) Investigative immunotoxicology. Taylor and Francis, New York, pp29-48, 2005.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Brousseau P, Pellerin J, Morin Y, Cyr D, Blakey B, Boermans H, *et al*. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following *in vitro* exposure to heavy metals. Toxicol. Lett. 142: 145-165, 2000.

Burmester V, Nimptsch J, Wiegand C. Adaptation of freshwater mussels to cyanobacterial toxins: Response of the biotransformation and antioxidant enzymes. Ecotoxicol. Environ. Saf. 78: 296-309, 2012.

Cheng TC. Hemocytes: Forms and functions. The Eastern Oyster Crassostrea virginica. Maryland Sea Grant Book, College Park, pp. 299-333, 1996.

Collé J, Davison IR. *In vivo* measurement of active oxygen production in the brown alga Fucus evanescens using 2′,7′-dichlorohydrofluorescein diacetate. J. Phycology 33: 643-648, 1997.

Contardo-Jara V, Pflugmacher S, Wiegand C. Multi-xenobiotic-resistance a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins. Toxicon 52: 936-943, 2008.

Das BK, Pradhan J, Sahu S, Marhual NP, Mishram BK, Eknath AA. *Microcystis aeruginosa (Kütz)* incorporated diets increase immunity and survival of Indian major carp Labeo rohita *(Ham.)* against *Aeromonas hydrophila* infection. Aquaculture Res. 44: 918-927, 2013.

Farcy E, Gagné F, Martel L, Fortier M, Trépanier S, Brousseau P, *et al*. Short-term physiological effects of a xenobiotic mixture on the freshwater mussel *Elliptio complanata* exposed to municipal effluents. Environ Res. 111: 1096-1106, 2011.

Fernandes S, Welker M, Vasconcelos VM. Changes in the GST activity of the mussel Mytilus galloprovincialis during exposure and depuration of microcystins. J. Exp. Zool. A Ecol. Genet. Physiol. 311: 226-230, 2009.

Gagné F, Bérubé E, Fournier M, Blaise C. Inflammatory properties of municipal effluents to *Elliptio complanata* mussels: Lack of effects from anti-inflammatory drugs. Comp Biochem Physiol C Toxicol Pharmacol. 141: 332-337, 2005.

Gagné F. Biomarkers of infection and diseases. Chapter 11 in: Biochemical Ecotoxicology: Principle and Methods, 1st Edition. New York: Elsevier Inc. pp197-207, 2014.

Gao J, Zuo H, Yang L, He JH, Niu S, Weng S, *et al*. Long-term influence of cyanobacterial bloom on the immune system of Litopenaeus vannamei. Fish Shellfish Immunol. 61: 79-85, 2017.

Gélinas M, Fortier M, Lajeunesse A, Fournier M, Gagnon C, Gagné F. Energy status and immune system alterations in Elliptio complanata after ingestion of cyanobacteria Anabaena flos-aquae. Ecotoxicology 22:457-468, 2013.

Gélinas M, Fortier M, Lajeunesse A, Fournier M, Gagnon C, Barnabé S, *et al*. Responses of freshwater mussel (*Elliptio complanata*) hemocytes exposed *in vitro* to crude extracts of Microcystis aeruginosa and Lyngbya wollei. Ecotoxicology 23: 260-266, 2014.

Hoiczyk E., Hansel A. 2000. Cyanobacterial Cell Walls: News from an Unusual Prokaryotic Envelope. J Bacteriol. 182: 1191-1199, 2000.

Hu M, Wu F, Yuan M, Li Q, Gu Y, Wang Y, *et al*. Antioxidant responses of triangle sail mussel *Hyriopsis cumingii* exposed to harmful algae Microcystis aeruginosa and hypoxia. Chemosphere. 139: 541-549, 2015.

Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CE, *et al.* Liver failure and death after exposure to microcystins at a

hemodialysis center in Brazil. N. Engl. J. Med. 338: 873-878, 1998.

Ottaviani E, Malagoli D, Franchini A. Invertebrate humoral factors: Cytokines as mediators of cell survival. Prog. Mol. Subcell. Biol. 34: 1-25, 2004.

Pham TL, Shimizu K, Kanazawa A, Gao Y, Dao TS, Utsumi M. Microcystin accumulation and biochemical responses in the edible clam Corbiculaleana P. exposed to cyanobacterial crude extract. J. Environ. Sci. 44: 120-130, 2016.

Prieto AI, Jos A, Pichardo S, Moreno I, Cameán A.M. Differential oxidative stress responses to microcystins LR and RR in intraperitoneally exposed tilapia fish (Oreochromis sp.). Aquat Toxicol. 77: 314-321, 2006.

Queiroga FR, Marques-Santos LF, Hégaret H, Sassi R, Farias ND, Santana LN, *et al*. Effects of cyanobacteria Synechocystis spp. in the host-parasite model *Crassostrea gasar*-Perkinsus marinus. Aquat. Toxicol. 187: 100-107, 2017.

Rice EW, Baird RB, Eaton AD, Clesceri LS. Standard Methods for the Examination of Water and Wastewater, 22nd edition. American Public Health Association, American Water Works Association, Water Environment Federation, 2012.

Sabatini SE, Brena BM, Luquet CM, San Julián M, Pirez M, Carmen Ríos de Molina MD. Microcystin accumulation and antioxidant responses in the freshwater clam Diplodon chilensis patagonicus upon subchronic exposure to toxic Microcystis aeruginosa. Ecotoxicol. Environ. Saf. 74: 1188-94, 2011.

Shimoda Y, Watson SB, Palmer ME, Koops MA, Mugalingam S, Morley A, *et al*. Delineation of the role of nutrient variability and dreissenids (Mollusca, Bivalvia) on phytoplankton dynamics in the Bay of Quinte, Ontario, Canada. Harmful Algae 55: 121-136, 2016.

Verdon CP, Burton BA, Prior RL. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by $NADP^+$ when the Griess reaction is used to assay for nitrite. Anal Biochem 224: 502-508, 1995.

Wiegand C, Pflugmacher S. Ecotoxicological effects of selected cyanobacterial secondary metabolites: A short review. Toxicol Appl Pharmacol. 203: 201-218, 2005.