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Changes in hemocyte and biochemical parameters in the soft-shell clams *Mya arenaria* from the St. Lawrence estuary

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ABSTRACT

The cumulative effects of urban pollution and the presence of toxic phytoplankton in bivalves are not well understood. The purpose of this study was to determine the impacts of urban and algal pollution on wild *Mya arenaria* clams in the St. Lawrence Estuary. Clams were collected during low tide at sites differing in urban population (S1: 100; S2: 675; S3: 2,500; and S4: 50,000 inhabitants) in areas susceptible to toxic algal blooms and analyzed for immunocompetence (hemocyte density, viability, phagocytosis and oxidative burst/oxygen reactive species), energy reserves (sugars and proteins) and neural activity (acetylcholinesterase). Total phytoplankton counts in surface waters at the four sites, in decreasing order, were S3>>S1>S4 and S2. However, toxic phytoplankton species counts at the four sites were different: S4>>S1>S2>S3. The most abundant toxic species was *Pseudo-nitzschia delicatissima*, which is responsible for amnesic shellfish poisoning. Multiple regression analysis showed that toxic phytoplankton counts were significantly correlated with urban population size (β = 0.89) and with total phytoplankton (β = -0.25), suggesting that large populations favored algal bloom proliferation. Factorial analysis showed that toxic phytoplankton counts and population size were closely associated with reactive oxygen species production in hemocytes and increased AChE activity in clams, which is consistent with the cholinergic properties of some toxic algae. Total phytoplankton loadings were associated with increased energy reserves and hemocyte density. The data suggest that urban pollution contributes to algal production and that the effects of both could combine in clams, especially in stimulating AChE activity and the production of ROS in hemocytes.

KEYWORDS: *Mya arenaria*, toxic phytoplankton, oxidative stress, acetylcholinesterase, energy reserves.

INTRODUCTION

The St. Lawrence estuary (Québec, Canada) is subject to recurrent blooms of harmful algae that forms characteristic “red tides.” Outbreaks of the toxic dinoflagellate *Alexandrium tamarense* could cause paralytic shellfish poisoning (PSP) from the production of saxitoxin, which presents a serious health risk to the human population and wildlife [1, 2]. Moreover, PSP can be transferred through the food web from bivalves up to beluga whales [3]. Indeed, PSP and other algal toxins have been implicated in the mortalities of marine mammal species around the world, including in the St. Lawrence Estuary. During an intense bloom of *A. tamarense* in 2008, an unusually high number of beluga whales (an endangered species) died. In addition, algal blooms often occur after rainfalls, and heavy rainfall events are expected to increase in temperate coastal zones due to global warming [4]. Algal toxins are powerfully toxic secondary metabolites produced by prokaryotic cyanobacteria in both marine and freshwater ecosystems and by eukaryotic dinoflagellates in marine water [5, 6].

Other shellfish toxins have been reported in the past 20 years, including those causing neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (domoic acid), diarrhetic shellfish poisoning (DSP; okadaic acid), ciguatera fish poisoning and azaspiracid shellfish poisoning. Most of these poisonings are caused by neurotoxins with highly specific effects. Voltage-gated sodium channels (saxitoxin, brevetoxins), glutamate receptors (domoic acid) and nicotinic acetylcholine receptors (anatoxins, domoic acid) are often involved during neurotoxicity [7]. Dinoflagellates, cyanobacteria, diatoms and ciliates have been causing harmful algal blooms in many regions of the world with increasing frequency, due to global distribution and water eutrophication resulting from human activities.

Recent evidence suggests that some algal toxins could affect the immune and neurological systems and feeding activity in bivalves. For example, okadaic acid, the toxin responsible for diarrhetic shellfish poisoning, was shown to influence the immune system in scallops [8]. Hemocyte superoxide dismutase and acid phosphatase were depressed by okadaic acid in scallops. Bivalves rely solely on the non-specific immunity which involves hemocyte proliferation, cytokine production and phagocytosis activity. In another study, mussels exposed to saxitoxin, which is responsible for PSP, showed elevated expression of genes involved in immunity, such as pattern recognition, Toll-like and tumor necrosis factor receptors [9]. Exposure of *Mytilus edulis* larvae to *Pseudo-nitzschia sp.* (1,400 cells/mL) increased phenoloxidase activity in hemocytes [10]. Exposure to *Prorocentrum lima* cells (okadaic acid) significantly reduced larval viability. Phagocytosis activity and esterase activity were decreased by okadaic acid in *Ruditapes decussatus* clam hemocytes exposed *in vitro* [11]. A transcriptomic analysis of *Mytilus edulis* feeding on *Pseudo-nitzschia* spp. (producing domoic acid) revealed a number of toxic effects [12]. Indeed, changes in solute carrier membrane transporters, detoxification processes (carboxylesterases, sulfotransferases), oxidative stress and altered immunological processes were observed in the digestive glands of mussels.

Domoic acid is a potent glutamate receptor agonist which could cause overexcitation of nerves leading to cell death. Downstream effects of domoic acid may include stimulation of cholinergic neurons at the neuro-muscular junction, and that may lead to increased induction of acetylcholinesterase (ACHE), which is involved in the inactivation of acetylcholine. Exposure of mussels to domoic acid by intramuscular injection (500 ng/g) led to increased AChE activity after 48 hours, but the activity returned to control values after 7 days [13]. Brevetoxins are known to activate voltage-dependent sodium gates, and anatoxins could stimulate nicotinic acetylcholine receptors [7, 14]. Some algal blooms were also shown to decrease bivalves' feeding activity and the quality of their food intake. For example, oysters feeding on brevetoxin-producing algae *Karenia brevis* showed evidence of gut damage and decreased gamete viability [15]. These studies revealed that both known and unknown toxins could produce a variety of toxic effects on the neurological and immune systems and on energy assimilation in bivalves. Given that human activity (urban areas) and climate (precipitation) could modulate algal proliferation and the release of rainfall overflows in wastewater treatment plants, the cumulative effects of algal proliferation and urban pollution on resident aquatic fauna require close scrutiny.

The purpose of this study was therefore to examine the influence of total phytoplankton and toxic phytoplankton levels in *Mya arenaria* clam populations at sites with varying anthropogenic influence (population density). Clam health status was measured by monitoring immunocompetence, neurological activity and energy reserves to determine the influence of total and toxic phytoplankton density. These studies will also make it possible to determine whether cumulative effects are likely to result from exposure to urban and algal pollution in wild clam populations in the St. Lawrence estuary.

MATERIALS AND METHODS

Study area

Mya arenaria clams were collected in August 2011 at three sites along the south shore of the St. Lawrence estuary (Québec, Canada) near the city of Rimouski and at one site in the Saguenay fjord located on the north shore of the Saint-Lawrence Estuary (https://goo.gl/maps/MuwSWCQGiB82). The first site was the bay at Métis-sur-Mer (MsM; 48.666551 Lat, -68.015351 Long), which serves a

municipality of about 675 residents (S2). The second site is the largest city in the area, Rimouski (RIM; 48.453056 Lat., -68.530141 Long.), with a population of about 50,000 (S4). The third site is the village of Bic (BIC; 48.324186 Lat., -68.856829 Long.), with 2,500 residents (S3). These sites are subject to algal growth during the summer that varies in intensity from year to year. The last site is located north of the St. Lawrence estuary, about 70 km upstream in the Saguenay fjord. Anse Sainte-Étienne (ASE; 48.221775 Lat., -69.916768 Long.) is a sandy bay in the fjord (located 20 km upstream of Anse Saint-Jean) with a small year-round population of circa 50, which can grow to include 100–200 tourists during the summer at a local camping and lodging facility (S1). The salinity is lower at this site (22 g/L) than in the St. Lawrence estuary (35 g/L). Because of its low salinity, this site is not expected to have major algal bloom events.

Clam collection and handling

Adult *Mya arenaria* clams of 5-7 cm in length were collected during low tide in the morning in August 2011. The clams were at the post-spawning/resting phase where active gametogenesis occurs in late May and late June. They were brought back to the laboratory in coolers at 4 °C for weighing and hemolymph collection and tissue preparation. Shell length and weight were measured to determine the condition index (CI): clam wet weight (g)/shell length (cm). The clams were maintained on ice for hemolymph collection. Hemolymph samples (0.5–1 mL) were collected through the posterior adductor muscle with a sterile 3-mL syringe and a 23G needle and transferred in microcentrifuge tubes on ice until immunocompetence assessment was performed the same day, as described below. The visceral mass, composed of the digestive system and gonad tissues, was dissected out and processed as described below for acetylcholinesterase (AChE) and total protein and sugar assessments.

Water sampling for chemical and phytoplankton analyses

Water sampling was also carried out at the collection site for chemical analysis and phytoplankton analysis. Water temperature was taken at the site of collection during low tide and was in the range of 5.8-7 °C. The levels of total nitrogen, nitrates, phosphates and total organic carbon were determined by standard methods for water analysis using four 100-mL grab samples per site [16]. The data for water chemistry are shown in Table 1. One litre of surface water was collected and brought back to the laboratory for filtration under a 0.7-µm membrane filter disk (cellulose acetate membrane filter). The filter disk was removed and washed with 1–3 mL of 3% NaCl solution and fixed by adding 0.2% paraformaldhyde. The levels of phytoplankton were determined using a hematocytometer under a microscope at 100–200 × enlargement. Species were identified based on morphological characteristics [17]. The phytoplankton species information is reported in Table 2. The phytoplankton counts were expressed as number of cells/L and the data are reported in Table 3.

Immunocompetence assessments

Immunocompetence was determined based on hemocyte concentration, viability, phagocytosis activity and the production of reactive oxygen species. Flow cytometry was used to evaluate hemocyte counts and viability: specifically, a 3-colour Guava EasyCyte Plus cytometer with a laser emitting at 488 nm, using the supplied Viacount kit (Guava Technologies, Hayward, CA, USA). Briefly, an aliquot of 20 µL of hemolymph was mixed with 80 µL of Viacount solution and incubated for 10 minutes at room temperature before instrument analysis. A total of 5,000 events were counted for hemocyte concentration and viability determination under a pre-determined volume sample (time of aspiration).

For phagocytosis activity, the methodology based on evaluating ingested fluorescently labeled latex beads was used [18]. Briefly, fluorescent latex beads (Polysciences, PA, USA) were added to the hemocyte suspension at a 30:1 (beads:cell) ratio in sterile RPMI 1640 cell culture media containing 50 µg/mL Gentamycin sulfate. The cell suspension was then incubated for 18 hours in a humidified incubator at 15 °C and 5% CO_2. After the incubation period, the hemocyte suspension was centrifuged at 150 × g for 5 minutes, resuspended in phosphate buffered saline (PBS: 145 mM NaCl, 5 mM KH_2PO_4, pH 7.4) and layered over 4 mL of RPMI cell culture medium supplemented with 3% bovine serum albumin (BSA) (Sigma, ON, Canada). Elimination of free or loosely bound beads was followed by centrifugation at 150 × g for 5 minutes at 4 °C.

Table 1. Basic physico-chemical characteristics of surface waters.

Sites	Nitrogen (mg/L)	Nitrates (mg/L)	Total phosphates (mg/L)	Total organic carbon (mg/L)
ASE	0.32 ± 0.04	0.1 ± 0.01	0.02 ± 0.002	4.3 ± 0.3
MsM	0.37 ± 0.03	0.07 ± 0.01	0.02 ± 0.002	3.9 ± 0.04
BIC	0.35 ± 0.03	0.05 ± 0.006	**0.59 ± 0.06***	**2 ± 0.2***
RIM	**0.24 ± 0.02***	**0.01 ± 0.005***	0.01 ± 0.005	4.7 ± 0.5

*Asterisk indicates significant difference from the other sites ($p < 0.05$).

Table 2. Phytoplankton characterization.

Species	Category	General properties
Prorocentrum cordatum	Dinoflagellates	A potentially toxic dinoflagellate that causes two kinds of shellfish poisoning: venerupin shellfish poisoning (VSP) and diarrheic shellfish poisoning. VSP causes liver damage and gastrointestinal illness in humans, which can lead to death. During blooms, this dinoflagellate can turn the water brown (from *Phytopedia*, University of British Columbia, Canada; [35]).
Scrippsiella trochoidea	Dinoflagellates	A non-toxic, marine dinoflagellate that can be found in both cold and tropic waters. It forms red-tide blooms and could deplete dissolved oxygen contents.
Pseudo-nitzschia delicatissima	Pennate diatoms	A toxic marine planktonic diatom genus capable of producing the neurotoxin domoic acid (DA), which is responsible for the neurological disorder known as amnesic shellfish poisoning.
Heterocapsa rotundata	Dinoflagellates	A non-toxic species for humans, but this species caused mass mortality of bivalves such as oysters, short-necked clams and pearl oysters in Japan [36]. Strains of *H. rotundata* have also been demonstrated to be toxic to *Artemia* sp.
Mesodinium rubrum	Ciliates	Not toxic, but could produce red tides and strange colours in animals feeding on them, making them less palatable.
Skeletonema costatum	Central diatoms	A non-toxic diatom with appreciable nutritive values in aquaculture of bivalves (oysters) and shrimps. At high density, it could produce discomfort (irritation) in fish.
Cylindrotheca closterium	Pennate diatoms	A non-toxic diatom that produces mucilage which could form aggregates and large flocs in water. However, no toxins have been detected.
Pseudo-nitzschia seriata	Pennate diatoms	A potentially toxic diatom that could produce domoic acid and produce symptoms of amnesic shell poisoning [37, 38].
Gyrodinium spirale	Dinoflagellates	The most distinctive species of *Gyrodunium* spp. and is commonly found in the Gulf coasts and the Atlantic. It occurs sometimes with other toxic dinoflagellates but no evidence of toxicity.
Dinophysis acuminata	Dinoflagellates	A toxic phytoplankton forming okadaic acid, leading to diarrhetic shellfish poisoning.
Scrippsiella lachrymosa	Dinoflagellates	Not toxic but could deplete dissolved oxygen.

The cell pellet was then suspended in 0.5 mL of 0.5% formaldehyde and 0.2% sodium azide (Sigma Chemicals, Canada) in PBS. 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 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 determined in order to assess the phagocytosis activity and efficiency, respectively.

The resting levels of intracellular reactive oxygen species (ROS) were measured as previously described [19]. The probe dihydrochlorofluorescein diacetate (H_2DCFDA; CAS 4091-99-0) was used to measure ROS levels in hemocytes. The final concentration of H_2DCFDA was prepared at 2 µM and allowed to stand for 45 minutes in the dark. In viable cells, the presence of intracellular ROS was determined by oxidation of H_2DCFDA dye; 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.

Neural activity and energy reserves

AChE activity was determined in the visceral mass as follows. First, clams were thawed on ice and the visceral mass (mainly composed of the digestive system and gonad tissues) was dissected out on ice. The tissues were homogenized in ice-cold 50 mM Tris-acetate buffer, pH 7.4, containing 150-mM NaCl, 0.1-mM EDTA and 0.1-mM dithiothreitol using a Teflon pestle tissue grinder (five passes). A portion of the homogenate was kept aside for analysis of total sugars and proteins and the other portion was centrifuged at 12,000 × g for 20 minutes at 2 °C. The supernatant was collected and maintained on ice for AChE activity assessment. AChE activity was determined using the acetylthiocholine substrate methodology [20]. The supernatant (50 uL) was mixed with 0.5-mM acetylthiocholine and Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid)) in 100-mM Tris-acetate buffer, pH 7.4. The formation of thiocholine was measured at 412 nm at each 5-minute interval for 30 minutes. Readings were taken in clear polystyrene microplates using a microplate reader (Synergy 4, Biotek Instruments, USA). A calibration curve using reduced glutathione was generated and the data were expressed as nmol thiol/min/mg proteins in the supernatant. Metabolic energy intake was determined by monitoring changes in total sugars and proteins in the visceral mass. Total sugars were determined using the anthrone reaction [21, 22]. Briefly, 10 µL of the homogenate was mixed in clear polystyrene microplates with 40 µL of water and 150 µL of the anthrone reagent (1mg/mL in concentrated H_2SO_4). The mixture was heated at 80 °C for 15 minutes, then cooled at room temperature, and the absorbance was measured at 620 nm. Standard sucrose solution was used for calibration and the data were expressed as mg sugars/g clam wet weight. Total protein concentration was determined using the protein-dye binding principle [23]. First, 10 µL of the homogenate was diluted in 200 µL 50-mM NaOH and incubated at 40 °C for 30 minutes. Then 10 µL of the mixture was used for total protein assays. The absorbance was measured at 595 nm in a microplate reader (Synergy 4, Biotek, USA), and bovine serum albumin was used for calibration. The protein levels were expressed as mg proteins/g clam wet weight.

Statistical analysis

The analyses were conducted for N = 12 clams at each site. Data normal distribution and homogeneity of variance was tested using the Shapiro–Wilk and Bartlett tests respectively. The data were subjected to one-way analysis of variance (ANOVA) and the least square difference (LSD) post-hoc test to determine differences between sites. Correlation was examined using the Pearson-moment correlation procedure. Factorial analysis was also performed to determine the most important biomarkers and examine the interrelationships between biomarkers. Significance was set at $p < 0.05$. All statistical analyses were performed using Statistica version 8.

RESULTS

Water samples were collected at each of the four sites during clam collection. Basic water chemistry analyses were performed for total nitrogen, nitrates, total phosphates and total organic carbon content (Table 1). Total nitrogen values were significantly lower at the RIM site (0.24 ± 0.02 mg/L) compared to the other sites (ASE, MsM and BIC), which ranged between 0.32 and 0.37 mg/L. Total nitrates followed the same pattern as total nitrogen values. Total phosphate levels were significantly higher at the BIC site (0.59 ± 0.06) compared to the other sites, which ranged from 0.01 to 0.02 mg/L. Total organic carbon levels were significantly lower at BIC (2 ± 0.2) compared to the other sites, which

ranged from 3.9 to 4.7 mg/L. Phytoplankton analysis was performed to determine the most dominant species (Table 2 and 3). We found 11 dominant species, of which 5 (45%) were considered toxic to aquatic life and vertebrates: *Prorocentrum minimum*, *Pseudo-nitzschia delicatissima*, *Pseudo-nitzschia seriata*, *Gyrodinium spirale* and *Dinophysis acuminata*. The remaining 6 species (55%) were considered non-toxic; they were *Scrippsiella trochoidea*, *Heterocapsa rotundata*, *Mesodinium rubrum*, *Skeletonema costatum, Cylindrotheca closterium* and *Scrippsiella lachrymosa*. The relative water concentrations of these organisms are reported in Table 3. At MsM (estimated population 675), the total phytoplankton and toxic phytoplankton loadings were 280 cells/L and 240 cells/L, respectively. The phytoplankton consisted of mainly 2 species: *P. minimum* (240 cells/L) and *S. trochoidea* (40 cells/L). At RIM, which is considered the most polluted of the four sites (estimated population 50,000), the total and toxic phytoplankton loads were 1,120 and 840 cells/L, respectively. The samples contained *Pseudo-nitzschia delicatissima* and *seriata*, *H. rotundata*, *P. minimum* and *M. rubrum*. At BIC (estimated population 2,500), the total and toxic phytoplankton loadings were 45,480 and 120 cells/L, respectively. *M. rubrum* was the main species (45,280 cells/L), followed by *Pseudo-nitzschia delicatissima* (80 cells/L), *S. trochoidea* (80 cells/L) and *P. minimum* (40 cells/L). This site has the highest density of phytoplankton, most of which is not deleterious to bivalves and vertebrates. The last site, ASE, located north of the St. Lawrence estuary in the Saguenay fjord, was considered the most remote (estimated population <100). The total and toxic phytoplankton loadings were 4,000 and 320 cells/L, respectively. This site had the greatest diversity of organisms, and *H. rotundata* (1,520 cells/L) and *S. costatum* (1,960 cells/L), which are not considered toxic, were the main species.

Clam condition, immunocompetence, AChE activity, levels of sugars and proteins were measured to determine the health status of the clams. Clam condition factor (CF) was determined by the clam weight/shell length ratio (Figure 1A). The condition factor was significantly higher at ASE and MsM than at BIC and RIM. No significant difference was detected between RIM and BIC. Multiple regression analysis showed that condition factor was correlated ($r = 0.71$; $p < 0.001$) with population ($\beta = -4.3$; $p < 0.05$) and with toxic phytoplankton load ($\beta = 4.3$; $p < 0.05$), but not with total phytoplankton load ($\beta = 0.50$; $p > 0.05$). Hemocyte density was determined by flow cytometry (Figure 1B). Hemocyte density was significantly higher at BIC and MsM than at RIM and ASE. Correlation analysis uncovered no significant changes correlated with condition factor (Table 4). Multiple regression analysis showed that hemocyte density was significantly correlated ($r = 0.83$; $p < 0.001$) with all three parameters: population ($\beta = 6.3$; $p < 0.05$), total phytoplankton load ($\beta = -1.3$; $p < 0.05$) and toxic phytoplankton load ($\beta = -7.3$; $p < 0.05$). According to this analysis, hemocyte density tends to increase with population size while toxic phytoplankton seems to reduce hemocyte density. Hemocyte viability (Figure 1C) did not change significantly (ANOVA at $p > 0.1$), and no significant correlations with site characteristics (population, total phytoplankton or toxic phytoplankton) were found (Table 4). Hemocyte viability was significantly correlated with condition factor ($r = -0.41$; $p < 0.05$).

Phagocytosis activity and efficiency were determined in hemocytes. Phagocytosis activity, defined by the proportion of hemocytes that ingested one bead or more, is shown in Figure 2A. Phagocytosis activity was significantly lower at MsM than at RIM and BIC. ASE was not significantly different from MsM. Multiple linear regression analysis showed a weak correlation ($r = 0.32$; $p < 0.05$) with non-significant partial correlations for population, total phytoplankton and toxic phytoplankton loads. Phagocytosis efficiency represents the proportion of hemocytes that engulfed at least three beads (Figure 2B). The same pattern of phagocytosis activity was observed, with MsM showing less stimulation than the other sites. Correlation analysis revealed that the only significant relationship was with phagocytosis activity and efficiency ($r = 0.70$; $p < 0.001$). Multiple regression analysis showed similar results for phagocytosis activity ($r = 0.35$; $p = 0.01$): there were significant partial correlations with total phytoplankton ($\beta = 0.93$; $p < 0.05$) and toxic phytoplankton ($\beta = 2.9$; $p < 0.05$) loadings. The production of reactive oxygen species (ROS) was also monitored (Figure 2C). ROS production was significantly higher at the RIM site than at the

Table 3. Phytoplankton analysis.

Site/ population	Central diatoms # L-1	Pennate diatoms # L-1	Dinoflagellates (unplated) # L-1	Dinoflagelates (plated) # L-1	Ciliates # L-1	Total phytoplankton # L-1	Toxin-producing
MsM/675	ND*	ND	ND	240 *P. minimum* 40 *S. trochoidea*	ND	280	240
RIM/50,000	ND	680 *P. delicatissima* 40 *P. pseudodelicatissima* 40 *P. seriata*	ND	40 *H. rotundata* 80 *P. minimum*	240 *M. rubrum*	1,120	840
BIC/2,500	ND	80 *P. delicatissima*	ND	40 *P. minimum* 80 *S. trochoidea*	45,280 *M. rubrum*	45,480	120
ASE/<100	1,960 *S. ostatum*	240 *C. closterium*	40 G. spirale	1,520 *H. rotundata* 40 *D. acuminata* 40 *S. lacrymosa*	240 *M. rubrum*	4,000	320

*ND: not detected.

Table 4. Pearson-moment correlation analysis of phytoplankton and biomarker data.

	Population	Total phytoplankton	Toxic phytoplankton	Hem dens	Hem via	CF	Phag act	Phag eff	ROS	Proteins	AchE	Sugars
Population	1	**-0.57** **p < 0.01**	**0.98** **p < 0.001**	**-0.53** **p < 0.01**	-0.23	-0.33	0.26	0.07	**0.47** **p < 0.05**	-0.21	**0.48** **p < 0.05**	-0.34
Total phytoplankton		1	**-0.73** **p < 0.001**	**0.75** **p < 0.001**	-0.08	-0.36 p < 0.1	0.02	-0.16	-0.25	**0.82** **p < 0.001**	**-0.54** **p < 0.01**	**0.82** **p < 0.001**
Toxic phytoplankton			1	**-0.65** **p = 0.001**	-0.17	-0.16	0.21	0.1	**0.47** **p < 0.05**	**-0.38** **p < 0.1**	**0.54** **p < 0.01**	**-0.48** **p < 0.05**
Hem dens				1	0.14	-0.32	-0.03	-0.09	**-0.59** **p < 0.01**	**0.54** **p < 0.01**	**-0.54** **p < 0.01**	**0.57** **p < 0.01**
Hem viab					1	**0.41** **p < 0.05**	-0.06	0.09	**-0.40** **p < 0.1**	-0.19	0.28	-0.29
CF						1	-0.16	0.21	0.11	**-0.65** **p = 0.001**	0.19	**-0.45** **p < 0.05**

Table 4 continued..

Phag act							1	**0.7 p < 0.001**	0.16	0.20	-0.29	0.02
Phag eff								1	0.07	-0.15	-0.09	-0.29
ROS									1	-0.14	0.30	-0.03
Proteins										1	**-0.48 p < 0.05**	**0.75 p < 0.001**
AchE											1	**-0.45 p < 0.05**

1. The significant correlations ($p < 0.05$) with toxic algae are in **bold**. Hemocyte density (Hem dens), hemocyte viability (hem via), condition factor (CF), phagocytosis activity (Phag act) and efficiency (Phag eff), reactive oxygen species (ROS), acetylcholinesterase (AChE).

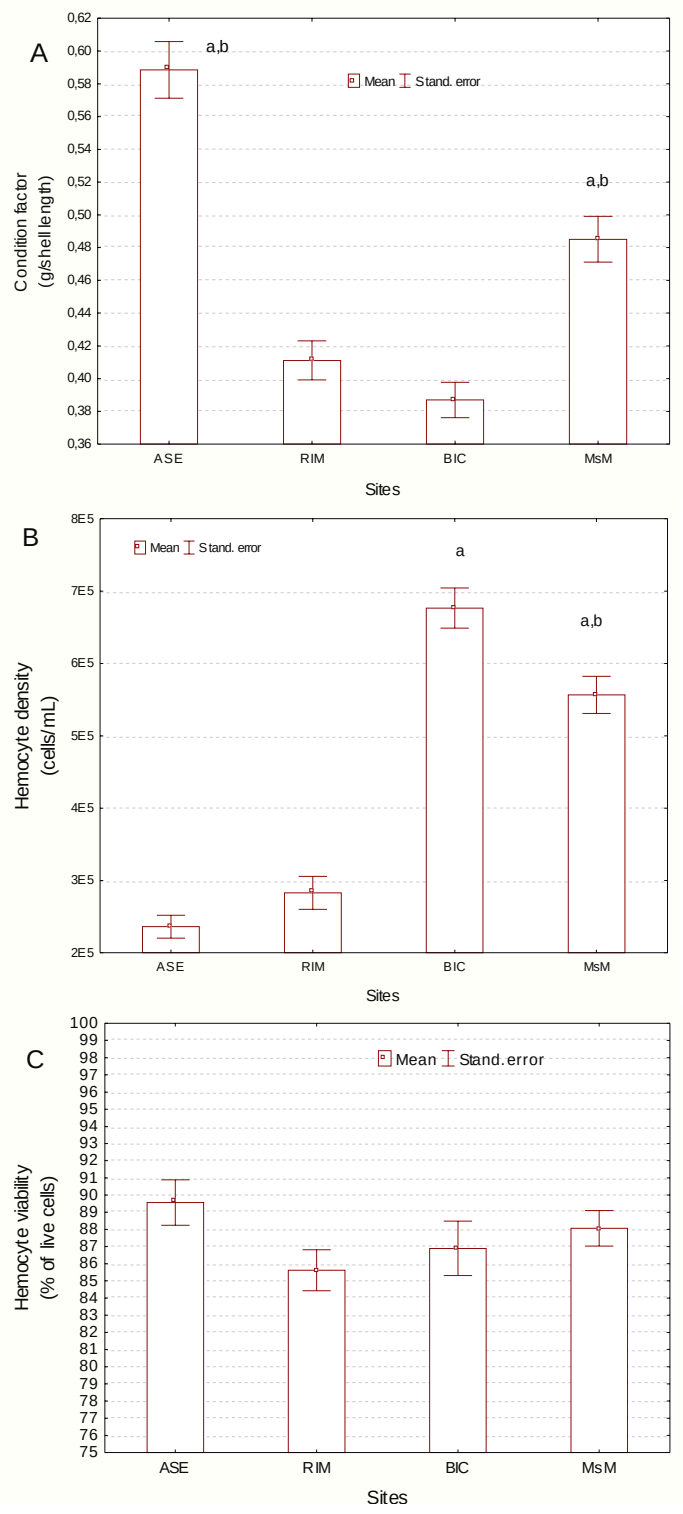

Figure 1. Condition factor and hemocyte density in *Mya arenaria* clams collected from the St. Lawrence estuary and Sagueny fjord. Clams were collected during low tide and processed for condition factor (**A**), hemocyte density (**B**) and viability (**C**) assessments. The letter 'a' indicates significant difference from the polluted RIM site (human activity with high levels of toxic phytoplankton). The letter 'b' indicates significant difference from the site containing the highest density of phytoplankton (BIC).

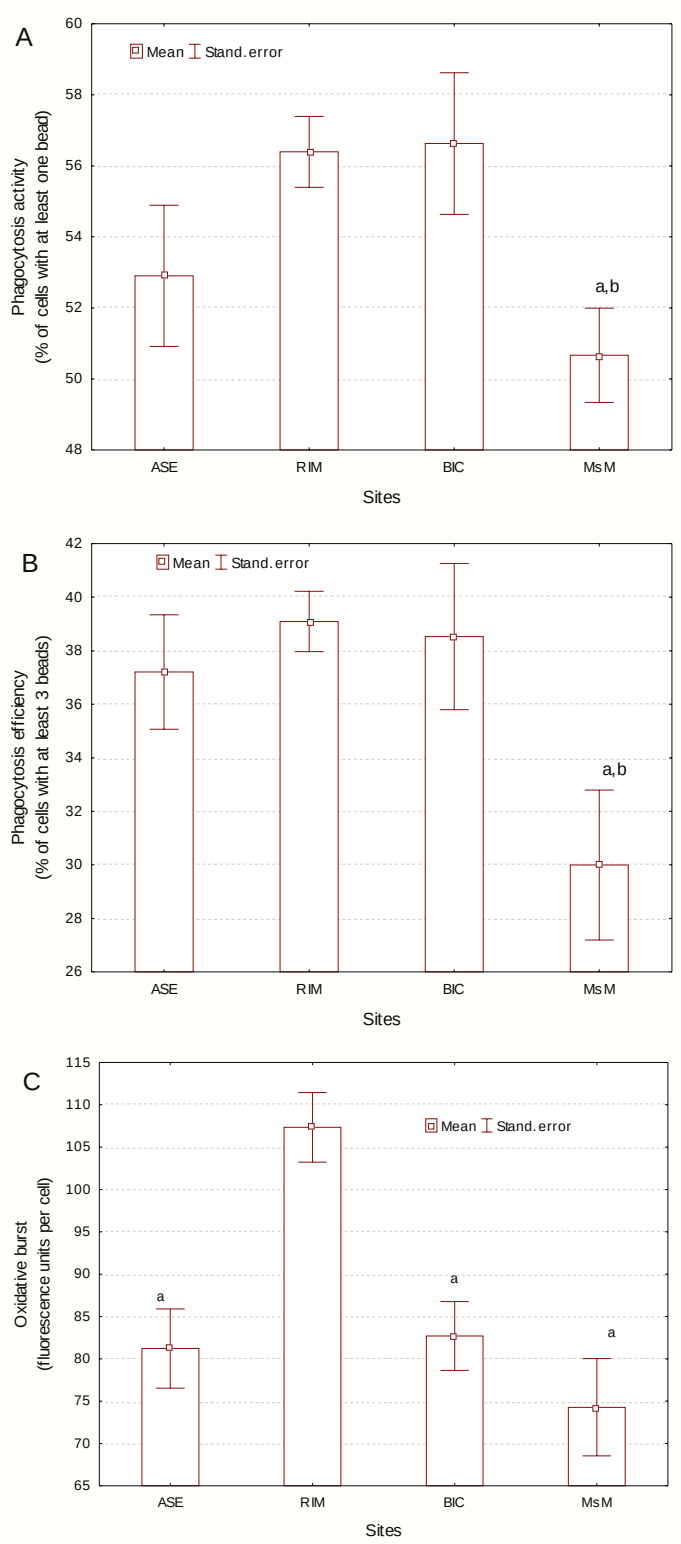

Figure 2. Change in immunocompetence in *Mya arenaria* clams. Clams were collected during low tide and processed for phagocytosis activity (**A**), efficiency (**B**) and oxidative burst (**C**). The letter 'a' indicates significant difference from the polluted RIM site (human activity with high levels of toxic phytoplankton). The letter 'b' indicates significant difference from the site containing the highest density in phytoplankton (BIC).

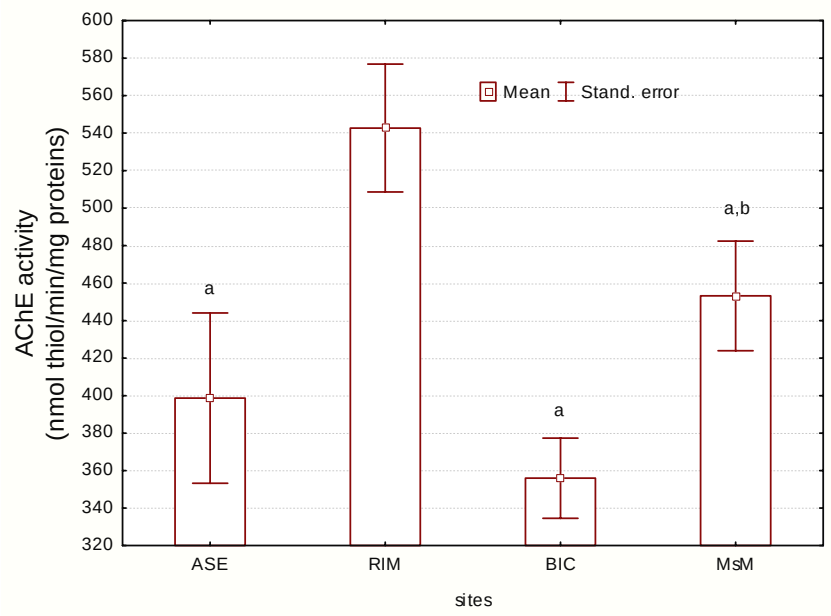

Figure 3. Change in neural activity in *Mya arenaria* clams.AChE activity was determined in the visceral mass of the clams. The letter 'a' indicates significant difference from the polluted RIM site (human activity with high levels of toxic phytoplankton). The letter 'b' indicates significant difference from the site containing the highest density of phytoplankton (BIC).

ASE, BIC and MsM sites. ROS production was significantly correlated with hemocyte density ($r = -0.59$; $p < 0.01$), suggesting that ROS levels were higher at low hemocyte concentration. Multiple regression analysis revealed that ROS production was correlated with the site characteristics ($r = 0.46$; $p = 0.01$), but there were no significant partial correlations with population size, total phytoplankton and toxic phytoplankton levels. This suggests that the site characteristics influence each together for this parameter.

Neural activity was determined in clams by monitoring changes in AChE activity in the visceral mass (Figure 3). AChE differed significantly by site; the highest activity was found at RIM compared to ASE, BIC and MsM. AchE activity at BIC site was the lowest. Multiple regression analysis showed that AChE activity was significantly correlated ($r = 0.43$; $p < 0.001$) with population size, total phytoplankton and toxic phytoplankton loadings. However, as with ROS activity, no significant partial correlations were found. The levels of total sugars and proteins in the visceral mass of clams were also examined to determine the energy state (Figure 4). The levels of sugars were significantly higher at BIC, which was the site with the highest density of total phytoplankton (Figure 4A). The sugar levels at RIM were higher than at MsM and ASE, which suggests that pollution from human activity was associated with increased energy uptake in the form of sugars. Indeed, multiple regression analysis showed that total sugars were significantly correlated ($r = 0.70$; $p < 0.001$) with total phytoplankton, population and toxic phytoplankton. Partial correlation analysis revealed that only total phytoplankton ($\beta = 0.81$; $p < 0.05$) was significant. The same pattern was obtained for total proteins in visceral mass (Figure 4B). The BIC and RIM sites had higher levels of proteins than the ASE and MsM sites. Multiple regression showed that protein levels were significantly correlated ($r = 0.78$; $p < 0.001$) with population ($\beta = 0.36$; $p > 0.1$), total phytoplankton ($\beta = 0.81$; $p < 0.05$) and toxic phytoplankton ($\beta = -0.1$; $p > 0.1$). This analysis is consistent with the observation that clams from the BIC site, which contained the highest amounts of algae, had increased energy reserves in the form of total sugars and proteins.

To obtain a global view of the responses, the data were subjected to factorial analysis (Figure 5), which showed that the first and second factors explained 30% and 25% of the variance, respectively. The

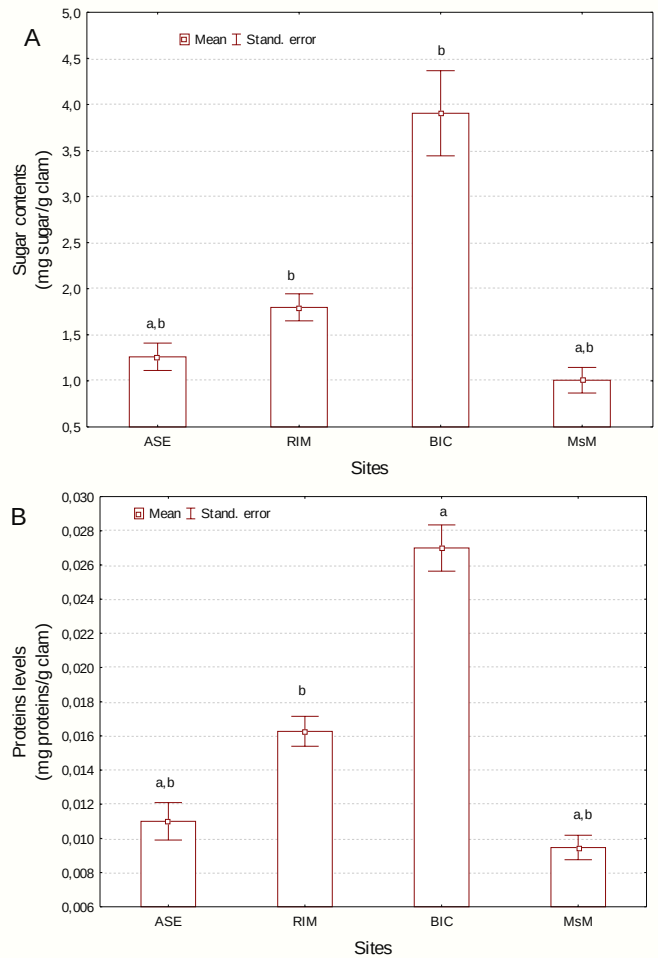

Figure 4

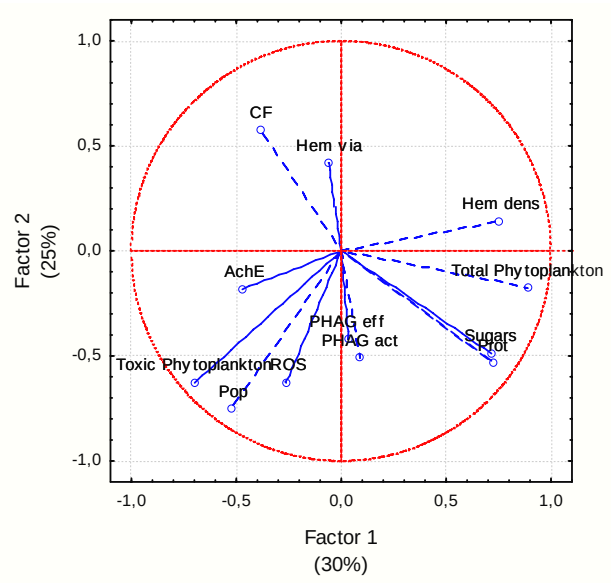

Figure 5

biomarkers that most explained those two factors were total phytoplankton, toxic phytophytoplankton, hemocyte density, total proteins, total sugars and population. Total phytoplankton levels were closely related to hemocyte density and energy reserves as total proteins and sugars. Toxic phytoplankton levels were closely related to AChE activity (a neurotoxicity marker), ROS levels and population density.

DISCUSSION

Toxic phytoplankton levels were the highest at the RIM site, which has higher input of urban pollutants such as metals, polyaromatic hydrocarbons (PAHs) and pesticides [24]. Clam condition factor was lower at RIM and BIC, while hemocyte densities were generally lower at RIM than at the other sites on the south shore of the St. Lawrence Estuary (BIC and MSM). Long-term exposure to municipal effluents in an urban area could be deleterious to bivalves. Urban pollution is known to increase hemocyte concentration and modulate phagocytosis activity where this response is considered a first response to the presence of suspended matter (and microorganisms) [25]. The concomitant exposure of mussels to a pathogen and urban pollution (effluents) had impacts on hemocyte counts, phagocytosis and ROS [26]. This suggests that urban pollution and pathogenic bacteria act through the same pathways, i.e., cumulatively. Oysters feeding on phytoplankton-rich *Nitzschia* spp. algae lost weight and eventually died due to infection by opportunistic microorganisms [27]. It was suggested that feeding on "inedible algae" during blooms of *Nitzschia* spp. starved the oysters, rendering them more susceptible to disease and leading to mortality. *Nitzschia* spp. are known to produce domoic acid, a potent glutamate receptor agonist which could lead to neural cell death. This algal species was also detected at RIM and BIC sites (Table 3). Exposure of oysters to paralytic shellfish toxins from *Alexandrium catenella* decreased resistance to a pathogenic strain of *Vibrio tasmaniensis* [28]. This study suggested that feeding on neurotoxic dinoflagellates could present a risk not only to the neurological system but also to immunocompetence, which could contribute to the severity of oyster mortality events.

In a study of snails feeding on toxic cysts of the dinoflagellate *Alexandrium minutum*, decreased hemocyte viability, phagocytosis and ROS production were observed [29]. Hemocyte viability was seemingly lower at RIM than at ASE but was not significantly different ($p > 0.05$). However, ROS production was found to be significantly higher at RIM than at ASE, BIC and MsM, suggesting increased oxidative burst (stress) in clams. Snails exposed to the toxic cysts showed abnormal behaviour, possibly due to muscle paralysis. In a study of scallops, exposure to *Alexandrium minutum* strain producing paralytic shellfish toxins (PST) led to decreased growth, reduced escape time from a predator (starfish), and signs of muscular damage consistent with increased acetylcholine signalling [30]. Interestingly, exposure to other extracellular bioactive compounds (in a strain of *A. minutum* not expressing PSP) could also damage scallops, causing reduced growth and increased reaction time to starfish predation. The CF in clams at RIM, where the highest levels of toxic algae occurred, was significantly lower than at ASE and MsM. The CF at BIC was also lower, although the total toxic algae loadings were small compared to those at the other sites. However, BIC and RIM consistently contained *Pseudo-nitzschia* species which were not found at the other sites (ASE and MsM). This is also consistent with the increase in AChE activity at RIM (where the highest number of toxic phytoplankton occurred) compared to the other sites. The neurotoxin anatoxin is a potent nicotinic acetylcholine receptor agonist which would increase acetylcholine signalling in tissues [14]. Other neurotoxins such as brevetoxin could

Legend to Figure 4. Change in relative protein and sugar levels in visceral mass. The levels of sugars (A) and total proteins (B) were determined in the visceral mass of the clams. The letter 'a' indicates significant difference from the polluted RIM site (human activity with high levels of toxic phytoplankton). The letter 'b' indicates significant difference from the site containing the highest density of phytoplankton (BIC).

Legend to Figure 5. Factorial analysis of biomarker data. Biomarker data were analyzed using principal component analysis. The biomarkers closest to the circle have the highest factorial weight.

stimulate sodium gate channels in persistent and repetitive firing, leading to the release of neurotransmitters from autonomic nerve endings [7]. In an attempt to reduce acetylcholine signaling, AChE activity could increase in order to hydrolyze (inactivate) acetylcholine. AChE could be an indicator of exposure to neurotoxins during toxic phytoplankton proliferation. *Nitzschia* spp. are producers of domoic acid, which is a potent glutamate receptor agonist. This stimulation could lead to tremors and downstream acetylcholine stimulation of muscular activity [31]. Indeed, glutamate signalling stimulates inositol lipid signalling in many neuronal cells, which is also involved in cholinergic-induced excitotoxicity. This could be compensated by increased turnover of acetylcholine (AChE). However, RIM (the largest urban site) could contain other pollutants that would increase acetylcholine stimulation, such as neonicotinoid pesticides [32]. Neonicotinoids could stimulate nicotinic sensitive acetylcholine receptors and produce cholinergic effects. More research is needed to confirm whether AChE activity could be increased by the release of acetylcholine from brevotoxins or the activation of gated sodium channels by neurotoxins in mollusks. Nevertheless, the cumulative effects of releases of neurotoxins during algal blooms and the use of neonicotinoid pesticides could eventually harm local clam populations. Exposure to metals could also contribute to biochemical responses against the dinoflagellate *A. minutum* in oysters [33]. Simultaneous exposure to Cd, Cu and *A. minutum* induced both antagonistic effects (increased hemocyte concentration and phagocytosis by *A. minutum*) and synergic effects (on lipid catabolism, neutral lipids and diacylglycerol levels). Interestingly, metals decreased ROS production in hemocytes while an *A. minutum* diet did not produce changes compared to controls feeding on *Tisochrysis lutea*. Exposure to the toxic dinoflagellate *A. catenella* (PST and extracellular bioactive compounds) in juvenile oysters leads to increased hemocyte density and ROS production in circulating hemocytes [34]. This is consistent with the observed responses in the present study, although *Alexandrium* sp. was not detected, suggesting that other toxic phytoplankton could have contributed to these non-specific effects.

CONCLUSION

In conclusion, clams collected at the most populated site, RIM, had low condition factor (clam weight/shell length) and showed elevated levels of toxic phytoplankton species (especially *Pseudo-nitzschia* spp.), ROS production and AChE activity. Hemocyte density, total proteins and sugars in the visceral mass were highest at BIC, which had the highest phytoplankton loadings in the water. The study suggests that the combined presence of toxic phytoplankton species and urban pollution could act at the same receptors (AChE activity and ROS levels), producing cumulative effects in local clam populations. However, this study is considered exploratory/preliminary given mussels were sampled spatially only and no-time related data in tissue toxin loadings were performed to more-directly determine the cumulative effects of urban pollution and the occurrence of potentially toxic phytoplankton.

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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