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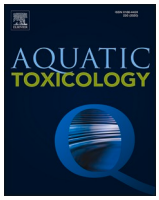

Hypoxemia as the mechanism of acute cationic polymer toxicity in rainbow trout and prevention of toxicity using an anionic neutralizing polymer

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ABSTRACT

Industrial operations such as surface mining, road building, and aggregate washing result in high concentrations of suspended particles (Total Suspended Solids; TSS) in surface waters which must be treated prior to discharge into fish-bearing waters. A common industrial practice is to add flocculants to improve the efficacy and speed of TSS sedimentation. A significant environmental issue even small amounts of uncomplexed cationic polymer coagulant/flocculant remaining in treated water is highly toxic to fish at very low concentrations (LC_{50} ~ 0.3 mg L^{-1}). Fingerling trout *(Oncorhynchus mykiss)* were exposed to (1) a cationic flocculant (Water Lynx 800 (WL800), (2) a Clearflow neutralizing polymer (CN369), and (3) a combination of WL800 and CN369 at various ratios with measured LC_{50} as an index of toxicity. Acute toxicity was entirely reversed by addition of the neutralizing polymer at WL800:CN369 ratios >1:1.5 mg/L.

Furthermore, we demonstrate that the proximal mechanism of acute cationic polymer toxicity is hypoxemia due to accumulation of polymer on the gill epithelia rather than gill damage. Exposure of 0.5 mg/L WL800 reduced oxygen consumption by >50% reduction by 12 h and this was accompanied by significantly increased blood, brain, and liver [lactate] and [glucose]. The development of an inexpensive amelioration technique preventing cationic polymer toxicity is a significant advancement in surface and industrial water treatment to prevent cationic polymer mediated fish kills.

1. Introduction

Industrial operations such as mining, textile industry, pulp and paper, agricultural and food processing, road building, and aggregate washing result in high concentrations of suspended particles (Total Suspended Solids; TSS) in surface waters which must be treated prior to discharge (Teh et al., 2016) due to the adverse effects of TSS on aquatic organisms (Newcombe and Macdonald, 1991; Bilotta and Brazier, 2008). While tailing/settling ponds are commonly used to passively treat surface waters by promoting sedimentation of TSS, some suspended particles do not settle rapidly. Industrial best practices to further reduce TSS discharge include using chemical flocculants and coagulants to agglomerate TSS and promote their sedimentation prior to the release of water (Chesters et al., 2009; Mahmudabadi et al., 2018). These flocculants include long-chain cationic, anionic, or neutral polymers (Bolto and Gregory, 2007; Mahmudabadi et al., 2018). Given that most fine clays are negatively charged, cationic polymers are amongst the most commonly used flocculating agents. Conversely, anionic polymers, including anionic polyacrylamide (PAM) are ineffective against negatively charged clay particulates, but are extremely effective in flocculating positively charged particles including residual metals (Barvenik, 1994; Entry et al., 2002; Lentz and Sojka, 1994; Sojka et al., 2005; Mahmudabadi et al., 2018; Vajihinejad et al., 2019).

One environmental issue with cationic polymers is their high toxic potential as demonstrated in standard toxicity assays. This is especially true if accidental releases occur in environments with receiving waters containing low total organic carbon (TOC) such as occurs in rivers primarily fed by glacial/snowmelt waters (e.g., mountain top mining). Reported LC50's for Magnafloc 368 range from 0.38 mg L^{-1} (rainbow trout; Kerr el al., 2014) to 2.08 mg/L (lake trout; Liber et al., 2005). These differences in LC50 are likely the result of differences in TOC of test media (Salinas et al., 2020). Conversely, anionic polymers have ~300-1000-fold lower toxicities than their cationic counterparts (Kerr et al., 2014; Liber et al., 2005) and therefore can be relatively safely used for treating industrial wastewaters.

Negatively charged substances like organic carbon (humic and fulvic

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acids) are known to significantly reduce the toxicity of cationic polymer flocculants (Cary et al., 1987; Goodrich et al., 1991; Hall and Mirenda, 1991; Muir et al., 1997; Salinas et al., 2020). One strategy to circumvent toxicity of water treated with cationic polymers is to release treated water to ponds or wetlands containing high levels of negatively charged organic carbon. While an appealing solution, the applicability of this strategy is limited by the availability of humic acids in the receiving environments. An alternative strategy to mitigate excess cationic polymer toxicity would be to apply specific neutralizing anionic polymers prior to release. We hypothesized these anionic polymers would bind to any residual cationic polymer present in the water column, thereby mitigating cationic polymer toxicity. While there is anecdotal evidence in the success of anionic polymers mitigating cationic polymer toxicity, the method's efficacy has not been empirically demonstrated. Furthermore, a direct demonstration of the effectiveness of the anionic mitigation method on preventing toxicity to fish has yet to be demonstrated. Thus, the first aim of this study was to investigate the potential for the amelioration of cationic polymer toxicity *via* charge neutralization using pre-treatment with anionic polymers.

While cationic polymers have been shown to be associated with minor gill damage (Kerr et al., 2014; Liber et al., 2005), the degree of damage is incongruent with the rapid (< 24 h) toxicity noted for most cationic polymers. There is evidence that cationic polymers coat the outer, negatively charged cell membranes of fish (Muir et al., 1997) and other organisms (Salinas et al., 2020, Simões et al.,2022). In fishes, polymer binding would increase the oxygen diffusion distance across the gill epithelium, thus reducing gill oxygen transport capacity and effectively suffocating the fish. Our second objective was to examine if the acute toxicity from cationic polymers results from the binding of the polymer to the gill membrane and induction of hypoxemia. We hypothesized that the hypoxemia resulting from inhibition of oxygen transport should be evidenced earlier changes in tissue metabolites lactate and glucose (Fromm, 1980). To test this hypothesis, we measured oxygen consumption and metabolite levels in plasma, brain, and liver tissues at 12 h and 48 h after exposure to either cationic polymer (0.5 mg/L), anionic polymers (0.75 mg/L), or a combination of anionic and cationic polymers (toxicity mitigation). Standard histological analysis was also performed to determine if anionic polymers used as neutralizing agents can also prevent any gill damage normally caused by cationic polymer exposure.

2. Materials and methods

2.1. Animal husbandry and ethics

Fingerling rainbow trout (*Oncorhynchus mykiss*; <4 cm, <2 g) were obtained from Sam Livingston Fish Hatchery (Calgary, Alberta) and shipped to the University of Alberta, where they were housed in 300 L tanks receiving flow-through dechlorinated Edmonton municipal tap water. The trout were housed on a 16:8 h light: dark cycle and were fed daily (~1% body weight) with commercial fish chow. Fish were fasted 24-48 h prior to experimental protocols as per OECD guidelines. Prior to experimentation, juvenile rainbow trout were transferred from main holding tanks to a large 60 L tank within the experimental room for overnight acclimation to the room where the tests were performed. All experiments were conducted at 15°C ±1°C in static 96 experiments. All tanks were individually aerated and monitored daily for dissolved oxygen, total ammonia, pH, nitrate, and nitrite. All experiments followed Canadian Council on Animal Care guidelines and were approved by the University of Alberta Animal Care Committee (AUP# 0001334 and AUP# 00002352).

2.2. Chemicals

One commercial cationic polymer, Water Lynx 800 (WL800), and a Clearflow neutralizing polymer (CN369) were provided by Clearflow Group Inc (Sherwood Park, Alberta). WL800 is a cationic polymer with >99% charge density and an average MW of >200 kDa. All other chemicals were supplied by Sigma-Aldrich.

2.3. Experimental protocol

2.3.1. 96 h NOEC toxicity tests for cationic: anionic toxicity mitigation

To determine a suitable mixture of cationic and anionic exposures for downstream sub-lethal toxicological effect experiments, comprehensive set of 96 h toxicity test series was conducted using different concentrations of cationic polymers and adding CN369 polymer to achieve different WL800:CN369 ratios. 72 exposure chambers could be tested simultaneously (24 exposure conditions x 3 replicates). For experimentation, glass exposure chambers were filled to 7 L with activated charcoal filtered and dechlorinated City of Edmonton municipal tap water. Cationic polymer was added to test tanks from a 1 g/L stock solution of WL800 to achieve the desired final [cationic polymer]. Multiple cationic polymer (WL800) concentrations (0, 0.25, 0.38, 0.5, 0.75, 1.0, 1.4, 2.4, 3.8, 4.2, 5.0, 5.6, 10 and 11.2 mg L^{-1}) were all tested in at least 3 LC50 replicates at each amelioration ratio. For each eachTCN369 neutralizing polymer was ratiometrically added to each test tank from a 1 g/L stock solution in ratios ranging from 1 part cationic:0 parts anionic (no amelioration), 1 part cationic: 0.1 part anionic, 1 part cationic:0.25 parts anionic, 1 part cationic:0.5 parts anionic, 1 part cationic:1 part anionic; 1 part cationic:1.5 parts anionic, 1 part cationic:2 parts anionic, 1 part cationic:5 parts anionic). In total, > 600 individual exposures were conducted. The goal of using ratios was to test for the efficiency of mitigation of cationic polymer toxicity regardless of actual cationic polymer concentration. Following the addition of both polymers to the experimental chambers, the water was mixed by continuous aeration for at least an hour before adding fish. Each 7 L test chamber trout contained ten fingerlings of ~ 0.5-1 g and mortality was monitored twice daily for the next 96 h according to OECD Test Guideline 203.

2.3.2. Effect of anionic toxicity mitigation on metabolic oxygen consumption

Initial experiments in Series 1 demonstrated that the lethality of cationic polymer could be fully mitigated when mixed in a ratio of 1-part cationic WL800:1.5 parts anionic polymer CN369. Thus, going forward, we utilized exposure concentrations of 0.5 mg/L for cationic polymers which was greater than the 96 h LC_{50} of 0.38 mg/L but causing less than 100% mortality during the first 48 hrs of exposure) and 0.75 mg/L of anionic polymer CN369 (therefore, a ratio of 1:1.5 when mixed), to examine the physiological implications of both cationic polymer alone, anionic polymer alone, or a combination of the two agents.

On the night prior to experimentation, tanks filled with dechlorinated tap water (7L) were prepared in duplicate with appropriate additions of cationic and anionic polymer stock solutions to achieve the following conditions: control (no polymer); cationic (0.5 mg/L); anionic (0.75 mg/L); and a polymer mixture (0.5 mg/L cationic + 0.75 mg/L anionic). Fingerling rainbow trout (1.5-2 g) were transferred from the main holding tank into a 60 L tank for overnight acclimation. Food was withheld 24 h prior to all experiments. On the morning of the experiment, groups of 5 fish were placed in each respective exposure tank (n = 10 or 2 tanks per treatment; Control, Anionic, and Mixture groups) or (n = 20 of 4 tanks of 5 fish for Cationic polymer alone exposure group to account for greater potential mortality) and mortality observed at 0, 12, 24, and 48 h exposure.

During the exposure, 4 fish from each replicate fish were individually removed from the exposure for measurement of routine oxygen consumption rate (MO2) at 12, 24, and 48 h. Fish were placed individually into individual air-tight containers with ~400 mL of normoxic treatment-specific water. A magnetic spin-bar ensured appropriate mixing of water. Chambers were hermetically sealed and verified for lack of air bubbles which would confound measurement. Oxygen tension was measured intermittently using fiber optic oxygen sensors

connected to a WITROX 4 oxygen meter (Loligo, Viborg, Denmark). After each flux period, the chambers were weighed to determine volume. Then, the animals were lightly anesthetized (80 mg/L TMS (Syndel) and 80 mg/L$NaHCO_3$) and then weighed prior to returning to their respective exposure condition. Oxygen tensions were converted to μmol L^{-1} using the oxygen solubility constants described in Boutilier et al. (1984), and MO_2 was calculated as previously described (Clifford et al., 2016).

Effect of anionic toxicity mitigation on fuel and metabolite levels. Rainbow trout were exposed to the cationic, anionic, mixture, and control conditions as in Series 2; however, in this experimental series, trout ($n = 8$ for each treatment) were sacrificed at either 12 or 48 h via MS222 overdose (1 g/L MS222 + 1 g/L $NaHCO_3$). Following cessation of opercular movement, animals were blotted dry, weighed and the posterior end of the animal (posterior to the cloaca) was severed to allow for a blood sample (mixture of arterial and venous blood) *via* capillary tube. Blood was transferred to a 1.5 mL Eppendorf tube and immediately centrifuged for 60 s at 12,000 x *g*. The resulting plasma supernatant was drawn off, and a 25 μL aliquot was deproteinated 1:2 vol: vol with 8% perchloric acid (PCA) and stored for later determination of fuel and metabolite levels (see below). Following collection of blood, the brain and liver (in that order) were removed and placed into a pre-weighed 1.5 mL Eppendorf tube, which was snap-frozen in liquid nitrogen and stored at -80 °C until processed for biochemical analysis. Altogether, the anaesthetization, blood sampling and dissection process took < 2 min to complete.

2.4. Tissue processing for fuel and metabolites

Tissues collected from Series 3 experiments were processed *via* techniques adapted from Clifford et al. 2012. Briefly, frozen tissues were weighed and diluted 4X with 4 °C homogenization buffer (8% PCA + 1 mM EDTA) and immediately homogenized using a battery-powered handheld homogenizer (Gerresheimer Kimble Kontes LLC, Dusseldorf, Germany). All PCA-processed tissues were left on ice for 10 min and then split into two subsamples; the first was used for glycogen analysis, and the second was designated for ATP, Phosphocreatine (PCr), and lactate quantification. All subsequent centrifugation occurred at 4 °C at a speed of 10,000 x *g*. Subsample one was vortexed, centrifuged for 2 min, and then neutralized by adding 0.5 volumes (volume: weight) of 2 mol L^{-1} KOH containing 0.4 mol L^{-1} imidazole and 0.4 mol L^{-1} KCL to the extract. The extract was centrifuged for 3 min and the resulting supernatant drawn off into a 1.5 mL Eppendorf tube which was snap-frozen in liquid nitrogen and stored at -80 °C for later determination of tissue lactate concentration, ATP, and Phosphocreatine.

Subsample two was thawed on ice and diluted 1:1 (vol: vol) with 2 mol L^{-1} $C_2H_3NaO_2$. An aliquot of this acetate /sample mixture was removed and frozen for determination of background glucose levels. At the same time, 40 i.u. of amyloglucosidase enzyme was added to the remainder of the acetate/sample mixture, followed by a 2 h incubation in a 37 °C water bath. The reaction was terminated with the addition of 70% PCA solution followed by neutralization with 3 mol L^{-1} K_2CO_3.

2.5. Characterization of tissue energy stores and metabolites

Tissue glucose and lactate, ATP, and phosphocreatine concentrations were measured *via* spectrophotometry (Spectramax 190; Molecular Devices, Sunnyvale, California) using established NAD-linked assays as per Bergemeyer (1983). Briefly, plasma glucose and tissue glucose (pre-and post-glycogen liberation *via* amyloglucosidase) were measured enzymatically using hexokinase and glucose-6-phosphate dehydrogenase (as a coupling enzyme). Plasma and tissue lactate were measured enzymatically using lactate dehydrogenase. Levels of tissue ATP and phosphocreatine were measured using hexokinase, glucose-6-phosphate dehydrogenase (as a coupling enzyme), and creatine kinase.

2.6. Gill morphology following acute transfer to polymer mixtures

Rainbow trout were exposed and euthanized as in Series 3 and processed for histological use as previously described (Clifford et al., 2017). Briefly, following cessation of opercular movement, the operculum was removed. The 3rd gill arch excised, rinsed in phosphate-buffered saline (PBS), then placed into fixative (4% Paraformaldehyde in 10 mmol L^{-1} PBS, pH 7.3) for 24 h. Tissues were thereafter washed 3 times in PBS, then dehydrated (1 h washes in 30, 50 and 70% ethanol followed by a second 1 h wash in 70% ethanol). Tissues then underwent standard paraffin embedding prior to being sectioned (5 μm sections) on a microtome. Tissues were stained using standard hematoxylin and eosin (H&E) staining protocols for structural reference. Stained sections were viewed under a Zeiss Scope.A1 microscope and images captured on an optronic camera.

2.7. Statistical analysis

All data are presented as means ± s.e.m. Differences in oxygen consumption and tissue/plasma fuel and metabolite levels were analyzed using a two-way analysis of variance (ANOVA), followed by Tukey's posthoc test with a fiducial limit of $p < 0.05$. Figures and all statistical analyses were produced/conducted using Prism 9.0 (Graphpad Inc. La Jolla, San Diego).

3. Results

3.1. 96 h toxicity tests in cationic: anionic polymer mixtures

3.1.1. LC50

In all experiments, we monitored water quality daily in 4–6 randomly selected test tanks (of 72 each week). A YSI multiprobe meter was used to measure dissolved oxygen and pH, while 1 sample of the common dilution water was taken prior to any polymer additions for TOC measurement. Total ammonia and nitrate were also monitored at 24, 48 h and 96 h using aquaria dip test strips in the same randomly selected 4-6 test tanks. DO was always greater than 95% saturation (>9 mg/L), pH varied between 7.85 and 8.05 and [total ammonia] in all cases was < 1 mg/L. Nitrate was always <10 mg/L in all tests. TOC in the test water varied slightly between 0.38 and 0.64 mg/L. We did not monitor hardness, but hardness as reported by EPCOR water utility varied between 166 and 183 mg/L during this period.

Rainbow trout exposed to increasing concentrations of WL800 cationic polymer showed an LC50 response of ~0.3 mg L^{-1} (Fig. 1). WL800 at 0.7 mg L^{-1} or greater resulted in 100% mortality of the trout fingerlings, and 0.7 mg L^{-1} was used to determine the efficacy of amelioration by neutralizing anionic polymers. When fish were exposed

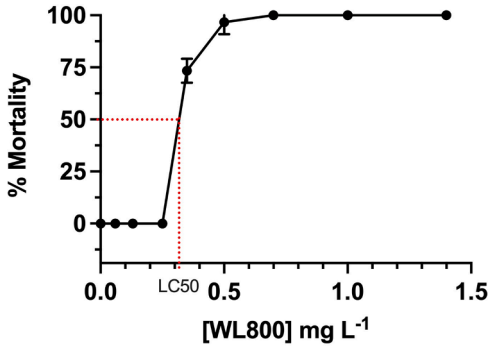

Fig. 1. Mortality following 96h of exposure to cationic polymer WL800 for juvenile rainbow trout (*Oncorhynchus mykiss*) showing LC50 of ~0.3 mg/L.

to varying ratios of cationic: anionic polymer mixtures, a ratio-dependent mortality rescue in fingerling rainbow trout was found. Amelioration effects were observed starting at cationic to anionic polymer ratios of 1:0.5 and greater. (Fig. 2). At cationic to anionic ratios of 1:1.5 and 1:2.0 (and at higher ratios), there were no mortalities (Fig. 2) nor observable toxic effects during the 96 h test period. This was true for all concentrations of cationic polymer tested: the concentrations of WL800 tested ranged from 0.5 mg L^{-1} to 25 mg L^{-1}. Anionic polymers alone did not have any observable toxicity at all concentrations tested. Based on these findings, a minimal 1:1.5 mixture ratio was selected for the remainder of our experiments with the concentrations of 0.5 mg L^{-1} for cationic polymer and 0.75 mg L^{-1} for the anionic polymer to demonstrate both the mechanism of toxicity (cationic polymer alone) and potential for mitigation (co-exposure of cationic: anionic at ratio of 1:1.5).

3.2. Effect of cationic polymer toxicity mitigation by anionic polymer amelioration on metabolic oxygen consumption

Rainbow trout were exposed to freshwater alone (Control group), or water spiked with cationic polymer (Cationic group 0.50 mg L^{-1}), anionic polymer (Anionic group 0.75 mg L^{-1}), or a mixture of the two polymers (combined polymer group 0.5 mg L^{-1} and 0.75 mg L^{-1} for cationic and anionic polymer, respectively) for up to 48 h. No mortalities were observed within the exposure period in all conditions except in the cationic group, which had 5% mortality (1 fish) at 12 h and reached 50% mortality by the end of the 48 h exposure period (Fig. 3A). Notably, all fish in the cationic alone group displayed significant coughing of the buccal cavity by 12 h while this behavior was absent in either the anionic or mixture treatments. Routine metabolic oxygen consumption (MO_2) was measured over 1 h periods in animals from each of the exposure groups prior to exposure (pre-exposure) and intermittently at 12, 24 and 48 h into the exposure duration (Fig. 3B). No differences among groups were observed during pre-exposure MO_2 determinations. Throughout exposure, MO_2 remained unchanged in all exposure groups except the cationic polymer alone, where MO_2 was > 50–60% reduced compared to pairwise trials in control animals and all other experimental groups at all exposure time points.

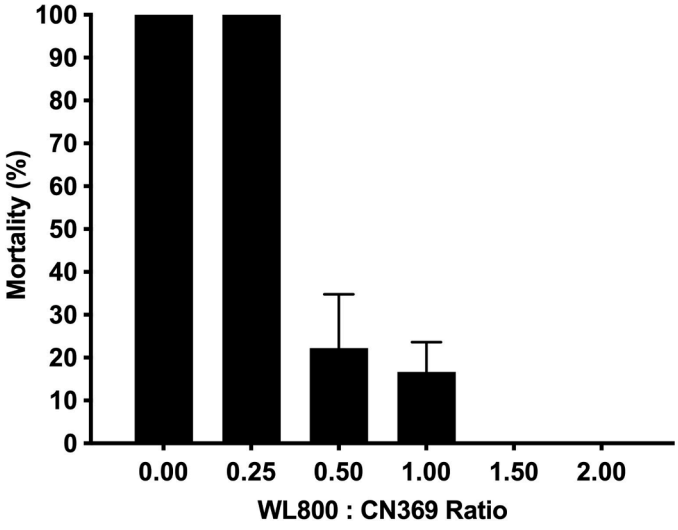

Fig. 2. Mortality of juvenile rainbow trout (*Oncorhynchus mykiss*) following 96h of exposure to a range of cationic WL800 polymer concentrations (0, 0.25, 0.38, 0.5, 0.75, 1.0, 1.4, 2.4, 3.8, 4.2, 5.0, 5.6, 10 and 11.2 mg L^{-1}) and the corresponding anionic (CN369) polymer ratio presented as WL800:CN369 ratios. Each WL800 concentration was tested at each amelioration ratio in at least 3 separate tests. There was total amelioration of WL800 induced mortality at CN369 ratios exceeding 1.5 (WL800:CN369). Data are presented as mean ± s.e.m. of each ratio, with $n = 10$ fish for each replicate.

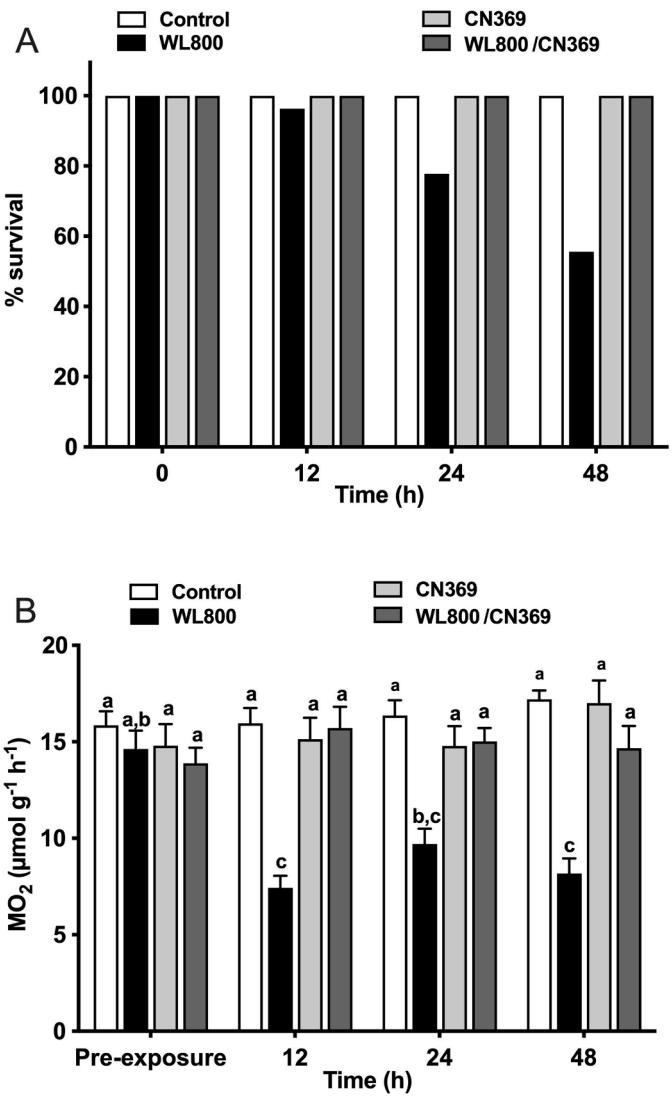

Fig. 3. Effects of cationic, anionic, and cationic/anionic mixtures on (A) survival and (B) routine metabolic oxygen consumption. Rainbow trout ($n = 70$) were exposed to either cationic (0.5 mg L^{-1}; black bars) or anionic (0.75 mg L^{-1}; light grey bars) polymers, or a mixture of cationic and anionic polymers (0.5 mg L^{-1}/0.75 mg L^{-1}; dark grey bars) for up to 48 h. Throughout the exposure period, **(A)** survival was quantified at intermittent time points, and **(B)** trout ($n = 8$) were randomly selected from each exposure condition with MO_2 determined over a 1 h period in their respective conditions. Following sampling, trout were returned to exposure conditions. Data presented as means ± s.e.m. $n = 8$ for each data point. Bars not sharing the same letter are significantly different; $p<0.05$, two-way ANOVA with Tukey's post-hoc analysis

3.3. Effect of cationic polymer toxicity mitigation by anionic polymer amelioration on energy stores and metabolites

Exposure of trout to cationic polymer, but not an anionic polymer, caused several significant alterations to fuel and metabolite levels in plasma, liver and brain tissue compared to control animals; when exposed to anionic polymer, or a combination of cationic and anionic polymers, these effects were generally absent. Plasma glucose concentrations in control animals averaged ~4-5 mmol $L^{-1}$and were unchanging throughout exposure (Fig. 4A). Animals in the cationic group had, albeit non-significantly, higher plasma glucose levels by 12 h of exposure than time-matched control animals ($p = 0.0799$). Plasma glucose levels in the cationic group were similar between 12 and 48 h exposure times; however, at 48 h, levels were more than 2-fold greater

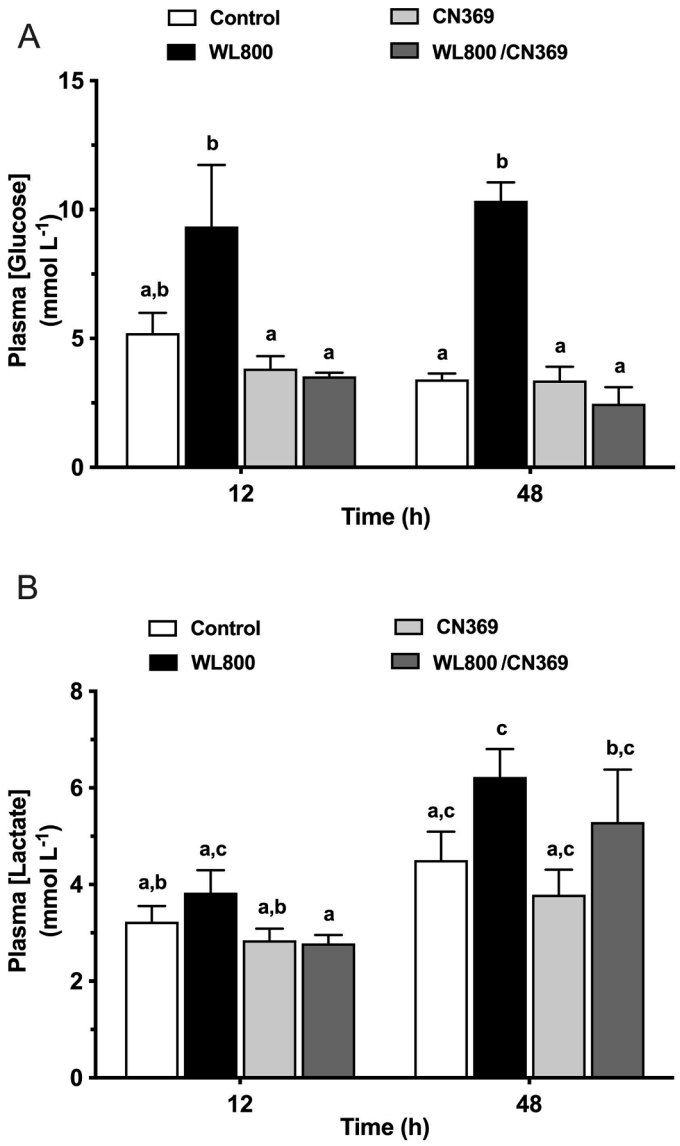

Fig. 4. Changes in plasma glucose **(A)** and plasma lactate **(B)** in rainbow trout following 12 and 48 h exposure to cationic (0.5 mg L^{-1}; black bars) or anionic (0.75 mg L^{-1}; light grey bars) polymers, or a mixture of cationic and anionic polymers (0.5 mg L^{-1}/0.75 mg L^{-1}; dark grey bars). Control animals (open bars) were held in freshwater containing neither polymer. Data are presented as mean ± s.e.m. Bars not sharing the same letter are statistically different; $p<0.05$, two-way ANOVA with Tukey's post-hoc analysis. $n = 8$ for each data point

than pair-matched control animals and animals from the other experimental groups at either time-point. No deviations in plasma glucose were observed in the anionic exposure group compared to control levels at either timepoint nor were there any differences observed in plasma glucose levels from control levels in animals exposed to a cationic /anionic mixture (Fig. 4A).

Plasma lactate levels averaged ~3 mmol g^{-1} wet tissue in 12 h control group animals and were not significantly different from time-matched experimental exposure groups ($p > 0.9946$; Fig. 4B). By 48 h, plasma lactate levels had more than doubled from 12 h control levels in the cationic group. Still, this elevation was not significantly different from levels measured in the 12 h cationic group. We did find a small but statistically significant elevation in plasma lactate present in the combined polymer group at 48 h when compared to the 12 h period group. (Fig 4). However, no differences were detected in this group against control animals at the time matched period.

Rainbow trout from all exposure groups displayed liver lactate levels that were not significantly different from control levels at 12 h. By 48 h, however, lactate levels in animals exposed to cationic polymer alone had risen ~2 fold, while animals in the control group, anionic group, and combined polymer group remained unchanged from 12 h exposure levels (Fig. 5A). Brain lactate levels averaged ~7 mmol g^{-1} wet tissue after 12 h in all control and treatment conditions (Fig. 5B). Levels remained unchanged after 48 h of exposure in animals from control, anionic, and combined polymer groups. In comparison, brain lactate levels in the 48 h cationic group were ~2-fold greater than in all other exposure treatments, regardless of sampling time (Fig. 5B). ATP and phosphocreatine were unaffected in any experimental group compared to levels in control animals at both 12 and 48 h (Table 1).

3.4. Gill morphology following acute transfer to polymer mixtures

H&E-stained gill sections from trout exposed for 12 h and 48 h to the

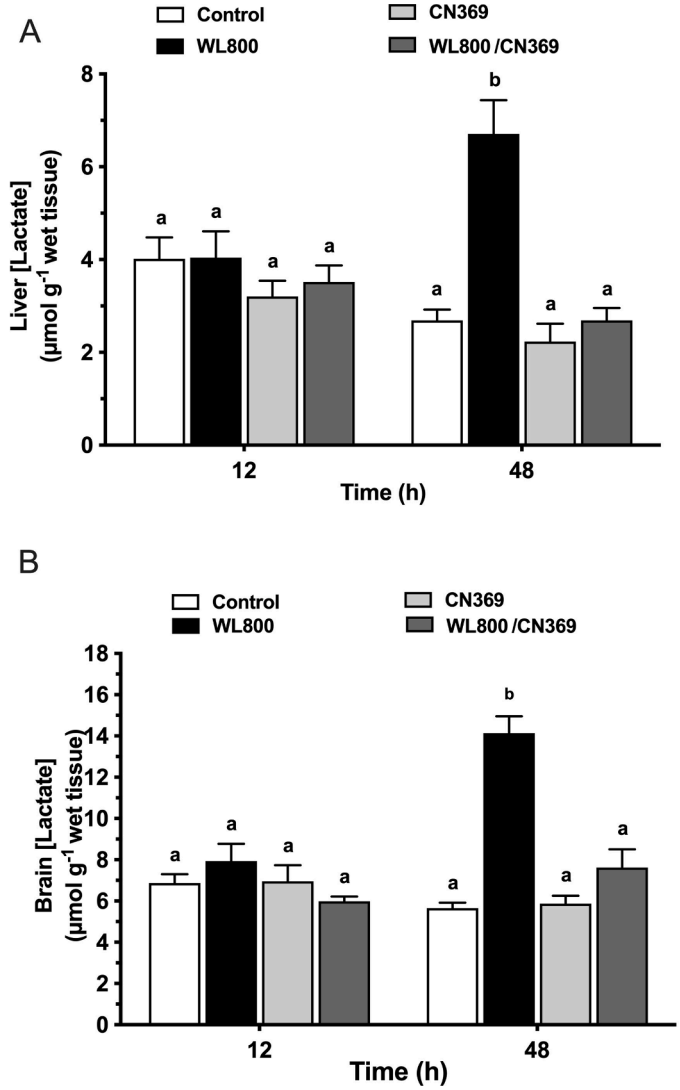

Fig. 5. Changes in liver lactate **(A)** and brain lactate **(B)** in rainbow trout following 12 and 48 h exposure to cationic (0.5 mg L^{-1}; black bars) or anionic (0.75 mg L^{-1}; light grey bars) polymers, or a mixture of cationic and anionic polymers (0.5 mg L^{-1}/0.75 mg L^{-1}; dark grey bars. Control animals (open bars) were held in freshwater containing neither polymer. Data are presented as mean ± s.e.m. Bars not sharing the same letter are statistically different; $p<0.05$, two-way ANOVA with Tukey post hoc analysis. $n = 8$ for each data point

Table 1
Characterization of tissue (liver and brain) ATP and Phosphocreatine in rainbow trout following 12 and 48 h exposure to cationic (0.5 mg L-1) or anionic (0.75 mg L-1) polymers, or a mixture of cationic and anionic polymers (0.5 mg L-1/ 0.75 mg L-1).

	Liver		Brain	
	12 h	48 h	12 h	48 h
	Tissue ATP (μmol g^{-1} wet tissue)			
Control	1.22 ± 0.21	0.99 ± 0.17	0.86 ± 0.14	0.92 ± 0.08
+ (0.5 mg L^{-1})	1.22 ± 0.12	0.83 ± 0.25	0.94 ± 0.13	0.79 ± 0.04
- (0.75 mg L^{-1})	1.29 ± 0.25	1.15 ± 0.12	0.85 ± 0.09	0.71 ± 0.16
+/- (0.5/0.75 mg L^{-1})	1.29 ± 0.30	0.50 ± .14	0.65 ± 0.13	0.45 ± 0.23
	Tissue Phosphocreatine (μmol g^{-1} wet tissue)			
Control	1.51 ± 0.18	1.85 ± 0.39	1.61 ± 0.40	6.68 ± 1.99
+ (0.5 mg L^{-1})	1.67 ± 0.31	1.48 ± 0.09	3.77 ± 1.18	5.18 ± 2.54
- (0.75 mg L^{-1})	1.45 ± 0.12	1.28 ± 0.07	4.81 ± 2.10	2.65 ± 0.78
+/- (0.5/0.75 mg L^{-1})	1.24 ± 0.23	1.33 ± 0.08	2.98 ± 0.93	6.57 ± 2.72

experimental conditions were examined for histological/morphological effects and images of 48 h exposed fish are found in Fig. 6. The gill filaments at both 12 h and 48 h had similar morphology when comparing the control, anionic polymer, and combined polymer groups (Fig. 6A, C and D). Gills of 12 h exposed juvenile trout did not show any differences from the other treatment groups while fish exposed to cationic polymer for 48 h showed some clumping/adhesions of gill filaments (Fig. 6B) but there was no epithelial lifting or other adverse histological effects noted.

4. Discussion

Industrial operations that involve land clearing have significant issues with meeting environmental discharge limits for TSS, especially when working in areas with fine negatively charged clays in the soils. Typically, tailing/settling ponds are used to promote sedimentation of TSS to meet environmental discharge limits, creating significant costs for companies as sedimentation ponds require both maintenance and testing. Cationic polymers are highly effective at removing negatively charged TSS but create additional compliance issues since cationic polymers cannot be directly released to fish-bearing waters due to their high toxicity (De Rosemond and Liber 2004; Kerr et al., 2014). The present study demonstrates that the LC_{50} for WL800 cationic polymer is ~ 0.3 mg/L in the absence of either added dissolved organic carbon (Salinas et al., 2020) or any mitigating anionic polymer. However, if CN369 anionic polymer was present and pre-mixed at a 1.5-fold higher

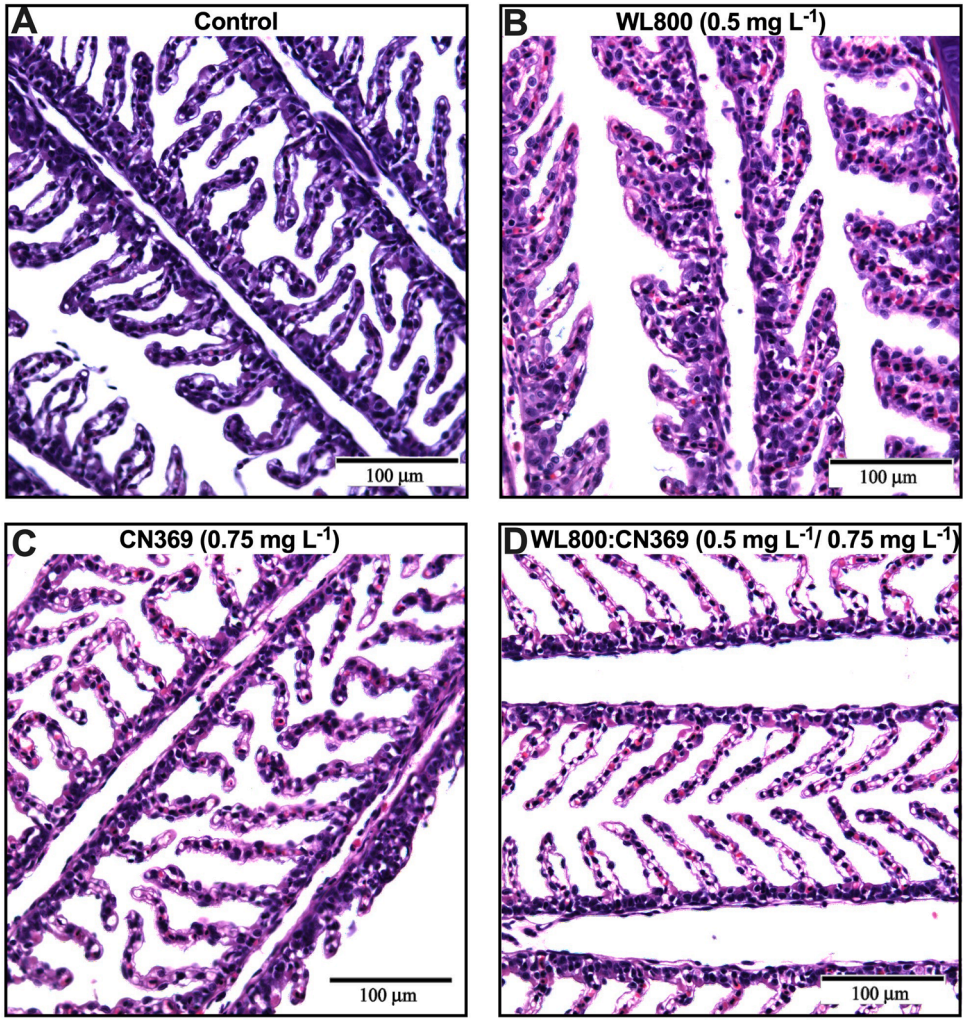

Fig. 6. Representative hematoxylin and eosin-stained gill filaments (40X magnification) from rainbow trout exposed for 48 h to **(A)** freshwater control, **(B)** cationic polymer (0.5 mg L^{-1}), **(C)** anionic polymer (0.75 mg L^{-1}), or **(D)** mixture of cationic and anionic polymers (0.5 mg L^{-1}/0.75 mg L^{-1}). Scale bar = 100μM.

concentration than the cationic polymer, the toxicity to rainbow trout was completely ameliorated. This ratio held true for both low concentrations (e.g., 0.5 mg/L cationic/0.75 mg/L anionic) and high concentrations (25 mg/L cationic, 37.5 mg/L anionic) of polymers. This finding creates the unique opportunity to treat cationic contaminated industrial wastewater by simple pre-application and mixing of appropriate concentrations of a cationic neutralizing anionic polymer such as CN369 prior to water release.

This study also revealed that the proximal cause of cationic polymer toxicity is the impairment of oxygen transport, resulting in reduced oxygen uptake, subsequent hypoxemia, and death. Fish exposed to water treated with neutralizing anionic polymers displayed none of the physiological indicators of impairment observed in fish exposed to untreated, cationic spiked water. The anionic surface of biological membranes provides a binding site for cationic polymers (Muir et al., 1997; Kerr et al., 2014). Upon binding to the gill, cationic polymers would significantly increase the boundary layer on the surface of the gill, increasing diffusion distance for oxygen and impairing oxygen uptake into the fish. A lack of oxygen is known to result in the generation of tissue lactate due to increased reliance on anaerobic glycolysis for metabolic processes (Hochachka, 1991) and result in an increase in glucose mobilization, likely due to mobilization of cortisol as a stress hormone (Jentoft et al., 2005). Previous research (Liber et al., 2005) suggested that gill damage was the cause of cationic polymer toxicity with significant lamellar fusion and epithelial lifting noted in fish exposed to cationic polymer at sublethal concentrations for 72 h. However, given the clear effect on gill oxygen transport at a much earlier time point (12 h) when there was a relative absence of any histological impairment, it is proposed that the proximal cause of cationic polymer toxicity is though impairment of oxygen transport. Addition of neutralizing polymer at merely 1.5 times the concentration of the cationic polymer eliminates these oxygen impairment effects and ameliorated toxicity. We proposed that the saturation binding of the anionic polymer to the cationic polymer created by the minimum 1.5X ratio prevents the binding of cationic polymers to the anionic gill surface and therefore prevents acute toxicity of the polymers.

While this procedure holds promise as a water treatment process for TSS contaminated in industrial waters, there remains a few opportunities for continued research that should be addressed. It must be noted that various cationic and anionic polymers have different charge densities (Tiravanti et al., 1985; Pereira et al., 2018; Salinas et al., 2020; Simões et al., 2022) and how these charge densities affect the relative ratios required for amelioration remains the subject of future investigation. A second significant issue with the implementation of this passive treatment process is the inability to measure cationic polymer concentrations easily and accurately in water to determine the required neutralizing polymer required. Industry, particularly where surface water treatment is concerned, adjusts dosing of cationic polymers based on volume and load factors of TSS and then stores this water in surface impoundments. Incoming TSS values are dramatically influenced based on operational requirements and climate/rain events (Mines et al., 2007)). This obviates the need for the development of a practical and rapid method to measure cationic polymer concentrations in real time to implement an anionic polymer mediated treatment protocol. Finally, field studies examining any potential longer-term effects of combined polymers on invertebrate and vertebrate biota in receiving waters should be conducted to ensure safety of this new method for treatment of cationic treated surface waters.

5. Conclusions

In summary, this study provides evidence that WL800 cationic polymer's mode of toxicity is through impairment of oxygen transport at the gill surface. Further, it is demonstrated that cationic polymer toxicity can be eliminated by the addition of CN369 anionic polymer at appropriate ratios if cationic polymer concentrations are known. Future research will focus on the impact of physicochemical properties such as charge density (Simões et al.,2022) of cationic and neutralizing polymers on toxicity, methods for measurement of cationic polymers in water, and potential longer-term effects of cationic: anionic mixtures on aquatic biota in receiving waters.

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CRediT authorship contribution statement

Alexander M. Clifford: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Edyta J. Jasinska:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Jesse Meints:** Resources, Writing – review & editing. **Jerry Hanna:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Greg G. Goss:** Conceptualization, Methodology, Validation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Jerry Hannah is the inventor of the Clearflow products and Jesse Meints is staff scientist at Clearflow Group. Alex M Clifford and Edyta J Jasinska were employed by Clearflow Group during this research as a condition of the funding from the National Research Council of Canada but it was conducted 100% of the time in GGG's laboratory at the University of Alberta. All research was under direct independent supervision by Greg Goss (U of Alberta). Greg Goss and Jerry Hannah were jointly responsible for conceptualization, but Greg Goss was solely responsible for supervision and conduct of the research, and final version of the MS. Greg Goss has no conflicts of interest to declare.

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