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The effects of dissolved petroleum hydrocarbons on benthic organisms: Chironomids and amphipods

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A R T I C L E I N F O

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A B S T R A C T

The oil sands industry in Canada, produces heavy unconventional oils, diluted for transport and called diluted bitumen. However, despite advances in our knowledge of the ecotoxicological risk that these products represent, their effects on benthic organisms following a spill are still largely unknown. In order to fill these gaps, this study aims to determine the lethal and sublethal effects of two diluted bitumens (Bluesky and Cold Lake) and one conventional oil (Lloydminster) for two freshwater benthic invertebrates: *Chironomus riparius* and *Hyalella azteca*. The objective of this study is to assess the toxicity of dissolved hydrocarbons, resulting from the physical dispersion of oil, immediately after a spill on the benthic invertebrates. To this end, organisms were exposed for 7 days for chironomids and 14 days for amphipods to a fraction containing soluble hydrocarbons (WAF: water accommodated fraction; 10 g/L, 18 h of agitation, followed by 6 h of sedimentation) with natural or artificial sediment. After exposure, the effects of hydrocarbons were determined using size, mortality, and antioxidant capacities. Dissolved hydrocarbons induced mortality for both species, but these hydrocarbons disappeared very quickly from the water column, regardless of the oil type. The amphipods were sensitive to both types of oil while the chironomids were only sensitive to diluted bitumens. The presence of a natural sediment seems to provide a protective role against dissolved hydrocarbons. The antioxidant enzymes measured (CAT, SOD and GPx) do not appear to be relevant biomarkers for the exposure of these organisms to diluted bitumen.

1. Introduction

Canada has the world's third largest reserve of oil, in the form of oil sands. Oil sand is a mixture of crude bitumen (a form of crude oil), sand, clay mineral, and water. These reserves are located in Alberta and Saskatchewan (Barron et al., 2018; Canadian Association of Petroleum Producers (CAPP), 2018; Douben, 2003). The extraction of this oil requires unconventional methods (Lee et al., 2015) and produces bitumen that is too dense and viscous to be transported as is by pipeline. Therefore, it is diluted with natural gas or naphtha-based condensates, and this process results in diluted bitumen (dilbits). As expansion projects increase (e.g., Kinder Morgan Trans Mountain), the number of pipelines crossing freshwater systems will increase as well as the risk of spills. Although spills have been studied extensively due to the impact on aquatic ecosystems of oil extraction and transportation disasters (e.g., the 1989 Exxon Valdez Oil Spill, Alaska, USA; the 2010 Kalamazoo River oil spill, Michigan, USA; the 2013 train derailment and spill of Bakken Light crude oil in Lac-Mégantic, Qc, Canada; the 2016 pipeline spill of diluted bitumen in the Saskatchewan River, Canada), many aspects of the environmental fate and impacts of diluted bitumen in aquatic

Abbreviations: BTEX;, Benzene Toluene Ethylbenzene Xylenes; CAT;, Catalase; CEAEQ;, Centre d'expertise en analyse environnementale du Québec; DFO;, Department of Fisheries and Oceans Canada; GPx;, Glutathione peroxidase; GST;, Glutathione-S-Transferase; HMW;, High molecular weight; IC25;, Inhibition concentration for 25% of the test organisms; LC50;, Lethal concentration for 50% of the test organisms; LMW;, Low molecular weight; LOD;, Limits of detection; OPA;, Oil particle aggregates; PAC;, Polycyclic aromatic compounds; PAH;, Polycyclic aromatic hydrocarbons; SARA;, Saturates aromatics resins asphaltenes; SOD;, Superoxide dismutase; TOC;, Total organic carbon; TPAC;, Total polycyclic aromatic compounds; TU;, Toxic unit; WAF;, Water accommodated fraction; WSF;, Water soluble fraction.

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environments are still unknown compared to conventional crude oil.

Diluted bitumens, like conventional oils, are composed of four fractions: saturates, aromatics, resins, and asphaltenes (SARA) (Dew et al., 2015; King et al., 2017; Lee, 2015). In addition to the SARA fractions, oils may contain other compounds such as metals, organometallic compounds, sulfur, naphthenic acids, mineral particles, and water (Lee, 2015; NAS, 2015). Diluted bitumens are characterized by 1) a high proportion (around 30%) of low molecular weight (LMW) compounds due to the use of diluents (naphtha or gas condensates) (Alsaadi et al., 2018; Lee et al., 2015), and 2) a high proportion of resins and asphaltenes, corresponding to 50% of their relative weight compared to 3% for the lightest oils (Dew et al., 2015; Lee et al., 2015). Asphaltenes affect its adhesive properties. Hence, after a spill and after evaporation of the lighter fractions, diluted bitumen generates more persistent residues that adhere to the shoreline (NAS, 2016).

During a diluted bitumen spill in an aquatic ecosystem, the lighter components of the diluent will rapidly volatilize and be dispersed in the system through dissolution (Dew et al., 2015; Lee et al., 2015) and diluted bitumen viscosity will increase. The concentration of dissolved hydrocarbons will be high in the area near the spill a few hours after the event. The oil droplets dispersed in water by waves, wind, and currents will form oil-particle aggregates (OPAs) with the particles suspended in the water (Fingas, 2013; Fitzpatrick et al., 2015; Lee et al., 2015). As they form, the density of the aggregates increases, which leads to their sedimentation. Although most of the diluted bitumen will be associated with sediments (Hua et al., 2018; Marcus et al., 1988; Varanasi et al., 1985), the LMW hydrocarbons dissolved in the water column or incorporated into OPAs could also have an impact on aquatic organisms, including fish and aquatic invertebrates. For conventional oils, Muschenheim and Lee (2002) estimate that 10–30% of spilled oil settles by aggregation with particulate matter. A higher proportion can be expected with diluted bitumen. This was observed in the 2010 Marshall, Michigan spill into the Kalamazoo River, where the pipeline rupture released dilbit into the river (FOSC, 2016; Hua et al., 2018). Eventually, the Line 6B oil became denser than water and/or became aggregated with sediment, submerged, and settled into areas of the Kalamazoo River with lower water velocity (FOSC, 2016).

Oil toxicity is mostly due to LMW and aromatic fractions. The most common monoaromatics are Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX), which are volatile organic compounds (VOCs). Polycyclic aromatic hydrocarbons (PAHs) are the second category of aromatic hydrocarbons of concern. Diluted bitumens contain high proportions of 3-to-5-ringed alkylated and non-alkylated PAHs (Alsaadi et al., 2018; Hodson, 2017). In addition to these compounds, diluted bitumens also contain heterocyclic aromatic hydrocarbons such as dibenzothiophenes (sulfur included in the benzene ring) and carbazoles (nitrogen included in the benzene ring), for which few toxicity data are available (Alsaadi et al., 2018). PAHs, alkylated PAHs, and heterocyclic aromatics compounds can be grouped under the term PACs for Polycyclic aromatic compounds (Hodson et al., 2020). PACs are problematic because of their diversity and complexity, with alkylated compounds and metabolites that are even more toxic than the parent compounds (Hodson et al., 2020; Wallace et al., 2020). Based on available studies, high molecular weight hydrocarbons, such as asphaltenes and resins, which are present in larger quantities in diluted bitumen and heavy oil, appear to be less toxic to aquatic organisms. They can have a physical impact on organisms through engulfment, but at lower concentrations, their direct toxic effects seem limited as they cannot cross biological membranes due to their size (Lee et al., 2015).

A few studies have been conducted on the toxicity of petroleum products on benthic invertebrates, but these have mainly focused on oil-contaminated sediment or assessed the effects of a spill in the natural environment (Dollhopf et al., 2014; Federal On Scene Coordinator (FOSC), 2016). Very few studies have focused on the effects of oil water-accommodated fractions (WAFs) on benthic organisms and even fewer on the effects of a complex mixture such as diluted bitumen WAFs. Everitt et al. (2020) when exposing the epibenthic amphipod *Hyalella azteca* to a water-soluble fraction (WSF, similar to WAF but excluding droplets or particulate oil) of a weathered diluted bitumen containing 1.08 μg/L total PAHs did not observe lethality. Overall, few studies have compared the toxicity of dissolved compounds in diluted bitumen with conventional oil to aquatic organisms (Berube et al., 2021; Lara-Jacobo et al., 2021) and, to our knowledge, there are none on benthic invertebrates. Most current literature on the toxicity of diluted bitumen on aquatic species is on fish (Wallace et al., 2020). The most recent studies have shown that diluted bitumen induce embryotoxicity, deformities, cardiotoxicity, DNA damage, and impaired reproduction in fish (Berube et al., 2021; Dew et al., 2015; Lara-Jacobo et al., 2021; Madison et al., 2017; NAS, 2016; Wallace et al., 2020).

The most recent studies on pelagic invertebrates focused on the toxicity of diluted bitumen dissolved compounds on two cladocerans and one amphipod (Barron et al., 2018; Robidoux et al., 2018). Organisms were exposed to WAFs or WSF obtained from unweathered and weathered diluted bitumen. Acute toxicity ranged from 7.6 to 25.4 μg/L total PAHs, and 480–8020 μg/L BTEX in *Ceriodaphnia dubia* and *Americamysis bahia*. Robidoux et al. (2018) showed that WAF at 32 g of oil per liter induced 27% mortality for *Daphnia magna*, and an LC_{50} of 7844 μg/L of total VOCs and 22.8 μg/L of total PAHs for *Ceriodaphnia dubia*. In this study, the WAF of unweathered oil induced more toxic effects than the weathered one. According to Robidoux et al. (2018), the difference in toxicity can be partially explained by the PAH concentration. In fact, as the oil is weathered, the lighter compounds, which are also the most bioavailable, will disappear. The remaining compounds, which are heavier, such as resins and asphaltenes, are less available and will cause less toxicity than the unweathered oil.

The general objective of our study is to improve knowledge of the impact of diluted bitumen on two benthic invertebrates, the amphipod *Hyalella azteca* and the chironomid larva *Chironomus riparius*, widely distributed in freshwater environments in Canada. *Hyalella azteca* is an epibenthic crustacean native to North America and common in various freshwater environments. It is a detritivore and grazing organism, feeding primarily on bacteria, diatoms and algae found on the sediment (Environment and Climate Change Canada (ECCC), 2018). *Chironomus riparius* is a non-biting midge in the Chironomidae family. It is widely distributed in the northern hemisphere at temperate latitudes in lentic and lotic aquatic environments, in the first ten centimeters of the sediment. It is a burrowing benthic detritivore. The larvae build tubes in the sediment in which they grow (ECCC, 1997). These organisms are an important food source for birds, fish, amphibians and aquatic macroinvertebrates (Tokeshi, 1995). In addition, this benthic invertebrate play an important role in the transfer of contaminants from sediments because of their intimate association with sediments and their importance as a food source for bottom-feeding fish (Clements et al., 1994) since they consume a large mass of detrital material relative to their individual mass. *Hyalella azteca* and *Chironomus riparius* are model species that have been studied extensively in ecotoxicology due to their ubiquity, rapid culture, and short life cycle (Al-Shami et al., 2012; Fisher et al., 2003; Gauthier et al., 2015). They are good biological indicators for contaminants that can adsorb to sediment, such as PAHs (Fisher et al., 2003, Fitzpatrick, 2015).

Our study aims to identify the delayed impacts of diluted bitumen, physically dispersed in the hours following a spill, on benthic organisms due to the presence of dissolved hydrocarbons in the water column and potentially including hydrocarbons bound to OPAs. This paper investigates the lethal and sublethal effects of physically dispersed hydrocarbons (WAF) of two diluted bitumens compared with those of a conventional heavy oil on *H. azteca* and *C. riparius*. WAFs were used in order to simulate the potential impact of the physically dispersed hydrocarbons in the water column a few hours after an oil spill. The protective role of the sediment in the early hours following a spill was explored by exposing organisms in the presence or absence of a non-contaminated natural sediment. We assume that physically dispersed hydrocarbons do not induce lethal effects for either organism, due to their high volatility and non-persistence in the aquatic environment and to the protection offered by the sediment, but induce sublethal effects, such as a smaller size and activation of defense mechanisms.

2. Materials and Methods

2.1. Sampling

Non-contaminated natural sediments were collected in June 2019 from the Chaudière River in the town of Saint-Ludger (45°43’ 14.9" N 70°44’ 00.6" W, Qc, Canada). The sediment was transported in polyethylene containers, stored in the laboratory at 4 °C, sieved at 4 mm, homogenized, distributed in 3 L polyethylene jars, and stored at − 18 °C until further analysis. Sediment was analyzed and showed no trace of contamination (Table A.1) for nitrogen, phosphorus, sulfur, extractible metals and mercury, C_{10}-C_{50} and alkylated PAHs, and pesticides such as organophosphates, glyphosate, and aminomethylphosphoenic acid (AMPA). The sediment is a sandy loamy sediment (6.6% clay, 32.5% silt and 66.3% sand) that presents a low content of total organic carbon (0.51%).

2.2. Culture of organisms

The *H. azteca* and *C. riparius* cultures were conducted under laboratory conditions following the standard methods SPE 1/RM/33 (Environment Canada, 1997, 2013) and XP T 90–33–1 (Association Française de Normalisation (AFNOR), 2003) for *H. azteca* and SPE 1/RM/32 (Environnement Canada, 1997) and XP T 90 339–1 (AFNOR, 2004) for *C. riparius*. *Hyalella azteca* was maintained in the M5 medium (Ca_2Cl_2.2 H_2O, 2.9150 g/50 mL; $NaHCO_3$, 1.68 g/50 mL; $MgSO_4$.7 H_2O, 1.2300 g/50 mL; KCl, 0.0745 g/50 mL; NaBr, 0.0206 g/50 mL; pH, 6.5–8.5; conductivity, 300–500 μS/cm; hardness, 120–140 mg $CaCO_3$/L). Amphipods were kept in static conditions with soft aeration, and 70% (v/v) of the water was changed weekly. Organisms were fed three times a week with algae wafers (HIKARI®tropical) (one wafer in each aquarium) and a suspension of 20 mL of algae (*Raphidocelis subcapitata*) in its exponential growth phase. Chironomids were maintained in aquaria with a mix of siliceous sand (Ø 106–250 μm and Ø 250–500 μm) and M5 medium. Cultures were kept in static conditions with soft aeration, and 70% (v/v) of the water was changed weekly. Organisms were fed with a suspension of TetraMin® fish food (Tetrawerke, Melle, Germany) three times a week (400–500 mg in each aquarium).

2.3. Water-accommodated Fractions

Following a spill, hydrocarbons will disperse horizontally and vertically in the water column, depending on their solubility. To study the toxicity of these physically dispersed hydrocarbons, we used a standard method which imitates at a small scale the physical dispersion of oils in water, called the Water-Accommodated Fractions (WAF) of petroleum. For the preparation of the WAF, non-unfiltered freshwater from the Beauport River (Québec, Canada) was used (pH=8.27, alkalinity=19.3 mg $CaCO_3$/L, conductivity=45 μS/cm, hardness=12.83 mg $CaCO_3$/L, total organic carbon (TOC)= 5.24 mg C/L, dissolved organic carbon (DOC)= 3.8 ± 0.11 mg/L, suspended particles (SS)= 1.30 ± 0.31 mg/L, PAHs = < 0.003 μg/L (Limits of detection, LOD)) in order to reflect natural conditions and consider the role of TOC in the fate of hydrocarbons. The resulting WAF contained water-soluble hydrocarbons, oil droplets, and presumably OPAs, since we used unfiltered natural water. WAFs were prepared according to Singer et al. (2000) and the recent Fisheries and Oceans Canada (DFO) recommendations (2017) by adding oil in freshwater at the concentration of 10 g/L (oil to water ratio 1:100), in a 9-L glass jar, with a headspace of 20–25% in order to optimize the solubilization of volatile compounds (Adams et al., 2017; Singer et al., 2000). The solution was gently stirred for 18 h (magnetic stirring bar 72.2 ± 12.7 mm, Thermolyne Maxi-stirrer Model 525535; 200 rpm), at room temperature (20 ± 2 °C), in the dark and settled for 6 h in order to allow oil droplets to float back to the surface. The aqueous fraction was recovered from the bottom of the jar. Dilutions of WAFs were immediately prepared at 3%, 6%, 12%, 25%, 50%, 75% and 100% V/V of WAF (nominal values) for test volumes of 350 mL including control (only river water).

2.4. Bioassays

To assess the protective role of natural sediment and organic matter on benthic exposure to physically dispersed hydrocarbons, two types of exposures were used: with natural sediment or with artificial sediment. Before the start of the test, the natural sediment was thawed at 4 °C. Bioassays were conducted according the standard procedures of Environment and Climate Change Canada (ECCC, 1997; ECCC, 2018) and AFNOR (Association Française de Normalisation (AFNOR), 2003). All toxicity tests were validated for control survival and exposure water quality according to Environment and Climate Change Canada standards (ECCC, 1997; ECCC, 2018). Glass test beakers of 500 mL were filled with sediment (70 g) and 350 mL of natural water (control) or WAF dilution. In the case of the "with artificial sediment" condition, a hydrophilic gauze was used for *H. azteca* and a silica sand bottom for *C. riparius* to allow them to build their larval tube. The artificial and natural sediments were 2 cm in thickness, and the same ratio of water to sediment (1:4) was used in both tests. At the beginning of the bioassays, 20 first-instar *C. riparius* larvae (aged 2 d after hatching, 1.8 mm± 0.3 mm) or 20 juvenile *H. azteca* (aged 7–9 d) were introduced randomly in each test beaker. Five replicates were prepared for each concentration (from control to 100%V/V of WAF). Chironomids and amphipods were fed three times a week with a suspension of TetraMin® fish food (4 g/L), with 6 mL for *C. riparius* and with 3.2 mL for *H. azteca.* Temperature, pH, conductivity, dissolved oxygen, nitrates, and ammonium contents were measured before the beginning of the test, and at the end of the assay. Bioassays were carried out at 23 ± 1 °C, with a continuous gentle aeration, and a 16:8 h light:dark photoperiod (1000 lux). At the end of the 7-d exposure for chironomids and 14-d exposure for amphipods, organisms that survived were recovered. Half of them were used to measure their body length (size) to the nearest 0.1 mm with a stereo microscope (Leica Wild M8, Richmond Hill, Canada). The other half was used for the measurement of biomarkers. The organisms used for the biomarkers were pooled together by concentration in Eppendorf tubes immersed in liquid nitrogen and stored at − 80 °C until analysis. The acceptability criteria are an average control of ≤ 20% and ≤ 30% mortality for the amphipod and chironomid larvae tests, respectively, based on Environment and Climate Change Canada standards (1997, 2017).

2.5. Defense biomarkers/ antioxidant activity

In this study, we analyzed the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), as well as GST (glutathione S-transferase), a member of the phase II detoxification enzyme family. Spectrophotometric enzyme assay kits were used to measure GPx (Cayman-703102), SOD (Cayman-706002), GST (Cayman-703302), and CAT activities (Cayman-707002). Samples obtained at the end of the assays were diluted to 1/10 with ice-cold HEPES buffer (20 mM) containing EDTA (1 mM) and 0.1% of Triton X-100 and pH adjusted to 7.5. Samples were homogenized using a vibratory mill (MM400 RETSCH). Two 4 mm glass beads were added to each tube. They were shaken for 30 s at a frequency of 30 s^{-1}. For the amphipods, it was necessary to repeat the operation twice to break their cuticles. As much supernatant as possible was collected after a centrifugation for 15 min at 10,000× g at 4 °C. The supernatant was diluted at 1/50 with the HEPES buffer to have a final volume of 325 μL. Aliquots were prepared for the different measurements (SOD, CAT, GST, and GPx) and stored at − 80 °C awaiting analysis. The enzyme activity was calculated according to the instructions in the kit and standardized for protein measured using the Bradford method (Bradford, 1976).

2.6. Chemical characterization of water and sediment

The water column and the sediment of the exposure systems (WAF dilutions 0%, 3%, 25%, 50%, 75% and 100% of WAF) were analyzed at the beginning of the assay and at the end of the assay. For water samples, PAHs, alkylated PAHs, volatile organic compounds (VOCs), C_6-C_{10} fraction, and C_{10}-C_{50} fraction were measured on water samples without filtration after total extraction. The extraction method consists in adding hexane, allowing the compounds to move from water to hexane. The hexane part is recovered and concentrated. For sediment samples, PAHs, alkylated PAHs, and C_{10}-C_{50} fraction were measured after total extraction. The PACs measured are listed in Table A.2. The analyses were performed by the CEAEQ (Centre d′expertise en analyse environnementale du Québec, Ministère de l′Environnement et de la Lutte contre les changements climatiques, Canada), whose laboratories are accredited by the Standards Council of Canada (ISO/CEI 17025). Detailed QA/QC are available in Table A.3. The PAHs were measured by Gas Chromatography/Mass Selective Detector (model Agilent 7890B GC and 5977 A MSD) (CEAEQ, 2019) . The C_{10}-C_{50} fraction was quantified by gas chromatography assay coupled to a flame ionization detector (GC-FID, model Agilent 7890 A) (CEAEQ, 2016). VOCs were analyzed using a purge and trap system (Teledyne Termar AtomX), coupled with a gas chromatograph (Agilent 7890B) and a mass spectrometer (Agilent 5977 A) (CEAEQ, 2014). The C_6-C_{10} fraction was quantified by using a purge and trap system (Teledyne Tekmar AtomX) coupled with a gas chromatograph and flame ionization detector (Agilent 7890B). The C_6-C_{10} fraction was obtained by subtracting the BTEX concentration from the result obtained (CEAEQ, 2021).

2.7. Calculations and statistical analysis

For size and mortality data, an analysis of variance (ANOVA) followed by a Tukey post hoc test was used to identify significant differences among concentrations. Where assumptions (normality distribution of residuals and homogeneity of variance) were not met, such as for biomarker results, a Kruskal-Wallis test, followed by Dunn's multiple comparison test, was performed. The statistical analyses were performed using Prism (GraphPad, version 9). An alpha level of 0.05 was used to determine significance for all tests. When possible, the median lethal concentration, LC_{50}, and IC_{25} (25% inhibition concentration relative to the control) were calculated using the CETIS software (V1.9.2.6, Tidepool Scientific). LC_{50} were measured using the Spearman-Kärber method. In the one case where IC_{25-14d} could be calculated, the linear interpolation method was used for the test with amphipods, with artificial sediment, exposed to the Bluesky WAF. LC_{50} are expressed as total PACs (TPAC), BTEX, and toxic unit (TU). One TU is the concentration at which there is a 50% effect on a given parameter.

3. Results

3.1. Chemical characterization of water and sediment

The TPAC concentration was higher in the Lloydminster WAF (84–97 μg/L) than in those of the two diluted bitumen (72–96 μg/L for Bluesky and 27–37 μg/L for Cold Lake) (100% v/v; Fig. A.1 and Fig. A.2). Comparing the profiles of PAHs and alkylated PAHs, we can note that the heaviest PAHs were not dissolved in the WAF (data not shown). The majority of VOCs are composed by BTEX in these WAFs, therefore BTEX will be mainly presented in the graphs. For VOCs, Bluesky WAF was more concentrated (10,000 μg/L) than those of Cold Lake (900 μg/L) and Lloydminster (6000 μg/L) when prepared for the amphipod test. In contrast, the Cold Lake WAF had the highest VOC concentration among those prepared for the chironomid larvae test (100% v/v; Fig. A.2). In these water columns, C_{10}-C_{50} concentrations were very low (between 0.5 and 3.1 μg/L) (Table A.4).

Regardless of the oil, WAFs contained a majority of 2- and 3-ring alkylated PACs (Fig. A.1 and Fig. A.2). In the WAFs, most of the VOCs were BTEX (97% for Bluesky, 99–100% for Cold Lake, 92–97% for Lloydminster) (Table A.4), mostly Benzene and Toluene (Fig. A.1 and Fig. A.2). Other VOCs included Isopropylbenzene, n-Propylbenzene, 1,3,5-Trimethylbenzene and 1,2,4-Trimethylbenzene, and Naphthalene (data not shown). The Lloydminster WAF also contained PACs that were absent from the WAFs of diluted bitumen that are represented in Table A.5. These are mainly alkylated groups, for examples, alkylated Dibenzothiophene, alkylated Dibenzopyrene.

At the highest concentration (100% WAF), 73% of the PACs in the Bluesky WAF disappeared from the water column in one day compared to 97% in the Lloydminster WAF (Fig. 1). The same rates were observed for BTEX. For the lowest concentrations of Lloydminster WAF, 3% and 25% of WAF, PACs and BTEX had completely disappeared from the water column after 24 h (Fig. 1). Unfortunately for technical reasons, the water column of organisms exposed to the Cold Lake WAF could not be analyzed one day after the start of exposures. Finally, at the end of exposure, C_{10}-C_{50} and PAC concentrations in sediment were below the detection limit of the analytical methods (0.15 μg/L and 0.001 μg/L respectively).

3.2. Mortality and size of *H. azteca*

Hyalella exposed to the Bluesky WAF, in the absence of sediment, exhibited a significantly higher percentage of mortality than the control, ranging from 80% to 100% mortality at concentrations above 25% of WAF (Fig. 2A). Mortality was also significantly higher in the absence of sediment than in its presence for concentrations ranging from 25% to 100% of Bluesky WAF (Fig. 2A). At 50% of WAF concentration, mortality was five times higher in the absence of sediment. Amphipods exposed to the Cold Lake WAF with artificial sediment showed significantly less mortality than those exposed to the Bluesky WAF with artificial sediment at the highest concentrations (Fig. 2A and 2B). Indeed, the Cold Lake WAF induced only 50% mortality at the two highest concentrations (75% and 100%) (Fig. 2B) compared to 86% and 100% for the Bluesky WAF. In addition, mortality with the Cold Lake WAF was lower in the presence of natural sediment, with a significant difference from the control only at 75% of WAF concentration. However, like for the Bluesky, less mortality was observed in the presence of natural sediment than in the presence of artificial sediment. This difference was significant at concentrations ranging from 3% to 25% and at 100% WAF, with 2–4 times more mortality in the presence of artificial sediment than in the presence of natural sediment (Fig. 2B). Lloydminster WAF induced a higher mortality compared to the two diluted bitumen WAFs (Fig. 2C). With artificial sediment, concentrations ranging from 6% to 25% WAF induced 60% mortality, significantly different from controls and 3% WAF, and 100% mortality from 50% of WAF. In the presence of natural sediment, only 75% and 100% WAF were significantly different from the control with 100% mortality. Finally, as with the two diluted bitumens, less mortality was observed in the presence of natural sediment, except at the two highest concentrations (Fig. 2C). However, this was only significant at 50% WAF.

In the presence of artificial sediment, the Bluesky WAF induced higher amphipod mortality than the Cold Lake one at the highest concentrations (ANOVA, $p < 0.001$, data not shown). In contrast, the Bluesky WAF was less toxic for the amphipod survival at 50% WAF than the Lloydminster's one (ANOVA, $p < 0.001$, data not shown). At the two highest concentrations (75% and 100%), there was no difference between both oils. In the presence of natural sediment, Bluesky WAF induced higher amphipod mortality than the Cold Lake one at 100% WAF (ANOVA, $p < 0.001$, data not shown), but was less toxic than the Lloydminster one at 50% and 75% WAF concentrations (ANOVA,

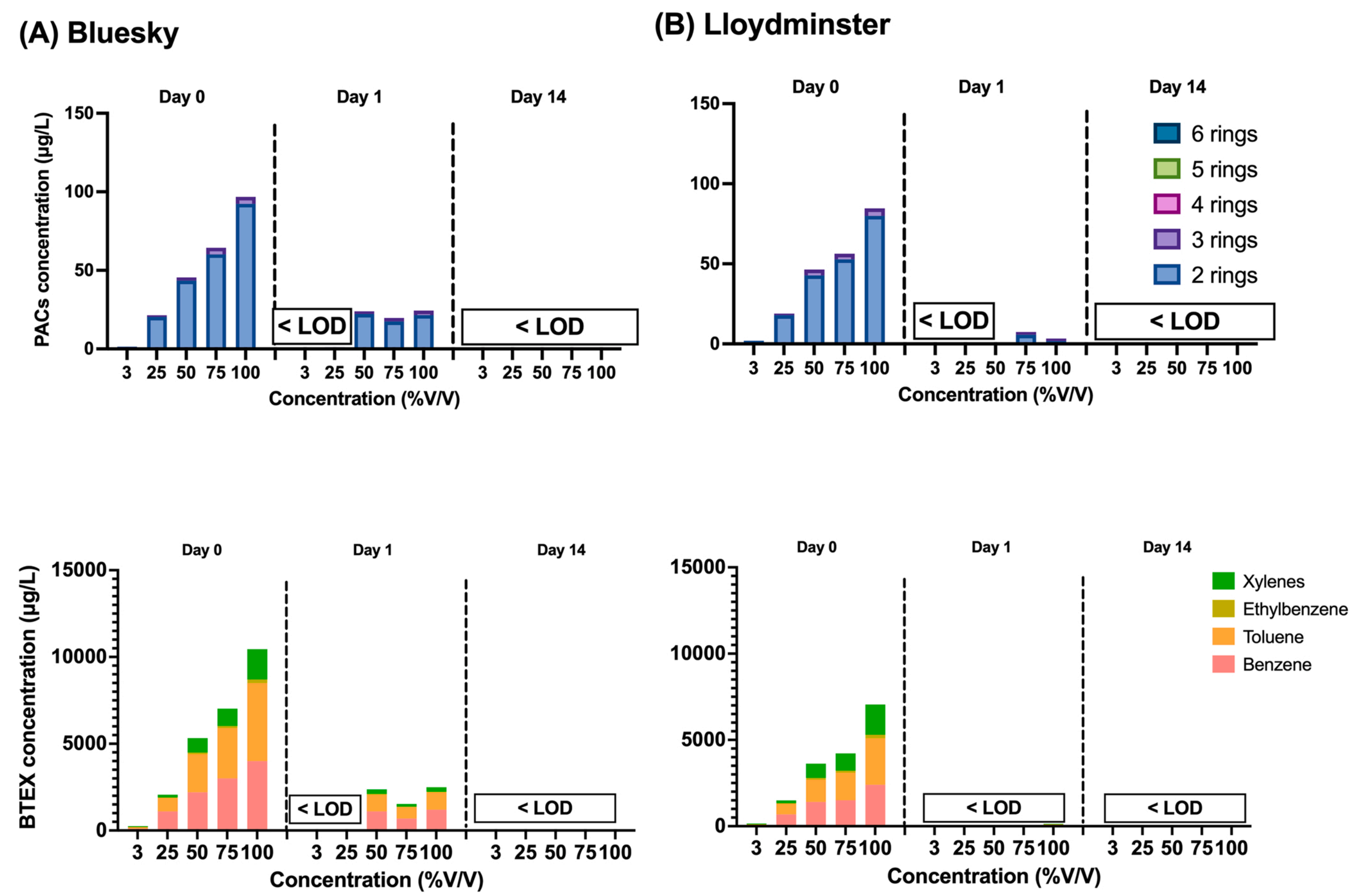


Fig. 1. Concentration of total polycyclic aromatic compound (PACs, top) and alkylated PACs, grouped by the number of aromatic rings (blue: 2 rings; purple: 3 rings; pink: 4 rings; green: 5 rings; turquoise: 6 cycles) and the concentration of benzene, toluene, ethylbenzene and xylenes (BTEX, bottom) (Xylenes: green, Ethylbenzene: persimmon, Toluene: orange, Benzene: pink) on Day 0, Day 1 and Day 14, as a function of WAF concentrations (% of WAF) of Bluesky oil (A) and Lloydminster oil (B), in exposure media of Hyalella azteca.

$p < 0.001$, data not shown).

Hyalella azteca size was not inhibited following exposure to WAFs from the diluted bitumen (Fig. A.3A and A.3B) mean size of amphipod exposed to Bluesky WAF with artificial sediment were 1.63 ± 0.16 mm, Bluesky WAF with natural sediment 2.02 ± 0.2 mm, Cold Lake WAF with artificial sediment: 1.48 ± 0.16 mm, Cold Lake WAF with natural sediment 1.52 ± 0.15 mm. Amphipods tended to be longer in the presence of natural sediment, although a significant difference was only observed in the Bluesky WAF at the concentrations of 3%, 6%, and 25% WAF (Fig. A.3A). For Lloydminster, a significant difference in amphipod length was observed in organisms exposed to WAF at the 25% concentration in the absence of natural sediment (20% inhibition relative to the control; Fig. A3C). In the presence of natural sediment, there was a significant difference in amphipods size compared to the control for concentrations ranging from 12% to 50%, with 20% and 35% inhibition (Fig. A3C). *Hyalella* exposed to Lloydminster WAFs in the presence of natural sediment tended to be slightly longer, but this difference was only significant at 6% of Lloydminster WAF. It was not possible to perform ANOVA at the highest concentrations (75% and 100%) because of mortality.

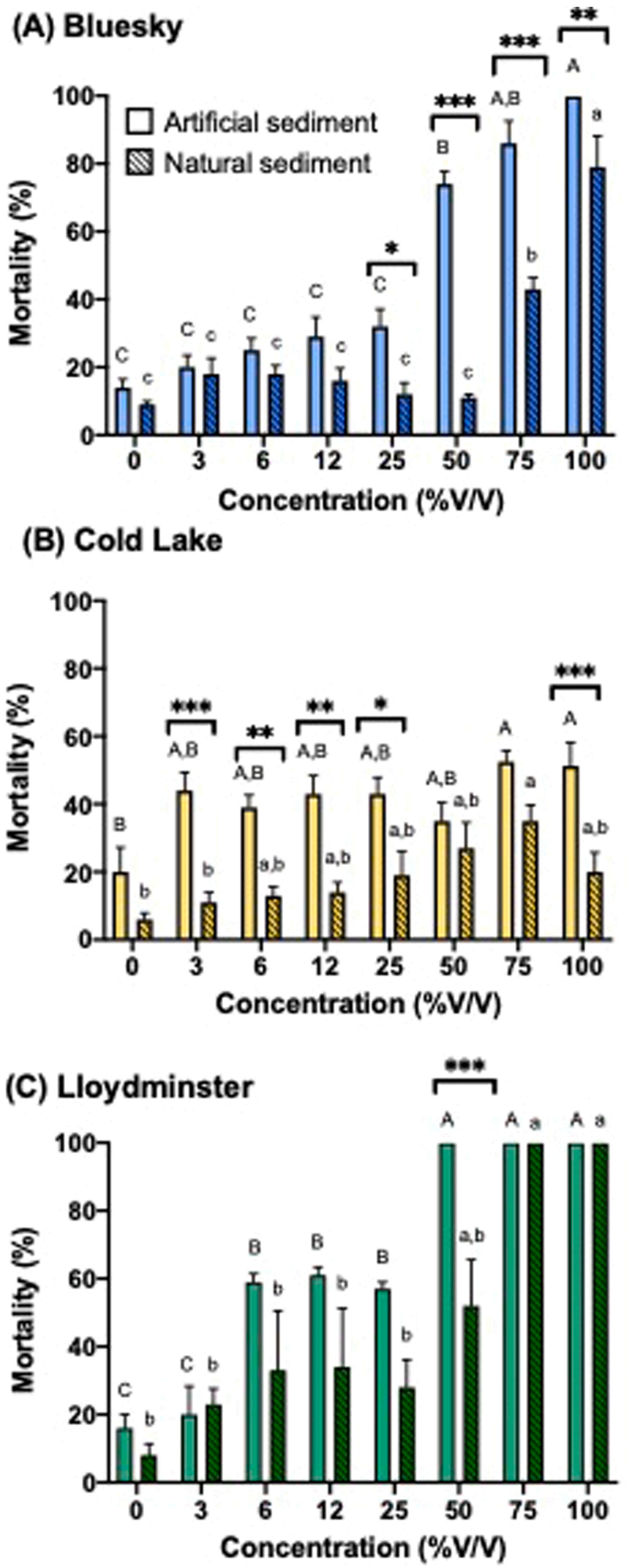
With artificial sediment, there was no difference in amphipod size, except at the 25% concentration where organisms exposed to Lloydminster WAF were smaller than those exposed to the Bluesky one (Fig. A.3A and B, ANOVA, $p < 0.01$, data not shown). In the presence of natural sediment, amphipods exposed to Lloydminster WAF were smaller than those exposed to the Bluesky WAF (ANOVA, <0.001, data not shown) but there were no differences with those exposed to the Cold Lake's one.

The LC_{50-14d} and IC_{25-14d} obtained are presented in Table 1. LC_{50-14d} for *H. azteca* exposed to the Bluesky WAF with natural sediment was 2.5 times higher than LC_{50-14d} with artificial sediment which confirms that the Bluesky WAF was more toxic in the presence of artificial sediment than in the presence of natural sediment to the amphipods. The Cold Lake WAF did not induce enough mortality to calculate an LC_{50-14d} in the presence and absence of natural sediment. In this way, it can be said that the Bluesky WAF is more toxic than the Cold Lake WAF. LC_{50-14d} for *H. azteca* exposed to Lloydminster WAF was 3 times higher in the absence of sediment than in the presence of natural sediment. Lloydminster WAF was the most toxic, followed closely by Bluesky WAF, and then Cold lake WAF. WAFs were systematically more toxic in the absence of natural sediment compared in the presence of natural sediment.

Concerning the IC_{25-14d} of amphipod size, only one could be calculated, an IC_{25-14d} of 104.9 µg/L of TPACs (or 6587 µg/L of BTEX) for the Bluesky WAF artificial sediment. Size reduction was too low, and probably not biologically significant, to be measured in the other experimental conditions.

3.3. *Mortality and size of C. riparius*

For chironomids, the Bluesky WAF induced 36% mortality on average in the presence of natural sediment and 32% in the presence of artificial sediment (Fig. A.4A). The Cold Lake WAF induced 41% mortality on average in the presence of sediment and 40% in the presence of artificial sediment (Fig. A.4B). Chironomid larval mortality was significantly different from the control of Cold Lake experiment, in the



(caption on next column)

Fig. 2. Mortality (%) of Hyalella azteca as a function of WAF concentration (in % of WAF) with natural sediment and with artificial sediment for each oil after 14 days of exposure. (A) Bluesky WAF, (B) Clearwater WAF, (C) Lloydminster WAF. Significant differences among concentrations were identified by one-way ANOVA or Kruskal-Wallis and Dunn's post hoc analysis and are represented by letters. Concentrations sharing the same letter are not significantly different ($\alpha > 0.05$). Upper case letters correspond to the condition with artificial sediment and lower-case letters to the condition with natural sediment. Significant differences due to the type of sediment for the same concentration were identified by two-way ANOVA and pairwise comparison and represented by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data is presented as mean $\pm$ standard deviation ($n = 5$).

presence of artificial sediment with 50% mortality at 100% WAF (Fig. A.4B) and in the presence of natural sediment with 60% mortality at 75% of WAF (Fig. A.4B). Overall, according to our results, the presence of natural sediment did not influence the mortality of chironomid larvae exposed to diluted bitumen's WAFs. Moreover, there was no difference between the two diluted bitumens (data not shown). The Lloydminster WAF induced 60% of mortality at 3% in the presence of artificial sediment, significantly different from the control (Fig. A4C). In the presence of natural sediment, there was no significant effect on mortality. Mortality was also significantly higher in the presence of artificial sediment at 3% WAF, with six times more mortality than in the presence of natural sediment. Moreover, at 3%, in the presence of artificial sediment, the Lloydminster-induced mortality was higher than with both diluted bitumen's WAFs (ANOVA, $p < 0.01$, data not shown). In the presence of natural sediment, the Lloydminster WAF was less toxic at 100% than with both diluted bitumen WAFs. Indeed, 0% mortality was observed in larvae exposed to 100% Lloydminster while 40% of larvae died following dilbit exposures at the same dilution and in the presence of natural sediment (ANOVA, $p < 0.01$, data not shown).

Exposure to diluted bitumen WAFs had no significant impact on chironomid larval size, except for a few concentrations where an inhibition compared to the control can be observed (75% of Bluesky WAF in the presence of artificial sediment; 25% and 50% of Bluesky WAF in the presence of natural sediment; 50% and 75% of Cold Lake WAF in the presence of artificial sediment; 50% of Cold Lake WAF with natural sediment, Fig. A.3D, E, F). Larvae exposed to the Bluesky WAF were significantly longer than those exposed to the Cold Lake and Lloydminster WAFs (ANOVA, $p < 0.01$, data not shown) but when comparing controls to each other, a difference between larvae can be seen. Indeed, the Bluesky exposure of the control larvae were significantly larger than the control larvae of the other two oils (ANOVA, $p < 0.001$, data not shown). Although chironomid larvae tended to be longer in the presence of natural sediment, no statistical differences were identified except at 75% for the Bluesky WAF, 50% for the Cold Lake WAF, and 3% and 75% for the Lloydminster WAF (Fig. A.3D, E, F).

The LC_{50-7d} for chironomids were all $> 100\%$ V/V, except for the Cold Lake WAF in the presence of natural sediment. In chironomid larval exposures, the Cold Lake WAF was the most toxic in the presence of natural sediment. No IC_{25-7d} could be estimated for size of chironomid larvae because, as for amphipods, inhibition was too low (Table 1).

3.4. Defense biomarkers/ antioxidant activity

Measurement of the enzymatic activity of catalase, superoxide dismutase and glutathione peroxidase in *H. azteca* and *C. riparius*, with natural or artificial sediment, showed no significant effect when the organisms were exposed to diluted bitumen or Lloydminster WAFs (data not shown).

The glutathione S-transferase (GST) activity of amphipods exposed

Table 1
Summary of acute and chronic toxicity expressed as percentage of WAF, TPAC or BTEX concentration: 1. LC50: medial lethal concentration (statistical analyses: Trimmed Spearman-Käber), 2. IC25: Inhibition concentration of 25% of the size (statistical analyses: linear interpolation or non-linear regression), 3. %V/V: Result with the confidence interval of 95%. 4. Toxic Unit.

Oil	Sediment	Species	LC50[1]				IC25[2]		
			WAF (%V/V)[3]	TU[4]	TPAC (μg/l)	BTEX (μg/l)	WAF (%V/V)	TPAC (μg/l)	BTEX (μg/l)
Bluesky	Natural	*H. azteca*	81 (75–87)	1	75	8192	> 100	> 92	> 10,075
Bluesky	Artificial	*H. azteca*	32 (29–37)	3	30	3320	65.38 (51.48–74.73)	65	6587
Cold Lake	Natural	*H. azteca*	> 100	< 1	> 26	> 1008	> 100	> 26	> 1008
Cold Lake	Artificial	*H. azteca*	> 100	< 1	> 26	> 1008	> 100	> 26	> 1008
Lloydminster	Natural	*H. azteca*	34 (28–40)	3	28	1872	> 100	> 82	> 5498
Lloydminster	Artificial	*H. azteca*	11 (10 – 13)	11	9	644	> 100	> 82	> 5498
Bluesky	Natural	*C. riparius*	> 100	< 1	> 69	> 6510	> 100	> 69	> 6510
Bluesky	Artificial	*C. riparius*	> 100	< 1	> 69	> 6510	> 100	> 69	> 6510
Cold Lake	Natural	*C. riparius*	46 (38–57)	6	16	3441	> 100	> 35	> 7359
Cold Lake	Artificial	*C. riparius*	> 100	< 1	> 35	> 7359	> 100	> 35	> 7359
Lloydminster	Natural	*C. riparius*	> 100	< 1	> 89	> 3980	> 100	> 89	> 3980
Lloydminster	Artificial	*C. riparius*	> 100	< 1	> 89	> 3980	> 100	> 89	> 3980

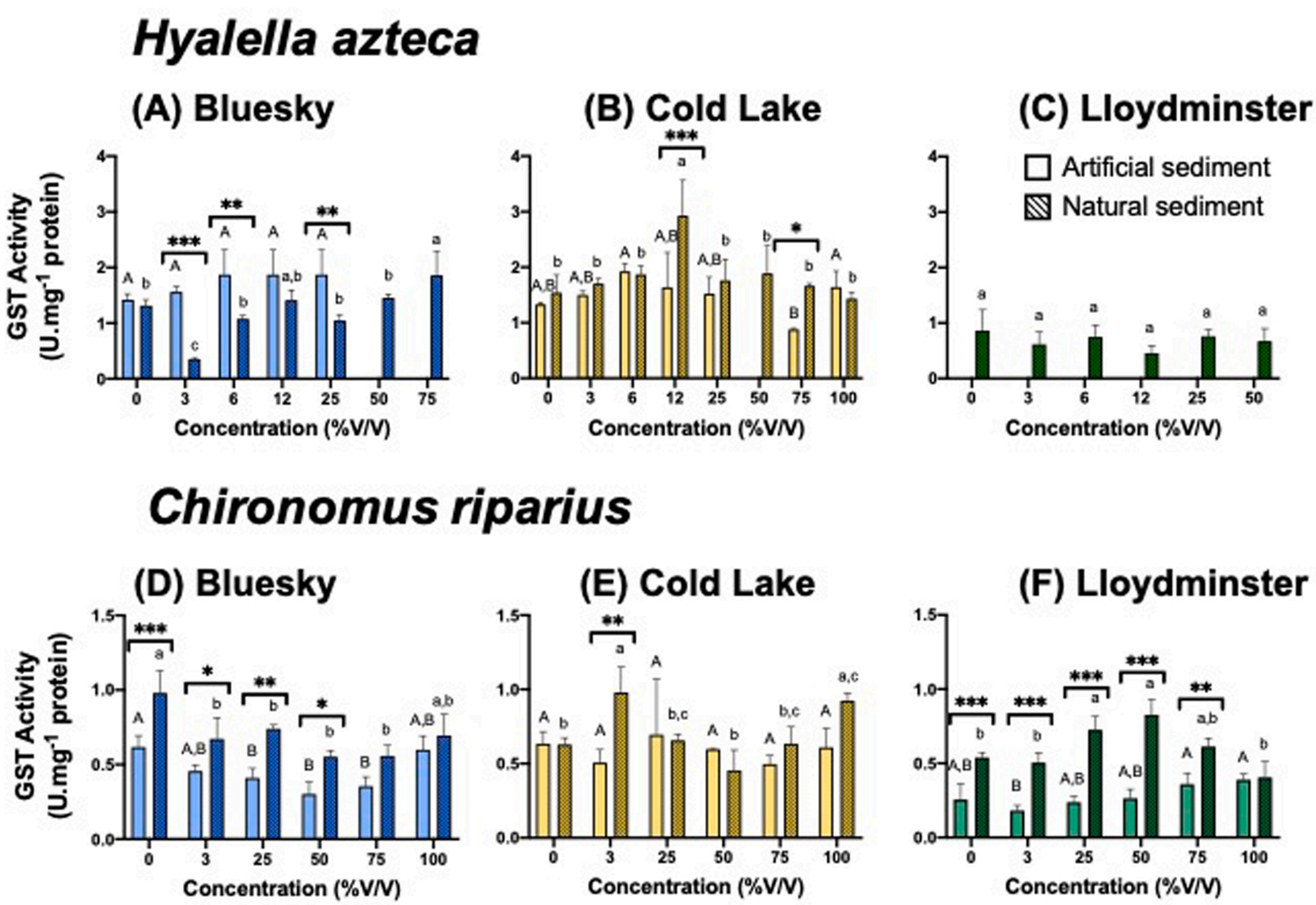


Fig. 3. Glutathione S-transferase (GST) in Hyalella azteca after 14 days of exposure (top) in enzyme unit (U. amount of enzyme required to process one micromole of substrate in one minute) per mg of protein as a function of WAF concentration (in % of WAF) for each oil, (A) Bluesky's WAF in blue with artificial sediment and with natural sediment in blue shaded, (B) Cold Lake 's WAF in with artificial sediment and with natural sediment in yellow shaded, (C) Lloydminster's WAF in green with artificial sediment and with natural sediment in green shaded, and of in Chironomus riparius after 7 days of exposure (bottom) in U.mg − 1 of protein as a function of WAF concentration (in % of WAF) for each oil, (D) Bluesky's WAF in blue with artificial sediment and with natural sediment in blue shaded, (E) Cold Lake 's WAF in yellow with artificial sediment and with natural sediment in yellow shaded, (F) Lloydminster's WAF in green with artificial sediment and with natural sediment in green shaded. Significant differences between each concentration were identified by one-way ANOVA or Kruskal-Wallis and Dunn's post hoc analysis and are represented by letter. Concentrations sharing the same letter are not significantly different ($\alpha = 0.05$. n = 3). Upper case letters correspond to the condition with artificial sediment and lower-case letters to the condition with natural sediment. Significant differences from the type of sediment for the same concentration were identified by two-way ANOVA and pairwise comparison and represented by an asterisk (*$p < 0.05$. **$p < 0.01$. ***$p < 0.001$). Data is presented as mean ± standard deviation.

to the Bluesky WAFs with artificial sediment showed no significant difference with controls or among WAF dilutions (Fig. 3A). In the presence of natural sediment, GST activity at 3% WAF was three-fold lower than the activity of the control (Fig. 3A). The activity rose to the same level as the control for the other concentrations up to the 50% of WAF concentration. At 75% of the Bluesky WAF, GST activity was highest (1.85 $U.mg^{-1}$ protein) and different from the control. At 3%, 6%, and 25% of WAF, GST activity was greater in amphipods exposed with artificial sediment than in those with natural sediment (Fig. 3A). This amphipod exposure to the Bluesky WAF is the only one where GST activity was greater in the presence of artificial sediment. The activity of GST in amphipods exposed to 75% of Cold Lake WAF with artificial sediment was only significantly lower than in those exposed to 6% and 100% WAF, with intermediate values for controls and other WAF dilutions (Fig. 3B). In the presence of natural sediment, a peak in GST activity was observed at 12% of WAF (Fig. 3B). GST activity was higher in the presence of natural sediment at 12% and 75% WAF than in the presence of artificial sediment (Fig. 3B). The WAF of Lloydminster in the presence of natural sediment did not appear to induce an effect on GST activity (Fig. 3C). The very high mortality in the presence of artificial sediment did not allow enough material to be harvested for GST measurement.

In chinonomid larvae, the presence of artificial sediment typically induced major and significant decreases in GST activity, even without WAF exposure (controls) (Fig. 3D-F). The phenomenon was also observed for WAF concentrations up to 50% (Bluesky) and 75% (Lloydminster) WAF. In contrast, for chironomids exposed to the Cold Lake WAF, GST activity decreased in the presence of artificial sediment only for larvae exposed to 3% WAF. In chironomids exposed to the Bluesky WAF in the presence or absence of natural sediment, GST activity was lower at concentrations of 3–75% WAF compared to the control (Fig. 3D). Exposure of chironomid larvae to Cold Lake WAF in the presence of artificial sediment had no effect on GST activity (Fig. 3E). For larvae exposed to Lloydminster WAF in the presence of artificial sediment, their enzyme activities were greater at 75% and 100% WAF but are not significantly different from the control (Fig. 3F). In the presence of natural sediment, there were an increase in GST activity at 25% and 50% WAF compared to the control and the 3% WAF concentration. The activity decreased at the 100% WAF concentration to return to control values (Fig. 3 F).

From the data collected, it appears that basal (control) GST activity in amphipods was higher than that measured in chironomid larvae. The data are inconclusive, and few effects are significant.

4. Discussion

The purpose of this study was to determine the toxicity of two physically dispersed diluted bitumens, the Bluesky and Cold Lake, on two species of benthic invertebrates, an amphipod and an insect larva. Their toxicity was compared to that of a conventional heavy oil, Lloydminster. Several endpoints were evaluated: oxidative stress, antioxidant activity, size, and mortality. The study focused on the impact of hydrocarbons present in dissolved form and in the form of microdroplets, as well as on the impact of OPA, which are the forms likely to be present in the environment in the hours following a spill.

4.1. Chemical composition of the WAFs

The Lloydminster WAFs contained more PACs than the two diluted bitumens, particularly in the form of 5- and 6-ring PACs and alkylated PACs. The Bluesky's WAF did not contain any 4-ring aromatic PACs, in contrast to the Cold Lake and Lloydminster WAFs. PACs with 2- and 3-aromatic rings were the most prevalent PAHs and alkylated PAHs in the WAFs of the diluted bitumens, especially the Bluesky one. The chemical profiles of the diluted bitumen in our study thus showed a profile of PAHs similar to other studies on this type of oil, such as Lara-Jacobo et al. (2021), Berube et al. (2021), and Schiano Di Lombo et al. (2021). Naphthalene and its alkyl derivatives were the compounds most present in the WAFs of both our study and Schiano Di Lombo et al. (2021). In addition, the conventional heavy oil WAF, Lloydminster, contained PACs that were absent from the diluted bitumen ones (Table A.5). These differences highlight the importance of measuring more than the 16 priority PAHs identified by the US Environmental Protection Agency (US-EPA, 2008), as well as alkylated PAHs and heterocyclic aromatic hydrocarbons (Dibenzothiophenes and Carbazole) to be able to understand and predict the fate and toxicity of oils in freshwater.

After their dispersion in the water column, hydrocarbons can evaporate or dissolve in water. As expected, PACs and VOCs rapidly disappeared from the water column of the experimental chambers. Several studies have also observed this rapid disappearance (e.g., Lara-Jacobo et al., 2021; Redman and Parkerton, 2015; Robidoux et al., 2018) due to the hydrocarbon volatilization, sedimentation, or adhesion to the test chamber glass. Volatilization concerns the very light fractions of the hydrocarbons (Fingas, 2013; NAS, 2016). Here, this is particularly the case for the hydrocarbons that constitute the diluent present in diluted bitumen, which facilitates their transport in pipelines. Indeed, these diluents consist of low molecular weight PAHs with low volatilization half-lives such as naphthalene (0.4–3.2 h) and anthracene (17 h) (Gouvernement du Canada, 1994). They will therefore tend to disappear quickly from the water column. In this study, for example, the concentration of naphthalene in the freshly prepared Bluesky WAF was 20 μg/L while it was below the detection limit (>0.001 ug/L) in the test chamber after 24 h. In contrast, a PAH with a high molecular weight has a longer volatilization half-life. This is the case for Pyrene, which has a volatilization half-life between 115 h and 3.2 years (Gouvernement du Canada, 1994).

Since the use of unfiltered natural water, oil droplets physically dispersed in the water, as well as dissolved oil, could interact with the suspended solids (clay minerals or organic matter) present in the WAFs and the water column to form OPAs (Fitzpatrick et al., 2015). Depending on the density of the aggregates, they may have sedimented and thus disappeared from the water column. However, the concentration of PACs measured in the sediment at the beginning and at the end of our tests was below the detection limit of the analytical method used (< 0.05 mg/kg). In some freshwater mesocosm studies, an increase in PACs in the sediment (Rodriguez-Gil et al., 2021) was observed following diluted bitumen contamination, 13–30 days after contamination, depending on the concentration of diluted bitumen applied (Stoyanovich et al., 2021). In our study, the organisms were only exposed to water contaminated with dissolved hydrocarbons whereas in Rodriguez-Gil et al. (2021) and Stoyanovich et al. (2021), the bitumen slick remained present in the test chamber (limnocorrals), leading to the presence of a continuous source of oil in the water column and possibly the sediments. Thus, the lack of observation of sedimentation in our study may be due to the absence of the oil slick, or to the bioturbation of the sediment by the organisms and the addition of nitrate/nitrite produced by the organisms and food (TetraMin®) which may have helped the microorganisms present to biodegrade the hydrocarbons more rapidly (Yang et al., 2018). In addition, the temperature (23 ± 1 °C) at which the tests were conducted may have favored this biodegradation.

4.2. Toxicity of the WAFs

We observed a specific response of both organisms to oil contamination in water, in the form of a dose-response relationship between our contaminants (PACs) and mortality as well as size of the organisms. However, the enzymatic biomarkers used did not express a dose-response relationship with oil exposure. The Bluesky WAF resulted in greater amphipod mortality than the Cold Lake WAF. In contrast, chironomid larvae were more sensitive to the Cold Lake WAF than to the Bluesky WAF. The difference in toxicity of the two diluted bitumens on those organisms can be explained by the chemical composition of the WAFs. The Bluesky WAF had the highest VOC concentration in the amphipod test (10,748 μg/L VOCs vs. 6926 μg/L VOCs in the chironomid larval test; Table A.4), while the Cold Lake WAF in the chironomid test had the highest VOC concentration (7909 μg/L VOCs vs. 994 μg/L VOCs in the amphipod test; Table A.4). This high VOC concentration may explain the higher chironomid mortality observed with the Cold Lake and not with the Bluesky. In the WAFs of this study, the VOCs were mainly composed of BTEX. They were probably responsible for the lethal effects measured in amphipods and chironomid larvae, in particular the high mortality at high concentrations of WAFs that contained the highest concentrations of LMW hydrocarbons. Indeed, monoaromatics and such as and Naphthalene are considered the main contributors to the acute toxicity of unweathered oils (Douben, 2003; Hodson, 2017; Philibert et al., 2016), more so than PAHs. However, as VOCs disappeared very quickly from the water column, they could have induced an important delayed mortality due to early exposure before disappearing, but they cannot be responsible for all the toxicity observed at the end of the assay (Redman and Parkerton, 2015).

Lloydminster WAF, the conventional heavy oil, resulted in greater amphipod mortality than WAFs from both diluted bitumens. This higher toxicity may be due to the higher concentration of PAHs and in particular alkylated products in these WAFs. The toxicity of alkylated PAHs is still poorly known, but available studies show that they seem to be more toxic than the parent molecules (Alsaadi et al., 2018). Moreover, PAHs with 2- and 3-aromatic rings, i.e. the most present in WAFs, have a higher bioavailability than those with 4- and 5-aromatic rings. WAF by definition contains oil droplets formed by the dispersion of oil in water (Lee et al., 2015). The presence of these droplets could explain the 60% mortality of chironomid larvae observed at 3% of the Lloydminster's WAF. Toxicity due to the presence of droplets could have been additional to that of dissolved hydrocarbons (Hansen et al., 2021; Nordtug et al., 2011; Redman and Parkerton, 2015). These droplets could have formed OPAs with the suspended matter of the unfiltered natural water that are deposited at the water-sediment interface or have been incorporated in the sediment, and thus come into contact with the larvae in the sediment. As the larvae burrow into the sediment, their exposure to the water column was limited, unlike the amphipods, which remain at the water-sediment interface, and were therefore more easily in contact with the physically dispersed hydrocarbons. The natural sediment still had a protective role for the amphipods since, in the presence of natural sediment, the amphipods showed less mortality than artificial sediment. The same is true for chironomid larvae: mortality was higher for larvae exposed to Cold Lake WAFs in the absence of sediment and the percentage of mortality obtained indicates a significant impact on this endpoint (>20%; MDDEFP and ECCC, 2013).

The difficulty of comparing the results of our study with other studies can be highlighted as the parameters used for the preparation of WAFs can vary from one laboratory to another, such as for the concentration of oil (25 and 50 g/L for Barron et al., 2018, 32 g/L in Robidoux et al., 2018), the type of water (filtered, UV and activated carbon treated water or river water), the agitation time (18 or 24 h), and the resting time (0, 4 or 6 h) (Adams et al., 2017). Furthermore, when studying the toxicity of benthic organisms, the presence of sediment is required which is not the case with pelagic invertebrates or fish. In addition, the chemical parameters measured are variable: PAHs, alkylated PAHs, C_6-C_{10}, C_{10}-C_{50}, PACs, VOCs, BTEX. Finally, among the existing studies, there are very few studies on the effects of diluted bitumen WAF on benthic invertebrates.

Some studies have been carried out on pelagic invertebrates, like Barron et al. (2018) who observed toxic effects on a freshwater crustacean *Ceriodaphnia dubia* after exposure to WAFs from two diluted bitumens, Cold Lake Blend (CLB) and Western Canadian Select (WCS). For *C. dubia*, the 48-h LC_{50} for CLB was 8020 μg/L BTEX or 14.5 μg/L total PAHs (WAF at 25 g/L dilbit). Western Canadian Select did not induce mortality on *C. dubia* at the WAF concentrations tested, the $LC_{50-48\,h}$ was > 29 μg/L PAHs or > 5860 μg/L BTEX (WAF at 25 g/L dilbit). Based on their results, Barron et al. (2018) concluded that the toxicity of CLB and WCS was broadly similar to that of other petroleum products. Robidoux et al. (2018) also obtained LC_{50} values after exposure of *Daphnia magna* and *C. dubia* to Cold Lake Blend's WAFs (10 g/L or 32 g/L): an $LC_{50-48\,h}$ of 8257 μg/L VOCs or 25.3 μg/L PAHs for *D. magna*; and an $LC_{50-6-7d}$ of 7844 μg/L VOCs or 22.8 μg/L PAHs for *C. dubia*. Furthermore, Robidoux et al. (2018) found that exposure of *Daphnia magna* to Cold Lake Blend's WAF at 10 g/L led to an immobility of 97%, which would likely have led to the death of organisms (Ucan-Marin and Dupuis, 2015). According to our mortality results, *H. azteca* would be more sensitive than *C. dubia* and *D. magna* when amphipods are exposed to WAFs from diluted bitumens without natural sediment. However, in the presence of natural sediment, amphipods seem to be less sensitive than *C. dubia* and *D. magna*. In our study, chironomid larval mortality was also more sensitive than *D. magna* and *C. dubia*, according to the results of Barron et al. (2018) and Robidoux et al. (2018), for Cold Lake WAF in presence of natural sediment. The toxicity of our WAFs was probably higher than that of these two studies due to the higher concentration of PACs and VOCs and the use of unfiltered natural water that could allow a higher concentration of hydrocarbons in the water due to the formation of OPAs.

As there is little data on the impact of diluted bitumen on invertebrates, it is of interest to identify measured effects on other ecological receptors, some of which could be observed in aquatic invertebrates. Numerous studies have been conducted on the toxicity of diluted bitumen for fish at different stages of development, particularly the embryonic stage, including model species such as zebrafish (Philibert et al., 2016) and medaka (Madison et al., 2015, 2017), and emblematic fish of Canadian freshwaters such as fathead minnow, white sucker, inland silverside, sockeye salmon, and rainbow trout (Alderman et al., 2017, 2018; Barron et al., 2018; Schiano Di Lombo et al., 2021). Beyond mortality with $LC_{50-96\,h}$ ranging from 5.9 to 16.3 mg/L total petroleum hydrocarbons for WCS and CLB on fathead minnow (Barron et al., 2018), the most frequently reported sublethal effect on fish is the blue sac disease (BSD). This syndrome brings together several symptoms such as craniofacial deformation, yolk sac, and pericardial edema, fin erosion and spinal curvatures, which, of course, cannot be observed in invertebrates. More specifically, the study by Berube et al. (2021) showed that diluted bitumens could be more toxic than conventional oils to *Pimephales promelas* larvae, which is also demonstrated in our study for *C. riparius.* The most toxic oil was found to be Clearwater McMurray diluted bitumen, while the Bluesky diluted bitumen and the conventional heavy oil (Lloydminster) were equally toxic. As in our study, the authors propose that toxicity was mainly due to LMW hydrocarbons such as VOCs and C_6-C_{10}, which were more present in the WAFs of Clearwater McMurray (2597–3508 μg/L VOCs/BTEX) compared to the Lloydminster WAFs (789 μg/L VOCs/BTEX) (WAF at 100 g/L). Philibert et al. (2016) found a greater toxicity for two conventional light/medium oils, mainly characterized by the light fractions of the oils, compared to a diluted bitumen, for the zebrafish *Danio rerio*. This result is consistent with the hypothesis that LMW hydrocarbons are likely to be responsible for the toxic effects of diluted bitumen to aquatic organisms, i.e. aliphatic and aromatic LMWs (<C_{10}; water soluble and volatile) as well as PAHs with 2- to 3-rings.

In our study, the size of surviving organisms was little or not at all impacted by exposure to WAFs. For size, the IC_{25-14d} on organism size was > 100% except when amphipods were exposed to the Bluesky WAF in the absence of sediment. Chironomid larvae exposed to the Bluesky WAF were significantly larger than those exposed to Cold Lake and Lloydminster's WAFs (ANOVA, <0.01, data not shown). Size depends on several factors such as the type of sediment (e.g., organic vs. mineral) as well as the food available. The use of natural sediment versus artificial sediment may indeed have influenced the size of surviving organisms in the artificial sediment, with individuals being smaller in size than in natural sediments due, in part, to the lack of organic matter. However, as oil induced mortality, more food was available for the surviving organisms, regardless of the sediment. This may have resulted in a larger size of these organisms in contrast to the concentrations where low mortality was observed or to the controls. It is therefore important to compare only what is comparable. However, there was no difference between the controls in either condition (natural sediment or artificial sediment) in terms of mortality or size.

Wiseman et al. (2013) still demonstrated growth inhibition in larvae exposed to oil sands process-affected (OSPW). Unfortunately, the authors did not provide a chemical analysis that would have allowed a comparison with our study. To our knowledge, no other studies have investigated the effects of diluted bitumen WAFs on the size of invertebrates. In fish, exposures of *Pimephales promelas* larvae to various diluted bitumens (Clearwater McMurray, Bluesky) did not either lead to any significant effect on organism weights (2597–3508 μg/L VOC/BTEX, 100 g/L WAF; 60.67–22.05 μg/L TPAC, 100 g/L WAF) (Lara-Jacobo et al., 2021; Berube et al., 2021). In contrast, Berube et al. (2021) demonstrated a decrease in the weight of *P. promelas* larvae exposed to a conventional heavy oil, Lloydminster (789 μg/L VOC/BTEX, WAF 100 g/L; 89.66 μg/L TPAC, WAF 100 g/L) but not to the diluted bitumens.

The metabolization of PACs induces the formation of reactive oxygen species (ROS). These ROS in turn induce oxidative damage (e.g., lipid peroxidation, DNA adducts) which can cause DNA damage, leading to carcinogenic effects, as well as lipid and protein peroxidation (Birben et al., 2012). Variations in the activity of antioxidant and detoxification enzymes may indicate exposure or an effect due to the presence of a contaminant and oxidative stress. These enzymes are commonly used in the study of defense mechanisms against xenobiotics and induced ROS. A useful biomarker should reveal the exposure of an organism to a past or present chemical and its associated effects (Hook et al., 2014). Few effects were measured on the biomarkers of antioxidant capacities measured in our study at the end of the exposures. This may be due to the fact that after 7 or 14 d of exposure, the detoxification system of the organisms has probably had time to return to basal levels as a result of the low persistence of the hydrocarbons in the water column or sediment. The study by Berube et al. (2021) conducted on *P. promelas* also showed that oxidative enzymes such as catalase and superoxide dismutase were not affected following exposure of larvae to diluted bitumens (Clearwater McMurray, Bluesky) and conventional heavy oil, Lloydminster. To our knowledge, no such study has been performed on invertebrates.

In our study, GST activity showed significant variation between the different exposure conditions in both species, in contrast to the other antioxidant enzymes measured. The GST activity measured in our study did not show saturation, unlike Berube et al. (2021). In their study, GST activity appeared saturated at 75% WAF (23.05–89.66 μg/L TPACs, 789.3–3509 μg/L VOCs,) leading to an increase of lipid peroxidation, presumably because an accumulation of PAH metabolites would lead to an overwhelming of the defenses against oxidative stress (Berube et al., 2021). In our study, the hydrocarbons did not accumulate and disappeared very quickly from the water column, as these were acute and not chronic exposures to WAF. It would have been interesting to measure biomarkers of effects such as lipid peroxidation or biomarkers of exposure such as EROD, which could not be done here due to the low availability of biological material. The expression of genes involved in detoxification such as *cyp1a* or *gst* would provide complementary insights into the mechanisms of toxicity and should be considered in future studies.

The toxicity data generated by studies like ours can be used to assess the environmental risks to ecosystems that may be exposed to a spill. Overall, data from our study highlight that in natural environments where water renewal is low, such as in lakes and bays, oil spills can induce in the short term an increase in petroleum hydrocarbons in the water column posing a risk to benthic invertebrates, in addition to the already better-known risks for fish, even in the absence of settling of undissolved spill residues on the sediment. Specifically, our study can contribute to predicting the risk of toxicity for freshwater benthic invertebrates following an oil spill after analysis of the contamination of the water column by PACs. However, the chemical complexity of crude oils increases the challenges of developing effective ecological risk assessment strategies (Wang et al., 2006). Crude oil is composed of a wide variety of organic compounds, from highly volatile hydrocarbons such as BTEX, to very complex compounds such as asphaltenes and resins, which are more difficult to measure. The Canadian Council of Ministers of the Environment (CCME) does not propose criteria for the protection of aquatic life for all PACs, but certain toxicity thresholds may highlight hydrocarbon concentrations in WAFs that are potentially toxic to organisms. For example, the quality criteria for Naphthalene is 1.1 μg/L, while the concentration in the WAFs from our study was 6–18 times higher. The same is observed for Phenanthrene (threshold value at 0.4 μg/L against values that were 1.3–4 times higher), Pyrene (present only in the Lloydminster's WAF being 1.1–5 times higher than the threshold at 0.25 μg/L), Benzo(a)anthracene (threshold at 0.018 μg/L against values that were 1.6–9 times higher in WAFs), Benzo(a)pyrene (threshold 0.015 μg/L against values that were 0.6–7 times higher), and for Fluoranthene (threshold of 0.018 μg/L compared to values 10 times higher in WAFs). On the other hand, for Fluorene, which has a threshold of 3 μg/L, the concentrations in WAFs were 3–7 times lower, which was also the case for Acenaphthene (threshold of 5.8 μg/L compared to values 5–11 times lower in WAFs). These hydrocarbons may also have additive or synergistic effects and it is therefore highly complex to determine which is/are responsible for the observed effects. Furthermore, alkylated PAHs are not or only minimally regulated, although they represent a higher fraction of total PAHs in crude oils than the 16 EPA PAHs (Yang et al., 2014) and can be toxic to aquatic organisms (Honda and Suzuki, 2020; Uno et al., 2010). By measuring only the 16 PAHs recommended by the US EPA, and not the alkylated products, the toxicity of these WAFs cannot be fully explained. Indeed, for Bluesky, for example, 80% of its PAHs were in the alkylated form.

Under natural spill conditions, the oil slick remains present, unlike during our laboratory study, which is closer to a chronic exposure. Rodriguez-Gil et al. (2021) and Stayonovich et al. (2021) showed, in mesocosms, that PAC concentrations in the water column increased before reaching a plateau, and that in parallel the PACs concentration in the sediment also increased. Consequently, benthic organisms could be exposed to higher concentrations of hydrocarbons compared to the ones of our study. Indeed, the protective effect of the sediment observed in our study is likely to be much weaker under real spill conditions. As a matter of fact, Black et al. (2021) observed a decrease in insect emergence, among which *Cladotanytarsus* sp. (Chironomidae), during mesocosm exposure similar to Rodriguez-Gil et al. (2021) and Stayonovich et al. (2021), as well as an alteration of the benthic invertebrate community structure. This calls for further studies to investigate the impacts of sediments contaminated by aged oil on benthic organisms, an important trophic link in aquatic ecosystems. Our study is only a first step to better understand the effects associated with physically dispersed hydrocarbons dissolved in the water column.

5. Conclusion

This study shows that physically dispersed hydrocarbons have a toxic effect on benthic organisms. The hydrocarbons present in the WAFs of the petroleum products selected for this study disappeared very quickly from the water column of the exposure chambers of the benthic organisms. Diluted bitumen WAFs were characterized by higher concentrations of BTEX whereas conventional oil WAFs contained more 5- and 6-ring aromatic PACs. While the majority of the oil present quickly disappeared from the water column, a small proportion of these hydrocarbons was able to be deposited on the sediments. Amphipods were as sensitive to WAF from diluted bitumen as to WAF from conventional oil whereas chironomid larvae were only sensitive to WAF from diluted bitumen. The natural sediment was protective for the amphipods only up to a certain concentration of WAFs. In contrast, the sheath in which chironomid larvae develop appeared protective against hydrocarbons in the water column for these organisms. After 7 and 14 days of exposure to WAFs and presumably due to the rapid disappearance of hydrocarbons from the test chambers, the measurement of antioxidant enzymes (CAT, SOD, and GPx) did not allow to distinguish the effects of the different oils. Antioxidant enzymes did not appear to be good biomarkers for these organisms and this type of contamination.

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CRediT authorship contribution statement

Nishodi Indiketi, Marie-Claire Grenon, Paule Émilie Groleau, Éloïse Veilleux, Gaëlle Triffault-Bouchet, Patrice Couture, Nishodi Indiketi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original draft, Writing - Review and Editing, Visualization, **Marie-Claire Grenon:** Formal analysis, **Paule Émilie Groleau:** Formal analysis, **Éloïse Veilleux:** Resources, **Gaëlle Triffault-Bouchet:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing - Original draft, Writing - Review and Editing, **Patrice Couture:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing - Original draft, Writing - Review and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113554.

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