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Use of a fluorescent molecular rotor probe for nanoplastics assessment in epiphytic biofilms growing on submerged vegetation of Lake Saint Pierre (St. Lawrence River)

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Abstract

Studies on plastic pollution conducted in freshwaters mainly focused on monitoring plastic debris in the water column or in the sediments. Few studies have investigated the occurrence of plastic debris in benthic biofilms (periphyton). Yet, algal biofilms may potentially act as a sink for plastic debris, trapping it within their mucilaginous or filamentous matrix. Biofilms may also represent a source of plastic debris by sloughing when they become senescent. In addition, plastic debris accumulated within biofilms may enter the food web via primary consumers. Considering these observations, this study aims to quantify nanoplastics (NPs) accumulated in biofilms growing on aquatic vegetation from Lake Saint Pierre (LSP), a fluvial lake of the St. Lawrence River, and its archipelago. Biofilms were removed from submerged plants and the presence of NPs was assessed by spectrophotometry using the fluorescent molecular rotor probe 9(dicyanovinyl)-julolidine (DCVJ). The results of this study confirm the biofilms' ability to act as a sink for NPs. Despite the fact that the determination of the absolute nanoparticle number and size distribution remains a challenge, we estimated a median concentration of 1.05×10^9 NP/mg of biofilm dry weight (DW) when using 100 nm polystyrene beads for calibration. Concentrations were significantly different between

water masses, with higher concentrations in samples collected in the two lateral water masses compared to the central water mass. Our study provides, for the first time, a quantitative assessment of NPs from epiphytic biofilms in a large river under the influence of anthropogenic sources.

Keywords: Plastic pollution, nanoplastics, freshwater, epiphytic biofilm, fluorescent probe

INTRODUCTION

Since the year 1950, when global plastic production was around 0.5 million tons[1], its annual production has grown steadily, reaching 400 million tons per year in 2022[2]. North America produced 17% of these plastics in 2022[2]. With increasing production, extensive usage of plastic products, as well as poor waste management combined with insufficient regulation, plastic pollution raised global concerns as debris [i.e., mesoplastics 5 to 25 mm, microplastics (MPs) 1 μm to 5 mm, and nanoplastics (NPs) ≤ 1 μm[3]] has been detected in all ecosystems worldwide[4]. For instance, hundreds of research articles assessed the presence and fate of plastic debris in the aquatic environment over the past decade, with a particular focus on MPs in marine ecosystems and in the water column[5]. In the water compartment, plastic debris of 0.45 to 505 μm in size has been detected in concentrations ranging between 3×10^{-8} to 2 particles per mL[6]. Several studies focusing on marine ecosystems highlighted the adverse effects of plastic debris on the biota. Plastic pollution can cause physical damage, such as the entanglement of animal appendages or the obstruction of their digestive tract[7]. Biological and chemical effects such as oxidative stress or DNA damage have also been reported[6], and have been related to the presence of additives compounds such as plasticizers used during plastic production or to the presence of sorbed contaminants[8].

Compared to the marine environment, there are fewer studies that investigated the occurrence of plastic debris in freshwater ecosystems and their ecotoxicity[6]. However, streams and rivers are the major pathways for plastic debris into marine ecosystems[9], and studies suggest that these freshwaters may be at least as contaminated as marine waters[10]. For example, the sediment compartment of the St. Lawrence River is among the 25% most polluted in the world, with concentrations ranging from 65 to 7,562 plastic particles per kg dry weight (DW)[11]. Concentrations ranging from 48 to 187 particles per L were recorded in the water column of the Amsterdam canal (the Netherlands), which was higher than concentrations recorded in the North Sea coast (the Netherlands), where the mean concentration recorded was 27 particles per L[12].

Studies conducted in freshwaters mainly focused on monitoring plastic debris concentrations in the water column or in the sediments, while their occurrence and abundance in periphytic biofilms (or periphyton) have been largely overlooked. Periphyton is a key compartment at the base of aquatic and terrestrial food webs; it is a consortium of viruses, archaea, bacteria, algae, protozoa, fungi, and meiofauna, embedded in a matrix of extracellular polymeric substances. Periphyton colonizes all types of substrates exposed to light[13]. This biological matrix may function as a sink for plastic debris as particles get trapped and incorporated and may also represent a source of plastic particles for higher trophic levels[14]. Trapped plastic debris may have several ecotoxicological consequences on the microorganisms composing the periphyton, as reviewed in Guasch *et al.* (2022)[15]. For example, a study by Merbt *et al.* (2022) conducted on periphyton exposed to virgin and aged polyethylene (PE) beads (1-4 μm, 0.96 g/mL) for 28 days revealed their incorporation in the periphyton and showed a significant shift in microbial community composition[16]. A study by Miao *et al.* (2019) observed that periphyton exposed for 3 h to polystyrene beads (0.1 μm, 0.1 mg/mL) led to a significant decrease in chlorophyll concentration and impaired activity of functional enzymes, such as β-glucosidase and leucine aminopeptidase[17]. Plastic debris also acts as vectors for contaminants and may consequently influence their bioavailability[18] for the microorganisms of the periphyton and for consumers.

Biofilms influence the degradation of plastic particles by promoting their fragmentation by microorganisms that secrete enzymes capable of breaking covalent bonds[14]. Plastic debris accumulated within the periphyton may enter the food web via primary consumers feeding on this basal resource[19]. Considering that periphytic biofilms might act as a sink of plastic particles, dietary exposure of consumers might be more important than direct exposition through the water column[15]. Despite the key role periphytic biofilms play in freshwaters, little is known about their ability to trap and accumulate plastic particles[14]. To our knowledge, the article by Wang *et al.* (2023) is the only field study that provides evidence supporting the hypothesis that periphyton can incorporate plastic debris[20]. They recorded MPs, mainly fibers, in concentrations ranging from 419 to 1,314 items/m^2 in the CaoE River, China[20]. The authors focused on particle sizes in the mm range (≤ 0.5, 0.5-1, 1-2, 2-5 mm) as plastic debris was handpicked under an optical stereomicroscope prior to identification through Raman microscope spectroscopy.

To this date, less research has focused on NPs as compared to MPs; nanosize particles require complex analytical methods to be accurately quantified[21] and only a few studies measured particles smaller than 10 μm in the environment[6]. However, the ecotoxicity of plastic debris is likely to increase with decreasing size. Indeed, surface area in contact with contaminants increases with decreasing particle size[22], thus making NPs a particularly concerning hazard to biota. In addition, tissue translocation of NPs has been observed in laboratory experiments where nanometer-sized particles can passively cross tissue membranes[23,24]. To our knowledge, no studies have investigated the presence of NPs accumulated in periphyton. The following keywords were used in a Web of Science search TI = [*plastics AND (biofilms OR periphyt* OR "microbial communit*") AND (fresh$water* OR river* OR stream*)] and returned 16 results with only 4 of them addressing periphytic biofilms as a sink for plastic debris. Among those four publications, two were related to experimental exposure of periphyton to MPs[16,25], and one study was related to the quantification of MPs larger than 500 μm in periphyton collected in the field[20]. The fourth article by Holzer *et al.* (2022) was the only study specific to NPs, in which the authors investigated the bioaccumulation and toxicity of NPs in stream periphyton, as well as their transfer and resulting effects on the aquatic snail *Physa acuta*[26]. In their experiment, they exposed periphyton grown on glass slides in microcosms containing palladium-doped polyacrylonitrile coated with a polystyrene shell (160 nm, 0.5 mg/L) for 24 h and performed a feeding trial with the contaminated periphyton for 14 days. Their study revealed a strong accumulation of NPs in periphyton, where 90% of the NPs added to the microcosms were no longer in the overlying media.

Based on the scarcity of studies conducted to address plastic pollution in freshwaters, especially within the biofilm compartment, additional knowledge is needed to assess the extent to which this primary resource may act as a sink for NPs and as a source of contamination to consumers. The main objective of this study was to estimate NPs accumulated in periphyton growing on aquatic vegetation (i.e., epiphyton) from Lake Saint Pierre (LSP), a fluvial lake of the St. Lawrence River, and its archipelago (Quebec, Canada). LSP is an exceptionally rich ecosystem, both biologically and economically[27]. This widening of the St. Lawrence River is recognized as an ecosystem of international importance under the Ramsar Convention and is identified as a World Biosphere Reserve by UNESCO[28]. Plant beds of submerged/rooted aquatic vegetation are one of the key components of LSP, as they play an essential role in regulating various physical parameters such as current velocity and sedimentation of suspended particulate matter[29]. These dense plant communities provide essential habitats for the survival of many species of invertebrates and fish[29], and the epiphyton growing on this vegetation is a key component of the food web as several consumers rely on this resource[30]. Aquatic plants also form the basis of waterfowl diets[31], which may be exposed to NPs accumulated within the epiphyton.

Seventy percent of the water flowing through LSP comes from Lake Ontario[27]. This water is of relatively good quality and mainly flows through the navigation channel[32]. The shallower areas of the lake are thus isolated by this central water mass and show degraded water quality due to certain highly impacted tributaries entering the lake[33]. Because the main sources of water coming into LSP mix very little[34], they form three distinct water masses with different physico-chemical characteristics. The different origins of the three water masses and their water quality led to the hypothesis that NP concentrations accumulating in the epiphyton will differ depending on sampling locations within the LSP system (north, south, and central water masses). A second hypothesis was formulated around the upstream-downstream longitudinal gradient, where NP concentrations were projected to be higher in the epiphyton collected at the upstream stations compared to downstream stations. This hypothesis was based on the assumption that dense aquatic vegetation will favor NPs accumulation in the epiphyton and thus act as a filter for plastic particles.

The secondary objective of this study was to explore the feasibility of assessing polystyrene-like NPs (PSNPs) in the epiphyton by spectrophotometry using the fluorescent molecular rotor probe 9(dicyanovinyl)-julolidine (DCVJ). In this study, NPs detection was based on the probe's sensitivity to changes in hydrophobicity[35,36]. Briefly, a hydrophobic interaction between the probe and polystyrene nanoplastic beads (PSN) occurs at the PSN's surface, which produces a fluorescent signal[36]. After calibration with PSN, this signal is correlated to the presence of PSN and/or PSNP polymers, and its intensity is proportional to their concentration. The method allows for relative quantification of NPs in biofilm samples with PSN as the reference polymer. This method was previously used to assess PSN in diverse biological samples such as mussels[35-37] or radish sprout[35] and was tested here for the first time in the complex and heterogenous periphyton matrix.

MATERIALS AND METHODS

Study area

Lake Saint Pierre [Figure 1], stretching from Sorel to Trois-Rivières, is a widening of the St. Lawrence River and is characterized by a lentic hydrology (except for the central water mass)[38]. The system is 32 km long and 13 km wide, covers an area of 500 km² and has an average depth of 3 m with a maximum depth of 11.3 m at the center due to a man-made navigation channel[27]. The channel forms a physical barrier between the northern water body, mainly fed by the Ottawa, Yamachiche, Maskinongé and du Loup rivers, and the southern water body of the lake with waters from Richelieu, Saint-François, Yamaska, and Nicolet rivers[27]. Those tributaries drain highly anthropized lands, such as agricultural areas, which account for 37.7% of the lake's watershed[32]. As for the central water body, it is mainly fed by Lake Ontario[27]. The water quality from the central water body differs from the lateral water masses that exhibit a lower quality[27] due to tributaries such as the Yamaska River which is among the most agriculturally and industrially polluted rivers in the Province of Quebec[39]. In addition, the northern water mass receives wastewater effluents from Montreal, the world's 3rd largest treatment plant of its kind[40]. The water masses of the lake also differ by the spatial distribution of the submerged vegetation[32], with greater abundances in the littoral zones and around the archipelago[34]. The floodplains of the lateral masses are exposed to heavy anthropization, such as dense road networks, sewer overflows, row crops, and several municipalities and industries[32,33].

Biofilm sampling and water physicochemistry

In August 2023, 14 stations were visited across LSP and its archipelago. To minimize secondary contamination of samples with plastic debris, field staff were dressed in cotton clothing and used sampling tools made of metal or glass. No gloves were used during sampling to avoid potential contamination with plastic-like material. Biofilm samples were collected on submerged plants such as *Vallisneria americana*, *Potamogeton pusillus*, and *Potamogeton perfoliatus*. Submerged vegetation was harvested using a metal rake and transferred to a steel bucket containing 750 mL of lake water. Plants were then manually vigorously

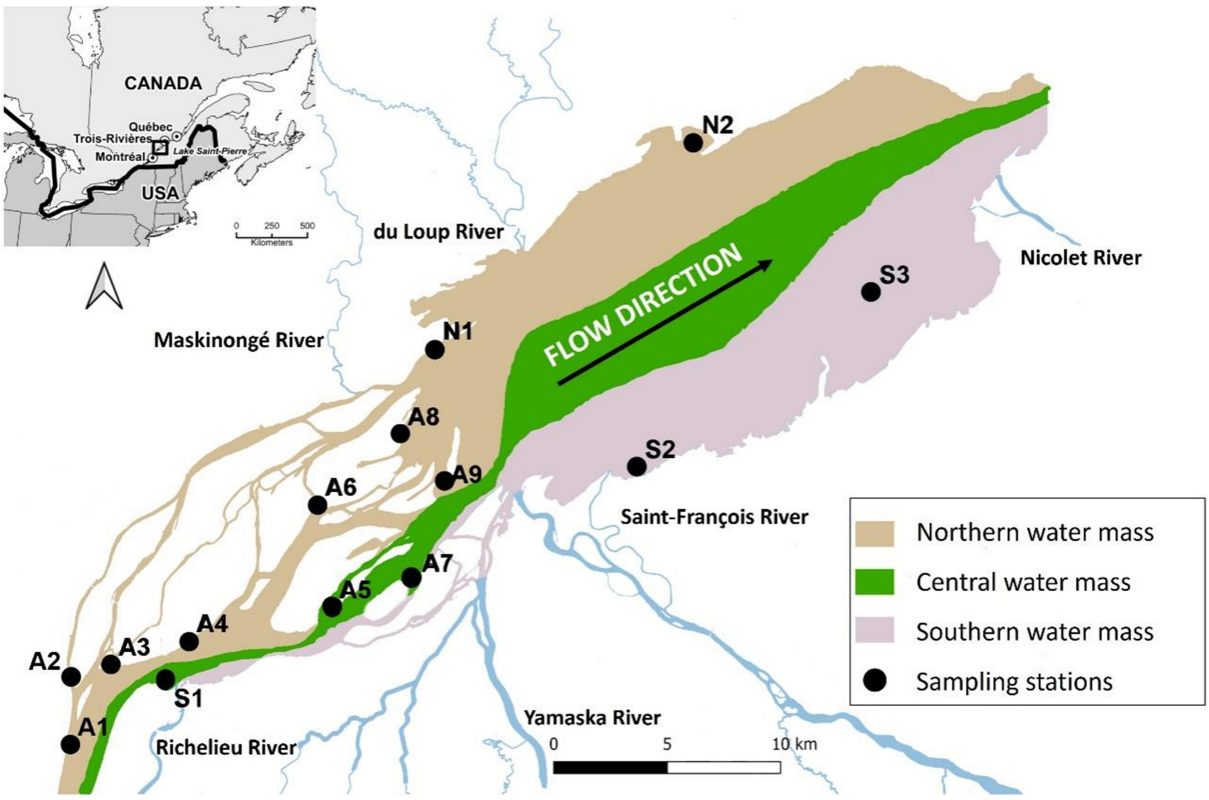

Figure 1. Distribution of water masses within Lake Saint-Pierre (Quebec, Canada) and the stations visited in 2023. Out of the 14 stations sampled, nine were located in the lake's archipelago area (stations A1 to A9), three on the south shore (stations S1, S2, and S3), and two on the north shore (stations N1 and N2). Nine stations (A1 to A4, A6, A8, A9, N1, and N2) were located in the northern water body, three (A5, A7, and S1) in the central water body and two (S2 and S3) in the southern water body.

shaken to detach the epiphython. The content was then transferred into 1-liter glass jars after filtration on a 0.5 mm pore size metal sieve to remove plant debris and large invertebrates. Samples were preserved in coolers in the field and stored at 4 °C in the dark until arrival at the laboratory. In order to obtain representative samples, each of the 14 stations was sampled along a transect of three sampling points that were 10-20 meters apart. Conductivity, temperature, and pH were measured on site using the ORION Thermo Scientific multi-probe field instrument [Supplementary Table 1]. Once in the laboratory, biofilm samples were oven-dried at 50 °C to preserve the physicochemistry of potential plastic polymers[41], and stored at room temperature in obscurity until further analyses.

Sample digestion and detection of PSNPs by spectrophotometry

A subsample (referred to as samples hereafter) from each of the 42 biofilm samples (14 stations × 3 points per transect) was rehydrated in ultrapure water (5 mL per gram of dry mass) and manually stirred with a stainless steel spatula. To ensure complete rehydration, samples were placed on an orbital shaker at 300 rpm for one week (room temperature in obscurity). Afterwards, potassium hydroxide (KOH 20%) was added to each sample (v/v for a 10% final KOH concentration) to digest organic content[42] without degrading PSN, and the samples were left for agitation for one additional week after thorough vortexing. After one week under agitation, the digestates were left to stand for 3 h to allow for microparticles to settle. The supernatants were then collected for analysis. Potassium dihydrogen phosphate (KH_2PO_4, pH 6.5, 100 mM) was added (0.5 v/v) to neutralize KOH and avoid interference with the quantification method[42]. Levels of NPs were then estimated by spectrophotometry using the fluorescent molecular rotor probe DCVJ.

The commercial DCVJ probe (Sigma Aldrich, CAS 58293-56-4) was dissolved in methanol at a concentration of 1 mM to serve as a stock solution. The stock solution was diluted at 0.4 µM in ultrapure water for a daily solution used for fluorescence analysis. A volume of 30 µL collected from the sample's supernatant was mixed with 160 µL of DCVJ probe and 10 µL of ultrapure water in 96-well microplates. The fluorescence intensity of the microplates was analyzed using a microplate reader (Agilent, BioTek, Synergy Neo 2) with the excitation wavelength at 450 nm and the emission wavelength at 620 nm. PSNPs detected in biofilm samples were assumed to have a maximum size of 100 nm as standard solutions of PSN beads of 50 and 100 nm (Polyscience, Polybead® Microspheres) were used for method calibration [Supplementary Figure 1]. Biofilm NP concentrations were calculated using the 100 nm low concentration calibration curve as they fit best the fluorescence intensity range, except for station S2 which had a higher fluorescence than the range provided by the 100nm low concentration curve, and as such levels were calculated with the 100 nm high concentration calibration curve (i.e., above 5,000). The influence of plastic material autofluorescence[43] on the detection of PSNPs with the fluorescent DCVJ probe was also investigated and no autofluorescence was detected at 620 nm [Supplementary Table 2].

Quality assurance and quality control of the DCVJ probe assay

Several procedural controls were implemented in the protocol and were analyzed with the samples to assess (1) the potential contamination during the sample treatment process (i.e., negative controls); and (2) the efficiency of PSN recovery during the digestion process (digestion controls). Negative controls consisted of ultra-pure water samples that were digested using the same protocol as for biofilm samples, and the digestion controls were suspensions of PSN of a known theoretical concentration suspended in deionized water (8.41×10^{13} 50 nm PSN/mL; 1.15×10^{13} 100 nm PSN/mL) and digested also using the same protocol as for biofilm samples. Deionized water samples (water blanks) were analyzed to ensure the specificity of the DCVJ probe with PSN and to assess the background noise. Biological controls were performed during the method development and involved spiked biofilm samples (pooled biofilm samples, and the addition of a known theoretical concentration of 8.35×10^{12} PSN/mL) to assess fluorescence signal in the presence of the biofilm matrix and potential interactions. As for the limit of detection (LOD), its theoretical value (blank standard deviation × 2) was estimated at 65 ng/mL and the blank standard consisted of the DCVJ probe diluted in water (Gagné, 2019). Standard curves using suspensions of PSN (50 and 100 nm) were generated to validate the linearity of the signal using the DCVJ probe (R^2 ranged between 0.92 and 0.99 depending on particle size; Supplementary Figure 1).

Data representation and analysis

Mapping of the sampling stations at the scale of LSP was carried out using QGIS-Server, QGIS software (version 3.26.1). Estimated levels of PSNPs in the biofilms growing on submerged vegetation at each station were graphically presented to allow for inter-station comparison, and a Pearson correlation was conducted to explore a potential upstream-downstream longitudinal gradient. Sampling stations were subsequently classified according to their respective water masses (i.e., northern, central, or southern water mass). Differences in terms of PSNP concentrations between the three water masses were assessed by a Kruskal-Wallis test followed by a Dunn post-hoc test using the FSA package. Significant differences were set at $P < 0.05$ and statistical analyses were conducted using the softwares R (version 4.3.0) and R studio (version 2024.04.0+735).

RESULTS

Quality assurance and quality control

Water blanks and negative controls used for the quality assurance and quality control (QAQC) assay did not emit any fluorescent signal (values not shown), contrary to samples containing PSN (i.e., digestion and biological controls). No autofluorescence from PSN controls was measured at 450:620 nm in the absence of

Table 1. Average concentrations of polystyrene nanoplastic beads (PSN/mL) of 50 and 100 nm detected in the sample used for quality assurance and quality control

PSN size	Sample type	Emission 620 nm (AU)	Initial theoretical concentration	Final measured concentration
50 nm	Water blanks	NA	-	NA
	Negative controls	NA	-	NA
	Digestion controls	5,615	8.41×10^{13}	3.29×10^{10}
	Biological controls	2,827	8.02×10^{13}	4.26×10^{10}
100 nm	Water blanks	NA	-	NA
	Negative controls	NA	-	NA
	Digestion controls	6,683	1.15×10^{13}	1.24×10^{12}
	Biological controls	1,792	8.35×10^{12}	4.16×10^{11}

Fluorescence intensities are expressed as AU and were determined at 450 nm excitation wavelength. Water blanks: deionized water; negative controls: digested deionized water; digestion controls: PSN suspensions of a known theoretical concentration; biological controls: spiked biofilm samples. PSN: Polystyrene nanoplastic beads; AU: arbitrary units; NA: not applicable.

DCVJ, indicating that the observed fluorescent signal in the presence of both PSN and DCVJ was specific to their interaction. Hence, biological controls emitted a fluorescent signal that was not related to the autofluorescence of plastic materials[44], as confirmed by the autofluorescence assay [Supplementary Table 2]. Samples containing PSN showed a loss, especially with PSN of 50 nm [Table 1]. For 50 nm PSN beads, there was a difference of a factor of 2,000 for digestion controls and of a factor of 1,000 for biological controls between the theoretical and the measured PSN concentrations. As for PSN of 100 nm, there was a ninefold and a twentyfold difference (digestion and biological controls, respectively) in PSN concentration between the initial theoretical concentration and the final measured concentration.

Detection of PSNPs in biofilms

Samples' fluorescence at 620 nm was mostly below 5,000 AU and ranged from 320 to 4,800 AU [Supplementary Table 3]. The three samples from the most contaminated station emitted a fluorescence signal above 5,000 at 620 nm (i.e., 5,316, 5,355, and 6,253). All samples' fluorescence signal fell along the linear part of the 100 nm PSN calibration curve [Supplementary Figure 1], implying that samples contained PSNPs. Estimated levels of PSNPs ranged from 1.05×10^{7} to 5.61×10^{9} PSNP/mg biofilm DW with a median concentration of 1.05×10^{9} PSNP/mg biofilm DW. The average (± standard-deviation, SD) concentration was $1.34 \times 10^{9} \pm 1.10 \times 10^{9}$ PSNP/mg biofilm DW. As a general trend, PSNP concentrations observed in the three samples collected along the transect at each station were relatively similar, except for A5, where PSNPs were in much lower concentrations in one sample. There was a twenty-eight-fold difference in PSNP concentration between the most contaminated station, S2, and the least contaminated station, A5 [Figure 2]. Overall, PSNP concentrations at the different sampling stations seemed to be mainly related to the three water masses rather than to an upstream-downstream longitudinal gradient. Indeed, no significant correlation was observed between the distance (km) from the most upstream station (A1) and PSNP concentrations ($r = 0.26$; $P > 0.05$).

Sampling stations were grouped according to their water mass (central, northern, and southern). The central water mass was the least contaminated with PSNPs, where a median concentration of 5.24×10^{8} PSNP/mg biofilm DW and an average concentration of $5.01 \times 10^{8} \pm 3.06 \times 10^{8}$ PSNP/mg biofilm DW were estimated. The southern water mass was the most contaminated, with a median concentration of 3.02×10^{9} PSNP/mg biofilm DW and a mean concentration of $3.18 \times 10^{9} \pm 1.78 \times 10^{9}$ PSNP/mg biofilm DW. As for the northern water mass, it was three times less contaminated than the southern water mass, and twice as contaminated as the central water mass. The median concentration in the northern water mass was 1.03×10^{9} PSNP/mg biofilm DW and the average concentration was $1.16 \times 10^{9} \pm 5.02 \times 10^{8}$ PSNP/mg biofilm DW.

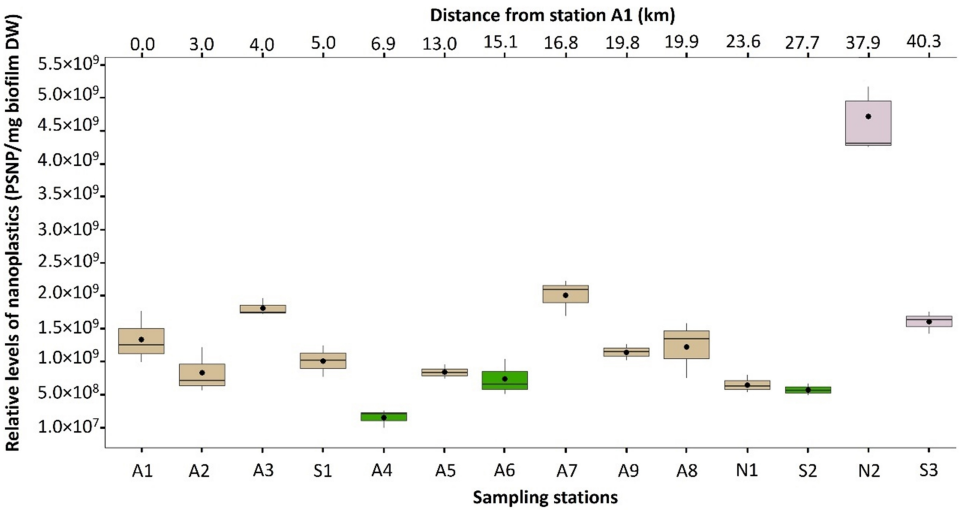

Figure 2. Estimated concentrations of nanoplastic (PSNP/mg DW) in the biofilm samples collected across the 14 stations (× 3 sampling points) visited at Lake St-Pierre. Codes on the X-axis represent station location where A = archipelago, N = north shore, S = south shore. Colors represent water masses, where brown for the northern water body, green for the central water body, and lilac for the southern water body. The solid black line represents the median concentration, and the black dot represents the mean concentration. Stations are presented from upstream to downstream, with station A1 being the most upstream station. Distances in km from station A1 are also indicated. PSNP: Polystyrene-like nanoplastics; DW: dry weight.

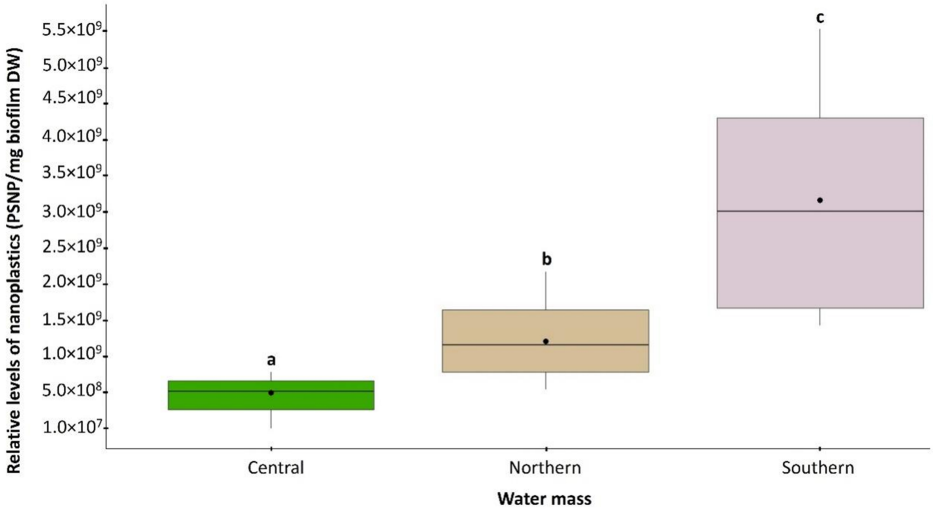

Figure 3. Estimated concentrations of nanoplastic (PSNP/mg DW) in the biofilm samples collected across the 14 stations grouped according to their respective water mass (central, northern and southern). The central water body includes three stations (A5, A7 and S1), the northern water body includes nine stations (A1 to A4, A6, A8, A9, N1 and N2), and the southern water body includes two stations (S2 and S3). The solid black line represents the median concentration, and the black dots represent the mean concentration. The letters a, b, and c indicate significant differences in estimated concentrations of nanoplastics between water masses. PSNP: Polystyrene-like nanoplastics; DW: dry weight.

A Kruskall-Wallis test performed to assess the difference in PSNP concentrations between the three water masses showed that there was a significant effect of water mass ($P < 0.001$) [Figure 3]. The Dunn post-hoc test revealed significant differences between central and northern water masses ($P = 0.002$), southern and central water masses ($P < 0.001$), and northern and southern water masses ($P = 0.015$). The epiphyton from the submerged vegetation collected in the southern water mass appeared to have accumulated six times more PSNP/mg biofilm DW than the epiphyton from the central water mass.

DISCUSSION

Methodological development of the DCVJ probe

Water blanks and digestion controls used in the QAQC assay did not exhibit a fluorescence signal at 620 nm contrarily to PSN suspensions, meaning that water, reagents, and sample processing did not contribute to the observed signal at 620 nm in biofilm samples, i.e., if plastic contamination occurred, it was below LOD. Digestion controls and biological controls showed a loss of PSN, especially for the smallest PSN size (50 nm), which might imply a size-dependent efficiency of the analytical method. However, given the NPs' tendency to aggregate, even more so as concentration increases[45] and particle size decreases[46], the particle loss was considered minor, given the overall PSN concentration and the fact that the primary goal was to compare contamination levels between sampling stations. Preliminary tests with biological controls helped confirm a potential interaction between KOH, biofilm organic matter, and spiked PSN, which slightly decreased the fluorescent signal at 620 nm for both particle sizes. The extent of this interaction remains to be quantified, as it is directly linked to (1) biofilm composition, (2) NP concentration, and (3) NPs size. Furthermore, the size and concentration of NP particles are well known to strongly influence their propension to aggregate in suspension, especially in the absence of surface surfactants[47,48].

Overall, the QAQC assay, in addition to the autofluorescence assay [Supplementary Table 2], demonstrated the method's ability to detect NPs resembling PSN in the complex epiphyton matrix, and enabled a comparison of NP contamination between stations and between water masses of LSP. However, there might be a marked underestimation of PSNPs detected in biofilm samples, given the loss of PSN used in the QAQC assay. Therefore, the DCVJ probe currently provides information on whether or not epiphythic biofilms can accumulate NPs from LSP and enables comparison between stations but cannot be used for absolute quantification. Further research and method development are needed to improve the quantitative assessment of PSN in periphyton samples, such as studying the interaction between organic matter and the DCVJ probe, and to link periphyton biochemical properties to potential interactions (such as lipid content). The use of the DCVJ probe could be coupled with other analytical methods, such as nanoparticle tracking analysis (NTA) or pyrolysis-GC/MS. These methods also require handling and sample processing, with the potential introduction of other methodological biases.

Developing protocols and methods to properly analyze and quantify NPs is an ongoing process with multiple challenges due to the complex nature of NPs contamination[49,50]. There are currently very few methodologies enabling rapid quantitative analysis of plastic particles, and most of them solely focus on MPs in environmental water samples[51-53], which are less complex than organic matrices such as periphytic biofilms. The DCVJ probe has been successfully applied to quantify PSN in diverse aquatic and terrestrial organisms[35,37,54], demonstrating promise for high-throughput analysis of environmental samples and organisms. The approach allows for effective means of comparison between treatments or sampling stations, although it still needs further development and adjustments to improve quantification depending on the nature and biochemical complexity of the samples being analyzed.

Presence of PSNPs in biofilms

Lower concentrations of PSNPs in the central water mass of LSP compared to the lateral water masses might be linked to the water residence time and, therefore, to the water flow regime. In this zone, the water flow regime is higher, with a residence time of 20 h, while it can reach up to 72 h and even several weeks during the summer low-water period in the lateral water masses[55]. High water flow regime, such as in the central water mass, may promote the dispersal and movements of particles[56] and thus prevent NP accumulation in biofilms. The lower PSNP concentrations observed in the central water mass might also be linked to the origin of the water coming from Lake Ontario, where this lentic environment favors particle sedimentation. Another factor that can drive NP contamination is point sources[57], and direct effluent

sources potentially containing plastic pollution may not be as present in this central zone of the lake compared to the lateral water masses. For example, station A5 (lowest estimated PSNP concentration) is surrounded by a protected area[27], which can help limit inputs of plastic debris.

High concentrations of PSNPs observed in the epiphyton collected in lateral water masses might be attributed to the proximity to sources of pollution along the lake shore[58]. This observation is consistent with the results by Crew *et al.* (2020), who assessed MPs in the sediments of the St. Lawrence River and found a fourfold difference between MP concentrations in sampling points located around the LSP archipelago (n = 3) and the one station located on the north shore of LSP[11]. In lateral water masses, sources of pollution seem mainly linked to agricultural land use and urban areas. In the southern water mass, elevated NP concentrations were mainly driven by station S2, where the average concentration reached $4.73 \times 10^9 \pm 7.56 \times 10^8$ particles/mg DW. This station is located downstream of the Yamaska and Saint-François rivers, which flow through agricultural watersheds[59,60]. In addition, the Yamaska River is among the most polluted rivers in the province of Quebec, partly due to the presence of industrial activities. Indeed, the Yamaska River runs through the municipality of Granby, which is home to 271 industries, including 28 plastic/rubber industries[61]. In the northern water mass, elevated PSNP concentrations were mainly driven by stations A8 and N1 with average concentrations of $2.02 \times 10^9 \pm 2.76 \times 10^8$ particles/mg biofilm DW and $1.24 \times 10^9 \pm 4.31 \times 10^8$ particles/mg biofilm DW, respectively. Both stations were located in agricultural areas, with station N1 located downstream (1.4 km) of a small agricultural stream that also drains large proportions of agricultural lands[62]. Agriculture is one major source of plastic debris into the environment[9]; therefore, it is not surprising to observe elevated PSNP concentrations in the epiphyton collected on the submerged aquatic vegetation growing along the south and the north shores.

Finally, differences in PSNP concentrations between the northern and southern water masses might reflect the general pollution level of their affluents. For instance, the water quality (i.e., nitrogen, phosphorus, suspended particle matter, and bacteriological loads) is poorer in the tributaries of the south shore compared to those of the north shore[63]. For example, at the St. Lawrence River scale, the annual total suspended particle load between 2013 and 2017 was 1.3 times higher in the tributaries of the south shore than in the tributaries of the north shore[63]. Higher concentrations of all other parameters (i.e., nitrogen, phosphorus and bacteriological loads) were also observed in water samples collected from the south shore than in samples collected from the north shore. For instance, Maskinongé River, which is in close proximity to station A8 in the northern water body, exhibited a better water quality than Yamaska and St-François rivers, which were the main tributaries discharging upstream of the most contaminated station (S2) in the southern water body.

Comparing PSNP concentrations in the biofilm growing on submerged vegetation from LSP with NP concentrations found in other systems around the world is difficult as, to our knowledge, there are no other studies focusing on NPs in this biological compartment. The same goes for other benthic compartments, such as the sediments, as empirical data are lacking due to technical limitations to retrieving NPs particles[64] or analytical limitations to detecting nanoscale particles[57]. However, laboratory experiments highlighted a continuum between MPs and NPs, where MPs subsequently degrade into NPs[3]. For example, Wagner and Lambert (2016) investigated the degradation of different plastic types under aqueous conditions and observed that PS of 1 × 1 cm squares size generated the highest number of particles (6.4×10^8 particles/mL) in the 30-2,000 nm size range[65]. Based on this, the elevated PSNP concentrations observed in the epiphyton of LSP seem plausible, considering the elevated concentrations of MPs found in the sediments of the St. Lawrence River[11].

CONCLUSION

The present study is the first to investigate NPs (resembling polystyrene) contamination in the biofilm growing on the submerged vegetation. The DCVJ fluorescent probe was used to detect and quantify these NPs. This analytical method provided insights into a rapid and cost-effective approach enabling the quantification of PSNPs and laid the foundations for the development of field monitoring protocols in a context where standardized and accessible protocols are lacking. However, the method needs further improvement, particularly in its ability to detect and quantify a broader range of plastic polymers. Moreover, the DCVJ probe can currently only be employed as a diagnostic tool for plastic pollution as the quantified particles need to be correlated with a calibration standard, in this case, PSN. Therefore, it is essential to compare the DCVJ method with other techniques, such as pyrolysis-GC/MS, to obtain absolute quantification and to provide valuable complementary information from a qualitative perspective. Finally, insights into samples' biochemical composition (e.g., lipids, amino acids) could help to better understand the potential *in situ* interactions between biofilms and NPs contamination.

Overall, the analysis of biofilm samples collected from submerged vegetation revealed an important accumulation of PSNPs, supporting the hypothesis that periphyton can act as a sink for plastic particles. Estimated levels of PSNPs were linked to the three water masses of LSP with higher concentrations in stations located in lateral waters compared to the central water mass, suggesting an important influence of land-based sources from the tributaries. Further research is needed to better assess the link between specific anthropogenic activities at the watershed scale and NPs pollution.

DECLARATIONS

Authors' contributions

Field sampling: Mouatchô LY, Ponton DE, Amyot M, Lavoie I
Sample preparation: Mouatchô LY
Laboratory analyses: Roubeau Dumont E
Data analysis: Mouatchô LY
Study design: Lavoie I, Ponton DE, Amyot M, Mouatchô LY
Funding acquisition: Lavoie I, Gagné F, Amyot M
Manuscript writing: Mouatchô LY, Roubeau Dumont E, Lavoie I
Manuscript review and editing: Mouatchô LY, Roubeau Dumont E, Ponton DE, Gagné F, Amyot M, Lavoie I
All authors have read and approved the final manuscript.

Availability of data and materials

The data are available in Supplementary Materials and can also be available from the corresponding author upon reasonable request.

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Conflicts of interest

Gagné F is an Editorial Board member of the journal *Water Emerging Contaminants & Nanoplastic*, while the other authors have declared that they have no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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