

MICROFIBER UPTAKE BY FRESHWATER MUSSELS IN TRIBUTARIES OF THE SAINT  
JOHN RIVER WATERSHED, NEW BRUNSWICK

BY

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## ABSTRACT

Recognized as a potential novel environmental stressor, microfibers are a widespread contaminant of anthropogenic origin. Many enter aquatic systems through wastewater treatment plant (WWTP) effluent and may persist in the environment indefinitely. Although the environmental impacts of microfibers are not well-understood, they are suspected to play a role in the dispersal of chemicals and other contaminants throughout aquatic systems and may impair feeding and respiration of aquatic filter-feeding organisms due to their tendency to accumulate on digestive and respiratory tissues. This study aimed to determine whether freshwater mussels in the Saint John River watershed acquire microfibers on or within their tissues, and if so, whether microfiber abundance was related to WWTP discharge points and other potential diffuse microfiber sources. Microfiber abundance was assessed in mussels of the species *Margaritifera margaritifera* L., which were collected both upstream and downstream of WWTP discharge points and at intermediate sampling points along the Kennebecasis and Tobique rivers. No spatial trends were observed in microfiber abundance in relation to WWTP discharge on either river. Mussels from the Tobique River had significantly ( $p < 0.01$ ) more microfibers per gram of soft tissue than those from the Kennebecasis. These results help reveal a potential pathway through which microfibers may be entering aquatic food webs and addresses the plausibility of using mussels as bioindicators of microfiber contamination.

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## INTRODUCTION

### *Microfiber Contamination*

In recent years, microplastics have become increasingly recognized as a ubiquitous contaminant, with microfibers accounting for the majority of microplastics found in the environment (Miller et al., 2017). Of the plastic waste generated globally, 79% accumulates in landfills or within terrestrial, freshwater, and marine environments, as most common plastics are not biodegradable (Geyer et al., 2017). However, little is known regarding the environmental impacts of non-plastic microfibers, which, despite comprising up to 50% of all observed microfiber contamination, are often disregarded due to the misconception that they will biodegrade and are therefore not a threat to the environment (Miller et al., 2017). For the purpose of this study, the term ‘microparticle’ will be used to refer to any particle (< 5 mm) believed to be of anthropogenic origin, while all filamentous microparticles, both plastic and non-plastic, are herein referred to as ‘microfibers.’ While it is true that non-plastic microfibers will not persist indefinitely, some may take up to 5 years to decompose (Arshad et al., 2014; Henry et al., 2019), and may be further preserved as a result of treatment with chemicals and blending with non-biodegradable materials during manufacturing (Xu et al., 2018; Zambrano et al., 2019).

Both plastic and non-plastic textiles are in high demand globally, with 100 million metric tons produced in 2016 (International Cotton Advisory Committee [ICAC], 2017; Dris et al., 2018). Of this, polyester accounts for about 50% of the global fiber market (IHS Markit, 2018), followed by cotton, which is the second highest in demand, at 30% of the market (Carr, 2017). Plastic textiles other than polyester account for another 10%, and semi-synthetic or regenerated cellulose fibers (such as rayon and viscose) and all other natural fibers including silk and wool account for the remainder (Carr, 2017).

Microfiber contamination primarily originates from clothing but may also be released by other textiles such as upholstery, bedding, and carpeting (Carr, 2017). This is apparent in the polyester industry, where apparel accounts for 50% of polyester produced annually, while home furnishings account for only half that amount (Carr, 2017). Transport to the environment begins following abrasion, either through wear over time or laundering (Hartline et al., 2016). During

laundering, a single article of clothing may release up to 1,900 microfibers, although these values may be affected by the age and material laundered (Browne et al., 2011). In some cases, non-plastic textiles such as cotton and rayon release more microfibers per wash than plastic textiles (Zambrano et al., 2019). Browne et al. (2011) also suggested there may be significant seasonal variability in the number of microfibers released from households as laundering may increase as much as 700% during colder months. Microfibers released during laundering are carried to WWTP facilities, which serve to filter and disinfect municipal wastewater before it is discharged into the environment. Although WWTPs capture more than 98% of microfibers from wastewater (Magnusson & Norén, 2014; Carr et al., 2016; Murphy et al., 2016), it is well established that WWTPs are a major point source of microfiber contamination (Ziajahromi et al., 2017; Xu et al., 2018). In addition to being directly discharged into aquatic systems in WWTP effluent, captured microfibers are often released into the environment through the application of sewage sludge as fertilizer on agricultural land and ultimately reach aquatic systems via runoff (Zubris & Richards, 2005).

Until recently, the laundering of clothing and discharge from WWTPs were assumed to account for the largest proportion of microfibers delivered to the environment, however new research demonstrates that atmospheric deposition may account for a much larger proportion of microfiber contamination than previously thought, including that of non-plastic microfibers, which were observed to account for 50% of those transported by atmospheric deposition (Dris et al., 2016; De Falco et al., 2020). The abrasion of clothing and other textiles directly releases microfibers into the environment which are then transported and deposited by atmospheric processes (Dris et al., 2016). A recent study demonstrated that in the span of a year the number of microfibers released by an individual to the environment by laundering is comparable to the number they will release into the air, suggesting that the contribution of atmospheric deposition of microfibers is underestimated (De Falco et al., 2020).

### ***Ecosystem Implications***

Although the environmental impacts of microfibers generally remain poorly understood, they may impair feeding and respiration of filter-feeding organisms due to their tendency to

accumulate on digestive and respiratory tissues, and are suspected to play a role in the dispersal of contaminants throughout aquatic systems (Scherer et al., 2018; Zambrano et al., 2019). The small size and high surface-to-volume ratio of microfibers makes them easily ingestible by a range of organisms, particularly filter-feeders (Dris et al., 2018). In addition, the shape of microfibers makes them more harmful than other microparticle contaminants (such as fragments or spheres), as they have the longest residence times in the guts of organisms (Au et al., 2015). Ingested microfibers may create digestive obstructions resulting in insufficient nutrient absorption and false sense of satiation (Cesa et al., 2017; Germanov et al., 2018), thereby disrupting feeding behaviours, which has been observed in both filter-feeding organisms and their predators (Besseling et al., 2013; Watts et al., 2015; Woods et al., 2018). As such, microfiber ingestion has been linked to weight loss, decrease in growth and reproduction rate, increase in mortality rate, and ultimately population decline of aquatic organisms (Besseling et al., 2013; Au et al., 2015; Jemec et al., 2016).

The ecological consequences of microfiber contamination are not limited to their accumulation on vital tissues and may also result from the bioaccumulation and biomagnification of the harmful chemicals they may be carrying (Ladewig et al., 2015; Rochman, 2016). Textiles, including plastic and non-plastic, are treated with various chemicals during manufacturing, including dyes and special coatings such as preserving resins and Polybrominated Diphenyl Ethers (PBDEs) or other flame retardants (Schreder & La Guardia, 2014; Miller et al., 2017; Xu et al., 2018). These chemicals often cause natural fibers such as cotton to take considerably longer to biodegrade in the environment and may also deliver harmful chemicals, such as carcinogenic PBDEs, to the environment (Schreder & La Guardia, 2014; Miller et al., 2017; Xu et al., 2018).

Additionally, microfibers may become vectors of other harmful contaminants within wastewater which may sorb to them (McCormick et al., 2014; Brennecke et al., 2016; Dris et al., 2018; Li et al., 2018). Plastic microfibers are known to sorb antibiotics (Li et al., 2018), bacterial pathogens (McCormick et al., 2014), heavy metals (Brennecke et al., 2016), and persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Mendoza & Jones, 2015). In contrast, little information exists as to which specific agents may sorb to non-plastic fibers, however a study by Ladewig et al. (2015) concluded that non-plastic microfibers do indeed sorb harmful chemicals. As the molecular composition of different fibers will determine how they interact with different chemicals, certain pollutants may

sorb more readily to natural fibers (Grancaric et al., 2005; Ladewig et al., 2015). It has also been suggested that chemicals sorbed to natural microfibers (which are susceptible to digestion) may enter food webs more readily than those sorbed to plastic fibers, which may pass through an organism without being released (Ladewig et al., 2015; Zhao et al., 2016).

Freshwater mussels are filter-feeding organisms and as such may be threatened by microfiber contamination in the same way as other aquatic filter-feeders. Microparticles captured from the water column may become trapped on or within their tissues, including on their gills and within their digestive system (Scherer et al., 2018). Although few studies exist regarding microparticle contamination in freshwater bivalves, there have been various studies conducted on marine bivalves, which may provide insight into the effects this form of contamination may be having on freshwater mussels (Li et al., 2016; Su et al., 2018; Woods et al., 2018; Li, J. et al., 2019). Ingestion of microparticles in marine mussels has been linked to decreases in filtration rates, growth and reproduction rates, and viability of offspring, and may result in population declines (Worm et al., 2017; Woods et al., 2018). Woods et al. (2018) also observed increased pseudofeces (captured material which is expelled prior to digestion) production in blue mussels (*Mytilus edulis* L.) exposed to elevated microfiber concentrations.

### ***Freshwater Mussels***

Freshwater mussels are macroinvertebrates belonging to the class Bivalvia and are considered of great ecological importance due in part to their ability to filter large volumes of water (Martel et al., 2010). Although present around the world, the greatest diversity of freshwater mussel species exists in North America (Martel et al., 2010). However, over the last century, 35 of North America's native mussel species were believed to have become extinct, and almost 75% are considered to be endangered, threatened, or of special concern, largely as a result of habitat loss and degradation (Natural Resources Conservation Service [NRCS], 2007; Martel et al., 2010). In addition to their capacity to filter and purify water, freshwater mussels are an important food source for a variety of aquatic and terrestrial species, making them foundational to food webs (Martel et al., 2010). Adult freshwater mussels have a host of predators, including various turtle

and fish species, as well as otters, minks, raccoons, and most notably, muskrats, which are their primary predators (Hanson et al., 1989; Martel et al., 2010).

Freshwater mussels are ubiquitous, relatively easy to sample, and directly interact with their environment as they filter large volumes of water daily, particularly during the summer months when their feeding intensifies (Goodsell et al., 2009; Martel et al., 2010). Contaminant levels within mussels are often reflective of surrounding environmental conditions due to their tendency to concentrate certain contaminants in their tissues, as well as their longevity and relatively sessile habit (Van Hassel & Ferris, 2007; Waykar & Deshmukh, 2012). As such, freshwater mussels and other bivalves are often used as bioindicators of environmental pollutants such as trace heavy metals (Van Hassel & Ferris, 2007; Waykar & Deshmukh, 2012; Rzymiski et al., 2014). Likewise, several characteristics of mussels may make them well suited as bioindicators of microparticle contamination, however their usefulness and reliability for this purpose is currently contested, as they are capable of discriminating among particles they filter and rejecting some as pseudofeces, creating a possible bias (Goodsell et al., 2009; Su et al., 2018; Li, J. et al., 2019; Ward et al., 2019). Additionally, unlike other contaminants, there is no current evidence that microparticles larger than 10- $\mu\text{m}$  are capable of translocating into mussel tissues at a cellular level, making the longevity of mussels irrelevant to their usefulness in this application (Browne et al., 2008).

Freshwater mussels may be a potential pathway for microfibers, and sorbed contaminants, to transfer to higher trophic levels. Of the microparticles found within a mussel, many will be located within the digestive organs, although significant microparticle adherence has also been observed on the mantle, foot, gills, adductor muscles, gonads, and visceral mass (Kolandhasamy et al., 2018). This demonstrates that microparticle uptake in mussels may occur both through ingestion as well as through adherence to other tissues, which, unlike ingestion, is not affected by the selective feeding capabilities of mussels (Kolandhasamy et al., 2018; Ward et al., 2019). This also suggests that it may be more useful to measure microparticles within the whole organism, rather than isolating the digestive organs, particularly when assessing trophic transfer of contaminants.

## ***Rationale***

This study aimed to determine if freshwater mussels act as a potential pathway for microfibers to enter and move through the food web, as well as to assess the viability of freshwater mussels as bioindicators of microfiber contamination in the environment. Although poorly understood, it is becoming increasingly clear that microfibers have the potential to adversely affect biota as well as ecosystem health (Scherer et al., 2018; Zambrano et al., 2019). As such, there is an increasing need to acquire a clearer understanding of this emerging stressor in urban and more remote watersheds, as well as its impact on key aquatic organisms. Furthermore, identifying useful and reliable bioindicators will be invaluable to monitoring efforts moving forward with respect to microfiber contamination.

The specific questions this study addressed were: 1) Are freshwater mussels within two tributaries of the Saint John River watershed acquiring microfibers on or within their tissues? and 2) Is there spatial variability in microfiber uptake by freshwater mussels? If so, does it relate to WWTP discharge volumes or other diffuse sources of microfibers? To address these questions, microfiber abundance was analyzed in freshwater mussels (*Margaritifera margaritifera* L.) collected from strategic locations along each river, including up and downstream from WWTP discharge points. The results of this study will be useful in assessing the scope of microfiber contamination within the Saint John River watershed (eastern North America's second largest watershed) and may reveal a potential pathway for microfibers to move through this important freshwater system to higher trophic levels.

## **METHODS**

### ***Study Sites***

#### *Kennebecasis River*

The Kennebecasis River is located within the Saint John River watershed in rural southern New Brunswick. With its headwaters originating from Hamilton Lake, near Mechanic Settlement

(Figs. 1, 2), it flows for approximately 95 km before joining the Saint John River just north of the city of Saint John. The substrate within this system varies and ranges from large cobble to fine silt in many locations, based on observations collected at each sampling location. Industries within the watershed include fish hatcheries, an abandoned potash mine, sawmills, and agriculture (which comprises 17% of the land area) (Kennebecasis Watershed Restoration Committee [KWRC], 2017). Most municipalities within the Kennebecasis River watershed have populations between 500 and 1,000 (Millar et al., 2019), although Sussex, which is the largest community located along the ~18 km of river that was sampled for this study, had a population of 5,298 as of 2016 (Statistics Canada, 2017b).

Six WWTPs discharge into the Kennebecasis, however only the upper three were sampled as a result of the marine influence toward the lower reaches of the river. This influence prevented sampling from taking place in the lower Kennebecasis, as site conditions (including flow direction and appropriate mussel habitat) are inconsistent with sites further upstream. As such, a total of eight sites were sampled on the Kennebecasis River, including two intermediate sites, and three upstream and downstream WWTP sites. The upper three WWTPs include the Sussex, Apohaqui, and Norton facilities, which are located within about 20 km of one another and differ in terms of discharge volume (Table 1), with the Sussex facility discharging the largest volume daily. Due to the amount and range of land use in the watershed and the number of WWTPs discharging into it, the Kennebecasis River was considered the more impacted of the two study rivers.

### *Tobique River*

The Tobique River is located within the Saint John River watershed in remote northwestern New Brunswick. Originating near Mount Carleton, it is approximately 148 km in length and enters the Saint John River just north of Perth-Andover (Figs. 1, 3). Most municipalities within the watershed have fewer than 500 residents (Millar et al., 2019), and the largest community along the river is Plaster Rock, which had a population of 1,023 as of 2016 (Statistics Canada, 2017a). Substrate along the Tobique primarily ranges from larger rocks to coarse cobble, based on observations collected at each sampling location. Sampling was unable to extend to the mouth of the river as a result of the presence of the Tobique Narrows Dam and its reservoir, which alter the

water flow to the point where samples would not have been comparable to those collected from other sites along the river. In total, 14 sites were sampled along the Tobique across ~82 km, including ten intermediate sites, and two upstream and downstream WWTP sites.

The Tobique was considered less impacted than the Kennebecasis as only two WWTPs discharge into it and it runs through relatively remote forested areas for most of its length. Additionally, silt accumulation in the river substrate was observed far less in the Tobique than in the Kennebecasis. Both WWTP facilities, Everett and Wapske, service the rural community of Plaster Rock, and discharge roughly equal volumes per day (Table 1).

## ***Sample Collection and Processing***

### *Field Sampling*

Freshwater mussels, of the species *M. margaritifera*, were collected (DFO licence # 354349) as they were the most abundant in both rivers and are a widespread species throughout the Saint John River watershed (Martel et al., 2010). All specimens were collected over a 4-week period (June 11 – July 10, 2019) following the spring freshet. At WWTP sites, the effluent pipe was first located to ensure sampling was conducted an equal distance (20 m) up and downstream from the discharge point. When possible, specimens downstream were collected from directly within the effluent plume. Intermediate sites, which capture more diffuse sources of microfibers, were determined prior to field sampling, in an attempt to sample at even intervals along the length of each river (Figs. 2, 3).

During fieldwork, cotton clothing was worn exclusively to reduce the risk of sample contamination. When possible, five mussels of approximately the same size were collected within a 5-m radius to ensure they were exposed to comparable environmental conditions, however this varied based on mussel abundance at each site. Specimens were located after surveying using an underwater viewing tool, and the desired species (*M. margaritifera*) was identified prior to disturbing, when possible, based on aperture characteristics and lateral compression (Nedeau et al., 2000). Identification was later confirmed by the distinct ventral curve present in *M.*

*margaritifera* and then later in the lab by internal features. Collected specimens were individually packaged in Whirl-Pak® bags placed into a cooler on ice for transport to the lab where they were then transferred to a freezer.

### *Digestion*

White cotton lab coats were worn at all times when working with samples. Glass apparatus were used instead of plastic whenever possible. All glassware and other apparatus were thoroughly rinsed three times with DI water before use and between samples to prevent cross contamination. All nearby work surfaces were cleaned with 100% ethanol prior to use. Specimens remained frozen until processed, at which time they were removed from the freezer and allowed to thaw for between 15 to 30 minutes, depending on the size of the mussel. Once thawed enough to be pried open, the species of each specimen was confirmed, and their length (longest axis of shell), width (perpendicular to length axis), and total mass were recorded. Shells were pried open using a clean slotted screwdriver, which was then used to scrape soft tissue into a clean beaker to be weighed. Any material remaining within the Whirl-Pak® bag was also rinsed into the beaker as it was assumed to have been adhered to or expelled by the specimen during transport. Samples were then covered with aluminum foil, and only uncovered when necessary and for as little time as possible. Soft tissue was submerged in a 10% KOH solution of approximately triple the volume of the tissue as per Rochman et al. (2017) and digested for 24 hours on a 60°C hotplate. This method is known to effectively digest organic material without degrading plastic particles (Dehaut et al., 2016). Following digestion, beakers containing digested tissue were submerged in an ultrasonic cleaner run for ~3 minutes in order to break up large chunks of remaining tissue. The solution was then poured onto a 90-µm sieve (thus determining the lower size limit of visual detection) and rinsed thoroughly with DI water before being backwashed into the beaker and re-covered. Sieving was done as efficiently as possible (< 2 minutes) to minimize interior contamination while still being thorough. Remaining tissue was re-submerged in 10% KOH and digested on the 60°C hotplate for an additional 24 hours, after which each sample underwent the same ultrasonic bath and sieving process as it had after the first treatment. One blank sample was run for every batch of five mussel samples processed (i.e., one blank per sample site) in order to account for potential interior sources

of laboratory microfiber contamination, which are considered unavoidable (Wesch et al., 2017). Blank samples were prepared in clean beakers with DI water and were not treated with KOH, however each was sieved and rinsed in the same manner and for the same duration as the corresponding mussel samples.

### *Visual Inspection and Microfiber Classification*

Processed and sieved samples were poured into clean glass petri dishes and examined under Zeiss Stemi 508 dissecting microscopes between 12.5× and 50× magnification. Each sample was inspected twice before being discarded to catch any particles that may have been missed in the first pass through. Particles were classified by colour and morphology following the protocol outlined in Rochman et al. (2019). Although microfibers were the focus of this study, all microparticles found within samples were identified and collected. Microparticles within each sample and blank were identified using visual inspection based on morphology and differences from observed biological material. Colouration and shininess are often considered to be indicative of non-biological origin (Fries et al., 2013). Blue microparticles, in particular, are almost certain to be of anthropogenic origin due to the lack of blue pigment in nature (Newsome et al., 2014). Conversely, fragility may indicate a biological origin, and microparticles which powdered or broke when touched with forceps were not counted (Masura et al., 2015). As the absence of three-dimensional bending in fibers may indicate biological origin, entirely straight fibers were closely inspected before counting (Norén, 2007). Although the chemical composition of each particle could not be confirmed with these visual methods, similar studies have found that about 50% of collected microfibers are composed of plastic (Miller et al., 2017). Once identified, microparticles were removed using forceps to prevent double counting and mounted onto glass slides using clear double-sided tape for long-term storage.

## *Data Analysis*

Prior to all analysis, microparticle counts were adjusted (or “corrected”) to account for potential contamination arising from the lab procedures. As a blank was run for each site, or for every five mussels processed, each blank was assumed to account for laboratory contamination that each of the associated samples was exposed to during processing. This correction was performed by categorically subtracting the microparticle counts (categorized by morphology and colour) within each blank from the counts of each associated sample as per the procedure outlined by Rochman et al. (2019). Following blank correction, it was determined that microparticles other than microfibers (such as fragments, spheres, pellets/nurdles, films or foams) were effectively negligible as they accounted for less than 1.7% of observed microparticles. As such, all further analysis was carried out excluding the non-fiber microparticles.

As with similar studies, mussel microfiber abundance was reported per gram of soft tissue (Su et al., 2018). To verify that soft tissue mass was an appropriate metric by which to standardize microfiber counts for all further analysis, the Pearson coefficient of correlation was calculated between various mussel size metrics to determine their relationship to each other. This included testing the correlation between the shell length and width, shell length and soft tissue mass, shell width and soft tissue mass, and soft tissue mass and total mass (combined soft tissue and shell mass). Thus, instead of comparing average microfiber abundance per mussel at each site, comparisons were made between the average microfiber abundance per gram of soft tissue at a given site.

Comparisons of microfiber abundance were made between the two rivers as well as between site types: intermediate, upstream WWTP, and downstream WWTP. The normality of each dataset was tested using the Shapiro-Wilk test. The comparison of standardized mussel microfiber abundance between the two rivers was conducted with the non-parametric Wilcoxon rank sum test via the statistical software R (version 3.5.1). A Welch two sample t-test was conducted to compare the mussel sizes between the two rivers. To determine whether there were differences in microfiber abundance per gram of soft tissue between mussels from different site types, a non-parametric Kruskal-Wallis rank sum test was used, and pairwise comparisons (with

Bonferroni-corrected p-values) were conducted using the Wilcoxon rank sum test to determine which site types differed significantly ( $p < 0.05$ ).

To assess the relationship between mussel size and microfiber abundance per gram of soft tissue, the highly skewed (+ 4.27) mussel microfiber abundance data required  $\log_{10}(x + 1)$  transformation to satisfy the linearity assumption of the Pearson test. The Pearson correlation coefficient ( $R$ ) and coefficient of determination ( $R^2$ ) were then calculated using the transformed data, and a scatterplot was constructed so the pattern could be examined visually. Finally, general trends from upstream to downstream in mussel microfiber abundance and colour were examined visually for each river.

## RESULTS

Non-fiber particles, including fragments, films, pellets/nurdles, spheres, and foams, accounted for less than 1.7% of the microparticles observed, and were thus excluded for all further analyses. Microfibers were found within all but 9 of the 110 mussels collected. Of the 92% of mussels that contained microfibers, the microfiber abundance ranged from 2 – 29 microfibers / individual and 1 – 163 microfibers / individual in the Kennebecasis and Tobique, respectively. Microfiber abundance also varied once standardized by soft tissue mass, ranging from 0.0 to 0.6 microfibers / g soft tissue in the Kennebecasis and from 0.0 to 10.9 microfibers / g soft tissue in the Tobique.

Positive correlations were observed between shell width and length ( $R = 0.96$ ,  $p < 0.01$ ), and shell length and soft tissue mass ( $R = 0.96$ ,  $p < 0.01$ ) (Figs. 4, 5). Shell width and soft tissue mass were also positively correlated ( $R = 0.93$ ,  $p < 0.01$ ), as was soft tissue mass and total mass ( $R = 0.99$ ,  $p < 0.01$ ), supporting the use of soft tissue mass as a size reference for all further analysis. A negative correlation ( $R = -0.47$ ,  $p < 0.01$ ) was observed between the  $\log_{10}(x + 1)$  transformed mussel microfiber abundance and mussel size (Fig. 6).

## *Spatial Distribution of Microfiber Abundance and Colours*

### *Kennebecasis River*

Blue fibers accounted for the largest proportion of microfibers at each of the eight sites along the Kennebecasis River, ranging from 43% to 83% (Fig. 7). Black and red microfibers were frequently found at most sites, whereas clear microfibers ranged from being entirely absent at three sites to the second most abundant colour at Apohaqui-DS and Coyote-Intermediate (Fig. 7). There were no significant ( $\chi^2 = 2.04$ ,  $df = 2$ ,  $p = 0.36$ ) differences in mussel microfiber abundance among site types (e.g., intermediate, upstream WWTP, downstream WWTP) on the Kennebecasis River (Fig. 8). Of the three WWTP sites sampled, microfiber abundance was higher in downstream mussels compared with upstream mussels only at the Norton WWTP (Fig. 7). In general, there was little variation in microfiber abundance across the Kennebecasis sites, with average microfiber abundance in mussels ranging from  $0.08 (\pm 0.07)$  –  $0.26 (\pm 0.09)$  microfibers / g soft tissue (Fig. 7). Additionally, no general trend in microfiber abundance was observed from upstream to downstream within the ~18 km of river sampled (Fig. 7).

### *Tobique River*

Blue was among the most abundant microfiber colours observed, accounting for more than 20% of the fibers present at all but two of the 14 sites sampled along the Tobique River (Fig. 9). Clear microfibers were also consistently in high abundance at all but two sites along the Tobique and accounted for as much as 75% at Three-Brooks-Intermediate (Fig. 9). Other microfiber colours found in high abundance along the Tobique River included black and red fibers (Fig. 9). A significant ( $\chi^2 = 8.97$ ,  $df = 2$ ,  $p < 0.05$ ) difference was found among the site types on the Tobique River, where microfiber abundance was higher at upstream WWTP sites when compared with intermediate sites (Fig. 10). Mussel microfiber abundance varied among the Tobique sites, ranging from  $0.01 (\pm 0.02)$  –  $5.03 (\pm 4.62)$  microfibers / g soft tissue (Fig. 9). Higher microfiber abundance in downstream mussels compared with upstream mussels was only observed at the Plaster Rock Everett WWTP. No trend in microfiber abundance from upstream to downstream was observed along the ~82 km of river sampled (Fig. 9).

## ***River Comparisons***

Across the study, mussel soft tissue mass ranged from 4.4 – 79.8 g (Fig. 11). This range was considerably smaller when looking at each river individually, where soft tissue masses ranged from 24.9 – 79.8 g in the Kennebecasis and 4.4 – 44.6 g in the Tobique (Fig. 11). A significant difference ( $t = -15.87$ ,  $df = 56.46$ ,  $p < 0.01$ ) in mussel mass was observed between the Kennebecasis and Tobique rivers, with larger mussels collected from the Kennebecasis (Fig. 11). Additionally, microfiber abundance was significantly ( $W = 742$ ,  $p < 0.01$ ) higher in mussels from the Tobique River compared to those from the Kennebecasis (Fig. 11).

## **DISCUSSION**

Broadly, the goal of this study was to assess microparticle contamination as an emerging environmental stressor within the Saint John River watershed through its presence within an ecologically important aquatic species. The presence of microfibers within freshwater mussels suggests that mussels may be a vector for microfibers and their associated contaminants to enter food webs. Results clearly indicate that mussels of the Saint John River watershed are indeed acquiring microfibers on or within their tissues, although they do not reflect the expected trends in microfiber exposure.

Prior to standardization by soft tissue mass, the abundance of microfibers observed within each mussel ranged from zero to 163. These results are similar to those of Berglund et al. (2019) which reported a range of four to 142 plastic microfibers / individual in freshwater duck mussels (*Anodonta anatina* (L., 1758)) from the Höje River in southern Sweden. The Höje watershed is more densely populated than either the Kennebecasis or Tobique watersheds, and receives urban and agricultural runoff, from areas occupying over 11% and 60% of the watershed, respectively (Berndtsson & Bengtsson, 2006). Mussel microfiber abundances also varied once standardized, ranging narrowly in the Kennebecasis from 0.0 to 0.6 microfibers / g soft tissue and widely in the Tobique River from 0.0 to 10.9 microfibers / g soft tissue. In contrast, a study by Su et al. (2018) of microparticle (primarily microfiber) abundance observed between 0.3 and 4.9 items / g soft tissue in freshwater Asian clams (*Corbicula fluminalis* (Müller, 1774)) collected from the Yangtze

River in China. This system is considered highly impacted, having about one-third of China's population within its basin, and as such, exposure levels would be expected to be dramatically higher than those experienced by organisms in the more remote and less densely populated watersheds of the Kennebecasis or Tobique rivers. Despite this, microfiber abundances in mussels from the Kennebecasis and Tobique rivers were within the same order of magnitude as those observed by Su et al. (2018). It should be noted, however, that as much as 50% of the microfibers observed in mussels of the Kennebecasis and Tobique rivers may be non-plastic (Dris et al., 2016; Miller et al., 2017), whereas both Su et al. (2018) and Berglund et al. (2019) focused exclusively on plastic microparticles. This uncertainty highlights the need for future research to confirm the plastic or non-plastic origin of microfibers.

### ***Spatial Distribution and Trends***

As WWTPs are recognized as one of the largest contributors of microfibers to the environment despite high removal rates (Murphy et al., 2016; Ziajahromi et al., 2017; Xu et al., 2018), microfiber abundances were expected to be highest in mussels downstream from discharge points compared to upstream or intermediate sites. However, trends in ambient microfibers (sampled from water and sediments) between the sampling sites were not reflected in the mussels (Labaj, A., pers. comm., 2020; LeBlanc, A., pers. comm., 2020). Although the expected trend in microfiber abundances downstream from WWTPs was observed in the surrounding water column (LeBlanc, A., pers. comm., 2020), and also generally in the sediment (Labaj, A., pers. comm., 2020), mussels sourced downstream from WWTPs did not have higher microfiber abundances than those observed at upstream or intermediate sites (Figs. 7, 9). Instead, only mussels from intermediate and upstream WWTP sites were found to differ significantly ( $p < 0.05$ ) in microfiber abundance, and only on the Tobique River (Fig. 10). Due to the unidirectional water flow at all study sites, the difference in microfiber abundance observed between upstream WWTP and intermediate sites along the Tobique cannot be explained by the proximity of upstream mussels to WWTP discharge points. Additionally, as this trend was not reflected in mussels along the Kennebecasis, where microfiber abundances were observed to not differ among site types (Fig. 8), this further suggests that mussel microfiber abundance does not reflect ambient levels in water or

sediments. These findings contradict those of other studies which have observed that mussels will contain more microfibers where exposure is higher (Li, L., et al., 2019).

Microfiber abundance was also expected to increase downstream on each river, due to greater microfiber exposure, as contaminants delivered by various point source and diffuse inputs along the length of each river may accumulate downstream. However, based on preliminary data collected by A. Labaj (pers. comm., 2020) and A. LeBlanc (pers. comm., 2020), this expected trend was not detected either in the surrounding sediments or the surrounding water column and suggests losses or dilutions of microfibers occur that cannot be explained by sample locations. As such, microfiber abundance was not higher in mussels sourced from downstream sites compared to those upstream, even at three of the five WWTP locations (Figs. 7, 9).

Higher microfiber exposure was expected in the Kennebecasis compared to the Tobique River, given the greater number of WWTPs discharging into it, and the higher levels of development and greater population size within its watershed (KWRC, 2017). Although neither river is considered highly impacted and most of each watershed is remote or rural, the Kennebecasis has more land-use change and agriculture within its watershed and passes through larger communities (KWRC, 2017). Despite this, microfiber abundance was found to be significantly ( $p < 0.01$ ) higher within mussels of the Tobique River (Fig. 11).

A possible explanation as to why mussel microfiber abundance may not reflect ambient environmental concentrations is the ability of mussels to eject unwanted particles, such as microfibers, as pseudofeces through selective feeding (Ward et al., 2019). This would remove microfibers from the body of the organism before they enter the digestive tract, thereby reducing their residence time within the body (Ward et al., 2019). Another possible explanation is that particle uptake in mussels may be affected by factors other than ambient environmental concentrations. Factors such as water flow rate (Newell et al., 2001), temperature and acidity (Loayza-Muro & Elías-Letts, 2007), food availability (Roper & Hickey, 1995), and organism morphology (Wagner, 1976; Jones et al., 1992) have been found to influence filtration rates, which could result in inconsistencies in microfiber abundance across sites. Clearly, complex relationships between exposure and contaminant levels can be observed in filter-feeding organisms such as freshwater mussels, especially those sampled directly from the field.

## ***Size, Filtration Rates, and Microfiber Abundance***

The negative correlation found between mussel size and microfiber abundance suggests that smaller mussels contained more microfibers per gram of soft tissue than larger mussels (Fig. 6). It is possible that this correlation could, in part, explain the higher microfiber abundance observed in mussels from the Tobique, as they were significantly ( $p < 0.01$ ) smaller than those collected from the Kennebecasis (Fig. 11). While these results differ from the observations of Berglund et al. (2019) in duck mussels, they are supported by a number of studies conducted on various marine bivalves which indicate that smaller individuals have higher filtration rates than larger individuals per gram of tissue (Fox et al., 1937; Jørgensen, 1943; Rice & Smith, 1962). This relationship between size and filtration rate has also been observed in a freshwater mussel species (*Elliptio complanata* (Lightfoot, 1786)) which frequently co-occurs with *M. margaritifera* throughout the Saint John River watershed (Wagner 1976; Martel et al., 2010). If small mussels are filtering larger quantities of water by weight, then it follows that they will capture and accumulate more microfibers per gram of tissue than larger organisms. One possible explanation for higher filtration in small individuals is that small bivalves have more gill surface area per gram of tissue than their larger counterparts (Rice & Smith, 1962). Another potential explanation is that smaller mussels may have a higher metabolic rate as they may be growing more rapidly than large mussels and therefore need to filter-feed more rapidly (Wagner, 1976). In addition to differing filtration rates, it is possible that larger mussels are more successful or more efficient at ejecting unwanted particles in the form of pseudofeces, which could account for the lower microfiber abundances observed in larger individuals. Since standardizing microfiber abundance by soft tissue mass, as was done in this study, operates on the assumption that larger mussels will accumulate proportionally more microfibers than their smaller counterparts, these results indicate that it may be necessary to also take into account differences in filtration rates in order to most accurately standardize microfiber abundances between individuals.

## ***Conclusion***

Freshwater mussels, of a common and widespread species, in two tributaries of the Saint John River watershed are acquiring microfibers within their soft tissues, as demonstrated by the

observation of microfiber contamination within >90% of mussels in this study. This indicates that microfiber contamination is present within these relatively unimpacted watersheds of New Brunswick and potential exists for these novel contaminants to interact with the health of aquatic organisms. While variability in microfiber uptake by freshwater mussels was observed, it did not relate to WWTP discharge volumes, as was expected, nor did it reflect ambient trends in microfiber contamination levels. Future studies should focus on acquiring a clearer understanding of the interaction between freshwater mussels and microfiber contamination, factors which may influence mussel filtration rates, and the extent to which microfibers and associated contaminants are being delivered to higher trophic levels via the predation of freshwater mussels. Despite its ubiquitous nature, microfiber contamination remains poorly understood, making the need for further laboratory and field research apparent.

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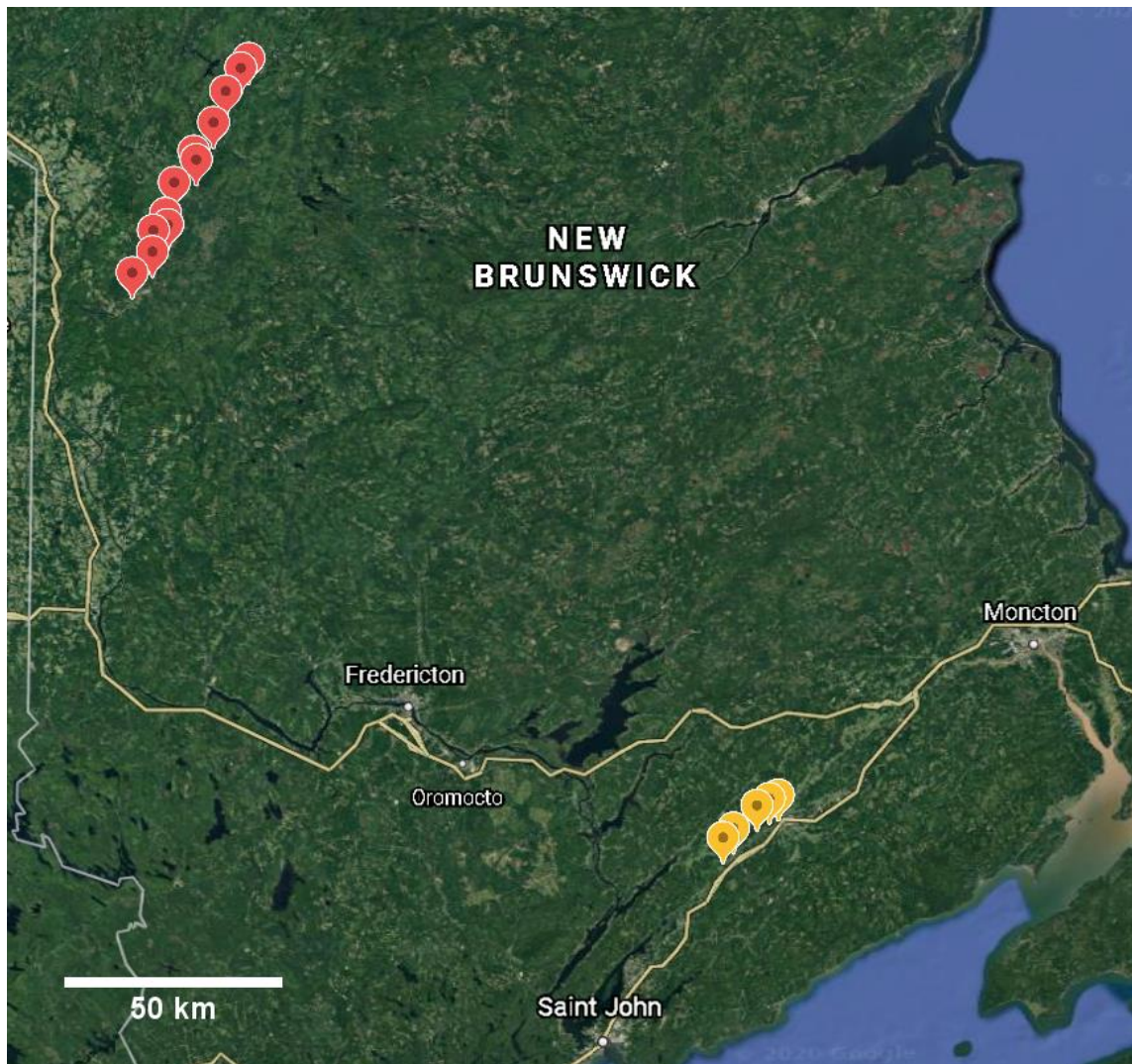
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## TABLES

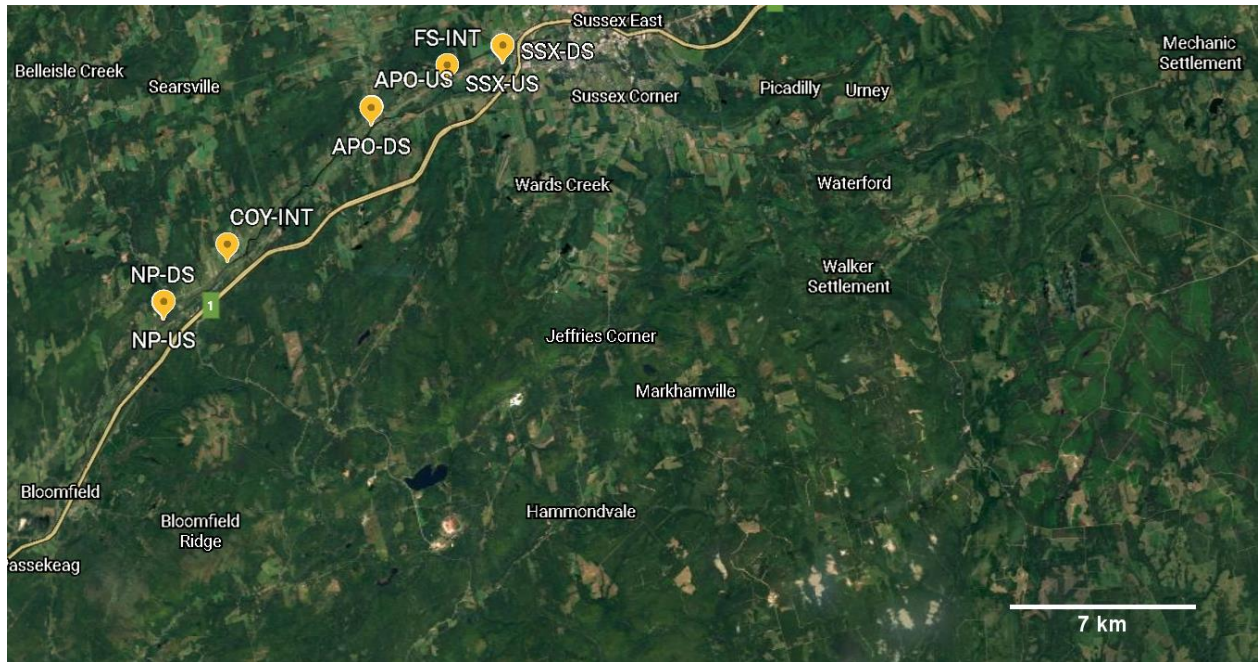
**Table 1.** Discharge volumes and locations of wastewater treatment plants (WWTPs) sampled along the Kennebecasis and Tobique rivers (WWTP discharge information provided by T. Leblanc, Department of Environment and Local Government, pers. comm., 2017).

WWTP	River	Latitude	Longitude	Discharge volume (m <sup>3</sup> /day)
Sussex	Kennebecasis	45.7201	-65.5414	5953
Apohaqui	Kennebecasis	45.6993	-65.6044	146
Norton	Kennebecasis	45.6338	-65.7039	15
Everett (Plaster Rock)	Tobique	46.9165	-67.3896	955
Wapske (Plaster Rock)	Tobique	46.8939	-67.3824	955

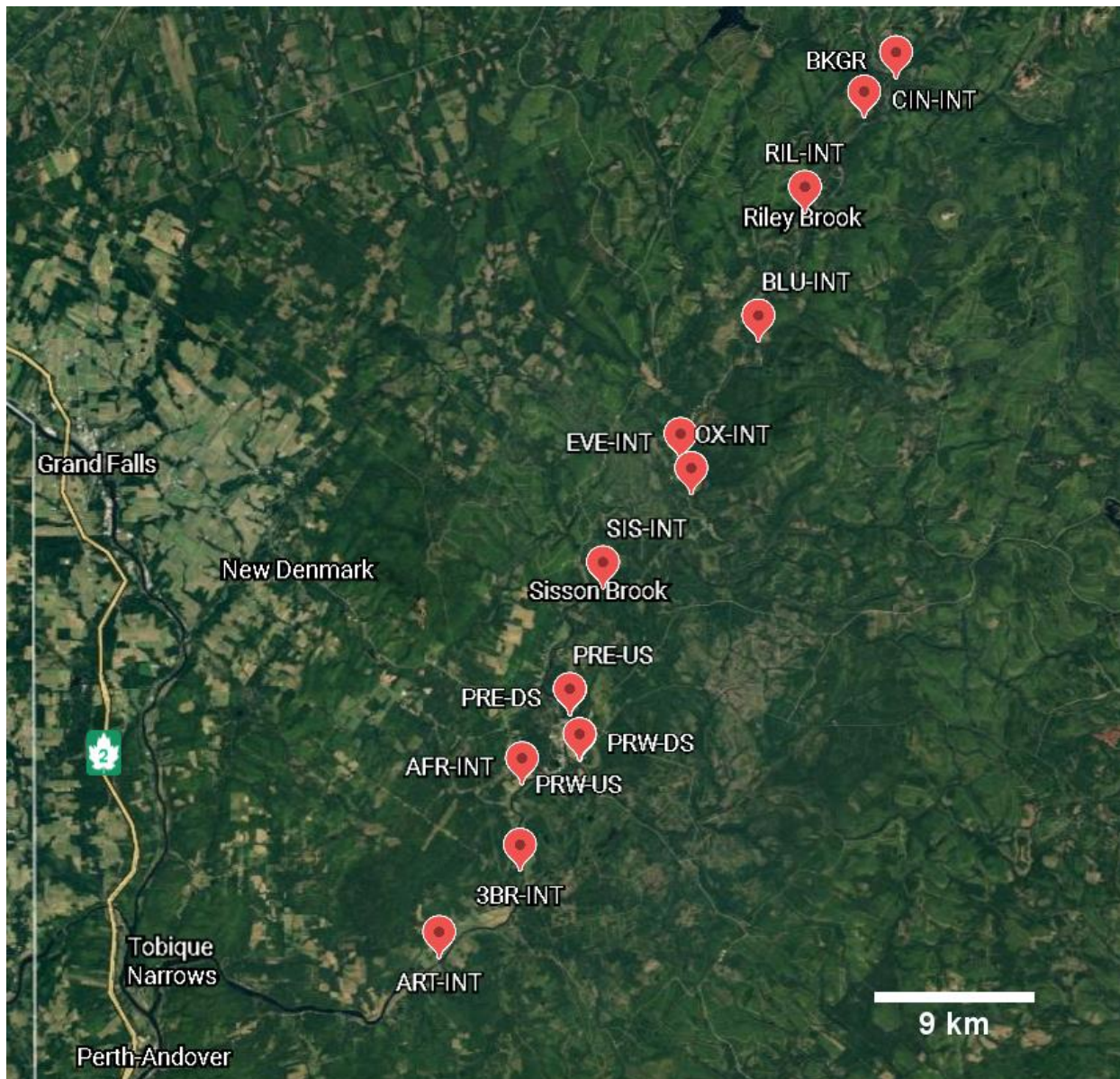
## FIGURES



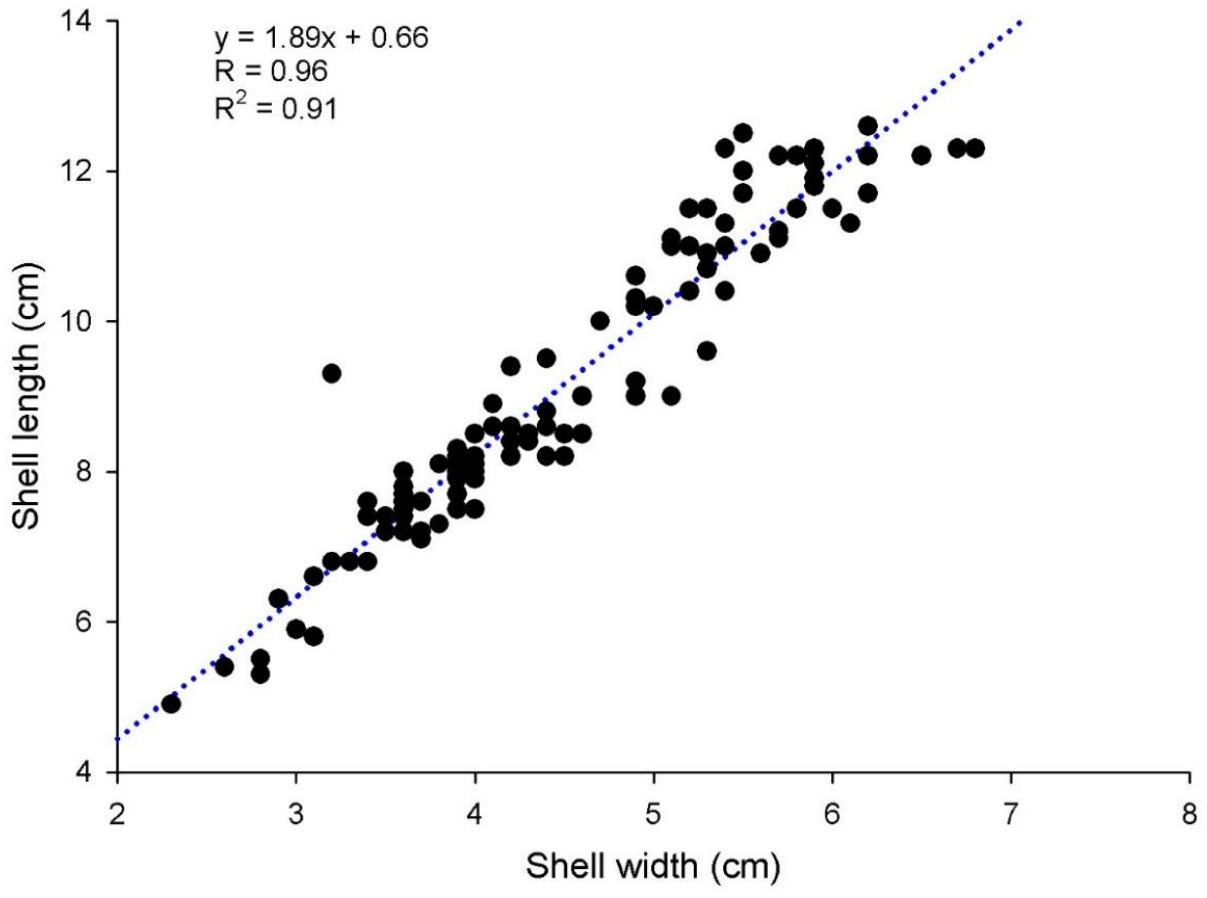
**Figure 1.** Map of study sites along Tobique (red markers) and Kennebecasis (yellow markers) rivers in New Brunswick. Map imagery from Google Earth.



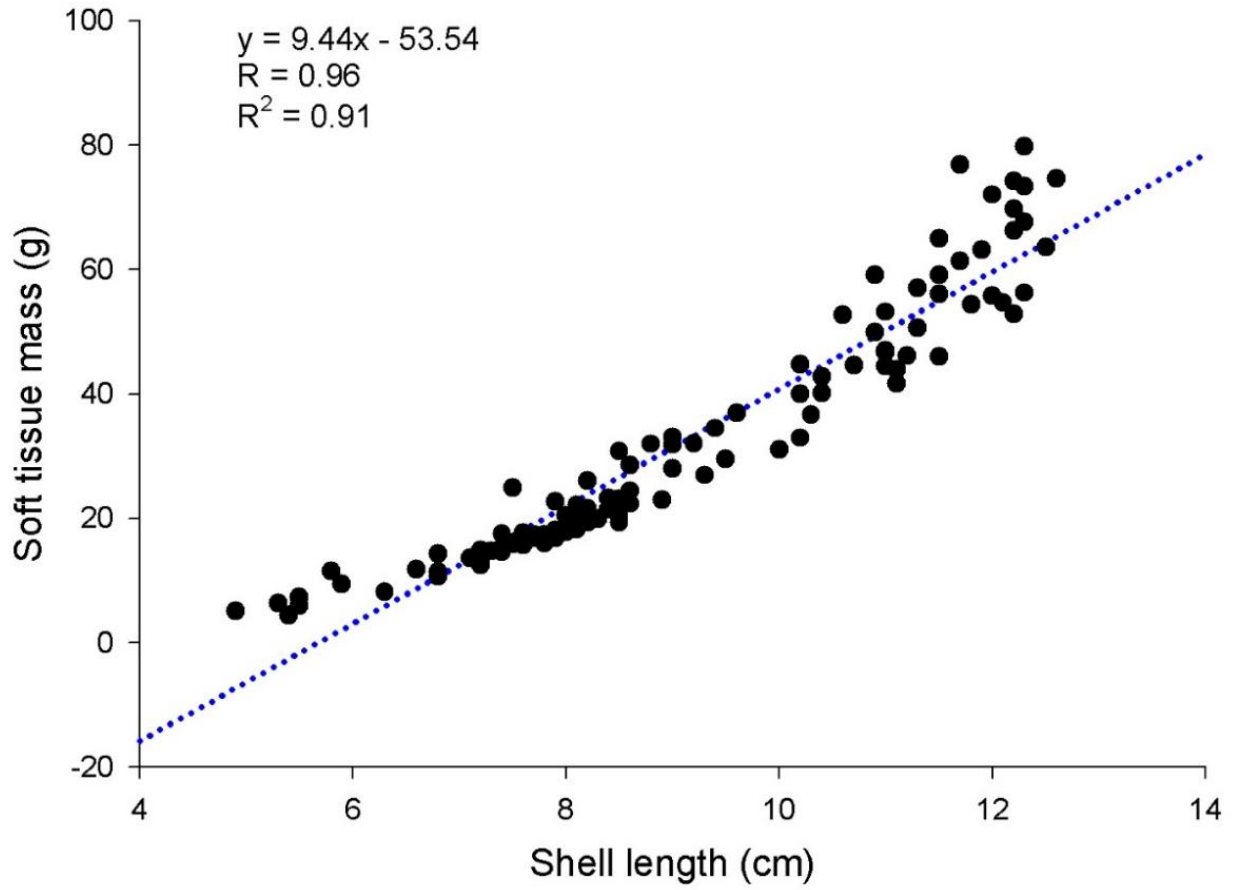
**Figure 2.** Map of sampled section of Kennebecasis River. Study sites are indicated by markers and site codes (Appendix 1). Map imagery from Google Earth.



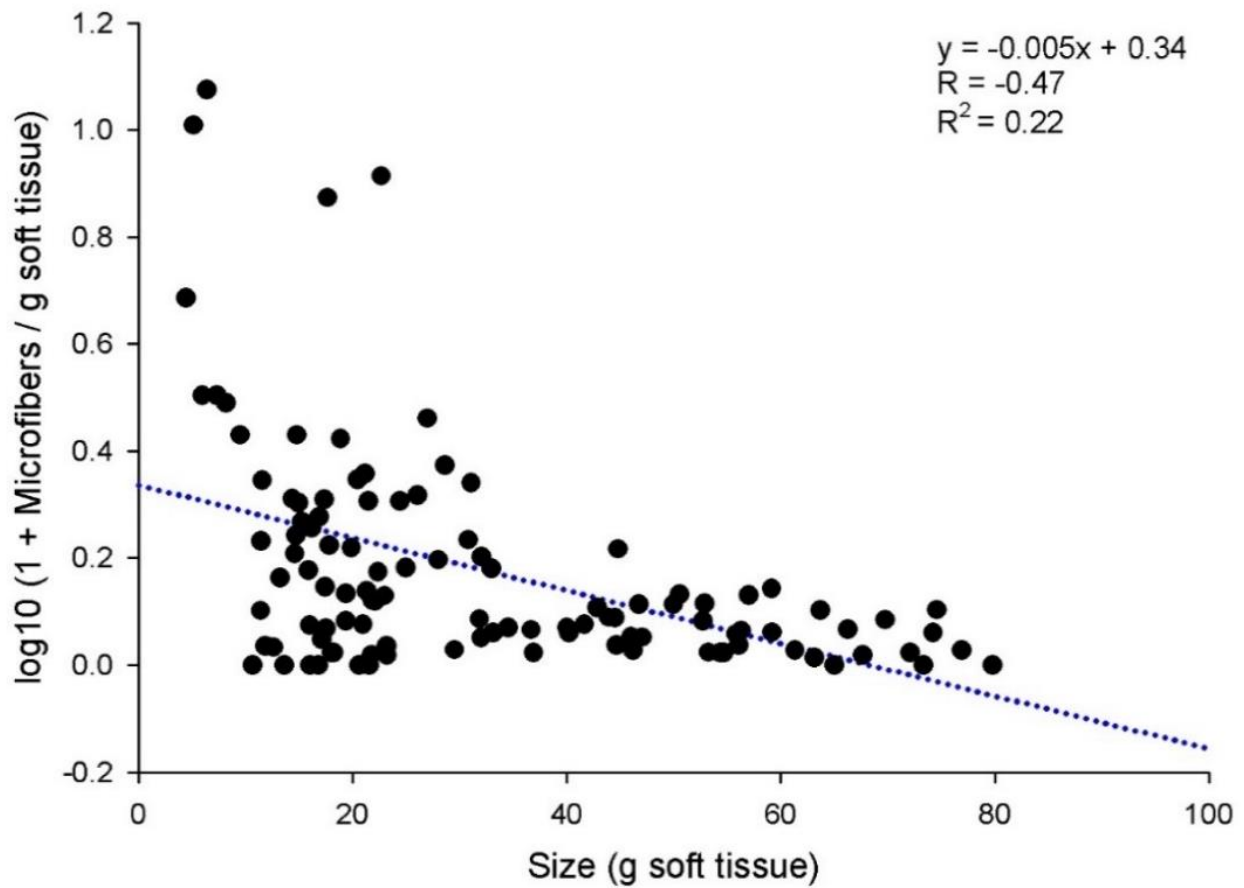
**Figure 3.** Map of Tobique River. Study sites are indicated by markers and site codes (Appendix 1). Map imagery from Google Earth.



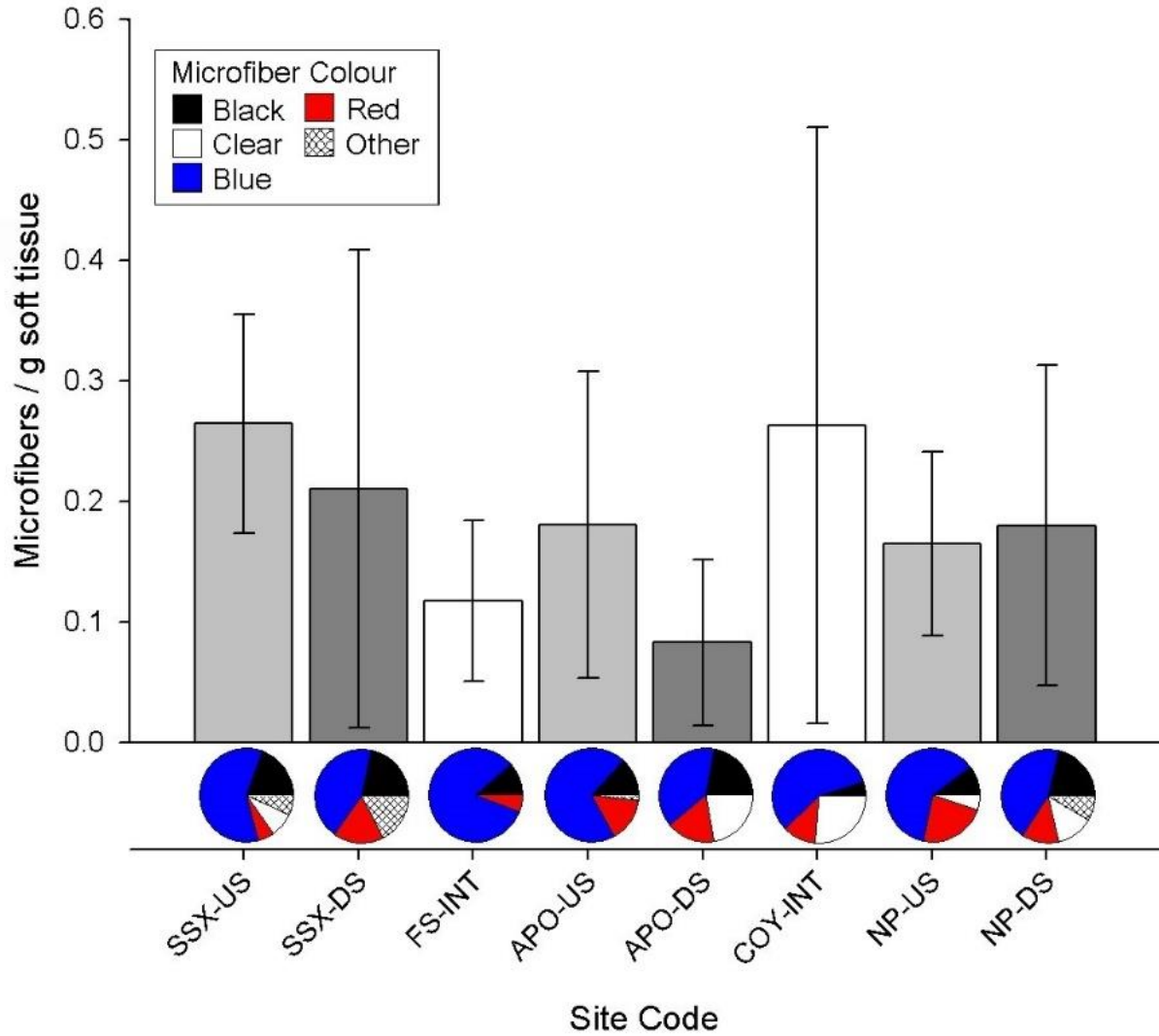
**Figure 4.** Plot of mussel shell width and length for all mussels analyzed. Trend line (blue) depicts the positive correlation between variables.



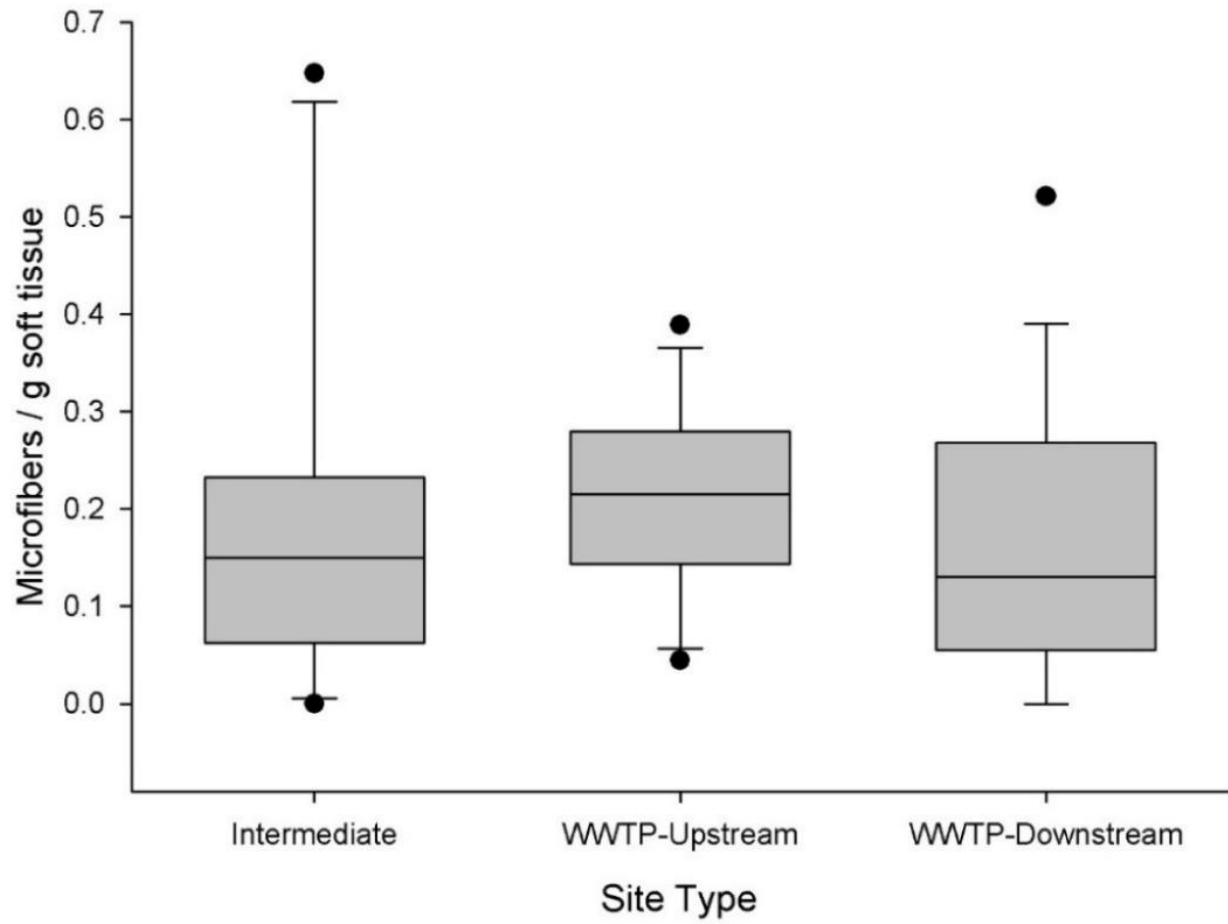
**Figure 5.** Plot of mussel shell length and soft tissue mass for all mussels analyzed. Trend line (blue) depicts the positive correlation between variables.



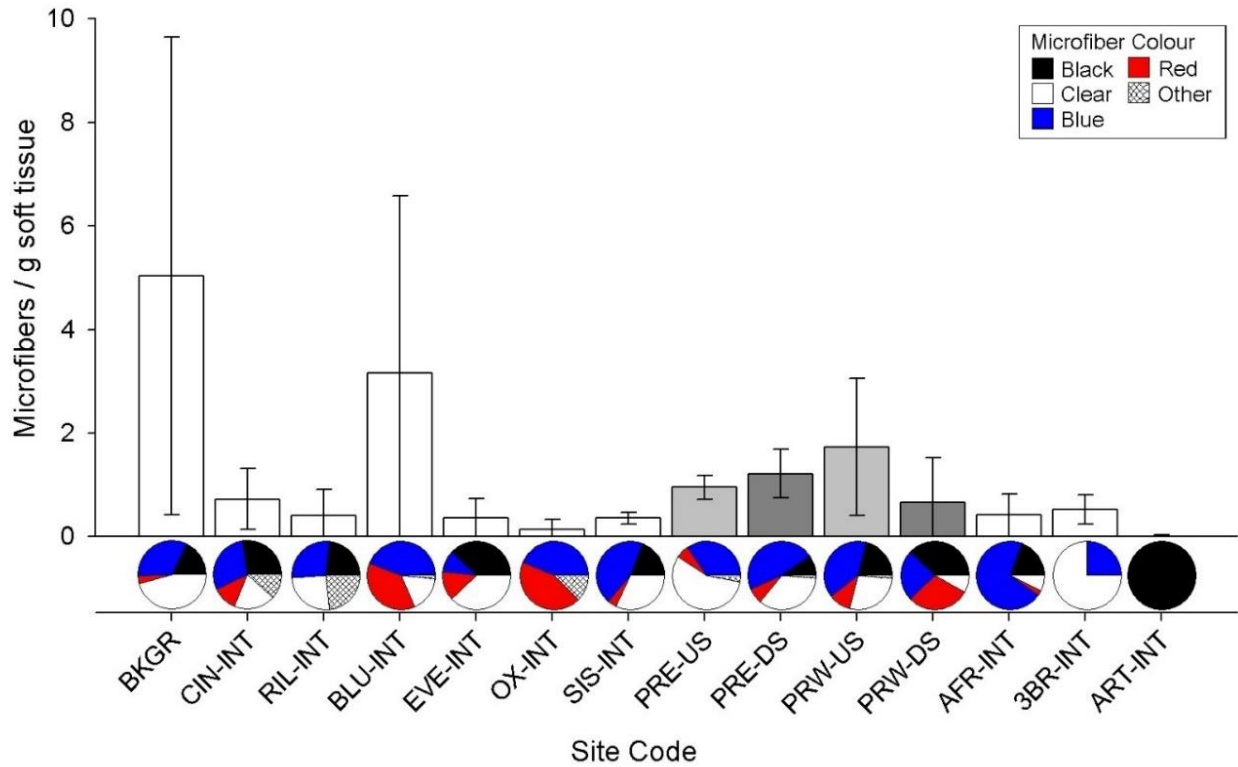
**Figure 6.** Plot of log-transformed mussel microfiber abundance [ $\log_{10}(1 + \text{microfibers} / \text{g soft tissue})$ ] and mussel size (by soft tissue mass) for all mussels analyzed in this study. Trend line (blue) depicts the negative correlation between variables.



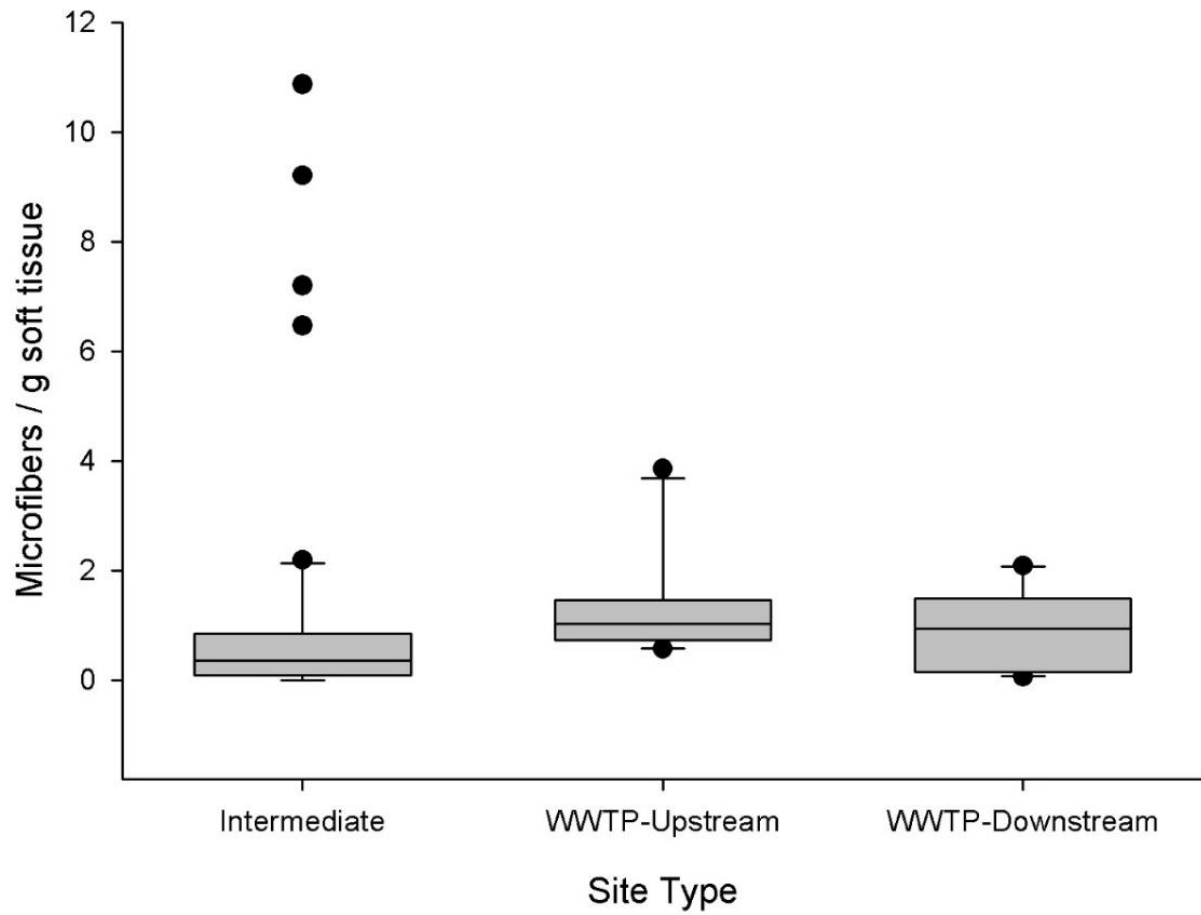
**Figure 7.** Spatial distribution of microfiber abundance per gram soft tissue at sites (intermediate – white; upstream WWTP – light grey; downstream WWTP – dark grey) along the Kennebecasis River ordered from upstream to downstream (left to right). Pie graphs below each bar indicate the distribution of microfiber colour present at each site.



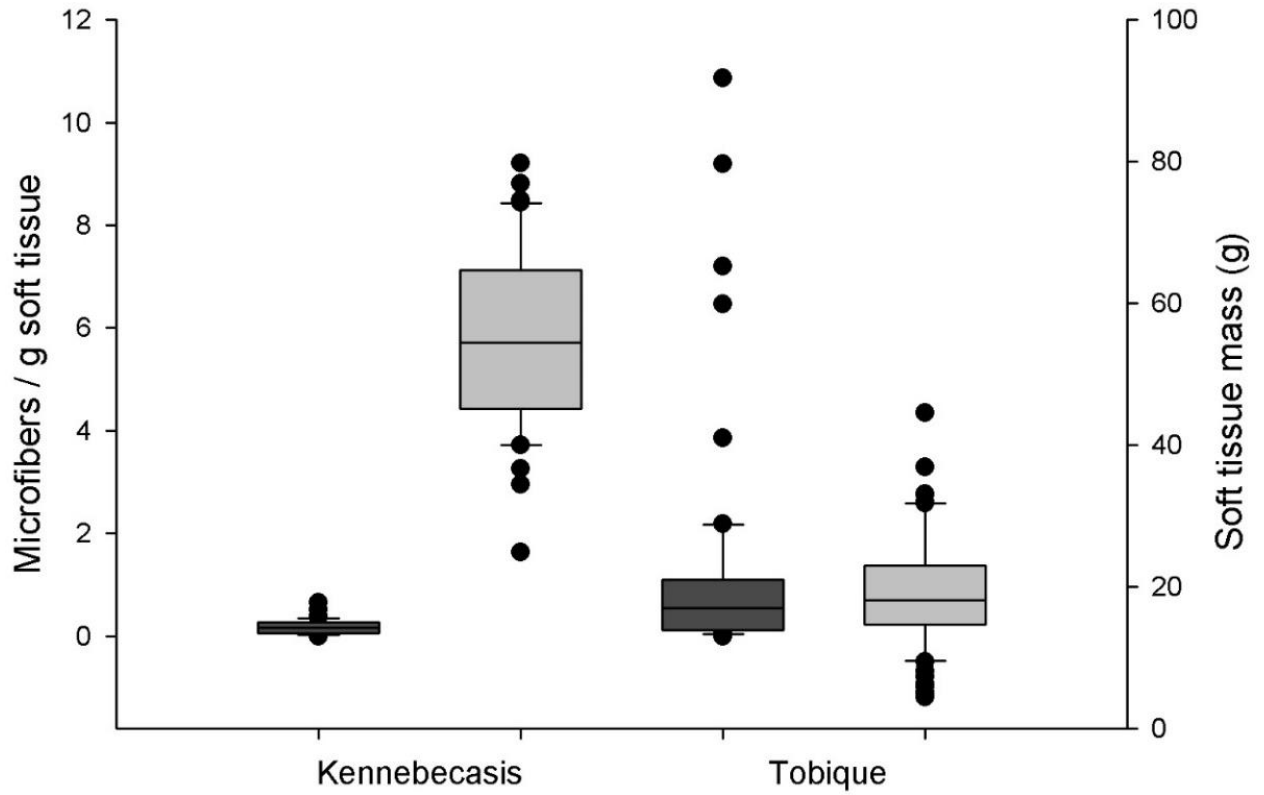
**Figure 8.** Comparison of microfiber abundance per gram soft tissue between intermediate, upstream WWTP, and downstream WWTP sites on the Kennebecasis River.



**Figure 9.** Spatial distribution of microfiber abundance per gram soft tissue at sites (intermediate – white; upstream WWTP – light grey; downstream WWTP – dark grey) along the Tobique River ordered from upstream to downstream (left to right). Pie graphs below each bar indicate the distribution of microfiber colour present at each site.



**Figure 10.** Comparison of microfiber abundance per gram soft tissue between intermediate, upstream WWTP, and downstream WWTP sites on the Tobique River.



**Figure 11.** Comparison of mussel microfiber abundance per gram soft tissue (primary axis; dark grey) and mussel size (secondary axis; light grey) between Kennebecasis and Tobique rivers.

## APPENDICES

### Appendix 1. Study sites.

Site Code	Site Name	River	Latitude	Longitude
SSX-US	Sussex-Upstream (WWTP)	Kennebecasis	45.72037	-65.5414
SSX-DS	Sussex-Downstream (WWTP)	Kennebecasis	45.72018	-65.5418
FS-INT	Farm Supply Intermediate	Kennebecasis	45.71360	-65.5683
APO-US	Apohaqui-Upstream (WWTP)	Kennebecasis	45.69944	-65.6049
APO-DS	Apohaqui-Downstream (WWTP)	Kennebecasis	45.69916	-65.6052
COY-INT	Coyote Intermediate	Kennebecasis	45.65282	-65.6749
NP-US	Norton Pipe-Upstream (WWTP)	Kennebecasis	45.63338	-65.7057
NP-DS	Norton Pipe-Downstream (WWTP)	Kennebecasis	45.63327	-65.7062
BKGR	Background Salmon Barrier	Tobique	47.24051	-67.1436
CIN-INT	Cinderblock Farm Intermediate	Tobique	47.22047	-67.1677
RIL-INT	Riley Brook Intermediate	Tobique	47.17203	-67.2125
BLU-INT	Blue Mountain Intermediate	Tobique	47.10658	-67.2479
EVE-INT	Everett Landing Intermediate	Tobique	47.04642	-67.3064
OX-INT	Oxbow Intermediate	Tobique	47.02899	-67.2985
SIS-INT	Sisson Brook Intermediate	Tobique	46.98117	-67.3646
PRE-US	Plaster Rock Everett-Upstream (WWTP)	Tobique	46.91671	-67.3896
PRE-DS	Plaster Rock Everett-Downstream (WWTP)	Tobique	46.91643	-67.3894
PRW-US	Plaster Rock Wapske-Upstream (WWTP)	Tobique	46.89368	-67.3823
PRW-DS	Plaster Rock Wapske-Downstream (WWTP)	Tobique	46.89346	-67.3819
AFR-INT	A-Frame Intermediate	Tobique	46.88148	-67.4253
3BR-INT	Three Brooks Intermediate	Tobique	46.83724	-67.4267
ART-INT	Arthurette Bridge Intermediate	Tobique	46.79286	-67.4870

### Appendix 2. Microfibers (blue and red) and undigested mussel tissue.

