

Prenatal Fluoride Exposure and Neurodevelopmental Outcomes in a National Birth Cohort

Rivka Green

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements
for the Degree of Master of Arts

Graduate Program in Psychology
York University
Toronto, Ontario

July 2018

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Abstract

Background: The potential neurotoxicity of exposure to fluoride, which has sparked controversy about community water fluoridation, is poorly understood.

Objective: To test the association between prenatal fluoride exposure and childhood IQ in 512 Canadian mother-child pairs.

Methods: We measured fetal exposure to fluoride using: (a) maternal urinary fluoride (MUF) during pregnancy; (b) fluoride concentration in water; and (c) fluoride intake estimated from beverage consumption. We evaluated children's IQ using the Wechsler Primary and Preschool Scale of Intelligence-III. Multiple linear regression analyses examined covariate-adjusted associations between fluoride predictors and IQ.

Results: Higher MUF levels predicted lower IQ in males ($B=-4.49, p=.02$) but not females. Higher levels of water fluoride and fluoride intake predicted a main effect of diminished IQ.

Conclusion: Exposure to fluoride during fetal development is associated with lower IQ scores. These findings, which suggest that fluoride is neurotoxic, underscore the need to critically evaluate the practice of water fluoridation.

Acknowledgements

First and foremost, thank you to my supervisor, Dr. Christine Till, for your constant support and encouragement throughout my MA career. You have provided me with the immense privilege of contributing to your NIH-funded project and have given me the opportunity to use it for my thesis. Thank you for allowing me to operate as the data manager on this protected dataset, and to participate on numerous aspects of the project, including Knowledge Translation. Thank you for bringing me along to international meetings, and for entrusting me to deliver oral conference presentations on this work. Your passion and enthusiasm for this research has sparked my own passion and excitement, and your never-ending knowledge pursuit has inspired me to reach for my potential and to never stop learning. Thank you for cultivating such a positive and nurturing lab environment and for providing me with many educational and professional opportunities that I would not have had otherwise so early on in my career.

Thank you to our NIH-project collaborators for all of your invaluable contributions, and for always welcoming my analyses and opinions. To Dr. Bruce Lanphear, thank you for your continuous positive feedback, encouragement, and inspiration. Your fierce and genuine commitment to this field has instilled in me an obligation to protect children on multi-national levels and to never give up the pursuit. To Dr. Rick Hornung, thank you for teaching me how to handle large-scale epidemiological datasets, and for never treating any statistical question as too basic. Thank you to both Drs. Lanphear and Hornung for visiting York University and for many, many, many stimulating and thought-provoking discussions. Thank you also to Dr. Martinez-Mier, Dr. Ayotte, and Dr. Muckle for your advice, guidance, and time on this project. I would be remiss not to mention Raichel Neufeld who meticulously collected the water data that was used in this project and Dr. John Grundy for all of his statistical support — a huge thank you.

To Dr. David Flora, thank you for your positive and constructive feedback regarding this thesis and other projects more generally. Your statistical approach had a great impact on how I conceptualized this project. I feel so privileged to have had such brilliant and esteemed researchers as my committee members – Drs. Debra Pepler and Hala Tamim, thank you for your stimulating questions and critical insight.

To my fellow “fluoride” colleagues who have paved the way – Ashley, you are an unbelievable mentor and your passion never ceases to inspire me; Julia, thank you for your countless impromptu debriefing sessions and pep talks. To my lab-mates: Elisea, your presence exudes positive energy, and I thank you for bringing wellness into my life! Emily, thank you for being an amazing source to bounce ideas off of and for always giving me reassurance. Tracy, thank you for being so supportive; your willingness and enthusiasm for all of our research projects is inspiring. Thank you to my incredible cohort! Your relentless support is unparalleled, and I always look forward to our profound conversations, both in and out of the classroom.

I would not be writing this today without the unwavering support of my entire family. Jesse, your commitment to my graduate career is unrelenting. You welcome every one of my opportunities with affirmation (even the out of town ones!) and celebrate my accomplishments as if they were your own. Orly and Jacob, thank you for giving me so much joy — and for letting me share the role of motherhood with my graduate studies! To my extended family, thank you for being there for my children when I couldn't, and for your constant support and encouragement. Specifically, Ima, words cannot express my appreciation for all of the unearned help that you have given me over the past two years, and for the innumerable presentations that you have not only listened to, but also provided instrumental feedback to. I feel tremendously grateful to all of you.

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List of Abbreviations

ADHD: Attention-deficit/hyperactivity disorder
ADA: American Dental Association
BMI: Pre-pregnancy body mass index
BPA: Bisphenol-A
CDC: Centers for Disease Control and Prevention
CNS: Central nervous system
CRE: Creatinine
CWF: Community water fluoridation
DNA: Deoxyribonucleic acid
EPA: Environmental Protection Agency
FC: The geometric mean of fluoride concentration added to the woman's respective WTP averaged across her pregnancy
FI: The geometric mean of fluoride concentration added to the woman's respective WTP averaged across her pregnancy multiplied by the number of water or water-based cups consumed per day to a litre scale and incorporating the amount of fluoride estimated from green and black tea to a litre scale
FSIQ: Full-Scale IQ
GFR: Glomerular filtration rate
HOME: *Home Observation for Measurement of the Environment (HOME) - Revised Edition*
IQ: Intelligence quotient
MIREC: Maternal-Infant Research on Environmental Chemicals cohort
MCL: Maximum contaminant level
MLR: Multiple linear regression
MUF: Maternal urinary fluoride
MUF_{SG}: Maternal urinary fluoride adjusted for specific gravity and averaged across three trimesters of pregnancy
NTP: National Toxicology Program
NRC: National Research Council
PCB: Polychlorinated biphenyl
PFOA: Perfluorooctanoic acid
PIQ: Performance IQ
PPM: Parts per million
SG: Specific gravity
VIF: Variance Inflation Factor
VIQ: Verbal IQ
WPPSI-III: *Wechsler Preschool and Primary Scale of Intelligence, Third Edition*
WTP: Water treatment plant

Background

Community water fluoridation (CWF) is the controlled addition of fluoridation chemicals to municipal drinking water for the purpose of preventing dental caries (cavities). This practice raises the naturally occurring fluoride level to the level of 0.7 mg/L, which is considered the optimal level to provide a balance of protecting dental caries while limiting the risk of fluorosis (discolouration of tooth enamel) (U.S. Department of Health, 2015). Concerns about the safety of CWF have been raised because the developing brain is vulnerable to the interruption of neurotoxins. Currently, the potential neurotoxic effects of fluoride during brain development are unclear, especially for exposure levels that are typical to the North American population. Because of the lack of high quality studies in this area, a report published by the National Research Council (NRC) in 2006 concluded that more research is warranted to examine the impact of early-life exposure to fluoride, such as during the prenatal period, and child neurodevelopmental outcomes. The overall aim of this project is to test the potential neurotoxicity of low-level, chronic exposure to fluoride during pregnancy.

Susceptibility of the prenatal period

The prenatal period has been identified as being more vulnerable to the adverse consequences of neurotoxins as compared to other developmental periods (Rauh & Margolis, 2016; Lanphear, 2015; Faustman, Silbernagel, Fenske, Burbacher, & Ponce, 2000; Landrigan, Kimmel, Correa, & Eskenazi, 2004). There are many basic biological reasons as to why the prenatal period is more vulnerable than other stages of life (Rauh & Margolis 2016). First, fetuses have higher exposures to chemicals in the environment due to their higher metabolic rate, which means that they are exposed to higher concentrations of chemicals per pound of body weight as compared to both children and adults. In addition to their faster metabolic rate, fetuses

are experiencing periods of rapid growth, especially in the central nervous system (CNS), and these developmental processes are accompanied by windows of plasticity and vulnerability (Faustman et al., 2000). Not only are growing cells more vulnerable to toxins, but plasticity makes the bodily organs, including the brain, particularly sensitive to chemical influences. In addition, the fetal brain is especially vulnerable to stress hormones and neurotoxins because the blood-brain barrier, which protects the brain from unwanted chemicals, is not fully formed prenatally; thus, it is more permeable to toxins than the mature brain. Lastly, fetuses have immature metabolic pathways and are missing certain enzymes that metabolize and excrete environmental toxicants, making it more difficult to rid the body of toxicity.

Additionally, there is a host of research that focuses on how fetal environmental exposures can lead to epigenetic regulation, causing long-term effects on gene expression, thereby leaving an increased risk of disease (Barouki, Gluckman, Grandjean, Hanson, & Heindel, 2012). Therefore, consequences as a result to toxic exposures in early life may have a lifetime influence that cannot be undone (Rauh & Margolis, 2016).

The mechanisms by which specific neurotoxins affect the developing brain have also been well established, allowing scientists to identify which developmental periods are the most susceptible to which toxins (Lanphear, 2015). Toxins, such as mercury, can cause cell death and alter cell migration and proliferation, and because these processes occur rapidly during fetal development, mercury likely has its greatest effect *in utero* (Rodier, 1995; Rice & Barone, 2000). This was the unfortunate consequence of the Minamata Disaster, when thousands of children suffered from a congenital neurological syndrome caused by maternal ingestion of mercury during pregnancy. Toxins, like lead, disrupt neurotransmission, synaptogenesis, and synaptic trimming, which are all crucial during early childhood. Of all the toxins, lead has been suggested

to pose the most serious threat to young children, in part because of its impact on multiple neurodevelopmental processes (Rodier, 1995; Rice & Barone, 2000; Schneider, Huang, & Vemuri, 2003). For example, in the 1970s, lead-based paints caused many children to suffer from acute lead poisoning when they ingested paint fragments during play. Some environmental toxins, like polychlorinated biphenyl (PCBs) and other pesticides, disrupt certain hormones, such as estrogenic hormones and thyroid hormones (Braun et al., 2012; Chevrier et al., 2012), thereby affecting their circuits and function. Lastly, other toxins, such as arsenic, tobacco, and diethylstilbestrol can alter epigenomic expression by modifying gene expression without changing DNA sequences (Baccarelli & Bollati, 2009; Pilsner et al., 2009).

Since early developmental periods, including the prenatal period, are highly susceptible windows to environmental exposures, the G8 Environmental Minister Meeting in 2009 encouraged more research on children's environmental health (World Health Organization (WHO), 2010). Much of the contributing research on children's environmental health has come from multiple prospective birth cohort studies examining the relationship between environmental exposures in early life and childhood health outcomes. Acknowledging that fetuses are more susceptible to toxic influences, and recognizing that these toxins can have a lifelong impact on brain function (Lanphear, 2015), it is vital to identify neurotoxins that children are exposed to in an effort to mitigate adverse health consequences.

Developmental neurotoxins

There have been over a dozen industrial chemicals classified as developmental neurotoxins, such as lead, mercury, PCBs, arsenic, solvents, and other pesticides (Grandjean & Landrigan, 2006; 2014). Developmental neurotoxins pose an insidious threat to children, leaving them at a greater risk for many neurodevelopmental disorders, including learning disabilities,

attention-deficit/hyperactivity disorder (ADHD), and autism spectrum disorder – all of which are on the rise and are thought to have an underlying environmental component that contributes to their etiology. Grandjean (2013) coined the term *chemical brain drain*, which begins with observations of adult clinical toxicity supported by subsequent findings of child or fetal subclinical toxicity, which may have occurred at exposure levels previously thought to be safe. For example, lead used to be considered safe at a level of 0.7 mg/L, but it is now advised that there is no safe level for lead (Center for Disease Control and Prevention (CDC), 2018).

Since the developing brain is particularly vulnerable to toxins, low doses of neurotoxins that might not have an adverse effect on adults could interfere with neurodevelopment in critical periods of brain development. Therefore, early identification and recognition of potential neurotoxins is crucial to protect children from harm posed by environmental toxins.

Community Water Fluoridation in Canada

Community water fluoridation (CWF), a practice which is endorsed by many health organizations, including Health Canada and the World Health Organization, occurs in certain municipalities across Canada (Health Canada, 2010). Currently, approximately 38% of Canadians are receiving artificially fluoridated water (Firsten-Kaufman & Quinonez, 2017), a value that has decreased over time as more and more municipalities opt against compulsory water fluoridation due to ongoing controversy. Municipalities in Ontario, Alberta, and Manitoba have the highest rates of CWF, whereas Quebec now has no regions which practice CWF. Despite the support by Health Canada for CWF, this public health practice has declined considerably across Canada since it was introduced over 60 years ago in 1945 in Brantford, Ontario at a time when fluoridated dental products were not yet mainstream.

Protection of CWF against dental decay

Although multiple studies have confirmed the benefits of water fluoridation for the prevention of dental caries (U.S. Department of Health, 2001), a recent Cochrane review (Iheozor-Ejiofor et al., 2015) concluded that the methodological quality of these studies is modest and may not reflect a contemporary lifestyle. This review identified 155 studies looking at CWF and caries prevention, and found many limitations, including the highly observational nature of the studies, high-risk of bias, and minimal applicability to current lifestyles. For example, currently over 95% of toothpastes contain fluoride – a major source of fluoride for caries prevention; yet, 71% of the reviewed studies were published prior to 1975, before the introduction of fluoridated dental products. Thus, Iheozor-Ejiofor et al. concluded that there was insufficient contemporary evidence to determine whether water fluoridation results in improved caries prevention.

Recent studies, published after the introduction of fluoridated toothpastes, have found that tooth decay rates are declining to the same extent in non-fluoridated areas and fluoridated areas in Western countries (Cheng, Chalmers, & Sheldon, 2007; Pizzo, Piscopo, Pizzo, & Giuliana, 2007; Neurath, 2005; Colquhoun, 1997; Diesendorf, Colquhoun, Spittle, Everingham, & Clutterbuck, 1997; Bratthall, Hansel-Petersson, & Sundberg, 1996; Diesendorf, 1986). Additionally, it is widely accepted that fluoride's predominant effect on preventing tooth decay comes from topical application and not from systemic exposure (NRC, 2006; Fejerskov, 2004).

Sources of fluoride exposure

There are many sources of fluoride in the environment, including drinking water, food, tea, beverages made with fluoridated water, fluoridated dental products, and naturally occurring fluoride from soil (Hirzy, Connett, Xiang, Spittle, & Kennedy, 2016). Among adults, artificially

fluoridated drinking water accounts for approximately 61% of their daily source of fluoride, whereas for children aged one to 11 years of age, drinking water accounts for about 42% of their daily source of fluoride (for those consumers using the 90th percentile age-related water consumption estimates) (Table B-3; United States Environmental Protection Agency (EPA), 2010). However, for infants fed powdered formula reconstituted with fluoridated tap water, fluoride from drinking water accounts for 71% of their total exposure (United States EPA, 2010). A recent study by our group found urinary fluoride levels in pregnant women living in fluoridated cities to be almost double the levels of women living in non-fluoridated cities (Till et al., *in revisions*), further supporting the idea that fluoridated water is a major source of fluoride exposure. Considering that the public is already exposed to fluoride through dental products and other naturally occurring fluoride sources (e.g. tea), the additional burden of fluoride exposure from the controlled addition of water fluoridation has attracted much attention, particularly among countries, such as Ireland, where most of the population drinks tea daily, and drinking water supplies are artificially fluoridated (Sutton, Kiersey, Farragher, & Long, 2015; Waugh, Potter, Limeback, & Godfrey, 2016).

According to Health Canada (2010), the recommended range for daily intake of fluoride – that is, to maximize protection against tooth decay while minimizing risk of fluorosis (considered an adverse health effect to too much fluoride ingestion early on in life) – is 50 to 70 µg/kg of body weight per day. However, this intake level is highly dependent on life stage, as a function of body size, and ingestion patterns, as a function of water consumption. Therefore, despite Health Canada’s recommendation, there is a tremendous variability of daily intake levels, resulting in certain groups getting a higher concentration per body weight per day, such as bottle-fed infants who ingest powdered formula mixed with fluoridated water who far exceed this

recommended level, with levels ranging from 80 to 120 µg/kg of body weight per day (EPA, 2010). Considering these variables, it is impossible to control one's dose of fluoride exposure from the tap, which is especially relevant for vulnerable populations, such as pregnant women.

Maternal-fetal transfer of fluoride

Human studies have shown that fluoride is transferred to the fetus from maternal ingestion of fluoride (Gedalia, Brzezinski, Portuguese, & Bercovici, 1964). Other studies have confirmed the passive diffusion of fluoride through the placenta (Ron, Singer, Menczei, & Kidroni, 1986; Montherrat-Carret et al., 1996). Moreover, studies suggest that when mothers are exposed to fluoride added to drinking water at greater than 0.7mg/L, fluoride uptake in tooth germs increases at least 10-fold (Gedalia, Zuckermann, & Leventhal, 1965; Gedalia, 1971; Blayney & Hill, 1964). Therefore, fetal exposure to fluoride depends on the dose of maternal ingestion. It is also important to note that although it was once thought that prenatal exposure to fluoride could be a beneficial method to prevent dental caries, this idea has been falsified (Takahasi et al., 2017) and fluoride's efficacy is predominantly effective post tooth eruption (Centers for Disease Control and Prevention (CDC), 2001). Thus, there is no benefit of prenatal fluoride exposure.

Neurotoxicity of fluoride

In 2014, fluoride was reported as a developmental neurotoxicant by experts in the field of developmental neurotoxicology (Grandjean & Landrigan, 2014). Although it is widely accepted that at high levels, fluoride is neurotoxic, concerns about the safety of CWF have been raised, including possible neurotoxic features at levels that are currently found in drinking water in North America (National Toxicology Program (NTP), 2016), sparking controversy with

proponents on both sides of the conundrum. A consensus has not been reached due to the dearth of high quality research studies examining human exposure to fluoride at levels typically found in North America. In light of the lack of recent data, there is an urgent need for studies on the potential detrimental effects of fluoride.

Neurotoxicity of fluoride: Animal studies

Animal studies, using various levels of fluoride exposure, have demonstrated that fluoride crosses the blood brain barrier and accumulates in brain tissues, including degeneration and neuronal deformations of the hippocampus, the central processor of memory (Bhatnagar, Rao, Jain, & Bhatnagar, 2002; Pereira et al., 2011; Gao, Liu, Wu, Long, & Guan, 2008). Fluoride has also been found to cause neurochemical changes in the brain, including decreases of certain receptors (Nabavi et al., 2013). These studies suggest that fluoride exposure can alter behaviour, affect learning and memory, and impact neurodevelopment, leading to cognitive deficits later in life (Mullenix, Denbesten, Schunior, & Kernan, 1995; Chioca, Raupp, Da Cunha, Losso, & Andreatini, 2008; Liu et al., 2014). Recently, a study looking at prenatal exposure to fluoride in rodents found histological changes in brain tissues suggesting a toxic effect of fluoride intake during early developmental stages (Guner, Uyar-Bozkurt, Haznedaroglu, & Menten, 2016).

While many of these studies used fluoride levels far greater than concentrations found in controlled drinking water, it is important to note that rodents require approximately five times more fluoride in their water to achieve the same level of fluoride in their blood as humans (NTP, 2016). Some studies found no effect of lower fluoride exposure levels of 2.26 to 4.52 ppm (or mg/L) (Zhu, Zheng, LV, Ma, & Zhang, 2012, Gao et al., 2008; Liu et al., 2014), others found learning deficits when the duration of levels of low fluoride exposure (0.9 - 2.26 ppm) was extended over a longer period of time (Liu, Gao, Wu, & Guan, 2010; Liu, Gao, Long, Yu, &

Guan, 2011; Liu et al., 2014; Dong, Wang, Wei, Zhang, & Guan, 2015; Niu, Sun, Wang, Cheng, & Wang, 2008; Chouhan, Lomash, & Flora, 2010; Wu, Zhao, Gao, & Li, 2008; Gao, Liu, Young, Huan, & Jin, 2009; Sandeep, Kavitha, Praveena, Sekhar, & Rao, 2013; Zhang, Xu, Shen, & Xu, 1999; Zhu, Zhang, & Zhang, 2011; Bhatnager et al., 2011; Banala & Karnati, 2015; Reddy et al., 2014; Lou et al., 2012; Lou, Guan, & Pei, 2014; Sun, Liu, Wu, Lu, & Yu, 2008; Han et al., 2014; Zhou, Luo, Wang, Niu, & Wang, 2014; Guner et al., 2016). Importantly, it is estimated that some children in high fluoridated areas receive the equivalent dosage of fluoride as rats drinking 0.9 ppm, the lower range of chronic fluoride levels administered to rodents in these studies (National Toxicology Program, 2016).

In a comprehensive review, the NTP recently concluded that there is insufficient laboratory evidence to support or refute the conclusion that low-level fluoride exposure is neurotoxic (NTP, 2016). Specifically, their systematic review found a low-to-moderate level of evidence for adverse effects on learning and memory in animals exposed to fluoride. Contrary to the human studies, the level of evidence was strongest (moderate) in animals exposed as adults (Gao, Liu, & Guan, 2009; Liu et al., 2010) and weaker in animals exposed during development (Liu et al., 2014; Du, 1992). To address the limitations of the studies published in this report, and to use exposure levels for rodents that approximate the Maximum Contaminant Level (4 ppm) for fluoride intake by humans, the NTP published a comprehensive study which found that while fluoride concentrations increased with age in the brain and bone, there were no exposure-related differences between the high fluoride dose group and low fluoride dose group in motor, sensory, or learning and memory performance (McPherson et al., 2018). Thus, the extant animal studies examining the neurotoxicity of fluoride offer mixed results.

Neurotoxicity of fluoride: Epidemiological studies

In 2006, the NRC affirmed that fluoride can interfere with brain function and that more studies were needed to determine the relationship between water fluoridation and cognitive developmental outcomes (NRC, 2006). Epidemiologic research has demonstrated that lowered IQ scores can occur in children with mild dental fluorosis (associated with excess fluoride intake), when children consumed water with fluoride concentrations that ranged from 0.75 mg/L (close to CWF levels found in North America) to 6.3 mg/L (Das & Mondal, 2016). This finding is especially concerning because the NRC has noted that 41% of American youth between the ages of 12 and 15 years demonstrated mild to severe dental fluorosis, despite being exposed to “optimal” levels of fluoridated water (NRC, 2006).

A meta-analysis of human epidemiological studies conducted in Eastern populations concluded that 26 of 27 studies found a negative relationship between fluoride exposure and children’s intelligence with a standardized weighted mean difference in IQ score of -0.45 between the exposed and reference groups (Choi, Sun, Zhang, & Grandjean, 2012). While many of the studies reviewed in this analysis included samples that were exposed to higher levels of fluoride than found in most parts of North America, 13 of the 18 waterborne fluoride studies included fluoride levels below 4 mg/L, considered the standard for the maximum contaminant level (MCL) goal by the EPA, or the level at which no adverse health effects are expected to occur. These studies had an average fluoride exposure of 2.3 mg/L and a range of 0.8 mg/L to 4.1 mg/L when including both groups.

Following Choi et al.’s (2012) review, several new studies have associated water fluoride levels that are less than 4 mg/L (0.7 mg/L - 3.9 mg/L) with reduced IQ (Sudhir, Chandu, Prashant, & Reddy, 2009; Zhang, Lou, & Guan, 2015; Das & Mondal, 2016; Choi et al., 2015;

Sebastian & Sunitha, 2015; Trivedi, Sangai, Patel, Payak, & Vyas, 2012; Khan et al., 2015; Nagarajappa et al., 2013; Seraj et al., 2012; Karimzade, Aghaei, & Mahvi, 2014). However, these studies are all ecological in nature, failing to control for relevant confounders, and are based on samples with varying levels of fluoride exposure, most of which exceed the recommended level for CWF. Furthermore, these studies reflect dental habits of Eastern populations, mostly Chinese, which may or may not generalize to Western populations. This difference is important to note when comparing Western populations to Asian populations because fluoridated product habits differ, and these practices can contribute to a child or mother's daily fluoride intake (Zohoori et al., 2013).

Of the Western population epidemiological studies, CWF has been linked to ADHD (Malin & Till, 2015) and hypothyroidism (Peckham, Lowery, & Spencer, 2015). In terms of prospective studies conducted, Broadbent et al. (2015) compared youth and adults in fluoridated versus non-fluoridated areas of New Zealand. While they found no effect on IQ, a large portion of the residents in the non-fluoridated areas used 0.5 mg/day fluoride tablets in addition to fluoridated toothpaste, and the exposures were not based on biomarker surveillance. Given the considerable exposure to fluoride through alternate sources in the “non-fluoridated” group, this study has since been criticized (Hirzy et al., 2016) because the expected difference between the “high” and “low” fluoride exposure groups was too small (less than 0.2 mg of fluoride per day) to detect a difference in IQ. Moreover, fluoride was not measured as a continuous measure with biomarkers, but instead relied on self-report measures asking about fluoride tablets and using residential status as a predictor (Broadbent et al., 2015). In addition to the aforementioned limitations, none of these studies had a prenatal component that objectively measured prenatal fluoride exposure.

A recent study, however, looked at maternal urinary fluoride as a biomarker for fluoride exposure in pregnant women in Mexico, and their children's IQ at age four and age six through 12 (Bashash et al., 2017). They found that an increase of 1 mg/L in maternal urinary fluoride predicted a lower General Cognitive Index and IQ score by 6.3 and five points among preschool-aged and school-aged children, respectively. While this study is the first of its kind to analyze prenatal fluoride exposure, and although it overcame many limitations in the literature thus far, it is limited by its smaller sample size ($N = 299$) relative to the current paper, and it fails to have urinary samples available at each trimester in order to reduce limitations associated with urinary spot samples and physiological changes throughout pregnancy. Further, the application of the results to a North American cohort have been questioned (American Dental Association (ADA), 2017) because the methods of ingestion differ (fluoride is added to the salt in Mexico as opposed to water fluoridation in North America), and their dental habits and socioeconomic status might not be comparable. To our knowledge, there is no longitudinal study that objectively measures prenatal fluoride exposure in drinking water and its association with children's neurodevelopmental outcomes with applicability to a North American sample.

Neurotoxicity of fluoride: Mechanisms

The mechanisms by which prenatal fluoride exposure affect the brain have been studied in humans with high fluoride exposure. Studies report that compared to aborted fetuses from areas of low fluoride exposure, aborted fetuses from high fluoride exposure areas corresponding with dental fluorosis and higher urinary fluoride values, had altered neurotransmitter and receptor changes (Yu et al., 1996; Dong, Wan, Zhang, & Liu, 1993). The aborted specimens from the high fluoride areas also contained higher fluoride content in brain and bone tissue, swollen mitochondria in the nerve cells, expanded granular endoplasmic reticula, damage to the

nuclear envelope, a lower number of synapses, fewer mitochondria, microtubules and vesicles within the synapses, and damage to the synaptic membrane; all consistent with fluoride slowing the growth and division of neurons and lessening the connections between neurons (He, Cheng, & Liu, 2008; Du, 1992). It is important to note that these aborted fetuses came from areas with endemic fluorosis with women having mean urinary fluoride levels greater than 4.0 mg/L, and currently, the mechanisms by which fluoride at low levels could affect the developing brain remain speculative.

Rationale

Approximately 38% of Canadians and nearly 75% of Americans are supplied with artificially fluoridated drinking water. Fluoride is listed as an “emerging neurotoxic substance” (Grandjean & Landrigan, 2014) that needs further in-depth studies, especially for exposures that occur during early brain development. Past human studies have associated higher levels of fluoride with lower IQ, reduced attention and working memory, and increased risk of developing ADHD. However, methodological concerns related to these studies reduce the quality of the evidence, and are coupled with many of the studies coming from areas with water fluoride levels that are much higher than what is found in North America. Given the widespread exposure to fluoridated water among millions of individuals, rigorous epidemiological research is urgently needed to address the current controversy about the safety of water fluoridation, with a particular emphasis on vulnerable populations.

Aims and Hypotheses

The overall goal of this study is to prospectively examine the potential association of prenatal fluoride exposure and neurodevelopmental outcomes in young Canadian children. Our primary (internal) measure of fluoride exposure will consist of maternal urinary fluoride (MUF)

concentrations, a urinary biomarker derived by taking serial urine samples obtained during pregnancy. A secondary (external) measure of fluoride exposure will consist of the fluoride levels found in drinking water among pregnant women living in fluoridated versus non-fluoridated cities, and an estimated fluoride intake measure. Considering that epidemiologic studies have found sex-specific effects and there is a call to address sex in both developmental neurotoxicological studies (Mergler, 2011; Arbuckle, 2006) and in studies examining fluoride specifically (NTP, 2016), sex will be tested as a moderator of the association between fluoride exposure and neurodevelopmental outcomes. The specific aims are below.

Primary aim

To measure the association between maternal urinary fluoride (MUF) levels during pregnancy and childhood Full-Scale IQ (FSIQ), Performance IQ (PIQ), and Verbal IQ (VIQ), while controlling for covariates and examining moderation by child sex.

Hypothesis: Higher levels of MUF exposure will be associated with lower childhood FSIQ, PIQ, and VIQ, while controlling for covariates.

Secondary aim

Given our prior findings showing that fluoride from drinking water is a major predictor of MUF level (Till et al., *in revisions*), we examined whether water fluoride levels and estimated fluoride intake, as measured from the water treatment plants at the time of pregnancy and from beverage consumption questionnaires, are associated with offspring IQ outcomes.

Hypothesis

Higher levels of fluoride exposure and intake from the water will be associated with lower childhood FSIQ, PIQ, and VIQ, while controlling for covariates.

Methods

Study Design

This is a prospective, birth-cohort study that followed pregnant women and their offspring over time.

Participants

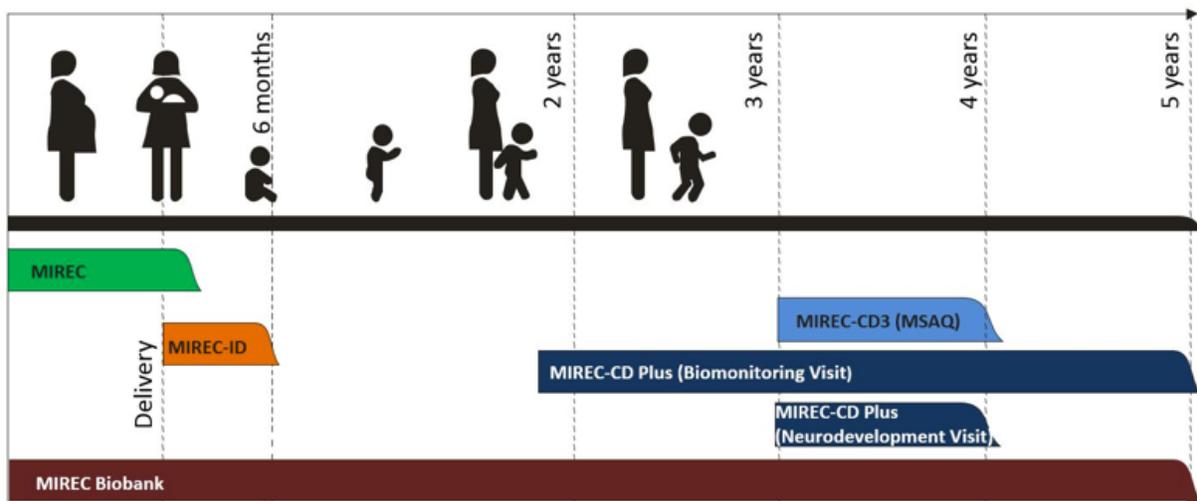
Mothers

Between 2008 and 2011, the Maternal-Infant Research on Environmental Chemicals (MIREC) program recruited a large sample ($N = 2001$) of pregnant women from ten distinct cities across different geographical regions of Canada, seven of which have water fluoridation (Toronto, Hamilton, Ottawa, Sudbury, Halifax, Edmonton, Winnipeg; $n = 1259$) and three of which do not (Vancouver, Montreal, Kingston; $n = 742$), to participate in a longitudinal environmental health study. Participants were recruited in prenatal clinics during their first trimester from hospitals with clinical obstetrical research infrastructures. Participants were included if mothers could consent, communicate in English or French, were older than 18 years of age, and their pregnancies were below 14-weeks of gestation. Participants were excluded if there was a known fetal abnormality, if they had any medical complications (i.e., cancer, renal disease, heart disease), or if there was known maternal alcohol or drug use during pregnancy. Of the overall sample, maternal mean age of the sample was 32.2 years ($SD = 5.10$) with a range of ages from 18 to 48. Eighty-three percent of the sample identified as white and 79% of the women were born in Canada. Over 95% were married or common-law and over 85% had a college diploma or university degree. At the time of pregnancy, 83% of the women were employed either full or part time. Further details can be found in Arbuckle et al. (2013).

Children

Subsequently, a subset of these women's children ($n = 654$), recruited from six of the ten cities included in the original cohort, was evaluated for the developmental phase of the study, with approximately half of the sample living in a non-fluoridated city ($n = 335$) and half living in a fluoridated city ($n = 275$). This phase occurred in numerous visits at six months, two years, and between three to four years of child age within different cohorts (Figure 1).

Figure 1. *MIREC cohort recruitment timeline*



Source: <http://www.mirec-canada.ca/en/about/some-facts-and-numbers/>

For the purpose of the current study, in the MIREC-Child Development (CD) Plus cohort, children were seen between the ages of three and four to give biomarkers and participate in a neurodevelopment assessment. Children were excluded if they were already four at the time of testing. A total of 610 children out of the 654 (93.3%) recruited from six distinct cities agreed to participate in the neurodevelopment portion (Figure 2).

Figure 2. Participation of the MIREC sites in the neurodevelopment visit

MIREC PLATFORM SITES PARTICIPATION

Site Number	City	Participating institutions	MIREC	MIREC-ID*	MIREC-CD3	MIREC-CD Plus		Neuro-development
					MSAQ**	MSAQ**	Bio-monitoring	
01	Vancouver	BC Children's and Women's Health Centre	162	34	45	38	83	55
02	Edmonton	University of Alberta	20		6			
03	Winnipeg	St. Boniface General Hospital/ Health Sciences Centre	90		23			
04	Toronto	Mount Sinai Hospital/ Sunnybrook Health Sciences Centre	325			70	108	72
05	Hamilton	McMaster University	275	114	47	75	112	85
06	Sudbury	Sudbury Regional Hospital	130		30			
07	Kingston	Kingston General Hospital	255	115	21	120	122	126
08	Ottawa	The Ottawa Hospital	119	12	50			
09	Montreal	CHU Sainte-Justine	300	127	58	115	175	145
10	Montreal	Jewish General Hospital	25	3	5	8	11	9
11	Halifax	IWK Health Centre	300	120	85	100	192	118
Total			2001	525	370	526	803	610

*Participants who completed at least one of the two visits in MIREC-ID (visits at birth & at 6 months)

** MSAQ (Mother self-administered questionnaire) was completed as part of MIREC-CD3 or CD Plus; the total number of MSAQ is 896.

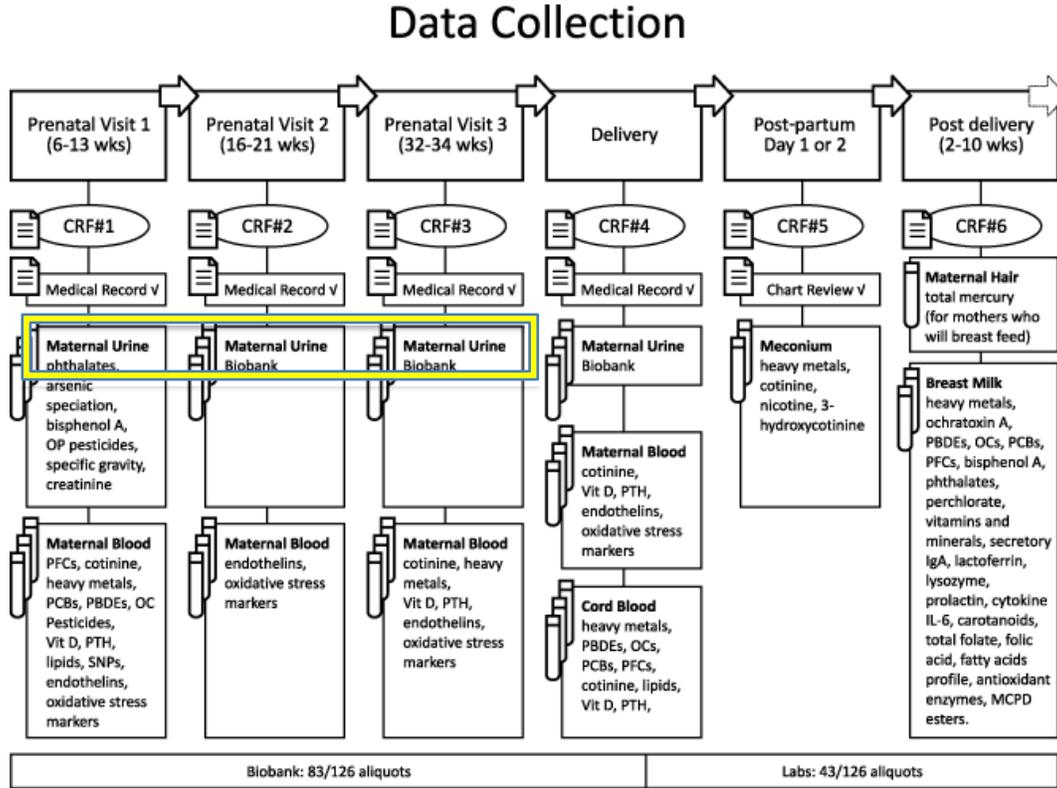
Source: <http://www.mirec-canada.ca/en/about/some-facts-and-numbers/>

Measures

Measure of fluoride

Archived urine samples were obtained from all three trimesters of pregnancy (Figure 3): trimester one at 11.57 ± 1.57 (mean \pm SD) weeks ($n = 1885$), trimester two at 19.11 ± 2.39 weeks ($n = 1738$), and trimester three at 33.11 ± 1.50 weeks ($n = 1660$).

Figure 3. MIREC data collection



Source: <http://www.mirec-canada.ca/en/about/some-facts-and-numbers/>

Due to the expected variability associated with urinary fluoride measurement and fluoride absorption in the fetus and mother across pregnancy, we only included women who gave all three urine samples and used the average urinary fluoride concentration taken over all three trimesters. Fluoride concentration was analyzed using diffusion analysis, the optimal measure of fluoride, which releases free and bound fluorine, concentrates it, and is used for samples in a covalent or complexed form. This method has been shown to yield the highest recoveries of fluoride for both diluted and undiluted samples (Martinez-Mier, Soto-Rojas, Buckley, Margineda, & Zero, 2009). This procedure was completed at Indiana University under the supervision of Dr. Angeles Martinez-Mier and further details on the technique are described in Till et al. (*in revisions*).

Urinary adjustments

To account for variations in urine dilution at the time of measurement, MUF concentrations were adjusted for both specific gravity (SG) and creatinine. Both adjustment methods were used since pregnancy can cause variations in creatinine metabolism and excretion. In addition, glomerular filtration rate (GFR) increases with greater tubular functioning causing more variations in urinary dilution. Currently, it is unclear whether adjustment for SG or creatinine is the optimal way to adjust for urinary dilution in pregnancy.

The Indiana University laboratory measured SG in all urine samples and methods for analysis can be found in Till et al. (*in revisions*). For the purpose of the current study, MUF samples were adjusted for SG using the following equation (Hauser, Meeker, Park, Silva, & Calafat, 2004):

$$MUF_{SGadj} (mg/L) = MUF_i * (SG_M - 1) / (SG_i - 1)$$

where $MUF_{SGadj} (mg/L)$ is the SG adjusted fluoride concentration, MUF_i is the observed fluoride concentration, SG_i is the SG of the individual urine sample and SG_M is the median SG for the sample. This method was used to adjust each urine sample per trimester using the median SG value for each trimester respectively.

There are many ways to adjust for creatinine (CRE) in urinary measurements. For the purpose of this paper and for comparing to other cohorts that analyzed fluoride during pregnancy, we used the same equation that was used recently in a published manuscript of a pregnant Mexican sample by Bashash et al. (2017). However, it is important to note that our group demonstrated that adjustment for CRE using various equations, or even using CRE as a covariate, all produce very similar results (Till et al., *in revisions*). For this paper, we adjusted for CRE using the following equation (Thomas et al., 2016, as cited in Bashash et al., 2017):

$$MUF_{CREadj} (mg/g) = (MUF/CRE_i) * CRE_{avg}$$

where $MUF_{CREadj} (mg/g)$ is the creatinine adjusted fluoride concentration (mg fluoride per g of creatinine), MUF_i is the observed fluoride concentration, CRE_i is the observed creatinine concentration for that individual, and CRE_{avg} is the average creatinine concentration of the sample available at each trimester.

Our group found that urinary adjustments for SG and CRE produced extremely similar results (Till et al., *in revisions*), suggesting that SG and CRE are interchangeable for adjusting for hydration status. Given that we retained more MUF samples corrected for SG than CRE (because some samples were missing creatinine measurements, whereas SG levels were available for 100% of the samples), our primary results will be presented with SG adjustment averaged across all three trimesters of pregnancy, referred to hereafter as MUF_{SG} (maternal urinary fluoride adjusted for SG and averaged across all three trimesters).

Measure of residential fluoridation status

Along with the amount of fluoride in maternal urinary samples, we used information on residential fluoridation status by linking municipal public water reports with the first three digits of participants' postal codes. Although natural fluoride may exist in some of the non-fluoridated areas included in the study, the mean concentrations of fluoride in these regions is less than 0.05 mg/L. Fluoridation was defined according to national drinking water guidelines (Health Canada, 2010), which include a recommended range of 0.6 to 0.8 mg/L fluoride in the water. In practice, fluoridated levels can correspond to a wider range with a Maximum Acceptable Concentration of 1.5 mg/L (Health Canada, 2010).

In order to link the water treatment plant's fluoride (WTP) values with participants, each participant's average fluoridated drinking water value was derived by matching the fluoride

concentration values that were added to the WTP over the duration of their pregnancy. We took the geometric mean of the WTP fluoride values corresponding to each trimester of pregnancy. We then took the average of these three geometric means to derive individualized fluoride concentration (FC) per mother. More information on this method can be found in Till et al. (*in revisions*). It is important to note that WTP boundary regions were predetermined for each city; 27.6% of participants fell outside that boundary.

Information on drinking-water habits and consumption of other beverages reconstituted with water (e.g., tea, coffee) were asked in questionnaires. Participants were asked the following question at the first and third trimesters: “Since the beginning of your pregnancy, how much did you drink the following: water (number of glasses; 1 glass = 8 oz); regular tea (cups); herbal tea (cups); green tea (cups); decaffeinated coffee (cups); caffeinated coffee (cups), (number of cups; 1 cup = 6 oz)?” Participants could answer *none* or insert a number of glasses or cups and select a frequency (day, week, or month). All responses were recoded to elicit a response of glasses/cups per day for each type of beverage (e.g., regular tea cups/day, caffeinated coffee cups/day).

In order to estimate total fluoride intake from tap water consumed per day, we multiplied each woman’s consumption of water and water-based beverages by their respective FC (averaged across pregnancy) and then multiplied by 0.2 (to equate the amount of fluoride for a 200mL cup) to equal the total fluoride content found across all of the cups of water consumed. Since black tea contains a high fluoride content level (USDA, 2005; Waugh, Potter, Limeback, & Godfrey, 2016), we also estimated the amount of fluoride women would be consuming from the cups of black tea by multiplying 2.6 mg F/L (average amount of fluoride found in black tea made with deionized water) by 0.2 for each cup of black tea and added this fluoride content to the fluoride intake variable for each woman (FI). Green tea has also been shown to contain

fluoride and varies depending on where it is imported from. Therefore, we took a conservative approach and used the average for the green teas listed in the USDA (1.935mg/L), (2005) which is lower than all estimates from the UK (Chan, Mehra, Saikat, & Lynch, 2013). We multiplied 1.935 (amount of fluoride found in green tea made with deionized water) by 0.2 for each cup of green tea and added this fluoride content to the FI variable for each woman.

Outcome variables

The IQ scores were derived from the *Wechsler Preschool and Primary Scale of Intelligence, Third Edition (WPPSI-III)* and included Full Scale IQ (FSIQ), a measure of global intellectual functioning, Verbal IQ (VIQ), a measure of acquired knowledge, verbal reasoning, and comprehension of verbal information, and Performance IQ (PIQ), a measure of nonverbal reasoning, spatial processing skills, attention to detail, and visual-motor coordination skills. FSIQ, VIQ, and PIQ on the *WPPSI-III* are normed to have a mean of 100 with a SD of 15.

Covariates

Potential covariates were chosen based on both a literature review (Buzalaf & Whitford, 2011) and specialist opinions from fluoride experts on what may influence fluoride intake and metabolism; neurodevelopmental experts on what may influence cognitive outcomes; and epidemiological experts on what to consider in developmental neurotoxicological studies. These potential covariates included the following: maternal characteristics: pre-pregnancy body mass index (BMI), maternal age, mother's prenatal smoking status, second hand smoke exposure during the prenatal period, prenatal alcohol consumption, prenatal caffeine consumption, gestational diabetes, chronic disease, medication taken during pregnancy, city (of participant's residence during pregnancy), marital status, maternal education, total household income, and maternal race. Maternal education and income were measured from a questionnaire asking

women to select their highest level of education and annual household income (on an interval scale) before taxes from January to December of the last year. Race was represented as a binary variable consisting of white or non-white because over 80% of the MIREC cohort were white. Alcohol and caffeine consumption were captured on questionnaires asking women to report consumption of specific beverages containing alcohol (beer, liquor, wine) and caffeine (coffee, tea, soda drinks) per day as specified above. Paternal covariates included paternal education and paternal smoking status.

Potential covariates also considered the following child characteristics: child sex, child age at time of testing, and the quality of child's home environment while growing up, measured by the *Home Observation for Measurement of the Environment (HOME) - Revised Edition* (Caldwell & Bradley, 1984).

Covariates also included urinary characteristics, including time of void and time since last void, and season of urine sample. Finally, other exposures during pregnancy were examined as potential covariates, including: prenatal exposure to lead, mercury, and arsenic. Lead analytes may react synergistically with silicofluorides to increase fluoride's uptake into the body (Masters & Coplan, 1999). Maternal and umbilical arsenic and mercury levels were considered because industrial-grade fluoride chemicals that are added to public water supplies have been shown to contain these metals (Rocha-Amador, Navarro, Carrizales, Morales, & Calder, 2007).

Some variables, although related to cognitive outcomes, were not considered because they may be on the pathway of the fluoride exposure to IQ relationship. These variables included gestational age (Diouf et al., 2012) and maternal thyroid levels (Ge et al., 2013), both of which may be influenced by fluoride exposure and can have an influence on neurodevelopmental

outcomes. These variables, with some evidence indicating them as consequences of fluoride exposure, could be considered independently of neurodevelopmental outcomes.

Ultimately, covariates were retained in the model using the change in estimate criterion procedure based on their significance and the change in estimate criterion: a variable is retained in the model if its *p* value falls at or below .2 or its inclusion changes the regression coefficient of the predictor by more than 10% for any of the IQ models (Kleinbaum, Kupper, & Morgenstern, 1982).

Statistical analyses

Descriptive statistics were calculated for demographics, exposure variables, other covariates, and outcome variables.

Associations were summarized using product-moment correlations and tabular analyses. Variance Inflation Factor (VIF) statistics were used to identify multicollinear variables that may be excluded in sensitivity analyses. A series of multiple linear regression models was estimated to represent the associations between MUF_{SG} and the outcomes, holding covariates constant. In addition to testing whether there was an interaction with baby sex, only those covariates that were significant in the final models were tested for potential interactions with MUF_{SG}.

Additional models were estimated to include certain covariates which were only available for a subset of the sample. These covariates included other neurotoxins, specifically lead, arsenic, and mercury. Further models were estimated to include additional variables that are known to interfere with development during pregnancy, specifically alcohol consumption. Next, sensitivity analyses included using MUF adjusted for creatinine as opposed to SG.

For the secondary aim of predicting IQ outcomes from water fluoride levels, associations between the fluoride concentration (FC) and fluoride intake (FI), IQ scores, and covariates were

first visualized using product-moment correlations and tabular analyses. VIF statistics were used to identify multicollinear variables that may be excluded in sensitivity analyses. A series of multiple linear regression models was estimated to represent the associations between FC and FI, holding covariates constant, and IQ outcomes. In addition to testing whether there was an interaction with baby sex, only covariates that were significant in the final models were tested for potential interactions with FC and FI.

We used a two-sided alpha of .05 for hypothesis testing. This study received ethics approval from Health Canada’s Research Ethics Board, York University Research Ethics Board, and Indiana University’s Ethics Board.

Results

Demographics

The distribution of the participants across the six cities in which the neurodevelopmental visits were conducted as part of MIREC Child Development–Age 3 Study (MIREC-CD Plus) is shown in Table 1.

Table 1. *MIREC recruitment table*

City adds fluoride to public water	Sites	Total neurodevelopmental visits (<i>n</i>)	% of MIREC sample ¹	Total <i>N</i>
No	Vancouver	55	72%	335
	Montreal ²	154	71%	
	Kingston ³	126	63%	
Yes	Toronto	72	57%	275
	Hamilton	85	54%	
	Halifax	118	82%	

¹ Proportion of eligible participants from the entire MIREC sample who completed neurodevelopmental testing

² Montreal has mixed fluoridation status with majority of women not receiving fluoridated water

³ Fluoridation to East Kingston (Canadian Forces Base) was discontinued by order of the Canadian Military on May 30, 2008

Of the women who had all three MUF_{SG} samples from each trimester ($n = 1566$), 526 children completed the neurodevelopment visit in its entirety. At delivery, these women had a mean age of 32.53 years ($SD = 4.52$, range = 18-46), 90% identified as white, and 83% were born in Canada. Almost 97% were married or common-law and 91% had a college diploma or university degree. At the time of pregnancy, 88% of the women were employed either full or part-time. The demographic characteristics of women included in the current study did not differ substantially from the original MIREC sample or the sample without all three urine samples (Table 2), but these women were more likely to be white, slightly more highly educated, more likely to be employed (than the overall sample only) and reported a slightly higher total household income.

Table 2. Comparison of current sample to other MIREC samples

	Participants in the MIREC cohort with:		
	Live births*	Women with 3 urine samples and child IQ scores	Women with fewer than 3 urine samples and child IQ scores
<i>n</i>	1983	526	75
Mean age (years) of mother at enrollment (SD)	32.2 (5.1)	32.53 (4.52)	32.43 (5.29)
Caucasian (%)	84.7	90.3	81.2
Married or Common law (%)	95.3	96.8	91.3
Born in Canada (%)	79	83.1	76.8
Maternal Education (%)			
High school or less	8.8	4.9	5.8
Some college	5.3	3.4	7.2
College diploma	23.6	23.6	29.0
University degree	62.3	67.7	58.0
Employed at time of pregnancy (%)	83	88.2	87.0
Net income household greater than \$70,000 (%)	64.0	70.7	65.2

*from a total of 2001 women who were recruited

Abbreviations: BMI = body mass index; SD = standard deviation

The 526 children (51.7% female) who were included in the current study were between the ages of three and four years old at the time of testing with an average age of 3.43 years ($SD =$

0.32). The mother-child pairs were distributed across the cities as follows: Vancouver ($n = 47$), Toronto ($n = 53$), Hamilton ($n = 60$), Kingston ($n = 116$), Montreal ($n = 143$), and Halifax ($n = 107$).

Fluoride analyses

The women had an average MUF_{SG} concentration of 0.51 mg/L ($SD = 0.36$ mg/L, range = 0.06-2.44 mg/L). Women receiving fluoridated drinking water had significantly higher levels of MUF_{SG} ($M = 0.69$ mg/L, $SD = 0.42$ mg/L) than women not receiving fluoridated drinking water ($M = 0.40$ mg/L, $SD = 0.27$ mg/L), $t = -7.31$, $p < .001$. Further descriptive information and results by sex can be found in Supplemental Table 2.

Covariates results

Numerous covariates were considered that are routinely used in neurotoxicologic studies or have been shown to be associated with fluoride exposure or childhood cognition. The following covariates were retained according to the strategy explained previously: quality of the child's home environment (HOME score), child sex, maternal education, race, and city (or site of testing). Of the 526 mother-child pairs, 512 mother-child pairs had available information for the HOME total score leading to a final sample size of $N = 512$ for estimating the main regression models (Supplemental Figure 1). The rest of the results with MUF_{SG} will be presented with the 512-sample size. The mean HOME score was 47.32 ($SD = 4.32$) and it ranged from 27 to 55 (Supplemental Table 2).

Interactions were tested individually for the following covariates as potential moderators of the association between MUF_{SG} and IQ: the HOME score, city, maternal education dichotomized, race (white or non-white), and child sex. Maternal education was dichotomized as

having an undergraduate degree or higher ($n = 348$; 68%) or a college/trade school diploma or lower ($n = 164$; 32%) since two thirds of the sample had an undergraduate degree or higher.

Additional regression models were estimated to include certain variables for which only a subset of participants had complete data. These variables included maternal blood lead level at trimester one ($n = 504$, $M = 0.03$ nmol/L, $SD = 0.02$ nmol/L), maternal blood mercury at trimester one ($n = 456$, $M = 5.24$ nmol/L, $SD = 4.49$ nmol/L), and maternal urinary arsenic from all possible arsenic biomarkers at trimester one and adjusted for SG ($n = 269$, $M = 0.67$ μ mol/L, $SD = 0.23$ μ mol/L).

IQ measures

Of the 610 children who underwent the neurodevelopmental assessment, two children were already four years old at the time of testing and seven children missed a subtest that disqualified the overall IQ score. As mentioned previously, of the remaining 601 children with possible IQ scores, 526 of their mothers (87.5%) had all three MUF_{SG} samples, and 512 (85%) mother-child pairs had complete IQ and covariate data. In our sample of 512, the mean FSIQ score was 107.16 ($SD = 13.26$ range = 51 to 143) (Supplemental Table 2). Females ($M = 109.56$) performed significantly better than males ($M = 104.61$), $t = -4.27$, $p < .001$. FSIQ also differed across cities, $F(5, 506) = 3.208$, $p = .007$.

Out of the 610 children who underwent the neurodevelopmental assessment, complete VIQ scores were derived for 509 (83.4%) children who also had three MUF_{SG} samples and complete covariate data. Mean VIQ was 109.65 ($SD = 13.01$, range = 58 to 144). Out of the 610 children who underwent the neurodevelopmental assessment, complete PIQ scores were derived for 507 (83.1%) children who also had three MUF_{SG} samples and complete covariate data. Mean PIQ was 103.24 ($SD = 14.59$, range = 55 to 144). Therefore, the sample sizes used to estimate

the VIQ ($n = 509$) and PIQ models ($n = 507$) were slightly smaller than for the FSIQ models. Information by sex can be found in Supplemental Table 2.

Two children had IQ scores that fell more than 2.5 standard deviations below the mean, so the regression models were also re-estimated with these two cases removed. No FSIQ scores or MUF values were higher than 2.5 standard deviations from the mean.

Multiple linear regressions for primary aim

Regression diagnostics confirmed that there were no collinearity issues in any of the IQ models with MUF_{SG} ($VIF < 2$ for all covariates). Residuals from the models had approximately normal distributions and the Q-Q plot revealed no extreme outliers. The plot of residuals against fitted values did not suggest any assumption violations and there were no substantial influential observations as measured by Cook's distance. Therefore, there was no need to transform MUF_{SG} (or any other variable), and we elected to present regression results for the linear model only. Finally, testing for non-linearity was also done by adding a quadratic term of MUF_{SG} to the linear model, which was not significant and therefore was not included in the final models.

The model predicting childhood FSIQ from MUF_{SG} , an interaction between baby sex and MUF_{SG} , and the remaining covariates accounted for 22% of the variance in FSIQ scores, $F(12, 499) = 11.71, p < .001$ (Table 3, Figure 4). The MUF_{SG} by sex interaction was significant ($B = 6.89, p = .02$). Simple slope analyses (e.g., Bauer & Curran, 2005) indicated that among males, higher levels of MUF_{SG} significantly predicted lower FSIQ scores ($B = -4.49, p = .02, 95\% CI: -8.38, -0.60$), but among females MUF_{SG} was not significantly associated with FSIQ ($B = 2.40, p = .34, 95\% CI: -2.53, 7.33$).

Table 3. Results of MUF_{SG} and FSIQ Multiple Linear Regression

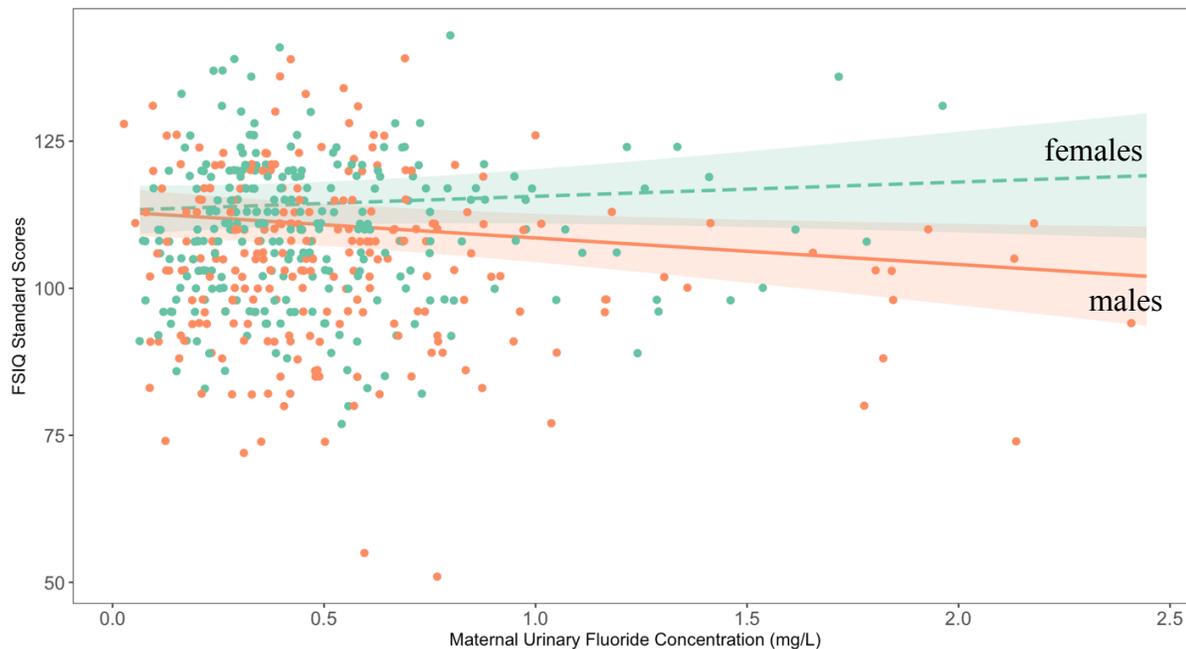
Variables	B	SE(B)	t	p	95% CI
<i>Simple model with sex-interaction*</i>					
MUF _{SG} males	-5.01	2.06	-2.43	.02	-9.06, -0.97
MUF _{SG} males x baby sex	7.24	3.27	2.21	.03	0.81, 13.67
<i>Model with all covariates and sex-interaction**</i>					
MUF _{SG} among males	-4.49	1.98	-2.27	.02	-8.38, -0.60
MUF _{SG} among females	2.40	2.51	0.96	.33	-2.53, 7.33
City:					
Vancouver***					
Toronto	-3.01	2.48	-1.22	.22	-7.89, 1.86
Hamilton	-4.59	2.42	-1.90	.06	-9.34, 0.17
Kingston	-8.05	2.14	-3.76	< .001	-12.25, -3.85
Montreal	-4.24	2.03	-2.09	.04	-8.23, -0.25
Halifax	-8.49	2.16	-3.93	< .001	-12.74, -4.24
HOME total score	0.89	0.14	6.54	< .001	0.62, 1.16
Maternal education	3.76	1.20	3.14	.002	1.41, 6.11
Race	4.09	1.87	2.19	.03	0.42, 7.77
MUF*Baby Sex	6.89	3.02	2.28	.02	0.96, 12.82

*Note. $N = 512$. $R^2 = 0.04741$, $F(3, 508) = 8.428$, $p < .001$.

**Note. $N = 512$. $R^2 = 0.2197$, $F(12, 499) = 11.71$, $p < .001$.

***Vancouver was the reference category for a set of 5 dummy variables used to represent the 6 cities.

Figure 4. Results of Multiple Linear Regression Predicting FSIQ from MUF_{SG}



The model predicting childhood VIQ from MUF_{SG} and controlling for covariates did not obtain a significant main effect of MUF_{SG} and there were no significant interactions with MUF_{SG}.

The model predicting childhood PIQ from MUF_{SG}, an interaction between baby sex and MUF_{SG}, and the remaining covariates accounted for 17% of the variance in FSIQ scores, $F(12, 494) = 9.71, p < .001$. The MUF_{SG} by sex interaction was significant ($B = 9.14, p = .007$). Simple slope analyses indicated that among males, higher levels of MUF_{SG} significantly predicted lower PIQ scores ($B = -4.63, p = .04, 95\% CI: -9.01, -0.25$), but among females MUF_{SG} was not significantly associated with PIQ ($B = 4.51, p = .11, 95\% CI: -1.02, 10.05$).

Sensitivity analyses, which involved (1) including lead, arsenic, mercury as covariates, (2) refitting the models with the two cases with extremely low IQ removed, and (3) using MUF adjusted for creatinine, did not substantially change the effect of MUF_{SG} among males (Table 4).

Table 4. *Sensitivity analyses*

MLR Models	<i>N</i>	<i>B (SE)</i> of MUF among males	<i>t</i>	<i>p</i>	<i>95% CI</i>
Model A	512	-4.49 (1.98)	-2.27	.02	-8.38, -0.60
Model A+lead	504	-4.61 (1.98)	-2.33	.02	-8.50, -0.71
Model A+mercury	456	-5.13 (2.05)	-2.50	.01	-9.16, -1.10
Model A+arsenic	269	-4.93 (3.58)	-1.38	.17	-11.97, 2.11
Model A+second hand smoke exposure	512	-4.18 (1.98)	-2.12	.03	-8.06, -0.30
Model A+alcohol consumption during pregnancy	512	-4.48 (1.98)	-2.26	.02	-8.38, -0.59
Model B	510	-4.11 (1.92)	-2.14	.03	-7.89, -0.33
Model C	407	-4.96 (1.83)	-2.71	.007	-8.56, -1.36

Model_A – MUF_{SG} controlling for city, HOME total score, race and maternal level of education with baby sex as effect modifier

Model_B – Model_A without two FSIQ outliers (males with FSIQ lower than 60)

Model_C – MUF adjusted for creatinine with same covariates as Model_A

Water fluoride analyses

As described earlier, fluoridation status was calculated by linking participants' residential status with an available water treatment plant (WTP), and not based on city fluoridation status because families' residence could fall outside the treatment plant's fluoridation distribution area. Of the 526 women with three MUF_{SG} samples whose child underwent neurodevelopmental testing, 479 (91%) reported a primary drinking water source of the tap, 45 (8.6%) reported well water, and 2 (0.4%) reported other, resulting in those reporting well or other being excluded from analyses. Of the 479 women who reported drinking tap water, 369 (77%) fell within the pre-determined WTP zones. Of these, 228 mother-child pairs lived in non-fluoridated regions and 141 mother-child pairs lived in fluoridated regions at the time of the pregnancy.

Among the final sample of 369 women with WTP data, the mean fluoride concentration (FC) was 0.31 mg/L ($SD = 0.23$ mg/L, range = 0.04-0.76 mg/L). After estimating fluoride intake (FI) by multiplying FC with beverage consumption data, the mean FI was 0.54 mg/L ($SD = 0.44$ mg/L, range = 0.01-2.10 mg/L). As expected, the levels differed largely between women receiving non-fluoridated water ($M = 0.30$ mg/L, $SD = 0.26$ mg/L) and women receiving fluoridated water ($M = 0.92$ mg/L, $SD = 0.4$ mg/L), $t = -16.32$, $p < .001$. FI was moderately correlated with MUF_{SG}, $r = 0.50$, $p < .001$, and was more strongly correlated than the correlation between FC and MUF_{SG}, $r = 0.38$, $p < .001$.

Relevant covariates, chosen by using the augmented backward elimination method, for the secondary aim included HOME score, dichotomized maternal education, race, second-hand smoke exposure, baby sex, and city. Regression diagnostics confirmed no collinearity issues with the exception of city. Because, as expected, city was strongly multicollinear with FC ($VIF > 20$), it was excluded from the model using FC as the focal predictor. Since city had questionable

multicollinearity with FI ($VIF > 2$), results will be presented with and without city. There were no substantial influential observations as measured by Cook's distance.

Multiple linear regressions for secondary aim

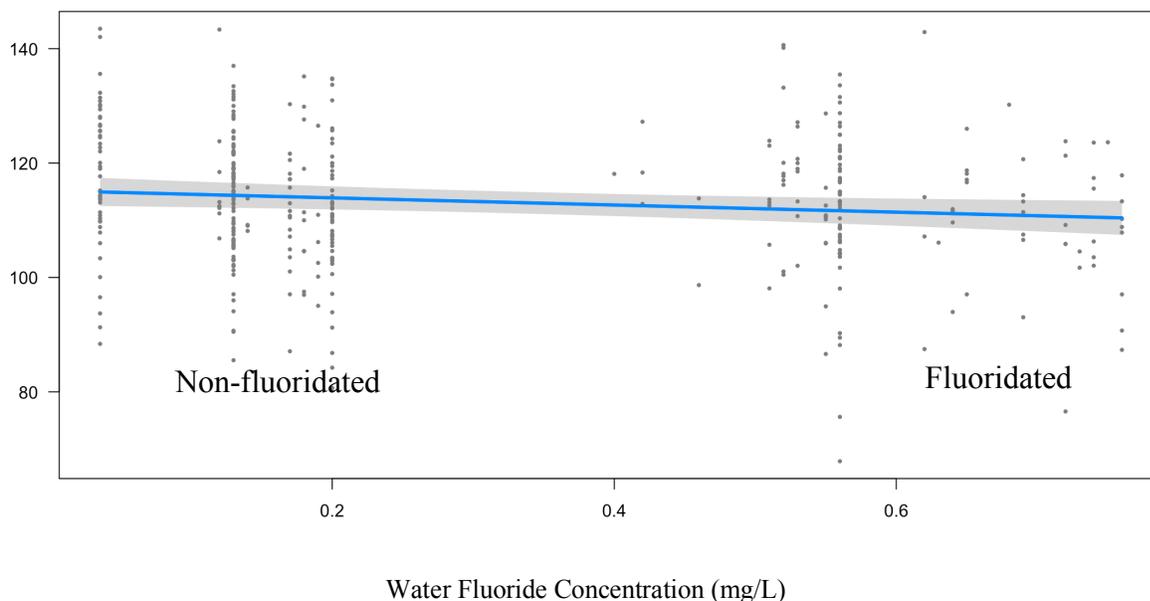
Holding the covariates constant, FC significantly predicted FSIQ scores ($B = -6.25$, 95% CI : -11.56 to -0.94, $p = .02$) (Table 5, Figure 5). The interaction between sex and FC was not significant ($p = .59$) and no other covariates significantly interacted with FC.

Table 5. Results of Fluoride Concentration (FC) and FSIQ Multiple Linear Regression

Variables	<i>B</i>	<i>SE</i>	<i>t</i>	<i>p</i>	95% <i>CI</i>
FC	-6.25	2.70	-2.32	.02	-11.56, -0.94
HOME total score	0.95	0.16	6.02	< 0.001	0.64, 1.26
Level education	6.06	1.42	4.28	< 0.001	3.27, 8.85
Race	3.95	2.13	1.86	.06	-0.24, 8.13
Baby sex	3.53	1.26	2.79	.006	1.04, 6.02
Second hand smoke	8.36	3.71	2.25	.02	1.06, 15.66

Note. $N = 369$. $R^2 = .21$, $F(6, 362) = 17.08$, $p < .001$.

Figure 5. Multiple Linear Regression predicting FSIQ from Fluoride Concentration (FC)

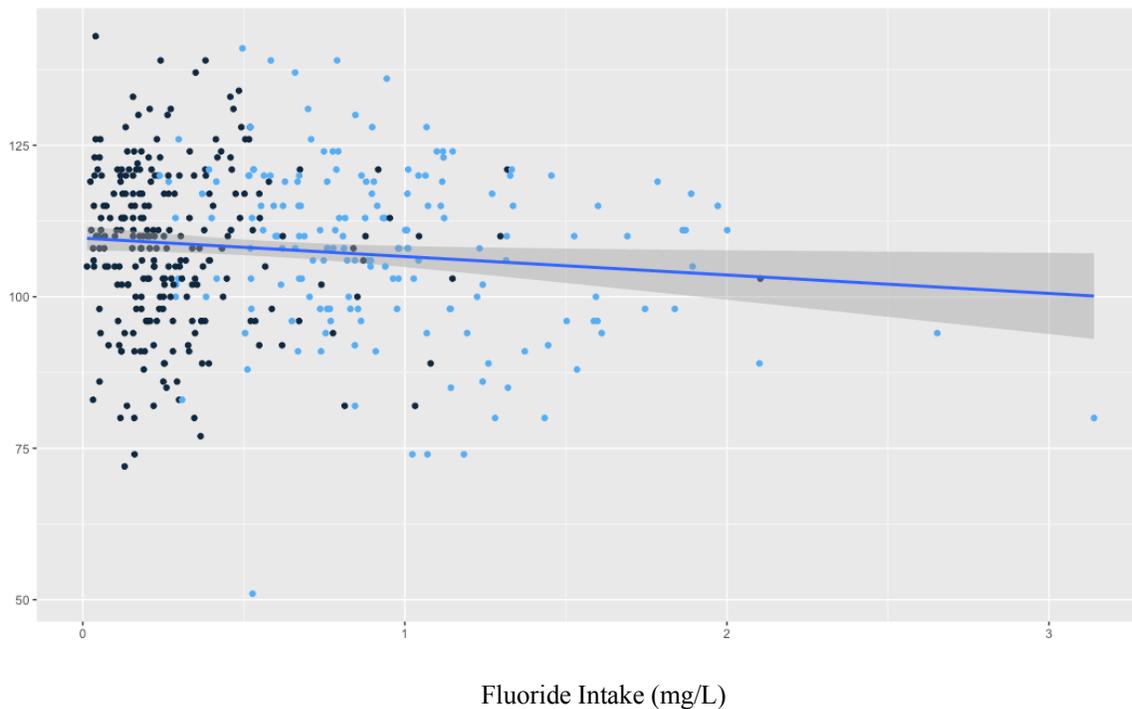


The model predicting childhood VIQ from FC and covariates did not demonstrate a significant main effect of FC and there were no significant interactions with FC.

Holding the covariates constant, FC significantly predicted PIQ scores ($B = -14.93$, 95% $CI: -21.11$ to -8.75 , $p < .001$). The interaction between sex and FC was not significant ($p = 0.39$) nor were there significant interactions between FC and the other covariates.

Holding all covariates constant, FI significantly predicted FSIQ scores without city in the model ($B = -4.03$, 95% $CI: -6.82$ to -1.25 , $p = .005$) (Figure 6). With city in the model, FI just missed significance ($B = -3.82$, 95% $CI: -7.65$ to 0.02 , $p = .05$). In both models, there were no significant interactions between FI and any of the covariates.

Figure 6. Multiple Linear Regression predicting FSIQ from Fluoride Intake (FI)



The model predicting childhood VIQ from FI and covariates did not demonstrate a significant main effect of FI and there were no significant interactions involving FI.

Holding all covariates constant, FI significantly predicted PIQ scores without city in the model ($B = -6.85$, 95% $CI: -10.13$ to -3.58 , $p < .001$), and did not reach significance with city included. There were no significant interactions between FI and any of the covariates.

Discussion

Primary aim findings

The current study examined the relationship between prenatal fluoride exposure and childhood IQ outcomes in a large national Canadian birth cohort. Results demonstrated that an increase in MUF_{SG} by 1.0 mg/L corresponded to a decrease in FSIQ by 4 and a half points in preschool aged males. It is important to note that MUF_{SG} ranged from 0.06 mg/L to 2.44 mg/L with an IQR of 0.34 mg/L across both fluoridated and non-fluoridated groups. A value of 1.0 mg/L is equivalent to the 86th and 96th percentile for a woman living in a fluoridated and non-fluoridated region, respectively. This effect was stable across all sensitivity analyses.

These findings are highly consistent with a recent Mexican birth cohort study that found a similar drop in IQ score (6.3 IQ points in preschool aged children) for every 1 mg/L of maternal urinary fluoride level during pregnancy (Bashash et al., 2017). In contrast to the Mexican study, we observed a sex effect such that only males' IQ scores were predicted to be affected by prenatal fluoride exposure as measured by MUF_{SG} concentration. Considering that women's MUF levels are almost double for those living in fluoridated communities compared with non-fluoridated communities in this sample (Till et al., *in revisions*), this study questions the safety of this widely accepted public health practice. These results suggest that fluoride levels in a North American population may have neurotoxic features for the developing male fetal brain. Although we did not observe a significant effect among females using MUF_{SG} as a biomarker, it could be that adverse effects of prenatal exposure to fluoride manifest biologically differently when it comes to females.

Sex differences

To date, sex has not been adequately accounted for in neurotoxicological or epidemiological studies and research (Gochfeld, 2007; Gochfield, 2010; Weiss, 2011). However, it is known that sex differences in response to chemical and environmental stressors, and differences in susceptibility are readily apparent (Gochfield, 2017; Mergler, 2011; Arbuckle, 2006). Generally, males and females differ by sex-specific organs, distinct hormonal axes, and differences in anatomical, physiological, and biological organ systems. In addition, males and females differ in terms of sociocultural factors and degrees of relative exposure and body mass.

While the examination of sex differences is sparse in neurotoxicologic research in general (Mergler, 2011), the NTP's (2016) systematic review of the effects of fluoride on learning and memory in animal studies reported a gap specifically regarding characterization of sex differences to fluoride exposure, and called for separate analyses of males and females. More specifically, they noted that the animal literature failed to evaluate sex differences due to pooling of males and females in one group, only measuring one sex, or having largely unequal group sizes between sexes.

Epidemiological studies of prenatal and early childhood fluoride exposure rarely report sex differences; of those that did, some have reported that no sex differences were observed (Lu et al., 2000; Zhao, Liang, Zhang, & Wu, 1996; Bashash et al., 2017), while one Chinese study's figures suggest that males' IQ scores show a sharper decline in response to fluoride exposure (Xiang et al., 2003). In a representative Canadian sample collected by Statistics Canada (Statistics Canada, Cycle 2 of CHMS from 2009-2011), 262 males between the ages of six and 11 years had *higher* urinary fluoride levels (530 µg/L) as compared to 252 females of the same age (470 µg/L). Sex-based data were not reported for children aged three to five years. Further, a recent report examining fluoride concentrations in water and plasma in the United States found

that while six to 11-year-old children were exposed to the same fluoride concentration in the tap water, males (0.41 $\mu\text{mol/L}$) had significantly higher fluoride plasma levels than females (0.38 $\mu\text{mol/L}$), ($p < .01$) (Jain, 2017). Sex-based data were not reported for children aged three to five years in both these samples. These sex differences in fluoride concentrations suggest that while males and females are exposed to the same amount of fluoride in the environment, they may be ingesting or metabolizing it differently. This difference is important to consider because U.S. national data on reports of human exposure to environmental chemicals have identified numerous chemicals for which sex differences in body burdens are noted, including lead, cadmium, and some phthalates (CDC, 2003).

Hormonal variations

Numerous hypotheses have been suggested, which attempt to explain why one sex might be more susceptible to neurotoxic exposures. Sex hormones have been shown to be involved in sex differences in terms of the transport of chemicals (Morris, Lee, & Predko, 2003). There are also hormonal differences in brain function and structure associated with sex-specific manifestations, and animal and epidemiologic studies have demonstrated that neurotoxic exposures can contribute to sex-specific behavioural changes (Paus, 2010). Gonadal hormones are important determinants of sexually dimorphic brain development, and neurotoxins can affect the production and metabolism of gonadal hormones, thus differentially affecting neurodevelopment in males and females.

Some examples of exposures fit within the classic sex hormone paradigm, and there is some evidence suggesting that males may be more neurologically vulnerable to environmental exposures. For example, perfluorooctanoic acid (PFOA) is almost universal in human and animal tissue (Gochfield, 2017), and its half-life is 70 times longer in male rats compared to female rats

(Worley & Fisher, 2015). Estradiol treatment can speed the elimination of PFOA in males, while testosterone increases its half-life.

Estradiol has also been shown to have neuroprotective properties in lead-exposed neurons in neuronal culture models (McEwen, Akama, Spencer-Segal, Milner, & Waters, 2012). The protective female effect (Chetty, Vemuri, Reddy, & Suresh, 2007) has been seen in many epidemiological studies of stroke, schizophrenia, and Parkinson's Disease (Amantea et al., 2005). This protective female effect could reflect the role of estradiol (Chetty et al., 2007), which has been seen to enhance cell proliferation and synaptic density, and can protect neurons from oxidative stress.

Sex differences are also seen in the stages of toxicokinetics (absorption, distribution, metabolism, and elimination) (Gochfield, 2017) and in xenobiotic concentrations in blood. Physiological differences between men and women can affect the rate and extent of the chemical distribution and sex hormones can influence the variations in pharmacokinetics of certain chemicals (Gandhi, Aweeka, Greenblatt, & Blaschke, 2004).

Neurological variations

Within the brain, neuroimaging studies have shown a complex pattern of sexual dimorphism beginning early in life (Goldstein et al., 2001). Specifically, sex steroids can modify brain development and exert effects during critical periods across development (NRC, 2005). In addition, remethylation of imprinted genes during gametogenesis varies, and among males, imprints are established in the germ line and maintained throughout mitotic divisions of spermatogenic stem cells, but among females, imprints are established during oocyte growth and stop during the meiotic prophase I (Perera & Herbstman, 2011). Further, gray matter has been shown to peak at 11 years of age for males and nine years of age for females (Lenroot et al.,

2007). As well, normal gray matter loss occurs at a faster rate in the frontal lobes of males than females (De Bellis et al., 2001; Giedd et al., 1999). There are also differences in organization of neural networks between the sexes, suggesting that males and females employ different strategies in problem solving and cognition (Pogun, 2001).

Taken together, the evidence demonstrating sex-based neurodevelopmental differences suggests that there may be distinct windows of vulnerability between males and females. The blood-brain barrier's permeability has also been shown to differ across sex depending on substances (Pakulski, Drobnik, & Millo, 2000; Saija, Princi, D-Amico, De Pasquale, & Costa, 1990; Minami, Sakita, Ichida, & Dohi, 2002). In neurotoxicologic studies, sex differences have been seen in regard to neurobehavioural and cognitive responses to exposures, such as lead and bisphenol-A (BPA) (Evans et al., 2014; Braun et al., 2011; Braun et al., 2009; Harley et al., 2013; Roen et al., 2015). For example, imaging studies of response to lead exposure in childhood have demonstrated that males have significant gray matter loss in numerous brain regions while females do not (Cecil et al., 2008), and stronger associations have been seen in males compared to females across all ages (Brubaker, Dietrich, Lanphear, & Cecil, 2010). In addition to these imaging studies, blood lead was more strongly associated with attention and visuoconstruction (Ris, Deitrich, Succop, Berger, & Bornschein, 2004) and executive function (Froehlich et al., 2007) among males than among females.

Our group is currently conducting a systematic review to examine whether males are intrinsically more vulnerable than females to the manifestation of neuropsychological sequelae as a consequence to neurotoxic exposures. However, it is important to note that the literature is inconsistent regarding associations between neurotoxins and global IQ. Bellinger (2000) notes that one sex could be more sensitive under various environmental circumstances which can be

attributed to chance or contextual factors (e.g., socioeconomic status) interacting with sex-specific genetic expressions. Considering that males have a higher prevalence of many neurodevelopmental disorders (ADHD, learning disabilities, Autism Spectrum Disorder, Tourette's Disorder, intellectual disabilities) (CDC, 2013), further research in this area is warranted.

Secondary aim findings

In addition to internal dosimeters (i.e., MUF_{SG}), our results found significant effects for external dosimeters of fluoride exposure. For fluoride concentration (FC), an increase of 1 mg/L of fluoride exposure from the tap water was associated with a decrease of 6.25 IQ points in young children. While this variable does not control for dose or source, it is an individualized measure linking the actual fluoride added to the respective woman's tap at her exact time of pregnancy. These consistent results with MUF_{SG} further question the safety of CWF.

With the estimated fluoride intake variable (FI), an increase of 1 mg/L of fluoride exposure from all beverages consumed with tap water and from tea was associated with a decrease of about 4 IQ points in young children. It is important to note that the range of fluoride exposure from water and water-based beverages (e.g., tea, coffee) was quite wide, and women who reported drinking eight total cups a day (75th percentile) could be consuming double the amount of fluoride as compared to women who reported drinking four total cups a day (just under 25th percentile). This variable, while strengthened by its more individualized measure of fluoride exposure, is limited by self-report of mothers' recall of cups of water, coffee, and tea consumed per day at two time points of pregnancy (trimesters one and three) as generalized to the entire pregnancy. It is possible that women had times of higher or lower beverage consumption. It is

also possible that women purchased their beverages (e.g., coffee) at regions of the city that do or do not receive fluoridated water.

While both these water fluoride measures predicted an effect on IQ consistent with MUF_{SG}, it was an overall effect collapsed across sex that was not specific to males. It could be that this dosimeter, especially fluoride concentration, represents a postnatal (cumulative) effect, including fluoride consumed during potential formula feeds, which occurs for females as well, as this is the amount of fluoride that the children are exposed to from drinking water postnatally. These results are consistent with a recent study, which found that postnatal fluoride exposure (as measured by fluoride in the tap water in Canada) was associated with an increase in attention problems (Riddell et al., *in preparation*) and an increase in the likelihood of learning challenges (Barberio, Quiñonez, Hosein, & McLaren, 2017).

Limitations

There are several limitations with using maternal urine as a biomarker of environmental exposures. First, urinary fluoride has a short half-life, about 5 hours, so information is lost after it leaves the biological matrix not long after the exposure has occurred. As well, there are various intra-individual variabilities in measurements impacted by fluctuations in everyday practices, which may affect the accuracy of urinary measures of fluoride. These varying practices include behaviours, such as the use of fluoridated toothpaste, diet, and drinking bottled versus fluoridated tap water, as well as metabolism and excretion rates. Urinary measures are further compromised in pregnant women because of differing habits, such as higher water intake, increased kidney size, and elevated GFR (Gordon, 2016). To account for these differences, urine is either adjusted for specific gravity or for creatinine. In pregnant women, however, this adjustment is

complicated because creatinine fluctuates based on trimester and is highly influenced by muscle mass, increased kidney size, and diet.

The current study used several strategies to overcome this limitation. First, this is the first study to take serial urinary measurements across three time points in pregnancy for each woman, and we have established in previous work that these time points are moderately correlated (Till et al., *in revisions*). As well, we adjusted for specific gravity in our primary model but also presented results of urine adjusted for creatinine and showed that they produced very similar results. We also had information about time of urine sample and time since last void which were tested as covariates to account for daily fluctuations.

Notwithstanding these strengths, while maternal urinary fluoride is meant to represent the fetus' prenatal fluoride exposure, these measurements do not necessarily provide accurate measures of fetal exposure because maternal biomarkers do not account for variability in placental transport and metabolism (Andra, Austin, Wright, & Arora, 2015). Finally, maternal urinary spot samples are not indicative of a fetus' exposure throughout the entire prenatal period, and do not control for the cumulative lifelong burden of fluoride exposure when assessing the prenatal contribution.

Despite these limitations, our study is the first to objectively measure fluoride in maternal urine using the largest birth cohort to-date to look at the association between prenatal fluoride exposure and IQ measures. It also consists of numerous covariates related to fluoride ingestion and IQ outcomes that were considered for all models. Additionally, our results were consistent across three measures of fluoride exposure – internal and two external dosimeters. Our study is also novel in that it consisted of a Canadian birth cohort exposed to water fluoridation using the “optimal” level of fluoride and is thus generalizable to other North American populations. Our

sample consisted of a national sample from women across Canada and was not limited to one city, and it also consisted of women from both fluoridated and non-fluoridated regions.

Future directions

It is important to note that our outcome variable consisted of IQ scores from preschool-aged children (three to four years old), and additional studies are needed to replicate findings in older children using cognitive outcomes appropriate for later stages of development. In addition to retesting cognitive outcomes at an older age, future studies may consider using a biomarker that can assess cumulative exposure and control both prenatal and postnatal exposure (e.g., tooth dentin) to better elucidate windows of susceptibility, in which urinary biomarkers cannot account for.

Considering that our external dosimeter measures of fluoride had an effect on children's IQ scores, it will also be important to examine the impact of postnatal exposure to fluoride. Specifically, infants who are fed formula reconstituted with fluoridated tap water are consuming six times the amount of fluoride as infants receiving formula from non-fluoridated tap water or breastfed infants (U.S. EPA, 2010; Table B-3), and research has shown that bottle-fed infants with fluoridated water have 100 times higher urinary fluoride than breast-fed infants at 1 ppm (Ekstrand, Fomon, Ziegler, & Nelson, 1994). Future studies are underway in our group to investigate the potential neurotoxic features of fluoride to this highly vulnerable population.

Conclusion and policy implications

Currently, there is an urgent need to identify the potential risks associated with water fluoridation, specifically in vulnerable time periods, such as the prenatal period, which is especially prone to neurodevelopmental consequences from toxic exposures. There is evidence

emerging from epigenetics that environmental influences can lead to permanent changes in brain development, especially during sensitive and critical periods of development (Tran & Miyake, 2017). Proponents of CWF have argued that the “dose makes the poison”; however, research has demonstrated that toxic chemicals can be biologically active even at very low levels (Lanphear, 2015), and because children are exposed to many neurotoxins at once, the cumulative burden of toxins can be profound. Since subclinical levels of toxins can be detrimental to children’s neurodevelopment, these findings call the safety of water fluoridation into question.

The current study’s findings may have major significance for public health policy. Considering the fact that fluoride’s use has a strong history of benefits for oral health, the evidence of its toxicity at low levels could introduce a public health conundrum. Our findings could lead to immediate educational dissemination to Canadians living in fluoridated cities on the potential neurotoxicity of fluoride exposure at levels found in Canada and the United States and help inform decisions made at the municipal level regarding the safety of this widely accepted public health practice.

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Appendix

Supplemental Table 1. Correlations between MUF, IQ scores and covariates

Measure	MUF _{SG}	FSIQ	V-IQ	P-IQ	WTP _F	WTP _{FxC}	HOME	Level Education
MUF _{SG}	—							
FSIQ	-0.07	—						
V-IQ	0.03	0.83*	—					
P-IQ	-0.14*	0.83*	0.38*	—				
FC	0.38*	-0.05	0.11	-0.20*	—			
FI	0.50*	-0.09	0.06	-0.21*	0.85*	—		
HOME	0.03	0.35*	0.33*	0.25*	0.12*	0.10	—	
Level Education	0.03	0.23*	0.26*	0.12*	0.10	0.05	0.25*	—

* $p < .05$

Supplemental Table 2. Descriptives of IQ scores, MUF, and HOME by sex

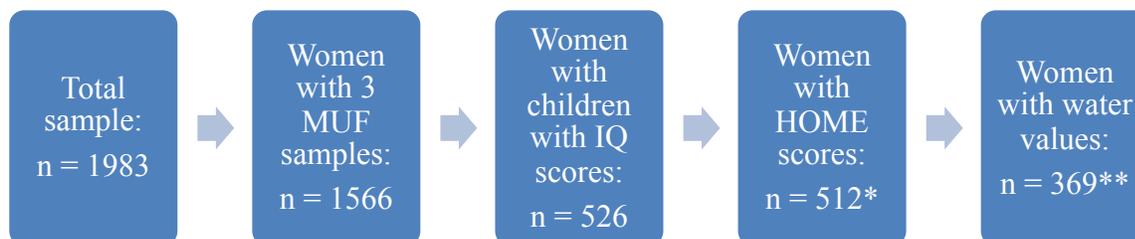
Variable	Males (Mean (SD)) <i>n</i> = 248	Females (Mean (SD)) <i>n</i> = 264	<i>p</i>
*MUF _{SG}	0.53 (0.40)	0.49 (0.31)	.13
HOME	46.58 (4.78)	48.01 (3.73)	< .001
FSIQ	104.61 (14.09)	109.56 (11.96)	< .001
VIQ [†]	107.04 (13.59)	112.10 (11.96)	< .001
PIQ [‡]	101.53 (14.99)	104.82 (14.05)	.01

*represents mothers pregnant with males compared to mothers pregnant with females

[†]*n*(males) = 246; *n*(females) = 263

[‡]*n*(males) = 244; *n*(females) = 263

Supplemental Figure 1. Flowchart of inclusion criteria



*final *n* for primary aim model (FSIQ and MUF_{SG});

**final *n* for secondary aim models (FSIQ and FC/FI)