In Vivo Detection of Trace Organic Contaminants in Fish Using Solid Phase Microextraction

by

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Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The feasibility of using solid phase micro-extraction (SPME) as an *in vivo* sampling tool for analysis of trace environmental contaminants in fish exposed to municipal wastewater effluents (MWWEs) was validated using controlled laboratory and field experiments. SPME was compared with traditional extraction techniques， including solid phase extraction (SPE) in water and solid-liquid extraction (SLE) in fish tissues to assess relative efficiencies. All three techniques were used to quantify the presence of eight compounds of interest in fish exposed to MWWEs in the laboratory (48-h static renewal), as well as in wild and field caged fish upstream and downstream of three wastewater treatment plants in the Grand River watershed (Guelph, Waterloo, Kitchener). Atrazine, carbamazepine, naproxen, diclofenac, gemfibrozil, bisphenol A, fluoxetine and ibuprofen were selected as target compounds due to their diverse chemical characteristics and frequent detection in surface waters and sediments around the world. Four fish species were used to determine the potential bioaccumulation of selected contaminants， including two lab-reared species, Rainbow Trout (*Oncorhynchus mykiss*) and Fathead Minnow (*Pimphales promelas*), and two wild species, Greenside Darter (*Etheostoma blennioides*) and Rainbow Darter (*Etheostoma caeruleum*). The distribution coefficients between various sample matrices (water, fish) and extraction phases (SPME fibers) were compared, as were extraction profiles and bioconcentration factors of target analytes in muscle of fish

exposed to MWWEs under laboratory conditions, during field caging studies, or collected (wild) from the Grand River. Poly(dimethylsiloxane) (PDMS) medical grade tubing was utilized as the SPME extraction phase, which when kinetically calibrated, were effective at extracting and quantifying the target analytes from both water and fish tissue relative to traditional techniques. Caged and in wild fish exposed to MWWEs from all three (Guelph, Waterloo, Kitchener) municipal treatment plants bio-accumulated detectable levels of several of the target chemicals. All target analytes (except for fluoxetine) were identified in the MWWEs and exposed fish by SPME at low concentrations (ng/L). Diclofenac, carbamazepine, ibuprofen, bisphenol A and gemfibrozil were the most frequently detected compounds in both surface waters and wild Greenside and Rainbow Darter within the Grand River watershed. Although, bisphenol A has relatively higher potential to bio-accumulate in fish muscle than the other targeted compounds, the concentrations of this analyte in MWWEs were typically low, resulting in relatively low body burdens in exposed fish. The presence and concentration of the targeted analytes in both water and wild fish living in the Grand River watershed varied with season and proximity to the wastewater outfalls. Results demonstrate that properly applied SPME can detect and quantify selected contaminants in fish tissues, surface water, and wastewater effluents. *In vivo* SPME allows for non-lethal sampling of fish, which creates the opportunity for monitoring contaminant exposure in receiving environments influenced by MWWEs or non-point-source runoff while minimizing the impact on the organisms.

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Finally, I would like to express my appreciation to all those who gave me the possibility to complete this thesis.

Dedication

I dedicate this thesis to my parents for their constant love, support and encouragement.

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Chapter 1

Introduction

Recently heightened concerns surrounding the use of experimental animals in research has encouraged researchers to investigate alternatives to lethally sampling animals for environmental analysis. *In vivo* solid phase micro-extraction (SPME) may be an important innovation allowing for non-lethal sampling of experimental or endangered animals with only minor, short-term effects on the sampled organism. Simultaneously, the near ubiquitous detection of pharmaceuticals and personal care products (PPCPs) in low concentrations in surface waters has raised considerable public concern. These environmental PPCPs have only recently been identified as contaminants of emerging concern, but their potential to exert subtle effects on reproduction and development on a wide variety of biological organisms, and their widespread detection in aquatic ecosystems, warrant further study into their environmental effects. In this study, the development and validation of SPME fibres for the *in vivo* sampling of fish for emerging contaminants will simultaneously address two research needs. First, *in vivo* SPME can be a rapid and facile tool to assess the uptake of environmental compounds in rigorous studies utilizing large numbers of fish; and second, demonstrating this relatively non-invasive approach that once optimized, may significantly reduce the need for lethal sampling in environmental monitoring studies.

1.1 Emerging Contaminants

Pharmaceuticals and personal care products (PPCPs) represent a large group of compounds which include drugs for human and veterinary use, and the active and inert ingredients in personal care products (Daughton and Ternes 1999). Examples of PPCPs include analgesics, lipid regulators, synthetic hormones, steroids, fragrances, sun screens, shampoos, and cosmetics. The active ingredients in pharmaceuticals are different from many conventional pollutants as they are designed to elicit specific biological effects through interactions with specific cellular effectors, many of which are highly conserved among most vertebrates and some invertebrates (Henschel et al. 1997). Most PPCPs and their biologically active metabolites tend to be more hydrophilic and less persistent than traditional contaminants such as PCBs. However, as they are continuously entering the environment through MWWEs, their effective persistence and resultant biological effects may be more pronounced than predicted by their chemical properties alone. Aquatic organisms resident in MWWE-receiving environments may be exposed to a variety of PPCPs for either their entire lifespan or at some critical point within their life history (Fent et al. 2006). PPCPs have been found in sewage effluents and surface waters around the world and they may be subjected to further transformation in the sewage treatment facilities, resulting in a wide diversity of potential environmental contaminants (Daughton and Ternes 1999). In Canada, the presences of PPCPs in the effluents of Canadian municipal treatment

plants and surface waters have been documented by several recent studies (Metcalfe et al. 2003, Servos et al. 2005, Lishman et al. 2006).

Personal care products, pharmaceuticals, and estrogens can reach surface waters, ground waters (Farré 2001), and sediments (Zuccato et al. 2000) through MWWEs, thereby posing a potential threat to aquatic organisms (Matthiessen and Sumpter 1998). Most conventional treatment process technologies used in MWWTPs are insufficient to completely remove these emerging contaminants from MWWEs. Although they can be degraded in the receiving environment by biotic (Winkler et al. 2001) or abiotic processes (Andreozzi et al. 2002), these compounds may still alter the normal endocrine function of exposed organisms for considerable distances below the outfall prior to their degradation (Daughton and Ternes 1999).

Synthetic and natural estrogens as well as other endocrine disrupting compounds (EDCs) such as pesticides, herbicides and other industrial chemicals are widely distributed in the Canadian environment (Servos 1999, Ternes et al. 1999, Hewitt and Servos 2001). Some EDCs exhibit estrogenic effects in cultured cells by binding directly to the estrogen receptors, ER_{α} and ER_{β} (Kuiper et al. 1998, Gaido et al. 2000, Safe et al. 2001, Safe et al. 2002, Scippo et al. 2004, Singleton et al. 2004, Singleton and Yuxin 2006). Researchers revealed that endocrine disruption was found to be widespread in fish populations exposed to environmental contaminants. Marine fish

exposed to endocrine disruptors showed disruption of hormone controlled physiological processes such as sexual differentiation, reproduction, growth, and immunity (Zhou et al. 2008a). Kidd et al. (2007) demonstrated recruitment failure of a population of Fathead Minnows exposed to low concentrations of 17α-ethinylestradiol (5 ng/L) in an experimental lake. Exposure to estrogens may have resulted in high levels of intersex observed below municipal effluent outfalls (Jobling et al. 1998, Örn et al. 2003, Zhou et al. 2008a).

1.2 Extraction Techniques

In order to detect environmental contaminants such as pharmaceuticals and pesticides in environmental matrices, these compounds need to be first extracted from samples (water, tissue, etc.). Widely used methods for separation of chemicals from environmental samples include solid-liquid extraction (SLE), solid-phase extraction (SPE), liquid-phase microextraction (LPME), membrane-assisted extraction, ultrafiltration, dialysis, microdialysis, supercritical fluid extraction (SFE), affinity sorbent extraction, and solid-phase microextraction (SPME) (Heringa and Hermens 2003, Walt 2005). Each of these methods has numerous advantages and disadvantages for environmental analysis. However, only SPME has the potential for rapid *in vivo* sampling which produces minimal impact on the sampled organism.

1. 3 Solid Phase Micro-extraction (SPME)

Solid phase micro-extraction was developed to facilitate rapid sample preparation and is an excellent alternative to traditional methods of separating target compounds from a complex matrix. SPME is not an exhaustive extraction method, and typically measures the free or bio-available fraction. A typical SPME fiber is made by dispersing a minute quantity of an extracting phase (such as fused silica or other appropriate material) on a fine stainless steel rod. The high concentrating ability and selectivity of the technique allows for direct, high sensitivity analysis of the extracted mixtures. As only small quantities of analyte are extracted, the overall disturbance is negligible, unless the sampled system's volume is relatively small.

1.3.1 SPME Fiber

One of the most common fibers used for SPME has a polydimethylsiloxane (PDMS) coating. PDMS is a highly permeable polymer with a high enrichment factor for many organic compounds (Westover et al. 1974, Freeman and Pinnau 1997). PDMS is suitable for bioanalysis due to its chemical inertness, low polarity, low conductivity and elasticity (Lide 1993), and ability to extract non-polar, hydrophobic analytes via absorption (Górecki et al. 1999). No toxic reactions or immunological rejection in living organisms has been observed from the use of PDMS (Chanard et al. 2003, Lipatova and Lipatov 2000).

1.3.2 Advantages of SPME

SPME eliminates the use of organic solvents, substantially shortens the total time of analyses and allows for convenient automation of the sample preparation. It is simple and economical, easy to deploy and retrieve. SPME is also suitable for on-site analysis and process monitoring, which reduces errors, and eliminates the possibility of sample change and time delays associated with both sample transport and storage, resulting in more accurate, precise and quickly available analytical data (Pawliszyn 2006).

The SPME technique overcomes the limitations posed by the solid-liquid extraction (SLE) technique allowing for rapid turn-around time from sample collection to the determination of target analyte concentrations. Solid-liquid extraction of fish tissue generally requires lethal sampling (or biopsy), and the amount of fish tissue collected has to be sufficient to produce a reliable result. In comparison, SPME is suitable for non-lethal on-site sampling, which not only allows for trace amounts of analytes in the living fish body to be extracted, but also creates the opportunity to study mechanisms of action in a more complex living biological system. *In vivo* monitoring of dynamic living systems by SPME results in minimal disturbance to the system and generates lower stress levels in the fish. Therefore, SPME technique makes it possible for non-lethal sampling of highly valued game fish or species at risk which could not be sampled by SPE or SLE. Furthermore, one fish can be used for

repeated temporal or spatial samplings, which creates the opportunity for a variety of *in vivo* experiments. Although SPME was initially applied only for the analysis of organic compounds from rather clean samples (air, water), it is now increasingly used in bioanalysis (*in vitro* and *in vivo*) because of its successful coupling with liquid chromatography (Jia et al. 1998, Pawliszyn 1999, 2003)

1.3.3 *In vivo* applications of SPME

To date, several *in vitro* applications of SPME have been developed, including the analysis of drugs from serum, plasma, whole blood, milk, urine, saliva and hair (Musteata and Pawliszyn 2007). For example, commercially available Carbowax™ fibers have been successfully used for direct extraction of chlorhexidine from saliva during a pharmacokinetic study (Musteata and Pawliszyn 2005). The cation-exchange diol silica can be used to extract two types of peptides (angiotensin 1 and 2) from whole blood (Musteata et al. 2005). Although most biological samples have been analyzed by *in vitro* SPME, many recent efforts are now directed towards *in vivo* analysis. Early *in vivo* investigations with SPME focused on fragrances emitted by insects, fungi and bacteria (Musteata and Pawliszyn 2007), and later applications focused on the detection of biogenic volatile organic compounds emitted by animals and plants. Recently, SPME has been used as an *in vivo* sampling tool for analyzing intravenous drug concentrations in a living animal (Lord et al. 2003). Most SPME coatings applied to date for *in vivo* sampling consist of PDMS or PDMS/DVB

(Polydimethylsiloxane/Divinylbenzene) (Musteata et al. 2006). For example, PDMS fibers have been used for the *in vivo* extraction of polycyclic aromatic hydrocarbons (PAHs) from earthworms (Jonker et al. 2007). In addition to PDMS or PDMS/DVB coatings, molecularly imprinted polymers (MIPs) with selective molecular recognition abilities have recently been developed. These coatings consist of polymeric matrix, which are complementary in size, shape, and chemical functionality to the target analyte, and are excellent sorbents to be used in SPME (Turiel et al. 2007). More examples about recent applications of SPME in bioanalysis are shown in **Table 1.1**

1.4 Calibration in Solid Phase Micro-extraction

When the volume to be sampled by SPME is very small, and the distribution coefficient of the analyte between the SPME fiber coating and the sample matrix is relative large, it is possible to extract all the analytes onto the fiber coating, so exhaustive extraction can be applied. However, SPME is not normally used as an exhaustive extraction method, so various calibration techniques have been developed to allow quantification of the analytes of interest (**Table 1.2).** When concentrations of target analytes in an unknown sample are predicted to be very low, or the instrumental sensitivity is poor, the standard addition method can be used. The internal standard calibration approach is often used when matrix effects have to be taken into account. When sample volume is very large, equilibrium extraction could be a good option,

because the analyte extracted by the coating is linearly proportional to the concentration of the analyte in the sample under equilibrium conditions. Diffusion based models are also available for predicting the absorption/adsorption and desorption processes of analytes traveling between the sample and extraction phase. Pre-equilibrium extraction was developed based on the theory of equilibrium extraction. This method suggested that since there is a linearly proportional relationship between the amount of analyte adsorbed and the initial concentration in the sample matrix, quantification of SPME is feasible under pre-equilibrium conditions if factors such as agitation, sampling time, and temperature are carefully held constant (Ai, 1997). If accuracy of quantification is imperative, isotopically-labeled surrogates can be pre-loaded onto the fiber coating to compensate for variations in experimental conditions. However, standard loading is inconvenient for short-term sampling when the losses of the standards are too small to detect. More recently, a standard-free kinetic calibration method was proposed for on-site and *in vivo* extraction.

Table 1.1 Selected recent applications of SPME in bioanalysis

Analytes	Biological sample	Extraction Method	Fiber	Reference(s)
Ibuprofen, warfarin, verapamil, propranolol, caffeine, valproic acid	Human plasma	DI	PPY, PDMS	Hsu, 2002; Musteata, 2006
7-aminofluitrazepam, benzodiazepines,ibuprofen, naproxen, angiotensin 2, and neurotensin	Human urine	DI	Alkyl diol silica, fiber coated with antibodies	Lord, 2006; Mullett, 2002; Guzman, 2003
Angiotensin 1 and 2	Whole human blood	DI	Exchange diol silica	Musteata, 2005
chlorhexidine and its degradation products	Human saliva	DI	Fibers coated with antibodies	Musteata, 2005
Diazepam and metabolites	Whole blood (beagle vein, *in vivo*)	DI	Hydrophilic PPY, PEG	Vas, 2004; Musteata, 2006
PAHs, cuticular hydrocarbons, fatty acids, volatile compounds, sex pheromones	Insects (*in vivo*)	HS and DI	PDMS, PDMS/DVB, pencil lead	Said, 2005; Tentschert, 2001; Tentschert, 2002; Peeters, 1999; Monnin, 1998; Gilley, 2006; Djozan, 2005; Anderbrant, 2005; Rochat, 2000
Antibiotics (linezolid)	Whole blood (pigs, *in vivo*)	DI	Hydrophilic PPY	Lord, 2006
Volatile organic emanations	Human skin (on arms, *in vivo*)	HS	PDMS/DVB	Zhang, 2005
Flavor compounds	Human noses (*in vivo*)	HS	PDMS	Pionnier, 2004; Pionnier, 2005
Pharmaceuticals	Fish (*in vivo*)	DI	PDMS	Zhou et al, 2008

Table 1.2 SPME calibration methods and their main advantages and disadvantages

	Calibration Method	Advantages	Disadvantages
Traditional Techniques	External standard	Easy sample preparation	Blank sample matrixes for calibration should be available. Sampling procedure and chromatographic conditions must remain constant
	Standard addition	Appropriate for unknown or complex samples	Extensive sample preparation and analysis; unwieldy for large number of samples
	Internal standard	Compensation of matrix effects, loss of analytes during sample preparation or irreproducibility in parameters	Suitable internal standards for complex samples are not easy to find. Isotope -labeled standards are expensive and not available for all analytes of interest
	Equilibrium extraction $n = \frac{K_{fs}V_fV_s}{K_{fs}V_f + V_s}C_0$ (Pawliszyn, 1997)	The concentration of analyte in the sample can be calculated by the amount of the analyte extracted by the fiber The amount of extracted analyte is independent on the sample volume when sample volume is very large	The distribution coefficients of the analytes between fiber coating and sample matrix (K) need to be known or determined
	Exhaustive extraction $n \approx V_sC_0$ (Ezquerro, 2003; Zhang 1995)	The concentration of the analyte can be easily calculated using the amount of analyte extracted by the fiber coating and the sample volume	Only appropriate for small sample volumes and analytes with very large distribution coefficients, or requires special devices or method to achieve exhaustive extraction

Table 1.2 SPME calibration methods and their main advantages and disadvantages (Continued)

	Calibration Method	Advantages	Disadvantages
Diffusion – based Techniques	Fick's first law of diffusion $n = R_s \int C(t)dt = D\frac{A}{Z}\int C(t)\,dt$ (Martos, 1999)	Suitable for time-weighted average (TWA) sampling. The sampling rate is independent of face velocity	The sorbent should be zero sink for target analyte. The sample rate for water sampling is very low
	Interface model $C_0 = \frac{nln((b+\delta)/b)}{2\pi D_a L t}$ (Augusto, 2001)	High sampling rate and short sampling time; minimized competitive effect for solid coating	The flow velocity of sampling matrix should be controlled or determined
	Cross-flow model $C_0 = \frac{n}{hAt}$ (Chen, 2003)	Suitable for on-site sampling where the construction of a calibration curve or addition of standard are difficult	Limited to the linear sampling regime
Kinetic Calibration Techniques	Kinetic calibration with standard $n = [1-\exp(at)]\frac{K_{fs}V_fV_s}{K_{fs}V_f + V_s}C_0$ (Chen, 2004)	Suitable for TWA sampling, especially where convective forces and concentrations of analytes are dynamic; particularly useful *for in vivo* determinations	Standard loading required; K values need to be known or determined
	Standard-free kinetic calibration $\frac{t_2}{t_1}\ln(1-\frac{n_1}{n_e}) = \ln(1-\frac{n_2}{n_e})$ (Ouyang, 2008)	Does not need standard loading. The concentrations of all extracted analytes in the sample can be calculated	Need two independent sampling occasions; sampling conditions should be kept constant. Unsuitable for long term monitoring. K value should be known or determined

1.5 In-fiber standardization technique

The calibration method used in the present research is referred to as in-fiber standardization. This technique assumes a linearly proportional relationship between the amount of analyte absorbed and the initial concentration in the sample matrix; thus, quantification of SPME is feasible under pre-equilibrium conditions only if factors such as agitation, sampling time, and temperature are constant (Ai 1997). Isotopically-labeled surrogates can be pre-loaded onto the fiber coating to compensate for variations in experimental condition. Isotropy of absorption and desorption in the SPME was demonstrated by Chen et al. 2004, so by measuring either absorption or desorption, the opposite process can be estimated. A kinetic calibration method, also referred to as in-fiber standardization technique, uses desorption of pre-loaded standards to calibrate the extraction of the analytes. To determine the concentration of an analyte in a sample matrix, the fiber is exposed to the sample matrix for a specific time during which a portion of the deuterated analogue is desorbed from the fiber while an unknown amount of analyte is absorbed into the fiber. Since extraction of the analyte and desorption of the preloaded standard occurs simultaneously, the effect of environmental factors, such as biofouling, temperature or turbulence, can be calibrated with this approach (Bragg et al. 2006, Ouyang et al. 2007, Zhao et al. 2006a). For sampling, desorption of the standard from a SPME fiber can be described by:

$$\frac{Q}{q_0} = \exp(-at) \qquad (1)$$

where q_0 is the amount of pre-added standard in the extraction phase and Q is the amount of the standard remaining in the extraction phase after exposure to the sample matrix for the sampling time, t. The constant a in Equation (1) from desorption also allows calibration of absorption.

Equation (1) can be expressed as

$$\frac{n}{n_e} = 1 - \exp(-at) \qquad (2)$$

where n is the amount of the extracted analyte in the extraction phase after exposure to the sample matrix for the sampling time, t, and n_e is the amount of the extracted analyte at equilibrium. According to the theory of in-fiber standardization technique, the constant a has the same value for the absorption of target analytes and the desorption of pre-loaded standards, so the sum of Q/q_0 and n/ne should be 1 at any desorption/absorption time:

$$\frac{n}{n_e} + \frac{Q}{q_0} = 1 \qquad (3)$$

The most well-established and widely used quantification method using SPME is the equilibrium extraction method. In this approach, a partitioning equilibrium between the sample matrix and extraction phase is reached. The amount of analyte absorbed by the coating at equilibrium (n_e) is linearly proportional to the initial concentration in the sample (C_0) at equilibrium (Ouyang et al. 2005, Zhao W et al. 2006b) and can be determined by equation (4)

$$n_e = \frac{K_{fs}\, V_f\, V_s\, C_0}{V_s + K_{fs}\, V_f} \qquad (4)$$

where K_{fs} is the fiber/sample distribution coefficient, V_f is the volume of the fiber coating, V_s is the volume of the sample matrix, and C_0 is the initial concentrations of target analyte in the sample. In general, the sample volume (V_s) does not have to be taken into account during analysis when the product of the fiber–sample partition coefficient (K_{fs}) and fiber volume (V_f) is much smaller than V_s. Assuming that the above condition is fulfilled when the value of KV_f is less than 1% of V_s, the sample volume must be greater than 100 KV_f. So the amount of extracted analyte will correspond directly to its concentration in the matrix, independent of the sample volume.

Equation (4) can be simplified to

$$n_e = K_{fs}\, V_f\, C_0 \qquad (5)$$

Thus,
$$C_0 = \frac{n_e}{K_{fs}\, V_f} \qquad (6)$$

The concentration of the target analyte can be determined by the amount of the analyte on the fiber under extraction equilibrium, by knowing the distribution coefficients of the analytes between the fiber coating and the sample matrix.

Since

$$n_e = \frac{q_0\, n}{(q_0 - Q)} \qquad (7)$$

C_0, can be calculated from Equations (6) and (7).

$$C_0 = \frac{q_0 n}{K_{fs} V_f (q_0 - Q)} \qquad (8)$$

K_{fs} can be calculated using the following equation (9), which is derived from equation (8). C_0 can be determined by doing an exhaustive extraction such as SPE or SLE.

$$K_{fs} = \frac{q_0 n}{C_0 V_f (q_0 - Q)} \qquad (9)$$

Therefore, the amount of the analyte extracted at equilibrium can be predicted under pre-equilibrium conditions, and the concentration of the analyte in the sample matrix can be calculated with equation (8) when values of K_{fs} are known.

1.6 The Grand River Watershed

The Grand River watershed is the largest watershed (6,800 km^2) in southern Ontario draining into Lake Erie. Fifty percent of the fish species (82 species) in Canada can be found in this watershed. The major tributaries comprising the upper Grand River include the Conestoga, the Eramosa, and the Speed Rivers (**Figure 1.1**). There are currently 925,000 people living in the Grand River watershed, and it is rapidly growing (Grand River Conservation Authority 2009). There are 28 sewage treatment plants of various designs in the Grand River watershed, serving approximately 700,000 people with the remaining population primarily on septic systems (Grand River Conservation Authority 2009). Many parts of the Grand River

aquatic ecosystem have been considerably stressed from urban and agricultural development, with excessive nutrient additions leading to elevated plant growth and extremely low dissolved oxygen levels (Grand River Conservation Authority, 2009). Agriculture and urbanization also result in a wide variety of environmental contaminants entering the surface waters. Despite considerable investment in municipal wastewater effluent treatment and application of best management practices, many issues remain and the Grand River continues to be degraded.

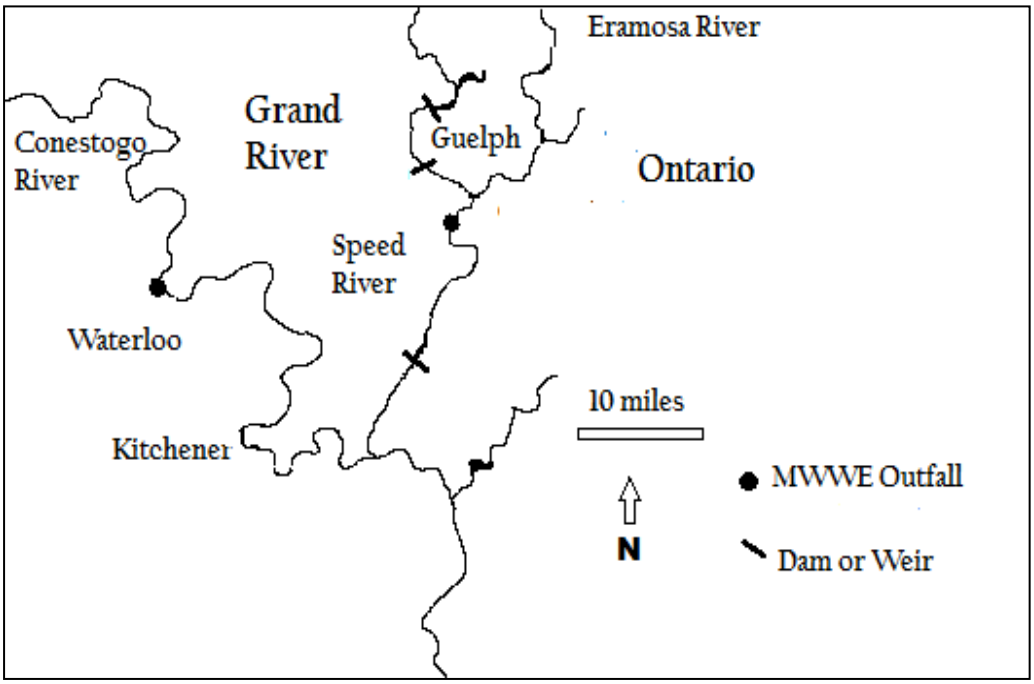

Figure 1.1 Map of The Central Portion of Grand River watershed

1.7 Fish Species Selected for Study

Rainbow Trout (*Oncorhynchus mykiss*), Fathead Minnow (*Pimphales promelas*), Greenside Darter (*Etheostoma blennioides* Rafinesque), and Rainbow Darter (*Etheostoma caeruleum*) were used in both laboratory exposures and field studies. Rainbow Trout and Fathead Minnow are easily cultured model organisms routinely used in toxicity assessments of xenobiotic compounds. Rainbow Trout (RBT), as a well studied, large-bodied fish species, provides sufficient tissue for determination of accumulation of chemicals in specific tissues and was therefore used for validating accumulation of target analytes in fish muscle. Fathead Minnow (FHM) can be readily cultured and exposed in the laboratory due to their small size, high reproduction rates and lower cost. In contrast with RBT, FHM can be caged in the field, even at elevated summer temperatures. Fathead Minnows are excellent field study species because their sizes are more representative of fish collected in the Grand River, and can be caged in sewage effluents to monitor the impacts of effluents on fish all year. Greenside Darter and Rainbow Darter are widely distributed species in the Grand River watershed, which makes them suitable for monitoring the water quality of the Grand River. The Darter species can be used as sentinels for monitoring contaminants in the river, and for demonstrating the utility of non-lethal fish sampling using SPME.

1.8 Thesis Objective

This thesis is composed of a series of experiments with the objective to determine the feasibility of *in vivo* SPME for environmental applications using fish for biomonitoring applications and includes:

1. Method development and validation of SPME *in vivo* detection of selected analytes in fish including controlled fish laboratory exposure of three fish species (Rainbow Trout, Greenside Darter, and Fathead Minnow) (Chapter 2).
2. Laboratory exposures of Rainbow Trout to municipal wastewater effluents to assess the suitability of kinetically calibrated SPME (Chapter 2).
3. Field caging of Fathead Minnows in municipal wastewater effluents to assess uptake of selected contaminants (Chapter 3).
4. Use of *in vivo* SPME to evaluate the concentrations of selected contaminants in two wild fish species (Greenside Darter and Rainbow Darter) collected adjacent municipal treatment plants outfalls in different seasons (Chapter 3).

Table 1.3 Summary of the experiments, species and sites used in the studies of *in vivo* SPME extraction of emerging contaminants

Exposure	**Species**			
	Rainbow Trout	Fathead Minnow	Greenside Darter	Rainbow Darter
Chemical Mixture Exposure	Waterloo (SP2009)	Waterloo (SP2009)	Waterloo (SP2009)	
Lab Effluent Exposure	Guelph (S2009) Waterloo (S2009)			
Caged Fish		Guelph (F2008) Waterloo (F2008) Kitchener (F2008)		
Wild Fish			Guelph (SP2009; S2009) Eramosa (SP 2009) Waterloo (S2009) Kitchener (SP2009; S 2009)	Guelph (SP2009; S2009) Eramosa (SP 2009) Waterloo (S2009) Kitchener (SP2009; S2009)

SP: spring; S: summer; F: fall; Chemical mixture exposure of eight selected contaminants of interest.

Chapter 2: Validation and Use of *In vivo* Solid Phase Micro-extraction (SPME) for the Detection of Emerging Contaminants in Fish

This chapter has been prepared for submission to *Chemosphere* with contributing authors Shuang Wang[1], Ken D. Oakes[1], Leslie M. Bragg[1], Janusz Pawliszyn[2], D. George Dixon[1], Mark R. Servos[1]

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Dr. Ken Oakes: Assisted with the research design, lab exposures, field work and editing.

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Prof Janusz Pawliszyn: Committee member and provided expertise and suggestions on SPME experiments.

Prof. George Dixon: Co-supervisor to Shuang Wang and provided valuable advice during the research design and preparation of the manuscript.

Prof Mark R. Servos: Co-supervisor to Shuang Wang and provided research direction and advice as well as assisting with field work.

Summary

Emerging contaminants of concern, including pharmaceuticals and personal care products (PPCPs), can contaminate surface waters and sediments via their continuous release from municipal effluents to receiving environments. The ability to rapidly determine *in vivo* concentrations of these contaminants in fish would be of significant advantage to facilitate research and assessment of their risk to the environment. The feasibility of solid phase micro-extraction (SPME) approaches to measure emerging contaminants in fish and municipal wastewater effluents (MWWEs) was assessed and validated against conventional extraction techniques including solid phase extraction (SPE) and solid liquid extraction (SLE). Rainbow Trout (*Oncorhynchus mykiss*), Greenside Darter (*Etheostoma blennioides* Rafinesque), and Fathead Minnow (*Pimphales promelas*) were exposed to a mixture of eight emerging contaminants (carbamazepine, naproxen, diclofenac, gemfibrozil, bisphenol A, fluoxetine, ibuprofen and atrazine) as well as two municipal wastewater effluents. This research determined the extraction profiles of target analytes from fish muscle using *in vivo* SPME, distribution coefficients (K_{fs}) between the extraction phase and sample matrices, and bioconcentration factors (BCF) of the selected compounds. Four target compounds were quantified in the MWWEs with maximum concentrations of 730 ng/L for naproxen, 140 ng/L for gemfibrozil, 490 ng/L for ibuprofen, and 170 ng/L for bisphenol A. These compounds were also bioconcentrated in fish, with BCF value ranges of 0.59-266 for Rainbow Trout, 0.34-94.1 for Greenside Darters, and 0.19-109

for Fathead Minnows. SPME was determined to be an excellent alternative extraction technique for quantitative determination of contaminants in MWWEs, with the advantage over conventional techniques due to its ability to non-lethally monitor *in vivo* bioconcentrated contaminants in tissues of living organisms.

2.1 Introduction

Emerging contaminates such as pesticides, industrial chemicals, pharmaceuticals and personal care products (PPCPs) can contaminate surface waters, ground waters, and sediments, thereby posing a potential threat to fish health (Farré 2001, Zuccato et al. 2000). Although they are used in large quantities throughout the world, these pollutants have, until recently, received little attention (Daughton and Ternes 1999). Pharmaceuticals and the active ingredients in personal care products are continually introduced into the environment via a number of routes such as MWWEs and agricultural runoff and can cause subtle effects on non-target organisms (Henschel et al. 1997, Halling-Sørensen et al. 1998, Daughton and Ternes 1999, Kolpin et al. 2002). MWWEs and biosolids are perpetual sources of PPCPs in the environment as the current treatment processes used by municipal wastewater treatment plants are insufficient to completely remove them from final effluents (Metcalfe et al. 2003, Lishman et al. 2006). PPCPs and their metabolites can induce subtle effects at very low concentrations by acting on specific cellular processes or receptors, many of which are poorly understood in non-target species such as fish (Henschel et al. 1997). Therefore, PPCPs may adversely affect fish in aquatic environments receiving MWWEs or runoff, even at very low concentrations (Matthiessen and Sumpter 1998).

Solid phase micro-extraction (SPME) is an efficient extraction method that

integrates sampling, isolation and enrichment in one step, allowing for quantitative determination of contaminants in a wide variety of matrices (Pawliszyn 1997). Several *in vitro* applications of SPME have been successfully developed for identification of pharmaceuticals in whole blood, urine, saliva and hair (Musteata and Pawliszyn 2007). *In vivo* analysis using SPME has recently been used for analysis of pharmaceuticals in living organisms (Jia et al. 1998, Pawliszyn 1999, Pawliszyn 2003, Musteata and Pawliszyn 2007, Jonker et al. 2007, Zhou et al. 2008c). In this study, conventional extraction techniques including solid phase extraction (SPE) and solid liquid extraction (SLE) were compared against SPME to validate *in vivo* SPME as a sampling tool for extraction of environmental contaminants (e.g., PPCPs) from live fish.

In-fiber standardization using pre-loaded deuterated surrogates was used to improve the accuracy and precision of experimental data (Chen et al. 2004). Once a fiber is inserted into a sample (e.g. fish), extraction of the analytes and desorption of the preloaded surrogates is assumed to occur simultaneously and at an identical rate. Consequently, the effects of biofouling, temperature, turbulence, or other environmental factors can be compensated for with this approach (Bragg et al. 2006, Ouyang et al. 2007, Zhao et al. 2006b). Concentrations of target analytes in fish were estimated under pre-equilibrium condition using: $n_e = \frac{K_{fs}V_fV_s}{K_{fs}V_f+V_s}C_0$

where n_e is amount of analyte absorbed by the coating at equilibrium, C_o is the initial concentration of target analyte in the sample, K_{fs} is the fibre/sample distribution coefficient, V_f is the volume of the fiber coating, V_s is the volume of the sample (Ouyang 2006).

The SPME fiber selected for fish experiments is composed of polydimethylsiloxane (PDMS), a permeable polymer which readily adsorbs many organic compounds (Westover et al. 1974, Freeman and Pinnau 1997). PDMS is a biocompatible material, suitable for bioanalysis due to its chemical inertness, low polarity, low conductivity and elasticity (Lide 1993). SPME, SPE, and SLE were used to quantify the presence of selected contaminants in dorsal-epaxial muscle of Rainbow Trout (*Oncorhynchus mykiss*), Greenside Darter (*Etheostoma blennioides* Rafinesque), and Fathead Minnow (*Pimphales promelas*) in controlled lab exposures to a mixture of spiked analytes in clean water as well as MWWEs. Liquid chromatography– tandem mass spectrometry (LC–MS/MS) was utilized to quantify analytes in samples. Eight compounds (atrazine, bisphenol A, carbamazepine, diclofenac, fluoxetine, gemfibrozil, ibuprofen, and naproxen) arising from three distinct sources (agricultural, industrial, and urban wastes) were spiked as target analytes for the clean water mixture exposure. These contaminants are near ubiquitous in surface waters, including those of the Grand River watershed (**Figure 2.1**), and have the potential to disrupt normal physiological functions in exposed biota. As such,

the quantification of these emerging contaminants of concern in water and exposed biota is of interest to managers and regulators globally.

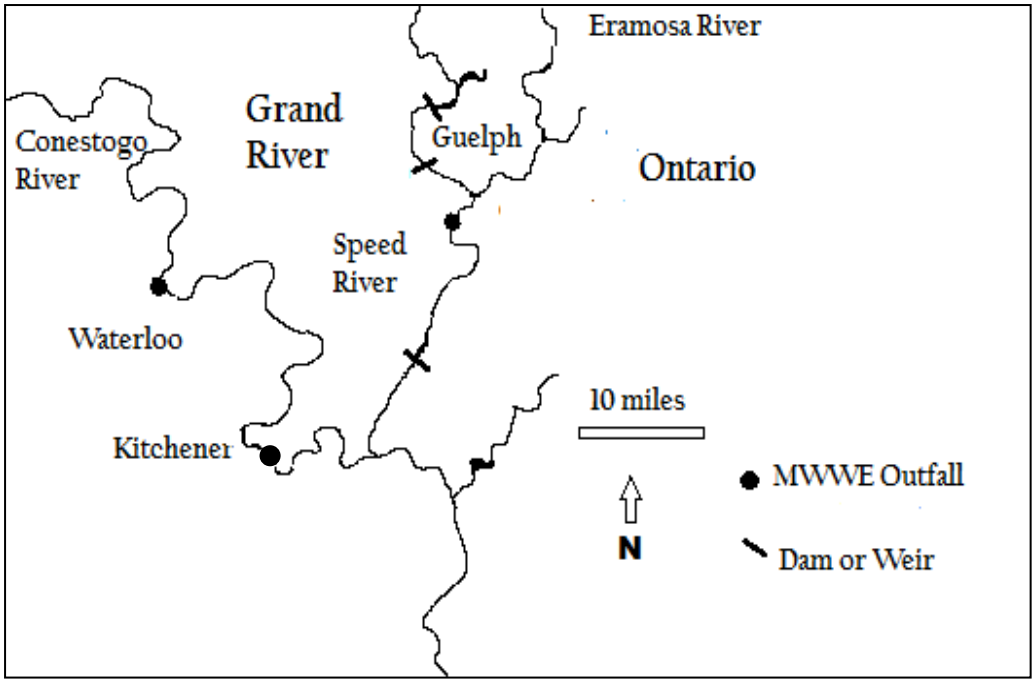

Figure 2.1 The central portion of Grand River watershed, Ontario, Canada.

2.2 Methods and Materials

2.2.1 Solid Phase Extraction (SPE) of Water Samples

SPE has been commonly utilized to extract analytes from samples by exhaustively extracting analytes from the aqueous matrices. Oasis HLB cartridge was selected for this research as it is suitable for determination of concentrations of both polar and non-polar compounds. The cartridge consists of lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone copolymers with modifications for ionic compounds which extends the pH range for good retention of acidic, basic and neutral compounds (Oasis HLB, SPE Technologies). Therefore, concentrations of target analytes with various polarities, LogKow, and pKa values can be determined by SPE. External standards and internal standards were used for calibration of SPE to increase the accuracy of quantitation by compensating matrix effects and variations in samples' volume. Water samples were collected in 500 mL amber glass bottles and were preserved using sodium azide (0.2 g/L) and ascorbic acid (0.01 g/L) prior to filtration using glass fiber filter paper (GFF#1825-070, Whatman, Maidstone, Kent, England). Oasis HLB cartridges (#186000115,Waters, Milford, MA, U.S.A) held by a 12-port Visiprep™ vacuum manifold (Supelco #57030-U, Bellefonte, PA) were preconditioned with 5 mL of HLPC grade tert-butyl methyl ether (MTBE) (Sigma-Aldrich, Oakville, ON), 5 mL of HLPC grade methanol (Fisher Scientific, Unionville, ON, Canada), 5 mL of HLPC grade water (Fisher Scientific Unionville,

ON, Canada) with a flow rate of 5 mL/min. A 50 µL volume of a 100 µg/L solution containing isotopically labeled standards then was spiked into each water sample as external standard to compensate for matrix effects. Two matrix-evaluating spikes were prepared by adding 50 µL of the 100 µg/L deuterated standard solution and 50 µL of the 100 µg/L non-deuterated standards into 500 mL HPLC grade water for determination of relative recoveries by SPE. Additional two bottles of 500 mL HPLC grade water were used as blank samples. Isotopically labeled standards (atrazine-d_5, bisphenol A-d_{16}, carbamazepine-d_{10}, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3, and $^{13}C_1$-naproxen-d_3) were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Gemfibrozil, ibuprofen, carbamazepine, diclofenac, naproxen, and bisphenol A were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fluoxetine, lorazepam, and chloramphenicol, and atrazine were obtained from Cerilliant Corp (Round Rock, TX). Water samples were drawn into Teflon tubing (3 mm i.d. x 4 mm o.d. x 60 mm, #57276) connected to stainless steel weights (#57278) and SPE tube adaptors (#57277, Supelco, Bellefonte, PA, U.S.A) under vacuum (20'' Hg; Gast oil-less diaphragm-type pressure/vacuum pump, # 01-092-29, Fisher Scientific, Unionville, ON, Canada) at a flow rate of 15 mL/min. SPE cartridges were rinsed with 5 mL HPLC grade water and dried under vacuum for 15 min prior to elution using 5 mL of HPLC grade methanol and 5 mL of 10/90 (*v/v*) methanol/MTBE. Eluted solutions were collected with disposable borosilicate culture tubes (15x85 mm, Fisherbrand #14-961-28, Fisher Scientific, Unionville, ON, Canada)

and blown dry under a gentle stream of nitrogen (~ 20 psi) using a Dionex SE 500 solvent evaporator modified with 40 mL scintillation vials to hold the culture tubes. Each dry sample was reconstituted in 500 µL of methanol (containing 75 µg/L of lorazepam and chloramphenicol as internal standards) in 2 mL amber glass vials (Target DP vials, National Scientific #C4000-2W), with vial caps (DP blue caps and TST septa, National Scientific #C4000-53B) and vial inserts (5 mm, 0.15 mL Kim spring, PP conical insert, National Scientific #C4012-530P). The reconstituted solution was then saved in an amber glass vial (2 mL) and stored at (-20 °C) until analyzed by LC-MS/MS.

2.2.2 Instrumental Analysis

An Agilent 1200 liquid chromatography (LC) and a Finesse Genesis C18 column (150 × 2.1 mm, 4 µm, (Chromatographic Specialties Inc., Brockville, ON, Canada) were used for separation. Mobile phases consisted of (A) 5 mM ammonium acetate in water and (B) methanol. The flow rate was set at 0.8 mL/min, and a 60% B gradient for positive compounds (10% B for negative compounds) was applied within the first 0.5 min. This was ramped to 100% B over 5 min for positive compounds (8 min for negative compounds), and finally returned to 60% B for positive compounds (10% for negative compounds) within 3.0 min. This resulted in a total run time of 8.0 min for positive compounds (11.0 min for negative compounds). A 10 µL injection volume

was used for each experimental sample. Eluted analytes were monitored by a triple-quadruple tandem mass spectrometer (MS/MS) using a API 3200 Qtrap system with a TurboIonSpray source (Applied Biosystems/MDS Sciex, ON, Canada) in positive/negative ion mode. Each transition was monitored for 200 ms. Compound specific mass spectrometer settings were determined for each compound separately by infusing a 0.5 µg/mL methanol solution at 3 µL/min using the integrated syringe pump. Transitions monitored in positive mode were as follows: atrazine, *m/z* 216.2/174.3; carbamazepine, *m/z* 237.1/193.3; fluoxetine, *m/z* 310.3/44.3; d5-atrazine, *m/z* 221.1/179.3; d10-carbamazepine, *m/z* 247.2/204.4; d5-fluoxetine, *m/z* 315.2/44.2; and lorazepam, *m/z* 321.1/275.1. Transitions monitored in negative mode were: ibuprofen, *m/z* 204.9/160.9; bisphenol A, *m/z* 227/211.9; naproxen, *m/z* 229/170; gemfibrozil, *m/z* 249.1/121.1; diclofenac, *m/z* 293.9/250; chloramphenicol, *m/z* 321/151.9; d-ibuprofen, *m/z* 207.9/164.1; d-bisphenol A, *m/z* 213/221; d-naproxen, *m/z* 233/169.9; d-gemfibrozil, *m/z* 255/120.7; d-diclofenac, *m/z* 298.2/253.8. Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples through the injection of 10 µL of a series of standards (0.5-500 µg/L) prepared in methanol containing the internal standard, lorazepam (75 µg/L). External calibration curves were performed with good precision (RSD < 5%) and linearity (R^2 > 0.999). Analyst version 1.4.2 software (Applied Biosystems) was used for data collection and analysis.

2.2.3 Method Detection Limit (MDL) and Instrument Detection Limit (IDL)

Detection limits were determined for the instrument and each of the different extraction methods – SPE (water) and SPME (fish and water) – and are shown in Table 2.1. The instrument detection limit (IDL) is the minimal amount of analyte that can generate meaningful signal with the instrument, normally with the peak height 3 times of the noise. To determine the IDL, a range of low concentration calibration curve standards were injected into the LC-MS-MS and the IDL was chosen to be the minimal concentration of standard that can produce a signal which is three times larger than the noise signal.

The method detection limit (MDL) is the minimum concentration that can be determined with 99% confidence that the true concentration is greater than zero. The MDL was calculated by multiplying the student's t-value by the standard deviation of the replicates. The MDLs were determined for the SPE method by extracting ten replicate samples at low concentrations (1, 5 and 10 ng/L) which were approximate 5 times greater than the estimated MDL and running these extracts on the LC-MS/MS. The MDL for SPME was determined by extracting seven samples from clean muscle tissue or HPLC grade water using the SPME method for extraction. Concentrations of 0, 0.1, 0.5, 1, 10, 25, 50, and 100 ng /g in muscle or 0, 0.5, 1, 10, 50, 100, 200, and 500 ng/L in HPLC grade water were used for the calculation of the SPME MDL.

Table 2.1 Method detection limit (MDL) and instrument detection limit (IDL)

Analytes	Fish SPME MDL (ng/g)	Water SPME MDL (µg/L)	Water SPE MDL (µg/L)	IDL (µg/L)
Bisphenol A	15	0.05	0.004	10
Ibuprofen	1	0.03	0.002	1
Diclofenac	2	0.05	0.007	0.15
Gemfibrozil	0.5	0.05	0.003	0.15
Naproxen	1.5	0.05	0.003	0.1
Atrazine	2.5	0.2	0.001	0.025
Carbamazepine	2	0.01	0.001	0.05
Fluoxetine	10	0.01	0.003	0.2

2.2.4 Liquid Extraction of Fish Muscle

Each sample of dorsal-epaxial muscle excised from fish was cut into fine pieces with a scalpel prior to homogenization at 540 rpm for 1 min by a Craftsman 10-in Drill Press (#137 28007, Fort Worth, TX, U.S.A). A 0.5 g subsample of the homogenized sample were mixed with 500 µL of extraction solution (95:5:0.1, methanol:water:formic acid, *v/v/v*) with 20 µg/L deuterated standards added in a microcentrifuge tube and agitated at 2400 rpm for 1.5 h at room temperature. SLE was calibrated using deuterated standards to improve the quantitation accuracy by compensating for tissue matrix effects. The fluid portion of the sample was transferred into a Microcon™ Centrifugal Filter Devices (Fisher, Millipore # 42 403) and centrifuged at 14 000 rpm for 30 min to allow the sample to pass through the filter for cleanup. The tissue was discarded and the filtrate was saved with a 100 µL portion mixed with 50 µL of methanol containing the internal standards (225 µg/L

lorazepam and chloramphenicol) and stored at -20 °C until analysis. After extraction by SLE, recoveries of deuterated standards were calculated to determine the recovery of each compound in the sample. In addition, 1 µg of isotopic standards were spiked into 0.5 g portions of homogenized control fish muscle (for Rainbow Trout, Greenside Darter, and Fathead Minnow) and extracted by SLE to determine the mean recovery for each analyte. Three sample blanks were made by spiking 1 µg of isotopic standards into 500 µL of extraction solvent and a 100 µL portion of subsample was reconstituted in 50 µL methanol containing internal standards (**Table 2.2**).

2.2.5 Solid Phase Microextraction of Water Samples and Living Fish

SPME Water Samples

SPME fiber coatings (165 µm PDMS fiber tubing, Helix Medical# 60-795-01, Carpinteria, CA) were cut into 1.0 cm lengths and mounted on 3 cm long 19 gauge (127 µm o.d.) stainless steel wires (Small Parts Inc, #GWX-0190-60-10, Miami Lakes, FL). The prepared fibers were sequentially sonicated in de-ionized water and acetone, methanol and then in Nanopure water for 15 min (respectively) to ensure low background contamination. After cleaning, fibers were exposed to solutions containing deuterated surrogates (500 µg/L) in 2 mL amber glass vials and agitated at 1500 rpm for 3 hrs to load the isotopic standards. Loaded fibers were wiped dry with Kimwipe™ tissues and stored at 4°C overnight prior to use. Water samples

extracted by SPME were simultaneously agitated in triplicate by mounting the stainless steel shafts (with packing tape) of three fibers to the wooden shaft of a dissecting probe secured in the chuck of a 12V battery operated drill (Ryobi, China), which provided constant agitation at 550 rpm for 10 min. Fibers were then removed from the sampled water, cut from the dissecting probe, and wiped dry with a Kimwipe™ prior to transfer into 100 µL of HPLC grade methanol in a 2 mL amber glass vial containing a 150 µL polypropylene insert. Sampled SPME fibers were initially placed in vials on ice, but upon returning to the lab, analytes extracted by each fiber were desorbed in methanol with agitation at 2000 rpm for 1 h. Fibers were then removed from vials using forceps and an additional 50 µL of methanol containing 225 µg/L lorazepam and chloramphenicol was added into the sample followed by agitation at 1000 rpm for 1 min to ensure mixing. Extracts were stored at -20°C until analyzed by LC-MS/MS.

2.2.6 Sampling Living Fish Using SPME

Greenside Darter and Fathead Minnow exposures to the 3 µg/L analyte mixture (Table 2.1) were conducted at the University of Waterloo using de-chloraminated municipal water, while Rainbow Trout exposures to Guelph and Waterloo's MWWEs were conducted at the University of Guelph's Hagen Aqualab using unmodified campus wellwater as both the MWWE diluent and for control fish. All fish procedures

were approved by local Animal Care Committees at both institutions (University of Waterloo AUPs#04-24, 07-16, and 08-08). Fish were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma-Aldrich, Oakville, ON) prior to a 20 gauge guide needle being gently inserted into the dorsal-epaxial muscle of the fish. A PDMS fiber (1 cm long) pre-loaded with deuterated surrogates was inserted into the same hole made by the needle for 20 min, and fish were placed in clean water to recover from the anaesthetic (<3 min). After 20 min, fish were re-anesthetized and fibers were removed using clean forceps and wiped dry with a Kimwipe™. Fibers were desorbed and stored in an identical manner as described for SPME water samples.

2.2.7 Lab Exposure of Fish to Chemical Mixture

Laboratory exposures of Greenside Darter, Fathead Minnow, and Rainbow Trout to a 3 µg/L mixture of selected contaminants (**Table 2.1**) spiked to clean water were conducted to determine the distribution coefficients between SPME fibers and fish muscle (Kfm) for each fish species, as well as distribution coefficients between SPME fibers and water (Kfw). Gemfibrozil, ibuprofen, carbamazepine, diclofenac, naproxen, and bisphenol A were purchased from Sigma-Aldrich (Oakville, ON). Fluoxetine and atrazine were purchased from Cerilliant Corp (Round Rock, TX). Stock solution were prepared in methanol and stored at -20°C. Greenside Darters were collected by

backpack electroshocking from the Eramosa River (43^{o}32’50” N; 80^{o} 11’49”W) using a Smith-Root 12A-POW unit (Smith-Root Canada, Merritt, BC), which temporarily stuns, but does not harm, captured fish. Three aquaria were setup containing 12 Greenside Darter per aquaria (one control aquaria and two chemical-exposure aquaria containing the 3 µg/L analyte mixture) and fish were exposed for a period of 8 d (with water renewals every 2 d) prior to SPME sampling to ensure adequate time to bioconcentrate the chemicals had elapsed. Each aquarium containing Greenside Darter were filled with 34 L of de-chloraminated municipal tap water, either with or without the chemical mixture. For the Rainbow Trout exposure, immature fish were obtained from Silvercreek Aquaculture (Erin, ON) and acclimated in the UW Wet Lab prior to the onset of the experiment. Four Rainbow Trout aquaria were set, each containing 3 fish (1 control aquarium and 3 chemical-exposure aquaria at 3 µg/L) and all 12 fish were exposed for a period of 8 d (with water renewals every 2 d). Fathead Minnow were obtained from Silhanek Baitfish (Bobcaygeon, Ontario) and were exposed to the chemical mixture for 6 d only, due to unexpected mortalities. Six 34 L aquaria were initially set containing 6 Fathead Minnows per tank (2 control tanks and 4 exposure tanks at 3 µg/L).

After exposing Rainbow Trout and Greenside Darter to the chemical mixture (nominal 3 µg/L) for 8 d, fish were *in vivo* extracted by SPME for 20 min, 1 d and 2 d on day 9-11. Fathead Minnows were only exposed to the SPME for 20 min due to

their smaller size and vulnerability. A small portion of fish fillet (dorsal-epaxial muscle) was surgically removed from each fish for comparison with the liquid extraction approach. Fish exposure aquaria water was extracted in triplicate (500 mL) by SPE and also by SPME using the drill mounted approach described previously. Exposure aquaria water from the Fathead Minnow exposure was used for Kfw determination, while water from the Greenside Darter exposure were extracted by both SPME and SPE to assess the precision and accuracy of SPME for water analysis.

2.2.8 Wild Fish Collection

To evaluate the reproducibility of fish SPME, contaminants in wild Greenside Darter were quantitated using distribution coefficients between fibre and fish muscle (Kfm) determined during the laboratory Greenside Darter exposure to the chemical mixture (**Table 2.1**). Wild Greenside Darters were collected from the Grand River downstream of Kitchener wastewater treatment plant outfall (43°23'53.65"N; 80°24'56.32"W) on August 18, 2008 and were sampled by both *in vivo* SPME and SLE to compare the closeness of results obtained from both techniques.

2.2.9 Determination of Lipid Content in Fish Muscle

The lipid content of fish muscle for each species was determined using the

method developed by Bligh and Dyer (1959). Each 2 g sample of tissue was homogenized at 540 rpm by a Craftsman 10-in Drill Press (Model No. 137 28007) for 2 min in a mixture of 2 mL of HPLC grade chloroform (Fisher Scientific Unionville, ON) and 4 mL of methanol. To this mixture, an additional 2 mL of chloroform was added and mixed for 30 sec, followed by 2 mL of and another 30 seconds of vortexing. The resultant homogenate was filtered through Whatman No. 1 filter paper under slight vacuum into a 25 mL graduated cylinder. After a period to achieve separation of the two phases (~ 15 min), the volume of the chloroform layer was recorded and the alcoholic layer removed by aspiration. The residue and filter paper were mixed with 20 mL of chloroform, and rinsed with an additional 10 mL of chloroform. Three 15x85 mm culture tubes (Fisher Scientific #14-961-28, Unionville, ON) were tared prior to adding the lipid extract, which was subsequently evaporated to dryness under a stream of nitrogen in a Dionex SE 500 solvent evaporator (P/N 063221, 120 v, Salt Lake City, UT). The residue brought to room temperature over phosphoric anhydride in a vacuum desiccator. The dry weight of the residue was determined and subtracted from the initial weight and the lipid content of the sample was calculated as follows:

$$\text{Total Lipid} = \frac{\text{weight of lipid in aliquot} \times \text{volume of chloroform layer}}{\text{volume of aliquot}}$$

2.2.10 Determination of pH in Muscle

The pH of fish muscle was determined using the method described by (Arashisar S. 2008). Approximately 0.02 g of fish muscle was combined with 2 mL distilled water and homogenized for 2 min. The homogenate pH was measured using a Schott model pH meter (Schott, Lab Star pH, Mainz, Germany).

2.2.11 Controlled Lab Exposures to Municipal Effluents

The bioconcentration potentials of the target compounds (**Table 2.1**) were determined in Rainbow Trout exposed to municipal wastewater effluents from the cities of Guelph and Waterloo during the summer of 2009 using 48 h static renewals over a 14 d exposure interval. The cities of Guelph and Waterloo are urban centers in the central portion of the Grand River watershed. Both WWTPs process domestic, institutional, commercial and industrial wastewater. The Guelph WWTP collects wastewater from the city of Guelph, as well as the community of the Township of Guelph/Eramosa (Village of Rockwood), serving a population of 118,000 (City of Guelph Wastewater Treatment Plant 2003). The Guelph WWTP provides tertiary treatment including preliminary screening, grit removal, sedimentation, secondary treatment by conventional and extended aeration activated sludge, two stage tertiary treatment utilizing rotating biological contactors, sand filtration, seasonal sodium hypochlorite disinfection, and sodium bisulphite dechlorination (City of Guelph

Wastewater Treatment Plant 2003). The Waterloo WWTP collects wastewater from the City of Waterloo serves a population of 121,700It consists of a regular secondary treatment system using conventional activated sludge and sodium hypochlorite disinfection(Regional Municipality of Waterloo 2008). Exposure troughs containing 54 exposure aquaria were constructed so collected MWWEs could be pre-chilled 12 h prior to water changes by having two aquaria tanks alternately holding the same group of fish. Each 34 L aquaria contained six Rainbow Trout exposed to control water, 20% or 90% MWWE in triplicate aquaria (note that an additional 50% exposure was added for the Waterloo effluent exposures) for intervals of 2, 8, and 14 d with static renewals every 2 d. Tanks were held in a water bath to maintain a constant nominal temperature of 15ºC and aerated to achieve water quality parameters consistent with good husbandry practices for this species. Final effluents used for exposures were collected immediately following the disinfection stage at each plant and immediately transported to the University of Guelph Aqualab where all Rainbow Trout exposures were conducted. At the end of each exposure interval, two fish per aquaria were anaesthetized and sampled for target analytes by *in vivo* SPME for 20 min. Therefore, concentrations of selected contaminants in fish at each sampling interval could be quantitative determined to monitor the dynamic bio-accumulation process. After removing fish from tanks, 2 L of exposure water were taken from each tank and extracted with 3 SPME fibers for 10 min as described earlier. Three additional water samples (1500 mL) were extracted by SPE.

2.2.12 Statistical Analysis

All analyses were performed using SPSS software version 18.0. Data were checked for homogeneity of variance and normality prior to analysis. For data that met these criteria, all statistical differences ($p < 0.05$) were detected by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc t-test. When more than two species or exposures were analyzed, differences were detected by Tukey's HSD. Nonparametric tests (Kruskal–Wallis H-test, followed by Mann–Whitney U-test) were performed on data that failed assumptions of normality and homogeneity of variance after log transformation. Statistical differences in contaminant concentrations in water and in fish determined by Tukey's HSD are indicated in the figures by different lower case or upper case letters plotted above standard derivation bars.

2.3 Results

2.3.1 Controlled Laboratory Exposure to Chemical Mixture

Rainbow Trout and Greenside Darter exposed to a chemical mixture of target analytes (**Table 2.1**) at a nominal concentration of 3 µg/L for 6-8 d were extracted by *in vivo* SPME for intervals of 20 min, 1d and 2 d on exposure days 9 through 11. Extraction time profiles for PDMS fibres were investigated to establish the time required for each analyte to achieve equilibrium between the fish muscle and the fiber. For both fish species, the time required to reach equilibrium varied by analyte from less than 20 min to more than 2 d. The protracted time required by some compounds to equilibrate with the fiber indicates that equilibrium SPME is not a realistic approach for field or on-site sampling (**Figure 2.2 (a**, **b)**). Higher amounts of fluoxetine, naproxen and atrazine were extracted from tissues of both fish species on day 11 ($P < 0.0001$), indicating either the properties of the PDMS fiber coating was more efficient in extracting these compounds, or the concentrations of these analytes in fish muscle tissue was relative high. Consequently, total analyte concentrations in fish muscle and partition coefficients between the fiber and fish tissue were later determined and compared.

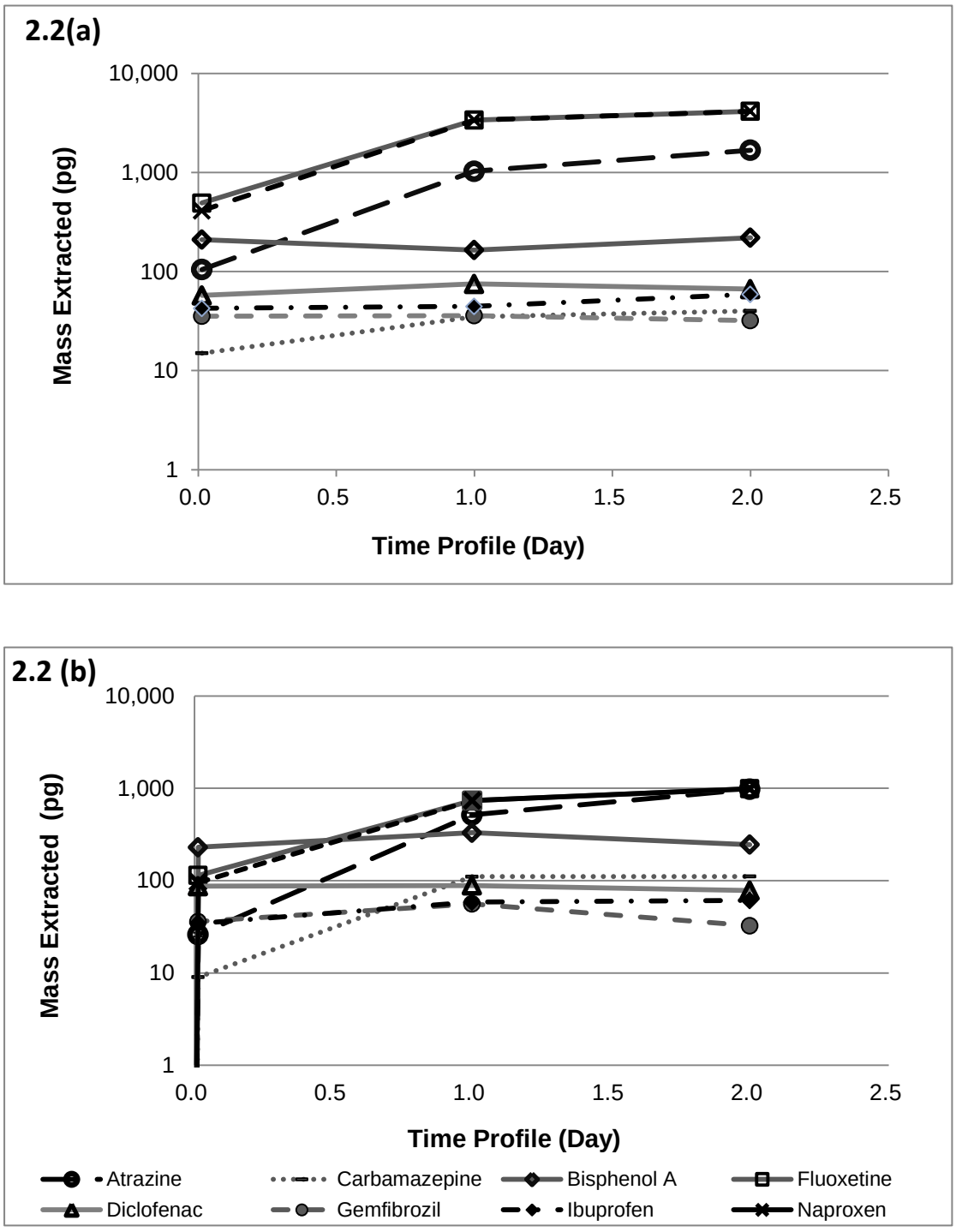

Figure 2.2 Extraction time profile of target analytes from muscle of Rainbow Trout (a) and Greenside Darters (b) exposed to chemical mixture (3 µg/L nominal) using SPME. n = 3.

After *in vivo* extraction of fish by SPME, fish tissues were extracted by solid-liquid extraction (SLE). SLE is a widely used extraction technique for tissue samples which allows soluble components to be removed from solids (tissues) using a liquid solvent. After *in vivo* extraction of fish by SPME, excised portions of fish muscle were extracted by SLE *in vitro* to determine the total concentrations of target analytes in all muscle of three fish species. Fathead Minnow with the smallest body size among the three fish species accumulated more carbamazepine ($P < 0.0001$), ibuprofen ($P < 0.0001$), and bisphenol A ($P < 0.003$) than the other two larger fish species did. As fish were pre-exposed to the chemical mixture for 8 d, most analytes would have achieved their steady states in the muscle tissues, as evidenced by relatively constant analyte concentrations over days 9 through 11 ($P > 0.65$) (**Table 2.2**).

Table 2.2 Analyte concentrations in fish muscle as determined by solid-liquid extraction

Species	**RBT**				**GSD**				**FHM**	
Method	**SLE (ng/g)**				**SLE (ng/g)**				**SLE (ng/g)**	
Days of Exposure	**8 d**	**9 d**	**10 d**	**Recov (%)**	**8 d**	**9 d**	**10 d**	**Recov (%)**	**6 d**	**Recov (%)**
Carbamazepine	2.84±0.35	2.56±0.24	2.27±0.31	47.7	4.46±0.65	4.90±0.57	5.01±0.54	50.5	156±20	54.4
Naproxen	1.86±0.47	1.62±0.43	1.30±0.36	73.3	1.46±0.34	1.32±0.28	1.38±0.31	78.8	1.14±0.02	78.8
Atrazine	1.69±0.23	1.38±0.11	1.31±0.21	46.5	1.38±0.43	1.54±0.38	1.76±0.41	45.3	1.63±0.23	53.9
Ibuprofen	1.14±0.13	1.18±0.12	1.05±0.11	78.4	1.25±0.13	1.26±0.12	1.24±0.15	79.1	275±16	71.4
Diclofenac	4.82±0.13	4.46±0.14	4.80±0.13	59.9	2.63±0.04	2.58±0.02	2.71±0.05	66.7	0.37±0.02	58.5
Gemfibrozil	0.98±0.32	0.95±0.28	0.90±0.25	76.4	0.58±0.16	0.69±0.12	0.53±0.14	94.7	0.32±0.03	75.8
Fluoxetine	150±20	175±26	181±23	58.4	55.7±2.4	54.3±2.1	57.2±1.8	61.3	32.5±0.8	83.4
Bisphenol A	83.5±0.4	47.7±0.5	34.5±0.3	74.3	52.4±1.8	34.7±2.1	32.2±2.3	81.6	145±26	74.0
N	3	3	3	9	3	4	3	10	11	11

RBT: Rainbow Trout; GSD: Greenside Darter; FHM: Fathead Minnow; SLE: solid-liquid extraction; Recov: Recovery of SLE

Bioconcentration factor (BCF) was calculated as the ratio of the chemical concentration in the organism (fish) to that in the surrounding water. Results indicated that potentials for all selected compounds to bioaccumulate in muscle of all three fish species are relative low (BCF < 266) (**Table 2.3**). BCF values were 0.59-266 for Rainbow Trout, 0.34-94.1 for Greenside Darter, and 0.19-109 for Fathead Minnows. Generally, BCF values of compounds with higher pKa values (fluoxetine, bisphenol A, carbamazepine) were higher than compounds with lower pKa. In addition, for compounds with LogKow ≥ 4.02 (e.g. diclofenac, gemfibrozil and fluoxetine), higher BCF values were often found in lipid-rich fish (Rainbow Trout) (**Table 2.3, 2.4**) ($P < 0.001$). The tissue pH values of the three fish species range from 6.68 to 7.58. Compounds with relatively high pKa (≥ 4.9) (e.g. ibuprofen, bisphenol A and carbamazepine) were more bioaccumulative in fish muscle with high pH (Fathead Minnow) ($P < 0.003$).

Table 2.3 Bioconcentration factors (BCF) of target compounds in muscle of fish

Species			RBT	GSD	FHM
Compound	**pKa**	**LogKow**	**BCF**		
Carbamazepine	13.4	2.25	2.20±0.31	4.38±0.58	32.8±0.3
Naproxen	4.15	3.10	1.58±0.16	1.36±0.34	0.52±0.01
Atrazine	1.7	2.82	1.37±0.22	1.36±0.40	0.78±0.12
Ibuprofen	4.91	3.79	1.21±0.14	1.37±0.15	57.2±3.3
Diclofenac	4.15	4.02	54.8±1.5	29.9±0.4	1.37±0.06
Gemfibrozil	4.7	4.77	0.59±0.19	0.34±0.09	0.19±0.02
Fluoxetine	10.1	4.65	266±34	94.1±23.9	16.5±3.2
Bisphenol A	9.59-11.30	3.41	55.9±9.3	39.9±1.8	109±20

pKa (acidic dissociation constant) values were searched from literatures (Cousins I.T. 2002, Kwon and Armbrust 2008, Packer et al. 2003, Queiroza et al. 2008, Radjenović et al. 2009, Shaner et al. 2007) Values of LogKow were obtained from databank of Syracuse Research Corporation (DEOWPC, 2010)

Table 2.4 Fish data and water chemistry for chemical mixture exposures

Species	RBT	GSD	FHM
Total Lipids (%)	2.61±0.01 (n=3)	2.28±0.02 (n=11)	1.33±0.03 (n=15)
Length (cm)	25.2±1.5	6.5±0.2	5.0±0.3
Weight (g)	159.2±29.5	2.7±0.5	0.9±0.2
Muscle pH	6.7±0.1 (n=9)	6.9±0.1 (n=10)	7.6±0.1 (n=11)
Water pH	8.3 ± 0.2	8.3± 0.1	8.5±0.3
Dissolved oxygen (mg/L)	10.01± 0.24	8.42±0.41	8.66±0.25
Conductivity (µs)	1490 ± 50	1475 ± 65	1490±35

RBT: Rainbow Trout; GSD: Greenside Darter; FHM: Fathead Minnow;

Results showed that Kfm values of most selected contaminants (except for fluoxetine and bisphenol A) were different among three fish species so Kfm values must be determined independently for each fish species ($P < 0.0001$). Kfm values were 2.41-736 for Rainbow Trout, 4.30-266 for Greenside Darters, and 2.04-381 for Fathead Minnows. Overall, Kfw values (71-1636) were generally greater than the corresponding Kfm values (2.04-736) (**Table 2.5**). In addition, highest Kfm values were frequently seen in Fathead Minnows and the lowest Kfm values were often detected in Rainbow Trout. Since target analytes are chemicals with a wide range of pKa values, so Log D (is the ratio of the equilibrium concentrations of all species (unionized and ionized) of a molecule in octanol to same species in the water phase at a given temperature, normally 25° C.) was used to compare with corresponding Kfs values. Results showed Log D dropped as pH decrease (from 8.3 to 7) for all the selected compounds. A relationship Kfm/Kfw and Log D was not evident.

Table 2.5 Distribution coefficients between fibre/water (Kfw) and fibre/fish muscle (Kfm)

Analyte	pKa	Log D (pH = 8.3)	Log D (pH = 7)	Kfm (RBT)	Kfm (GSD)	Kfm (FHM)	Kfw (Water)
Carbamazepine	13.4	1.34	1.34	10.3	17.5	34.1	20.6
Naproxen	4.15	3.10	0.32	212	215	252	282
Atrazine	1.7	2.82	0.18	736	263	381	547
Ibuprofen	4.91	3.79	0.42	10.3	6.66	6.75	261
Diclofenac	4.15	4.02	0.32	3.82	7.29	29.3	1640
Gemfibrozil	4.7	4.77	0.40	8.49	12.8	6.32	71.0
Fluoxetine	10.1	1.97	1.94	23.6	15.4	29.5	1100
Bisphenol A	9.59-11.30	1.69	1.68	2.41	4.30	2.04	1090
N (SLE/SPME)				9/27	10/30	11/33	

RBT: Rainbow Trout; GSD: Greenside Darter; FHM: Fathead Minnow: Log D (pH dependent partition coefficient) values for acidic compounds were calculated using equation: Log D =1/[Log([AH]/[A^-] +1) + 1/Log Kow] while Log D values for basic compounds were calculated Log D =1/[Log([B]/[BH^+] +1) + 1/Log Kow].

Concentrations of analytes determined by SPME and SPE were statistically the same ($P > 0.06$) (**Figure 2.3 (a)**). Results of wild fish sampling showed that eight target analytes were detected in low concentrations in wild Greenside Darters by SLE and five of compounds were detected by SPME **(Figure 2.3 (b)**). For compounds detected by SPME, results of SPME and SLE are statistically the same as well ($P > 0.15$). Gemfibrozil, bisphenol A, and fluoxetine were not extracted from wild fish likely due to their low Kfm values and possibly because their free concentrations in fish muscle are low as well (total concentrations determined by SLE were low). The standard deviations observed in results of wild fish were relatively high, likely due to differences in mobility and diet between captured wild fish.

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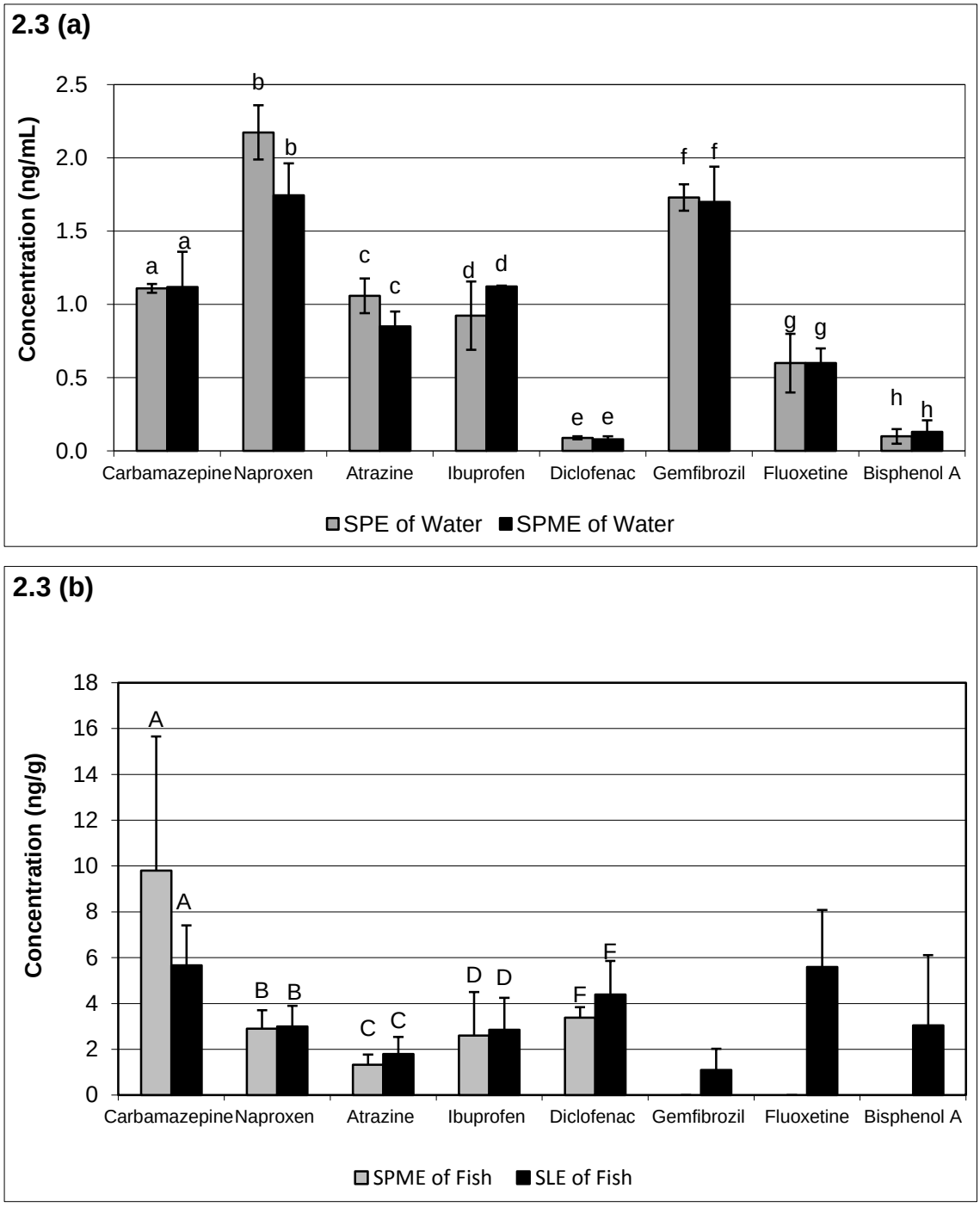

Figure 2.3 (a) Comparison of concentrations of target analytes in water determined by SPME and SPE; Figure 2.3 (b) Comparison of concentrations of analytes in muscle of wild Greenside Darter determined by SPME and SLE

n (fish SPME) = 7; *n* (fish SLE) = 21;Fish sampling time: July, 2008; Fish data: Length (cm): 8.2±0.4; Weight (g):6.2±0.9

2.3.2 Controlled Lab Exposures to Municipal Effluents

Since SPME has been validated as an efficient tool for extraction of selected contaminants from fish and water, it was then applied to detect the waterborne and fish muscle accumulated concentrations of contaminants from sewage effluents. Four selected contaminants including ibuprofen, naproxen and bisphenol A were detected in effluents and the same compounds were also identified in Rainbow Trout exposed to effluent from Guelph and Waterloo (**Table 2.6, 2.7**). Bisphenol A is mainly used for manufacturing plastics and gemfibrozil is a lipid regulator. Ibuprofen and naproxen are active ingredients in non-steroidal anti-inflammatory drugs. Results showed higher concentrations of selected contaminants (e.g. ibuprofen, gemfibrozil) detected in effluents from Waterloo WWTP compared to that of the Guelph WWTP (**Table 2.6**, **2.7**) ($P < 0.002$). The concentrations in the fish were generally related to the exposure concentrations in the effluents in a dose dependent manner (**Table 2.6**, **2.7**). Gemfibrozil concentrations declined during both effluent exposures in a similar manner. This suggests that the fish may have been depurating this chemical from a previous exposure (food or water) rather than accumulating the chemical during the experimental exposures. Bisphenol A was initially detected in fish exposed to City of Guelph's effluent on day 2, but was depurated very quickly as the exposure concentration declined (**Figure 2.5**). Bisphenol A bioaccumulated in fish very quickly, which could be related to its relatively high BCF as well as its low degree of ionization at basic pH, and it was then depurated by fish as its effluent concentrations

dropped off to zero. Ibuprofen bioaccumulated in fish muscle during the exposure, even though the effluent concentrations varied over time (**Table 2.6**, **2.7**, **Figure 2.4, 2.5**). Although naproxen was detected in the effluents of Waterloo, it did not bioaccumulate in fish tissue, likely due to its low BCF and the high ionization degree at basic pH.

In the Guelph effluent exposure, the effluent concentrations varied considerably during the exposure period (**Figure 2.4**), likely due to changes occurring in the treatment plant. In contrast, concentrations of contaminants in the effluents of the Waterloo WWTP were much more consistent over time (**Table 2.6**, **Figure 2.5**). Contaminant concentrations in the fish reflected changes in effluent water quality as steady state was quickly achieved (< 8 d) for most compounds (**Table 2.2**).

Table 2.6 Concentrations of selected analyte in Guelph's sewage effluent exposures and in muscle of exposed Rainbow Trout determined by SPME and SPE

Days of Exposure	% Effluent	Bisphenol A			Ibuprofen			Gemfibrozil		
		Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)	Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)	Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)
Day 2	0%	N/D	N/D	N/D	N/D	11±2	N/D	N/A	N/A	4.36±3.33
	20%	95.7 ± 4.2	80±16	180±12	56.2 ± 0.2	56.2±20.7	2.06±0.01	N/A	22.6±0.5	21.3±9.1
	90%	167.5 ±3.6	172±80	93.6±16.6	112 ± 1	92.3±23.3	6.01±0.01	73.2 ± 29.6	110±21	25.4±1.2
Day 8	0%	N/D	N/D	N/D	N/D	14.2±2.7	N/D	N/D	N/A	0.974±0.482
	20%	N/D	N/A	N/D	77.2± 3.3	41.1±16.0	5.66±1.80	N/A	N/A	0.551±0.184
	90%	N/D	45.0±33.8	N/D	90.9±1.7	86.7±18.3	10.1±4.0	N/A	N/A	2.11±1.67
Day 14	0%	N/D	N/D	N/D	N/D	16.7±2.2	N/D	N/D	N/A	N/D
	20%	N/D	N/A	N/D	59.1 ± 0.6	44.9±7.5	9.58±0.16	N/D	N/A	0.265±0.101
	90%	N/D	N/A	N/D	131 ± 2	134±33	11.3±0.5	N/D	N/A	1.35±0.12

pH: 8.79±0.25; Temperature: 11.7±0.2; Dissolved oxygen: 10.50±0.75 mg/L; n (fish SPME) =6, n (water SPME) = 9; Fish data: Length (cm): 13.7±1.0; Weight (g): 23.4±4.4; N/D: not detected; N/A: concentration of the compound was not reported

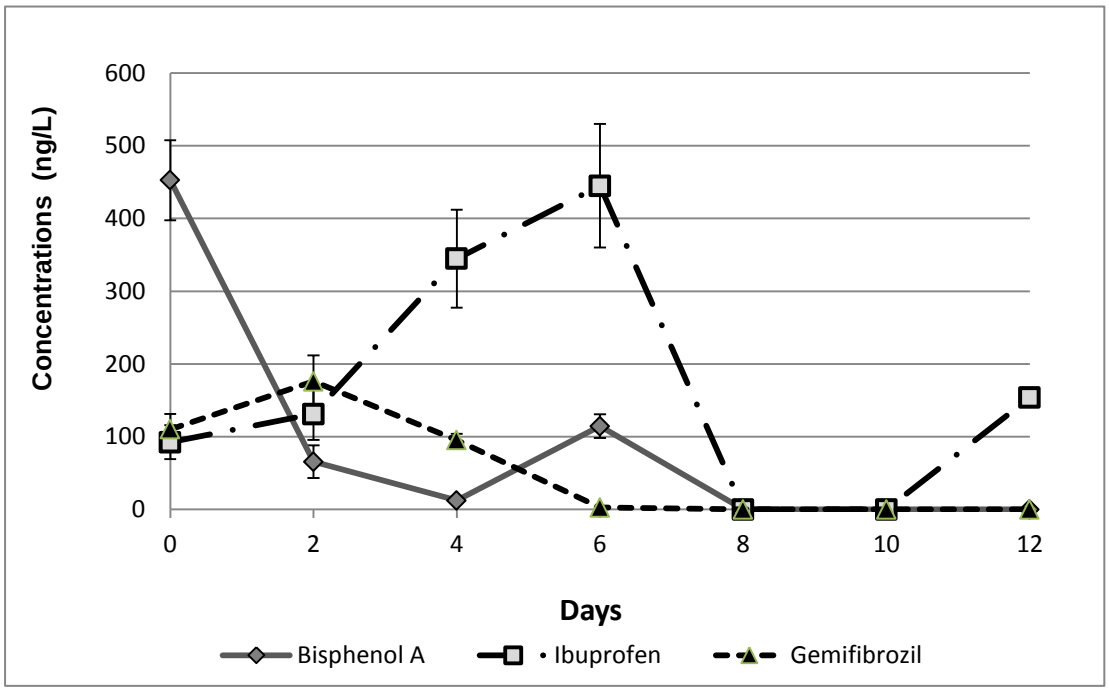

Figure 2.4 Concentrations of selected analytes in City of Guelph's 100% effluents collected on different days.

Table 2.7 Concentrations of selected analytes in Waterloo sewage effluent exposures and in muscle of exposed Rainbow Trout

Days of Exposure	% Effluent	Ibuprofen			Gemfibrozil			Naproxen		
		Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)	Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)	Water SPME (ng/L)	Water SPE (ng/L)	RBT SPME (ng/g)
Day 2	0%	N/D	45.1±13.6	N/D	N/A	N/A	2.74±0.90	N/D	N/A	N/D
	20%	N/A	72.2±16.8	N/D	30.4±28.5	32.9±10.4	2.38±0.63	74.6±18.5	74.6±25.5	N/D
	50%	N/A	162±33	N/D	128±15	116±5	8.07±2.21	185±83	157±28	N/A
	90%	N/A	231±25	6.99±0.11	174±12	139+27	2.39±0.52	597±12	651±98	N/A
Day 8	0%	N/A	27.4±4.5	N/D	28.1±12.3	N/A	0.83±0.02	N/D	N/A	N/A
	20%	265±10	110±80	N/D	31.3±4.5	52.3±15.5	0.88±0.39	145±59	80.4±8.9	N/A
	50%	398±12	383±88	2.29±0.07	139±37	150±11	0.85±0.31	510±36	401±98	N/A
	90%	424±10	397±81	10.8±0.7	133±13	128+29	0.88±0.05	731±48	726±14	N/A
Day 14	0%	N/A	65.2±17.0	N/D	N/D	N/D	N/D	N/D	N/D	N/D
	20%	259±7	231±34	N/A	62.5±20.9	22.4±6.5	2.65±0.55	57.9±7.2	27.4±5.4	N/A
	50%	274±18	300±29	10.2±0.2	128±12	146±15	1.39±0.48	305±36	339±65	N/A
	90%	496±3	N/E	No fish	142±1	N/E	No fish	731±49	N/E	No fish

pH: 8.65±0.25; Temperature: 12.3±0.2 ° C; Dissolved oxygen: 10.50±0.75 mg/L;n (fish SPME) =6, n (water SPME) = 9; Fish data: Length (cm): 13.7+1.0; Weight (g): 23.4+4.4; n (fish SPME) = 6, n (water SPME)=3; n (water SPE) =3; N/D: not detected; N/A: not reported because concentration of the analyte is below method detection limit. N/E: not extracted.

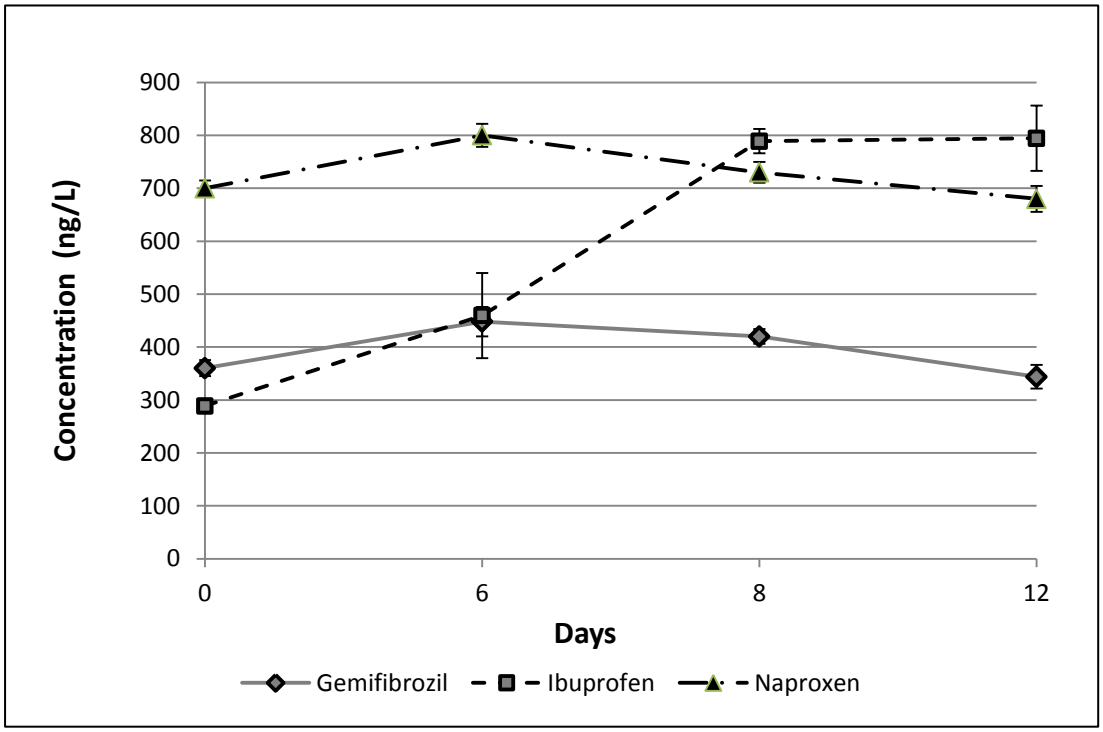

Figure 2.5 Concentrations of selected analytes in City of Waterloo's 100% effluents collected on different days. n (water SPE) = 9

2.4 Discussion

In controlled laboratory exposures to chemical mixtures, *in vivo* SPME was able to extract a selected group of emerging contaminants of concern from the tissues of several fish species with similar results to traditional monitoring methods (i.e. solvent extraction). SPME fibres were also very effective as an alternative to solid phase extraction (SPE), for extraction of water or municipal effluents, although the detection limits were slightly better for SPE. *In* vivo SPME was also effective at extracting the target analytes in fish exposed under laboratory conditions to treated municipal effluents. The chemicals used in these studies were relatively hydrophilic and therefore had low BCFs. In addition, because of their physical/chemical properties, their bioavailability is related to their extent of ionization (pKa) as well as hydrophobicity (Log Kow). The biological characteristics of the fish may also alter bioavailability, resulting in differences in the uptake into tissues as well as partitioning between the fish tissue and the SPME fibres (Kfs). However, with careful calibration, *in vivo* SPME can be used to measure trace levels of environmental contaminants and has many advantages compared to traditional extraction approaches.

In the chemical mixture used for exposure (nominal 3 µg/L), all target analytes were detectable in water using SPME and SPE as well as in all three fish species using *in vivo* SPME and SLE. Kfw values were statistically greater than the corresponding

Kfm values ($P < 0.0001$) suggesting PDMS fibre was more efficient in extracting contaminants from water than from fish. One explanation is that the pH of fish muscle (pH= 6.7-7.6) was lower than pH of water samples (pH = 8.3). Thus, the dependent octanol-water partition coefficients (Log D) in fish tissue were generally lower than that of in water which makes PDMS fibre less efficient in extracting target analyte from fish tissue than that from water. In addition, relative to water, lipids in fish muscle have a greater affinity and retention ability towards non-polar and lipid soluble compounds, which makes it more difficult for PDMS to extract the analytes, resulting in lower Kfs values. In addition, highest Kfm values were often seen in lipid-poor Fathead Minnows while lowest Kfm values were frequently detected in lipid-rich Rainbow Trout. Previous literature reported that the distribution coefficients in muscle tissue (low lipid content) were generally higher than adipose tissue (high lipid content) which consistent with results of this study (Zhang et al. 2010). Several studies have indicated that the distribution of non-polar analytes between PDMS fibre and the sample correlates with the pH independent octanol:water partition coefficient (Kow), and the lipid content of fish (Kopinke et al. 1995, Popp and Paschke 1997, Tuduri et al. 2001). However, since the eight target analytes with various functional groups are different in polarities, and hydrophobicities their Kfw or Kfm values may not follow a similar pattern. Expressing Kfs as a function of LogKow may therefore be misleading because it ignores the chemical properties and the nature of partitioning phenomena (Mackay and Seth 1999). Determining Kfs experimentally

therefore is important as actual Kfs values are currently difficult to predict. Kfs values must be determined for each species and Kfs may be varied slightly by sites and seasons (e.g. variations in lipid content, sex etc.). The distribution coefficients were determined for each fish species under the lab exposure conditions and applied to field experiments.

The physical/chemical properties of the chemicals and physiology of the fish may alter the relative bioconcentration factors. Bisphenol A and fluoxetine were detected at higher concentrations in muscle of three fish species than other selected contaminants examined using both SLE and *in vivo* SPME. A possible explanation for this observation may be the high pH (mean 8.3-8.5) of the water used for the chemical mixture laboratory exposure. Since the selected chemicals were chosen to span a wide range of pKa (1.7-13.4) and the degree of ionization of a compound is controlled by pH, the basic compounds (bisphenol A, carbamazepine, fluoxetine) would be present predominantly in the non-ionized form while very small portions of the acidic compounds would be present in the non-ionized form. The uptake of acidic or basic compounds is significantly affected by the degree of ionization as regulated by water pH (Hunn and Allen 1974, Hamelink and Spacie 1977, Call et al. 1980). Previous studies indicated that fish gills were much more permeable for the non-ionized form than ionized form of contaminants (Pärta 1989, Pärt et al. 1992). Thus, basic compounds (e.g. bisphenol A and fluoxetine) in this study are expected to be more

bioavailable than acidic compounds for fish exposed to the same total concentrations.

Rainbow Trout, Greenside Darter and Fathead Minnow accumulate the target analytes at different rates. The hydrophobic compounds with relatively high LogKow (e.g. ≥ 4.02) values were more bioaccumulative in lipid-rich fish. Other researchers have demonstrated that compounds with high LogKow have a relatively high potential to bioconcentrate because hydrophobic compounds are preferentially distributed to hydrophobic compartments such as lipid bilayers of cells (EPA 1999). The bioaccumulating potential of a hydrophobic contaminant is therefore positively related to the lipid content of fish tissue (Liu et al. 1996, Ossiander L. 2008, Zhang et al. 2010, Jahnke et al. 2009). For compounds with low LogKow (≤ 3.79 e.g. ibuprofen and carbamazepine), BCF values appear to be more dependent on muscle pH of the fish rather than lipid content. Relatively higher concentrations of carbamazepine and ibuprofen were detected in Fathead Minnows which had higher muscle pH compared to the other two fish species. This suggests that these compounds were possibly more bioavailable to Fathead Minnows for uptake and possibly more difficult to depurate. Fish with muscle soft texture have higher pH values compared to fish with firm texture (Dunajski 1979). Hence, the differences in muscle texture might lead to different bioaccumulation potentials of the same compound in muscle of different fish species. Additionally, factors such as sexual maturity and age, uptake and depuration kinetics, metabolic and diffusion rates, and

affinity of the chemical may all affect BCF values (ECETCO 1996).

In controlled lab exposures SPME compared well with SPE for extraction of the chemicals of interest from water. *In vivo* SPME also compared well with SLE, indicating that this new technique is suitable for quantitative determination of emerging contaminants in fish tissues. SPME has several advantages over SPE or SLE in extracting from complex matrices as it eliminates the need for extensive sample cleanup which substantially shortens analysis time. In addition, SPME fibres calibrated by preloading standards are less affected by complex sample matrices. Exposure of the SPME fibre to fish tissue did not impair (e.g. biofouling) the extraction ability (Zhang et al. 2010). SPME has the important advantage that it is suitable for *in vivo* monitoring of contaminants in live fish without the need for lethal sampling. Kinetic calibration of the SPME enhances accuracy and reduces variability by taking into account differences in environmental conditions, while presenting a minimal risk to the sampled organisms.

In municipal effluent laboratory exposures, SPME were very effective at extracting contaminants from municipal effluents and results of SPME were comparable with that of SPE. Concentrations of contaminants detected in the effluents of the Waterloo WWTP (secondary treatment) were generally higher compared to that of Guelph WWTP (tertiary treatment). Previous studies have demonstrated that

tertiary treatment eliminates additional contaminants compared to secondary treatment (Polar 2007). The removal of PPCPs, pesticides, and industrial chemicals from wastewater mainly occurs through abiotic transformation, biodegradation and sorption. Biodegradation and sorption to suspended solids are the main factors affecting the removal of PPCPs, although sources and composition of the wastewater, and operational parameters of the wastewater treatment facility, including biomass concentration, sludge retention time, pH and temperature of wastewater all modify removal of specific compounds (Cirja et al. 2008). For example, the removal of naproxen, ibuprofen, gemfibrozil, and diclofenac from MWWEs is reported to be mainly due to adsorption of those compounds to sludge particles present in the aeration treatment stages (Carballa et al. 2005). As the Guelph WWTP uses conventional and extended activated sludge treatment while Waterloo WWTP only has conventional activated sludge treatment, micropollutants would be expected to be removed from wastewater more extensively by the Guelph facilities. This may help explain why lower levels of ibuprofen and naproxen were detected in City of Guelph's effluents compared to those of the City of Waterloo's effluents. In addition, the Guelph WWTP provided higher solid retention time which may further improve the contaminant removal performance of the facility (Tartakovsky et al. 1996, City of Guelph Wastewater Treatment Plant 2007, Polar 2007, Zhang and Farahbakhsh 2007). Carballa et al.(2005) demonstrated removal of polar compounds with low LogKow values (e.g. ibuprofen and naproxen) from MWWEs were relatively low due to their

low rates of adsorption to the sludge. Further, compounds with complex structures (e.g. compounds with two aromatic rings such as bisphenol A, or alkyl chain branching such as . gemfibrozil) were more resistant to biodegradation processes (Kimura et al. 2005, Cirja et al. 2008). Consequently, compounds such as naproxen, ibuprofen, gemfibrozil, and bisphenol A would be predicted to be difficult to completely remove by contemporary treatment approaches, as evidenced by their detection in both Waterloo and Guelph MWWEs.

Several of the contaminants detected in municipal effluents were also identified in exposed fish by *in vivo* SPME. Ibuprofen, gemfibrozil, and bisphenol A were detected in Rainbow Trout by *in vivo* SPME, but at very low concentrations. This is due to their low concentrations in the effluents as well as their low BCF values in Rainbow Trout. However, ibuprofen was found to be bioaccumulated in fish muscle by *in vivo* SPME in a dose dependent manner in both effluent exposures. The gemfibrozil appears to have not bioaccumulated from the effluent exposure but was depurating a previous exposure as the concentration declined during the experiments. Bisphenol A was not detected in fish exposed to the Waterloo effluent. Bisphenol A was detected in Rainbow Trout exposed to 20% and 90% effluent of City of Guelph, but only on day 2. The effluent concentration varied considerably such that the exposure concentrations declined to below detection limits after day 2. Bisphenol A in fish reflected this pattern in exposure with concentrations declining from172 ng/g to

levels below detection limits after day 8. This result was consistent with results of chemical mixture exposure as contaminants approached steady states in fish very quickly. *In vivo* SPME was only able to detect a few selected contaminants in Rainbow Trout. Although several of the compounds (diclofenac, atrazine) were detected at very low concentrations by SPE (not SPME) in effluent they did not bioaccumulate to detectable levels using *in vivo* SPME. This could be due to low concentrations, low BCFs and the ionization of the compounds. Therefore, modification of SPME fibers are needed to improve the extraction efficiencies in the future experiments.

In conclusion, SPME has been validated as a simple, rapid and promising method for quantitative *in vivo* analysis of selected contaminants in fish (*in vivo*) as well as in surface waters impacted by municipal effluents. Although SPME is an excellent extraction tool, it has limitations. Environmental factors such as pH, salt content, biological molecules, organic matter, and temperature may affect the extraction efficiencies of PDMS fibers (Pan et al. 1995, Rodriguez et al. 2004, Sheu et al. 2006). In addition, pre-equilibrium SPME needs to be calibrated by fiber-loaded isotope surrogates which are not always available. Distribution coefficients between the SPME fiber and sampled matrix must be known *a priori* for kinetic calibration. Although some animals need to be sacrificed to determine the distribution coefficients, the number of animals which must be sacrificed is still far less than the number

required for monitoring by conventional extraction techniques (e.g. SLE). *In vivo* SPME has many potential applications for research and monitoring where animals cannot be sacrificed (threatened or recreationally important species), or to minimize the number and impact on animals needed in research or monitoring programs.

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Chapter 3: *In vivo* monitoring of emerging contaminants in wild fish exposed to municipal effluents using solid phase micro-extraction technique

This chapter has been prepared for submission to Environmental Toxicology and Chemistry with contributing authors Shuang Wang[1], Ken Oakes[1], Leslie Bragg[1], Janusz Pawliszyn[2], George Dixon[1], Mark R. Servos[1]

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Shuang Wang: M.Sc. candidate who researched, collected, analyzed and wrote the manuscript.

Dr. Ken Oakes: Provided valuable advice and assisted with lab exposures and field work.

Leslie Bragg: Provided expertise for developing and optimizing the LC/MS-MS method.

Prof Janusz Pawliszyn: Committee member and provided expertise and suggestions on SPME experiments.

Prof. George Dixon: Co-supervisor to Shuang Wang and provided valuable advice during the design and preparation of the research.

Prof Mark R. Servos: Co-supervisor to Shuang Wang and provided valuable advice during the design and preparation of the research

Summary

An *in vivo* solid phase micro-extraction (SPME) technique was applied to determine the bioaccumulation of selected emerging contaminants in fish exposed *in situ* to municipal effluents. Samples of effluent from three municipal wastewater treatment plants (2 secondary and 1 tertiary) in the Grand River watershed, Ontario, Canada, were analyzed for residues of selected pharmaceuticals, personal care products and industrial chemicals in three seasons. Wild Greenside Darters (*Etheostoma blennioides*) and Rainbow Darters (*Etheostoma caeruleum*) collected from areas near the three municipal effluent outfalls, as well as Fathead Minnows (*Pimephales promelas*) caged upstream and downstream of the outfalls, were analyzed for selected emerging contaminants using *in vivo* pre-equilibrium solid phase micro-extraction (SPME) calibrated by an in-fibre standardization technique. Effluents and surface water were extracted by SPME and solid phase extraction (SPE), followed by analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Several neutral and acidic compounds were detected in effluents at ng/L concentrations, including analgesic/anti-inflammatory agents, carbamazepine, gemfibrozil, and bisphenol A. Effluent concentrations differed among the three treatment plants and with seasons such that the highest concentrations were frequently found in summer (low flow) compared to that of spring and fall. Compounds detected in effluents were also identified in fish at low ng/g concentrations and generally reflected the effluent concentrations to which they were exposed. Concentrations of target analytes in river water and in exposed fish were related to the chemical and physical properties of compounds, treatment facilities, fish species, and environmental factors. *In vivo* SPME in fish was determined to be an effective approach for detection of contaminants in municipal effluents that may limit the need for lethal sampling.

3.1 Introduction

Solid phase micro-extraction (SPME) is a relatively simple and time efficient extraction method that integrates sampling, isolation and enrichment in one step (Pawliszyn 1997). It eliminates the use of organic solvents, substantially shortens the total time of analysis and allows convenient automation of sample preparation. SPME is very useful for analysis of a variety of chemicals in the environment, such as volatile organic compounds, semi-volatile organic compounds, amines, pesticides, herbicides, and organometallic compounds in a wide range of environmental matrices including air, water, soil and sediment samples (Nilsson et al. 1998, Gorlo et al. 1999, Basheer and Lee 2004, Centineo et al. 2004, Parkinson et al. 2004, Zimmermann et al. 2004, Dong et al. 2005, Dungan 2005, Kataoka 2005, Nakamura and Daishima 2005, Fidalgo-Used et al. 2006, Larroque et al. 2006). SPME has been recently utilized for *in vivo* analysis of human skin and noses, insects, beagles, and pigs, which not only allows extraction of trace amounts of analytes in the living organisms, but also creates the opportunity to study mechanisms of action in a more complex living biological system (Rochat et al. 2000, Tentschert et al. 2001, Tentschert et al. 2002, Djozan et al. 2004, Pionnier et al. 2004, Said et al. 2005, Anderbrant et al. 2005, Gilley et al. 2006, Lord et al. 2006, Musteata et al. 2006). Although the feasibility of *in vivo* analysis of living fish using SPME has been demonstrated (Zhang et al. 2010), it has not yet been validated fully in the field.

Micropollutants such as pesticides, industrial chemicals, pharmaceuticals and personal care products (PPCP) which arise from industrial, agricultural and urban sources are now widely detected in surface waters world-wide. Municipal wastewaters and agricultural runoff have been identified as substantial sources of these chemicals being released into aquatic environments (Ternes 1998, Halling-Sørensen et al. 1998, Daughton and Ternes 1999, Kolpin et al. 2002). In Canada, the presence of micropollutants in effluents and surface waters has been documented by several recent studies (Metcalfe et al. 2003, Brun et al. 2006, Lishman et al. 2006, Lissemore et al. 2006). Micropollutants, in their original or biologically altered form, are discharged into wastewater and make their way to municipal wastewater treatment plants (MWWTPs) where they are subjected to further transformation (Daughton and Ternes 1999). Unfortunately even modern wastewater treatment plants cannot completely remove these contaminants of concern from wastewater (Halling-Sørensen et al. 1998, Ternes 1998, Cirja et al. 2008). Therefore, aquatic organisms, including fish, are exposed to a variety of these contaminants of concern from MWWTP effluents for either their entire lifespan or during critical points within their life history (Fent et al. 2006).

Eight selected micropollutants (atrazine, bisphenol A, carbamazepine, fluoxetine, gemfibrozil, diclofenac, ibuprofen, and naproxen) were chosen as target analytes due to their ubiquitous distribution in river water and their potential to harm aquatic

species (Fent et al. 2006). Contaminants are accumulated by fish through direct uptake from water or from ingested food which is highly dependent on the physical chemical properties of the chemicals (Hamelink and Spacie 1977). These diverse chemicals selected for study can impact aquatic organisms through a wide variety of mechanism. Bisphenol A is an industrial chemical that has been widely used for making plastics. Studies have shown that bisphenol A is an endocrine disruptor as it can mimic the action of natural estrogen and cause effects such as an increase in the concentration of the yolk protein, vitellogenin, in male fish (Benjonathan N. 1998, Christiansen et al. 2000, Lindholst et al. 2000). Ibuprofen, diclofenac, and naproxen are used as non-steroidal anti-inflammatory drugs. Acute exposure to low levels of ibuprofen may induce heat shock protein 70, which works in defense against stressor-mediated toxicity in fish (Schwaiger et al. 2004, Gravel and Vijayan 2006). Schwaiger (2004) demonstrated that diclofenac can bio-accumulate in fish and cause histopathological organ lesions following long-term exposure (Hoeger et al. 2005). Although naproxen can be metabolized or degraded in the environment, the acute and chronic toxicity of its photo-degradation products in algae, rotifers and microcrustaceans have been shown to be significantly greater than naproxen itself (Isidori et al. 2005). Gemfibrozil is a lipid regulator which may elicit endocrine responses in fish by regulating blood lipid levels (Mimeault et al. 2005). Atrazine is a commonly used herbicide that may potentially alter normal endocrine, neuroendocrine and immune responses in fish (Suzawa and Ingraham 2008). Fluoxetine is a widely

prescribed antidepressants and it may induce ecotoxiclogical effects in aquatic organisms, such as aggressive behavior in crustaceans (Huber et al. 1997, Stanley et al. 2007). Carbamazepine, as a prescribed antieplieptic, has the potential to inhibit antioxidant enzyme activities of fish (Li et al. 2009).

The objective of this study was to apply and validate a kinetic calibrated pre-equilibrium *in vivo* SPME technique to monitor the bioaccumulation of the eight target compounds in fish exposed *in situ* to municipal effluents. Bioaccumulation in fish exposed to he effluents discharged from three municipal wastewater treatment plants (Guelph, Kitchener and Waterloo) in the Grand River watershed of southern Ontario was investigated using caged and wild fish exposed near the outfalls. The Grand River watershed ecosystems are considerably stressed from rapid urbanization and intensive agricultural development (Grand River Conservation Authority 2009). The current human population that exceeds 925,000 people is concentrated in the central section of the watershed and is predicted to continue to grow rapidly over the next two decades. Fathead Minnows (*Pimphales promelas*) were used in caging studies, while Greenside Darter (*Etheostoma blennioides*) and Rainbow Darter (*Etheostoma caeruleum*) as common fish species widely distributed in the Grand River watershed were collected from reference and exposed sites in the river. The spatial and seasonal variability of fish bioaccumulation determined using *in vivo* pre-equilibrium SPME was contrasted to water concentrations determined using both

SPME and conventional solid phase extraction (SPE) techniques.

3.2 Methods and Materials

3.2.1 Chemicals

Isotopically labeled standards (atrazine-d_5, bisphenol A-d_{16}, carbamazepine-d_{10}, diclofenac-d_4, fluoxetine-d_5, gemfibrozil-d_6, ibuprofen-d_3, and $^{13}C_1$-naproxen-d_3) were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Gemfibrozil, ibuprofen, carbamazepine, diclofenac, naproxen, and bisphenol A were purchased from Sigma-Aldrich (Oakville, ON, Canada). Fluoxetine, lorazepam, and chloramphenicol, atrazine were obtained from Cerilliant Corp (Round Rock, TX).

3.2.2 Sample Extraction

SPME Fish and Water Samples

Live fish and water samples were extracted by SPME on-site using a method adapted from Zhou et al. (2008) and Zhang et al. (2010). Details about SPME fibre preparation and methods of sample extraction are described in Chapter 2. Basically, a guide needle was first inserted into the dorsal-epaxial muscle of an anesthetized fish, then a SPME fibre with a 1.0 cm portion of PDMS tubing was inserted into the tissue. After extracting the tissue *in vivo* for 20 min, the fibre was removed and immediately desorbed in 50 μL methanol. Water samples collected at each site were extracted by five SPME fibres attached to a rod which was placed in a portable electrical drill for

agitation for 10 min. Fibres were then extracted with methanol, followed by addition of internal standards (lorazepam and chloramphenicol (225 µg/L).

Solid Phase Extraction of Water Samples

Three 500 mL bottles of water were collected from every site, preserved using sodium azide and ascorbic acid, and then extracted by solid phase extraction (SPE). Raw water samples were filtered through glass fiber filters (GF/F) to remove most of the solid particles. Deuterated surrogates (100 µL of 100 µg/L) were spiked into 500 mL of filtered water samples to correct for of matrix effects and to aid in quantitation of analyte concentration. Oasis HLB cartridges (#186000115, Waters, Milford, MA) were preconditioned sequentially with methyl-tert-butyl-ether (MTBE), methanol and HPLC grade water. Samples were then passed through to the cartridges under vacuum. The analytes retained on the cartridge were eluted with methanol and MTBE, evaporated to dryness under a gentile stream of nitrogen gas and reconstituted with a methanol solution of internal standards (75 µg/L of lorazepam and chloramphenicol). The final extract was transferred into 2 mL amber glass vials and stored in the freezer at -20 °C until analysis.

3.2.3 Instrumental Analysis

Instrument analysis was described previously in Chapter 2. An Agilent 1200

liquid chromatrography (LC) with a Finesse Genesis C18 column (150 × 2.1 mm, 4 μm, (Chromatographic Specialties Inc., Brockville, ON, Canada) was used for separation using a gradient of 5 mM ammonium acetate in water and methanol. Detection was done with an API 3200 Qtrap mass spectrometer (MS/MS) (Applied Biosystems MDS Sciex, Mississauga, ON, Canada) using electrospray ionization (ESI) in positive and negative ion mode and multiple reaction monitoring (MRM). Mass spectrometer response sensitivity and linearity were monitored before and after each set of experimental samples through the injection of 10 μL of a series of standards (0.5-500 ng/mL) prepared in methanol containing the internal standard, lorazepam (75 μg/L). External calibration curves were performed with good precision (RSD < 5%) and linearity (R^2 > 0.999). Analyst version 1.4.2 software (Applied Biosystems) was used for data collection and analysis. MDLs were determined by seven samples of concentrations near the expected limit of detection (**Table 3.1**) (Refer to Chapter 2 for details of calculation).

Table 3.1 Method detection limit (MDL) and instrument detection limit (IDL)

Analytes	Fish SPME MDL (ng/g)	Water SPME MDL (µg/L)	Water SPE MDL (µg/L)	IDL (µg/L)
Bisphenol A	15	0.05	0.025	10
Ibuprofen	1	0.03	0.01	1
Diclofenac	2	0.05	0.01	0.15
Gemfibrozil	0.5	0.05	0.01	0.15
Naproxen	1.5	0.05	0.01	0.1
Atrazine	2.5	0.2	0.01	0.025
Carbamazepine	2	0.01	0.01	0.05
Fluoxetine	10	0.01	0.01	0.2

3.2.4 Site Selection

The focus of this work was on sites adjacent to municipal effluent outfalls on the main branch of the Grand River as it flows through Waterloo and Kitchener, Ontario, on a major tributary, the Speed River near Guelph, as well as reference sites upstream in the Eramosa River (**Figure 3.1**). The characteristics of the treatment plants are summarized in **Table 3.2**. Waterloo and Kitchener WWTPs are secondary treatment plants that process wastewater with conventional activated sludge which integrates chemical phosphorus removal, anaerobic sludge digestion and sodium hypochlorite disinfection (Kitchener Wastewater Treatment Plant 2010, Regional Municipality of Waterloo 2004). Guelph WWTP provides complete tertiary treatment, including conventional and extended aeration activated sludge, two stage tertiary treatment utilizing rotating biological contactors (nitrification), sand filtration, seasonal sodium hypochlorite disinfection, and sodium bisulphite dechlorination (City of Guelph

Wastewater Treatment Plant 2003). Generally, in a conventional treatment plant (e.g. Waterloo and Kitchener WWTPs), influent is first treated by removing large particles from wastewater by mechanical screens, followed by sorption in a biological activated sludge tank, and finally separation by gravity sedimentation in an external clarifier. In contrast, in the tertiary treatment plant at Guelph WWTP, wastewater is also subjected to extended aeration activated sludge treatment, additional nutrient removal which incorporates nitrification processes for the removal of ammonia nitrogen, followed by chlorination, and sand filtration (Laws 2000, City of Guelph Wastewater Treatment Plant 2007).

Table 3.2: Description of municipal wastewater treatment plants for Guelph, Kitchener, and Waterloo (Guelph Wastewater Treatment Master Plan, 2009; City of Guelph, 2003; Conestogo Wastewater Treatment Plant, 2009; Waterloo Wastewater Treatment Plant, 2009; Region of Waterloo, 2009; Kitchener Wastewater Treatment Plant, 2009; Clara, 2005)

Parameters	**Guelph**	**Waterloo**	**Kitchener**
Population Served	118,000	121,700	190,000
Capacity m^3/day	55000	72,730	122,745
Discharge m^3/day	54,400	23,802	77,768
Secondary Treatment	Conventional and Extended activated sludge	Conventional Activated Sludge	Conventional Activated Sludge
Tertiary Treatment	Describe	None	None
Nitrification and Denitrification	Yes	No	No
Solids Retention Time	15-28 d	2 d	2 d
Combined Sewers	Sanitary wastewater and Storm or Surface water runoff	No	Some Foundation Drains
Disinfectant	Sodium Hypochlorite	Sodium Hypochlorite	Sodium Hypochlorite

3.2.5 Field Cage Exposures

Fathead Minnows from a commercial bait fisherman, Silhanek Baitfish (Bobcaygeon, ON), were caged in sewage effluents downstream and upstream of the Guelph, Waterloo and Kitchener (Doon) wastewater treatment plants in the fall of 2008. Five sites were chosen for fish caging adjacent to wastewater treatment plants (2 upstream and 3 downstream) as outlined in **Table 3.3**. Thirty fish were placed in two plastic minnow pails and these were then placed inside two inverted plastic laundry baskets that were attached together and anchored with concrete blocks at each site. Fish were caged in Guelph and Waterloo for 14 d, while fish caged at the Kitchener site were exposed to the effluent for 7 d.

Table 3.3 Locations for Fathead Minnow caging

Location	Site Description	Distance from Outfall (meter)	Dates of Sampling	GPS
Waterloo Caging	100% Effluent		Sept.26/08	43°28'46.26" N; 80°28'55.14" W
	WCUS1	860		43°29'0.40" N; 80°28'35.48" W
	WCUS2	580		43°29'1.09" N; 80°28'46.96" W
	WCDS1	350		43°28'39.01" N; 80°28'48.34" W
	WCDS2	920		43°28'28.26" N; 80°28'27.46" W
	WCDS3	1590		43°28'16.39" N; 80°28'2.364" W
Kitchener Caging	100% Effluent		Sept.23/08	43°24'3.38" N; 80°25'12.13" W
	KCUS1	4160		43°24'59.88" N; 80°25'1.91" W
	KCUS2	2800		43°24'30.11" N; 80°25'33.75" W
	KCDS1	24		43°24'3.34" N; 80°25'11.13" W
	KCDS2	70	Sept.24/08	43°24'3.54" N; 80°25'9.083" W
	KCDS3	240		43°24'0.44" N; 80°25'2.65" W
Guelph Caging	100% Effluent		Sept.22/08	43°31'18.40" N; 80°15'51.97" W
	GCUS1	20		43°31'18.74" N; 80°15'50.69" W
	GCUS2	33		43°31'18.74" N; 80°15'50.07" W
	GCDS1	19		43°31'17.80" N; 80°15'51.32" W
	GCDS2	33		43°31'17.33" N; 80°15'51.58" W
	GCDS3	60		43°31'16.53" N; 80°15'51.72" W

US: upstream; DS: downstream

3.2.6 Wild Fish Collections

Ten wild Greenside Daters and 10 Rainbow Darters were collected from each site, as identified in **Figure 3.1** and **Table 3.4** using a backpack electroschocker (Smith-Root Model 12, LR-24, or HT-2000). Fish of each species were extracted on site using *in vivo* SPME by holding the fish for 20 min in 6-8 L of river water in plastic buckets with an air stone. River water from each site was extracted with 5 SPME fibres on-site and an additional three bottles (500 mL) of water were collected and extracted by SPE.

One site upstream and one site further downstream of the Kitchener wastewater treatment plant were sampled in May of 2009, as well as in July of 2009. Fish and river water from the immediate upstream site and form one site further downstream of Waterloo wastewater treatment plant were sampled in August of 2009. Four sites upstream, including Rockwood, Eden Mills, Watson Road upstream of Guelph wastewater treatment plant and two sites in the downstream (Immediate downstream, Niska Road) were sampled in May of 2009. Watson Road, Gravel Pit, Immediate Downstream, and Niska Road were sampled in July of 2009. The dilution of effluent was calculated based on conductivities using the equation:

$$\% \text{ Effluent} = [\text{conductivity}_{(\text{downstream})} - \text{conductivity}_{(\text{reference})}]/[(\text{conductivity}_{(100\% \text{ effluent})} - \text{conductivity}_{(\text{reference})}]$$

3.2.3 Statistical Analysis

All data were checked for homogeneity of variance and normality prior to analysis. For parametric analysis data were tested for statistical differences ($p < 0.05$) by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc t-test. Nonparametric tests (Kruskal–Wallis H-test, followed by Mann–Whitney U-test) were performed on data that failed assumptions of normality and homogeneity of variance after log transformation. When more than two exposure sites were analyzed between sites, differences were detected by Tukey's HSD. Statistical differences in contaminant concentrations in river water and in fish between two exposure sites were determined by Tukey's HSD and are indicated in the figures by different lower case or upper case letters above standard derivation bars. All analyses were performed using SPSS software version 18.0.

Table 3.4 Information on sites selected for wild fish collection (2009)

	Waterloo Wild Fish Collection		**Kitchener Wild Fish Collection**		**Guelph Wild Fish Collection**				
Date	N/S	N/S	May 2009	May 2009	May 2009	May 2009	May 2009	May 2009	May 2009
Sites	N/S	N/S	**Upstream (KWUS)**	**Downstream 5%Effluent (KWDS)**	**Rockwood (GWUS1)**	**Eden Mills (GWUS2)**	**Watson Road (GWUS3)**	**10% Effluent (GWDS2)**	**Niska Road 5% Effluent (GWDS3)**
GPS	N/S	N/S	43°24'03.41"N 80°25'15.74"W (elv.920 ft)	43°23'53.67"N 80°24'56.37"W (elv.909 ft)	43°36'52.68"N 80°08'27.27"W (elv.1118 ft)	43°34'42.18"N 80°08'47.93"W (elv.1054 m)	43°32'49.79"N 80°11'50.07"W (elv.1018 ft)	43°31'16.51"N 80°15'51.78"W (elv.991 ft)	43°30'07.83"N 80°15'14.35"W (elv.990 ft)
Distance (m)	N/S	N/S	90	460	18750	13360	7000	70	3220
Date	August 2009	August 2009	July 2009	July 2009	N/S	July 2009	July 2009	July 2009	July 2009
Sites	**Upstream (WWUS)**	**Downstream 20% Effluent (WWDS)**	**Upstream (KWUS)**	**Downstream 17% Effluent (KWDS)**	**N/S**	**Watson Road (GWUS3)**	**Gravel Pit (GWUS4)**	**60% Effluent (GWDS1)**	**Niska Road 20% Effluent (GWDS3)**
GPS	43°28'53.18"N 80°28'55.37"W (elv.979 ft)	43°28'25.52"N 80°28'24.19"W (elv.977 ft m)	43°24'03.41"N 80°25'15.74"W (elv.920 ft)	43°23'53.67"N 80°24'56.37"W (elv.909 ft)	N/S	43°32'49.79"N 80°11'50.07"W (elv.1018 ft)	43°31'33.72"N 80°15'40.38"W (elv.1001 m)	43°31'18.26"N 80°15'50.45"W (elv.991 ft)	43°30'07.83"N 80°15'14.35"W (elv.990 ft)
Distance (m)	240	960	90	460	N/S	6700	550	24	3220

N/S: not sampled; Elv: elevation; Distance: distance from outfall

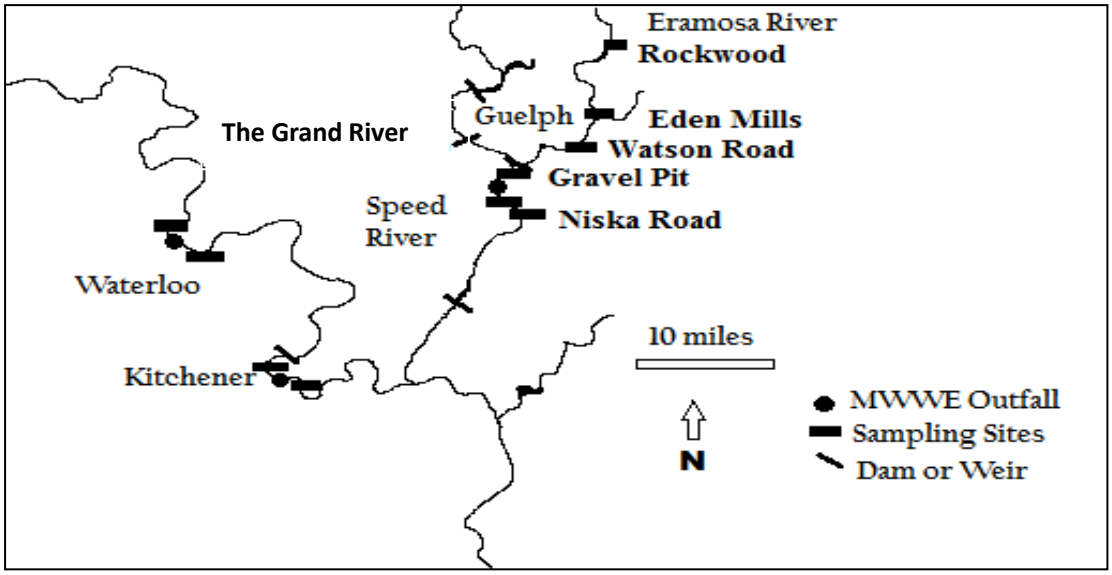


Figure 3.1 Sampling locations within the Grand River watershed.

3.3 Results

3.3.1 Controlled field cage exposures to municipal effluents

Based on conductivity, the exposure ranged from 15-50% at Waterloo, 10-60% at Kitchener, and 15-80% at Guelph. Surface water pH ranged from 7.8- 8.0 at Waterloo, 7.5-7.6 at Kitchener, and 7.2-7.9 at Guelph. Levels of dissolved oxygen (DO) were high at all sites Lengths and weights of Fathead Minnows selected for caging exposures were similar across sites (mean length 5.13-5.26 cm; mean weight 1.00-1.05 g) (**Table 3.5**). Fathead Minnows caged upstream of the of all three treatment plants outfalls all survived during the exposures (0% mortality). Fish caged downstream of Guelph WWTP had only 3% mortality at GCDS1 (80% effluent) and GCDS2 (50% effluent) and no mortality at the farthest downstream site (GCDS2). In contrast, fish caged downstream of the Waterloo outfall had relatively higher mortality of 13% and 40% at WCDS1 (50% effluent) and WCDS2 (25% effluent) respectively. Highest mortalities were seen in Kitchener exposure where fish caged at KCDS1 (60% effluent) and KCDS2 (30% effluent) had 100% mortality compared to 7% mortality farther downstream at KCDS3 (10% effluent).

Table 3.5 Water chemistry and fish data for Fathead Minnow caging

	Location	Exposure Days	Conductivity (µs)	Fraction of Effluent	pH	DO (mg/L)	WaterTemp.(°C)	# Fish	Length (cm)	Weight (g)	% Mortality
Waterloo	100% Effl.	0	4280	1.00	7.9	N/S	N/S	N/S	N/S	N/S	N/S
	US 1	14	1066	0.00	7.9	11.43	17.7	30	5.13±0.35	1.02±0.34	0
	US 2	14	1070	0.00	8.0	11.6	17.9	30	5.18±0.26	1.03±0.36	0
	DS 1	14	2680	0.50	7.7	9.6	18.3	30	5.25±0.22	1.02±0.27	40
	DS 2	14	1869	0.25	7.7	10.05	18.2	30	5.21±0.24	1.01±0.34	13
	DS 3	14	1507	0.15	7.8	10.34	18.3	30	5.26±0.23	1.02±0.32	0
Kitchener	100% Effl.	0	4630	1.00	7.6	N/S	N/S	N/S	N/S	N/S	N/S
	US 1	6	1250	0.00	7.6	9.11	15.9	30	5.16±0.22	1.01±0.34	0
	US 2	6	1262	0.00	7.6	9.3	16.6	30	5.20±0.31	1.03±0.24	0
	DS 1	6	3150	0.60	7.6	9.4	17.3	30	N/A	N/A	100
	DS 2	6	2160	0.25	7.6	10.23	17.0	30	N/A	N/A	100
	DS 3	6	1530	0.10	7.5	9.56	16.7	30	5.14±0.18	1.00±0.36	7
Guelph	100% Effl.	0	3787	1.00	7.1	8.91	19.2	N/S	N/S	N/S	N/S
	US 1	14	1173	0.00	7.9	10.81	16.3	30	5.30±0.12	1.03±0.34	0
	US 2	14	1156	0.00	7.6	10.01	16.3	30	5.24±0.26	1.02±0.28	0
	DS 1	14	3160	0.80	7.2	9.48	18.2	30	5.20±0.24	1.05±0.24	3
	DS 2	14	2450	0.50	7.4	10.25	16.5	30	5.23±0.23	1.03±0.14	3
	DS 3	14	1568	0.15	7.4	10.03	16.9	30	5.18±0.32	1.00±0.31	0

US: upstream; DS: downstream; N/S: not sampled; Effl.: effluent;

Three compounds were detected by SPME in water downstream of the Waterloo WWTP's outfall at low ng/L concentration (**Figure 3.2 (a)**). Concentrations of bisphenol A, ibuprofen, and carbamazepine in river water increased significantly at the first downstream site (WCDS1) ($p < 0.0001$) and then gradually declined further downstream (WCDS2, WCDS3). Levels of bisphenol A and carbamazepine detected at the furthest downstream site (WCDS3, 15% effluent) were still higher, compared to upstream river concentrations ($p < 0.0001$), while ibuprofen levels were similar between WCDS3 and WCUS2. Contaminant concentrations detected in Fathead Minnows were generally related to the concentrations detected in the in surface water where they were caged (**Figure 3.2 (b)**). Significant increases in concentrations of ibuprofen and bisphenol A were found in Fathead Minnows caged at WCDS1 ($p < 0.0001$ (ibuprofen); $p < 0.005$ (bisphenol A)) with a gradual decrease in concentrations further downstream (WCDS2 and WCDS3) as effluent exposure decreased. Levels of ibuprofen and bisphenol A in fish caged at WCDS3 were still statistically higher than concentrations in fish caged at upstream sites ($p < 0.0001$ (ibuprofen); $p < 0.01$ (bisphenol A)). Concentrations of carbamazepine in fish caged at WCDS1 and WCDS2 were higher ($p < 0.05$) than those in fish caged at upstream sites, but the standard derivations were relatively high, so a clear trend is not evident.

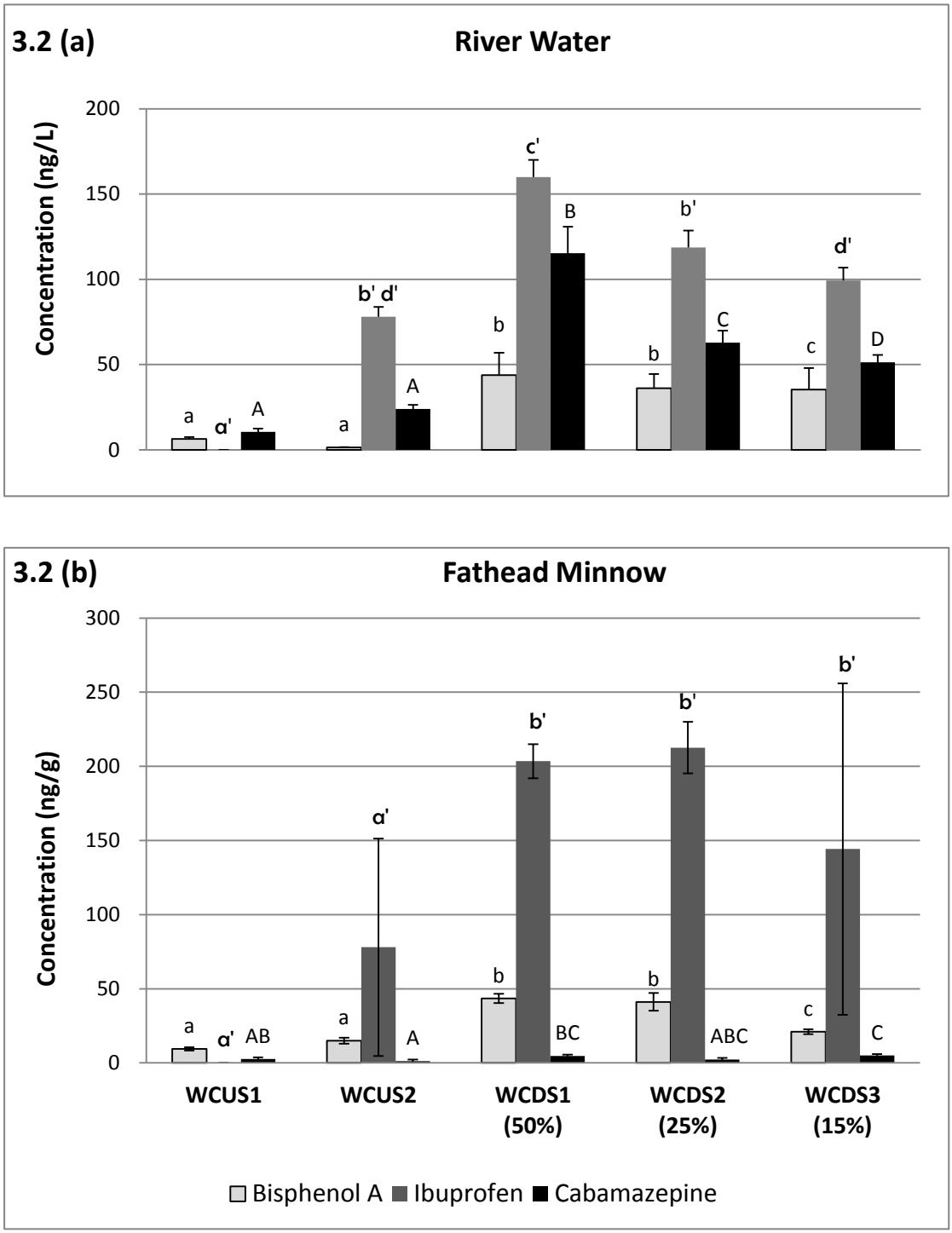


Figure 3.2 (a) Concentrations of selected analytes in river adjacent to Waterloo wastewater treatment plant (a) and in caged Fathead Minnows caged in the upstream and downstream of Waterloo wastewater treatment plant (b) determined by SPME. Sampling time: Fall 2008; 50%, 25%, and 15% are percent of effluent

All three sites (KCDS1, 2, 3) downstream of the Kitchener outfall all showed significant higher levels of ibuprofen compared to upstream sites ($p < 0.0001$). Carbamazepine and bisphenol A both have a trend of elevated concentrations in fish from the first downstream site (KCDS1) ($p < 0.0001$ for carbamazepine; $p < 0.002$ for bisphenol A) and then declining concentrations at KCDS2 and KCDS3 as the influence of effluent became less significant (**Figure 3.3 (a)**). Fathead Minnows caged in the first two downstream sites (KCDS1, KCDS2) had 100% mortality and therefore could not be sampled (**Figure 3.3 (b)**). Although effluent was highly diluted (< 10% effluent) at the furthest downstream site (KCDS3), caged fish showed statistically higher levels of bisphenol A and ibuprofen, compared to fish caged upstream of the outfall (KCUS1, KCUS2) ($p < 0.0001$). The Kitchener upstream sites are several kilometers downstream of the Waterloo outfall and showed similar river water concentrations of ibuprofen and carbamazepine, but relatively higher levels of bisphenol A.

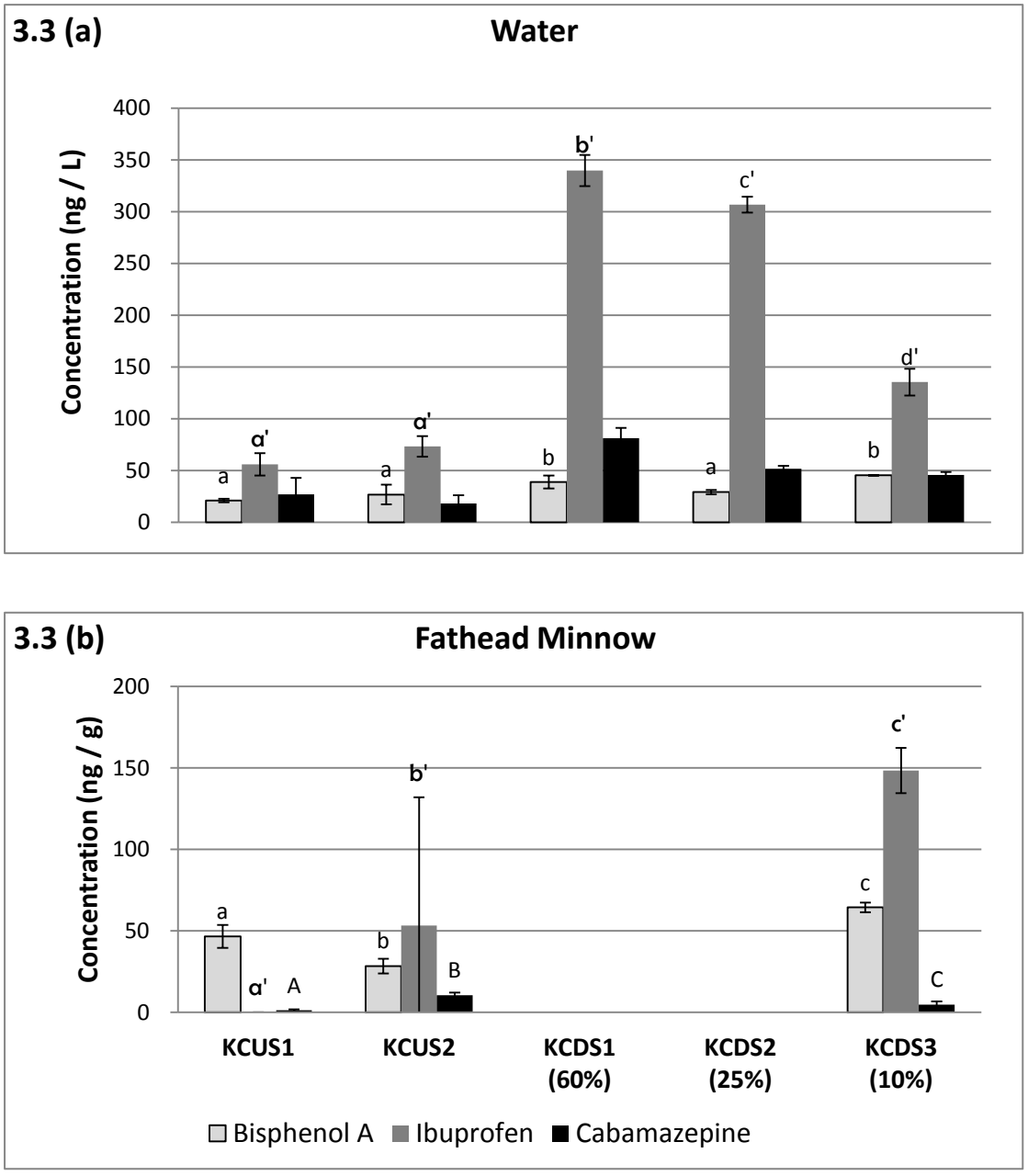


Figure 3.3 Concentrations of selected analytes in river adjacent to Kitchener wastewater treatment plant (a) and in Fathead Minnows caged in the upstream and downstream of Kitchener wastewater treatment plant (b) determined by SPME. Sampling time: Fall, 2008.

Carbamazepine was the only contaminant detected by SPME in river water and in Fathead Minnows caged adjacent to Guelph WWTP (**Figure 3.4 (a, b)**). Levels of carbamazepine in river water increased significantly at GCDS1 ($p < 0.0001$) and then gradually decreased further downstream such that river water concentrations detected at GCDS3 were still higher than upstream ($p < 0.0001$). The average concentrations of carbamazepine in Fathead Minnows caged at downstream sites were generally greater than that in fish caged of upstream sites, but the standard deviations of concentrations in fish were relatively high.

Overall, levels of contaminants detected in water and in caged Fathead Minnows at sites near all three WWTPs by SPME showed a similar pattern, with concentrations elevated in river water and in caged fish downstream of effluent outfalls (**Figure 3.2, 3.3, 3.4**). Lowest concentrations were observed in water and fish downstream of the tertiary treated wastewater outfall at Guelph, even at higher effluent exposures (e.g. 80% effluent).

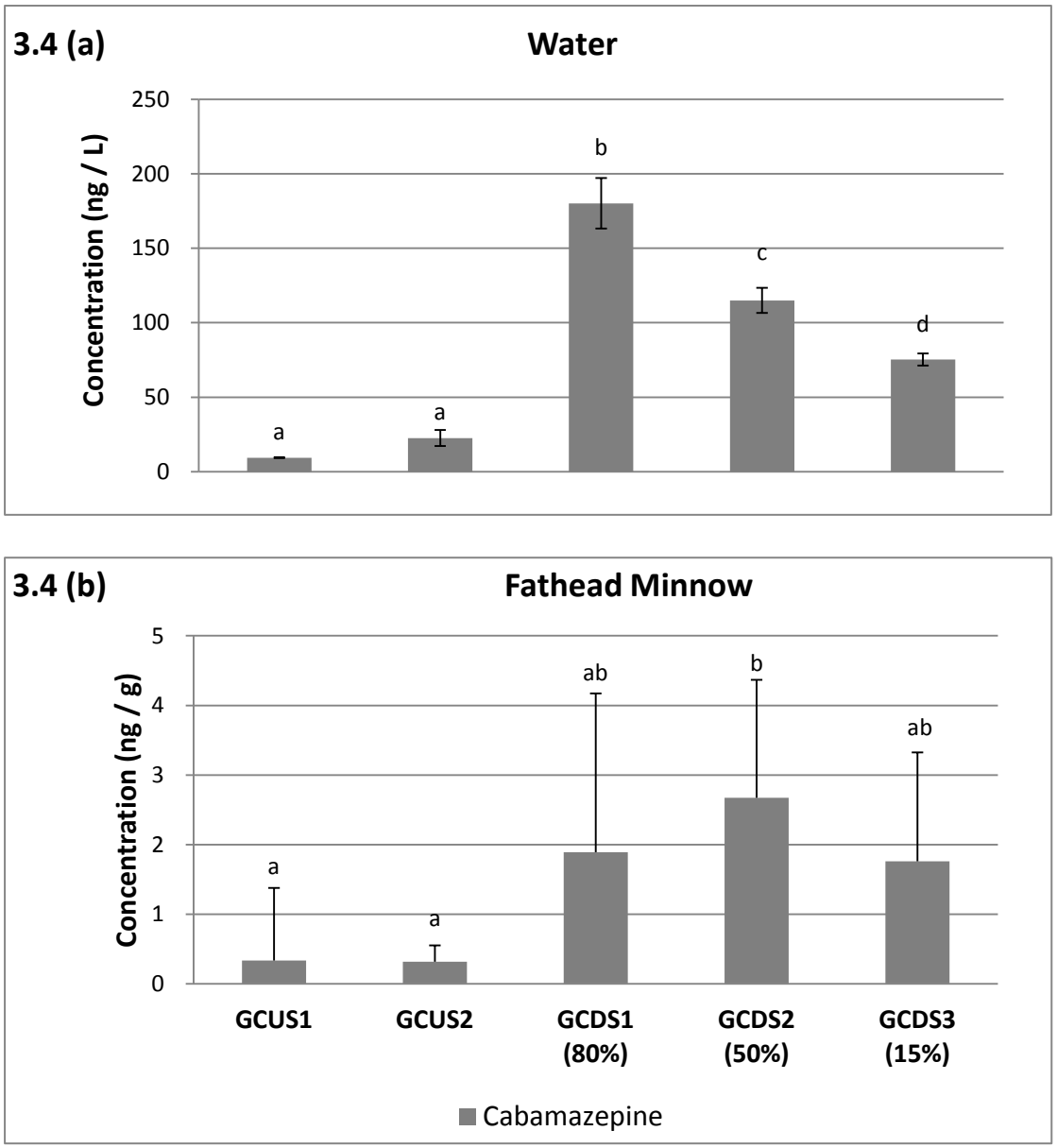


Figure 3.4 Concentrations of carbamazepine in river (a) and in Fathead Minnows (b) caged in the upstream and downstream of Guelph wastewater treatment plant as determined by SPME Sampling time: Fall, 2008

River water extracted by SPE, the several target contaminants were detected at low ng/L concentrations levels downstream of the Waterloo and Kitchener MWWEs, while few were detectable downstream of the Guelph MWTP (**Table 3.6**). Concentrations determined by SPE were similar to those determined by SPME, although several acidic compounds (e.g. gemfibrozil, diclofenac, atrazine) detected at very low concentrations were only detected by SPE, and not by SPME. Six compounds were detected by SPE in river water downstream of the outfalls of the two secondary treatment plants (Kitchener and Waterloo). Concentrations of bisphenol A, diclofenac, gemfibrozil, ibuprofen, and carbamazepine all followed similar patterns, with levels being significantly higher near the outfalls (50-60% effluent) ($p < 0.0001$ for Waterloo; $p < 0.002$ for Kitchener) and showing a general decline downstream (10-15% effluent). In comparison, only three compounds were detected in river water downstream of the outfall of the more advanced tertiary treatment plant of Guelph. Concentrations of atrazine in river water upstream and downstream of all three effluent outfalls were similar. Bisphenol A, gemfibrozil and ibuprofen occurring in surface water near Waterloo and Kitchener WWTPs all have an increasing trend in river water downstream of effluent outfalls while these compounds were absent or below quantification limits in river water adjacent to the Guelph WWTP outfall. Among the selected contaminants detected by SPE, carbamazepine was the only compound occurring at relatively higher concentrations downstream of the Guelph treatment plant compared to concentrations detected at upstream sites.

Table 3.6 Concentrations of target analytes in upstream and downstream of Guelph, Waterloo, and Kitchener wastewater treatment plant determined by SPE (ng/L)

	Compound	Further Upstream (US 1)	Immediate Upstream (US 2)	Immediate Downstream (DS 1)	Further Downstream (DS 2)	Further Downstream (DS 3)
Waterloo	Bisphenol A	N/A	N/A	36.9±1.3	35.0±2.3	44.1±2.3
	Diclofenac	20.7±3.1	16.3±1.8	42.8+5.1	23.0+2.6	32.3+4.9
	Gemfibrozil	N/D	N/D	16.5±1.4	28.7±2.2	N/A
	Ibuprofen	N/D	84.5±8.8	183±31	111±19	80.7±7.6
	Atrazine	275±67	186±48	179±29	250±73	194±15
	Carbamazepine	12.7±5.6	14.9±11.1	109±13	72.4±9.3	55.0±3.4
Kitchener	Bisphenol A	N/A	N/A	35.2±5.7	28.1±4.33	41.5±0.4
	Diclofenac	N/D	N/A	37.2±6.1	41.3±9.1	31.6±3.7
	Gemfibrozil	N/D	N/D	33.4±1.6	24.5±4.2	13.1±4.4
	Ibuprofen	66.9±10.6	69.9±15.3	340±42	305±13	150±10
	Atrazine	65.4±3.1	50.5±11.9	29.3±8.7	54.9±11.6	52.5±2.4
	Carbamazepine	10.0±0.1	12.0±1.4	85.1±16.4	60.3±13.4	57.4±2.3
Guelph	Bisphenol A	N/D	N/D	N/D	N/D	N/D
	Diclofenac	35.2±4.0	24.0±5.0	6.10±0.45	2.36±1.25	6.24±0.49
	Gemfibrozil	N/D	N/D	N/D	N/D	N/D
	Ibuprofen	N/A	N/A	N/A	N/A	N/A
	Atrazine	92.3±21.1	113±46	99.8±32.7	106±24	80.3±6.50
	Carbamazepine	33.1±11.8	18.2±0.2	171±16	125±7	82.8±6.6

N/D: not detected; N/A: not analyzed because concentration of the analyte was under detection limit

3.3.2 *In vivo* Detection of Emerging Contaminants in Wild Fish Collected from Sewage Effluent Using Solid Phase Micro-extraction

3.3.2.1 Wild Fish Collection Upstream and Downstream of the Waterloo Effluent Outfall

The upstream site (WWUS) chosen for Waterloo wild fish collection was between the WCUS2 (Fathead Minnow caging) and the effluent outfall, while the downstream site (WWDS) selected was between WCDS2 and WCDS3. Water conductivity, temperature, and pH in August 2009 were approximate the same as those measured in September, 2008. However, DO levels of river water observed in August 2009 (DO: 6.7-7.03 mg/L) were slightly lower than for September 2008 (DO: 9.6-11.6 mg/L) (**Table 3.3**, **3.5 (a)**). From the wild Greenside Darters and Rainbow Darters collected were fish selected to have similar sizes for *in vivo* extraction by SPME.

Table 3.7 (a) Water chemistry and morphometric data for fish collected in the upstream and downstream locations near the Waterloo wastewater treatment plant (August, 2009)

Sites		Upstream		Downstream (20% Effluent)	
Water Chemistry	**pH**	7.9		7.8	
	Conductivity(μs)	1005		1606	
	DO (mg/L)	6.70		7.03	
	Temperature(°C)	19.9		20.2	
Fish Data		**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**
	GSD	5.42±1.39	2.21±1.76	5.92±1.10	3.75±1.61
	RBD	5.41±0.85	1.99±0.31	5.62±0.29	2.31±0.50

GSD: Greenside Darter; RBD: Rainbow Darter; % Effluent was calculated according to conductivities. n (fish) =5

Six compounds were identified in river water extracted by SPME and SPE in August 2009, and five of there compounds were also detected in September, 2008, but at different concentrations. Low levels of naproxen were detected in August 2009, but there were no differences in concentrations between river water upstream and downstream of the outfall (**Table 3.7 (b)**). Unlike the results of field sampling in September 2008, bisphenol A was not detected the river water adjacent to the Waterloo treatment plant and it was not found in either Greenside Darters or Rainbow Darters (**Table 3.7(b)**). Concentrations of diclofenac (1080 ng/L) in water from the upstream sites were much higher than concentrations detected in water from upstream sites in September, 2008 (16.3 ng/L) and levels of this compound slightly increased at

the downstream site (20% effluent) ($p < 0.0001$) (**Table 3.6**, **3.7(b)**). Despite the relatively high concentrations of diclofenac found in water, concentrations of diclofenac in fish were low. Atrazine and carbamazepine occurred at lower concentrations in river water in August 2009 compared to concentrations in river water in September 2008. Atrazine concentrations declined as river water flowed downstream. Concentrations of carbamazepine were higher in river water downstream of the outfall compared to that of upstream ($p < 0.0001$). Concentrations of ibuprofen in river water were similar between August 2009 and September 2008. All of the contaminants identified in wild fish in August 2009 were at low concentrations (< 15 ng/g), which is likely due to the low concentrations in river water. Rainbow Darters seemed to have a higher ability to bioaccumulate contaminants than Greenside Darter at the same sites.

Table 3.7 (b) Selected analytes detected in water and in fish collected in the upstream and downstream of Waterloo wastewater treatment plant using SPME and SPE

Compounds	WWUS				WWDS			
	Water SPME (ng/L)	**Water SPE (ng/L)**	**GSD (ng/g)**	**RBD (ng/g)**	**Water SPME (ng/L)**	**Water SPE (ng/L)**	**GSD (ng/g)**	**RBD (ng/g)**
Bisphenol A	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Diclofenac	937±30	1080±10	4.19±1.56	14.67±1.52	1110±10	1260±20	6.95±0.71	13.5±3.6
Ibuprofen	63.9±12.4	80.5±22.5	3.75±0.07	5.28±0.54	51.2±0.4	75.4±25.9	3.20±0.10	5.30±0.06
Gemfibrozil	156 ±20	163±26	0.45±0.31	1.24±0.43	160.4±16.0	151±31.5	0.72±0.56	0.87±0.18
Atrazine	N/D	104±5	N/D	N/D	N/D	66.8±7.1	N/D	N/D
Carbamazepine	1.00±0.10	6.19±1.78	0.28±0.05	0.42+0.07	10.4±0.6	12.7±1.0	0.82±0.10	2.59±0.85
Naproxen	81.3±11.6	83.2±15.6	0.69±0.16	1.19±0.38	83.7±26.6	84.6±28.4	0.36±0.16	1.27±0.61

GSD: Greenside Darter; RBD: Rainbow Darter; n(GSD SPME)=5, n(RBD SPME)=5, n (water SPME) = 5, n (water SPE) = 3

3.3.2.2 Wild Fish Collection Upstream and Downstream of Kitchener Effluent Outfall

One site (KWUS) immediately upstream of the Kitchener effluent outfall as well as one site (KWDS) further downstream of KCDS3 (Fathead Minnow caging) were selected for wild fish collection. Water pH, conductivity, DO of river water upstream and downstream the outfall measured in September 2008, May 2009, and July, 2009 were similar (**Table 3.6**, **3.8 (a)**). Water temperature was warmest in July compared to that of May and September. Downstream sites had generally higher conductivity than upstream sites.

Table 3.8 (a) Water chemistry and fish data of spring and summer sampling in the upstream and downstream of Kitchener wastewater treatment plant (May and July, 2009)

Sites		Upstream (KWUS) (May, 2009)		Downstream 5% Effluent (KWDS) (May, 2009)		Upstream (KWUS) (July, 2009)		Downstream 17% Effluent (KWDS) (July, 2009)	
Water Chemistry	**pH**	8.40		8.14		7.6		7.7	
	Conductivity(µs)	1805		1456		1385		1850	
	DO (mg/L)	10.26		8.76		9.3		8.80	
	Temperature(°C)	15.9		15.4		21.5		22.8	
Fish Data		**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**
	GSD	7.40±1.60	4.84±3.33	6.80±1.10	3.68±1.98	7.89±0.24	5.8±1.56	8.21±0.44	6.20±2.08
	RBD	5.10±0.60	1.73±0.65	6.00±0.70	3.18±0.82	N/S	N/S	N/S	N/S

GSD: Greenside Darter; RBD: Rainbow Darter**;** n (RBD) =10, n (GSD) =10; N/S: not sampled; Refer to **Table 3.2** for locations of sites.

Lower levels of contaminants were detected in river adjacent to Kitchener outfall in May, 2009 relative to September 2008 (**Table 3.6**, **3.8 (b)**). Bisphenol A and carbamazepine were the only compounds that could be quantitated in river water in May and this corresponded with low concentrations in wild Greenside Daters and Rainbow Darters (**Table 3.8 (b)**). Concentrations of these two compounds detected in river water and fish in May were similar between upstream and downstream (5% Effluent) sites. In contrast, four compounds (bisphenol A, diclofenac, gemfibrozil, carbamazepine) were identified downstream of the Kitchener outfall September, 2008, and highest concentrations were found in July 2009 (**Table 3.6**). Diclofenac was found in wild fish and river water downstream of the outfall, but not in the upstream site. Concentrations of gemfibrozil and carbamazepine showed a significant increase in river water downstream ($p < 0.0001$) and their concentrations in fish followed a similar pattern ($p < 0.0001$). Concentrations of atrazine declined in both river water and wild fish moving downstream. Although ibuprofen was not identified in river water at the time of sampling, it was detected in fish caught downstream.

Overall, concentrations of selected contaminants detected in river near Kitchener effluent outfall changed over time. Carbamazepine appeared to be the most persistent compound which was been found most frequently. Ibuprofen, gemfibrozil, diclofenac, and bisphenol A were also frequently detected in the river and these contaminants often occurred at higher concentration levels downstream of the outfall.

Table 3.8 (b) Concentrations of target analytes in the Grand River and in fish collected in the upstream and downstream of Kitchener wastewater treatment plant in May and July using SPME (2009)

Site	KWUS (May)			KWDS (May)			KWUS (July)		KWDS (July)	
Analyte	Water SPME (ng/L)	GSD (ng/g)	RBD (ng/g)	Water SPME (ng/L)	GSD (ng/g)	RBD (ng/g)	Water SPME (ng/L)	GSD (ng/g)	Water SPME (ng/L)	GSD (ng/g)
Atrazine	N/D	N/D	N/D	N/D	N/D	N/D	260±65	1.54±0.23	233±83	1.34±0.04
Bisphenol A	1.1±0.2	35.7±7.2	22.8±7.0	0.65±0.22	8.26±6.21	7.11±1.58	N/D	N/D	N/A	N/A
Carbamazepine	N/A	0.23±0.16	N/A	N/A	0.37±0.12	N/A	23.5±7.4	1.32±0.53	146±60	8.63±4.63
Diclofenac	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	160±20	1.45±0.57
Fluoxetine	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Gemfibrozil	N/D	N/D	N/D	N/D	N/D	N/D	250±26	N/D	450±41	N/D
Ibuprofen	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/A	7.00±4.00
Naproxen	N/A	N/A	N/D	N/A	N/A	N/A	N/D	N/D	N/D	N/D

N/D: not detected; N/A: not analyzed because concentration of analyte was below method detection limit; Sampling time: Spring, 2009;

3.3.2.3 Wild Fish Collection Upstream and Downstream of Guelph Effluent Outfall

Three sites (GWUS1, 2, 3) located considerably upstream of the Guelph outfall in the Eramosa River (a major tributary), a site immediately upstream (GWUS4) and two sites downstream (GWDS2 and GWDS3) downstream of GCDS3 were selected for wild fish collection in May, 2009 (**Table 3.9 (a)**). Water conductivity and temperature measured in September (2008), May (2009), and July (2009) were similar. However, water pH and DO levels were higher in May and July 2009 (8.01-8.4) compared to that of September 2008 (7.1-7.9). No Greenside Darters were caught at Rockwood site.

Table 3.9 (a) Water chemistry and fish data of spring sampling in the upstream and downstream of Guelph wastewater treatment plant (May, 2009)

Sites		Rockwood (GWUS1)		Eden Mills (GWUS2)		Watson Road (GWUS3)		10% Effluent (GWDS2)		Niska Road 5% Effluent (GWDS3)	
Water Chemistry	**pH**	8.40		8.41		8.17		8.01		8.02	
	Conductivity(µs)	1058		1042		1189		1951		1780	
	DO (mg/L)	10.13		11.64		11.50		11.39		11.42	
	Temperature(°C)	14.1		14.4		13.4		14.2		14.1	
Fish Data		**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**	**Length (cm)**	**Weight (g)**
	GSD	No GSD	No GSD	7.39±0.39	4.21±0.81	5.60±1.17	2.23±1.31	5.31±0.68	1.85±0.90	5.21±0.48	2.34±1.02
	RBD	6.33±0.61	4.55±1.63	5.93±0.48	3.25±0.64	5.70±0.52	2.75±0.77	5.03±0.58	1.97±0.38	5.25±0.58	2.16±0.64

GSD: Greenside Darter, RBD: Rainbow Darter; DO: dissolved oxygen; % Effluent was calculated according to conductivities. n (fish) =10

Diclofenac and atrazine which have been identified in river water at fish caging sites in September, 2008 were also detected at sites selected for wild fish collection in May, 2009 using SPME (**Table 3.9 (b)**). Similar to previous observations, concentrations of diclofenac showed a gradual declining trend in the river water downstream, but concentrations were slightly higher (~20 ng/L) than that detected in September 2008 (~ 6 ng/L) (**Table 3.6**). Concentrations of atrazine in river water upstream May, 2009 were relative lower than Sept 2008 and dropped off to non-detectable at the downstream site. Two compounds including naproxen and bisphenol A, which were not detected in 2008, were found in May, 2009. Low levels of naproxen were detected in river water upstream and there was a small increase in concentrations observed downstream of the Guelph outfall. Bisphenol A was not detected in upstream in water or fish t but it was present in the river water and wild fish downstream of the outfall.

Overall, concentrations of contaminants present in river water downstream (10% effluent) of the outfall in May 2009 were low (< 41.1 ng/L) and there was little difference between upstream and downstream. In addition, none of the contaminants occurring in the river water bioaccumulated significantly in wild Greenside Darters or Rainbow Darters in May 2009 (**Figure 3.4 (b**), **3.5 (b**), Table **3.9 (b)**). Among all the contaminants detected in wild fish, bisphenol A was found at highest concentrations in muscle of wild Greenside Darters and Rainbow Darters.

Table 3.9 (b) Concentrations of target analytes in the Eramosa River and in Greenside Darters collected adjacent to Guelph wastewater treatment plant using SPME in May 2009

Sites	GWUS1		GWUS2		GWUS3		GWDS2 (30%)		GWDS3 (20%)	
Analyte	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**
ATR	N/F	0.06±0.01	0.10±0.01	0.14±0.01	0.03±0.01	N/A	0.03±0.01	N/A	N/D	N/D
BPA	N/F	N/D	N/D	N/D	N/D	N/D	37.9±10.9	N/A	11.4±3.3	3.64±1.04
CAR	N/F	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
DIF	N/F	18.6±0.1	6.02±0.05	2.24±0.02	N/D	N/D	15.3±0.4	10.3±0.2	N/D	N/D
FLX	N/F	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
GEM	N/F	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
IBP	N/F	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
NPR	N/F	0.29±0.01	N/D	N/D	0.25±0.01	0.20±0.01	0.31±0.21	0.20±0.09	N/D	N/D
	Water (ng/L)		**Water (ng/L)**		**Water (ng/L)**		**Water (ng/L)**		**Water (ng/L)**	
ATR	48.3±11.7		36.4±19.9		45.9±14.0		N/A		N/D	
BPA	N/D		N/D		N/D		41.1±21.8		19.5±1.6	
CAR	N/D		N/D		N/D		N/A		N/D	
DIF	134+4		14.4±0.2		N/D		20.2±0.7		N/D	
FLX	N/D		N/D		N/D		N/A		N/D	
GEM	N/D		N/D		N/D		N/D		N/D	
IBP	N/D		N/D		N/D		N/D		N/D	
NPR	N/D		N/D		5.76±1.75		8.55±2.55		N/D	

N/D: not detected; N/A: not analyzed because concentration of analyte was below method detection limit

ATR: atrazine; BPA: bisphenol A; CAR: carbamazepine; DIF: diclofenac; FLX: fluoxetine; GEM: gemfibrozil; IBP: ibuprofen; NPR: naproxen

Similar to May, river water pH upstream and downstream Guelph effluent outfall in July (2009) was higher than that of in September (2008) (**Table 3.10 (a)**). Other environmental factors such as conductivity, water temperature, DO of river water in were at relative constant levels during all three sampling periods, but values obtained in July (2009) were slightly higher compared to May (2009) and September (2008).

Seven compounds were identified in river water upstream and downstream of the Guelph effluent outfall in July (2009) by SPME and SPE (**Table 3.10 (b)**). Comparing to results of sampling in May (2009) and September (2008), contaminants occurred at higher concentration levels in river water and wild fish in July (2009). Concentrations of naproxen, ibuprofen, gemfibrozil, diclofenac, carbamazepine, and bisphenol A in river water all exhibited a similar trend with levels being increased at downstream of the outfall ($p < 0.001$). Compound concentrations in fish were general related to concentrations in the river water. Ibuprofen, diclofenac, carbamazepine and bisphenol A in wild Greenside Darters and Rainbow Darters showed an significant increase at immediately downstream(50% effluent) ($p < 0.0001$) and then a gradual declining downstream 2 (20% effluent). Gemfibrozil and atrazine were not detected in wild fish likely due to their low concentrations in river water. Both Greenside Darters and Rainbow Darters caught downstream (GWDS1) accumulated a large amount of bisphenol A and carbamazepine although Greenside Darters seemed to accumulate more ibuprofen than Rainbow Darters.

Table 3.10 (a) Water chemistry and fish data of summer sampling in the upstream and downstream of Guelph wastewater treatment plant (July, 2009)

Sites		Watson Road (GWUS3)		Gravel Pit (GWUS4)		50% Effluent GWDS1		Niska Road 25% Effluent GWDS3	
Water Chemistry	pH	8.51		8.45		8.27		8.51	
	Conductivity(µs)	1245		1267		2550		1860	
	DO (mg/L)	11.23		11.08		10.72		12.85	
	Temperature(°C)	19.0		18.0		17.7		18.3	
Fish Data		Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)	Length (cm)	Weight (g)
	GSD	5.97±0.48	2.23±0.88	5.95±0.57	2.16±0.50	5.97±0.48	2.23±0.88	5.85±0.43	2.16±0.50
	RBD	5.24±0.37	1.76±0.43	5.34±0.38	1.77±0.37	5.24±0.37	1.76±0.43	5.26±0.58	1.83±0.45

GSD: Greenside Darter, RBD: Rainbow Darter; DO: dissolved oxygen, % Effluent was calculated according to conductivities; n (fish) =10;

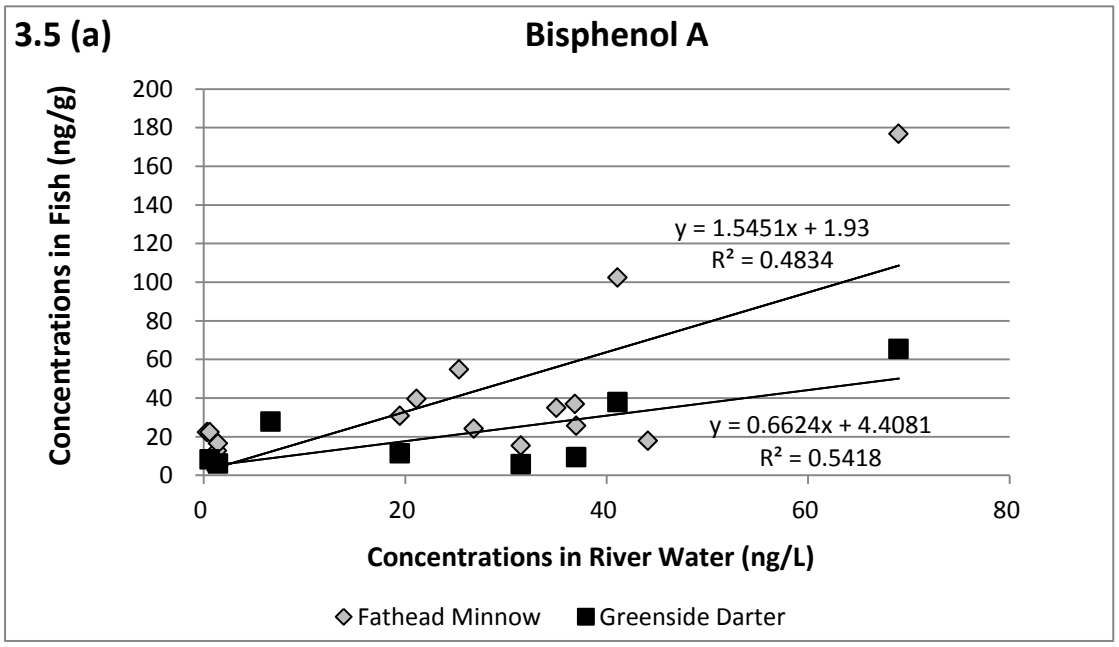
Table 3.10 (b) Concentrations of target analytes in the Eramosa River and in fish collected adjacent to the upstream and downstream of Guelph wastewater treatment plant in July 2009

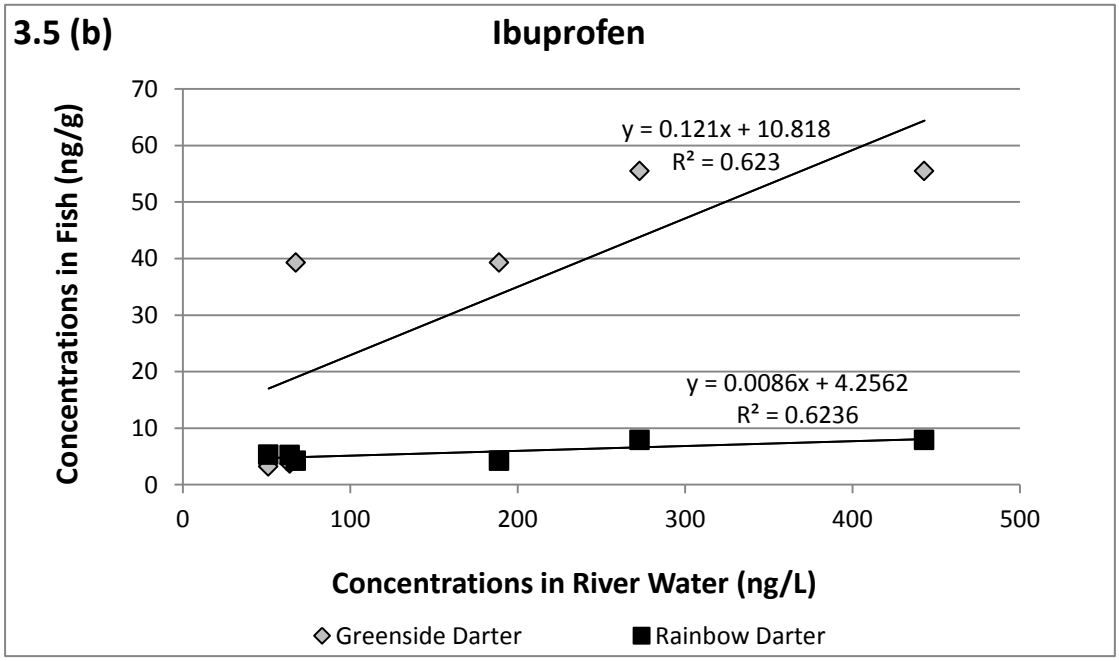
Sites	UGWUS3		GWUS4		GWDS1 (50%)		GWDS3 (25%)	
Analyte	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**	**GSD (ng/g)**	**RBD (ng/g)**
ATR	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
BPA	27.8±4.5	12.7±0.6	9.50±0.15	N/D	286±107	389±213	65.5±15.2	N/D
CAR	N/D	N/D	N/D	N/D	221±113	98.6±62.8	13.2±6.7	10.3±7.7
DIF	1.02±0.19	2.84±0.39	3.02±0.17	3.26±0.43	5.43±4.32	13.8±2.3	7.83±0.76	4.17±0.03
GEM	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
IBP	N/D	N/D	N/D	N/A	55.5±4.3	7.90±0.27	39.3±2.8	4.24±0.04
NPR	N/A	N/A	1.01±0.28	0.30±0.01	N/A	0.24±0.08	1.31±0.36	0.47±0.02
	Water SPE (ng/L)	**Water SPME (ng/L)**	**Water SPE (ng/L)**	**Water SPME (ng/L)**	**Water SPE (ng/L)**	**Water SPME (ng/L)**	**Water SPE (ng/L)**	**Water SPME (ng/L)**
ATR	5.12±0.91	N/D	N/A	N/D	N/A	N/D	N/D	N/D
BPA	6.66±1.47	6.66±0.03	36.6±7.4	37.7±1.2	53.7±3.2	48.7±2.3	68.8±4.2	59.9±5.3
CAR	N/D	N/D	N/D	N/D	100±32	120±40	127±23	130±32
DIF	73.1±10.1	82.1±3.9	104±40	80.3±11.7	273±29	309±19	67.6±6.3	98.5±22.9
GEM	N/D	N/D	N/D	N/D	14.9+0.4	N/D	11.6±2.4	N/D
IBP	176.8±1.9	163.9±11.6	86.1+1.9	60.3+0.2	443±45	450±36	189±30	153±45
NPR	28.2±10.2	20.9±2.7	38.2+4.3	34.7+4.8	47.1±0.6	39.7±3.1	35.4+0.7	35.4±3.7

N/D: not detected; N/A: not analyzed because concentration of analyte was below method detection

ATR: atrazine; BPA: bisphenol A; CAR: carbamazepine; DIF: diclofenac; GEM: gemfibrozil; IBP: ibuprofen; NPR: naproxen

Combined results of Fathead Minnow caging and wild fish collection concentration data for two selected compounds were summarized for comparison (**Figure 3.5**). There was a weak positive linear correlation between the concentrations of bisphenol A in both wild Greenside Darter ($R^2 = 0.5418$) and caged Fathead Minnow ($R^2 = 0.4834$) and river water concentrations. In addition, ibuprofen in the two wild fish species, Greenside Darter and Rainbow Darter, were also positively correlated ($R^2 = 0.623$; $R^2 = 0.6236$) with river water concentrations. The slopes of the trend lines varied with fish species suggesting bioaccumulating potential is different for each fish species


3.5 (a)
Bisphenol A
Concentrations in Fish (ng/g)
200
180
160
140
120
100
80
60
40
20
0
y = 1.5451x + 1.93
R² = 0.4834
y = 0.6624x + 4.4081
R² = 0.5418
0
20
40
60
80
Concentrations in River Water (ng/L)
Fathead Minnow
Greenside Darter


3.5 (b)
Ibuprofen
Concentrations in Fish (ng/g)
70
60
50
40
30
20
10
0
y = 0.121x + 10.818
R² = 0.623
y = 0.0086x + 4.2562
R² = 0.6236
0
100
200
300
400
500
Concentrations in River Water (ng/L)
Greenside Darter
Rainbow Darter

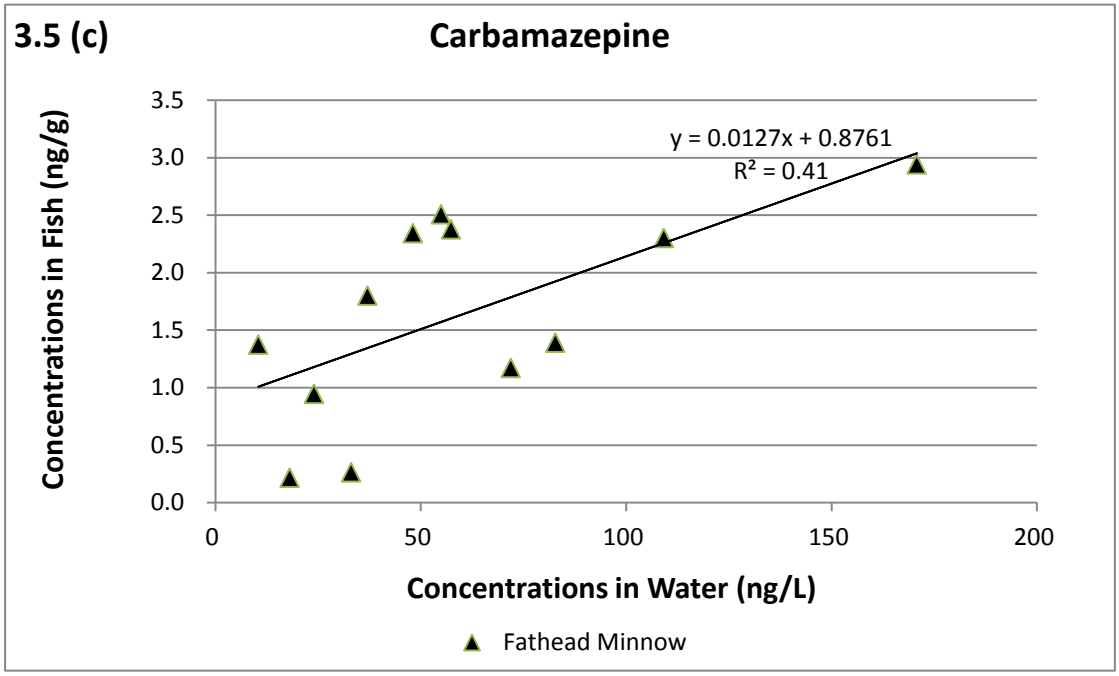


Figure 3.5 Correlation between concentrations of selected contaminants (Bisphenol A (a) Ibuprofen (b), Carbamazepine (c)) in fish and in river water

3.4 Discussion

Several recent studies have demonstrated that selected contaminants, including pharmaceuticals, can be measured in water and *in vivo* in fish muscle using SPME (Barrionuevo and Lanças 2002, Zhou et al. 2008b, Zhang et al. 2010). However, most of these studies have been conducted under controlled laboratory conditions. This study is one of the first to comprehensively apply *in vivo* SPME to measure a variety of microcontaminants in fish exposed to several municipal effluents in a watershed. One of the major advantages of *in vivo* SPME is that it minimizes the needs for lethal sampling. Only a small number of fish need to be sacrificed for determination of distribution coefficients between SPME fibre and fish muscle (Kfm), which is a prerequisite for kinetic calibration (values of Kfm used by this study were determined in Chapter 2). In the future it may be possible to develop a surrogate for fish tissues for determination of Kfm.

The selected contaminants extracted from river water by SPME and SPE did not differ significantly, supporting the use of SPME as an alternative extraction technique for quantitative determination in complex samples (e.g., river water, effluent). SPME has several advantages, including that it is time efficient by integrating sample cleanup and preparation into one step, and it is less expensive, which makes it more practical for analysis of a large number of samples. However, SPE was more sensitive,

detecting several acidic compounds (e.g., atrazine, diclofenac, gemfibrozil) that were not detected by SPME in the same sample. The reason may be related to the ionization of acidic compounds in river water with high pH (>7) which is then poorly extracted by the PDMS fibres. The SPE method uses a much greater mass of sorbent, allowing for greater extraction volumes and therefore sensitivity. SPME sensitivity may be significantly improved by increasing volumes of extraction phases (e.g. thicker/larger membrane coating), increasing the extraction rate (thin film, turbulence) or by extending extraction time (Loughrin 2006, Ouyang and Pawliszyn 2006, Ouyang et al. 2007).

Results of fish sampling showed that concentrations of selected contaminants in caged fish or in wild fish were general reflections of concentrations in river water, although the R^2 values were relatively low. SPE and SPME extraction only provides for a snapshot in time within a highly dynamic system and therefore does not reflect the long-term average water concentrations to which the fish are exposed to. Fathead Minnows may be able to bioaccumulate bisphenol to a greater extent than Greenside Darters when they were exposed to the same river water concentrations. In laboratory studies BCF values of bisphenol A in Fathead Minnows were 2.7 higher than that of Greenside Darter (Chapter 2). The reason for this difference is not clear, but may be related to the physiology of fish.

Results of field sampling showed that four selected contaminants, including carbamazepine, ibuprofen, diclofenac, and gemfibrozil were often detected in the surface water downstream of effluent outfalls. Carbamazepine was the most frequently detected compound and concentrations of this compound often showed an increasing trend in river water and fish (caged and wild fish) downstream of effluent outfalls, even after tertiary treatment. Carbamazepine has been widely reported as a contaminant in municipal effluents in Canada (Metcalfe et al. 2003; Lishman et al. 2006). The highest removal efficiency reported for carbamazepine in a treatment plant was only 53% and the removal efficiencies by conventional activated sludge were mostly below 10%, as the treatment of wastewaters by activated sludge usually did not result in any practical removal of neutral carbamazepine (Paxéus 2004, Zhang et al. 2008). Micropollutants (e.g. pharmaceuticals, pesticides, and industrial chemicals) are mainly removed during the activated sludge treatment through sorption, air stripping, biotransformation and phototransformation (Clara et al. 2004, Clara et al. 2005, Ivashechkin et al. 2005a, b, Joss et al. 2005, Zhang et al. 2008). The low rate of reduction of carbamazepine in a wastewater treatment plant is due to its low biotransformation (or biodegradation) rates, high polarity, and low sorption to sludge which relates to its low partition coefficient between water and sludge (Stamatelatou et al. 2003, Ternes et al. 2004). In addition to carbamazepine, several acidic compounds (e.g. ibuprofen, diclofenac, gemfibrozil) were often identified at quantifiable levels in river water downstream of the effluent outfalls. Previous

research has identified pH as a critical factor that affects the removal of micropollutants (Urase et al. 2005). Disturbances in the conventional activated sludge process usually resulted in lower removal rates for all acidic drugs, e.g., diclofenac (<10% removed), gemfibrozil (<55% removed), and ibuprofen (<60% removed) (Paxéus 2004, Zorita et al. 2009). Kimura et al. (2005) demonstrated that compounds with a complex structure (e.g. diclofenac with aromatic groups and chlorine groups) may make the compound more resistant to biodegradation. Similar results were also found by Andreozzi et al. (2006) who concluded that increasing amounts of nitro- and chlorine-groups in aromatic compounds lead to a decreasing degradation rate (Andreozzi et al. 2002). Atrazine was found across the sites, including upstream of the effluent outfalls. This herbicide is likely coming from the intensive agriculture areas upstream. It serves as a good reference compound that is found in both upstream and effluent impacted sites in the watershed. For compounds with relatively high hydrophobicities such as fluoxetine, naproxen, and bisphenol A, they were either absent or occurred at very low concentrations in river water downstream of all three WWTPs. Data in the literatures have shown high removal rates for these hydrophobic compounds, with more than 90% of bisphenol A, fluoxetine, and naproxen being eliminated in activated sludge treatment plants (Balest et al. 2008, Sun et al. 2008, Zorita et al. 2009). Since adsorption involves interactions of the hydrophobic groups (e.g. aliphatic and aromatic groups) of compounds with the organic fractions of the sludge and the lipophilic cell membranes of microorganisms,

hydrophobic compounds found in influent are therefore eliminated more effectively through sorption processes and transferred to the sludge of treatment plants rather than the final effluent (Carballa et al. 2005).

Seasonal variations in concentrations of contaminants were also observed in river water and in wild fish most likely as a result of variations in seasonal flow in the river with the highest contaminant exposure in the summer. Generally, highest levels of contaminants were detected in river water in July (2009), followed by September (2008) and May (2008). Frequent rainfalls in spring and fall month results in greater dilution of the effluents compared to mid-summer. Average river flow in the Speed River in July (2009) was 3.05 m^3/s which was much lower than that of in May (2009) (10.05 m^3/s) and September (2008) (7.73 m^3/s) (Grand River Conservation Authority 2009). Similar flow changes were also found in the river near Waterloo and Kitchener outfalls. Therefore, the influence of municipal effluent on the river was more significant in July and September compared to that of in May, which at least partially explains the higher levels of contaminants detected at downstream of outfalls in July and September. Temperature may be another factor that affects contaminant solubility, partitioning and biotransformation during treatment and in the environment to alter the distribution of microcontaminants. Seasonal changes may also alter habitat, movement and diet of fish species, changing bioavailability.

Overall, water quality, in terms of the contaminants examined, was better downstream of the tertiary treatment plant (Guelph). Several parameters during treatment processes are important factors that affect the removal efficiencies of micropollutants such as sludge retention time (SRT), hydraulic retention time (HRT), biomass concentration, pH, and temperature (Cirja et al. 2008). Previous studies have revealed that acidic compounds cannot be efficiently removed from wastewater at pH higher than 6 (Kim and Yu 2005, Urase et al. 2005). Since conventional WWTPs (e.g. Waterloo and Kitchener WWTPs) require pH control in the range of 7 to 9, many acidic compounds escape from sorption to activated sludge, resulting in low removal rates (Shammas et al. 2009). In comparison, the nitrification process provided by a tertiary treatment plant (e.g., Guelph WWTP) decreases pH and may lead to higher removal rates of acidic compounds. Urase et al. (2005) demonstrated pH values changed from neutral to acidic as a result of nitrification. Hence, reductions of acidic analytes by tertiary treatment plants (e.g. diclofenac, naproxen, gemfibrozil, ibuprofen) were generally better than removals by secondary treatment plants. For example, gemfibrozil was only detected in effluents of secondary treatment plants (e.g. Kitchener and Waterloo WWTPs). In addition, tertiary treatment plants generally provide much higher solids and hydraulic retention times than a secondary treatment plant. The Guelph WWTP has an SRT of 15-28 d compared to < 2 d for both Waterloo and Kitchener WWTPs (Tartakovsky et al. 1996, City of Guelph Wastewater Treatment Plant 2007, Zhang and Farahbakhsh 2007). The Guelph WWTP uses

extended activated sludge and extended aeration which resulted in higher biomass removal. Previous research found that higher solid retention time improved the performance of a treatment plant in removing micropollutants (Clara 2005, Polar 2007).

Higher levels of carbamazepine, diclofenac, ibuprofen, and carbamazepine were frequently detected in river water downstream of Guelph, Waterloo, and Kitchener outfalls, suggesting many of these contaminants reached surface water via municipal effluents. However, all the contaminants detected in municipal effluents were at low concentration levels and were quickly diluted downstream. Predicted no effect concentrations (PNEC) have been determined for a number of micropollutants: 0.13 µg/L for naproxen, 1 µg/L for gemfibrozil, 11-71 µg/L for bisphenol A, 0.6 µg/L for atrazine, 0.1 µg/L for diclofenac, 0.004 µg/L for fluoxetine, 7.1 µg/L for ibuprofen, 0.5 µg/L for carbamazepine (ERI 2007, FHI 2005, Ort et al. 2009, Staples et al. 2008). Based on these PNECs, the river water in this study was at concentration levels that would not cause acute toxicity to wild fish. However, these chemicals may exert subtle effects on fish by targeting enzymes or receptors at much lower concentrations (Tarazona et al. 2009). Concentrations of selected contaminants in caged Fathead Minnows or wild Greenside Darters and Rainbow Darters downstream of the effluent outfalls all showed an increasing trend in contamination immediately below the outfalls. However, concentrations of some contaminants detected were different

among Fathead Minnows, Greenside Darter and Rainbow Darter (e.g. bisphenol A, ibuprofen) collected from the same sites or from sites with similar water concentrations. Brown et al. (2010) demonstrated that although the physical habitats were similar upstream and downstream of the outfall at the Guelph WWTP the Rainbow Darter had higher condition factors downstream. In addition, the analysis of stable isotopes ($\delta^{15}N$, ^{13}C) suggested that there was a shift in diet of either the Rainbow Darter or Greenside Darter downstream in response to effluent inputs (Brown 2010). Many factors can influence the relative bioavailability of chemicals among species. Differences in sizes, physiology, transport, uptake and depuration kinetics, metabolisms, lipid content, diffusion rates through cell membranes, may all alter chemical uptake in fish species (ECETCO 1996).

Both ibuprofen and carbamazepine bioaccumulated in caged Fathead Minnows likely due to their higher concentrations in river water and their relatively high BCF values relative to other contaminants in the study (Chapter 2). Levels of bisphenol A and carbamazepine detected in wild Greenside Darters and Rainbow Darters caught downstream of the Guelph effluent outfall in July were much higher than concentrations of other compounds. Concentrations of bisphenol A in river water downstream of the outfall are highly variable in wild fish which may reflect differences in exposure resulting from mobility, habitat selection, etc. Chapter 2 showed that concentrations of bisphenol A and carbamazepine in the effluent of the

Guelph WWTP can be highly variable over a period of days. Overall, all the selected contaminants in this study did not bioaccumulated in wild Greenside Darters and Rainbow Darters significantly. The low bioaccumulating potential of these compounds is likely due to their low BCF values, their high degree of ionization in effluents with high pH (>7), and relative low water concentrations.

Although, SPME has many advantages, there are some limitations. The mass of the SPME fibre limits the amount of analytes that can be extracted. Hand-made PDMS fibres vary slightly in sizes and volumes which introduces relative higher analytical variability. Although this can be reduced by careful standardizing or calibrating the SPME fibres in advance, additional effort is required. Distribution coefficient (Kfs), are a prerequisite for pre-equilibrium kinetic SPME and have to be determined under laboratory controlled conditions prior to field sampling. There are a lot of variations between laboratory and field conditions such as pH, temperature, salinity, the types and amounts of analytes present, as well as differences in biology condition (lipid content etc.) of the fish, which may affect the Kfs values. Thus, Kfs values need to be carefully adjusted using fibre loaded deuterated standards to compensate for changes in environmental factors. *In vivo* detection of selected contaminants in fish (caged fish and wild fish) exposed to municipal effluents is possible with SPME fibres Thus, use of *in vivo* SPME creates opportunities for experimentation in the future that allows for rapid and accurate analysis and

monitoring in living organisms with minimal impact on their health.

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Chapter 4

Summary

A major advantage with solid phase micro-extraction (SPME) is its suitability for *in vivo* analysis of compounds in fish on-site without the need of lethal sampling. Although SPME has many advantages over the conventional extraction techniques, further development may enhance the utility. Development and testing of additional SPME coatings for polar and ionic analytes, such as endogenous peptides, pharmaceuticals, and their metabolites that are robust enough (less fragile) to be used *in vivo* applications are future opportunities for improvement. In addition, the interactions between SPME fibers and the diversity of analytes is still not fully understood, which makes it difficult to select the best fibers for compounds of interest. Extraction efficiencies of SPME coatings are affected by factors such as pH, temperature, salinity, and complexities of the matrix which need further development. Although kinetic calibration allows for compensation for variations in environmental factors using isotopic standards, it is only applicable to the cases when isotopic standards are available and the losses of the standards within sampling time are measurable. Hence, more work is needed to optimize the extraction efficiencies of SPME fibres especially for *in vivo* extraction or field sampling.

In this study, SPME has been validated as an excellent extraction technique for quantitative determination of low levels of contaminants in fish, surface waters and municipal effluents. Results indicated that wild fish in Grand River watershed were exposed to a variety of weakly bioaccumulative compounds from municipal effluents. Trace concentrations of selected contaminants were detected in municipal effluents from Guelph, Kitchener and Waterloo wastewater treatment plants, but at concentrations that do not pose a significant acute toxicity threat to exposed wild fish. However, the complexity of emerging contaminants in effluents and the diversity of potential biological mechanism that may cause subtle adverse effects need further study.

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