A SCREENING APPROACH TO ASSESS THE

IMPACTS OF MUNICIPAL WASTEWATERS ON AQUATIC SYSTEMS

Karen Kidd (McMaster University), Mark Servos (University of Waterloo), Markus Hecker (University of Saskatchewan) and François Gagné (Environment and Climate Change Canada)

Research conducted 2013-2015

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WHY DID WE DO THIS RESEARCH?

Municipal wastewaters contain diverse mixtures of chemicals, including hormones, pharmaceuticals, household chemicals and additives from personal care products. A number of these contaminants are not or only partially removed through conventional wastewater treatment and are continuously released into receiving waterbodies. There is concern and uncertainty about whether these chemicals, either individually or as mixtures, pose risks to aquatic ecosystems. In fact, there is growing evidence that certain groups of these chemicals in wastewater are affecting the fitness and/or health of fish and other aquatic life.

Hormones, other chemicals that mimic hormones, and compounds that can affect the production or elimination of hormones are found in these effluents, are taken up into fish and other aquatic life, and can interfere with their endocrine systems. The endocrine system controls key biological processes such as early development, growth, sexual development and reproduction. For example, natural and synthetic estrogens, which are frequently detected in municipal wastewaters, have been associated with the feminization of male fish living downstream of wastewater discharges (Routledge et al., 1998). Many other endocrine-disrupting chemicals can also remain in wastewater effluent, and the extent of their impacts on downstream organisms for any given discharge varies, depending on the chemical constituents, type of treatment used and characteristics of the waterbody.

Municipalities across Canada will be upgrading their wastewater treatment plants (WWTPs) to meet Canada's Wastewater Systems Effluent Regulations, which came into force in 2015, and the upgrades will remove more contaminants. As such, some co-benefits of the upgrades are anticipated for endocrine-related impacts on downstream fish and other aquatic life (Canadian Water Network, 2018). For example, recent research conducted in Ontario's Grand River watershed showed that when the estrogenicity of wastewater effluents decreased because of upgrades, the presence of feminized male fish declined downstream (Hicks et al., 2017). However, there are uncertainties that remain regarding the overall ability of traditional and modern wastewater treatment technologies to remove endocrine-disrupting chemicals from raw sewage. Prioritizing actions to identify and subsequently address the most significant concerns requires an ability to efficiently screen wastewater effluents and receiving environments to determine where clear impacts are occurring.

HOW DID WE APPROACH THE CHALLENGE?

Canadian Water Network (CWN) convened a workshop in February 2013 with a group of international experts to compile and prioritize a suite of elements that would be effective in assessing endocrine system impacts on fish as a direct result of exposure to contaminants in municipal wastewaters. The workshop was developed in partnership with the Water Environment Research Foundation (WERF) as part of a research collaboration to assess the capacity of existing tools to determine the impacts of contaminants in wastewater on aquatic ecosystems in Canada and the United States (WERF, 2010; Water Environment & Reuse Foundation, 2017).

The elements selected for the toolbox included: testing wild fish to assess effluent exposure and impacts; measuring fish and cell lines exposed to effluents in the laboratory to determine how reproductive endocrine pathways are affected; as well as measuring endocrine-disrupting chemicals in wastewater effluents (see Table 1). Some of the elements in the toolbox represent general indicators of fish health, whereas others were selected because they are more specific to endocrine-related impacts of concern.

Using reproductive endocrine system impacts as the key indicators, the main goal of this study was to assess whether this toolbox provides a clear indication of biological disruption in fish due to exposure to wastewater discharges. The study was also designed to provide a practical basis for distinguishing between sites of higher and lower concern for impacts and for prioritizing next steps.

Table 1: Elements selected for the toolbox.

TESTS CONDUCTED ON WILD FISH TO IDENTIFY IMPACTS

- Gene expression (a measure of molecular responses to exposure, which provides insights into the specific mechanisms by which chemicals can affect an organism)
- Vitellogenin induction (a measure of egg yolk protein produced by male fish, which is a sensitive marker for exposure to estrogens)
- Condition index and organ size, which provide information on the general health status of an organism [relative liver size index (LSI), relative gonad size index (GSI)]
- Measures of reproductive health (e.g., histology of the gonads, presence of intersex [a condition where gonads contain both male and female tissue], secondary sex characteristics, sex ratios, sex steroid hormone levels)
- Relative levels of stable isotopes of carbon (C) and nitrogen (N) to assess effluent exposure

LAB TESTS TO IDENTIFY ENDOCRINE ACTIVE COMPOUNDS IN WASTEWATERS AND THEIR IMPACTS ON FISH OR CELL LINES

- Short-term fish bioassays (laboratory exposures to effluents)
  - Assessment of endpoints described above for wild fish studies
  - Characterization of fecundity (i.e., the number of eggs produced by females)
- *In vitro* cell lines
  - Identification of compounds in wastewater influents (i.e., raw sewage) and treated effluents with the following properties:
    - Estrogenicity
    - Antiestrogenicity
    - Androgenicity
    - Antiandrogenicity
    - Disruption of estrogen production (steroidogenesis)
- Chemical analysis of the effluent

WHAT DID WE DO?

The research was conducted from 2013 to 2015 by three teams at the University of Waterloo, University of Saskatchewan and l'Institut national de la recherche scientifique (INRS). Each research team selected a study location within a local watershed (Grand River, Ontario; Wascana Creek and Saskatchewan River, Saskatchewan; St. Lawrence River, Québec). Within each location, the teams selected a reference site upstream of municipal wastewater discharges, and one or more corresponding downstream sites characterized as being of lower or higher concern for expected endocrine system impacts from municipal wastewater effluent. The selection of sites at each location was done based on the risk-screening criteria described by WERF in 2010. The criteria included WWTP input and treatment characteristics, and receiving waterbody characteristics (Table 2).

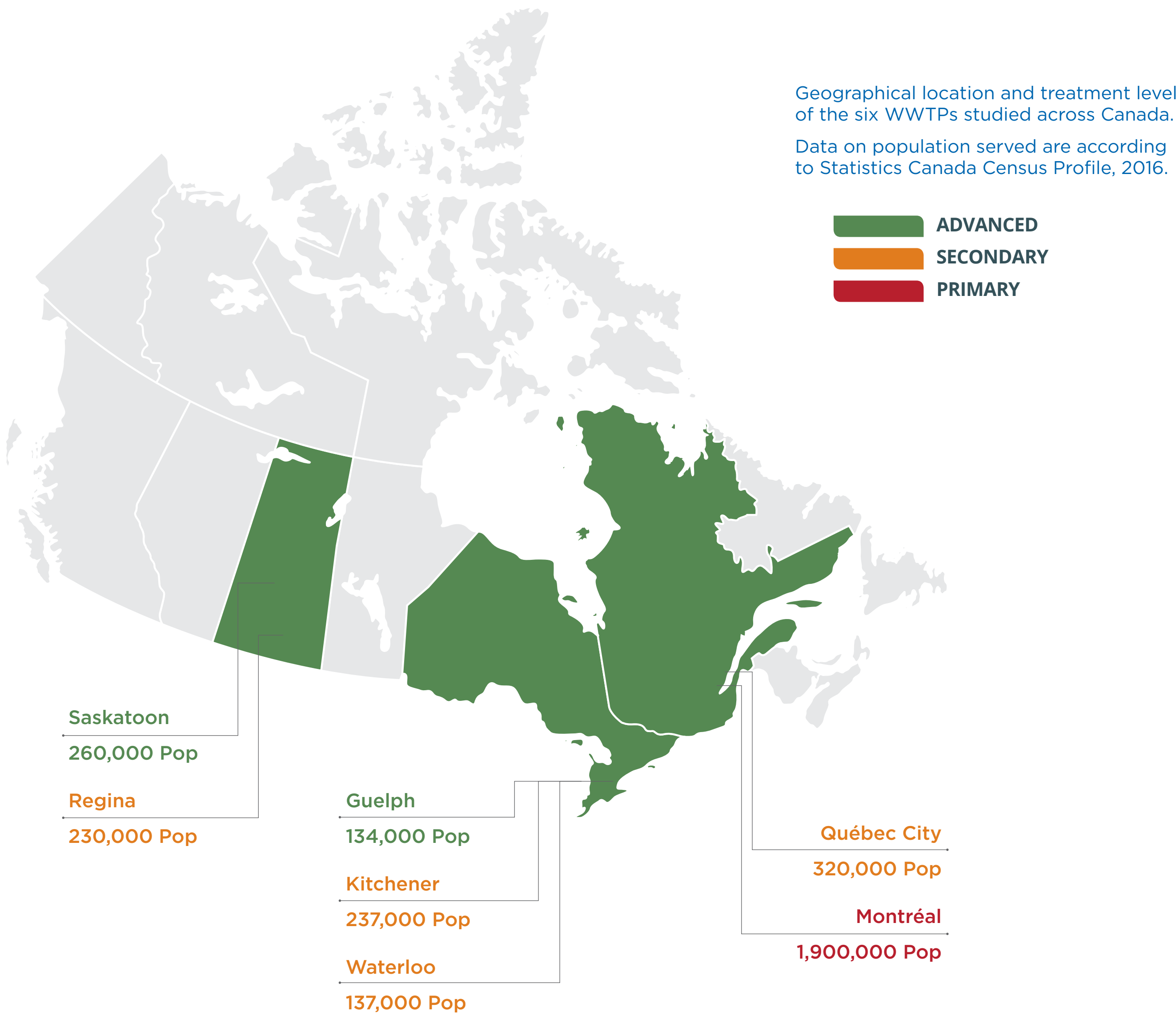

Table 2: Characteristics of the study sites, including the characterized level of concern* from impacts of wastewater effluent.

	GRAND RIVER			WASCANA CREEK AND SASKATCHEWAN RIVER		ST. LAWRENCE RIVER	
	Waterloo	Kitchener	Guelph	Regina	Saskatoon	Montréal	Québec City
Level of concern* (based on criteria from WERF 2010)	Higher	Intermediate (recovering)	Lower	Higher	Lower	Higher	Lower
Upstream watershed	Urban	Urban	Urban	Urban	Urban	Heavily urbanized	Suburban
Wild fish species sampled	Darters	Darters	Darters	Fathead minnow	Fathead minnow	Yellow perch	Yellow perch
Population served by the WWTP	~ 137,000	~ 237,000	~ 134,000	~ 230,000	~ 260,000	~ 1.9 million	~ 320,000
Level of treatment at the WWTP (at time of study)	Secondary, Activated sludge	Secondary, Activated sludge	Advanced, Activated sludge	Secondary, Lagoon	Advanced, Activated sludge	Advanced primary	Secondary, Biofiltration
Dilution of effluents at discharge site†	Low	Low	Low	Very low	High	Very high	High
Barriers to migration of fish	No	No	Yes	Yes	Yes	No	No

†Very low < 10%, low < 20%, high > 90%, very high > 99%

Elements from the toolbox in Table 1 were used at each location and customized to the area and expertise of the researchers. The general design included wild fish collections and lab bioassays that exposed whole fish and cell lines to effluents. The methods are described below:

WILD FISH TESTS

At each location, a local fish species was collected at a reference site upstream of the point of wastewater discharge and a study site downstream of one or more WWTPs. These fish were weighed, measured and dissected to assess sex and relative gonad and liver weights, and to obtain tissues for various biochemical and tissue measures. A suite of molecular markers (gene expression) was applied in conjunction with physiological responses such as hormone production and occurrence of histological anomalies such as intersex, delayed maturation, tissue degeneration, etc., as applicable for the fish species. Changes in stable isotope ratios of carbon and nitrogen in fish were also assessed, which reflect exposure to compounds in effluent via their diet.

LAB TESTS TO IDENTIFY ENDOCRINE ACTIVE COMPOUNDS IN WASTEWATERS AND THEIR POTENTIAL IMPACTS ON FISH

Effluents from the WWTPs were tested in controlled lab studies with widely-used laboratory fish species, and the effects on different hormonal pathways were examined using *in vitro* cell bioassays. In addition, analyses were conducted to identify differences in effluent chemistry. In brief, whole fish bioassays with effluents were used to determine the potential for changes in egg production, histological alterations of the gonads, hormone production and gene expression in selected effluents. Furthermore, influents and effluents of all of the WWTPs were tested for the presence of endocrine-active compounds (e.g., selected pharmaceuticals, personal care products and pesticides) to assess the efficiencies of current wastewater treatment processes to remove these chemicals, and to pinpoint constituents in wastewater potentially affecting the reproductive health of fish. Specifically, *in vitro* cell-based assays were used to identify compounds with (anti) estrogenic, (anti) androgenic and estrogen production-disrupting properties (steroidogenesis). High resolution mass-spectrometry was used to characterize the presence of chemicals in the wastewater influent (i.e., raw sewage) and treated effluent.

Overall, the results of these analyses were compared: 1) between reference (i.e., unimpacted upstream) sites and downstream sites, 2) between sites of higher vs. lower concern within the same watershed location and 3) across regions to develop an overall understanding of the toolbox elements useful for distinguishing sites of higher and lower concern.

WHAT DID WE FIND?

GRAND RIVER, ONTARIO (LED BY MARK SERVOS, UNIVERSITY OF WATERLOO)

Rainbow darter (*Etheostoma caeruleum*) and greenside darter (*E. blennioides*) were collected in the fall of 2013 using backpack electrofishers at reference sites upstream of urban wastewater discharges and at study sites downstream of the WWTP in Waterloo (characterized as higher concern), Kitchener (characterized as intermediate concern due to upgrades in 2012) and Guelph (characterized as lower concern).

The fish collected downstream of the effluent discharges, especially WWTPs with lower treatment levels, showed detectable impacts (Table 3). They showed responses in steroid production, intersex, gene expression increases or decreases (vitellogenin, gene microarray) and stable isotope composition when compared with fish upstream. In the laboratory, a model fish species (zebrafish, *Danio rerio*) exposed to effluents had reduced egg laying and altered gene expression following exposure to the Waterloo WWTP effluent, as well as some lesser impacts from exposure to Kitchener WWTP effluent (Tables 3 and 6). Cell line bioassays also showed that there are chemicals that act like androgens, anti-androgens and anti-estrogens in effluents from the Waterloo WWTP and chemicals that act like anti-estrogens in the effluents from the Guelph WWTP (Table 6). The other toolbox elements did not prove useful at this location in distinguishing impacts of exposure to contaminants in wastewater effluent.

(left)
Male rainbow darter with eggs in testes (intersex) collected from the Grand River in 2011
Photo credit: Mark Servos

Table 3: The ability of elements in the toolbox to distinguish responses in fish at sites of higher and lower concern in the Grand River in Ontario (2013).

Yes or no indicates whether the reference site upstream versus the study site downstream of the WWTP discharge were significantly different. An up arrow indicates a higher value at the downstream site compared to the reference site. A down arrow indicates a lower value at the downstream site compared to the reference site. Dashes indicate no data. It should be noted that since 2013, considerable improvements have been seen in the key indicators in response to upgrades of the treatment plants in the Grand River (Hicks et al. 2017).

TOOLBOX ELEMENT	Waterloo (higher concern)	Kitchener (intermediate concern; recovering)	Guelph (lower concern)
Condition (Effect size 10%) [1]			
Males	No	No	No
Females	No	No	No
Relative liver size (LSI; Effect size 25%) [1]			
Males	No	No	No
Females	No	Yes ↓	No
Relative gonad size (GSI; Effect size 25%) [1]			
Males	No	No	No
Females	No	No	No
Secondary sex characteristics[1]			
Males	No	No	–
Sex ratios (F:M)[1]	No	No	–
Circulating steroids [2]			
Males – 11-KT	No	Yes ↓	
Females – E2	No	–	
Tissue steroids/ *in vitro* [1]			
Males – 11-KT	Yes ↓	No	No
Males – T	Yes ↓	Yes ↓	No
Females – E2	No	Yes ↑	No
Intersex incidence [1]			
Males	Yes ↑	Yes ↑	No
Stable isotopes [1]			
Nitrogen	Yes ↑	No	No
Carbon	Yes ↑	No	No
Liver vitellogenin gene expression [2]			
Males	Yes ↑		
Gene microarray data [2]	Yes ↑ ↓	Yes ↑ ↓	–
Zebrafish reproduction bioassay (lab)	Yes ↓	No	No

Rainbow darter[1] or greenside darter[2]

WASCANA CREEK AND THE SASKATCHEWAN RIVER, SASKATCHEWAN (LED BY MARKUS HECKER, UNIVERSITY OF SASKATCHEWAN)

Fathead minnows were collected in the summers of 2014 and 2015 at reference sites upstream and study sites downstream of the Regina WWTP in Wascana Creek, which is considered a higher concern site due to the outdated lagoon-based WWTP (at the time of sampling, and which has been upgraded to an advanced secondary treatment system in 2016/17) and the very low dilution of effluent in the receiving water body. Several attempts were made to collect wild fish from the Saskatchewan River, near Saskatoon, which is considered a lower concern site due to the advanced WWTP and the high dilution of effluent in the receiving water body. These attempts were unsuccessful due to unusually high water levels during the study years.

Wild fish in Wascana Creek showed increased liver size, decreased gonad size and differences in stable isotopes of nitrogen and carbon in individuals exposed to wastewater effluent (Table 4). Lab studies with effluents from both WWTPs found that egg production was lower in fathead minnows exposed to the Regina (but not Saskatoon) effluents, and that there was disruption of gonadal maturation and general health status in fish exposed to both effluents (Tables 4 and 6). In general, the effects were greater and more severe in fish exposed to the Regina effluents, and impacts occurred at dilutions as low as 10% effluent. Wascana Creek can be up to 99% effluent during the dry season. *In vitro* analyses using cell lines indicated that Regina effluents contained more contaminants that acted like estrogens, albeit the effects were considered mild. Furthermore, all treatment plants in Saskatchewan, Ontario and Québec, with the exception of Montréal, were highly efficient in removing chemicals with androgenic properties. In general, the weak estrogenic and androgenic effects were outweighed by compounds that blocked the action of estrogens and androgens (Table 6).

Table 4: The ability of toolbox elements to distinguish responses in fathead minnows at sites of higher and lower concern in Wascana Creek and the Saskatchewan River in Saskatchewan.

Yes or no indicates whether the reference site upstream versus the study site downstream of the WWTP discharge were significantly different. An up arrow indicates a higher value at the downstream site compared to the reference site. A down arrow indicates a lower value at the downstream site compared to the reference site. Dashes indicate no data.

TOOLBOX ELEMENT	Regina (higher concern)	Saskatoon (lower concern) [a]
Condition (Effect size 10%)		
Males	No	No
Females	No	No
Relative liver size (LSI; Effect size 25%)		
Males	Yes ↑	No
Females	Yes ↑	Yes ↑
Relative gonad size (GSI; Effect size 25%)		
Males	Yes ↓	No
Females	Yes ↓	No
Fecundity (females) [a]		
Inhibition of maturation	Yes	No
Histology (males)		
Inhibition of maturation	Yes	Yes*
Circulating steroids		
Males – 11-KT	No	No
Females – E2	No	No
Vitellogenin gene expression		
Males	No	No
Female	No	No
Estrogen receptor alpha gene expression		
Males	No	No
Female	Yes ↑	Yes ↓
Stable isotopes		
Nitrogen	Yes ↓	–
Carbon	Yes ↑	–

[a] Laboratory fish studies only
* Effects were less severe than in fish exposed to Regina effluent

ST. LAWRENCE RIVER, QUÉBEC (LED BY FRANÇOIS GAGNÉ, ENVIRONMENT AND CLIMATE CHANGE CANADA AND MICHEL FOURNIER, L'INSTITUT NATIONAL DE LA RECHERCHE SCIENTIFIQUE)

Yellow perch were collected using seine nets in the summer and fall of 2014 at a site downstream of Québec City's WWTP (characterized as lower concern), and three sites downstream of Montréal's WWTP (characterized as higher concern) (at 4 km, 6 km and 12 km, respectively). Yellow perch were also collected at a common reference site upstream of both major cities. The wild fish results showed that female fish caught downstream of the Montréal plant had higher immune system responses (Table 5). The condition of female perch increased downstream of Montréal relative to the reference site. The condition of male perch decreased downstream of Québec City, and relative liver size was also smaller in females downstream of Québec City. In the lab, fathead minnows exposed to effluents from the Montréal WWTP showed higher levels of impact in vitellogenin (a 20-fold increase) and steroid inactivation genes (a 4-fold increase; CYP3A1) than exposure to effluents from the Québec City WWTP (Table 6). The fish bioassays also showed that exposures to effluents from the site of lower concern stimulated egg production, whereas effluents from the site of higher concern decreased egg production by 10-fold. Cell lines exposed to effluents indicated the presence of chemicals that acted like androgens and anti-estrogens in Montréal effluents and those that acted like anti-estrogens in Québec City effluents. In addition, effluents from both plants contained numerous pharmaceuticals and other compounds, some of which act as endocrine disruptors or perturb sex steroids (e.g., estrone, progesterone, testosterone, cholesterol and bisphenol A).

Table 5: The ability of the elements in the toolbox to distinguish responses in wild yellow perch at sites of higher and lower concern in the St. Lawrence River in Québec.

Yes or no indicates whether the reference site upstream versus the study site downstream of the WWTP discharge were significantly different. An up arrow indicates a higher value at the downstream site compared to the reference site. A down arrow indicates a lower value at the downstream site compared to the reference site. Dashes indicate no data.

TOOLBOX ELEMENT	Montréal (higher concern)	Québec City (lower concern)
Condition (Effect size 10%)		
Males	No	Yes ↓
Females	Yes ↑	No
Relative liver size (LSI; Effect size 25%)		
Males	No	No
Females	No	Yes ↓
Sex ratios (F:M)	No	No
Immune system responses		
Males – Plasma leucocyte #s	No	No
Female – Plasma leucocyte #s	Yes ↑	No
Vitellogenin gene expression		
Males	No	–
Female	No	–
Stable isotopes		
Nitrogen	Yes ↓	No

WHAT DID THIS PROJECT TELL US?

This project addressed the need for a practical approach for distinguishing between sites of higher and lower concern regarding the impacts of municipal WWTP effluent discharges on fish. The goal was to examine whether the suite of elements measured across sites in wild fish and lab bioassays provide clear indication of biological impacts — specifically endocrine disruption — as a result of contaminants in treated wastewater effluent discharges. Across the three locations in Ontario, Saskatchewan and Québec, the responses observed in wild fish and fish exposed to effluents in the lab were variable, reflecting the different composition and treatment of municipal wastewater and the different receiving waterways. This initial research suggests that some elements have promise for distinguishing sites of lower concern from those of higher concern and for prioritizing sites for further work (Table 6).

Table 6: Summary of the utility of elements in the toolbox to distinguish sites of higher and lower concern within and across watershed study locations.

Yes means that this element responded more strongly at the sites of higher concern, compared to sites of lower concern. Dashes indicate that an element was not assessed at that location.

	ONTARIO	SASKATCHEWAN	QUÉBEC	RECOMMENDED?
WILD FISH TESTS				
Gene expression	Yes	—	—	Highly variable; useful in combination with other endpoints
Steroids	Yes	—	—	Highly variable; not recommended
Histology/Intersex	Yes	—	—	Important indicators of specific pathologies; yes
Condition, LSI, GSI	No	—	No	Important indicators of overall health; yes
C or N isotopes	Yes	—	Yes	Indicator of effluent exposure; yes
LAB TESTS TO IDENTIFY IMPACTS ON FISH				
Reproductive assay[1]	Yes	Yes	Yes	Indicator of impacts on reproductive success; yes
LAB TESTS TO IDENTIFY IMPACTS ON CELL LINES				
Receptor based assays[2]	Yes	Yes	Yes	Strong links to endocrine disruptors and effects in fish; yes
Steroidogenesis	Yes	Yes	Yes	Strong links to endocrine disruptors and effects in fish; yes
EFFLUENT CHEMISTRY	Yes	Yes	Yes	Yes

[1] Of the elements measured, egg production was a sensitive indicator in the assay but not specific to endocrine disruption, so it is recommended to add other endocrine-specific endpoints such as vitellogenin and estrogen receptor gene expression
[2] Includes estrogenicity, antiestrogenicity, androgenicity, antiandrogenicity

WHAT DO THESE RESULTS MEAN FOR UTILITIES, RESEARCHERS AND OTHER STAKEHOLDERS?

A COMBINATION OF FIELD AND LABORATORY ELEMENTS CAN BE USED TO IDENTIFY LOCATIONS WHERE FISH ARE IMPACTED BY CONTAMINANTS IN TREATED WASTEWATER.

An assessment of municipal wastewater for its potential impacts on the environment requires an informed selection of elements from the toolbox that address the specific characteristics of the effluent, and site, spatial and temporal variability, as well as the specific questions facing wastewater managers. Applying a suite of well-established elements that characterize effluent and assess fish responses can indicate areas of impact and provide information about the effectiveness of wastewater treatment processes in reducing contaminants. Overall, it is important to examine the responses of wild and lab fish across levels of biological organization (molecular, tissue, whole organism), because together they provide a holistic assessment of effects of contaminants in wastewater effluents.

- The results of this study indicate that wild fish sampling, cell line bioassays and fish reproductive tests in the lab can be effective at detecting endocrine-related effects of contaminants in wastewater effluent.
- Gene expression can be highly specific, providing insights into mechanisms. However, gene expression changes over time and can be confounded by compensatory responses, especially under field conditions, and should be used in conjunction with other elements.
- Sex steroid production and plasma levels are useful for characterizing impacts of effluents, but can be highly variable among individual fish, depending on their age or the season.
- The effects of wastewater contaminants on histopathology (such as intersex and delayed maturation) can be consistent and meaningful, but are not always detectable, depending on the site, effluent and timing of sampling.
- Whole organism elements such as organ size, histopathology and condition integrate responses to a variety of stressors, and are therefore much more difficult to link to specific contaminant exposures. However, they are more ecologically relevant, reflect important changes that are more closely tied to ecosystem responses than molecular-level responses such as gene expression, and are more common endpoints in biological monitoring programs. As such, they are important to keep in the toolbox.
- Elements to define exposure such as chemistry and stable isotopes are also very helpful.
- Fish and cell laboratory-based bioassays are well-developed and powerful. They provide complementary information that is predictive of the biological alterations observed in wild fish. These bioassays are useful in identifying WWTPs for further study and for separating the effects of municipal effluents from other wastewaters that discharge into the same receiving environment.

GIVEN THE DIVERSITY OF WASTEWATER TREATMENT SYSTEMS AND AQUATIC ENVIRONMENTS, ELEMENTS IN THE TOOLBOX SHOULD BE CUSTOMIZED.

By further refining this approach, one can better identify locations where impacts are actually occurring to support an appropriate remedial response (CWN, 2018). Further work should expand our understanding of the most useful elements of an effective monitoring program, as well as on how to select the best elements for a given site, rather than prescriptive lists of tests and criteria.

The design of any future studies needs to consider the characteristics of the receiving environment and the fish species present to ensure that sampling is conducted at a time that will maximize the interpretation of the results. Some elements, like hormone measures, require certain background knowledge to interpret the results against the natural hormone cycles that exist in fish. Multiple reference sites and recovery sites may be needed to understand and separate impacts from natural variation. There can also be considerable annual or seasonal variability in both effluent exposure and receiving water conditions, and ideally multiple years of monitoring are needed to adequately assess impacts.

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