

ECOTOXICITY OF SELECTED PHARMACEUTICALS OF URBAN ORIGIN DISCHARGED TO THE SAINT-LAWRENCE RIVER (QUÉBEC, CANADA): A REVIEW

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ABSTRACT

Blaise, C.; Gagné, F.; Eullaffroy, P. & Férard, J-F. 2006. Ecotoxicity of selected pharmaceuticals of urban origin discharged to the Saint-Lawrence River (Québec, Canada) : a review. *Braz. J. Aquat. Sci. Technol.* 10(2):29-51. ISSN 1808-7035. Twelve pharmaceuticals comprising anti-inflammatory agents (ibuprofen, naproxen), lipid regulators (bezafibrate, gemfibrozil), an anti-convulsant (carbamazepine), antibiotics (sulfamethoxazole, sulfapyridine, oxytetracycline, novobiocin, trimethoprim), a stimulant (caffeine) and a nicotine metabolite (cotinine) were identified in the final effluent of a major wastewater treatment plant (WWTP) based in the Montreal area (Québec, Canada) discharging to the Saint-Lawrence River. Their measured concentrations ranged from 33 (carbamazepine) to 22187 ng/L (caffeine) in the Montreal WWTP effluent and from 58 (cotinine) to 85000 (ibuprofen) ng/L based on maximum concentrations found in other municipal effluents as reported elsewhere. In general, elimination of these drugs by wastewater treatment is purported to be fairly efficient for ibuprofen, naproxen, bezafibrate, gemfibrozil and caffeine. In contrast, carbamazepine, sulfamethoxazole, sulfapyridine and oxytetracycline appear more resistant to treatment whereas removal efficiency for novobiocin, trimethoprim and cotinine is either unclear or unknown. Despite the degree to which they are eliminated in WWTPs, their presence in urban effluents discharging to surface waters is common. To estimate their potential risk to aquatic biota, we undertook acute/chronic toxicity testing with a suite of small-scale bioassays representing four taxonomic groups (bacteria, algae, invertebrates and fish) and complemented our results with those published in the literature. When all acute and chronic toxicity tests were combined, their toxicity responses spanned over five orders of magnitude. Barring caffeine and cotinine, all others are clearly recognized as toxic based on the EU-Directive 93/67/EEC classification, but their concentrations measured in WWTPs are too low to cause acute or chronic effects on an individual basis. Sulfamethoxazole, naproxen and ibuprofen, however, were found to produce toxic effects at concentrations that are \leq one order of magnitude to those found in municipal effluents. In general, bioassay responses displayed a wide range of sensitivity that justifies continuing use of representative species within test batteries to properly appraise the toxic potential of pharmaceuticals. We also showed that exposure of *Hydra attenuata* to caffeine for 48 h induced xanthine oxidoreductase activity which may in turn lead to production of oxidative stress at concentrations close to those present in urban effluents. Future studies on the ecotoxicity of pharmaceuticals should therefore also seek to include appropriate biomarkers to assess possible adverse long-term effects to biota resulting from the biotransformation of drugs.

Keywords : pharmaceuticals, acute and chronic toxicity, municipal effluents, bioassays, biomarkers, Saint-Lawrence River.

INTRODUCTION

Historically known for introducing biological agents (bacteria, viruses, parasites, etc.), as well as several types of «classical» contaminants (*i.e.*, metals and organics) to surface waters, municipal effluents are also now regarded as important sources of so-called «emerging chemicals» including household products, surfactant and pharmaceuticals (drugs and hormones).

The latter, either through original molecules or metabolites, are then released into the aquatic environment where their biological effects can potentially be exerted on biota. Clearly a source of concern with respect to environmental protection, this issue has been remarkably evoked in several recent reviews relating to drugged waters (Cunningham *et al.*, 2006; Garric & Ferrari, 2005; Halling-Sorensen *et al.*, 1998; Kümmerer, 2001; Jones *et al.*, 2001).

Indeed, the presence of pharmaceuticals and personal care products is now documented for sewage, wastewater treatment plants and other (non)point sources of pollution (Stumpf *et al.*, 1999; Jones *et al.*, 2001; Kümmerer, 2001; Pedersen *et al.*, 2005), as well as for surface (Dietrich *et al.*, 2002; Metcalfe *et al.*, 2003a; Gross *et al.*, 2004; Kolpin *et al.*, 2002) and even potable waters (Stackelberg *et al.*, 2004). In the short term, pharmaceutical strains on aquatic systems will likely not diminish and may even be expected to increase owing to an aging human population and continuing efforts to develop new classes of therapeutic chemicals (Servos *et al.*, 2002). In the long term, improved technology and efficiency of sewage wastewater treatment plants should eventually prevail to curb the entrance of undesirable drug products in receiving waters, as suggested by marked research presently on-going in this area (Heberer & Feldmann, 2004; Perez *et al.*, 2005; Roberts & Thomas, 2006; Batt *et al.*, 2006a,b; Jasim *et al.*, 2006). For now, ecotoxicological studies, still relatively sparse, must continue to document hazard and risk potential for chemicals released to aquatic destinations by reporting their acute and chronic toxicities, both individually and in combination (Crane *et al.*, 2006). Drugs displaying persistent characteristics will also merit additional investigations to assess their bioaccumulation and biomagnification profiles via trophic chain experimentation (Gagné *et al.*, 2006c).

In the work presented herein, we report on several aspects linked to the ecotoxicity of 12 recently-quantified pharmaceuticals discharging to the freshwater portion of the St-Lawrence River in the Greater Montreal area via a major municipal wastewater treatment plant (WWTP) effluent. This WWTP, offering primary treatment with phosphorus removal, handles close to three million m³/d of wastewater (sewage combined with 10-20 % industrial loading input), is known to release (in)organic contaminants (Pham & Proulx, 1997; Quémerais *et al.*, 1998) and has been shown to be toxic (Environment Canada, 1998), genotoxic (White, 1996), immunotoxic (Blaise *et al.*, 2002), as well as being a source of estrogenic substances causing feminization effects in freshwater bivalves (Gagné *et al.*, 2001; Blaise *et al.*, 2003).

Our study first investigated the effects of these 12 pharmaceuticals with a suite of small-scale toxicity tests (microbiotests) representing different levels of biological organization. In so doing, we also considered all other acute and chronic toxicity test data reported for these substances in the literature to determine their relative hazard potential in relation to risk for aquatic species. Beyond bioassays, we also discuss the more subtle adverse effects that can be associated with uptake and metabolization of pharmaceuticals. In this

area, aspects of the biotransformation of caffeine by the cnidarian *Hydra attenuata* were explored.

MATERIALS AND METHODS

Sampling of municipal effluent and pharmaceutical analysis

Three 60 L grab samples were collected from the final wastewater treatment plant effluent (Montreal, Canada) in June 2003 and placed in Rubbermaid plastic containers (High density poly-ethylene; Rubbermaid commercial products, Mississauga, Ontario, Canada) lined with polyethylene bags. The samples were maintained at 4°C during transport to our laboratories with commercial ice-packs placed between the container and bag lining.

The effluent was analyzed for a total of 42 products: six neutral pharmaceuticals (trimethoprim, cotinine, pentoxifylline, carbamazepine, cyclophosphamide, and caffeine); nine acidic pharmaceuticals (bezafibrate, gemfibrozil, naproxen, clofibrate, diclofenac, fenoprofen, ibuprofen, indomethacin and ketoprofen); 18 sulfonamide antibiotics (sulfacetamide, sulfaguanidine, sulfapyridine, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfasoxazole, sulfamoxole, sulfamethizole, sulfabenzamide, sulfamethazine, sulfamethoxypyridazine, sulfamer, sulfachloropyridazine, sulfadimethoxine, sulfaphenazole and sulfaquinoxaline); four quinolone antibiotics (norfloxacin, piperidic acid, oxolinic acid, ofloxacin); three tetracyclines (oxytetracycline, chlorotetracycline and tetracycline) and two macrolide antibiotics (roxithromycin, novobiocin). Analytical techniques made use of high performance liquid chromatography-mass spectrometry (LC-MS/MS). They were performed by Enviro-Test Laboratories/Xenos Division, located in Ottawa (Ontario, Canada).

Toxicity tests

Six well-standardized bioassays representing four trophic levels were employed to assess the toxic potential of pharmaceutical products detected in the municipal wastewater treatment plant effluent. Their features are reported in Table 1. Listed references are links to the respective methods and statistical analyses that were followed to undertake testing.

Biomarker measurement and statistical analysis

Hydra attenuata was cultivated under previously reported conditions (Blaise and Kusui, 1997) and exposed to concentrations of caffeine for 48 h at 20°C at a density of 50 animals per treatment. At the end of

the exposure period, 25 *Hydra* were collected with a Pasteur pipette, centrifuged at 100 *g* for 5 min and the *Hydra* pellet homogenized with a Teflon pestle tissue grinder in 1 mL of 50 mM NaCl containing 10 mM HEPES-NaOH, pH 7.4 containing 0.1 mM EDTA. The homogenate was then centrifuged at 15000 *g* for 20 min at 4°C and the supernatant (S₁₅ fraction) collected for analysis. The oxidative metabolism of xanthines was measured by following xanthine oxidoreductase (XOR) activity in the S₁₅ fraction by the method of Zhu et al. (1994). The mixture contained 50 µL of S₁₅ fraction, 1 mM hypoxanthine, 0.1 mM aminotriazole (a catalase inhibitor), 2 µM dichlorofluorescein and 0.1 µg/mL horseradish peroxidase in 25 mM KH₂PO₄, pH 7.4, containing 10 µM molybdate. The reaction proceeded at 30°C for 0, 15, 30 and 60 min and resulting production of fluorescein was measured under 485 nm excitation

and 520 nm emission. Standard solutions of fluorescein were used for calibration. Enzyme activity was normalised against total protein in the S₁₅ fraction using serum bovine albumin as standard (Bradford, 1976). Enzyme activity was expressed as nmole of fluorescein formed/min/mg proteins. Differences between caffeine concentrations exposed to *Hydra* over controls were shown by ANOVA followed by the least square difference (Fisher) using Statistica software (Version 7).

RESULTS AND DISCUSSION

Effluent concentrations of pharmaceuticals

Of 42 pharmaceutical products analyzed for, 12 were found to be above instrumental detection limits in the municipal effluent. Their characteristics and

Table 1 - Characteristics of the small-scale bioassays used by the authors to determine the toxic potential of the 12 pharmaceuticals.

<i>Trophic level</i>	<i>Toxicity test</i>	<i>Assessment endpoint</i>	<i>Measurement endpoint</i>	<i>Reference</i>
Decomposer	<u>Bacterial test</u> <i>Vibrio fischeri</i> (Microtox® toxicity test)	Acute sublethal light inhibition (after a 15-min exposure)	15min-IC50	Environment Canada, 1992
Decomposer	<u>Bacterial test</u> <i>Escherichia coli</i> (SOS Chromotest genotoxicity assay)	SOS gene DNA repair induction with and without metabolic activation (after a 2-h exposure)	2 h-TEC ^a	Legault <i>et al.</i> , 1996
Primary producer	<u>Algal test</u> <i>Pseudokirchneriella subcapitata</i> microplate assay)	Chronic sublethal growth inhibition (after a 72-h exposure)	72h-IC50	Blaise and Vasseur, 2005
Primary consumer	<u>Micro-crustacean test</u> (ThamnoToxkit assay)	Acute lethality (after a 24-h exposure)	24h-LC50	Microbiotests Inc., http://www.microbiotests.be/
Secondary consumer	<u>Cnidarian test</u> (<i>Hydra attenuata</i> assay)	Acute sublethality indicated by morphology changes (after a 96-h exposure)	96h-EC50	Blaise and Kusui, 1997
Secondary consumer	Fish cell test (rainbow trout primary hepatocyte test)	Acute cytotoxicity (after a 48-h exposure)	48h-TEC ^b	Gagné, 2005

a) TEC (threshold effect concentration) for SOS gene induction as manifested by significant production of β-galactosidase = (NOEC x LOEC)^{1/2}, where NOEC = no observed effect concentration and LOEC = lowest observed effect concentration.

b) TEC (threshold effect concentration) for cytotoxicity as manifested by a significant reduction in cell viability = (NOEC x LOEC)^{1/2}, where NOEC = no observed effect concentration and LOEC = lowest observed effect concentration.

concentrations are reported in Table 2. In general, measured concentrations are in agreement with those quantified in urban effluents elsewhere. Ibuprofen, a commonly prescribed non steroidal cyclooxygenase (COX) inhibitor preventing prostaglandin synthesis and hence blocking pain and inflammation (Vane & Botting, 1998), has been reported in other Canadian and European municipal wastewaters at concentrations ranging from 1300–85000 ng /L (Andreozzi *et al.*, 2003; Metcalfe *et al.*, 2003b; la Farré *et al.*, 2001; Stumpf *et al.*, 1999; Ternes, 1998; Roberts & Thomas, 2006). Effluents from hospitals can be important sources of ibuprofen and other drugs to WWTPs via municipal sewage collection systems (Gomez *et al.*, 2006). Despite marked biodegradation (75-99%) following its passage in wastewater treatment plants (Tixier *et al.*, 2003; Buser *et al.*, 1999; Paxéus, 2004; Roberts & Thomas, 2006), ibuprofen is nevertheless very present in North American and European surface waters (41 – 9500 ng/L), suggesting significant use by human consumers (Heberer *et al.*, 2001; Ternes, 1998; Buser *et al.*, 1999; Stumpf *et al.*, 1999; la Farré *et al.*, 2001; Kolpin *et al.*, 2002). Its elimination after urban wastewater treatment has also been estimated to be 77.8% in South Korean wastewater sewage treatment

plants (Han *et al.*, 2006) and between 12-100% for a series of plants located in cities of several countries where treatment, process and season of assessment differed (Fent *et al.*, 2006).

Similarly, naproxen, also a COX inhibitor, has been found in urban effluents of large cities at concentrations between 168 – 33900 ng/L (Andreozzi *et al.*, 2003; Metcalfe *et al.*, 2003b; la Farré *et al.*, 2001; Ternes, 1998; Tixier *et al.*, 2003; Jones *et al.*, 2002). Elimination of naproxen after urban wastewater treatment is estimated to be 66% (Ternes, 1998) owing to biodegradation and photo-transformation processes (Tixier *et al.*, 2003). Again, its removal rate following passage through sewage plants based on varying treatment options has been shown to vary between 15-100% (Fent *et al.*, 2006). Overall, less consumption of naproxen compared to ibuprofen (*e.g.*, 35 tons versus 162 tons annually in the UK, respectively : Jones *et al.*, 2002) might somewhat explain the lower concentrations observed in municipal effluent sources.

Acting as lipid regulators, gemfibrozil and bezafibrate control blood levels of cholesterol and triglycerides to minimize cardiovascular risk. Both were detected in similar concentrations in the urban effluent under study (59 and 72 ng/L, respectively). Gemfibrozil

Table 2 - Classes and concentrations of the 12 pharmaceutical products detected in a major municipal wastewater treatment plant effluent located in the Montreal area.

Class	Substance	Biological effect	Detected concentration (ng/L) ^a
Anti-inflammatory agent	Ibuprofen	COX inhibitor	1191
	Naproxen	COX inhibitor	217
Lipid regulator	Gemfibrozil	PPAR ^b agonist	59
	Bezafibrate	PPAR ^b agonist	72
Anti-convulsant	Carbamazepine	Narcosis (membrane depolarisation)	33
Antibiotics	Sulfamethoxazole	Tetrahydrofolate synthesis inhibitor	99
	Sulfapyridine	Competitive inhibitor of bacterial PABA ^c	46
	Oxytetracycline	Bacterial protein synthesis inhibitor	440
	Novobiocin	DNA synthesis inhibitor	330
	Trimethoprim	Prevents reduction of dihydrofolate	63
Stimulant	Caffeine	CNS ^d , cardiac and respiratory stimulant	22187
Nicotine metabolite	Cotinine	Nicotine metabolite	236

a) These recently-published data (Gagné *et al.*, 2006a, Table 1, page 331) are reproduced by permission of Elsevier.

b) Peroxisome proliferator-activated receptor.

c) Para-aminobenzoic acid.

d) Central nervous system

concentrations found in comparable wastewaters of other countries have been reported to be somewhat higher (1200 – 4760 ng/L) than that measured in our effluent (Metcalf *et al.*, 2003b; Tixier *et al.*, 2003; Andreozzi *et al.*, 2003; Ternes, 1998). In their study of 18 Canadian-based wastewater treatment plants, Metcalfe *et al.* (2003b) failed to detect gemfibrozil in the same Montreal effluent (< 20 ng/L detection limit). Elimination of this drug by wastewater treatment has been purported to be in the order of 46% and 69% according to studies conducted in Brazil (Stumpf *et al.*, 1999) and Germany (Ternes, 1998), respectively. Citing varied sources, Fent *et al.* (2006) report gemfibrozil removal rates to lie between 10-75%. Variations in treatment plant performance may, among other factors, explain this discrepancy with respect to % elimination. The bezafibrate concentration reported by Metcalfe *et al.* (2003b) in the same effluent was 600 ng/L, a value eight-fold higher than the one we report herein (72 ng/L). Other concentrations measured elsewhere in similar effluents range from 259 – 4600 ng/L (Metcalf *et al.*, 2003b; Ternes, 1998; Stumpf *et al.*, 1999). Again, treatment plant efficiency to eliminate this product appears to vary between 50% (Stumpf *et al.*, 1999) and 83% (Ternes, 1998). A more recent investigation indicates that bezafibrate removal, all factors confounded, can vary between 10-100% (Fent *et al.*, 2006).

Carbamazepine possesses anti-convulsant properties proven useful for treating epilepsy. It is also used to ease chronic pain (Hansen, 1999) and in psycho-pharmaco therapy to treat bipolar disorder (Bowden, 1997). Unlike most other medications, carbamazepine appears resistant to wastewater treatment and its elimination, 3 % (Jones *et al.*, 2002), 7% (Ternes, 1998), and < 10% (Paxéus, 2004) is rather negligible. More recent studies, however, suggest that removal of this pharmaceutical by sewage treatment plants is improving : 0-53% (Fent *et al.*, 2006); 91.3% (Han *et al.*, 2006). Several of its chemical properties including lipophilicity ($K_{ow} = 2.93$, Kasim *et al.*, 2004), half-life of 100 days (Ternes, 1998; Andreozzi *et al.*, 2003) and biocentration factor (15.36, Jones *et al.*, 2002) suggest it could be persistent once it enters an aquatic environment and may have a potential for food chain bio-magnification that will merit further attention (Gagné *et al.*, 2006c). In their study on the same Montreal effluent, Metcalfe *et al.* (2003b) reported a concentration for carbamazepine of 400 ng/L, a value 12-fold higher than ours (33 ng/L). Values determined elsewhere in similar effluents range from 126 – 6900 ng/L (Metcalf *et al.*, 2003b; Tixier *et al.*, 2003; Andreozzi *et al.*, 2003; Ternes, 1998; Zwiener & Frimmel, 2004).

Five antibiotics were detected in the municipal effluent investigated (Table 2). Designed to inhibit

microbial growth or to be biocidal, antibiotics are widely used for human and veterinary purposes. Examples of reported consumption indicate 10000 and 14000 tons per annum in Europe (Kummerer, 2001) and the United States of America (Sanderson *et al.*, 2004). With regards to sulfonamide antibiotics, sulfamethoxazole concentrations determined in similar effluents in European cities ranged from 20 – 2000 ng/L (Hirsch *et al.* 1999; Andreozzi *et al.* 2003), a spread clearly influenced by factors such as population, consumption as well as wastewater treatment process and efficiency. For final WWTP effluents in Canada, median and maximum values of 243 and 871 ng/L have been reported, respectively (Miao *et al.*, 2004). A more recent investigation conducted at three WWTPs with varying treatment technologies and design in the United States detected sulfamethoxazole concentrations ranging from 370 to 6000 ng/L (Batt *et al.*, 2006a). A 1985 study suggests that sulfamethoxazole is poorly degradable (Richardson & Brown, 1985), while a more recent one indicates the contrary based on its half-life of 2.4 days (Andreozzi *et al.*, 2003). Other studies corroborate its poor degradability by sewage treatments (Halling-Sorensen, 1998) and its persistence in the environment (Jjemba, 2006). Our reported value of 46 ng/L in sewage effluent for sulfapyridine, a second sulfonamide antibiotic, could only be spatially compared with two other published studies. In WWTP effluents located in five Canadian cities, its concentrations averaged 81 ng/L with a maximum value of 228 ng/L (Miao *et al.*, 2004). Concentrations ranging from 40-350 ng/L were also reported for two municipal WWTPs in Switzerland (Göbel *et al.*, 2005). This same study also showed that elimination of sulfapyridine following activated sludge treatment is incomplete likely owing to the low sorption capacity of sulfonamides with solid particles (*i.e.*, sorption constants for activated sludge below 500L/kg).

Three other antibiotics were quantified in the Montreal urban effluent for which little or no other spatial data exist in terms of their presence in similar waste or surface waters. Oxytetracycline, a tetracycline antibiotic, widely used in the UK at 30 tons per annum (Jones *et al.*, 2002; Schowanek & Webb, 2000), is known to be resistant to biodegradation (Richardson and Brown, 1985; Jones *et al.*, 2002), but likely not bioaccumulative owing to its low bioconcentration factor value of three (Jones *et al.*, 2002). Its half-life under anoxic conditions is reported to be one of nine days (Halling-Sorensen *et al.*, 1998). While there is a paucity of data on the presence of oxytetracycline in other sewage effluents, maximum concentrations of 340 and 1630 ng/L have been reported for oxytetracycline in surface waters of the U.S.A. (Kolpin *et al.*, 2002) and the U.K. (Schowanek & Webb, 2000), respectively. Total removal of this antibiotic by WWTP processes

(biodegradation, sorption to sludge, etc.) has been estimated to be in the order of only 2% (Jones *et al.*, 2002).

No spatial data were found for similar effluents with which to compare our reported concentration of 330 ng/L for the macrolide antibiotic, novobiocin (Table 2). Data on its persistence and degradability potential also appear absent in the literature. For the bacteriostatic antibiotic trimethoprim, quantified at 63 ng/L in our municipal wastewater (Table 2), an average concentration of 154 ng/L for other sewage treatment plant effluents has been reported (Hernando *et al.*, 2006), as have maximum values of 660 ng/L in German WWTP effluents (Zwiener & Frimmel, 2004) and of 1288 ng/L in U.K. WWTP effluents (Ashton *et al.*, 2004). In another investigation, three grab samples taken at different times of the year in one WWTP effluent in North America revealed trimethoprim concentrations of 94, 313 and 344 ng/L (Hua *et al.*, 2006). This study also demonstrated the marked influence that seasonality can have in modulating WWTP effluent pharmaceutical concentrations for trimethoprim and other substances owing to changes in water conditions. The fate of trimethoprim in WWTPs is unclear, but its environmental half-life has been reported to lie between 20-100 days (Zuccato *et al.*, 2001). A recent study reports a WWTP removal efficiency of only three percent for trimethoprim based on a comparison of median concentration values measured in raw and final effluents (Roberts and Thomas, 2006). In contrast, nitrifying bacteria activated sludge was shown to enhance removal of trimethoprim in WWTP effluent up to 50% over conventional activated sludge at only 1% owing to higher solid retention time in the former sludge (Batt *et al.*, 2006b).

As an alkaloid present in more than 60 plant species, *e.g.* tea, coffee, cacao (IARC, 1991), caffeine is likely the most consumed drug worldwide (Ogunseitan, 1996). Clearly beneficial as a cardiac, cerebral and respiratory stimulant, among other pharmaceutical properties, its presence appears ubiquitous in wastewaters, surface waters and groundwaters (Buerge *et al.*, 2003). Indeed, it has been suggested as a suitable marker of anthropogenic inputs of domestic origin to aquatic ecosystems (Buerge *et al.*, 2003; Siegener & Chen, 2002). Wastewater treatment plant effluents in Switzerland are reported to discharge between 30-9500 ng/L of caffeine (Buerge *et al.*, 2003) while concentrations emitted by similar effluents to Boston harbor lie between 370-6700 ng/L (Siegener & Chen, 2002). The higher concentration found in the Montreal municipal effluent investigated (22187 ng/L, Table 2) may be linked to its limited treatment (primary treatment with phosphorus removal only), as caffeine appears to be more efficiently removed in domestic wastewaters receiving activated sludge

treatment (Buerge *et al.*, 2003) where it is readily biodegradable (Halling-Sorensen *et al.*, 1998).

The nicotine metabolite cotinine was the 12th pharmaceutical product detected in the Montreal-based wastewater treatment plant (WWTP) effluent investigated at a concentration of 236 ng/L (Table 2). Cotinine is generally present in WWTP effluents and surface waters contaminated by drugs, and levels of 22 (mean) and 58 ng/L (maximum) have been previously reported for Canadian WWTP effluents (Metcalf *et al.* 2004). We have not found data relating to its persistence/degradability potential nor removal efficiency by WWTPs in the scientific literature.

Biological effects of pharmaceuticals

Toxicity data determined in our study on the 12 pharmaceuticals integrated with those reported in the literature reflect a wide spectrum of sensitivity that is biological level-, test- and endpoint-specific (Table 3). Overall, toxicity testing performed elsewhere with acute/chronic bioassays similar to ours essentially display commensurate levels of responses for these chemicals. Table 3 highlights their available acute and chronic effects that have been thus far generated worldwide at various levels of aquatic biological organization. Included are toxicity results for bacteria (acute), blue-green algae (chronic), algae (chronic), macrophytes (chronic), invertebrates (acute and chronic), as well as fish/fish cells (acute) and bivalve cells (acute). Notable is the recurrent use of several well-documented and popular bioassays (*e.g.*, *V. fischeri* luminescence inhibition test, *P. subcapitata* growth inhibition test, *D. magna* immobilization and reproduction tests) that have proven valuable to screen chemical toxicity in past decades, as well as more recent bioassays that show promise for such purposes (*e.g.*, macrophyte growth inhibition tests, *Hydra* morphology and behavioral tests, fish and bivalve cell assays). With all acute and chronic tests confounded for these 12 pharmaceuticals, toxicity responses span over five orders of magnitude (least sensitive response : 24h LC50 of the invertebrate *T. platyurus* = 4661 mg/L for caffeine; most sensitive response : 96 h IC50 of the cyanobacterium *S. leopolensis* = 0.027 mg/L for sulfamethoxazole). A similar spectrum of responses has been reported in a recent study of ecotoxicity data compiled for over 100 pharmaceuticals where acute toxicity effects spanned over six orders of magnitude (Webb, 2004).

Classifying bioassay data in Table 3 according to EU-Directive 93/67/EEC (CEC, 1996) offers some estimate of hazard potential for the pharmaceuticals studied (Table 4). While the spread of toxicity responses places these 12 compounds in all cut-off classes, some notable trends appear in their ecotoxic potency. Caffeine and cotinine are clearly less hazardous than all others.

Table 3 - Acute (A) and chronic (C) bioassay responses to the 12 pharmaceutical products investigated. Toxicity data are from our study and from those reported in the literature.

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Ibuprofen					
Bacteria	12.3	5-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Knoll, 1995
	19.1	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	la Farré <i>et al.</i> , 2001
	57.9	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
Algae	1	5d-IC25 (C)	Growth inhibition	<i>Synechosystis sp.</i> (cyanobacterium)	Pomati <i>et al.</i> , 2004
	90.5	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	315	72h-IC50 (C)	Growth rate inhibition	<i>Desmodesmus subspicatus</i>	Cleuvers, 2003
	342	72h-IC50 (C)	Growth rate inhibition	<i>Desmodesmus subspicatus</i>	Cleuvers, 2004
Macrophytes	>1	7d-NOEC (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> , 2004
	4.0	7d-IC50 (C)	Growth inhibition	<i>Lemna gibba</i>	Pomati <i>et al.</i> , 2004
	22	7d-IC50 (C)	Growth rate inhibition	<i>Lemna minor</i>	Cleuvers, 2003
Micro-invertebrates	>0.01	7d-LOEC (C)	(Sub)lethal toxicity and feeding inhibition	<i>Hydra vulgaris</i>	Pascoe <i>et al.</i> , 2003
	9.1 to 11.5	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Knoll, 1995
	17.5	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	20	21d-NOEC (C)	Reproduction inhibition	<i>Daphnia magna</i>	Han <i>et al.</i> , 2006
	108	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Cleuvers, 2003
	132.6	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Han <i>et al.</i> , 2006
	>200	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	Nalecz-Jawecki and Persoone, 2006
	471	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	173	96h-LC50 (A)	Mortality	<i>Lepomis macrochirus</i> (bluegill sunfish)	Knoll, 1995
	>228	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	248	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
	1194	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) gonad cells	Caminada <i>et al.</i> , 2006

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Naproxen					
Bacteria	>12.5	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	35.6	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	la Farré <i>et al.</i> , 2001
Algae	19.3	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	31.8	96h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Isidori <i>et al.</i> , 2005
	>320	72h-IC50 (C)	Growth rate inhibition	<i>Desmodesmus subspicatus</i>	Cleuvers, 2003
	626	72h-IC50 (C)	Growth rate inhibition	<i>Desmodesmus subspicatus</i>	Cleuvers, 2004
Macrophytes	1	7d-LOEC (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> 2004
	24.2	7d-IC50 (C)	Growth rate inhibition	<i>Lemna minor</i>	Cleuvers, 2003
Micro-invertebrates	0.33	7d-EC50 (C)	Reproduction inhibition	<i>Ceriodaphnia dubia</i>	Isidori <i>et al.</i> , 2005
	0.56	48h-EC50 (C)	Reproduction inhibition	<i>Brachionus calyciflorus</i>	Isidori <i>et al.</i> , 2005
	2.6	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	>25	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
	62.5	24h-LC50 (A)	Mortality	<i>Brachionus calyciflorus</i>	Isidori <i>et al.</i> , 2005
	66.4	48h-LC50 (A)	Immobilization	<i>Ceriodaphnia dubia</i>	Isidori <i>et al.</i> , 2005
	84.1	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	Isidori <i>et al.</i> , 2005
	140	24h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Webb, 2004
	166	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Cleuvers, 2004
	174	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Cleuvers, 2003
	>200	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	Nalecz-Jawecki and Persoone, 2006
	383	96h-LC50 (A)	Mortality	<i>Hyalella azteca</i>	Webb, 2004
Fish/fish cells	>230	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	585	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
	560	96h-LC50 (A)	Mortality	<i>Lepomis macrochirus</i>	Webb, 2004
	690	96h-LC50 (A)	Mortality	<i>Oncorhynchus mykiss</i>	Webb, 2004
	1032	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) cells	Caminada <i>et al.</i> , 2006

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Gemfibrozil					
Bacteria	>15.6	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	31.5	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	la Farré <i>et al.</i> , 2001
	45.1	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Zurita <i>et al.</i> , 2006
Algae	7.3	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	150	72h-IC50 (C)	Growth inhibition	<i>Chlorella vulgaris</i>	Zurita <i>et al.</i> , 2006
Micro-invertebrates	0.8	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	10.4	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Han <i>et al.</i> , 2006
	30	72h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Zurita <i>et al.</i> , 2006
	>31.3	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	219	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
	>250	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	986	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) cells	Caminada <i>et al.</i> , 2006
Bezafibrate					
Bacteria	>15.6	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
Algae	>31.3	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Micro-invertebrates	5.3	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	30.3	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Han <i>et al.</i> , 2006
	>62.5	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	>362	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	445	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) cells	Caminada <i>et al.</i> , 2006
	941	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
Carbamazepine					
Bacteria	78.5	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Jos <i>et al.</i> , 2003
	>125	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	>810	30-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Ferrari <i>et al.</i> , 2004

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Algae	31.6	96h-IC50 (C)	Growth inhibition	<i>Cyclotella meneghiniana</i> (diatom)	Ferrari <i>et al.</i> , 2004
	33.6	96h-IC50 (C)	Growth inhibition	<i>Synechococcus leopoldensis</i> (cyanobacterium)	Ferrari <i>et al.</i> , 2004
	36.6	48h-IC50 (C)	Growth inhibition	<i>Chlorella vulgaris</i>	Jos <i>et al.</i> , 2003
	74	72h-IC50 (C)	Growth rate inhibition	<i>Desmodesmus subspicatus</i>	Cleuvers, 2003
	79.4	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	>100	96h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Ferrari <i>et al.</i> , 2004
Macrophytes	>1	7d-EC10 (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> 2004
	25.5	7d-IC50 (C)	Growth rate inhibition	<i>Lemna minor</i>	Cleuvers, 2003
Micro-invertebrates	12.1	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	>13.8	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Ferrari <i>et al.</i> , 2004
	77.7	48h-EC50 (A)	Immobilization	<i>Ceriodaphnia magna</i>	Ferrari <i>et al.</i> , 2004
	97.8	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Jos <i>et al.</i> , 2003
	>100	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Cleuvers, 2003
	111	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Han <i>et al.</i> , 2006
	140	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	Nalecz-Jawecki and Persoone, 2006
	>250	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	21.3	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	>591	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
	>591	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) cells	Caminada <i>et al.</i> , 2006
Sulfamethoxazole					
Bacteria	65.3	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	>84	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Ferrari <i>et al.</i> , 2004

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Algae	0.027	96h-IC50 (C)	Growth inhibition	<i>Synechococcus leopolensis</i> (cyanobacterium)	Ferrari et al., 2004
	0,146	96h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Ferrari et al., 2004
	1.53	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Eguchi et al., 2004
	2.4	96h-IC50 (C)	Growth inhibition	<i>Cyclotella meneghiniana</i> (diatom)	Ferrari et al., 2004
	13.4	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Micro-invertebrates	>0.1	6d-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Flaherty and Dodson, 2005
	19.3	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	>100	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Ferrari et al., 2004
	>100	48h-EC50 (A)	Immobilization	<i>Ceriodaphnia magna</i>	Ferrari et al., 2004
	>250	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	>253	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
Bivalve cells	70.9	24h-TEC ^e (A)	Phagocytosis inhibition	Hemocytes of <i>Elliptio complanata</i> (bivalve)	Gagné et al., 2006b
Sulfapyridine					
Bacteria	>125	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
Algae	10.2	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Micro-invertebrates	144.4	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
	>200	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
Fish/fish cells	>249	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
Bivalve cells	7.98	24h-TEC ^e (A)	Phagocytosis inhibition	Hemocytes of <i>Elliptio complanata</i> (bivalve)	Gagné et al., 2006b
Oxytetracycline^c					
Bacteria	3.0 to 6.9	4h-IC50 (A)	Growth rate inhibition	Activated sludge micro-organisms	Christensen et al., 2006
	66	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Christensen et al., 2006
	135.9	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Algae	0.2	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	0.207	7d-IC50 (C)	Growth inhibition	<i>Microcystis aeruginosa</i> (cyanobacterium)	Holten-Lützhof <i>et al.</i> , 1999
	0.342	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Eguchi <i>et al.</i> , 2004
	0.47 to 2.0	48h-IC50 (C)	Growth rate inhibition	<i>Pseudokirchneriella subcapitata</i>	Christensen <i>et al.</i> , 2006
	4.18	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	De Liguoro <i>et al.</i> , 2003
	4.5	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Holten-Lützhof <i>et al.</i> , 1999
	5	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Webb, 2004
	6.4	48h-IC50 (C)	Growth inhibition	<i>Chlorella vulgaris</i>	Pro <i>et al.</i> , 2003
	7.05	72h-IC50 (C)	Growth inhibition	<i>Chlorella vulgaris</i>	Eguchi <i>et al.</i> , 2004
Macrophytes	0.79	7d-EC10 (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> , 2004
	1.01	7d-IC50 (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> , 2004
	4.92	7d-IC50 (C)	Growth inhibition	<i>Lemna minor</i>	Pro <i>et al.</i> , 2003
Micro-invertebrates	41.5	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	46.2	21d-EC50 (C)	Reproduction inhibition	<i>Daphnia magna</i>	Wollenberger <i>et al.</i> , 2000
	100	48h-EC10 (A)	Immobilization	<i>Daphnia magna</i>	Wollenberger <i>et al.</i> , 2000
	337.6	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	200	96h-LC50 (A)	Mortality	<i>Salvelinus namaycush</i> (Lake trout)	Webb, 2004
	>200	24h-LC50 (A)	Cytotoxicity	RTG-2 (rainbow trout gonad cells)	Babin <i>et al.</i> , 2005
	>200	24h-LC50 (A)	Cytotoxicity	RTL-W1 (rainbow trout liver cells)	Babin <i>et al.</i> , 2005
	>497	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
Novobiocin					
Bacteria	0.08	5h-IC50 (A)	Growth inhibition	<i>Vibrio harveyi</i>	Thomulka <i>et al.</i> , 1993
	30.1	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	>100	1h-IC50 (A)	Luminescence inhibition	<i>Vibrio harveyi</i>	Thomulka <i>et al.</i> , 1993
Algae	130	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Micro-invertebrates	135.2	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	168.1	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Fish/fish cells	34.9	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
Bivalve cells	0.635	24h-TEC ^e (A)	Phagocytosis inhibition	Hemocytes of <i>Elliptio complanata</i> (bivalve)	Gagné <i>et al.</i> , 2006b
Trimethoprim					
Bacteria	183.3	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
Algae	80.3	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Eguchi <i>et al.</i> , 2004
	96.7	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
	110	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Halling-Sorensen <i>et al.</i> , 2000
	112	7d-IC50 (C)	Growth inhibition	<i>Microcystis aeruginosa</i> (cyanobacterium)	Holten-Lützhof <i>et al.</i> , 1999
	130	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	Holten-Lützhof <i>et al.</i> , 1999
Macrophytes	>1	7-d EC10 (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> , 2004
Micro-invertebrates	>85.3	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	123	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Halling-Sorensen <i>et al.</i> , 2000
	161.2	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	>290	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	>100	72h-LC50 (A)	Mortality	<i>Brachydanio rerio</i>	Halling-Sorensen <i>et al.</i> , 2000
Caffeine^d					
Bacteria	569	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
	920	24h-MTC* (A) *microbial toxic concentration	Growth inhibition	This assay uses an array of 11 microbial strains	Gabrielson <i>et al.</i> , 2003
	2100	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	Ekwal <i>et al.</i> , 1998
Algae	339.3	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Macrophytes	>1	7d-EC10 (C)	Growth inhibition	<i>Lemna gibba</i>	Brain <i>et al.</i> , 2004

Table 3 - (continuation).

Substance	Reported value (mg/L)	Measurement endpoint	Assessment endpoint	Species	Reference
Micro-invertebrates	160	48h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Ekwal <i>et al.</i> , 1998
	>200	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	Nalecz-Jawecki and Persoone, 2006
	530.3	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	684	24h-EC50 (A)	Immobilization	<i>Daphnia magna</i>	Lilius <i>et al.</i> , 1994
	>1500	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
	4661	24h-LC50 (A)	Mortality	<i>Brachionus calyciflorus</i>	Calleja <i>et al.</i> , 1994
Fish/fish cells	151	96h-EC50 (A)	Mortality	<i>Pimephales promelas</i> (fathead minnow)	Russom <i>et al.</i> , 1997
	>194	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study
	>485	24h-EC50 (A)	Cytotoxicity	PLHC-1 (<i>Poeciliopsis lucida</i>) hepatoma cells	Caminada <i>et al.</i> , 2006
	>485	24h-EC50 (A)	Cytotoxicity	RTG-2 (rainbow trout) cells	Caminada <i>et al.</i> , 2006
Cotinine					
Bacteria	369.3	15-min IC50 (A)	Luminescence inhibition	<i>Vibrio fischeri</i>	This study
Algae	589.5	72h-IC50 (C)	Growth inhibition	<i>Pseudokirchneriella subcapitata</i>	This study
Micro-invertebrates	>375	96h-EC50 (A)	Morphology perturbation	<i>Hydra attenuata</i>	This study
	>700	24h-LC50 (A)	Mortality	<i>Thamnocephalus platyurus</i>	This study
Fish/fish cells	88.1	48h-TEC ^a (A)	Cytotoxicity	<i>Oncorhynchus mykiss</i> (primary hepatocytes)	This study

a) Rainbow trout primary hepatocytes.

b) Threshold Effect Concentration (see Table 1, footnote b).

c) SOS Chromotest positive indicating DNA damage (without S9 mix) at a threshold effect concentration of 22.1 mg/L.

d) SOS Chromotest positive indicating DNA damage (without S9 mix) at a threshold effect concentration of 354 mg/L.

e) TEC (threshold effect concentration) for phagocytosis as manifested by a significant reduction in internalization capacity of fluorescent bacteria = (NOEC x LOEC)^{1/2}, where NOEC = no observed effect concentration and LOEC = lowest observed effect concentration.

While caffeine has been placed in the «toxic» category based on an indeterminate value of >1 mg/L reported for the macrophyte *L. gibba* (Table 3), this may be an unrealistic overestimate of its toxic potential and the real value may in fact lie in the «harmful» or «not toxic» categories. Other than toxicity data generated in our

study for the nicotine metabolite cotinine (Table 3), none were found in the literature. In contrast, the parent molecule nicotine appears generally more toxic judging from aquatic toxicity values reported elsewhere : fathead minnow 96h-LC50 = 13.8 mg/L (Russom *et al.*, 1997); fathead minnow 96h-LC50 = 19.7 mg/L; goldfish 96h-

LC50 = 13.1 mg/L; rainbow trout 96h-LC50 = 7.31 mg/L; bluegill 96h-LC50 = 4.31 mg/L; *D. magna* 48h-EC50 = 3.25 mg/L (Holcombe *et al.*, 1987); rainbow trout yolk-sac stage post-hatch fry 96h-LC50 = 4 mg/L (Edsall, 1991); *D. pulex* 48h-EC50 = 0.24 mg/L (Smith *et al.*, 1988). After metabolic transformation, toxic potency therefore decreases from «very toxic» (nicotine) to one of «harmful» or «not toxic» (cotinine), based on EU-Directive classification (Table 4).

The anti-inflammatory agents (ibuprofen, naproxen) and lipid regulators (gemfibrozil, bezafibrate) display a somewhat similar risk profile ranging from «very toxic» to «not toxic». Algae, cyanophytes, invertebrates and macrophytes appear particularly more sensitive to these classes of pharmaceuticals than other groups owing to their responses in the «very toxic» and «toxic» class ranges (Table 4). Toxic responses for the anti-convulsant carbamazepine are surprisingly homogeneous as they all fall in the «harmful» category, thereby suggesting a lower risk for aquatic life than that of other groups of substances. Acute toxicity results on the ecotoxicity of pharmaceuticals also show reported effects for carbamazepine ranging from 12.7 mg/L and higher (Fent *et al.*, 2006).

The spectrum of toxicity responses for the five antibiotics is the most diversified ranging from «extremely

toxic» to «not toxic» (Table 4). More susceptible to their action are algae, bacteria, bivalve cells, cyanophytes and macrophytes whereas invertebrates and fish/fish cells appear less sensitive. For example, sulfamethoxazole is shown to be «extremely toxic» to the cyanophyte *S. leopolensis* and, likewise, novobiocin to the luminescent bacterium *V. harveyi* (Tables 3 and 4). Respective exposure of these two micro-organisms to sulfamethoxazole (96h-IC50 = 0.027 mg/L) and novobiocin (0.08 mg/L) resulted in the highest toxicity values reported in Table 3. Again, sulfamethoxazole, novobiocin, oxytetracycline and sulfapyridine produce «very toxic» and «toxic» responses in algae, bacteria, bivalve cells or macrophytes. Of the five antibiotics, trimethoprim appears less bioactive as it proved to be possibly «toxic» to macrophytes, «harmful» to algae and «not toxic» to all other taxonomic groups (Table 4).

Overall, fish/fish cells were the least sensitive of all biotic groups when challenged with the 12 pharmaceuticals. Barring a «harmful» hazard potency for carbamazepine and cotinine, all other responses fell in the «not toxic» category (Table 4). An acute toxicity data base constructed for over 100 pharmaceuticals showed that fish were less sensitive than algae and invertebrates (Webb, 2004). The apparent lesser sensitivity of fish to pharmaceuticals was also pointed

Table 4 - Toxicity of the pharmaceutical groups found in the municipal wastewater treatment plant effluent according to European Union Commission Guideline 93/67/EEC classification and based on measurement endpoint values (LC50, EC50, IC50, threshold effect concentration, etc.) reported for the most sensitive bioassay listed in Table 3.

Group	Extremely toxic (< 0.1 mg/L)	Very toxic (0.1-1 mg/L)	Toxic (1-10 mg/L)	Harmful (10-100 mg/L)	Not toxic (> 100 mg/L)
Anti-inflammatory agents : ibuprofen (ibu), naproxen (nap)		C-ibu I-nap M-nap	I-ibu M-ibu	A-ibu/nap B-ibu/nap	F-ibu/nap
Lipid regulators : gemfibrozil (gem), bezafibrate (bez)		I-gem	A-gem I-gem/bez	(A-bez) ^a (B-bez) ^a	F-gem/bez
Anti-convulsant : carbamazepine (cbz)			(M-cbz) ^a	A-cbz B-cbz C-cbz F-cbz I-cbz	
Antibiotics : sulfamethoxazole (smx), sulfapyridine (spy), oxytetracycline (oxy), novobiocin (nov), trimethoprim (tri)	B-nov C-smx	A-smx/oxy ^b Bi-nov C-oxy M-oxy	A-spy B-oxy Bi-spy (M-tri) ^a	A-tri B-smx Bi-smx I-smx/oxy	A-nov B-spy/tri C-tri F-smx/spy/oxy/nov/tri I-spy/nov/tri
Stimulant : caffeine (caf)			(M-caf) ^a		A-caf B-caf F-caf I-caf
Nicotine metabolite : cotinine (cot)				F-cot	A-cot B-cot I-cot

Taxonomic groups : A (algae); B (bacteria); Bi (bivalve cells); C (cyanobacteria); F (fish/fish cells); I (invertebrates); M (macrophytes).

a) Based on an indeterminate value of «greater than» (>) for this class (see Table 3).

b) Example : For antibiotics (oxytetracycline), the green alga *P. subcapitata* gave the most sensitive response for reported algal toxicity tests (IC50 = 0.2 mg/L, Table 3), thereby placing this compound in the «Very Toxic» category.

out in a review undertaken by Carlsson *et al.* (2006) on the environmental risk assessment of 27 active pharmaceutical ingredients. The authors reported that acute toxicities of 24 of these were shown to differ markedly based on taxonomic group (micro-organisms, algae, invertebrates and fish) with algae being the most sensitive (effects at µg/L for antibiotics) and fish generally the least sensitive (effects greater than 1000 mg/L for some pharmaceuticals). That fish appear less prone to the adverse effects of antibiotics was also noted by Crane *et al.* (2006). In their own review, Fent *et al.* (2006) state that fish were, in general, less sensitive to pharmaceuticals than other trophic levels.

Comparing the highest and lowest sensitivity responses of bioassay data reported in Table 3 for each pharmaceutical offers further insights regarding their toxicity (Table 5). Indeed, the toxic spread index (tsi) gives some information about whether toxicity is generic (low index value) or specific (high index value). On the one hand, cotinine, whose calculated tsi value is >7.9, is indicative of a narrow span of toxic effects towards all taxonomic groups. Sulfamethoxazole, on the other hand,

whose tsi value is >9370, indicates toxic effects that are more or less intense depending on the exposed biotic group. Cotinine's low tsi value is also clearly reflected by its hazard potential essentially confined to the «not toxic» category, whereas sulfamethoxazole's high tsi value spreads across all categories of toxicity (Table 4). This suggests that pharmaceuticals with large tsi values are likely more hazardous to aquatic environments if they feature a broad range of effects on a large number of species across several biological levels. However, drugs having low tsi index values (as per Table 5) but categorized in the «toxic» to «extremely toxic» categories (as per Table 4) would also present greater risk. For the non-antibiotics studied, cotinine, caffeine, bezafibrate and carbamazepine display lower tsi values than ibuprofen, naproxen and gemfibrozil. For the five antibiotics, tsi values for novobiocin, oxytetracycline and sulfamethoxazole are markedly above those of sulfapyridine and trimethoprim (Table 5). The most sensitive endpoint values reported in Table 5 are also informative about pharmaceutical concentrations at which the most susceptible species can be affected.

Table 5 - Toxic spread index (ratio of least sensitive endpoint value ÷ most sensitive endpoint value) based on the comparative sensitivity responses of bioassays reported in Table 3 to the 12 pharmaceuticals.

Pharmaceutical class	Toxic spread index
Non-antibiotics	
Cotinine: >700 mg/L (<i>T. platyurus</i>) ÷ 88.1 mg/L (Fish primary hepatocyte cells)	>7.9
Caffeine: 4661 mg/L (<i>B. calyciflorus</i>) ÷ 151 mg/L (<i>P. promelas</i>)	30.9
Bezafibrate: 941 mg/L (Fish PLHC-1 cells) ÷ 5.3 mg/L (<i>H. attenuata</i>)	178
Carbamazepine: >591 mg/L (Fish RTG-2 and PLHC-1 cells) ÷ >1 mg/L (<i>L. gibba</i>) ^a	>296
Ibuprofen: 1194 mg/L (Fish RTG-2 cells) ÷ 1 mg/L (<i>Synechosystis</i> sp.) ^b	1194
Naproxen: 1032 mg/L (Fish RTG-2 cells) ÷ 0.33 mg/L (<i>C. dubia</i>)	1233
Gemfibrozil: 986 mg/L (Fish RTG-2 cells) ÷ 0.8 mg/L (<i>H. attenuata</i>)	3127
Antibiotics	
Sulfapyridine: >249 mg/L (Fish primary hepatocyte cells) ÷ 7.98 mg/L (Bivalve cells)	>31.2
Trimethoprim: >290 mg/L (Fish primary hepatocyte cells) ÷ >1 mg/L (<i>L. gibba</i>) ^a	>145
Novobiocin: 168.1 mg/L (<i>T. platyurus</i>) ÷ 0.08 mg/L (<i>V. harveyi</i>)	2101
Oxytetracycline: >497 mg/L (Fish primary hepatocyte cells) ÷ 0.2 mg/L (<i>P. subcapitata</i>)	>2485
Sulfamethoxazole: >253 mg/L (Fish primary hepatocyte cells) ÷ 0.027 mg/L (<i>S. leopolensis</i>)	>9370

a) The value of ">1 mg/L" reported for *Lemna gibba* by Brain *et al.*, (2004) in Table 3, their highest experimental concentration, was set at "2" for the purpose of this exercise.

b) The value of «>0.01 mg/L» reported for *Hydra vulgaris* by Pascoe *et al.*, (2003) in Table 3, their highest experimental concentration, was not considered for the purpose of this exercise.

Notably, and depending on the substance being considered, one species of each of the taxonomic groups tested was always found to be the most sensitive. This pleads in favor of applying future test batteries for toxicity assessment of existing and new pharmaceuticals that should incorporate sensitive species from representative biological levels so as to circumscribe their full ecotoxic potential. Based on our study of 12 substances that encompass several classes of pharmaceuticals (Table 2), the alga *P. subcapitata*, the bacterium *V. harveyi*, the cyanophytes *Synechosystis* sp. and *S. leopolensis*, the invertebrates *C. dubia* and *H. attenuata*, as well as the macrophyte *L. gibba*, all appear to be good candidates for this purpose (Table 5).

The relationship between bioassay effect concentrations for the pharmaceuticals investigated and those found in WWTPs offers notions about their risk potential towards aquatic biota (Fig. 1). Based on maximal concentrations of pharmaceuticals reported in either the Montreal WWTP or those reported elsewhere, the most sensitive bioassay response differs by anywhere from less than one to more than six orders of magnitude. Owing to their toxic effects determined at levels close to the range of wastewater concentrations, sulfamethoxazole, naproxen and ibuprofen would appear

to pose greater risk to aquatic life, while less risk would be expected from oxytetracycline, gemfibrozil and novobiocin, whose biological effects are two orders of magnitude higher than concentrations emitted by WWTPs. The remaining compounds appear to pose little threat because of the wide gap separating their most sensitive bioassay response and reported WWTP concentrations (*i.e.*, three to six orders of magnitude difference).

Beyond the unquestionable use of acute and chronic toxicity tests for screening of emerging chemicals such as pharmaceuticals, more subtle adverse effects possibly resulting from the biotransformation of drugs uptaken by biota deserve attention.

In particular, oxidative stress owing to the release of reactive oxygen species can be a consequence of metabolism following xenobiotic accumulation (Winston *et al.*, 1996). In fact, acute exposure of the cnidarian *Hydra attenuata* to carbamazepine for 48 h induced oxidative metabolism at a threshold level of 7.1 µg/L with a concomitant increase in lipid peroxidation (Quinn *et al.*, 2004). This concentration is similar to the maximum value of 6.3 µg/L reported in WWTPs for this substance (Ternes, 1998), suggesting that oxidative

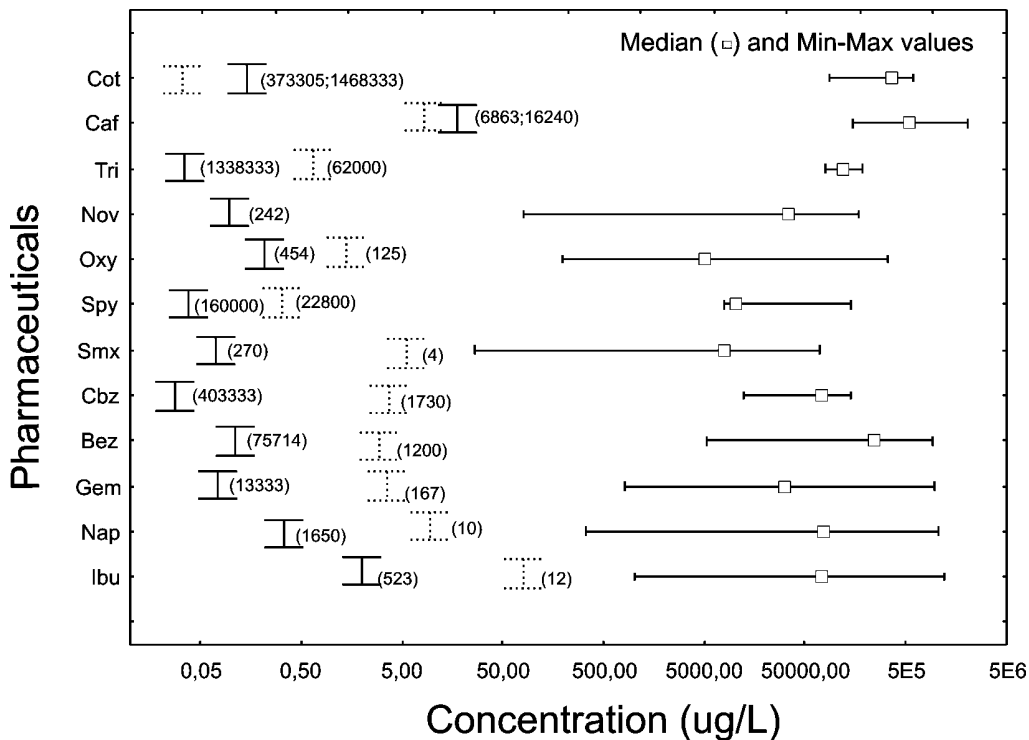


Figure 1 - Acute and chronic toxicity of the 12 pharmaceuticals (all biological levels confounded) and relationship with maximal concentrations found in WWTP effluents. Only determinate bioassay endpoint values in Table 3 were used to construct this scale. The vertical bars refer to maximal WWTP effluent concentrations found in the Montreal-based plant (full bars) and elsewhere (dotted bars). Values in parentheses are respective ratios of the maximum WWTP effluent concentration reported for each pharmaceutical and the corresponding most sensitive bioassay response obtained.

stress could occur at levels close to those discharged by municipal effluents. Since the sensitivity of *Hydra* has been shown to increase 10-15 fold under conditions of chronic exposure (21 days versus 4 days) to toxic samples (Arkhipchuk *et al.*, 2006), effect concentrations producing oxidative stress after metabolization of carbamazepine or other xenobiotics may even decrease further under situations of prolonged interaction.

Of the 12 pharmaceuticals studied, caffeine consistently yielded bioassay responses that were >100 mg/L (Table 3) and was therefore classified as «not toxic» according to EU-Directive 93/67/EEC (Table 4). However, following a 48h exposure of caffeine to *Hydra attenuata*, a significant activity of xanthine oxydoreductase, indicative of oxidative biotransformation, was measured (Fig. 2). Since this enzyme transforms hypoxanthine (and caffeine) into uric acid and hydrogen peroxide, oxidative stress occurs and reactive oxygen species may cause cellular damage. Results show that xanthine oxidative metabolism is induced in *Hydra* at = 0.2 mg/L which is only 10-fold the 0.022 mg/L concentration measured in the Montreal-based WWTP effluent (Table 2).

Since oxidative stress resulting from metabolization of uptaken drugs could realistically be

manifest at even lower concentrations under scenarios of chronic or continuous exposure to caffeine and other pharmaceuticals, such effects certainly warrant future study. More subtle effects owing to uptake of pharmaceuticals have also been highlighted for gemfibrozil where exposure to environmental levels by *Carassius auratus* (goldfish) leads to bioconcentration of the drug in plasma with consequent endocrine disrupting effects evidenced by a decrease in plasma testosterone (Mimeault *et al.*, 2005).

CONCLUSIONS

Chemical analysis of a Montreal-based WWTP effluent discharging to the Saint-Lawrence River identified the presence of 12 pharmaceuticals comprising anti-inflammatory agents, lipid regulators, an anti-convulsant, antibiotics, a simulant and a nicotine metabolite. Bioanalytical assessment of these substances with our own suite of tests complemented by acute and chronic toxicity data reported in the literature confirmed that most of these substances can be classified as toxic according to EU-Directive 93/67/EEC, although their measured concentrations in WWTPs are essentially too

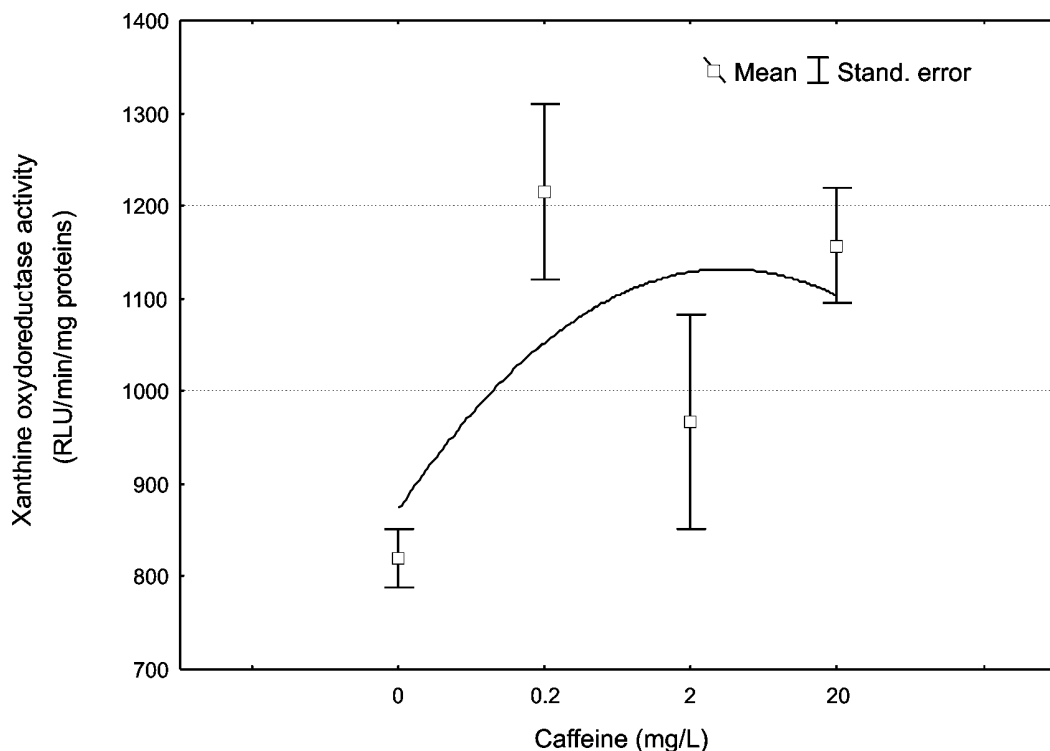


Figure 2 - Xanthine oxydoreductase activity after a 48h exposure of *Hydra attenuata* to caffeine. The data represent the mean with standard error from N=3 replicates. Curve adjustment was performed using the exponential negative ponderation model (Statistica software, version 7.1).

low to cause acute or chronic effects on an individual basis. Exceptions are perhaps sulfamethoxazole, naproxen and ibuprofen whose toxic effects were found to occur at concentrations close to those found in municipal effluents.

Overall, bioassay responses to the pharmaceuticals showed a wide range of sensitivity that advocates the use of tests representing diverse taxonomic levels to properly appraise their full toxic potential. Some species from each biotic group employed in toxicity testing were shown to be particularly sensitive toward individual drugs and certainly merit being part of future test batteries to evaluate other emerging substances such as pharmaceuticals. Beyond acute and chronic toxicity testing of pharmaceuticals, we intimated that metabolization of drugs uptaken by biota (e.g., induced activity of xanthine oxydoreductase in *Hydra* by caffeine, an essentially non toxic molecule) may lead to more insidious outcomes such as oxidative stress. These effects may be manifest at drug concentrations close to those present in urban effluents particularly under conditions of chronic or continuous exposure.

As recommended by others, we emphasize that future appraisal of the ecotoxicity of pharmaceuticals should continue to include bioassays (acute and chronic testing) of individual products and of mixtures (determination of interactive effects) at various trophic levels. Biomarker measurements are also required to assess the consequences associated with the biotransformation of drugs by biota. Fish, bivalves and other invertebrates, for example, are particularly suited for this purpose. Finally, recalcitrant drugs with lipophilic activity (e.g., $K_{ow} \geq 2$) need to be investigated to assess their potential to bio-accumulate and to bio-magnify up the food chain. We are presently developing simple experimental trophic chains in the laboratory for this goal.

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Effluent concentrations of pharmaceuticals in Table 2 are reprinted from Gagné et al., 2006a, Table 1, page 331, with permission from Elsevier.

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