Environmental monitoring of urban streams using a primary Fish Gill cell Culture System (FIGCS).

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Highlights

- A primary gill cell cultures system tolerates filtered urban river water
- The gill cells showed an increase in metallothionein gene expression
- The gill cells showed an increase in cyp1a1 and cyp3a27 gene expression
- The response of the gill cell culture system could be used for environmental monitoring purposes

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Abstract

The primary FIsh Gill Cell culture System (FIGCS) is an *in vitro* technique which has the potential to replace animals in whole effluent toxicity tests. In the current study FIGCS were transported into the field and exposed to filtered (0.2 µm) river water for 24 hrs from 4 sites, on 2 different sampling dates. Sites 1 and 2 are situated in an urban catchment (River Wandle, London, UK) with site 1 downstream of a sewage treatment work; site 3 is located in a suburban park (River Cray, Kent, UK), and site 4 is more rural (River Darent, Kent, UK). The change in transepithelial electrical resistance (TER), the expression of the metal responsive genes metallothionein A (*mta*) and B (*mtb*), cytochrome P450 1A1 (*cyp1a1*) and 3A27 (*cyp3a27*), involved in phase 1 metabolism, were assessed following exposure to sample water for 24 hrs. TER was comparable between FIGCS exposed to 0.2 µm filtered river water and those exposed to synthetic moderately soft water for 24 hrs. During the first sampling time, there was an increase in *mta*, *cyp1a1* and *cyp3a27* gene expression in epithelium exposed to water from sites 1 and 2, and during the second sampling period an increase in *cyp3a27* gene expression at sites 1 and 4. Urban river water is a complex mixture of contaminants (e.g., metals, pesticides, pharmaceuticals and polyaromatic hydrocarbons) and the increase in the expression of genes encoding *mta*, *cyp1a1* and *cyp3a27* in FIGCS is indicative of the presence of biologically active pollutants.

Introduction

A large number of fish are used each year for waste effluent toxicity testing, with an estimated 3 million being used in the US alone (see Tanneberger et al., 2013). There is a desire worldwide to reduce the number of fish used in toxicity testing and thus reliable alternatives are being investigated. A number of studies have assessed fish cell lines as alternative methodologies with success (Davoren et al., 2005; Dayeh et al., 2009; Kinani et al., 2010; Schnell et al., 2013). However, a drawback to using cell lines for waterborne toxicity is that they are often unable to tolerate hypoosomotic water. To overcome this, water has to be modified by the addition of osmolytes to ensure osmotic tonicity between the external medium and the intracellular compartment. An alternative approach is the use of a primary FIsh Gill Cell culture System (FIGCS; Walker et al., 2008; Minghetti et al., 2014, Bury et al., 2014). This method uses a double seeding technique and ensures that the epithelium contains the different cell types characteristic of an intact gill (Fletcher et al., 2000; Walker et al., 2007). When grown on permeable supports, the membrane forms a polarised tight epithelium with transepithelial electrical resistance (TER) measurements exceeding 10K Ω. At this stage the epithelium is able to tolerate the application of water on the apical surface for up to 48 hrs. The property of tolerating freshwater has led to the use of the system for physiological studies (see Wood et al., 2002), the assessment of pharmaceutical uptake (Stott et al., 2015) and toxicity of pollutants within the aquatic environment (Sandbacka et al., 1999, Bury et al., 2014).

A recent study also explored the potential for FIGCS to be used for environmental monitoring of natural waters (Minghetti et al., 2014). In this study the cells were transported 1000km in a temperature controlled container and were exposed in the field to metal-contaminated river water under non-sterile conditions. The membrane maintained integrity, showing comparable changes in TER after 24 hrs between those exposed to river water and those exposed to reconstituted sterile water. The cells also showed no signs of cell mortality, as measured by the Methylthiazol Tetrazolium (MTT) assay, but they did show an increase in expression of

the genes encoding for the metal binding proteins metallothionein A and B (Minghetti et al., 2014), demonstrating the presence of bioreactive metals.

The previous study (Minghetti et al., 2014) specifically targeted rivers in Cornwall, South West England as they are known to have elevated metals with very little other pollutant load and in the laboratory the primary gill cells are known to respond to metals with increased expression of *mta* and *mtb* genes (Walker et al., 2007). The FIGCS output (gene expression) is an integrative response that takes into account the over lying water chemistry, which determines metal speciation, and the ability of the metal to enter the cell and bind to intracellular receptors in sufficient quantities to cause an effect. If this system is to be used more widely to detect the presence of compounds that may elicit a biological effect, it is necessary to evaluate the response of the cells to more complex aquatic matrices. Thus, the aims of the current study are to expose the primary gill cell culture to a further 3 sites on urban rivers in London, UK, and one site on a more rural river in Kent, UK, that potentially have a far complex mixture of pollutants than the metal contaminated rivers in Cornwall (Minghetti et al., 2014) and to measure the expression of genes encoding for *mta* and *mtb*, as well as *cyp1a1* and *cyp3a27*, enzymes which are involved in phase 1 organic compound metabolism (Uno et al., 2012), as well as TER following 24 hrs of exposure to the river water. An increased transcription of *mta* and *mtb* indicate transactivation through metal-responsive transcription factor-1 (Mtf1) (Olsson et al., 1995; Samson and Gedamu, 1995), whilst increased levels of mRNA for *cyp1a1* and *cyp3a27* are indicative of increased activity of the aryl hydrocarbon receptor (Ahr) and the pregnane-X-receptor /retinoic acid-X-receptor (Pxr/Rxr) heteroduplex, respectively (Uno et al., 2012).

Materials and Methods

Study Sites and water chemistry.

87 The 4 study sites were on the River Wandle at Colliers wood (site 1, latitude 51.420368;
88 longitude -0.181487) and Beddington (site 2, 51.370284; -0.125072), the river Cray at
89 Sidcup (site 3, 51.428425; 0.132730), all in South East London, and the River Darent at
90 Lullingstone (site 4, 51.362372, 0.196315) in Kent, UK. Site 1 on the River Wandle is highly
91 urbanised and is approximately 4.5 km downstream of Beddington Sewage Treatment
92 Works (STW) which receives wastewater from approximately 360,000 people. Site 2 is
93 above the input from Beddington STW, but is still within a heavily urbanised catchment,
94 receiving drainage from the Borough of Croydon. Site 3 is within a suburban park, whilst Site
95 4 is within a rural setting; however the River Darent flows through suburban area of
96 Sevenoaks, Kent. The first sampling date was 2.12.2013 and the second sampling on
97 11.12.2013.

98 Water pH, conductivity, temperature and suspended solids were measured using a Hanna
99 Hi991300 probe. For chemical analysis water samples were collected in the field in low
100 density polyethylene bottles and immediately frozen and stored at -20^{o}C on returning to the
101 laboratory. Total and Mg hardness and alkalinity were measured colourimetrically, and for
102 Cu and Zn analysis water samples were filtered (0.2 µm filters) and acidified prior to
103 measurement via inductively coupled plasma mass spectrometer (Aglient 7700x ICP-MS).
104 For analysis of pharmaceuticals, sample clean-up and pre-concentration was achieved by
105 solid phase extraction (SPE) on Waters Oasis mixed-mode hydrophilic lipophilic balanced
106 (HLB) cartridges, 6 cc, 200 mg sorbent (Waters Corporation, Milford, MA, USA), similarly to
107 our previous works (Lacey et al., 2008; Barron, et al., 2008, 2009; Miller et al., 2015). Briefly,
108 100 mL aliquots of surface water samples were adjusted to pH 6.5 with ammonium acetate
109 (1 mL of a 1 M solution). SPE cartridges were conditioned with 6 mL of MeOH and ultra-pure
110 water followed by sample loading. Cartridges were then washed with 1 mL ultra-pure water
111 and dried for ~30 min under a vacuum. Cartridges were eluted in 10 mL of 50:50 ethyl
112 acetate:acetone and dried under N_2 and at 30°C using a TurboVap (Biotage, Uppsala,
113 Sweden). The dried extract residues were reconstituted in 0.5 mL of 90:10 (v/v) 10 mM

ammonium acetate in water:acetonitrile and transferred to a septum capped vial.. Analysis was performed on an Agilent 1100 high pressure liquid chromatography system interfaced to Waters Quattro triple quadrupole mass spectrometer according to the conditions listed in (Miller et al 2015). Separations were performed on a C_{18} reversed-phase column (Waters Sunfire $C_{18,}$ 2.1x150 mm, 2.5 μm). Multiple reaction monitoring was used to detect characteristic transitions of all targeted pharmaceutical compounds. Concentrations of all pharmaceuticals are expressed as single-shot quantitation measurements based on comparison to a single matrix matched calibrant at 200 ng L^{-1} spiking level (in triplicate). Therefore concentrations should be considered as semi-quantitiative.. These were extracted alongside unspiked samples (n=3) for background correction purposes.

Cell culture and field exposures

Primary gill cell culture techniques and exposure methods followed the methods described in Minghetti et al. (2014). Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local trout farm. Primary gill cell cultures were prepared from fish of 80-100 g. All fish were housed at King's College London where they were maintained in fibreglass tanks (1000 L) with flowing and aerated de-chlorinated City of London tap water ($[Na^+]$=0.53 mM; $[Ca^{2+}]$=0.92 mM; $[Mg^{2+}]$=0.14 mM; $[K^+]$= 0.066 mM; $[NH_4^+]$=0.027 mM), which was passed through activated carbon, mechanical and biological filters. Water temperature was maintained at 14°C, while photoperiod was held constant (12 hrs light, 12 hrs dark). Fish were fed daily a one-percent (w/w) ration of trout pellets. The primary gill cells were isolated and cultured as described in Fletcher et al. (2000) and prepared using the double seeding technique as described in Kelly et al. (2000) and Walker et al. (2007). Sterile techniques were used throughout all cell culture procedures. Briefly, for each seeding, 2 fish were sacrificed (following local UK Home Office schedule 1), the gills were dissected out and the gill filaments were subject to cleaning and tryptic digestion (0.05 % Trypsin-EDTA; Invitrogen).

Isolated rainbow trout gill cells were seeded onto cyclopore polyethylene terephthalate membrane (cell , surface area 0.9 cm^2, pore size 0.4 µm, Falcon) at a cell density of 1.2 x 10^6 per insert, in Leibovitz (L-15) medium (Invitrogen) supplemented with antibiotics (5 % fetal bovine serum (FBS); Sigma, 2 % penicillin and streptomycin (PEST); Invitrogen and 2 % gentamicin; GIBCO *v*/*v*). After 24 hrs incubation at 18°C in an air atmosphere cool incubator (Sanyo Mir-253), the cells were washed twice in phosphate-buffered saline (PBS) to remove debris and another seeding of primary gill cells was added at the same density per insert, and cultured in supplemented L-15 medium. After a further 24 hrs incubation another PBS wash followed and supplemented L-15 was replaced at a volume of 1.5 mL in the apical chamber of the insert and 2.0 mL in the basolateral chamber. Cultures were grown at 18^{o}C. After 96 hrs the gill cell system was cultured using L-15 medium + 5% FBS, but without antibiotics with complete medium changes every 48 hrs. The development of an intact gill epithelium was monitored daily through 'blank'-corrected measurements of transepithelial electrical resistance (TER) using a custom-modified epithelial tissue voltohmeter (EVOMX; World Precision Instruments) fitted with chopstick electrodes (STX-2). Inserts with a TER > 10 KΩ (range 10 to 32.2 KΩ) were used for the study.

To avoid any potential alterations in water chemistry when transporting samples from the field to the laboratory for toxicity testing we chose to transport the primary gill cell cultures to the field in a Labcold portable medical refrigerator (Model RPDF0012D) at 18^{o}C and expose to water directly taken from the sites, before being transported back to the laboratory. Two field trips were conducted in early December 2013 and inserts used in each trip had been derived from 4 biological replicates. Prior to travelling into the field the TER was measured for each insert. In the field, media was removed and the cells washed with PBS. To the basolateral compartment fresh L15 media was added and to the apical compartment either unfiltered or filtered (0.2 µm filters) river water or filtered medium-soft water (MSW: $[Na^+]$=0.770 mM; $[Cl^-]$=0.757; $[Ca^{2+}]$=0.340 mM; $[Mg^{2+}]$=0.152 mM; $[K^+]$= 0.077 mM;

166 [HCO_3]=0.771; [SO_4]= 0.152). The inserts were transferred back to the lab and remained in
167 the Labcold portable medical refrigerator at 18^{o}C for 24 hrs.

168 QPCR

169 Total RNA was extracted from cells using TRIzol® Reagent (Ambion, UK) and phase
170 separation performed using Phase Lock heavy tubes (5prime, USA). The purified total RNA
171 was DNase treated (TURBO DNase kit, Ambion, UK) and cDNA synthesis was performed
172 following the manufacturer's instruction (Advantage RT for PCR kit, Clontech) from 0.5 μg of
173 total RNA. Primers for qPCR of target genes metallothionein A (*mta*) and B (*mtb*) and
174 cytochrome P450 1A1 (*cyp1a1*) and 3A27 (*cyp3a27*), as well as the reference gene
175 elongation factor 1 beta (*eef1b*) were designed using Primer-BLAST
176 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), see Table 1 for details. Only 1 reference
177 gene (*eef1b*) was tested because the invariability of its expression has previously been
178 established in the gill cell culture (Minghetti et al., 2014). All amplified cDNA had been
179 previously sequence verified (Minghetti et al., 2014; Schnell et al unpublished data). For
180 each sample, qPCR was run in triplicate on an ABI-prism 7900 HT qPCR thermocycler using
181 SYBR-green Premix Taq II (Takara, RR820A). QPCR conditions followed those suggested
182 by Takara, except for *mta* and *mtb*, where a 3 step program was applied, 95°C for 5 sec,
183 55°C for 30 sec and 72°C for 30 sec. After 40 cycles, specificity of reactions was checked by
184 inspecting melting curve profiles. Gene expression of target genes were normalised to *eef1B*
185 and expressed as fold change relative to those in the control MSW controls.

186 Statistics

187 All data are presented as means + SEM. TER data (expressed as a % of pre-exposed
188 conditions) and gene expression, expressed as a ratio of the expression measured in MSW
189 controls. Differences (p<0.05) between the gene expression levels at each site and the
190 water controls was assessed via a Student's *t*-test (SigmaPlot v 12.0) on log transformed
191 data.

Results

Site 1 is downstream of Beddington STW discharge and receives input from a catchment that has a greater proportion of impervious cover compared to the other sites. At this site the highest measured pH, conductivity, total dissolved solids (TDS), hardness alkalinity, metal content and pharmaceutical concentrations were recorded (Table 2 and 3), with the pH reaching 8.77, conductivity 801 µS/cm, total hardness 233 mg $CaCO_3$ L^{-1} and alkalinity 170 mg $CaCO_3$ L^{-1}. Dissolved copper levels exceeded the United Kingdom Environmental Quality Standards (EQS) for waters with a hardness of between 200-250 mg $CaCO_3$ L^{-1} [Dissolved Cu EQS 10 µg L^{-1}] at both sample points with concentrations of 26 and 14.1 µg L^{-1}, the EQSs were not exceed at the other sites. Dissolved zinc concentrations were also highest at site 1, but these did not exceed the EQS (Table 2 – For waters of 200-250 mg $CaCO_3$ L^{-1} Zn EQS 300 µg L^{-1}]). Pharmaceuticals were detected at each site on both of the sampling times with the highest concentrations determined at site 1. The highest of these was carbamazepine at 552 and 298 ng L^{-1}, followed by ranitidine at 359 and 179 ng L^{-1}, propranolol at 207 and 212 ng L^{-1}, and trimethroprim at 156 and 162 ng L^{-1}. Of the other compounds, atentolol, cimetidine and bezafibrate were also detected at site 1 at both sampling points. At sites 2, 3 and 4 caffeine was also present along with carbamazepine and propranolol. Of the other drugs measured at sites 2, 3 and 4 atenolol, ranitidine, metoprolol, temazepam and diazepam were occasionally detected (see Table 3 for full details). The pharmaceutical concentrations were generally lower at the second sampling point (Table 3).

On both sampling days the TER of FIGCS exposed to filtered (0.2 µm) river water following 24 hrs exposures were comparable to the TER of FIGCS exposed to MSW (Figure 1). FIGCS exposed to unfiltered urban river water showed a drop in TER of between 75- 90% (data not shown).

Gene expression levels were only performed on those cell cultures exposed to the filtered river water, because the large drop in TER on exposure to unfiltered water was presumed to

be due to a loss of membrane integrity and potentially cell death. The gene expression levels of *mta* were significantly elevated at site 1 and 2 on the first sampling date. At all other sites and on the second sampling date *mta* was not induced compared to the controls. *mtb* did not differ from the MSW treatment during either of the two field trips (Figure 2 a and b). Expression of *cyp1a1* was also elevated at sites 1 and 2 on the first sampling, but not at the other sites and not on the second sampling date. *Cyp3a27* expression was elevated also at sites 1 and 2 during the first trip and during the second exposure at sites 1 and site 4 during the second trip. Expression of *cyp3a27* was unaffected at the other sites at the other sampling time (Figure 2).

Discussion

The present study showed that the primary fish gill cell culture system (FIGCS) can be transported to the field to sample urban rivers, withstands filtered urban river water for 24 hrs and exhibits altered expression of genes encoding the metal binding protein metallothionein A and two phase 1 enzymes cytochrome P4501A1 and 3A27. Previous work had demonstrated the ability of the primary gill cells to be transported to the field for environmental monitoring of rivers contaminated with metals in the South-West of England (Minghetti et al., 2014). The current work corroborates the findings that these cells show increased *mta* expression when exposed to natural waters with elevated Cu and also extends this observation to show that the cells can also detect chemicals capable of inducing *cyp1a1* and *cyp3a27* expression. This supports the use of the primary gill cells as a potential tool for detecting biologically active chemicals in natural waters.

The transepithelial electrical resistance (TER) is a measure of membrane integrity and the gill cultures form exceedingly tight epithelia with TERs >10KΩ (Fletcher et al., 2000; Bury et al., 2014). In the laboratory the application of water to inserts where the TER is still rising

causes a further rapid rise in the TER before dropping to around or below the starting TER (Schnell personal observation): this can be maintained for 24 to 48 hrs (Walker et al., 2007; Stott et al., 2015). Previously, the application of natural water from metal contaminated rivers in the field, whether unfiltered or filtered (0.2 µm filter), had no significant effect on the TER after 24 hrs if compared to inserts that received synthetic water or media change in the field (Minghetti et al., 2014). Similarly, FIGCS transported into the field and exposed to (0.2 µm) filtered urban river waters for 24 hrs show a comparable TER to FIGCS exposed to MSW under the same conditions (Figure 1). However, exposure of the cells to unfiltered urban river water caused a rapid decline in TER (data not shown). Urban streams receive a considerable amount of particulate matter, including bacteria from faecal contamination and the assumption is that this is toxic to the cells.

The most prominent molecular response measured in the cultured cells was an induction of *cypa3a27* at sites 1 and 2 during the first sampling trip (Figure 2). The rainbow trout *cyp3a27* belongs to the CYP3A subfamily of cytochrome p450 monooxygenases involved in xenobiotic phase 1 metabolism (Uno et al., 2012); the trout sequence is similar to the human CYP3A4 (Lee et al., 1998). Cyp3a27 is associated with steroid and other xenobiotic metabolism in hepatocytes (Buhler et al., 2000), but it is also highly expressed, >2 fold higher than the liver, in the pyloric caeca and anterior intestine of rainbow trout, suggesting an important role in first phase metabolism of xenobiotics present in the diet (Lee et al., 2001). Cyp3A27 is also expressed in rainbow trout gills and increases during a salinity challenge and facilitates the metabolism of the pesticide fenthion (Lavado et al., 2009).

It is estimated that the *hCYP3A4* gene is induced by an estimated 50% of all therapeutic drugs, primarily via the pregnane-X-receptor signalling, and potentially other pathways (Luo et al., 2002; Luo et al., 2004). Consequently, the study chose to analyse for the presence of pharmaceuticals using previously established methods (Lacey et al., 2008; Barron, et al., 2008, 2009; Miller et al., 2015). Of the pharmaceuticals measured in the river, carbamazepine was the highest at Site 1 on both sampling times (Table 3) and this drug is

known to induce *CYP3A* gene expression in humans (Oscarson et al., 2006) and rats (Tateishi et al., 1999). However, the concentrations of carbamazepine often used in the mammalian studies (e.g. Usui et al., 2003; Kamiguchi et al., 2010) are around the known clinical plasma concentrations of 25.39 μmol L^{-1} (reported in Usui et al., 2003) which is equivalent to 5992 μg/L, a value in excess of an order of magnitude higher than that measured at site during the first sampling time point ([carbamazepine] = 552 ng/L). It is difficult to associate an elevation in *cyp3a27* expression solely with carbamazepine, as the expression of this gene was also seen at site 2 (first sampling period) and site 4 (second sampling period), where there were significantly lower pharmaceutical concentrations (Table 3). In addition, within the mixture of pharmaceuticals measured at site 1 there are compounds such as cimetidine and ranitidine (Table 3), which are inhibitors of *CYP3A4* gene expression (Martínez et al., 1999). The CYP3 family of enzymes are known for their oxidative transformation of xenobiotics including, in addition to pharmaceuticals, pesticides (Lavado et al., 2009) and polyaromatic hydrocarbons (Zanette et al., 2013). For example, in the bivalve *Mytilus edulis*, *cyp3-like 1* and *cyp3-like 2* genes were induced by beta-naphthalene and PCB126, respectively (Zanette et al., 2013). The current results would suggest that, besides pharmaceuticals, there are other compounds present in these urban rivers that are inducers of *cyp3a27* gene expression in the primary gill cell cultures.

The induction of *CYP1A1* gene occurs via the arhyl-hydrocarbon receptor (AHR) and is associated with the detoxification of polyaromatic hydrocarbons (Billard et al., 2006). The catchment of the river Wandle (sites 1 and 2) is heavily urbanised and the induction *cyp1a1* in the primary gill cell at sites 1 and 2 during the first sampling period may reflect the presence of PAHs (Figure 2), which, also may influence *cyp3a* expression levels (Zanette et al., 2013). However, again the situation may be more complicated because caged rainbow trout exposed to a complex mixture of compounds from STW effluent, including pharmaceuticals, in Uppsala, Sweden show a large increase in the expression of various isoforms of *cyp1* (*cyp1c3, 1c2, 1c1, 1a3 1a1*) (Jonsson et al., 2010), and in the gills of

stickleback exposed to pharmaceutical production waste effluent also show an increase in *cyp1a1* expression (Biejer et al., 2013). In contrast, carbamazepine has been shown to inhibit Cyp1a1 enzyme activity in fish hepatocytes (Laville et al., 2004). Thus, this increase in *cyp1a1* and *cyp3a27* expression is better described as the detection of CYP1a and CYP3a-active pollutants, than specific classes of compounds.

The induction of *mta* was only observed at sites 1 and 2 during the first sample period (Figure 2). The lack of induction of *mtb* may reflect the fewer number of metal response elements (MREs) present in the promoter region of this gene (Olsson et al., 1995; Samson and Gedamu, 1995). At site 1 at this time there is elevated Cu concentrations above the EQS and high Zn, although this does not exceed the EQS (Table 2). The concentrations of Cu are similar to those measured at Relubbus (30.5 µg /L) and St Erth (20.2 µg /L) on the river Hayle that induced *mta* expression in FIGCS (Minghetti et al., 2014). However, at site 2 the Cu and Zn levels are less than at site 1 and are similar to those at the other two sites, and the concentrations of Cu (2.45 µg/L) and Zn (6.38 µg/L) are not expected to induce *mta* expression in the primary gill cells, based on previous work with natural waters and addition of metals to synthetic waters in the laboratory (Walker et al., 2007; Minghetti et al., 2014). Consequently, either other metals that have not been measured or other inducers of *mta* gene expression are present in these water samples. MT may also act as a free radical scavenger (Kling and Olsson, 2000). Some pharmaceuticals and personal care products have been shown to affect reactive oxygen species(ROS) production in rainbow trout gonad cell line (RTG-2) (Fernandez et al., 2013) and PAHs are known to induce free radical production (e.g. Wells and Winn 1996; Zhu et al., 2014). The distal region of the rainbow trout *mta* promoter possess both activator protein 1 (AP1) and a nuclear factor interleukin-6 (NF-IL6) elements that have been shown to play a direct role in *mta* induction in response to paraquat, an herbicide that induces ROS (Kling et al., 2013). In addition, FIGCS treated with H_2O_2, that produces ROS, show an increase in *mta* and *b* gene expression, as well as other antioxidant genes (Chung et al., 2005). The antioxidant gene expression is attenuated by the

zinc chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), suggesting that the response to ROS in FIGCS is in part mediated by an increase in intracellular zinc concentrations (Chung et al., 2005).

The study further demonstrates the potential for the primary Fish Gill Cell culture System (FIGCS) to be used for environmental monitoring and shows that that genes associated with metal exposure and biotransformation of organic compounds are present in FIGCS and are induced on exposure to filtered (0.2 µm) urban river water. Natural waters contain a mixture of pollutants, for example at site 1 elevations in dissolved Cu and Zn, along with the identification of the presence of 16 pharmaceuticals were made (Table 2 and 3), but, this is only likely to be a fraction of the chemicals present with urban rivers likely to possess PAHs from road run-off as well as pesticides and herbicides. The primary gill cell *mta*, *cyp1a1* and *3a27* gene expression is thus an integrated measure of the bioactive compounds, whether they are inducers or inhibitors of gene expression, present in a complex natural river matrix.

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476 **Figure 1**. Transepithelial electrical resistance (TER) of inserts exposed to filtered (0.2µm)
477 river water from the 4 sites from the first (white bars, 2.12.13) and second (black bars,
478 11.12.13) sampling period and medium soft water (MSW). Values represent an average of 4
479 – 6 inserts ± SEM and are expressed as a % of the starting TER prior to changing the media
480 for water after 24 hrs exposure.

481

482 **Figure 2.** Fold induction of primary gill cell culture (A) metallothioenin a and (B) a, as well as
483 (C) cytochrome p4501a1 and (D) 3a27 genes exposed to filtered (0.2µm) river water from
484 the 4 sites from the first (white bars, 2.12.13) and second (black bars, 11.12.13) sampling
485 period. Values are expressed as fold induction of expression in cells exposed to moderately
486 soft water (MSW). Values represent an average of 4 – 5 inserts ± SEM and asterisks
487 indicates significance difference to MSW (Student's *t*-test, $P<0.05$).

Table 1. Primer details and GenBank (http://www.ncbi.nim.nih.gov/) accession number

Gene name	Forward primer 5' – 3'	Reverse primer 5'-3'	GenBank accession number
mta	ACACCCAGACAAACTACTAC	GGTACAAAAGCTATGCTCAA	M18103
mtb	GCTCTAAAACTGGCTCTTGC	GTCTAGGCTCAAGATGGTAC	M18104
cyp1a1	TGACCCGGAGCTGTGGAAGGAG	CAGCCTTTGGAGCAGGATGGCC	U62796
cyp3a27	ACATGGAGACGGATCCGCAGTG	AAGCTGTGCTGGTGACCACGTC	U96077
eef1b	TTGGCGGCATAGGCTGCGATTC	TGGGCCAGTATGGTCCTTCCGG	FP321654

Table 2. Water chemistry and metal concentrations at the 4 study sites

	Site 1		Site 2		Site 3		Site 4	
	2/12/13	**11/12/13**	**2/12/13**	**11/12/13**	**2/12/13**	**11/12/13**	**2/12/13**	**11/12/13**
pH	8.77	8.49	7.33	7.26	7.6	7.92	7.63	7.61
Conductivity (µS/cm)	790	801	709	609	626	645	555	556
TDS (ppm)	403	403	308	310	316	325	284	286
Total hardness	203	233	162	166	149	141	152	158
Calcium hardness	195	206	145	141	139	121	138	135
Alkalinity	170.2	165.2	130.1	115.1	120.1	120.1	110.1	95.1
Cu (µg L^{-1})	26	14.1	2.45	1.55	1.11	3.71	2.3	1.74
Zn (µg L^{-1})	36.3	15.5	6.38	4.81	0.6	3.25	1.51	2.61

Hardness and alkalinity expressed as (mg $CaCO_3$ L^{-1})

Table 3. Semi-quantitative results for pharmaceutical concentrations measured in surface waters across 4 study sites

	Site 1		Site 2		Site 3		Site 4	
	2/12/13	**11/12/13**	**2/12/13**	**11/12/13**	**2/12/13**	**11/12/13**	**2/12/13**	**11/12/13**
Atenolol	143 (6)	81 (71)	ND	ND	ND	2.6 (0.5)	ND	ND
Caffeine	251 (47)	111 (22)	247(100)	321 (70)	463(119)	127 (23)	89 (36)	87 (9)
Cimetidine	103 (12)	44 (13)	ND	ND	ND	ND	ND	ND
Rantidine	359 (51)	179 (152)	ND	ND	16.7 (29)	ND	ND	16 (23)
Sulfamehtazine	1.6 (2.8)	6.3 (10.9)	ND	ND	ND	ND	ND	ND
Antipyrin	ND	21 (35)	ND	ND	ND	ND	ND	ND
Trimethoprim	156 (3)	162 (6)	ND	ND	ND	ND	ND	ND
Metoprolol	22 (0.6)	9.7 (5)	0.7 (1.2)	ND	5.3 (0.7)	1.4 (1.2)	ND	ND
Ketaprofen	27 (5)	43 (35)	ND	ND	ND	ND	ND	ND
Bezafibrate	108 (32)	102 (7)	ND	ND	ND	ND	ND	ND
Propranolol	207 (40)	212 (54)	71 (35)	181 (108)	105 (29)	72 (14)	63 (46)	105 (10)
Carbamazepine	552 (22)	298 (136)	7.9 (3)	8.3 (0.2)	4.8 (0.1)	9.1 (5.1)	3.7 (3)	2.7 (0.2)
Indometacin	50 (12)	ND	ND	ND	ND	ND	ND	ND
Naproxen	561 (9)	339 (144)	ND	ND	ND	ND	ND	ND
Warfarin	9.1 (16)	59 (63)	ND	ND	ND	ND	ND	ND
Gemfibrozil	ND	ND	ND	ND	ND	ND	ND	ND
Nimesulide	ND	ND	ND	ND	ND	ND	ND	ND
Temazepan	69 (5)	55 (3.6)	2 (0.3)	ND	ND	ND	ND	ND
Diazepam	3.9 (4.5)	ND	28 (19)	ND	9.4 (12)	7.4 (7.1)	ND	ND
Nifedipine	ND	ND	ND	ND	ND	ND	ND	ND

Values in ng/L. Values represent average + SD in parentheses of triplicate measurement from a single water sample. NB The same water was used for the exposure. ND: not detected.

Figure

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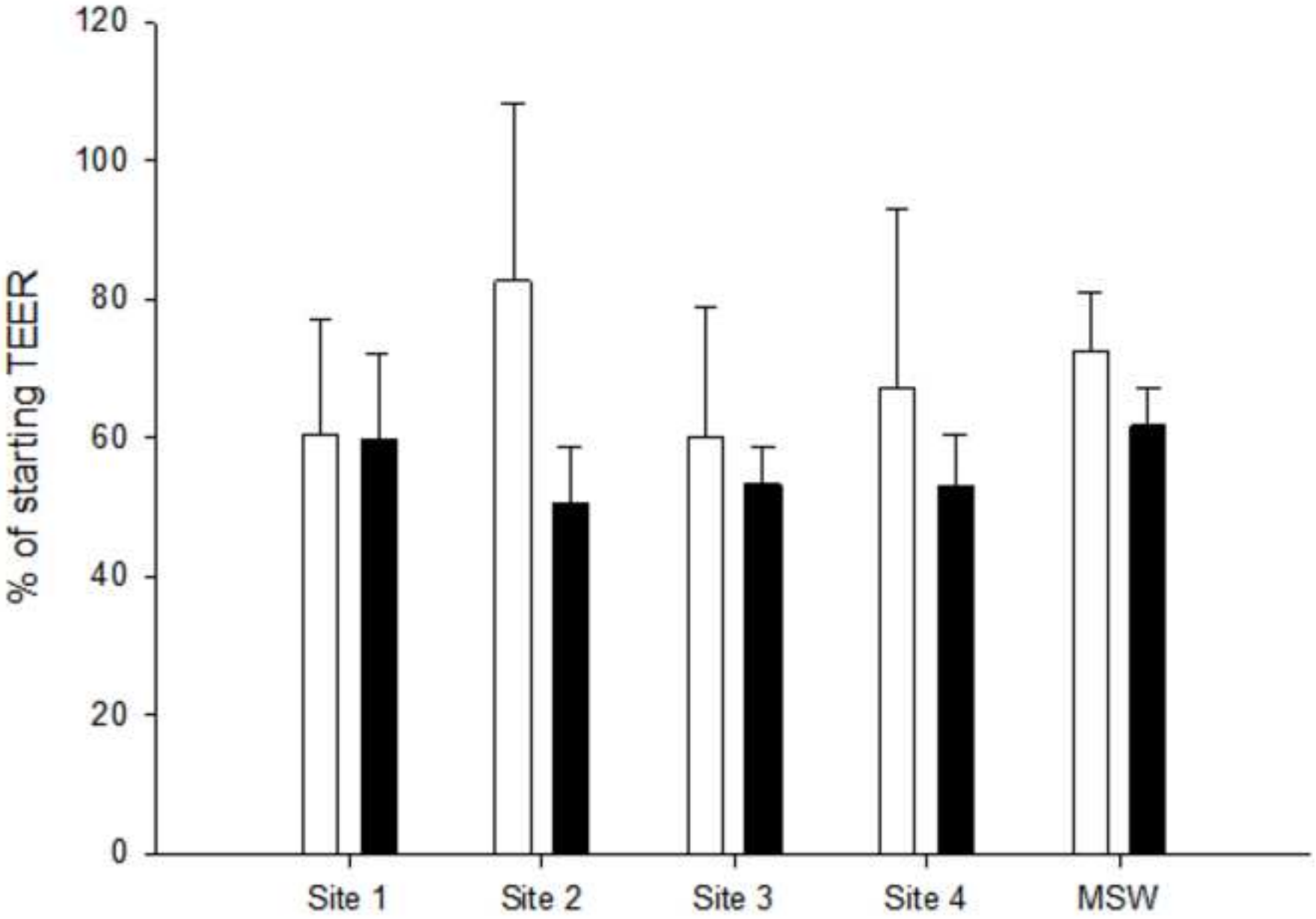

Figure

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