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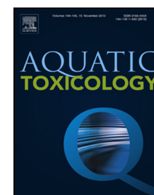

A primary FIsh Gill Cell System (FIGCS) for environmental monitoring of river waters

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ABSTRACT

Studies were conducted to assess the feasibility of a primary FIsh Gill Cell culture system (FIGCS) for both laboratory and field based environmental monitoring of rivers known to be affected by metal contamination. FIGCS were exposed in the laboratory and in the field to water from the River Hayle, a metal-contaminated system in Cornwall, United Kingdom. Water chemistry, including transition metal concentrations, changes in transepithelial electrical resistance (TEER), cell viability and the expression of metal responsive genes, metallothionein A and B were measured. FIGCS tolerated river water in the laboratory showing no loss in TEER or cell viability following 24 h exposure. The cells also tolerated transport to the field (∼1000 km and 30 h) and exposure to unfiltered and filtered river water. Metallothionein A and B, a measure of intracellular biologically active metals, expression was induced in the laboratory and field on exposure to water from sites with elevated metal concentrations compared to those sites where metal levels were below water metal Environmental Quality Standards. This demonstrates that FIGCS detects bioreactive metals in river waters on exposure in the laboratory or field and can be used for on-site environmental monitoring as well as investigations into bioavailability and toxicity of contaminant mixtures in natural waters.

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1. Introduction

Passive sampling methods for environmental pollutants in water, such as the diffusive gradients in thin films (DGT), incorporate the properties of different binding agents to determine labile metals (Davison and Zhang, 2012). For the detection of organic pollutants, several systems use polymers with absorbent properties for hydrocarbons (Pejcic et al., 2007), whilst recent developments in screen-printed electrodes have incorporated enzymes involved in toxicant biotransformation into polymers (Liu et al., 2013). These approaches are extremely encouraging in determining the presence of potentially harmful compounds. They, however, do not replicate the dynamic complex response of organisms, which are mediated or regulated by cellular uptake and detoxification mechanisms, and determine whether a compound in natural waters is toxic. A number of cell based biosensors for aquatic environmental pollutants have been developed (Liu et al., 2013) that use reporter gene-based assays (e.g. Routledge and Sumpter, 1997) or cell viability assays based on electrochemical properties (Kafi et al., 2013). These take into account cellular uptake or response to a pollutant and have been very successful in detecting the presence of endocrine disrupting chemicals (Routledge and Sumpter, 1996), pesticides (Liu et al., 2013), DNT explosives (Garmendia et al., 2008), volatile organic compounds (Paitan et al., 2004), and PCBs (Kafi et al., 2013).

A drawback, with these cell-based monitoring systems is that they often require some form of organic extraction procedure to concentrate the toxicant, which is then applied to the culture media. To overcome this issue Dayeh et al. (2002) demonstrated that the fish gill cell line, RTgill-W1, can tolerate simplified media and thus could be exposed to natural waters supplemented with salts, galactose and pyruvate to maintain the osmolality of the culture media. However, adding salt and sugars to the media will modify water chemistry and reduce or enhance the representative response of these systems to pollutants.

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The fish gill is an important multifunctional organ that provides gas exchange, osmotic and ionic regulation, acid–base regulation, and excretion of nitrogenous wastes (Evans et al., 2005). Moreover, the gills are constantly bathed in water and damage to gill function is the direct cause of death in many situations of acute toxicity to fish. Thus, on exposure to aquatic pollution the fish responds to protect this vital organ. The development of primary fish gill cell culture offers an in vitro model for the study of the branchial epithelial response to aquatic toxicants (see review by Bury et al., 2014). These cells when cultured on permeable filter supports (Fletcher et al., 2000; Kelly et al., 2000; Walker et al., 2007) develop a polarized tight epithelium with the formation of tight junctions (Sandbichler et al., 2011) resulting in high transepithelial electrical resistance (TEER) (Fletcher et al., 2000). Moreover, in the laboratory they tolerate water on the apical surface (Fletcher et al., 2000).

This double seeded FIsh Gill Cell System (FIGCS) has been used in a number of ecotoxicological studies (Zhou et al., 2005, 2006; Walker et al., 2007, 2008; Farkas et al., 2011). For example, FIGCS in laboratory conditions responded to metals similarly to intact animals; specifically, exposure to 0.076 μM silver resulted in a reduction in whole body Na^+ influx by 50% in vivo and induced comparable metallothionein (MT) expression, a metal response gene, in FIGCS (Walker et al., 2008). Both in vivo and in vitro responses were shown to be modulated by altering water chemistry, with the addition of dissolved organic carbon, increasing chloride and Na^+ concentration ameliorating the toxicity of Ag to whole organisms and reducing the FIGCS MT expression (Walker et al., 2008). This implies that FIGCS MT expression is indicative of the bioreactive metal concentration in water, e.g. that fraction of total metal concentration in water that is bioavailable to the cells and induces a response.

The current study aimed to extend previous laboratory studies to investigate the feasibility of FIGCS to detect bioavailable metals in natural waters. The study site was the River Hayle, Cornwall, UK that has a history of metal mining in its catchment and despite cessation for this mining activity in the late 19th Century there is a polymetal gradient, and very little other pollution sources (Durrant et al., 2011). FIGCS was exposed to river waters in both the laboratory and the field with an aim to determine whether cells were robust enough to tolerate non-sterile (unfiltered) river water, transport to the site and respond in a predictable way to metals. If successful, this strategy would demonstrate an in vitro system capable of identifying bioreactive compounds in natural waters that contain a complex mixture of contaminants. Moreover, there is a desire worldwide to reduce the numbers of animals used in toxicity testing and research. With an estimated 3 million fish used in whole organism waste effluent toxicity (WET) tests in the US (reported in Tanneberger et al., 2013) identifying an alternative biological based test method, such as FIGCS, that can be taken to the field for site specific environmental monitoring would be desirable.

2. Materials and methods

2.1. Animal husbandry

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local trout farm. Primary gill cell cultures were prepared from fish of 40–120 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 14–16 °C, while photoperiod was held constant (16 h light, 8 h dark). Fish were fed daily a 1% (w/w) ration of trout pellets.

2.2. Fish Gill In vitro Cell culture System (FIGCS) and cell viability

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. Equipment, containers and solutions were autoclaved or sterile filtered (0.2 μm, Sartorius stedim Minisart® filters). Briefly, for each seeding two fish were sacrificed following UK Home Office schedule 1 procedures, the gills were dissected out and the gill filaments were subject to cleaning and tryptic digestion (0.05% Trypsin-EDTA; Invitrogen) (see Kelly et al., 2000 for more detailed methods). 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 1.5×10^6 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 h incubation at 18 °C in an air atmosphere cooler incubator (LMS), the cells were washed twice in phosphate-buffered saline (PBS) to remove debris and another seeding of primary gill cells was added at 1×10^6 per insert, and cultured in supplemented L-15 medium. After further 24 h 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 °C, but do tolerate temperatures below this and can be stored for prolonged periods at 4 °C until required (Stott et al., unpublished data). After 96 h the gill cell system was cultured using L-15 medium +5% FBS, but without antibiotics with complete medium changes every 48 h. The development of an intact gill epithelium was monitored daily through 'blank'-corrected measurements of transepithelial electrical resistance (TEER) using a custom-modified epithelial tissue voltohmeter (EVOMX; World Precision Instruments) fitted with chopstick electrodes (STX-2). A TEER > 5 kΩ was used as a criterion for the presence of a tight epithelium (Fletcher et al., 2000; Walker et al., 2008) and this was formed after approximately 7 to 10 days of culture.

2.3. Environmental sample sites and water chemistry

Environmental samples were taken from the River Hayle, Cornwall, a catchment affected by metal mining previously studied and described (and see Durrant et al., 2011 for a map of the region). Five sites were chosen; Drym (N50 9 24.28; W5 19 57.5), Binner Bridge (N50 8 50.67; W5 20 6.25), Godolphin (N50 8 37.67; W5 21 37.57), Relubbus (N50 8 23.9; W5 24 29.95) and St. Erth (N50 14 0.98; W5 19 30.85), representing a polymetal gradient. Samples for water chemical analysis were collected in the field in low density polyethylene bottles that had received a 24 h acid-wash (10% HNO_3), were then rinsed three times with reverse osmosis water and left to air dry. At each site samples taken for metal analysis where either filtered through 0.45 μm (Experiment 1), 0.2 μm (Experiment 2) filters or left unfiltered and then acidified to 1% HNO_3 (Fisher Chemicals for trace metal analysis). Samples taken for total and dissolved organic carbon were filtered (0.45 μm) and those for cation and anion analysis (0.2 μm filtered) placed at ∼4 °C before freezing at −20 °C on arrival in the laboratory prior to analysis. Water transition metal concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS; E:AN 6100DRC, Perkin Elmer, Cambridge, UK), with calibration standards (Sigma-Aldrich) and a reagent blank were analyzed every ten samples. Other water cation (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anion

(Cl^-, SO^{4-}, PO^{4-}) were measured via ion chromatography (Dionex). Total and Mg Hardness and alkalinity were measured colourimetrically (Hache). Total and dissolved organic carbon (following 0.2 μm filters (Chromafil PET-20/25)) was measured on a Shimadzu TOC-Vcph analyser. Water pH, conductivity, temperature and suspended solids were measured using a Hanna Hi991300 probe.

2.4. Experiment 1—Laboratory testing

Water was collected from each site and brought back to the laboratory for testing. All tests took place within 24 h of the water being sampled from the river. A minimum of 4 FIGCS per site from two biological replicates (e.g. different double seeded insert preparations) with an average TEER of 20.3 ± 8.6 kΩ (range 8 to 31 kΩ) were used. The apical L15 media was replaced with either sterile fresh L15 media, termed symmetrical conditions; sterile 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; [HCO_3] = 0.771; [SO_4] = 0.152), termed asymmetrical condition; or unfiltered or 0.45 μm filtered river water. The later represents the dissolved metal fraction. The basolateral media was replenished with L15 supplemented with 5% foetal bovine serum (FBS). Cells were exposed for 24 h, an exposure period that induced maximum MT mRNA expression in previous studies (Walker et al., 2007, 2008) and changes in TEER, cell viability (see Section 2.6) and metallothionein A and B mRNA expression assessed (see Section 2.7).

2.5. Experiment 2—In field testing

The FIGCS with an average TEER of 19.1 ± 4.9 kΩ (range 5 to 27 kΩ) were transported from the Franklin Wilkins Building, King's College London, London, in a Labcold portable medical refrigerator (Model RPDF0012D) at 18 °C to the River Hayle. On site, under non-sterile conditions, the apical media was exchanged for either L15 media, MSW, river water from Drym, Relubbus or Godolphin sites, or 0.2 μm filtered river water to remove particulate bound metals and for sterilization from Drym and Godolphin sites. The basolateral media was replenished with L15 supplemented with 5% FBS. The FIGCS were returned to the 18 °C chamber and driven back to the laboratory. The duration of fieldtrip (e.g. the time the FIGCS remained in the portable container travelling) was 30 h and the distance travelled ∼1000 km. Exposures lasted 24 h, which included ∼12 h travelling and ∼12 h in the laboratory. MTA and B mRNA expression (Section 2.7) and TEER (measured prior to the travel and following the 24 h exposure) were measured and compared to those FIGCS in symmetrical or asymmetrical conditions. Each exposure condition contained 4–5 FIGCS derived from two biological replicates.

2.6. Cell viability assay

Cell viability was measured through MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay which measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes, as described in Berridge et al. (2005).

2.7. RNA extraction, cDNA synthesis and quantitative PCR (qPCR)

Total RNA was extracted from FIGCS using TRIzol® Reagent (Ambion, UK) and phase separation performed using Phase Lock heavy tubes (5prime, USA). The purified total RNA was DNase treated (TURBO DNase kit (Ambion, UK). Between 0.6 and 2.3 μg of total RNA was purified from each FIGCS. Moreover, 20% of the samples, randomly selected, were analyzed by bio-analyzer (Agilent Technologies) and RIN (RNA integrity number) values between 8 and 10 were measured. cDNA synthesis was performed following the manufacturer's instruction (Advantage RT for PCR kit, Clontech) from 0.5 μg of total RNA. Primers for qPCR of target genes metallothionein A (MTA) and B (MTB), chosen because they are known to respond to metals in the FIGCS (Walker et al., 2007, 2008), and five reference genes were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) see Table 1 for details. All amplified cDNA were sequence verified. For each sample, QPCR was run in triplicate on an ABI-prism 7900 HT qPCR thermocycler using SYBR-green Premix Taq II (Takara). QPCR conditions followed those suggested by Takara, except for MTA and MTB, where a 3 step program was applied, 95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s. After 40 cycles, specificity of reactions was checked by inspecting melting curve profiles. All amplification efficiencies, measured using a dilution series of a pool of cDNA, were over 93%. To determine the most stable reference gene combination under metals exposure geNorm analyses was run on a set of FIGCS (n = 8) apically exposed to MSW or MSW containing 10 μM $CuSO_4$ or 14 μM $ZnSO_4$. All reference genes showed acceptable gene mRNA expression stability by geNorm (data not shown). However, ARP and eef1b had the least variable expression with an M value of 0.038 and were chosen for normalisation purposes determined using geNorm software (Vandesompele et al., 2002). Quantification was achieved by running a parallel set of reactions containing standards consisting of a serial dilution of linearized plasmid (TOPO® TA Cloning® Kits, Invitrogen, UK) containing the cDNA sequences.

2.8. Statistical analyses and biotic ligand model

All data are presented as means + SEM. TEER data (expressed as a % of pre-exposed conditions) and MTA and B expression (expressed as a ratio of the expression measured in asymmetrical condition) were log-transformed and a one-way ANOVA followed by a Tukey's post-hoc test was used to compare values between treatments using SPSS v. 22. Toxicity modelling of single metals was based on the biotic ligand model (BLM) using the measured water chemistry (Table 2) and BLM Version 2.2.3 from Hydroqual Inc.

Table 1
Primers used for qPCR.

Gene name	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Repository ID
MTA	ACACCCAGACAAACTACTAC	GGTACAAAAGCTATGCTCAA	M18103[a]
MTB	GCTCTAAAACTGGCTCTTGC	GTCTAGGCTCAAGATGGTAC	M18104[a]
EF1a[b]	ATATCCGTCGTGGCAACGTGGC	TGAGCTCGCTGAACTTGCAGGC	NM_001124339[a]
ARP[b]	GCCCTGGCCAGCGTAGACATTG	GACCGAAGCCCATGTCGTCATCG	TC205875[c]
Ubiquitin	GCTGCGTCTTCGTGGAGGCATT	TTGGGGCGCAGGTTGTTTGTGT	NM_001124194[a]
eef1b[b]	TTGGCGGCATAGGCTGCGATTC	TGGGCCAGTATGGTCCTTCCGG	FP321654[a]
18s[b]	GGCGCCCCCTCGATGCTCTTA	CCCCCGGCCGTCCCTCTTAAT	TC117754[c]

[a] GenBank (http://www.ncbi.nim.nih.gov/).
[b] EF1α, Elongation factor 1 alpha; ARP, Acidic Ribosomal Protein; eef1b, eukaryotic translation elongation factor 1 beta; 18s, 18s ribosomal RNA.
[c] Rainbow trout gene index (http://compbio.dfci.harvard.edu/tgi/).

Table 2
Water chemistry measurements.

Experiment 1

	Cl^- (mg/L)	NO_3^- (mg/L)	SO_4^- (mg/L)	Na^+ (mg/L)	K^+ (mg/L)	Mg^{2+} (mg/L)	Ca^{2+} (mg/L)	Tot Hard (mg/L)	Ca Hard (mg/L)	Alk (mg/L)	pH	μSi	ppm (mg/L)	TOC (mg/L)	DOC (mg/L)
Drym	30.2	2.75	15.0	18.7	2.90	5.75	11.6	108	60	60	6.68	199	97	3.44	3.32
Binner	34.0	3.76	17.8	20.4	3.37	7.39	12.7	132	68	64	6.93	242	124	3.12	2.93
Godolphin	39.2	2.81	43.2	23.6	3.98	10.9	14.2	158	71	52	6.67	269	140	1.44	1.32
Relubbus	42.8	2.78	36.6	25.2	4.69	11.0	15.1	167	84	64	6.70	294	146	2.00	1.63
St Erth	44.3	2.87	37.7	26.1	4.84	11.8	17.0	178	82	70	6.78	312	159	1.93	1.71

	Cu (μg/L)		Zn (μg/L)		Cd (μg/L)		Ni (μg/L)		Pb (μg/L)		Cr (μg/L)		As (μg/L)		Fe (μg/L)	
	Tot	0.45	Tot	0.45	Tot	0.45	Tot	0.45	Tot	0.45	Tot	0.45	Tot	0.45	Tot	0.45
Drym	3.7	3.1	15.3	11.9	0.07	0.05	0.88	0.79	1.00	0.38	0.26	0.25	4.08	3.44	137	67
Binner	12.8	10.4	103	97.1	0.43	0.39	2.11	1.99	0.84	0.33	0.29	0.26	13.1	11.8	134	70
Godolphin	86.1	76.1	935	907	1.95	1.90	36.9	36.4	0.51	0.17	0.16	0.17	5.68	4.28	152	29
Relubbus	33.6	31.4	680	701	1.29	1.28	28.4	28.9	0.14	0.11	0.18	0.16	6.79	5.39	124	64
St Erth	23.9	20.6	726	682	1.21	1.16	28.0	26.2	0.22	0.15	0.17	0.15	8.98	7.49	125	66

Experiment 2

	Cl^- (mg/L)	NO_3^- (mg/L)	SO_4^- (mg/L)	Na^+ (mg/L)	K^+ (mg/L)	Mg^{2+} (mg/L)	Ca^{2+} (mg/L)	Tot Hard (mg/L)	Ca Hard (mg/L)	Alk (mg/L)	pH	μSi	ppm (mg/L)	TOC (mg/L)	DOC (mg/L)
Drym	28.1	2.98	12.8	17.9	2.42	5.04	13.3	103	97	57	6.80	202	103	2.26	2.15
Binner	33.9	4.79	23.3	19.5	2.66	7.29	14.8	142	Nm	77	6.8	243	125	1.81	1.45
Godolphin	36.5	4.67	35.6	22.1	3.52	10.3	16.2	148	145	88	6.20	283	142	1.010	1.21
Relubbus	37.7	4.99	25.8	24.0	3.79	10.7	17.2	157	156	84	6.40	292	148	1.13	0.84
St Erth	39.2	4.92	31.2	26.9	4.31	12.8	21.0	125	Nm	85	6.96	307	157	1.42	1.03

	Cu (μg/L)		Zn (μg/L)		Cd (μg/L)		Ni (μg/L)		Pb (μg/L)		Cr (μg/L)		As (μg/L)		Fe (μg/L)	
	Tot	0.2	Tot	0.2	Tot	0.2	Tot	0.2	Tot	0.2	Tot	0.2	Tot	0.2	Tot	0.2
Drym	3.4	2.9	39.8	38.7	0.14	0.11	1.24	1.17	0.84	0.48	0.36	0.30	2.36	1.81	139	49
Binner	10	8.8	120	129	0.4	0.4	2.52	2.41	0.73	0.26	0.38	0.29	5.81	4.75	141	62
Godolphin	139	112	1018	968	1.87	1.76	35.3	34.5	2.67	0.10	0.28	0.25	7.19	2.75	478	45
Relubbus	60.0	49.2	910	869	1.58	1.51	30.2	29.9	0.88	0.11	0.21	0.23	7.87	4.43	272	72
St Erth	44.9	34.9	751	706	1.3	1.21	25.7	25.1	0.9	0.17	0.31	0.27	9.95	6.24	254	77

Table 3
United Kingdom statutory guidance for metal Environmental Quality Standards (EQS) for the protection of aquatic life.

Tot Hard (mg/L)	Cu (μg/L)		Zn (μg/L)	Cd (μg/L)	Ni (μg/L)	Pb (μg/L)	Cr (μg/L)	As (μg/L)	Fe (μg/L)	
	Tot	Dis								
0–50	5	1	30[a]	8[b]	5	50	4	5	50	1000
50–100	22	6	200[a]	50[b]	5	100	10	10	50	1000
100–150	40	10	300[a]	75[b]	5	150	10	20	50	1000
150–200	40	10	300[a]	75[b]	5	150	20	20	50	1000

All values given are for the protection of sensitive freshwater aquatic life (e.g. salmonid fish) and were obtained from the United Kingdom K Environment Agency.
For Cu, Dis refers to dissolved copper values and represent the annual average; Tot represents values or 95% of samples and are for total copper concentrations.
For Zn, [a] it represents the values for 95% of samples and are for total zinc; [b] it represents those values for total zinc and represent the annual average. For Cd, the value represents annual mean value including both soluble and insoluble forms. For Ni, all vales are for dissolved nickel and represent the annual average. For Pb, all values are for dissolved lead and represent an annual average. For Cr, all values are for dissolved chromium and represent the annual average. For As, the values represent an annual mean. For Fe, the values are for dissolved iron and represent the annual average.

3. Results

3.1. Water chemistry

Within the River Hayle catchment there are a number of disused mines and the river receives a major input of metals via a mine adit at Godolphin (see Durrant et al., 2011 for a site map). The upper reaches at Drym and Binner Bridge are relatively clean with metal concentrations less than the UK Environmental Quality Standards (EQS), whereas below Godolphin the water has elevated Cu, Zn that exceed the EQS values as well as elevated, as well as Cd and Ni concentrations (Tables 2 and 3). Sites where metals were elevated coincided with increased hardness and a decrease in total and dissolved organic carbon (Table 2).

3.2. Experiment 1—FIGCS laboratory tests

Exposure of FIGCS for 24 h to natural unfiltered water caused no significant effect on cell viability compared to those FIGCS exposed to sterile MSW (Fig. 1b). None of TEERs dramatically dropped on exposure to MSW or natural freshwater, but there was some variation in the % change of TEER (Fig. 1a), with a significant increase in % change for those FIGCS exposed to water from Drym and Binner Bridge compared to all other treatments (Fig. 1a).

The metallothionein A and B mRNA expression in FIGCS exposed to unfiltered or filtered natural waters from Godolphin, a site where metal levels are elevated (Table 2), were significantly increased when compared to inserts that were exposed to MSW, asymmetrical conditions (Fig. 2). In the case of MTB the mRNA expressions in FIGCS exposed to waters from Godolphin were also significantly greater than those measured in FIGCS exposed to water from two sites on the river Hayle where metal concentrations are lower [Drym and Binner Bridge (Fig. 2c and d)]. Metal levels were elevated downstream of Godolphin at Relubbus and St Erth and expression of MTA was also significantly elevated compared to asymmetrical controls, except in the case of FIGCS exposed to filtered water from Relubbus (Fig. 2b). mRNA expressions of MTB in FIGCS exposed to unfiltered waters from Relubbus and St Erth are significantly elevated compared to those exposed to water from Drym, but not the asymmetrical controls (Fig. 2c and d).

3.3. Experiment 2—FIGCS field deployment

In the field, a change of apical L15 media (symmetrical conditions), or media replaced with MSW (asymmetrical conditions), unfiltered or filtered natural waters and subsequent exposure for a further 24 h did not cause a dramatic drop in TEERs (Fig. 3). However, the TEER for those inserts exposed to filtered Godolphin water were significantly lower than those exposed to Drym filtered or Relubbus unfiltered, but not the symmetrical or asymmetrical controls.

MTA mRNA expressions in FIGCS exposed to unfiltered natural water from Relubbus or filtered water from Godolphin were significantly elevated (Fig. 4A and C) compared to asymmetrical controls (MSW). MTB mRNA expression was significantly elevated in FIGCS exposed to unfiltered Godolphin and Relubbus and unfiltered Godolphin water (Fig. 4B and D).

4. Discussion

The results from the current study show that a primary fish gill cell culture system, can withstand unfiltered and filtered (0.2 μm

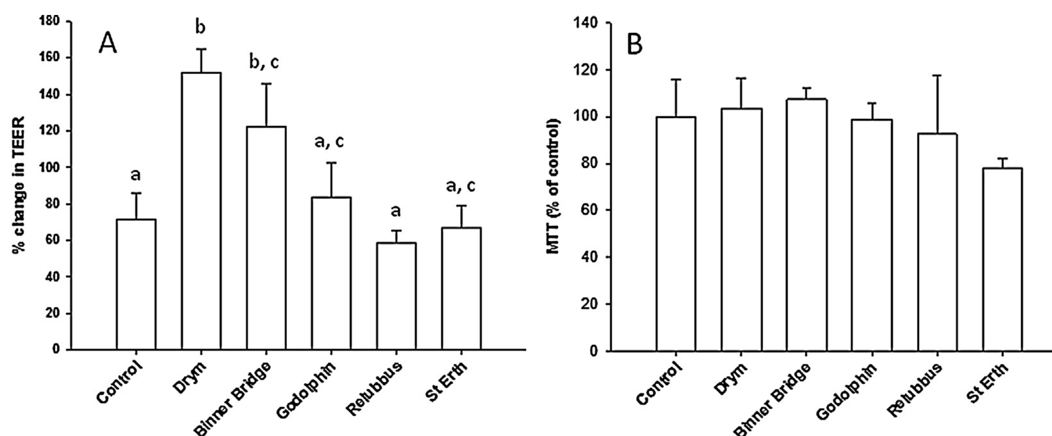

Fig. 1. The change in transepithelial electrical resistance (TEER) as percentage of pre-exposed conditions (A) and cell viability (B) on exposure for 24 h in the laboratory to sterile medium soft water (Asym) or unfiltered river Hayle water (Drym, Binner Bridge, Godolphin, Relubbus and St Erth). Values represent mean + sem ($n = 4$). Bars bearing different letters are significantly different (one-way ANOVA followed by a Tukeys post-hoc test).

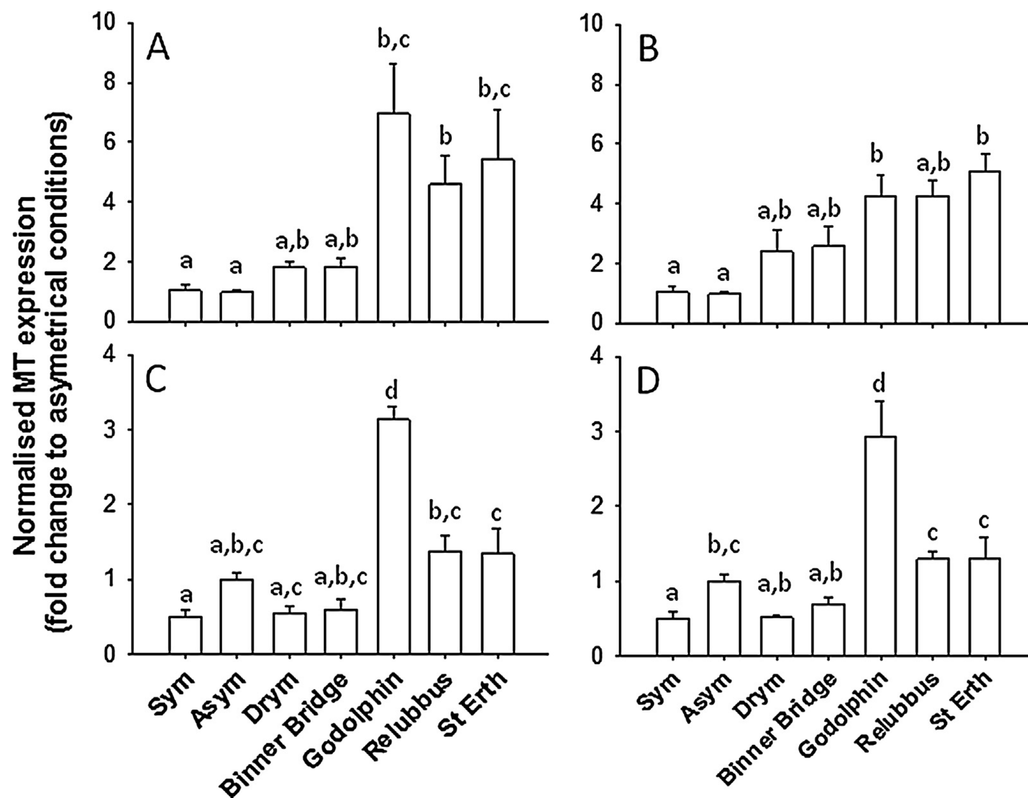

Fig. 2. Metallothionein A, MTA (A) and (B) and metallothionein B, MTB (C) and (D) mRNA expression following 24-h exposures in the laboratory to L15 media (Sym), medium soft water (Asym), unfiltered (A) and (C) or 0.45 μm filtered (B) and (D) water from the River Hayle (Drym, Binner Bridge, Godolphin, Relubbus and St Erth). All values are expressed as a ratio of the expression in those inserts exposed to medium soft water (Asym). Values represent average of 3–4 inserts + SEM. Bars bearing different letters are significantly different (one-way ANOVA followed by a Tukeys post-hoc test).

Sartorius Minisart® filters) river waters in the laboratory or field. The gill cells responded in a predictable way to natural metal contaminated water by increasing the mRNA expression of the metal responsive genes encoding metallothionein A and B (MTA and MTB); demonstrating the presence of intracellular bioreactive metals. Results from these experiments show the feasibility for FIGCS as a biomonitoring tool, either in the laboratory or field and

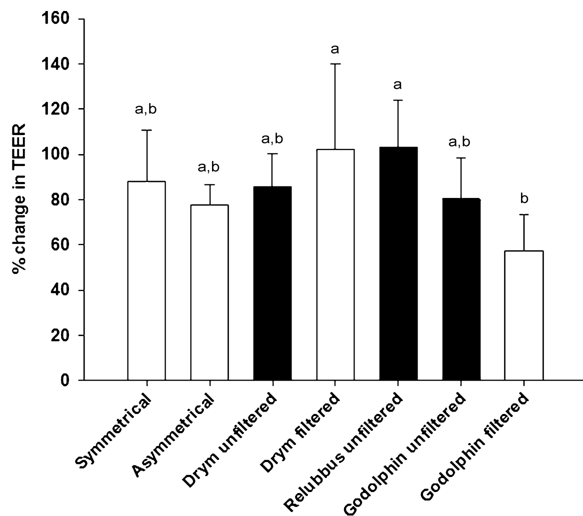

Fig. 3. The percent changes in transepithelial electrical resistance (TEER) prior to travel to the field and after 24 h exposure to L15 media (Sym), medium soft water (Asym), unfiltered or 0.2 μm filtered (dark columns) water from the River Hayle (Drym, Godolphin and Relubbus). Values represent mean + sem (n = 5). There is no significant deference between values (one-way ANOVA).

the potential for its use in assessing factors that determine contaminant bioavailability in complex natural water where mixtures of contaminants are present.

The primary gill cells were cultured in symmetrical condition for approximately 7 days prior to use in the field and by this time they have formed an extremely tight epithelium, >5 kΩ, as previously observed (see review by Bury et al., 2014). The addition of MSW in the laboratory or field caused a slight reduction in TEER (Figs. 1 and 3), and similar responses were seen with river water when FIGCS were exposed in the field or from a number of sites in the laboratory (Figs. 1 and 3). Previous studies using the double seeding insert (DSI) technique have produced inserts with exceedingly high TEERs (Fletcher et al., 2000) and when sterile water was added to the apical surface there was either no change or a slight decrease in TEER over time (reviewed by Bury et al., 2014). It is only in DSI where the TEER were still rising, or in inserts seeded with cells first grown in flasks (single seeded inserts, SSI), that the application of water caused a rapid increase in the TEER (Bury et al., 2014). Thus, the significant increase in TEER in inserts exposed to water from Drym and Binner Bridge in Experiment 1 (Fig. 1a) are likely explained by the lower starting TEERs compared to the other sites: Drym 10.1 ± 2.5 kΩ; Binner Bridge 12.6 ± 0.6 k, asymmetrical conditions 24.4 ± 5.9 kΩ, Godolphin, 19.8 ± 8.2 kΩ, Relubbus 29.7 ± 1.3 kΩ and St Erth 26.1 ± 4.8 kΩ. The criteria of using an insert with a TEER in >5 kΩ was based on our previous work (Walker et al., 2007, 2008) and also had a practical component because for each study we have a number of different batches of cells that that are all growing at slightly different rates.

Replacing media with water creates a large osmotic stress for the cells (media ~300 mOsm to freshwater ~1 mOsm) that induces adjustments in lipid composition of the cell membrane (Hansen et al., 2002). An initial concern was that this osmotic stress would

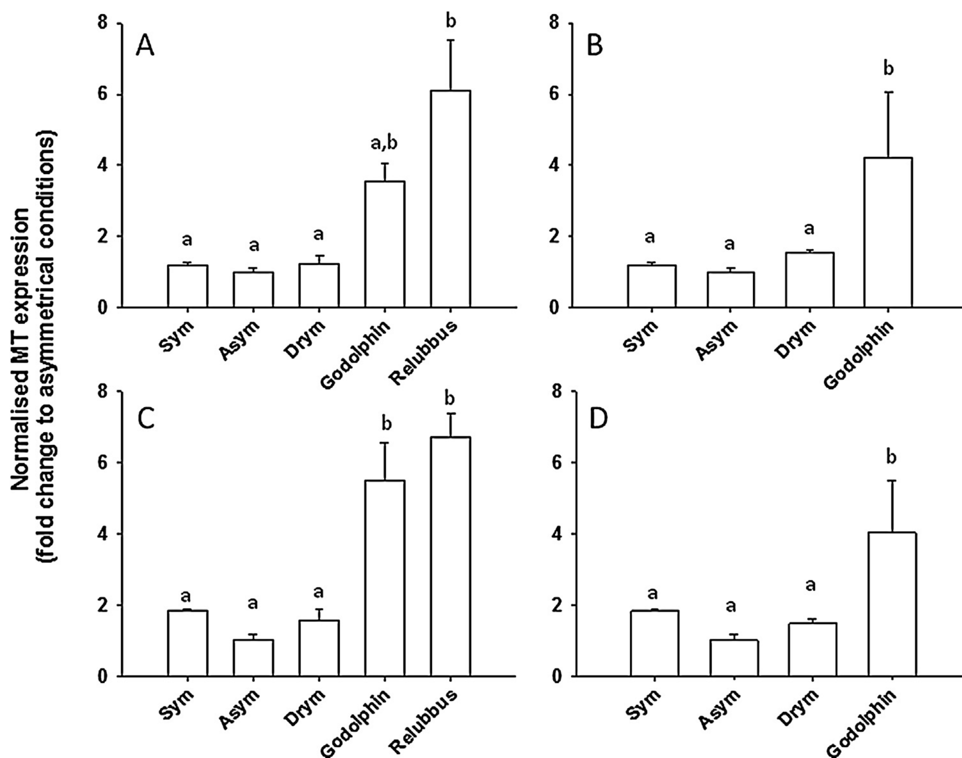

Fig. 4. Data collected from FIGCS exposed to water in the field. Metallothionein A, MTA (A) and (B) and metallothionein B, MTB (C) and (D) mRNA expression following 24-h exposures to L15 media (Sym), medium soft water (Asym) or unfiltered (A) and (C) or 0.2 μm filtered (B) and (D) river water from the River Hayle (Drym, Godolphin and Relubbus). Values are expressed as a ratio of the expression in those FIGCS exposed to medium soft water (Asym). All values represent average + sem [n = 3–5] Bars bearing different letters are significantly different (one-way ANOVA followed by a Tukey's post-hoc test).

induce a large change in the expression pattern of the selected genes (MT and B and five reference genes). However, a comparison between the expression of MTA and B between those inserts exposed to symmetrical or asymmetrical conditions showed no significance difference (Figs. 2 and 4). These results confirm previous studies demonstrating that gene expression levels are stable for 24 h following apical addition of water (Walker et al., 2007, 2008). In addition, gene expression (in this article, whenever the phrase gene expression is used, it is used as a synonym to gene transcription, although it is acknowledged that gene expression can also be regulated at, e.g., translational and protein stability level) analysis of a further nine genes showed no difference in gene expression between inserts cultured in media and those exposed to water for 24 h, irrespective of the starting TEER and whether the exposure was in the laboratory or in the field (data not shown).United Kingdom EQSs for Zn and Cu are exceeded at, and downstream, of Godolphin and the Hydroqual Inc Biotic Ligand Model (BLM) Version 2.2.3 also predicts that the waters of the river Hayle are extremely toxic to fish (Table 4). The waters at Godolphin did cause an inhibition of Na^+ influx rates of rainbow trout measured over 3 h, whilst the waters from the other sites had no significant effect (data not shown and see also Durrant et al., 2011). The predicted accumulation of Cu onto the gill in the water at Godolphin would exceed the BLM lethal accumulation (LA), e.g. the level of branchial Cu predicted to cause 50% mortality, by 200%; thus, a longer exposure period to this water may have produced clearer inhibition of Na^+ influx (Durrant et al., 2011). The predicted branchial Zn accumulation greatly exceeded that of Cu (Table 4) and assessing branchial Ca^{2+} influx rates, a known target of acute zinc exposure (Hogstrand et al., 1995) may have been a better indicator of whole organism toxicity. However, it would appear that the BLM overprotects in this instance, because there are migratory brown trout present in this river (Durrant et al., 2011). The reasons for the presence of this thriving population may be due to local adaptation and an increase in expression of genes associated with metal detoxification and ionoregulation in the gills and kidney (Uren Webster et al., 2013).

Metalliferous rocks are seldom rich in a single metal and as a result sites of mine pollution, such as the River Hayle, are affected by a number of metals simultaneously (Table 2). The present study did not set out to assess whether MT expression in metal mixtures from natural waters follows Toxic Unit (Playle, 2004), concentration addition (CA) or independent action models (Kortenkamp et al., 2007) for mixture toxicity and which combination of metals are additive, antagonistic or synergistic. There are, however, likely to be a combination of metals where competition at uptake sites reduces intracellular concentrations. The gills respond dynamically to metal exposure, altering metal metabolism to maintain intracellular homeostasis that enables the cell to retain its metabolic function. Pivotal to this response to metals is the metal transcription factor (MTF1) (Colvin et al., 2010). On exposure to a metal the

Table 4
Biotic ligand model (Version 2.2.3 from Hydroqual Inc.) predictions for single metal toxicity.

		Drym	Binner	Godolphin	Relubbus	St Erth
Exp. 1	Cu	0.3	1.1	114	16.1	7.2
	Zn	8.1	62.8	574	418	419
	Cd	0.4	1.9	66.1	45.6	40.8
Exp. 2	Cu	0.4	2.6	200	86.9	28.4
	Zn	28.2	90	457	469	413
	Cd	6.9	17.8	55.1	50.0	39.4

Values represent the predicted biotic ligand metal concentrations as a % of the biotic ligand lethal accumulation (LA) value predicted by the model at each site. The calculation were performed using the Biotic Ligand Model Windows Interface, Version 2.2.3 from Hydroqual Inc. (http://www.hydroqual.com/wr_blm.html) with the water chemistry values from Table 2.

intracellular metal concentration rises and causes the displacement of Zn from Zn binding sites on proteins. MTF-1 acts as an intracellular zinc sensor mopping up this excess zinc (Colvin et al., 2010) and the zinc-MTF1 complex interacts with *cis* element metal responsive element (MRE) in the promoter of metal responsive genes, such as the metal binding protein MT, to elicit a response to the metal stressor. The biological output (MT expression) from the cells is thus an integrated measure of aquatic speciation, uptake and intracellular active metal.

During the exposure to natural water in the laboratory (Experiment 1) MTA mRNA expression was elevated at all sites downstream of the mine adit (Fig. 2A), whereas MTB mRNA expression was only elevated at Godolphin (Fig. 2B). In contrast, in Experiment 2 during the field trial the MTB mRNA expression is elevated at both sites (Godolphin and Relubbus) downstream of the mine adit. The difference in nRNA expression between MTA and B may reflect differences in the response to different metal profiles. In *Drosophila*, the MTF-1 possess copper-binding motives (Marr et al., 2012; Sims et al., 2012) and single nucleotide changes in MREs can confer copper-specific gene expression patterns. Interestingly, copper concentrations vary between the sites in the two experiments, with Cu being generally higher in Experiment 2 compared to Experiment 1 (Table 2). This may explain the difference in MTB mRNA expression response, but further research is necessary to assess metal concentration-dependent MTA and MTB mRNA expressions. But, differences in the response of FIGCS to different metals in laboratory experiments have been observed, which suggests that cellular gene expression profiles may be useful way to identify specific metal contaminants (Walker et al., 2008).

5. Conclusions

The present study demonstrates that FIGCS tolerates natural river waters. It also shows that the cells are capable of detecting metals in complex mixtures and highlights the potential of FIGCS as a biomonitoring tool. In the United States over three million fish are used for waste effluent toxicity (WET) testing (reported in Tanneberger et al., 2013). The societal drive to reduce the number of animals used in toxicity tests necessitates a search for alternative methods to whole organism testing. A number of studies have assessed the use of the rainbow trout gill cell line RTgill-W1 for environmental monitoring (Lee et al., 2009; Dayeh et al., 2002, 2009). In these studies, the water samples either go through an extraction process and resuspension of the extract in a solvent prior to administration to the cells, or the water is used to dilute double strength media to maintain the exposure media isoosmotic to the cells. The latter method has been used to assess the toxicity of paper mill (Dayeh et al., 2002) and industrial (Dayeh et al., 2009) effluents with results demonstrating the cell line as a potential alternative test methods. However, because this procedure requires the water sample to be processed and placed in isoosomtic media this exposure route does not replicate the chemical speciation of natural freshwaters. The current study circumvents this issue and demonstrates a system where water can directly be placed on the cells. The potential to reduce fish in testing procedure is large because the harvesting of cells for primary cell culture is not a legislated procedure under UK Home Office regulation. However, for clarity, 2 fish will produce between 40 and 72 inserts. In the current study there was no difference between the responses in cells exposed in the laboratory or field. This suggests that for metal contaminated waters laboratory exposures are sufficient to detect a response in FIGCS. However, by demonstrating the ability of these cells to withstand transport to the field and to respond to the environmental sample means that in situ exposures are possible if there are concerns that water chemistry may alter during sampling, storage and transport back to the laboratory.

This is the first assessment of the use of FIGCS as a biomonitoring tool. Further studies are required to identifying other biomarkers of exposure to difference classes of contaminants (e.g. organic contaminants, pharmaceuticals), as well as an assessment of exposure to different water types (e.g. urban rivers, end-of-pipe effluent) and repeated seasonal exposures from the same source to further demonstrate the versatility of FIGCS for environmental monitoring.

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