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Long-term storage and impedance-based water toxicity testing capabilities of fluidic biochips seeded with RTgill-W1 cells

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ABSTRACT

Rainbow trout gill epithelial cells (RTgill-W1) are used in a cell-based biosensor that can respond within one hour to toxic chemicals that have the potential to contaminate drinking water supplies. RTgill-W1 cells seeded on enclosed fluidic biochips and monitored using electric cell-substrate impedance sensing (ECIS) technology responded to 18 out of the 18 toxic chemicals tested within one hour of exposure. Nine of these chemical responses were within established concentration ranges specified by the U.S. Army for comparison of toxicity sensors for field application. The RTgill-W1 cells remain viable on the biochips at ambient carbon dioxide levels at 6 °C for 78 weeks without media changes. RTgill-W1 biochips stored in this manner were challenged with 9.4 μM sodium pentachlorophenate (PCP), a benchmark toxicant, and impedance responses were significant ($p < 0.001$) for all storage times tested. This poikilothermic cell line has toxicant sensitivity comparable to a mammalian cell line (bovine lung microvessel endothelial cells (BLMVECs)) that was tested on fluidic biochips with the same chemicals. In order to remain viable, the BLMVEC biochips required media replenishments 3 times per week while being maintained at 37 °C. The ability of RTgill-W1 biochips to maintain monolayer integrity without media replenishments for 78 weeks, combined with their chemical sensitivity and rapid response time, make them excellent candidates for use in low cost, maintenance-free field-portable biosensors.

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1. Introduction

The protection of drinking water supplies from the threat of accidental or deliberate contamination with toxic chemicals is a concern for both the military and civilian sectors. Modern methods of analytical chemistry can detect and identify a wide array of inorganic and organic compounds with accuracy and precision, but thorough analysis requires complex instrumentation used away from the site of field sampling, and there is usually a time delay of hours to days until results can be obtained. These methods are usually not practical, timely, or cost-effective for field testing of water supplies where time-to-results may be critical.

A wide array of biosensors are available that utilize antibodies, enzymes, and nucleic acids for detection of chemicals in water, (Pancrazio et al., 1999; States et al., 2003; Kelly et al., 2008; Reardon et al., 2009) but they are analyte-specific, thus requiring a large number of biosensors for responsiveness to a wide range of chemicals. Toxicity sensors offer an alternative approach to evaluating drinking water for the presence of chemical contaminants. A review of vertebrate cell-based biosensors indicates that metabolism, impedance, and intra- and extra-cellular potentials can be used for detection of chemical toxicity (Pancrazio et al., 1999; van der Schalie et al., 2006). Some of these sensors have been shown to respond to a broad range of chemical contaminants, including mixtures and unknown compounds, and thus have great potential for use as rapid indicators of chemical contaminants in drinking water supplies (O'Shaughnessy et al., 2004; Iuga et al., 2009; Eltzov and Marks, 2010).

The concept of using the measurement of electrical impedance of cell monolayers as a possible toxicity sensor, which is also known as electric cell substrate impedance sensing (ECIS), was first described by Giaever and Keese (1992). Cellular impedance has been shown to be a sensitive indicator of cell viability and cytotoxicity (Giaever and Keese, 1993; Curtis et al., 2009a; Keese et al., 1998; Xing et al., 2005, 2006). Other investigators have found excellent correlation between cellular impedance and other toxicity endpoints such as tetrazolium dye and neutral red uptake assays (Xing et al., 2006).

ECIS is based on the principle that a confluent monolayer of cells impedes the flow of electricity. Impedance measurements correlate with changes in cellular morphology, movements and functions, and activation of signaling pathways, and has been

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described extensively (Giaever and Keese, 1993; Xiao and Luong, 2003; DeBusschere and Kovacs, 2001; Narakathu et al., 2010). When the integrity of the cell monolayer is compromised (as by a toxic chemical injury), the ECIS sensor records a change in electrical impedance (Curtis et al., 2009a; Keese et al., 1998; Xing et al., 2005, 2006). Although mammalian cell lines have successfully detected toxic compounds and may more closely reflect human physiology than non-mammalian cells, they are more difficult to maintain since they require an incubation temperature of 37 °C, artificial levels of 5–10% carbon dioxide, and frequent media replenishment (Curtis et al., 2009a,b). Curtis et al. (2009b) describe a compact, self-contained disposable cell maintenance system (CMS) for supporting the health of mammalian cells on fluidic biochips for up to 30 days. The CMS eliminates the need for a cell culture facility to be on-site where testing is being done. Although fully automated, the CMS has limitations for field use, including relatively short viability of cells on the biochips, and the large size and weight of the device.

Rainbow trout gill epithelial cells (RTgill-W1) are a promising alternative to mammalian cells for use in toxicity sensor applications. Lee et al. (2009) provides a review of the extensive applications of RTgill-W1 cells in toxicology as well as basic research. These cells form a monolayer on fluidic biochips (Glawdel et al., 2009; Hondroulis et al., 2010), can be cultured at ambient CO_2 levels (Bols et al., 1994), and can survive over a wide range of temperatures (4–25 °C), with optimal growth occurring at 20 ± 2 °C (Dayeh et al., 2005b). RTgill-W1 cells have been utilized extensively in *in vitro* toxicity testing with fluorescent indicator dye assays in traditional multiwell plates (Dayeh et al., 2005a,c), as well as in microfluidic biochips (Glawdel et al., 2009). RTgill-W1 cells have been found to be sensitive to different classes of compounds, including polybrominated diphenyls (Shao et al., 2008, 2010), ammonia (Dayeh et al., 2009), copper (Bopp et al., 2008), polycyclic aromatic hydrocarbons (Schirmer et al., 1998), industrial effluents (Dayeh et al., 2002), and nanoparticles (Kühnel et al., 2009). Recently, the RTgill-W1 cells on ECIS biochips were applicable in real time cytotoxicity of nanoparticles such as silver, gold, single walled carbon nanotubules and cadmium oxide. (Hondroulis et al., 2010). Applications of RTgill-W1 cells in toxicology as well as in the basic research arena were recently reviewed by Lee et al. (2009).

Here we demonstrate the potential for using ECIS technology and RTgill-W1 cells that have been seeded on fluidic biochips as a rapid screening test for potential chemical contaminants in drinking water. The 18 chemicals selected for testing were those recommended by an Army user group (van der Schalie et al., 2006) based on the criteria that they represented different modes of toxicity and had the potential to be water contaminants. The RTgill-W1 cells have toxicant sensitivity that is comparable to BLMVECs, while having culturing characteristics more suitable than BLMVECs for water testing. Most notably, the RTgill-W1 cells can survive for extended periods on fluidic biochips and still be responsive to the benchmark toxicant, PCP, when used in the ECIS assay. This long-term survival capability opens possibilities for low cost, low maintenance field-portable biosensors.

2. Materials and methods

2.1. Seeding of fluidic biochips with RTgill-W1 cells

Sterile fluidic biochips (Fig. 1) used for these studies were assembled at Applied BioPhysics, Troy, NY from two components; an upper polycarbonate layer with two separate fluid channels, and a lower electronic layer that contained four electrode pads per channel for impedance sensing. There were 10 working electrodes per pad; each electrode was 250 μm in diameter. The assembled biochips had gold electrode connections for acquiring impedance readings when inserted into the ECIS test unit, which is described below. The biochips are commercially available from Agave BioSystems, Austin, TX, and are described in detail by Curtis et al. (2009b).

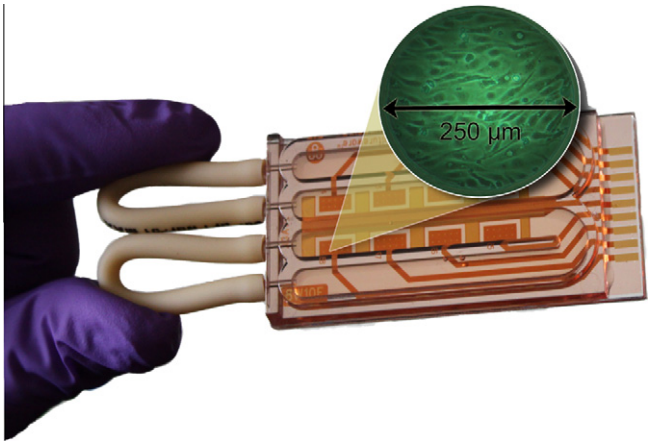

Fig. 1. Fluidic ECIS biochip seeded with RTgill-W1 cells. Fluidic biochips are sealed and able to be stored for over one year without feeding and maintenance.

Rainbow trout gill epithelial (RTgill-W) cells, an immortalized fish cell line, were obtained from American Type Tissue Culture Collection, Manassas, VA (ATCC CRL-2523). The cells were cultured in 75 cm^2 polystyrene flasks in complete Leibovitz-15 (L-15) media containing 10% fetal bovine serum (FBS) (*v/v*), 1% 200 mM GlutaMAX-1 supplement (*v/v*), and 100 U/mL penicillin and 100 U/mL streptomycin (Lonza, Walkersville, MD) in a 20 °C incubator with ambient carbon dioxide (CO_2). The cells were used at passages 5–55 for seeding the fluidic biochips.

Prior to being seeded with cells, the channels of the fluidic biochips were coated with 0.01% fibronectin (Calbiochem, Gibbstown, NJ) solution in L-15 media for 1 h in order to facilitate cell attachment and the formation of a continuous epithelial monolayer. After 1 h incubation at room temperature, the fibronectin solution was aspirated off and each biochip channel was seeded with 2.5 mL of trypsinized cells (2.5×10^5 cells/mL). Sterile Pharmed® Biopharm tubing (McMaster-Carr, Santa Fe Springs, CA) was used to form closed loops on the ends of the biochips (Fig. 1). Bare electrodes that contained culture media and no cells had impedance values of 300–400 ohms (Ω). Once the RTgill-W1 cells were introduced to the fluidic biochip and a monolayer was formed, impedance values ranged from approximately 1200–2000 Ω. Seeded biochips were then incubated at 20 °C for 7 days. During this time, the media in the fluidic channels was replenished on days 4 and 7 prior to storing the biochips at 6 °C on day 7.

Biochips were used at anywhere from 11 to 18 days post-seeding for chemical testing. Several hundred biochips were seeded and stored in this manner over the course of the testing period for use in chemical testing.

Nine more biochips were seeded using the same procedure described above for studying the effects of temperature on impedance levels during long-term storage. These chips were not, however, fed after seeding but rather went directly into a thermoelectric unit as described in 2.6 below.

An additional 36 biochips were seeded with RTgill-W1 cells in the same manner as the biochips for long-term storage chemical testing with PCP. These biochips were held at 20 °C, received media replenishments on days 4 and 7, and were then stored at 6 °C with no further media replenishments or manipulation for the entire storage period. Sets of three individual chips were removed from

6 °C after 2, 3, 4, 8, 12, 16, 20, 24, 26, 28, 30 and 39 weeks of storage, and were tested with 9.4 μM PCP as described in 2.7 below.

2.2. Seeding of fluidic biochips with BLMVEC cells

Seeding of the fluidic biochips with BLMVEC cells was described previously (Curtis et al., 2009b). The BLMVECs and the MCDB-131C complete media with 10% FBS for culture were obtained from VEC Technologies (Rensselaer, NY). The cell culture flasks and fluidic biochips were maintained in a 37 °C incubator with 5% CO_2. The BLMVECs were used for biochip seeding at passages 5–11 2.5×10^5 cells/mL. Unlike the RTgill-W1 cells, the BLMVEC cells are a finite cell line and were not used for ECIS experiments beyond passage 11. Prior to being seeded with cells, the channels of the fluidic biochips were coated with 0.2% gelatin solution in 0.15 M NaCl for 1 h in order to facilitate cell attachment and the formation of a continuous endothelial monolayer. Pharmed® Biopharm tubing was used to form closed loops on the ends of these biochips in the same manner as with the RTgill-W1 biochips. Cell monolayers were allowed to grow on the biochips in a tissue culture incubator for at least 7 days prior to being used for toxicity testing. Media changes were done on the fluidic biochips three times a week under sterile conditions, with the last media changes being completed 24 h prior to using the biochips for chemical testing.

2.3. Test chemicals – preparation and analysis

Eighteen chemicals were tested in the fluidic biochips using ECIS. Chemical test concentration ranges (van der Schalie et al., 2006) were selected to be at or above the Military Exposure Guideline (MEG) concentration (based on consumption of 15 L per day of water for 7–14 days) (USAPHC, 2010) and at or below the estimated Human Lethal Concentration (HLC; based on the consumption of 15 L of water per day for a 70 kg person) (TERA, 2006). The concept of MEG was used to provide a reference point above which adverse health effects may be expected in deployed military personnel (USAPHC, 2010). The HLC concentrations were derived based on data available on accidental human poisonings and rodent LD50 data. Both the MEGs and HLCs are only available for single compounds and not mixtures (van der Schalie et al., 2006). Table 1 lists the MEG and HLC values for the test chemicals. Acrylonitrile, aldicarb, sodium arsenite, fenamiphos, methamidophos, methyl parathion, nicotine, and paraquat dichloride were obtained from Chem Service (West Chester, PA). Ammonium chloride, copper sulfate, sodium cyanide, sodium fluoroacetate, mercuric chloride, sodium azide, phenol, thallium sulfate, and toluene were obtained from Sigma–Aldrich (St. Louis, MO). Pentachlorophenol was obtained from Mallinckrodt Baker (Phillipsburg, NJ).

Test compound stock solutions were prepared in deionized water (DI) with the exception of sodium pentachlorophenate (PCP), which was prepared from pentachlorophenol in 5 mM phosphate buffer, pH adjusted to 7.5. Stock concentrations of test compounds were analyzed in-house using the following analytical methods. Acrylonitrile, aldicarb, fluoroacetate, methyl parathion, nicotine, paraquat, PCP, and phenol were analyzed by high performance liquid chromatography (HPLC). Ammonia was measured colorimetrically using a LaMotte 1200 Colorimeter (Chestertown, MD). Arsenic, copper, mercury, and thallium were measured by inductively coupled plasma-mass spectrophotometry (ICP-MS). Fenamiphos and toluene were measured with a gas chromatograph (GC). An ion probe was used to measure cyanide. Azide was measured using ion chromatography. Methamidophos was tested at nominal concentrations because suitable methods for analysis were not available. Volatile chemicals (acrylonitrile and toluene) were stored in zero headspace vials at 4 °C. All test chemicals, except azide, were verified previously as stable for two weeks (van der Schalie et al., 2006). Azide stability testing was done by US Center for Environmental Health Center chemists and was found to be stable for at least 2 weeks (unpublished data). On the day of ECIS testing, stock solutions of the selected compounds were diluted with DI water to obtain desired concentrations.

2.4. Procedure for ECIS chemical testing with the RTgill-W1 fluidic biochips

The protocol for toxicant testing of the fluidic biochips was based upon the statistical approach used by the Joint Chemical Biological Radiological Agent Water Monitor (JCBRAWM) program for

Table 1
Toxicant sensitivity of fluidic biochips seeded with either BLMVEC or RTgill-W1 cell monolayers using ECIS. Target detection level was ⩾ the Military Exposure Guidelines (MEG) and ⩽ Human Lethal Concentration (HLC) in ⩽one hour.

Compound	Chemical Abstract Service (CAS) number	MEG[a] (μM)	HLC[b] (μM)	BLMVEC detection level (μM)	RTgill-W1 detection level (μM)
Acrylonitrile	107-13-1	8.85	79.1	***3615.8***	7627.1
Aldicarb	116-06-3	0.025	0.9	***893.5***	3484.5
Ammonia	7664-41-7	1762	54257	20786.8	***5872.0***
Arsenic (sodium arsenite)	7784-46-5	0.267	60.1	60.1	60.1
Azide (sodium)	26628-22-8	2.856	1111.4	not tested	285.6
Copper (sulfate)	7758-98-7	0.74	1621.0	786.9	***15.7***
Cyanide (sodium)	143-33-9	76.86	538.0	>538	***538.0***
Fenamiphos	22224-92-6	0.014	1.8	18.5	18.5
Fluoroacetate (sodium)	62-74-8	0.009	50.6	25792.2	***6623.4***
Mercury (chloride)	7487-94-7	0.050	123.1	24.9	***1.2***
Methamidophos	10265-92-6	0.002	9.9	***3543.1***	>7100.3
Methyl parathion	298-00-0	0.532	127.8	127.8	***97.3***
Nicotine	54-11-5	0.080	103.6	***1035.6***	>1615
Paraquat (dichloride)	1910-42-5	0.132	17.9	1944.2	***1788.6***
Pentachlorophenate (sodium)	131-52-2	0.526	270.0	9.4	9.4
Phenol	108-95-2	29.76	972.3	***972.3***	3910.3
Thallium (sulfate)	7446-18-6	0.016	66.0	132.1	132.1
Toluene	108-88-3	100.9	9117.6	2713.6	***1085.4***
Chemicals detected ⩽ HLC using 16/16 biochips (more sensitive cell line in *bold italics*).				8/17	9/18
Chemicals detected > HLC using 3/3 biochips.				9/17	9/18
Total number of chemicals detected.				17/17	18/18

[a] Military Exposure Guidelines (MEG) concentration (based on consumption of 15 L per day of water for 7–14 days; USAPHC, 2010).
[b] Human Lethal Concentration (HLC) concentration (based on consumption of 15 L per day of water for a 70 kg person; TERA, 2006).

determining a minimum detection limit (MDL) for each test chemical. The MDL is the lowest tested concentration at which there is a 90% probability of detection with 80% confidence (Hogan et al., 2007). Based on binomial probabilities, this required that a minimum of 16 of 16 samples be detected with no false negatives. Therefore, 16 out of 16 individual biochips had to respond to a compound at the level being tested in order for the chemical to be considered positive, or toxic. Selection of concentrations of test chemicals for ECIS testing was established based on previous range-finding tests done in our laboratory on all 18 chemicals. The range-finding was done in a step-wise fashion with 3 out of 3 biochips having to respond to a chemical concentration in order to establish the lowest level of response. If the level of response was within the MEG and HLC, then this concentration level was used to test 16 biochips and 16 out of 16 chips. If the level of response was above the HLC, then the upper limits of response were determined using 3 out of 3 chips.

Each fluidic biochip had two channels for testing; one channel was used for a control, and the other for the test compound. The results of previous ECIS open-well range find studies (unpublished data, USACEHR) were used to determine the test concentrations. All ECIS testing for the RTgill-W1 cells was done at 25 °C. L-15ex powdered media with phenol red (Cat # L1501, US Biological, Swampscott, MA) was used as the test media for both the control and treatment injections. The L-15ex media recipe formulated by Schirmer et al. (1997) has been used extensively for *in vitro* toxicity assays with the RTgill-W1 cells for metals and poly aromatic hydrocarbons (Schirmer et al., 1998, 2001; Dayeh et al., 2005a). L-15ex is a modified version of L-15 media and contains the same concentrations of salts, galactose, and pyruvate as basal L-15, but no vitamins or amino acids. For ECIS testing purposes, a 2× solution of L-15ex was prepared with Millipore water. This was used for both control (2× L-15ex diluted 1:1 with Millipore water) and test media solutions (2× L-15ex diluted 1:1 with the desired test chemical concentration). A pre-exposure period was initiated by injecting each channel of the biochip with 10 mL of 25 °C L-15ex control media over a one minute interval using a 10 mL syringe. Since the media environment surrounding the monolayers of RTgill-W1 cells within the biochips was being changed within the biochips from complete growth media to test media, this pre-exposure period allowed the impedance levels of the cells to reach equilibrium before introducing the test compound. Each biochip was then inserted into the 25 °C test unit (Fig. 2) and the ECIS software was started to collect 30 min of pre-exposure data with one minute readings immediately after the injections. The hardware and software of the ECIS test unit were described previously (Curtis et al., 2009b).

During the pre-exposure period, desired concentrations and volumes of the test chemical were prepared in the 2× L-15ex media along with replicate control solutions and equilibrated to 25 °C. Inclusion of the pH indicator phenol red in the media facilitated visual comparisons between the color of the control and test samples. Differences in pH that were greater than 0.20 pH units caused changes in media color that could be detected visually. In these instances, test samples were adjusted with 20% HCl or 1.0 N NaOH until the pH of the test sample was the same visually as the control sample to ensure ECIS responses were due to chemical toxicity and not pH shifts.

At the end of the 30 min pre-exposure, one channel of each of the biochips was injected with 10 mL of control media, and the other channel was injected with 10 mL of test media solutions. This was done while the biochips were in place in the test unit and impedance data was still being collected (Fig. 2). The ECIS software continuously collected the average raw impedance values from each of the four electrode pads in each channel every 60 s. The data was normalized and displayed on the ECIS reader monitor as a real-time graph. Since there was a range of starting impedance values on each electrode due to variability of the cell layers covering the electrodes, normalizing the data allowed for a more uniform comparison of the impedance values between the electrodes, and subsequently, between experiments. The normalized impedance values at each time point were calculated by dividing by the initial impedance values just prior to the exposure period on each electrode. After one, four, and eight hours of exposure, the impedance data was analyzed using the curve discrimination software to compare differences between the control channels and the treated channels (see Section 2.8 analysis, below).

Fig. 2. Multiple fluidic biochips inserted into the multichip reader test unit for 1 h exposure testing. The picture depicts a manual exposure injection with attached syringes of control and test samples in L-15ex media.

2.5. Procedure for ECIS chemical testing with the BLMVEC fluidic biochips

The procedure for ECIS chemical testing with the BLMVEC fluidic biochips was described in detail in Curtis et al. (2009b). The procedure was similar to the testing done with the RTgill-W1 biochips; the main differences were use of a 37 °C testing temperature because the BLMVECs are mammalian cells, a 60 min pre-exposure period instead of 30 min, 5 mL injection volumes instead of 10 mL, and the use of a different test media. The BLMVEC testing was done using powdered serum-free MCDB-131 (Sigma–Aldrich, St. Louis, MO) with added sodium bicarbonate as the test media for both the control and treatment injections. A pre-exposure period was initiated by injecting each channel of the biochip with 5 mL of pre-warmed 37 °C MCDB-131 control media using a 5 mL syringe over a one minute period. Each biochip was then inserted into the test unit and the ECIS software was started to collect 60 min of pre-exposure data with one minute readings. The raw impedance values of the confluent biochips ranged from approximately

1800–2000 Ω. At the end of the 60 min pre-exposure, one channel of each of the biochips was injected with 5 mL of control media, and the other channel was injected with 5 mL of test compound. This was done while the biochips were in place in the test unit and impedance data was still being collected. Collection and statistical analysis of ECIS data was the same as described for the RTgill-W1 biochips.

2.6. Effects of temperature on long-term storage impedance levels of RTgill-W1 fluidic biochips

To determine the length of cell viability at temperatures above 6 °C, nine fluidic biochips seeded with RTgill-W1 cells were placed in a thermoelectric ECIS unit where impedance values were recorded every hour for up to 20 weeks. Three biochips each were held at 12, 20, and 25 °C. No media replenishments were done on the biochips. Viability of the cell monolayer was determined by monitoring impedance readings over the specified time period. Another set of 36 fluidic biochips were also seeded with RTgill-W1 cells and placed at 6 °C with no media replenishments, and impedance readings were taken from three biochips over the course of 78 weeks at 2,3, 4, 8, 12.16, 20, 24, 32 38, 52, and 78 weeks.

2.7. Long Term Storage PCP Evaluations of RTgill-W1 fluidic biochips

In order to study the effects of cold storage with no media replenishment on the viability and sensitivity of the RTgill-W1 cells, fluidic biochips were initially seeded, held at 20 °C, and had media changes on days 4 and 7 as described above. The biochips were then held at 6 °C with no media replenishment for up to 39 weeks. Three biochips were removed for viability assessment and ECIS testing using the 9.4 μM detection level for PCP (Table 1) at 2,3,4,8,12,16,20,24,26,28,30 and 39 weeks using the chemical exposure procedure described above. PCP was selected as the benchmark toxicant due to its chemical stability, relative ease of analysis, and because changes in cellular impedance in response to PCP were very reproducible.

To verify RTgill-W1 cell viability after long-term storage, a live/dead cell stain was done on 39 week-old fluidic biochips after PCP exposures using a Live/Dead Viability Cytotoxicity Kit ™ (Invitrogen, Carlsbad, CA). The chips were flushed with 5 mLs of phosphate buffered saline (PBS) per channel and then flushed with 5 mLs per channel of 4 μM ethidium homodimer-1 and 2 μM calcien AM that was prepared in PBS, and incubated for 30 min. Fluorescent photomicrographs were taken using a Nikon Ti-S Eclipse with a CFI Plan Fluor DLL 10X NA 0.3 WD 16 mm phase contrast objective and Nikon C-FL B-2E/C fluorescein isothiocyanate and C-FL Y-2E/C Texas Red filters.

2.8. Statistical analysis

Previous work by Curtis et al. (2009b) describes a method for determining the statistical significance of the impedance response of an individual fluidic biochip to an unknown water sample. Each biochip contains a control and a treatment channel, and these impedance responses were statistically compared to a known database of control exposed fluidic biochips. The method developed by Dr. Steve Schwager of Cornell University using MATLAB (The MathWorks, Inc., Natick, MA) was incorporated into a curve discrimination program software package. The program was used to analyze the fluidic biochip impedance responses and is described in detail by Curtis et al. (2009b). A total of 107 individual RTgill-W1 and 53 BLMVEC control water exposed fluidic biochips were used in the respective control models for the curve discrimination analysis. A confidence level of 99.9% ($p < 0.001$) was used to establish statistical significance. Functional data analysis techniques (Ramsay and Silverman, 2005) were used to extend the standard single time point analysis of variance approach (Ott and Longnecker, 2000) to a curve consisting of approximately 60 points. If at least ten consecutive time intervals over a 60 min period were statistically different from the control model then an individual chip response was considered positive.

3. Results

3.1. Toxicity Testing of BLMVEC and RTgill-W1 fluidic biochips

Table 1 compares and summarizes the ECIS toxicity test results for both the RTgill-W1 and the BLMVEC seeded fluidic biochips. With the RTgill-W1 biochips, toxicity was detected for 9 of the 18 test chemicals after a one hour exposure at concentrations that were greater than or equal to the MEG, and less than or equal to the HLC. These chemicals were ammonia, arsenic, azide, copper, cyanide, mercury, methyl parathion, pentachlorophenate, and toluene. All of these compounds caused a significant decrease ($p < 0.001$) in impedance in 16 out of 16 biochips at the levels shown as compared to controls, with the exception of copper, which caused an increased impedance compared to the controls. Acrylonitrile, aldicarb, fenamiphos, fluoroacetate, methamidophos, nicotine, paraquat, phenol, and thallium were detected in 3 out of 3 biochips, but at levels above the HLC.

With the BLMVEC biochips, toxicity was detected for 8 of 17 tested chemicals after one hour of exposure at concentrations that were greater than or equal to the MEG, and less than or equal to the HLC. The chemicals detected within these ranges were the same as those detected with the RTgill-W1 cells, with the exception of cyanide, which the RTgill-W1 cells detected, but the BLMVECs did not. The BLMVECs also detected phenol, while the RTgill-W1 cells did not. Azide was not tested with the BLMVECs. The BLMVECs responded to acrylonitrile, aldicarb, cyanide, fenamiphos, flouroacetate, methamidophos, nicotine, paraquat, and thallium in 3 of 3 biochips, but at levels that were above the HLC. In summary, both cell lines were equally sensitive to arsenic, fenamiphos, pentachlorophenate, and thallium. The BLMVECs were able to detect acrylonitrile, aldicarb, methamidophos, nicotine and phenol at lower concentrations than the RTgill-W1 cells. The RTgill-W1 cells, however, were more sensitive to ammonia, copper, cyanide, mercury, fluoroacetate, methyl parathion, and toluene. A representative graph of normalized average impedance responses of BLMVEC and RTgill-W1 cells to a one hour exposure to control water and 60.1 μM arsenic (sodium arsenite) is shown in Fig. 3. Both cell lines had significant ECIS responses ($p < 0.001$) to arsenic, but the patterns of response were different. Sixteen out of 16 biochips were tested for control and aresenic exposures for each cell line. The BLMVECs typically had an inital spike in impedance levels immediately after the injection of the test media. This occured in both the control and treatment channels of the biochip subsequent to the one hour pre-exposure of both channels to the serum-free media. The RTgill-W1 cells typically did not have an initial spike in impedance levels after either the control or treatment injections. Both cell lines were able to detect arsenic with equal sensitivity, as evidenced by decreased impedance. The RTgill-W1 cell monolayers, however, appear to be less responsive to disturbances related to control media injections.

3.2. Effects of temperature on long term storage of RTgill-W1 fluidic biochips

As stated previously, fluidic biochips that contained media but no cells had raw impedance values ranging from 300 to 400 Ω. Once a cell monolayer had been established on the biochips, the

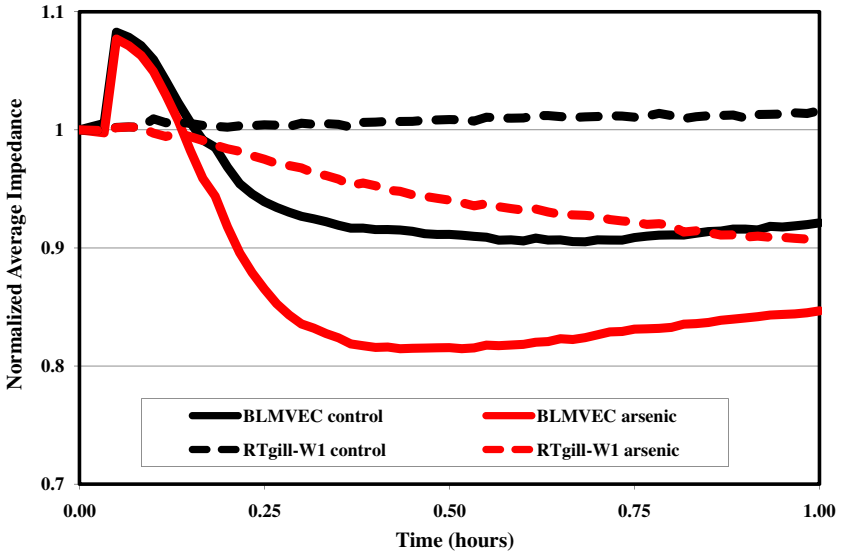

Fig. 3. Representative graphs of normalized average impedance values of BLMVEC and RTgill-W1 seeded fluidic biochips that were exposed to either control water (black lines) or 60.1 μM arsenic (red lines).

impedance values for RTgill-W1 cells ranged from 1200 to 2000 Ω when stored at 20 °C, which is the ATCC-recommended culture temperature for the RTgill-W1 cells. When impedance values approached 500 Ω, it was visually observed that the cells had become detached from the electrodes and the monolayer was no longer viable. As can be seen in Fig. 4, these biochips maintained a viable monolayer of cells for over 16 weeks without media renewal. Biochips held at 6 °C without media replenishment maintained high impedances even after 78 weeks of storage, with impedance levels increasing to ~2400 Ω at 8 and 52 weeks. A similar bimodal pattern of changing impedance levels can be seen at 12, and 20 °C. Impedance levels at 12 °C were also higher than 20 °C impedances and remained viable for over 20 weeks. A storage temperature of 25° led to decreased impedance and a shelf life of less than 4 weeks. At 30 °C, RTgill-W1 seeded fluidic biochips were viable for less than 24 h (data not shown).

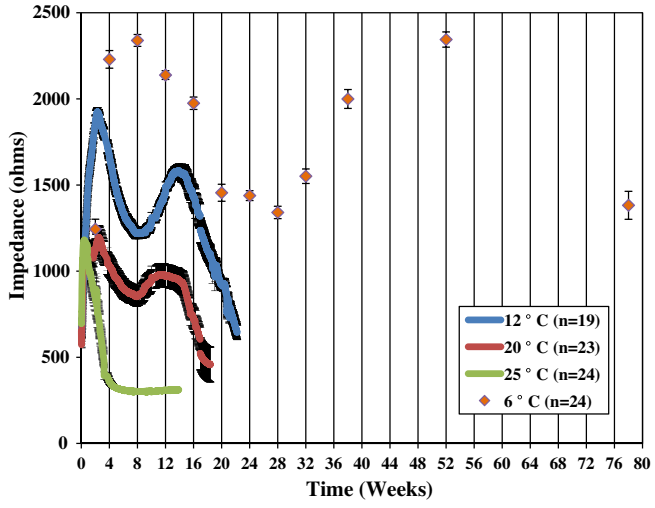

Fig. 4. Effect of temperature on RTgill-W1 impedance readings from long term fluidic biochip storage evaluations. The 12, 20, and 25 °C data were from biochips seeded on the same day and represent continuous hourly impedance readings from replicate fluidic biochips at different temperatures. There were three individual biochips for each temperature. The 6 °C data depicts fluidic biochips that were seeded in a separate study. Error bars represent standard error of mean of impedance for three individual biochips at each time point. Cells were not fed during the monitoring period.

3.3. Long Term Storage PCP Evaluations of RTgill-W1 fluidic biochips

In order for the RTgill-W1 cells to be useful in biosensors for field toxicity tests, it is essential that cell monolayers on the fluidic biochips respond to toxic insult after prolonged storage. Fig. 5 illustrates the toxicity responses as the average normalized impedance difference between the control and treatment channels of fluidic biochips that were stored at 6 °C. Three individual biochips were tested at each time point. The cells responded significantly ($p < 0.001$) to a one hour 9.4 μM PCP exposure at all the time points indicated (2, 3, 4, 8, 12, 16, 20, 24, 26, 28, 30 and 39 weeks of storage).

An example of the graphic impedance responses of the one of the 39 week-old biochips is illustrated in Fig. 6, as well as the corresponding live/dead stain photomicrographs of the same chip. In Fig. 6A, the ECIS response of the RTgill-W1 cells to 9.4 μM PCP was significant ($p < 0.001$) within one hour of exposure. The black lines on the graphs represent the control channel normalized impedance responses, and the red lines represent the PCP channel normalized. Starting impedance values on the biochip ranged from 1636–1914 Ω, which, in our experience, was indicative of an intact monolayer of RTgill-W1 cells. Fig. 6B and C are photomicrographs of the resultant live/dead stains of representative sections of the control channel and PCP-treated channels, respectively. Live cells stain green, while the nuclei of dead cells stain red.

4. Discussion

One of the objectives of this research was to optimize storage conditions for cell monolayers on a fluidic biochip to produce a potentially field-portable biosensor that required little or no maintenance. Preliminary observations in our laboratory indicated that the RTgill-W1 cell line could withstand long-term storage in refrigerated temperatures, which was not achievable with mammalian lines requiring frequent media renewal in addition to 37 °C storage temperatures (Curtis et al., 2009b). It is well known that fish can withstand relatively long periods of starvation without severe consequences, and water temperature influences long-term fish

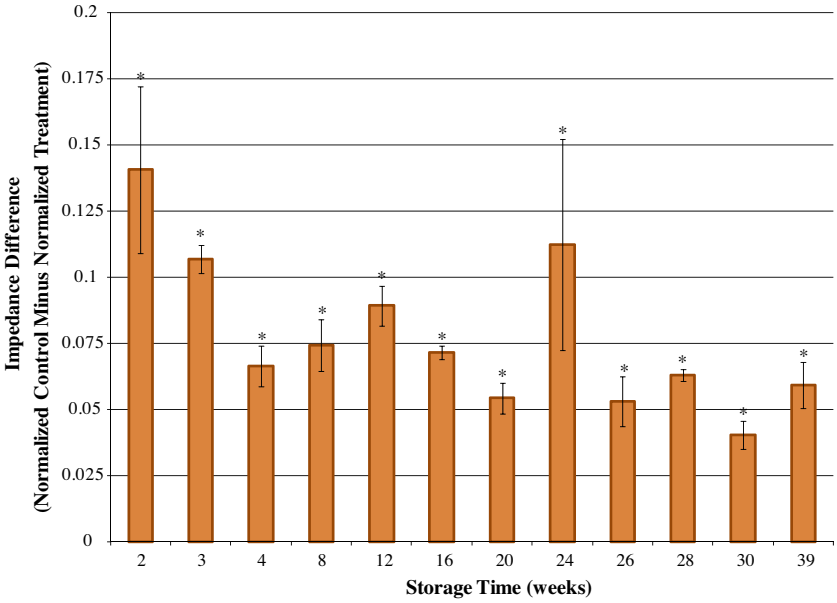

Fig. 5. Responsiveness of RTgill-W1 cells seeded on ECIS biochips to PCP with time of storage. RTgill-W1 cells on fluidic biochips maintained at 6 °C with no media replenishments for different storage durations were tested with 9.4 μM PCP at indicated storage time points. Data depicts response mean differences (±S.E.) between the normalized control and treatment channels at each storage time for three fluidic biochips. Significance at each time point ($p < 0.001$) is depicted by an * and required a positive detection for all three biochips at each time point using the curve discrimination program.

survival by altering their metabolism. Rainbow trout, *Oncorhynchus mykiss*, have been shown to survive in waters between 0 and 29.8 °C, dependent upon on temperature adaptation and fish strain (Currie et al., 1998). Consequently, the ability of the RTgill-W1 cells to survive for 78 weeks at 6 °C on a biochip with no media replenishments is not surprising for a cell line derived from fish that thrive in cold waters. This observation is not unique to RTgill-W1 cells; RTG-2, a rainbow trout gonad-derived cell line, was reported to retain cellular viability for up to 2 years at 4 °C without media changes (Wolf and Mann, 1980), and was reported to survive at 0 °C for 28 days with little change in cell number (Mosser et al., 1986). Another salmonid cell line, CHSE-sp, a subline of Chinook salmon embryo cells, was also shown to maintain viability and retain the ability to support viral growth at 4 °C for 6 months (Araki et al., 1994). Since the intrinsic physiology of a poikilothermic organism, such as the rainbow trout, allows these organisms to survive at relatively low ambient temperatures *in vivo*, we theorized that extended low temperature survival might be possible for cells derived from such organisms. The impedance data shown in Fig. 4 supports this hypothesis, demonstrating that the RTgill-W1 cells could be maintained at reduced temperatures for extended periods of time without feeding or maintenance. In fact, there was an increase in the shelf-life of the seeded biochips stored at decreased temperatures. Decreasing the storage temperature to values below the recommended culture temperature of 20 °C provided two distinct advantages for long-term biosensor development. First, the shelf-life of the biochips increased to over 20 weeks when held at 12 °C, and to 78 weeks when held at 6 °C. Second, an overall increase in monolayer impedance was observed at the 12 and 6 °C storage temperatures as compared to biochips held at 20 and 25 °C. The reason for the increased impedance levels during storage time still needs further investigation, but it is well known that cell membrane fluidity decreases with decreasing temperatures (Los and Murata, 2004). These changes in membrane fluidity can cause a signaling event in which cold inducible proteins may become expressed, which in turn, regulate cell metabolism so that the cells can survive at colder temperatures (Guschina and Harwood, 2006). Cell membrane lipids of poikilothermic organisms are modified *in vivo* in order for the fish to survive temperature extremes (Guschina and Harwood, 2006). The increased monolayer impedance observed at colder temperatures with the RTgill-W1 cells in this paper may be related to these cellular adaptation mechanisms.

One interesting observation from this data is the bimodal pattern of rising and declining impedance levels that was recorded over time for all storage temperatures. Natural biological oscillations have been noted in many life forms, mostly in response to endogenous, and sometimes rhythmic, metabolic cycling that is not well understood (Tu and McKnight, 2006). Schweizer et al. (2011) noted that in rainbow trout liver cells, calcium oscillations were present in 40% of the cells that were unrelated to ATP concentrations, indicating that these cells have some endogenous metabolic rhythm. The replication of these impedance level patterns at all four storage temperatures in the RTgill-W1 cells in this paper seems to indicate that the effect may be linked to the metabolic machinery of the cells, which is reduced at lower temperatures.

Although the chemical sensitivity of the BLMVEC mammalian cell culture was comparable to that of the RTgill-W1 fish cell line when using ECIS as the indicator of toxicity, the overall advantages of using the fish cell line for water supply testing becomes evident when considering the maintenance and storage of the cells on the fluidic biochips. As shown here, the fluidic biochips seeded with RTgill-W1 cells were able to maintain a confluent monolayer for 78 weeks when stored at 6 °C without media replenishment and were still responsive to the benchmark toxicant, PCP, when used in the ECIS assay. In contrast, BLMVEC cells were viable for at least 16 weeks when stored on the biochips in a 37 °C incubator, but the media on the biochips had to be replenished three times per week (Curtis et al., 2009b). The sensitivity of the BLMVECs to challenges of 38 μM PCP in ECIS assays also decreased over the 16 week period. The BLMVEC ECIS toxicity sensor (Curtis et al., 2009b) was considerably larger and heavier than the current ECIS sensor that is described here and was not very practical for field use, especially when combined with the complexity of the automated cell maintenance system that was required for media replenishment of to feed the mammalian cells on the biochips. Using the RTgill-W1 cells

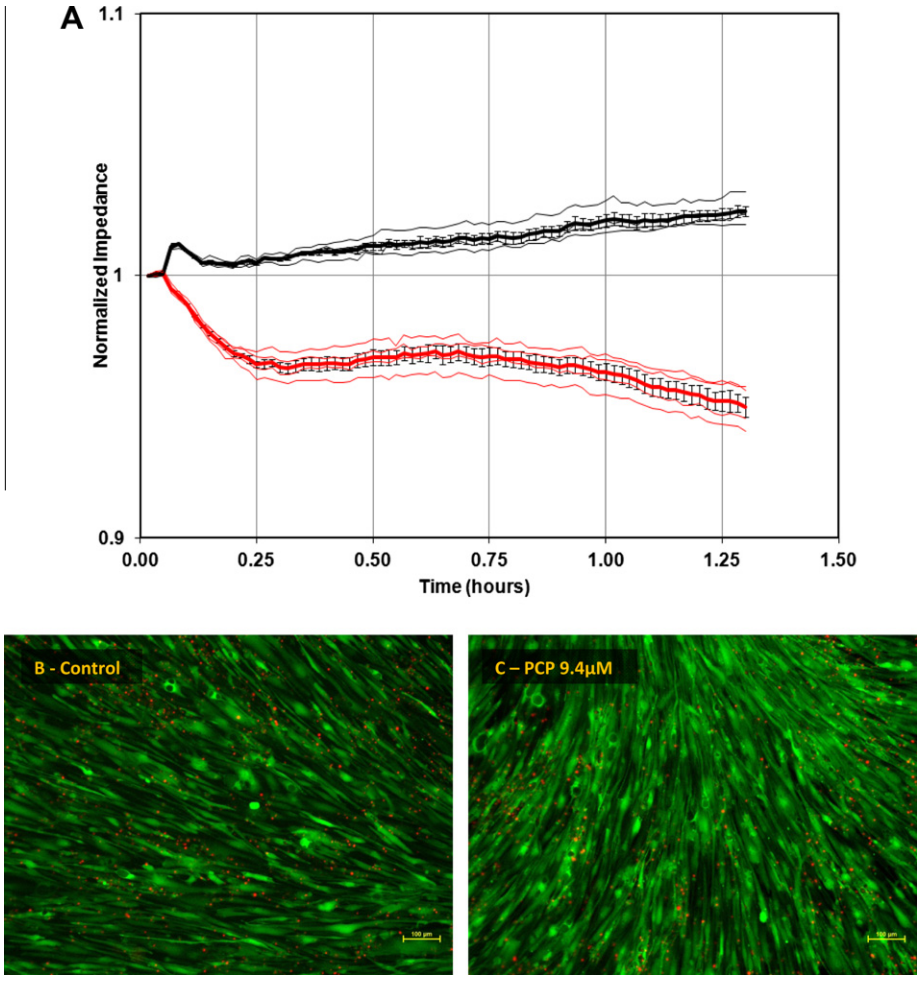

Fig. 6. Top: (A) Normalized impedance response of RTgill-W1 fluidic biochip maintained at 6 °C with no media replenishments for 39 weeks, and then exposed to 9.4 μM PCP. Thick black lines depict control channel average withstandard error of mean and individual control electrodes (thin black lines). Thick red lines depict PCP channel average with standard error of mean and individual control electrodes (thin red lines). Starting impedances ranged from 1636 to 1914 ohms and significant differences ($p < 0.001$) between control and treatment channels were detected using the curve discrimination program. (B) and (C) Bottom: Live/Dead Cell Stain of monolayers of Rtgill-W1 cells within the channels of the fluidic biochip after control and PCP exposures. (B) depicts control channel and (C) depicts PCP exposed channel. Live cells are shown in green (2 μM calcein AM) and dead cells in red (4 μM ethidium homodimer-1). Live/dead images demonstrate that the majority of cells are living and an intact monolayer exists, which correlates with the high impedance measurements recorded.

allowed us to design a toxicity sensor that was more compact since the cell maintenance system was no longer needed. Research with the RTgill-W1 cells on the fluidic biochips is continuing to determine the maximum shelf-life of these biochips at 6 °C and to further elucidate why there are cycles in the impedance values of the stored biochips over time.

Portable ECIS readers are currently being developed by Biosentinel, Inc. (Austin, TX) for use in field situations (Fig. 7). These portable readers are being designed for hand-held operation and to withstand the rigors of field use. The RTgill-W1 fluidic biochips that have been developed in parallel with the ECIS reader show great promise as field-portable biosensors for broad-based screening of drinking water supplies because of their rapid response (less than one hour) and sensitivity to chemicals, combined with their low maintenance and long-term cold storage capabilities. For the testing of drinking water for Army purposes, this biosensor is intended for use as a screening device to be used in conjunction with other complementary field water testing devices.

Broad spectrum chemical water screening biosensors, such as the RTgill-W1 fluidic biochips described here, are invaluable in the field as first-alerts to potential contamination of drinking water that may warrant further investigation and analysis. Although one could argue that only single compounds were tested with this

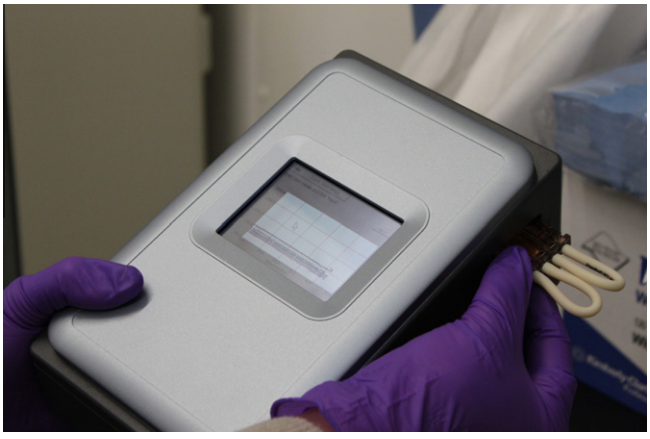

Fig. 7. Biosentinel prototype portable ECIS fluidic biochip reader, showing fluidic biochip insertion. The portable reader includes battery operation, touch screen display, and built in temperature control for field analysis of water samples.

biosensor and the most likely scenario encountered under realistic environmental conditions will be mixtures, the lack of human toxicity data for mixture scenarios makes this data difficult to interpret. There are few, if any, benchmarks (such as the MEG and HLC) available for chemical mixtures in regards to human health effects. Broad spectrum rapid toxicity sensors, such as the RTgill-W1 fluidic biochips, that are becoming available available for testing drinking water under field use conditions, will provide rapid rapid information as to the to the potential safety of a drinking water supply.

As stated earlier in this paper, there are a variety of chemical toxicity sensors available, but these are analyte-specific. The RTgill-W1 biochips, however, are designed as a screening tool to rapidly respond to the presence of chemicals, either alone or as mixtures, which may be present in the drinking water. The strength of this biosensor is that not only does it respond to specific chemicals in the water at levels that are relevant to human health, as defined in this paper, but it also has the potential to respond to a much larger set of chemicals with similar toxicological properties. Work is on-going to explore the feasibility of using additional cell lines and methods for storing the seeded biochips to prolong the lifespan of the biochips without reducing chemical sensitivity. Improvements to the portable ECIS hand-held reader are also on-going.

Conflicts of interest statement

I wish to confirm that there are no known conflicts of interest associated with this publication.

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