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Multixenobiotic resistance (MXR) inhibition and interactive toxic effects of the cationic surfactant, benzalkonium chloride, in embryonic medaka (*Oryzias latipes*)

A Thesis Presented

by

John Gondek

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the degree of

Master of Science

in

Marine and Atmospheric Science

Stony Brook University

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2010

Stony Brook University

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Abstract of the Thesis

Multixenobiotic resistance (MXR) inhibition and interactive toxic effects of the cationic surfactant, benzalkonium chloride, in embryonic medaka (*Oryzias latipes*)

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Contaminated environments represent a challenge to resident species. Organisms must possess underlying mechanisms of resistance to protect against a broad range of environmental insults. One mechanism likely conferring resistance is expression of low substrate specificity membrane efflux transporters termed multixenobiotic resistance (MXR). MXR transporters confer resistance by preventing toxics from entering into cells and also by expelling potentially cyto- and genotoxic metabolites. Chemicals known as chemosensitizers, which can inhibit MXR function thus represent a threat to aquatic organisms. This study used embryos of medaka (*Oryzias latipes*) to measure MXR activity and inhibition through a dye exclusion assay. This assay was used to investigate the chemosensitizing potential of the emerging contaminant, benzalkonium chloride (BAC), representative of a class of quaternary ammonium compounds, which are high production volume cationic surfactants. BAC was found to be a potent inhibitor of MXR at high concentrations in early life stage medaka. BAC was also determined to be developmental toxicant as evidenced by the occurrence of morphological deficits in medaka fry in a long term developmental toxicity test. Most interestingly, there was a greater than additive toxic effect when embryos were co-exposed to BAC and the common organic contaminant, benzo[a]pyrene. As an environmentally relevant source of BAC and other contaminants, chlorinated effluent was also assessed and found to both inhibit MXR and be embryotoxic. These results highlight the need to further investigate the mechanisms of BAC induced toxicity. This study is among the first to demonstrate sub-lethal effects of this important group of widely used chemicals which may have significant ecological consequence to aquatic organisms by enhancing the toxicity of co-occurring contaminants.

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1. Introduction

It is important to approach ecological problems from new perspectives in order to identify previously overlooked hazards. Because contaminated environments, particularly around urban estuaries, contain complex mixtures of chemicals, interactive effects are often unaccounted for by traditional toxicology studies.

When considering how organisms survive in contaminated environments, it is important to understand the underlying mechanisms of resistance that marine organisms must have to a variety of chemicals. Xenobiotic metabolizing enzymes and sequestration molecules such as metallothionein have been studied extensively as detoxification systems (DiGiulio, 1995). Also important, yet less well understood, are the processes that influence how exogenous chemicals enter into the body at the cellular level. Organisms manifest general protection of cellular targets via efflux transporters, such as P-glycoproteins (P-gp) and multidrug resistance proteins (MRP), on cell membranes (Ambudkar et al., 1999; Cole and Deeley, 1998, Endicott, 1989). Known substrates share some general chemical properties; this includes having low molecular weight, moderate hydrophobicity, a basic nitrogen atom and being either cationic or neutral (Smital et al., 2004). Through broad substrate recognition, transporters limit the permeability of xenobiotics into the cell, and recognize and expel potentially cyto- and genotoxic metabolites produced through the metabolism and detoxification of contaminants (Deeley et al., 2006). This phenotypic expression has been termed multixenobiotic resistance (MXR) in aquatic organisms (Bard, 2000; Kurelec, 1992). Furthermore, efflux transporters are the primary defense in early developmental stage embryos against xenobiotics, prior to the development of transformation and metabolism systems that appear after tissue differentiation (Hamdoun and Epel, 2007).

MXR is the first line of defense an organism has against a broad group of potentially toxic chemicals.

Immunochemical analysis has been used to detect the presence of P-gp in fish (Bard et al. 2002a,b, Cooper et al. 1997). Because P-gps are highly conserved, these studies utilized a mammalian monoclonal antibody to cross react with fish P-gp counterparts. Expression of MXR proteins is reflective of xenobiotic levels to which an organism is exposed. These studies looked at expression of P-gp in aquatic organisms known to thrive in contaminated environments, such as killifish (*Fundulus heteroclitus*). It was found that killifish collected from polluted sites had higher levels of P-gp compared to fish collect from clean environments, indicating that their resistance to environmental toxics is likely conferred by the overexpression of these proteins.

Another common method to detect MXR activity is through dye exclusion assays. This utilizes specific fluorescent dyes (such as rhodamine and calcein) to test the ability of cells to expel a known MXR substrate. As seen in Figure 1, under normal conditions where transporters are functional (left), the dye is recognized by the transporters and expelled, leading to a low cell fluorescence. Conversely, the figure on the right would be an example of a cell where MXR function is compromised through the use of an MXR inhibitor. In this scenario, where efflux function is inhibited, the fluorescent dye accumulates within the cell. Dye exclusion assays are important for evaluating chemicals, such as environmental contaminants, for their ability to inhibit MXR.

MXR inhibition, or reversal, as it is sometimes described, can occur when there is an abundance of substrates present, leading to saturation of the efflux system. With only so many pumps to bind and expel these substrates, the pumps become overwhelmed and this defense is

Model for detection of MXR via dye exclusion assay

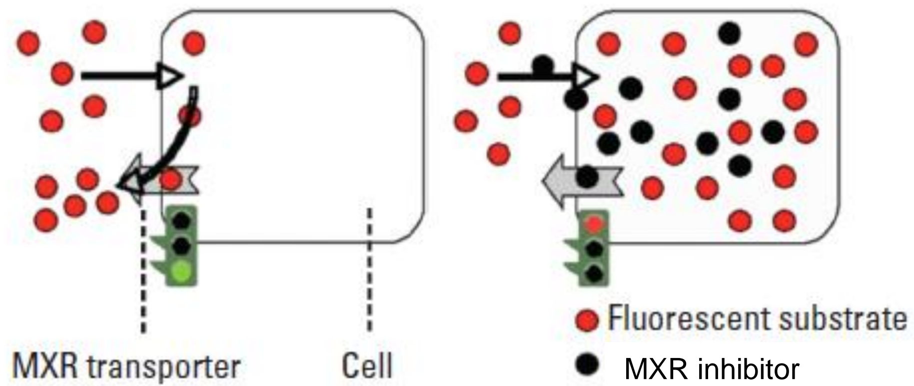


Figure 1.

Under normal MXR function (left), transporters recognize the fluorescent dye and expel it from cells which have a low fluorescence. When cells are exposed to an MXR inhibitor or chemosensitizer, the fluorescent dye begins to accumulate leading to higher cell fluorescence. Modified from Epel et al. 2008

rendered ineffective as chemicals begin to accumulate within the cell (Epel et al., 2008). Another way is through chemicals known as “chemosensitizers” which actively disrupt transporter function or otherwise increase the permeability of other xenobiotics into cells (Smital and Kurelec, 1998). Inhibition of MXR may increase the toxicity of xenobiotics by enhancing uptake and slowing down removal after metabolism, leading to accumulation of toxic metabolites. The low substrate specificity of MXR proteins make them vulnerable to having their function disrupted by an equally broad variety of substrate inhibitors. The large number of environmental contaminants in this group makes identification and testing of potential MXR inhibitors an arduous task.

Environmental contaminants that have known chemosensitizing potential include: diesel-oil (which contains more soluble PAHs) (Kurelec, 1995), moderately hydrophobic pesticides (Bain and LeBlanc, 1996; Buss and Callaghan, 2008; Cornwall et al., 1995; Toomey and Epel, 1995), perfluoroalkyl acids used in nonstick cookware (Wania, 2007), lipophillic pharmaceuticals such as those used in chemotherapy (Hofsli and Nissenmeyer, 1990), fragrance musks found in soaps and lotions, (Luckenbach and Epel, 2005; Luckenbach et al., 2004), and natural toxins taken from extracts of invasive and harmful algal blooms like *Caulerpa sp.* and *Alexandrium sp.* (Eufemia et al., 2002; Schroder et al., 1998; Smital, 1996). In the case of pesticides, optimal binding to P-gp was most associated with having a cyclical structure, moderately hydrophobic (log K_{ow} 3.6-4.5) and a molecular weight between 391-490 kDa (Bain and LeBlanc, 1996). While not all of these chemicals may be inherently toxic, their ability to inhibit MXR activity may compromise an organism’s defense in an already contaminated environment.

The main ecological risk that chemosensitizers may pose is that they may enhance the toxicity of other co-occurring contaminants. This has been extensively studied by Kurelec who addressed the potential of chemosensitizers to enhance the genotoxicity of known toxicants (Kurelec, 1993). In studies of a freshwater clam, *Corbicula fluminea,* exposed to the model mutagen, acetylaminofluorine (AAF), Kurelec found that in the presence of an MXR inhibitor, clams had an increased number of single strand DNA breaks, a classic endpoint for genotoxicity (Kurelec et. al, 1996). Furthermore, the addition of an inhibitor lowered the no observable effect concentration of AAF by an order of magnitude. Similar results were observed in a freshwater fish, *Leuciscus idus*, where co-exposure to an MXR inhibitor increased the genotoxicity of aminoanthracene by measure of single strand DNA breaks and DNA adducts (Kurelec, 1992). In addition, exposure to the MXR inhibitor triggered a behavior change where fish exposed to inhibitor alone made escape attempts from their tanks and the addition of aminoanthracene increased the frequency of this suicidal behavior.

Considering the large pool of chemicals that may be chemosensitizers, high production volume chemicals (HPVC) should be among the first evaluated for their chemosensitizing potential. Occurrence of such chemicals would most likely be bodies of water near densely populated land, or receiving waters or downstream of STPs or manufacturing plants. An optimal area would be in urban estuaries where both the number of point sources and number of chemicals entering the system are substantial. One class of HPVC chemicals which should be investigated are quaternary ammonium compounds (QACs), a group of cationic surfactants. Economically important, their uses are many and include biocides, fabric softeners, surfactants, pesticides, disinfectants, lubricants and components of personal care products, among a multitude of other applications (Cross, 1994).

QACs are common environmental contaminants that have been gaining attention in recent years, though data on their toxicity to aquatic organisms remains fragmentary. Major point sources for QACs to the environment include wastewater treatment plants, and hospital and laundry effluent (Kreuzinger et al., 2007). Because of their strong affinity for particles, QACs become enriched in sewage sludge and marine sediments, leading to high environmental concentrations. One class of QAC, alkyldimethylbenzylammonium chloride, commonly known as benzalkonium chloride (BAC), has become of concern because of its heavy usage and appreciable concentrations found in the environment (Li and Brownawell, 2010). Direct toxicity testing at environmentally relevant concentrations with relevant co-stressors is needed to evaluate the consequences of potential MXR inhibition.

QACs' basic structures consist of a positively charged nitrogen atom bonded to four alkyl groups. These structural properties, including BAC's cyclical structure (Figure 2), are shared with other chemosensitizers making QACs potential candidates to cause cellular efflux disruption. The mechanism by which QACs act as biocides is by binding to target membranes which changes hydrophobicity, causing the cell to leak. This is believed to be done by the lipophillic alkyl chain of QACs penetrating within the lipid bilayer with the positive quaternary ammonium group remaining on the outside of the outer membrane (Thorsteinsson et al., 2003, Figure 3). QACs alter the structure and polarity of the lipid bilayer which changes the permeability of the membrane, causing cells to leak electrolytes and other cytoplasmic constituents in addition to disrupting membrane enzyme activity. (Eich et al., 2000, El-Falaha et al., 1985, Maillard, 2002). The potential for QACs to bind to cellular membranes and disrupt function may make them chemosensitizers by compromising membrane integrity.

Basic structure of benzalkonium chloride (BAC)

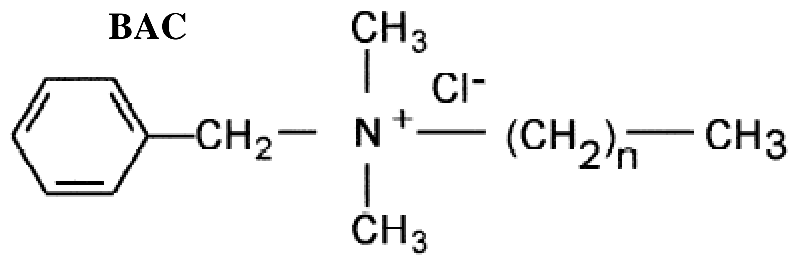


Figure 2.

Structure of benzalkonium chloride. Modified from Kummer et al. 1997

Biocide action of quaternary ammonium compounds (QACs)

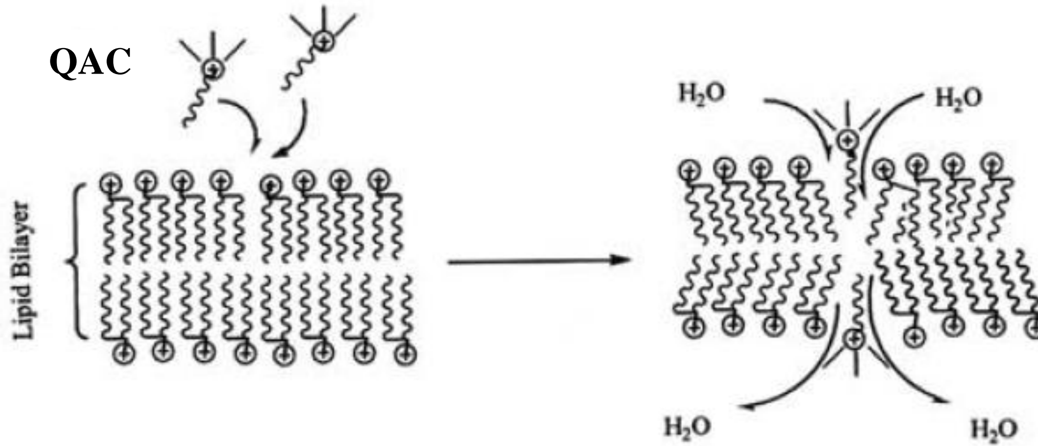


Figure 3.

Possible mode of action for QAC toxicity. The lipophillic alkyl chain penetrates within the lipid bilayer with the positive quaternary ammonium group remaining on the outside of the outer membrane. This changes the structure and polarity of the target membrane causing a change in permeability

Using embryos of medaka (*Oryzias latipes*), this study sought to develop a method to measure MXR inhibition in early life stage embryos and test the potential of BAC to inhibit these systems. Due to the widespread use of QACs, BAC was also tested to see if it is a developmental toxicant, and to determine whether it acts synergistically with a common organic contaminant, the polycyclic aromatic hydrocarbon benzo(a)pyrene (BaP). Finally, as an environmentally relevant source for QACs, chlorinated sewage effluent was also assessed for its chemosensitizing potential and embryotoxicity.

2. Methods

2.1 Fish care and egg collection

Medaka were maintained at 26 ±1 °C on a 16:8 hour light:dark cycle, and fed Aquatox (Aquatic Ecosystems, Apopka, FL) flake food twice daily and newly hatched brine shrimp in the morning to stimulate egg production. Eggs were collected every day two hours after feeding by removing eggs by hand from each female. Eggs were separated from each other by gently rolling eggs over a fine mesh and placed in embryo rearing medium (ERM) and stored at 25 °C until ready for use.

2.2 MXR inhibition assay

To test for MXR activity and inhibition, a calcein-AM exclusion assay originally designed for zebrafish (*Danio rerio*) (Langsner et al, 2010)., was modified for use in embryos of medaka Calcein-AM is an ideal probe for measuring MXR because it is a known MXR transporter substrate and only when it enters into a cell and becomes hydrolyzed by intracellular esterases does it form the fluorescent by-product calcein. Under normal MXR function, embryos exposed to calcein-AM will actively expel the dye from cells and the embryos will have a low

fluorescence. Conversely, embryos exposed to MXR inhibitors are less able to transport calcein from inside the embryo, and show increased fluorescence.

Embryos were treated individually in 1.5 mL microcentrifuge tubes. Each tube contained 1 μM calcein-AM (Invitrogen, Carlsbad, CA). MK 571 (Sigma-Aldrich St. Louis, MO), a model inhibitor of multidrug resistance proteins (MRP) was run as a positive control at a concentration of 10 μM. A DMSO control (1%) was run alongside all treatments. Preliminary studies assessing activity from 6 hours post fertilization (hpf) to 9 days post fertilization (dpf) demonstrated maximum activity at 6hpf (data not shown). After 3 days of development, pigmentation in the embryo made imaging calcein fluorescence problematic. Based on preliminary studies MXR activity was assessed at this 6hpf in all subsequent work. In order to have all medaka embryos analyzed before the mid blastula transition (MBT -around 8 hpf in medaka), dosing began roughly at 4 hpf and the embryos allowed to incubate one hour in the dark on a shaker plate. At 6 hpf embryos were removed from their solutions and triple washed in fresh ERM to remove any external calcein. Accumulation of calcein was assessed by quantifying fluorescence using a Nikon Eclipse TE-2000S inverted microscope equipped with a Spot Insight QE camera (Ex 495 nm/Em 535 nm at 40X magnification). Images of each embryo were taken and analyzed using ImageJ. The mean grey value for the each embryo's blastula was used as semi-quantitative measurement of fluorescence and as a proxy for MXR inhibition.

2.2.1 BAC dose response

To evaluate the potential of BAC to inhibit MXR activity, a dose response test was performed. A 14C alkyl chain length BAC (BAC-14 also known as miristalkoniumchloride) was used as a representative BAC because it is the most soluble and primarily used as a biocide.

Starting at the critical micelle concentration 12,000 μg/L, and at 1,200, 120 and 12 μg/L with five embryos per treatment group.

2.2.2 Interactive effects of sewage exposure and organic contaminants

Sewage inputs represent a common source of contaminants in the environment but little work has been done to investigate their potential impacts and interactive effects on early life stage aquatic organisms. Chlorinated sewage effluent was collected from the Bergen Point Sewage Treatment Plant (STP) in West Babylon, NY, the largest STP on Long Island which processes around 30M gallons per day. The MXR calcein assay was also used to assess the influence of effluent and the potential interactive effects with BaP. Exposures were done using 100% effluent, BaP at 10 μg/L, and a combination of 100% effluent spiked with 10 μg/L BaP with 10 embryos per treatment group.

2.3 Early life stage toxicity

2.3.1 BAC toxicity

To determine the early life stage toxicity of BACs and examine the potential interactive effects of BACs and a well known developmental toxicant such a BaP, a long term developmental exposure was conducted evaluating the presence of developmental defects. Treatment groups (10 embryos per treatment) were set up to test both BaP and BAC individually and in combination with one another. Embryos were first exposed to concentrations of BaP and BAC at 10, and 100 μg/L, and 12 and 120 μg/L respectively. In addition, embryos were also exposed to each BaP concentration at each concentration of BAC (ex. BaP 10 μg/L + BAC 12 μg/L and BaP 10 μg/L + BAC 120 μg/L). In a subsequent experiment, embryos were dosed at lower BaP concentrations 2 and 10 μg/L) individually and in co-exposure with BAC.

Development toxicity experiments were conducted with embryos exposed individually in 2 mL glass vials and allowed to incubate for 10-11 days in an incubator at 25 °C and under 12:12 day night cycle to promote hatching. Red lights were used for illumination to minimize phototoxicity of BaP.

After exposure, individuals were visually scored for developmental defects under a dissecting microscope. Scoring was based on a 3 point system for each potential defect where 0=not present, 1=present and 2=severe and a toxic score was given to each embryo as the cumulative score of its toxic deficits. Originally a set of 10 defects was chosen (skeletal deformity, cranial deformity, pericardial edema, yolk sac edema, cardiac deformity, cardiac arrhythmia, hemorrhage, underdeveloped, delay of hatch, failure to hatch, and death). However most of these deficits were not observed, or only observe rarely, so data from only the four most commonly observed (skeletal deformity, cranial deformity, pericardial edema, and cardiac deformity) are reported here. Skeletal deformity was scored for fry with a bent spine or a hooked tail. Cranial deformity was scored for fry that had a short snout that did not extend far past the eyes and for those that were unable to open their jaws fully. Cardiac deformity was scored for embryos with a broad range of observable defects such as deformed heart chambers, tube heart, and poor cardiac blood flow. Examples of these deficits are shown in Figure 4.

2.3.2 Effluent toxicity

Embryos were also exposed to chlorinated effluent at 25% and 100% concentrations. To test whether exposure to effluent in combination with BaP would lead to increased toxicity, BaP was also tested by itself at 2 and 10 µg/L and also in combination with 25% or 100% effluent (n=

Examples of development defects scored for toxicity in medaka fry

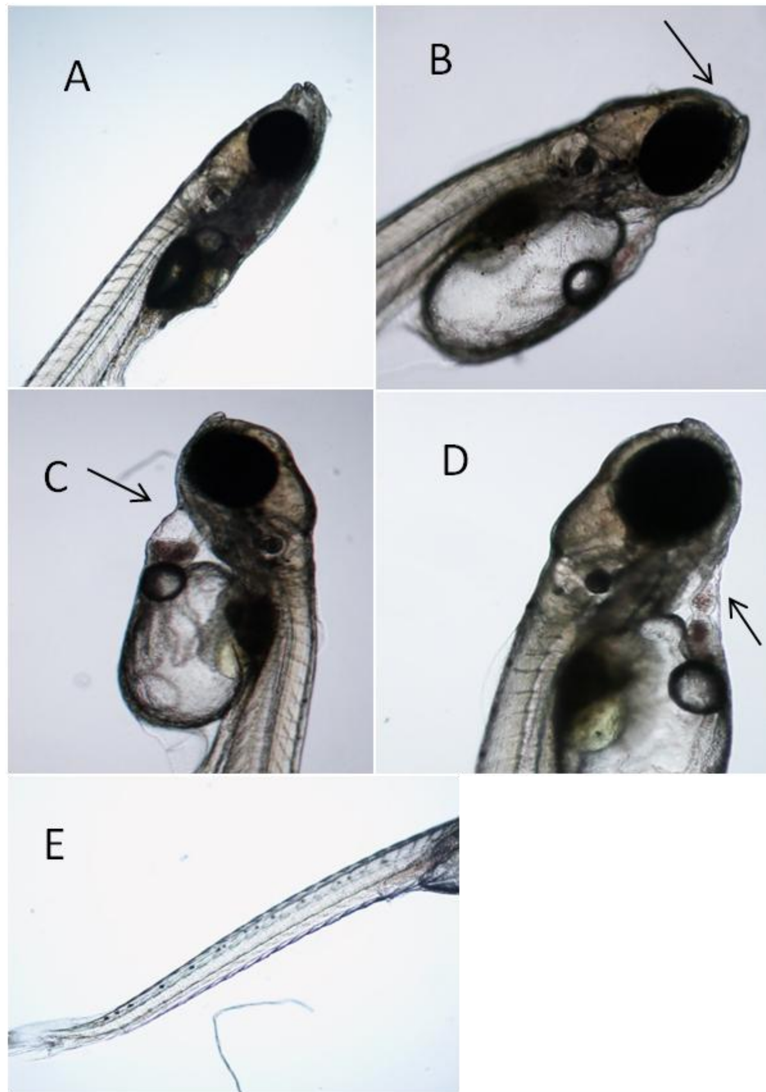


Figure 4.

Deformities scored for newly hatched fry were a. control, b. cranial deformity, c. pericardial edema, d. cardiac deformity, e. skeletal deformity

10). Because the test solution was not replaced over the course of the experiment, the vials were kept on a shaker plate (~30 oscillations/min) while incubating to maximize oxygen exchange.

2.4 BAC and 14C BaP accumulation

Two possible mechanisms by which BAC might enhance the toxicity of other contaminants is through increasing bioaccumulation due to its affect on membrane permeability, or through inhibition of MXR transporters. To evaluate whether or not BAC altered embryos bioaccumulation of other contaminants, a bioaccumulation study was done where embryos (n=8 per treatment) were exposed to 7,10 ^{14}C radiolabeled BaP (Amersham Piscataway, NJ, 60 mCi/mMol) at 2 µg/L either alone or in combination with BAC at 1.2, 12 and 120 µg/L using 1 embryo per 20 mL scintillation vial. Dosing began at 1 day post fertilization (dpf) and vials were incubated on a shaker plate at 25 °C on 12:12 hr red light cycle.

At 6dpf, embryos were removed from solution and tripled washed in ERM to remove external BaP. Embryos were moved to individual clean vials and 0.5 mL of Soluene (Packard, Meridin, CT) was added to solubilize the embryos and vials were placed in a 50 °C water bath to accelerate dissolution. Once completely dissolved, embryo solutions were suspended in 10 mL of Ultima Gold XR (Perkin Elmer, Waltham, MA) liquid scintillation cocktail and allowed to sit overnight in the dark. Degradations per minute (DPM) were measured using a Packard Tricarb liquid scintillation counter.

2.5 Statistical analysis

For MXR assays, either parametric or non-parametric ANOVAs were used to test for overall treatment effects with post hoc means tests used for multivariable analysis using SigmaStat statistical software (Aspire Software International, Ashburn, VA). For toxicity

experiments, data were evaluated using binomial logit analysis (comparing no effect to some effect) or multinomial (toxicity score 0=no effect, 1=minimal effect, 2=moderate effect, 3= high effect, and ≥ 4 = severe effect) logit analysis in either a categorical or ordinal distribution using Statistica software (Statsoft, Tulsa, OK). Categorical analysis takes into account the different possible scores for toxicity but does not rank the scores. Ordinal distribution treats the scores low to high based on a general linear model for toxicity.

3. Results

3.1 MXR inhibition assays

Results indicated clear MXR activity in 6hpf medaka embryos as evidenced by the enhanced accumulation of the model substrate, calcein in response to the model inhibitor, MK 571 (Figure 5a & b). Fluorescence was minimal in the control embryos exposed only to the carrier, DMSO, and significantly enhanced in the blastulas of the MK 571 inhibited embryos (Figure 6 & 7). Fluorescence observed using the calcein-AM assay (where the fluorescent product is only formed intracellularly) was localized in the blastula. This made it easier to quantify fluorescence compared to using a generally fluorescent MXR substrate such as rhodamine B, where general staining of the embryo was problematic. (data not shown).

Exposure to BAC appeared to increase fluorescence in a dose dependent manner, though this effect was only statistically significant at the 1200 µg/L concentration (Tukey test, $P < 0.05$), where mean fluorescence was elevated over controls by a factor of 3 (Figure 6). These results demonstrate the potential of high doses of BAC alter MXR function or change the permeability of early life stage embryos. As with MK 571, accumulated fluorescence was concentrated in the blastula of the embryos (Figure 5c). It was not possible to determine MXR inhibition in embryos

Accumulation of fluorescent calcein in medaka embryos

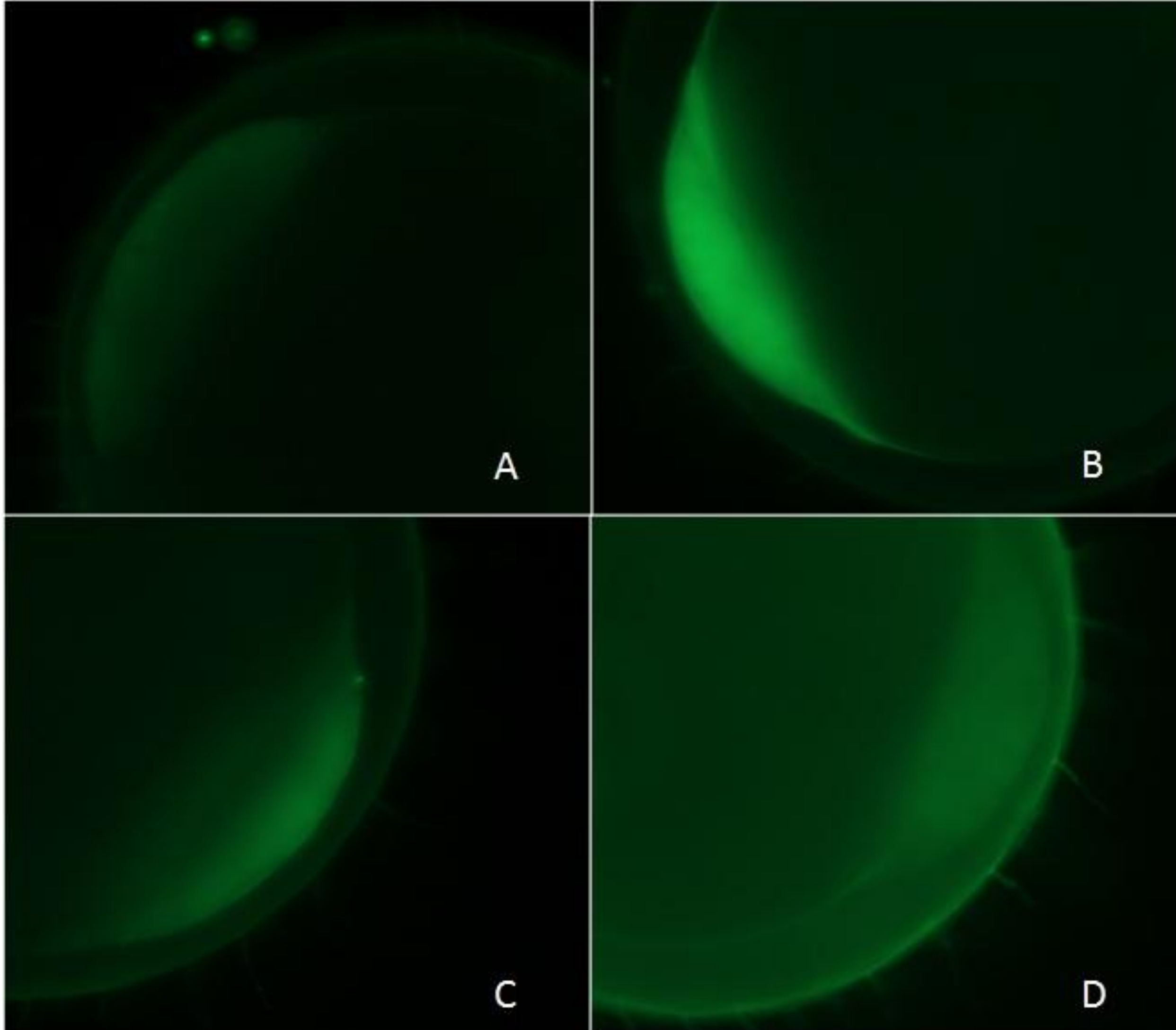


Figure 5.

6 hpf medaka embryos dosed with 1uM of the fluorescent dye calcein and a. DMSO control b. 10 µM MK 571 c. 1200 µg/l BAC d. 100% effluent.

Chemosensitzing potential of MK 571 and benzyalkonium (BAC)

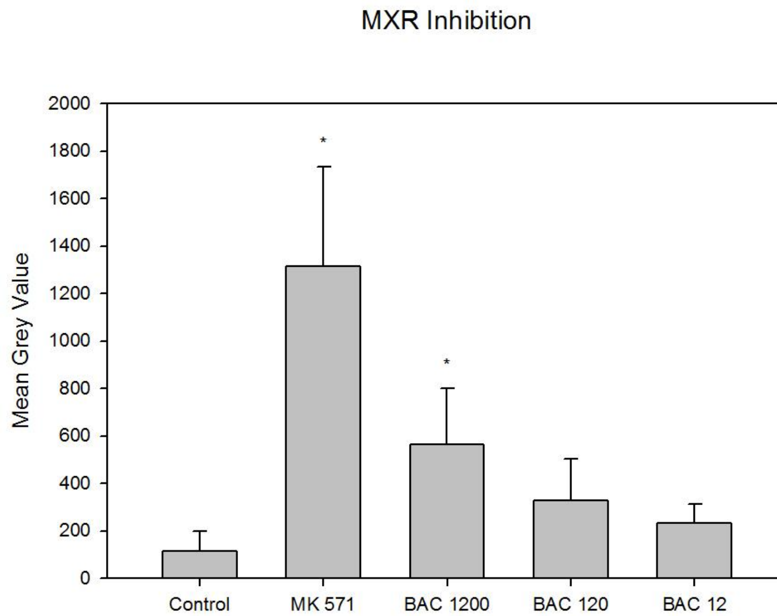


Figure 6.

Fluorescence of 6 hpf embryos (n= 5) exposed to BAC in tenfold dilutions. Mean +95% CI (µg/L). * Significant against control at $P < 0.05$

Chemosensitizing potential of MK 571, 100% effluent and benzo(a)pyrene (BaP)

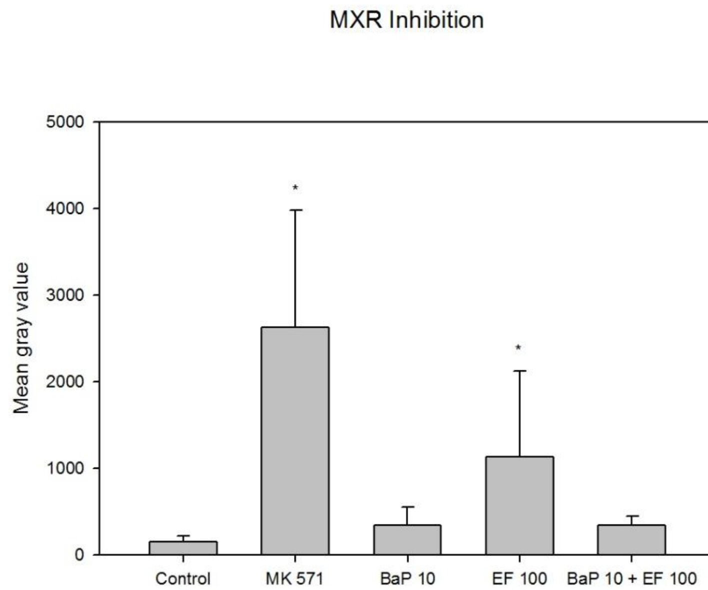


Figure 7.

Fluorescence of 6hpf embryos (n=10) exposed to BaP (µg/L) and 100% effluent mean +95% CI
*Significant against control at $P < 0.05$

exposed to BAC at 12,000 µg/L, as this treatment dose was 100% lethal to medaka embryos, and development was aborted.

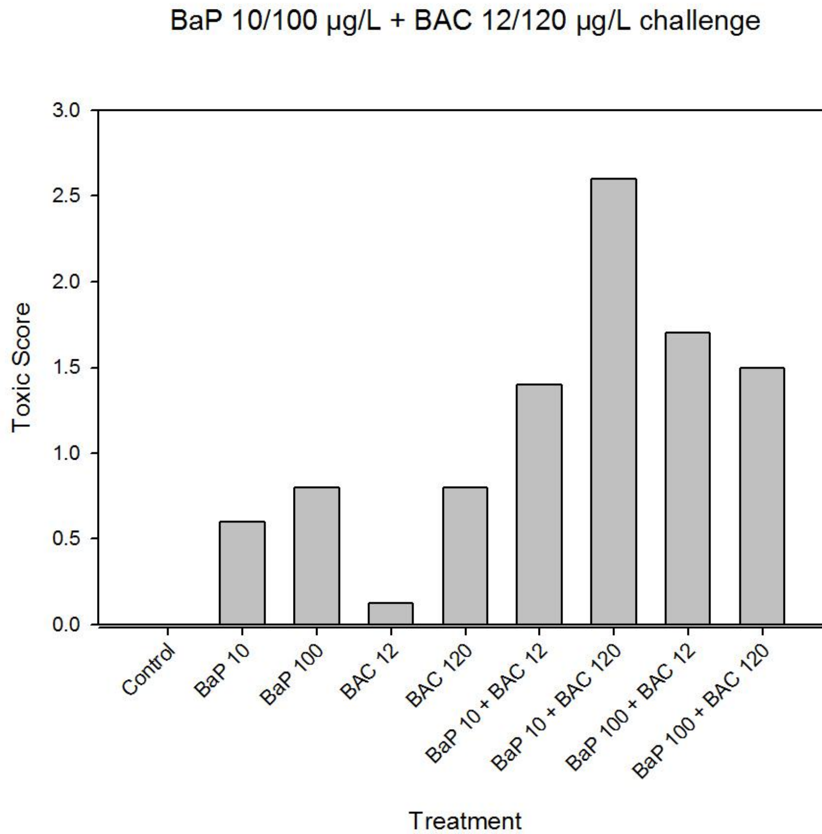
Embryos exposed to sewage effluent had increased accumulation of dye over control embryos, but fluorescence was more general and not concentrated within the blastula (Figure 5d). Embryos also exhibited a highly variable response to the sewage. Because of the high coefficient of variation within the effluent group (1.13), non-parametric statistics were used to calculate significance. In this experiment, both MK571 and effluent significantly inhibited MXR (Kruskal-Wallis ANOVA, ($P < 0.001$) with both effluent and MK 571 being significantly different from the control groups at the $P < 0.05$ level - Figure 7). BaP did not act as an inhibitor of MXR activity, nor did mixtures of BaP and effluent.

3.2 Developmental toxicity

The four endpoints chosen, skeletal deformity, cranial deformity, pericardial edema and cardiac deformity were the most commonly observed deficits in these experiments and could be easily scored (Figure 4). Development deficits were only scored in live individuals. Because scoring was based on discrete values for each endpoint (0, 1, or 2), the data are not continuous, nor are they normally distributed, so calculation of a standard deviation is invalid, therefore only the mean values of the toxic score of the replicate treatments are shown in Figures 8-10. Imbedded in the figures are the results of the binomial and multinomial analysis.

In the first experiment, comparing toxicity of BaP, BAC and mixtures of BaP and BAC, both chemicals increased toxicity in apparently a dose dependant manner. Mixtures of BaP and 12 µg/L BAC yielded mean toxic scores that appear to be greater than additive. Mixtures of higher concentrations did not always yield enhanced effects (e.g. the mean toxic score of BaP

Toxicity- Experiment 1, interactive effects of BaP and BAC

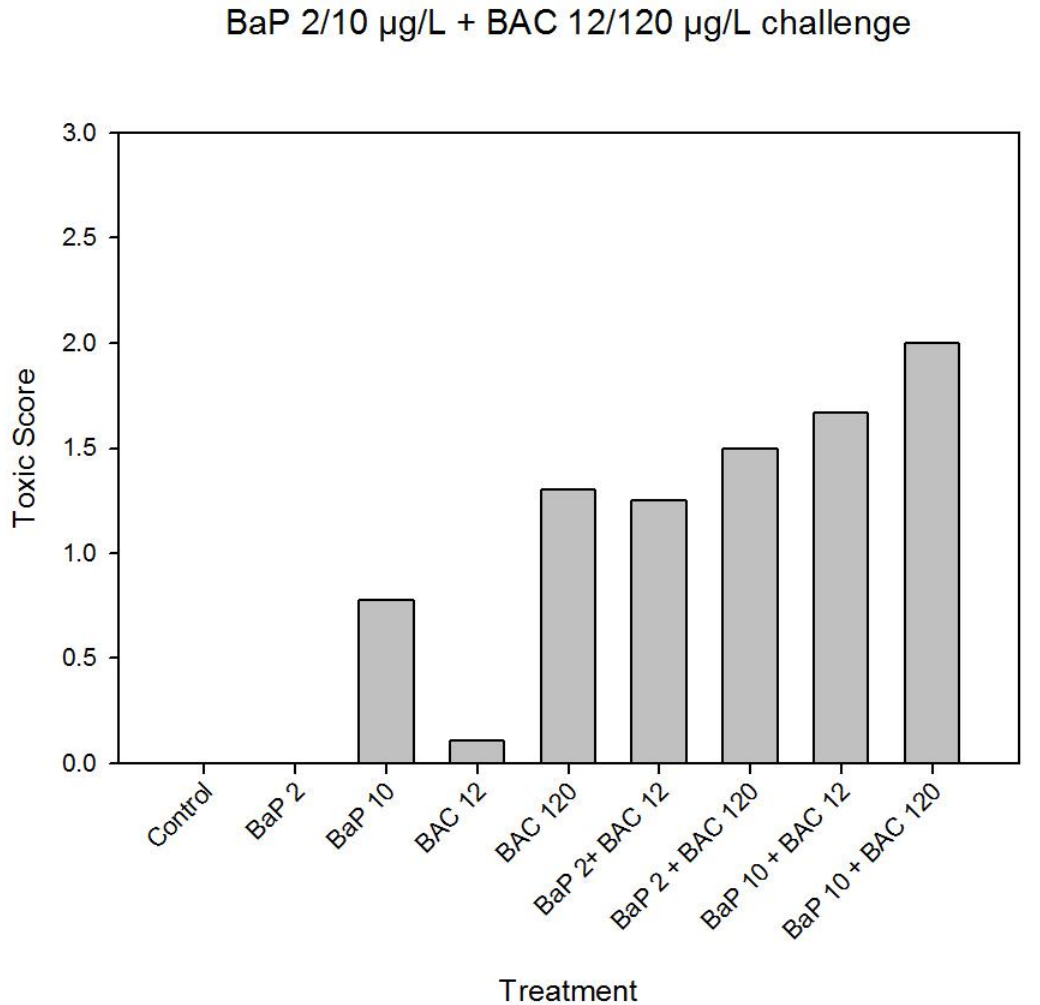


Experiment 1	Binomial Analysis		Multinomial Analysis (categorical)		Multinomial Analysis (ordinal)	
Effect	Chi-Square	p	Chi-Square	p	Chi-Square	p
BaP Factor	*26.6848*	*0.000002*	*31.244*	*0.00012*	*25.86404*	*0.000002*
BAC Factor	*14.0155*	*0.000905*	*21.8911*	*0.00512*	*18.1392*	*0.000115*
BaP Factor x BAC Factor	*11.0177*	*0.004057*	*14.4606*	*0.00596*	*6.87894*	0.142426

Figure 8.

Mean toxic score of each treatment, BaP and BAC values in µg/L. Statistical analysis for each factor listed, significance at $p < 0.05$ indicated by italic text

Toxicity – Experiment 2, interactive effects of BaP and BAC

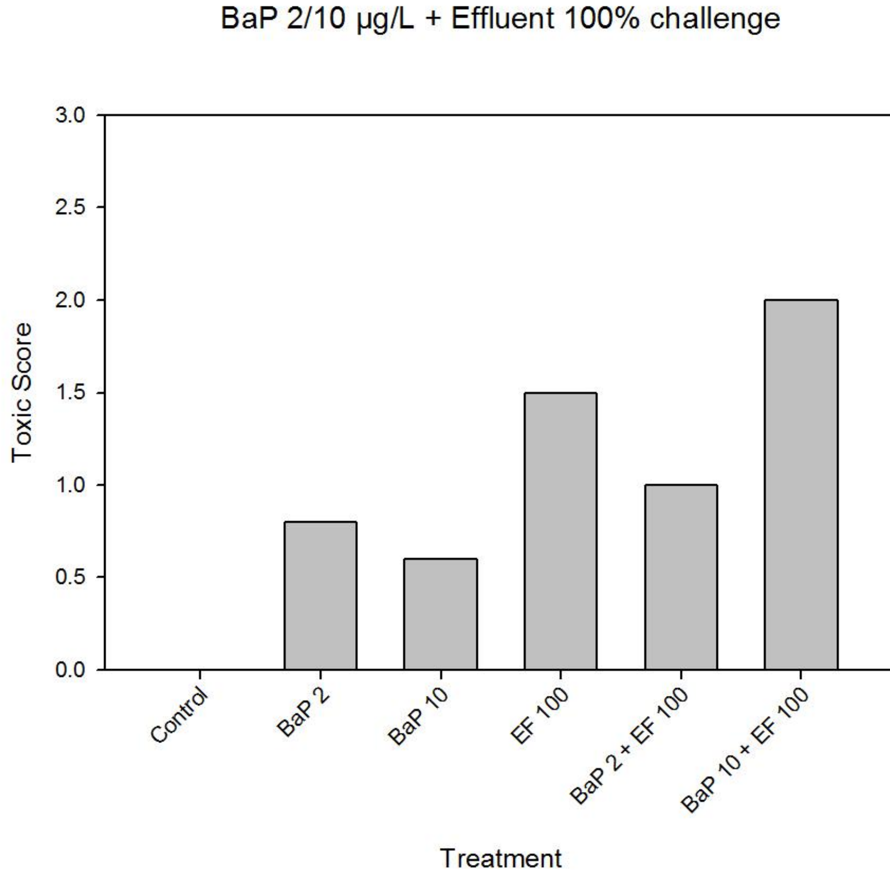


Experiment 2	Binomial Analysis		Multinomial Analysis (categorical)		Multinomial Analysis (ordinal)	
Effect	Chi-Square	p	Chi-Square	p	Chi-Square	p
BaP Factor	*18.8987*	*0.000079*	*31.43854*	*0.000117*	*18.5563*	*0.00009*
BAC Factor	*22.47197*	*0.000013*	*27.13082*	*0.000671*	*24.4476*	*0.00005*
BaP Factor x BAC Factor	6.12919	0.189706	*17.6686*	*0.023853*	7.6273	0.05437

Figure 9.

Mean toxic score of each treatment, BaP and BAC values in µg/L. Statistical analysis for each factor listed, significance at $p < 0.05$ indicated by italic text

Toxicity – Experiment 3, interactive effects of BaP and 100% effluent



Experiment 3	**Binomial Analysis**		**Multinomial Analysis (categorical)**		**Multinomial Analysis (ordinal)**	
Effect	Chi-Square	p	Chi-Square	p	Chi-Square	p
BaP Factor	3.80975	0.148842	*17.99186*	*0.021288*	3.336	0.188624
Effluent Factor	*18.34159*	*0.000018*	*19.76982*	*0.000554*	*15.93281*	*0.000066*
BaP Factor x Effluent Factor	3.54799	0.169654	5.51246	0.238636	*6.88187*	*0.032035*

Figure 10.

Mean toxic score of each treatment, BaP values in µg/L, effluent is 100%. Statistical analysis for each factor listed, significance at $p < 0.05$ indicated by italic text

100 + BAC 12 mixture was similar to the mean toxic score of the BaP 100 + BAC 120 mixture). Using the binomial model both BaP ($p=2x10^{-6}$) and BAC ($p=9x10^{-4}$) treatments were highly significant and the interaction between treatments was also significant ($p=4x10^{-3}$) indicating a more than additive effect. When a multinomial analysis was applied in a categorical format, similar results was obtained, but when the multinomial analysis was performed in an ordinal format, only the treatment effects remained significant, indicating the interactive effect was only additive when severity was considered in a linear analysis.

Based on the BaP toxicity observed in the first experiment, a lower dose of BaP was evaluated (2 and 10 μg/L instead of 10 and 100 μg/L) in the second experiment. Exposure to 2 μg/L BaP yielded no toxicity, yet the mixture of 2 μg/L BaP and 12 μg/L BAC yielded a mean toxic score 11x times larger than that observed in embryos exposed to 12ug/L BAC alone (Figure 9). Similar to the first experiment, all mixture treatments had a higher mean toxic score than the treatments for individual chemicals. Using binomial analysis, both BaP ($p=7.9x10^{-5}$) and BAC ($p=1.3x10^{-5}$) were significant, while the interactive effect was not. Using multivariable analysis with a categorical distribution, the interactive effect was however significant (p=0.02) and nearly significant under ordinal distribution (p=0.054). This indicates that the interaction of BaP and BAC at these concentrations did not significantly increase the frequency of toxic deficits but rather increased the severity, as can also be seen in the distribution of toxic scores in Figure 11b.

The third experiment examined the effect of sewage effluent on developmental toxicity with and without added BaP. There was an extremely variable response to effluent at both concentrations tested. Diluted effluent (25%) proved to be extremely toxic in which 40% of the embryos exposed to diluted effluent alone completely disintegrated so that only eye fragments

Distribution of toxic scores in each toxicity experiment

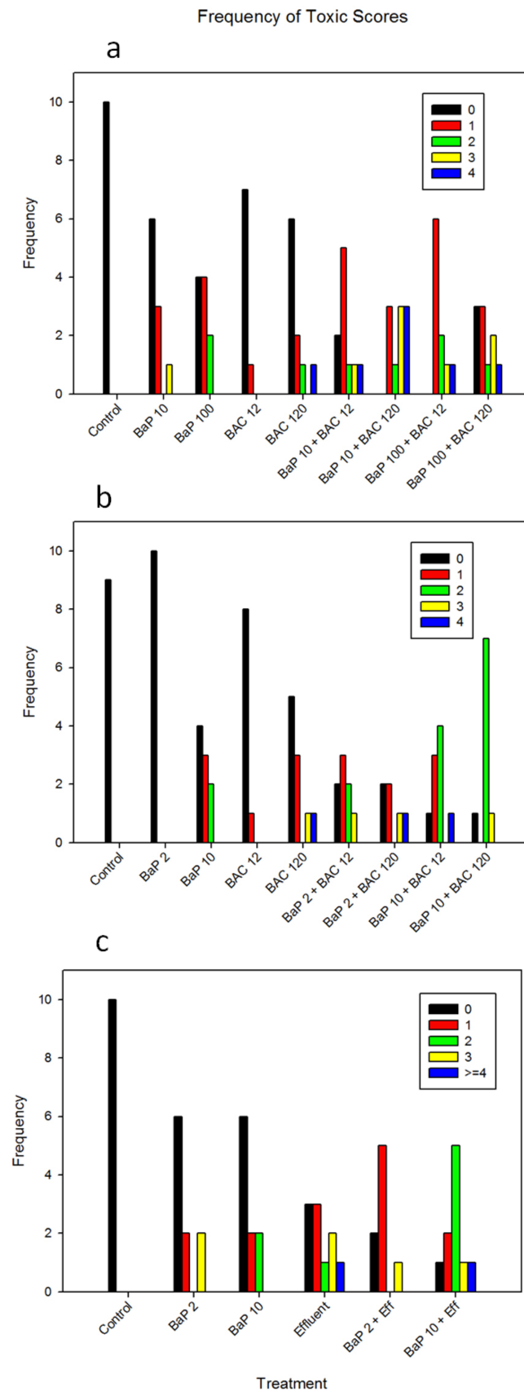


Figure 11.

Frequency of toxic scores for each experiment, a. experiment 1 b. experiment 2 c. experiment 3. Dosing in μg/L

were visible and total dissolution was higher (up to 80%) in the 25% effluent treatments spiked with BaP (data not shown). Due to the large number of dead or disintegrated embryos, results from the 25% effluent treatments were not included in the analysis of developmental toxicity. 100% effluent was toxic though not lethal with treatment effects being significant ($p=1.8x10^{-5}$) based on a binomial analysis (Figure 10). In comparison to the second experiment, 2 µg/L appeared to be more and 10 µg/L was less toxic when applied alone. Because of this, BaP was a significant factor only using multinomial analysis with a categorical distribution (p=0.02). Only the BaP 10 + EF 100 treatment group had a mean toxic score greater than the combined scores of the treatments alone. The interactive effect was only significant using ordinal multinomial analysis (p=0.03) indicating a greater than additive when dose is considered.

Figure 12 shows data for the individual deficits scored. In this figure the data from all three experiments were combined and for treatment groups which were repeated the data was pooled. The most commonly observed defect among BaP exposed embryos was pericardial edema followed by cardiac deformities. BAC was not toxic at the lowest concentration tested, 12 µg/L but at the higher concentration, 120 µg/L, was a moderate inducer of cardiac defects and edema. Mixtures of BaP and BAC produced a more than additive interactive effect; this can be more clearly seen in Figure 13 where the proportion of deficits found in each treatment are depicted in stacked bar plots. This is most notable in the increased frequency of cranial deformities and cardiac defects. The most toxic group tested was the BaP 10 µg/L + BAC 120 µg/L group, which had the highest toxic score. This group also had the highest percentage of cardiac defects and cranial deformities, and was the highest of the non-effluent groups for pericardial edema.

Frequency of toxic deficits observed

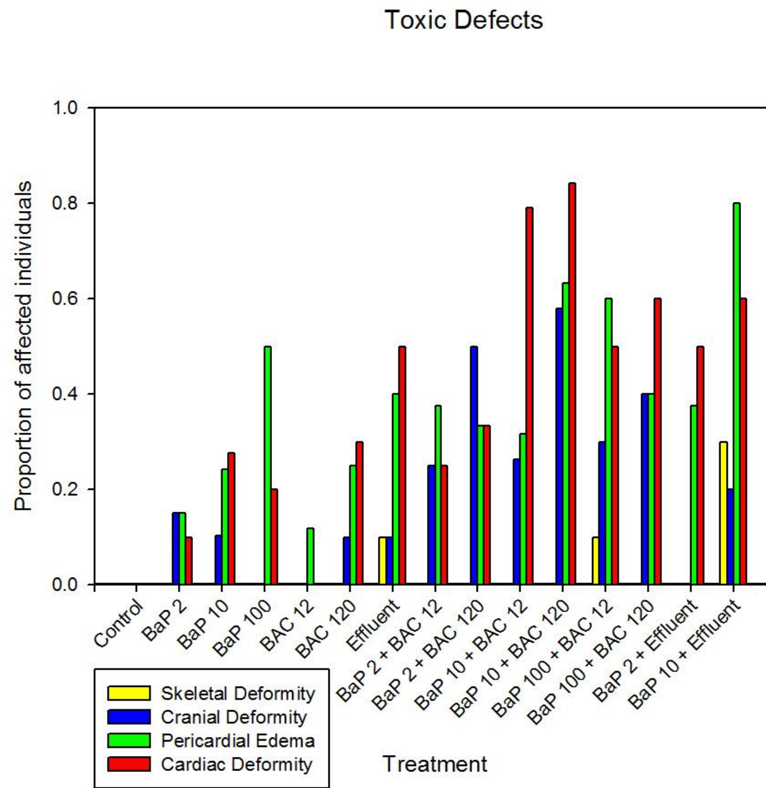


Figure 12.

The frequency of each deficit measured for each treatment. Dosing in µg/L. For treatment groups which were repeated between toxicity experiments, data were pooled

Stacked Frequency of toxic deficits observed

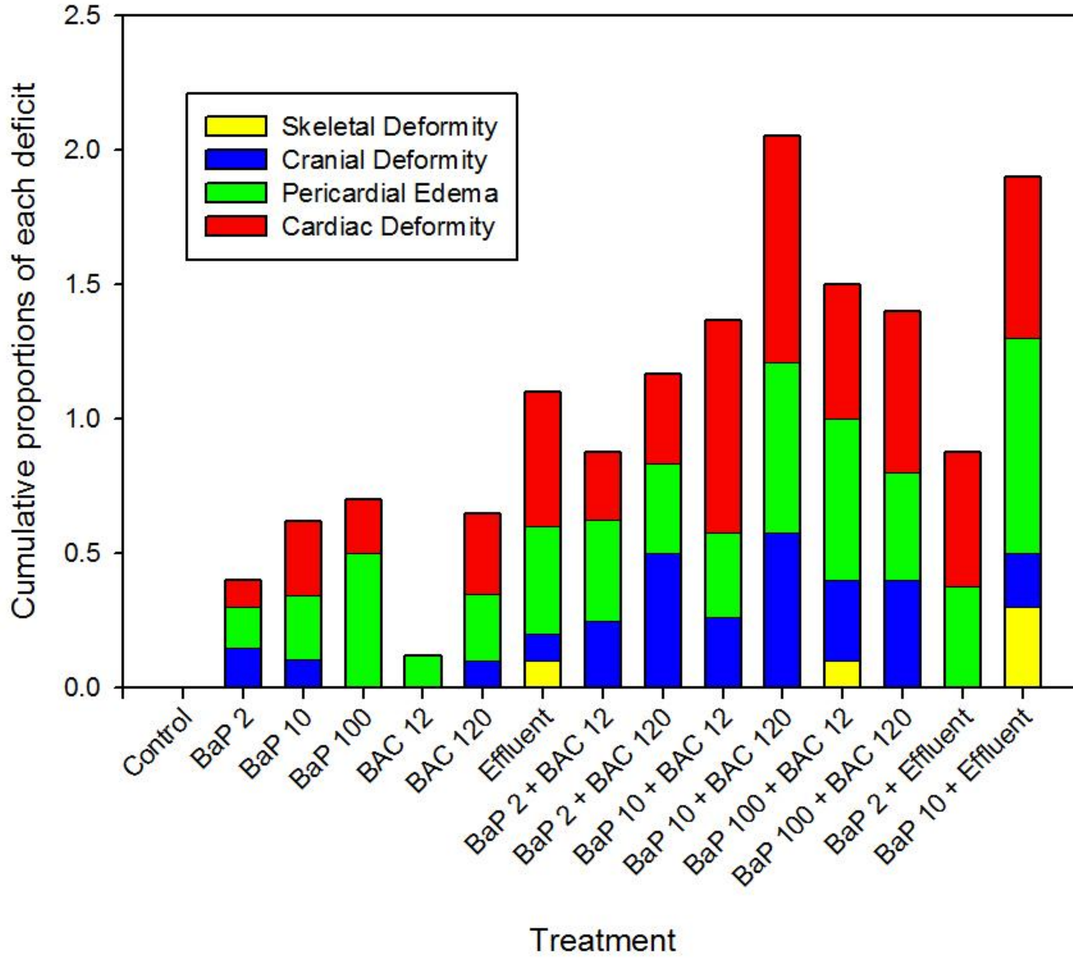


Figure 13.

Stacked data from Figure 12 showing the proportion of individuals from each treatment afflicted with each deficit, dosing in μg/L.

Skeletal deformities were only observed in one embryo that was not exposed to effluent, while skeletal deformities were present in all effluent groups tested but BaP 2μg/L + Effluent. Effluent spiked with BaP 10 μg/L had a 30% incidence of skeletal deformities while effluent alone was only 10%. Of the intact embryos exposed to diluted effluent, skeletal deformities were observed in as much 50% of the treatment group (data not shown).

3.3 14C BaP bioaccumulation

BAC did not increase the uptake of radioactive BaP. Using a Tukey ANOVA test, the overall difference between treatment groups was significant (P=0.019) but using post hoc mean tests, the accumulation of ^{14}C BaP in BAC dosed treatments was not significantly different from the BaP control at any concentration tested (Fig 14).

4. Discussion

The MXR assay adapted for medaka was able to demonstrate the chemosensitizing potential of both a known inhibitor (MK 571) and the emerging contaminant, BAC. The use of calcein-AM ensures that the activity of cellular membrane transporters is being tested for their ability to expel substrate dyes while minimizing general staining. Using only 6 hpf embryos also ensures the reagents and test solutions were only being transported through cellular efflux transporters since other mechanisms of active resistance have not yet arisen during development, such as transformation through hepatic cytochrome P450. In medaka, the liver is first visible at 2 dpf (Iwamatsu, 1994). Although there is the possibility of limited metabolic ability prior to liver formation, certainly without an associated biliary system, removal of toxic metabolites would remain a problem. Inhibition was achieved despite the short incubation time and during imaging, embryo fluorescence remained constant indicating embryos and MXR transporters are both very

Bioaccumulation of ^{14}C BaP in embryos exposed to BAC

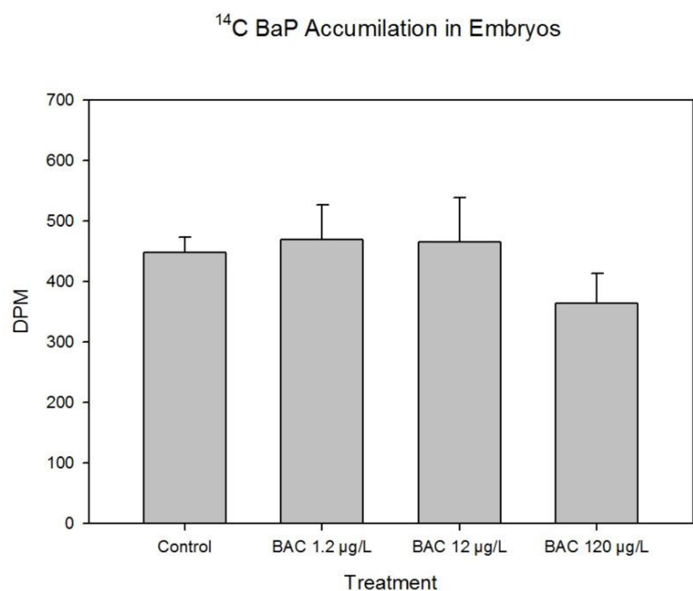


Figure 14.

Accumulation of radiolabeled BaP. All treatments were dosed in a 2 µg/L BaP solution. Those dosed with BAC had no significant change in embryo BaP accumulation. Mean +95% CI

sensitive to inhibition and normal function does not quickly return even in the absence of any inhibitors. There was however a large difference in MK 571 induced fluorescence between experiments, likely due to the age and storage of the reagents. Due to the cost of MK 571 stock solutions were frozen in multiple individual ampules for single use. However freshly made up and never frozen MK571 was most effective as an inhibitor.

While there have been preliminary reports on using rhodamine to test MXR in zebrafish embryos (Epel et al., 2008; Scholz et al., 2008), to our knowledge this is the first time this calcein AM assay was used for medaka and only the second time for a teleost embryo (Langsner, 2010). Medaka embryos have the advantage of not developing as quickly as zebrafish embryos, thereby providing more time to carry out experiments, but still evaluate effects at early developmental stages.

Our data indicates BAC can cause reversal of MXR while BaP did not appear to affect MXR activity. This resulting difference in response is consistent with previous data showing that extremely hydrophobic compounds such as BaP are not good substrates for MXR transporters (Toomey and Epel, 1993). The dye assay used however, cannot discriminate between mechanisms by which BAC may be inhibiting MXR. BAC could act directly on the MXR transporters or the increase in fluorescent dye could be through passive membrane alteration, or through disputing the supply of ATP needed for transporter function.

The most interesting property of BAC is its chemosensitizing potential as demonstrated by the clear increase of toxicity when embryos were exposed to a relevant co-stressor. While this study was only able to investigate binary mixtures of contaminants, it is more representative of the mixtures an organism would be exposed to in a contaminated environment than single

compound assessments. The findings of this study also supports the results of previous research showing the complexity of the exposure enhances the toxicity of surfactants to aquatic organisms (Lewis, 1992). Recent studies have also implicated another common surfactant, linear alkylbenzene sulfonate (LAS), as a chemosensitizer (Tan et al., 2010). LAS was shown to increase dietary accumulation of BaP in catfish. However these studies are not directly comparable. It is possible that LAS stimulates other processes during dietary absorption such as micelle formation that might enhance absorption of lipophilic contaminants such as BaP. BACs and other surfactants should be more closely scrutinized for their inhibitory potential and should be included among the growing list of known chemosensitizers (Smital et al., 2004).

BaP induced toxicity in this study was in agreement with other studies for fish embryo toxicity using PAHs and polychlorinated biphenyls (PCBs) at similar concentrations (Table 1). In addition, the types of defects commonly measured, cranial-facial deformity, pericardial edema and cardiac deformity, are classical endpoints for what's called "blue sac disease," first described in trout fry exposed to tetrachlorodibenzo-p-dioxin (TCDD) (Spitsbergen et al., 1991). Observation of these particular deficits in fish embryo and fry are typical of exposure to halogenated organic contaminants like dioxins and PCBs and also for PAHs (Carls et al., 2008, Hornug et al., 1999, Wassenberg et al., 2004). It is important to remember that the endpoints observed are not always independent of each other, such as the occurrence of pericardial edema and tube heart. So the frequency of each endpoint measured cannot be used as an independent measure of toxicity. Results from this study are also in agreement with Incardona et al. 2004 where it was found cardiac related deficits were the most sensitive measure of PAH induced toxicity and preceded all other morphological endpoints in zebrafish. In the BaP groups tested in this study, there was an increase in pericardial edema as concentration increased but not a

Morphological defects observed in fish fry exposed to organic contaminants

Chemical	Effect	Class	Dose	Species
β-naphthoflavone	Heart deformities, pericardial edema, tail shortening, hemorrhaging	PAH	10-100 µg/L	*Fundulus heteroclitus* (Wassenberg et al., 2005)
Benz[b]anthracene	Cranial-facial, pericardial edema, malformed heart chambers and poor circulation	PAH	456 µg/L	*Danio rerio* (Incardona et al., 2006)
Pyrene			1 mg/L	
Retene	Hemorrhage, yolk and pericardial edema, cranial and skeletal deformity	PAH	9-31 µg/L	*Oncorhynchus mykiss* (Brinkworth et al. 2003)
PCB 77	Tube heart, lesions, blood pooling (EC_{50})	PCB	1.48 µg/L	*Oryzias latipes* (Kim and Cooper, 1999)
PCB 126		PCB	0.43 µg/L	
TCDD		Dioxin	10.1 ng/L	

Table 1. Toxicity to fish fry from exposure to organic contaminants

dramatic increase in overall toxicity between groups. This may be due to the fact that these concentrations were well beyond solubility concentrations and only the dissolved fraction is bioavailable to the embryos.

The mechanism by which BAC acts as chemosensitizer is still unknown. The MXR assay used in this study only demonstrates a functional response to exposure to BAC. The assay does not discriminate whether the increase in fluorescence is due to BAC acting as a competitive MXR substrate, attaching to cell membrane and changing permeability or through some other unknown mechanism such as interference with ATP production within the cell. BAC induced similar cardiac related defects as BaP, but similar deficits do not necessarily mean BAC is working through the same mechanisms. Besides changing the fluidity of membranes, BAC toxicity may also be through interaction with intracellular components. BAC has been shown to decrease the pH in mammalian cells and exposure concentration of BAC was found to be inversely proportional to the ratio of ATP/ADP, a measure of metabolic integrity of cells (Grant et al., 1996). A similar result was seen in bacteria, where exposures to a *bis*quaternary ammonium salt completely inhibited ATP synthesis (Mlynarcik et al., 1981). It is possible that BAC is acting on mitochondria, which could starve MXR efflux pumps of the energy needed to expel toxic compounds, it could also deprive embryos of the energy needed to repair cellular damage. Or by causing a change in pH, BAC may affect enzyme activity, limiting energy production. These speculations require further research to see how ATP levels change in medaka embryos exposed to BAC and whether there is a relationship to toxicity.

This study represents to our knowledge the first to assess BAC toxicity in an early life stage aquatic organism. BACs were shown to be acutely toxic to medaka embryos and fry at concentrations as low as 120ug/L. The EPA reports BAC to be acutely toxic to fish at 280 µg/L

and chronically toxic at 32.2 µg/L (Environmental Protection Agency, 2006). In addition, BACs are reported to be highly toxic to marine invertebrates and non-target protists and bacteria (Environmental Protection Agency, 2006; Kummerer et al., 1997; Nalecz-Jawecki et al., 2003), so the sub-lethal environmental impact of BAC and other surfactants should not be overlooked (Lewis, 1991).

BACs are commonly used as antifouling agents, antialgal agents and as disinfectants and antibiotics in aquaculture (His et al., 1996, Hoskins et al., 1984, Perez et al., 2009). These all represent direct routes into the environment and more direct pathways to induce sub-lethal effects on aquatic organisms. In a study assessing the growth of nine species of marine diatom, it was found that exposure to 10^{-3} % concentrations of BAC prevented nearly all growth within each species measured (Beveridge et al., 1998). Diatoms in this high dose treatment also had little to no pigment, suggesting BAC may interfere with cell metabolism or otherwise cause cell damage. The effect BAC has on reducing chlorophyll-α concentrations has also been reported for two monoclonal cultures along with a natural assemblage of marine phytoplankton (Perez et al., 2009).

The toxic values for BAC measured in this study also fall within the range of environmental concentrations. The sources of most interest for environmental exposure would be through sediments, wastewater effluent and combined sewer overflows. Because of BACs' positive charge, they strongly sorb to sediments and sludges with water concentrations being low (Martinez-Carballo et al., 2007). For urban estuary sediments, median total QAC concentrations were 26 µg/g and median concentration of BACs been measured at 1.5 µg/g and at concentrations as high as 8.9 µg/g for BAC 14 (Li and Brownawell, 2010). STP influent concentrations for individual BAC homologues has been measured as high as 170 µg/L but

because BACs are nearly all removed through treatment (<99% at some STPs), sewage effluent concentration are much reduced (630 ng/L) (Clara et al., 2007). Despite sewage treatment plants being one of the most common point sources for contaminants to the aquatic environment, sewage effluent toxicity has been poorly characterized Investigation of CSO effluent toxicity should also be assessed, as Li and Brownawell (2010), have shown concentrations of more water soluble, shorter chain length BACs dramatically increase in CSO impacted sediment compared to the surrounding areas.

Bergen Point sewage treatment plant which was sampled for this study operates both primary and secondary wastewater treatment which includes the use of activated sludge. Because of this, it is expected that most of the QACs are be removed before effluent is discharged into the environment (Clara et al., 2007). The sludge at Bergen Point has been analyzed for QACs, where total QAC concentrations have been measured as high as 1.7 mg/g and BAC as high as 0.36 mg/g (Brownawell, unpub.) Preliminary analysis of the influent (0.948 µg/L) and effluent (0.064 µg/L) sampled for toxicity in this study however indicated very low BAC14 concentration (Brownawell, unpub.). Thus the toxic potential of effluent-related BAC, or QACs in general, may not have been adequately evaluated in this preliminary study.

It is possible that the toxic effects of effluent observed in this study may be due to the myriad other contaminants or mixtures of contaminants found is sewage effluent, such as other organic contaminants such as PAHs and heavy metals. Wastewater represents a major source of anthropogenic metals into the marine environment, including very toxic heavy metals, such as copper (Karvelas et al., 2003). As with other contaminants, developing embryo and fry are very sensitive to heavy metal exposure, and skeletal deformities are one of the more common signs of toxicity (Dave et al., 1991,Weis and Weis 1991). This may account for why in this study,

skeletal deformities were almost exclusively found in effluent dosed treatments. Further characterization of the effluent from this STP is needed to determine the composition and concentrations of metals and other contaminants typically found in its effluent.

Nevertheless, the inhibition of MXR and genotoxicity observed in response to effluent exposure are significant findings. Due to the limited availability of sublethal effects of sewage effluent, further work is warranted. One surprising results of this study was the complete embryo disintegration observed in diluted effluent treatments. Such complete tissue destruction is consistent with a fungal of viral attack of the cell. It is possible that disinfection byproducts or other contaminants in the 100% effluent minimized microbial proliferation, and when the effluent was diluted, microbes were able to flourish and destroy the embryos (Cooper, *pers. comm.*). In addition, incubating embryos on a shaker plate during this experiment may have also increased the sensitivity of embryos to toxicants in the effluent and BaP treatments, although no increase in control toxicity was observed in this experiment. This has been seen in medaka in previous studies where agitation increases PAH sensitivity (Farwell et al., 2006) which may explain why in this experiment, 2 μg/L BaP treated embryos had a higher toxic score than in previous experiments.

5. Conclusions

In order to perform proper ecological risk assessment, the complexity of environmental exposures must be taken into account. This includes the understanding of how organisms defend themselves against environmental insults and what factors may compromise their resistance response. The ability of chemicals to act as chemosensitizers needs to be considered. This study identifies QACs, a HPVC, as having significant chemosensitizing potential, and further

demonstrates that sewage effluent can also act in this way. These effects may lead to significantly increased toxicity due to other contaminants. These compounds were also shown to be potent developmental toxicants in this study, with mixtures demonstrating more than additive toxicity. While the mechanism for enhanced toxicity may not be related to their inhibition of MXR, the developmental toxicity of BAC and the enhanced toxicity of BAC/BaP mixtures and effluent are noteworthy. The development deficits observed in this study would likely sufficiently compromise these individuals resulting in high mortality in the lab if experiments had been carried out longer, or most certainly in the field, where fry need to compete for food and escape predation. The data from this study highlights the need for further investigation of QACs and other surfactants for MXR reversal effects and their ability to enhance the toxicity of well recognized environmental toxicants. Finally the results of this study indicate that further study is needed into the sub-lethal effects of sewage effluent, which in this case was shown to not only be a strong developmental toxicant, but to act as a chemosensitizing agent likely enhancing the toxicity of other co-occurring contaminants.

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Appendix I: MXR assay

Note: The target of this experiment is to image the embryos before the mid-blastula transition (MBT). This occurs around 8 hpf in medaka. Because dosing, incubation, washing and especially imaging can take a significant amount of time, it is essential to begin dosing around 4hpf so embryos can begin to be imaged at 6 hpf.

Materials:

- 1.5 mL microcentrifuge tubes
- MK 571: Sigma-Aldrich (MRP inhibitor) + control – product #: **M7571**
- Verapamil: Sigma-Aldrich (P-gp inhibitor) + control– product #: **V 4629**
- Calcein-AM: Invitrogen – cat #: **C3100MP**
- Benzalkonium chloride(BAC14)
- Benzo(a)pyrene (BaP)
- DMSO
- Glass Petri dish
- Plastic Petri dishes
- Flexible forceps
- Disposable glass pipettes
- Microcentrifuge tube rack
- Foil
- 2 well depression slide

Stock solutions and preparation:

Embryo rearing medium (ERM):

Medaka egg rearing medium (ERM)

$NaCl$ 1.0g

KCl 0.03g

$CaCl_2 \cdot 2H_2O$ 0.04g

$MgSO_4 \cdot 7H_2O$ 0.163g

=> 1L distilled water

Treatment groups:

Control | Verapamil (10 µM) | MK571 (10 µM) | BAC 1200 | BAC 120 | BAC 12 µg/L | BaP 10 µg/L

Calculations: Calcein-AM: MW: 994.87 g so 1 M = 994.87 g/L (~1000 g/L). Comes in 50 µg vials. Target is a 1 mM stock and 1 µM concentration in our test vials.

- 1 mM = 1 g/L = 1 mg/mL = 1 µg/µL
- Adding 50 µL DMSO to one of the vials is 50 µg/50 µL = 1 mM
- 1 µl of this solution to 1mL ERM would be 1 µL/mL which is equal to 1 µM
- Since we get 50 reactions per vials we would need at least two.
- Make up two vials of calcein and transfer the full volume (100 µL) to a new vial which is easier to pipette from

Verapamil: MW: 491.06 so 1 M = 491.06 g/L Target is 1 mM stock: 491 mg/L or 491 µg/mL

- Measure out for 10 mL DMSO
- 491 µg/mL = 4.91 mg/10mL

- Each 1 μL pipetted will contain 491 ng. Reactions are in 1 mL: 491 ng/mL = 1 μM
- So each 1 μL added to 1 mL is 1 μM

MK 571: In a 1 mM stock

- Same dosing as Verapamil

BAC: our stock is 1.2 mg/mL

BaP: our stock is 1 μg/μL: our target is 10 μg/L so this is too concentrated for our volumes (meaning adding 1 μL of our stock to 999μL ERM would give a 1 mg/L concentration). Need to make a secondary stock. If we add 1 μL to 10 mL ERM: that would be 100 μg/L

Make up fresh ERM and prepare a stock for each reaction (make enough for 13 1 mL reactions)

Control: 12857 μL ERM + 13 μL calcein AM (1 μL x 13) + 130 μL DMSO (10 μL x 13) (12857 + 13 + 130 = 13000 μL = 13 mL)

MK 571 & Verapamil: Same as control but 10 μL of their respective solutions will be added instead of pure DMSO

BAC secondary stock: make enough for 20 mL: so to get a 1200 μg/L (= 1200 ng/mL) concentration in 20 mL that would be 24000 ng/20 mL or 24 μg/20 mL. Each μL of our stock contains 1.2 μg. We would need to add 20uL to 20 mLs ERM to get 24 μg/20mL (1200 μg/L) concentration.

- For the BAC 1200 group, use 13 mL of the above solution and 13 μL calcein-AM
- For BAC 120 use 1.3 mL of the BAC solution + 11.7 mL ERM + 13 μL calcein-AM (10x dilution)

- For BAC 12 use 130 µL of secondary BAC solution + 13 µL calcein-AM + 12857 µL ERM

BaP add 1.3 µL of our secondary BaP stock (1.3 µL contains 130 ng BaP and 130 ng/13 mL = 13 µg/1.3 L = 10 µg/L) + 13 µL calcein 12985 µL ERM

Embryo collection:

Eggs are collected everyday two hours after feeding by removing eggs by hand from each female. Separate eggs from each other by gently rolling eggs over a fine mesh and place in embryo rearing medium (ERM) in a glass Petri dish and store at 25 °C until ready for use. Eggs are checked for viability under a dissecting microscope shortly before dosing begins.

Dosing:

Because both MK 571 and BaP are photosensitive, all dosing should be done under red light. Using the above calculations, pipette out 1 mL of each treatment solution into a 1.5 mL plastic centrifuge tube (n=10). Using the forceps, gently place 1 embryo on the side of each tube without touching the solution. Slightly tip each tube or tap on the side to dislodge the embryo into the solution making sure it sinks to the bottom and not stuck within surface tension on the surface. Cap all tubes and cover tube rack in foil. Place rack on a shaker plate on “low” setting for 60 min.

Washing:

After 60 min, remove tube rack from the shaker plate. Set up 3 empty plastic Petri dishes and fill with clean ERM. Have an empty beaker to use as a waste disposal and another beaker

filled with ERM. Using a glass pipette, remove the egg from vial and place in the first ERM dish. Discard all remaining solution from vial. Using forceps, move the embryo from the first dish to the second and to the third to wash off any external calcein. Add the embryo back to its original vial and refill with clean ERM. Repeat this doing one of from each treatment (ex. Control-A, MK 571-A etc…) before moving on to the next row of vials so no treatment is incubating longer on average than any other.

Imaging:

Under minimal light, embryos are imaged using a Nikon Eclipse TE-2000S inverted microscope equipped with a Spot Insight QE camera (Ex 495 nm/Em 535 nm at 40X magnification).

Place each embryo on a depression slide with a little ERM. Rotate the embryo until a clear profile few of the blastula can be imaged. This is the most essential part because a clear image of the blastula is the raw data for this experiment. Like in the washing stage, repeat imaging for one of each treatment before moving to the next row of replicates.

Analysis:

Images are loaded in ImageJ and using the 'Freehand selection' tool, outline the blastula of the embryo. Under the 'Analyze' bar select 'Set Measurments' and check the box for 'mean gray value.' With the embryo highlighted, hit Ctrl+M for "Measure" and record the mean gray value of the selection into an Excel Worksheet.

Appendix II: Toxicity Assessment

Materials:

- 2 mL glass vials
- Benzalkonium chloride (BAC14)
- Benzo(a)pyrene (BaP)
- DMSO
- MS-222
- Flexible forceps
- Glass pipettes
- Glass Petri dish
- 2 well depression slide

Stock solutions and preparation:

Embryo rearing medium (ERM):

$NaCl$	1.0g
KCl	0.03g
$CaCl_2 \cdot 2H_2O$	0.04g
$MgSO_4 \cdot 7H_2O$	0.163g

=> 1L distilled water

Treatment groups:

Control, BaP (2, 10, 100 µg/L), BAC (12, 120 µg/L), BaP + BAC (2 + 12/120, 10 + 12/20, 100 + 12/120 µg/L)

Embryo collection:

Eggs are collected everyday two hours after feeding by removing eggs by hand from each female. Separate eggs from each other by gently rolling eggs over a fine mesh and place in embryo rearing medium (ERM) in a glass Petri dish and store at 25 °C until ready for use. Eggs are checked for viability under a dissecting microscope shortly before dosing begins.

Dosing:

Embryos are exposed in 2 mL glass vials with 1 embryos per vial, n=10 per treatment, filled with 1 mL of dosing solution. Dosing begins at 0 dpf.

Embryos are placed in an incubator set to 25 °C which should also be equipped with a red lamp set on a 12:12 hr light/dark schedule. It was found in preliminary work that incubating embryos in the dark delayed hatching in control embryos. Medaka hatch occurs between 9-14 days though in some cases much longer. For this experiment, the test was declared over between 10-11days, or when the greater majority (≥80%) of control embryos had hatched.

Toxicity assessment:

Embryos were scored individually for toxic deficits under a dissecting microscope. Scoring was based on a 3 point system for each potential defect where 0=not present, 1=present and 2=severe and a toxic score was given to each embryo as the cumulative score of its toxic deficits. Originally a set of 10 defects were chosen (skeletal deformity, cranial deformity,

pericardial edema, yolk sac edema, cardiac deformity, cardiac arrhythmia, hemorrhage, underdeveloped, delay of hatch, failure to hatch and death), but only the four most commonly observed and easy to score (skeletal deformity, cranial deformity, pericardial edema, and cardiac deformity) are covered below.

Fry are anesthetized with MS-222 before scoring. It is best to first score one or two control fry to give the observer a clear idea what normal development should look like. And on the 2 well depression slide, a control fry should be kept in one of the wells to act as a comparison when scoring other treatment groups.

Skeletal deformity:

Observe the fry both lengthwise and from a top-view to see if there are any bends in the body/tail. The body should be straight to the tip of the tail. The common area for skeletal deformity is towards the end of the tail where it may hook slightly or a slight curve to the overall body.

Cranial deformity:

A normal medaka fry should have a snout that extends well beyond the eyes. Cranial deformities should be scored where the jaw ends shortly after the eyes and/or when there is a smaller distance between the eyes. It should also be scored in cases where the jaw cannot fully open.

Pericardial Edema

This is scored when the area around the heart extends beyond the profile of the body and can be seen to be filled with fluid.

Cardiac deformity

Cardiac deformity covers a broad range of observable defects such as deformed heart chambers, and blood not clearing the chambers. In some of the most severe cases the individual will have tube heart where the heart chambers are elongated and stretched apart from another.

The total toxic score for each individual is the cumulative score of the above four deficits.