# THE CONNECTION BETWEEN HORMONE EXTRUSION AND LACK OF BIODEGRADATION IN WASTEWATER TREATMENT

by

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# STATEMENT OF DISSERTATION APPROVAL

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# ABSTRACT

Endocrine disruption in aquatic animals caused by treated wastewater effluent has been extensively studied. Endocrine disruption describes the alteration of normal endocrine functions due to exposure by endocrine disrupting chemicals (EDCs). Estrogenic activity was frequently detected in wastewater treatment effluent, fresh water, or sedimentation due to incomplete degradation of EDCs in the wastewater treatment facilities. These endocrine disruptors can be degraded by microorganisms but at time scales of a few days to a hundred days. Biological processes only can degrade EDCs to a limited degree as indicated in treatment plant mass balance studies. Microorganisms utilize oxidation enzymes to breakdown EDCs to lower toxic level products during wastewater treatment, while metal and drug resistance proteins interact with the biodegradation process by exporting chemicals out of the cell. This mechanism reduces EDCs' availability and contact time with oxidation enzyme, resulting higher extracellular contaminant concentrations. The objective of this research is to find out if drug resistant proteins contribute EDCs' biopersistence and lack of EDCs biodegradation in wastewater treatment.

The substrates of drug resistant proteins were selected among various estrogenic chemicals including natural and synthetic estrogens, phytoestrogens, plasticizers, flame retardants, and nonionic surfactants. Besides steroids, the synthetic estrogen 17α-

ethynylestradiol, phytoestrogens of nonylphenol, octylphenol, and plasticizer bisphenol-A are substrates of the major multidrug resistant proteins AcrAB-TolC in *E. coli* and MexAB-OprM in *Pseudomonas aeruginosa*. As endocrine disruptors enter the bacterium, they are exported from the cell, limiting their contact time with oxidizing enzymes.

The enzyme oxygenase was employed in this study to remove these endocrine disruptors. The results showed that toluene dioxygenase degrades bisphenol-A efficiently. Both multidrug resistant and oxygenase genes were transformed into *E. coli*, and batch bioreactors were used to determine the biodegradation ability. The presence of a drug resistant protein reduced the degradation rate of bisphenol-A to 0.146 $hours^{-1}$ from the rate of 0.184 $hours^{-1}$ without a resistant protein.

To find out which proteins function at environmentally relevant concentrations found in wastewater treatment plants and surface water, efflux and oxidation gene induction was measured. In *E. coli*, hormone-resistant genes *acrB* and *yhiV* and peroxidase *katE* were expressed the greatest; in *Pseudomonas*, ring cleavage oxygenases and certain drug-resistant genes were up-regulated in the presence of EDCs. To apply this study for water reclamation and wastewater treatment plants, the bacteria population was monitored, and endocrine disrupting chemicals analysis was performed. Biological treatment only removed 75% of E2 (17β-estradiol) equivalent estrogenic activity in the Central Valley Water Reclamation Facility in Salt Lake City, Utah. Meanwhile, drug resistant bacteria were detected in the effluent of this wastewater treatment plant after all treatment processes, including UV disinfection.

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# CHAPTER 1

# INTRODUCTION

Hormones and hormone mimics have the ability to disrupt the endocrine system of aquatic species at levels of ng/L (Purdom et al., 1994). Estrogenic activity has been frequently detected throughout wastewater treatment systems, in fresh water, and in sediments due to the incomplete degradation of endocrine disrupting chemicals (EDCs) during wastewater treatment. These endocrine disruptors can be degraded by microorganisms in the environment on the order from a few days to 100 days depending on the chemical, concentrations, sites, and conditions. Fujii et al. had previously demonstrated (Fujii et al., 2002) that 60% of the human hormone 17β-estradiol (E2) can be biodegraded in 14 days with activated sludge samples from a Tokyo wastewater treatment plant (WWTP). The strain isolated from this sample degraded 40% of estrone in 14 days and removed estriol almost completely within 10 days. The synthetic hormone 17α-ethynylestradiol (EE2) was shown to be degraded by *Pseudomonas, Acinetobacter, and Nitrosospira* (Pauwels et al., 2008; Gaulke et al., 2008). It was reported that bisphenol-A (BPA) and nonyphenol (NP) can be oxidized in rivers, sediments, active sludge and marine sediment samples, and *Pseudomonas sp.* was isolated as degradation bacteria in many studies (Bradley et al., 2009; Ike et al., 2000; Klecka et al., 2001; Yuan et al., 2004).

Ring cleavage on aromatic rings was involved in the degradation pathway of EDCs. As shown in Figure 1.1, the complex proteins of toluene dioxygnease in *Pseudomonas sp.* can add atoms of oxygen to the aromatic nucleus to form cis-dihydroxy and initiate ring cleavage and further degradation (Coombe et al., 1966; Gibson et al., 1970; Jeffrey et al., 1975; Jerina et al., 1971; Kobal et al., 1973; Ziffer et al., 1973). This oxygenase can oxidize the multirings structure of EDCs.

Although most endocrine disruptors are removed during secondary wastewater treatment, biological processes can only degrade EDCs to a limited degree (Fatta-Kassinosa et al., 2011). The removal efficiency of natural estrogens, such as 17β-Estradiol, is 50 to 90% (Fujii et al., 2002) and is mainly due to adsorption of E2 into activated sludge (Pentreath, 1997; Rougledge et al., 1999).

Drug resistance proteins in microbes have the potential to reduce bio-availability and contribute to biopersistence of EDCs. In this research, it was determined that endocrine disruptors, including 17α-ethynylestradiol, bisphenol-A, and nonylphenol, are substrates of major multidrug resistant pumps in *E. coli* and *Pseudomonas aeruginosa*. These two resistant proteins belong to resistance-nodulation-division (RND) protein families, which are present only in gram negative bacteria as they span the inner and outer membranes.

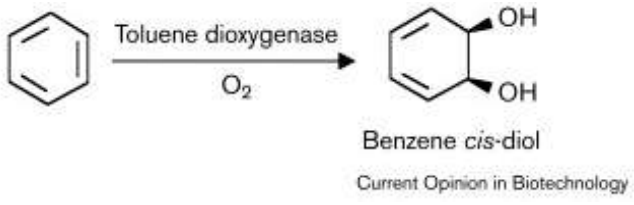



Figure 1.1 Toluene dioxygenase oxidize benzene to benzene cis-dihydroxy. (Gibson & Parales, 2000)

The improper disposal, overprescription, and incomplete degradation of antibiotics during wastewater treatment are causing an increase in drug-resistant bacteria in the environment (Baquero et al., 2008; Bönemann et al., 2006; Hassani et al., 1992; Marth et al., 2003; Reinthaler et al., 2003; Volkmann et al., 2004; Walter and Vennes, 1985). Such bacteria have been frequently detected throughout wastewater treatment facilities and in treated effluent and sludge (Akiyama and Savin, 2010; Batt et al., 2006; Pruden et al., 2006; Schwartz et al., 2003). Surface water bodies that receive treated wastewater effluent are showing elevated levels of drug-resistant genes. In addition, groundwater, river sediment, and land-applied biosolids contain such bacteria (Brooks et al., 2007; Mckeon et al., 1995).

Bacteria express different classes of efflux pumps in response to toxic chemical accumulation (Piddock, 2006). When toxins accumulate within a cell, response mechanisms are activated that result in production of efflux proteins, specifically drug-resistant pumps. These membrane-bound proteins facilitate export of toxic chemicals from the cytoplasm or periplasm of a bacterium. They are classified as ATP driven pumps, proton/sodium ion antiporters, or multi-unit proton antiporters, and they include the ATP-binding cassette (ABC) efflux, the multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR), major facilitator superfamily (MFS), and resistance-nodulation-division (RND) protein families. As drugs are exported from the bacterium, a cation ($H^+/Na^+$) is taken up, or ATP is hydrolyzed (Piddock, 2006).

Multicomponent RND pumps are present only in gram negative bacteria as they span the inner and outer membranes, while other efflux proteins are present in both gram-negative and gram-positive bacteria (Lomovskaya et al., 2007; Nikaido, 1996; Nikaido

and Takatsuka, 2009). RND efflux pumps consist of an inner membrane protein, a membrane fusion protein, and an outer membrane channel, which pumps antibiotics or other compounds from the cytoplasm or periplasm of a bacterium. For example, the RND pump complex AcrAB-TolC in *E. coli* is formed by AcrB (inner membrane protein), AcrA (membrane fusion protein), and TolC (outer membrane channel) (Ma et al., 1993; Tikhonova et al., 2002). During export, an antibiotic (or other) compound enters the protein complex and is pumped out of the bacterium. Similar RND proteins are found in *Pseudomonas*, *Salmonella*, and *Cupriavidus* genera (Grass et al., 2000; Nies, 1995; Pontel et al., 2007; Poole, 2008).

The substrate range of RND proteins varies diversely. RND complexes in *E. coli* (e.g., AcrAB-TolC, AcrEF-TolC) export dyes, detergents, antibiotics, bile salts, human hormones, metals, and solvents (Elkins & Mullis, 2006; Elkins & Nikaido, 2002; Pos, 2009). Elkins and Mullis (2006) had previously demonstrated that human hormones 17β-estradiol, progesterone, and hydrocortisone are substrates of AcrAB-TolC. Substrates of pumps found in *Pseudomonas aeruginosa* (MexAB-OprM for example) are limited to antibiotics, and include the macrolides, fluoroquinolones, polyketides, and β-lactams classes (Chuanchuen et al., 2002; Jeannot et al., 2005; Mao et al., 2002; Poole, 2008). Other *Pseudomonas* strains are resistant to solvents and polynuclear aromatic hydrocarbons (e.g., TtgGHI in *Pseudomonas putida*) (Hearn et al., 2003; Hearn et al., 2006; Kieboom and de Bont., 2001; Terán et al., 2007). Bacteria that export metals include *Salmonella enterica* (GesABC and SilABC), and *Cupriavidus metallidurans*, in addition to *E. coli* (CusCFBA), *Pseudomonas putida* (CzcCBA), and *Pseudomonas aeruginosa* (CzrABC) (Conroy et al., 2010; Grass et al., 2000; Gupta et al., 1999; Hassan

et al., 1999; Leedjärv et al., 2008; Nies, 1995; Pontel et al., 2007). While the substrate range of certain metal-specific RND-proteins is quite specific, multidrug efflux proteins export a broad range of chemicals (Conroy et al., 2010).

Here we determined that endocrine disrupting chemicals (EDCs) are substrates of major multidrug efflux pumps found in gram-negative bacteria. Our focus is on the RND complexes AcrAB-TolC in *E. coli* and MexAB-OprM in *Pseudomonas aeroguinosa*. We used BPA as an example to determine if endocrine disrupting chemicals are substrates of toluene dioxygenase and napthalene dioxygenase genes and whether multidrug resistant proteins influence EDC degradation. At environmental conditions, the drug resistant proteins and oxygenase enzymes respond to the low level EDCs, and the estrogenic activities and drug resistant genes were monitored in local water reclamation and fresh water bodies in Utah.

# CHAPTER 2

# RESEARCH HYPOTHESES AND OBJECTIVES

## Problem Statement

Endocrine disrupting chemicals are emerging contaminants with the ability to mimic hormones and are shown to be biologically active at low levels. Endocrine disruption in aquatic animals has long been attributed to anthropogenic and natural sources of pollution such as release of treated sewage effluent into the environment. These chemicals are expected to be removed during biological treatment process along with other organic compounds. The primary mechanisms at this stage are biodegradation/biotransformation and biosorption. However, trace levels of EDCs can be found in wastewater treatment effluent, fresh water, and sedimentation. It has been found that interacting with the biodegradation and biosorption mechanisms is the occurrence of chemical efflux of EDCs that would contribute to incomplete degradation. The research here explores if the drug resistant protein interferes with the EDC biodegradion process and reduce EDCs' removal rate.

## Research Objective

The overall objective is to determine if the presence of drug-resistant proteins affect the biodegradation of EDCs. First, the substrate range of exporting proteins was

investigated. Secondly, with EDC induction, expressions of functional genes were screened in the selected strains. Third, selected oxygenases were investigated for their ability to degrade endocrine disruptors, and the degradation rates were determined with and without the influence of resistance proteins. Finally, estrogenic activities and the existence of resistant genes in the effluent of local wastewater treatment plants and fresh water were monitored.

The following section defines the hypotheses and approaches that were tested in this research.

**Hypothesis 1:** Hormones and endocrine disrupting chemicals are exported by multi-drug efflux pumps.

- Task 1: The Minimum Inhibitory Concentration (MIC) of various hormones and hormone mimics were tested on *E. coli* strains with drug-resistance proteins, such as AcrAB-TolC and MexAB-OprM. Steroids, including estrone, 17β-estradiol, and estriol, synthetic hormones, alkylphenols, and plasticizers were evaluated for substrate specificity.

**Hypothesis 2:** Environmental concentrations of EDCs induce gene expression of RND pump and oxygenase genes in bacteria.

- Task 2: The gene expression induced by EDCs was measured in strains of Escherichia coli, Pseudomonas aeruginosa, and Pseudomonas putida, which are able to degrade steroid using quantitative polymerase chain reaction (q-PCR).

**Hypothesis 3:** Resistance genes affect hormone oxidization efficiency.

- Task 3: Oxygenase enzymes were tested for their ability to degrade EDCs. Bisphenol-A was selected as a representative chemical.

- Task 4: The interference of drug resistance on hormones degradation was quantified by conducting batch reactors. Estrogenic activities were measured before and after the biodegradation process.

**Hypothesis 4:** Incomplete hormone removal will be traced to the presence of drug resistant genes in wastewater treatment and river water.

- Task 5: EDCs concentration was monitored in local wastewater treatment plants, Utah Lake and Jordan River.
- Task 6: Bacteria possessing drug resistance genes were collected from the local WWTPs, and then isolated, classified, and quantified in the laboratory.

# CHPATER 3

# LITERATURE REVIEW

## Environmental Impact of Endocrine Disrupting Chemicals

Endocrine disruptor chemicals (EDCs) are chemicals that interfere with the endocrine system's function. They are defined, by U.S. Environmental Protection Agency (EPA), "as external compounds that interfere with or mimic natural hormones in the body that are responsible for the maintenance, reproduction, development, and/or behavior of an organism" (Crisp et al., 1997). Endocrine disrupting chemicals comprise human and synthetic hormones, pharmaceuticals, personal care products, surfactants, pesticides, detergent products and various industrial additives (Ashfield et al., 1998; Bolong et al., 2009; Kojima et al., 2005; Meerts et al., 2001). Natural hormones are produced by the endocrine system to regulate metabolism, blood sugar levels, growth, the reproductive system, brain development, and the nervous system. Estrogenic endocrine disrupting chemicals can mimic natural hormones or inhibit the hormone effects to disrupt the endocrine system, which could cause population and reproductive problems for aquatic animals and other wildlife. Concentrations of EDCs found in wastewater, ground water, and drinking water range from several ng/L to hundreds of ng/L (Kashiwada et al., 2002; Kuch et al., 2001; Ying et al., 2002). Human activities also introduce endocrine disruptors in environmental hazards. For example, the oil spill from Deepwater Horizon and

production wells contaminated sea water with dispersants. Some dispersants contain nonylphenol ethoxylates, which are degraded to nonylphenol, a well known endocrine disrupting chemical (Jobling & Sumpter, 1996; Judson et al., 2010).

Researchers have suggested that EDCs may disrupt the endocrine system of humans and other animals in low concentrations (Bolong et al., 2009; Gomes & Lester, 2003). Fish and birds living near wastewater treatment plant effluent have abnormal sexual characteristics, which have been positively correlated with water discharge or EDCs residual in the environment. Male fish located downstream of a wastewater treatment plant effluent produce vitellogenin, an egg yolk protein normally produced by females (Purdom et al., 1994). Harries et al. (1996) conducted an experiment that placed adult male trouts at the varying distances downstream of wastewater treatment effluent for several weeks. The fish located 15 km downstream showed an increase in plasma vitellogenin concentration and with significant increases closer to the treatment plant. In the Columbia River, U.S., which contains high levels of EDCs, juvenile male otters have smaller reproductive organs than normal (Henny et al., 1996). Bird populations that feed on fish exposed to EDCs may produce thin eggshells, have low fertility rates, and display intersex characteristics (Colburn, 1996). There is no evidence showing that endocrine system disease in humans is directly caused by the exposure of environmental contaminants. Some research suggests that EDCs exposure may cause breast and vaginal cancer, male sperm count decrease, testicular and prostate disease (Michael, 2001; Sharpe & Skakkebaek, 1993; Tarone et al., 1997).

In 1996, the EPA was authorized by the Food Quality Protection Act and the Safe Drinking Water Act Amendments to screen chemicals and pesticides for possible

endocrine disrupting effects. The initial chemical list was issued on April 15, 2009, and the Second list of chemicals for Tier 1 Screening was published on November 17, 2010. Starting in the 1990s, the U.S. Geological Survey (USGS) also executed a national investigation of many rivers and lakes in the U.S. to study EDCs' impact on the aquatic ecosystem. In this study, they examined carp and bass at 26 stream sites in 11 river basins to determine if endocrine disruption is widespread in the US. The results showed that estrogen and testosterone in the blood of carp coincide with the concentration of waterborne pesticides and sex steroid hormones. The chemicals appearing in this investigation included organochlorine compounds, phenols, polyaromatic hydrocarbons, phthalates, and dissolved pesticides. Sites with high concentrations of contaminants included the Connecticut River, CT; Housatonic River, MA; Hudson River Basin, NY; and the Potomac River Basin, WV and DC (USGS FS-081-98, 1998).

Most wastewater treatment plants are not designed to treat the high portion of emerging compounds and their metabolites (Petrovic et al., 2003). Hence, they are discharged to the environment (Aerni et al., 2004). Studies show that hormones could be oxidized in a typical bioreactor, but cannot be completely mineralized (Li, 2005). Snyder et al. documented how advanced treatment technologies can remove EDCs from water, suggesting that adsorption and revise osmosis (RO) membrane could remove some EDCs effectively. Ozone and chlorine dioxide are able to breakdown endocrine disruptors (Snyder et al., 2003). However, ozone treated effluent is toxic in many of *in vivo* tests, and post-treatment is needed to reduce the toxicity (Stalter et al., 2010). Additionally, Alice et al. employed sand filtration, chlorine dioxide, and granular activated charcoal (GAC) to treat activated sludge associated effluent in a wastewater treatment plant in

Ilkeston, Derbyshire, UK. Chemical analyses with LC-MS/MS, the Yeast Estrogen Screen, and biological analyses of vitellogenin measured from the fish raised in the treated effluent were conducted. Interestingly, compared to chlorine dioxide, sand filtration and GAC greatly removed the intersex induction, but the effluent of sand filtration showed higher estrogenic activity than either GAC or chlorine dioxide effluent in steroid estrogen measurement. It was suggested that both biological and chemical analysis should be employed to assess new treatment technologies (Alice et al., 2012).

Current wastewater treatment facilities employ biodegradation processes to remove the majority of endocrine disrupting chemicals. To understand the reasons of EDCs' incomplete removal and leading residue estrogenic activities in wastewater effluent, comprehensive studies on enzymes and mechanisms involved in the degradation process were conducted in this research.

## Oxygenases

Oxygenases are enzymes responsible for the aerobic degradation of organic compounds in secondary wastewater treatment. Their function is to add hydroxyl groups to the carbon backbone, thus oxidizing organic chemicals (Coombe et al., 1966). Monooxygenases add one hydroxyl group, and the dioxygenase inserts two cis-hydroxyls onto an aromatic ring (Gibson & Parales, 2000). Oxygenases play a key role in degradation of the aromatic compounds in activated sludge process. In this stage, degradation was achieved by oxidizing the aromatic ring, making it more susceptible to cleavage by ring-cleaving dioxygenases (Phale et al., 2007). Many oxygenases were common in different wastewater treatment plants in different location (Jadeja, 2014).

Catechol-2,3-oxygenase from *Pseudomonas putida* could accelerate the phenol degradation process (Fujita et al., 1993).

Research on hormones degradation products have verified the 17 β-estradiol (E2) oxidation pathways, which are summarized and shown in Figure 3.1 (Coombe et al., 1966; Kurisu et al., 2010; Lee & Liu, 2002). Two pathways were proposed in these studies. As shown in Figure 3.1, one pathway is E2 degradation to E1, followed by the hydroxyl addition on ring A or D. The other pathway shows hydroxylation of C-4 on the A-ring to produce V 4-OH-E2, or the B ring to have intermediate product I, followed by metacleavage.

Besides natural hormones, the biodegradation pathways of many endocrine disrupting chemicals were studied. The degradation intermediate, bacteria, and pathways of bisphenol-A have been well documented (Ike et al., 2000; Kang et al., 2006). The strains involved in bisphenol-A degradation include *Psudomonas paucimobilis FJ-4*,

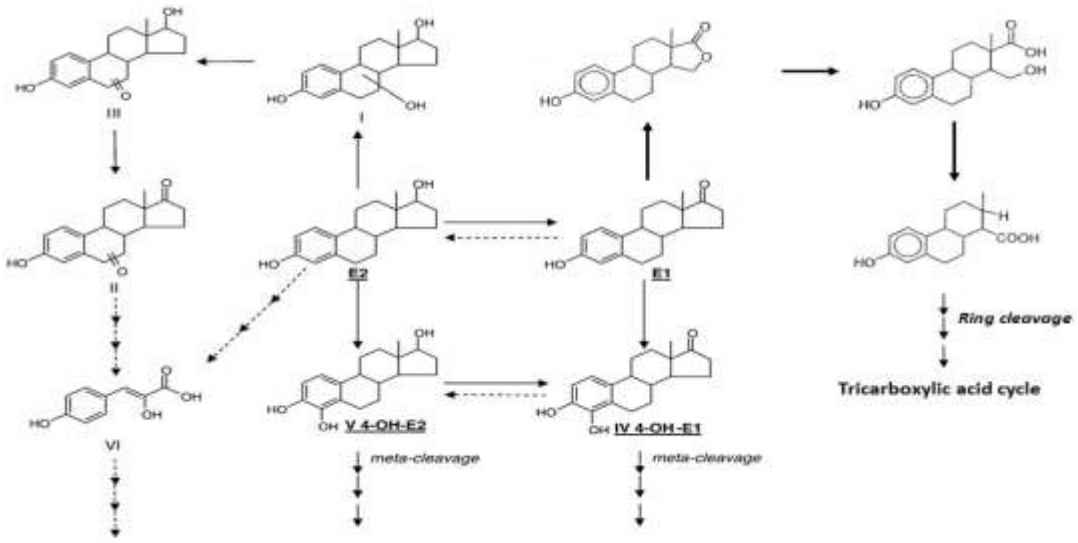



Figure 3.1 17 β-estradiol (E2) biodegradation pathways. Identified compounds with authentic chemicals are shown underlined. Uncertain pathways are shown with the dotted line. (Kurisu et al., 2010; Lee & Liu, 2002)

*Pseudomonas sp., Pseudomonas putida, Streptomyces sp.,* and *Sphingomonas sp. strain AO*. The pathway of nonylphenol degradation is also involved in ring cleavage (Gabriel et al., 2005).

The microbes potentially involved in oxidizing endocrine disrupting chemicals include *Stenotrophomonas maltophilia, Novosphingobium tardaugens sp. nov., Sphingomonas sp., Rhodococcus zopfii, Rhodococcus equi, Achromobacter xylosoxidans, Ralstonia sp., Sphingomonas,* and *Pseudomonas aeruginosa*, which were isolated from activated sludge. The enzymes contributing to this process are unknown yet, but based on oxygenase's mechanism, ammonia monooxygenase, aliphatic monooxygenases, and aromatic monooxygenases or dioxygenases may contribute to the degradation of human hormones and endocrine disrupting chemicals during secondary wastewater treatment.

Most EDCs are degraded incompletely in WWTP, but with longer sludge retention time, more efficient removal could be approached (Kurisu et al., 2010; Li & Li, 2011; Suzuki & Maruyama, 2006). The reason for a lack of hormone biodegradation is unknown, and here it is hypothesized that multidrug efflux proteins play a role in limiting the contact time of EDCs with oxidizing enzymes. Drug efflux pumps may remove antibiotics and other compounds from the cell before degradation by oxygenases can occur.

## Antibiotic Resistance

Elkin and Mullis showed that the major multiresistant antibiotic pumps AcrAB-TolC and AcrAD-TolC in *E. coli* can export human hormones from bacteria after chemical exposure (Elkin & Mullis, 2006). Antibiotic "pumps" are drug efflux proteins

that are responsible for drug resistance mechanisms. Drug efflux pumps are proteinaceous transporters localized in the cytoplasmic membrane of many cells and associate with uptake of protons or sodium ($H^+$/$Na^+$) or ATP hydrolysis. There are five classes of the efflux protein, including ATP-binding cassette (ABC) efflux, the multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR), major facilitator superfamily (MFS), and resistance-nodulation-division (RND) protein families. The resistance pumps studied in this research, AcrAB-TolC from *E. coli* and MexAB-OprM from *Pseudomonas aeruginosa*, are RND transporters that span the inner and outer membranes of gram-negative bacteria. As shown in Figure 3.2(A), this protein has three essential parts: TolC – the outer membrane channel; AcrA – the periplasmic adaptor protein; and AcrB – the pumpsof the RND super family. The absence of any one of these three proteins will make the drug efflux complex entirely nonfunctional (Nikaido & Takatsuka, 2009).

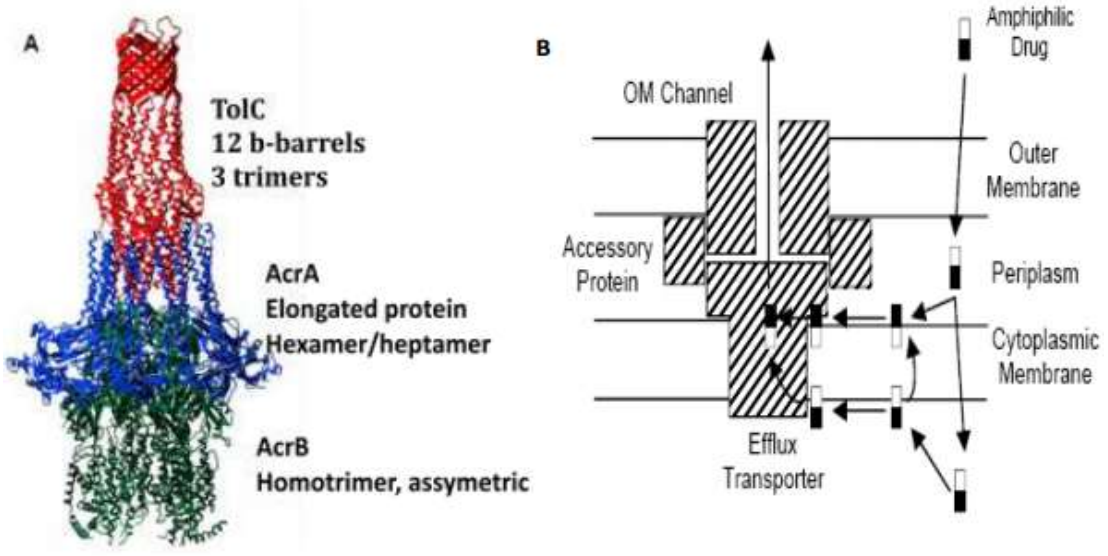



Figure 3.2 The structure of AcrAB-TolC proteins. (A) Crystal structure of TolC, AcrA, and AcrB. (B) Schematic of the tripartite pump complex (Nikaido & Takatsuka, 2009).

Figure 3.2(B) shows the mechanism of the AcrAB-TolC drug resistance pump, with a similar mechanism observed in MexAB-OprM of Pseudomonas. Nikaido and Takatsuka's genetic studies of chimeric transporters showed that much of the substrate specificity is determined by their periplasmic domains (Nikaido & Takatsuka, 2009). The substrates are captured either from the periplasm or cytosol after it enters the cell. Next, two possible pathways may happen: either the substrate is flipped over to the outer surface of the membrane first and then follows the regular periplasmic capture pathways, or it follows a different capture pathway from the cytosol (Nikaido, 1996).

Antibiotic resistant genes could be produced by strains occurring naturally in environments (Waksman & Woodruff, 1940) and has become a major concern in clinic and environment with pharmaceutical production of antibiotics. There are two main mechanisms involved in the development of antibiotic resistance: mutation and horizontal gene transfer (Davies, 1994; Martinez, 2011; Martinez & Baquero 2000; Wellington et al., 2013). The selection factors of drug resistance genes in environments include the protection against endogenous antibiotics and heavy metals, pharmaceutical production consumption of antibiotics, and antibiotic application and consumption in agriculture and animals. The drug resistant genes are transmitted in wind, water run-off, human activities, animals, insects and birds (Allen et al., 2010). Human activities dramatically increase the selection pressure of antibiotic-resistant genes. The same resistant gene present in human pathogens has been found in environments with a history of antibiotic contamination (Pallecchi et al., 2008), and the prohibition of employing some antibiotics in farming reduced antibiotic resistance in animals and humans (Aarestrup et al., 2001). From many studies, the drug resistant bacteria in wastewater treatment plants increased with the

pharmaceuticals present in the environment (Baquero et al., 2008). Wastewater effluent and biosolids contain high levels of drug resistant genes and bacteria (Reinthaler et al., 2003). Szczepanowski detected 140 clinically related antibiotic-resistance genes in wastewater effluent with 192 resistance-gene-specific PCR primer pairs. About 64% of the 192 reference resistance genes were detected in bacteria obtained from wastewater effluent (Szczepanowski et al., 2009). Enterobacterial *ampC* resistance genes encoding β-lactamase was detected from wastewater, surface water and drinking water biofilms in the study conducted in Germany (Schwartz et al., 2003).

# CHAPTER 4

# MATERIALS AND METHODS

Endocrine disrupting chemicals used in this research (Table 4.1 and Figure 4.1) include 17β-estradiol (E2), estrone (E1), estriol (E3), 17α-ethynylestradiol (EE2), bisphenol-A (BPA), octylphenol (OP), and nonylphenol (NP), were obtained from Sigma-Aldrich. Strains and plasmids information were listed in each section.

## Methods to Test Hypothesis 1 (Task 1): Hormones and Endocrine Disrupting Chemicals Are Exported by Multidrug Efflux Pumps

*E. coli* containing deletions of the multidrug efflux genes *acrA* and *acrB* was transformed with plasmids encoding either AcrAB or MexAB-OprM. The minimum

Table 4.1 Physiochemical properties and EDCs used in this research.

| **Category** | **Name** | **Abbreviation** | **EDC Classification** | **MW, g/mol** | **Log $K_{ow}$** |
|---|---|---|---|---|---|
| Hormones: | Estrone | E1 | Estrogen | 270.37 | 3.13 |
| | 17β-estradiol | E2 | Estrogen | 272.38 | 4.01 |
| | Estriol | E3 | Estrogen | 288.38 | 2.45 |
| Synthetic hormones: | 17α-ethynylestradiol | EE2 | Estrogen | 296.40 | 3.67 |
| Alkylphenols: | Nonylphenol | NP | Estrogen | 220.35 | 5.76 |
| | Octylphenol | OP | Estrogen | 206.32 | 5.50 |
| Plasticizers | Bisphenol-A | BPA | Estrogen | 228.29 | 3.32 |

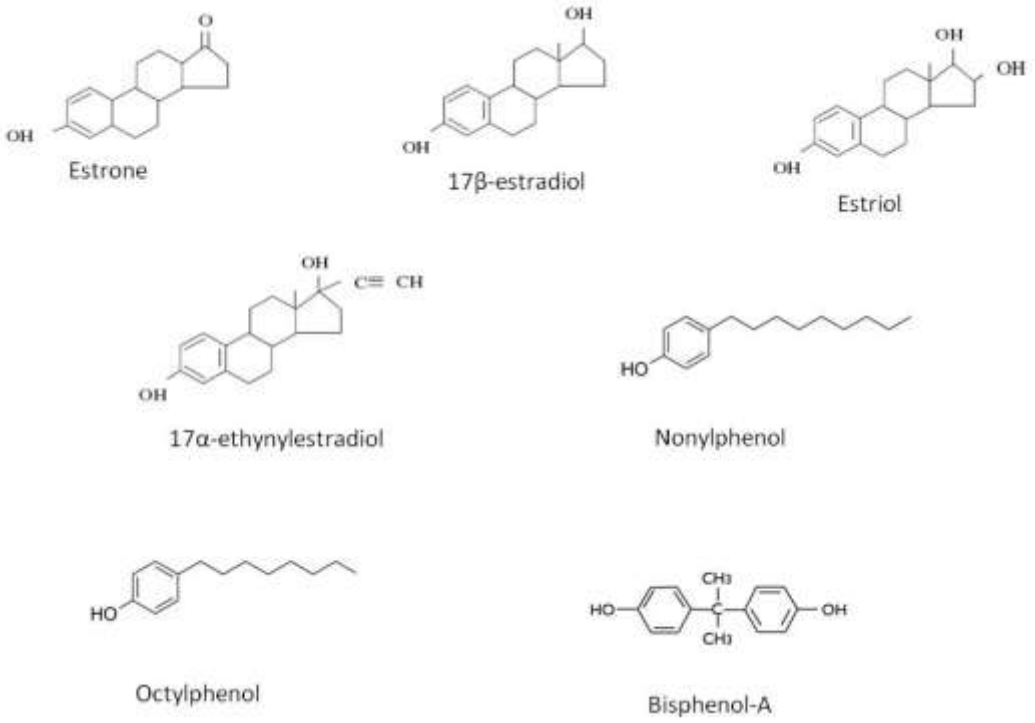



Figure 4.1 The chemical structures of EDCs used in this research.

inhibitory concentration (MIC) of estrogenic endocrine disrupting chemicals was then obtained by growing strains on increasing concentrations of E2, EE2, BPA, NP, OP, E1, and E3. MICs were then used to select for EDC-resistant bacteria in two local wastewater treatment facilities and their presence in treated effluent was quantified as CFU/mL (colony forming units per mL). Finally, DNA from viable colonies was amplified to detect the presence various RND genes, in addition to AcrAB-TolC and MexAB-OprM.

Strains and Plasmids

Genotype details of strains and plasmids may be found in Table 4.2. *E. coli* possessing deletions of major efflux proteins (W4680A-ΔacrAB, ECM2112-ΔacrAB-ΔtolC) were used as host cells. Electrocompetent cells were prepared according to

Table 4.2 Bacteria strains and plasmids used to test for substrate specificity of major multidrug efflux pumps in *E. coli* and *Pseudomonas aeruginosa*. $Amp^R$ = ampicillin resistant; ::*Kan* = kanamycin resistant.

| | Relevant genotype | Source or reference |
|---|---|---|
| Strains: | | |
| W3110 | W3110 | Bachmann, 1972 |
| W4680A | K-12 Δ*acrAB::Kan* | Ma et al., 1995 |
| ECM 2112 | MC4100 Δ*acrAB* Δ*tolC::Kan* | Lomovskaya et al., 2007 |
| Plasmids: | | |
| pAB | pUC19-*acrAB*; $Amp^R$ | Tikhonova & Zgurskaya, 2004 |
| pMABO | pUC18-mexAB-oprM; $Amp^R$ | Tikhonova et al., 2002 |
| pUC18 | Empty vector control, $Amp^R$ | Yanisch-Perron et al., 1985 |
| pUC19 | Empty vector control, $Amp^R$ | Yanisch-Perron et al., 1985 |

standard methods (Sambrook & Russel, 2006) and stored at -80 ℃. Plasmids encoding either acrAB (pAB) or mexAB-OprM (pMABO) were transformed into W4680A and ECM2112 and grown for 24 hours on Luria Bertani (LB) agar containing ampicillin (100 mg/L). Empty vectors pUC18 and pUC19 served as controls.

Minimum Inhibitory Concentration Tests

The minimum inhibitory concentration (MIC) is the concentration at which a chemical is toxic to a bacteria strain, as measured by $OD_{630}$. This test is a simple method used to determine substrates of drug resistant proteins and was carried out on either solid or liquid media. Bacteria expressing either acrAB or mexAB-OprM were exposed to increasing concentrations of EDCs (0-100 μg/mL of each of 17β-estradiol, estrone, estriol, 17α-ethynylestradiol, bisphenol-A, octylphenol, and nonylphenol) containing no more than 0.1% ethanol. The smallest concentration at which no bacteria grew was labeled the MIC (μg/mL).

## Methods to Test Hypothesis 2 (Task 2): Environmental Concentrations of EDCs Induce Gene Expression of RND Pump and Oxygenase Genes in Bacteria

### Strains

Wild-type *E. coli* W3110 and *Pseudomonas aeruginosa* K784 were obtained from Dr. Helen Zgurskaya at the University of Oklahoma and Dr. R. Keith Poole, at Queen's University. *Pseudomonas putida* F1 (ATCC # 700007) was purchased from American Type Culture Collection (ATCC).

### Primer Design and PCR Verification

The genes of interest are listed in Table 4.3. The primers for PCR amplification of cDNA were designed using Primer 3 provided by National Center for Biotechnology Information GenBank database (NCBI) (http://www.ncbi.nlm.nih.gov/). The primer was designed for amplifying about 100–400 base-pairs (bps) fragments with a melting temperature around 60 ℃. Primers were clustered with a genomic DNA sequence of *E. coli* W3110 (GeneBank ID: AP009048.1), *Pseudomonas putida* F1 (GeneBank ID: NC_009512.1), and *Pseudomonas aeruginosa* K784 (GeneBank ID: NC_002516.2) for examining the PCR targets. To ensure there was only one single fragment for each primer, regions larger than 400 bps were acceptable for primer sets (Appendix A). To enable an accurate quantification of the targets, all the primers were amplified with the genomic DNA, and the PCR products were separated on a 1.6% agarose gel to verify the target size. As shown in Figure 4.2, primer concentration and annealing temperature used in the RT-PCR program were optimized.

Table 4.3 Drug resistant genes and oxidation genes in *E. coli*, *Pseudomonas putida F1*, and *Pseudomonas aeruginosa* tested in this research. Resistance proteins were selected from http://www.tcdb.org/. Oxygenase proteins were selected from the complete gene list of each strains in http://www.ncbi.nlm.nih.gov/. (Strain ID: *E. coli* W3110: Accession #AP009048.1; *Pseudomonas putida* F1: Accession #AP009048 NC_009512.1; *Pseudomonas aeruginosa* K784: Accession #AP009048 NC_002516.2).

| Gene name | Function |
|---|---|
| ***E. coli* W3110** (Musso et al., 1977) | |
| **Resistance protein** | |
| cusA | Cu+ / Ag+ |
| acrD | Multidrug (Acrflavin, doxorubicin, ethidium, rhodamine 6G, SDS, deoxycholate) |
| acrB | Multidrug, dye, detergent, bile salt, orgainc solvent, steroid hormones, signaling molecules |
| acrF | Multidrug (aminoglycosides) anionic detergents, steroid hormones, copper, zinc, |
| mdtBC | Heteromeric multidrug, detergent, copper, zinc |
| yhiV (MdtF) | Multidrug, dye, detergent, steroid hormones (estradiol, progesterone) |
| **Oxidation protein** | |
| katE | Hydroperoxidase HPII(III) (Catalase), mine drainage metagenome, Oxidoreductase; Peroxidase . |
| katG | catalase/hydroperoxidase HPI(I), hydrogen peroxide, oxidoreductase |
| **Housekeeping** | |
| mdoG | Cell wall synthesis (housekeeping gene) (Heng et. al ., 2011) |
| ***Pseudomonas putida* F1**(Copeland et al., 2007) | |
| **Resistance protein** | |
| arpB | Multidrug (tetracycline, chloramphenicol, carbenicillin, streptomycin, erthromycin, novobiocin) |
| ttgB | Solvent (toluene, styrene, m-xylene, ethbenzene, propylbenzene) |
| **Oxygenase** | |
| Pput_1893 | Monooxygenase, FAD-binding protein, oxidation-reduction process |
| Pput_2363 | DNA-N1-methyladenine dioxygenase, oxidoreductase activity |
| Pput_2880 | Benzene 1,2-dioxygenase subunit beta, toluene catabolic process, xylene catabolic process |
| Pput_2897 | Aromatic-ring-hydroxylating dioxygenase |
| Pput_3846 | Extradiol ring-cleavage dioxygenase, cellular aromatic compound metabolic process |
| Pput_5066 | 2OG-Fe(II) oxygenase |
| **Housekeeping** | |
| rpoD | RNA polymerase sigma factor, (housekeeping gene) (Liu et. al ., 2011) |
| ***Pseudomonas aeruginosa* K784** (Winsor et al., 2009) | |

Table 4.3 continued

| | |
|---|---|
| **Resistance protein** | |
| czrA | $Cd^{2+}$, $Zn^{2+}$ |
| mexB | Multidrugs, tea tree oil |
| mexD | Multidrug (β-lactams, fluoroquinolones, tetracycline, macrolides, chloramphenicol, biocides, etc.) |
| mexF | Multidrug (fluoroquinolones, chloramphenicol, biocides, xenobiotics and chloramphenicol) |
| mexK | Multidrugs, Triclosan (without OprM) |
| mexY | Multidrugs (aminoglycosides, β-lactams, fluoroquinolones, macrolides, chloramphenicol, tetracycline, erythromycin, ofloxacin, etc.) |
| triC | Triclosan |
| mexI | Multidrug (fluoroquinolones) |
| mexW | Multidrug (fluoroquinolones, microlides, chloramphenicol, tetracycline) |
| mexQ | Multidrug (fluoroquinolones, microlides, chloramphenicol, tetracycline) |
| mexN | Multidrug (chloramphenicol) |
| muxB | Multidrug (aztreonam, macrolides, novobiocin and tetracycline) (2 RND proteins) |
| **Oxygenase** | |
| alKB1 | alkane-1 monooxygenase, lipid metabolic process |
| alkB2 | alkane-1 monooxygenase, lipid metabolic process, monooxygenase activity, Oxidoreductase |
| PA0817 | Probable ring-cleaving dioxygenase |
| PA0880 | Probable ring-cleaving dioxygenase |
| PA1538 | Probable flavin-containing monooxygenase |
| PA2024 | ring-cleaving dioxygenase |
| PA2083 | Probable ring-hydroxylating dioxygenase subunit, aromatic compound catabolic process |
| PA2085 | Probable ring-hydroxylating dioxygenase subunit, cellular aromatic compound metabolic process |
| PA2097 | Probable flavin-binding monooxygenase |
| PA2355 | Probable FMNH2-dependent monooxygenase |
| PA2546 | Probable ring-hydroxylating dioxygenase subunit |
| PA3389 | Probable ring-cleaving dioxygenase |
| pqsH | FAD-dependent monooxygenase, oxidation-reduction proces |
| pqsL | Putative monooxygenase, oxidation-reduction process |
| **Housekeeping** | |
| rpoD | RNA polymerase sigma factor, (housekeeping gene) (Savli et al ., 2003) |

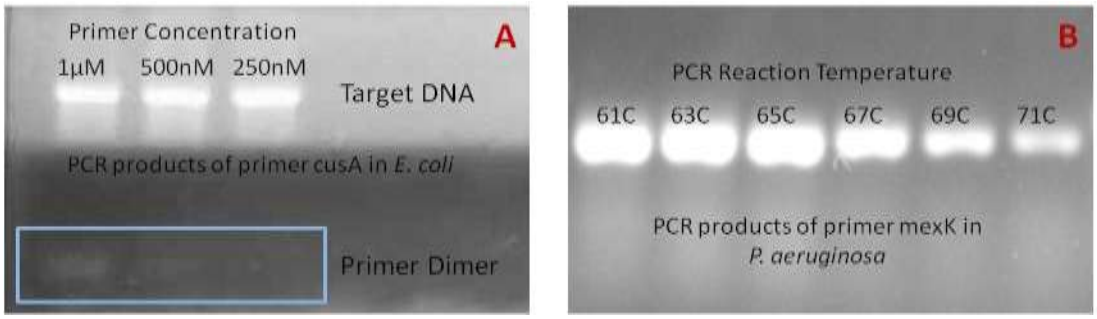



Figure 4.2 Bands of PCR products in primer optimization test. (A) Variation of primer concentration. At primer concentration of 250 nmol/L, primer dimer was disappeared. (B) Variation of PCR reaction temperature. At 71 ℃, the PCR efficiency was greatly reduced with a weak band. RT-PCR reaction was conducted at 70 ℃ annealing temperature with 250 nmol/L of primer concentration.

Biodegradation Reaction

To detect the gene expression levels when EDCs were introduced to bacteria strains, batch bioreactors were used. *E. coli* W3110, *P. aeruginosa* K784, *P. putida* F1 were grown in 5 ml of LB Broth (Sigma) at 37 ℃ for 5 hours until the preculture reached log growth (between 1.0 to 1.5 $A_{630}$). The 5-mL test culture was seeded with 100 μl of preculture and environmentally relevant concentration of estrogenic EDCs. Initial EDC concentrations were 10 ng/L of EE2, 10 μg/L of NP and 1 μg/L of BPA. Bacterial growth was determined by optical density at 600 nm ($OD_{600}$). To establish the number of viable bacteria (as CFU/mL), cultures were serially diluted in sterilized media and 100 μl was spread on LB agar plates. Plates were incubated at 37 ℃ for 24 hours. The plates with colonies between 20 and 200 were counted for cfu/ml calculation.

Total RNA Isolation and Reverse Transcription

To measure gene expression with chemicals induction, RNA was isolated from each bioreactor and synthesized to cDNA for PCR. RNA was extracted with Qiagen

RNeasy Protect Bacteria Mini Kit (Cat. 74524). Total RNA was isolated by transferring a 0.3 ml aliquot of the culture, corresponding to $2 \times 10^8$ cells, to 1 ml of RNAprotect Bacteria Reagent, according to the manufacturer. After removal of genomic DNA by RNase-free DNAase, RNA was precipitated and resuspended in 100 µl of RNAse-free $H_2O$. RNA was dispensed into small aliquots of 20 µl and store at -20 ℃.

Complementary DNA (cDNA) was synthesized according to the instructions of Qiagen QuantiTect Reverse Transcription kit (Cat. 205311). Before synthesis of cDNA, a reaction of mixing 2 µg of RNA and DNA elimination buffer was incubated at 42 ℃ for 2 minutes to remove trace DNA remaining from the RNA extraction steps. Reverse transcription was performed with 1.5 µg of DNA elimination mixture and incubated at 42 ℃ for 30 minutes by using random hexamer primers with reverse transcriptase in 30 µl total volume. Reverse transcriptase was inactivated by incubating at 42 ℃ for 3 minutes. The remaining 0.5 µg RNA from the DNA elimination reaction was used as the non-reverse-transcription control (NRT control). NRT control was the negative control samples with no reverse transcription process employed. To synthesize NRT control samples, RNA was treated same as reverse transcription reaction except RNase-free water was used instead of reverse transcriptase enzyme.

RT-PCR

The primers used for real-time PCR were verified and optimized by PCR and RT-PCR reaction. The reaction was performed on 384-well plates with 20 ng of cDNA, with 250 nM of primer, and 2.5 µl of 2X QuantiFast SYBR Green PCR master mixes (Qiagen Cat. 204054) in a total volume of 5 µl. PCR was accomplished after a 5-min activation at

95 ℃, followed by 50 cycles of denaturation at 95 ℃ for 10s, and annealing and extension at 70 ℃ or 68 ℃ for 30 s. Each sample was tested in triplicate.

Real-time PCR was performed with a Life Technologies QuantStudio 12k Flex instrument. Results were read and analyzed by QuantStudio 12K Flex software. Primer dimers and other effects were evaluated by melting curve analysis (Figure 4.3), and only dimer-free reactions were considered valid. To check the residual contaminant genomic DNA, no-RT-control (NRT) and no-template control (NTC) were performed for each primer set. The No-RT-control contains RNA, which was used to examine contamination in RNA samples, DNA elimination process, and reverse transcription process. The no-template control contains only water, primer, and PCR master mix reagent, which is used to test for DNA contamination in primer solution, water, and PCR mater mix solution. Expression of the target genes was normalized by the housekeeping genes (Table 4.3).

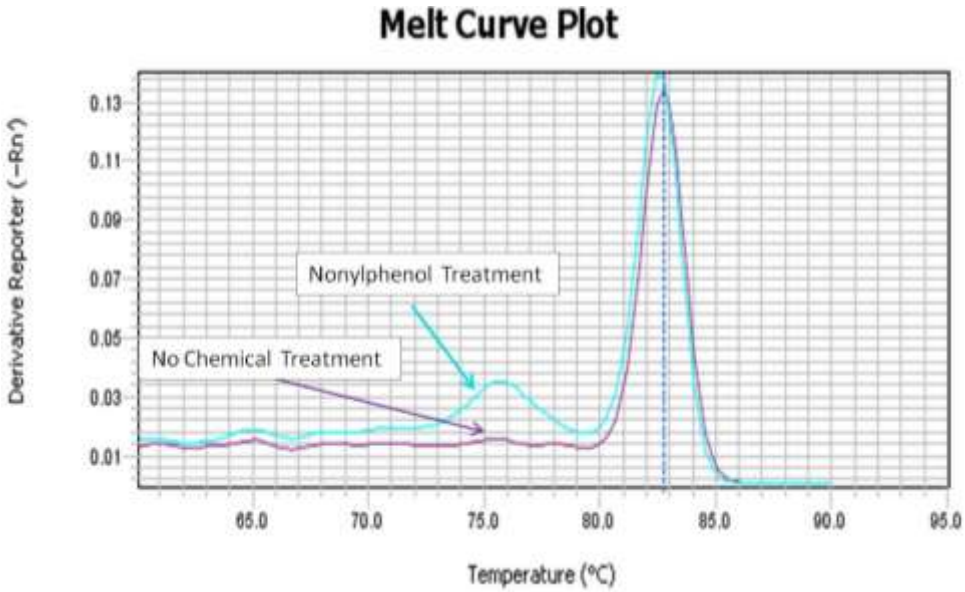



Figure 4.3 RT-PCR melting curves of primer acrF products in *E. coli*. The blue line represents the PCR product in nonylphenol treatment reactor. The purple line represents the PCR product in a no-chemical reactor. Primer acrF amplified the target at 83 ℃. The peak at 76 ℃ was primer dimer.

## Methods to Test Hypothesis 3 (Tasks 3 and 4): Resistance Genes Affect Hormone Oxidization Efficiency

To measure the effect of resistance genes on hormone oxidation efficiency, wild type *E. coli* and *E. coli* containing deletions of the multidrug efflux protein AcrAB were transformed with plasmids encoding either toluene dioxygenase todC1C2BADE or its empty vector pkk223-3. The oxidation rate of estrogenic endocrine disrupting chemicals was then obtained by growing strains on minimal media spiked with 11 mg/L of bisphenol-A. BPA concentrations were measured using the Yeast Estrogen Screen.

### Strains and Plasmids

Genotype details of strains and plasmids are given in Table 4.4. Wild type of *E. coli* (W3110) and *E. coli* possessing deletions of major efflux proteins (W4680A - ΔacrAB) were used as host cells. Electrocompetent cells were prepared according to standard methods (Sambrook & Russell, 2006) and stored at -80 ℃. Plasmids encoding toluene dioxygenase todC1C2BADE (pDTG603) were transformed into W3110 and W4680A and grown on LB media. The empty vector pKK223-3 served as the control.

Table 4.4. Bacteria strains and plasmids used to test for substrate degradation with *E. coli*. $Amp^R$ = ampicillin resistant; ::Kan = kanamycin resistant.

| | **Relevant genotype** | **Enzymes** | **Reference** |
|---|---|---|---|
| **Strains** | | | |
| W3110 | W3110 | Wild type *E. coli* | Bachmann,1972 |
| W4680A | K-12 *ΔacrAB*::Kan | Resistance gene deletion | Ma et al, 1995 |
| **Plasmids** | | | |
| pDTG603 | *todC1C2BADE*; $Amp^R$ | Toluene dioxygenase | Zylestra, 1989 |
| pKK223-3 | Empty vector control, $Amp^R$ | | Zylestra, 1989 |

Plasmid pDTG603 was engineered by inserting about 5000 base pairs of *todC1C2BADE* gene into a commercial vector of pKK223-3 (Zylstra & Gibson, 1989).

EDC Degradation Tests

Bacteria expressing various combinations of oxygenase genes and RND pumps were used in this experiment (Figure 4.4). EDC degradation was tested in bacteria possessing either the oxygenase or RND pump, bacteria with both sets of the genes, or bacteria without either gene. Bacteria were exposed to 11 μg/mL of BPA in a 10 ml minimal media reactor and were incubated at 37 ℃ for 25 hours. Minimal media solution was adopted from Racz (2010; Appendix B). The ethanol concentration was no more than 0.1% in the reactors.

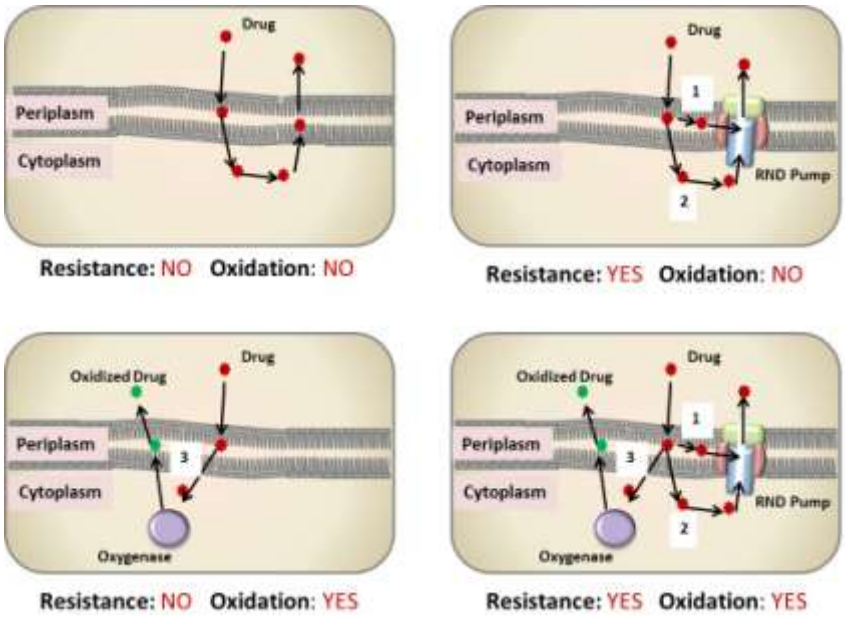



Figure 4.4 Bacteria engineered to determine if lack of degradation is linked to hormone resistance. 1: first hypothesis pathway in the drug is exported from periplasm by RND pump; 2: second hypothesis pathway in the drug is exported from cytoplasm by RND pump; 3: third hypothesis pathway in the drug is oxidized to lower toxic level compound by oxygenase.

Samples were collected every 4 hours to measure bacteria growth. Endocrine disrupting activity was measured by Yeast Estrogen Screen (Conroy, 2006), a recombinant yeast screen integrated with human estrogenic developed to detect estrogenic activity in water samples (Figure 4.5). In the presence of the hormone, the altered yeast produces β-galactosidase, an enzyme that induces a color change in the substrate CPRG. Hormonal activity was determined by measuring color production at wavelengths of 570 and 600 nm.

## Methods to Test Hypothesis 4 (Tasks 5 and 6): Drug Resistant Genes Present in Wastewater Treatment and River

Water samples from the major treatment processes in the Snyderville Water Reclamation Plant and the Central Valley Water Reclamation Facility and water from

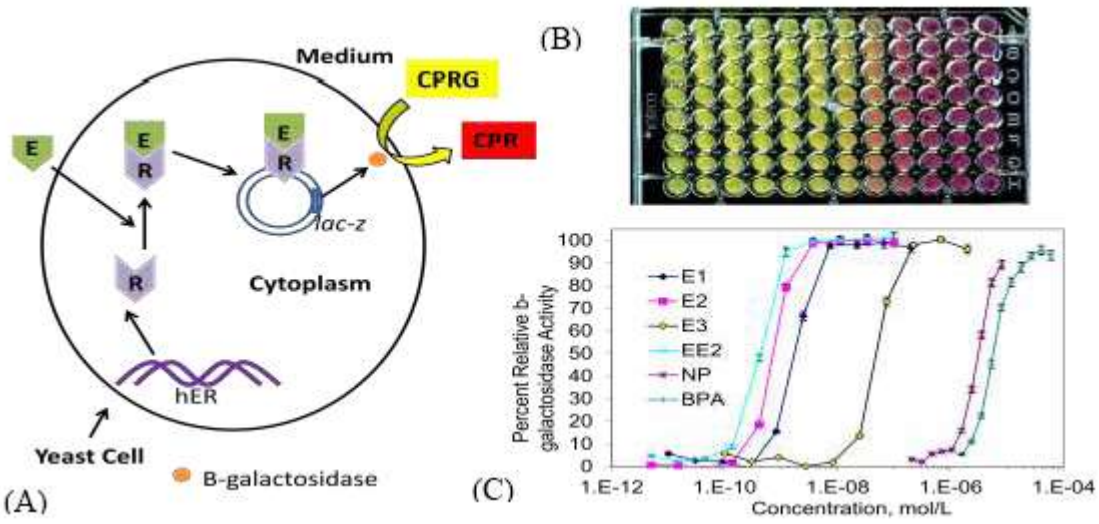



Figure 4.5 Yeast estrogen screen. (A) Physical and biochemical events in the yeast estrogen screen process. (B) Yeast assay screen plates with color change according to the concentration of CPR, which represents the estrogenic activities in Figure (C). More intense red color represents higher estrogenic activities in water samples. (C) Standard curves of steroids and endocrine disrupting chemicals.

Utah Lake and the Jordan River were collected and quantified of estrogenic activity by the Yeast Assay Screen (YES). Meanwhile, RND membrane fusion protein genes were screened using PCR.

Wastewater and Fresh Water Estrogenic Measurement

Samples were collected from each of the liquid treatment processes in the Snyderville Water Reclamation Plant and the Central Valley Water Reclamation Facility. The Snyderville Water Reclamation Plant (4 MGD) utilizes an oxidation ditch followed by media filtration and UV disinfection prior to discharge into a local freshwater stream. The Central Valley Water Reclamation Facility uses a trickling filter for biological treatment, followed by aerated solids contact tanks and disinfection with UV light prior to discharge in the Jordan River. For the fresh water body, Utah Lake and Jordan River, water samples were collected along the river flow from south of Utah Lake to North of the Jordan River to the Great Salt Lake. The lake and river samples were collected in various seasons except winter, when the water body was frozen.

Water samples were processed within 24 hours of collection. One liter of water was filtered through glass fiber filters and followed by solid phase extraction. Organic compounds in water samples were extracted with a C18 disk and were eluted by 20 ml of 100% ethanol. Samples were stored in ethanol up to 1 month at 4 ℃. Ethanol was evaporated under nitrogen at 40 ℃. Autoclaved C-18 water (2 ml), which was prefiltered through a C18 disk to remove residual organic chemicals from Millipore water, was used to redissolve organic compounds in the water samples. The final concentration of the samples were 500-fold concentrated (500X) from the original 1 litter water samples. The

estrogen 17β-estradiol was used as standard.

Wastewater Sample Collection for PCR

Water samples were collected to detect the drug-resistance bacterial efflux proteins. Secondary treated effluent was collected in 1-L amber glass bottles (burned at 550 ℃ for 5 hours) from two local wastewater treatment facilities in Utah. Samples were centrifuged at 8000 rpm for 15 minutes in 50-mL centrifuge tubes. After decanting the supernatant, the pellet was resuspended in 5 mL of LB media. Seventy-five microliters of sample were spread on LB agar plates containing individual antibiotics (ampicillin, kanamycin, novobiocin, ciprofloxacin, doxycycline, erythromycin, cefmetazole, chloramphenicol, and sulfamethoxazole) or endocrine disrupting chemicals. The range of antibiotics was selected based on the individual mode of action and their presence in wastewater effluent, using standard resistance concentrations (Table 4.5). The final concentration of EDC diluted into agar was equal to or greater than the MIC determined for *AcrAB-TolC* and *MexAB-OprM*. Viable colonies grown on each chemical were recorded and counted as CFU/mL.

Amplification of RND Membrane Fusion Protein Gene Products

DNA was extracted from culturable bacteria colonies using Qiagen tissue kits (Qiagen Cat. No. 69504). Primers were designed to amplify a 400–600 base pair region of the inner membrane protein of common RND complexes in gram-negative bacteria using the Primer Design Tool provided by National Center for Biotechnology Information GenBank database (NCBI) (http://www.ncbi.nlm.nih.gov/). The genes *acrB*, *adeB*, *mtrD*,

Table 4.5 Antibiotics and estrogenic endocrine disrupting chemicals used to select for chemical-specific resistance in bacteria samples.

| Antibiotic | Chemical Class | Resistance Concentration |
|---|---|---|
| Ampicillin | β-lactam | 100 μg/mL |
| Kanamycin | Aminoglycoside | 50 μg/mL |
| Novobiocin | Aminocoumarin | 100 μg/mL |
| Ciprofloxacin | Fluoroquinolone | 1 μg/mL |
| Doxycycline | Tetracycline | 10 μg/mL |
| Erythromycin | Macrolide | 200 μg/mL |
| Cefmetazole | Cephalosporin (β-lactam) | 10 μg/mL |
| Chloramphenicol | Bacteriostatic | 25 μg/mL |
| Sulfamethoxazole | Sulfonamide | 80 μg/mL |
| | | |
| Endocrine Disruptors | | |
| Bisphenol-A (BPA) | Plasticizer | 100 μg/mL |
| Nonylphenol (NP) | Surfactant | 75 μg/mL |
| Octylphenol (OP) | Surfactant | 75 μg/mL |
| β-Estradiol (E2) | Estrogenic hormone | 75 μg/mL |
| Estrone (E1) | Estrogenic hormone | 75 μg/mL |
| Estriol (E3) | Estrogenic hormone | 75 μg/mL |
| 17α-Ethynylestradiol (EE2) | Synthetic hormone | 75 μg/mL |

*mexB, ttgH, emhB, eefB, cmeB, bpeB, adeJ, and gesB* were selected to be amplified. Primers are shown in Table 4.6, with a common $T_m$ around 60 ℃. Each DNA sample was amplified against 11 pairs of primers to determine if the strain possessed the particular inner membrane component. The PCR reaction mixture was used as follows: 3 L of DNA, 27.8 L PCR master mix (Qiagen Cat. No. 201445), 1.1 L each of forward and reverse primers, 22.2 L molecular grade water. The thermocycle (ThermoScientific) was operated at 94 ℃ for 3 minutes; 30 cycles of 94 ℃ for 1 minute; 58 ℃ for 30 seconds; 72 ℃ for 1 minute; and 10 minutes at 72 ℃. Amplified DNA products were separated on a 1.7% agarose gel.

Table 4.6 Primer sequences used to amplify a 400–600 base pair region of the inner membrane protein of the RND chemical efflux pump. The bacteria from which the sequence was obtained is listed under the gene. BTEX = benzene, toluene, ethylbenzene, and xylenes; CAM = chloramphenicol; PAHs = polynuclear aromatic hydrocarbons

| Gene<br>Bacteria Strain | Substrates | Primer Sequence | Product Size (bp) |
|---|---|---|---|
| ***acrB*** (Elkins & Mullins, 2006; Elkin & Nikaido, 2002; Pos, 2009) | | | |
| *E. coli* | Dyes, detergents, antibiotics, bile salts, steroids | For 5'-GGCTTTGCGGGACGTGGTCA-3'<br>Rev 5'-GCAGCGCCCAGAGTGGTGTT-3' | 412 |
| ***adeD*** (Chau et al., 2004) | | | |
| *Acinetobacter sp.* | Antibiotics | For 5'-GGTTGCGTACCCACGCGACA-3'<br>Rev 5'-CGAGCGTTACCGAGAACTGGCG-3' | 457 |
| ***mtrD*** (Kamal et al., 2007) | | | |
| *Neisseria gonorrhoeae* | Steroids, bile salts | For 5'-AAGGCGGCTCGCAAACCCTC-3'<br>Rev 5'-ACACGGACAAGAGCGTGCCG-3' | 475 |
| ***mexB*** (Chuanchuen et al., 2002; Jeannot et al., 2005; Mao et al. 2002; Nehme & Poole, 2007; Poole, 2008) | | | |
| *Pseudomonas aeruginosa* | Antibiotics | For 5'-GGCGGCTTCTTCGGCTGGTT-3'<br>Rev 5'-ACACGCTGTTCTCGCCACCG-3' | 405 |
| ***ttgH*** (Kieboom & de Bont, 2001; Terán et al., 2007) | | | |
| *Pseudomonas putida* | Antibiotics, solvents (BTEX, styrene) | For 5'-AGAGAGCGCCTCTGACGGCA-3'<br>Rev 5'-CGCGTCTTGCCCAGAACGGT-3' | 476 |
| ***emhB*** (Hearn et al., 2003) | | | |
| *Pseudomonas fluorescens* | PAHs | For 5'-ACCTGGGTAACGCCACCGGT-3'<br>Rev 5'-CGCCGTTGTAACGGGCCAGT-3' | 443 |
| ***eefB*** (Masi et al., 2005) | | | |
| *Enterobacter aerogenes* | Antibiotics | For 5'-CCAACCGCGCCACCAGTTCT-3'<br>Rev 5'-TATCCAGCGCGTTAGCGCCG-3' | 467 |
| ***cmeB*** (Lin et al., 2005) | | | |
| *Campylobacter jejuni* | Antibiotics, bile salts | For 5'-GCCGCAACTGCAAAAATGCCAGAT-3'<br>Rev 5'-CTGCTGTATGCAATGCGTTTGCCC-3' | 577 |
| ***bpeB*** (Chan et al., 2004) | | | |
| *Burkholderia pseudomallei* | Antibiotics, dyes | For 5'-TCGCGTCGCACGTGAAGGAC-3'<br>Rev 5'-GCGTTGGTCGCGAGCTGGAT-3' | 418 |
| ***gesB*** (Pontel et al., 2007) | | | |
| *Salmonella enterica* | $Au^{3+}$, CAM | For 5'-ACGGTAAACACGCTGGGCCG-3'<br>Rev 5'-AGGTGCCGACCAGCGAAACG-3' | 441 |
| ***adeJ*** (Damier-Piolle et al., 2008) | | | |
| *Acinetobacter baumannii* | Antibiotics | For 5'-AGGTGGTGCTCCGGCTGTACA-3'<br>Rev 5'-GCGTCGTCGACCAGAAGACCG-3' | 569 |

# CHAPTER 5

# RESULTS AND DISCUSSION

## Task 1: EDCs as Substrates of RND Efflux Pumps in *E. coli* and *Pseudomonas*

Estrogenic endocrine disruptors were classified as substrates of RND-type multidrug efflux proteins if the chemical was toxic to the bacteria with empty vector control (pUC18) compared to bacteria expressing the efflux pump. The xenoestrogens 17α-ethynylestradiol, nonylphenol, and bisphenol-A were determined to be substrates of both AcrAB-TolC and MexAB-OprM. The measured MIC values for W4680A-pAB, W4680A-pMABO, and ECM2112-pMABO were greater than their respective empty vector controls (pUC18), indicating the presence of the efflux pump preventing intracellular toxicity. ECM2112-pAB exhibited toxicity because tolC was not present, which is essential for chemical efflux. Minimum inhibitory concentrations of 17α-ethynylestradiol, octylphenol, nonylphenol, and bisphenol-A are summarized in Table 5.1.

As shown in Figure 5.1 (A), the human estrogens and metabolites 17α-estradiol, estrone, and estriol did not inhibit cell growth of either AcrAB-TolC or MexAB-OprM. Elkins and Mullis showed that the natural estrogens are in fact substrates based on radioactive uptake assays (Elkins & Mullis, 2006). Growth was similar for the control

Table 5.1. Minimum inhibitory concentrations (mg/L) of estrogenic endocrine disrupting chemicals. A MIC value greater for pAB (which contains AcrAB) or pMABO (which contains MexAB-OprM) than for the empty vector control indicates that the chemical is a substrate of the overexpressed efflux pump (**difference in bold**). 17β-estradiol (E2), estrone (E1), and estriol (E3) are not substrates of either AcrAB or MexAB-OprM; 17α-ethynylestradiol (EE2) is a substrate of MexAB-OprM and not of AcrAB; nonylphenol (NP), octylphenol (OP), and bisphenol-A (BPA) are substrates of AcrAB and MexAB-OprM. Note: Data for pUC19 are not shown as results were identical to pUC18.

| Strain | E2 | E1 | E3 | EE2 | NP | OP | BPA |
|---|---|---|---|---|---|---|---|
| W3110 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
| W4680A | | | | | | | |
| pAB | >100 | >100 | >100 | >100 | **>100** | **>100** | **>100** |
| pMABO | >100 | >100 | >100 | >100 | **>100** | 25 | **>100** |
| pUC18 | >100 | >100 | >100 | >100 | 25 | 25 | 50 |
| ECM 2112 | | | | | | | |
| pMABO | >100 | >100 | >100 | **>100** | **>100** | **>100** | 50 |
| pUC18 | >100 | >100 | >100 | 50 | 25 | 25 | 50 |

plasmid and the strain expressing either AcrAB-TolC or MexAB-OprM, indicating that EDCs do not alter the toxic response. This could be explained by the presence of an efflux pump or the human estrogens and because metabolites are not toxic to cell. Analyte concentrations greater than 100 mg/L were not tested due to solubility limitations. Natural estrogens may still be substrates of AcrAB-TolC and MexAB-OprM, but are not toxic to the cell. A different end point would need to be measured to evaluate the full substrate range of natural estrogens.

The inhibitory concentrations of 17α-ethynylestradiol, bisphenol-A, and nonylphenol were determined to be 50, 50, and 25 mg/L, respectively (Figure 5.1). With 50 mg/L of 17α-ethynylestradiol, strains with MexAB-OprM could grow without the influence of this toxic level compounds, and the same phenomena were observed in strain W3110 (Figure 5.1(B)). These results indicated that 17α-ethynylestradiol is substrate of AcrAB-TolC and MexAB-OprM. However, the growth of strain ECM 2112-PAB was

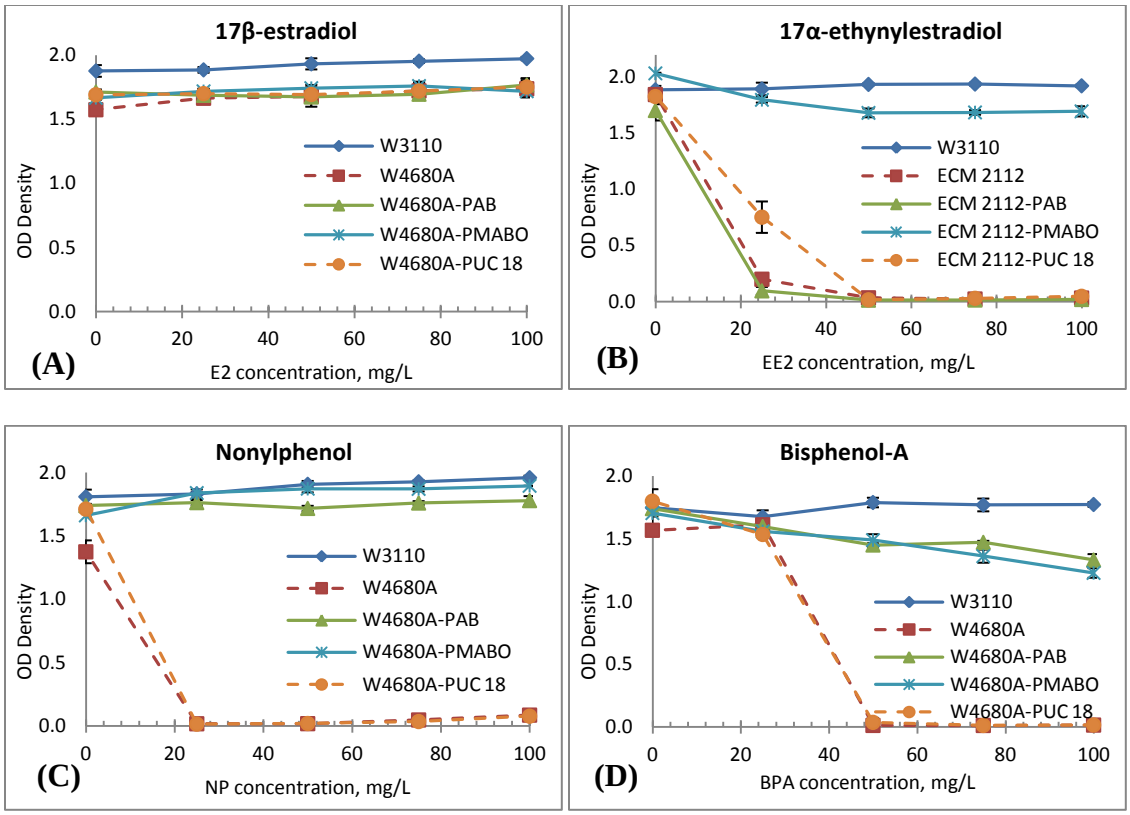



Figure 5.1 Growth curves used to determine the minimum inhibitory concentration of each chemical acting on either pAB (AcrAB) or pMABO (MexAB-OprM). Strain W3110 is wild-type E. coli (no gene deletions); W4680A has a deletion of acrAB; ECM2112 has a deletion of acrAB-tolC.

inhibited at the same level, proving TolC is the essential part for the arcAB-TolC efflux protein function. With the absence of acrAB, nonylphenol was toxic to *E. coli* strains of W4680A and W4680A-PUC18 at 25 mg/L (Figure 5.1(C)). When the *acrAB* gene was inserted back to the cell as plasmid pAB, efflux protein exported nonylphenol to protect *E. coli* growth. Nonylphenol is also the substrate of MexAB-OprM. As shown in Figure 5.1(D), even with the efflux protein AcrAB-TolC and MexAB-OprM, *E. coli* growth showed a slight decrease at the bisphenol-A concentration above 50 mg/L, while wild type *E. coli* W3110 was not affected by this toxic condition. Both strains of W3110 and W4680A-pAB contained complete genes of *acrAB-TolC* and exported toxic bisphenol-A,

so the growth difference was due to the mutation process. The same conclusion was made for strain W4680A-pMABO. *E. coli* strains may be more sensitive to bisphenol-A compared to 17α-ethynylestradiol and nonylphenol.

Task 2: Estrogenic EDCs Induce Resistant and Oxygenase Genes

To determine if chemical resistance or oxidation is preferentially expressed in wastewater bacteria, genes were induced by environmental levels of endocrine disruptors found in wastewater. Reactors with no EDCs were used as control samples, and housekeeping genes *modG* and *rpoD* were used to normalize the results for *E. coli* and *Pseudomonas*, respectively (Liu et al., 2011; Savli et al., 2003; Sean et al., 2011). Bacteria were exposed to 10 ng/L of EE2, 10 μg/L of NP, and 1 μg/L of BPA.

Real time polymerase chain reaction (RT-PCR) detects and quantifies DNA amplification at each cycle with a fluorescent signal. The results present as a threshold cycle, which is the cycle number at the fluorescent signal baseline. Relative expression quantification measures the Ct (threshold cycle) difference between the target samples and reference samples for a given gene and presents as the fold difference (Hellemans et al., 2007; Willems & Vandesompele, 2008, IDT). Calculations followed the following steps and equations:

1. Ct values were determined. Each sample and control was run in triplicate to ensure the constant expression.
2. Ct values were normalized by calculating the difference (ΔCt) between target gene and endogenous control (housekeeping gene).

$$\Delta Ct = Ct\ (\text{target gene}) - Ct\ (\text{reference gene}) \qquad \text{Equation (1)}$$

3. To find the gene expression base on the control samples (ΔΔCt), subtract the control samples from the chemical treated samples.

$$\Delta\Delta Ct = \Delta Ct \text{ (target sample)} - \Delta Ct \text{ (control sample)} \quad \text{Equation (2)}$$

4. To calculate the gene expression change (R) of the target sample to the control sample, the negative value $\Delta\Delta Ct$ is the exponent of 2. Since PCR doubles the DNA fragment in each cycle, so this step calculation corrects the difference in the number of cycles to threshold.

$$R = 2^{-\Delta\Delta Ct} \quad \text{Equation (3)}$$

RND genes were selected from the Transporter Classification Database (http://www.tcdb.org/) with the list of verified drug-resistant proteins. By screening the complete gene list for each strain, oxygenase, oxidase, and peroxidase genes were chosen when the protein function had been reported on the NCBI database.

Gene expression levels induced by EE2, NP, and BPA are shown in Table 5.2. In *E. coli*, drug resistant genes of *cusA, acrB, acrF, mdtB, yhiV*, and the oxidases *katE* and *katG* were up-regulated by EDCs. With BPA induction, *katE* expression level increased 45.3-fold relative to the control sample, and the *acrB* expression level increased 36.8-fold. Gene *yhiV*, which is a chemical transporter, also was expressed in the BPA-treated sample. These three genes exhibited high expression in NP and EE2 treated samples as well, which means the presence of endocrine disruptors trigger the proteins' function to export EDCs and degrade EDCs. It is not surprising that *acrB* had high expression in all the three samples, which was been verified in Task 1 that these three EDCs are substrates of *acrB*. Elkins and Mullis (2006) reported that steroid hormones are substrate of *yhiV*.

From this experiment, it is very likely that endocrine disruptors of EE2, NP, and

Table 5.2 Gene expressions of *E. coli*, *P. putida*, and *P. aeruginosa* in the LB broth reactors with 10 ng/L of EE2, 10 µg/L of NP, or 1 µg/L of BPA. Presented as fold increase relative to control samples.

| *Escherichia coli* | | | | *Pseudomonas putida* | | | |
|---|---|---|---|---|---|---|---|
| | EE2 | NP | BPA | | EE2 | NP | BPA |
| cusA | **2.6** | **3.7** | **4.6** | arpB | 0.9 | 0.4 | 0.3 |
| acrD | 0.0 | 0.3 | 0.6 | ttgB | **1.5** | 0.8 | 0.9 |
| acrB | **6.5** | **18.4** | **36.8** | Pput_1893 | 0.4 | 0.1 | 0.2 |
| acrF | 0.8 | **1.3** | **3.5** | Pput_2363 | 0.4 | 0.1 | 0.1 |
| mdtB | **3.0** | **3.7** | **3.2** | Pput_2880 | 0.7 | 0.2 | 0.3 |
| yhiV | **8.0** | **18.4** | **19.7** | Pput_2897 | **1.6** | 1.0 | **1.5** |
| katE | **8.6** | **22.6** | **45.3** | Pput_2897 | 0.7 | 0.5 | 0.4 |
| katG | **2.1** | **3.2** | **7.0** | Pput_5066 | 0.6 | 0.2 | 0.2 |

| *Pseudomonas aeruginosa* | | | | | | | |
|---|---|---|---|---|---|---|---|
| | EE2 | NP | BPA | | EE2 | NP | BPA |
| mexB | 0.4 | 0.4 | 0.9 | PA0817 | **1.1** | 0.9 | **1.3** |
| mexD | **1.2** | **1.1** | **1.6** | PA0880 | 0.6 | 0.6 | **1.2** |
| mexF | **1.4** | **1.9** | **2.3** | PA2024 | 0.4 | 0.3 | 0.4 |
| mexK | 0.9 | **2.8** | **5.4** | PA2083 | 0.4 | 0.4 | 0.4 |
| mexY | 0.4 | 0.5 | 0.9 | PA2085 | 0.8 | 1.0 | **1.3** |
| triC | 0.9 | **1.2** | **1.4** | PA2097 | **1.2** | **2.0** | **1.5** |
| mexI | 0.8 | 0.7 | 0.8 | PA2355 | 0.4 | 0.4 | 0.4 |
| mexW | 0.7 | **1.0** | **1.6** | PA2546 | 1.0 | **1.3** | 0.9 |
| mexQ | 0.1 | 0.3 | 0.6 | PA3389 | 0.7 | 0.7 | 0.9 |
| mexN | 0.5 | 0.4 | 0.3 | pqsH | 0.7 | **1.3** | **2.0** |
| alkB1 | 0.7 | **1.6** | **2.6** | pqsL | 0.4 | 0.5 | 0.6 |
| alkB2 | 0.3 | 0.4 | 0.8 | | | | |

BPA are the substrates of *yhiV* as well, as the gene was upregulated. Among the oxidases, *katE* expression levels increased 45.3-fold in BPA samples, 22.6-fold in NP samples, and 8.6-fold in EE2 samples. *KatE* is a hydrogen peroxidase, which may contribute mostly to EDCs degradation in the reactors. Other than these 3 genes, *cusA, acrF, mdtB,* and *katG* showed a noticeable elevated expression, meaning they responded in the presence of EDCs, but were not as upregulated as *acrB*, *yhiV,* and *katE*. *CusA* is a metal resistant gene, which responds to EDCs in this experiment. Conroy et al. previously showed that

this metal transporter has a slight affinity for organic molecules (Conroy et al., 2010). Before antibiotics were applied to the clinic, heavy metal was used to treat diseases; perhaps this is the reason that some metals and antibiotics share same drug resistant gene. Comparing the 3 different chemicals, BPA induced gene expression the most, and EE2 has the weakest activity to trigger gene expression.

RND efflux genes *ttgB* and oxyenase *Pput_2897* in *P. putida* showed slightly increased expression after EE2 and BPA exposure. *TtgB* is a solvent efflux protein that exports toluene, styrene, m-xylene, ethylbenzene, and propylbenzene (Teran et al., 2007). Protein *Pput_2897* is an aromatic-ring-hydroxylating dioxygenase (Copeland et al., 2007). The substrate range for both proteins are aromatic-ring compounds, which is the basic structure of EDCs.

In *P. aeruginosa*, efflux genes of *mexD, mexF, mexK, triC,* and *mexW* showed a trace amount of increasing expression. These genes have similar substrates. The oxygenases *alkB1, PA0817, PA0880, PA2085, PA2097, PA2546,* and *pqsH* in *P. aeruginosa* responded to the presence of EDCs. Among these oxygenases, *PA0817, PA0880, PA2085,* and *PA2546* are ring cleaving/hydroxylating dioxygenase, *alkB1, PA2097* and *pqsH* are monooxygenase. Except *alkB1*, all other oxygenases showed increased expression involved in breaking down the aromatic-ring structure chemicals.

In *Pseudomonas putida* both RND genes and oxygenase genes were expressed only slightly increasing beyond the control, and similar phenomena were observed in *Pseudomonas aeruginosa*. *Pseudomonas sp.* is often isolated in steroid hormone and EDC degradation studies from activated sludge and river sediments (Pauwels et al., 2008; Yuan et al., 2004) and is the bacteria widely used to degrade carbon ring structure

introduce the significant change on the high level gene expression background. MexB in *pseudomonas aeruginosa* was identified as an EE2, NP, and BPA transporter gene, but it did not show elevated expression with these 3 compounds presence in this experiment. If *mexB* is already expressed in the cell by the complex media, estrogenic EDCs that are substrates of the protein will be exported. In the case of *P. putida*, and *P. aeruginosa* in this experiment, the data reported in Table 5.2 were significantly underestimated.

## Tasks 3 and 4: Toluene Dioxygenase Degradation of BPA and BPA Resistance

To measure the influence of resistance genes on EDCs' oxidation efficiency, degradation will be compared in the *E. coli* strain with and without the oxygenase. The parent *E. coli* strains used in this test are wild type *E. coli* W3110 (w/ acrAB) and the strains with acrAB deletion W4680A (ΔacrAB). Toluene dioxygenase pDTG603 (todC1C2BADE) was selected to degrade bisphenol-A in this research. The DNA fragment encoding of *todC1C2BADE* was inserted on the commercial vector of pKK223-3 (ΔTOD), and transformed into W3110 (w/ acrAB), a wild type of *E. coli*, and W4680A (ΔacrAB), a W3110 mutation containing the deletion of the resistant gene of acrAB. Toluene dioxygenase is a complex unit that includes iron-sulfur protein, ferredoxin, reductase, cis-toluene dihydrodiol dehydrogenase, and 3-metheylcatechol 2,3-dioxygenase genes. From the NCBI database, toluene dioxygenase (todC1C2BDE) is present in the genomic DNA of *Pseudomonas putida* and shares a 100% similarity sequence with a DNA fragment in *Pseudomonas monteilii,* which belongs to the *Pseudomonas putida* group. Based on the toluene and benzene oxidation ability of this

strain, *Pseudomonas putida* was commonly used in oil sites for bioremediation purposes (Raghavan, 1999).

Degradation tests were conducted in minimal media containing peptone and sodium acetate as major organic nutrient, a trace amount of citric acid and hippuric acid, mineral salts, and 11 mg/L BPA. *E. coli* strains, harboring either toluene dioxygenase (TOD) or the empty vector (ΔTOD), served as the degrading microorganism. The degradation level was measured as BPA equivalents using the Yeast Estrogen Screen. In this method, human estrogenic receptor was employed as a biological sensor to detect the estrogenic level caused by BPA.

As shown in Figure 5.2, BPA activities decreased 2 to 20 % in the first 2 hours in all the reactors, followed by 41 to 76% increase in the next 4 or 9 hours. This significant increase of estrogenic activities indicates that BPA degradation intermediates are more estrogenic than the parent compound. For the strains with TOD, after 6 hours reaction, BPA levels decreased, and reached to 30 to 40% of initial level at 15 hours and remained at the same level until 25 hours. For the strains absent of toluene dioxygease, BPA activities decreased to the same level as strains with TOD after a 25 hours reaction. Ike et al. demonstrated biodegradation can reduce the toxic effects of BPA (Ike et al., 2002). However, the intermediates may express higher level estrogenic effects compared with BPA.

Initial 2 Hours: BPA Levels Slightly Decreased

In the initial 2 hours, BPA activity in ΔacrAB reactors did not decrease, while the activities in strains with acrAB reactors were reduced by 20%. This is because the strain

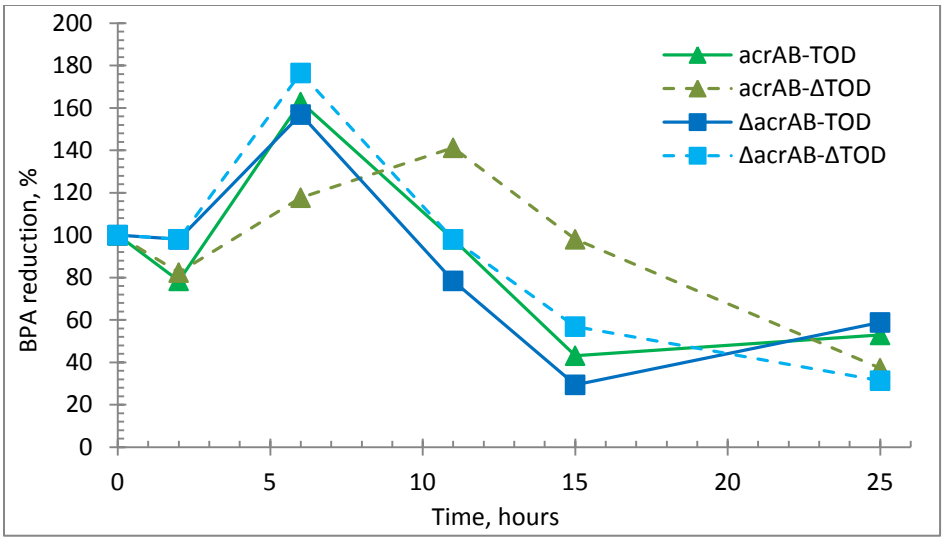



Figure 5.2 BPA levels measured by the yeast estrogen screen. acrAB-TOD: strain with both acrAB drug-resistant protein and toluene dioxygenase protein; acrAB-ΔTOD: strain with only acrAB drug-resistant protein; ΔacrAB-TOD: strain with only toluene dioxygenase protein; ΔacrAB-ΔTOD: strain with neither drug-resistant protein nor toluene dioxygenase protein.

with acrB is wild type E. coli, while the strain with the acrAB deletion (ΔacrAB) is an engineered mutant from wild type E. coli. Strains with mutations tend to grow and adapt to the environment slower than wild type bacteria (Hoffmann & Rinas, 2004; Kurland & Dong, 1996). In the growth test of this experiment, the optical density did not show a noticeable difference in the first 2 hours (data not shown) since the starting culture's concentration was small; but in previous experiments, the mutation strains exhibited a slower growth rate. The strain with ΔacrAB needed a longer time to adapt to the environment and induce the degradation process properly, as shown by a lack of degradation in the first 2 hours.

2 Hours to 6 Hours: BPA Activity Increased

Between 2 to 6 hours in this experiment, estrogenic level increase was observed in all samples. Higher estrogenic activity of the intermediates or perhaps chemicals acting in a synergistic or additive may explain the estrogenic increase. The intermediates and pathways of oxidizing BPA have been reported in several studies with various oxidation method and bacteria (Katsumata, 2003; Lobos, 1992; Suzuki, 2004; Zhang, 2007). However, BPA degradation pathway and intermediates with toluene dioxygenase is unknown, and further tests on the intermediates' structure with mass spectrometry (MS) need to be pursued. Yeast Assay Screen method is a good approach to detect the estrogenic activities in an aquatic environment. However, it can not specify the chemical structures in this method. To verify the intermediate products and study the BPA degradation at an environmental level, liquid chromatography-mass spectrometry (LC-MS/MS) should be introduced in the experiment.

Chemical transport by the efflux protein was also observed during the increasing-estrogenic stage in the first 6 hours. The strain acrAB (acrB-ΔTOD) extended this stage to 11 hours (Figure 5.3) due to the chemical efflux, which reduced the BPA-degradation rate and intermediate production rate. However, the same phenomenon was not observed in *E. coli* with toluene dioxygenase. In the control, *E. coli* does not encode mono- or di-oxygenase, which has limited oxidation ability. Gene expression studies in Task 2 showed that *katG* and *katE* are produced in response to BPA. These two enzymes belong to hydrogen peroxidase and catalase enzyme families. Peroxidase could break down phenol (Ghioureliotis & Nicell, 1999) during wastewater treatment, but the mechanism of BPA oxidation by the function of *katG* and *katE* is unknown. Besides *katG* and *katE*,

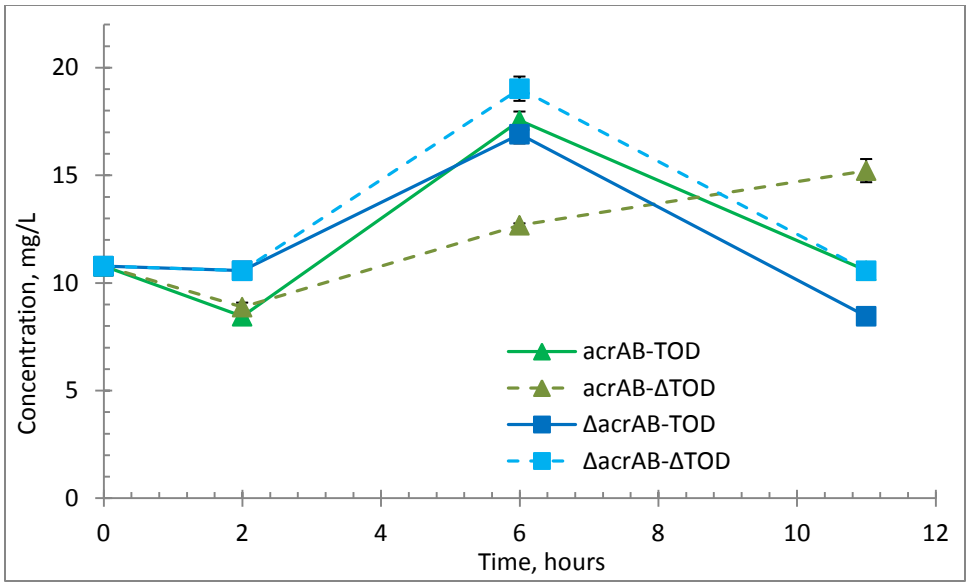



Figure 5.3 The estrogenic increasing stage in BPA degradation process. AcrAB-TOD: strain with both acrAB drug-resistant protein and toluene dioxygenase protein; acrAB-ΔTOD: strain with only acrAB drug-resistant protein; ΔacrAB-TOD: strain with only toluene dioxygenase protein; ΔacrAB-ΔTOD: strain with neither drug-resistant protein nor toluene dioxygenase protein.

other oxidases may be involved in BPA degradation. Previous results showed BPA degradation was accelerated with the addition of toluene dioxygenase. Compared to the control, TOD increased the intermediate production rate and minimized the delay. Deletion of the drug-resistant protein in strain ΔacrB-ΔTOD eliminated the delay on the biodegradation process. This would explain why only the strain with the efflux protein and absent toluene dioxygenase produced intermediates at a slower rate.

6 Hours to 15 Hours: BPA Levels Decreased

The BPA degradation rate between 6 hours to 15 hours (between 11 hours to 25 hours for strain acrAB-ΔTOD) in Figure 5.2 was calculated and is shown in Figure 5.4. The reaction rates followed first order elimination. The BPA degradation rate increased

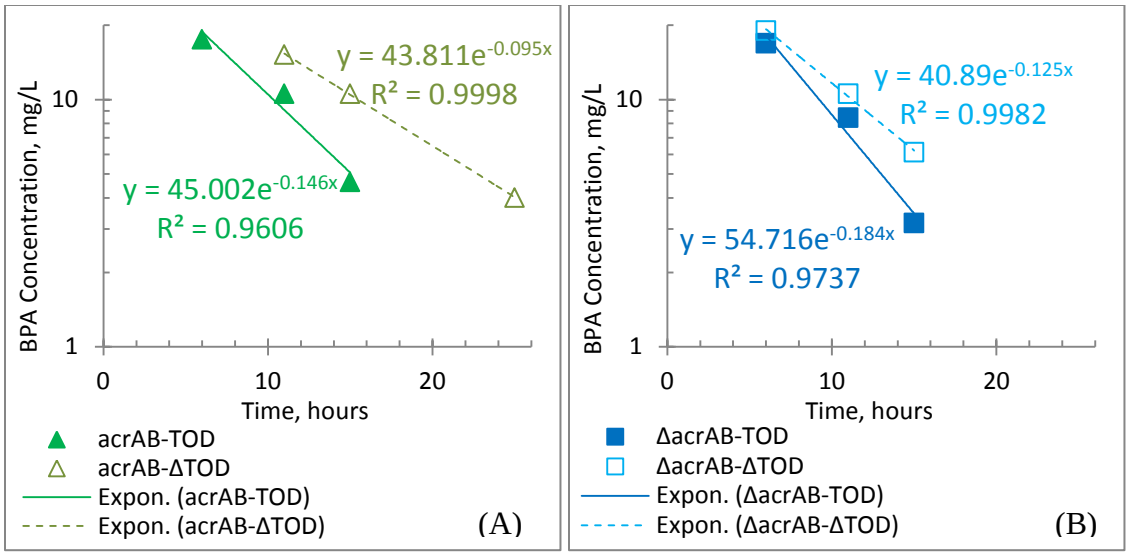



Figure 5.4 Kinetic analysis of the enzymatic reduction of BPA activity. AcrAB-TOD: strain with both acrAB drug-resistant protein and toluene dioxygenase protein; acrAB-ΔTOD: strain with only acrAB drug-resistant protein; ΔacrAB-TOD: strain with only toluene dioxygenase protein; ΔacrAB-ΔTOD: strain with neither drug-resistant protein nor toluene dioxygenase protein.

from 0.095 hour$^{-1}$ with the absence of toluene dioxygenase to 0.146 hour$^{-1}$ with the presence of toluene dioxygenase in the wild type *E. coli* with acrAB (Figure 5.4 (A)), and the degradation rate was increased from 0.125 hour$^{-1}$ to 0.184 hour$^{-1}$ with the additional toluene dioxygenase in the mutation *E. coli* with ΔacrAB (Figure 5.4(B)). These results indicated that toluene dioxygenase is able to degrade BPA.

When toluene dioxygenase was absent (ΔTOD), strain W3110, which possesses drug-resistant protein (acrAB), degraded BPA slower than the strain without acrAB, indicating the presence of the efflux protein exporting the chemical out of the cell and limiting the contact time between the oxidase and chemical. This extrusion process was when toluene dioxygenase was absent (ΔTOD), strain W3110, which possesses drug-resistant protein (acrAB), degraded BPA slower than the strain without acrAB, indicating the presence of the efflux protein exporting the chemical out of the cell and limiting the

contact time between the oxidase and chemical. This extrusion process was competing with the chemical oxidation process and resulted in a reduction of the BPA biodegradation rate from 0.125 $hour^{-1}$ to 0.095 $hour^{-1}$. During degradation when BPA enters the cell, the efflux protein acrAB transports BPA from the periplasm or cytoplasm. BPA is exported out before it interacts with oxidation enzymes, and the extrusion process reduces the chemical degradation rate.

With the presence of toluene dioxygenase (TOD) in strain ΔacrAB and strain acrAB, the oxidation rates are 0.184 $hour^{-1}$ and 0.146 $hour^{-1}$, respectively. Even with increasing BPA oxidation by toluene dioxygenase, drug resistant proteins still influence the BPA degradation process in that efflux proteins export the chemical from the cell, slowing degradation. In large scale biological treatment facilities, this influence could affect the effluent water quality with the limited hydraulic retention time (HRT).

15 Hours to 25 Hours: Evaporation and Further Degradation

During the reaction from 15 hours to 25 hours (Figure 5.2), BPA levels slightly increased in strains with TOD while the control strains (ΔTOD) decreased BPA further. Although the chemicals remaining in the reactors and metabolic mechanisms were unknown in this 10-hour period, the concentration increase was possibly due to evaporation. From our control test with no culture presence, the evaporation rate was 18.6% in 25 hours reaction under 37℃. The evaporation process contributes to the BPA concentration increase more obviously at low levels and low volumes. The lowest BPA concentrations of each strain are all around 3.17 to 4.65 mg/L which equal to 30–40% of initial concentration. The estrogenic activities in toluene dioxygense reactors reached this

level at 15 hours, and BPA cannot be degraded further. However, without toluene dioxygenase, BPA was degraded much slower and reached to a similar level at 25 hours. In this 10 hours gap, evaporation played a major role in the BPA concentration change in toluene dioxygenase reactors, and oxidation was the dominant process in the reactors without toluene dioxygenase. These results indicated that estrogenic activity cannot be completely removed at the ppm level, and toluene dioxygenase could accelerate the degradation process.

Cell Growth Associate with BPA Degradation

Optical density was plotted in Figure 5.5 to analyze the influence of toluene dioxygenase and drug-resistant protein acrAB on cell growth. Growth rate was calculated for the exponential phase, and it followed first order kinetics. The growth rates are 0.22 $hours^{-1}$ for the strain possessing both acrAB/ TOD and the strain with only acrB, 0.27 $hours^{-1}$ for the strain with only TOD, and 0.26 $hours^{-1}$ for the strains absent both acrB and TOD. The cell growth rates were essentially the same with toluene dioxygenase present or absent, indicating toluene dioxygenase did not make a difference. Here, peptone and sodium acetate served as major organic sources in the reactors. This organic matter could be metabolized by *E. coli* easily and utilized to support cell growth so that the addition of toluene dioxygenase as a carbon source did not affect the growth rate. The BPA minimal inhibition concentration was measured as 50 mg/L in Task 1, which is the toxic BPA level for *E. coli*, and at the concentration of 25 mg/L in the previous test, cell growth was not inhibited by this toxic compound (Figure 5.1(D)). In this experiment, BPA concentration was initially 11 mg/L, and with the elevated estrogenic activities at 6 hours, the highest

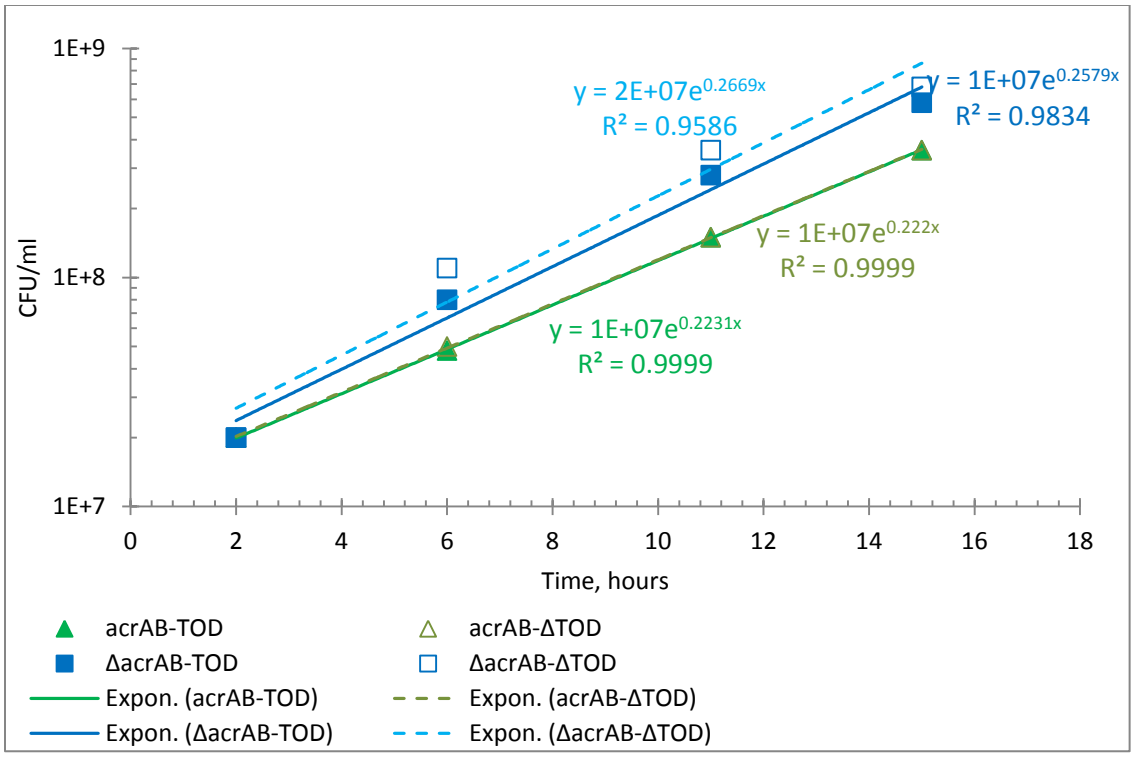



Figure 5.5 Kinetic analysis of the culture growth. acrAB-TOD: strain with both acrAB drug-resistant protein and toluene dioxygenase protein; acrAB-ΔTOD: strain with only acrAB drug-resistant protein; ΔacrAB-TOD: strain with only toluene dioxygenase protein; ΔacrAB-ΔTOD: strain with neither drug-resistant protein nor toluene dioxygenase protein.

BPA equivalent concentration was 19 mg/L, which was far below the inhibition concentration, and BPA toxicity did not affect cell growth and the degradation rate in this experiment.

Comparing the growth between acrAB to ΔacrAB, cells without acrAB grew faster than the cells with the protein. This supports the results that RND resistant proteins export BPA and perhaps other organic matter out of the cell, thus removing the beneficial electrons and carbon from the metabolic pathway. The strain ΔacrAB, with deletion of drug export protein, allowed BPA to stay within the cell and was used as nutrients or electrons to support the *E. coli* growth. From previous gene expression studies, BPA

could trigger oxidoreductase proteins *katE* and *katG*, which essentially functions as hydrogen peroxide (Musso et al., 1977). These two proteins can contribute the BPA degradation in this test.

## Task 5: EDCs Were Detected in Local Wastewater Treatment Plants, Utah Lake and Jordan River

While drug resistant bacteria can be eliminated after the disinfection process and introduced into receiving water, endocrine disrupting chemicals cannot be completely removed from wastewater treatment facilities and discharged to surface water. To monitor the containment level of EDCs and drug-resistant bacteria in local wastewater treatment plants, rivers, and lakes, estrogenic activities and drug-resistant bacteria and genes were measured. Estrogenic activities were monitored in the East Canyon Water Reclamation Facility (ECWRF) and the Central Valley Water Reclamation Facility (CVWRF). The two local treatment plants differ in size, treatment processes, and water quality. The East Canyon Water Reclamation Facility, located in Park City, Utah, is a 4 MGD plant that utilizes primary treatment, activated sludge, secondary sedimentation, media filtration, and UV disinfection prior to discharge into the East Canyon Creek. The Central Valley Water Reclamation Facility (75 MGD) uses primary treatment, trickling filter, solids contact, secondary sedimentation, and UV disinfection prior to discharge into the Jordan River.

In ECWRF, the estrogenic level measured in the influent was 0.25 nM E2 equivalents and was removed by the activated sludge process to an undetectable concentration. At CVWRF, estrogenic level in influent was 0.83 nM E2 equivalent,

which is 3.3 times higher than ECWRF. Grit tank and primary sedimentation reduced the estrogenic level by 20%, and after trickling filtration treatment, the estrogenic activity was reduced by 75% (Figure 5.6). Biological treatment is the major process to remove organic compounds including endocrine disruptors and other emerging contaminants; however, the drug-resistant mechanism competes with the degradation process and reduces the degradation rate. In our test here, estrogenic activities were not completely removed after biological treatment. Although the estrogenic activity was not detected in

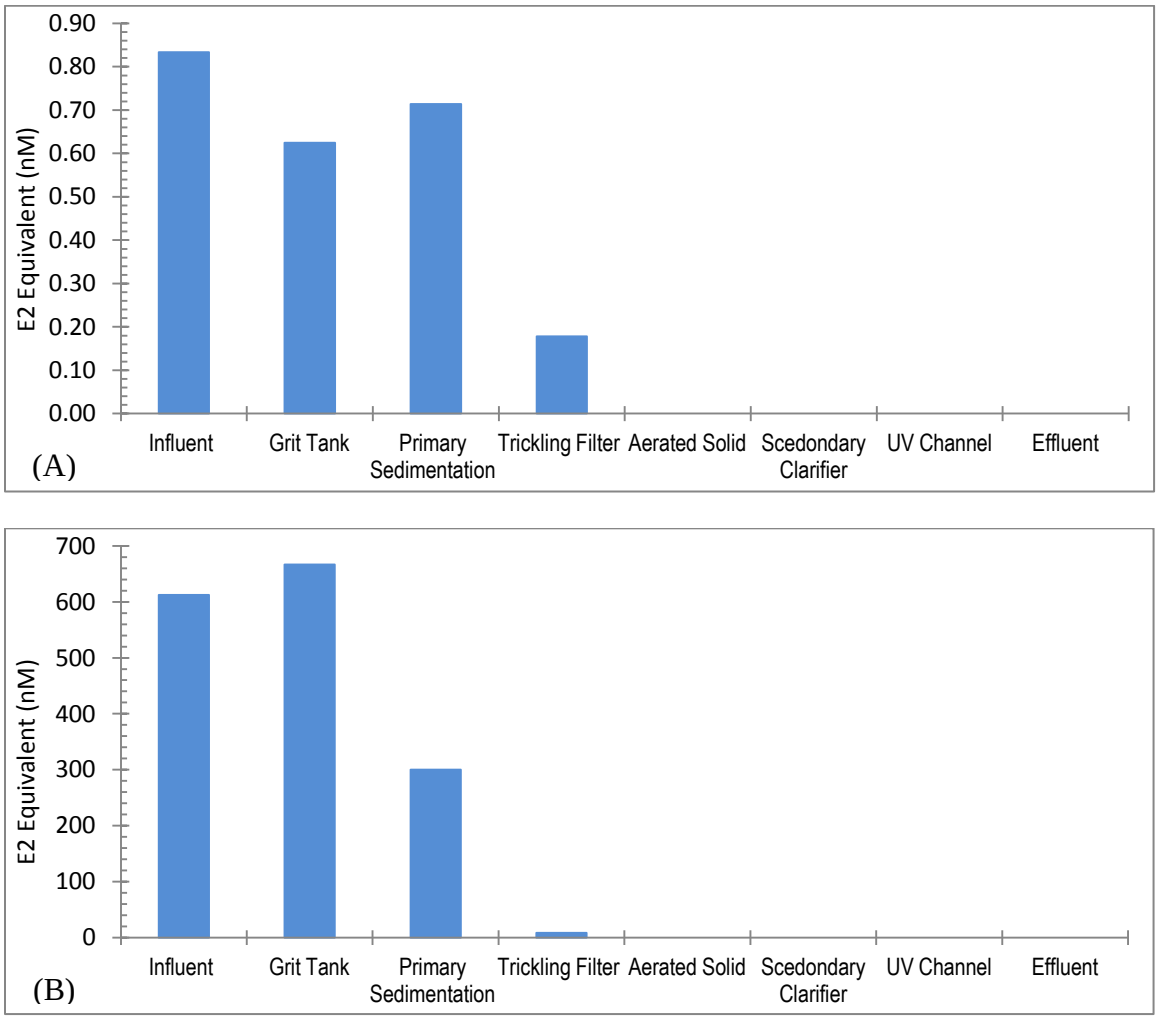
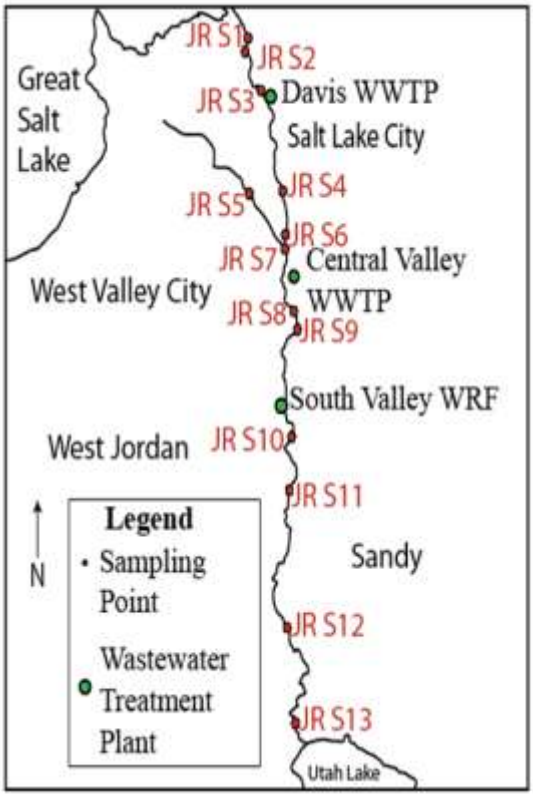



Figure 5.6 E2 equivalent estrogenic (A) and androgenic (B) activities in Central Valley Water Reclamation Facility (CVWRF).

the effluent water with the yeast estrogen screen, the level of estrogenic compound could be enough to disrupt endocrine systems in fish. For example, 17α-ethynylestradiol could induce vitellogenin produced in male fish at the concentration as low as 1 to 10 ng/L (Purdom, 1994). The detection limit of 17α-ethynylestradiol in YES is a few hundred ng/L.

Estrogenic activities were also detected in fresh water bodies in Utah. Thirteen sampling sites were selected from south of the Jordan River at Utah Lake outfall to the north where the Jordan River merges into the Great Salt Lake (Figure 5.7). Among these 13 sampling sites, water samples collected at the sites near I-80 (JRS4), Big Cottonwood Creek (JRS8), and the Utah Lake outlet showed elevated estrogenic activities. The results




| | |
|---|---|
| JR S1 | State Canal Bridge XING |
| JR S2 | South Davis South WWTP |
| JR S3 | Sewage Canal at Cudahey Lane XING |
| JR S4 | Surplus Canal at I-80 XING (USGS Gage) |
| JR S5 | 1300 S. Storm Drain at Mouth |
| JR S6 | Jordan River at 1100 W. 2100 S. below Diversion (USGS Gauge) |
| JR S7 | Big Cottonwood Ck. above Jordan River at 500 W. 4200 S. (SLCo Gage) |
| JR S8 | Little Cottonwood Ck. at 4900 S. 600 W. (SLCo Gage) |
| JR S9 | Jordan River at 9000 S. XING (SLCo Gage) |
| JR S10 | Jordan River at Bluffdale Road XING |
| JR S11 | Jordan River at Jordan Narrows Pump Station |
| JR S12 | Jordan River at Utah Lake Outlet at U-121 XING |

Figure 5.7 Sampling points along the Jordan River. Flow is from South to North.

cannot be correlated to the effluent of the wastewater treatment plant, but may be attributed to other point pollution sources by human activities. As shown in Figure 5.8, for Utah Lake samples, water is collected from the outfall of Spanish Fork, the Provo River, the inlet of the Jordan River, and at the bank of Utah Lake State Park. Estrogenic activities were detected in all the sites. EDC sources for the Utah Lake region have not been tracked in this study, but farm fields lay near the west bank of Utah Lake where the Spanish Fork and Provo River flow. Farms have a long history as the source of EDCs contaminated soil and leaching water due to the usage of pesticides, which are estrogenic compounds (Andersen et al., 2001; Baquero et al., 2008; Colucci et al., 2001). The influence of human activities in Provo city on the west of Utah Lake cannot be excluded as a potential factor for the high estrogenic level in this area. It is not surprising that high estrogenic activities were detected in Spanish Fork, the Provo River and their receiving water of Utah Lake and the Jordan River.


Figure 5.8 Sampling points of Spanish Fork, the Provo River, the Jordan River, and Utah Lake.

## Task 6: EDC and Antibiotic Resistant Genes and Bacteria in WWTP Effluent and Jordan River

Plate counts of culturable bacteria collected from two local wastewater treatment facilities showed that selection for EDC resistant bacteria is possible. After determining the MIC for each chemical, inhibitory concentrations of estrogen mimics were prepared in LB agar. As Table 5.1 shows in task 1, the inhibitory concentrations of 17α-ethynylestradiol, BPA, octylphenol, and nonylphenol were determined to be 75, 100, 75, and 75 mg/L, respectively. While 17β-estradiol, estrone, and estriol were not substrates of AcrAB-TolC or MexAB-OprM, they may be substrates of other RND efflux pumps expressed in wastewater bacteria. Subsequently, all wastewater samples were plated on agar containing 100 mg/L of 17β-estradiol, estrone, or estriol to test for selectivity even though the chemicals were not inhibitory.

Results showed that the effluent water quality of the East Canyon Water Reclamation Facility (ECWRF) was superior to that of the Central Valley Water Reclamation Facility (CVWRF) (Table 5.3). More antibiotic-resistant and EDC-resistant bacteria were present in CVWRF effluent than in ECWRF for all tested chemicals, with the exception of chloramphenicol resistance, which was not detected. Kanamycin, novobiocin, and doxycyline-resistant bacteria were detected in the effluent of CVWRF but not in ECWRF. EDC-resistant bacteria were detected at both facilities. Within the ECWRF, the levels of bisphenol-A and nonylphenol resistant bacteria were comparable (at 2.5 cfu/mL), while a higher concentration of OP, E2, and EE2 resistant bacteria was detected (at 5.5-6.5 cfu/mL). Within CVWRF, elevated concentrations of OP, NP, E2, and EE2-resistant bacteria were present compared to BPA-resistant ones. Overall, the

Table 5.3 Concentration (as CFU/mL) of antibiotic or EDC-resistant bacteria found in the effluent of two local wastewater treatment facilities (ECWRF = East Canyon Water Reclamation Facility; CVWRF = Central Valley Water Reclamation Facility).

| Chemical Resistance | ECWRF (CFU/mL) | CVWRF (CFU/mL) |
|---|---|---|
| Ampicillin | 0.5 | 6 |
| Kanamycin | 0 | 1 |
| Novobiocin | 0 | 1 |
| Ciprofloxacin | 0.5 | 2.5 |
| Doxycycline | 0 | 0.5 |
| Erythromycin | 0.5 | 4 |
| Cefmetazole | 0.5 | 0.5 |
| Chloramphenicol | 0 | 0 |
| Sulfamethoxazole | 3 | 10 |
| Bisphenol A | 2.5 | 6.5 |
| Nonylphenol | 2.5 | 32 |
| Octylphenol | 6 | 18 |
| 17β-Estradiol | 5.5 | 67 |
| 17α-Ethynylestradiol | 6.5 | 48 |

prevalence of EDC-resistant bacteria in CVWRF effluent was at least 2.6-fold greater than in ECWRF for BPA, OP, NP, EE2, and E2. Finally, growth results on agar verified that E2 was not toxic to *E. coli* or *Pseudomonas*, as excessive amounts of bacteria grew on high concentrations of E2 (at 100 mg/L), indicating the concentration was not inhibitory.

A comparison of both wastewater treatment facilities shows that polishing techniques such as media filtration may affect the concentrations of drug and EDC-resistant bacteria in the effluent. The ECWRF performs better than CVWRF at removing drug-resistant and EDC-resistant pathogens; the effluent from the former is pumped to a media filter unit prior to UV disinfection and discharge, while the effluent from the latter uses only UV disinfection after secondary treatment and sedimentation. These results are expected as the filtration media removes particulates such as bacteria. The CVWRF also

treats a larger volume per day with a more diverse sewage fingerprint than the ECWRF. The burden of a larger population may contribute to an increased pharmaceutical loading during secondary treatment, leading to acclimation of more drug and EDC-resistant bacteria.

EDC-resistant bacteria were found in all sampling sites of the Jordan River with higher concentrations than that found in wastewater effluent (Figure 5.9). Wastewater treatment facilities discharge the effluent water after disinfection processes that eliminate bacteria. In fresh water bodies such as lakes and rivers, antibiotics and EDCs could be degraded by microorganisms over time. However, the elevated estrogenic level was detected in the Jordan River and Utah Lake from Task 5. These compounds are persistent in water and sedimentation for days to months, a microorganism developed resistance mechanism to adopt the increasing level of emerging contaminants.

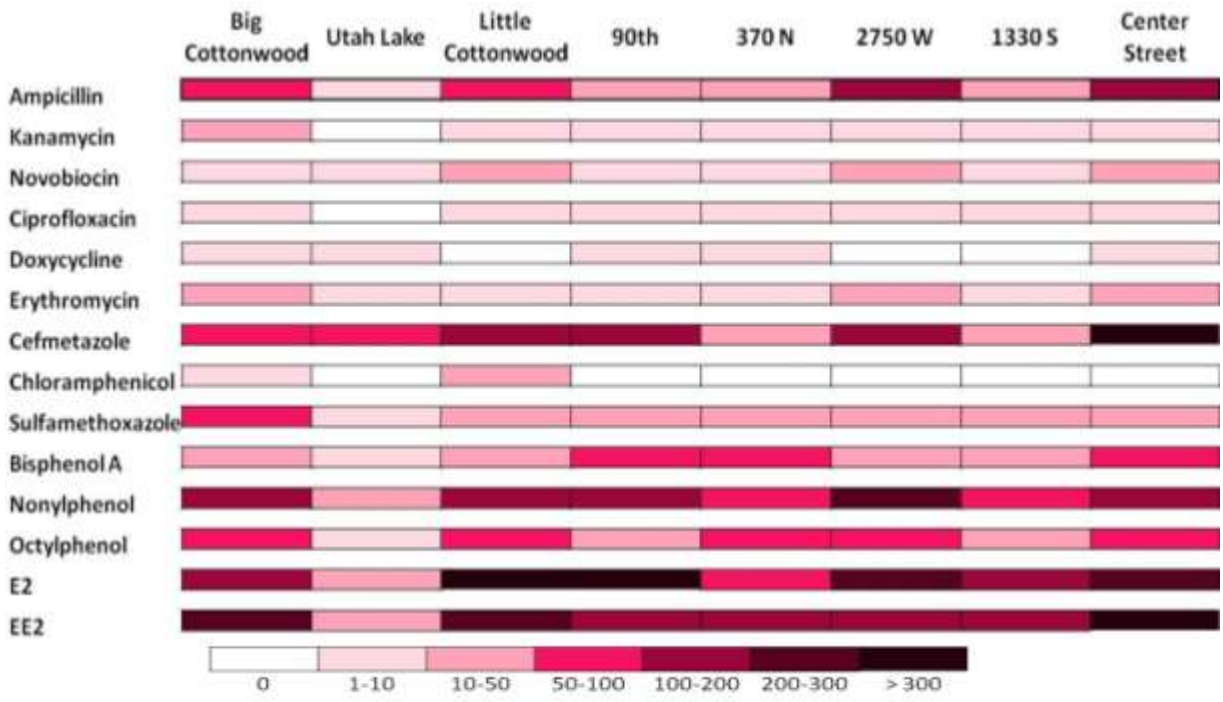



Figure 5.9 Antibiotic or EDC-resistant bacteria found in the Jordan River.

EDCs as Substrates of Other RND Efflux Pumps

Culturable bacteria strains grown on inhibitory concentrations of antibiotics or EDCs from the two wastewater treatment facilities were analyzed for the presence of RND efflux proteins through DNA amplification. A short segment of DNA encoding the inner membrane protein was amplified, with 11 different RND genes targeted in total (*acrB, mexB, adeJ, mtrD, cmeB, gesB, ttgH, bpeB, adeD, emhB,* and *eefB*), as shown in Table 4.6. Identification of the RND gene in each bacteria strain grown on antibiotics or EDCs provides more information on the substrate specificity of each pump. Figure 5.10 shows amplification of acrB found in bacteria colonies grown on 100 mg/L nonylphenol. EE2 resistance was frequently detected in bacteria possessing the gene products *mexB, bpeB, emhB, acrB, gesB, adeJ,* and *adeD* (Table 5.4). Nonylphenol resistance was most frequently detected in strains carrying *acrB, mexB,* and *emhB* (*Pseudomonas fluorescens*), although *cmeB, ttgH, bpeB,* and *adeD* gene products were positively identified in isolated colonies. NP is a by-product of nonylphenol ethoxylate and is generated during

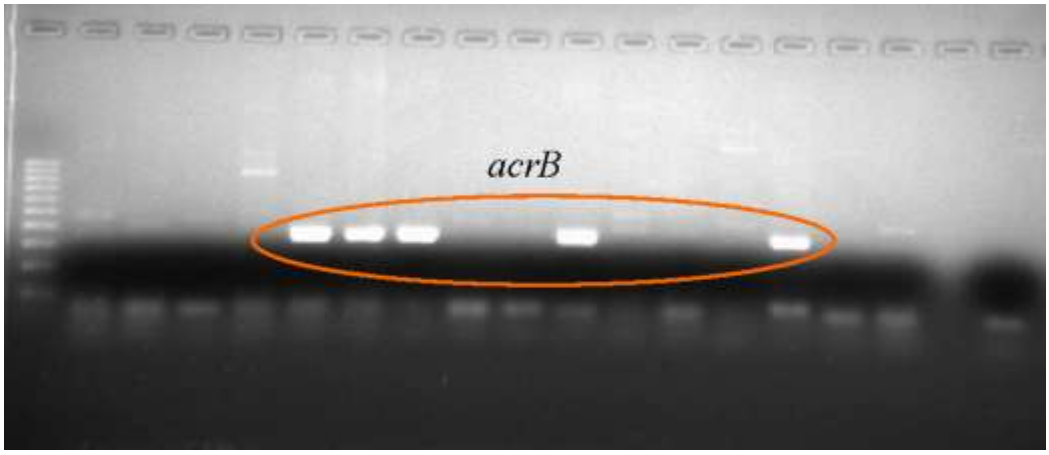



Figure 5.10 Amplification of a segment of *acrB* in pure colonies isolated from treated wastewater effluent. Samples were grown on 100 mg/L of nonylphenol. Ladder (lane 1) ranges from 100 to 1000 base pairs, at 100 bp intervals.

Table 5.4 Gene products identified from culturable antibiotic and EDC-resistant bacteria collected from two local wastewater treatment plants. Antibiotics and estrogenic EDCs may be substrates of RND proteins other than AcrAB-TolC or MexAB-OprM. Note: Not all DNA from culturable bacteria was amplified by the 11 RND inner membrane primers.

| Bacteria Grown On: | PCR Products Detected: |
|---|---|
| Ampicillin | *acrB, mexB, adeD* |
| Novobiocin | *emhB* |
| Ciprofloxacin | *acrB, mexB, adeJ, adeD, emhB* |
| Sulfamethoxazole | *acrB, mexB,emhB* |
| Bisphenol A | *acrB, mexB, bpeB, adeD, emhB* |
| Nonylphenol | *acrB, mexB, cmeB, ttgH, bpeB, adeD, emhB* |
| 17α-Ethynylestradiol | *acrB, mexB, adeJ, gesB, bpeB, adeD, emhB* |

wastewater treatment. It is not surprising that NP is a substrate of multidrug efflux proteins in gram-negative bacteria as surfactants such as Triton-X-100 are exported by AcrAB-TolC upon entering the cell. Like nonylphenol, bisphenol-A resistance was primarily limited to strains possessing *emhB, acrB,* and *mexB,* with additional efflux traced to strains expressing *bpeB* and *adeB.* Due to the high concentration of bacteria grown on E2-agar plates, amplification of the gene products listed was not attempted. The human estrogen may be a substrate of other antibiotic pump classes such as the MATE, ABC, and SMR; however, the concentration tested here was not inhibitory, and selection of E2-resistant bacteria was not possible. DNA from other antibiotic resistant bacteria (ampicillin, novobiocin, ciprofloxacin, and sulfamethoxazole) were amplified against the PCR primer set for verification.

These results indicate the presence of substrate specificity of various RND efflux pumps in gram-negative bacteria. In *E. coli*, the protein complex AcrAB-TolC exports a wide range of chemicals of varied structures; that endocrine disruptors EE2, BPA, and NP have been identified verifies the broad substrate range of this pump. The substrate

specificity of MexAB-OprM in *Pseudomonas aeruginosa* is limited to antibiotics: β-lactams, macrolides, fluoroquinolones, and chloramphenicol. These results show that additional substrates of MexAB-OprM include endocrine disrupting chemicals BPA, NP, and EE2. EmhB, expressed in *Pseudomonas fluorescens*, is a transporter of antibiotics and polynuclear aromatic hydrocarbons. Here, bacteria encoding emhB (Table 5.4) also conferred resistance to BPA, nonylphenol, and EE2, all containing aromatic functional groups. It should be noted that *Pseudomonas fluorescens* also expresses EmhB, and they may be responsible for exporting BPA, NP, and EE2. Substrates of other RND proteins followed the general classes of chemicals they export. For instance, substrates of TtgGHI in *Pseudomonas putida* encompass small aromatic solvents (Kieboom de Bont, 2001; Terán et al., 2007). It appears that only nonylphenol is recognized as a substrate, and larger organics with aromatic moieties such as EE2 and BPA are not.

PCR products from genes encoding other RND chemical efflux pumps were not identified (Table 5.4). *MtrD*, an exporter of steroids, bile salts, and fatty acids in the complex *MtrCDE* of *Neisseria gonorrhoeae* (Kamal et al., 2007) and *EefABC*, a multidrug exporter found in *Enterobacter aerogenes* (Masi et al., 2005), were not detected in strains growing on select antibiotics or estrogen mimics. This may indicate the antibiotics or endocrine disruptors are not substrates of efflux pumps in these strains, but more plausibe is that the bacteria were simply not present in the collected wastewater samples or that they were not culturable on LB media. The same argument could be made for *adeJ* from *Acinetobacter baumannii*, *gesB* in *Salmonella enterica*, and *ttgH* in *Pseudomonas putida* as these gene products were identified in only a few colonies. However, to clearly understand if emerging contaminants are substrates of RND proteins,

the entire complex must be cloned into a vector, expressed, and tested in a viable host such as *E. coli*.

Previous results in Task 5 showed that the estrogenic activity as measured by the yeast estrogen screen was higher for CVWRF effluent than for ECWRF, while CVWRF contains more drug-resistant bacteria in the effluent than ECWRF. The additional filtration process in ECWRF could remove microorganisms and EDCs (Alice et al., 2012; Bolong et al., 2009), leading a lower level of EDCs and drug resistant bacteria in the effluent. Antibiotic resistant genes were developed to enhance bacteria tolerance to toxic environments. With high selection conditions—chemical toxicity and a complex matrix—bacteria produce resistance genes to survive in the nonfavorable condition. CVWRF has higher estrogenic activities in the influent and effluent and receives sewage from more complicated sources comparing with ECWRF. These factors will introduce higher pressure to microorganisms to produce resistance genes and discharge to the river. Meanwhile, CVWRF receives a variety and larger amount of pathogens from human waste, which introduces more categories of drug-resistant bacteria. These drug-resistant genes could transfer from the same species and different species. Horizontal gene transfer powers the drug resistant ability for the entire microorganism community, which leads to more chemical extrusion and a higher concentration of EDCs in the effluent in CVWRF.

# CHAPTER 6

# CONCLUSIONS

## Implications in Cycling of Emerging Contaminants

Two significant conclusions can be drawn from this research. First, the substrate range of major multiresistant efflux proteins in bacteria includes the endocrine disrupting chemicals 17α-ethynylestradiol, nonylphenol, octylphenol, and bisphenol-A. After continuous exposure to wastewater, common bacteria such as *E. coli* and *Pseudomonas* have adapted and expanded their substrate range. Plating of wastewater bacteria on inhibitory levels of EDCs also revealed that RND proteins in other classes of bacteria may export estrogenic emerging contaminants.

Second, RND chemical efflux pumps play a role in the cycling of emerging contaminants in wastewater. The fates of endocrine disruptors, antibiotics, and other pharmaceuticals in wastewater and the environment are beginning to be understood. Presently, most estrogenic EDCs are only moderately degraded during secondary treatment, with longer sludge retention time (SRT) leading to more extensive removal of emerging contaminants. EDCs are not likely biodegraded because they are exported prior to degradation. In other words, chemical efflux competes with biodegradation in wastewater treatment, leading to discharge of EDCs into the environment through wastewater effluent. A longer SRT supports the efflux theory that EDCs have

insufficient contact time with oxidative enzymes and thus are not completely removed. During wastewater treatment, competing processes (or lack of) dictate the liquid-phase concentration of environmental contaminants. Efflux from microorganisms is just one response that chemicals are subjected to in the environment. Different physical, chemical, and biological processes are responsible for water quality. Sorption, biotransformation, and biodegradation generally contribute to an improvement in water quality by eliminating harmful chemicals. Hydrophobic organic contaminants sorb to membrane lipids and are wasted with the biomass that enters anaerobic digestion. Antibiotics are simply exported from the cell if the appropriate machinery is present. While common organics are completely mineralized, certain emerging contaminants such as the synthetic hormone 17α-ethynylestradiol are only partially oxidized. Here, we consider that the competition between efflux and oxidation determines the extent of degradation of organic molecules, EDCs particularly.

Other arguments could be postulated for the lack of degradation during biological treatment. Oxidizing enzymes such as oxygenases or oxidases may not recognize estrogenic EDCs as substrates. Toluene/o-Xylene monooxygenase (touABCDEF) from *Pseudomonas stutzeri* was transformed into *E. coli* strains to test for EDC degradation. Estrogenic actives did not show a noticeable decrease, indicating EDCs are not substrates of this enzyme. Partial degradation of the hormone 17β-estradiol has been attributed to general oxygenase activity in wastewater studies. Degradation of 17β-estradiol includes hydroxylation of the aromatic ring, attributed to oxygenase activity, or oxidation of the 17β-alcohol to ketone. The synthetic hormone 17α-ethynylestradiol is also oxidized at the aromatic ring, typical of oxygenase activity. BPA is degraded by *Pseudomonas*, with

a primary oxidation product of 4-hydroxyacetophenone (Kang & Kondo, 2002; Lobos et al., 1992). Nonylphenol, the degradation product of the detergent nonylphenol ethoxylate, is hydroxylated with concurrent removal of the alkyl side chain. The removal efficiencies of these EDCs are incomplete, as these chemicals enter the environment at biologically active concentrations.

## Implications for Other Emerging Contaminants

What remains to be discovered is the ability of chemical efflux pumps to transport other classes of persistent contaminants such as androgenic hormones, drugs of abuse, prescription pharmaceuticals, biocides, nanoparticles, and flavors and fragrances. The survival of emerging contaminants and wastewater indicators vary as they progress through different wastewater processes. As this research focuses on persistence through biological treatment, those that survive aerobic treatment should be further examined for chemical efflux. If the chemical is organic and resistant to degradation or is only partially degraded, it may be a substrate of a chemical efflux protein. To evaluate if an organic emerging contaminant is exported prior to biodegradation, similar experiments described thus far may be implemented, in which bacteria growth is compared between strains lacking the chemical efflux pump versus a strain overexpressing the pump. Of particular interest would be contaminants that are water soluble, such as flavors, fragrances, and drugs of abuse.

Improved water quality and wastewater pollution prevention are nationwide priorities. Water quality related to emerging contaminants is dependent on the presence of chemical-efflux proteins. This research showed that the major multidrug efflux pumps

in two bacteria strains are capable of exporting persistent anthropogenic endocrine disrupting chemicals from the cell. Natural estrogenic hormones (17β-estradiol, estrone, and estriol) may be substrates of these two specific RND proteins, but may be exported by other classes of chemical efflux pumps. When RND proteins are expressed, substrates are simply recycled back into the aquatic surroundings by efflux pumps before they fully interact with degrading enzymes. This competition between efflux and biodegradation may be responsible for environmental endocrine disruption, antibiotic resistance, and metal pollution.

# CHAPTER 7

## SCIENTIFIC CONTRIBUTION AND DISSEMINATION OF RESULTS

This research provides a better understanding of the biodegradation process of hormone and hormone mimics. Endocrine disruptors of 17α-ethynylestradiol (EE2), bisphenol-A (BPA), octylphenol (OP), and nonylphenol (NP) were verified as substrates of the drug-efflux pump of AcrAB-TolC in *E. coli* and MexAB-OprM in *Pseudomonas aeruginosa*, extending the substrates of these two proteins. Enzymes contributing to hormone degradation have been defined in this research. The oxygenase enzymes selected in this study cannot illustrate and explain the entire hormone degradation mechanism, but it will benefit the future hormone biodegradation studies.

Gene expression experiments provided the list of genes that respond to the presence of EE2, NP, and BPA, providing information for future EDCs biodegradation studies. The drug-resistant genes responding to EDCs should be tested in the future to confirm the EE2, NP, and BPA substrates. These drug-resistant genes have substrates of antibiotics and may extended substrate categories to steroid hormones and endocrine disruptors.

With the biodegradation kinetics' study, we discovered that the drug resistant process reduced EDCs biodegradation rate. This competition between organic uptake and extrusion affected the chemical degradation rate constant. The extrusion mechanism contributes to EDCs' biopersistence and incomplete removal during the wastewater

treatment process. This study is the one of the first to indicate the impact of drug-resistant in chemicals' oxidation and provides a critical factor that should be considered during chemical degradation studies and water treatment process design. In addition, the intermediates of BPA oxidation were more estrogenic than the parent compound, which has never been reported.

This research will allow environmental engineers to better understand why biological treatment can not completely degrade EDCs. With the effect of endocrine disrupting chemicals on aqueous animals found in the environment, we will make a contribution to future EDCs bioremediation studies and wastewater treatment of emerging contaminants.

APPENDIX A

PRIMER INFORMATION FOR THE GENE EXPRESSION EXPERIMENT

Table A.1 Primer information for the gene expression experiment

| Name | | Sequence | Tm | Product Length | Strain |
|---|---|---|---|---|---|
| ***E. coli*** | | | | | |
| **Resistance protein** | | | | | |
| cusA | **F** | GCGATTATTTCGTTGCCGCT | 60.0 | 396 | W3110 |
| | **R** | GATCACTACGATCGCCAGCA | 60.0 | | |
| acrD | **F** | CCCGTTGAACAATACCCCGA | 60.0 | 314 | W3110 |
| | **R** | GTATCGCCGGTTTTACGCAC | 60.0 | | |
| acrB | **F** | CATCCCCGTCCAGTCATAGC | 60.0 | 681 | W3110 |
| | **R** | GATCGTCCGGGCGAAGAAAA | 60.7 | | |
| acrF | **F** | TGTGCAACGCTGCTTAAACC | 60.0 | 132 | W3110 |
| | **R** | GGATCCGAGGATTTTGCCGA | 60.2 | | |
| mdtBC | **F** | CATTCAGGTGGTCACGCTCT | 60.0 | 529 | W3110 |
| | **R** | GAGGCTACCTTTTGCCGAGT | 60.0 | | |
| yhiV | **F** | CAGAACCGGCACCATGACT | 60.0 | 943 | W3110 |
| | **R** | CTGCTGGACAACGGTAACCT | 60.0 | | |
| **Oxidation protein** | | | | | |
| katE | **F** | TTGCTGAAAACGAACAGGCG | 59.97 | 242 | W3110 |
| | **R** | GTTCGGTTCGTAATTCGCCG | 59.98 | | |
| KatG | **F** | ATGCCGCCACGAAGTCTTTA | 60.04 | 266 | W3110 |
| | **R** | CAGCAAAAACGGCGTCTTCA | 59.97 | | |
| **Housekeeping** | | | | | |
| mdoG | **F** | CCCAAAAGCAACTTGCCCTC | 59.97 | 474 | W3110 |
| | **R** | ATCAGTCGGTTTTGGACGCT | 59.97 | | |
| ***Pseudomonas putida*** | | | | | |
| **Resistance protein** | | | | | |
| arpB | **F** | AACCGCTTAAGAAGGGCGAA | 59.96 | 135 | F1 ATCC#700007 |
| | **R** | GCAGGAATGGCACCTTGTTG | 60.04 | | |
| ttgB | **F** | GTATCAGATCGACCCTGCCG | 60.04 | 738 | F1 ATCC#700007 |
| | **R** | CCTTCCTCGATCAGCCAGTC | 59.90 | | |

Table A.1 continued

| | | | | | |
|---|---|---|---|---|---|
| **Oxygenase** | | | | | |
| Pput_1893 | **F** | ACGAGGCCAACCGTAAAGAG | 60.04 | 116 | F1 ATCC#700007 |
| | **R** | CTGCCCATAGAGGTCGTAGC | 59.68 | | |
| Pput_2363 | **F** | GCACATGTACACCCCAGGAG | 60.39 | 94 | F1 ATCC#700007 |
| | **R** | GTACCGGTAGCCATGGTGAT | 59.24 | | |
| Pput_2880 | **F** | GGACTCGCGCCTTGAATACT | 60.18 | 226 | F1 ATCC#700007 |
| | **R** | CTCCAGACGATTGCGGTACA | 59.83 | | |
| Pput_2897 | **F** | TGCCGAAGAGATTCAGGTGG | 59.75 | 86 | F1 ATCC#700007 |
| | **R** | AGTGCGTGCTACCGACATAG | 59.9 | | |
| Pput_3846 | **F** | CCCAGCCTGTTCATTTCCCA | 60.25 | 200 | F1 ATCC#700007 |
| | **R** | GGTGGGAAGCCGTAGAAGTC | 60.11 | | |
| Pput_5066 | **F** | CACCTTGGGCCTCTCAGAAC | 60.32 | 100 | F1 ATCC#700007 |
| | **R** | GAAGTGCCTCACGAAGTCCT | 59.68 | | |
| **Housekeeping** | | | | | |
| rpoD-F | **F** | TTGGCGGGAAATACGGTTGA | 59.96 | 191 | F1 ATCC#700007 |
| | **R** | AGGAAGGCAACATCGGTCTG | 60.04 | | |

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***Pseudomonas aeruginosa***

| | | | | | |
|---|---|---|---|---|---|
| **Resistance protein** | | | | | |
| czrA | **F** | GATCCTCTCGGTGACCTTCG | 59.6 | 634 | PAO1 |
| | **R** | CTTCTCCGCAGTGTCGTTGA | 60.3 | | |
| mexB | **F** | GGGATCGACAATCTGCGCTA | 60.0 | 558 | PAO1 |
| | **R** | GTCGGGATTGACCTTGAGCA | 60.0 | | |
| mexD | **F** | TTGGCGAGATCCAGACCAAC | 60.0 | 320 | PAO1 |
| | **R** | TTCCCATTTCACGCTGACGA | 60.0 | | |
| mexF | **F** | CCACCCTGATCAAGGACGAA | 59.4 | 471 | PAO1 |
| | **R** | ATTCGCTGATGGGTAGCTGG | 59.9 | | |
| mexK | **F** | GCCCTCTTCGAAGCCGTATT | 60.2 | 297 | PAO1 |
| | **R** | GTGCACCTGGATTGGGAAGA | 60.0 | | |
| mexY | **F** | TGGACGAGATCAACACCACC | 59.7 | 429 | PAO1 |
| | **R** | ACACGATCAACACCGAGAGG | 59.8 | | |
| triC | **F** | CGAATCGACGGTCTTCGTCT | 59.9 | 508 | PAO1 |
| | **R** | GGACAGCCGGTAGAAACGAT | 59.8 | | |
| mexI | **F** | TGGAAAGCTCGACCATCACC | 60.04 | 136 | PAO1 |
| | **R** | ACCGAGGTCGAGGAAAGGTA | 59.96 | | |
| mexW | **F** | GGTGAGCCGGCAGAACTATT | 60.1 | 607 | PAO1 |
| | **R** | GATGCCGATGTAGACCGAGG | 60.0 | | |
| mexQ | **F** | CGGTGAACGGTTTCGTCAAC | 60.4 | 479 | PAO1 |
| | **R** | CTGGGCAGTGACCTTGTAGG | 60.4 | | |
| mexN | **F** | CGCAGGACATCAACCTCAGT | 60.0 | 367 | PAO1 |
| | **R** | CGCAGGTAGAACCAGTCCAG | 60.1 | | |

Table A.1 continued

| | | | | | |
|---|---|---|---|---|---|
| muxB | **F** | GGATTGTTCGGCATACCGCT | 60.8 | 872 | PAO1 |
| | **R** | CATCCATCCGGTGACCATCC | 60.3 | | |
| **Oxygenase** | | | | | |
| alKB1 | **F** | GCGAGGATTCTCTGCCGAAT | 60.25 | 301 | PAO1 |
| | **R** | ATGACGATAGCCCGCAGATG | 60.04 | | |
| alkB2 | **F** | GCTACTACCGCGTACTGTCC | 59.97 | 520 | PAO1 |
| | **R** | CCGAGGAAGAAGATCGCTCC | 59.97 | | |
| PA0817 | **F** | GCTTCGTGATCGAGCCCTAC | 60.6 | 71 | PAO1 |
| | **R** | GTCGAAGAGAAACAGCGTGG | 59.22 | | |
| PA0880 | **F** | GTGCGCAGAAGATCAACCTG | 59.55 | 94 | PAO1 |
| | **R** | ACGATGAAACAGAGGTCGGC | 60.39 | | |
| PA1538 | **F** | AACGAATACCTCCAGCGCAA | 60.04 | 175 | PAO1 |
| | **R** | GTGTCGTGGTGAGGTGGTAG | 60.04 | | |
| PA2024 | **F** | GGTTTCGTAGAAGGCGATGC | 59.35 | 100 | PAO1 |
| | **R** | GCTATGCTTGTCCGTGGAGG | 60.81 | | |
| PA2083 | **F** | TTTCCCCGTGCTAGGTTTCC | 59.96 | 103 | PAO1 |
| | **R** | AAGTAACTTGCGTCGTCGGT | 59.97 | | |
| PA2085 | **F** | GGGTCAGCGATTTCCTCGTC | 60.81 | 74 | PAO1 |
| | **R** | GAACAGCGCGAACCAGGTAT | 60.74 | | |
| PA2097 | **F** | AAACGGGAGCACAACAATGC | 59.97 | 236 | PAO1 |
| | **R** | TCGGCTCGAAGGAAAACGAA | 59.97 | | |
| PA2355 | **F** | TGAGTAGAAGGTCGGTTGCG | 59.76 | 76 | PAO1 |
| | **R** | CCCGCTGGACTACAAGATCC | 59.89 | | |
| PA2546 | **F** | CGATAGAAAGCGATGCTGCG | 59.84 | 108 | PAO1 |
| | **R** | TAGTCCACGGTCCAAGAGGT | 59.89 | | |
| PA3389 | **F** | CCCAACCTGGAAGAATGCGA | 60.32 | 116 | PAO1 |
| | **R** | CCGAGGGAAAGGTTGTCGTT | 60.25 | | |
| pqsH | **F** | GGGACTTCTCCATAGCGACC | 59.61 | 161 | PAO1 |
| | **R** | GAGGAATACCCTCGTTCGCC | 60.25 | | |
| pqsL | **F** | GGCTGGCCTACTTCTATCCG | 59.68 | 212 | PAO1 |
| | **R** | CAGGTTCAGGTAGCCGATGG | 60.18 | | |
| **Housekeeping** | | | | | |
| rpoD-P | **F** | CGCAACAGCAATCTCGTCTG | 59.91 | 319 | PAO1 |
| | **R** | TCCACGGTACCCATTTCACG | 60.04 | | |

# APPENDIX B

## MINIMAL MEDIA SOLUTION

Table B.1 Minimal media solution

| **Feed** | **Final Concentration** | |
|---|---|---|
| $NaHCO_3$ | 44.6 | g/L |
| Peptone | 6 | g/L |
| Sodium Acetate | 1.25 | g/L |
| $NH_4Cl$ | 2.26 | g/L |
| $MgCl_2\ 6H_2O$ | 6.86 | g/L |
| $CaCl_2\ 2H_2O$ | 1.72 | g/L |
| $KH_2PO_4$ | 0.6675 | g/L |
| Citric Acid | 0.1092 | g/L |
| Hippuric Acid | 0.08 | g/L |
| $Na_3NTA\ 2H_2O$ | 0.0144 | g/L |
| $Na_3EDTA\ 4H_2O$ | 0.006 | g/L |
| $FeCl_3\ 6H_2O$ | 0.06 | g/L |
| $H_3BO_3$, | 0.01 | g/L |
| $ZnSO_4\ 7H_2O$ | 0.006 | g/L |
| $MnCl_2\ 4H_2O$ | 0.0048 | g/L |
| $CuSO_4\ 5H_2O$ | 0.0028 | g/L |
| KI | 0.0012 | g/L |
| $Na_2MoO_4\ 2H_2O$ | 0.0012 | g/L |
| $CoCl_2\ 6H_2O$ | 0.0012 | g/L |
| $NiCl_2\ 6H_2O$ | 0.0012 | g/L |
| $Na_2WO_4\ 2H_2O$ | 0.0012 | g/L |

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