DISPARATE OUTER MEMBRANE EXCLUSIONARY PROPERTIES AND INTRINSIC RESISTANCE TO TRICLOSAN IN *PSEUDOMONAS* SPECIES ISOLATED FROM OKLAHOMA SURFACE WATERS

By

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Abstract: Outer membrane exclusionary properties of *Pseudomonas aeruginosa* underlie its intrinsic resistance to the hydrophobic biocide triclosan, but environmental bacteria have not been analyzed for similar properties. Bacterial communities were sampled by directly plating surface waters from three locations onto Reasoner's 2A agar (R2A) either lacking or containing triclosan. Two isolates from each plating method were chosen for detailed examination based on their relationships to triclosan and phylogenetic similarities to *P. aeruginosa*. Macrobroth dilution bioassays and batch cultural growth kinetics were initially employed to assess the degree to which isolates were intrinsically resistant to the mechanistically-disparate hydrophobic molecules novobiocin and triclosan. Minimal inhibitory concentrations (MICs) for novobiocin and triclosan for susceptible organisms were predictably low with the exception of LD7A being resistant to novobiocin. In contrast, MICs for organisms selected for with triclosan were high. Triclosan titrations of batch cultures revealed growth kinetics for isolates obtained in the absence of triclosan were inhibited in a concentration-dependent manner, while growth kinetics were similar to respective controls at all concentrations for resistant isolates selected for with triclosan. These data were confirmed by outer membrane accessibility to the hydrophobic probe 1-N-phenylnapthylamine (NPN) by measuring relative fluorescence of treated isolates. The results revealed susceptible isolate LD8B possessed an exceptionally accessible outer membrane in comparison to LD7A, although both susceptible isolates had outer membranes significantly less accessible to NPN than triclosan-resistant isolates. The degree to which isolates could be sensitized to novobiocin and triclosan in the presence of the outer membrane permeablizer compound 48/80 was also determined using growth kinetics. The triclosan-susceptible isolates were permeable to both novobiocin and triclosan regardless of compound 48/80 concentrations. Triclosan-resistant isolate HD33 was able to resist sensitization to novobiocin and triclosan in the presence of compound 48/80, whereas isolate HD36 was slightly sensitized to both novobiocin and triclosan. These results support the notion that outer membrane exclusion underlies intrinsic resistance to hydrophobic substances in some, but not all *Pseudomonas* spp. selected for on the basis of triclosan resistance from municipal surface waters and that multiple triclosan resistant mechanisms work in concert in these refractory bacteria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Gram-negative bacteria possess a unique feature in the outer cell envelope known as the outer membrane. It is composed of phospholipids lining the inner leaflet and an outer leaflet consisting primarily of highly negative-charged lipopolysaccharides or lipooligosaccharides, which preclude surface association with and permeability of nonpolar molecules (Nikaido and Vaara, 1985; Silhavy *et al.*, 2010). Water-filled channels called porins restrict periplasmic entry to polar molecules on the basis of size, while all nonpolar molecules are excluded regardless of size (Nikaido, 1976; Nikaido and Vaara, 1985; Delcour, 2009). The outer membrane of gram-negative bacteria thus confers intrinsic resistance to many antibiotics and biocides on the basis of outer cell envelope exclusion.

The hydrophobic biocide triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is increasingly utilized as an antiseptic or preservative in many common household and healthcare products due to its broad-spectrum antibacterial activity for both gram-negative and gram-positive organisms (Singer *et al.*, 2002; Dhillion *et al.*, 2015). Despite its hydrophobic nature, it is atypically able to reach its cytoplasmic enoyl-acyl carrier protein (ACP) reductase target (McMurry *et al*., 1998) by passively partitioning through the outer membrane of almost all gram-negative organisms into the periplasm. *Pseudomonas aeruginosa* is a ubiquitous soil organism that is an important opportunistic nosocomial pathogen in immunocompromised individuals. It is intrinsically resistant to many disparate antibacterial agents (Li *et al.*, 2000b) including high

concentrations of triclosan (Jones *et al.*, 2000).

Research in our laboratory has centered on the gram-negative cell envelope of opportunistic pathogens of primarily nosocomial relevance and the molecular mechanisms underlying their relationships with nonpolar antimicrobial agents. Champlin and coworkers (2005) reported that chemical modification of the *P. aeruginosa* outer membrane sensitized the organism to triclosan when using the disparate outer membrane permeabilizers polymyxin B nonapeptide, compound 48/80, or ethylenediaminetetraacetate (EDTA). They reasoned that intrinsic low-level triclosan resistance was due to the exclusionary function of the outer membrane for nonpolar molecules in the environment. Likewise, *in vitro* studies with high concentrations of the hydrophobic biocide implicated the same exclusionary properties in exceptional intrinsic resistance to triclosan.

Subsequent work in this laboratory with research strains of *P. aeruginosa* (Ellison *et al*., 2007) revealed synergy between compound 48/80 and triclosan to be transient, yet not due to the repair of a temporal diffusion pathway in the outer membrane specific for hydrophobic molecules. They went on to implicate the expression of triclosan-recognizing active efflux pumps capable of nullifying the effects of outer membrane permeabilization and subsequent biocide sensitization. This work established that a structurally and functionally intact outer membrane (Champlin *et al*., 2005) in concert with an active efflux pump system (Ellison *et al*., 2007) representing a second line of defense are responsible for intrinsic resistance to the hydrophobic biocide triclosan in *P. aeruginosa*. Further work showed the outer membrane of *Pasteurella multocida*, a zoonotic gram-negative opportunistic bacterium, is naturally permeable to hydrophobic molecules in general, and that its exceptionally high susceptibility to triclosan is dependent on concentration of the biocide. (Ellison and Champlin, 2007). Capsulated and noncapsulated variants were equally susceptible indicating that the rate of triclosan permeation was unaffected by the capsular phenotype.

Bullard and coworkers (2011) next implicated changes in cell envelope unsaturated fatty acid composition in response to triclosan exposure as factors possibly contributing to resistance. A study by Clayborn *et al*. (2011) showed that triclosan methylation mitigated its ability to inhibit the growth of both the triclosan-susceptible gram-negative organism *P. multocida*, as well as outer membrane-permeabilized *P. aeruginosa*. They concluded that the ability to covalently inactivate the biocide might represent an additional resistance mechanism in these organisms. More recent work has shown that the outer membrane of the phylogenetically-related opportunistic pathogen *Burkholderia multivorans* resists the effect of outer membrane permeabilization and subsequent sensitization to triclosan through outer membrane modifications (McDonald *et al*., 2017) under conditions that render *P. aeruginosa* susceptible (Ellison and Champlin, 2007).

Municipal water treatment processes incompletely remove triclosan from wastewater (Bester, 2005). Due to its overuse over several decades, triclosan and its metabolic residues have been released into surface waters where they accumulate as components of effluent (Singer *et al*., 2002; Bester, 2005). The U.S. Geological Survey performed a study on the presence of wastewater contaminants in U.S. streams from 1999 to 2000, which revealed triclosan to be among the most common organic wastewater contaminants in 57.6% of streams sampled (Kolpin *et al.*, 2002). Recent studies suggest residue concentrations and exposure duration potentiate the impacts of triclosan on resistance selection and decreases in microbial diversity (Lubarsky *et al*., 2012; Nietch *et al.*, 2013). Moreover, selective isolation of triclosan-resistant bacteria from feedlot and residential soil samples revealed *Pseudomonas* spp. to be highly prevalent (Welsch and Gillock, 2011). However, these workers did not investigate the mechanism(s) underlying such high-level resistance, or the possibility that surface water bacteria are triclosan-resistant by virtue of exclusionary properties similar to what our laboratory has shown for *P. aeruginosa* (Champlin *et al*., 2005; Ellison and Champlin, 2007) has not been addressed in the literature.

Several major pitfalls exist due to the indiscriminate use of triclosan. First, nonspecific efflux pumps are found extensively in *P. aeruginosa*, thereby contributing to its exceptional resistance to a wide variety of antibacterial molecules (Schweizer, 2001). Furthermore, a study by Ellison and coworkers (2007) revealed the lack of these efflux pumps resulted in more sustained susceptibility to the hydrophobic biocide triclosan in outer membrane-permeabilized cells. It is conceivable that cross-resistance could result when, for example, there is an overexpression of a non-specific multidrug efflux pump in response to the presence of triclosan. The second potential pitfall is more controversial. Triclosan has been implicated as being an endocrine system-disrupting chemical with the potential to be harmful to a wide range of the general population (Wang and Tian, 2015). Triclosan exposure may result in adverse effects associated with the production of androgens in male rats (Kumar *et al*., 2007) and in disruption of responses regulated by estrogen in female rats (Stoker *et al*., 2010). Triclosan also has been implicated as negatively impacting thyroid hormone production, leading to abnormal levels of thyroxine in the blood of female rats (Crofton *et al*., 2007). However, a conflicting study evaluated the function of the thyroid in humans exposed to triclosan-containing toothpaste for an extended period of time and revealed toothpaste containing 0.3% triclosan does not alter thyroid function (Cullinan *et al*., 2012). Finally, a major negative consequence of environmental triclosan contamination may be the subsequent formation of dioxin (2,8-dichlorodibenzo-*p*-dioxin), a toxic and even more stable derivative of the biocide found in wastewater produced in the presence of UV light irradiation from triclosan deterioration (Latch *et al*., 2003; Aranami and Readman, 2007). This problem was shown to be exacerbated in seawater when compared to the longevity of dioxin in freshwater (Aranami and Readman, 2007).

More recent research in our laboratory has focused on bacteria obtained from surface waters in three Oklahoma locations by directly plating samples onto Reasoner's 2A agar (R2A) either lacking or containing triclosan (DeGear *et al*., 2017). Sequencing of 16S rDNA gene

sequences revealed diverse genera to be present in the absence of triclosan, while only *Pseudomonas* species were selected for on triclosan-containing R2A. Two isolates from each sampling method were chosen based on their extreme susceptibility and resistance phenotypes regarding triclosan and their degrees of sequence similarity to *P. aeruginosa*. This allowed for the determination of the extent to which the selected organisms shared intrinsic outer membrane impermeability properties with closely-related *P. aeruginosa* for the hydrophobic molecules novobiocin and triclosan. Co-resistance to other hydrophobic substances was expected if in fact outer membrane impermeability properties could be implicated as being responsible for intrinsic resistance to triclosan in surface water bacteria.

DeGear *et al*. (2017) demonstrated that the isolates obtained both in the absence or presence of triclosan were gram-negative organisms. Triclosan resistance was confirmed by streak inoculating the bacteria on R2A and R2A supplemented with triclosan (40.0 µg/ml). Only bacteria isolated in the presence of triclosan and one *Rheinheimera* sp. isolated in its absence were able to initiate growth at this high concentration of the hydrophobic biocide, while all other bacteria isolated in the absence of triclosan failed to initiate growth. Novobiocin and triclosan minimal inhibitory concentrations (MICs) determinations revealed bacteria isolated in the absence of triclosan to possess disparate relationships with novobiocin, but all were susceptible to triclosan with the exception of the *Rheinheimera* sp. mentioned above. These data suggest that some indigenous gram-negative bacteria have outer membranes permeable to some hydrophobic molecules by virtue of an innate hydrophobic diffusion pathway, thereby allowing entry of hydrophobic molecules into the periplasmic space. In contrast, exceedingly high MICs for both novobiocin and triclosan were observed in *Pseudomonas* spp. and the *Rheinheimera* sp. bacteria isolated in the presence of triclosan. These data are consistent with MICs for the highly triclosan-resistant and phylogenetically-related *P. aeruginosa* (Champlin *et al.*, 2005). Finally, the hydrophobic fluorescence probe 1-N-phenylnapthylamine (NPN) was employed to further

evaluate the relative accessibility of isolate cell surfaces to the partitioning of hydrophobic molecules into outer membranes (DeGear *et al*., 2017). Isolates obtained in the absence of triclosan were more permeable than isolates obtained in the presence of triclosan. Taken together, this work suggests that gram-negative bacteria selected for on the basis of their intrinsic resistance to triclosan from municipal surface waters are closely related members of the genus *Pseudomonas*. Moreover, outer membrane exclusionary properties appear to underlie their intrinsic resistance to hydrophobic molecules in general, and triclosan in particular, in a manner similar to that seen in *P. aeruginosa*.

I hypothesized that *Pseudomonas* spp. selectively isolated for in the presence of large concentrations of triclosan from municipal surface waters are intrinsically resistant to the biocide by virtue of outer membranes which possess impermeability properties for hydrophobic substances in general, and triclosan specifically. Therefore, the purpose of this study is to test this hypothesis by determining if the chemical permeabilization of the outer membranes of selected *Pseudomonas* spp. results in sensitization to mechanistically disparate hydrophobic molecules novobiocin and triclosan.

CHAPTER II

METHODOLOGY

Bacterial Isolates

Organisms used in the present study represent a model system, which are described in Table 1. *P. aeruginosa* PAO1 is retained in this laboratory as a reference organism, while *Escherichia coli* ATCC 25922 was obtained from the American Type Culture Collection (Manassas, VA). Environmental surface water isolates LD7A, LD8B, HD33, and HD36 were isolated and provided by Dr. R.V. Miller (Oklahoma State University). Surface water samples from three independent sites receiving treated wastewater from the Oklahoma towns of Holdenville, Lawton, and Weatherford, OK were aseptically plated directly onto R2A (Becton Dickinson Difco, Sparks, MD) or R2A containing triclosan (R2A- TCS; 20 or 40 μg/ml; Irgasan DP 300; Ciba Specialty Chemical Corp., High Point, NC) and incubated for 48 h at ambient temperature (DeGear *et al.*, 2017). Isolates LD7A and LD8B were isolated in the absence of triclosan, while isolates HD33 and HD36 were isolated in the presence of triclosan and were therefore considered to be intrinsically resistant. Maintenance of all cultures occurred under cryopreserved conditions at -80°C as previously described (Darnell *et al*., 1987).

Table 1. Control organisms and model system isolates.

Organism	Identification to genus[a]	Description	Reference
Controls			
E. coli ATCC 25922	n/a	CLSI control strain for antibacterial susceptibility testing.	American Type Culture Collection
P. aeruginosa PAO1	n/a	Wild-type strain; contains an exceptionally impermeable outer membrane for nonpolar molecules.	Champlin *et al*., 2005
R2A[b]			
LD7A	*Pseudomonas*	Environmental surface water isolate.	DeGear *et al*., 2017
LD8B	*Pseudomonas*	Environmental surface water isolate.	DeGear *et al*., 2017
R2A-TCS[c]			
HD33	*Pseudomonas*	Environmental surface water isolate.	DeGear *et al*., 2017
HD36	*Pseudomonas*	Environmental surface water isolate.	DeGear *et al*., 2017

[a] Based on comparisons of 16S rRNA sequence determinations with most clearly related genera in GenBank. DeGear *et al*., 2017.
[b] Organisms isolated in the absence of triclosan.
[c] Organisms isolated in the presence of triclosan at a concentration of either 20.0 or 40.0 μg/mL.

Cultivation Conditions

Working cultures were prepared by streaking cells from cryopreserved cultures onto R2A or R2A-TCS (20 μg/ml) in accordance with the method of isolation, incubated at 25°C or 37°C (*E. coli* ATCC 25922 only) for 18 h (*E. coli* ATCC 25922 only) or 24 h, and stored at 4°C for later use. These cultures were employed to provide inocula for overnight starter cultures consisting of about 20 ml of Mueller Hinton Broth (MHB; Becton Dickinson Difco) in 125-ml culture flasks which were incubated in an Excella® E24 environmental shaker incubator (New Brunswick Scientific Co., Edison, NJ) for 15-18 h at 25°C with rotary aeration at 180 rpm.

Chemical Solutions

Ethanol (95%; Decon Laboratories Inc., King of Prussia, PA) was used as solvent to prepare triclosan stock solutions in order to potentiate its solubility in aqueous test solutions. They were prepared to desired concentrations, sealed tightly in Teflon-lined screw-capped tubes, and stored at 4°C for later use. Novobiocin (Sigma- Aldrich Chemical Co., St. Louis, MO) and compound 48/80 (Sigma-Aldrich Chemical Co.) stock solutions were dissolved in MHB to obtain desired concentrations, sterilized with the aid of a filter (Fisherbrand 0.22-μm syringe filter assemblies; Thermo Fisher Scientific Inc., Pittsburgh, PA), and stored at 4°C for later use.

MIC Susceptibility Bioassay

Susceptibilities to the hydrophobic antibacterial agents novobiocin and triclosan were determined using a conventional two-fold macrobroth dilution bioassay (Darnell *et al*., 1987) per the modified method by Ellison *et al.* (2007). Sterile novobiocin and triclosan stock solutions were prepared as described above to final concentrations of 1,024 μg/ml and 128 μg/ml, respectively. Overnight starter cultures were prepared as described above. Test cultures were prepared in culture tubes (18 x 150 mm; Kimax) by aseptically inoculating 5.0 ml of sterile MHB with starter culture cells in stationary phase to an initial OD_{620} of 0.05 with the aid of a Spectronic

20D+ optical spectrophotometer (Thermo Fischer Scientific Inc., Waltham, MA) and employing incubation methods as described above until an OD_{620} of 0.1 was reached. A 50-ml polypropylene centrifuge tube (Corning® disposable centrifuge tubes; Sigma-Aldrich Co.) containing 31.1 ml of sterile MHB was used to dilute 0.1 ml of a test culture cell suspension to yield approximately 5.0 x 10^5 CFU/ml as the final cell density. Twofold serial dilutions of novobiocin and triclosan were performed using MHB as diluent in culture tubes (13 x 100 mm, Pyrex). Each culture tube in the dilution series was inoculated with 1.0 ml of test culture cell suspension to obtain a final volume of 2.0 ml and final cell density of 2.5 x 10^5 CFU/ml. Cultures were incubated for 24 h at 25°C with rotary aeration at 180 rpm (Excella® E24 environmental shaker incubator) and visually scored for turbidity to determine MICs on the basis of the lowest antibacterial agent concentration to completely inhibit growth initiation.

Effect of Triclosan Titration on Cultural Growth Kinetics

The effect of different concentrations of triclosan on growth of the environmental isolates was determined by titration of batch cultures using a 10-fold dilution series and monitoring total cultural cell density turbidimetrically to determine growth kinetics as previously described by Ellison and Champlin (2007). For a representative photo taken at 6 h post inoculation for each experiment, see Figure 1. Starter culture cells in stationary phase were used to inoculate about 50 ml of sterile MHB to an initial OD_{620} of 0.05 using a Spectronic 20D+ optical spectrophotometer (Thermo Fischer Scientific Inc.). Test cultures were acquired by distributing 5.0 ml aliquots of inoculated MHB into each of six sterile culture tubes (18 mm x 150 mm, Kimax) and immediately adding experimental treatments by aseptically pipetting 20 µl of appropriate triclosan stock solutions in ethanol (95%) into each culture tube such that final concentrations of 0.02, 0.2, 2.0, and 20.0 µg/ml were obtained after brief mixing with vortex agitation. Control cultures received either no treatment or 20 µl of ethanol (95%), the maximal volume used which yielded a final concentration of less than 0.4%. Cultures were incubated using the above method

and growth was measured by reporting OD_{620} at 30-minute intervals for 6 hours. Resultant OD_{620} measurements were plotted semi-logarithmically as a function of time to visualize triclosan titration effects on batch cultural growth kinetics.

NPN Uptake Chemical Assay

The hydrophobic fluorescent probe 1-*N*-phenylnapthylamine (NPN; Sigma-Aldrich Chemical Co.) was employed to assess the degree of accessibility of cell surface hydrophobic regions to nonpolar substances using modifications (Ellison and Champlin, 2007; McDonald, *et al.*, 2017) of the Helander and Matilla-Sandholm (2000) method. Starter culture cells in stationary phase were used to inoculate MHB (control) to an initial OD_{620} of 0.025 as determined using a Spectronic 20D+ optical spectrophotometer and incubated in an Excella® E24 environmental shaker incubator at 25°C with rotary aeration at 180 rpm until late exponential-phase was reached (OD_{620} of approximately 0.3 to 0.4). Cells were harvested by centrifugation at 12,000 x g and 4°C for 15 minutes (Sorvall Legend XRT Centrifuge; Thermo Fisher Scientific, Waltham, MA) and resuspended in HEPES buffer (5.0 mM) (pH 7.2; Sigma-Aldrich Chemical Co.) to an OD_{620} of 0.5. An acetone solution (Certified ACS; Fisher Scientific, Fair Lawn, NJ) of NPN (500 µM) was diluted in HEPES buffer (5.0 mM) to a final concentration of 40 µM (NPN stock solution). Experimental treatments consisted of 100 µl of cell suspension, 50 µl of HEPES buffer, and 50 µl of NPN, each loaded into the appropriate wells of a 96-well microtiter plate (Costar 96-well black, clear bottom microtiter plates; Corning Inc., Lowell, MA). The wells were loaded as follows: 200 µl of HEPES buffer (HEPES blank), 150 µl of HEPES buffer plus 50 µl of NPN (NPN control), 100 µl of HEPES buffer plus 100 µl of cell suspension (organism control), and 50 µl of HEPES buffer plus 50 µl of NPN and 100 µl of cell suspension (organism with NPN). Fluorescence was measured immediately (Ellison and Champlin, 2007) with the aid of a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT) with excitation

wavelength at 340 nm and emission wavelength at 420 nm. Relative fluorescence (X) was calculated using the following equation:

$$X = \frac{(\text{Organism with NPN}) - (\text{Organism control})}{(\text{NPN control}) - (\text{HEPES blank})}.$$

Statistical Analysis

Microsoft® Excel® for Mac 2011 and GraphPad Prism 7 for Mac OS X (GraphPad Software; La Jolla, CA) were employed for the statistical analysis of the NPN relative fluorescence of the environmental isolates. A bar graph was constructed to visually represent the mean of the relative fluorescence ± standard error. Each mean represented the value of triplicate data (n=3). A one-way ANOVA with Tukey's post-hoc comparison was applied to determine the outer membrane accessibility to NPN ($\alpha = 0.05$).

Outer Membrane Permeabilization Bioassay

The ability of the outer membrane permeabilizer compound 48/80 (Sigma-Aldrich Chemical Co.) to sensitize cells to the hydrophobic antibacterial agents novobiocin and triclosan was determined turbidimetrically by measuring batch cultural growth kinetics as described above (Champlin *et al*., 2005 as modified by Ellison and Champlin, 2007). Slight modifications of this protocol included supplementation of compound 48/80 such that its final concentration ranged from 5.0 μg/ml to 20.0 μg/ml in the indicated test cultures. The final concentrations of novobiocin and triclosan were 10.0 μg/ml and 2.0 μg/ml, respectively.

CHAPTER III

FINDINGS

Susceptibility to Antibacterial Agents

MICs were obtained for the mechanistically-disparate hydrophobic molecules novobiocin and triclosan in order to initially ascertain the potential role of outer membrane impermeability in high-level intrinsic resistance of environmental isolates to triclosan (Table 2). *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 were employed as control strains for comparative purposes. *E. coli* ATCC 25922 was susceptible to triclosan but resistant to novobiocin, whereas *P. aeruginosa* PAO1 was highly resistant to both molecules. Isolates LD7A and LD8B, which were isolated in the absence of triclosan selection, were both predictably susceptible to triclosan. However, they differed with regard to their relationship with novobiocin in that LD7A was resistant, while LD8B was atypically susceptible given its gram-negative nature. In contrast, isolates HD33 and HD36, which were selected for with triclosan, were highly resistant to both hydrophobic molecules suggesting their respective outer membranes are intrinsically impermeable to hydrophobic molecules in a general manner.

Table 2. Susceptibility of control organisms and model system isolates to the hydrophobic antibacterial agents novobiocin and triclosan.

Organism	MIC (µg/ml)[a]	
	Novobiocin	Triclosan[b]
Control		
E. coli ATCC 25922	512.0	0.5
P. aeruginosa PAO1[c]	512.0	>64.0
R2A		
LD7A[c]	>512.0	2.0
LD8B[c]	4.0	2.0
R2A-TCS		
HD33[c]	256.0	>64.0
HD36[c]	512.0	>64.0

[a]Values obtained from three-to-six individual twofold serial dilutions.
[b]Ethanol (95%; <0.4% final concentration) was used to facilitate triclosan solubilization and no effect on control growth was observed (data not shown).
[c]Values taken from DeGear *et al*., 2017.

Effect of Triclosan Titration on Cultural Growth Kinetics

In order to determine the effect of triclosan on total cell density cultural growth kinetics, turbidimetric growth curves of batch cultures were constructed for controls *E. coli* ATCC 25922 and *P. aeruginosa* PAO1, and surface water isolates LD7A, LD8B, HD33, and HD36 (Figures 1 and 2). Growth kinetics of *E. coli* ATCC 25922 revealed a concentration-dependent susceptibility over four orders of magnitude as the triclosan concentration was increased from 0.02 to 20 μg/ml. It should be noted that triclosan may have precipitated out of solution at the highest concentration (20 μg/ml of triclosan) resulting in an immediate increase in initial turbidity, however, biomass remained inhibited throughout the 6 h bioassay period. In comparison, *P. aeruginosa* PAO1 was not affected by triclosan with the exception of a slight decrease in final biomass obtained at the highest concentration (20 μg/ml).

As can be seen in Figure 2, the growth kinetics for organisms isolated in the absence (LD7A and LD8B) and presence (HD33 and HD36) of triclosan were drastically different. Maximal growth inhibition was observed at 20 μg/ml for the susceptible isolates LD7A and LD8B with inhibition appearing to be concentration-dependent from 0.2 to 20 μg/ml. Resistant isolates HD33 and HD36 remained largely unaffected by all concentrations of triclosan with the exception of slightly decreased biomass yield at the highest concentration (20 μg/ml). The concentration of ethanol required for the initial solubilization of triclosan (0.4%) did not affect growth kinetics in any case when compared with the growth kinetics of the MHB control.

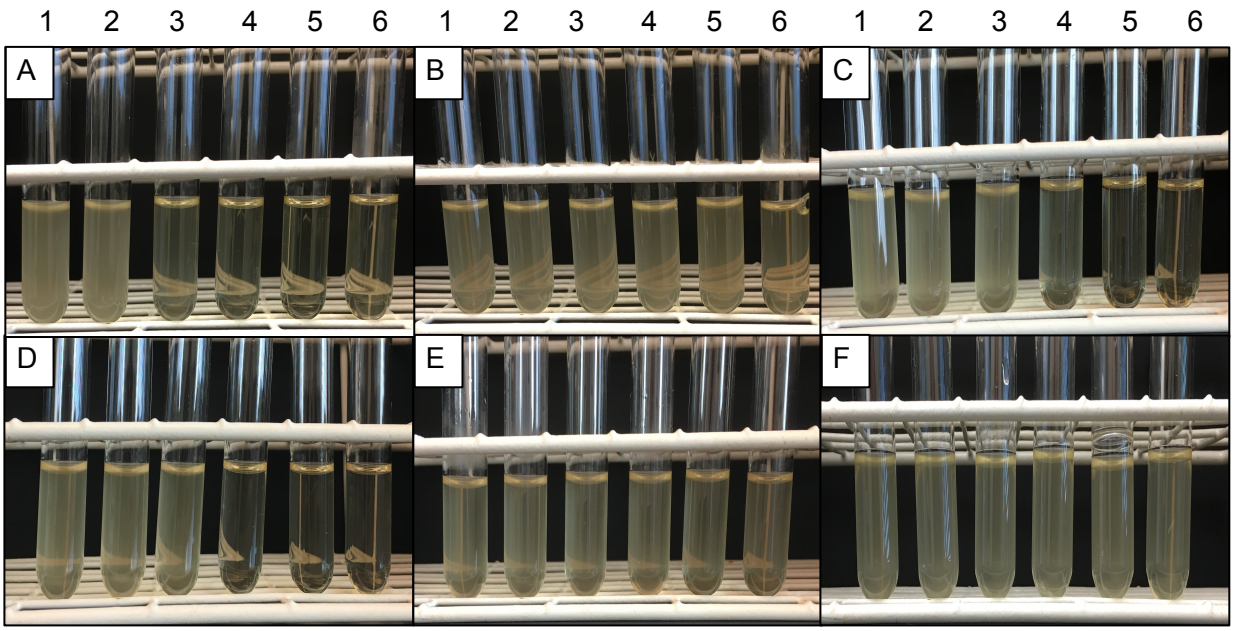

Figure 1. Representative triclosan titration bioassays. *E. coli* ATCC 25922 (A), *P. aeruginosa* PAO1 (B), LD7A (C), LD8B (D), HD33 (E), and HD36 (F). Culture numbers: growth control (1), 0.4% ethanol control (2), 0.02 µg/ml triclosan (3), 0.2 µg/ml triclosan (4), 2.0 µg/ml triclosan (5), and 20.0 µg/ml triclosan (6).

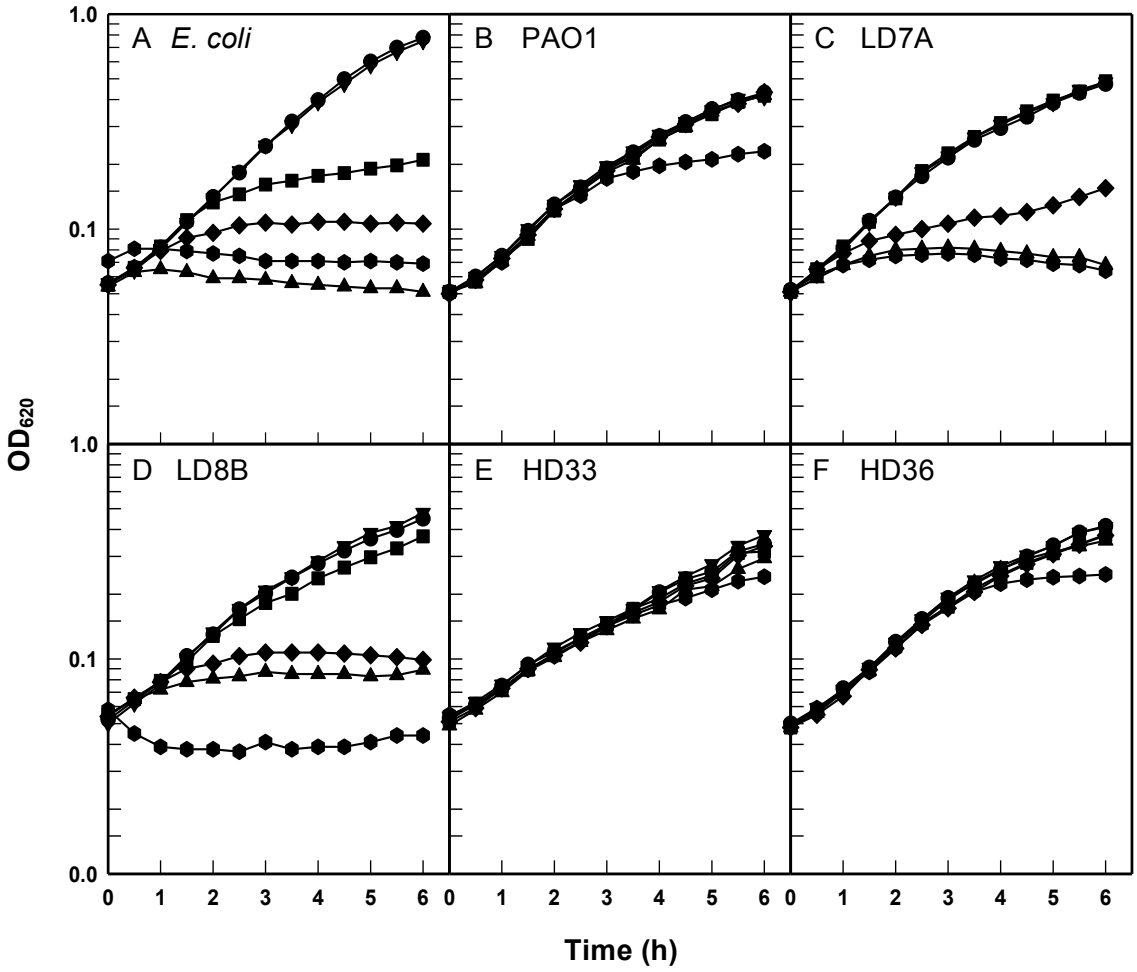

Figure 2. Total cell density growth kinetics when titrated with triclosan. *E. coli* ATCC 25922 (A), *P. aeruginosa* PAO1 (B), LD7A (C), LD8B (D), HD33 (E), and HD36 (F). Each value represents the mean of at least three independent determinations. Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, triclosan (0.02 μg/ml); ◆, triclosan (0.2 μg/ml); ▲, triclosan (2.0 μg/ml); and ⬢, triclosan (20.0 μg/ml).

Outer Membrane Accessibility to NPN

The degree to which the hydrophobic probe NPN was able to partition into hydrophobic regions of the outer membrane of surface water isolates was measured as a function of relative fluorescence intensity (Figure 3). *Pasteurella multocida* ATCC 11039 was employed as a positive control, because its outer membrane has been shown to be markedly permeable to hydrophobic molecules in general (Ellison and Champlin, 2007). In contrast, *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 provided negative controls by virtue of their refractory outer membrane permeability properties for hydrophobic molecules (Nikaido, 1976; Nikaido and Vaara, 1985; Champlin *et al*., 2005; Ellison and Champlin, 2007; Delcour, 2009; Silhavy *et al.*, 2010). No statistical significant difference was observed between *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 with a p value = 0.9888 (p value > 0.05). The outer membrane of *P. multocida* ATCC 11039 was significantly more accessible than those of *E. coli* ATCC 25922 and *P. aeruginosa* PAO1 with a p value < 0.0001.

The outer membranes of the environmental isolates LD7A, LD8B, HD33, and HD36 exhibited different permeability properties reflecting their relationships to the disparate hydrophobic molecules novobiocin and triclosan (Table 2). Statistical analysis revealed isolate LD8B to have an outer membrane significantly more accessible to NPN than LD7A in a manner consistent with their respective novobiocin resistance and susceptibility (p value < 0.0001). In contrast, statistical analysis of resistant isolates revealed no significant difference between isolates HD33 and HD36 (p value = 0.9967; p value > 0.05). Overall, NPN was able to partition into the outer membranes of the susceptible isolates to a greater degree than the outer membranes of the resistant isolates (p value < 0.05).

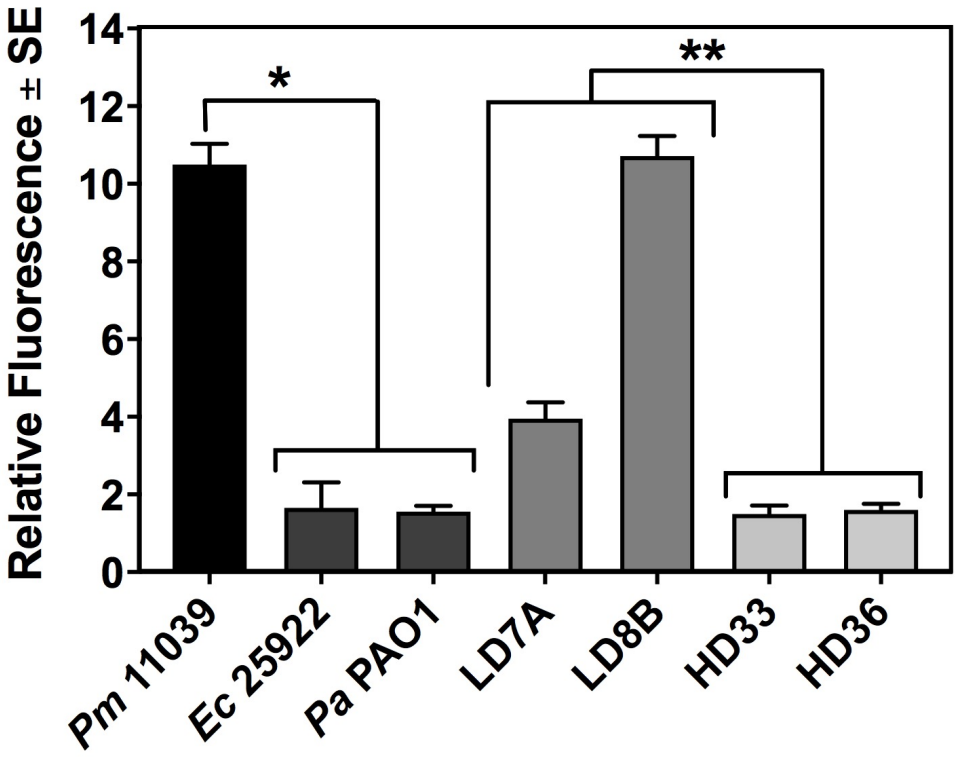

Figure 3. Cell surface accessibility to NPN. *P. multocida* (*Pm*) 11039 (taken from Boyina *et al*., unpublished data) (positive control), *E. coli* (*Ec*) ATCC 25922 (negative control), and *P. aeruginosa* (*Pa*) PAO1 (negative control) were employed as control strains for comparative purposes. Each value represents the mean of three independent determinations ± standard error. *, $P < 0.0001$ between positive control strain *P. multocida* and negative controls *E. coli* and *P. aeruginosa*. **, $P < 0.05$ between triclosan-susceptible isolates LD7A and LD8B and triclosan-resistant isolates HD33 and HD36. Determined using a one-way ANOVA with Tukey's post-hoc pairwise comparisons.

Outer Membrane Permeabilization

In order to more conclusively implicate involvement of outer membrane exclusivity as the cellular property underlying intrinsic resistance to triclosan, the effect of outer membrane permeabilizer compound 48/80 on intrinsic novobiocin and triclosan resistance was investigated using turbidimetric measurements of batch cultural growth kinetics per the methods of Champlin *et al*., 2005 (Figures 4-11). The control strain *E. coli* ATCC 25922 can be seen to be moderately susceptible to both novobiocin and triclosan sensitization alone with further sensitization occurring in the presence of compound 48/80 at a concentration of 2.5 μg/ml (Figures 4 and 5). Synergy was seen between compound 48/80 and both novobiocin and triclosan in the control strain *P. aeruginosa* PAO1 in a manner consistent with that seen previously (Champlin *et al*, 2005) (Figures 6 and 7).

Environmental isolates LD7A and LD8B, which were respectively resistant and susceptible to novobiocin (Table 2), were susceptible to both novobiocin and triclosan sensitization in the presence of compound 48/80 at a concentration of 2.5 μg/ml (Figures 8 and 9). In contrast, HD33 was resistant to novobiocin and triclosan sensitization in the presence of compound 48/80 at concentrations ranging up to 20.0 μg/ml, while HD36 was moderately sensitized to novobiocin and triclosan in the presence of compound 48/80 at concentrations ranging up to 15.0 μg/ml (Figures 10 and 11). Growth was unaffected by ethanol required for the initial solubilization of triclosan when compared to the MHB control in all control strains and environmental isolates.

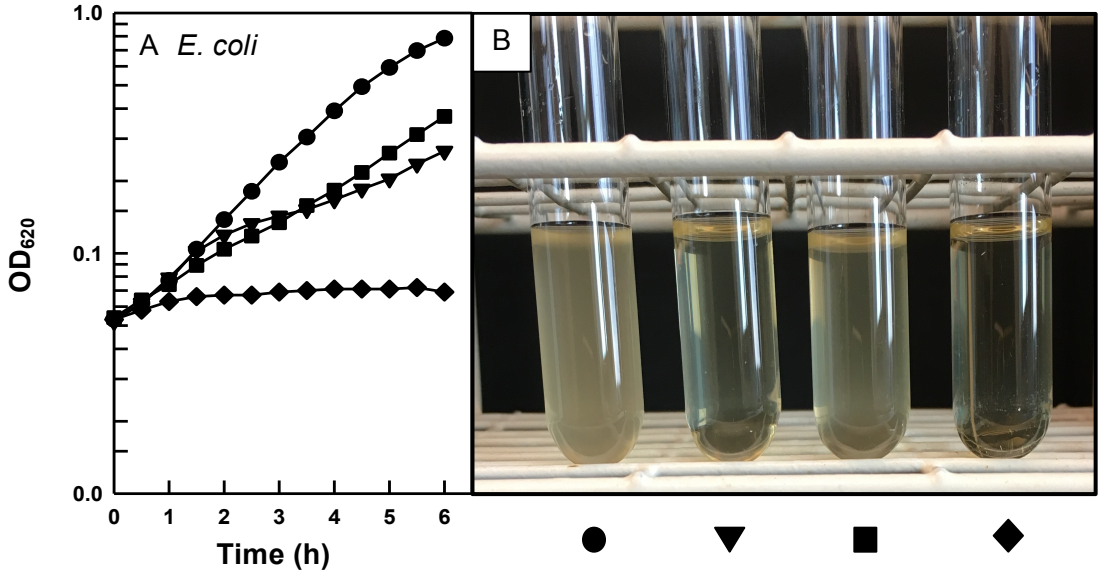

Figure 4. Total cell density growth kinetics of *E. coli* ATCC 25922 in the presence of compound 48/80 and novobiocin (NOV). Each value represents the mean of at least three independent determinations. (A) Symbols: ●, growth control; ▼, compound 48/80 (2.5 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (2.5 µg/ml) plus NOV (10.0 µg/ml). (B) Representative batch cultural kinetics bioassay results.

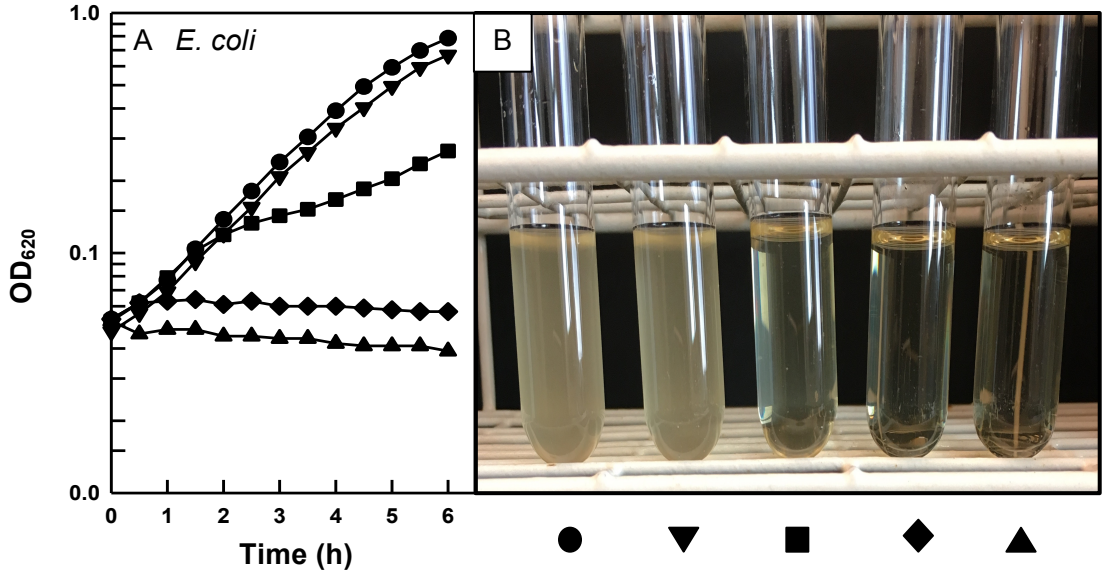

Figure 5. Total cell density growth kinetics of *E. coli* ATCC 25922 in the presence of compound 48/80 and triclosan (TCS). Each value represents the mean of at least three independent determinations. (A) Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (2.5 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (2.5 µg/ml) plus TCS (2.0 µg/ml). (B) Representative batch cultural kinetics bioassay results.

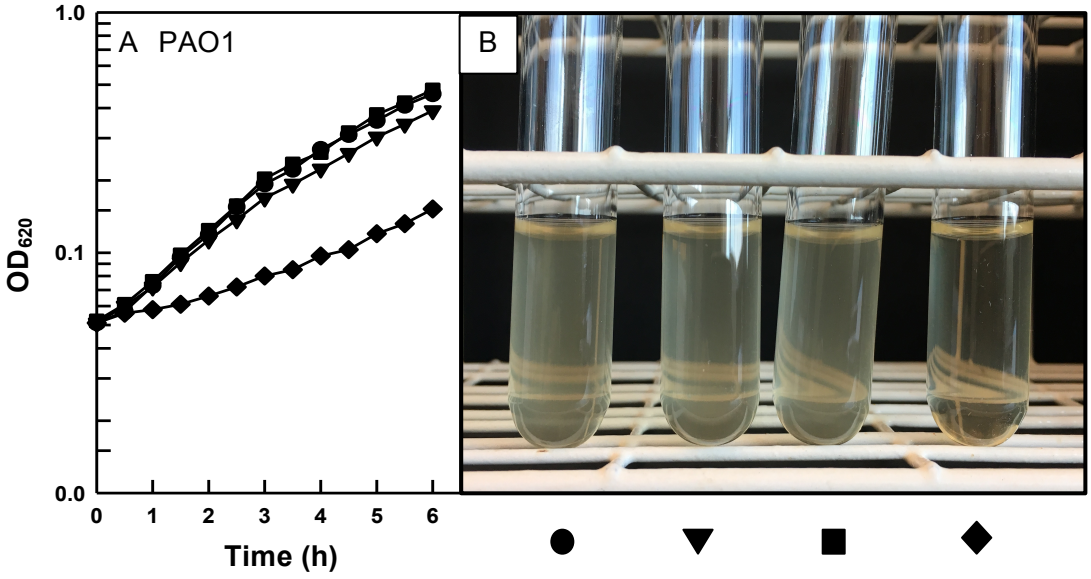

Figure 6. (A) Total cell density growth kinetics of *P. aeruginosa* PAO1 in the presence of compound 48/80 and novobiocin (NOV). Each value represents the mean of at least three independent determinations. (A) Symbols: ●, growth control; ▼, compound 48/80 (5.0 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (5.0 µg/ml) plus NOV (10.0 µg/ml). (B) Representative batch cultural kinetics bioassay results.

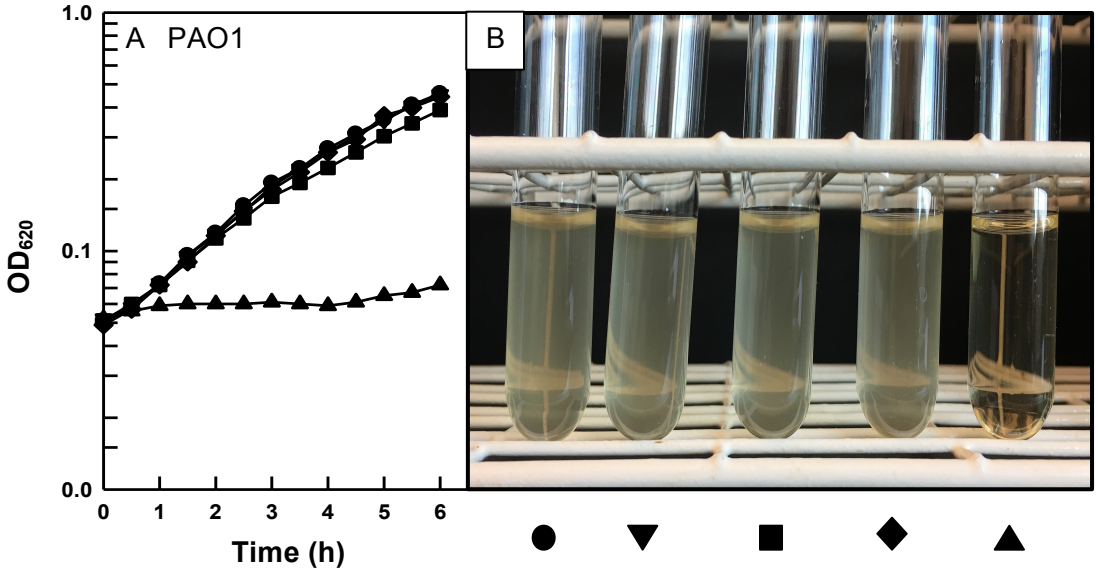

Figure 7. Total cell density growth kinetics of *P. aeruginosa* PAO1 in the presence of compound 48/80 and triclosan (TCS). Each value represents the mean of at least three independent determinations. (A) Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (5.0 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (5.0 µg/ml) plus TCS (2.0 µg/ml). (B) Representative batch cultural kinetics bioassay results.

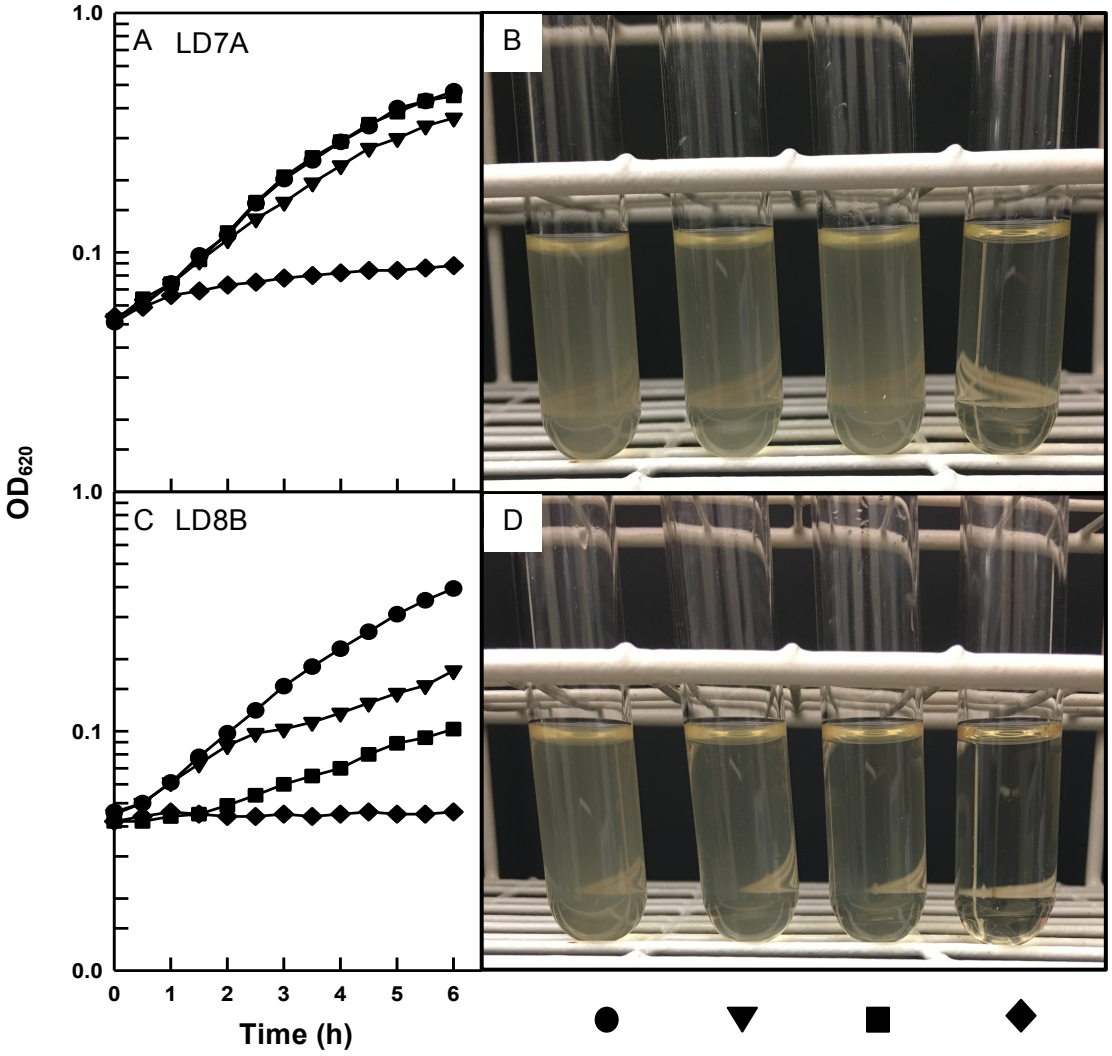

Figure 8. Total cell density growth kinetics of environmental *Pseudomonas* spp. isolates LD7A and LD8B in the presence of compound 48/80 and novobiocin (NOV). Each value represents the mean of at least three independent determinations. (A) LD7A. Symbols: ●, growth control; ▼, compound 48/80 (2.5 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (2.5 µg/ml) plus NOV (10.0 µg/ml). (B) Representative batch cultural kinetics bioassay results. (C) LD8B. Symbols: ●, growth control; ▼, compound 48/80 (2.5 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (2.5 µg/ml) plus NOV (10.0 µg/ml). (D) Representative batch cultural kinetics bioassay results.

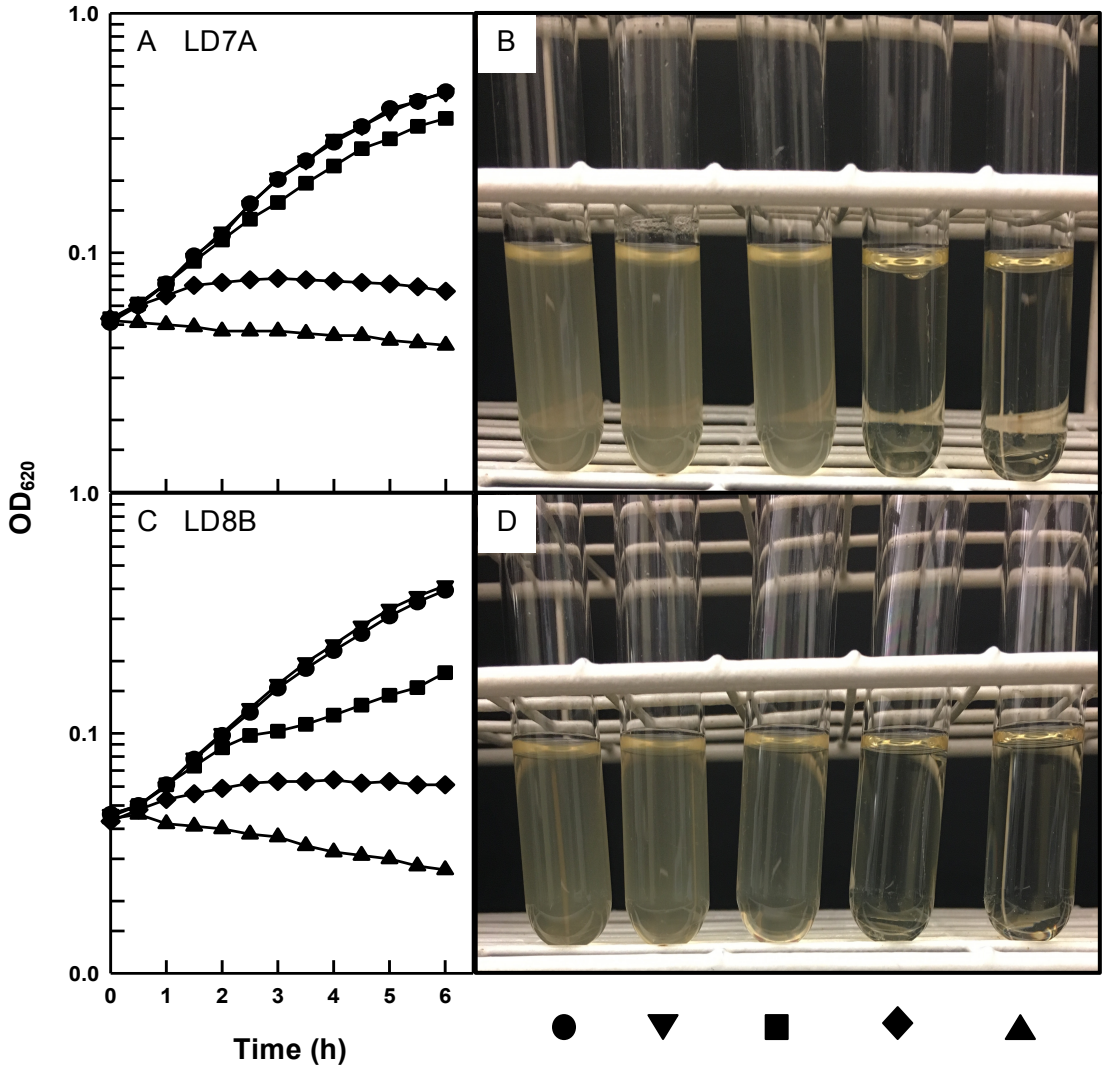

Figure 9. Total cell density growth kinetics of environmental *Pseudomonas* spp. isolates LD7A and LD8B in the presence of compound 48/80 and triclosan (TCS). Each value represents the mean of at least three independent determinations. (A) LD7A. Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (2.5 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (2.5 µg/ml) plus TCS (2.0 µg/ml). (B) Representative batch cultural kinetics bioassay results. (C) LD8B. Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (2.5 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (2.5 µg/ml) plus TCS (2.0 µg/ml). (D) Representative batch cultural kinetics bioassay results.

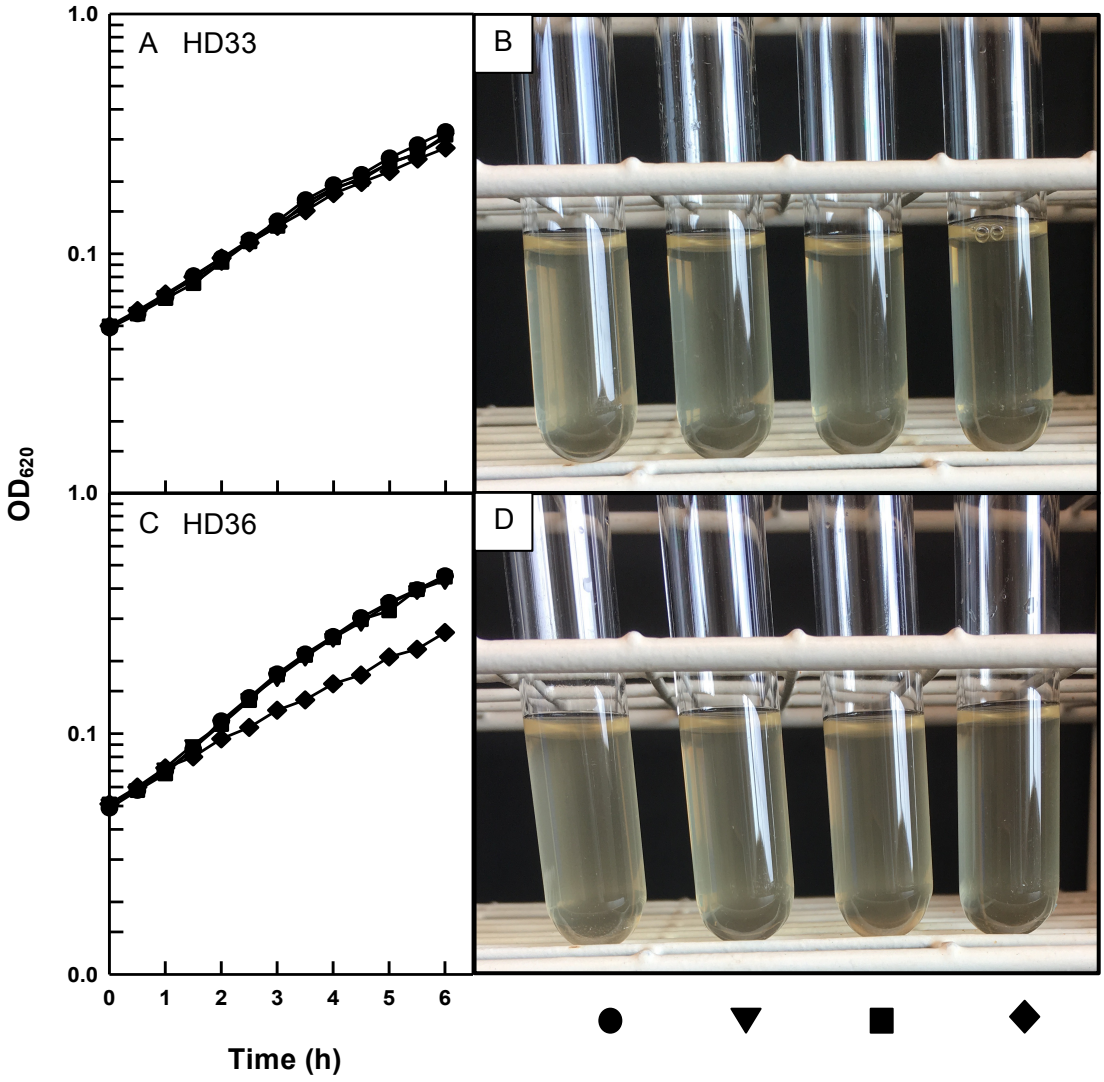

Figure 10. Total cell density growth kinetics of environmental *Pseudomonas* spp. isolates HD33 and HD36 in the presence of compound 48/80 and novobiocin (NOV). Each value represents the mean of at least three independent determinations. (A) HD33. Symbols: ●, growth control; ▼, compound 48/80 (15.0 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (15.0 µg/ml) plus NOV (10.0 µg/ml). (B) Representative batch cultural kinetics bioassay results. (C) HD36. Symbols: ●, growth control; ▼, compound 48/80 (15.0 µg/ml); ■, NOV (10.0 µg/ml); ◆, compound 48/80 (15.0 µg/ml) plus NOV (10.0 µg/ml). (D) Representative batch cultural kinetics bioassay results.

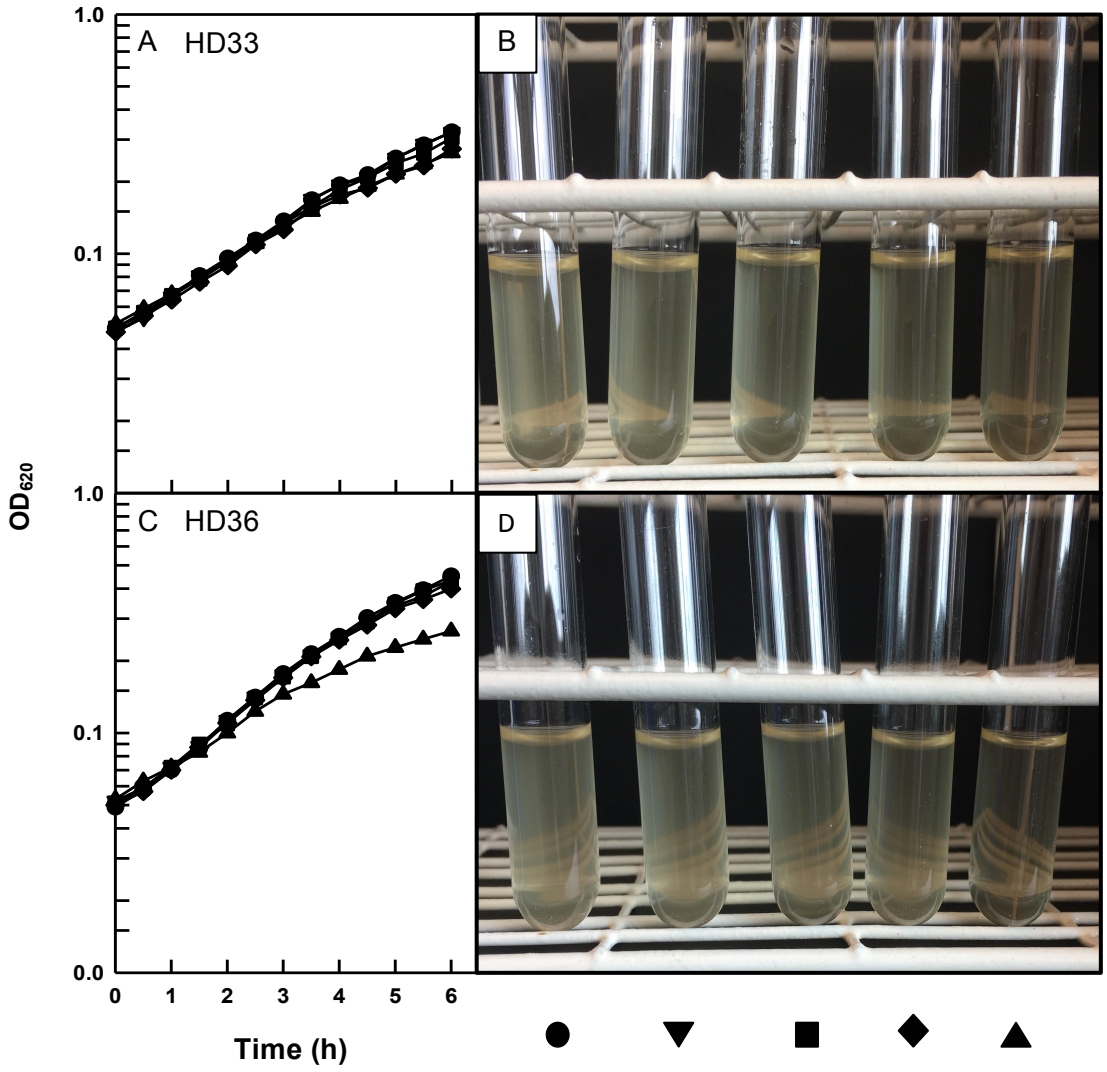

Figure 11. Total cell density growth kinetics of environmental *Pseudomonas* spp. isolates HD33 and HD36 in the presence of compound 48/80 and triclosan (TCS). Each value represents the mean of at least three independent determinations. (A) HD33. Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (15.0 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (15.0 µg/ml) plus TCS (2.0 µg/ml). (B) Representative batch cultural kinetics bioassay results. (C) HD36. Symbols: ●, growth control; ▼, ethanol control (0.4%); ■, compound 48/80 (15.0 µg/ml); ◆, TCS (2.0 µg/ml); ▲, compound 48/80 (15.0 µg/ml) plus TCS (2.0 µg/ml). (D) Representative batch cultural kinetics bioassay results.

CHAPTER IV

DISCUSSION

Unlike other gram-negative bacteria, which are typically susceptible to the hydrophobic biocide triclosan, *P. aeruginosa* is resistant intrinsically due to its marked outer membrane exclusionary properties for nonpolar substances (Champlin *et al*., 2005; Ellison *et al*., 2007) and active multidrug efflux pumps (Schweizer, 2001). Environmental isolates examined in the present study have been identified as members of the genus *Pseudomonas*, thereby suggesting that similar outer membrane impermeability properties may be at play with regard to hydrophobic molecules in general. The purpose of this study was to determine if the outer membrane impermeability properties of *Pseudomonas* spp. selected for with triclosan from Oklahoma surface waters underlie intrinsic resistance to hydrophobic substances in general, and triclosan specifically.

The MICs for isolates LD7A and LD8B, which were obtained in the absence of triclosan, revealed variable permeability properties for low concentrations of the disparate hydrophobic molecules novobiocin and triclosan. Interestingly, LD7A was resistant to novobiocin, but susceptible to triclosan, whereas LD8B was susceptible to both hydrophobic substances. These data suggest the outer membrane of LD7A contains a mechanism(s) that allows for triclosan to permeate into the periplasmic space, but not novobiocin in a manner similar to that seen for the control strain *E. coli* ATCC 25922 and most other gram-negative bacteria. In contrast, isolate LD8B has an outer membrane possessing atypical permeability for hydrophobic molecules in

general. In comparison, the MICs for isolates HD33 and HD36, which were selected for in the presence of triclosan, were able to initiate growth at high concentrations of both novobiocin and triclosan, thereby supporting the hypothesis that their outer membranes are impermeable to disparate hydrophobic molecules in general and, atypically triclosan.

Data obtained from titrations of the model system organisms with triclosan confirm conclusions reached for the MIC data in that isolates obtained in the absence of triclosan are permeable to the hydrophobic biocide resulting in concentration-dependent growth inhibition. In contrast, environmental isolates obtained from surface waters and selected for on the basis of intrinsic triclosan resistance exhibited no growth inhibition except for isolate HD36, which was slightly susceptible at only the highest concentration (20 μg/ml). These results, in combination with the above MIC data, strongly support the hypothesis that the triclosan-susceptible isolates have outer membranes which allow for permeation of hydrophobic substances through their outer cell envelopes, whereas the outer membranes of the intrinsically resistant isolates contribute at least a degree of impermeability for disparate hydrophobic molecules in general, and triclosan specifically.

The examination of outer membrane accessibility to the hydrophobic probe NPN revealed significantly higher relative fluorescence values for the susceptible isolates than for the resistant isolates. These data support the conclusion that the outer membranes of triclosan-susceptible isolates LD7A and LD8B are more permeable to nonpolar substances in general, while the outer membranes of HD33 and HD36 are more refractory. It can be concluded from these data that the susceptible isolates LD7A and especially LD8B have outer membranes that allow passive diffusion of disparate hydrophobic molecules into the periplasmic space and susceptibility, through the cytoplasmic membrane. In comparison, hydrophobic molecules cannot readily partition through the outer membrane of the resistant isolates HD33 and HD36 in a manner similar to that seen for the control organism *P. aeruginosa* PAO1.

Disruption of outer membrane exclusion properties for hydrophobic molecules using outer membrane permeabilizer compound 48/80 revealed further sensitization to both hydrophobic molecules novobiocin and triclosan in the already susceptible isolates LD7A and LD8B. This suggests an active role for the outer membrane as a rate-limiting step for the diffusion of disparate hydrophobic substances. In stark contrast, clear, cogent sensitization was not seen with compound 48/80 for either novobiocin or triclosan in isolate HD33, thereby suggesting that factors other than outer membrane impermeability may be contributing to intrinsic novobiocin and triclosan resistance, unlike what has been previously reported for *P. aeruginosa* (Champlin *et al*., 2005; Ellison *et al*., 2007). However, slight compound 48/80 sensitization was observed for both novobiocin and triclosan in isolate HD36, thereby suggesting a more prominent role for the outer membrane in their intrinsic resistance. These data suggest that the outer membrane functionally contributes as a protective barrier to hydrophobic compounds in general and triclosan specifically in the intrinsically resistant isolate HD36, but not for isolate HD33.

While these data strongly suggest that the outer membrane regulates entry of hydrophobic substances in general and triclosan specifically into the periplasmic space in some, but not all *Pseudomonas* spp. isolates, it is clear that outer membrane impermeability is not the sole mechanism at play. Other mechanisms most likely work in concert with outer membrane impermeability to confer the ability of environmental *Pseudomonas* spp. isolates to grow in the presence of high concentrations of triclosan. These may include (a) expression of an enzyme(s) which covalently modifies the biocide, thereby further inactivating it (Clayborn *et al*., 2011); (b) constitutive and inducible active multidrug efflux systems capable of removing any incoming biocide, thereby inhibiting bactericidal or growth-inhibitory effects (Schweizer, 2001); or (c) catabolic processes that deactivate the molecule, and possibly converting it into a useable energy source, thus supporting intermediary metabolism (DeGear *et al*., 2017).

Future directions of this work should include the use of other outer membrane permeabilizers such as EDTA or polymyxin B nonapeptide (Champlin *et al*., 2005) to confirm the conclusions reached in the present study. Efflux pump inhibitors can be employed along with the analysis of spent culture media for triclosan-inactivating factors to determine the ancillary mechanism(s) at work to supplement the exclusionary capabilities of the outer membranes in these environmental isolates. Furthermore, because of the recent ban of triclosan in many consumer products (U.S. Food and Drug Administration, 2016), the replacement of triclosan by equally or more harmful substances on our health and the environment is possible. An extension of this study could evaluate the outer membrane of these environmental isolates in the presence of other wastewater contaminants using similar methodologies.

Conclusions

Some, but not all environmental *Pseudomonas* spp. isolated from surface waters using triclosan selection in three disparate locations in Oklahoma appear to be intrinsically resistant to hydrophobic substances in general, and triclosan specifically, by virtue of outer membrane exclusion in a manner similar to that seen in the phylogenetically closely-related nosocomial opportunist *P. aeruginosa*. However, this property was unable to be shown to be due exclusively or even in large part to outer membrane impermeability. It is likely that it is working in concert with multidrug efflux systems and/ or triclosan-modifying enzymes to confer intrinsic resistance in some *Pseudomonas* spp. This research facilitates a better understanding of the cellular and molecular physiology of environmental isolates selected for on the basis of intrinsic triclosan resistance, and contributes to our appreciation of the impacts of environmental triclosan contamination. Moreover, it is anticipated that the data collected from this research will be useful for elucidating mechanisms underlying biocide resistance in *Pseudomonas* spp. These strategies could lead to comparison of environmental *Pseudomonas* isolates and the nosocomial opportunist

P. aeruginosa to facilitate the discovery of more efficient techniques for treating infections associated with the bacterium.

REFERENCES

1. Aranami K, Readman JW. 2007. Photolytic degradtion of triclosan in freshwater and seawater. Chemosphere. 66:1052-1056.
2. Bester K. 2005. Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters. Arch Environ Contam Toxicol. 49:9-7.
3. Bullard JW, Champlin FR, Burkus J, Millar SY, Conrad RS. 2011. Triclosan-induced modifcation of unsaturated fatty acid metabolism and growth in *Pseudomonas aeruginosa* PAO1. Curr Microbiol. 62:697-702.
4. Champlin FR, Ellison ML, Bullard JW, Conrad RS. 2005 Effect of outer membrane permeabilisation on intrinsic resistance to low triclosan levels in *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 26:159-164.
5. Clayborn AB, Toofan SN, Champlin FR. 2011. Influence of methylation on the antibacterial properties of triclosan in *Pasteurella multocida* and *Pseudomonas aeruginosa* variant strains. J Hosp Infect. 77:129-133.
6. Crofton KM, Paul KB, DeVito MJ, Hedge JM. 2007. Short-term *in vivo* exposure to the water contaminant triclosan: Evidence for disruption of thryoxine. Environ Toxicol Pharmacol. 24:194-197.
7. Cullinan MP, Palmer JE, Carle AD, West MJ, Seymour GJ. 2012. Long term use of triclosan toothpaste and thyroid function. Sci Total Enviro. 416:75-79.

8. Darnell KR, Hart ME and Champlin FR. 1987. Variability of cell surface hydrophobicity among *Pasteurella multocida* somatic serotype and *Actinobacillus lignieresii* strains. J Clin Microbiol. 25:67-71.
9. DeGear MA. 2017. PhD thesis. Oklahoma State University Center for Health Sciences, Tulsa, OK. In preparation.
10. Delcour AH. 2009. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta. 1794(5):808-816.
11. Dhillion GS, Kaur S, Pulicharla R, Brar SK, Cledón M, Verma M, Suampalli RY. 2015. Triclosan: current status, occurrence, environmental risks and bioaccumulation potential. Int J Environ Res Public Health. 12:5657:5684.
12. Ellison ML, Roberts AL, Champlin FR. 2007. Susceptibility of compound 48/80-sensitized *Pseudomonas aeruginosa* to the hydrophobic biocide triclosan. FEMS Microbiol Lett. 269:295-300.
13. Ellison ML, Champlin FR. 2007. Outer membrane permeability for nonpolar antimicrobial agents underlies extreme susceptibility of *Pasteurella multocida* to the hydrophobic biocide triclosan. Vet Microbiol. 124:310-318.
14. Helander IM and Mattila-Sandholm T. 2000. Fluorometric assessment of gram-negative bacterial permeabilization. J Appl Microbiol. 88:213-9.
15. Jones RD, Jampani HB, Newman JL, Lee AS. 2000. Triclosan: a review of effectiveness and safety in health care settings. Am J Infect Control. 28:184-196.
16. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater

contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ Sci Technol. 36:1202-1211.

17. Kumar V, Chakraborty A, Kural MR, Roy P. 2009. Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with triclosan. Reprod Toxicol. 27:177-185.
18. Latch DE, Packer JL, Arnold WA, McNeil K. 2003. Photochemical conversion of triclosan to 2,8-dichlorodibenzo-*p*-dioxin in aqueous solution. J Photochem Photobiol A Chem. 158:63-66.
19. Li X-Z, Barré N, Poole K. 2000a. Influece of the MexA-MexB-OprM multidrug efflux system on expression of the MexC-MexD-OprJ and MexE-MexF-OprN multidrug systems in *Pseudomonas aeruginosa*. J Antimicrob Chemother. 45:885-893.
20. Lubarsky HV, Gerbersdorf SU, Hubas C, Behrens S, Ricciardi F, Paterson DM. 2012. Impairment of the bacterial biofilm stability by triclosan. PloS one. 7:e31183.
21. McDonald AA. 2017. M.S. thesis. Oklahoma State University Center for Health Sciences, Tulsa, OK. In preparation.
22. McMurry LM, Oethinger M, Levy SB. 1998. Triclosan targets lipid synthesis. Nature. 394:531-532.
23. Nietch CT, Quinlan EL, Lazorchak JM, Impellitteri CA, Raikow D, Walters D. 2013. Effects of a chronic lower range of triclosan exposure on a stream mesocosm community. Environ Toxicol Chem. 32:2874-2887.

24. Nikaido H. 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of sme hydrophobic substances. Biochim Biophys Acta. 433:118-132.
25. Nikaido H, Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol Rev. 49:1-32.
26. Schweizer HP. 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol Lett. 202:1-7.
27. Silhavy TJ, Kahne D, Walker S. 2010. The bacterial cell envelope. Cold Spring Harb Perspect Biol. 2:a000414.
28. Singer H, Müller S, Tixier C, Pillonel L. 2002. Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. Environ Sci Technol. 36:4998-5004.
29. Stoker TE, Gibson EK, Zorrilla LM. 2010. Triclosan exposure modulates estrogen-dependent responses in the female Wistar rats. Toxicol Sci. 117(1):45-53.
30. U.S. Food and Drug Administration, U.S. Department of Health and Human Services. FDA issues final rule on safety and effectiveness of antibacterial soaps, 2016.https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm517478.htm. (11 April 2017, date last accessed).
31. Wang C-F, Tian Y. 2015. Reproductive endocrine-disrupting effects of triclosan population exposure, present evidence and potential mechanisms. Environ Pollut. 206:195-201.

32. Welsch TT, Gillock ET. 2011. Triclosan-resistant bacteria islated from feedlot and residential soils. J Environ Sci Health A Tox Hazard Subst Environ Eng. 46:436-440.

VITA

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