Molecular and Phenotypic Assessment of Antimicrobial Resistance Potential and Mechanisms by Mixtures of Environmental Pollutants In Wastewater

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Abstract

Antibiotic resistance poses a potential risk to human health. It has been proved that a variety of environmental pollutants from low environmentally relevant concentrations to relatively high minimum inhibitory concentrations (MICs), can induce antibiotic resistance. However, previous studies were mainly focused on the effect of single pollutants on antibiotic resistance induction, while the combined effects of the mixture of pollutants at environmentally relevant concentrations remain largely unknown. In this study, the effect of organic pollutants in wastewater effluents on inducing antibiotic resistance is investigated, and a library of transcriptional fusions of green fluorescent protein (GFP) that monitors the activity of promoters for 130 antibiotic resistance genes (ARGs) and antibiotic resistance (AR) pathway related genes in E. coli K12 is employed to evaluate the gene expression change induced by wastewater effluent samples. The results demonstrate that wastewater effluent extracts, at both the original concentration and concentrations factors of 500x, both before and after the disinfection process wastewater extracts, can induced significantly increased resistance to streptomycin, while the increased resistance to tetracycline is only induced by the disinfected wastewater exhibited sample at a concentrations factors of 500x. The differential expression of genes associated with outer membrane permeability change, alteration of efflux pump, and drug inactivation in exposure to effluent samples has been identified. The difference of antibiotic resistance induction potential between samples before and after disinfection has also been evaluated. This study provides evidence that continuous exposure to wastewater effluents can induce

antibiotic resistance in E. coli, and the potential mechanisms of antibiotic resistance induction of wastewater effluents can be evaluated by the GFP-fused ARG library.

BIOGRAPHICAL SKETCH

Yinmei Feng was born in Shanghai, China in 1997. She graduated with a bachelor's degree in Environmental Science from China Pharmaceutical University in 2019. She had a lot of research experience in the environment fate of endocrine disruptors using radioactive tracer technology.

In August 2019, she entered Cornell University and started her study towards the M.S. Degree in Environmental Engineering in the department of civil and environmental engineering working with Professor April Gu.

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Second, I give sincere thanks to all my lab mates. Thanks Jingyi Wu for teaching me lab rules and all the lab skills needed in my project. Thanks Zhenghao Li for helping start my project with experiment setup. Thanks Annand Patel for his analysis help. Thanks Stephanie Rich for helping me about the chemical analyze. I could not finish the thesis without your help.

Last, but not the least, thanks my friends, Nan Wang, Yi Sang, Rui Huang and Pris Zhang, and my cat Yuanbao for giving me mental support, and my family, for their unconditional support and love all the time.

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Chapter 1 Introduction

The spread and development of antibiotic resistance is an emerging global public health concern because it is associated with the effectiveness of antibiotics, with consequent impacts on morbidity and mortality. (Allen et al., 2010; Ashbolt et al., 2013; Taubes, 2008; Witte, 1998) Traditionally, the occurrence of antibiotic resistance is attributed to the overuse or misuse of antibiotics in both clinical and agricultural realms for human, livestock and aquatic purposes.(Witte, 1998; Xiong et al., 2015; Zhu et al., 2013) However, the ability and potential mechanisms of various categories of non-antibiotic environmental contaminants, such as disinfectants, water disinfection byproducts, heavy metals, nano materials, exhaust particles, pesticides, and other non-antibiotic drugs, to select for antimicrobial resistance have been recently evidenced and discussed.(Li et al., 2016; Rangasamy et al., 2018; Verma et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b; Zhang et al., 2017a) These chemicals have been reported to have antibiotic-like effects and led to antibiotic resistance genotype and phenotype changes under both high (above minim inhibition concentration, MIC) and low (sub-MICs) or environmentally relevant exposure concentrations. (Li et al., 2016; Rangasamy et al., 2018; Verma et al., 2018; Zhang et al., 2018a; Zhang et al., 2018b; Zhang et al., 2017a) Recent studies suggested that these environmental pollutants can induce bacteria to develop or acquire antibiotic

resistance via a number of mechanisms, not only including commonly recognized pathways like activation of efflux pump, changes in permeability of cellular membranes, modification of antibiotic target sites, and deactivation of antibiotics(Blair et al., 2015), pathways via killing sensitive bacteria and inducing intracellular ROS, which could cause oxidative stress and DNA damage, activating the SOS response and repair systems and consequently promote horizontal gene transfer were discussed and mutations related to transcription and translation, membrane structure and transport were also identified.(Alonso et al., 2001; Baquero et al., 2008; Li and Gu, 2019) Previous studies with the aims to reveal detailed pathways that were involved in the resistance induction by these antibiotic-like environmental chemicals suggested that they involve not only genes in commonly recognized antibiotic resistance pathways but also genes that have not yet been previously identified to be associated with antibiotic resistance(Li et al., 2016). Due to the multiple functions and involvement of these cellular metabolism-relevant genes and pathways, it is conceivable that there are many other chemicals that may lead to resistance.

Natural water body is a critical agent in the genetic evolution of antibiotic resistance, not only because pathogenetic bacteria which harbor antibiotic-resistance genes are constantly released into it, environmental pollutants can also contribute the evolution and spread of resistance mutants in the water environment. (Alonso et al., 2001; Baquero et al., 2008) Most of the contaminants which can have antibiotic

resistance induction potential are widely distributed in the water system. For instance, as waste water is the main source of heavy metal in environment, heavy metal concentration in waste water range from 1ug/L to 10mg/L, while the concentration in surface water and drinking water was at ug level(Flores et al., 2020; Islam et al., 2015; Zhang et al., 2016), nano particles have relatively low concentration in all environment water media (lower than 1 ug/L)(Gottschalk et al., 2013), pesticides and non-antibiotic drugs environment concentration were detected in diverse water media and their concentration were normally at ng/L to ug/L level(Hashim and Khan, 2011; Lee et al., 2004; PESTICIDES, 2011; Westlund and Yargeau, 2017), disinfectants and DBPs mainly occurred in drinking water, at mg/L and ug/L level, respectively(Wei et al., 2013) .

Antibiotics have been detected in wastewater treatment plant and in wastewater effluents (Göbel et al., 2005; Xu et al., 2007) Previous study evidenced that wastewater effluent contains various categories of antibiotic resistance-inducing contaminants (i.e. metals, disinfectants, DBPs, pesticides etc.), and this would inevitably affect the spread and dissemination of antibiotic resistance when the wastewater effluents are discharged to water bodies and further impact the public security in drinking water treatment systems. There has been rising interests in the impact of various environmental pollutants on the antimicrobial resistance phenomena. Most of the studies have focused on one or few chemicals individualy. Considering that there is a mixture of a large number of, and variety of chemicals in

natural environments, particularly in wastewater, their mixture and overall combined effects on inducing resistance is of interest and relevance. The evaluation of the combined effects of mixture of environmentally relevant chemicals, such as those in the wastewater effluent that contain both recalcitrant residual chemicals originated from the influent and transformation products generated through the treatment processes, has not been performed and warrants investigation due to its far-reaching implications in environmental and human health.

In this study, we, for the first time, evaluated the potential effect of mixture of organic pollutants in wastewater effluent on inducing antimicrobial resistance. First, we determined the phenotypic changes (MIC) in *E coli* strain in exposure to mixture of pollutants in wastewater effluents. MIC is one of the central concepts in the research on bacterial susceptibility, conventionally, the change of MIC is considered as a typical phenotypic change to indicate an antibiotic resistance.(Li et al., 2016; Mouton et al., 2012) We then systematically explored the possible mechanisms underlying the antimicrobial resistance induction by the wastewater with regards to the oxidative stress and intracellular ROS production, the cell membrane permeability changes and impact on horizontal gene transfer. This was base on the understanding from since recent studies that certain pollutants in wastewater (i.e. metals, disinfectants, DBPs, pesticides etc.) can induce oxidative stress, increase intracellular ROS levels and lead to damage of cell membranes that cause accelerated horizontal gene transfer, consequently induce antibiotic resistance. Furthermore, to reveal the

molecular mechanisms underlying the phenotypic change, we proposed and employed a novel antimicrobial resistance potential screening library, using a GFP-fused antimicrobial resistance gene ensemble library that targeting over 130 genes that were reported to be involved in various antibiotic resistance pathways. Similar to the previously proposed TELI concept, we, for the first time, proposed a new antimicrobial resistance induction potential index (ARIPI) as a quantifier to enable screening and quantification of the resistance induction potential from mixture of chemicals or any environmental samples. The high-resolution molecular disturbance profiling also reveals the molecular mechanisms underlying the cause of resistance phenotype.

Chapter 2 Materials and Method

2.1 Sampling sites

Water samples were collected from Ithaca Wastewater treatment plant, as shown in Figure S1. Previous studies suggested that water disinfections may play some role in the removal of antibiotics, (Gulkowska et al., 2008; Li et al., 2008) and both disinfectants and disinfection by-products can have antibiotic-like effect. We hypothesized that disinfection process may have some impact on the induction of antibiotic resistance. Tertiary effluent (final effluent before disinfection process) and final effluent (effluent after disinfection process) was taken from the treatment plant

(Figure S1), and store at 4 C samples were stored at 4 ºC until extraction (within one week). before subsequent treatment.

2.2 Sample preparation and extraction

Water samples were firstly filtered with 0.45 um membrane to remove insoluble particles and bacteria, then the cartridges (Oasis HLB (0.2 g, 6 ml, SILICYCLE INC., Canada) were used to extract the soluble organic chemicals in the samples. The cartridges were firstly conditioned with HCl to remove residual bonding agents, followed by methanol flew through the cartridges under very low vacuum to ensure that the sorbents were soaked. Then, deionized water was passed through the cartridges at a rate of 1–2 ml/min before water samples were extracted at a flow rate less than 5 ml/min. The analytes were eluted to 20 ml vials from the sorbents with 10 ml of methanol at a flow rate of 1 ml/min. (Li et al., 2013)The solvents were blown down to dry under a flow of nitrogen, and then the residue was redissolved by DI water to make the final concentration of 5000x extract stock.

2.3 Antibiotics

The six antibiotics examined in this study included Enoxacin (ENO) (AESAR), chloramphenicol (CHL) (Acros Organics, USA), tetracycline (TC) (Sigma Chemical Company, USA), streptomycin (STR) (MP Biomedical InC, USA), ampicillin (AMP)

(Fisher BioReagents, USA), trimethoprim (TRI) (MP Biomedical InC, USA) and neomycin (NEO) (Aesar, USA). These seven antibiotics were chosen to represent all categories of antibiotics , particularly those that have been detected in water and wastewater(Anjali and Shanthakumar, 2019; Karthikeyan and Meyer, 2006).

2.4 Exposure to Wastewater extract

The −80°C stock E. coli K12 MG1655 strain (Thermo Scientific E. coli Promoter Collection, PEC 3876) was employed to evaluate the phenotypic changes in exposure to water samples. In a 96-well-plate, added 180 ul of fresh Luria–Bertani (LB) media, then added a bit of (20 ul) ice of frozen strain solution from the original stock culture and incubated at 37°C overnight. The overnight culture of the isogenic E. coli K12 (approximately 10E8–10E9 CFU/mL) was diluted in LB broth (130ul LB + 5 ul bacteria) in a 96 well plate containing environmental water samples extracts at various concentrations (with final concentration of 1x,10x,100x,500x), along with control groups that were exposed to DI water. After 24 hours incubation at 37 °C, the culture was transferred to a new plate contain fresh LB broth with wastewater extracts concentration listed above and this sub-culture was repeated for 30 days. Each treatment had three replicates.

2.5 Determination of MICs:

To determine the MIC, the initial over-night bacterial cultures and those after 30 subculture cycles were diluted by fresh LB broth to an initial bacterial concentration of about 10E6 CFU/ml. Then, 5 ul of the above cultures, 15 ul of serially two-fold diluted antibiotics, as well as 130 ul of fresh LB broth were dispensed per well in a 96-well microplate. Following 20 h cultivation at 37 °C, a micro-plate reader was used to determine the bacterial growth by monitoring the optical density at 600 nm (OD600). Sterilized PBS solution was also tested as control. All of the MICs experiments were performed at least in triplicate. The MIC was calculated as the concentration of antibiotics that inhibit 90% of growth in LB.(Li et al., 2016)

2.6 Targeted Organic Micropollutants Analyses

Organic extracts from the wastewater samples were first diluted with DI water to achieve the final enrichment factor of 1000x, which was filtered with 0.22-μm polytetrafluoroethylene (PTFE) membrane, and then subjected to target screening for 200 organic micropollutants by means of high-performance liquid chromatography (HPLC) coupled to high-resolution mass spectrometry (HRMS, quadrupole-orbitrap, Thermo Scientific). The analytical HPLC–HRMS method was previously developed and validated for a broad range of micropollutants.(Carpenter and Helbling, 2018; Gao et al., 2019; Lin et al., 2020)

2.7 Measurement of intracellular ROS level

The bacterial cultures at a density of OD600 of ~0.3 was washed in PBS to remove the LB, then bacterial cultures were exposure to water samples extracts at various concentration factors (1x,10x,100x,500x) for 2 h at 37 °C, along with un-treated controls (PBS as control). The cells were washed with PBS to remove the chemicals before the culture was incubated with 10 μM of DCFH-DA at 37 °C for 0.5 h. Cells were then washed with PBS three times to remove the extra cellular DCFH-DA, and transferred into a 96-well plate. The fluorescence intensity (FI, 488 nm/525 nm) was measured by microplate reader. The FI values of the treatment groups were divided by the values of the untreated control to indicate enhanced ROS formation as "relatively elevated level". (Zhang et al., 2018a) All samples and controls were performed in triplicates.

2.8 Measurement of membrane permeability changes

To evaluate the membrane permeability of bacteria culture after exposure to wastewater extracts, DNA-intercalating fluorescent dye, propidium iodide (PI) was used to correlates fluorescence intensity with cell membrane permeability. The bacterial cultures at a density of OD600 of ~0.3 was washed in PBS twice to remove the LB, then bacterial cultures were exposure to water samples extracts at various

concentration factors (1x,10x,100x,500x) for 4 h at 37 °C, along with un-treated controls (PBS as control). The cells were then washed with PBS twice to remove the chemicals before the culture was incubated with PI at 37 C for 20 min. After the PI exposure, the culture was washed again with PBS three times to remove the extra cellular PI and transferred into a 96-well plate and, the fluorescence intensity (FI, 535 nm/617 nm) was measured by microplate reader.(Luo et al., 2014) The FI values of the treatment groups were divided by the values of the untreated control to indicate decreased membrane permeability as "relatively change level". All samples and controls were performed in triplicates.(Zhang et al., 2018a)

2.9 Measurement of Horizontal Gene Transfer Rate

A detailed method description of the measurement of horizontal gene transfer rate is available in Text S1.

2.9 Transcriptional Analysis of Selected Biomarkers in *E.coli* Cells for Antimicrobial resistance potential assessment

A library of 130 transcriptional fusions of GFP that includes promoters controlling the expression of genes involved in outer membrane permeability, efflux pump, drug inactivation, targets alternations, SOS response and detoxifications in E. coli K12, MG1655 was employed in this study (Table S1). The rationale for the biomarker selection, their roles involved in antimicrobial resistance are described in our previous publication ((Gou et al., 2014)) GFP-fused E. coli strains/reporters

selected were grown with M9 medium in clear bottom black 384-well plates for 4–6 h at 37 °C to reach early exponential growth stage (OD600 value of 0.15- 0.25). Freshly prepared water samples extracts (7x and 700x, final concentration 1x and 100x in the tested well) or PBS control were added at 10 ul per well. A microplate reader was used to read plates for absorbance (OD600 for cell growth) and GFP signal (filters with 485 nm excitation and 535 nm emission for gene expression) every 5 min for 2 h. All tests were performed in the dark in triplicate.(Lan et al., 2014)

2.10 Data Analysis and Statistics

Data processing of gene expression profiling and calculation of molecular toxicity endpoints were detailed in Text S2.

Unpaired t test was performed in R to compare the phenotypic change between wastewater extracts and control and $p < 0.05$ was considered as significant difference. Pearson correlation analysis was carried out in R to identify the potential relationships between the molecular toxicity quantifiers and micropollutant concentrations in water samples ($p < 0.05$ considered significant).

Chapter 3 Results and Discussion

3.1 Mixture of Organic pollutants In wastewater lead to antimicrobial resistance

3.1.1 MIC values comparison between wide strain and those with exposure to pollutants

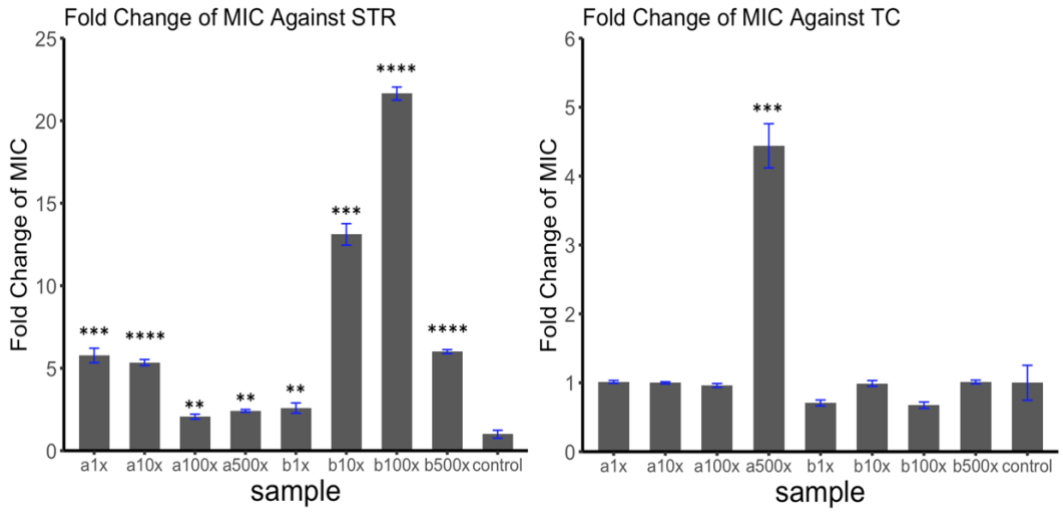

Figure 1. Fold change of MICs against Streptomycin and Tetracycline induced by exposure to different concentrations of wastewater effluents extracts after 30-day incubation. X-axis: sample names, a: after disinfection effluent, b: before disinfection ; Y-axis: fold change in MIC values compared to the control. Significant differences in fold change of MICs between test groups and control (DI water) were tested with unpaired t-test and shown with * ($p<0.05$), **($p<0.01$), ***($p<0.001$), and ****($p<0.0001$).

To determine whether the mixture of organics in the wastewater could induce the antibiotic resistance phenotype, we evaluated the impact of wastewater treatment

plant effluent on the MIC change in E.coli strain K12 MG1655. The MICs of the wild-type strain, before and after the exposure to various concentrations of organic extracts from the wastewater samples were determined against six antibiotics, namely Neomycin, Ampicillin, Streptomycin, Tetracycline, Chloramphenicol and Trimethoprim. The results showed that wastewater organic extracts induced significant changes in the MIC values and therefore induces antimicrobial resistance against streptomycin and tetracycline, with a fold change range from 1 to more than 20, compares to the control (Figure 1). MIC changes against the other four antibiotics were not significant (results not shown).

The results showed that the organics pollutants mixture in the wastewater samples prior to the disinfection process exhibited different impact on the antimicrobial resistance induction compared to those after the disinfection. In addition, the dose-response patterns varied depending on the specific antibiotics. As shown in figure 1, for STR, the samples before disinfection and at middle-range concentration factors (10x and 100x) led to the highest and significant changes in MIC with more than 4-fold changes. Here, we chose 4-fold as a high fold increase, which may be considered as a signal of antibiotic resistance. ((Li et al., 2016; Singh et al., 2012))Interestingly, the fold change in MIC decreased at the highest concentration factor of 500x. Our pre-screening cytotoxicity test (24-h cell growth inhibition) indicated that no observed cytotoxicity was observed for exposure concentration factor from 10x to 100x. In comparison, the water samples after the disinfection (which supposed to contain

higher level of DBPs) led to overall less extent of changes in MIC. And the lower concentrations (1x and 10x) led to more STR resistance that those samples at higher concentrations (100x and 500x). In contrast to the responses to SRT as discussed above, only one water sample after the disinfection at the highest concentration factor of 500x led to significant changes in the MIC value to TC. All other samples did not lead to significant antimicrobial resistance to TC.

Previous studies in our group proved that both disinfectants and water disinfection byproducts can induce antibiotic resistance, we hypothesized that the disinfection process in real water treatment plant can impact the induction of antibiotic resistance.((Li et al., 2016; Zhang et al., 2017a)). However, the results suggested that the tertiary effluent before disinfection led to significantly higher MIC fold changes and higher antimicrobial resistance induction potential, implying that the organic pollutants residual and their transformation products in the effluent likely contributed more to antimicrobial resistance induction against STR ($p<0.05$). Although disinfection process is expected to generate more DBPs, it also oxidize and transform other organic pollutants, leading to overall lesser resistance indication potential against SRT. These results indicated that disinfection is a complicated process, as it may impact the concentration and constructure of pollutants in wastewater, the mechanisms of the disinfection process in the induction of antibiotic resistance by wastewater remain unknown.((Liberatore et al., 2020; Postigo and

Richardson, 2014)) Potential correlation of resistance phenotypes and detectable microbial pollutants will discussed in later section.

3.1 2 Hereditary stability of the antimicrobial resistance induced my wastewater

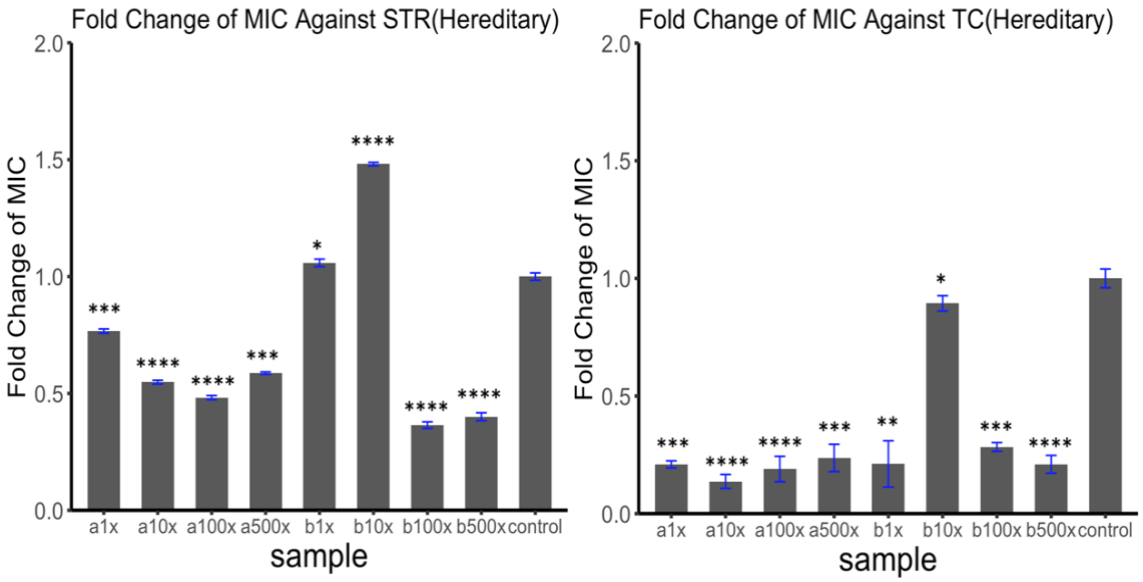

Figure 2. Fold change of MICs against Streptomycin and Tetracycline in wastewater effluents-induced resistance cells after sub-sequent 5 sub-culture cycles with LB media without wastewater extract exposure. X-axis: sample names, a: after disinfection effluent, b: before disinfection; Y-axis: fold change in MIC values compared to the control. Significant differences in fold change of MICs between test groups and control (di water) were tested with unpaired t-test and shown with *($p<0.05$), **($p<0.01$), ***($p<0.001$), and ****($p<0.0001$).

To test the hereditary stability of antibiotic resistance acquired from exposure to wastewater, the change in MICs of selected antibiotic-resistant mutants after 5 sub-

culture cycles in LB media with no wastewater exposure over 5 days were determined. As shown in figure 2, the resistant mutants had poor hereditary stability, since most of the tested groups shown significantly lower MIC than the control group, meaning that these pre-exposured cells become more susceptible to STR and TC after five subsequent growth cycles with LB media. Although for the 10x concentrated tertiary effluent before disinfection, there was a significantly increase of MIC against STR compared to the control, the 1.5-fold increase cannot be considered as a signal of antibiotic resistance. These results indicate that even though the antimicrobial resistance traits were not inheritable, there are certain traits (ie. Genetic changes) resulted from the wastewater exposure was inheritable and they contributed to their susceptibility to antibiotics. However, research proved that even non-inherited antibiotic resistance can also cause the failure of antibiotic treatment. Levin et al. introduced several models to demonstrate that non-inherited resistance can contribute to the microbiological outcome of antibiotic treatment. (Levin and Rozen, 2006)

3.2 Mechanisms of the antimicrobial resistance led by the mixture of pollutants

To investigate the potential mechanisms involved in the resistance, we evaluated the oxidative stress, membrane damage and antimicrobial resistance-relevant molecular pathways-perturbation. We assessed the involvement of oxidative stress by measuring the intracellular and extracellular ROS production by the water extracts. We evaluated the impact of mixture pollutants on cellular membrane integrality by

measuring cell membrane permeability. Furthermore, we developed a novel transcriptomics assay using GFP-fused biomarkers ensemble library targeting known genes that were reported to be involved in antimicrobial resistance.

3.2.1 Assessment of intracellular ROS levels induced by wastewater

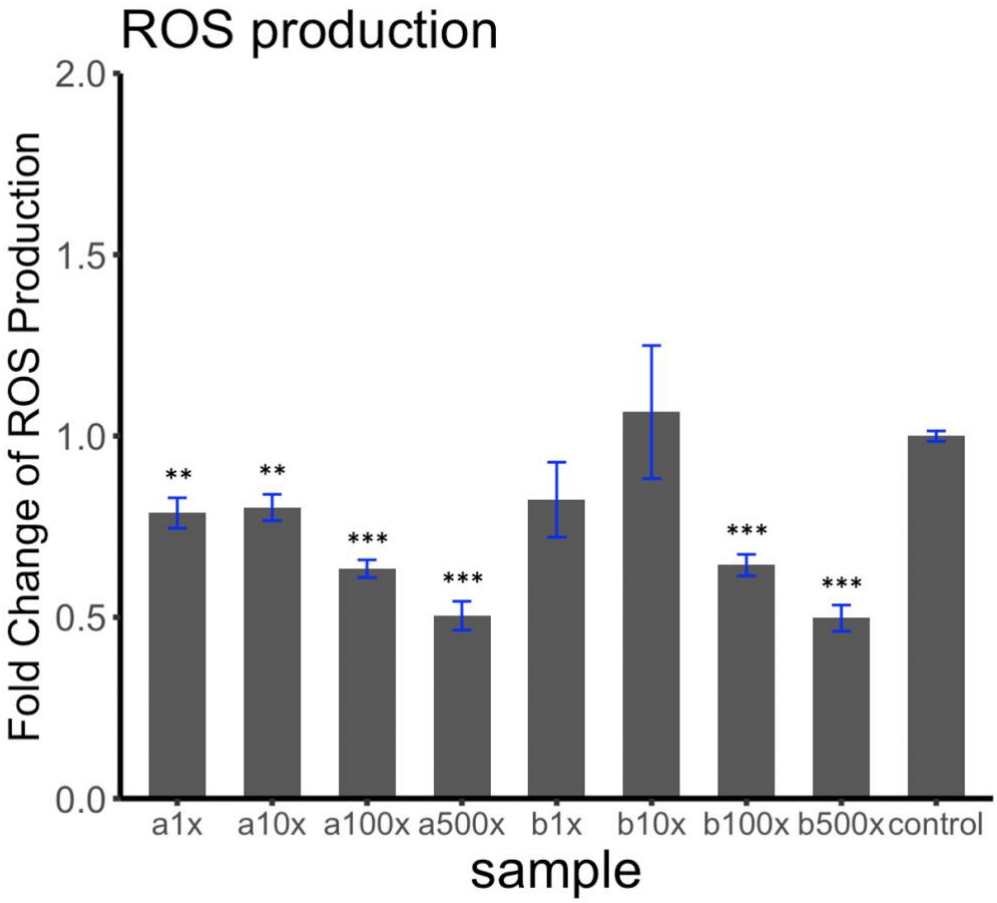

Figure 3. Fold change of ROS production levels compared to control induced by different concentration of wastewater samples extracts. X-axis: sample names, a: after disinfection effluent, b: before disinfection; Y-axis: fold change in ROS production compared to the control. Significant differences in fold change of ROS production

levels between test groups and control (di water) were tested with unpaired t-test and shown with *($p<0.05$), **($p<0.01$), ***($p<0.001$), and ****($p<0.0001$).

Antibiotics induce changes in metabolism can promote the formation of reactive oxygen species, which is responsible for damage to cellular structures and play a role in cell death(Lemire et al., 2013; Van Acker and Coenye, 2017) Previous studies have proved that disinfectants, disinfection by-products, and heavy meals can lead to intracellular ROS generation, and consequently induce oxidative stress and SOS response, which can promote mutagenesis and emerging of antimicrobial resistance. (Chapman, 2003; Li et al., 2016; Zhang et al., 2017a) Here, we hypothesis that wastewater may be able to raise intracellular ROS production, induce oxidative stress and SOS response, and therefore have the induction potential of antibiotic resistance.

To test the effect of wastewater on the ROS levels in E.coli, DCFH-DA was used to measuring intracellular ROS. The result shown that both tertiary effluent before disinfection and final effluent after the disinfection led to consistent decrease in ROS level compared to the control (Figure 3). The result of toxicogenomic assay was similar to the ROS production. As shown in Figure 6, the significant analyze of SOS response and detoxification related genes shown that there was no significant difference between the gene expression level of control and tested groups. This result was surprising since precious study proved a positive correlation between intracellular ROS and transconjugant.(Zhang et al., 2018b)

3.2.2 Assessment of cell membrane permeability affected by wastewater

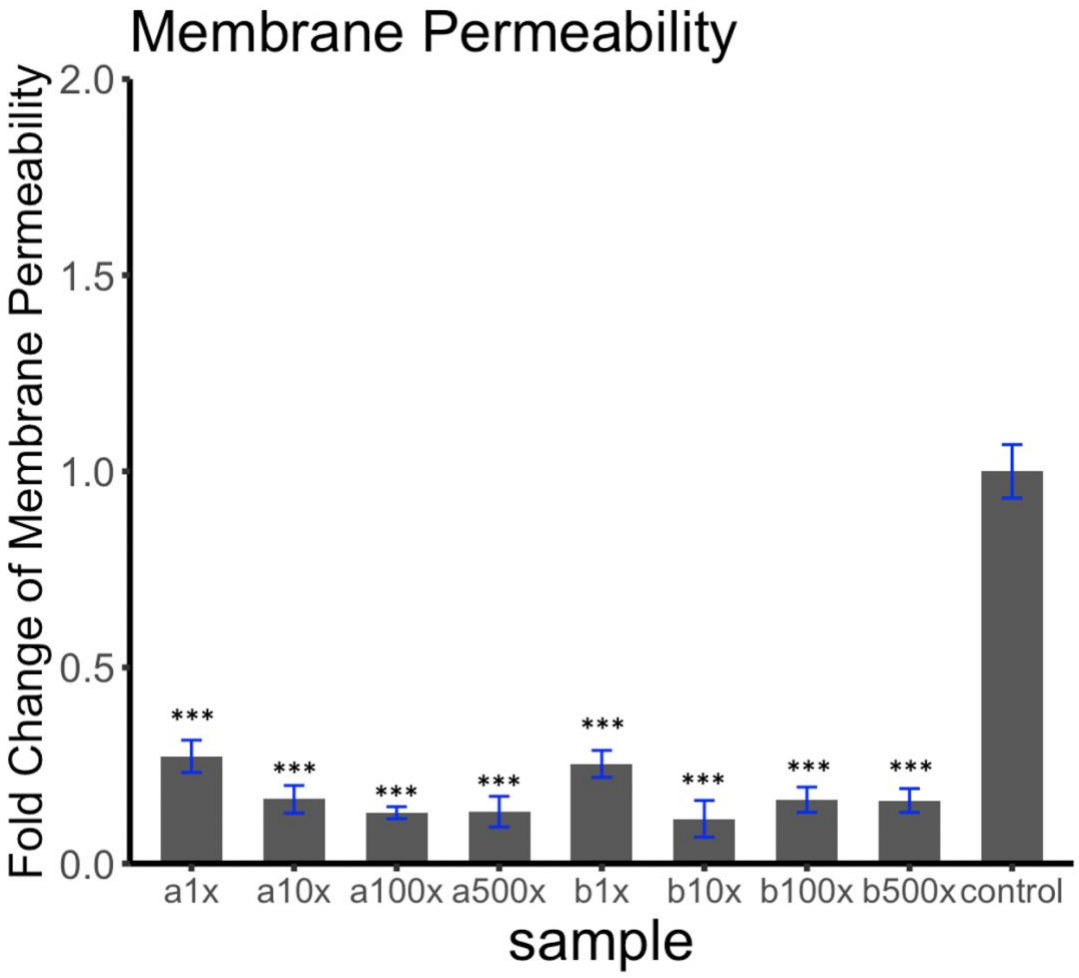

Figure 4. Fold change of membrane permeability compared to control induced by different concentration of wastewater samples. X-axis: sample names, a: after disinfection effluent, b: before disinfection; Y-axis: fold change in membrane permeability compared to the control. Significant differences in fold change of membrane permeability between test groups and control (di water) were tested with unpaired t-test and shown with *($p<0.05$), **($p<0.01$), ***($p<0.001$), and ****($p<0.0001$).

The outer membrane is a selective barrier that contains specialized protein and lipid, and have a strong impact on the sensitivity of bacteria to antibiotics, and drug resistance modifications.(Delcour, 2009) Previous studies demonstrated that chemicals like antibiotics, disinfectants, nanomaterials and ionic liquid can affect bacterial membrane permeability. (Guo et al., 2015; Li and Nikaido, 2009; Luo et al., 2014; Qiu et al., 2012) Therefore, we hypothesis that wastewater may be able to affect the cell membrane permeability, and thus induce antibiotic resistance. The changes in cell membrane permeability of E.coli strains treated with wastewater effluent extracts were determined with PI staining. All the test groups showed a significant decrease in the PI-positive cells, which indicate a decrease of cell membrane permeability (Figure 4).

Furthermore, the E.coli toxicogenomics assay data showed a significant increase of gene expression level in outer membrane permeability related genes (Figure 6). This suggests that wastewater can affect cell membrane permeability on both phenotypic and genotypic level, and the potential antibiotic resistance induction mechanisms of chemicals in wastewater effluent were likely related to the decrease of cell membrane permeability.

3.2.3 Assessment of horizontal gene transfer affected by wastewater

The horizontal transfer of antibiotic resistance genes is acknowledged as an important pathway to acquire ARGs, and thus develop and spread antibiotic resistance(Alekshun and Levy, 2007; Andersson and Hughes, 2014; Bengtsson-Palme and Larsson, 2015) Previous evidence suggested that various chemicals can promote conjugative transfer, therefore, we assume it might be a potential mechanism for the antibiotic resistance induction by wastewater.(Zhang et al., 2018b; Zhang et al., 2017a)

To determine whether the wastewater could facilitate the horizontal antibiotic resistance genes transfer, we evaluated the impact of wastewater effluent extracts on the conjugative transfer of ARGs from E.coli S17-1 to E.coli K12, and from P.putida to E.coli K12. Surprisingly, no test groups can promote conjugative transfer either cross genera or within genera.(Table S2) These results are consistent with the decrease or marginal impact of the wastewater extract on the cell membrane permeability as shown in Figure4. The increase of cell membrane permeability may explain why, since the bacteria outer membrane is an important barrier against horizontal gene transfer. (Zhang et al., 2017a) Another potential explanation for the low HGT rate might be related to the non-significant changes in the intracellular ROS level in exposure to the wastewater extracts. Previous study proved that concentration-dependent increase in ROS levels is likely responsible for the promotion of conjugative ARGs transfer. (Zhang et al., 2018b) However, in this

study, wastewater effluent extracts did not exhibit significantly affect on intracellular ROS, which are consistent with the observation that wastewater extract did not promote horizontal gene transfer.

3.3 Molecular insights into the potential mechanisms underlying the antimicrobial resistance induced by mixture of pollutants

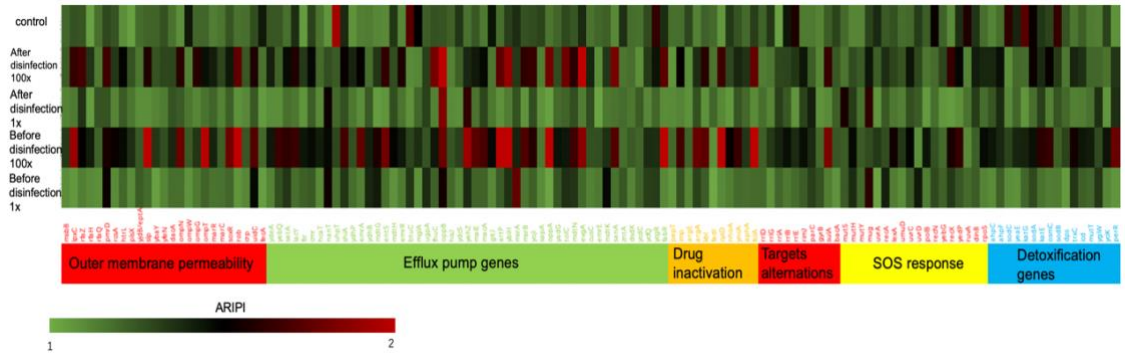

Figure 5. Heat map of Antimicrobial resistance induction potential index (ARIPI) (similar to the previously proposed Transcription Effect Level Index (TELI)) profiles representing the expression levels of antibiotic resistance pathway-relevant genes in the E. coli toxicogenomics assay in exposure to wastewater effluent extracts collected at Ithaca WWTP. TELIgene values targeting 129 genes associated with outer membrane permeability, efflux pump, drug inactivation, targets alternation, SOS response and detoxification genes are scaled by black-red color spectrum at the bottom.

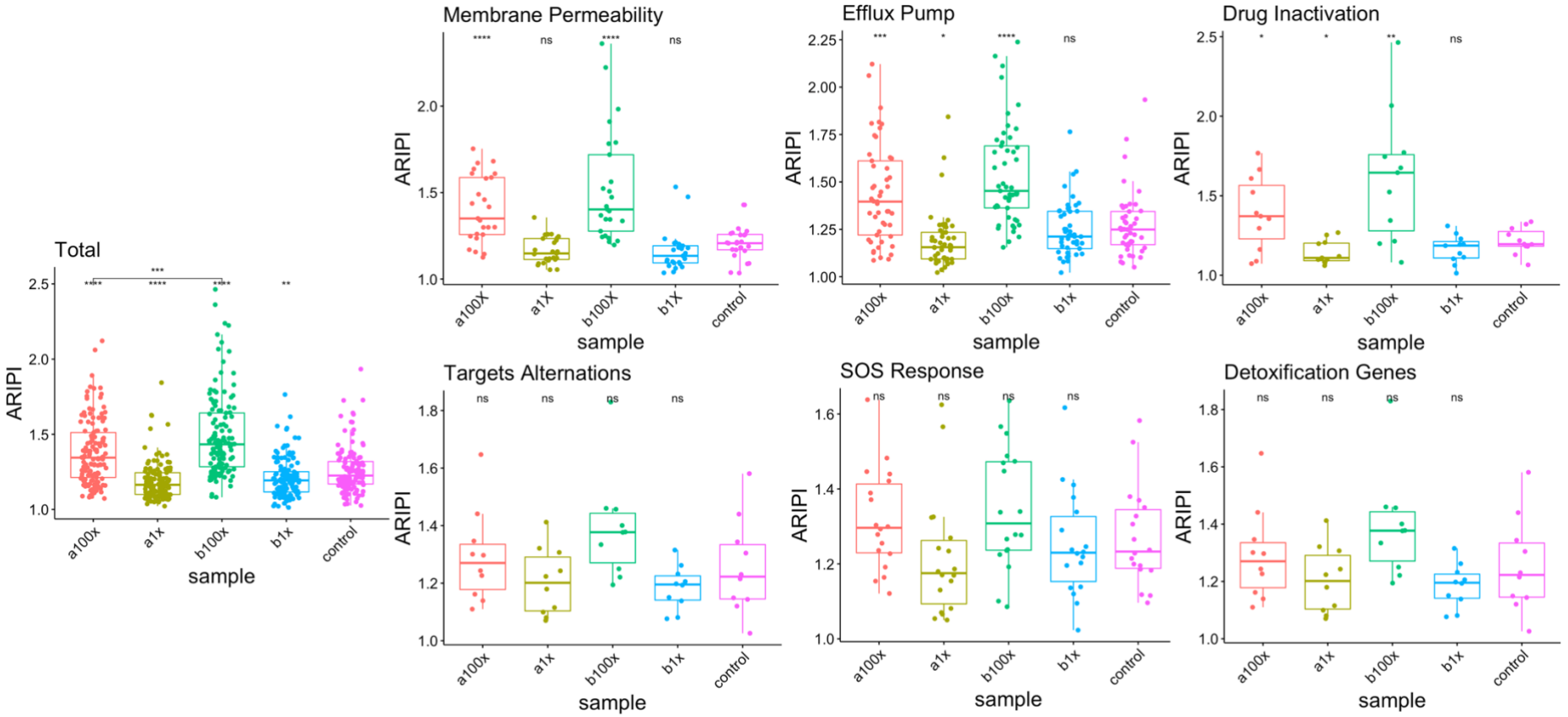

Figure 6. Box plots showing statistics of Antimicrobial Resistance Induction Potential Index (ARIPI) of all 129 genes and genes associated with six antibiotic resistance pathways in the E. coli toxicogenomics assay in exposure to wastewater effluent samples. The line within each box, the box top and the box bottom indicate the median, 75th percentile and 25th percentile of data, respectively. Wastewater samples with significantly

changed gene expression compared to the blank control (DI water) are highlighted with asterisks, *($p<0.05$), **($p<0.01$), ***($p<0.001$), and ****($p<0.0001$), respectively

A toxicogenomics assay using a GFP-fused E coli gen ensemble library, targeting six antibiotic resistance pathways, including outer membrane permeability, efflux pump, drug inactivation, targets alternation, SOS response and detoxification, was developed to quantitively evaluate the impact of wastewater effluent on antibiotic resistance induction. To quantify the chemical-induced gene expression level changes of a treatment, Transcriptional Effect Level Index (TELI) for E. coli were proposed and derived as quantitative molecular endpoints.(Gou and Gu, 2011) In this study, we employed the similar concept and refer it as antimicrobial resistance induction potential index (ARIPI). Through this toxicogenomics assay, detailed information on mechanisms of the antibiotic resistance induction potential by wastewater effluent extracts was obtained, and quantitative comparison among responses in multiple antibiotic resistance pathways was performed. Figure 5 shows the gene expression profiles of biomarkers indicative of different antibiotic resistance pathways upon exposure to wastewater extracts, which provide a general view of the disturbance resulted from the wastewater mixture on the antibiotic resistance pathway-relevant biomarkers. We further quantified the response of E.coli in exposure to different concentration of wastewater samples before and after the disinfection process, with ARIPI values for each of the six antimicrobial resistance pathways.. The differences in the ARIPI values between each wastewater sample and the blank control (DI water) were evaluated using t-tests (Figure 6).

As shown in figure 5 and 6, both before disinfection and after disinfection wastewater extracts led to disturbances in the expression level of the biomarkers involved in all six mechanistic pathways. They showed concentration-dependent pattern, with higher level disturbances at higher concentration. In overall, samples before the disinfection led to higher level of disturbance than those after the disinfection, which is consistent with previous phenotypic MIC evaluation results discussed above, suggesting that residual pollutants and their transformation products through the wastewater treatment processes seemed to exhibit higher antimicrobial resistance reduction potential than DBPs in the disinfected effluent. The results show that the E. coli toxicogenomics assay is sensitive enough to reveal the pathway- level antibiotic resistance induction difference in response to wastewater effluent. The toxicogenomics assay-derived molecular toxicity endpoint quantifiers (TELI or here referred as ARIPI) revealed that wastewater effluent has more pronounced impact on the biomarkers related to membrane permeability, efflux pump system and drug inactivation. Less impacts were exerted by the wastewater effluent on target alternation, SOS response and detoxification pathways. These are generally consistent with the phenotypic evaluations results of membrane permeability, intracellular ROS and horizontal gene transfer rate.

We examined all the individual biomarkers in further depth, and identified those who were impacted the most (ARIPI values >1.5) and they are listed in Table 1. For outer membrane permeability related biomarkers and efflux pump related biomarkers,

the average ARIPI value of tertiary effluent and final effluent is extremely significantly higher than control ($p < 0.001$), which is consistent with the results shown in figure 4. This suggests that wastewater effluent extracts can impact the antibiotic resistance induction at both phenotypic and genotypic level. For drug inactivation related biomarker, the result reveals a significantly difference gene expression level between wastewater effluent extracts and control. However, the MIC results suggests that these samples can't induce Beta-lactam antibiotic resistance, which potentially indicate that the effluent extracts of Ithaca WWTP can only affect the genotypic change on Beta-lactam category antibiotic resistance. For targets alternation, SOS response and detoxification related genes, there were no significantly difference of average ARIPI values between effluents and control. Meanwhile, our previous results also suggest that wastewater effluents have no impact on the intracellular ROS level and horizontal gene transfer rate, which is similar with the toxicogenomics assay data. This suggests that the tertiary effluent and final effluent of Ithaca WWTP may have no effects on oxidative stress, SOS response and ROS formation, and therefore, these three mechanisms may not be the major mechanisms under the antibiotic resistance induction by wastewater effluents.

Table 1. Summary of mechanic pathways and genes that showed altered expression level with ARIPI > 1.5 in *E.coli* strains in exposure to wastewater effluent extracts (REF=100).(Lan et al., 2014)

mechanism	gene	Before disinfection samples ARIPI	After disinfection samples ARIPI	Pathway	function	Ref.
Outer membrane permeability	*lpxC*	1.91	1.64	Lipid-mediated antibiotic resistance	catalyzes the second reaction and the first committed step in lipid A biosynthesis	(Vaara and Nurminen, 1999)
	rfaZ	1.51	1.68	Lipid-mediated antibiotic resistance	biosynthesis lipopolysaccharide	(Vaara and Nurminen, 1999)
	pmrD	1.56	1.67	Lipid-mediated antibiotic resistance	confers resistance to polymyxin B	(Vaara and Nurminen, 1999)
	rcsA	1.52	1.34	Lipid-mediated antibiotic resistance		(Yu et al., 2012)
	slp	2.23	1.59	protein-related		(Zhang et al., 2019)
	ompN	1.78	1.61	Porin-mediated antibiotic permeability	a porin with single-channel conductance properties	(Vaara and Nurminen, 1999)
	ompG	1.34	1.61	Porin-mediated antibiotic permeability	nonspecific channel for mono-, di, and trisaccharides less than 600 Daltons	(Viveiros et al., 2007)

	ompT	1.98	1.46	Porin-mediated antibiotic permeability	outer membrane protease with specificity for paired basic residues	(Viveiros et al., 2007)
	soxR	1.79	1.44	Porin-mediated antibiotic permeability	Superoxide Response protein, controls the transcription of the regulon involved in defense against redox-cycling drugs	(Ruiz, 2003)
	rob	2.36	1.75	Porin-mediated antibiotic permeability	Regulate outer porin expression in response to salicylate	(Chubiz and Rao, 2011)
	uidC	1.72	1.58	Porin-mediated antibiotic permeability		(Wang et al., 2019)
Efflux pump	*yebQ*	1.66	1.21	MSF (Major facilitator superfamily)	proton-driven drug efflux system	(Van Bambeke et al., 2003)
	setA	1.62	1.47	MSF (Major facilitator superfamily)	Efflux sugars and sugar analogues, proton/sugar antiporter	(Van Bambeke et al., 2003)
	lacY	1.66	1.58	MSF (Major facilitator superfamily)	A secondary transporter	(Radestock and Forrest, 2011)
	shiA	1.68	1.52	MSF (Major facilitator superfamily)	A proton/shikimate symporter	(Saidijam et al., 2006)
	emrA	1.76	1.51	MSF (Major facilitator superfamily)	membrane fusion protein for the EmrB multidrug efflux pump	(Lomovskaya and Lewis, 1992)
	mdtG	1.57	1.34	MSF (Major facilitator superfamily)	overexpressed in the fluoroquinolone resistance strain	(Nishino and Yamaguchi, 2001)

	entS	1.80	1.63	MSF (Major facilitator superfamily)	enterobactin exporter	(Crouch et al., 2008)
	emrR	1.43	1.57	TF (TetR family regulators) - MFS	efflux pump EmrAB	(Du et al., 2018)
	fhuC	1.43	1.81	ABC (ATP-binding cassette)		(Köster, 1991)
	oppB	1.78	2.06	ABC (ATP-binding cassette)	integral membrane component of ABC transporter	(Moussatova et al., 2008)
	yehZ	1.91	1.39	ABC (ATP-binding cassette)	periplasmic binding component of ABC transporter	(Moussatova et al., 2008)
	malE	1.73	1.43	ABC (ATP-binding cassette)	periplasmic substrate-binding component of the maltose ABC transporter	(Licht et al., 2019)
	macA	1.69	1.47	ABC (ATP-binding cassette)	macrolide efflux transport system - membrane fusion protein	(Greene et al., 2018)
	artP	2.24	1.75	ABC (ATP-binding cassette)	arginine transporter ATPase	(Chaudhari et al., 2015)
	ybiH	2.11	1.89	TF (TetR family regulators) - ABC	DNA-binding transcriptional dual regulator CecR	(Van Bambeke et al., 2000)
	msrB	1.71	1.62	ABC (ATP-binding cassette)	methionine sulfoxide reductase	(Kumar et al., 2010)
	yojI	1.60	1.64	ABC (ATP-binding cassette)	microcin J25 efflux protein	(Delgado et al., 2005)
	oppA	2.05	1.81	ABC (ATP-binding cassette)	oligopeptide transport, quorum sensing;	(Jin et al., 2018)

	tolC	1.44	1.78	RND (Resistance-nodulation-cell division)	cationic antimicrobial peptide (CAMP) resistance, macrolide resistance (MacAB-TolC transporter)	(Bina et al., 2008)
	mdtN	1.66	1.61	RND (Resistance-nodulation-cell division)		(Nishino and Yamaguchi, 2001)
	evgA	1.86	2.2	RND (Resistance-nodulation-cell division)	efflux pump MdtEF-TolC	(Hirakawa et al., 2003)
	sanA	1.72	1.82	uncategorized	encode an inner membrane protein involved in cell envelope barrier functions	(Rida et al., 1996)
	fabR	2.16	1.73	uncategorized - TF (TetR family regulators)	influence membrane lipid homeostasis	(Van Bambeke et al., 2000)
Drug inactivation	*imp*	1.68	1.52	Beta-lactam enzyme genes		(Vázquez-Ucha et al., 2020)
	argA	1.77	1.37	Beta-lactam enzyme genes		(Zhang et al., 2021)
	kbl	1.75	1.61	Beta-lactam enzyme genes	glycine C-acetyltransferase	(Zhang et al., 2017b)
	cysD	2.07	1.67	Beta-lactam enzyme genes	sulfate adenylyltransferase	(Ramirez and Tolmasky, 2010)
	nhoA	1.52	1.30	Beta-lactam enzyme genes		(Yamamura et al., 2000)

	yncA	1.65	1.39	Beta-lactam enzyme genes	Have L-amino acid N-acyltransferase activity	(Hentchel and Escalante-Semerena, 2015)
	folA	2.46	1.77	Drug deactivation (Trimethoprim) & SOS		(Zywno-van Ginkel et al., 1997)
Targets alternation	*sulA*	1.83	1.65	Target alteration (sulfonamides) & SOS		(Wang et al., 2019)
SOS response	*lexA*	1.55	1.30	SOS response	transcriptional repressor for SOS response	(Mo et al., 2017)
	recX	1.56	1.64	SOS response		(Zhang et al., 2018a)
	yedP	1.64	1.48	SOS response		(Zhang et al., 2019)
detoxification	*katE*	1.61	1.38	oxidative stress		(Zhang et al., 2019)
	sodA	1.45	1.53	oxidative stress		(Lu et al., 2020)
	osmC	1.64	1.52	oxidative stress		(Lu et al., 2020)
	trxC	1.55	1.40	oxidative stress		(Zhang et al., 2019)
	perR	1.78	1.34	oxidative stress		(Jin et al., 2018)

Our previous study showed that TELI exhibited dose–response patterns for both a single gene and a gene ensemble. Based on the dose-response curve, the specific or overall toxicity obtained by the toxicogenomics-based approach was represented by the corresponding REF that causes the TELI value to reach 1.5 (termed EC-TELI1.5), therefore, Gou et al. defined positive as TELI value greater than 1.5.(Gou et al., 2014) Table 1 detailed summarize all the genes that showed altered expression level with ARIPI > 1.5 after 2 hours exposure to the wastewater effluent extracts, which provide the insight into the antibiotic resistance induction potential of these wastewater samples on molecular level.

For the tertiary effluent before disinfection, outer membrane permeability, efflux pump and drug inactivation are the three main mechanisms underlying the antimicrobial resistance induction. For the membrane permeability related biomarkers, *lpxC*, *rfaZ*, *pmrD* and *rcsA* are related with lipid-mediated, either inhibit the biosynthesis or modify the lipid A on outer membrane, which is required for bacterial growth and virulence.(Clements et al., 2002; Klena et al., 1992; Rubin et al., 2015; Szczesny et al., 2018) Slp is membrane protein, and omp, rob and sox work to regulate the expression of outer membrane porin, which control the diffusion of chemicals across the membrane.(Ananthan and Subha, 2005; Chubiz and Rao, 2011; Tondera et al., 2009) Among the 10 selected biomarkers, four were lipid-mediated antibiotic resistance genes and five were porin-mediated antibiotic permeability related genes, while *rob* showed the highest altered gene expression level up-

regulation, which is function as the regulation of outer porin expression in response to salicylate. (Chubiz and Rao, 2011)

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment.(Webber and Piddock, 2003) Seven of the 20 selected genes were belong to the Major facilitator superfamily, which is a superfamily of transmembrane secondary transport proteins. (Wang et al., 2020) Nine of them were ATP-binding cassette superfamily, which proteins translocate substrates, hydrophobic compounds and metabolites across extra- and intracellular membranes. (Dean et al., 2001) Two were Resistance-nodulation-cell division, and two were TetR family regulators, which are widely associated with antibiotic resistance. (Cuthbertson and Nodwell, 2013)

Similar to effluent before disinfection, for final effluent after disinfection, outer membrane permeability, efflux pump and drug inactivation are the three main mechanisms underlying the antimicrobial resistance induction. 8 biomarkers were selected under membrane permeability, three were lipid-mediated antibiotic resistance and four were porin-mediated antibiotic permeability related, *rob* was also the highest updated one. This suggested that before and after disinfection wastewater extracts had similar effect on genotypic outer membrane permeability change. For efflux pump related biomarkers, five were belong to Major facilitator superfamily, seven were ATP-binding cassette superfamily, three were TetR family regulators and three were Resistance-nodulation-cell division related. This may indicate that although MFS and ABC were the major pathways for both samples, TF and RND may play a relatively

more effect under the antimicrobial resistance induction by after disinfection effluent extracts.

For both tertiary and final effluent, biomarkers related to Beta-lactam enzyme, trimethoprim deactivation and sulfonamides target alteration showed altered expression. However, our previous results didn't show any resistance against any antibiotics belong to these three categories. This may suggests that wastewater effluent may only affect the genotypic resistance change against Beta-lactam, trimethoprim and sulfonamides antibiotic.

Genes indicative of SOS response and oxidative stress were less affected by the wastewater exposure than those involved in the membrane permeability and efflux pump systems in this study. Selected biomarkers in these two functional categories were classified into several subcategories based on the oxidative stress and SOS response related function, including those related to DNA damage and repair (*recX*), mRNA expression genes (*lexA*), catalase (*katE*), and superoxide dismutases (*sodA*). However, genes such as *ahpC*, *oxyR*, and *ahpF*, which were proved by various studies to be related to SOS response and oxidative stress didn't exhibit higher than 1.5 fold threshold-level changes in their expression level in this study. (Gou et al., 2014) This was consistent with the phenotypic evaluation that these samples did not promote intracellular ROS formation.

3.4 Insights into the association of detected micropollutants with antimicrobial resistance induction potential (ARIP)

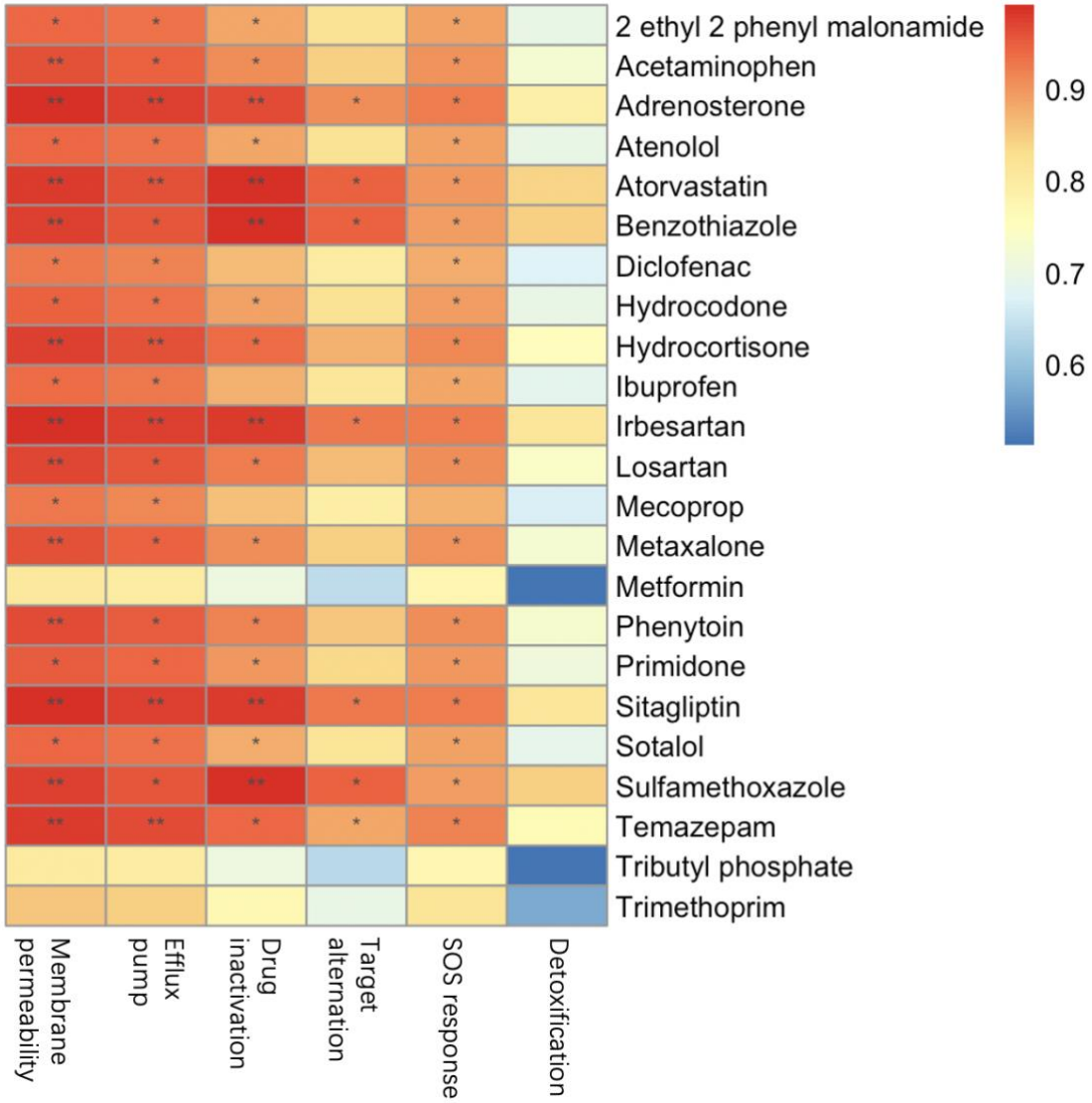

Figure 7. Pearson correlation analysis between chemical concentrations and pathway-level ARIPI values in wastewater effluent extracts. Correlation coefficients at 95% significance level are scaled with color spectrum on the right, zeros are assigned to the non-significant correlations. Asterisks indicating a significant correlations *($p<0.05$), and **($p<0.01$).

Quantifiers and organic micropollutants detected in the wastewater effluent extracts was conducted to gain insights into the potential association or candidate contributors that lead to the antibiotic resistance at pathway level. Among over 180 tested micropollutants, 23 were detected at a relatively high concentration, and were chosen to run the correlation analyze between the average ARIPI values of each of the antibiotic resistance pathways. A chemicals with a concentration more than 100 ug/L in the extracts was considered as a relatively high concentration, since 1 ug/L is an environment relevant concentration of contaminants with antibiotic-like effect in wastewater (which is100 ug/L in extracts)(Fang et al., 2012; Gottschalk et al., 2013; Petrasek Jr and Kugelman, 1983), and the lowest concentration level of these chemicals that can induced antibiotic resistance was 100 ug /L(Zhang et al., 2017a; Zhang et al., 2018c). As shown in Figure 7, 20 of them were significantly correlated ($p < 0.05$) with at least one antibiotic resistance pathway. Mechanisms related to outer membrane permeability and efflux pump system are the two pathways significantly correlated with all the highlight chemicals. Noticeably, the average ARIPI value of drug detoxification genes did not related to any of the evaluated chemicals.

Most of the chemicals listed above are non-antibiotic drugs, while adrenosterone and benzothiazole are transformation productions of drugs, mecoprop is a pesticide, trimethoprim is an antibiotic and Tributyl phosphate is a widely used solvent. Whether these non-antibiotic chemicals can potentially lead to antimicrobial resistance potential remain largely unknown. Previous study provided evidence that exposure to acetaminophen and ibuprofen can induce both phenotypic and genotypic

antibiotic resistance by increase the efflux pump.(Verma et al., 2018) In our study, the correlation between the genotoxicity biomarkers with acetaminophen and ibuprofen is therefore consistent with previous reports. As the correlation heatmap suggests that drugs with relatively high concentration in wastewater all strongly related with at least one antibiotic resistance pathway, indicating most of the drugs listing above may have the genotypic antibiotic resistance induction potential. Moreover, 2,4-D, a widely used pesticide, is also proved to have antibiotic resistance induction increase in the expression of efflux pumps, a reduced synthesis of outer membrane porins.(Kurenbach et al., 2015) Mecoprop is a pesticide that have a similar chemical constructure with 2,4-D, and is significantly related with the average ARIPI value of both membrane permeability and efflux pump related genes (Figure 7).

Chapter 4 Conclusion

In this study, the antimicrobial resistance induction potential and mechanisms of before and after disinfection process wastewater effluent extracts were systematically investigated, and the results showed that both two effluents can induce antimicrobial resistance in both phenotypic and molecular level. Results from toxicogenomics assay suggested outer membrane permeability, efflux pump, and drug inactivation related biomarkers reveals a significantly difference in the gene expression level between wastewater extracts and control, indicated that these three pathways may be the main

mechanism underlying the induction of antimicrobial resistance by wastewater effluents.

A new antimicrobial resistance induction potential index (ARIPI) was employed to screen and quantify the resistance induction potential and mechanisms of mixture of chemicals or any environmental samples. The correlation between commonly detected organic pollutants in the wastewater and ARIPI raise the possibility that many organic pollutants in the wastewater system and their mixture may have ability to lead antimicrobial resistance. In addition, further research on the antimicrobial resistance effects of different environmental samples is much needed, to elucidate the correlation between antimicrobial resistance induction potential and the mixture of different micropollutants.

Appendices

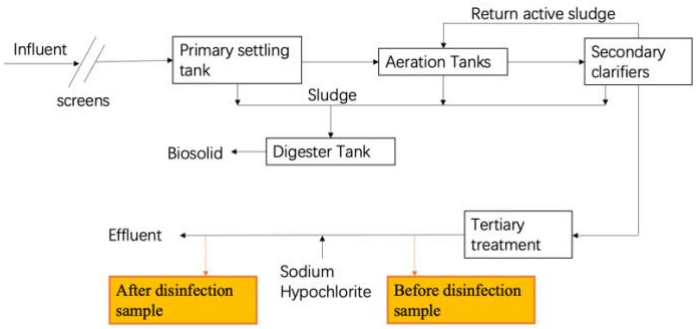

Figure S1. Diagram of wastewater treatment processes at Ithaca Wastewater Treatment Plant, Ithaca, New York, USA; Sampling locations are labeled.

Table S1, List of selected genes from *E.coli* antibiotic resistance gene library and their related antibiotic resistance pathways

Mechanism	Gene selected	Functions
Outer membrane permeability	*msbB, lpxC, rfaZ, rfaH, rfaQ, pmrD, rcsA,htrL, plsX, yjbB/eptA, slp, ybaY, yeN, dacA, ompN, ompW, ompG, ompT, marR, marC, soxR, rob, crp, uidC, fecA*	Lipid-mediated related, porin related, PBP related, Porin-mediated antibiotic permeability
Efflux pump	*ydeA, yebQ, setA, lacY, fsr, cmr, hcaT, nanT, uhpT, shiA, ydhP, emrA, ydhB, mdtG, entS, mdtH, emrR, btuC, mglA, ugpA, fhuC, oppB, hisJ, ydcS, yehZ, malE, macA, gltJ, artP, ybiH, msrA, msrB, yojI, sapA, oppA, yadG, tolC, mdtN, evgA, cusC, emrE, mdtK, sanA, zntA, dsdX, ycdC, ycfQ, ycgR, fabR*	Major facilitator superfamily, TetR family regulators, ATP-binding cassette, Resistance-nodulation-cell division, Small multidrug resistance family
Drug inactivation	*pepT, imp, argE, argA, kbl, fadI, cysD, nhoA, yncA, aphA, folA*	Beta-lactam enzyme genes, Trimethoprim deactivation
Targets alternation	*rrlD, rrlG, rrlA, rrlB, rrlE, rimJ, parC, gyrB, sulA, bacA*	Erythromycin-resistant targets, Aminoglycosides targets, Quinolones targets
SOS response	*mutS, mutH, mutY, mug, uvrA, recA, lexA, umuD, uvrC, uvrD, polB, recN, yebG, recX, yedP, ruvA, dinB, rpoS*	SOS response
Detoxification	*ahpC, ahpF, sodC, yeaE, katG, sodA, katE, osmC, sodB, dps, trxC, icd, mutT, ygiW, yciK, perR*	oxidative stress

Table S2 Horizontal gene transfer rate cross genera and within genera (CFU/uL)

sample	Cross genera *E.coli S17-1* & *P.putida*	Within genera *E.coli K12* & *E.coli S17-1*

control	0	0	0	0
b1x	0	0	0	0
b10x	0	2	0	0
b100x	1	0	0	0
b500x	0	0	0	0
a1x	3	0	0	0
a10x	0	0	0	0
a100x	0	0	0	2
a500x	0	0	0	0

Text S1. Measurement of horizontal gene transfer

Cross genera

The agar plate was made by adding 25 g/L LB and 15 g/L agar, autoclaved, then add 80mg/L STR and 50 mg/L AMP when media is cool at around 45 C. *E.coli S17-1* and *P.putida* was firstly wash with PBS twice to remove LB, then was 1:1 mixed at *E.coli S17-1* OD~0.25, *P.putida* OD~0.5(to make the CFU equal). Then bacterial cultures were exposure to wastewater samples at various concentrations for 24 h at 37 °C. Add 40ul of each sample to the plate and spread it. Culturing the plate for 48 h at 37 C.

Within genera

The agar plate was made by adding 25 g/L LB and 15 g/L agar, autoclaved, then add 80mg/L STR and 50 mg/L KN when media is cool at around 45 C. *E.coli K12* and *E.coli S17-1* was firstly wash with PBS twice to remove LB, then was 1:1 mixed at the same CFU (OD~0.25). Then bacterial cultures were exposure to wastewater samples at various concentrations for 24 h at 37 °C. Add 40ul of each sample to the plate and spread it. Culturing the plate for 48 h at 37 C.

Text S2. Gene expression profiling data processing and quantitative molecular toxicity endpoints derivation

Temporal raw data of optical density (OD) and green fluorescent protein (GFP) signals are first corrected by OD and GFP signals of medium controls and promoterless bacteria controls with or without sample, respectively. The induction factor I, which represented the alteration in gene expression for a given gene at each time point due to exposure to effluent water samples compared to untreated control, is calculated as I = Pe/Pc, where, Pe = (GFP/OD)experiment as the normalized gene expression GFP level in the experiments condition with sample exposure, and Pc = (GFP/OD)control in the blank control condition without any sample exposure.

To quantify the chemical-induced gene expression level changes of a treatment, Transcriptional Effect Level Index (TELI) was proposed and derived as a molecular toxicity quantifier. The accumulative altered gene expression change over the 2 h exposure period was calculated as:

$$TELI_{gene\ i} = \frac{\int_{t=0}^{t} e^{|\ln I|} dt}{exposure\ time}$$

Where, t is the exposure time.

The pathway level responses are calculated by intergrading the expression change of all the genes in a pathway, as:

$$TELI_{pathway} = \frac{\sum_{i=1}^{n} w_i \times TELI_{gene\ i}}{n}$$

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