Addressing the Global Crisis of Antibiotic Resistance: Developing an Accessible Framework to Monitor Antibiotic-Resistant Bacteria in Watersheds

In partnership with non-profit organization WILDCOAST

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Abstract:

The proliferation of antibiotic resistance is a pressing global threat to the health of plants, animals, and humans. Antibiotic resistance occurs when bacteria gain the ability to assimilate an antibiotic resistance gene (ARG), rendering traditionally used antibiotics ineffective. Due to this resilience, antibiotic resistant bacteria (ARB) can be extremely difficult to treat, making infections harder to treat and potentially deadlier. Interestingly, ARGs can build up in watersheds, posing a public health concern. Despite the rapid propagation of ARB, especially in aquatic areas, no set standardized methods exist to quantify their existence in the environment. Lengthy and expensive testing methods leave individuals unaware of potential health risks. This study aims to determine the suitability of the modified IDEXX-Colilert 18 method (modified IDEXX) as a potential standardized ARB measurement protocol and simultaneously quantify the amount of antibiotic resistance in the Tijuana River Watershed (TJR), a primary site of concern to public health. This research was conducted in two areas in California: various watersheds in Los Angeles (LA) and TJR. Water samples were collected from the rivermouth to inland areas; these were then analyzed in the lab to compare two different methods/techniques for antibiotic resistance quantification: traditional culturing (plate filtration) and modified IDEXX. Results showed the modified IDEXX method produced similar AR E.coli colony counts to plate filtration (0.96 and 0.98 Pearson correlation coefficient with MTEC and TBX plates, respectively), suggesting a directly proportional relationship. It was also found TJR sites were significantly more ARB-infested than LA, and inland sites were also more contaminated than rivermouth sites. YMCA and Tijuana River, public waterways, were found to have a high Antibiotic Resistance: 8-10% resistance. Use of the modified IDEXX method could be an effective qualitative ARB indicator to measure water quality and aid policy-making in public waterways in order to prevent infections and protect overall public health. Metagenomics and qPCR methods in comparison to modified IDEXX are still ongoing.

1. Introduction

1.1 What is antibiotic resistance?

Antibiotic resistance arises as bacteria gain resistance to previously effective antibiotics. These antibiotics are often ones administered to humans and animals for disease treatment and to plants for growth enhancement (discussed further in Section 3: How ARB Spread in the Environment). Antibiotic resistant bacteria (ARB) attain resistance through the expression of antibiotic resistance genes (ARG). These genes can encode for various defensive traits against antibiotics, such as enzymes that break them down or cellular membranes that prevent or limit their entry (Mutuku et al., 2022). As a result, ARB are incredibly difficult to treat with current antibiotics and persist in the aquatic environment, despite water-purifying efforts from wastewater treatment plants (WWTPs). Though not always the case, ARB can often be infectious, posing an acute threat to public and environmental health because of their resilience against treatment (Discussed further in Section 4: Concerns of ARB Spread: Health and the Economy).

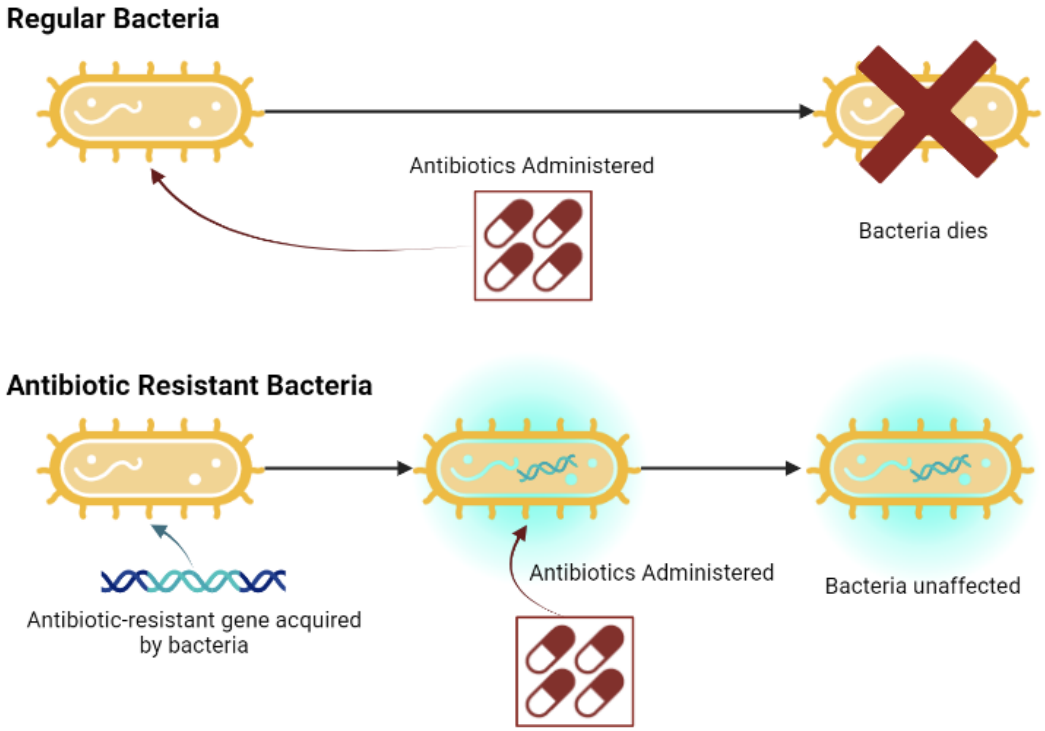

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Figure 1. Regular bacteria vs antibiotic resistant bacteria

1.2 How bacteria become antibiotic-resistant

As unicellular organisms, bacteria have short generational turnover times leading to rapid evolution and an ability to interact readily with their surrounding environment via cellular membranes (Gibson et. al., 2018). Consequently, there are ample opportunities for genetic code to change and thereby increase bacterial fitness in adverse environments. Bacteria can obtain new genetic code through two distinct mechanisms: vertical and horizontal gene transfer (VGT and HGT). These mechanisms have facilitated rapid bacterial evolution over the course of millions of years, allowing bacteria to acquire new advantageous traits and adapt to novel environmental pressures. ARB assimilate ARGs into their genetic code via VGT and HGT.

a. Horizontal gene transfer

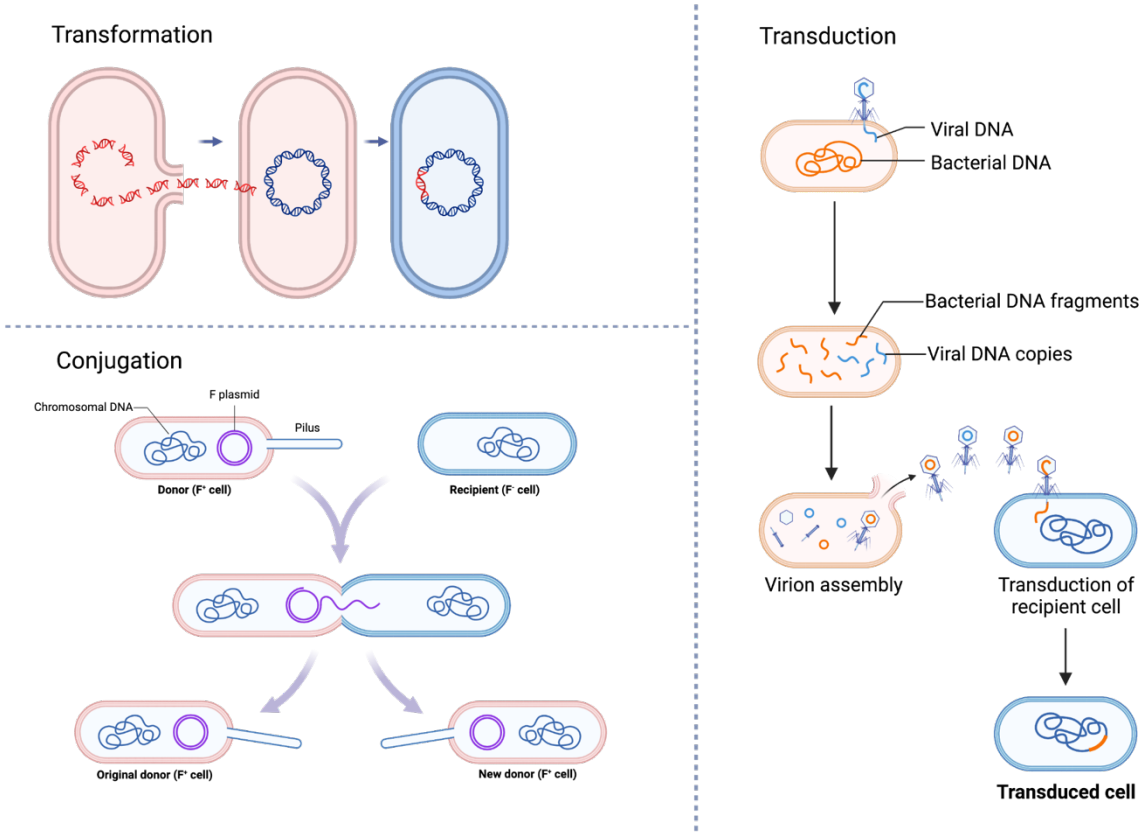

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Figure 2. Schematic of horizontal gene transfer

Horizontal gene transfer significantly contributes to bacterial resistance (Lerminiaux & Cameron, 2018). Mechanisms of HGT include obtaining DNA from other bacteria (transformation and conjugation) or viruses (transduction) in the surrounding environment.

i. Transformation:

Transformation occurs when extracellular DNA (eDNA) from the surrounding environment is selected through the cellular membrane of a bacteria then integrated into its DNA (Burmeister, 2015).These eDNA are released by other bacteria, and can linger in aquatic settings (discussed further in Section 1.3: How ARB spread in the environment).

ii. Conjugation:

Conjugation, also known as bacterial sex, is the transfer of DNA from one microbe to the next via direct contact (Burmeister, 2015). One bacteria extends a pilus, which acts like a bridge that joins into the cellular membrane of another bacteria to exchange plasmids (ring-shaped strands of DNA– not part of the main genetic code– found in bacteria that can be readily replicated and exchanged) and other DNA fragments.

iii. Transduction:

Transduction is the use of bacteriophages (bacteria-infecting viruses) to transfer new genetic code (Burmeister, 2015).

b. Vertical gene transfer

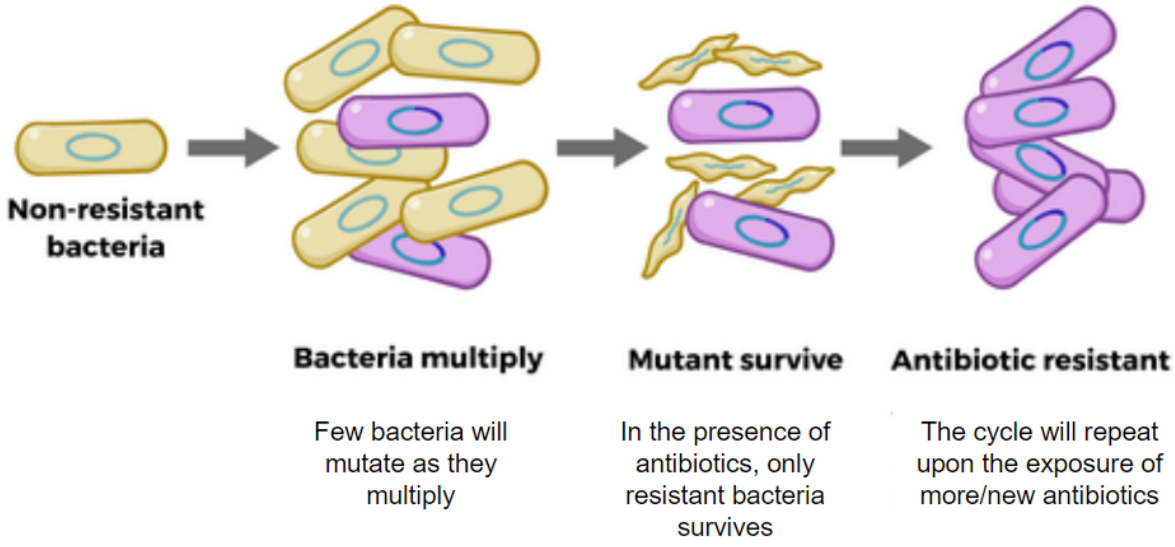

Image taken from Labster Theory. (Edited)

Figure 3. Schematic of vertical gene transfer

Vertical gene transfer refers to transmission of genes from one generation to the next (Bethke et al., 2023). The haploid nature of bacterial DNA combined with short generation turnover provides numerous opportunities for mutations. Sometimes, the mutations can result in new genetic code encoding for antibiotic resistance.

1.3 How AR spreads in the environment

Selection pressure plays a critical role in the propagation of advantageous traits like antibiotic resistance (Skalet et al., 2010). It is an environmental stressor that can favor the expression of certain phenotypes over others in a given environment. As a result, selection pressure is instrumental in driving evolutionary changes that organisms experience over time. In the case of antibiotic resistance, aquatic environments with heavy antibiotic presence promote the prevalence of ARB. Consequently, areas with high antibiotic usage such as hospitals, agricultural plots, and aquaculture sites would increase the likelihood of ARB presence. These industries use antibiotics as a means of treatment, growth, and disease prevention (Pruden et al, 2013).

Aquatic environments serve as a major reservoir for ARGs due to their widespread mixing and distribution. Marine environments, in particular, are the largest and most diverse breeding pool for ARGs. Previously, a common assumption was that marine environments primarily received ARGs from terrestrial sources by way of antibiotic-infused and contaminated discharge from terrestrial sources (Amarasiri et al., 2019). A survey conducted by Miranda et al. in 2018 demonstrates the environmental leaking of antibiotics from Salmon farms in Chile to the surrounding environment and sediments. A contrasting study by Muziasari, however, suggested an alternative explanation. Results from the metagenomic study found gut microbiota of fish carried similar levels of ARGs as sediment from both inside and outside the Salmon farm. This suggests that animal waste could be a driver of ARG and ARB prevalence in marine environments in addition to large-scale agricultural or aquacultural antibiotic leaks(Muziasari et al., 2017). There is also a possibility that microbes can naturally produce antibiotics as a defense mechanism. Thus, other bacteria can develop ARGs against these natural antibiotics to counteract them. Detectable amounts of antibiotic resistance genes were found as far as 522 kilometers away from shore, and at depths of 8,200 meters. The very turbulent, constantly-moving marine environment has a natural proclivity for mixing and distributing small particles far and wide, so the equilibrium of ARG concentration across the ocean is a natural progression.

Apart from marine and freshwater environments, wastewater treatment plants (WWTPs) are man-made structures that provide ample resources for the dissemination and proliferation of ARGs. WWTPs receive water contaminated with antibiotic-dense material from sources such as farms, residential areas, hospitals, and industrial plots. Although WWTPs have been designed to remove harmful bacteria through various filtration and disinfection processes, it has been found they are not entirely successful at removing ARGs (Amarasiri, 2019. These ARGs persist in treated water and are even found in drinking water sourced from these plants (Pruden et al. 2006). This contaminated water subsequently ends up in rivers, lakes, groundwater, and agricultural irrigation systems, threatening human safety. The population can be exposed to this ARG-contaminated water through numerous outlets: drinking, bathing, agricultural occupational

exposure, consumption of food requiring irrigation, and even aquatic sports. (Leonard et al., 2018; Amarasiri et al. 2019).

The agricultural industry is also a significant source of antibiotics and ARB in the environment. Subtherapeutic doses of antibiotics given to livestock create selective pressure for resistance genes in gut bacteria to proliferate. Concentrated animal feeding operations (CAFOs) hold large numbers of animals and create high amounts of agricultural waste that ultimately serve as an important reservoir for ARB and ARGs. Additionally, the presence of nondegradable heavy metals in manure exerts a continuous selection pressure on ARGs allowing them to persist (Lima et al., 2020). Contaminated manure is then applied to other agricultural land uses, providing a pathway for ARB and ARGs to enter the environment. In a 2020 study, He et al. found that ARGs can persist in manured soils for more than 120 days, taking three to six months to return to original levels. When livestock farms discharge waste containing ARBinto the environment, HGT may occur between ARB and native bacteria. The resistant bacteria can then enter the human body through food and water ingestion or inhalation and reproduce to cause infections (He et al., 2020). Through experimentation, manure application has been demonstrated to increase the abundance and diversity of ARGs in soil by 10^5-fold, resulting in an ARG content up to 28,000 times higher than un-manured soils (He et al., 2020).

Besides anthropogenic sources, the environment itself also plays a role in selection for and distribution of antibiotic resistance. Features of the soil such as temperature, pH, moisture, and water movement can all mediate the rate of ARG distribution. In bodies of water, the presence of heavy metals can create additional selection pressure. For example, metals like iron found in pipes can increase levels of ARB in municipal water (Mohan Amarasiri et al., 2019). Studies also show they can survive longer when adhered to a solid surface. eDNA can adhere well to negatively-charged particles found in clay, quartz, feldspar, and bacterial sludge (Amarasiri et al., 2019). eDNA and mobile genetic elements (MGEs) play a key role in AR proliferation (Aminov, 2011). MGEs are the conduits that facilitate the assimilation of eDNA into a bacteria during transformation. The presence of both is heavily determined by environmental conditions. These tiny compounds are delicate and can break down easily in harsh conditions. Thus, antibiotic resistance is most often selected for in nutrient-rich, stable environments with low fitness costs. They especially thrive in certain antibiotic resistance hotspots: adhered to solid surfaces, on biofilms, and in areas with high levels of mixing.

Biofilms are microbiota interconnected through a matrix, and they can also consolidate bacterial colonies and establish an ideal inhabitance for ARGs. Biofilms are considered hotspots for the spread of ARGs and eDNA due to their increased access to more condensed bacteria, coupled with protection from external stressors. It is also worth noting that they are bountiful in various types of environments, especially aquatic ones (Aminov, 2011). They are often found in WWTPs.

1.4 Health Concerns of ARB spread

The modern age has brought an increase in the human population and a subsequent demand for antibiotics to respond to the harmful bacteria present in the environment. However, bacteria's ability to succeed in an evolutionary arms race has been largely underestimated. Antibiotics that previously prevented illnesses can no longer consistently kill antibiotic resistant strains. With the rise of antibiotic resistance, human health is at risk of more life-threatening and prolonged infections from bacterial pathogens.

The rapid emergence of antibiotic resistance is a rising global crisis; already responsible for about 700,000 deaths per year worldwide, it is projected to cause more deaths per year than cancer by 2050 (Zalewska et al., 2021). This is a major concern across a multitude of societal sectors, such as healthcare and agriculture. Antibiotic resistant genes and bacteria that enter the ecosystem through the aquatic environment are passed to humans through the food chain and urban water cycle system, thereby increasing human ARGs abundance and raising health risks (Pruden, 2014). According to the World Health Organization (WHO), deadly infections like pneumonia, tuberculosis,blood poisoning, gonorrhea and food borne illnesses like salmonella are all projected to be on the rise due to antibiotic resistance.

1.5 Purpose

Measurement of antibiotic resistance in the environment is not currently standardized and consists of a patchwork of methods. The purpose of this research was to provide cross validation between four different methods of antibiotic resistance quantification (discussed in the "Methods" section below) to ascertain whether or not the modified IDEXX Colilert-18 method (modified IDEXX) is an appropriate tool for widespread standardized use. Modified IDEXX is a more accessible alternative to typical culturing methods as it is cheaper and faster. Our research can further elucidate the benefits and weaknesses of each AR quantification method and thus inform concerned parties which tool is the best to utilize. If found useful and accurate enough, modified IDEXX could potentially be a good option as a global AR monitoring protocol.

<u>Cost/Time Breakdown of Antibiotic Resistance Monitoring Methods</u>

Besides Modified IDEXX, there are 3 other methodologies for AR quantification (discussed later in Section 3: Methods): culture-based, qPCR, and Metagenomics. Traditional culture-based methods require a laboratory setting and feature a fairly laborious protocol. qPCR and Metagenomics are costly as a result of the need for specialized lab equipment and training. The modified IDEXX Colilert-18 method is a type of culture-based method that is considerably cheaper and requires much less labor than the traditional methods. As a result of the quick and easy procedure as well as the fact that this method does not require a full lab space or expensive lab equipment, this novel method has immense potential for global use. Time estimates as well as relative cost are shown in Table 1.

	Traditional culturing	Modified IDEXX Colilert-18	qPCR	Metagenomics
Time	6-8 days	~ 22-24 hours	8 hours	2 days
Relative Cost	$$	$	$$$$	$$$$$

Table 1. Time and relative cost of AR monitoring methods

Tijuana River Watershed

The Tijuana River Watershed is an approximately 1,750 mi^2 trans-national watershed flowing from Tijuana, Mexico to the Tijuana River Estuary in San Diego, United States (Figure 5). This location was selected as the primary site of interest by WILDCOAST due to the poor, outdated sewage infrastructure resulting in frequent spills of unfiltered, contaminated water. In early 2023 alone, around 13 billion gallons of contaminated water flowed from Tijuana into the ocean (CNS, 2023). There are countless reports of individuals falling ill after making contact with waters either in Tijuana or nearby areas. According to a California Water Board report, the Tijuana River Estuary's water quality was tested in 2022 and *E. coli* levels did not meet the "Water Quality Objective" standard designated for the bacteria (Figure 4). In January and February of that year, 15 sewage spills occurred and over 100 cases of leakage and excess flow were reported in 2021 (*Sewage Pollution within the Tijuana River Watershed and San Diego Regional Water Quality Control Board*). As a result, AR is a significant concern in this specific location. Imperial Beach locals frequently report illnesses even without going in the water. Atmospheric-oceanic exchange places the same contaminants from the water in the air that people breathe. Testimonies given to our team from two locals stated that although they never swam in Imperial Beach, they had to get sinus and ear surgeries as a result of the contaminants. In addition, a study from the Scripps Institute of Oceanography at UC San Diego found that sewage-contaminated ocean waters in Imperial Beach (a public waterway near TJR) can aerosolize and expose nearby beachgoers (CNS, 2023). Three quarters of the air near Imperial Beach emerges from the aerosolization of sewage water. The study also mentions the presence of Hepatitis A in coastal waters originating from TJR (Pendergaft et al., 2023).

There is also immense risk of illness on the Mexican side of the border. Researchers conducted an interdisciplinary study investigating the intersectionality of homelessness and hazardous environmental exposure in *El Bordo* near Tijuana (Calderon-Villarreal et al,, 2022). They reported that the river "has a green-brown color, has a strong odor of wastewater, and is highly turbid due to suspended organic matter and sediment". Reportedly during the study, there was also an influx of untreated sewage, trash, and dead animals. It was found that the *E. coli* concentration was four orders of magnitude above the Mexican limits for treated water, and the water quality has actually worsened over time, based on historical *E. coli* values (Calderon-Villareal et al., 2022).

Table of Results from the January 16, 2020, Tijuana River Valley Sampling Event (continued)

Parameter	Units	Smugglers Gulch Canyon Collector	Goat Canyon Canyon Collector	Tijuana River at Border	Basin Plan Water Quality Objective	Meets Basin Plan Water Quality Objective?	Typical values for Weak Wastewater[1]	Typical values for Medium Wastewater[1]	Typical values for Strong Wastewater[1]
pH	standard units	7.56	7.87	7.65	6.5-8.5	Yes			
Turbidity	NTU	95.5	210	64.9	20	No			
E. Coli	MPN/ 100 ml	≥2,420,000	≥2,420,000	≥2,420,000	33-576	No			
Total Coliforms	MPN/ 100 ml	≥2,420,000	≥2,420,000	≥2,420,000	1,000 - 10,000	No	1 Million – 100 Million	10 Million - 1 Billion	10 Million - 10 Billion

Figure 4: Values from TJR sampling (2020)

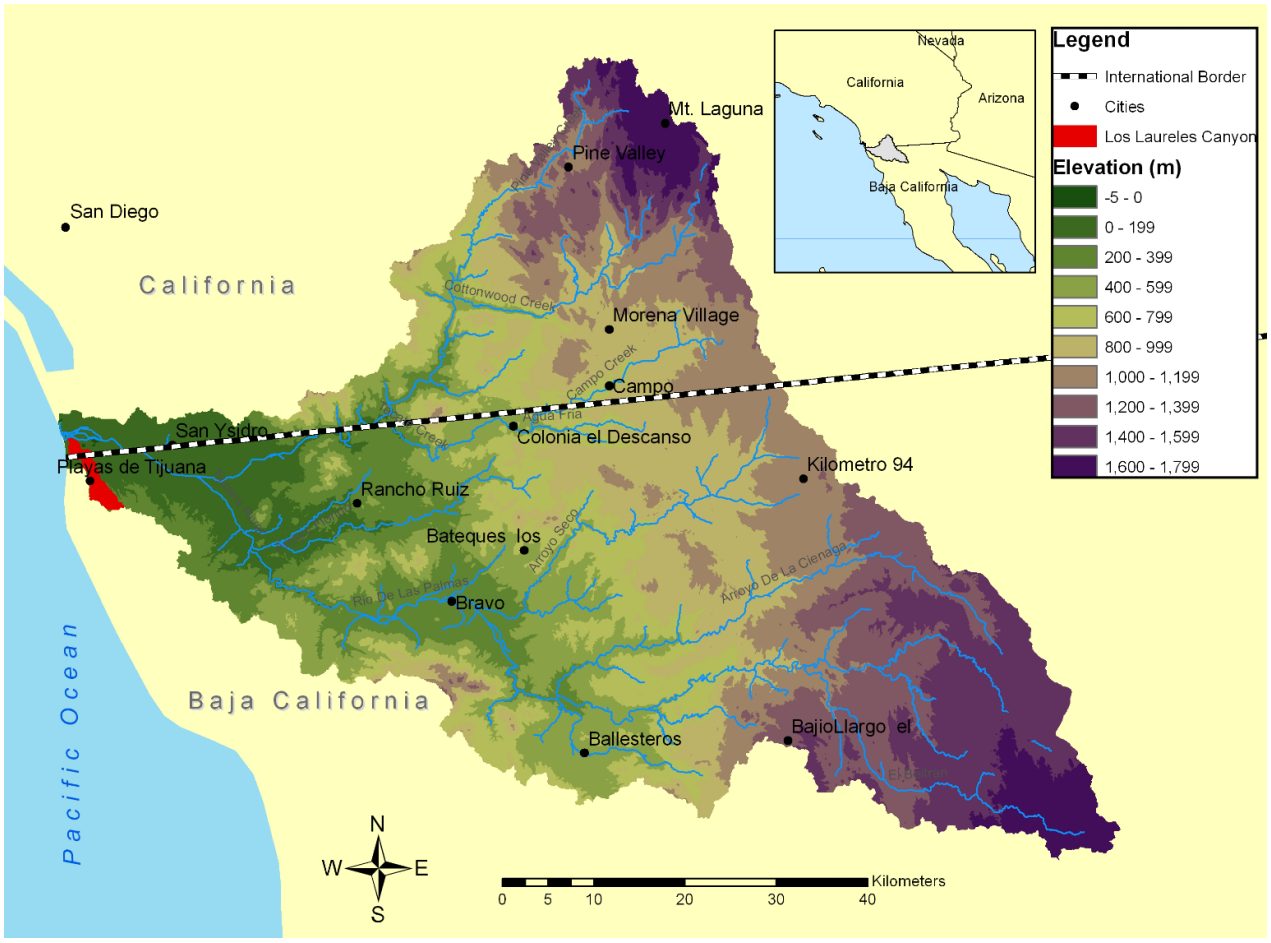

Figure 5: Map of the Tijuana River Watershed (source: ca.gov)

2. Methods

2.1 Study Sites and Sample Collection

This research focused on the southern California region; 9 sites were chosen in the city of Los Angeles and 8 sites in the Tijuana River Estuary region near San Diego. The study consisted of field sampling and analyses to (i) characterize *E. coli* levels in inland and coastal waters. (ii) characterize the percentage of antibiotic resistant *E. coli* present in inland and coastal waters. (iii) identify *E. coli* levels in source samples using modified IDEXX and culture based analysis methods. All water samples were acquired during dry weather. Accordingly, at least one inland creek sample and its paired coastal sample were taken each sampling day.

Sampling was done weekly at Los Angeles locations from 2/8/2023 to 4/19/2023 at Malibu Creek, Malibu Beach, Ballona Creek, Venice Beach, Inglewood Boulevard, Cochran Ave, Ballona Creek, Topanga Creek, and Topanga Beach (Figure 6). The LA sites were chosen for their accessibility and to allow team members to practice and master lab techniques prior to the San Diego trip. Some of the LA sites were a continuation of a previous study by the Jay Lab and served as a baseline to compare to the Tijuana River sites.

Sampling trips for the Tijuana River Watershed were conducted on 4/14/23 and 5/22/23. Water samples were collected from the following locations: YMCA Camp Surf, Imperial Beach Pier, Seacoast Beach, TJR Seacoast Drive, TJR Fork Beachside, TJR Fork, Smuggler's Gulch, and Butterfly Garden (Figure 7). These sites were commonly found in literature and/or sites of direct interest for WILDCOAST.

Site name:	**Site region:**	**Longitude:**	**Latitude:**	**Sampling Frequency (days)**
Ballona Creek	LA	33.9742250	-118.4334373	7
Venice Beach	LA	33.9655806	-118.4594108	5
Malibu Creek	LA	34.0429114	-118.6842356	3
Malibu Beach	LA	34.0315635	-118.6817465	1
Topanga Creek	LA	34.0393233	-118.5831340	2
Topanga Beach	LA	34.0383632	-118.5814171	2
Inglewood Boulevard	LA	33.9898973	-118.4115344	4

Cochran Ave	LA	34.0442555	-118.3539550	4
Santa Monica Beach	LA	34.0123206	-118.5010185	1
Will Rogers State Beach	LA	34.0350728	-118.5361699	1
Tijuana River Mouth Ocean Site	SD	32.5548144	-117.1292262	2
Tijuana River Mouth	SD	32.5536631	-117.1266479	2
Tijuana River Seacoast Drive Ocean Site	SD	32.5663882	-117.1331389	3
Tijuana River Seacoast Drive	SD	32.5664190	-117.1317545	3
YMCA Camp Surf	SD	32.5856986	-117.1329662	2
Imperial Beach Pier	SD	32.5836527	-117.1330903	2
Butterfly Garden/Smuggler's Gulch	SD	32.5514297	-117.0839287	2

Table 2. Coordinates of LA and TJR sampling locations and sampling frequency

Figure 6: Los Angeles Watershed Sampling Map

Figure 7: Tijuana River Estuary Sampling Map

Figure 8: Ofelia taking sand and ocean water sample from Tijuana River Seacoast Drive, Ocean site

Figure 9: Sampling tools (self-assembled collection bucket, sampling bottles, Turbidimeter™)

2.2 Description of AR Monitoring Methods and Protocols

Two lab-based methodologies were utilized in order to provide standardization and cross-validation between different types of AR monitoring methods: traditional culture-based methods and the modified IDEXX Colilert-18 method (a culture-based method of particular interest). Data obtained from plate filtering (culture-based) was compared with modified IDEXX measurements to determine the accuracy of the modified IDEXX method and its possible future applications.

I. Modified IDEXX Colilert-18

The technology utilizes Defined Substrate Technology (DST) nutrient indicators to detect the presence of both total coliforms and *E. coli* by binding to their β-galactosidase enzyme. Non-coliform do not contain β-galactosidase and therefore remain colorless and do not interfere. Nutrient indicator ONPG metabolizes to this enzyme to change the solution from colorless to yellow to indicate the presence of coliform. Nutrient indicator MUG metabolizes this same enzyme to produce a fluorescence to indicate the presence of *E. coli.* In the case of ESBL *E. coli*, we expect to see this fluorescence in Cefotaxime-dosed IDEXX trays.

Preparation

1 mg/mL Cefotaxime (CTX) solution was prepared using a syringe filter. All Nalgene bottles (used for preparing samples poured into the trays) and IDEXX Quanti-Trays were labeled with site name, sample date, dilution factor, and containing CTX (if applicable).

IDEXX Trays and Counts

The modified IDEXX procedure described by the manufacturer was followed with one exception: designated Quanti-Trays were spiked with 100 µL of CTX solution. The novelty of the modified IDEXX method is in the spiking of the samples used to fill the IDEXX trays with antibiotic (Cefotaxime, or CTX, in our case). After incubation, Colilert and ESBL *E. coli* counts were taken and recorded. The non-CTX trays were representative of the amount of total coliforms and *E. coli* present, while the CTX trays were representative of the ESBL total coliform and ESBL *E. coli* present. To calculate antibiotic resistance rates, the Most Probable Number (MPN) for the number of fluorescent wells on CTX trays was divided by the MPN for the amount of fluorescent wells on non-CTX trays.

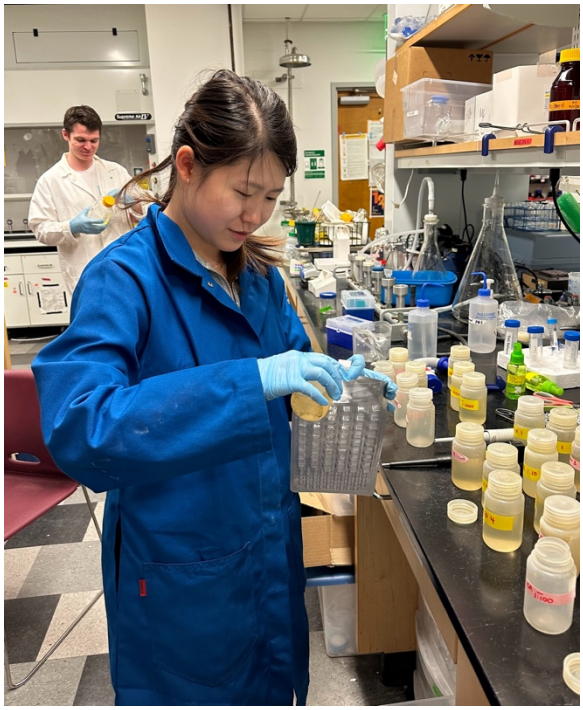

Figure 10: Yuhui pouring IDEXX tray

II. Culture-based

Two different types of agar were used during *E. coli* culturing: mTEC (BD Difco™ Chromogenic Dehydrated Culture Media: Modified mTEC Agar)- the agar type utilized as recommended by the World Health Organization (WHO)- and TBX (Oxoid™ Tryptone Bile X-glucuronide Agar (TBX) Medium (Dehydrated))- the agar type utilized as recommended by EPA. Both agar media were filtered for the same volume and the same number of samples during each sample analysis, thus allowing a cross-sectional comparison through the number of *E. coli* grown. To calculate the antibiotic resistance rate of *E. coli* at a given location, the number of

colonies on plates with antibiotic were divided by the number of colonies grown in antibiotic-free agar medium. The results obtained from this method will allow us to quantify the *E. coli*/ESBL-EC present in the water samples at the various sampling locations.

Agar Preparation and Plate Pouring

Phosphate-buffered saline (PBS) solution as well as mTEC and TBX agar plates were prepared approximately 1-3 days prior to each sampling day. Half of the mTEC and TBX plates were spiked with CTX.

Membrane Filtration

TBX and mTEC plates were labeled with the sample date, site name, and dilutions. 0.45µm Fisherbrand™ Water-Testing Membrane Filters were used and placed grid-side upward onto the cotton pad in the center of the funnel cup (see filtering setup in Figure 11). Blanks were processed to ensure no external contamination. Depending on the sample source, appropriate pore size MilliporeSigma™ Nylon Net Filters were placed on top of the gridded filters to prevent clogging and remove large particles. Small volume samples required use of a pipette or syringe. After filtering, PBS was used to rinse the walls of the funnel cup. Once completed, the 0.45µm filters were placed on their respective agar plate. Based on the agar type (mTEC or TBX), the plates were incubated accordingly and counted approximately 24 hours later.

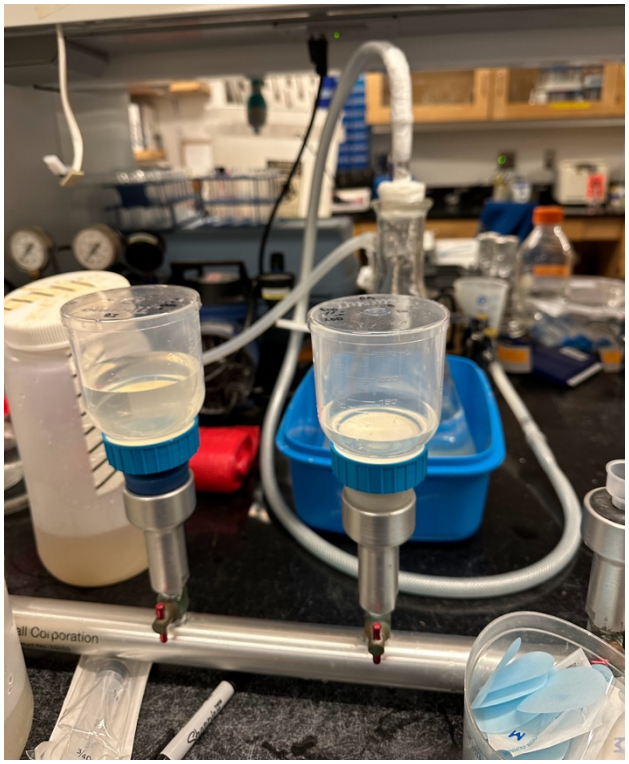

Figure 11: Membrane filtration experiment setting.

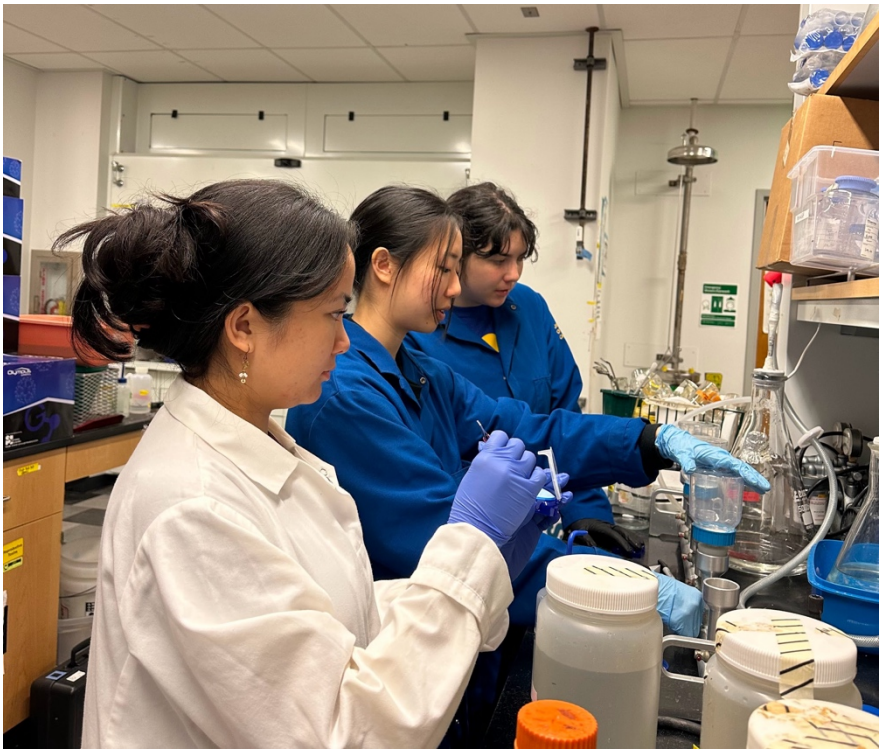

Figure 12: Anastasia, Julie and Brianna conducting membrane filtration.

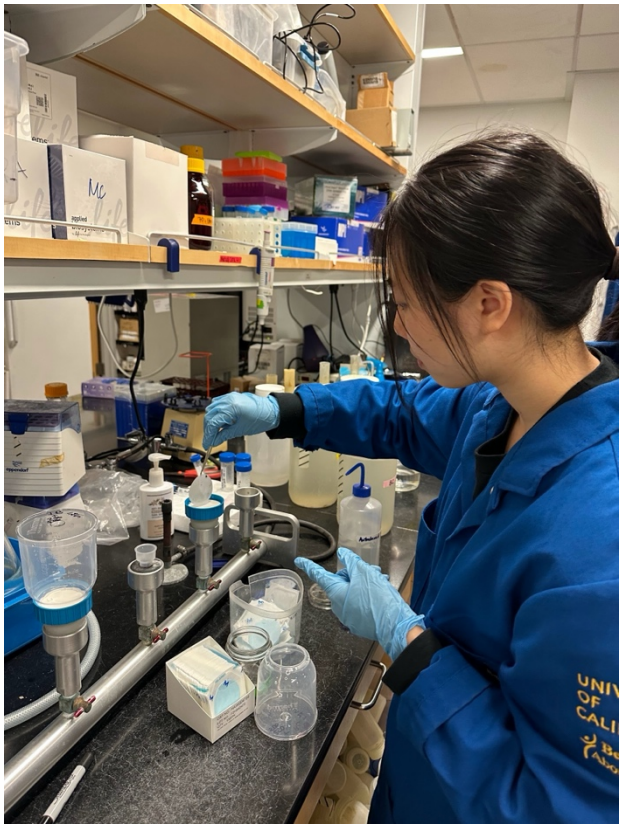

Figure 13: Julie removing nylon filter

Figure 14: Sana doing membrane filtration

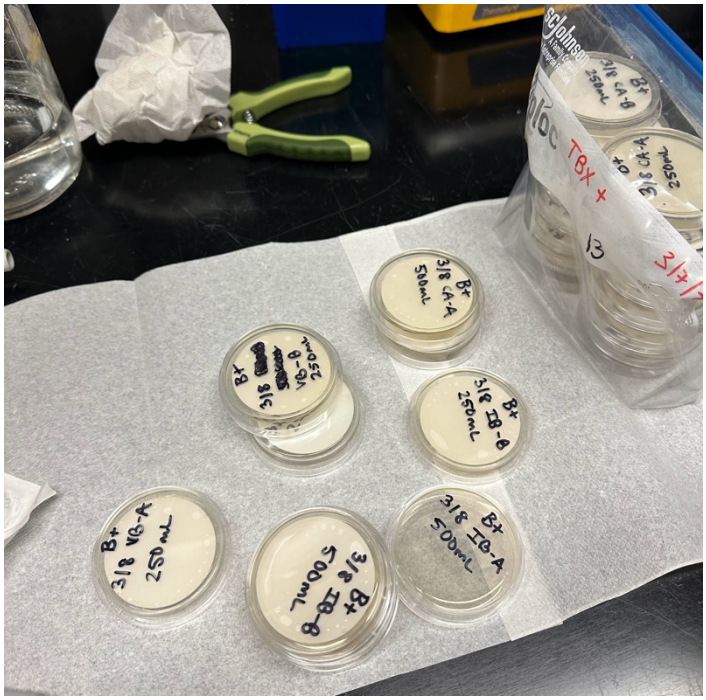

Figure 15: Filtered TBX plates.

Figure 16: Taylor organizing filtered agar plates.

Plate Counting

Colonies were manually counted on each plate. Each distinct colored dot on the plate was counted as one colony. Ideal dilutions would have between 10 and 100 colonies per plate.

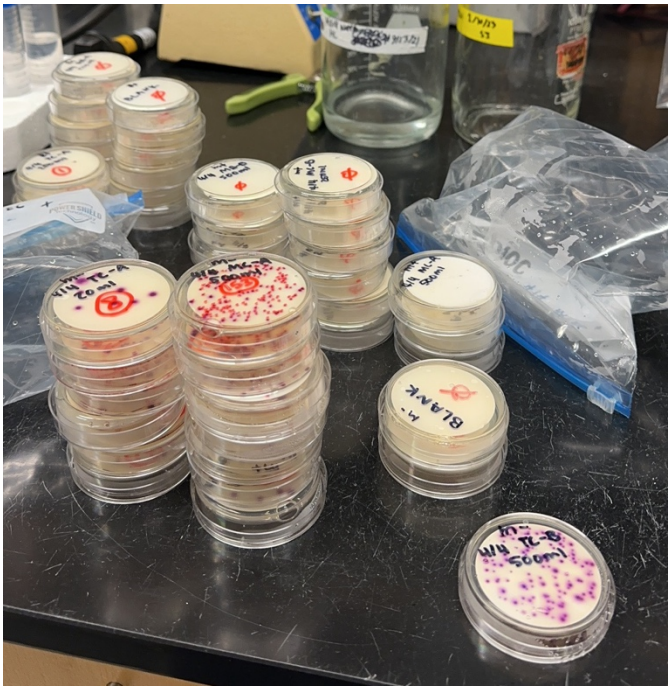

Figure 17: Counted plates after 24 hours of incubation.

Figure 18: Drew counting plates

DNA Filtration

For each site, triplicates were performed for DNA filtering. DNA filtration followed a similar procedure to membrane filtration with the exception that Isopore polycarbonate membrane 0.4 µm filters were used. With the manifold off, the selected volume of sample water (depending on whether the sample was from a beach or inland site) was poured and filtered through. The filters were subsequently folded up and placed in labeled tubes containing a 50% pure ethanol-50% DNA free water solution. This solution was prepared by volume. The samples were then stored in the -40ºC freezer.

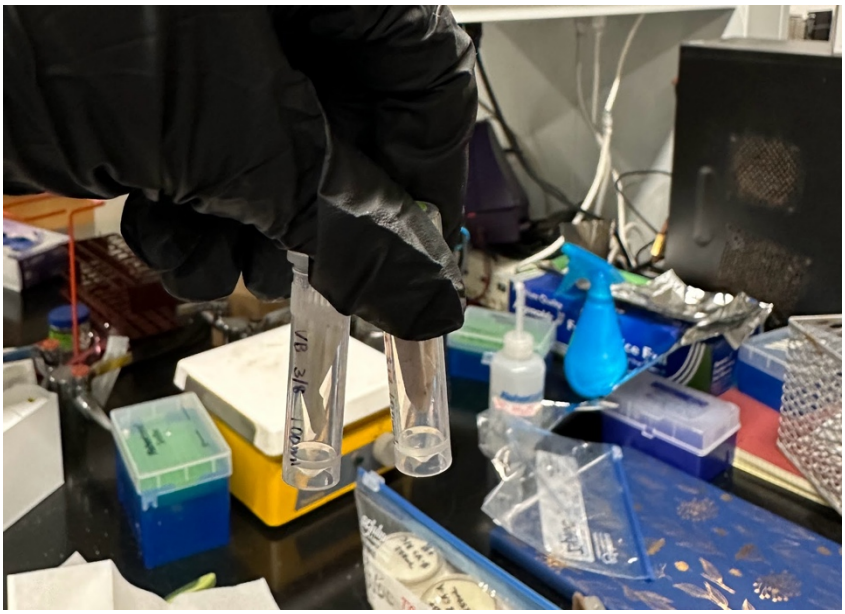

Figure 19: DNA filtration filters in tubes

Streaking

Prior to streaking, Tryptic Soy Agar (TSA) plates were prepared. In order to streak each isolated colony, the colony was gently scraped with the tip of the plastic inoculating loop. Standard streaking procedures were then followed. Setup and steps are shown in the flowchart below (Figure 20, 21, and 22).

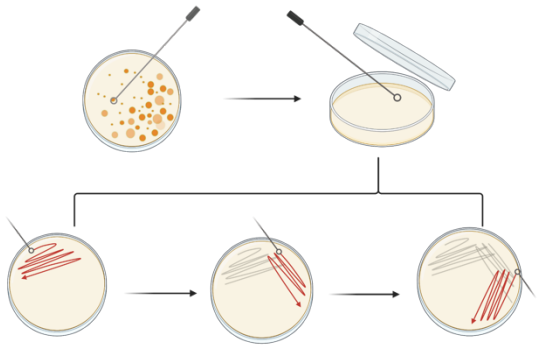

Figure 20: Streaking flow chart
Image made in Biorender

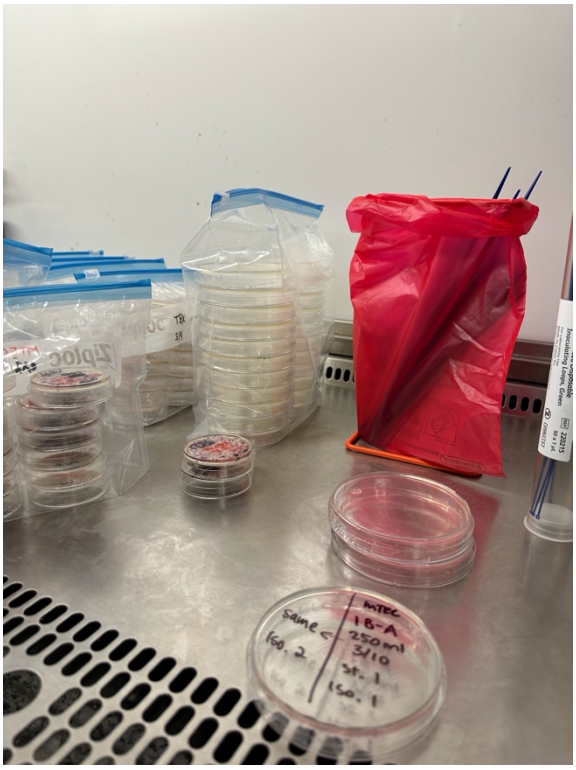

Figure 21: Streaking set up

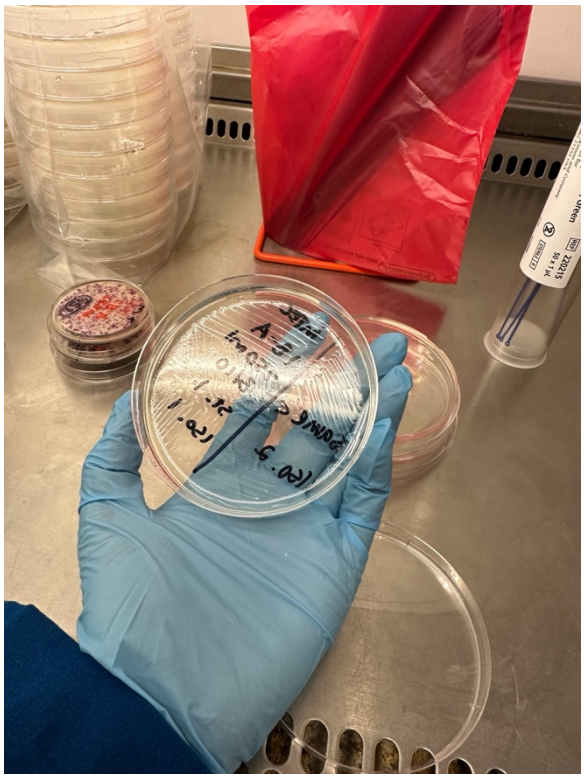

Figure 22: Streaking example

Preservations

Previously prepared Tryptic Soy Broth (TSB) was pipetted into screw cap tubes. Using plastic inoculating loops, the desired isolate (the one lowest on the plate) was touched and dipped into the respective tube containing TSB. This process was repeated with all the isolates before incubation. The following day, 50% glycerol was pipetted into each tube. After vortexing and centrifuging, the tubes were stored in the -80 ºC freezer until needed.

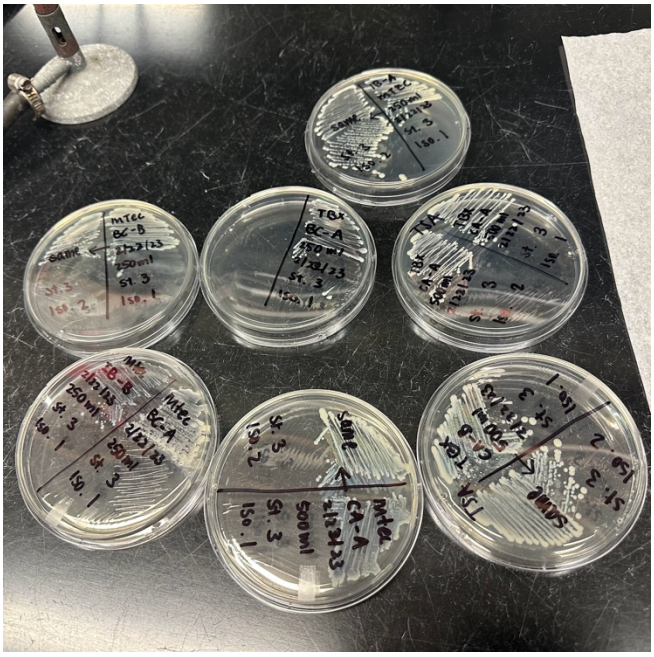

Figure 23: Streaking day 3 TSA plates (use for preservation)

3. Results

Comparison across site types of initial counts of CTX resistant *E. coli* on a log scale using modified IDEXX are shown as box plots in Figure 24, where Tijuana Rivershed beach sites, Tijuana Rivershed inland sites, and LA sites are grouped together. The minimum counts/100mL for Tijuana beach sites, Tijuana inland sites, and LA sites were 31, 100, and 1 respectively. The maximum counts/100mL for Tijuana beach sites, Tijuana inland sites, and LA sites were 81640, 1200000, and 1553.1 respectively and the median counts/100mL were 630, 17628, and 10 respectively. Our results show that LA sites are much cleaner than Tijuana Rivershed as a whole. Additionally, beach sites in Tijuana Rivershed tended to be cleaner on average compared to inland sites, as expected due to dilution.

Raw counts of CTX resistant *E. coli* using the modified IDEXX method are compared to the raw counts of resistant total coliform. As expected, for all sites the total coliform count is higher than the *E. coli* count. All sites surpass the EPA recommended *E. coli* threshold for water except for Sea Coast Beach (SCB).

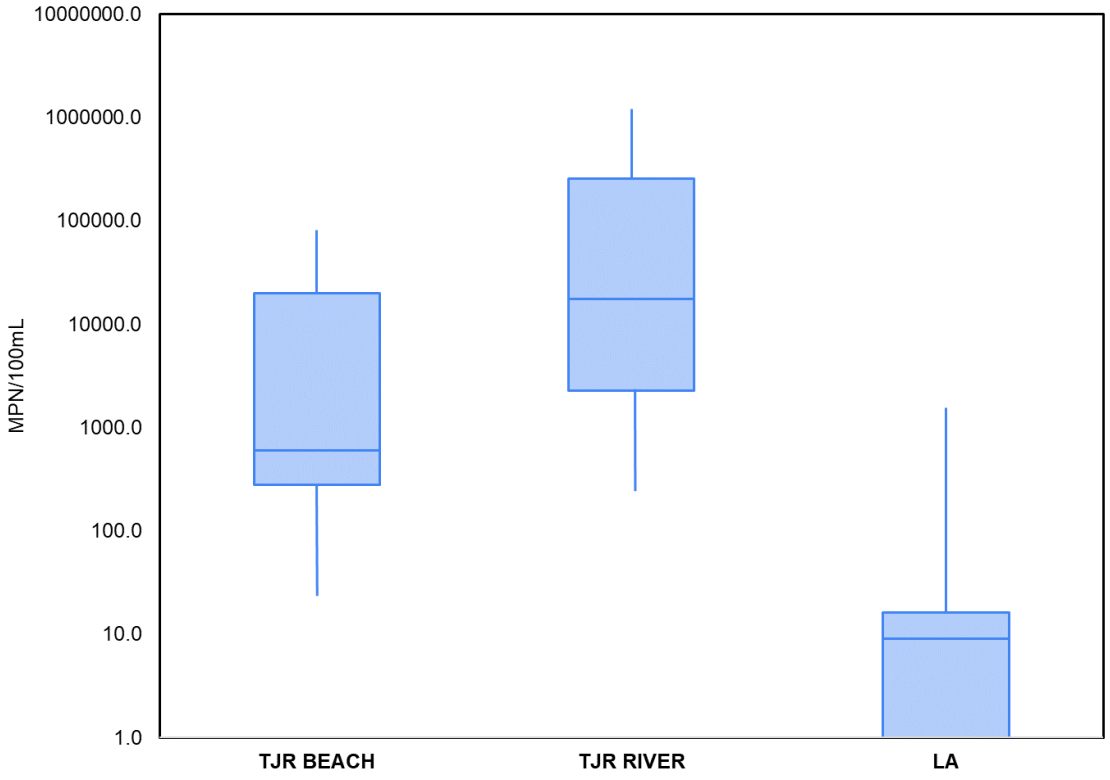

Figure 24: Box plots of Tijuana River beach sites, Tijuana River inland sites, and Los Angeles sites comparing the most probable number (MPN) of positive (resistant) *E. coli* on a log scale using modified IDEXX method.

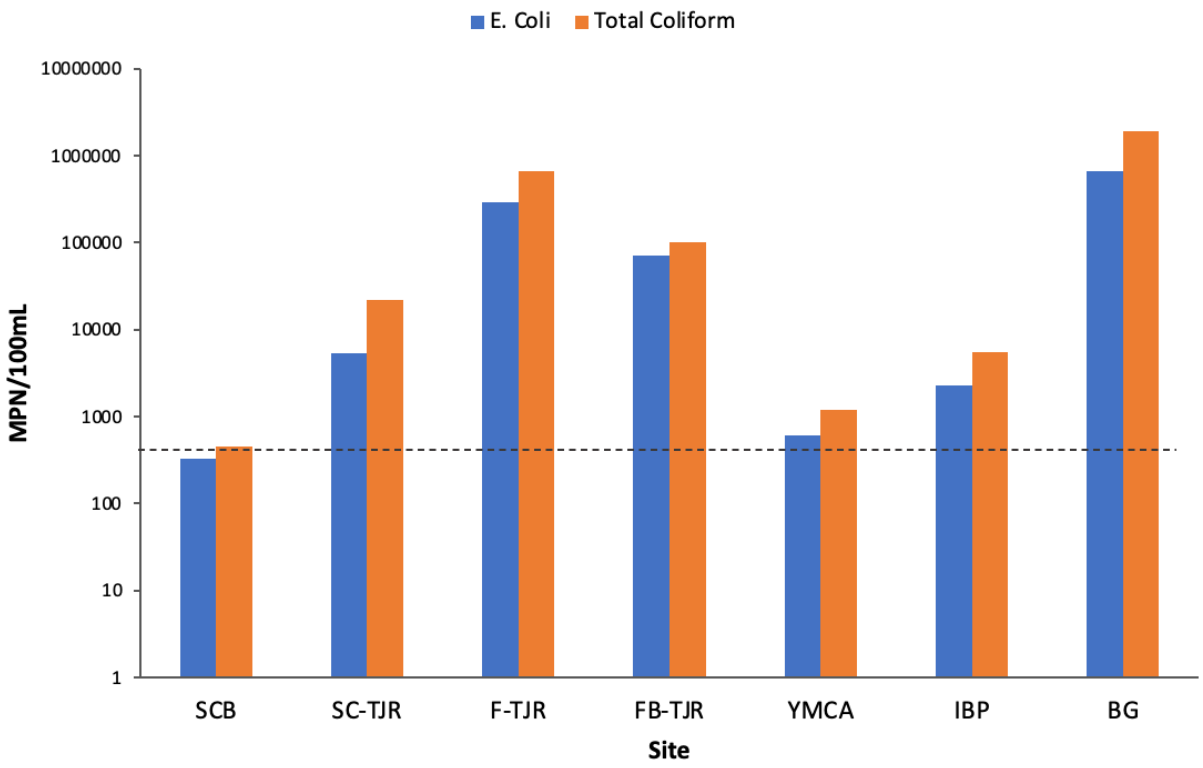

Figure 25: Most probable number (MPN) of CTX resistant *E. coli* and total coliform per 100 mL for TJR sites using the modified IDEXX method. The dotted line indicates the U.S. EPA's recommended threshold for *E. coli* counts per 100 mL.

Comparison between the culture-based method (TBX and mTEC) and modified IDEXX using raw counts for positive *E. coli* are shown in Figure 26. Modified IDEXX differed from TBX by a minimum of 156.3 counts/100mL to a maximum of 350594 counts/100mL, with the highest deviation being for Tijuana River Fork I and II. For mTEC, Modified IDEXX differed by a minimum of 46.8 counts/100mL to a maximum of 275,782 counts/mL, with the highest deviation also being for Tijuana River Fork I and II. Sites with higher counts generally had greater deviation between plate types and modified IDEXX. These sites tended to be inland such as Tijuana River Fork and Butterfly Garden, while beach sites only differed by a magnitude of 10-100 such as YMCA, Seacoast Drive, and Imperial Beach. This could potentially be due to error when counting high counts for plates by the naked eye as well as dirtier sites being less likely to be well mixed and homogeneous, resulting in variation between samples despite shaking the sample bottles.

The Spearman correlation coefficient for raw counts between the variables `TBX` and `IDEXX` was 0.9333333. This indicates a strong positive monotonic relationship between the two variables. The correlation coefficient between the variables `mTEC` and `IDEXX` was 0.9666667. This suggests an even stronger positive monotonic relationship between the two variables compared to the first correlation. The Spearman correlation coefficients confirm the

strong associations between the variables. However, using a two-tailed t-test, the p-value for mTEC and Modified IDEXX was 0.13 and the p-value for TBX and Modified IDEXX was 0.14, meaning that the differences are not statistically significant. Additionally, since actual count values vary up to the order of 10^5, we do not have sufficient evidence to recommend that modified IDEXX can replace the culture-based method for direct counts, though it has potential to serve as an initial screening tool.

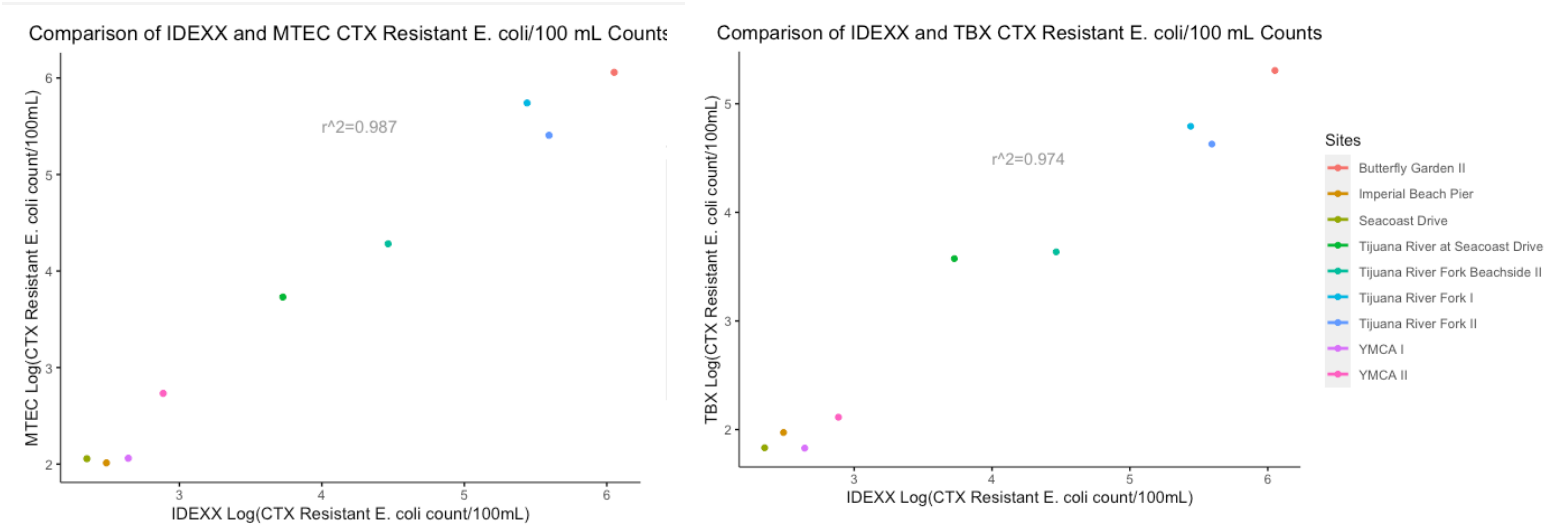

Figure 26: Correlation plot that compares CTX Resistant E.coli/100mL between the modified IDEXX method and the two plate types for the culture-based method. The data points of the counts are shown on a logarithmic scale. Only Tijuana River watershed samples were used since LA samples were mostly clean and below 2%. Sites with multiple points indicate that these locations were sampled twice: (I) indicates sampled on 4/14/2023 and (II) indicates sampled on 5/22/2023.

Site	Sample Date	mTEC Resistance %	TBX Resistance %	IDEXX EC Resistance %
Butterfly Garden	5/22/2023	7.30	2.68	8.57
Tijuana River at Seacoast Drive	4/14/2023	5.67	11.19	8.80
Tijuana River Fork I	4/14/2023	NA	1.17	2.78
Tijuana River Fork II	5/22/2023	9.75	2.64	6.24
Tijuana River Fork Beachside*	5/22/2023	7.46	NA	6.86

YMCA I*	4/14/2023	NA	7.76	12.22
YMCA II*	5/22/2023	6.49	NA	18.05
Imperial Beach Pier*	4/14/2023	5.02	12.65	11.12

Table 3: Average antibiotic resistance percentages across different San Diego sites with various methods: TBX, mTEC, and IDEXX E.Coli. * indicates beach sites. Sites lacking * are river sites.

Table 3 shows a comparison of average percent resistance across the two plate types and modified IDEXX. Modified IDEXX varied from a minimum of 0.61% to a maximum of 11.6% when compared to mTEC. Using a Pearson's correlation test, we found a correlation coefficient of -0.47, with a non-significant p-value of 0.34. Modified IDEXX varied from a minimum of 1.5% to 5.9% from TBX. As seen in Table 3, this caused a higher correlation between modified IDEXX and TBX compared to mTEC, with an R^2 value of 0.5341. Using a Pearson's correlation test, we found a correlation coefficient of 0.73, which indicates a strong, positive relationship, but the p-value was not statistically significant either (0.099). Due to both R^2 values being below 0.7 and the finding of no statistically significant correlation, we do not have enough evidence to conclude that modified IDEXX would suffice for providing direct counts in place of the culture-based method. However, due to there being an average deviation of 4.0% between Modified IDEXX and both plates, we suggest that modified IDEXX would be a good indicator of the presence of antibiotic resistant bacteria in highly contaminated sites (high percent resistance) and could potentially serve as an initial qualitative indicator. Low correlation could also be attributed to unusable negative plates causing minimal data points and error when counting.

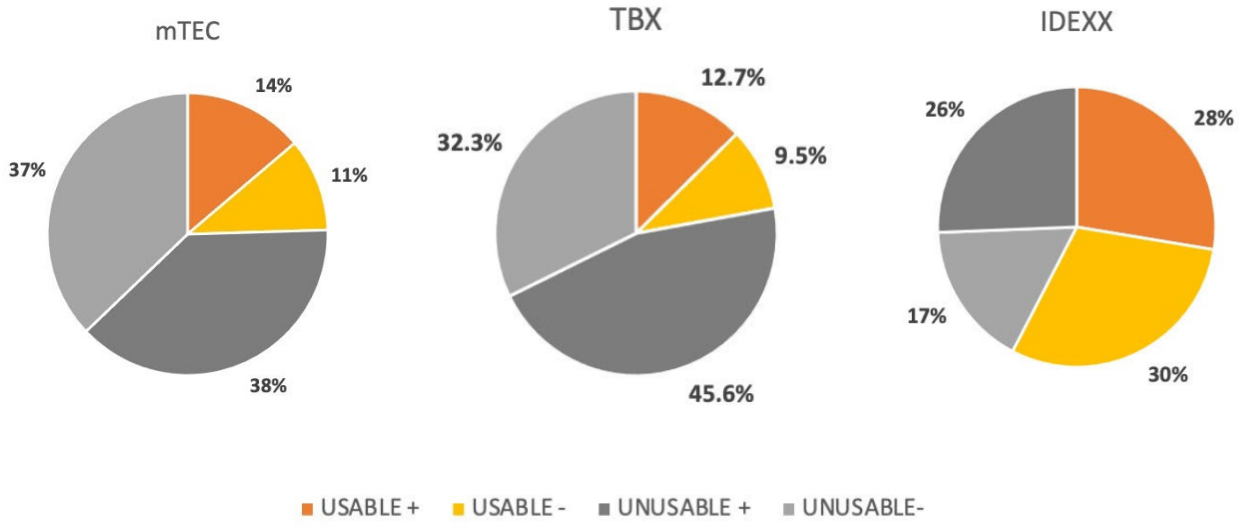

Figure 27: Percent of usable TBX plates, mTEC plates, and modified IDEXX counts throughout the span of our project. The (+) sign indicates the presence of antibiotics or positive plates/wells, while the (-) sign indicates the absence of antibiotics or negative plates/wells. Plates had to have a colony count of 1-100 in order to be considered usable. IDEXX counts have to be greater than 1 and less than 2419.6 to be considered usable.

Figure 27 indicates the loss of data points for each of three methods (TBX plates, mTEC plates, and modified IDEXX) as a result of potential counting error and dilution error. Modified IDEXX has a higher total percentage of usable data which is 58% compared to both plate types, which have less than half of the plates as usable. Only 22.2% of TBX plates were valid and only 25% of mTEC plates were valid. This suggests that IDEXX may present as a more accessible method, with a wider range for usable counts and less human uncertainty when counting.

4. Discussion

4.1 Future Implications / Significance

There is an urgent need for an accessible and affordable AR monitoring procedure that can be widely used on an international scale, a significant issue this study aims to address. To successfully and effectively create policy, it is pivotal to standardize the methods of monitoring AR in the environment. With additional sampling to confirm the values, the calculated AR ratios could be used to drive policymaking by highlighting which locations are more likely to pose threats to public health.

The ability for modified IDEXX to detect antibiotic resistance on average within 4.0% of traditional culture-based methods presents a potential way to carry out frequent and accessible environmental surveillance of ARGs worldwide. The modified IDEXX method was tested in multiple locations and compared with plate culturing using mTEC and TBX agars. Based on statistical analysis, the modified IDEXX method presented similar results to mTEC and TBX plating. Our results prove to be similar to other cross-validation studies for IDEXX such as in Hornsby et al., which found *E. coli* detection in modified IDEXX to be highly similar to TBX and MacConkey agars.

Typically, surveillance of ARGs has been limited to agricultural and health sectors despite their prevalence in environmental compartments humans come into contact with through recreational activities. The limitations of surveillance in such watersheds have disproportionately affected low income and underdeveloped populations. This is because culture-based, metagenomics, and qPCR methods are procedure heavy and require expensive equipment and lab space, meaning many communities cannot afford to carry out regular monitoring. Modified IDEXX proves to be a useful way to serve as an initial estimate and indicator of the presence of antibiotic resistant *E. coli* in highly contaminated waters, informing the need for further testing. Further modified IDEXX can be performed outside of the laboratory setting and has low

equipment costs. The overall accessibility of this method gives it immense potential as a screening tool with global applications.

Figure 29: Smuggler's Gulch – the driest site, unable to take water sample

Figure 30: Warning sign in Imperial Beach, San Diego

4.2 Limitations

The Tijuana River watershed spans across both the United States and Mexico. Although there were initial intentions to cross the border, various constraints did not allow for this option. Samples would have required immediate processing yet acquiring lab space in Mexico was not possible due to budget constraints. Additionally, it was not feasible to obtain the necessary permits to transport water and soil samples across the border within the time frame of the project. Consequently, our data for the Tijuana River was limited to sites in San Diego, downstream of the sewage source. Furthermore results from sampling are reliant on seasonality and weather conditions, particularly for more inland sites. Excessive rainfall can lead to increased runoff or cause samples to be more diluted; conversely, drier conditions may cause watersheds to dry up, eliminating potential sampling sites or reducing the availability of water for collection. Some inland sites such as Smuggler's Gulch were too dry during our sampling campaign. This posed a challenge as inland sites are often dirtier, thus providing a better comparison between AR detection methods.

Multiple visits to San Diego were expected to gather more accurate data, but due to the long distance and travel time, we were only able to complete two trips. Due to limited sampling time and knowledge of the locations, the dilution curves for modified IDEXX and plate filtration were not always optimal. For San Diego sampling, we had to prepare a wide range of dilutions as

a result of unfamiliarity with the area. Being able to focus on the most optimal couple dilutions could have given us more and stronger data. Another major limitation was not being able to sample the wastewater treatment plant near Imperial Beach. Sewage spills are often reported in the area and pose a major health threat to the nearby population. However, due to the need for permits and the logistic difficulties of sampling near WWTPs, we were not able to find the point source upstream of the Tijuana River.

Another limitation to the study stemmed from the lack of sufficient literature and relevant background information available on the TJR watershed. This upstream portion of the Tijuana River is located on the Mexico side of the border, and is presumed to be the main source of pollution. Moreover, numerous studies done on the Tijuana River are written in Spanish, which were difficult to assess due to the language barrier.

4.3 Uncertainty / Error

When performing small dilutions for plate filtering (0.1 µL - 1 mL), some lower dilutions had higher plate counts than higher dilutions. This was likely the result of random error from using such small sample volumes.

Additionally, for plate counting, if there were more than 200 colonies, we had to take a rough estimate by dividing the plate into quadrants, counting the number of colonies in one quadrant, and multiplying that number by four to receive the total number of colonies for the plate. Moreover, there can be considerable human error introduced when counting plates with counts above approximately 70 with the naked eye. To counter this, we had multiple team members count any questionable plates or plates with high counts.

Water turbidity could pose a potential error for TBX and mTEC agar because of the membrane filtration step and causing extra growth on the plates and difficulty distinguishing colonies (Hornsby et al., 2022). This likely caused the higher discrepancy between modified IDEXX counts and TBX and mTEC plates particularly in turbid waters such as the dirtier inland sites.

4.4 Further Research

Our research shows that modified IDEXX can be used as a reliable qualitative indicator for antibiotic resistance in highly contaminated sites through sampling the Tijuana Rivershed and LA Rivershed twice. In order to further validate the reproducibility of this method, these sites should be revisited and resampled, potentially adding more sampling locations. Ideally, these samples should also be used to validate modified IDEXX across the other standard methods including qPCR and metagenomics.

<u>Quantitative Polymerase Chain Reaction (qPCR)</u>

To verify the validity of the modified IDEXX method on *E. coli* profiling and quantification, quantitative polymerase chain reaction (qPCR) will be integrated into sample

analysis and is currently in progress in the Jay Lab. qPCR methods refer to the process of amplifying and quantifying genes of interest. qPCR is necessary for further validation as it is the most commonly accepted method for *E. coli* detection in environmental samples as well as healthcare and livestock surveillance efforts. Also, qPCR-based methods are valuable due to their ability to collect data over a log scale, which can allow analysis of trends and patterns in a certain environment (Liguori et al., 2022). Tracking trends can help gain an understanding of anthropogenic influences on ARGs. The basis of qPCR lies in increasing the target gene's concentration until detectable (Stewart, 2021).

For ESBL *E. coli*, we are interested in amplifying the *sul1, bla*$_{CTX\text{-}M\text{-}1}$, 16S-rRNA for water samples and *intl1* for soil samples. *Sul1* and 16S-rRNA can be chosen for water samples as they show total antibiotic resistance in aquatic environments, while *bla*$_{CTX\text{-}M\text{-}1}$ is an indicator gene for ESBL *E. coli*. Lastly, *intl1* is commonly found in soil and freshwater bacteria and frequently serves as a proxy for generic pollution.

Metagenomics

Metagenomics-based methods can analyze the complete genetic makeup of a sample, meaning there is no isolation of a single bacteria or specific selection of genes required (Duarte et al., 2021). Typically, when carrying out a metagenomics study, sample processing, DNA extraction, and how data will be shared, stored, and analyzed must all be planned in a carefully crafted experimental design (Thomas et al., 2012). Collecting additional data from genome sequencing would complement the modified IDEXX method well by helping strengthen validation and providing a more thorough surveillance of ESBL *E. coli* in the environment.

5. Glossary

ARB (Antibiotic-Resistant Bacteria): Bacteria that have developed resistance to previously effective antibiotics through the expression of antibiotic resistant genes

ARG (Antibiotic-Resistant Genes): Genes that encode defensive traits against antibiotics, such as resistant membranes or enzymes that break antibiotics down

CTX (Cefotaxime): Antibiotic with the ability to kill *E. coli*

***E. coli* (*Escherichia coli*):** A rod-shaped bacterium found in the lower intestines; present in fecal matter

EPA (Environmental Protection Agency): Government organization that protects the environmental and establishes baselines for *E. coli* contamination

ESBL (Extended-spectrum β-lactamase): Bacteria can become resistant to certain types of antibiotics if they produce enzymes (proteins) called β-lactamase

HGT (Horizontal Gene Transfer): Transmission of genes from DNA in other bacteria (transformation and conjugation) or viruses (transduction) in the surrounding environment.

Modified IDEXX Colilert-18: Novel, accessible methodology for measuring antibiotic resistance

mTEC (BD Difco™ Chromogenic Dehydrated Culture Media): Medium recommended by the WHO for culturing *E. coli*

PBS (Phosphate-Buffered Saline): Salt solution that has buffering capabilities; commonly used in research

qPCR (Quantitative Polymerase Chain Reaction): Process of amplifying and quantifying genes of interest; methodology for measuring antibiotic resistance

TBX (Oxoid™ Tryptone Bile X-Glucuronide Agar): Medium recommended by the EPA for culturing *E. coli*

TBS (Tryptic Soy Broth): Broth used during preservations of ESBL *E. coli* colonies

VGT (Vertical Gene Transfer): Transmission of genes from one generation to the next

WHO (World Health Organization): Global organization that monitors *E. coli* outbreaks, provides scientific assessment, and develops international baselines for food safety

WWTP (Wastewater Treatment Plants): Facility that removes contaminants from sewage water and releases it into oceans or land after treatment

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