NARMS
National Antimicrobial Resistance Monitoring System

Implementing a Pilot Environmental Effort for NARMS

Development, Status, and Next Steps in the Surface Water Monitoring Project

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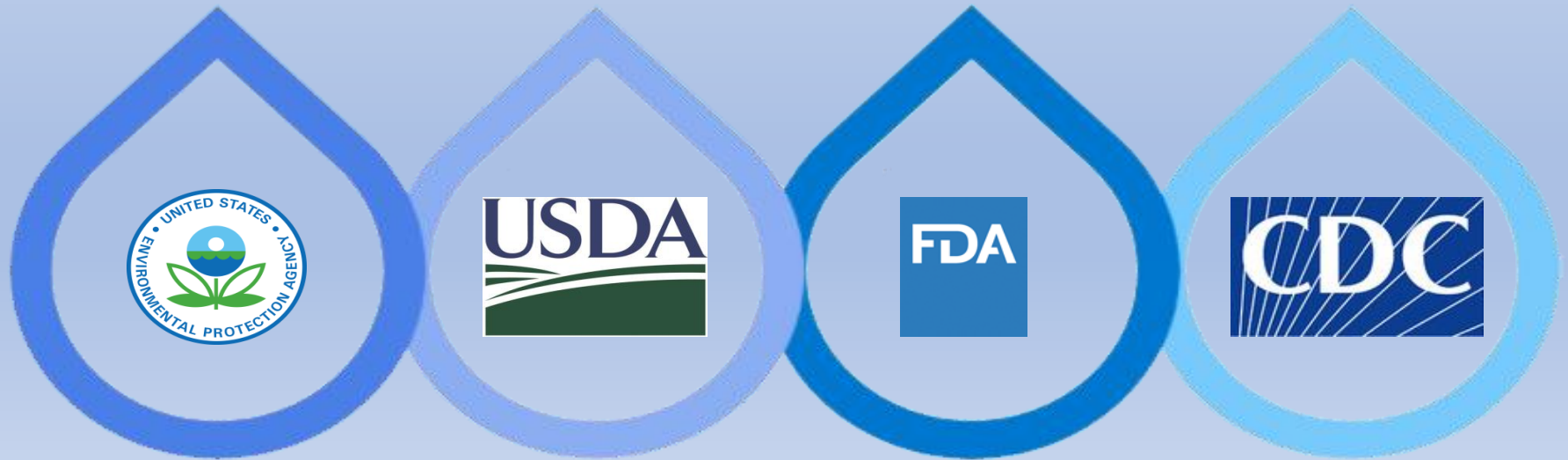

Interagency Collaboration

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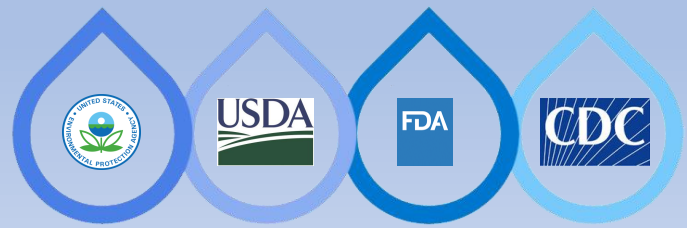

Outline

- Background on environmental dimensions of AMR
- Rationale & Development of the surface water pilot
- Review of Field Sampling & Analytical Methods
- Update on Progress to Date & Next Steps in Data Collection
- Plans for Data Publication and Use

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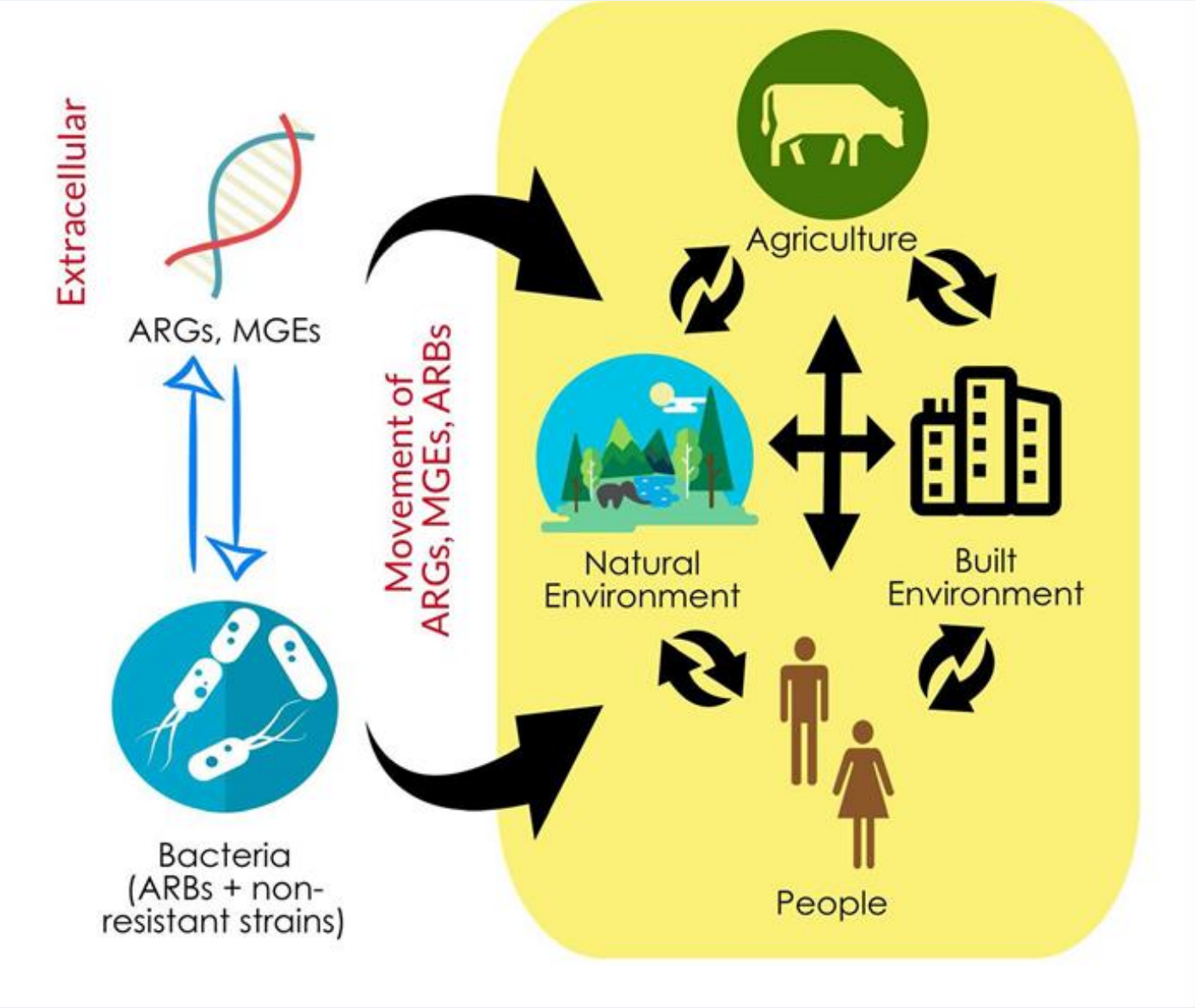
A Complex Environmental Contaminant



Vikesland et al. 2017. EST

Larson et al. 2018. ***Critical Knowledge Gaps and Research needs related to the environmental dimensions of antibiotic resistance.***
Environment International 117, 132-138

Relative Contributions of Different Sources

Role of Environment on Evolution of Resistance

Human/Animal Health Impacts from Environmental Exposures

Efficacy and Feasibility of Interventions

National-Scale Surface Water Efforts

Initiatives for Addressing Antibiotic Resistance in the Environment*: Current Situation and Challenges*
https://wellcome.org/sites/default/files/antimicrobial-resistance-environment-report.pd (2018)

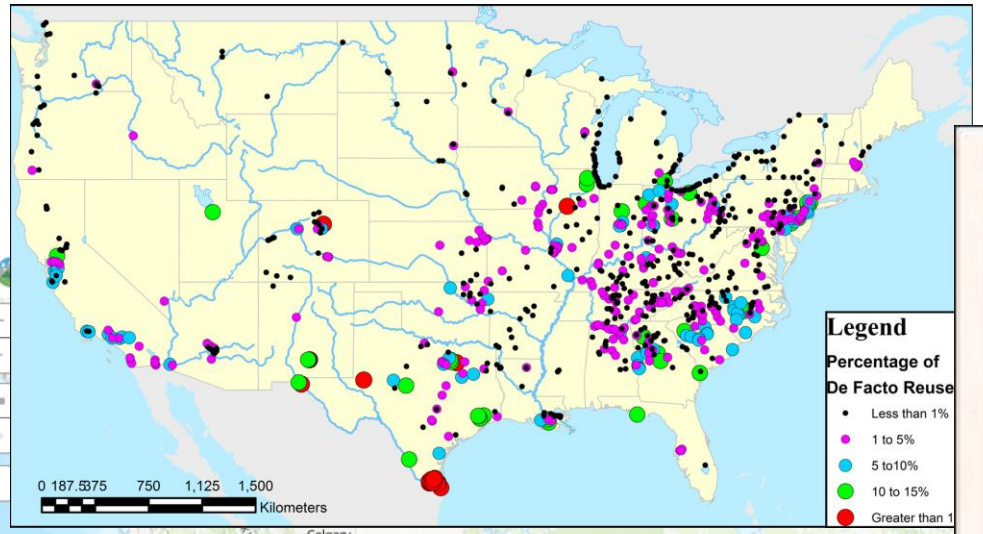
- **Environmental waters one of the areas in the report**
  - Geospatial distribution of resistance to inform risk
  - Sources & selective pressures for amplification/transmission
  - Define & standardize sampling/analysis methods

"Following the NARMS Review Subcommittee recommendations to incorporate the three major domains of the One Health model (humans, animals, environment), an important theme of this strategic plan is the expansion of testing to examine resistance in animal pathogens and the environment. For environmental monitoring, what constitutes the best sampling points will be refined over time. Surface waters as confluence points of ecosystems differentially affected by built environments is a starting point."

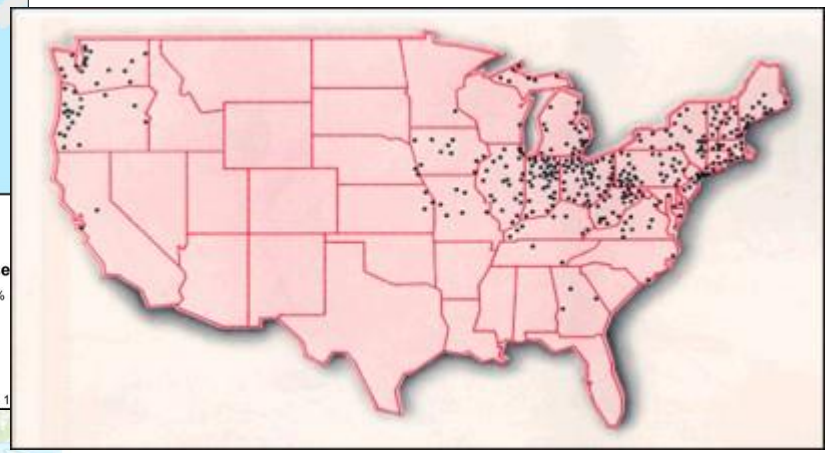
NARMS Strategic Plan 2020-2025

Why Water?

Rice J. and P. Westerhoff. 2015. Spatial and temporal variation in de facto Wastewater reuse in drinking water systems across the USA ES&T 49, 982



Human Wastewater



Animal Manure

https://enviroatlas.epa.gov/enviroatlas/DataFactSheets/pdf/ESN/Manureapplication.pdf

Multiple Inputs to Watersheds

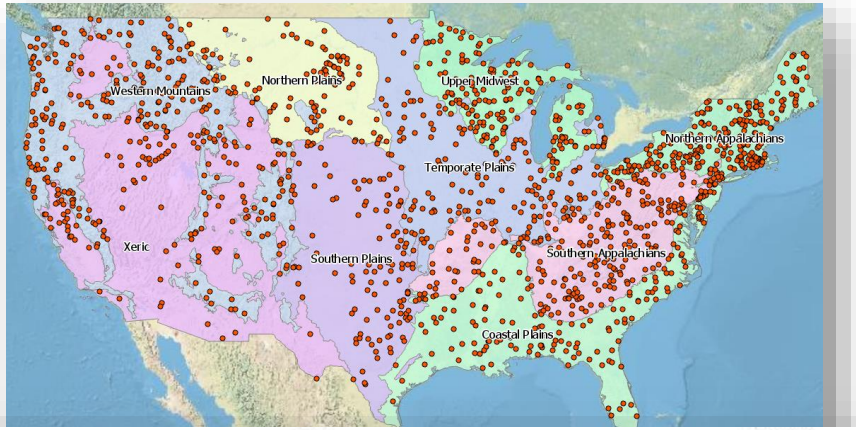
Surface Water AMR Monitoring (SWAM) *Objectives*

- A pilot environmental effort within a One Health focused NARMS

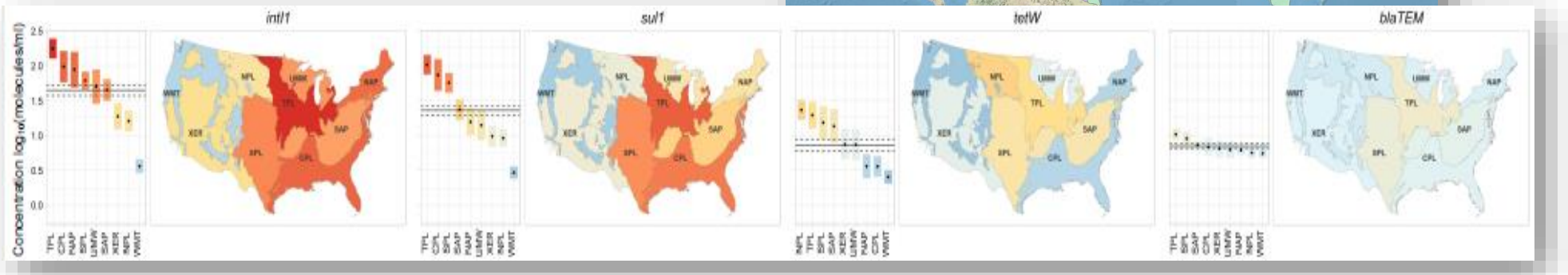
- Develop a national-scale, quantitative assessment of AMR within surface water:
  A. Standardized measure (and library of samples) to monitor trends as part of NARMS
  B. Input to models of AMR risks for various end uses of water (recreational, drinking, agricultural, water reuse).
  C. Help quantify drivers of occurrence and selective pressures for potential amplification
  D. Identify critical control points and assess current and new mitigation strategies

Designing the Study

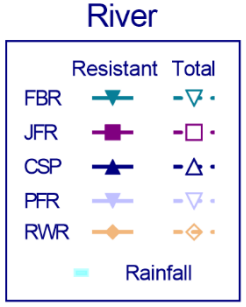
Go Big and Slow?

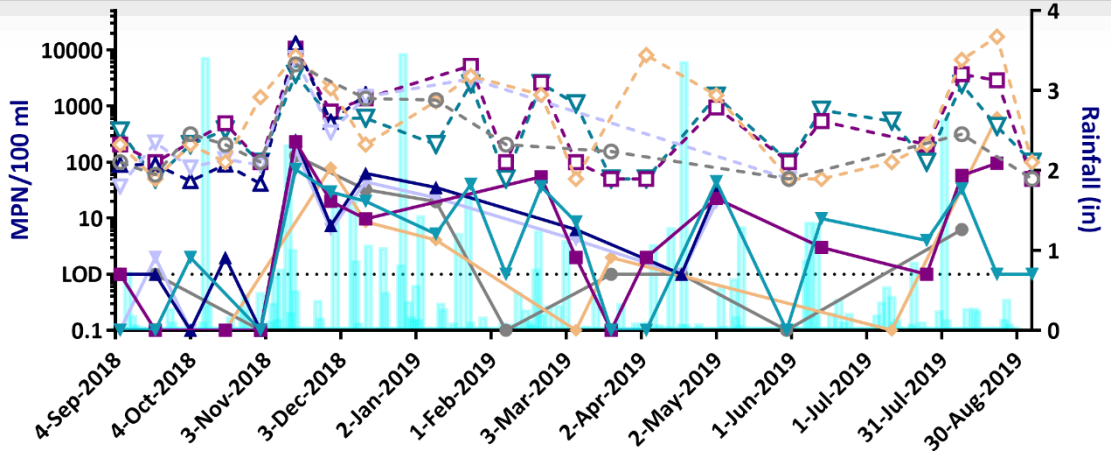


EPA National Rivers and Streams Assessment
5 year, probabilistic survey of aquatic resource



Or Small and Fast?





CDC Preliminary Surface Water Study in Chattahoochee River

Phased design for SWAM

Phase 1	SWAM Pilot	Statistical Design Subgroup discussions	Initial testing of methodologies	FY21-1st half FY22
Phase 2			Watershed based assessment to evaluate methodologies before national sampling and serve as a demonstration project for future watershed studies	Spring FY22-Spring FY23
Phase 3			Probabilistic national survey to provide statistically valid estimates of AMR status and trends in surface water, using methods tested in the other phases	Summers 2023-24
Phase 4			Continued probabilistic national monitoring together with expanding number of (partner-led) intensive watershed studies across the country	2024+

Analytical Targets

- **Culture**
  - *Enterococci, E.coli:* Links to existing water quality methods
    - Will quantify and determine resistance to specific antibiotics
  - *Salmonella:* Links to food cycle & NARMS
    - Presence/absence

- **Targeted Gene Analysis**
  - Defined panel of antibiotic resistance genes important to human, animal, and environmental health, including fecal source trackers (~90-100 genes)

- **Metagenomics**
  - Define environmental resistome in surface waters
  - Determine new genes to quantify via targeted gene analysis

Environmental Science & Technology

pubs.acs.org/est

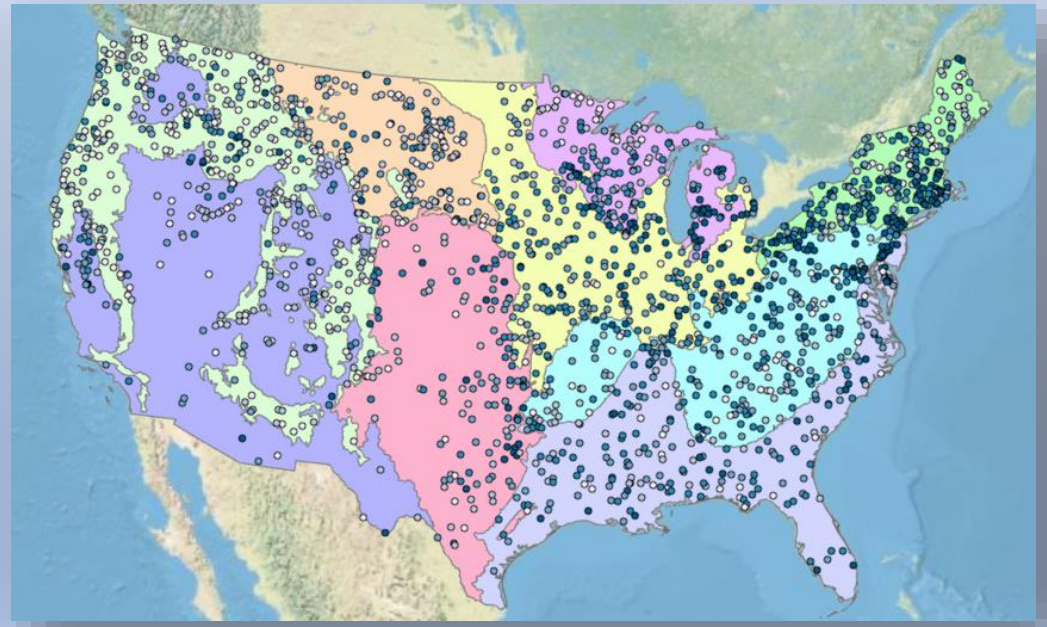
Article

Geospatial Patterns of Antimicrobial Resistance Genes in the US EPA National Rivers and Streams Assessment Survey

Scott P. Keely,*[,||] Nichole E. Brinkman,[||] Emily A. Wheaton, Michael A. Jahne, Shawn D. Siefring, Manju Varma, Ryan A. Hill, Scott G. Leibowitz, Roy W. Martin, Jay L. Garland, and Richard A. Haugland

Cite This: https://doi.org/10.1021/acs.est.2c00813

Read Online



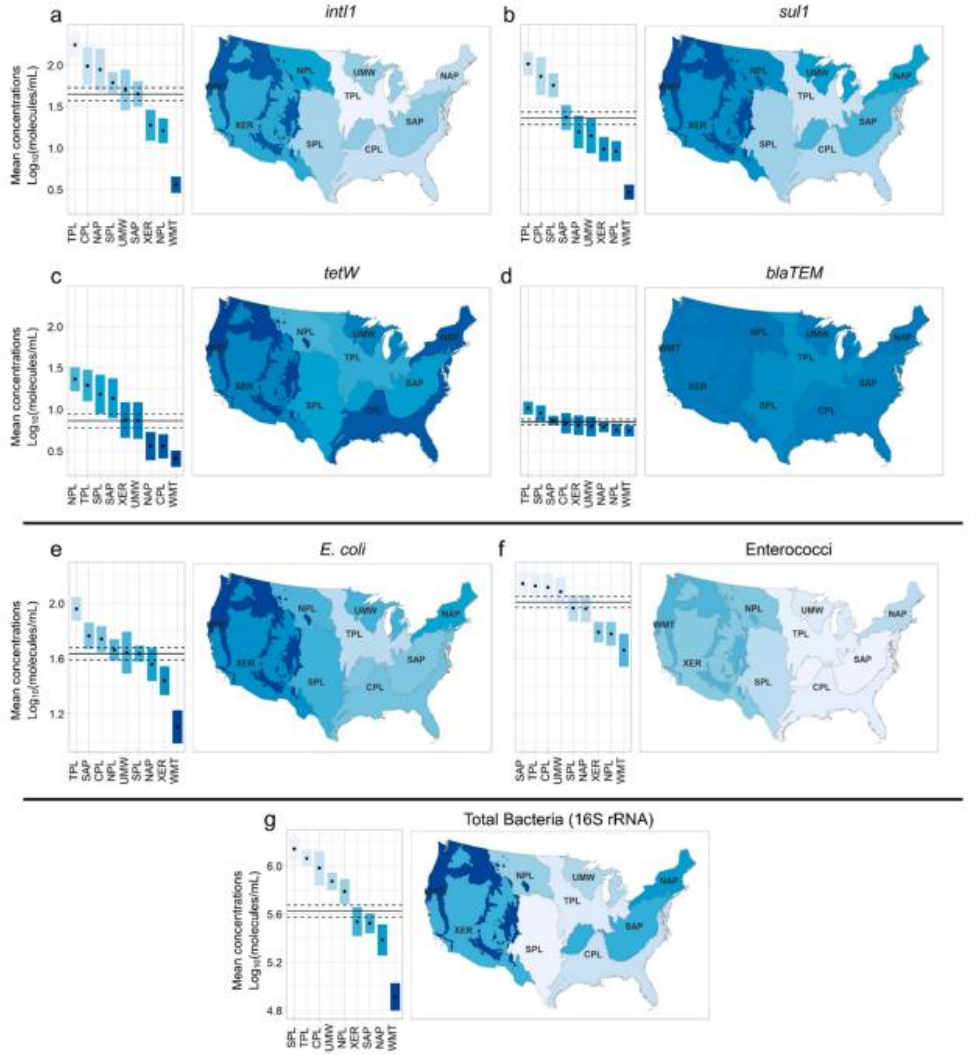
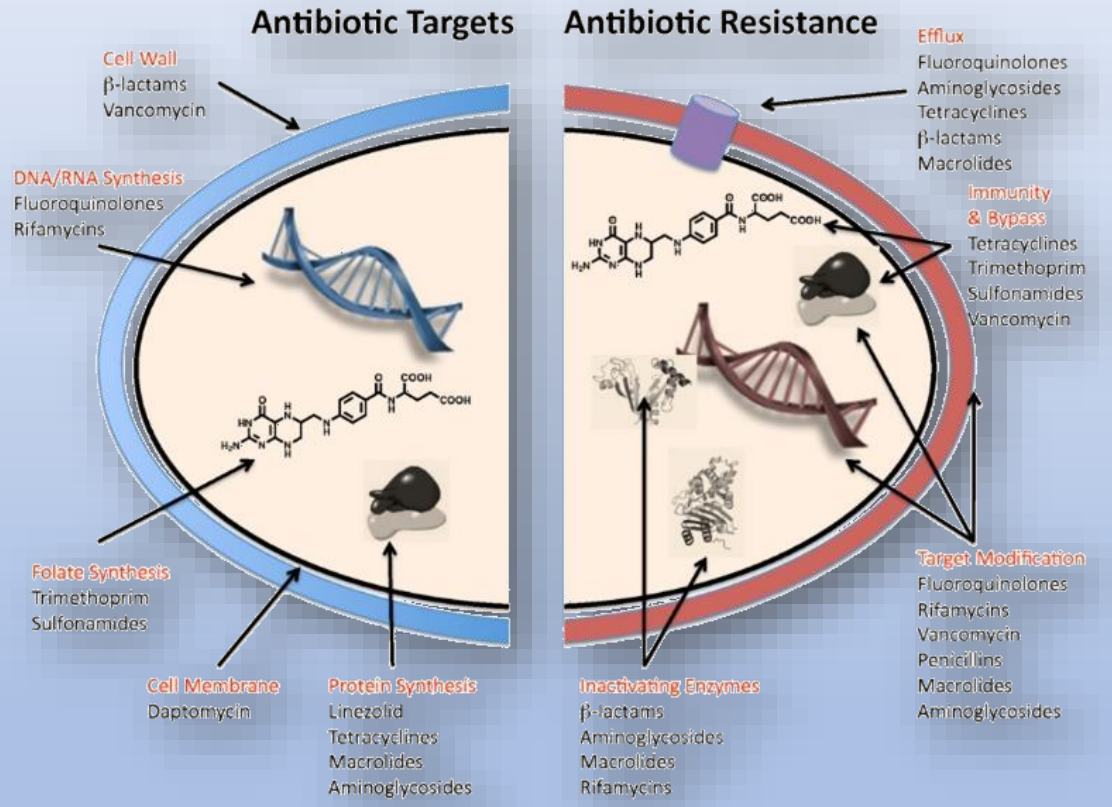


Figure 2. Geospatial distribution of genes among the nine ecoregions by weighted mean concentrations of (a) *intI1*, (b) *sul1*, (c) *tetW*, and (d) *blaTEM*, and ribosomal genes for (e) *E. coli*, (f) enterococci, and (g) 16S rRNA gene (total bacteria). The horizontal lines represent the national weighted mean and lower and upper 95% confidence intervals.

Genes Included in the Pre-Pilot



Wright, G. D. (2010)

- class 1 integron-integrase (*intI1*)
- sulfonamide resistance (*sul1*)
- tetracycline resistance (*tetW*)
- beta-lactam resistance (*blaTEM*)
- *Klebsiella pneumoniae* carbapenemase (*KPC*)
- vancomycin resistance (*vanA*)
- colistin resistance (*mcr-1*)
- 16S and 23S rRNA for total and fecal indicator bacteria (enterococci and *E. coli*)

Baseline Analysis

Published March 1, 2016
J. Environ. Qual. 45:420–431 (2016) doi:10.2134/jeq2015.06.0327
Journal of Environmental Quality SPECIAL SECTION
ANTIBIOTICS IN AGROECOSYSTEMS: STATE OF THE SCIENCE

How Should We Be Determining Background and Baseline Antibiotic Resistance Levels in Agroecosystem Research?

Michael J. Rothrock, Jr.,* Patricia L. Keen, Kimberly L. Cook, Lisa M. Durso, Alison M. Franklin, and Robert S. Dungan

Hypothesis: ARGs are associated with environmental impairment

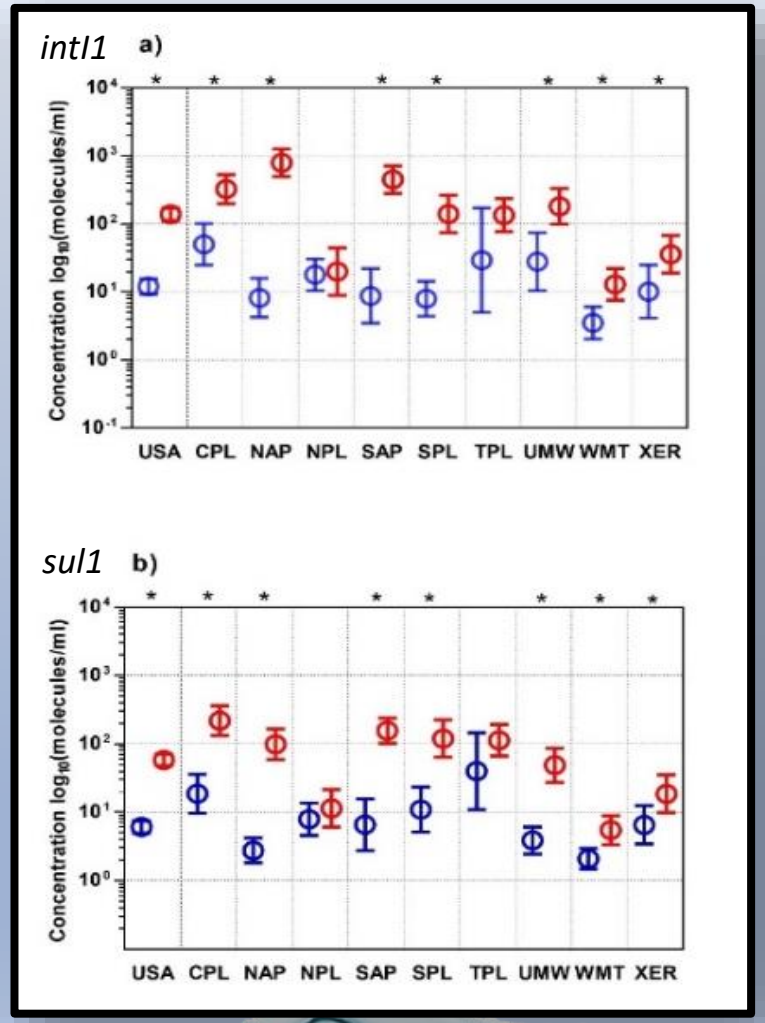
- Good condition (Least Disturbed Sites) associates with low gene concentrations
- Poor condition (Most Disturbed Sites) associates with high gene concentrations

https://www.epa.gov/national-aquatic-resource-surveys/national-rivers-and-streams-assessment-2013-2014-report

Least Disturbed Sites (LDS)	Ranges	
Total P (µg/L)	≤20	≤150
Total N (µg/L)	≤750	≤4500
Cl^- (µeq/L)	≤200	≤2000
$SO4^{2-}$ (µeq/L)	≤200	≤400
ANC (µeq/L)+ DOC (mg/L)	≥50 + ≥5	≥50 + ≥5
Turbidity (NTU)	≤5	≤50
Riparian Disturbance Index	≤0.5	≤2
% fine substrate	≤15	≤90

Most Disturbed Sites (MDS)	Ranges	
Total P (µg/L)	>100	>500
Total N (µg/L)	>1500	>15000
Cl^- (µeq/L)	>1000	>10000
$SO4^{2-}$ (µeq/L)	>1000	>4000
ANC (µeq/L) + DOC (mg/L)	<0 <5	<0 <5
Turbidity (NTU)	>10	>100
Riparian Disturbance Index	>3	>4
% fine substrate	>50	>100

Baseline Results: LDS versus MDS



Least Disturbed Sites

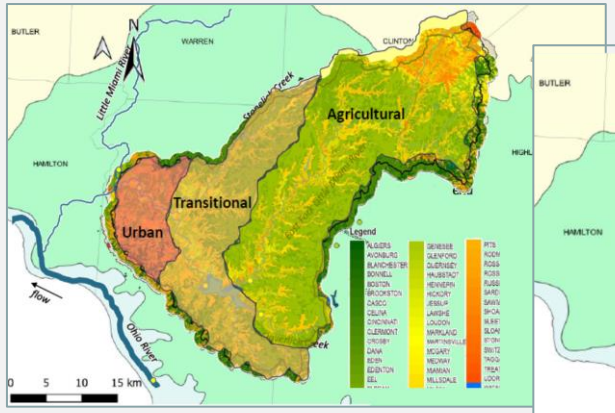
Most Disturbed Sites

* Credible differences

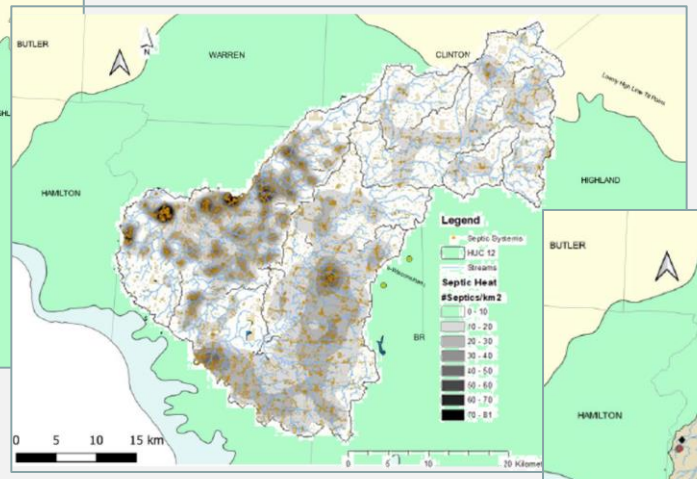
Conclusions

- ARGs showed significant geospatial patterns at national scale
- Good quality rivers/streams had lower ARG concentrations than poor quality ones
- These data suggest *intI1* can be used as an *operational* ecological condition indicator, but more research is needed
- Baseline analysis findings:
  - Urbanization and poor watershed integrity were significantly associated with high concentrations of *intI1* and *sul1*
  - Poor watershed integrity, but not urbanization, was associated with high concentrations of *tetW*
  - Urbanization and poor watershed integrity were not associated with *blaTEM*
- 2023-24 NRSA cycle: same statistical design but expanded analytical targets, larger volumes

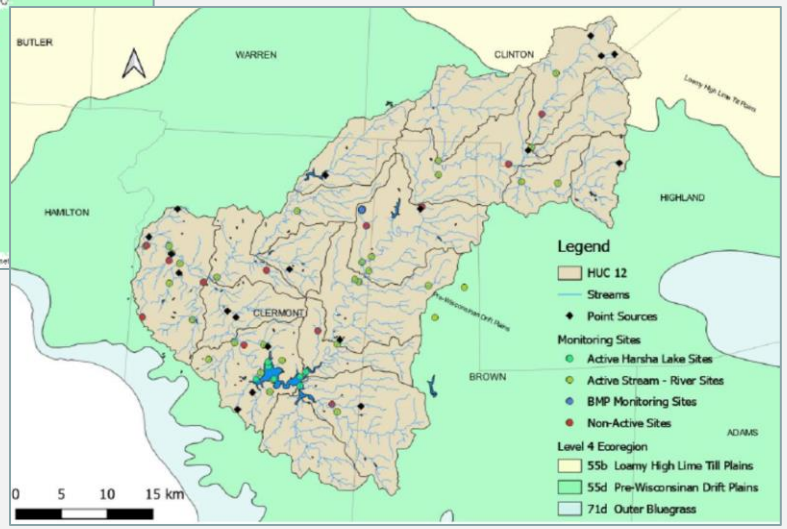
East Fork Little Miami Watershed AMR Pilot



Urban- Ag transition



12K septic systems mapped



Point sources and rec waters in relation to sample sites

Will determine minimum reporting and data quality objectives for comparisons to NRSA and future watershed studies

Additional watershed studies needed:

- High livestock inputs
- Highly urbanized systems
- Regional variation

Watershed studies complement NRSA design

Is there temporal/seasonal variation in antimicrobial resistant bacteria and genes?

Are there environmental reservoirs of AMR?

What are the relative contributions of different AMR sources (e.g., septic, WWTP, livestock, wildlife)

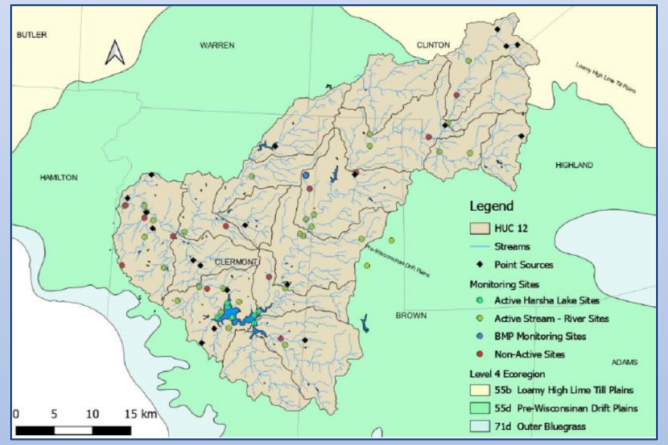
What are the watershed-scale drivers and attenuators of AMR?

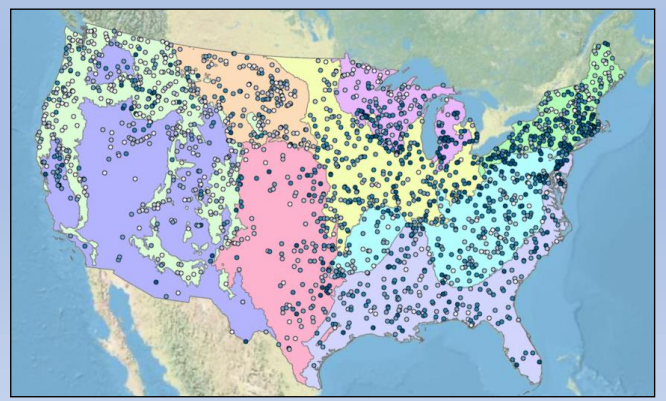
How can we mitigate AMR at local scales?

Developing an environmental component within the National Antimicrobial Resistance Monitoring System (NARMS)

Goal: Create a One Health Model (humans, animals, & environment)

"Following the NARMS Review Subcommittee recommendations to incorporate the three major domains of the **One Health model (humans, animals, environment), …Surface waters as confluence points of ecosystems differentially affected by built environments is a starting point."** NARMS Strategic Plan 2020-2025





Planning and Method Development	NARMS Surface Water Pilot	Development of the environmental assessment & Initial testing of methodologies	FY20 - FY22
Watershed Study		Watershed based assessment to evaluate methodologies before national sampling and serve as a demonstration project for future watershed studies Utilizing East Fork Watershed – 35 locations	FY22 - FY23
National Study		Probabilistic national survey to provide statistically valid estimates of AMR status and trends in surface water, using methods tested in the other phases Utilizing NRSA - ~2,000 locations	Summers FY23 - FY24,
Future Studies		Continued probabilistic national monitoring together with expanding number of (partner-led) intensive watershed studies across the country	FY25+

Overall Study Design

Sample site Location Selection

In-situ measurements

Field Sample Collection

Transportation/Shipping Protocols

Primary Sample Processing EPA

Laboratory Analysis

Whole Genome Seq– FDA

Water chemistry – EPA Coll. Lab, third-party lab

Culturing – EPA & Collaboration Labs

Targeted Gene Assays - EPA/Coll. Labs

Metagenomics - FDA

Analytics

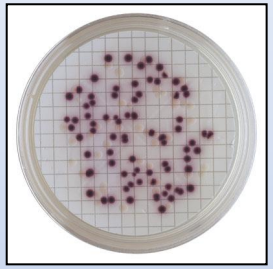
Publications/Reports

Slide design by Chris Grim (FDA)

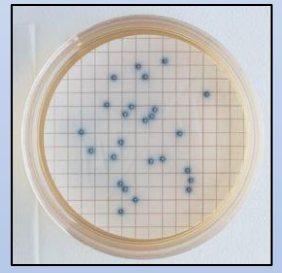
Culture Work Methods

Methods Selected

1. *E. coli* – Modified mTEC method (Modification of EPA Standard Method 1603)
   - Cefotaxime resistance



2. *Enterococcus spp.* – Modified mEI method (Modification of EPA Standard Method 1600)
   - Vancomycin resistance



3. *Salmonella* – Modified Standard Method
   - Presence/Absence

Reasoning

- ✓ Selection of standard methods that are being utilized by similar efforts
  - WRF Effort – Pruden et al. using same E. coli and Enterococcus methods
  - EPA Beaches Study – Used same E. coli method
- ✓ Need isolates for susceptibility testing and whole genome sequencing
  - IDEXX required additional second step to obtain isolates

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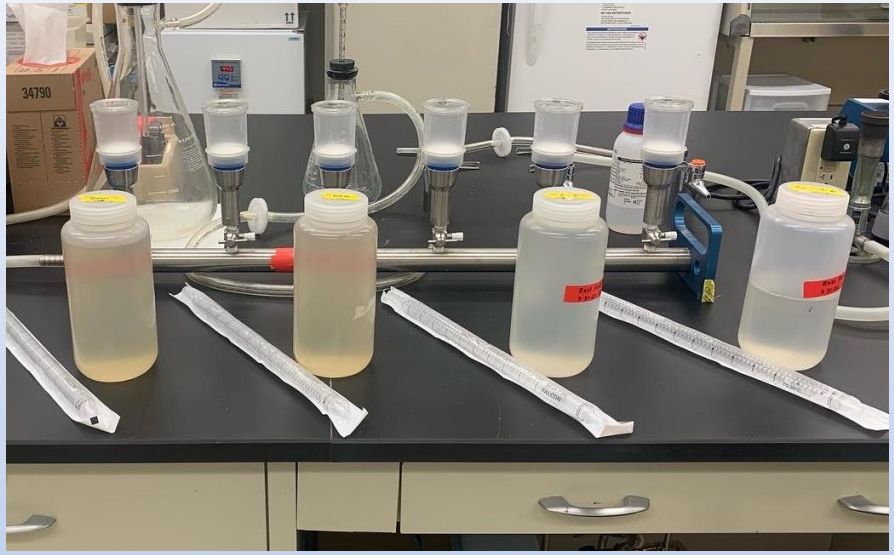
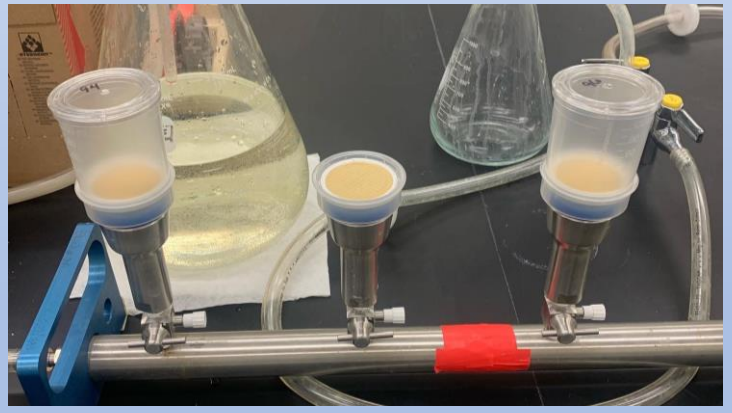
E. Coli & *Enterococcus* Workflow

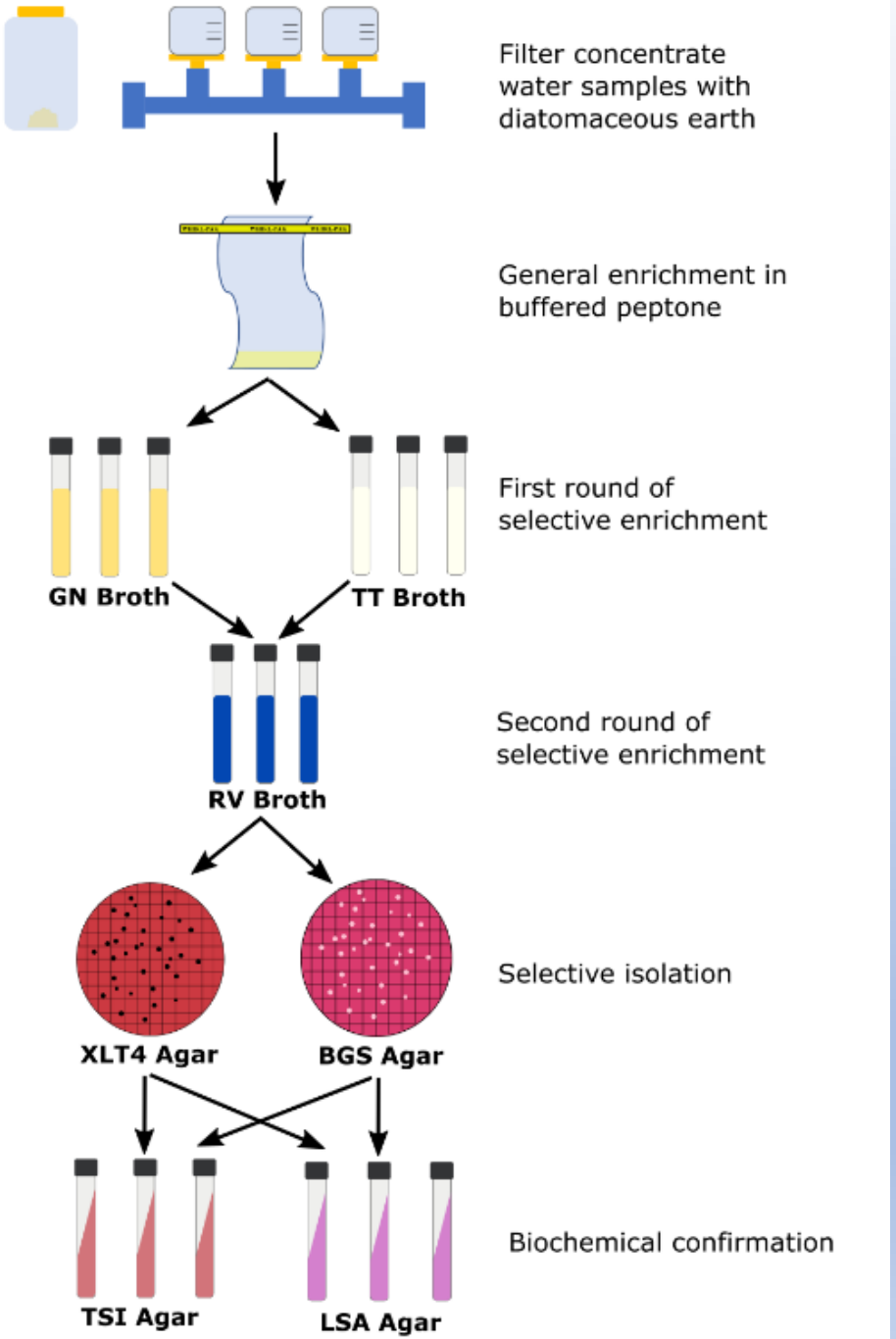
1. Membrane Filtration
    - Total *E. coli* & *Enterococcus* Quantification Serial Dilutions: 100 mL, 10 mL, 1 mL
    - Resistant *E. coli* & *Enterococcus*: 400 mL
2. Place filter on respective media plates
    - *E. coli* – mTEC & mTEC+Cefotaxime
    - *Enterococcus* – mEI & mEI+Vancomycin
3. Incubate
    - *E. coli* – 2 hours in dry incubator @ 37 C & 22 in water bath for 22 hours @ 45 C
    - *Enterococcus* – 24 hours in dry incubator @ 41 C
4. Plate Counting
5. Re-streaking up to 5 presumptive resistant *E. coli* & *Enterococcus* per sample
6. Further confirmations





Salmonella Workflow

1. Water sample (~1 L) filtered with diatomaceous earth & 3 uM filter.
2. Filter with diatomaceous earth cake added to BPW - Incubated 18-24 hours @ 37 C
3. BPW enrichment (1 mL) added to Gram Negative broth (9 mL) and Tetrathionate broth (9 mL) – Incubated for 24 hours (GN) and 48 hours (TT) @ 37 C
4. GN and TT enrichments (100 uL) added to Rappaport Vassiliadis broth (9.9 mL) – Incubated 24 hours @ 37 C
5. GN/RV and TT/RV enrichments (1 uL) plated on XLT-4 and Brilliant Green Sulfa agars
6. Re-streaking up to 4 presumptive isolates per plate (16 per sample)
7. Biochemical confirmation with Triple Sugar Iron agar and Lysine Iron agar.



Molecular Work Methods

1. Metagenomics – Whole Water Sample (FDA)

2. Targeted Gene Analysis (EPA)
   - Fluidigm – High throughput PCR (relative abundance; presence/absence)
   - ddPCR – Quantify select genes of interest

3. Whole Genome Sequencing (FDA)
   - All *Salmonella*
   - Subset of resistant *E. coli* and *Enterococcus spp.*

4. Quasimetagenomics – Culture Enrichment (FDA)

Reasoning

- ✓ Variety of molecular methods to characterize presence of AMR and microbial populations in surface waters.
- ✓ Targeted gene analysis to determine relative and absolute numbers of genes of interest.
- ✓ Metagenomics to determine the resistome of environmental microbial populations.
- ✓ Whole genome sequencing of select isolates to create a genetic database of resistant organisms in surface waters.

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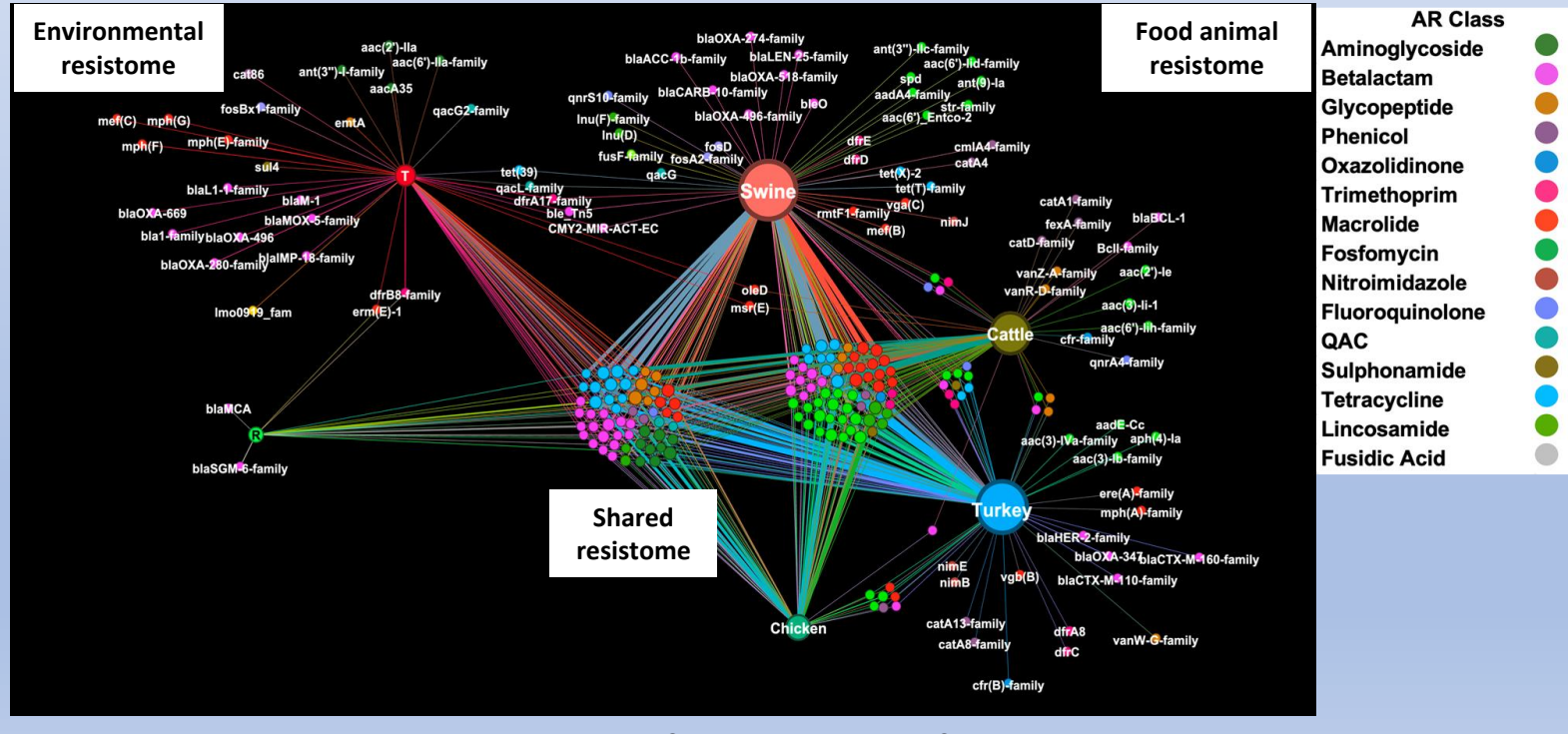
Metagenomics

Decisions Made to Aid Metagenomics Work

- Adequate Sample Volume – 500 mL
  - Ensure enough DNA to perform work.
- DNA Extraction with PowerWater
  - Broad Recovery
  - Highest Yield and Quantity
- Whole Cell Standards
  - Zymo
  - ATCC

Major Advantages for Pilot Study

- Characterize the full complement of environmental microbiome and resistome
- Identify early signal of emerging resistance genes



Resistome comparison between surface water and food animal

Figure courtesy of Daniel Tadesse (FDA)

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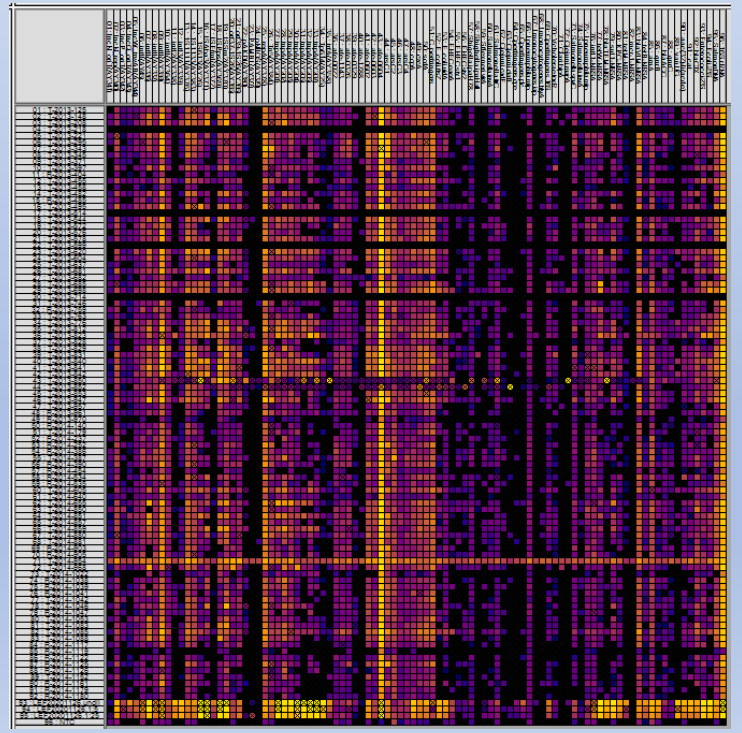
Targeted Gene Analysis

- Fluidigm – 96.96 IFC with nanoliter reactions and ability to screen 96 samples for 96 targets
  - Antimicrobial Resistance Genes
    - Aminoglycosides, Betalactams, cephalosporins, MLS, Quinolones, sulfonamides, tetracyclines, trimethoprim, vancomycin
  - Fecal Indicators:
    - Human, Cow/Ruminants, pig
  - Mobile Genetic Elements



- ddPCR – Quantification of select genes without need for standard curves
  - Antimicrobial Resistance Genes previously monitored during NRSA
    - *intI1*, *sul1*, *tetW*, *blaTEM*
  - Genes detected via Fluidigm and/or metagenomics

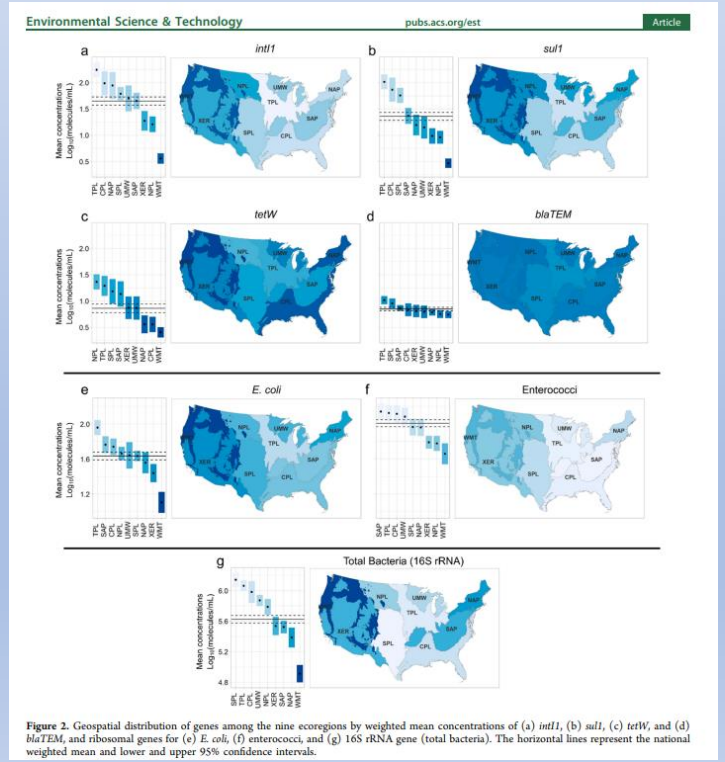


Figure 2. Geospatial distribution of genes among the nine ecoregions by weighted mean concentrations of (a) *intI1*, (b) *sul1*, (c) *tetW*, and (d) *blaTEM*, and ribosomal genes for (e) *E. coli*, (f) enterococci, and (g) 16S rRNA gene (total bacteria). The horizontal lines represent the national weighted mean and lower and upper 95% confidence intervals.

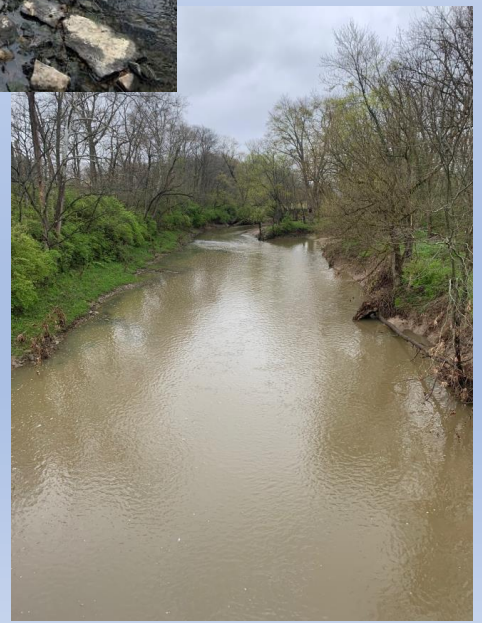
Keely et.al. https://pubs.acs.org/doi/10.1021/acs.est.2c00813

Field Methods

- Sample Type: Whole Water Grab
- Sampling location: Middle of surface water/confluence (if possible)
- Watershed Study:
  - Sampling occurs either by walking/wading in or bridge sampling
- National Study:
  - Sampling occurs at the X Site(kept on ice after collection) and either by wading in or by boat (non-wadeable)

Watershed Study Sampling Locations

Walk In Location

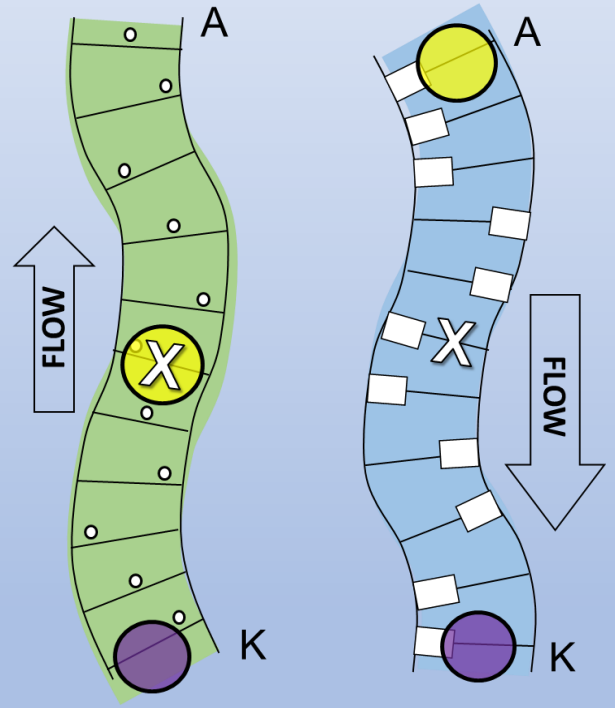


Bridge Site Location

National Study Sampling Schematics

Wadeable: sample **against** the flow

Non-Wadeable: sample **with** the flow



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Where are we now?

- Method Development was completed June 2022
- Watershed Scale study completed May 2023
- Sampling for first year of National Scale Study started May 2023 and ended Sept. 29, 2023.

Method Development	NARMS Surface Water Pilot	Initial testing of methodologies	FY21-1st half FY22
Watershed Study		Watershed based assessment to evaluate methodologies before national sampling and serve as a demonstration project for future watershed studies	Summer FY22-Summer FY23
National Study		Probabilistic national survey to provide statistically valid estimates of AMR status and trends in surface water, using methods tested in the other phases	Summers 2023-24,
Future Studies		Continued probabilistic national monitoring together with expanding number of (partner-led) intensive watershed studies across the country	2024+

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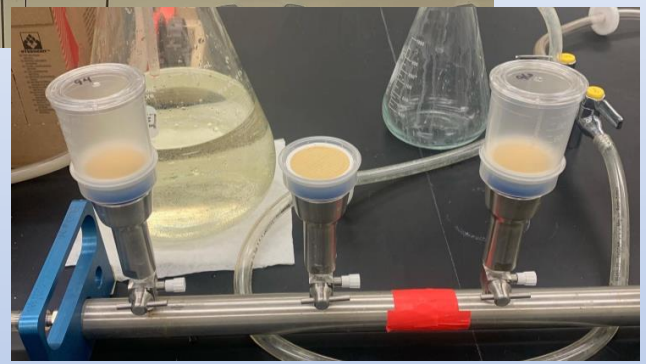
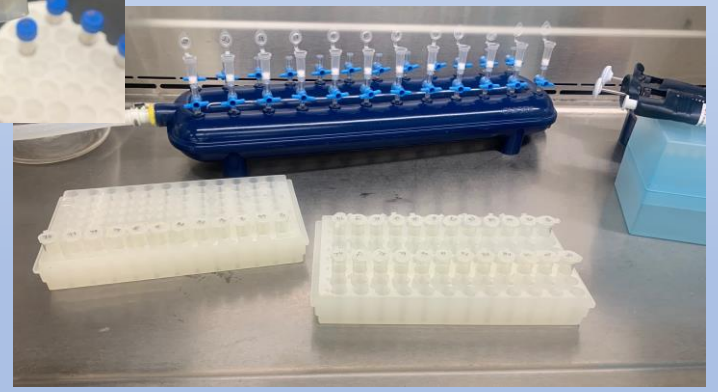
Watershed Study Overview

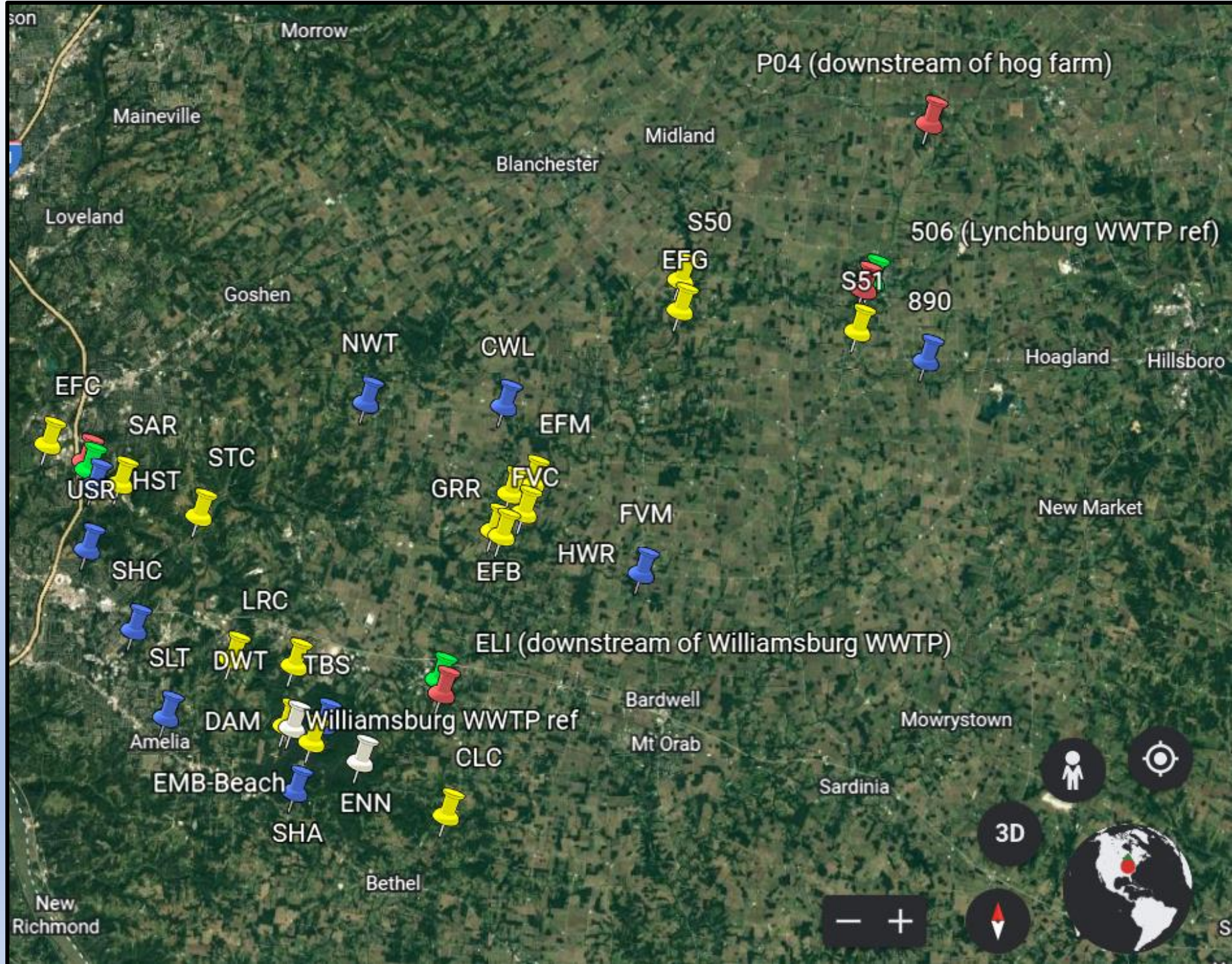
1. Almost a year long study: July 2022 – May 2023
   - 41 weeks of sampling.
   - 35 sites sampled
     - 31 sites sampled every 3 weeks (~12 times)
     - 4 site sampled weekly (41 times)
2. Analyses Performed
   - Molecular: All samples filtered & DNA extracted
   - Culture: *E. coli,* ESBL *E. coli, Enterococcus,* vancomycin resistant *Enterococcus* (VRE)*, Salmonella*
     - All presumptive ESBL, VRE, and Salmonella saved away
3. Upcoming
   - Confirmations of isolates
   - Whole Genome Sequencing of confirmed isolates
   - Metagenomics
   - High Throughput Targeted Gene Analysis





Sampling Locations in East Fork Watershed

- Upstream & Downstream of three WWTPs
- Downstream of a pig farm
- Leaky septic systems
- Agriculture, urban, and suburban areas
- Recreational (Lake and Dam)
- Drinking water intake

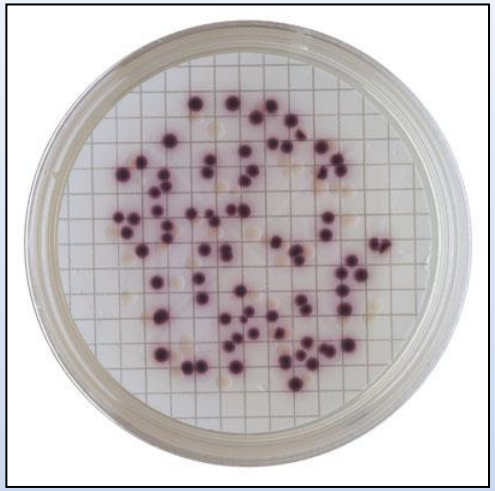


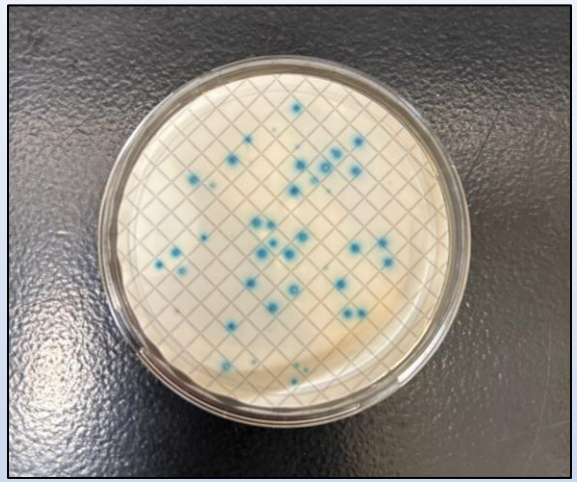
Watershed Study Data Overview

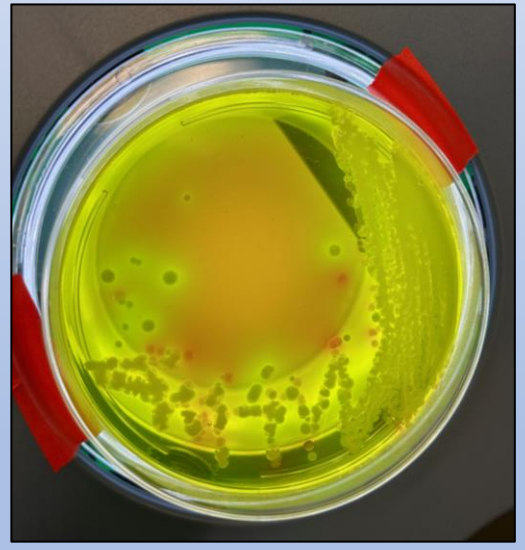
1. Number of Isolates
   - Presumptive ESBL E. coli: 420
   - Presumptive VRE: 167
   - Biochemically Confirmed Salmonella: 203

2. Percent Samples Positive
   - Presumptive ESBLE E. coli: 39%
   - Presumptive VRE: 29%
   - Biochemically Confirmed Salmonella: 50%

3. Bacterial Counts (CFU/100 mL):
   - Total E. coli: No detection – 30,000
   - Percent ESBL E. coli: 0-25%
   - Total Enterococcus: 1 – 12,600
   - Percent VRE: 0-5%





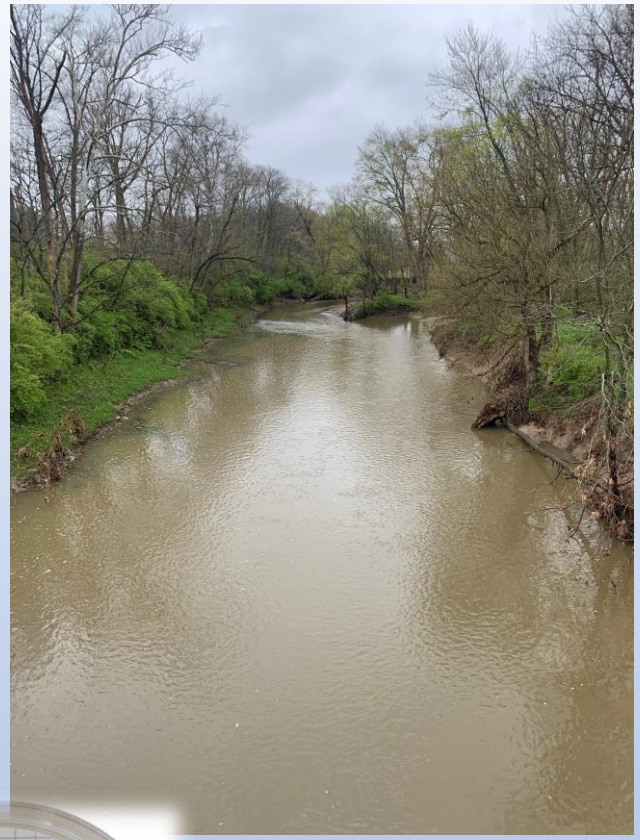


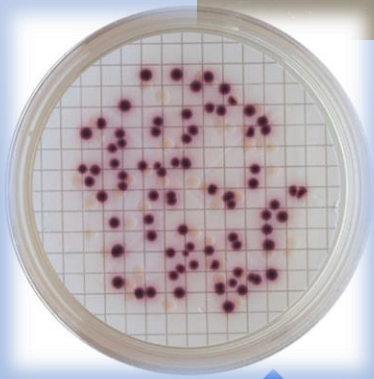
Overview of *E. Coli* Results

- Levels of *E. coli* are higher than EPA Water Quality Limits in 142 out of 566 samples collected (25%).
  - Frequently over limits around WWTPs, downstream of pig farm, and near suburban areas.
  - Recreational areas were always below Water Quality Limits

- ESBL *E. coli* detected in 220 out of 566 samples collected (39%).
  - Percent of *E. coli* that were ESBL ranged from 0 – 25%.
    - NOTE: In WWTP effluent, it was up to 77%.
  - Frequently around WWTPs and downstream of pig farm.
  - Highest % of ESBL *E. coli* was found in beach and dam samples (recreational areas).

- Rain events lead to significantly higher numbers of total *E. coli*
  - Precent ESBL *E. coli* only slight increased.





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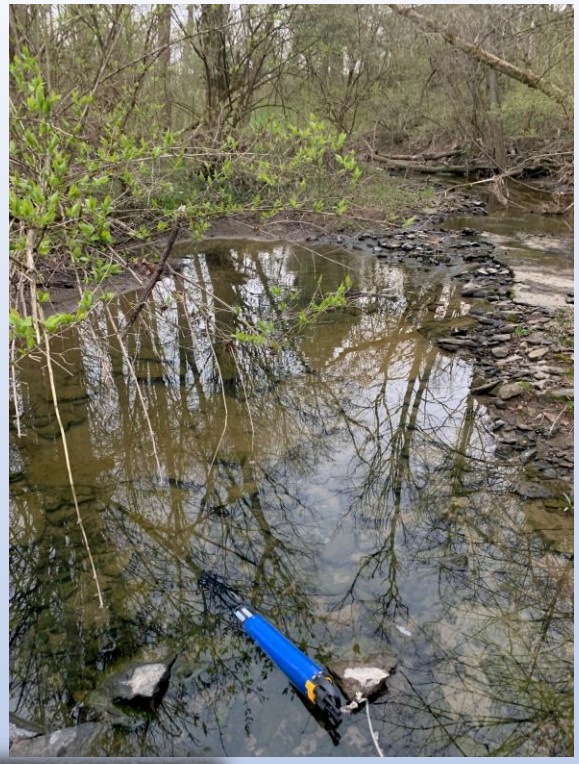
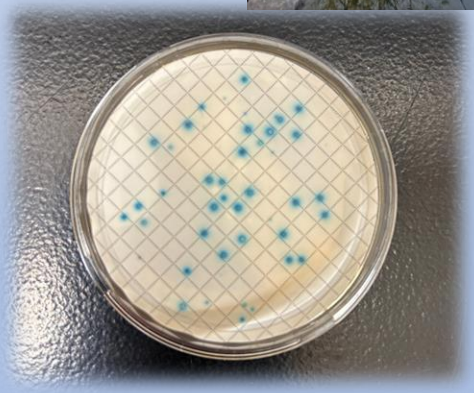
Overview of *Enterococcus* Results



- Levels of *Enterococcus* are higher than EPA Water Quality Limits in 148 out of 268 samples collected (~55%).
  - Frequently over limits around WWTPs, downstream of pig farm, and near suburban areas.
  - 33% of samples taken from recreational areas were above Water Quality Limits
- VRE detected in 60 out of 211 samples (29%).
  - Percent of *Enterococcus* that were VRE ranged from 0 – 5%.
  - Frequently around WWTPs and downstream of pig farm.
  - Highest % of VRE was found in WWTP effluent and downstream of WWTPs
- Rain events lead to significantly higher numbers of total *Enterococcus*
  -



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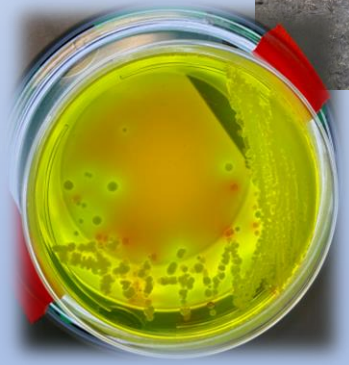
Overview of Salmonella Results

- Salmonella was most frequently detected in waters samples collected around WWTP.
  - Specifically, one WWTP – Williamsburg
- Typically, number of samples per week positive for *Salmonella* ranged from 28 – 50%.
- Number of samples per week positive for *Salmonella* spiked after rain event.
  - 86% of samples positive for Salmonella after rain event.
- *Salmonella* was detected in WWTP effluent prior to start of UV disinfection.



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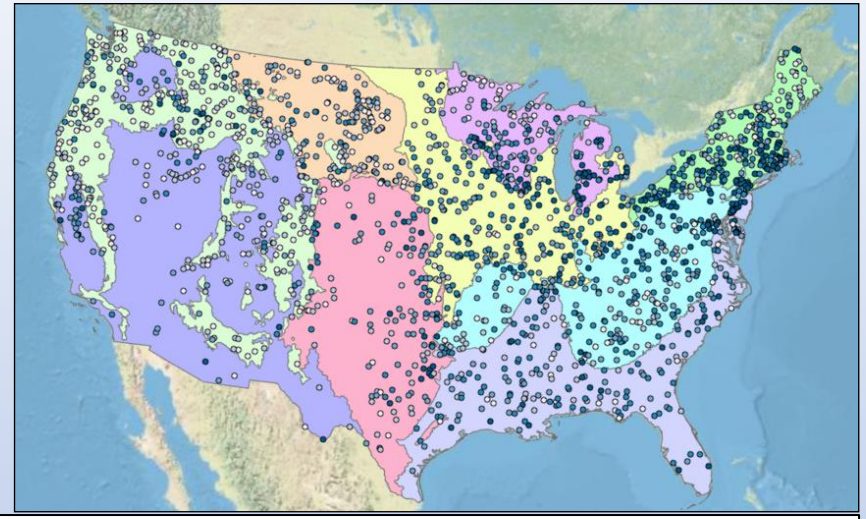
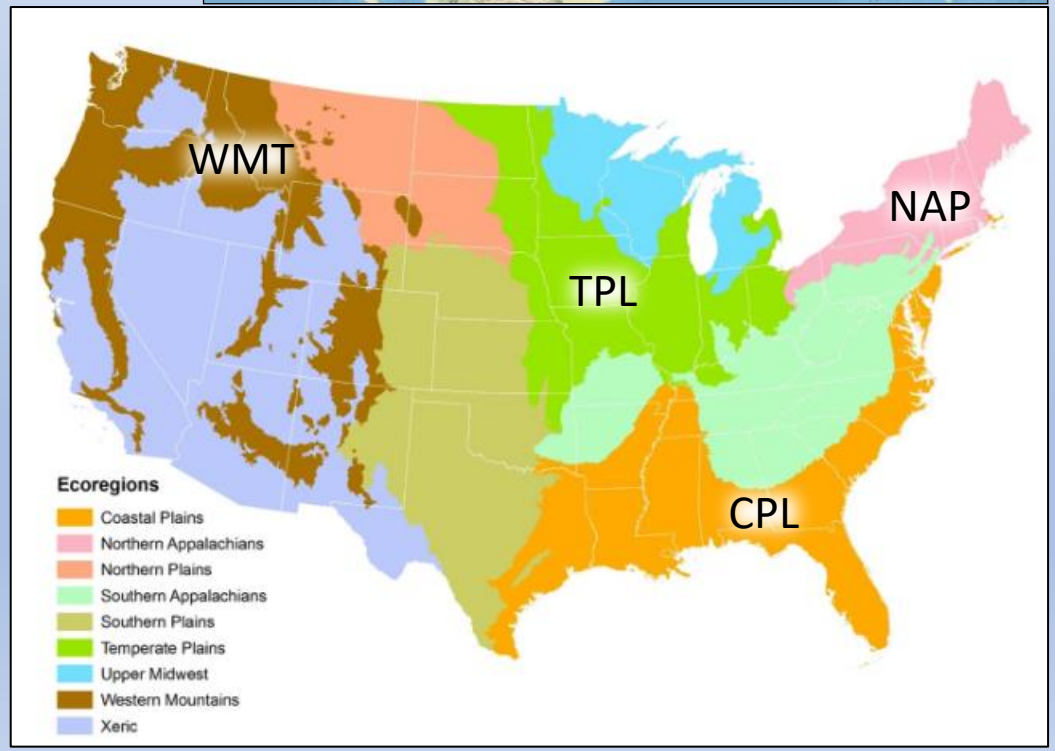
National Scale Study Status

1. First Year: May 2023 - Sept. 2023
   - Received 1,067 samples

2. Work Performed to Date
   - Molecular: All samples filtered and saved away in -80C to be extracted later
   - Culture: All samples processed for *E. coli*, ESBL *E. coli*, *Enterococcus* and VRE
     - *Salmonella* work performed on a subset (612 samples)
       - Selected ecoregions: Northern Appalachian (NAP), Coastal Plains (CPL), Temperate Plains (TPL), and Western Mountains (WMT)

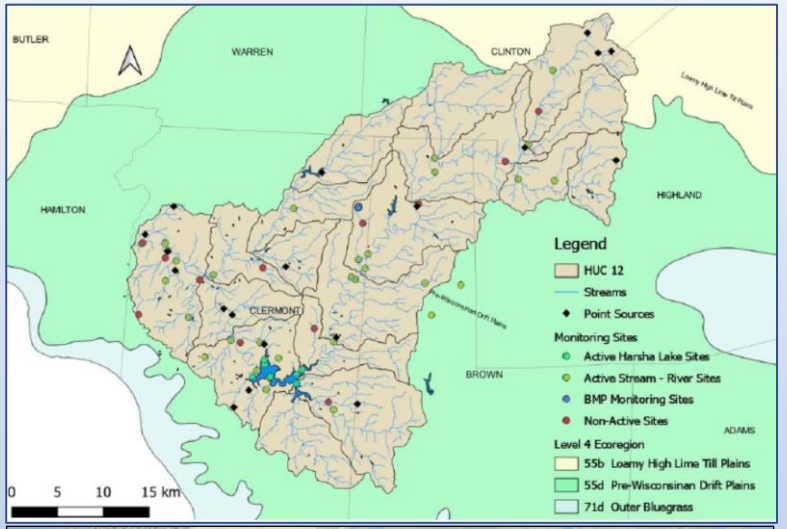
3. Number of Isolates
   - Presumptive ESBL *E. coli*: 215
   - Presumptive VRE: 201
   - Presumptive Salmonella: >1,000

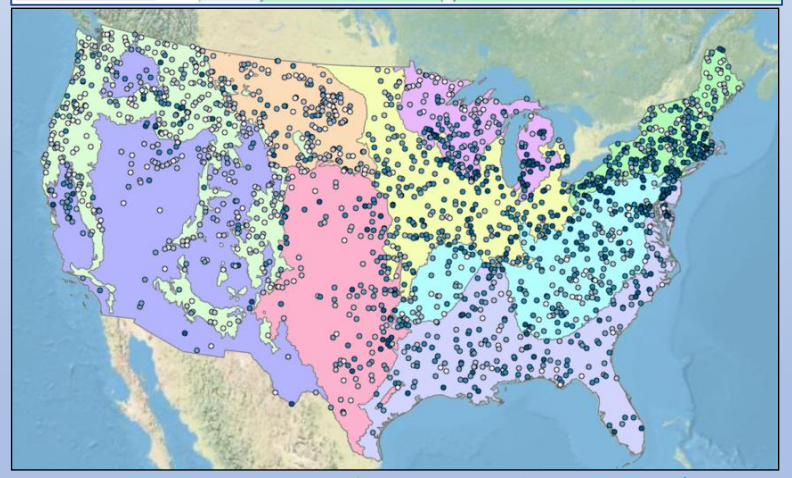




Upcoming Work

1. Data Analysis of Watershed Study Culture Work
   - Anticipated publication Spring 2024
2. Hold time study – Culture Work
   - Had varying hold times for E. coli, Enterococcus, and Salmonella work
   - Want to determine role of holding samples for extended periods of time.
3. Molecular work for Watershed Study
   - Metagenomics, targeted gene analysis, whole genome sequencing
4. Second year of National Study will start April/May 2024





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Data Reporting Plan

- East Fork Watershed Study publications
  - Submittal planned for April-December 2024
  - Separate publications on different analytical approaches
  - Integrated paper assessing the best approaches for assessing watershed level spatiotemporal distribution & drivers
- NRSA publications
  - Submittal planned for late 2025 into 2026
  - National scale drivers for distributions (as reported earlier for initial efforts with NRSA)
  - Relate to human and agricultural domains for Integrated OneHealth monitoring

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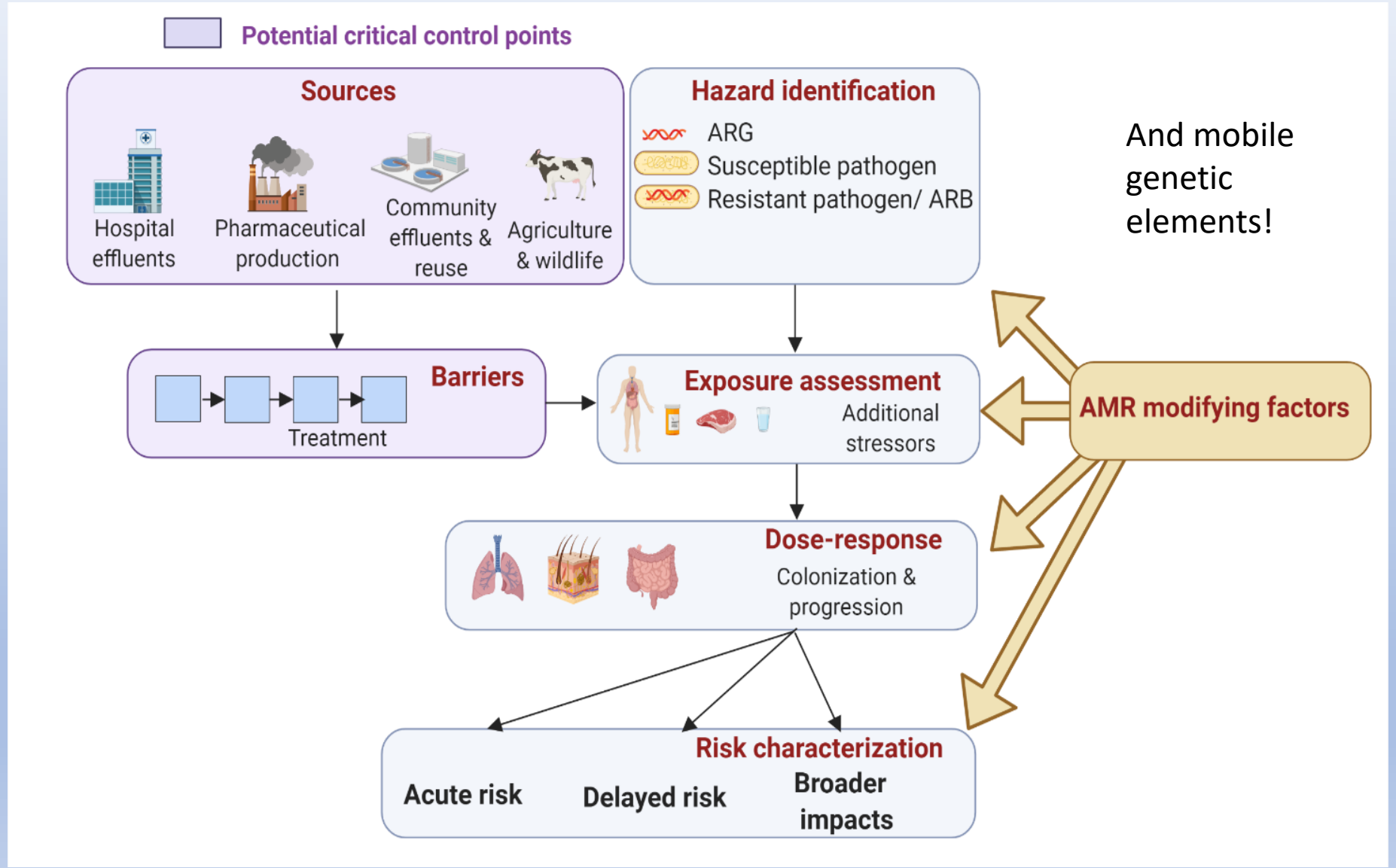
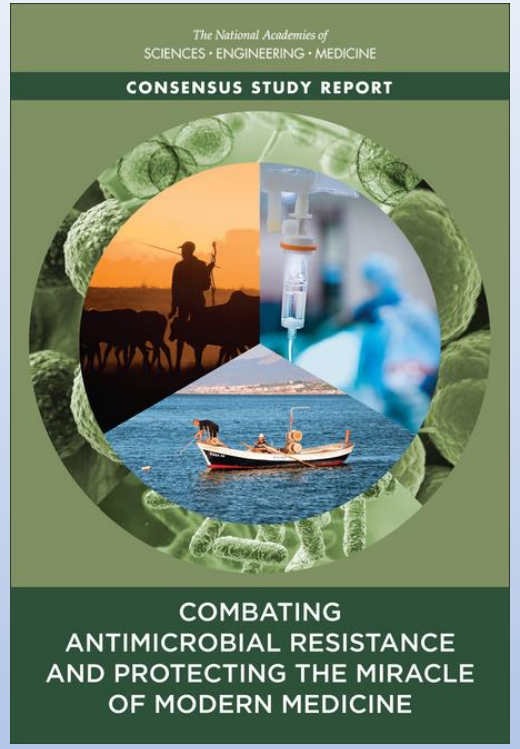
What factors do we need for “risk assessment +”?



And mobile genetic elements!

Hamilton et al. ES&T in revision

2021 National Academy of Sciences Report



The challenge for environmental monitoring is to determine what factors amplify resistance in the environment and what factors encourage their transmission

Water treatment plants are…. not equipped to eliminate resistance traits or drug residues….an important bridge between human made contamination and the natural environment

Strengthening - Combating Antimicrobial Resistance and Protecting the MiracSurveillancele of Modern Medicine - NCBI Bookshelf (nih.gov)

Recommendation 4.2 The EPA should provide guidance and resources to states for testing point source discharges at wastewater treatment plants for antimicrobial resistance traits and integrating these data with other surveillance networks”

National Priorities: Evaluation of Antimicrobial Resistance in Wastewater and Sewage Sludge Treatment and Its Impact to the Environment

This RFA will solicit research on selection and removal efficiency of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in wastewater treatment plants. It will also request research on the relative significance of wastewater as a source of ARB and ARGs in receiving waters…Proposals submitted and currently under review

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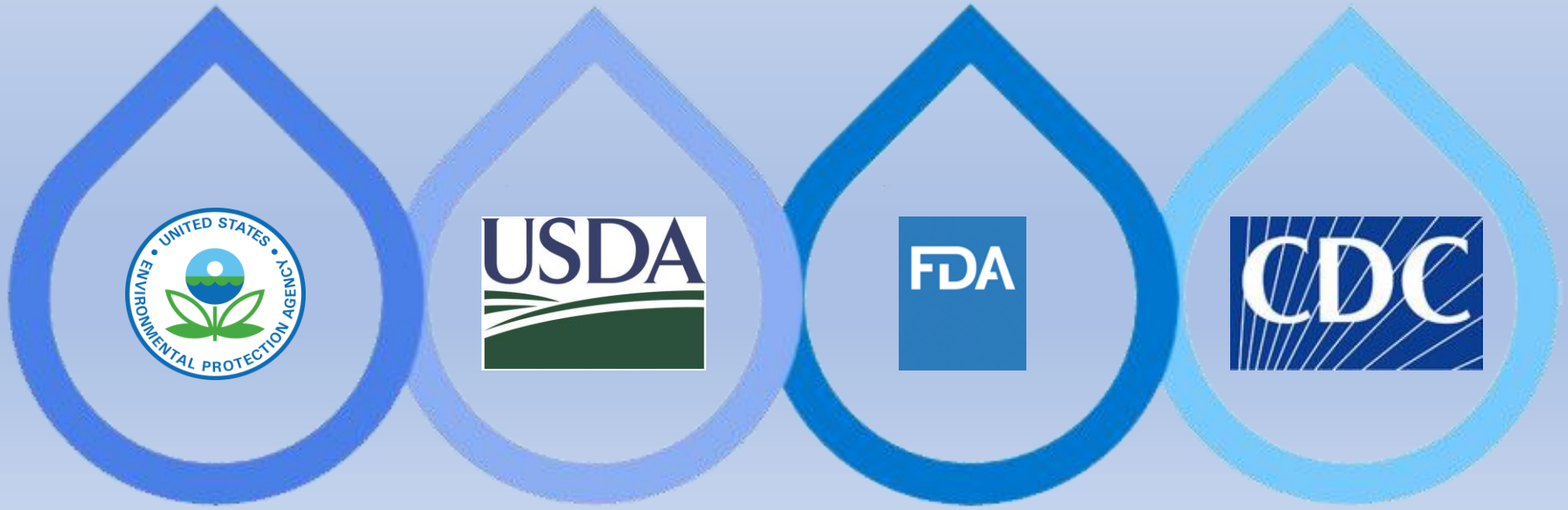
Thank You!

Any Questions?



Ancillary Slides

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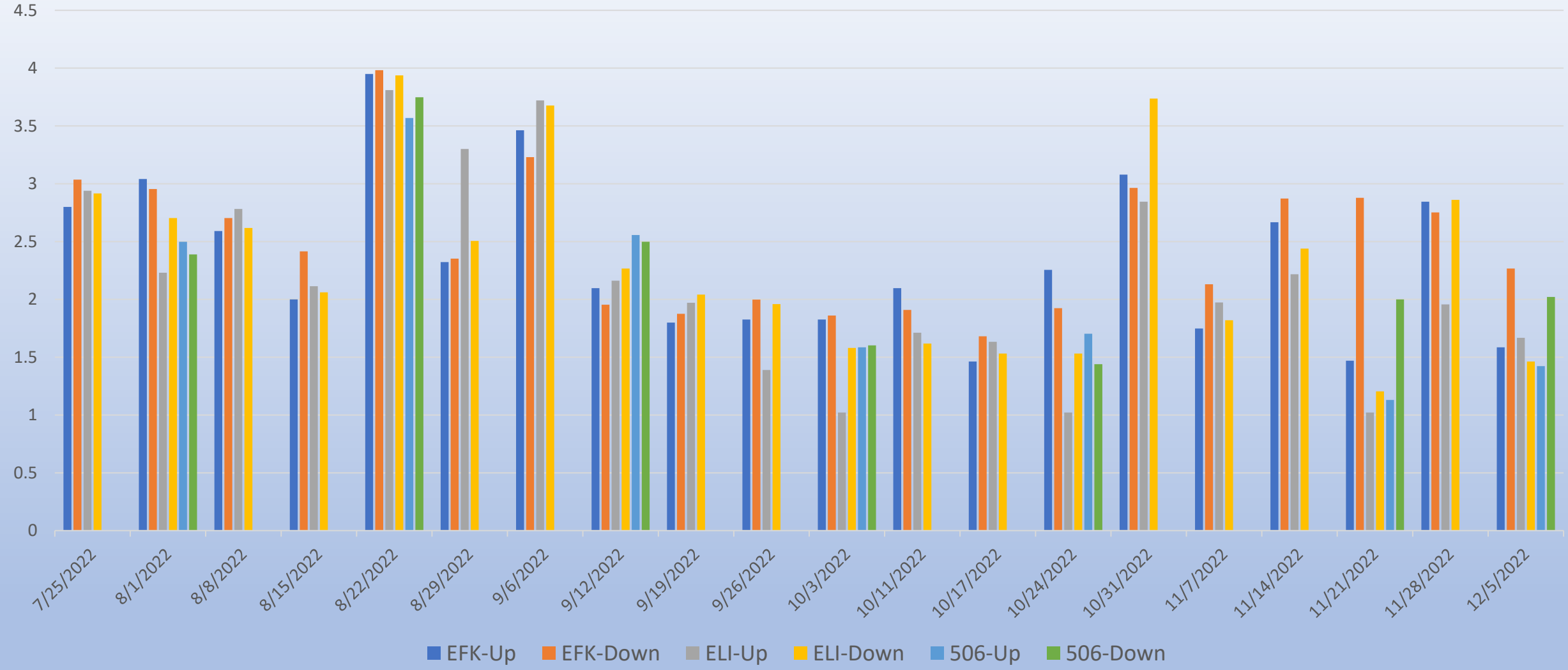
E. coli Counts (log-cfus/100ml) Upstream and Downstream of three WWTPs (July - December 2022)



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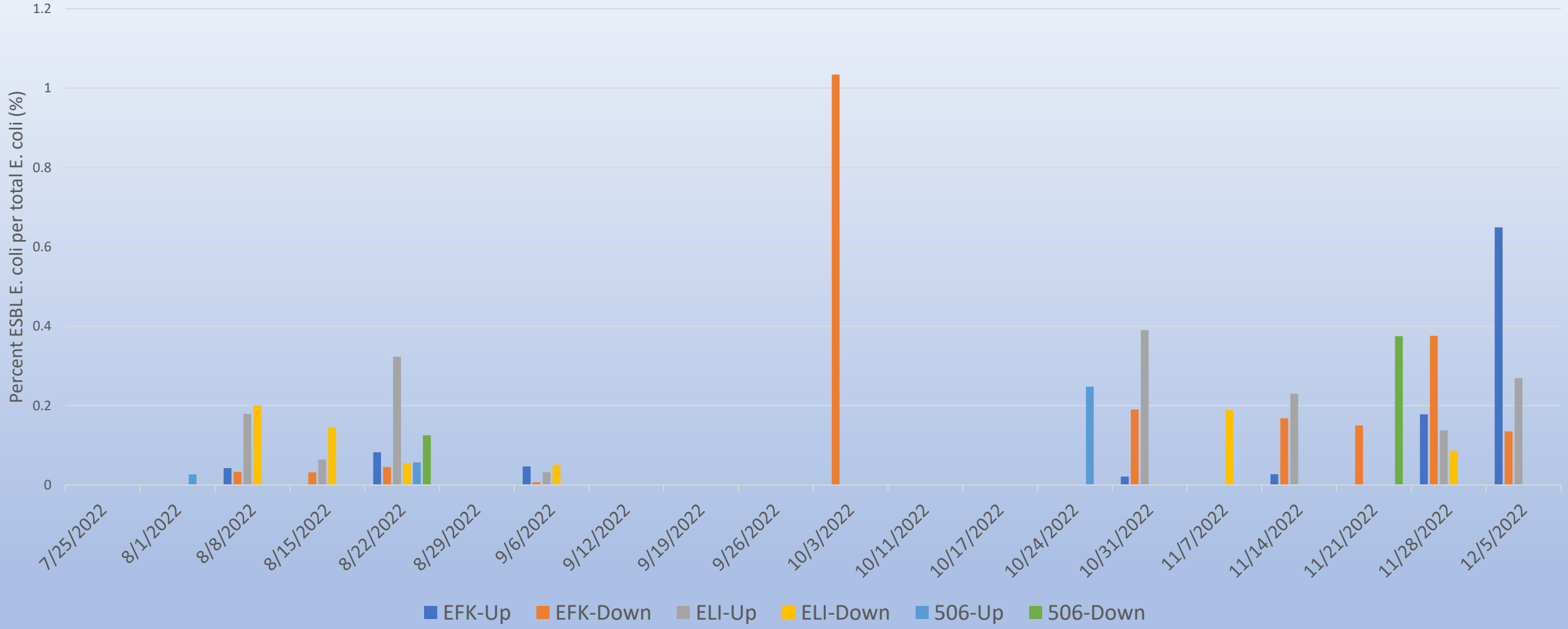
Percent ESBL E. coli per total E. coli in samples upstream and downstream of three WWTPs (July - December 2022)



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