

Effects of salinity reduction on the rate of decay of marine- adapted *Enterococcus*

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Abstract

Enterococcus is a bacteria found in the gastrointestinal tracts of animals and is commonly used as an indicator of fecal contamination in recreational waters, making it vital to understanding how changes in coastal conditions influence its prominence and impact on public health. This study investigated the relationship between decreasing salinity and *Enterococcus* concentrations in coastal seawater in order to simulate freshwater infiltration of coastal beaches. It was hypothesized that *Enterococcus* concentrations would decrease alongside salinity levels. Seawater collected from Virginia Key Dog Beach in Miami, Florida, was prepared at five salinity treatments (35, 25, 15, 5, and 0 PSU) and tested using the IDEXX Enterolert/Quanti-Tray/2000 procedure. Eight independent trials were conducted with three replicates per treatment, and *Enterococcus* were measured as Most Probable Number (MPN)/ 100 mL. At 24 hours, mean concentrations decreased from 168.21 MPN/ 100mL at 35 PSU to 113.96, 72.04, 27.92, and 0 MPN/ 100 mL at 25, 15, 5, and 0 PSU respectively. Statistical analysis showed a significant overall difference among salinity treatments (Freidman $X^2(4) = 27.18$, $p < 0.001$) and a significant trend in degreasing *Enterococcus* concentrations with decreasing salinity (Page trend test, $L = 422.5$, $p < 0.001$). These results supported the predicted relationship between lower salinity treatments and lower *Enterococcus* concentrations. Therefore, it is plausible that the findings demonstrate a consequential relationship between freshwater infiltration and detectable *Enterococcus*. Understanding this relationship is irrefutably essential to the interpretation of recreational water quality measurements in coastal environments affected by freshwater infiltration.

1. Introduction

1.1 Background

Enterococcus is a type of bacterium largely found in the intestinal tracts of both humans and animals. *Enterococcus*' bacterial concentration is widely used as an indication of fecal matter in recreational waters. Its detection does not necessarily mean that pathogens are present.

Alternatively, elevated concentrations can indicate unsafe levels of fecal contamination. The U.S. Environmental Protection Agency uses *Enterococcus* as an indicator of recreational water quality in marine waters.

The abundance of *Enterococcus* can be influenced by environmental conditions. Previous studies have established that sunlight can cause substantial photoinactivation of *Enterococcus* in marine environments as well as increase bacterial decay rates under high UV conditions. Salinity may also interact with environmental stressors, which potentially affect fecal indicator bacteria. Thus, making coastal environments particularly complex to investigate and interpret bacterial measurements.

1.2 Scientific Problem

Coastal environments frequently experience changes in salinity as freshwater enters marine systems through runoff, rainfall, and other sources. Because *Enterococcus* concentrations are used to determine recreational water quality, understanding how environmental conditions influence these concentrations is fundamental to monitoring results.

A decrease in measured *Enterococcus* following freshwater infiltration could reflect several processes, including the dilution of contaminated water, bacterial responses to rapid changes in the environment, or sunlight exposure. Distinguishing these conditions is important when interpreting water quality.

1.3 Research Question

How are decreasing salinity conditions, produced through progressive dilution of coastal seawater with distilled water, associated with concentrations of marine-adapted *Enterococcus* measured using the IDEXX Enterolert/Quanti-Tray/2000 method?

1.4 Hypothesis

Decreasing salinity levels from 35 PSU to 0 PSU will increase the decay rate of marine-adapted *Enterococcus* populations. This will result in significantly lower MPN/100 mL outcomes over 48 hours compared with the 35 PSU treatment.

1.5 Significance of study

Understanding the importance of *Enterococcus* concentrations is environmentally and publicly relevant because of its indication of fecal contamination in recreational waters to assess potential health risks depending on concentration.

Coastal waters can have lasting effects from rapid freshwater infiltration. Researching the relationships of freshwater dilution, salinity, and *Enterococcus* can therefore contribute to understanding how environmental conditions influence bacterial concentration and guide future studies of bacterial persistence in the presence of freshwater infiltration.

2. Materials and Methods

2.1 Study and Design

An experimental study was conducted to investigate the effect of reduced salinity on the viability of *Enterococcus* in coastal marine water. Seawater was collected during eight independent sampling trials and experimentally diluted to produce five salinity treatments: 35, 25, 15, 5, and 0 practical salinity units (PSU). The 35 PSU treatment represented undiluted seawater, while progressively lower salinity treatments simulated increasing freshwater infiltration. The 0 PSU treatment consisted entirely of distilled water and represented an extreme freshwater treatment.

Three replicate measurements (A, B, and C) were prepared for each salinity treatment. *Enterococcus* concentrations were quantified at 24 and hour marks and expressed in most probable number per 100 mL (MPN/ 100mL). The study consisted of eight sampling trials conducted between July and September 2026.

2.2 Sample Collection

Coastal water samples were collected from the Virginia Key Dog Beach area in Miami, Florida, across from MAST Academy. Each sample was collected manually in knee deep water at least 12 inches below the surface using sterile 100- mL Whirl- Pak sampling bags. The samples were kept over ice in a cooler to be transported to the lab. Ten Whirl- Pak bags were collected during each sampling event to provide sufficient water for preparation of the experimental treatments, allowing for excess seawater.

During the initial trials, logistical constraints required samples to be collected during the afternoon, generally between approximately 3:00 PM and 4:00 PM. Following repeated observations of low *Enterococcus* concentrations throughout these trials, the collection protocol was altered and standardized to 7:00 AM during later trials. This modification in collection time reduced the variability of collection time and significantly limited the period of exposure to UV rays experienced by the bacterial population prior to sampling.

2.3 Salinity Treatments

Five salinity treatments were prepared to represent progressive freshwater infiltration: 35, 25, 15, 5, 0 PSU. The 35 PSU treatment consisted of 100mL of collected seawater without any freshwater dilution and served as a reference for un-infiltrated seawater.

The salinity treatments were prepared using the following solutions:

- 35 PSU: 100.0 mL seawater and 0.0 mL distilled water
- 25 PSU: 71.4 mL seawater and 28.6 mL distilled water
- 15 PSU: 42.9 mL seawater and 57.1 mL distilled water
- 5 PSU: 14.3 mL seawater and 85.7 mL distilled water
- 0 PSU: 0.0 mL seawater and 100.0 mL distilled water

Collected seawater was transferred using a glass pipette into a sterile mixing bottle, after which distilled water was added to a final treatment volume of 100 mL. Treatment salinity was verified using a salinity refractometer prior to bacterial quantification.

2.4 Experimental Procedure

After preparing and verifying the salinity treatments, each treatment was assigned to replicates A, B, and C.

Enterococcus was enumerated in each replicate following a 1:10 dilution using 10 mL of the corresponding salinity treatment and 90 mL of distilled water. 1 packet of IDEXX Enterolert reagent was added to each diluted sample. The samples were then transferred to labeled IDEXX Quanti-Tray/2000 trays for processing and incubation.

To reduce potential temperature variation within the incubator chamber, each sample tray's location was randomized by using a random number generator to assign each tray a number 1-4. These numbers represented different corners within the chamber. The sample trays were stacked with the wells facing up and alternating based on large or small wells facing the incubator door. The sample trays were incubated at 41 degrees Celsius for exactly 24 hours, read, and then incubated for another 24 hours. *Enterococcus* concentration was quantified according to the Enterolert/ Quanti Tray procedures. A UVA lamp was utilized to enumerate the number of positive wells in each tray. This number was converted using the IDEXX Quanti-Tray/2000 MPN Table to produce the final MPN/100 mL of *Enterococcus* present in each sample tray.

To clarify, the 1:10 dilution in each sample was for the purpose of *Enterococcus* enumeration, rather than salinity manipulation. Salinity conditions were established in the samples prior to 10 mL being extracted for bacterial quantification.

2.5 Experimental Deviations

“During [trial], [issue] was observed. The experimental procedure was subsequently modified by [change] to reduce the likelihood of recurrence

Several methodological issues were identified during the initial experiment trials and were documented rather than used as grounds for removing the resulting observations from the study.

During earlier trials, sample collection occurred primarily in the afternoon, between 2:00 PM and 3:00 PM, due to logistical constraints associated with the researcher’s schedule. These samples repeatedly produced very low concentrations of *Enterococcus*. Exposure to the sun is a recognized environmental factor that affects the persistence of *Enterococcus*. Therefore, collection time was identified as a potential contributor to the variability observed between sampling events. Due to the fact that solar irradiance was not directly measured during this study; sun exposure cannot be established as the sole source of low *Enterococcus* concentrations observed in earlier trials.

The second methodological concern involved the homogenization of the prepared salinity treatments before subsampling. During the earlier experimental procedures, treatments were not shaken/ mixed exactly prior to extracting 10 mL for bacterial enumeration. Seawater is denser than distilled water and was likely remaining stagnant at the bottom of the mixing bottle, meaning that the 10 mL of the treatments being drawn could have potentially not been entirely representative of the *Enterococcus* colonies present in each treatment.

These observations prompted modification and standardization of the original experimental procedure. The data recorded during earlier trials were retained in the complete experimental record rather than being excluded on the basis of low bacterial concentrations.

2.6 Methodological Optimization

After analyzing the initial trials, two methodological changes were made to the experimental procedure in order to optimize consistency throughout the sampling events.

First, water collection time was shifted from afternoon sampling to approximately 7:00 AM. Standardizing the collection time to the early morning helped reduce variability among samples and reduce the seawater’s prolonged solar exposure.

Second, each salinity treatment was thoroughly mixed immediately before removing the 10 mL of solution used for bacterial enumeration. This modification was implemented to improve homogenization and reduce the possibility of producing nonrepresentative concentrations of *Enterococcus*.

All other experimental conditions remained the same according to the original experimental protocol.

2.7 Bacterial Quantification

Enterococcus concentrations were measured using the IDEXX Enterolert/Quanti-Tray/2000 method implemented by the Surfrider Foundation Blue Water Task Force.

Following incubation, with a UVA lamp, wells that lit up to a pronounced neon blue (in opposition to the muted yellow, which means that no bacteria are present in that well) were enumerated as positive wells – as either large or small wells. The combination of positive wells was converted to Most Probable Number using the IDEXX Quanti- Tray/ 2000 MPN reference table. Final bacterial concentrations were recorded as MPN of *Enterococcus* per 100 mL.

The MPN approach enumerates culturable *Enterococcus* rather than total bacterial cells. Therefore, values under detection should be interpreted as a lack of detection under analytical conditions rather than definitive evidence of a complete absence of bacteria.

2.8 Experimental Time Points

Bacterial concentrations were recorded at exactly 24 hours and 48 hours. The MPN calculations from each of these time points were used to detect changes in *Enterococcus* concentrations across 5 salinity treatments and determine whether there was a positive relationship between salinity and bacterial concentration over time.

All salinity treatments remained the same throughout all experimental trials to establish a viable comparison between 24- and 28-hour results.

2.9 Replication

Each of the 5 salinity treatments (35, 25, 15, 5, 0 PSU) was represented with 3 within-treatment replicates: A, B, and C. Each trial contained a total of 15 salinity treatment replicates.

Eight sampling trials were conducted during the study. Each replicate was treated as a replicate observation. This distinction served to **avoid** treating multiple measurements originating from the same sampling event as fully independent sampling events.

2.10 Quality Control

Several procedures were implemented to ensure consistency and mitigate experimental variability. First off, sterile Whirl-pak 100 mL bags were used to collect the seawater samples, and sterile mixing bottles were used to create salinity treatments. We verified each salinity treatment using a salinity refractometer rather than relying solely on calculated salt-to-distilled-water ratios.

The volume used to calculate the bacterial concentration was standardized to 10 mL of treatment sample diluted with 90 mL of distilled water. Incubation temperature remained constant at 41 degrees Celsius, and Quanti-Trays were placed in randomized positions within the incubator to reduce potential spatial effects within the incubator chamber.

Following methodological optimization, seawater samples were collected at a standardized early morning time, and salinity solutions were mixed promptly before subsampling. Raw observations from all experimental trials, including zero to low concentrations, were retained to preserve the transparency of experimental variability.

2.11 Statistical Analysis

Raw *Enterococcus* concentrations were recorded as MPN/ 100 mL for each replicate, salinity treatment, sample trial, and record points at 24 and 48 hours.

Replicates A, B, and C originated from the same sampling event and were therefore not treated as independent sampling events. Results were evaluated at both the individual trial level and across all independent trials and averaged to produce a one-trial-level value for each salinity condition. This prevented replicates from being interpreted as independent sampling events.

Mean, median, standard deviation, and interquartile range were calculated across the eight trials for each salinity condition. Because the data set contained numerous values of zero, substantial differences in bacterial concentrations across sampling events, and a strong right-skewed distribution, non-parametric repeated measures tests were selected rather than standard parametric ANOVA (Analysis of Variance). The non-parametric test was essentially used because the experimental data did not meet the homogeneity assumptions of traditional analysis of variance.

A Friedman test was used to determine whether *Enterococcus* differed overall among the five salinity treatments while accounting for repeated measurements within the sampling trials. Since the hypothesis predicted a comprehensive decrease in *Enterococcus* concentration with decreasing salinity, a Page trend test was additionally used to test for a trend associated with salinity. Wilcoxon signed-rank tests paired with Holm correction for multiple comparisons were used for post hoc comparisons (a follow-up test to see which specified groups differ). Statistical significance was evaluated at $\alpha = 0.05$.

The 24-hour Enterolert measurement was treated as the primary analytical endpoint because the validated IDEXX and Surfrider protocols specify incubation and bacterial readings after 24 hours at 41 degrees Celsius. The recorded 48-hour data were retained as exploratory observations and were analyzed separately but with correspondence to 24-hour data. They are not to be interpreted as a validated measurement for bacterial presence over an additional 24 hours.

3. Results

3.1 Overview of Findings

Enterococcus concentrations varied significantly among the 8 independent trials. Early trials generally produced low or undetectable concentrations, whereas trials 3, 7, and 8 contained

substantially greater detectable bacterial concentrations across multiple salinity treatments. Trial eight contained the record-high concentrations in the study, including replicate values of 1,401, 1,017, and 990 MPN/100 mL at 35 PSU after 24-hour incubation.

Despite the irrefutable variation in absolute bacterial concentrations among sampling dates, the higher detection trials generally exhibited a pattern in which treatments with higher salinity contained greater measured *Enterococcus* concentrations across 35- 5 PSU, while all 0 PSU replicates were zero, signifying absolutely no cross-contamination and a representation of complete freshwater infiltration.

3.2 Raw Data

The complete data set consists of eight sampling trials, five salinity treatments per trial, three replicates per treatment, and two recorder incubation readings per trial. This produced 120 replicate-level observations at each reading time across the full study.

All recorded observations, including zero to low concentration trials, were left in the dataset. No sampling trial was excluded because it failed to follow the predicted outcome or because bacterial concentrations were low. The complete replicate-level dataset is provided in Supplementary Table S1.

3.3 Descriptive Statistics

Descriptive statistics were calculated from trial-level treatment means after averaging the A, B, and C replicates within each trial.

Salinity	24 hr. Mean $\pm$ SD	24 hr. median	48 hr. Mean $\pm$ SD	48 hr. median
35 PSU	168.21 $\pm$ 392.71	10.00	237.12 $\pm$ 545.92	20.00
25 PSU	113.96 $\pm$ 271.73	3.33	137.83 $\pm$ 323.51	3.33
15 PSU	72.04 $\pm$ 183.41	0.00	83.25 $\pm$ 202.84	1.67
5 PSU	27.92 $\pm$ 63.31	0.00	39.50 $\pm$ 85.47	0.00
0 PSU	0.00 $\pm$ 0.00	0.00	0.00 $\pm$ 0.00	0.00

At the initial 24- hour endpoint, mean concentrations decreased in order from 168.21 MPN/100 mL at 35 PSU to 113.96 MPN/100 mL at 25 PSU, 72.04 MPN/100 mL at 15 PSU, 27.92 MPN/100 mL at 5 PSU, and 0 MPN/100 mL AT 0 PSU.

Standard deviations were largely relative to corresponding means, establishing a clear variation among independent sampling dates. The difference between means and medians further reveals the right-skewed nature of the data, more specifically due to the much greater bacterial concentrations observed during the eighth trial.

Descriptive statistics were calculated using trial-level treatment means after averaging replicates A, B, and C within each trial. Mean, standard deviation, median, and interquartile range were calculated for each salinity treatment at 24 and 48 hours.

3.4 Figures

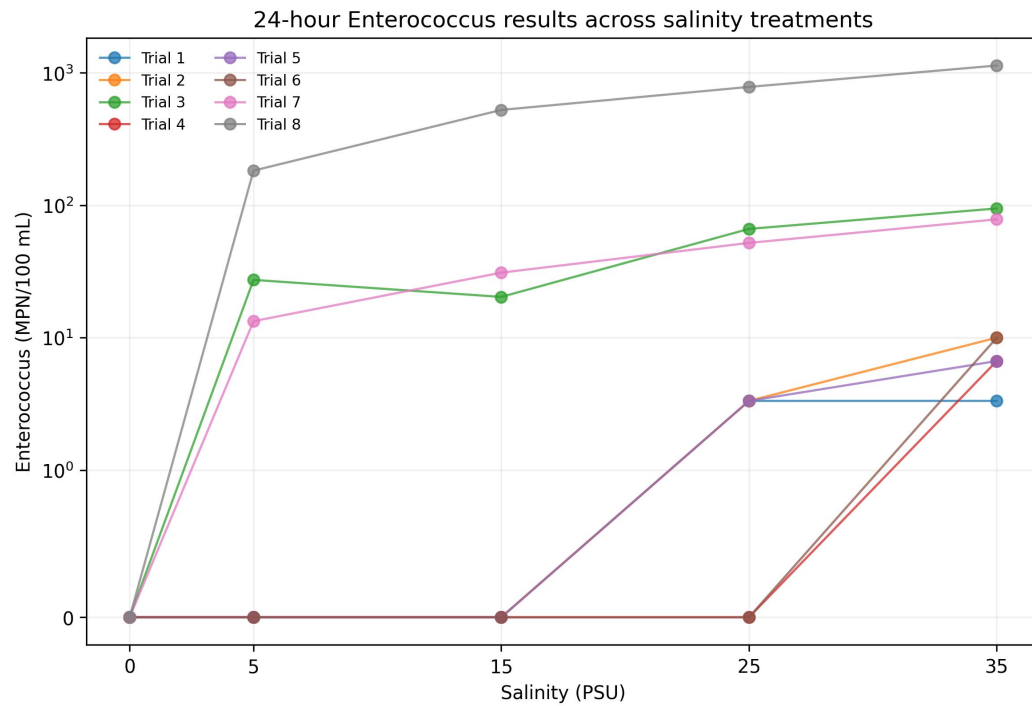


Fig. 1

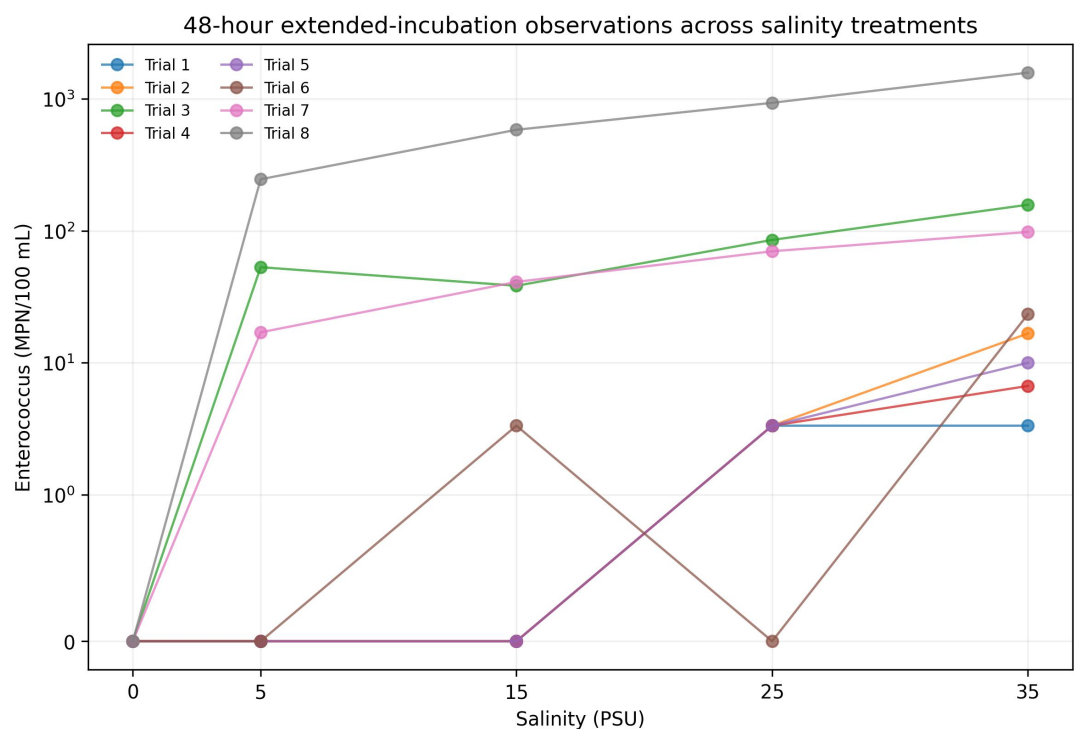


Fig. 2

Salinity- PSU	Time-h	Mean-MPN	SD	Median-MPN	Q1	Q3
0	24	0	0	0	0	0
0	48	0	0	0	0	0
5	24	27.92	63.31	0	0	16.83
5	48	39.5	85.47	0	0	26
15	24	72.04	183.41	0	0	23
15	48	83.25	202.84	1.67	0	39
25	24	113.96	271.73	3.33	2.5	55.58
25	48	137.83	323.51	3.33	3.33	73.83
35	24	168.21	392.71	10	6.67	82.42
35	48	237.12	545.92	20	9.17	112.92

Table 1.

- At 24 hours, mean *Enterococcus* concentrations decreased from 68.21 MPN/100 mL at 35 PSU to 0 MPN/100 mL at 0 PSU. The large standard deviations, particularly at higher salinities, indicate substantial variations in *Enterococcus* concentrations throughout trials.

Figure 1. *Enterococcus* concentrations across salinity treatments at 24 hours.

Overall, bacterial concentrations were highest at higher salinity levels and decreased as salinity decreased. Variation was observed among trials; trials 3, 7, and 8 showed the most pronounced variation.

Figure 2. *Enterococcus* concentrations across salinity treatments at 48 hours. Similar to Figure 1, higher concentrations generally occurred at higher salinities. The 48-hour measurement was considered exploratory because it is beyond the standard 24-hour Enterolert protocol reading period.

Together, both figures present an overall relationship between decreasing salinity/ freshwater dilution and decreasing *Enterococcus* concentrations, despite considerable differences in the amount of bacteria within sampling trials.

Table 1. Descriptive statistics for *Enterococcus* concentrations (MPN/ 100 mL) across salinity treatments at 24 and 48 hours.

3.5 Statistical Results

A Friedman test showed a significant overall difference in *Enterococcus* concentrations among the five salinity treatments at 24 hours ($X^2(4) = 27.18, p < 0.001$). A Page trend test also showed a significant pattern of higher *Enterococcus* concentrations at higher salinities ($L = 422.5, p < 0.001$).

The exploratory 48-hour results showed a similar pattern (Friedman $X^2(4) = 26.96, p < 0.001$).

4. Discussion

4.1 Interpretation of Findings

In summary, *Enterococcus* concentrations decreased as salinity decreased. This pattern was most prominent in Trials 3, 7, and 8, where sufficient bacteria were detected to clearly compare the salinity treatments.

4.2 Possible Mechanisms

Previous research has proven that *Enterococcus* survival in marine environments can be influenced by multiple environmental factors, including sunlight, salinity, temperature, water conditions, and relative precipitation. These studies support the idea that changes in coastal water conditions may affect the amount of *Enterococcus* detected in the water.

The present study showed a clear relationship between decreasing salinity and decreasing *Enterococcus* concentrations.

4.3 Environmental Significance

There are a multitude of factors that could explain the observed results. Distilled water's addition directly diluted the original seawater and therefore reduced the initial quantity of bacteria introduced into lower salinity treatments.

4.4 Public- Health Relevance

Enterococcus is a commonly used indicator of fecal contamination because it is found in the gastrointestinal tracts of animals and humans. High concentrations can indicate increased fecal contamination and associated pathogens in recreational waters.

Increasing public awareness of *Enterococcus* and the conditions that affect it can significantly improve coastal community safety and prevent disease. Understanding how environmental conditions influence the levels of these bacteria can therefore improve public interpretation of recreational water quality, particularly after freshwater infiltration.

Understanding how environmental conditions influence *Enterococcus* concentrations can

4.5 Limitations

Initial *Enterococcus* concentrations varied substantially between trials. Several early trials had zero to extremely low bacterial concentrations.

Collection time changed during the experiment from approximately 2:00 –3:00 PM to 7:00 AM. Although this change in experimental protocol standardized later trials and reduced potential exposure to UV// sun exposure before collection, it also created methodological differences between early and later trials.

Finally, the 48-hour readings exceeded the intended incubation period standard to the IDEXX Quanti-Tray/2000 procedure. That being said, the 48-hour results can only be validated as exploratory.

4.6 Sources of Experimental Error

Possible sources of experimental error include differences in initial bacterial concentrations between different trials, variation in collection time, and the stagnation of salinity treatments (no mixing before extraction) during early trials.

To improve consistency, later samples were collected at the same time in the early morning, and treatment bottles were vigorously mixed before extracting 10 mL for processing as replicates. Differences in coastal water conditions between sampling events could add another source of variation.

4.7 Future Research

Future research should keep the initial bacterial concentration equal across all salinity treatments. Potentially, the same number of bacteria could be added to prepared solutions of 25, 25, 15, 5,

and 0 PSU. This would allow salinity to be diluted without having the variable of initial bacterial concentration.

Future experiments should use a consistent collection time and record environmental conditions for each individual sampling event such as sunlight, UV, temperature, rainfall, and tides. Additional independent trials would strengthen statistical analysis.

5. Conclusion

To conclude, across eight independent trials, decreasing salinity was associated with progressively lower numerated concentrations of culturable *Enterococcus*. At the standard 24-hour Enterolert recording, trial-level mean concentrations declined from 169.21 MPN/ 100 mL at 25 PSU to 0 MPN/ 100 mL at 0 PSU. Statistical analysis demonstrated both an overall difference among treatments and a highly significant trend across the salinity treatments.

The study establishes a reproducible association between progressive freshwater dilution/ lower salinity and measured *Enterococcus* concentration, while identifying a significant experimental confound that should be controlled in future work.

The variation in sampling dates additionally demonstrates the importance of environmental and methodological factors in studies using naturally occurring fecal indicator bacteria. Standardizing early-morning collection and homogenizing samples improved consistency in the later experimental procedure. Overall, the study provides introductory evidence and a methodological foundation for more controlled investigation of how rapid freshwater infiltration influences *Enterococcus* in coastal environments.

6. Data Availability

All data collected during the study were retained, including all zero- to low-concentration results. The complete dataset is provided in the Results under Descriptive Statistics and Figures.

7. Acknowledgments

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- OpenAI's ChatGPT was used to assist with statistical analysis and the generation of figures from the author's original experimental dataset. All experimental data were collected by the author, and the resulting analyses and figures were reviewed by the author prior to inclusion in this paper.

10. Supplementary Materials

Supplementary Materials include: the complete dataset from all eight trials, including salinity treatment, replicate 24-hour MPN, and exploratory 48-hour MPN concentrations.