Effect of Sodium Pyruvate Supplement on Concentrations of Seawater-Injured *Escherichia coli* Using the Aerobic Plate Count Method

Julie Keller

Abstract The U.S. Environmental Protection Agency specifies that one of the elements of a state water monitoring and assessment program is the development of a monitoring design that will efficiently and effectively generate data to serve management needs. Part of this directive is to ensure regular monitoring of recreational waters so that they may remain free from sewage contamination. The fecal coliform, *Escherichia coli*, is commonly used as an indicator of fecal contamination in fresh and marine waters. Yet upon exposure to stresses such as high salinity, *E. coli* enters a "viable but non-culturable" state, in which the coliform is able to infect animal hosts but cannot be detected by standard methods. The addition of sodium pyruvate to solid medium has been found to increase recovery of *E. coli* stressed by artificial seawater. This study investigates whether the addition of sodium pyruvate to the E. coli-specific solid medium, MacConkey agar, will significantly increase recovery of *E. coli* that have been stressed by sterilized natural seawater. A longitudinal study was performed and testing conducted after each 24 hour period of seawater exposure. The Aerobic Plate Count method was used to determine numbers of *E. coli* colony forming units per milliliter on the solid MacConkey agar plates. Using the paired t-test, results showed a significant increase in *E. coli* colony forming units per milliliter of seawater-stressed *E. coli* on MacConkey agar supplemented with sodium pyruvate, compared to unsupplemented MacConkey agar. The results of this study are helpful in recognizing the link between the effectiveness of a water quality method and ensuring adequate detection of fecal coliforms in preventing possible illness.

Introduction

Sources of fecal contamination in marine waters include wastewater from sewage treatment plants, sewer outfalls, drainage from septic tanks, runoff from agricultural fields and feedlots, effluents from food processing plants and storm water runoff carrying animal droppings (Darakas 2002). The survival rate of fecal bacteria is dependent on environmental stresses such as temperature, solar radiation, dilution, osmotic pressure, presence of toxins, and salinity (Darakas 2002). About 40% of the wastewater in the United States is dumped into saltwater outfalls (Chang 2003, pers. comm.). An accurate and dependable method to estimate the number of living bacteria in the environment is necessary and the issue is an important public health concern (Braux *et al.* 1997). The U.S. Environmental Protection Agency specifies that one of the elements of a state water monitoring and assessment program is the development of a monitoring design that will efficiently and effectively generate data to serve management needs (USEPA 2003). Part of this directive is to ensure regular monitoring of recreational waters so that they may remain free from sewage contamination.

Coliform bacteria are commonly used as indicators of fecal contamination in fresh and marine waters (Pisciotta *et al.* 2001, Calabrese and Bissonnette 1990b). The fecal coliform, *Escherichia coli,* is considered the most reliable indicator since it lives inside the human gastrointestinal tract and the presence of this bacteria in water is most directly associated with fecal contamination (Geissler *et al.* 1999).

A problem of concern is the ability of *E. coli* to enter a "viable but non-culturable" (VNC) state in marine waters (Davies *et al.* 1995). These VNC *E. coli* bacteria are metabolically active and capable of infecting animal hosts, but cannot be cultured in laboratory media. Exposure of *E. coli* to environmental stresses such as high salinity results in sublethal injury of some surviving bacteria, which creates difficulties in quantifying bacteria on selective media suitable only for normal non-stressed cells (Calabrese and Bissonnette 1990a). The stress on the injured bacteria caused by saltwater reduces detection of the organism using classical plate count methods (Braux *et al.* 1997), and bacteria may escape detection by standard tests such as Colitag, Colilert, and Multiple Tube Fermentation.

Difficulties may arise when evaluating the quality of marine water using *E. coli* indicators due to the presence of these saltwater-injured bacteria. Recovery procedures for coliform detection are most often based on aerobic incubation, which requires the cell's respiratory system

to function properly (Calabrese and Bissonnette 1990a). If these cells are not detected, test results may yield an overly optimistic view of the safety of water, when pathogens may still exist (Calabrese and Bissonnette 1990a). If some of these cells retain their infective potential, they may pose a serious public health risk to people in contact with contaminated water (McFeters 1986, Braux *et al.* 1997). Chang *et al.* developed a new methodology for resuscitating seawater-injured but metabolically-active *E. coli* bacteria (1993). They found that adding sodium pyruvate to *E. coli*-infected artificial seawater yielded greatly improved recovery of *E. coli* on freshly made plates of solid Nutrient agar medium (Chang *et al. 1993)*. Others have found that addition of sodium pyruvate to laboratory media resulted in the recovery of stressed bacteria by way of degrading toxic H_2O_2 that builds in bacteria cells (Martin *et al.* 1976).

This study investigates whether the addition of sodium pyruvate yields higher recoverability of *E. coli* stressed by natural seawater when tested on the *E. coli*-specific and selective medium, MacConkey Agar. Using a source of natural seawater provides insight into actual environmental processes, as opposed to processes observed when using artificial seawater. Though MacConkey Agar counts alone may be a particularly rough method for determining water quality, the medium plays an instrumental role in the laboratory in culturing *E. coli*. Examining the parameters of sodium pyruvate as a supplemental ingredient for standard water quality methods is necessary for determining its overall potential in increasing *E. coli* yield and reducing the threat of false negative results in natural waters.

Methods

Data Collection This experiment was conducted from March 21-May 5, 2003 for a total of six trials. *Escherichia coli* ATCC 25922, from the American Type Culture Collection, was maintained at 4° C in nutrient broth (Difco, Sparks, MD), a medium containing peptone and beef extract used for cultivating non-fastidious microorganisms (Difco Laboratories 1998). The strain was subcultured into a 16 mm test tube containing 9 ml fresh nutrient broth, then incubated at 35°C for 18-24 hours. After incubation, 0.1 ml of *E. coli* in nutrient broth from test tube was then inoculated into a 250 ml sterile glass bottle of 99.9 ml of natural seawater to make a 10^{-3} dilution. The bottle of seawater inoculated with *E. coli* was tested immediately after inoculation at Day 0 exposure to seawater, then incubated under conditions described above for significant stress and injury of bacteria. Samples from the 10^{-3} dilution were tested after 24 hours (Day 1)

and 48 hours (Day 2) exposure to seawater. Seawater was collected at Bodega Bay, California, by the Office of Live Animal Care at the University of California, Berkeley, then filter-sterilized in the laboratory using a 0.22-μm membrane filter.

Testing of inoculated seawater for *E. coli* counts was performed by serially diluting the inoculated seawater at Day 0, Day 1, and Day 2 using Butterfield's Buffer, a phosphate-buffered dilution solution. Serial dilutions were prepared by aseptically pipetting 0.5 ml of the 10^{-3} dilution into a 16 mm test tube of 4.5 ml Butterfield's Buffer to make a 10^{-4} dilution. Then, 0.5 ml of this dilution was added to a 16 mm test tube of 4.5 ml Butterfield's Buffer to make a 10^{-5} dilution and so on, until a 10^{-7} dilution was achieved. Using a sterile spreader, 100 μl from each dilution, 10^{-3} to 10^{-7}, was spread onto freshly made plates of MacConkey Agar media and an identical volume was spread onto freshly made plates of MacConkey Agar media supplemented with 0.1% sodium pyruvate. MacConkey Agar was prepared according to manufacture instructions (Mikrobiologie, Germany). In preparing experimental plates of MacConkey, 0.5 g sodium pyruvate (Sigma Chemical Co., Denver, CO) was added to 500 ml MacConkey Agar prior to autoclaving for a 0.1% supplement.

Plates were incubated at 35°C for 18-24 hours. Colonies were then enumerated using the Aerobic Plate Count method, the major application of the colony count method (Swanson *et al.* 1992). For each day of exposure to seawater, an experimental value and a control value of *E. coli* Colony Forming Units (CFU) per ml was calculated for the original 10^{-3} solution of inoculated seawater through enumeration of plates with quantifiably confident numbers of bacteria (30-300 colonies).

Statistical Techniques Colony forming units per ml for the six trials were converted into Log base 10 values. These values were compared between supplemented and unsupplemented plates using a paired t-test on Days 0,1, and 2 for a total of three tests.

Results

The P-value for the first t-test for Day 0 was 0.895, indicating that there was no significant difference in mean CFU per ml of *E. coli* bacteria recovered from the bottle of inoculated seawater at the beginning of the experiment using unsupplemented MacConkey Agar and MacConkey Agar supplemented with sodium pyruvate (Table 1). The t-test analysis from Day 1, however, resulted in a P-value of 0.011, a value less than 0.05, indicating that after 24 hours of

exposure to seawater, MacConkey Agar supplemented with sodium pyruvate yielded significantly higher counts of CFU per ml of *E. coli* than unsupplemented MacConkey Agar. The P-value from Day 2 was 0.136, showing no significant difference between values of CFU per ml of *E. coli* exposed to seawater after 48 hours on supplemented and unsupplemented agar (Table 1). Overall, results showed that MacConkey Agar supplemented with sodium pyruvate yielded higher numbers of *E. coli* counts than did plates of unsupplemented MacConkey for Day 1. However, the P-values greater than 0.05 resulted in statistically insignificant differences between supplemented and unsupplemented agar for Day 0 and Day 2 exposure (Fig. 1).

Day	Mean CFU/ml MacConkey	Mean CFU/ml MacConkey Plus Sodium Pyruvate	% Percentage Difference	P-value
0	4.68×10^5	4.65×10^5	0.64	0.895
1	1.28×10^3	2.14×10^3	40.2	0.011
2	6.29×10^2	6.49×10^2	3.08	0.136

Table 1. Mean values from 6 trials of colony forming units (CFU) per ml of *E. coli* for unsupplemented MacConkey Agar and MacConkey Agar supplemented with sodium pyruvate for the 10^{-3} dilution of *E. coli* seawater. P-value from paired two-tailed t-test, $P \leq 0.05$ is statistically significant.

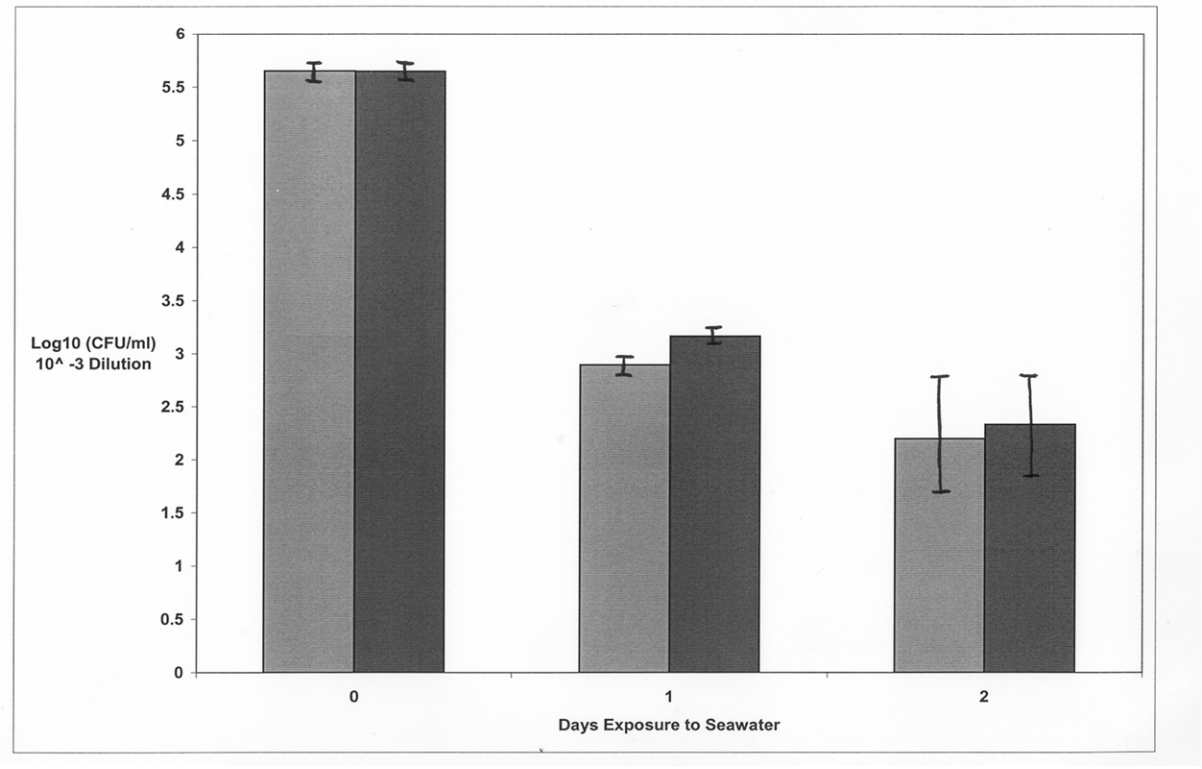

Figure 1. Columns represent log 10-transformed data from six trials for *E. coli* colony forming units (CFU) per ml for unsupplemented and supplemented MacConkey Agar over days of *E. coli* exposure to seawater. Light columns= unsupplemented MacConkey, Dark columns= MacConkey supplemented with sodium pyruvate.

Discussion

Results from this study show that after six trials, MacConkey agar supplemented with sodium pyruvate yielded significantly higher recovery values of *E. coli* colony forming units per ml than did unsupplemented MacConkey after exposure to seawater for Day 1 (P-value= 0.011). Yet, Day 0 and Day 2 of seawater exposure showed no significant differences between the two methods. The P-value of 0.895 for Day 0 is consistent with our expectations since very little injury of *E. coli* was anticipated in the short time between adding the *E. coli* and enumerating the results the same day. However, the P-value of 0.136 for Day 2 indicates no significant difference in recoverability of *E. coli.*

The insignificant results from Day 2 indicate that after 48 hours of exposure to sterilized natural seawater, there was not a significant increase in *E. coli* CFU/ml using MacConkey Agar supplemented with sodium pyruvate, compared to unsupplemented agar. This may be due to biofilm development on the sides of the bottle containing the 10^{-3} dilution, referred to as the "bottle effect." Bacteria can adhere to surfaces and form a slippery coat of biofilm, allowing survival in a stressed environment (Costerton *et al.* 1999). Halfway through the experiment, I began vigorously shaking the bottle prior to sampling, and results from these specific trials yielded marked differences between CFU/ml of *E. coli* when comparing supplemented and unsupplemented MacConkey Agar.

Errors in enumeration must be considered when analyzing results of this study. With many colonies on one plate, it is difficult to obtain a pure count since there may be colony overlap that may not be visible, which may result in an underestimation of bacteria colonies. Since this factor is complicated to account for, the assumption that all colonies counted in the study resulted in equal underestimation may be reasonable, and in this case, the significant differences found above may still hold true.

Another consideration is the strength of sodium pyruvate. Halfway through the study, a new bottle of sodium pyruvate was purchased from Sigma Chemical Company. This may have had an effect on data collected, resulting in lack of uniformity in data collected for experimental supplemented plates. However, since no data values appeared unusual, it is difficult to say what effect this could have had on results obtained.

Significant data in this study show that sodium pyruvate acts to enrich even a very selective and *E. coli*-specific media such as MacConkey agar. Chang *et al.* established the positive effect

of sodium pyruvate on seawater-injured *E. coli* when it was added as a supplement to nutrient agar, a solid medium used for cultivating a wide variety of microorganisms and containing peptone and beef extract (1993). Yet the effect sodium pyruvate might have on other types of media remained unclear. The possible applications of the supplement sodium pyruvate to future studies involving water quality and VNC bacteria cells remains promising. A better evaluation of the role of sodium pyruvate in the recovery of seawater-injured bacteria would incorporate an advanced method such as the rapid and accurate Colitag™ method, which is in the process of approval by the United States Environmental Protection Agency (Chang and Hsieh 2002).

Presence of VNC bacteria in recreational waters is a threat to public health if current water quality detection methods are limited to identifying only non-stressed normal bacteria cells. As water quality methods sharpen, specificity and selectivity measures improve the accuracy with which indicator bacteria such as *E. coli* are positively identified. Yet as these methods improve, they may be simultaneously excluding certain flawed and injured bacteria from proper identification. With further investigations into supplements like sodium pyruvate, which may act to include VNC bacteria in enumeration methods, the risk of negative health effects in response to sewage outfall may be decreased with new and innovative water quality detection measures.

Acknowledgements

I would like to thank George Chang from the Nutritional Sciences Department at UC Berkeley, Abby Jang, Cam Yuen, Catherine Fan, Lisa Wu and Enrica Fung for their constant support, advice and use of Chang Lab materials and space. Thank you to Matt Orr, John Latto, and Manish Desai for their very helpful suggestions and time.

References

Braux, A.S., J. Minet, Z. Tamani-Shacoori, G. Rious, and M. Cormier. 1997. Direct enumeration of injured *Escherichia coli* cells harvested onto membrane filters. Journal of Microbiological Methods. 31:1-8.

Calabrese, J.P. and G.K. Bissonnette. 1990a. Improved detection of acid mine water stressed coliform bacteria on media containing catalase and sodium pyruvate. Canadian Journal of Microbiology. 36:544-550.

Calabrese, J.P. and G.K. Bissonnette. 1990b. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. Applied and Environmental Microbiology. 56:3558-3564

Chang, G.W. 2003. Nutritional Science Professor. University of California, Berkeley, personal communication.

Chang, G.W., J.K. Fujii and R.A. Lum. 1993. Coliforms in ocean waters: new methodology for resuscitating seawater-injured *E. coli*. 66th Annual Conference of the Water Environment Federation, Anaheim, California.

Chang, G.W. and J.J. Hsieh. 2002. A comparison of the Colitag™ method and the standard method for the detection of fecal coliforms and *Escherichia coli* in urban creeks. Water Environment Federation 2002 Conference.

Costerton, J.W., P.S. Stewart, and E.P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. Microbes, Immunity, and Disease. 284:1318-1322.

Darakas, E. 2002. *E. coli* kinetics: effect of temperature on the maintenance and respectively the decay phase. Environmental Monitoring and Assessment. 78:101-110.

Davies, C.M., S.C. Apte, and S.M. Peterson. 1995. ß-D-glucuronidase activity of viable, non-culturable coliform bacteria in marine waters. Letters in Applied Microbiology. 21:99-102.

Difco Laboratories. 1998. Difco Manual. Becton Dickinson and Company, Maryland.

Geissler, K., M. Manafi, I. Amoros and J.L. Alonso. 2000. Quantitative determination of total coliforms and *Escherichia coli* in marine waters with chromogenic and fluorogenic media. Journal of Applied Microbiology. 88:280-285.

Martin, S.E., Flowers, R.S. and Ordal, Z.J. 1976. Catalase: its effect on microbial enumeration. Applied Environmental Microbiology. 32:731-734.

McFeters, G.A., J.S. Kippin, and M.W. LeChevallier. 1986. Injured coliforms in drinking water. Applied Environmental Microbiology. 51:1-5.

Pisciotta, J.M., D.F. Rath, P.A. Stanek, D.M. Flanery, and V.J. Harwood. 2002. Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for *Escherichia coli* in Florida waters. Applied and Environmental Microbiology. 68:539-544.

Swanson, K.M., F.F. Busta, E.H. Peterson, and M.G. Johnson. 1992. Colony Count Methods. Pp. 75-92 *In* Compendium of methods for the microbiological examination of foods. C. Vanderzant and D.F. Splittstoesser, ed. American Public Health Association, Washington D.C.

United States Environmental Protection Agency. 2003. Elements of a State Water Monitoring and Assessment Program. Publication EPA-841-B-03-003, Washington D.C.