PANAMJAS
Pan-American Journal of Aquatic Sciences

Enterobacterial growth in coastal groundwater wells of Cabo Polonio (Uruguay): an experimental approach

MARTINA SOUMASTRE[1,2], LORENA RODRÍGUEZ-GALLEGO[2,3] & CLAUDIA PICCINI[1,2*]

[1]*Departamento de Microbiología. Instituto de Investigaciones Biológicas Clemente Estable (IIBCE). Avenida Italia 3318. Montevideo 11600, Uruguay. Phone: ++59824871616.*
[2]*Grupo de Ecología Funcional de Sistemas Acuáticos. Comisión Sectorial de Investigación Científica (CSIC). Universidad de la República, Uruguay.*
[3]*Centro Universitario Regional Este (CURE), Rocha. Universidad de la República, Uruguay.*

[*]*Corresponding author: cpiccini@iibce.edu.uy*

Abstract: Groundwater access through wells is an important water source for human use along the coastline in Uruguay. We selected a coastal site, Cabo Polonio National Park (CP), as experimental area since it is characterized by high visitors loading during summer, sandy soil and absence of space planning that results in the construction of septic tanks nearby groundwater wells. In this study, we experimentally assessed the short-time dynamics of inoculated enterobacteria (*Escherichia coli*) in groundwater-containing microcosms, which were periodically inoculated with fresh pre-filtered groundwater through 8 h incubation at *in situ* temperature. We found that *E. coli* growth, as assessed by *in situ* hybridization using a probe for enterobacteria, was significantly stimulated concomitant to organic matter decrease. Our results suggest that concentration of organic matter in groundwater would be enough to support bacterial growth implying that water quality protection in CP is a major challenge, and encourage further studies comprising the whole area. Owing to similarities in water management practices along the Atlantic coast in Uruguay, these findings could help to design new strategies to protect water quality.

Keywords: Enterobacteria, groundwater, *Escherichia coli*, Cabo Polonio National Park

Resumen: Crecimiento de enterobacterias en agua subterránea del Cabo Polonio (Uruguay): una aproximación experimental. El acceso al agua subterránea a través de perforaciones es una importante fuente de agua para consumo humano en la zona costera de Uruguay. En este estudio seleccionamos al Parque Nacional Cabo Polonio (CP) como área experimental, ya que se caracteriza por una alta carga de visitantes durante el verano, terreno mayormente arenoso y ausencia de planeamiento territorial, lo que resulta en la construcción de pozos de agua demasiado cercanos a los pozos sépticos. Se analizó experimentalmente la dinámica a corto plazo de enterobacterias (*Escerichia coli*) inoculadas en microcosmos conteniendo agua de pozo, que fueron periódicamente alimentadas con agua subterránea durante 8 horas de incubación a la temperatura in situ. Se encontró que el crecimiento de *E. coli*, evaluado mediante hibridación *in situ* usando una sonda para enterobacterias se estimuló significativa y concomitantemente a una disminución en la concentración de materia orgánica. Nuestros resultados sugieren que el agua subterránea del sitio de estudio contiene una concentración de materia orgánica suficiente para sostener el crecimiento bacteriano. Estos hallazgos revelan la fragilidad de la calidad del agua en el CP y estimulan a realizar estudios que

comprendan toda el área. Debido a las similitudes de esta zona con el resto de la costa atlántica en Uruguay, los resultados obtenidos podrían contribuir al diseño de estrategias de manejo del agua para consumo humano.

Palabras clave: Enterobacteria, agua subterránea, *Escherichia coli*, Parque Nacional Cabo Polonio

Introduction

The coastal strip of Uruguay comprises 714 km of sandy beaches and has been one of the areas of South America suffering from major urbanization processes during the last 20 years. As urban pressure advances through the coast the lack of sanitation is an impediment for water access and use. Detection of bacterial pollution in water is usually performed through culture-dependent techniques targeting predominantly coliform bacteria (a group of facultative, Gram-negative bacilli from Enterobacteriaceae family). The use of coliforms as indicators of bacterial pollution can also be restricted to fecal coliforms, which in turn can be even limited to *E. coli* and enterococci (Buckalew et al. 2006, Figueras & Borrego 2010, Paruch & Mæhlum 2012). However, it was reported that a large proportion of viable Enterobacteriaceae is not detected by classic methods (Korzeniewska & Harnisz 2012), suggesting a considerable underestimation of bacterial abundance in environmental samples. It is well known that cultivation-based methods do not capture a complete view of aquatic bacterial abundance, since ca. 1% of the known microbes can be cultivated under laboratory settings (Jannasch & Jones 1959, Xu et al. 1982, Ferguson et al. 1984, Eilers et al. 2000). Therefore, molecular approaches addressing the abundance of aquatic bacterial populations have become a preferred choice. Fluorescence *in situ* hybridization (FISH) is a powerful, microscopy-based technique that analyses the composition of microbial communities in several environmental samples (Amann et al. 1990, Pernthaler et al. 2002). Thus, FISH has been extensively used in ecological studies to address composition and dynamics of aquatic bacterial communities (Bouvier & del Giorgio 2003) and has been useful to understand microbial pollution of aquatic systems (Wagner 2003).

Combined to the molecular ecology methods, experimental approaches involving enclosures or microcosms have been also extensively used in microbial ecology. It has been reported that microcosms are useful to study interactions between organisms, to evaluate effects of physical and chemical disturbance and to quantify nutrient cycling in ecosystems (Teuben & Verhoef 1992).

Cabo Polonio National Park (CP) is located on the Atlantic coast of Uruguay. It is characterized by the presence of important sand dunes, wetlands, open beaches and is a wildlife sanctuary for several birds and marine mammal species (Oficina de Planeamiento y Presupuesto 2000 , Chouhy 2013). Freshwater access in CP is restricted to groundwater wells and due to the absence of urban and space planning, most of the wells are located nearby septic tanks. In addition, because of the high permeability of the sandy soil (Haitjema 1995), is very likely that groundwater of CP receives important amounts of microorganisms, dissolved organic matter and nutrients inputs from septic tanks (Rhymes et al. 2014). We have selected CP as experimental area to determine if under a regime of frequent water extraction potentially pathogenic bacteria, such as *E. coli*, are able to survive and grow in groundwater associated to organic matter (OM) supply from nearby septic tanks.

Materials and methods

Experimental setup: For the experimental approach we extracted groundwater from a well located in a sand-dune zone of CP (34°23'51.03" S, 53°47'1.53" W; Figure 1), surrounded by autochthonous plant species *Juncus acutus* and *Androtrichum trigynum* (Delfino & Masciadri 2005) and whose distance to the nearest septic tank was ca. 5 m (distance of septic tanks to groundwater wells in the whole area is 10 ± 4 m). Before performing the experiment the *in situ* abundances of fecal coliforms and enterobacteria were determined. Fecal coliforms were assessed by the membrane filtration method (APHA 1995) using mFC media (HiMedia) (Geldreich *et al.* 1965). Enterobacteria abundance was determined by a double-labeled FISH approach (DOPE-FISH) using the EnterobactB probe (Ootsubo *et al.* 2002) labeled at both ends with the cyanine dye Cy3 (red signal). We selected this probe in order to detect not only *E. coli*, but also other enterobacteria that could be present in groundwater. Water from the selected well was extracted to fill six 1 L sterile glass flasks (microcosms). Each microcosm was inoculated with an overnight culture of *E. coli* XL1-Blue grown in Luria-Bertani at 37 °C, to reach a final abundance of 10^4 CFU mL^{-1}. Then, two treatments were performed: 1) water extraction and refilling treatment (hereafter WER),

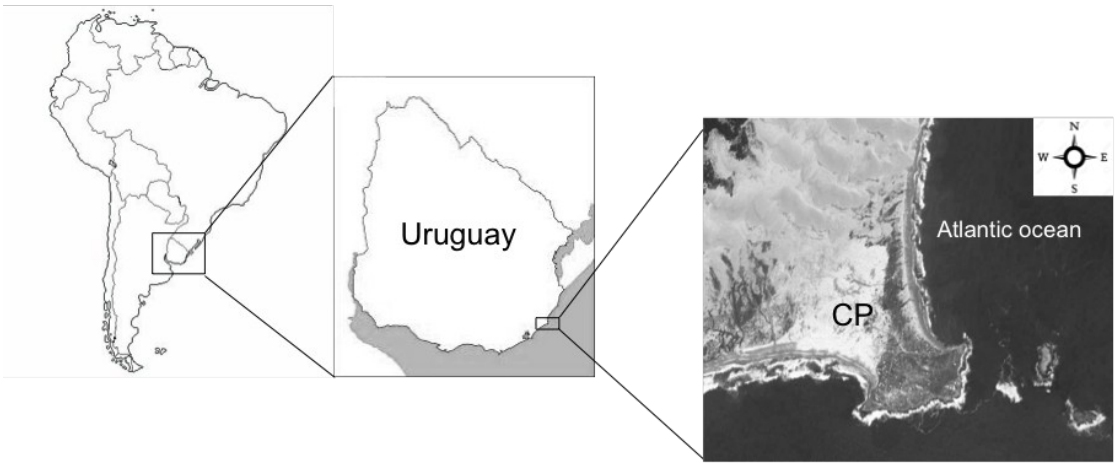

Figure 1. Map of the study area showing the geographic location of Cabo Polonio (CP).

simulating the extraction of water and re-infilling with underground water. By this treatment we intended to measure the effect of groundwater input on the inoculated bacteria. It consisted in the removal of 100 mL water from each microcosm at 0, 2, 4 and 8 hours of incubation that were replaced by 100 mL of groundwater pre-filtered by 0.22 µm filter pore size (Millipore) in order to exclude bacteria. Treatment 2) was considered a control (hereafter CTRL) without water refilling. Previous studies showed that incubation of groundwater in 1 L microcosms for longer than 10 h resulted in bacterial lost due to predation (Germán Perez, personal communication*). Thus, we decided to perform the incubation for 8 h at *in situ* temperature (17 ºC). In all cases, extracted water was fixed immediately with freshly prepared paraformaldehyde (PFA) solution to a final concentration of 1% (w/v), and stored at 4 ºC until reaching the laboratory. Once in the laboratory, samples were fractionated, 50 mL were used for DOPE-FISH and the remaining 50 mL were analyzed to determine the content of organic matter.

EnterobactB probe specificity: In order to address the EnterobactB probe specificity to hybridize *E. coli* and close relatives, including those belonging to the coliforms group (e.g. *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia*), an *in silico* analysis of its sequence was performed using Silva database (http://www.arb-silva.de) and its TestProbe 3.0 tool (Gurevich *et al.* 2013).

Optimization of hybridization conditions: DOPE-FISH conditions were tested using different formamide concentrations (from 25 to 45 %) and a culture of *E. coli*. Briefly, an overnight culture of *E. coli* XL1-Blue growing on Luria-Bertani agar was harvested and bacterial colonies were suspended in sterile 1x PBS to reach a concentration of 10^6 cells mL^{-1}. Aliquots of 1 mL of the suspension were fixed with PFA (1% final concentration, w/v), stored for 1 h at room temperature and filtered through polycarbonate filters (GTTP, 25 mm diameter, 0,22 µm pore size, Millipore). Filters were subjected to FISH using the Cy3 double-labeled EnterobactB probe and increasing formamide concentration (Stoecker *et al.* 2010). Hybridizations were checked by epifluorescence microscopy (IX81 Olympus), and the number of hybridized cells was addressed. Formamide concentration in the hybridization buffer to obtain a hybridization ≥ 99% was chosen for subsequent FISH assays.

Abundance of E. coli and close relative Enterobacteriaceae: A volume of 4 mL of PFA-fixed samples was filtered through white polycarbonate filters (GTTP, 25 mm diameter, 0,22 µm pore size, Millipore). Aliquots of the filtered water thereof were used in FISH. The filters were counterstained with DAPI, final concentration 1 μgmL^{-1} (Pernthaler & Pernthaler 2007), washed with distilled water and ethanol 70 % (v/v), and mounted on glass slides using Citifluor™. The identification of the fluorescent signals was performed manually using an epifluorescence microscope (IX81 Olympus) equipped with filter sets for DAPI and Cy3. For each sample and probe, more than 500 DAPI-stained bacteria were enumerated in 30 randomly chosen fields on each filter.

Organic matter content: The content of OM (specially particulate) from WER and CTRL water samples was determined by loss-on-ignition (Dean 1974); using glass fiber filters (GF/F, 0.7 µm pore size, Whatman). OM was determined in pools

generated from the triplicates of each treatment.

Statistical Analysis: Two-way ANOVAs were performed to test differences between treatments. If required, data were log- transformed prior to analyses in order to approximate normality (Kolmogorov–Smirnov test). Post hoc comparisons between samples were performed using Bonferroni test. Significant differences were considered at $p \leq 0,05$. Statistical analyses were conducted with STATISTICA 7 software.

Results

EnterobactB probe specificity: The *in silico* specificity check of the EnterobactB probe retrieved ca. 40000 sequences sharing 0 mismatch, 99% of them were from Enterobacteraceae family. Among this group, 31.6% of enterobacterial sequences belonged to *Escherichia* and *Shigella* genera, 14% to *Serratia*, 12% to *Enterobacter*, 8% to *Klebsiella*, 5% to *Citrobacter*, 3% to *Salmonella* and a remaining 13% were unclassified enterobacteria. These genera accounted for 86.6% of found Enterobacteraceae. The unclassified percentage was distributed among other enterobacterial genera, such as *Pantoea*. Since the EnterobactB probe showed a high specificity for coliform genera, we used it for subsequent quantification of the *E. coli* culture in the microcosm incubations. The formamide concentration in the hybridization buffer to obtain optimum astringency conditions was 35%, allowing a hybridization efficiency ≥ 99% using an *E. coli* culture.

Enterobacteria dynamics: Fecal coliforms, assessed by the membrane filtration method, were not detected in the *in situ* groundwater samples. Similarly, at the beginning of incubation (time 0) the abundance of EntB-hybridized bacteria (which included fecal coliforms such as *E. coli*) was 8.59 x 10^3 bacteria mL^{-1}, roughly the expected abundance after inoculation of the *E. coli* culture. Therefore, we assumed that *E. coli* abundance in the groundwater used to fill the microcosms was negligible and that most EntB-hybridized bacteria detected during incubation came from the inoculated culture.

The abundance of EntB-hybridized bacteria in WER significantly increased along the incubation period when related to time zero (ANOVA, $p \leq 0,05$) (Figure 2A and 2C). In the case of CTRL treatment, abundance of EntB-hybridized bacteria showed a significant increase related to time zero only after 4 h (ANOVA, $p \leq 0,05$) and then decreased significantly towards the end of the incubation time (ANOVA, $p \leq 0.05$) (Figure 2C).

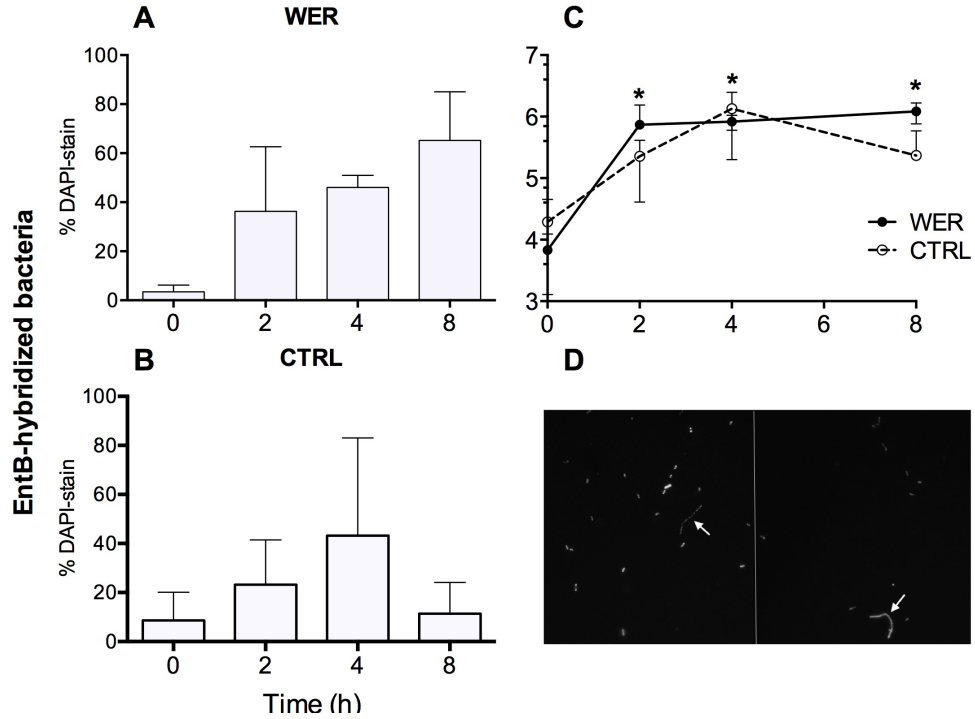

Figure 2. Relative abundance of EnterobactB-hybridized (EntB-hybridized) bacteria in WER (A) and CTRL (B) treatments related to DAPI-stained bacteria. In (C) total abundance of EnterobactB-hybridized bacteria is shown as $\log_{10}$ of bacteria per mL. Asterisks indicate significant differences related to time 0 for WER treatment. (D) Filaments found in CTRL treatment are shown (arrows).

The OM content in both microcosms showed different trends through the incubations (Table I). In WER, organic matter concentration was highest at 2 h incubation, decreasing towards the end and reaching similar concentration as in CTRL. On the other hand, in CTRL treatment showed a peak at 4 h and then decreased towards the end of the incubation period.

Table I. Organic matter content (in mg L^{-1}) measured in each treatment through the incubation. Fifty mL water samples from each flask were taken at each sampling time and pooled.

Time (h)	WER (mg L^{-1})	CTRL (mg L^{-1})
0	17.0	8.6
2	25.4	7.3
4	6.0	20.0
8	4.2	5.6

Discussion

In this study we selected a specific zone of the Uruguayan Atlantic coast to test the vulnerability of groundwater in the current management framework. We found that water extraction regime simulated in WER treatment stimulated the growth of enterobacteria targeted by EnterobactB probe, which included the inoculated *E. coli* population and suggested the presence of an additional source of carbon and nutrients in the groundwater used for refilling. In an attempt to elucidate this hypothesis we measured OM concentration in both treatments. The dynamics of OM showed different trends between treatments. In WER treatment the OM concentration did not follow the trend of the enterobacterial abundance, since a peak was detected at 2 h and then continuously decreased through the end of the experiment. This was probably due to the treatment setup where entire water was removed and pre-filtered water was added, provoking a dilution of the detected particulate OM. In this case, the enterobacteria growth would be mostly attributed to the dissolved organic matter content of the pre-filtered groundwater added, although dissolved organic carbon content was not analyzed. On the other hand, in CTRL treatment the OM peak found in the samples after 4 h incubation could be consequence of the rise in enterobacteria counts observed at that time (since bacteria are particles that can be retained in GF/C filters used for OM determination), also reflecting the treatment setup without added groundwater. Therefore, enterobacteria growth towards the end of the incubation in the CTRL was possibly constrained by a shortage in dissolved organic carbon. Another factor that may influence the population dynamics of EntB-hybridized bacteria during the CTRL treatment is predation by protists. An evidence of bacterivory in CTRL treatment was the detection of filamentous bacteria, which were not observed in WER (Figure 2D). Most filaments exceed the sizes that protists can ingest and their formation is considered a bacterial evasion mechanism to escape predation (Pernthaler 2005). Therefore, in the CTRL treatment a combination of predation and organic matter depletion could have been the controlling factors, not only for enterobacteria but also for the whole bacterial community.

Altogether, our results suggest that groundwater harbors a load of dissolved OM, probably from nearby septic tanks, which was enough to sustain the growth of potentially pathogenic bacteria such as *E. coli*. Thus, management efforts seeking to minimize these factors in groundwater should be taken. In this sense, several mitigation solutions have been proposed, including the growth of native vegetation or artificial wetlands to ensure nutrient and coliform bacteria retention (Fajardo *et al.* 2001, Iasur-Kruh *et al.* 2010, Cardoso *et al.* 2012). Since in Uruguay, as well as in several countries, conflicts associated with water quality are widespread we expect that the information obtained from this study site may also be applied to sites with similar conditions.

Acknowledgments

This work was partially financed by the Programa para el Desarrollo de las Ciencias Básicas (PEDECIBA, Universidad de la República) and a scholarship (INI_X_2011_1_3924) given to Martina Soumastre to fulfill her Bachelor in Sciences degree, from the National Agency for Research and Innovation (ANII). We also thank Dr. Luis Aubriot for his assistance in the determination of organic matter content.

References

Amann, R. I., Krumholz, L. & Stahl, D. A. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. **Journal of Bacteriology**, 172(2)**:** 762-770.

APHA. 1995. Standard methods for the examination of water and wastewater. Washington.

Bouvier, T. & del Giorgio, P. A. 2003. Factors influencing the detection of bacterial cells

using fluorescence in situ hybridization (FISH): A quantitative review of published reports. **Fems Microbiology Ecology**, 44(1)**:** 3-15.

Buckalew, D. W., Hartman L. J., Grimsley G. A., Martin, A. E. & Register, K. M. 2006. A long-term study comparing membrane filtration with Colilert® defined substrates in detecting fecal coliforms and *Escherichia coli* in natural waters. **Journal of Environmental Management,** 80(3): 191-197.

Cardoso, F., Shelton, D. Sadeghi, A. Shirmohammadi, A. Pachepsky, Y. & Dulaney, W. 2012. Effectiveness of vegetated filter strips in retention of *Escherichia coli* and *Salmonella* from swine manure slurry. **Journal of Environmental Management**, 110: 1-7.

Chouhy, M. 2013 Cabo Polonio, área protegida: conservacionismo en diálogo con cosmovisiones salvajes. **Anuario de Antropología Social y Cultural en Uruguay,** 11**:** 87-102.

Dean, W. E. 1974. Determination of carbonate and organic matter in calcareous sediments and sedimentary rocks by loss on ignition: comparison with other methods. **Journal of Sedimentary Petrology** 44: 242-248.

Delfino, L. & Masciadri, S. 2005. Relevamiento florístico en el Cabo Polonio, Rocha, Uruguay. **Iheringia, Ser. Bot., Porto Alegre**, 60: 119-128.

Eilers, H., Pernthaler, J. Glöckner, F. O. & Amann, R. 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. **Applied and Environmental Microbiology**, 66(7): 3044-3051.

Fajardo, J. J., Bauder, J. W. & Cash, S. D. 2001. Managing nitrate and bacteria in runoff from livestock confinement areas with vegetative filter strips. **Journal of Soil and Water Conservation**, 56(3): 185-191.

Ferguson, R. L., Buckley, E. N. & Palumbo, A. V. 1984. Response of marine bacterioplankton to differential filtration and confinement. **Applied and Environmental Microbiology,** 47(1): 49-55.

Figueras, M. & Borrego, J. J. 2010. New perspectives in monitoring drinking water microbial quality. **International journal of environmental research and public health,** 7**(**12**):** 4179-4202.

Geldreich, E. E., Clark, H. F. Huff, C. B. & Best, L. C. 1965. Fecal-coliform-organism medium for the membrane filter technique. **Journal American Water Works Association,** 208-214.

Gurevich, A., Saveliev, V. Vyahhi, N. & Tesler, G. 2013. QUAST: quality assessment tool for genome assemblies. **Bioinformatics,** 29(8): 1072-1075.

Haitjema, H. M. 1995. **Analytic element modeling of groundwater flow.** Academic Press.

Iasur-Kruh, L., Hadar, Y. Milstein, D. Gasith, A. & Minz, D. 2010. Microbial population and activity in wetland microcosms constructed for improving treated municipal wastewater. **Microbial Ecology,** 59(4): 700-709.

Jannasch, H. W. & Jones, G. E. 1959. Bacterial Populations in Sea Water as Determined by Different Methods of Enumeration1. **Limnology and Oceanography,** 4(2)**:** 128-139.

Korzeniewska, E. & Harnisz, M. 2012. Culture-Dependent and Culture-Independent Methods in Evaluation of Emission of Enterobacteriaceae from Sewage to the Air and Surface Water. Water, Air, and Soil Pollution. **Water Air Soil Pollution,** 223(7)**: ,** 4039-4046.

Oficina de Planeamiento y Presupuesto, Banco Iberoamericano de Desarrollo. 2000. Uruguay-Estudio Ambiental Natural. Accesible at http://www.oas.org/. Accessed 03/03/2015.

Ootsubo, M., Shimizu, T. Tanaka, R. Sawabe, T. Tajima, K. Yoshimizu, M. Ezura, Y. Ezaki, T. & Oyaizu, H. 2002. Oligonucleotide probe for detecting Enterobacteriaceae by in situ hybridization. **Journal of Applied Microbiology**, 93(1): 60-68.

Paruch, A. M. & Mæhlum, T. 2012. Specific features of *Escherichia coli* that distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. **Ecological Indicators,** 23:140-142.

Pernthaler, A. & Pernthaler, J. 2007. **Fluorescence *in situ* hybridization for the identification of environmental microbes**. Protocols for Nucleic Acid Analysis by Nonradioactive Probes. Springer.

Pernthaler, A., Pernthaler, J. & Amann, R. 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the

identification of marine bacteria. **Applied and Environmental Microbiology,** 68(6)**:** 3094-3101.

Pernthaler, J. 2005. Predation on procaryotes in the water column and its ecological implications. **Nature Reviews of Microbiology**, 3: 537-546.

Rhymes, J., Wallace, H. Fenner, N. & Jones, L. 2014. Evidence for sensitivity of dune wetlands to groundwater nutrients. **Science of the Total Environment,** 490: 106-113.

Stoecker, K., Dorninger, C. Daims, H. & Wagner, M. 2010. Double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. **Applied and Environmental Microbiology,** 76(3): 922-926.

Teuben, A. & Verhoef, H. A. 1992. Relevance of micro-and mesocosm experiments for studying soil ecosystem processes. **Soil Biology and Biochemistry,** 24(11)**:** 1179-1183.

Wagner, M., Horn, M., & Daims, H. 2003. Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. **Current Opinion in Microbiology, 6**(3)**:** 302-309.

Xu, H.-S., Roberts, N. Singleton, F. L. Attwell, R. W. Grimes, D. J. & Colwell, R. R. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. **Microbial Ecology**, 8(4)**:** 313-323.

Received: July 2015
Accepted: November 2015
Published: November 2015