OYSTER-RELAYING AND DEPURATION IN AN OPEN-WATER LOCATIONS

Indigenous Species Development/Experiment/ (07IND04UH)

Guillermo Rodríguez Domínguez
Eladio Gaxiola Camacho
Universidad Autonoma de Sinaloa (UAS)
Culiacan, Sinaloa, Mexico

Maria Haws
University of Hawaii Hilo
Hilo, Hawaii

John Supan
Louisiana State University
Baton Rouge, Louisiana

ABSTRACT

Oysters (*Crassostrea corteziensis*) were harvested at Boca de Camichin, Nayarit, Mexico and relayed to two sites (La Palicienta and Pozo Chino) to test whether oysters could be depurated in this way. Fecal and total coliform bacteria and *E. coli* in water and oyster tissues were monitored for ten days during July 2008. Bacterial levels generally exceeded legal limits during most of the ten day period at both sites, although levels were lower at La Palicienta during the neap tides. Relay and depuration is therefore not feasible at these sites during this time of year, although it is known that bacteria levels are significantly lower during winter months, suggesting that this experiment should be repeated during the winter.

INTRODUCTION

Bivalves are particularly susceptible to contamination due to their filter feeding habits. Pathogens acquired in this way can subsequently infect human consumers. This may be particularly serious in areas where waste water treatment is rare, shellfish sanitation plans non-existent and consumers lack awareness of the possible risks. This is generally the case in Mexico, where the risk is exacerbated by the preference for consuming bivalves raw and lack of good post-handling methods. Shellfish-borne disease that can affect humans include: *Salmonella* spp. (typhoid fever, Salmonellosis) *Shigella* sp. (dysentary and Shigellosis), *Vibrio cholerae* (cholera), *E. coli*, enteroviruses y rotavirus (gastroenteritis), *Clostridium perfringes* (gas gangrene), *Hepatitis A* (infectious hepatitis), *Entamoeba histolytica* (amoebiasis), *Ascaris lumbricoide*s (ascariasis), *Enterobius vermicularis* (enterobiasis), *Taenia sp.* (taeniasis), *Aeromonas spp* (gastroenteritis and skin infections) (Araujo et. al, 1989; Metcalf and Eddy, 2003).

Specific tests to detect each pathogen are required, making comprehensive testing for the purpose of shellfish sanitation economically unfeasible. Coliform bacteria are therefore generally used as indicator organisms. In Mexico, Regulation NOM-031-SSA1-1993 establishes the maximum legal permissible levels for total coliforms as 70 MPN/100 ml and 14 MPN/100 ml for fecal coliforms for shellfish growing waters. The maximum permissible level for oyster tissues is 230 MPN/100 g.

Oysters also have the capacity to depurate themselves if they are kept in clean waters for specific periods of time, although not all pathogens (e.g. some viruses) can be reduced to safe levels. There are generally two methods for depuration. One is to use land based, flow-through systems with water that is free of contaminants. In these systems, the required depuration time is 24-48 hours. The other is to move oysters after harvest to an open-water setting where water quality monitoring has demonstrated that pathogen levels are below legally permissible levels. Depuration in these cases usually lasts from 7-10 days, although the length of this period is a precautionary measure.

In the Boca de Camichin Estuary, Nayarit, oyster culture using the native “Pleasure Oyster”, *Crassostrea corteziensis*, has been practiced for over 30 years and is an important economic activity in this impoverished area. The oysters are sold locally and also in Tepic, Guadalajara and Mazatlan. A recent study indicated that the estuary waters have coliform levels above the legally permissible levels for an area approved for shellfish culture (Olivo-Garcia, 2007). This study only tested water, not oyster tissues.

In this study, oysters were obtained from farms in Boca de Camichin which had coliform tissue levels of 233 MPN/100g (slightly above the legal permissible level of 230 MPN/100g) and taken to two distant sites with water which was presumable cleaner than the oyster culture site, based on previous water quality testing. The water at each site and the oyster tissues were tested for coliform bacteria for ten days with the goal of determining the suitability of the sites for depuration and the rate of depuration. The sites were chosen due to their proximity at the mouth of the estuary and distance from potential sources of contamination.

Coliform bacteria counts have been utilized as indicators of fecal contamination in water (Orosco et al. 1983; Araujo et al., 1989; Barrera-Escorcia et al, 1999; Yap y Kahoru, 2001; Ravagnani et al., 2005, Ruiz García 2007, Olivo Rojas, 2007, Pérez González, 2009) and in oyster tissue (Rosas et al. 1985, Rodríguez, 1986, Leyva Castillo 1996, Barrera-Escorcia et al, 1998). Fecal coliforms are particularly appropriate indicators for recent contamination; these are distinguished using brilliant green bile broth or EC broth (Anónimo, 1987, Araujo et al. 1989). The association of fecal coliform bacteria in residential waste waters with other pathogens which cause gastrointestinal illness, bacterial infections and other disease has been amply documented (Araujo et al., 1989, Metcalf y Eddy, 2003).

Marine and estuarine waters appear to contain bacterial communities which may act to eliminate coliform bacteria so that that proliferation of coliforms appears to be low (Vallaro et al. 1950, Romero Jarero 1982 y Romero Jarero et al., 1986). In the Boca de Camichin estuary, the highest fecal coliform counts are during periods of low salinity due to input by rivers, while in Santa Maria Bay, Sinaloa, Ruiz Garcia (2007) attributed low coliform counts to high salinities and high exchange rate between the Bay and ocean.

Haws et al. (2006) noted that in Sinaloa and Nayarit waste waters do not receive adequate treatment before being released into coastal water bodies and represent a high public health risk, particularly through consumption of aquatic organisms. In Nayarit, this assertion was confirmed by Olivo Rojas (2007) who found that during the year concentrations of total and fecal coliforms were above legal permissible levels. In the case of Sinaloa, Ruiz Garcia (2007) found that total and fecal coliforms were on the average below the permissible level. For example, in Santa Maria Bay and Altata Bay, Mendez et al (1990) reported low concentrations with a maximum of 23 MPN/100 ml in May, which is below the legal limit of 70 MPN/100 ml for oyster culture waters. In Altata Bay, Sinaloa, Perez Gonzalez (2009) found total and fecal coliform levels well below permissible levels and attributed this to the mixing of freshwater and marine water by tides.

Contamination of oyster tissue with fecal coliform has been documented in the Gulf of Mexico (Rosas et al, 1985; Rodríguez, 1986) where levels much higher than the legal limit of 230 MPN/100g for tissue. Pathogens such as *Salmonella*, *Escherichia* coli and *Plesiomonas shigelloides* were also found. These results from Mexico are in contrast to those found in other Latin American countries such as Cuba (Leyva Castillo et al., 1996) and Puerto Rico (Fontanez Barris, 2005), where coliforms and other pathogens were well below permissible levels.

Study Site

The Boca de Camichin Estuary is at the mouth of the San Pedro River and is located in the northwestern part of Nayarit State in the Municipality of Santiago Ixcuintla. The estuary is located between 24° 48' al 24°44' north and 105˚30´al 105˚29' west (Figure 1). This estuary is part of a larger coastal system known as the Marismas Nacionales (National Wetlands) which are comprised of 200,000 ha of water area including 157 coastal lagoons, barrier islands and mangroves. It is considered to be a national treasure with incomparable biodiversity and conservation value (Garcia-Carmona, 2003). Additionally, it is economically important for its fisheries and aquaculture.

The water quality of Boca de Camichin is influenced by freshwater from the Lerma Santiago River 11 kilometers to the south. Nine kilometers to the north, the San Pedro river has another narrow outlet, La Palicienta, before continuing on to form the Boca de Camichin Estuary. Additionally, the southern part of Boca de Camichin runs inland for a short distance creating a narrow water body called Pozo Chino which is isolated from the ocean by a narrow peninsula.

Figure 1. The study site-Boca de Camichin Estuary, Nayarit.

METHODS

Oysters spat is collected on oyster shells strung on lines ("sartas") which are hung from rafts and subsequently grown out in this manner. For the purposes of this experiment, six sartas with mature oysters were taken from the main farming area at Boca de Camichin and taken to La Palicienta and Pozo Chino. These are the closest possible potential depuration sites and are presumably free from contamination due to their distance from human settlements. La Palicienta is a small mouth of the San Pedro River north of Boca de Camichin with a strong oceanic influence. Pozo Chino is located to the southeast of Boca de Camichin which does not have any other freshwater influence.

Oysters were left at these sites for ten days during which total coliforms, fecal coliform and *E. coli* were measured periodically in the water and oyster tissues. The experiment began during the neap tide with a tidal range of 50-70 cm and ended with a spring tide with a range of 170 cm.

Sample collection and preparation

Water samples were taken using sterilized BOD bottles which were submerged with the lids on, and opened at a depth of 20 cm. Each bottle was wrapped in aluminum foil, placed in a plastic bag and transported in an ice chest with frozen gel packs. At each site, 15-20 oysters were collected in order to obtain a total of 50 g of oyster tissue. Each oyster was carefully brushed to remove mud and biofouling, washed with distilled water and then opened with a knife cleaned with distilled water. Tissue was removed from the shell using sterilized forceps. To make a 1:10 solution using the 50 g of tissue, 450 ml of phosphate buffer solution was added and the mixture was then macerated in a sterilized blender. The tissue solution was then stored in a sealed, sterilized flask.

Laboratory analysis

The Most Probable Number Presumptive and Confirmative tests were used.

Presumptive Test

Water samples and the flask with the tissue samples were agitated using 25 rapid up and down motions in a 30 degree arc to homogenize the samples. As each container was opened, the mouth was flamed. Ten ml and 1 ml of the sample were each used to inoculated three test tubes containing the culture media. From the tubes inoculated with 1 ml of sample, a 1 ml sample was then taken and used to inoculate test tubes containing 9 ml of media, thus obtaining a dilution of 0.1 ml. Durham vials were used for gas collection. This procedure was conducted four times for each water and tissue sample. All were incubated at 35 ^{o}C and examined after 24 hours. If gas was not observed, incubation was continued for an additional 24 hours. A standard MPN table was used to determine the concentration of total coliforms.

Confirmative Test

Samples were taken from each test tube that showed gas formation in the presumptive test and inoculated into tubes containing brilliant green lactose bile (BGLB) broth which were incubated at 44.5 ^{o}C for 24 hours. Tubes were examined at 24 hours; if no gas formation was observed, incubation was continued for an additional 24 hours. A standard MPN table was used to determine the concentration of fecal coliform.

RESULTS

The concentrations of total and fecal coliforms and *E. coli* in water and oyster tissues taken from La Palicienta and Pozo Chino are summarized in Table 1. In the case of La Palicienta, total and fecal coliforms were under the upper limit for shellfish growing waters as established by regulation NOM031-SSA1-1993. In the case of Pozo Chino, levels exceeded the legal limits.

Table 1. Geometric mean and median for coliforms and *E. coli* in water and oyster tissues from La Palicienta and Pozo Chino.

Site	Mean or median	Date	Total coliform in water MPN/100 ml)	Fecal coliform in water (MPN/100 ml)	*E. coli* in water (MPN/100 ml)	Total coliform in oyster tissue MPN/100 g	Fecal coliform in oyster tissue (MPN/100g)	*E. coli* in oyster tissue MPN/100 g
Palicienta	Mean	7/13/09	65	2	0	711	233	163
		7/15/09	145	40	33	486	251	223
		7/16/09	460	156	39	885	200	200
		7/17/09	144	114	52	1100	1100	885
		7/18/09	37	17	12	1100	1100	1100
		7/19/09	605	20	13	1100	1100	1100
		7/22/09	1100	1100	1100	1100	1100	1100
	Median	7/13/09	87	2	0	780	305	195
		7/15/09	150	43	43	460	240	240
		7/16/09	460	122	36	1100	210	210
		7/17/09	122	93	43	1100	1100	1100
		7/18/09	43	18	14	1100	1100	1100
		7/19/09	780	32	18	1100	1100	1100
		7/22/09	1100	1100	1100	1100	1100	1100
Pozo Chino	Mean	7/13/09	96	29	13			
		7/15/09	81	53	0	885	585	585
		7/16/09	263	39	32	477	87	40
		7/17/09	28	28	19	1100	1100	1100
		7/18/09	137	50	45	1100	1100	1100
		7/19/09	1100	1100	439			
		7/22/09	1100	1100	1100	1100	1100	1100
	Median	7/13/09	93	43	24			
		7/15/09	93	68	0	1100	780	780
		7/16/09	305	49	33	780	122	57
		7/17/09	33	33	19	1100	1100	1100
		7/18/09	158	43	43	1100	1100	1100
		7/19/09	1100	1100	1100			
		7/22/09	1100	1100	1100	1100	1100	1100

The variation in the mean concentration of total and fecal coliform, and *E. coli*, in water and tissue samples was statistically significant between different days of the experiment. There was also significant variation between samples as indicated by the standard deviation (Table 2).

Table 2. Mean and standard deviation for total and fecal coliforms, and *E. coli* in water and oyster samples from Palicienta and Pozo Chino.

Site		Date	7/13/09	7/15/09	7/16/09	7/17/09	7/18/09	7/19/09	7/22/09	P
Palicienta	Water	Total coliforms	108.5±106	150.75±48	460±0	342.75±507	60.75±62	725±442	1100±0	P<0.001
		Fecal coliform	2.25±2	61.25±61	199±176	172.25±193	21.25±15	27.75±19	1100±0	P<0.001
		E. coli	0	42.5±27	46.25±32	55.5±25	18.5±18	15.75±9	1100±0	P<0.001
	Tissue	Total coliforms	780±370	565±371	940±320	1100±0	1100±0	1100±0	1100±0	P=0.018
		Fecal coliform	290.75±197	272.5±132	202.5±38	1100±0	1100±0	1100±0	1100±0	P<0.001
		E. coli	223.25±177	258.25±151	202.5±38	940±320	1100±0	1100±0	1100±0	P<0.001
Pozo Chino	Water	Total coliforms	166.75±198	111.75±92	305±179	31±14	204.5±191	1100±0	1100±0	P<0.001
		Fecal coliform	45.75±36	62.5±36	51.5±38	31±14	64.75±58	1100±0	1100±0	P<0.001
		E. coli	23.5±23	0	39±27	22.5±15	62.75±60	832±536	1100±0	P<0.001
	Tissue	Total coliforms		940±320	688.25±498	1100±0	1100±0		1100±0	P<0.001
		Fecal coliform		940±320	688.25±498	1100±0	1100±0		1100±0	P<0.001
		E. coli		717.5±453	61.25±54	1100±0	1100±0		1100±0	P<0.001

Results from La Palicienta

The initial concentration of total coliform in water from La Palicienta was 65 MPN/100 ml (the legal upper limit for shellfish growing grounds is 70 MPN/100 ml), and stayed below 200 MPN/100 ml during the first six days of the experiment, with the exception of day 4 when the value increased 400 MPN/100 ml (Figure 2A and 2 D). Towards the end of the experiment, levels rose to 1100 MPN/100 ml. In contrast, the concentration of total coliform bacteria in oyster tissues was between 500 and 1000 MPN/100 g in the first four days and in following days, was above 1100 MPN/100 g (Figure 2A and 2B). The initial concentration of fecal coliform in water from La Palicienta was less than 2 MPN/100 ml, but at day four increased to between 122 (median) and 156 (mean) MPN/100 ml, then dropped to between 20 (mean) and 32 (median) MPN/100 ml on the seventh day, then the experiment ended with 1100 MPN/100 ml (Figure 2B and 2E). In contrast, concentrations of fecal coliform was between 200 and 300 MPN/100 g during the first four days and beginning on the fifth day, concentrations rose to 1100 MPN/100 g (Figure 2B and 2 E).

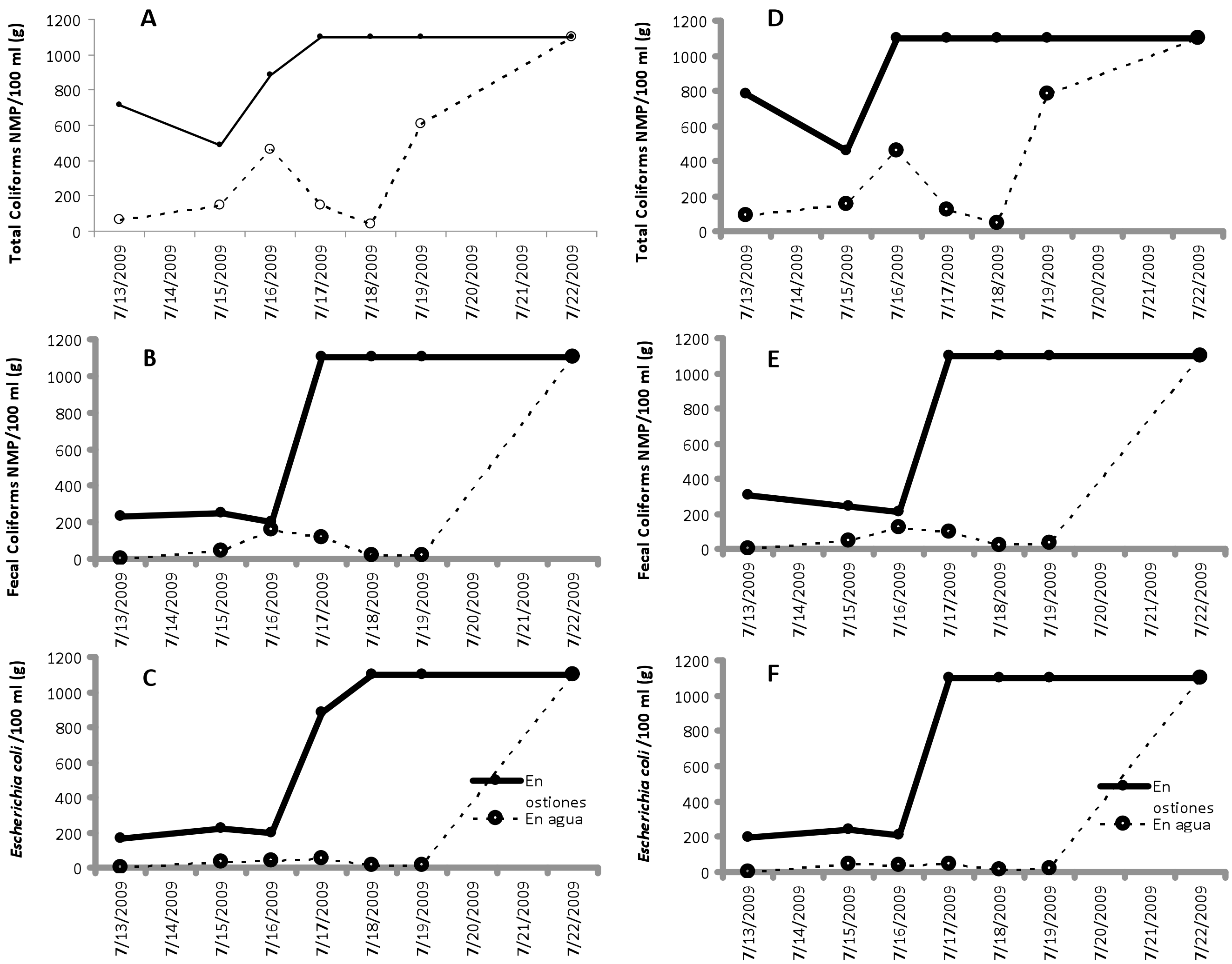

Figure 2. Geometric mean (A,B, C) and median (D,E, F) of total coliforms (above), fecal (middle) and *E. coli* (below) in water (dotted line) and oyster tissues (solid line) from La Palicienta.

At the beginning of the experiment, *E. coli* was not detected in water samples, but between the second and seventh day, the concentration varied between 12 and 52 MPN/100 ml and ended at 1100 MPN/100 ml (Figure 2C and 2F). In oyster tissues, *E. coli* was found at the beginning of the experiment at concentrations less than 200 MPN/100 g and increased in following days, reaching 1100 MPN/100 ml (Figure 2C and 2F).

From the third through fifth days, a significant amount of fresh water entered the system as shown by the low salinities between 13 and 19 ppt (Figure 3 A). This influx of freshwater coincided with the neap tides that are characterized by low tidal amplitudes and low current velocities (Figure 3B). After the sixth day, when the tides increased, the salinity also increased to 33-35 ppt indicating increased marine influence. The concentration of coliforms and *E. coli* during the first five days were associated with the predominant influence of freshwater during the neap tides. At the end of the experiment, coliform and *E. coli* levels arose during the spring tides and increases in salinity to 33-35 ppt.

The concentrations of coliforms and *E. coli* in oyster tissues stayed relatively low during the neap tides and increased to more than 1100 MPN/100 g when the spring tides occurred after the fifth day of the experiment (Figure 3).

The concentration of total coliform in water at La Palicienta stayed below 200 MPN/100 ml during the first six days of the experiment, with the exception of the fourth day when levels increased temporarily to 400 MPN/100 ml (Figure 2A and 2D). Levels later increased to greater than 110 MPN/100 ml. In contrast, oyster tissue levels stayed between 500 and 1000 MPN/100 g during the first four days and then rose to greater than 1100 MPN/100 g (Figure 2A and 2 B).

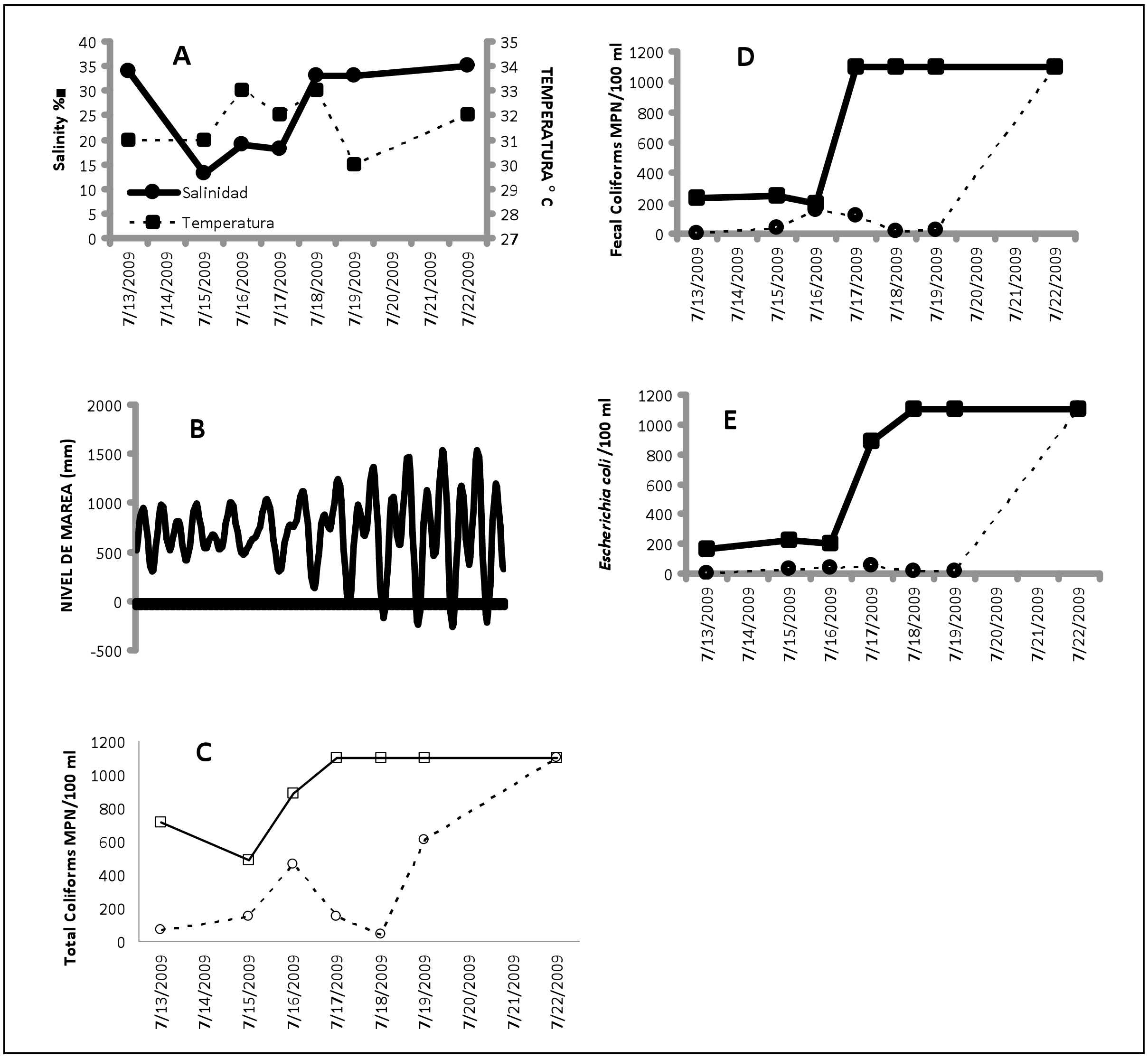

Figure 3. Temperature, salinity (A), tide level (B), total coliforms (C), fecal coliform (D) and *E. coli* in water (dotted line) and in tissues (solid line) for the experiment in La Palicienta.

<u>Experiment in Pozo Chino</u>

In contrast to the La Palicienta site, at Pozo Chino the initial concentration of total coliforms in water was 95 MPN/100 ml (the upper limit for shellfish growing grounds is 75 MPN/100 ml) and stayed between 80 and 140 MPN/100 ml during the first six day of the experiment. It then increased to more than 1100 MPN/100 ml during the last four days of the experiment (Figure 4A and 4 D). Levels in oyster tissues were always above 400 MPN/100 g and rose to over 1100 MPN/100 g beginning at day five and continuing until the end (Figure 4A and 4 D).

The initial concentration of fecal coliform in water at Pozo Chino was 28 MPN/100 ml, well above the maximum legal limit of 14 MPN/100 ml. Levels stayed between 28 and 52 MPN/100ml during the first six days of the experiment and arose to more than 1100 MPN/100 ml during the last four days. In the oyster tissues, the concentration of fecal coliform was 584 MPN/100g at day 3, then fell to 87 MPN/100g on the fourth day. Beginning on day 5 and continuing until the end of the experiment, concentrations were above 1000 MPN/100 g (Figure 4 B and 4E).

E. coli was detected in Pozo Chino waters at a concentration of less than 45 MPN/100 ml during the first six days, with the exception of the third day, then rapidly rose beginning on the seventh day to 1100 MPN/ml and stayed at this level until the end of the experiment. *E. coli* was detected on day three in oyster tissues at a concentration of 584 MPN/100 g, but then fell to 40 MPN/100 g the following day. The concentration then rose on the fifth day to 1100 MPN/100 g and continue at this level until the end of the experiment (Figure 4C and 4 F).

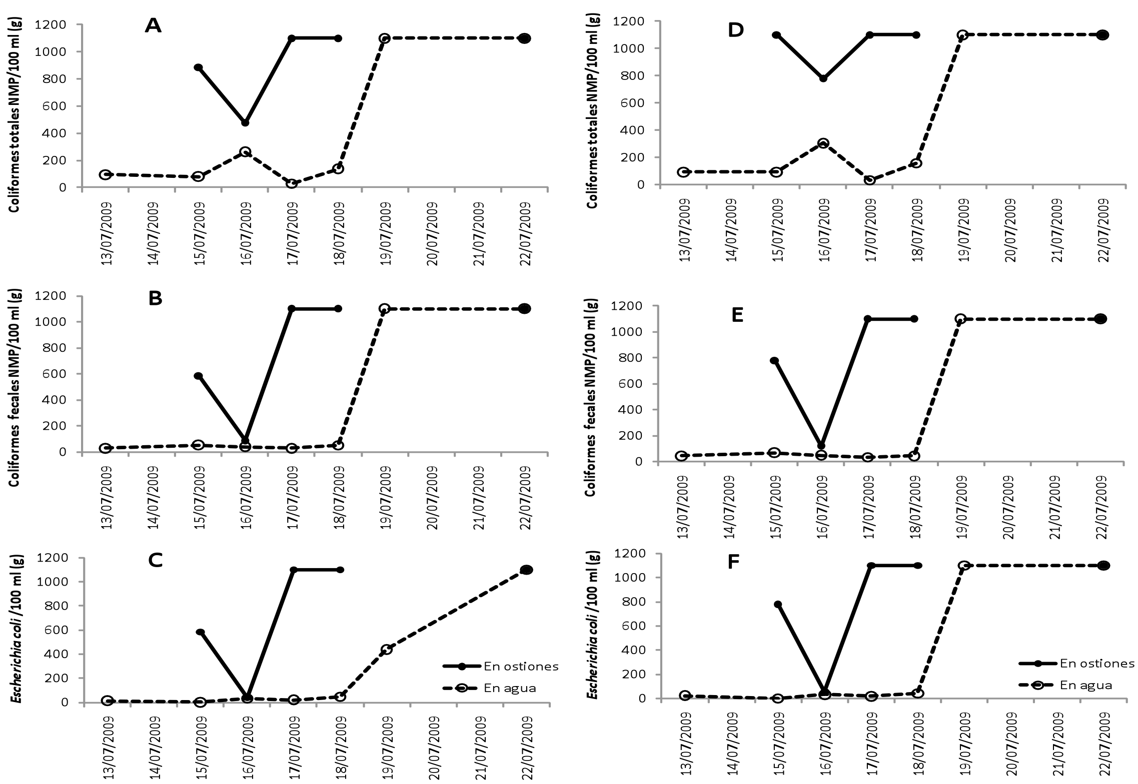

Figure 4. Geometric mean (A, B and C) and median (D, E and F) of the concentration of total coliform (above), fecal coliform (middle) and *E. coli* (below) in water (dotted line) and oyster tissue (solid line) from Pozo Chino.

The influx of freshwater at Pozo Chino is shown by the salinities of less than 35 ppt throughout the experimental period. The lowest salinity occurred on the seventh day when salinity was 26 ppt (Figure 5A). It is evident that the concentrations of total and fecal coliforms and *E. coli* maintain relatively low levels during neap tides and then show dramatic increases on the fifth day during the spring tides (Figures 5B, C and D).

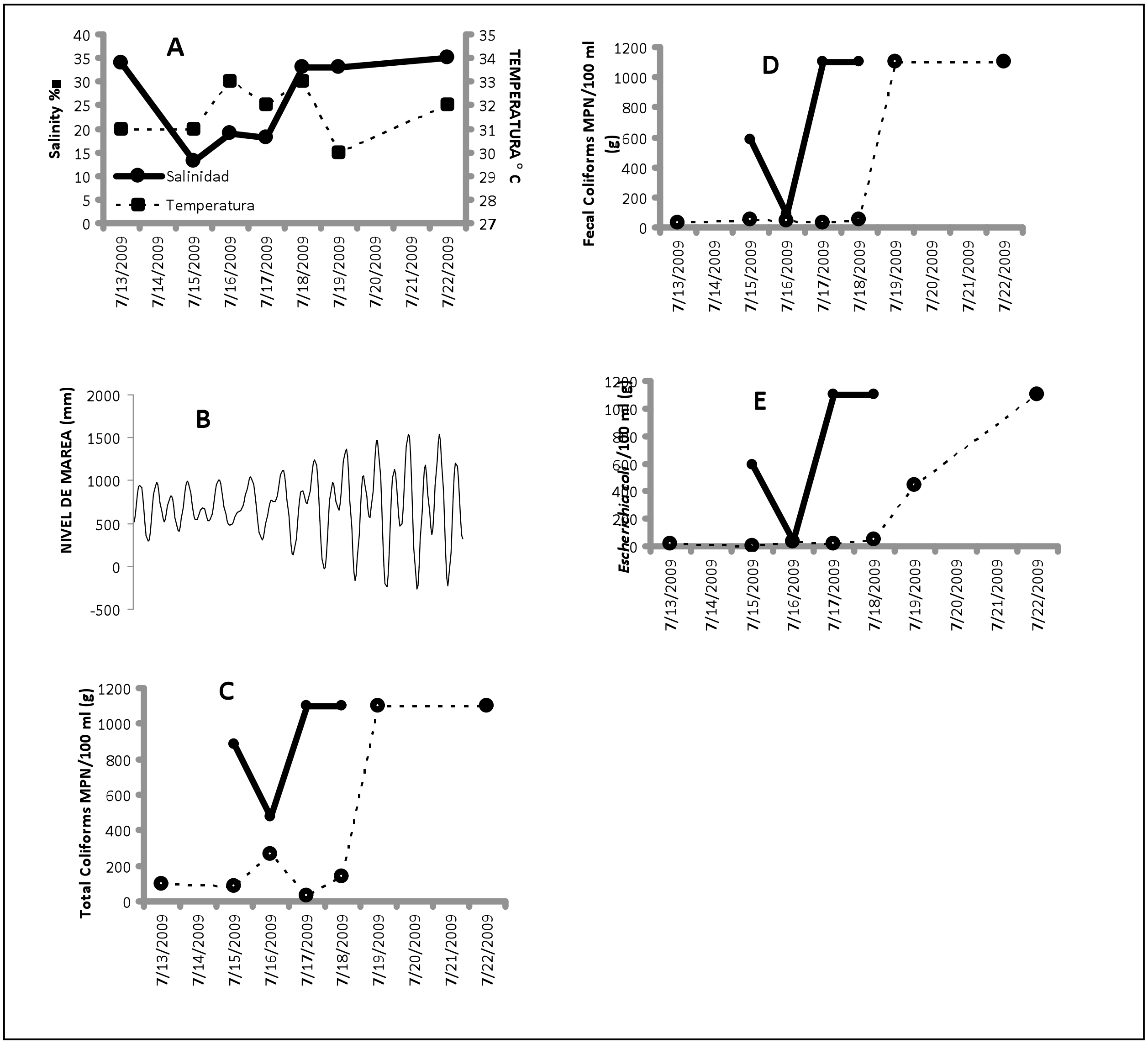

Figure 5. Temperature, salinity (A), tide level (B), total coliform (C), fecal coliform (D) and E. coli (E) in water (solid line) and oyster tissue (dotted line) at Pozo Chino.

DISCUSSION

The depuration experiment was not successful at La Palicienta since bacterial levels were not within legally permissible levels for the entire ten day period. Depuration was also not successful at Pozo Chino, although at this site, bacterial levels rose sooner than at La Palicienta

The concentration of total coliforms at both sites showed a similar pattern. Bacterial levels showed a spike at day 4, then dropped, only to rise again beginning on day 7 when they rose to high levels and remained so until the end of the experiment. The relationship between bacterial levels and environmental conditions did not show a clear correlation. For example, on day 4, the rise in bacterial levels at la Palicienta appear to be correlated with the influx of freshwater at that time, but the same occurred at Pozo Chino despite the lack of freshwater influx. The rise in bacterial levels at both sites on day 7 occurred when salinity was dropping at Pozo Chino, but there was no change in salinity at La Palicienta at that time. The first rise in the bacterial levels coincided with the neap tides, and the second rise coincided with the spring tides, but increased more with the latter. It may be that that the stronger currents generated during the spring tides re-suspend organic material from the bottom that fosters bacterial growth. Vallaro *et al*., (1950), Araujo *et al*., (1989) y Pérez González (2009) noted that higher levels of organic matter in water tend to favor the proliferation of pathogenic and coliform bacteria. In the case of La Palicienta, the first increase in bacterial levels corresponds to an influx of freshewater, thus suggesting a riverine source of contamination.

The increases in the concentration of coliforms and *E. coli* in oyster tissues during the spring tides at both sites even though bacterial levels in the water were low suggests that oysters may be concentrating bacteria during their filter feeding. Rajagopal et al (1998) notes that increased water flows may affect oyster growth rates through increased filtration rates; thus filtration rates may be higher during the spring tides leading to an increased accumulation of pathogens during that time.

In general, the concentration of fecal coliform in oyster tissues exceeded the legal limits during most of the duration of the experiment. Only on the third day did levels fall to acceptable levels. One factor to consider is that this experiment took place during the period during which coliform levels are highest in the Boca de Camichin Estuary (Olivo Rojas 2007). It may be possible that if the experiment were to be conducted during the winter months when bacterial levels are lower, bacterial levels in tissues might be lower. It is recommended that this experiment be repeated during the cooler months.

Another option may be to investigate the use of land-based tanks for depuration, although this is complicated by the lack of electricity in areas around the estuary where water quality is highest. In a similar case in the Aserradores Estuary, Nicaragua, use of solar electric power is being tested to power a small depuration system. This may allow for use of depuration in remote areas.

CONCLUSION

The concentration of total and fecal coliform, and *E. coli* in water exhibited two cycles during the experimental period at both sites: a cycle of lower magnitude during the neap tides and one with large magnitude during the spring tides. Bacterial concentrations were relatively low during the neap tides but increased rapidly with the spring tides, possibly due to increased rates of filtration. With the exception of the first day at La Palicienta, total and fecal coliform levels always exceeded legally permissible levels. *E. coli* in oyster tissues also exceeded legal limts during the spring tides, while during neap tides, it was below legal limits at La Palicienta. At Pozo Chino, legally acceptable levels occurred only during the fourth day during the neap tide. Depuration is not therefore possible at either site during the summer months, although it may be possible during the cooler months or through use of land-based tank systems.

BENEFICIARIES

Approximately 700 people who culture oysters at Boca de Camichin benefitted from this study as well as five institutions which participated or which received the results.

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