MICROBIOLOGICAL QUALITY OF BIVALVE GROWING WATERS AND TISSUES

Human Health Impacts of Aquaculture/ Experiment/ 07HHI05UH

Erick José Sandoval Palacios, Laura Yasica Arias Armas, Nelvia Hernandez, Eufrecia Balladares, Juan Ramón Bravo, Carlos Rivas, and Rodolfo Lazarich Gener
Centro de Investigación y Desarrollo de Ecosistemas Acuaticas (CIDEA)/Universidad Centroamericana (UCA)
Center for Aquatic Ecosystems Research and Development/Central American University
Managua, Nicaragua

Maria C. Haws
Pacific Aquaculture and Coastal Resources Center
University of Hawaii Sea Grant College Program
University of Hawaii Hilo
Hilo, HI 96720

John Supan
Louisiana State University Sea Grant Program
Baton Rouge, Louisiana

ABSTRACT

Black cockles, (*Anadara similis* and *A. tuberculosa*) are an important fisheries resources throughout Latin America. Women, children and the poor are particularly dependent upon bivalve collection for food and income. Developing shellfish sanitation plans that including water quality monitoring and relay and depuration strategies are key elements of an on-going integrated bivalve fisheries management effort in Nicaragua. *E. coli*, *Salmonella* sp. and *Vibrio parahaeolyticus* levels were monitored monthly over a one year period in the Aserradores Estuary, Nicaragua. *E. coli* levels were highest during the rainy season (May-October) at three out of six stations for cockle tissues and water samples. *Salmonella* sp. was found sporadically, mostly commonly during the dry season (November-April). This indicates the need for on-going water quality monitoring to assure food safety. Depuration in the laboratory and at an open water site indicated that *E. coli* levels are reduced to legally permissible levels in cockle tissues in 8-12 hours. *Vibrio parahaemolyticus* was always within legally permissible levels both in water and tissue samples. Relay and depuration proved to be technically simple with minimal cost, suggesting that cockle gatherers may use this strategy to improve cockle safety, and potentially, product value.

INTRODUCTION

This work had three objectives:

- Monitor the presence of *Salmonella* sp., *Echerichia coli* and *Vibrio parahaemolyticus* in the waters of the Aserradores Estuary at six stations and in the tissues of *Anadara tuberculosa* (black cockle) taken from the estuary; and
- Establish a depuration site in the estuary and conduct controlled depuration trials in the laboratory for two species of *Anadar*a cockles.

Black cockles are an important fisheries resource throughout Latin America hold aquaculture potential as well. The most common species of black cockle are *Anadara similis*, *A. tuberculosa and A. grandis.* These inhabit mangrove ecosystems and are widely distributed along the Pacific Coast of Latin America, ranging from Laguna Ballenas in Baja California to Tumbes, Peru (Keen, 1971). Cockles are generally found in muddy, or sandy-muddy substrates (Cruz and Jimenez, 1994). *A. similis* and *A. tuberculosa* now comprise the bulk of the fishery since the large *A. grandis* is only rarely found due to overfishing. Fishing is primarily conducted by women and children, in part because the cockles are a resource they can access without boats or gear. Cockles are an important daily and emergency food source for inhabitants of poor coastal communities, with women and children being particularly reliant upon bivalve collection to supply basic protein and income needs. Between 1600 and 2000 people gather cockles on a regular basis. Approximately 30 million *A. tuberculosa* and *A. similis* are harvested from Aserradores and three nearby areas, Kilaca, Padre Ramos and El Realejo (CIDEA 2005, 2009). Some of this product is exported to El Salvador and Honduras.

Throughout the LAC region, management of the cockle fishery is a chronic issue. Cockle populations have been overfished through most of their range to the extent that some countries have a permanent ban on cockle collection, although this rarely effectively enforced. Nicaragua has a closed season for cockles from April to July, coinciding with the months of highest demand, rather than any biological basis. Given that enforcement is relatively ineffective, a ban on fishing during times of peak demand has not resolved the fisheries issues. Additionally, since cockles are one of the most important resources for coastal communities, particularly since they are a daily food for many of the poor, when authorities do try to enforce the law, it poses hardship for many coastal residents. One result of enforcement is that the poorer collectors can not commercialize their product, although larger vendors manage to more successfully evade the law.

Since 2004, CIDEA and international partners have employing multiple approaches to addressing the fisheries management issues for cockles in Nicaragua as well as in Tanzania under USAID funding under the SUCCESS program (Sustainable Coastal Communities and Ecosystems) and since 2007, CRSP has provided additional support to this effort. To date, establishment of community-based no-take zones has proven effective in increasing cockle abundance and size in the estuary, including areas outside of the no-take zones.

At the same time, efforts were made to find ways to improve both the safety and value of the cockles for the benefit of consumers and fishers. Although highly nutritious, black cockles, like most filter feeding bivalves, can also represent a serious health issue. Filter feeding bivalves are able to pump water over the gills and mantle tissue, and remove food particles from the water column (Cantelmo et al. 1992). Pathogens that cause human disease may be present in the water, primarily due to contamination by sewage, and these may be ingested as the bivalve filter feeds (up to 50 liters of water per day) (Fernandez et al., 1971; Martinez et al., 1991; Wanke and Guerrant, 1987). Shellfish-borne diseases present serious public health issues around the world (Dsenclos, 1991). Among the most serious and potentially fatal illnesses that can result from eating contaminated bivalves are gastrointestinal illness associated with *E. coli*, *Salmonella*, *Hepatitus* and several types of Vibrios, including *Vibrio vulnificus* and *V. parahaemolyticus*.

This work had two objectives: 1) quantify the depuration rate for cockles in the laboratory to have a baseline for comparison with depuration in the field; and 2) test relay and depuration methods in the field. If inexpensive and simple depuration methods are developed that villagers can use, it would have multiple benefits including reducing the risk associated with consuming cockles, increase stakeholder engagement in the broader management efforts and potentially add value to the product. Additionally, the water quality monitoring not only would allow identification of possible depuration sites and high risk sites, it could inform future management decisions about where to establish no-take zones for the cockle fisheries management efforts. Ideally, the no-take zones could be re-located in the most contaminated areas to both reduce human health risks but also increase the probability of community cooperation with this management strategy.

In cases where shellfish growing grounds are contaminated to the extent that consuming bivalves gathered from these areas is hazardous to human health, three options exist. In some cases, contamination is seasonal and thus shellfish may be safely harvested periodically from the area. In the case of Asserradores, this option was judged not to be viable due to the short period of time in which cockles could be harvested and because the ability to constantly monitor water quality throughout the large estuary was not assured. Depuration is another option; this involves keeping contaminated bivalves in clean water until they can purge themselves of pathogens. Depuration can reliably remove bacteria, but not all viruses, although virus load may be reduced. Two depuration options could have been feasible in Asserradores. One would be to use land-based tanks to hold the cockles during the depuration period. A second option is to conduct the depuration in an area of the estuary which has been shown to be clean and which can easily be monitored on a regular basis to assure that its water quality remains at an acceptable level. In this case, the latter option was chosen as the being the least expensive, most viable for the community to maintain with minimal technical assistance and because larger numbers of cockles could be depurated at any one time. The process of harvesting cockles from a contaminated area and conducting depuration at another site is referred to as, “relay and depuration”. This is a common practice in other shellfish fisheries, for example, in the Gulf of Mexico oyster industry.

Generally *E. coli* is used as an indicator organism for shellfish sanitation since its presence is correlated with contamination from human or animal wastes (Fernández and Brunker 1977, McNeely 1979). Additionally, levels of *V. parahaemolyticus* and Salmonella sp. were measured in this study. Although several species of cockles are fished, only *A. tuberculosa* was studied due to its size and importance in the fishery.

MATERIALS AND METHODS

I. Baseline analysis of the presence of pathogens in cockle tissues and growing ground waters

Six sampling stations were established within the Aserradores Estuary for collection of water samples and cockle tissue samples

Figure 1. Location of sampling stations in the Aserradores Estuary

Stations	Coordinates (UTM)	
Station 1	UTM	0462964
		1393996
Station 2	UTM	0465682
		1393984
Station 3	UTM	0464418
		1396710
Station 4	UTM	0463631
		1397458

Station 5	UTM	0466130
		1396366
Station 6	UTM	0465416
		1399132

Cockles (*A. tuberculosa*) and water samples were collected from each station monthly from August 2008 to August 2009. Cockles and water samples were placed in sterilized plastic bags after collection and transported to the laboratory. The water samples were immediately stored on ice and transported at a temperature of <10ºC.

Analysis of *E. coli* levels in water was conducted using the Most Probable Number (MPN) method according to standard protocols (Standard Methods, 2005). The same methods was used for the tissue analysis as specified by the FDA (U.S. Food and Drug Administration) (BAM, 1998). Permissible *E. coli* levels were established by the EPA (1976) and are the same as those established by the Nicaraguan Government (CEPIS, 1986), i.e. < 43 MPN/100 ml water. Permissible levels for *E. coli* in molluscan bivalves have been established as $<1.0x10^2$ MPN/g (NTON, 2008). *Salmonella* spp. analysis was conducted by a isolation method, a modification of the standard method specified by the FDA (BAM, 1998). Permissible levels of *Salmonella* spp. in frozen or fresh seafood are zero (NTON, 2008). Dectection of *V. parahaemolyticus* in water and cockle tissue was conducted using TCBS agar plates with 5% NaCl. The permissible level established by NTON is $<1.0x10^3$ UFC/g. The CIDEA/UCA laboratory is certified by the National Accreditation Office under regulation NTN 04 001 05 which is the equivalent of ISO/IEC 17025-05.

II. Comparison of cockle depuration rates in the field and laboratory
This study was conducted using 504 cockles collected from the Aserradores Estuary. To monitor depuration rates in the laboratory, 6-24 gallon aquaria were used. The water supply was recirculated through a sand filter and was monitored to assure bacteriological quality. Twenty-four cockles were placed in each aquarium and left for periods of 4, 8, 12, 24, 48 and 72 hours respectively before being removed for bacteriological analysis of their tissues. Assays were conducted as noted above for *E. coli*, *V. parahaemolyticus* and *Salmonella* spp.

The field depuration site was selected based on previous water quality monitoring which demonstrated that E. coli levels were well below permissible levels. Cockles were suspended in net bags at this site at 1 meter depth. In order to also evaluate whether the number of cockles in a net bag affected the depuration time, comparisons were made between bags with 12 or 24 cockles. Cockles were kept at the site for 4, 8, 12, 24, 48 and 72 hours before being removed for bacteriological analysis.

RESULTS

I. Baseline analysis of the presence of pathogens in cockle tissues and growing ground waters
All sampling stations except for Station 1 were located within the internal branches of the estuary. Station 1 was located at near the mouth of the estuary. Cockle for tissue analysis were not collected at this point due to the difficulty in obtaining sufficient numbers for the analysis.

Six water and sixty cockle samples (randomly selected from among the cockles collected) were analyzed each month for a total of 72 water samples and 720 cockles over the year long monitoring period.

**Figure . ***E. coli* levels in water samples (MPN/100 ml).
Highlighted data indicates levels above legally permissible levels.

	Station					
Sampling period	**1 Estuary mouth**	**2 Los Tornos**	**3 Rio Viejo**	**4 Rio Viejo**	**5 La Chanchera**	**6 La Chanchera**
August 2008	<1.8	4.5	79	17	33	11
September 2008	7.8	17	14	7.3	130	<20
October 2008	920	0	540	50.4	130	170
November 2008	2	25	45	80	8	<20
January 2009	4	13	49	49	4	<20
February 2009	<1.8	23	2	17	6.8	<20
March 2009	<1.8	6.8	7.8	4.5	4.5	<20
April 2009	2	2	<1.8	2	2	20
May 2009	7.8	33	4.5	13	13	20
June 2009	46	33	49	1600	49	<20
July 2009	2	6.8	17	23	49	20
August 2009	11	23	2	11	6.8	20

Figure 2. *E. coli* levels in tissue samples (MPN/g)
Highlighted data indicates levels above legally permissible levels.

	Station					
Sampling period	**1 Estuary mouth**	**2 Los Tornos**	**3 Rio Viejo**	**4 Rio Viejo**	**5 La Chanchera**	**6 La Chanchera**
August 2008	NA	330	80	<20	20	50
September 2008	NA	<20	20	80	80	<20
October 2008	NA	<20	20	50	130	170
November 2008	NA	<20	<20	<20	<20	<20
January 2009	NA	<20	<20	<20	<20	<20
February 2009	NA	20	<20	<20	<20	<20
March 2009	NA	<20	<20	<20	<20	<20
April 2009	NA	3500	<20	20	<20	20
May 2009	NA	<20	<20	<20	<20	20
June 2009	NA	<20	20	700	20	<20
July 2009	NA	20	<20	<20	<20	20
August 2009	11	20	<20	40	<20	20

Figure 3. Presence or absence of *Salmonella* spp.
ND= not detected, W= in water samples; T= in tissue samples

	Station					
Sampling	**1**	**2**	**3**	**4**	**5**	**6**

period	Estuary mouth		Los Tornos		Rio Viejo		Rio Viejo		La Chanchera		La Chanchera	
	W	T	W	T	W	T	W	T	W	T	W	T
August 2008	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
September 2008	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
October 2008	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
November 2008	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
January 2009	ND	NA	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
February 2009	ND	NA	ND	ND	ND	ND	X	ND	X	X	X	ND
March 2009	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	X	ND
April 2009	ND	NA	X	ND	ND	ND	X	ND	ND	ND	ND	ND
May 2009	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
June 2009	ND	NA	ND	ND	ND	ND	ND	ND	X	X	ND	ND
July 2009	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	X
August 2009	ND	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

V. parahaemolyticus was either not detected or levels were always within permissible levels for both tissue and water samples from all stations during all sampling periods.

II. Comparison of cockle depuration rates in the field and laboratory

Laboratory depuration

Table 1 summarized the results of cockles held in aquaria with recirculating, filtered seawater for varying periods of time. Initially cockle tissues exhibited high levels of fecal coliform at 330 MPN/g and *E. coli* at 330 MPN/g. Salmonella was not detected and *V. parahaemolyticus* was below permissible levels at < 1.0×10^3 UFC/g. After 12 hours of depuration, both fecal and total E. coli levels fell to permissible levels.

Table 1; Bacteriological results for cockle tissues held in laboratory depuration system. Highlighted text indicates reduction to legally permissible rates for coliforms.

Time in aquarium	Fecal coliform (MPN/g)	*E. coli* (MPN/g)	*Salmonella* sp. UFC/ 25 g	*Vibrio parahaemolyticus*
0 hrs	330	330	Ausencia	< 1.0×10^3 UFC/g
4 hrs	78	78	Ausencia	< 1.0×10^3 UFC/g
8 hrs	78	78	Ausencia	< 1.0×10^3 UFC/g
12 hrs	20	20	Ausencia	< 1.0x103 UFC/g
24 hrs	< 20	< 20	Ausencia	< 1.0×10^3 UFC/g
48 hrs	< 20	< 20	Ausencia	< 1.0×10^3 UFC/g
72 hrs	< 20	< 20	Ausencia	< 1.0×10^3 UFC/g

Relay and depuration

A depuration site was selected across from the coast of Aserradores Island. Water quality at this site was monitored for three months prior to initiation of the tests. Coliform levels were always lower than the permissible levels. Cockles were put into 2 containers with wood frames and mesh sides with 408 cockles in the first container, and 240 cockles in the second container. Both containers were submerged to a depth of 1 meter. Samples from each container were removed at 4, 8, 12, 24, 48 and 72 hours. At the end of 72 hours, samples were transported back to the CIDEA laboratory for bacteriological analysis.

Table 2. Bacteriological analysis of cockle tissues from relay and depuration trials (408 cockles/container)

Depuration tiem	Fecal coliform (MPN/g)	*E.coli* (MPN/g)	*Salmonella* sp UFC/ 25 g	*Vibrio parahaemolyticus*
0 hrs	170	130	Re-testing	< $1.0x10^2$ UFC/g
4 hrs	350	130	Re-testing	< $1.0x10^2$ UFC/g
8 hrs	50	20	Re-testing	< $1.0x10^2$ UFC/g
12 hrs	20	20	Re-testing	< $1.0x10^2$ UFC/g
24 hrs	<20	<20	Re-testing	< $1.0x10^2$ UFC/g
48 hrs	65	50	Re-testing	< $1.0x10^2$ UFC/g
72 hrs	170	<20	Re-testing	< $1.0x10^2$ UFC/g

Similar to the depuration rates observed in the laboratory, the field trials showed adequate depuration rates after 8 hours.

DISCUSSION

The results of this study indicate the need for caution in harvesting black cockles because of high levels of pathogens at some stations during certain months, but also indicates that they can be gathered during certain time and from specific locations with minimal risk to the consumer. *E. coli* levels in waters were highest during the rainy season (May-November), most likely due to run-off from neighboring residential and cattle grazing areas. Earlier studies indicated that the most contaminated areas are closest to cattle grazing areas (SUCCESS, 2008). Contamination was also found in some areas during the June-July period, thus suggesting that some risk is present during all times of year. *E. coli* levels in cockle tissues, however, only exceeded permissible levels three times during the year indicating that *E. coli* levels in tissues and water from the same site are not necessarily correlated. Most shellfish sanitation programs however, monitor only *E. coli* in water and prohibit collection of shellfish at particular sites when *E. coli* levels exceed the legal standard as a precautionary measure. Although several more years of data may be necessary to reliably confirm temporal patterns in *E. coli* presence and concentrations, it would be reasonable to exercise more caution in designating areas where shellfish could be collected during the rainy season. At the same time, the monitoring did reveal that several areas appear to be relatively safe for shellfish collection over the entire year, particularly Stations 1 and 6. Shellfish can also be moved to these areas for depuration.

Salmonella was detected in water at Stations 2, 4, 5 and 6 but in tissues only at Stations 4, 5 and 6. Given the zero tolerance in regulations for seafood in the case of *Salmonella* and its potential lethality, caution would be indicated in these areas. *Salmonella* occurrence exhibited a different pattern than *E. coli*, being most commonly found during the dry season. The alternation of high levels of *E. coli* and *Salmonella* during the year suggested that in some sites, it may be unsafe to harvest shellfish at any time of year.

V. parahaemolyticus was either not detected or was within permissible levels at all stations at all sampling periods and may therefore not be a pathogen of concern in this estuary.

CONCLUSION

This work indicates that cockles can be depurated rapidly enough in both the laboratory and field that either tank systems or an open water depuration site could help improve cockle safety. A tank depuration system is being tested in 2010 using solar electric power funded by a grant from the European Union. The field experiment also demonstrated that as long as technical assistance is provided to routinely water quality, the Aserradores community can use open water depuration sites to improve cockle safety with a minimum of delay and cost. Although a longer period of monitoring is required for validation of the water quality results, this work along with a previous year's data of water quality monitoring in the Aserradores Estuary is assisting researchers to develop a database that will allow prediction of annual patterns of contamination. Aside from the obvious benefits to human health, this will also allow for improved site selection for no-take zones to support cockle fisheries management since ideally the no-take zones will coincide with the most heavily contaminated areas.

ANTICIPATED BENEFITS

Approximately 700 people in the Aserradores Estuary community collect or consume cockles. This work lays the ground work for efforts to improve human health, protect the cockle resource and improve the economic status of community residents.

ACKNOWLEDGEMENTS

The Aquaculture and Fisheries Collaborative Research Support Program is gratefully acknowledged for the support which made this effort possible. The USAID SUCCESS program is also due thanks for their technical and financial support.

LITERATURE CITED

Bacteriological Analytical Manual (BAM) Edition 8, Revision A /1998. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm.

Cantelmo, F. and T.Carte. 1992. A physiological indicator of hard clam commercial depuration. MTS Journal 23:9-13.

Centro Panamericano de Ingeniería Sanitaria (CEPIS) (1986). *Calidad del agua en el medio marino.* Historia y aplicación de normas bacteriolólogicas. 27 pp.

CIDEA. 2005. *Estudio de mercado de moluscos, ostras, mejillones y almejas en Centroamericaa.* Technical report.

CIDEA. 2009. Estudio de mercado de la concha negra (Anadara similis and Anadara tuberculosa) in Nicaragua. Technical report.

Cruz, R. and J. Jiménez. 1994. Moluscos asociados a las áreas de manglar de la Costa Pacífica de América Central. Editorial Fundación UNA. 180 p.

Dsenclos J.C.A., K.C. Klontz, M.H. Wilder, O.V. Nainan, H.S. Margolis and R.A. Gunn. 1991. A multistate outbreak of Hepatitis A caused by the consumption of raw oysters. Am. J. P. Health 81:1268-1272.

Environmental Protection Agency. 2005. Standard methods for examination of wastewater. Washington: American Public Health Association.

Fernández, B., T. Brunker and C. González. 1971. Calidad Sanitaria de las Aguas de la Playa de Puntarenas. Acta Med. Cost. 14:91-100.

Fernández, B. and T. Brunker. 1977. Estudio bacteriológico de bivalvos del Golfo de Nicoya, Costa Rica. Condición del molusco recién recolectado. Rev. Biol. Trop. 25:101-107.

Keen, A. 1971. Sea shells of tropical West America. 2 ed. California. Stanford University Press.

Martínez, E., F. Egea, D. Castro, M. Morinigo, P. Romero and J. Barrigo. 1991. Accumulation and depuration of pathogenic and indicator microorganisms by the mollusk *Chlamelea gallina,* under controlled laboratory odnditions. J. Food Protection. 54:612-618.

MIFIC (2009) *NTON 03 080 08: Criterios microbiológicos para la inocuidad de alimentos*: Comisión Nacional de Normalización Técnica y Calidad, Ministerio de Fomento Industria y Comercio.

McNeely R.N. V.P. Neimanis and L. Dwyer. 1979. Water Quality Sourcebook. A Guide to Water Quality Parameters. Inland Waters Directorate. Water Quality Branch, Ottawa, Canada. p. 17-57.

Sustainable Coastal Communities and Ecosystems. 2008. United States Agency for International Development (USAID). Annual report by the Coastal Resources Center, University of Rhode Island. Narragansett, RI.

Wanke C.A. and R.L. Guerrant. 1987. Viral hepatitis and gastroenteritis transmitted by shellfish and water. Inf. Dis. Clin. N. 1:649-664.