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Coliform risk assessment through use of the clam *Anomalocardia brasiliana* as animal sentinel for shellfish harvesting areas in Brazil's northeast

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Abstract

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Introduction

The long Brazilian coastline favors natural occurrence of many species of marine shellfish with high economic value. However, the quality and quantity of contaminating microorganisms in shellfish meats can vary under the influence of extrinsic factors such as tides and rainfall regimes (Solic et al. 1999; Brands et al. 2005; Band and Salvesen 2009). Human consumption of raw or undercooked shellfish is often associated with gastroenteritis episodes and intoxication by enteric human viruses as well as bacterial pathogens such as *Salmonella*, *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus* (Bauer et al. 2006; Ueki et al. 2007; Nappier et al. 2008). Infectious outbreaks have been reported in the USA, UK, Australia, Japan, Spain, Italy, China, Canada, Malaysia, Singapore, France and New Zealand (Rippey 1994; Potasman et al. 2002), but not in Brazil, possibly due to deficient reporting.

Here we investigate the suitability of the clam *Anomalocardia brasiliana* as an animal sentinel for coliform monitoring in shellfish harvesting areas of Brazil's northeast. According to the World Register of Marine Species (WoRMS), *A. brasiliana* (Gmelin 1971; Veneridae family) is a synonymized taxa for *A. flexuosa* (Gmelin 1791), also recognized with different scientific names: *A. rugosa* (Schumacher 1817), *Cryptogramma flexuosa* (Linnaeus 1767), *Venus brasiliana* (Gmelin 1791), *Venus flexuosa* (Linnaeus 1767), *Venus punctifera* (G.B. Sowerby II 1853) (WoRNS 2012). This shellfish and its synonymized taxa is distributed in countries on the Atlantic seaboard and Caribbean, including Belize, Colombia, Costa Rica, Cuba, Jamaica, Lesser Antilles, Puerto Rico and Venezuela, as well as in Madagascar (WoRNS 2012). Therefore, the potential of these shellfish as bioindicators has broad reach.

Rainfall levels are known to affect seafood quality. Due to the irregularity of precipitation in Brazil's northeast, samples of shellfish meats of *A. brasiliana* were collected at random in the bay area of Mangue Seco (state of Pernambuco), and the population of thermotolerant coliforms was investigated. The contamination levels were tentatively correlated with the precipitation recorded on the day of sampling or 24s to 48 h beforehand, during a 1-year-study. Additionally, frozen samples of shellfish meats obtained from local retail shops were also investigated. According to the results, we

recommend the use of the clam *A. brasiliana* as an animal sentinel in harvesting areas of Brazil's northeast.

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Material and methods

Study area

Clams were harvested along a 1600 m stretch of Mangue Seco Beach (7° 50′ 03.90″ S; 34° 50′ 39.52″ W), in the town of Igarassu, from April 2009 to March 2010 (Fig. 1a). This area is part of a highly productive ecosystem with extensive mangrove estuaries, segments of plains covered by coconut trees, coral reefs and small islands. The weather is usually hot and sunny, except for a period from April to June, when the rainfall reduces temperatures to approximately 24–26 °C, 3–6 °C degrees below those registered in the rest of the year. This beach is a natural breeding place for many species of mollusks, and particularly *A. brasiliana*. There are no structures near the shore, except for a group of small restaurants in the first 200 m of the beach (Fig. 1a, Section S1). At low tide, the beach is crowded with people from local coastal communities who harvest clams. The shellfish gathered are intended for personal consumption and to supply local restaurants and markets. Commercial shellfish were obtained at retail shops in the metropolitan area of Recife from August 2010 to June 2011. The precipitation throughout the study period is shown in Fig. 1b.

Fig. 1

Study area (**a**) and mean precipitation recorded from April/ 2009 through July/ 2011 (**b**). Satellite images: Google Earth Software

Sampling procedures

Mature *A. brasiliana* specimens (40 clams per sample; $n = 8$ per collection) measuring 20 to 25 mm in length were harvested during low tide from eight sampling points along Mangue Seco Bay, separated by 200 m (Fig. 1a; sections S1 to S8). The samples were collected in isothermal plastic bags from April through August 2009 (rainy season), and from September 2009 through March 2010 (dry season). Random samples of frozen shelled clam meat (250 g per sample/ $n = 3$ per market) were obtained from retail shops from August through November 2010 (dry season), and from April through June 2011 (rainy season).

Determination of the most probable number (MPN) of thermotolerant coliforms

The presence of thermotolerant coliforms in *A. brasiliana* meat was evaluated through the most probable number technique (MPN), following the recommendations of the National Sanitary Surveillance Agency, ANVISA (Brasil 2011). Briefly, the samples

were diluted to 10^{-1} to 10^{-3} and then 0.1 mL aliquots from each dilution were added to a set of nine tubes (triplicate) with lactose broth (LB) for presumptive identification of coliforms. Coliform bacteria were confirmed after incubation for 24–48 h at 35 °C in brilliant green bile broth (BGB). Then, 0.1 mL of material from the BGB-positive tubes was added to tubes with *E. coli* broth (EC) selective media, which were incubated for a further 24 h at 44.5 °C. After this time, bacterial growth plus gas production into Durham tubes was interpreted as indicative of thermotolerant coliforms. Results were expressed as mean geometric density of thermotolerant coliforms (MPN/ g).

Hypothesis and statistical analyses

The bacterial populations in shellfish meats of *A. brasiliana* were recorded during a 1-year-study to test the following null hypotheses:

a. No correlation between recent rainfall events and contamination levels by thermotolerant coliforms in shellfish meats of *A. brasiliana* gathered along Mangue Seco Beach.
b. No correlation between the local rainfall regime and contamination levels by thermotolerant coliforms in shellfish meats of *A. brasiliana* sold in retail shops.

For statistical evaluation of the contamination levels of thermotolerant coliforms, the values bellow < 3 MPN/ g were normalized as "2", and those above > 1100 MPN/ g as "1200". In this case, the mean geometric density of coliforms was calculated among samples collected on a single day, and then tested for Pearson's correlation to the precipitation recorded on day of collection and 24 to 48 h beforehand. The contamination levels of coliforms in shellfish meat obtained in local retail shops discriminated among samples sold in the dry and rainy seasons. Simple comparisons between populations of bacteria of two different samples were made by the Student *t*-test followed by the Mann-Whitney test. In all situations, the level of significance was set at $P < 0.05$. The Prism GraphPad (version 5.1) program was used for calculations. Data on precipitation was provided by the local official agency (National Institute of Meteorology, Recife).

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Results and discussion

Previous data have shown the suitability of animal sentinels for monitoring seafood quality (Martinez and Oliveira 2010). Here, the presence of thermotolerant coliforms in shellfish meats of *A. brasiliana* was higher following rainfall events and lower in the dry season of the year (Fig. 2). The likelihood of the rainfall regimen affecting the quantity of coliforms in contaminated samples was positive (Table 1). This was especially clear when these contamination levels were compared to the precipitation data 1 day before sampling ($P < 0.0001$). Moreover, a large number of shellfish meat samples collected in rainy periods were above the Brazilian legal threshold of 3×10^2 MPN/ 100 g determined for production areas (Table 2).

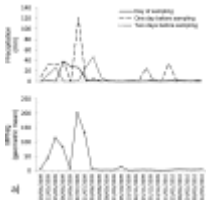

Fig. 2
Contamination levels by thermotolerant coliforms in shellfish meats of *A. brasiliana* during 1-year-study

Table 1

Pearson correlation analyses between precipitation and the contamination levels of the shellfish meats of *A. brasiliana* during 1-year study

Precipitation (mm) versus contamination by thermotolerant coliforms [a]	Day of sampling	One day before sampling
Number of XY Pairs	23	23
Pearson r	0.5069	0.7641
95 % confidence interval	0.1197 to 0.7603	0.5136 to 0.89
P value (two-tailed)	0.0136	*P* < 0.0001
Is the correlation significant? (alpha= 0.05)	Yes	Yes
R squared	0.2570	0.5838

[a]Geometric mean of MPN/ g was calculated for eight samples per day of collection. Pre

Table 1
Pearson correlation analyses between precipitation and the contamination levels of thermotolerant coliforms in shelfish meats of *A. brasiliana* during 1-year study

Table 2

Contamination levels of thermotolerant coliforms above the threshold determinate by t standards set forth for shellfish meats

	Apr/ 09	Mai/ 09	Jun/ 09	Jul/ 09	Aug/ 09	Sep/ 09	Oct/ 09	Nov/ 09	Dec/ 0
No. of samples of shellfish meats above 3×10^2 MPN/ 100 g of thermotolerant coliforms									
Number	3 out of 8	9 out of 16	10 out of 16	15 out of 16	7 out of 16	7 out of 16	7 out of 16	5 out of 16	3 out o 16
%	37.5	56.2	62.5	93.7	43.7	43.7	43.7	31.2	18.7

* The Brazilian official standards set forth establish a maximum of < 3×10^2 MPN/ 10 coliforms for shellfish meats in harvesting areas (BRASIL 2011)

Table 2
Contamination levels of thermotolerant coliforms above the threshold determinate by the Brazilian official standards set forth for shellfish meats

Rainfall causes suspension of bottom sediments and input of nutrients into seawater, favoring eutrophication and shellfish bioaccumulation. Therefore, seawater quality degradation in estuaries often increases from 1 to 2 days after rainfall events greater than 10 mm/day (Bougeard et al. 2011). Trials carried out in Hawaii also showed that fecal coliforms deposited in the soil can survive on substrates and eventually be detected in the water column (Fujioka et al. 1998). *A. brasiliana* can filter an average of 19 to 50 L of water per hour (Barnes 1984). The presence of mucus in the gill filament assists in capturing the phytoplankton used as food, and microorganisms that accumulate in the visceral mass hepatopancreas and intestinal lumen (Cook 1991). Our results show that *A. brasiliana* was very sensitive to detect contamination by coliforms in the water column after recent rainfall events.

To prevent outbreaks and ensure the quality of shellfish for human consumption, classification of bivalve mollusk production areas in Europe establishes a maximum of 2.3×10^2 MPN of *E. coli* per 100 g of shellfish meat (CEFAS 2010). In this case, depuration processes are not necessary. In Brazil, current policies establish a threshold 2.3×10^2 MPN of *E. coli* or 3×10^2 MPN of thermotolerant coliforms per 100 g of shellfish meat in harvesting areas (BRASIL 2011), and 1×10^2 MPN of thermotolerant coliforms per *g* of shellfish meat processed for human consumption (BRASIL 2001). Since the level of thermotolerant coliforms in shellfish meats of *A. brasiliana* increased

with rainfall, we also investigated clam meat sold at retail shops. We confirmed that the risk to human health was higher for frozen meat sold in the rainy season than in the dry season of the year ($P < 0.0001$) (Table 3).

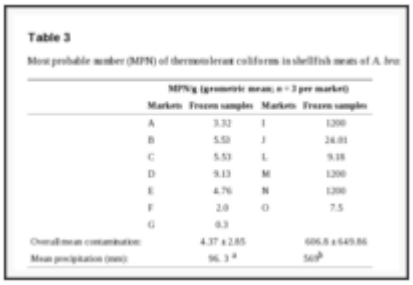

Table 3

Most probable number (MPN) of thermotolerant coliforms in shellfish meats of *A. bra*

	MPN/g (geometric mean; n = 3 per market)			
	Markets	Frozen samples	Markets	Frozen samples
	A	3.32	I	1200
	B	5.52	J	24.01
	C	5.53	L	9.18
	D	9.13	M	1200
	E	4.76	N	1200
	F	2.0	O	7.5
	G	0.3		
Overall mean contamination:		4.37 ± 2.85		606.8 ± 649.86
Mean precipitation (mm):		96. 3 [a]		569[b]

Table 3

Most probable number (MPN) of thermotolerant coliforms in shellfish meats of *A. brasiliana* traded in retail shops

Bacterial contamination of commercial shellfish is commonly affected by the season and tidal cycle (Lee and Morgan 2003). As a measure to diminish the risk to public health, closure of shellfish harvesting areas has been adopted after heavy rainfall events in Canada (Canadian Food Inspection Agency 2011). Recently we showed that several coagulase-negative staphylococcal species detected in shellfish meat of *A. brasiliana* were multi-drug resistant and positive for the presence of the *mec*A gene (Batista et al. 2013). Moreover, the enterotoxin-encoding genes *seg* and *seh* were detected among isolates from both environmental samples and those from retail shops (Batista et al. 2013). Taken together, our data provide further evidence that shellfish collected at Mangue Seco Bay area are prospective reservoirs of bacterial pathogens.

The results indicate the suitability of *A. brasiliana* as an animal sentinel for shellfish harvesting areas of Brazil's northeast. Although a previous study suggested *A. brasiliana* for use as a bioindicator (Barros 2009), until the present work this was not tested in a long-term study. We conclude that depuration of shellfish collected in the bay area of Mangue Seco is crucial to prevent outbreaks, regardless of the rainfall regimen. Also, closure of shellfish harvesting areas for at least 24 h following heavy rainfall events is recommended.