Evaluation of *Escherichia coli* concentrations in *Crassostrea gigas* and seawater in two oyster growing areas in the Gulf of Nicoya, Costa Rica

Evaluación de las concentraciones de *Escherichia coli* en *Crassostrea gigas* y agua de mar en dos zonas de cultivo de ostras ubicadas en el Golfo de Nicoya, Costa Rica

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ABSTRACT

Costa Rica does not have references on the health quality of *Crassostrea gigas* growing areas, which can pose a potential public health risk. This study evaluated the temporal trends of *Escherichia coli* concentrations in the *C. gigas* oyster and in the seawater of Punta Cuchillo and Punta Morales growing areas as a basis for the classification and monitoring of bivalve mollusc production sites in the Gulf of Nicoya, Costa Rica. Monthly samples of seawater and *C. gigas* were collected from each site from July 2011 to June 2012, and their levels of *E. coli* were determined using the most probable number method. Temperature and salinity were recorded at a one meter depth. Precipitation data was provided by *Instituto Meteorológico Nacional* (the National Meteorological Institute). Results indicated that the evaluated areas are probably being affected by wastewater discharge. Punta Cuchillo and Punta Morales could be classified as class A or authorized areas for the production of bivalve molluscs. A significant seasonal variability of *E. coli* concentrations was determined in seawater and oysters from the Gulf of Nicoya, defined primarily by salinity. National legislation should be created to establish controls and implement a monitoring system ensuring the bacteriological quality of the areas used for the cultivation of bivalve molluscs in the country.

Keywords: Marine pollution, mariculture, *Escherichia coli*, *Crassostrea gigas*, Costa Rica.

RESUMEN

Costa Rica no cuenta con referencias sobre la calidad sanitaria de las zonas de cultivo de *Crassostrea gigas* y esto puede representar un riesgo para la salud pública. Se evaluó la tendencia temporal de las concentraciones de *Escherichia coli* en la ostra *C. gigas* y en el agua de mar de las zonas de cultivo de Punta Cuchillo y de Punta Morales como base para la clasificación y la vigilancia de los sitios de producción de moluscos bivalvos en el Golfo de Nicoya, Costa Rica. Muestras mensuales de agua de mar y de *C. gigas* de cada zona fueron recolectadas de julio de 2011 a junio de 2012 y se les determinó los niveles de *E. coli* por la técnica del número más probable. La temperatura y la salinidad fueron registradas a un metro de profundidad. Los datos de precipitación fueron suministrados por el Instituto Meteorológico Nacional. Los resultados indicaron que las áreas evaluadas podrían estar siendo afectadas por el vertido de aguas residuales. Punta Cuchillo y Punta Morales podrían clasificarse como zonas autorizadas o tipo A para la producción de moluscos bivalvos. Se determinó una variabilidad estacional significativa de las concentraciones de *E. coli* en agua de mar y en ostras del Golfo de Nicoya, definida principalmente por la salinidad. Se debe generar una normativa nacional que establezca controles e implemente un sistema de vigilancia que asegure la calidad bacteriológica de las áreas destinadas para el cultivo de moluscos bivalvos en el país.

Palabras claves: Contaminación marina, maricultura, *Escherichia coli*, *Crassostrea gigas*, Costa Rica.

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INTRODUCTION

Marine ecosystems are vulnerable to anthropogenic pollution. According to Halpern *et al.* (2008), there are no marine coastal areas that have not been impacted by human influence. The discharge of wastewater in coastal areas is considered the main cause of degradation of bivalve mollusc growing areas (Oliveira *et al.* 2011). Evaluating bacteriological contamination in these areas is important since bivalve molluscs can accumulate bacteria potentially pathogenic to humans (Lee *et al.* 2008).

International regulations for the evaluation of the bacteriological quality of bivalve mollusc growing areas are based on the quantification of *Escherichia coli* as a fecal contamination indicator (Lee *et al.* 2008; Oliveira *et al.* 2011). Recent studies have shown that concentrations of *E. coli* in bivalve mollusc growing areas depend on, among many other factors, seasonal and climatic conditions of the area (Campos *et al.* 2013b; Derolez *et al.* 2013; Lee & Silk, 2013; Mignani *et al.* 2013).

Costa Rica does not have health regulations regarding mariculture, and production areas for oysters (*Crassostrea gigas*) do not have any references to evaluate their bacteriological quality. Oyster growing areas have been developed on a small scale in the Gulf of Nicoya. These areas are vulnerable to microbial contamination because they receive wastewater. This represents a potential risk to public health and to the development of this economic activity.

The objective of this research was to evaluate the temporal trend of *E. coli* concentrations in the *C. gigas* oyster and in the seawater in the growing areas in Punta Cuchillo and Punta Morales as a basis for the classification and monitoring of bivalve mollusc growing areas in the Gulf of Nicoya, Costa Rica.

MATERIALS AND METHODS

E. coli contamination was assessed in the oyster growing areas in Punta Cuchillo (9° 49' 48" N and 84° 52' 48" W) and Punta Morales (10° 4' 84" N and 84° 58' 3" W), located in the Gulf of Nicoya, Costa Rica (Fig. 1). A systematic random sampling plan was applied. Monthly samples of seawater and *C. gigas* were collected from each site from July 2011 to June 2012. With the same frequency, temperature and salinity were recorded for each site at a one meter depth using a YSI-556 multiparameter. Daily and monthly accumulated precipitation data during the sampling period was provided by *Instituto Meteorológico Nacional de Costa Rica* (the National Meteorological Institute of Costa Rica) and corresponded to the average existing records at the weather stations located in the study area (Puntarenas Weather Station: 09° 58' N and 84° 49' W, altitude = 15 masl; Paquera Weather Station: 09° 49' N and 84° 56' W, altitude = 15 masl) (Fig. 1).

Seawater samples were collected at a one meter depth using a Niskin bottle. Between 15 and 25 oyster samples were collected directly from culture lantern nets. Samples were transported to the laboratory in ice (< 9° C) and analyzed within 8.5 h. The determination of the most probable

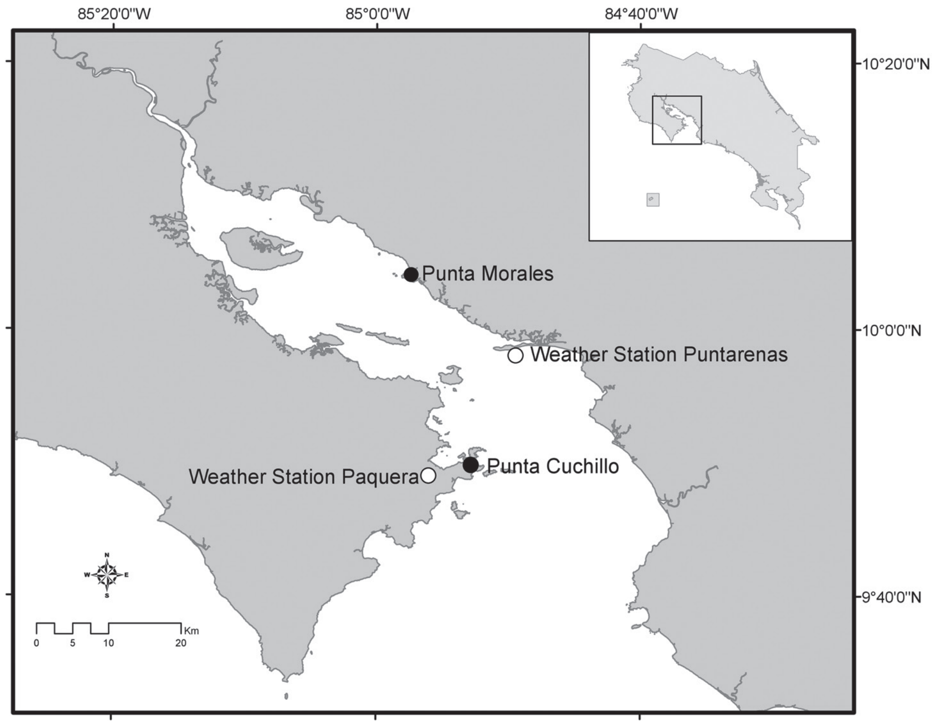

Fig. 1. Geographic location of oyster growing areas (●) and weather stations (○) in the Gulf of Nicoya, Costa Rica

Fig. 1. Ubicación geográfica de las zonas de cultivo de ostras (●) y de las estaciones meteorológicas (○) en el Golfo de Nicoya, Costa Rica

number (MPN) of *E. coli* (series of five tubes and three dilutions) was performed based on Clesceri *et al.* (1989) and Downes & Ito (2001).

For the presumptive phase, each series of five Lauryl Tryptose Broth (BD®) tubes was inoculated with 10, 1 and 0.1 mL of seawater sample. For oyster samples 25 g of flesh and intravalvular liquid were homogenized in 225 mL of Buffered Peptone Water (BD®). Dilutions of 1:100 and 1:1000 were prepared from this mixture. From each dilution 5 Lauryl Tryptose Broth tubes were inoculated.

The confirmatory phase for both samples (seawater and oysters) was done in Brilliant Green Bile Broth (OXOID®) for total coliforms and EC Broth (OXOID®) for *E. coli*. *E. coli* was isolated using Levine Agar (EMB) (OXOID®) and identified by IMViC (OXOID®) biochemical tests and miniaturized API® 20 E (bioMérieux®) test. *E. coli* concentrations per type of sample were obtained from the MPN table (Clesceri *et al*. 1989; Downes & Ito, 2001).

The geometric mean and the 90th percentile of the *E. coli* concentration was calculated by sample type to classify the growing areas based on the criteria established in the United States of America (Lee *et al.* 2008) and the European Union

(Oliveira *et al.* 2011) (Tables 1 and 2). These regulations were chosen since both systems are important in terms of international trade and set regulations to be met by countries exporting to these regions (Lee *et al*. 2008).

The Pearson correlation coefficient was determined between monthly accumulated rainfall and salinity and temperature for each growing site. A Generalized Linear Model (GLM) with log link function and a Poisson error distribution was applied to evaluate the effect of seasonal variability in the *E. coli* concentrations in the study area.

Results were plotted and analyzed using the R programming language (R Core Team, 2013). Values below the MPN method detection limit were categorized and plotted as non-detectable.

RESULTS

The presence of *E. coli* was determined in 42% of the seawater and oyster samples collected in the Punta Cuchillo growing area. In the Punta Morales growing area this bacterium was found more frequently in the seawater samples (83%) than in the oyster samples (25%). The geometric mean and the 90th

Table 1. Classification criteria for bivalve mollusc growing areas based on seawater samples
Cuadro 1. Criterios de clasificación de zonas de producción de moluscos bivalvos con base en muestras de agua de mar

Classification category	Fecal coliforms (MPN 100 mL^{-1})		Treatment required
	Geometric mean	**90% compliance**	
Approved areas	≤ 14	≤ 43	None.
Restricted areas	≤ 88	≤ 260	Depuration or relaying in an approved area.
Prohibited areas	No sanitary surveys or conditions for approved/restricted areas are met.		Harvesting not permitted.

Reference: Lee *et al*. (2008)

Table 2. Classification criteria for bivalve mollusc growing areas based on flesh and intravalvular liquid samples
Cuadro 2. Criterios de clasificación de zonas de producción de moluscos bivalvos con base en muestras de carne y líquido intravalvar

Classification category	*E. coli* (MPN 100 g^{-1})	Treatment required
A	≤ 230	Direct human consumption.
B	[230; 4,600]	Depuration or relaying, to meet category A.
C	[4,600; 46,000]	Protracted relaying to meet category A. Relaying to meet category B and depuration.
D	> 46,000	Harvesting prohibited.

Reference: Oliveira et al. (2011)

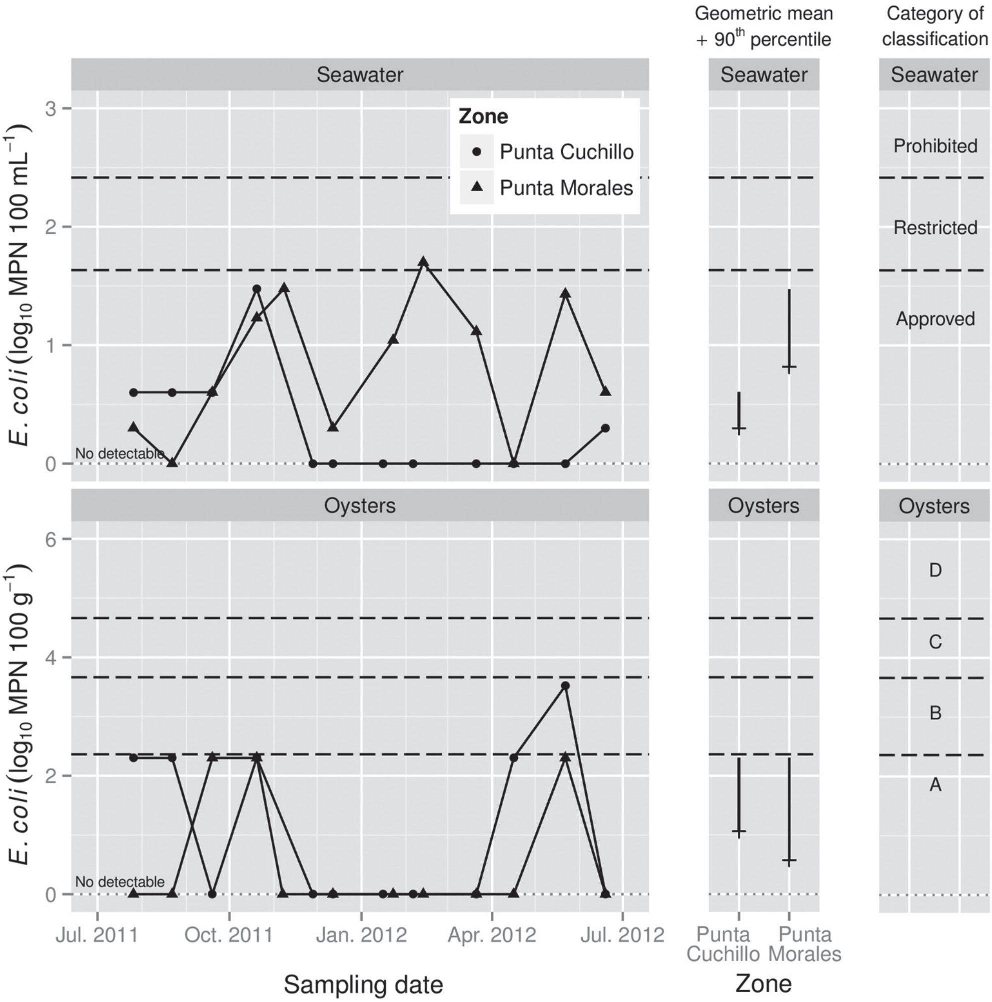

Fig. 2. *Escherichia coli* concentrations in seawater samples ($\log_{10}$ MPN 100 mL^{-1}) and oyster samples ($\log_{10}$ MPN 100 g^{-1}) collected on a monthly basis from July 2011 to June 2012 in the growing areas in Punta Cuchillo and Punta Morales, Gulf of Nicoya, Costa Rica. The geometric mean (+) and the 90th percentile are represented by sample type for each growing area. Dashed lines indicate the classification categories (90th percentile) for bivalve molluscs growing areas (Lee *et al*. 2008; Oliveira *et al*. 2011)

Fig. 2. Concentración de *Escherichia coli* en muestras de agua ($\log_{10}$ NMP 100 mL^{-1}) y de ostras ($\log_{10}$ NMP 100 g^{-1}) recolectadas mensualmente de julio de 2011 a junio de 2012 en las zonas de cultivo de Punta Cuchillo y de Punta Morales, Golfo de Nicoya, Costa Rica. Se representa la media geométrica (+) más el percentil 90 por tipo de muestra para cada zona de cultivo. Las líneas discontinuas indican las categorías de clasificación (percentil 90) para zonas de producción de moluscos bivalvos (Lee *et al*. 2008; Oliveira *et al*. 2011)

percentile of the *E. coli* concentration in the seawater and oysters for the two growing areas did not exceed the limit of the best sanitary quality classification category. Therefore, Punta Cuchillo and Punta Morales could be classified, taking into consideration only the bacteriological criteria established in the United States of America (Table 1) and in the European Union (Table 2), as class A or authorized areas for the production of bivalve molluscs (Fig. 2).

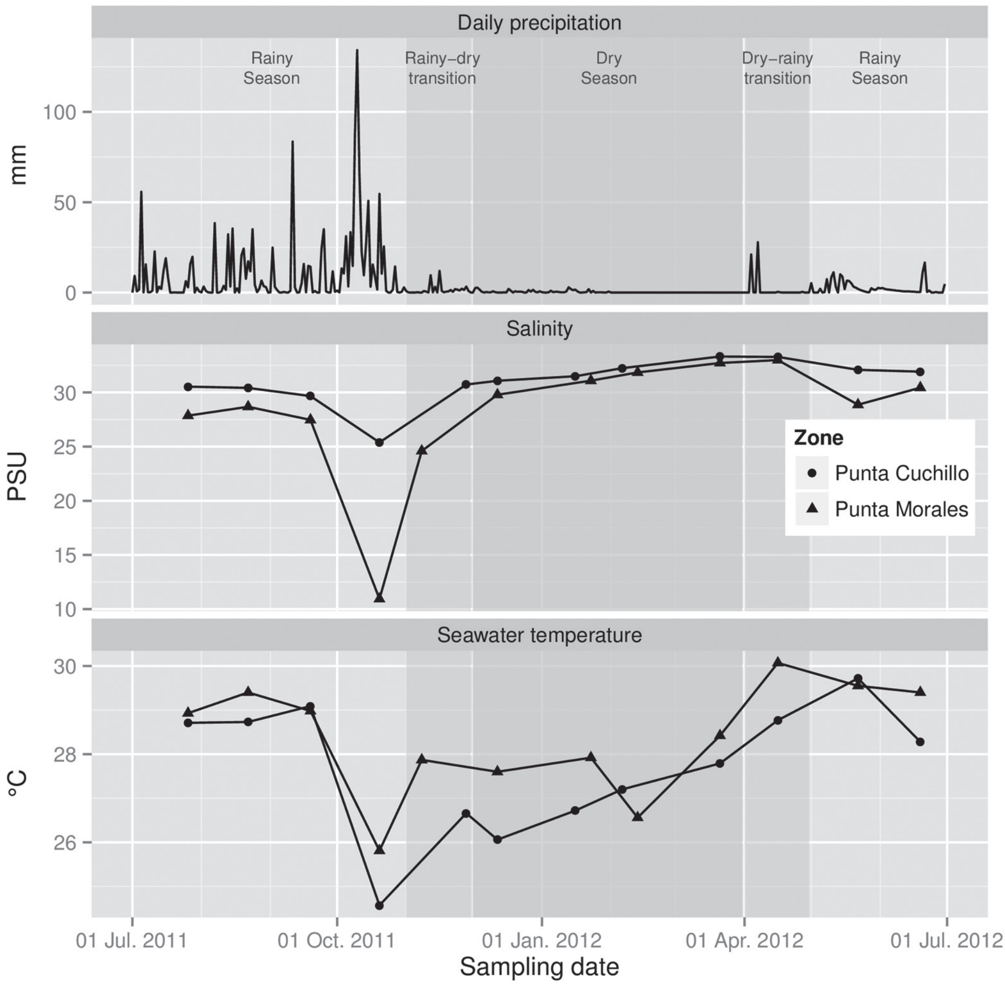

Fig. 3. Daily precipitation (mm) and salinity (PSU) and temperature (°C) recorded monthly from July 2011 to June 2012 in the *Crassostrea gigas* growing areas in Punta Cuchillo and Punta Morales, Gulf of Nicoya, Costa Rica. The dry and rainy seasons and the transition months established for the study area by *Instituto Meteorológico Nacional de Costa Rica* (the Meteorological Institute of Costa Rica) are represented here. Precipitation source: Meteorological Institute of Costa Rica
Fig. 3. Precipitación diaria (mm), salinidad (UPS) y temperatura (°C) mensual registradas de julio de 2011 a junio de 2012 en las zonas de cultivo de *Crassostrea gigas* de Punta Cuchillo y de Punta Morales, Golfo de Nicoya, Costa Rica. Se representan la estación seca, la estación lluviosa y los meses de transición establecidos para la zona de estudio por el Instituto Meteorológico de Costa Rica. Fuente precipitación: Instituto Meteorológico Nacional de Costa Rica

Daily precipitation in the study area, according to data provided by *Instituto Meteorológico Nacional de Costa Rica* (IMN) (the National Meteorological Institute of Costa Rica), confirms that the country's Pacific Regime has a well defined dry season (December to March) and rainy season (May to October). April and November are considered transition months (IMN, 2014). Salinity showed minimum values in the month of October for the two growing sites. During the dry season, this parameter increased in both areas due to the decreased precipitation in the Gulf of Nicoya. The minimum temperature was recorded in October and the maximum in April (Punta Morales) or May (Punta Cuchillo) (Fig. 3).

Monthly accumulated precipitation and salinity presented high negative correlation for both study areas. The Pearson correlation coefficient was -0.92 (95% CI: -0.98, -0.74) in Punta Cuchillo and -0.89 (95% CI: -0.97, -0.65) in Punta Morales. The recorded temperature in the growing areas did not show a clear tendency determining the seasonal variability in the Gulf of Nicoya (Punta Cuchillo: Pearson's r = -0.34, $P > 0.05$; Punta Morales: Pearson's r = -0.36, $P > 0.05$).

The GLM applied between salinity and *E. coli* concentration was significant for both types of samples. Coefficients estimated by the model indicated a negative trend between recorded *E. coli* concentrations and salinity (Table 3). As salinity increased in the growing areas *E. coli* concentration decreased in both types of samples. The GLM determined, for the study period, a significant seasonal variability in the *E. coli* concentration in seawater and oysters in the Gulf of Nicoya. During the dry season *E. coli* was not detected in the oyster samples from either growing area. Only the seawater samples from Punta Morales showed *E. coli* during this period (Fig. 4).

DISCUSSION

Total coliforms have been used to assess sanitary quality of coastal areas. However, *E. coli* is considered a more specific indicator of fecal contamination, mainly in tropical

Table 3. Coefficients estimated using the Generalized Linear Model ± 95% confidence interval and *P* value between *Escherichia coli* concentration (MPN for 100 mL or g) and salinity (PSU) recorded from July 2011 to June 2012 in the Gulf of Nicoya, Costa Rica
Cuadro 3. Coeficientes estimados por el Modelo Lineal Generalizado ± intervalos de confianza al 95% y el valor de *P* entre la concentración de *Escherichia coli* (NMP por 100 mL o g) y la salinidad (UPS) registradas de julio de 2011 a junio de 2012 en el Golfo de Nicoya, Costa Rica

Sample	Coefficients	GLM		
		Estimate	95% CI	*P*
Seawater	Intercept	2.012	0.426, 3.4	< 0.05
	Salinity	-0.065	-0.112, -0.007	< 0.05
Oysters	Intercept	2.388	0.99, 3.503	< 0.001
	Salinity	-0.061	-0.101, -0.13	< 0.01

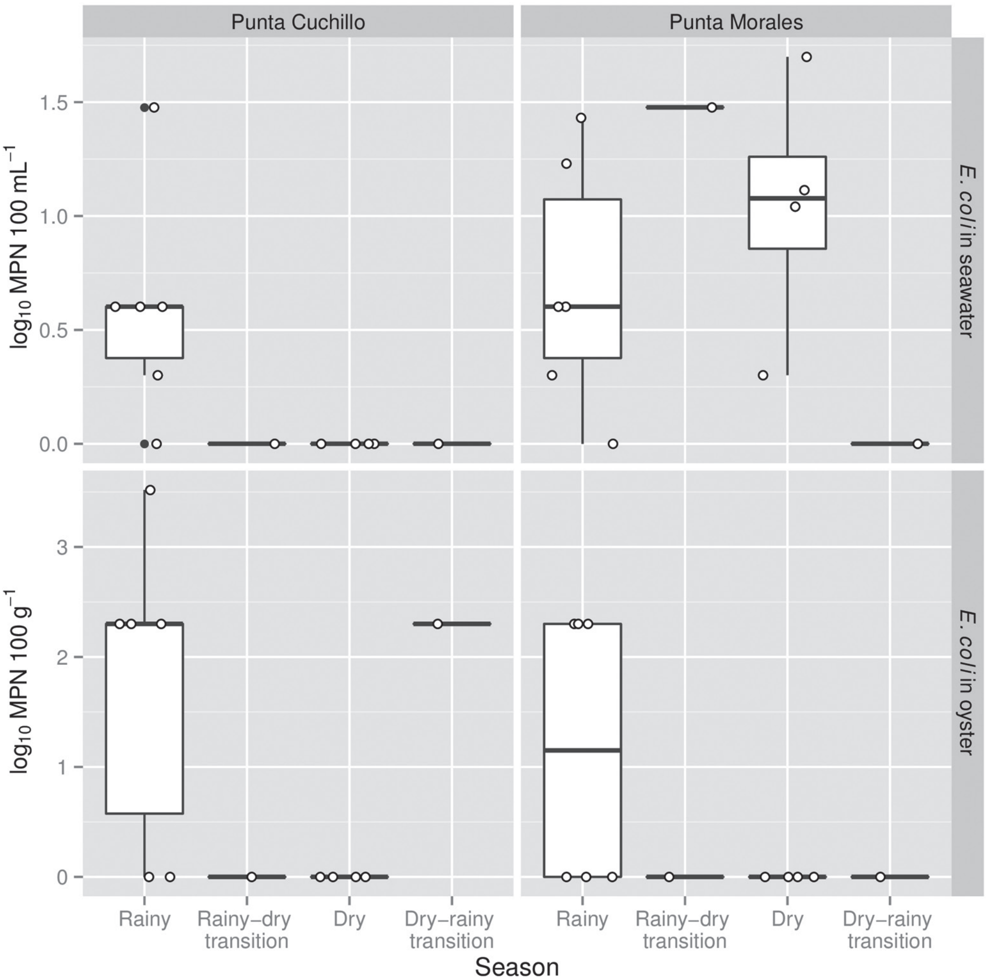

Fig. 4. Seasonal variability of the *Escherichia coli* concentration in samples of seawater (log_{10} MPN 100 mL^{-1}) and oysters (log_{10} MPN 100 g^{-1}) collected monthly from July 2011 to June 2012 in the growing areas in Punta Cuchillo and Punta Morales, Gulf of Nicoya, Costa Rica. Observations (○) and outliers (●) are represented here.

Fig. 4. Variación estacional de la concentración de *Escherichia coli* en muestras de agua de mar (log_{10} NMP 100 mL^{-1}) y de ostras (log_{10} NMP 100 g^{-1}) recolectadas mensualmente de julio de 2011 a junio de 2012 en las zonas de cultivo de Punta Cuchillo y de Punta Morales, Golfo de Nicoya, Costa Rica. Se representan las observaciones (○) y los extremos o atípicos (●)

coastal areas where the values of total coliforms may include bacteria that do not constitute a health risk (Lee *et al*. 2008). *E. coli* is also used for the bacteriological monitoring and classification of the bivalve mollusc growing areas (Almeida & Soares, 2012; Bettencourt *et al*. 2013).

Previous studies reported the presence of fecal coliforms or *E. coli* in samples of seawater (Acuña *et al*. 1998; García *et al*. 2006) and mollusc (Vega *et al*. 2013) from the Gulf of Nicoya; however, there are no references on the bacteriological classification of growing sites or their seasonal variability. *E. coli* concentrations in seawater and oyster samples analyzed during the study period allowed to classify, on a preliminary basis, Punta Cuchillo and Punta Morales growing areas as authorized (category A) areas for the cultivation of *C. gigas* in Costa Rica. This classification category establishes that produced molluscs do not require any post harvest treatment and can be directly used for human consumption (Lee *et al.* 2008; Oliveira *et al.* 2011).

The salinity variation recorded in this study reinforces the observations by Peterson (1960) and Voorhis *et al.* (1983), who established that this parameter is a seasonality indicator in the Gulf of Nicoya. Salinity in Punta Cuchillo and Punta Morales growing areas increased during the dry season. Increased precipitation and surface runoff during the rainy season reduce salinity and, usually, increase concentrations of organic matter and microorganisms in coastal environments (Barrera-Escorcia *et al.* 1999). Rain is, therefore, the parameter most commonly associated with the maximum levels of organisms indicating fecal contamination in coastal areas (Campos *et al*. 2013b).

The applied GLM estimated a significant negative trend between salinity and *E. coli* concentrations in seawater and oysters samples. In this way, precipitation in the Gulf of Nicoya seems to define a seasonal pattern in the variability of salinity and the registered *E. coli* contamination. Results of this study are consistent with other research projects. The inverse relationship between salinity and fecal coliform bacteria in water has been demonstrated in laboratory tests (Šolić & Krstulović, 1992) and in coastal marine environments (Barrera-Escorcia & Namihira-Santillán, 2004; Mignani *et al*. 2013). Anacleto *et al.* (2013) indicated that *E. coli* concentrations in seawater and sediments of the Targus Estuary (Portugal) were significantly higher during the rainy season. The same seasonal trend was found in the Thau Lagoon in France (Derolez *et al*. 2013).

Levels of thermotolerant coliforms in bivalve molluscs may differ with fecal contamination in the surrounding water (Martínez & Oliviera, 2010) and may change from one time period to another (Campos *et al*. 2013a; Soegianto & Supriyanto, 2008). Derolez *et al*. (2013) noted an inverse relationship between *E. coli* concentrations in seawater and in *C. gigas* oysters. In contrast, Anacleto *et al.* (2013) found a positive correlation between *E. coli* concentrations in water and in the *Venerupis pullastra* clam. The present study showed that *E. coli* concentrations in seawater and in *C. gigas* showed the same trend as Derolez *et al*. (2013). During the dry season, Punta Morales growing area presented higher levels of *E. coli* in the seawater than in oysters, possibly because the oyster farm is located at the mouth of Estero Morales. This could suggest that Punta Morales growing area receives direct contamination from the surrounding towns.

E. coli accumulation percentages among bivalve molluscs species in the same area may also show differences. Younger & Reese (2013) concluded that the percentage of accumulation between *Cerastoderma edule, Tapes philippinarum* and *Mytilus* spp. were equivalent in general terms, and that each of these species showed greater levels of accumulation than *C. gigas* and *Ostrea edulis*. In a controlled field study, *E. coli* accumulation was significantly higher in *Pecten maximus* than in *Mytilus* spp. and *C. gigas* (Lee & Silk, 2013). Programs assessing the microbiological quality of bivalve molluscs growing areas should monitor all cultivated species or select an indicator species that presents levels of pollution accumulation equivalent to or higher than the species they represent (Younger & Reese, 2013).

It is concluded that Punta Cuchillo and Punta Morales *C. gigas* growing areas are possibly being affected by wastewater discharge. This contamination is attributed to the coastal towns nearby and the river systems flowing into the Gulf of Nicoya from major urban, industrial and agricultural areas of the country. However, the bacteriological quality of seawater and oysters determined in this study suggests that these sites may be suitable for growing *C. gigas* in Costa Rica.

National legislation should be created to establish controls and implement a monitoring system ensuring the bacteriological quality of the areas used for cultivation of bivalve molluscs in Costa Rica. It is also important to supervise the treatment of wastewater to reduce the vulnerability of this estuarine system.

Depuration and proper handling of harvested molluscs is recommended to decrease potential dangers that may arise from the *E. coli* levels detected and sporadic contamination of the evaluated areas.

These provisions, in addition to protecting the consumer and public health in general, promote the mariculture development in the country.

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