Dear Author,

Please, note that changes made to the HTML content will be added to the article before publication, but are not reflected in this PDF.

Note also that this file should not be used for submitting corrections.

Marine Pollution Bulletin xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary

Luciano F. La Sala [a,c,*], Leandro M. Redondo [b,c], Juan M. Díaz Carrasco [b,c], Ana María Pereyra [b], Marisa Farber [c,d], Helen Jost [e], Mariano E. Fernández-Miyakawa [b,c]

[a] *Cátedra de Parasitología Clínica, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina*
[b] *Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25, 1712 Castelar, Buenos Aires, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas, Rivadavia 1917, 1033 Ciudad Autónoma de Buenos Aires, Argentina*
[d] *Instituto de Biotecnología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25, 1712 Castelar, Buenos Aires, Argentina*
[e] *Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721, USA*

ARTICLE INFO

Article history:
Received 26 February 2015
Revised 22 May 2015
Accepted 29 May 2015
Available online xxxx

Keywords:
Sewage
Pollution
Clostridium perfringens
Crabs
Monitoring organisms
Indicator bacteria

ABSTRACT

The Estuary of Bahía Blanca (EBB), Argentina, is an important wetland under intense sewage pollution. We investigated the occurrence of *Clostridium perfringens* (CP) in populations of two benthic crabs (*Neohelice granulata* and *Cyrtograpsus angulatus*) and in sediment from the EBB. CP was found in 49.1% of the crabs and all of the isolates were identified as type A. The alpha (cpa) and enterotoxin (cpe) encoding genes were identified. Genetic analyses identified 13 novel sequence types, and found no clustering among isolates, suggesting that CP is not part of the crabs' commensal flora. CP carriage was 51 times more likely in crabs from the area nearest sewage outfalls compared with crabs from an off-shore site. Our *in vitro* experiments suggest that the carriage of CP in crabs is transient, and the use of these benthic crabs as monitoring organisms of sewage pollution in coastal habitats is proposed.

1. Introduction

Oceans and human health are becoming increasingly related, in part because of the constantly growing proportion of humans living near marine coasts (Tibbetts, 2002). Estuaries, in particular, offer a wide variety of ecosystem services that generate value and contribute to human welfare (Barbier et al., 2011).

The microbiological quality of coastal and estuarine waters is a major public health concern (Shibata et al., 2004). In estuaries, as in other natural aquatic ecosystems, the levels of fecal-indicator bacteria and enteric pathogens are mainly influenced by point sources (*e.g.* discharge of effluents from wastewater treatment plants [WWTP]) and by the nature of the associated watershed. Although grazing animals are a major cause of microbial pathogen contamination for freshwater worldwide (Belsky et al., 1999), human fecal contamination is the most globally relevant source of human illnesses.

Various specific bacterial indicator systems have been used to distinguish fecal pollution in aquatic systems (Elliott and Colwell, 1985). Among them, the standard fecal indicator bacteria (SFIB) *Escherichia coli* and intestinal enterococci have long been used to monitor total fecal pollution, but their reliability has been questioned because these microorganisms can establish "naturalized" populations outside their natural hosts (Ishii and Sadowsky, 2008; Byappanahalli et al., 2012), their populations decay more rapidly due to predation by protozoans (Davies et al., 1995), and can be short-lived in the aquatic environment (Byamukama et al., 2005).

Clostridium perfringens (CP), a spore-forming rod, offers an alternative to SFIB and it has proven excellent as indicator for point source emissions from WWTP in rivers and other lotic systems (Fujioka and Shizumura, 1985; Sorensen et al., 1989; Skanavis and Yanko, 2001; Lisle et al., 2004; Byamukama et al., 2005; Cox et al., 2005; Farnleitner et al., 2010; Vierheilig et al., 2013) and as a tracer for sewage sludge pollution (Hill et al., 1993, 1996; Skanavis and Yanko, 2001). Moreover, studies indicate that the

* Corresponding author at: Cátedra de Parasitología Clínica, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina.
E-mail addresses: lucianolasala@yahoo.com.ar, lucianolasala@conicet.gov.ar (L.F. La Sala).

http://dx.doi.org/10.1016/j.marpolbul.2015.05.066
0025-326X/

concentrations of CP are correlated with certain pathogens such as *Cryptosporidium* spp. and with the risk of infection associated with recreational activities (Brookes et al., 2005; Viau et al., 2011). This has encouraged the use of CP as an alternative to SFIB for recreational water quality monitoring (Boehm et al., 2009).

Environmental monitoring for human pathogens can be problematic, partly because of the dilution effect that occurs as bacteria are disseminated from terrestrial to aquatic ecosystems. Although a combination of sedimentation, sorption, and extended survival in sediments favor higher concentrations of indicator and pathogenic bacteria in this compartment (see Burton et al., 1987 and references therein), the bioaccumulation of pathogens in some filter feeding invertebrates has proven useful as indicator of sewage pollution. For example, bivalve molluscs can act as a natural concentration systems (McMahon, 1991) which can be tested for pathogens and provide an indication of water quality (Miller et al., 2005, 2006a). The use of benthic organisms as a monitoring tools may provide integrated data over a period of time, and has clear advantages over other commonly used abiotic monitoring compartments (Miller et al., 2005, 2006a; Fayer et al., 2002; Freire-Santos et al., 2000; Graczyk et al., 2003). To be reliable, these indicators should have the capacity to accumulate pollutants from their surroundings and/or food so that an analysis of their tissue provides an estimate of the environmentally available concentrations of the pollutants.

The Estuary of Bahía Blanca (EBB) is a vast wetland ecosystem located in southwest Buenos Aires province, Argentina. The city of Bahía Blanca, located on the northern coast of the estuary, is served by two WWTP that dispose sewage with only pretreatment into this wetland, thus leading to chronic microbiological pollution (Brezina and Baldini, 2008; Streitenberger and Baldini, 2010), with bacteria levels above those allowed for bathing waters (US Environmental Protection Agency, 2003).

With this background, a study was conducted in populations of *Neohelice granulata* and *Cyrtograpsus angulatus* crabs in the EBB to test the hypotheses that (1) these crabs are non-commensal carriers of *C. perfringens*, and that (2) they constitute potentially reliable indicators of sewage pollution in estuarine ecosystems.

2. Methods and materials

2.1. Study area, sampling procedures and studied species

The EBB is a mesotidal coastal plain of 2300 km^2 located in a temperate zone (38°40′S; 62°09′W) in Argentina, formed by tidal channels, extensive tidal flats, salt marshes, and islands (Piccolo et al., 2008). Freshwater inputs originate from a drainage basin of 19,000 km^2 with three freshwater tributaries. Recreational and artisanal fishing activities are very important in the area. Various ports and industries are located along the northern shore of the estuary and have been identified elsewhere as important sources of pollutants.

Two species of bottom feeding crabs, *N. granulata* and *C. angulatus*, represent the most abundant and largest benthic macroinvertebrates in the estuary. *N. granulata* is the most abundant and largest (~40 mm in carapace width) crab on intertidal areas of the South Western Atlantic (Iribarne et al., 2000). It dwells in vegetated and non-vegetated areas along the coast from Rio de Janeiro (Brazil) to Patagonia (Argentina) (Bass et al., 2005; Boschi, 1964) and is considered a key species in the energy transfer in mudflats and salt marshes (D'Incao et al., 1990). In the EBB their density can be as high as 70 crabs m^{-2}, removing between 2.5 kg m^{-2} (in the marsh) and 6 kg m^{-2} (in the mud flat) of sediment per day (Iribarne et al., 1997). *C. angulatus* has a similar geographical distribution to *N. granulata* and it lives in intertidal and infralittoral habitats (Olivier et al., 1972).

A study was conducted in populations of these crabs in the EBB. Eight sampling sites were selected based on accessibility and varying distance from two major known sewage outfalls from WWTP, and they were: Puerto Cuatreros (Site A), Puerto Ingeniero White (Site B), Puerto Colonia (Site C), Isla Bermejo (Site D), Isla Verde (Site E), Villa del Mar (F), Balneario Los Pocitos (G), and Bahía San Blas (H) (Fig. 1).

A total of 110 crabs (86 *N. granulata* and 24 *C. angulatus*) were collected in 2009 and 2011. The number of specimens collected on each site and year are presented in Table 3. All the crabs from the same site were collected the same day. Captures were conducted on intertidal mudflats either by hand (during ebb tide) or using baited fish cast nets (at high tide). Crabs were transported live to the laboratory, humanely killed by stunning (cooling at 4 °C) followed by destruction of nerve centers and dissected using sterile scissors and tweezers. The gut of each animal was removed aseptically and processed for bacteriological assays as described below.

Environmental samples, consisting of 5 cm deep cores of surface sediment, were collected from sites A, B, E, F, G, and H. A superficial section of each sample was removed and the remaining was transported to the laboratory in anaerobic conditions in sealed polyethylene bags.

2.2. In vitro carriage of CP

An *in vitro* experiment was conducted to further test the hypothesis that crabs can carry CP in their gastrointestinal tracts, and to estimate the length of time during which crabs can carry the pathogen. Here, the simultaneous treatment of crabs with CP followed by random samplings at fixed time intervals was used as an approximation to the length of carriage state, under the assumption that carriership susceptibility and duration are approximately equal among individuals.

For this experiment, apparently healthy *N. granulata* crabs ($n = 40$) were collected in Bahía San Blas, where point sewage discharges are not reported. Crabs were conditioned in an aquarium containing 5 l of artificial seawater (Instant Ocean; Aquarium Systems, Mentor, Ohio). After initial feeding, the water was changed weekly and aerated using an air pump. Water salinity was adjusted to 25%, pH was 7.5, and temperature was 22–25 °C. A strain of CP previously isolated from *N. granulata* gut was used to treat the crabs. Before treatment, CP were grown in brain-heart infusion broth at 37 °C under anaerobic conditions for 24 h. Bacteria cells were harvested from grown cultures and washed with PBS before being used as inoculum. Before the experiment begun, crabs were fasted for 4–5 days (Huq et al., 1986), after which they were fed lyophilized krill (Aqua Stock Inc., Bayonne, N.J.) that had been presoaked in the bacteria suspension. Crabs ($n = 5$) were sampled and sacrificed at days 0, 10, 20, 30, 40, and 50 post-treatment. The gastrointestinal tract of each animal was removed aseptically and processed for bacteriological assays as described below.

2.3. Bacteriological analysis

Tubes containing sterile chopped meat broth (CMM) were boiled with their cap screwed loosely to create anaerobic conditions. After 10 min, the tubes were chilled in a water bath with ice and then they were immediately capped. The stomach and intestine of each crab were separated, placed into tubes containing chopped meat broth, and incubated for 18 h at 37 °C. Chopped meat cultures were seeded in blood agar plates supplemented with 100 mg/l of neomycin (Marshall et al., 1965). The inoculated plates

Please cite this article in press as: La Sala, L.F., et al. Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.05.066

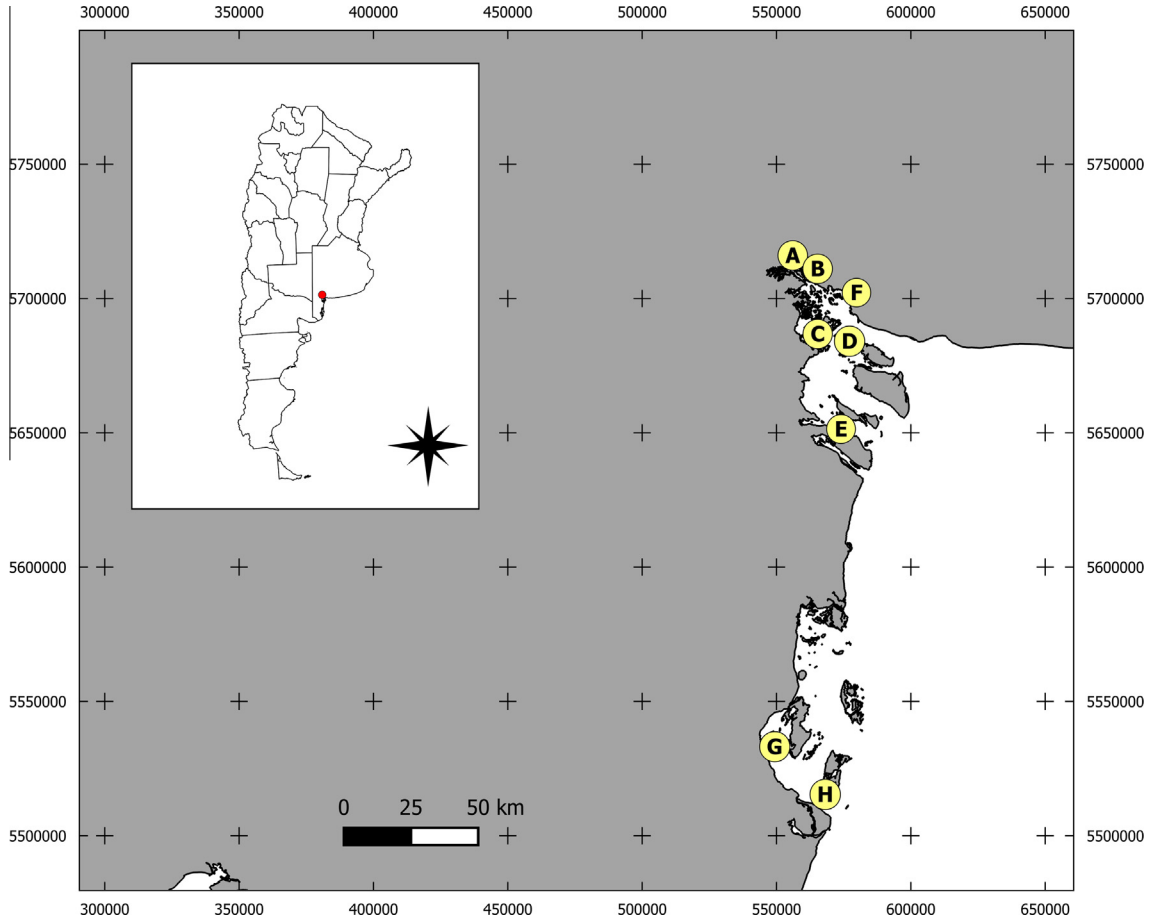

Fig. 1. Location of study sites in the Estuary of Bahía Blanca (A–F) and in Bahía San Blas (G and H), Argentina. The inset shows the location of Bahía Blanca (red dot). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were incubated in anaerobic jars (Oxoid) with H_2 10%: CO_2 10%: N_2 80% for 24 h. Following, colonies were analyzed according to their shape, color, and type of hemolysis. Bacterial morphology was microscopically assessed in Gram-stained smears. Colonies presenting CP characteristics were isolated, cultured in CMM and incubated at 37 °C for 18–24 h. These cultures were further assayed using biochemical tests (production of catalase, lecithinase and gelatinase, fermentation of glucose and lactose, and skim milk coagulation) for species identification. All the strains were incubated in CMM for 18–24 h at 37 °C, and cultures were stored at room temperature.

Sediment samples were diluted serially in sterile PBS (1/2; 1/5; 1/10; 1/20), and 0.1 ml of each suspension were inoculated into blood agar plates supplemented with 100 mg/l of neomycin. Plates were incubated in anaerobic jars at 37 °C for 18 h. The number of colonies compatible with CP was recorded.

2.4. PCR genotyping

Colonies compatible with *C. perfringens* were scraped from the plates, resuspended in water and boiled for 20 min. Cell debris were removed by centrifugation at 13,000g for 5 min. and the supernatant was used as template DNA. For the detection of CP major toxin genes, a multiplex PCR targeting cpa, cpb, etx, iap, cpe and cpb2 (Table 1) was performed (Meer and Songer, 1997).

Table 1
Oligonucleotides used for multiplex PCR in this study.

Gene	Primer	Sequence	Amplicon size
Cpa	F	GCTAATGTTACTGCCGTTGA	324
	R	CCTCTGATACATCGTGTAAG	
Cpb	F	GCGAATATGCTGAATCATCTA	196
	R	GCAGGAACATTAGTATATCTTC	
Etx	F	GCGGTGATATCCATCTATTC	655
	R	CCACTTACTTGTCCTACTAAC	
iA	F	ACTACTCTCAGACAAGACAG	446
	R	CTTTCCTTCTATTACTATACG	
Cpe	F	GGAGATGGTTGGATATTAGG	233
	R	GGACCAGCAGTTGTAGATA	
Cpb2	F	AGATTTTAAATATGATCCTAACC	567
	R	CAATACCCTTCACCAAATACTC	

2.5. CP multilocus sequence typing

A Multilocus Sequence Typing (MLST) scheme previously described (Jost et al., 2006) was used for genotyping the CP isolates, including eight genes (Table 2). PCR products were submitted to the Genomic Unit from the Institute of Biotechnology, INTA, for sequencing (DNA Analyzer 3130 xL, Applied Biosystems®).

An automatic pipeline was set up in order to overcome manually intensive steps including raw sequence data processing and downstream analysis. For the MLST-pipeline, the string processing was called from dedicated python wrappers designed according to the Galaxy open web-based platform (http://galaxyproject.org/), as described by Guillemi et al. (2015). The process generates an HTML report including the isolate haplotype and the set of strain alleles sequences.

Please cite this article in press as: La Sala, L.F., et al. Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.05.066

Table 2
Oligonucleotides used for MLST in this study.

Gene	Primer	Sequence	Amplicon size
plc	plcF	ATATGAATGGCAAAGAGGAAAC	544
	plcR	AGTTTTTCCATCCTTTGTTTTG	
ddlA	ddlAF	ATAATGGGGGATCATCAGTTGC	429
	ddlAR	TTATTCCTGCTGCACTTTTAGG	
dut	dutF	TTAAGTATTTTGATAACGCAAC	441
	dutR	CTGTAGTACCAAATCCACCACG	
glpK	glpKF	TGGGTTGAGCATGATCCAATGG	547
	glpKR	CACCTTTTGCTCCAAGGTTTGC	
gmk	gmkF	TAAGGGAACTATTTGTAAAGCC	475
	gmkR	TACTGCATCTTCTACATTATCG	
recA	recAF	GCTATAGATGTTTTAGTTGTGG	475
	recAR	CTCCATATGAGAACCAAGCTCC	
sod	sodF	GATGCTTTAGAGCCATCAATAG	475
	sodR	AATAATAAGCATGTTCCCAAAC	
tpi	tpiF	AAATGTGAAGTTGTTGTTTGCC	451
	tpiR	CATTAGCTTGGTCTGAAGTAGC	

For the MLST-database (MLST-DB), an interface application website was built using Web2py framework (http://www.web2py.com/). The MLST-DB was built using our own data (Redondo et al., 2013 and this work) and the nucleotide sequences from the CP MLST scheme from Jost et al. (2006). The MLST-DB can be explored in a friendly way to search and download the complete set of alleles for all gene targets and the ST numbers and haplotypes of the stored strains. The pipeline and MLST-DB are freely available at: http://bioinformatica.inta.gob.ar/galaxy/ and http://bioinfoinformatica.inta.gob.ar/mlst/, respectively, under username and password request at: ibiotecno.bioinfo@inta.gob.ar. In addition, the sequences for the 8 genes from each isolate were deposited in GenBank under accession numbers KP264126–KP264245.

2.6. Statistical analysis

Samples (crab and sediment) were considered positive if they grew colonies compatible with CP and if PCR was positive.

In 2009, crabs were sampled only in two sites (A and B). Therefore, two generalized linear models (logistic) were built using different subsets of data. Model 1 was constructed to assess the inter-year (2009 vs. 2011) difference in prevalence in sites nearest to sewage pollution (Sites A and B). These sites are located at a relatively short distance from each other and carriage prevalence was not different between them (results not shown) in 2009. Therefore, 2009 data from Site A and B were combined (henceforth denoted as A/B) and compared with 2011 data from Site A. The saturated model included carriage state (yes/no) as the response variable, and year (2009/2011) as independent variable.

Model 2 was used to test the hypothesis that prevalence of CP carriage is higher nearer sewage pollution sources. Because only two sites were sampled in 2009, this model included data from 2011 only. Therefore, the saturated model included Site (four factors: A–D) as the only independent variable.

The models were constructed by first fitting a full model which included independent variables, and then comparing this model with the intercept-only (null) model. The full and null models were compared using the Akaike information criterion (AIC) (Akaike, 1974), and the independent variable was retained in the model if its inclusion reduced AIC by more than two units (Burnham and Anderson, 2002).

The association between carriage status and independent variables was quantified by estimation of the odds ratio (OR) and their 95% confidence intervals (95% CI). The OR for independent variables (sampling site), which was computed as the exponential of the regression coefficient, indicates how much more likely it is for the outcome (carriage) to be present in individuals of the reference group (exposed) compared to those in the baseline group (unexposed). For the *in vitro* experiment, the proportion of positive crabs at each sampling time was calculated.

All analyses were performed using the statistical package R (R Development Core Team, 2014) and the package "epitools" of R (Aragon, 2012).

2.7. Phylogenetic analysis

Characterization of CP isolates by MLST was done, and phylogenetic relationships were analyzed and illustrated using the PHYLOViZ software (Francisco et al., 2012). This software was used for representing the possible evolutionary relationships between strains identified by allelic profiles. The program uses the goeBURST algorithm, a refinement of eBURST algorithm proposed by Feil et al. (2004) and its expansion to generate a complete minimum spanning tree (MST) for visualizing the possible evolutionary relationships between isolates.

3. Results

3.1. CP isolates, spatial distribution and in vitro carriage

C. perfringens was isolated in 49.1% (54/110) of crabs, and prevalence varied by sampling site (Table 3). All 54 isolates were positive for the alpha toxin encoding gene (*cpa*), corresponding with type A strains. One isolate was also positive for the *cpe* gen, corresponding with type A, enterotoxin-producing CP. Thirteen novel sequence types (ST) were identified, of which 10 were from *N. granulata*, one from *C. angulatus* crabs, and two from sediment samples.

The model evaluating between-year difference in carriage prevalence in sites nearer sewage outfalls showed that the odds of carriage were almost 12 times greater in 2011 (95% CI: 2.5–53.1; $P < 0.05$). When analyzing data from 2011 only, the odds of carriage were 51 (95% CI: 9.2–464.3; $P < 0.0001$) times greater in crabs from Site A/B, 7.9 (95% CI: 2.0–41.0; $P < 0.01$) times greater in crabs from Site C, and 3.4 (95% CI: 0.7–19.1; $P > 0.05$) in crabs from Site E, compared with crabs from Site D (baseline/unexposed category) (Fig. 2). The enumeration of CP in sediment samples ranged between undetectable levels and 400 cfu g^{-1} of sediment (Table 3).

In the *in vitro* experiment, carriage prevalence was 0% (0/5) before treatment. Post-treatment, prevalence was 60% (3/5) at days 10 and 20, 40% (2/5) by day 30, and 0% (0/5) at days 40 and 50.

3.2. Genetic relationship among isolates

MLST isolates obtained from crabs and sediment differed markedly from the 130 CP isolates reported by Jost et al. (2006) in 10 animal host species, showing different alleles in 4–7 of the eight genes analyzed (Fig. 3). New alleles were identified for each gene. The isolated STs did not cluster together, but rather they were found scattered throughout the MST (Fig. 3). No association was observed between isolates and the clonal complexes described by Jost et al., nor with any particular host species in Jost's MLST-DB (results not shown). Occasionally, isolates from the same site were identical (ST-88 and ST-94) or showed a close relation (ST-85 and ST-86, ST-87 and ST-89, ST-88 and ST-90, ST-91 and ST-93).

4. Discussion

To our knowledge, there is only one previous work assessing the use of CP in a wildlife host as indicator of sewage pollution (Miller

Please cite this article in press as: La Sala, L.F., et al. Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.05.066

Table 3
Clostridium perfringens carriage prevalence (%) followed by 95% confidence intervals in *N. granulata* and *C. angulatus* crabs, and *C. perfringens* concentration (cfu/g) in sediment samples southwest Buenos Aires province in 2009 and 2011.

Year		A	B	C	D	E	F	G	H
2009	Crabs	46.7 (24.8–69.9) (15)	40.0 (19.7–64.3) (15)	NA NA	NA NA	NA NA	NA	NA	NA
	Sediment	NA	NA						
2011	Crabs	90.0 (68.7–98.4) (20)	NA	58.3 (38.8–75.6) [a] (24)	15.0 (4.4–36.9) (20)	37.5 (18.4–61.5) (16)	NA	NA	NA
	Sediment	400	20	NA	20	ND	200	ND	ND

[a] *C. angulatus*; NA = samplings were not conducted. Sediment (cfu/g). Samples with counts below the detection limit are reported as ND.

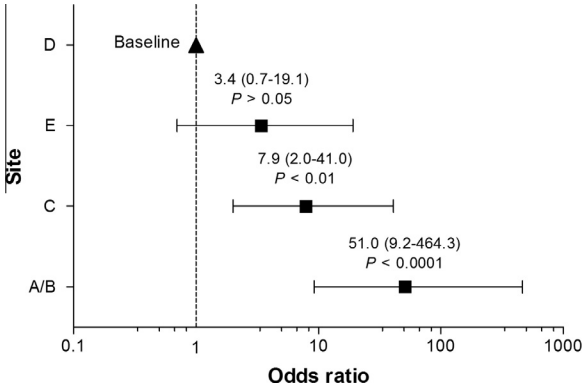

Fig. 2. Odds ratios (squares) and their 95% CI (whiskers) for *C. perfringens* carriage in *N. granulata* by sampling site: A/B: Puerto Cuatreros; C: Puerto Colonia; D: Isla Bermejo (reference site); E: Isla Verde. The dotted line represents odds of carriage in crabs from the unexposed site (baseline level or site D: Isla Bermejo) against which all other sites are compared.

et al., 2006b). The capacity of the studied crabs to act as indicators of sewage pollution should depend on the nature of the host-pathogen relationship, and their use as indicators would be useless if CP were part of the crabs' commensal flora. Under a host-pathogen commensal scenario, MLST analyses would be expected to yield some degree of clustering of isolates in the form of a clonal complex, as was found by Jost et al. (2006) in the analyses of 132 CP isolates from 10 host species and comprising all five toxin types.

Contrarily, the 13 novel ST found in crabs from our study were not genetically clustered, but followed a dispersed pattern throughout the MST. This finding suggests that these CP isolates were neither associated among themselves nor with any of the host species or clonal complexes described by Jost et al. (2006), and supports our first hypothesis that CP is not part of the crabs' commensal flora. Also, this lack of association among isolates agrees with the species' bottom-dwelling and feeding habits, which would favor a random collection of the repertoire of CP strains present in the environment.

The alpha-toxin, encoded by the *cpa* gene, is the major virulence determinant in cases of gas gangrene in humans and other mammals (McClane, 1996), also being associated with necrotic enteritis in calves (Verherstraeten et al., 2013), horses (Wierup and DiPietro, 1981), and poultry (Shojadoost et al., 2012). Recent studies have linked this toxin and non-enterotoxigenic CP type A with human diseases other than gas gangrene, such as necrotic enteritis (Iwanaka et al., 2004) and sudden infant death syndrome (SIDS) (Kamaras and Murrell, 2001a,b). Concerning the *cpe* gene, it encodes the enterotoxin responsible for food poisoning associated with CP type A, which is the third most common food-borne illness in the United States (Adak et al., 2002).

Here, CP type A was isolated in natural populations of two benthic crabs that occur across areas with varying degrees of sewage pollution. The fifty one-fold increase in the odds of carriage in crabs from the most polluted sites (Site A/B) compared with crabs from a more distant, off shore island (Site D), coupled with MLST results (*i.e.* lack of isolates clustering) supports our second hypothesis of a sewage-related origin for this pathogen in crabs from the EBB. It is worth noting that all positive crabs carried the *cpa* gene that encodes the alpha toxin in all strains of CP, and one crab (ST-84 in Fig. 3) carried the *cpe* gene encoding the enterotoxin responsible for food-borne illnesses, sporadic diarrhea, antimicrobial drug-associated diarrhea, and food poisoning in humans (Sparks et al., 2001; Harrison et al., 2005). *N. granulata* and *C. angulatus* crabs are key components of complex trophic webs in the EBB, being consumed by several species of teleost and chondrichthyes fish, such as Stripped Weakfish (*Cynoscion guatucupa*) and Narrownose Smoothhound (Mustelus schmitti), respectively. Considering that these represent important target species for fisheries and sport fishing, they could represent potential routes of CP transmission to the human population.

The genetic findings in the present study, coupled with the high prevalence of CP carriers and considerably high concentration of CP colonies observed in sediment from the innermost area of the estuary (Site A/B) raises awareness about the health risks involved in recreational uses of this area. Our conclusions are in agreement with those from recent official monitoring activities (Baldini and Streitenberger, 2014) which reported maximum counts of 8000 UFC g^{-1} for *E. coli* and 6500 UFC g^{-1} for *Enterococcus* spp. in sediment (dry weight), and 48,000 UFC 100 ml^{-1} for *E. coli* and 20,000 UFC 100 ml^{-1} for *Enterococcus* spp. in the water column from an area very near to Site A/B in our study. These levels are considerably above the maximum level of 126 and 35 UFC 100 ml^{-1} allowed for *E. coli* and *Enterococcus* spp., respectively, in bathing waters (US Environmental Protection Agency, 2003).

In Site E, the most distant one with crab samplings and arguably the least exposed to sewage pollution, carriage prevalence was 37.5%. Interestingly, the ST 83 isolated in one crab from this site had 3 alleles (out of the 8 analyzed) in common with ST 84 from a crab in Site D. Although more data would be necessary to draw a sound conclusion, this result could indicate the sewage origin of the CP strains found in the most distant sampling area. Surprisingly, enumeration of CP in sediment from site E yielded no positive results, suggesting that *N. granulata* crabs represent better monitoring compartments for sewage pollution than sediment samples.

Alternatively, CP originating from ranching activities and wildlife populations in the watershed could eventually reach the estuary via runoff across the land surface, by seepage through the surface soil layers or through natural creeks, thus explaining their presence in this distant site. However, as suggested by Vierheilig et al. (2013), CP would not confidently reflect fecal pollution from ruminant wildlife, being associated with excreta from human sewage and non-herbivorous animals such as carnivorous wildlife. Although the feces of the latter can contain very high concentrations of CP, it seems unlikely that they play a significant role in

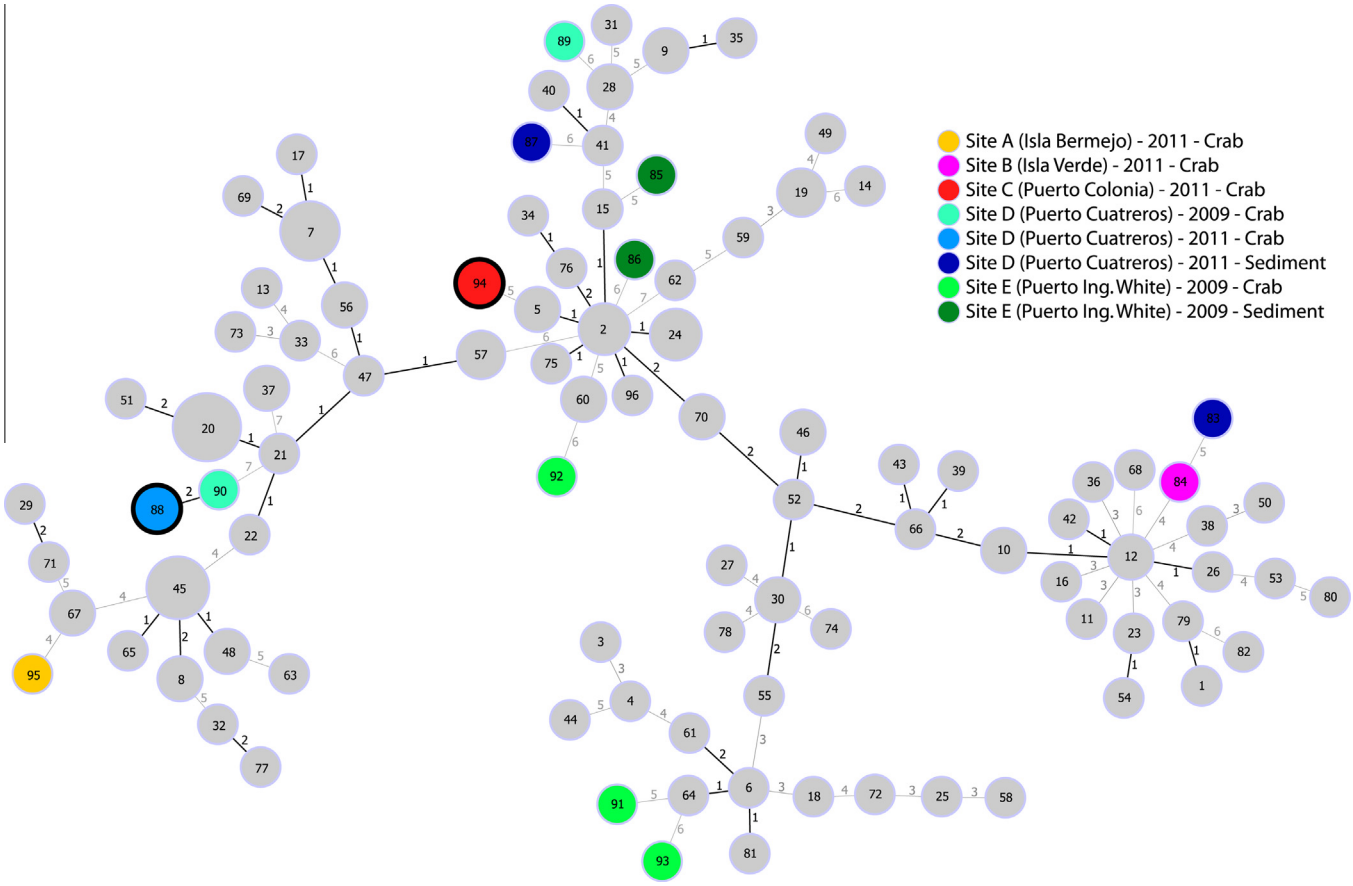

Fig. 3. Graphic representation of intraspecific relationship between CP isolates using PHYLOViZ. Each circular node represents a unique allele combination as determined by MLST. The diameter of the circles is proportional to the number of isolates with the corresponding ST and colors indicate the origin of the isolates according to the reference in the top right. Gray circles correspond to isolates described by Jost et al. (2006). Numbers next to the edges indicate the number of alleles differing between the connected nodes from a total of eight genes. The black edges correspond to single and double locus variants, indicating a close relationship. ST 88 and 94 include two isolates with the same characteristics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

water pollution with this pathogen as was postulated by the same authors. With this in mind, and considering the absence of human population in this distant area, our results suggest that sewage pollution originated on the northern coast of the EBB disperses through currents reaching the most distant sampling sites.

CP can be extremely resistant to environmental factors such as heat, low water availability, radiation, or disinfection procedures (Bisson and Cabelli, 1980; Tyrrell et al., 1995; Payment, 1999). Unlike other bacteria (*e.g.* fecal coliforms), CP does not reproduce in aquatic systems (Wright, 1989; Desmarais et al., 2002), and the spores become inert in temperate marine sediments (Rippey and Watkins, 1992; Davies et al., 1995). Therefore, spores facilitate the survival of this pathogen under suboptimal conditions and allow for their detection during long periods of time in aquatic environments (Edwards et al., 1998) and far from the source (World Health Organization, 2008). Whereas the levels of most bacteria can decrease significantly over relatively short time frames (Davies et al., 1995), CP spores not only survive but also accumulate in sediments (Characklis et al., 2005; Cizek et al., 2008).

Here, the only crab positive for *cpe* enterotoxin was also from Site E, the most distant site. The majority of human illnesses caused by CP are associated with this enterotoxin (McClane, 1996), but less than 5% of the global CP population apparently carries this gene (Czeczulin et al., 1996). Although the environmental or animal source of enterotoxin-positive strains has not been fully determined, freshwater suspended sediments and sewage influent are considered the main reservoirs for *cpe*-carrying CP (Mueller-Spitz et al., 2010). Then, it is likely that the long-term sewage disposal in the EBB, followed by bottom deposition and posterior large-scale dredging operations adjacent to sewage input points (Zilio et al., 2013) play a role in the desorption, resuspension and dispersal of CP spores in this vast wetland. Also, as explained before, the highest resistance of CP spores in general, and of vegetative cells of *cpe*-carrying CP in particular, to environmental conditions might explain the occurrence of these CP in crabs from the most distant area.

Results from the *in vitro* experiment suggest that after exposure to the bacteria, negative crabs become positive relatively fast and remain carriers for 30–40 days. One plausible explanation for this is that once prime exposition occurs, individuals remain positive until vegetative cells or spores are eliminated back into the environment. Also importantly, the cycling of CP (becoming negative after a carrier state) observed between the crabs and the environment under *in vitro* conditions reinforces our hypothesis that CP is not a commensal of these crabs.

In conclusion, our *in vitro* and field results suggest that benthic crabs represent useful monitoring organisms, offering a reasonable time window post-exposure during which CP could be detected. We suggest the capacity of these crabs to act as transient carriers for CP spores and vegetative cells present in the environment, and present this host-pathogen system as a promising tool for the monitoring of sewage pollution in coastal habitats.

Please cite this article in press as: La Sala, L.F., et al. Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.05.066

5. Uncited references

Ewing et al. (1998), Ewing and Green (1998), Fleming et al. (2006), Huang and Madan (1999) and Touron et al. (2007).

Acknowledgements

We thank Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for institutional support, and Agencia Nacional de Promoción Científica y Tecnológica for financial support of this Project (PICT 0861/2008).

References

Adak, G.K., Long, S.M., O'Brien, S.J., 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992–2000. Gut 51, 832–841.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Autom. Control 19, 716–723.

Aragon, T.J., 2012. epitools: Epidemiology Tools. R package version 0.5-7. <http://CRAN.R-project.org/package=epitools>.

Baldini, M.D., Streitenberger, M.E., 2014. Análisis bacteriológico en aguas y sedimentos en inmediaciones del balneario Maldonado, en los afluentes del estuario y en el efluente de la planta depuradora de líquidos cloacales de la 3ª Cuenca Bahía Blanca. <http://www.bahiablanca.gov.ar/areas-de-gobierno/medio-ambiente/comite-tecnico-ejecutivo/informes-medioambientales> (accessed on 14.05.15).

Barbier, E.B., Hacker, S.D., Kennedy, C., Koch, E.W., Stier, A.C., Silliman, B.R., 2011. The value of estuarine and coastal ecosystem services. Ecol. Monogr. 81, 169–193.

Bass, C.C., Luppi, T., Spivak, E.D., 2005. Population structure of the South American estuarine crab, *Chasmagnathus granulata* (Brachyura: Varunidae) near the southern limit of its geographical distribution: comparison with northern populations. Hydrobiologia 537, 217–228.

Belsky, A.J., Matzke, A., Uselman, S., 1999. Survey of livestock influences in stream and riparian ecosystems in the western United States. J. Soil Water Conserv. 54, 419–431.

Bisson, J.W., Cabelli, V.J., 1980. *Clostridium perfringens* as a water pollution indicator. J. Water Pollut. Control Fed. 52, 241–248.

Boehm, A.B., Ashbolt, N.J., Colford Jr, J.M., Dunbar, L.E., Fleming, L.E., Gold, M.A., Hansel, J.A., Hunter, P.R., Ichida, A.M., McGee, C.D., Soller, J.A., Weisberg, S.B., 2009. A sea change ahead for recreational water quality criteria. J. Water Health 7, 9–20.

Boschi, E.E., 1964. Los crustáceos decápodos Brachyura del litoral bonaerense (R. Argentina). Boletín Instituto Biología Marina (Mar del Plata) 6, 1–99.

Brezina, S.S., Baldini, M.D., 2008. Detection of somatic coliphages as indicators of faecal contamination in estuarine waters. Revista Argentina de Microbiología 40, 72–74.

Brookes, J.D., Hipsey, M.R., Burch, M.D., Regel, R.H., Linden, L.G., Ferguson, C.M., Antenucci, J.P., 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. Environ. Sci. Technol. 39, 8614–8621.

Burnham, K.P., Anderson, D.R., 2002. Information and likelihood theory: a basis for model selection and inference. In: KP, Burnham, DR, Anderson (Eds.), Model Selection and Multimodel Inference: a Practical Information-Theoretic Approach. Springer-Verlag New York Inc., pp. 49–97.

Burton Jr., G.A., Gunnison, D., Lanza, G.R., 1987. Survival of pathogenic bacteria in various freshwater sediments. Appl. Environ. Microbiol. 53, 633–638.

Byamukama, D., Mach, R.L., Kansiime, F., Manafi, M., Farnleitner, A.H., 2005. Discrimination efficacy of fecal pollution detection in different aquatic habitats of a high-altitude tropical country, using presumptive coliforms, *Escherichia coli*, and *Clostridium perfringens* spores. Appl. Environ. Microbiol. 71, 65–71.

Byappanahalli, M.N., Roll, B.M., Fujioka, R.S., 2012. Evidence for occurrence, persistence, and growth potential of *Escherichia coli* and enterococci in Hawaii's soil environments. Microbes Environ. 27 (2), 164–170.

Cizek, A.R., Characklis, G.W., Krometis, L.A., Hayes, J.A., Simmons, O.D., Di Lonardo, S., Alderisio, K.A., Sobsey, M.D., 2008. Comparing the partitioning behavior of *Giardia* and *Cryptosporidium* with that of indicator organisms in storm water runoff. Water Res. 42, 4421–4438.

Cox, P., Griffith, M., Angles, M., Deere, D., Ferguson, C., 2005. Concentrations of pathogens and indicators in animal feces in the Sydney watershed. Appl. Environ. Microbiol. 71, 5929–5934.

Czeczulin, J.R., Collie, R.E., McClane, B.A., 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally cpe-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. 64 (8), 3301–3309.

Davies, C.M., Long, J.A.H., Donald, M., Ashbolt, N.J., 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. 61, 1888–1896.

Desmarais, T.R., Solo-Gabriele, H.M., Palmer, C.J., 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl. Environ. Microbiol. 68, 1165–1172.

D'Incao, F., Silva, K.G., Ruffino, M.L., Braga, A.L., 1990. Hábito Alimentar do caranguejo *Chasmagnatus granulata*, Dana, 1851, na Barra do Rio Grande, RS (Decapoda, Grapsidae). Atlântica 12 (2), 85–93.

Edwards, D.D., McFeters, G.A., Venkatesan, M.I., 1998. Distribution of *Clostridium perfringens* and fecal sterols in a benthic coastal marine environment influenced by the sewage outfall from McMurdo Station, Antarctica. Appl. Environ. Microbiol. 64, 2596–2600.

Elliott, E.L., Colwell, R.R., 1985. Indicator organisms for estuarine and marine waters. FEMS Microbiol. Rev. 32, 61–79.

Ewing, B., Green, P., 1998. Basecalling of automated sequencer traces using phred. II. Error probabilities. Genet. Res. 8, 186–194.

Ewing, B., Hillier, L., Wendl, M., Green, P., 1998. Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genet. Res. 8, 175–185.

Farnleitner, A.H., Ryzinska-Paier, G., Reischer, G.H., Burtscher, M.M., Knetsch, S., Kirschner, A.K.T., Dirnböck, T., Kuschnig, G., Mach, R.L., Sommer, R., 2010. *Escherichia coli* and enterococci are sensitive and reliable indicators for human, livestock and wildlife faecal pollution in alpine mountainous water resources. J. Appl. Microbiol. 109, 1599–1608.

Fayer, R., Trout, J.M., Lewis, E.J., Xiao, L., Lal, A., Jenkins, M.C., Graczyk, T.K., 2002. Temporal variability of *Cryptosporidium* in the Chesapeake Bay. Parasitol. Res. 88, 998–1003.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., Spratt, B.G., 2004. EBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. 186, 1518–1530.

Fleming, L.E., Broad, K., Clement, A., Dewailly, E., Elmir, S., Knap, A., Pomponi, S.A., Smith, S., Solo Gabriele, H., Walsh, P., 2006. Oceans and human health: emerging public health risks in the marine environment. Mar. Pollut. Bull. 53, 545–560.

Francisco, A.P., Vaz, C., Monteiro, P.T., Melo-Cristino, J., Ramirez, M., Carriço, J.A., 2012. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics 13, 87.

Freire-Santos, F., Oteiza-Lopez, A.M., Vergara-Castiblanco, C.A., Arees-Mazas, M.E., Alvarez-Suarez, E., Garcia-Martin, O., 2000. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854.

Fujioka, R.S., Shizumura, L.K., 1985. *Clostridium perfringens*, a reliable indicator of stream water quality. J. Water Pollut. Control Fed. 57, 986–992.

Graczyk, T.K., Conn, D.B., Marcogliese, D.J., Graczyk, H., de Lafontaine, Y., 2003. Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*). Parasitol. Res. 89, 107–112.

Guillemi, E.C., Ruybal, P., Lia, V., Gonzalez, S., Lew, S., Zimmer, P., Lopez Arias, L., Rodriguez, J.L., Rodriguez, S.Y., Frutos, R., Wilkowsky, S.E., Farber, M.D., 2015. Development of a multilocus sequence typing scheme for the study of *Anaplasma marginale* population structure over space and time. Infect. Genet. Evol. 30, 186–194.

Harrison, B., Raju, D., Garmory, H.S., Brett, M.M., Titball, R.W., Sarker, M.R., 2005. Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional regulation of the beta2-toxin-encoding gene. Appl. Environ. Microbiol. 71, 8362–8370.

Hill, R.T., Knight, I.T., Anikis, M.S., Colwell, R.R., 1993. Benthic distribution of sewage sludge indicated by *Clostridium perfringens* at a deep-ocean dump site. Appl. Environ. Microbiol. 59, 47–51.

Hill, R.T., Straube, W.L., Palmisano, A.C., Gibson, S.L., Colwell, R.R., 1996. Distribution of sewage indicated by *Clostridium perfringens* at a deep-water disposal site after cessation of sewage disposal. Appl. Environ. Microbiol. 62, 1741–1746.

Huang, X., Madan, A., 1999. CAP3: a DNA sequence assembly program. Genome Res. 9, 868–877.

Huq, A., Huq, S.A., Grimes, D.J., O'Brien, M., Chu, K.H., Capuzzo, J.M., Colwell, R.R., 1986. Colonization of the gut of the blue crab (*Callinectes sapidus*) by *Vibrio cholerae*. Appl. Environ. Microbiol. 52, 586–588.

Iribarne, O., Bortolus, A., Botto, F., 1997. Between-habitat differences in burrow characteristics and trophic modes in the southwestern Atlantic burrowing crab *Chasmagnathus granulata*. Mar. Ecol. Prog. Ser. 155, 137–145.

Iribarne, O., Botto, F., Martinetto, P., Gutierrez, J.L., 2000. The role of burrows of the SW Atlantic intertidal crab *Chasmagnathus granulata* in trapping debris. Mar. Pollut. Bull. 40, 1057–1062.

Ishii, S., Sadowsky, M.J., 2008. *Escherichia coli* in the environment: implications for water quality and human health. Microbes Environ. 23 (2), 101–108.

Iwanaka, T., Kawashima, H., Kishimoto, H., Kakinuma, M., Arai, K., Sakurai, J., Nagahama, M., 2004. Enteritis necroticans caused by *Clostridium perfringens* type a. J. Pediatrics 144, 410.

Jost, B.H., Trinh, H.T., Songer, J.G., 2006. Clonal relationships among *Clostridium perfringens* of porcine origin as determined by multilocus sequence typing. Vet. Microbiol. 116, 158–165.

Kamaras, J., Murrell, W.G., 2001a. The effect of bacterial enterotoxins implicated in SIDS on the rabbit intestine. Pathology 33, 187–196.

Kamaras, J., Murrell, W.G., 2001b. Intestinal epithelial damage in SIDS babies and its similarity to that caused by bacterial toxins in the rabbit. Pathology 33, 197–203.

Lisle, J.T., Smith, J.J., Edwards, D.D., McFeters, G.A., 2004. Occurrence of microbial indicators and *Clostridium perfringens* in wastewater, water column samples, sediments, drinking water, and Weddell seal feces collected at McMurdo Station, Antarctica. Appl. Environ. Microbiol. 70, 7269–7276.

Marshall, R.S., Steenbergen, J.F., McClung, L.S., 1965. Rapid technique for the enumeration of *Clostridium perfringens*. Appl. Microbiol. 13, 559–563.

McClane, B.A., 1996. An overview of *Clostridium perfringens* enterotoxin. Toxicon 34, 1335–1343.

McMahon, R.F., 1991. Mollusca: bivalvia. In: Thorp, J.H., Covich, A.P. (Eds.), Ecology and Classification of North American Freshwater Invertebrates. Academic Press, New York, pp. 315–390.

Meer, R.R., Songer, J.G., 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am. J. Vet. Res. 58, 702–705.

Miller, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Fritz, H.M., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int. J. Parasitol. 35, 673–684.

Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2006a. Evaluation of methods for improved detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). J. Microbiol. Methods 65, 367–379.

Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Byrne, B.A., Jang, S., Harris, M., Ames, J., Jessup, D., Paradies, D., Worcester, K., Melli, A., Conrad, P.A., 2006b. *Salmonella* spp., *Vibrio* spp., *Clostridium perfringens*, and *Plesiomonas shigelloides* in marine and freshwater invertebrates from coastal California ecosystems. Microb. Ecol. 52, 198–206.

Mueller-Spitz, S.R., Stewart, L.B., Klump, J.V., McLellan, S.L., 2010. Freshwater suspended sediments and sewage are reservoirs for enterotoxin-positive *Clostridium perfringens*. Appl. Environ. Microbiol. 76 (16), 5556–5562.

Olivier, S.R., Escofet, A., Penchaszadeh, P., Orensanz, J.M., 1972. Estudios ecológicos de la región estuarial de Mar Chiquita. II. Relaciones tróficas interespecíficas. Anales de la Sociedad Científica Argentina 194, 89–104.

Payment, P., 1999. Poor efficacy of residual chlorine disinfectant in drinking water to inactivate waterborne pathogens in distribution systems. Can. J. Microbiol. 45, 709–715.

Piccolo, M.C., Perillo, G.M.E., Melo, W., 2008. The Bahía Blanca estuary: an integrated overview of its geomorphology and dynamics. In: Neves, R., Baretta, J.W., Mateus, M. (Eds.), Perspectives on Integrated Coastal Zone Management in South America. IST Press, Lisboa, pp. 219–229.

R Development Core Team, 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.

Redondo, L.M., Farber, M., Venzano, A., Jost, B.H., Parma, Y.R., Fernandez-Miyakawa, M.E., 2013. Sudden death syndrome in adult cows associated with *Clostridium perfringens* type E. Anaerobe 20, 1–4.

Rippey, S.R., Watkins, W.D., 1992. Comparative rates of disinfection of microbial indicator organisms in chlorinated sewage effluents. Water Sci. Technol. 26, 2185–2189.

Shojadoost, B., Vince, A.R., Prescott, J.F., 2012. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: a critical review. Vet. Res. 43 (1), 74.

Skanavis, C., Yanko, W.A., 2001. *Clostridium perfringens* as a potential indicator for the presence of sewage solids in marine sediments. Mar. Pollut. Bull. 42, 31–35.

Sorensen, D.L., Eberl, S.G., Dicksa, R.A., 1989. *Clostridium perfringens* as a point source indicator in non-point polluted streams. Water Res. 23, 191–197.

Sparks, S.G., Carman, R.J., Sarker, M.R., McClane, B.A., 2001. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. J. Clin. Microbiol. 39, 883–888.

Streitenberger, M.E., Baldini, M.D., 2010. Deterioro de un área recreacional por efectos del volcado de líquidos cloacales. Revista Argentina de Microbiología 42, 307–310.

Tibbetts, J., 2002. Coastal cities: living on the edge. Environ. Health Perspect. 110 (11), A674–A681.

Touron, A., Berthe, T., Gargala, G., Fournier, M., Ratajczak, M., Servais, P., Petit, F., 2007. Assessment of faecal contamination and the relationship between pathogens and faecal bacterial indicators in an estuarine environment (Seine, France). Mar. Pollut. Bull. 54 (9), 1441–1450.

Tyrrell, S.A., Rippey, S.R., Watkins, W.D., 1995. Inactivation of bacterial and viral indicators in secondary sewage effluents, using chlorine and ozone. Water Res. 29, 2483–2490.

US Environmental Protection Agency, 2003. Bacterial water quality standards for recreational waters (freshwater and marine waters). Status report. US Environmental Protection Agency Report EPA-823-R-03-008, Washington, DC.

Verherstraeten, S., Goossens, E., Valgaeren, B., Pardon, B., Timbermont, L., Vermeulen, K., Schauvliege, S., Haesebrouck, F., Ducatelle, R., Deprez, P., Immerseel, F.V., 2013. The synergistic necrohemorrhagic action of *Clostridium perfringens* perfringolysin and alpha toxin in the bovine intestine and against bovine endothelial cells. Vet. Res. 44, 45.

Viau, E.J., Lee, D., Boehm, A.B., 2011. Swimmer risk of gastrointestinal illness from exposure to tropical coastal waters impacted by terrestrial dry-weather runoff. Environ. Sci. Technol. 45, 7158–7165.

Vierheilig, J., Frick, C., Mayer, R.E., Kirschner, A.K.T., Reischer, G.H., Derx, J., Mach, R.L., Sommer, R., Farnleitner, A.H., 2013. *Clostridium perfringens* is not suitable for the indication of fecal pollution from ruminant wildlife but is associated with excreta from nonherbivorous animals and human sewage. Appl. Environ. Microbiol. 79 (16), 5089–5092.

Wierup, M., DiPietro, J.A., 1981. Bacteriologic examination of equine fecal flora as a diagnostic tool for equine intestinal clostridiosis. Am. J. Vet. Res. 42 (12), 2167–2169.

World Health Organization, 2008. Guidelines for drinking-water quality, third ed. Incorporating the First and Second Addenda, vol. 1. <http://www.who.int/water_sanitation_health/dwq/fulltext.pdf> (accessed on 28.11.14).

Wright, R.C., 1989. The survival patterns of selected faecal bacteria in tropical fresh waters. Epidemiol. Infect. 103, 603–611.

Zilio, M.I., London, S., Perillo, G.M.E., Piccolo, C.M., 2013. The social cost of dredging: the Bahia Blanca Estuary case. Ocean Coast. Manag. 71, 195–202.

Please cite this article in press as: La Sala, L.F., et al. Carriage of *Clostridium perfringens* by benthic crabs in a sewage-polluted estuary. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.05.066