Science of the Total Environment 579 (2017) 646–656

Contents lists available at ScienceDirect

Science of the Total Environment

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

Biogeochemical and hydrological drivers of the dynamics of *Vibrio* species in two Patagonian estuaries

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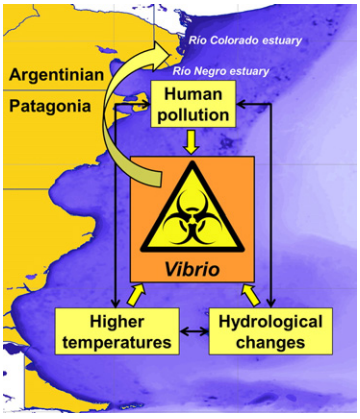
HIGHLIGHTS

- *Vibrio* species with virulence genes were reported in the Argentinian Patagonia.
- Salinity and ammonium were important factors explaining bacterial distribution.
- Salinisation and eutrophication of estuaries favour *Vibrio* abundance.
- Changing baselines are expected to increase the *Vibrio* impacts on ecosystem health.
- Adaptation strategies should sustain a good water quality.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 6 November 2016
Accepted 7 November 2016
Available online 18 November 2016

Editor: D. Barcelo

Keywords:
Organic matter
Nutrients
Hydrology
Sewage

ABSTRACT

The ecology of the most relevant *Vibrio* species for human health and their relation to water quality and biogeochemistry were studied in two estuaries in Argentinian Patagonia. *Vibrio cholerae* and *Vibrio parahaemolyticus* were reported in >29% of cases at the Río Colorado and Río Negro estuaries. Neither the pandemic serogroups of *Vibrio cholerae* O1, *Vibrio cholerae* O139 nor the cholera toxin gene were detected in this study. However, several strains of *V. cholerae* (not O1 or O139) are able to cause human disease or acquire pathogenic genes by horizontal transfer. *Vibrio vulnificus* was detected only in three instances in the microplankton fraction of the Río Negro estuary. The higher salinity in the Río Colorado estuary and in marine stations at both estuaries favours an abundance of culturable *Vibrio*. The extreme peaks for ammonium, heterotrophic bacteria and faecal coliforms in the Río Negro estuary supported a marked impact on sewage discharge. Generally, the more pathogenic strains of *Vibrio* have a faecal origin. Salinity, pH, ammonium, chlorophyll *a*, silicate and carbon/nitrogen ratio of suspended organic particulates were the primary factors explaining the distribution of culturable bacteria after distance-based linear models. Several effects of dissolved organic carbon on bacterial distribution are inferred.

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http://dx.doi.org/10.1016/j.scitotenv.2016.11.045

Cholera
Changing baseline

Global change is expected to increase the trophic state and the salinisation of Patagonian estuaries. Consequently, the distribution and abundance of *Vibrio* species is projected to increase under future changing baselines. Adaptation strategies should contribute to sustaining good water quality to buffer climate- and anthropogenic- driven impacts.

1. Introduction

Within the *Gammaproteobacteria*, the genus *Vibrio* comprises several well-known species that are threats to animal and ecosystem health. *Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the most important species responsible for emerging or re-emerging infectious disease in humans (Harvell et al., 1999; Morens et al., 2004; Wetz et al., 2014). *Vibrio cholerae* generally enters the human host via contaminated food or water and causes intense watery diarrhoea, which leads to severe dehydration and even to death in certain cases of cholera gravis. *Vibrio parahaemolyticus* provokes acute gastroenteritis through haemolysin production and is generally characterised as a seafood-borne disease. *Vibrio vulnificus* can follow a gastrointestinal route but is also widely known for wound infection and septicaemia in humans. These bacteria are native to estuarine and coastal environments and are hazards for coastal ecological and socioeconomic systems, principally in developing countries (Grimes, 1991; Harvell et al., 1999; Binsztein et al., 2004; Costa et al., 2010). Despite these water safety implications, *Vibrio* species play a key role in biogeochemical processes of aquatic ecosystems.

The ecology of microorganisms is intimately linked to the dynamics of organic and inorganic nutrients. The interactions between organic matter and bacteria are crucial for biogeochemical cycles in aquatic systems (Jiao et al., 2010; Amaral et al., 2016). Bacteria incorporate, metabolise and produce organic matter, changing its chemical properties and bioavailability. Organic matter has a strong effect on *Vibrio* ecology and cell metabolism (Grimes et al., 2009; Lara et al., 2011; Neogi et al., 2011). Heterotrophic bacteria can directly take up inorganic nutrients for metabolic processes (Bradley et al., 2010; Hitchcock and Mitrovic, 2013), and some inorganic nutrients have a direct effect on *Vibrio* abundance, cell motility, stationary phase changes and survival (Jahid et al., 2006; Lara et al., 2009). Likewise, nutrients have a bottom-up effect on the abundance and distribution of planktonic organisms.

Close interactions have been found between plankton abundance and pathogenic *Vibrio* in the aquatic environment (Epstein, 1993; Lipp et al., 2002; Seeligmann et al., 2008; Martinelli Filho et al., 2011). Bacteria, phytoplankton and zooplankton are temporally and spatially associated. The relation of *V. cholerae* with zooplankton has been inferred to be a factor in the transmission of human epidemic cholera. Zooplankton not only forms a suitable hard substrate for *Vibrio* biofilm formation but also offers protection against environmental stress and is an important source of nutrients (Thomas et al., 2006; Lara et al., 2011). Moreover, a variety of physicochemical parameters, such as water temperature, salinity and turbidity affect *Vibrio* abundance and distribution (Neogi et al., 2011; Johnson et al., 2012; Mookerjee et al., 2014; López-Hernández et al., 2015). The numerous implied biotic and abiotic factors, as well as their interactions, increase the complexity of studies on *Vibrio* ecology in changing estuaries.

Estuaries are areas of high productivity that provide habitat to a large number of species, their wetlands offer several ecosystem services such as nutrient retention, sediment accretion and environmental stabilisation. As areas of organic matter production, estuaries also play an important role in carbon cycling and export to the oceans (Wu et al., 2007; Canuel and Hardison, 2016). Several highly populated megacities are located at coastlines, and human activities are changing their hydrological and biogeochemical features. Eutrophication and environmental pollution, as a consequence of industrial, agricultural and domestic runoff, are major threats to coastal and estuarine ecosystems (Nixon, 1995; de Jonge et al., 2002; Fricke et al., 2016). The combination of anthropogenic and climate driven alterations shifts coastal ecosystem baselines and affects environmental restoration (Duarte et al., 2009; Kopprio et al., 2015).

Cholera invaded Argentina in epidemic waves during the second half of the 19th century (Carbonetti and Rodríguez, 2007). Penna (1897) reported the first cases in a regiment from an Indian ship at the Bahía Blanca estuary, near the northern limit of the Patagonian region. The epidemic form of cholera (serogroup O1) reappeared dramatically at the end of the 20th century in South America and several hundred cases were reported in the north of Argentina. *Vibrio cholerae* has been detected in the Río de la Plata Estuary and rivers of Tucumán in the central and northern regions of Argentina (Binsztein et al., 2004; González Fraga et al., 2007; Seeligmann et al., 2008). Recently, two cases of human deaths by *V. vulnificus* infections were reported for two elderly men after recreational activities in Uruguayan waters of the Río de la Plata Estuary. Studies contributing to the understanding of links between hydrological factors and ecological disease agents are urgently needed.

To date, there is no investigation on *Vibrio* abundance and its potential biogeochemical drivers in the temperate estuaries of Argentinian Patagonia. We hypothesise that *V. cholerae* and *V. parahaemolyticus* are present in the studied systems and that *Vibrio* abundance and distribution are strongly influenced by temperature, salinity and nutrient concentrations. The aims of the present study are as follows: I) to identify *Vibrio* species and their relationship to environmental factors in two contrasting Patagonian estuaries, II) to evaluate the origin and fate of organic matter and inorganic nutrients and their links with culturable bacteria and anthropogenic impacts, and III) to estimate the effect of global change on the ecology of *Vibrio* and ecosystem health based on the current baseline of Patagonian estuarine systems.

2. Methods

2.1. Study areas

The Río Colorado and Río Negro rivers (Fig. 1) present a nival regime altered by the presence of several dams. They originate at the confluence of upland tributaries, and both watercourses traverse >600 km across Northern Patagonia to flow into the Southwestern Atlantic Ocean. The Río Colorado and Río Negro rivers are vital resources for this semiarid region and suffer from anthropogenic impacts, mainly driven by agricultural activities and urban settlements (Kopprio et al., 2015). More than half of the human population of Argentinian Patagonia, which comprises a terrestrial surface of >800,000 km^2, is located in the Río Negro basin. The annual mean discharge of Río Negro is 930 $m^3\ s^{-1}$; while the discharge of Río Colorado is about four-fold lower, at 150 $m^3\ s^{-1}$. The Río Colorado estuary is characterised by a microtidal regime and has been significantly modified over decades by irrigation works, which shaped its current deltaic form and changed its hydrological characteristics, including an increase in salinity. The Río Negro is a macrotidal estuary with sand banks and marsh islets. The wetlands of both estuaries, particularly the southern wetland of Río Negro, offer several ecosystem services and are crucial for fish, crustaceans, birds and marine mammals. Moreover, the discharge of both rivers influences the marine protected area of Bahía San Blas, a bay with important fisheries and a nursery area and refuge for endangered species.

2.2. Sampling strategy

Sampling was carried out monthly from late spring (November 2013) to early autumn (March 2014) in the Río Colorado and Río Negro estuaries (Fig. 1). The estuaries were sampled across a salinity gradient from the river mouth during high tide (station 1, salinity 33–25) to upstream waters along seven and ten sampling stations in Río Colorado and Río Negro basins (salinity 0.70–0.1), respectively. The stations RN7 (Río Negro estuary at station 7) and RN8 are affected by the sewage discharge of the cities of Viedma and Carmen de Patagones. The samples were collected from a motor boat in Río Negro and from the coastline in Río Colorado because the latter was non-navigable. In November, the roads to reach RC1 (Río Colorado estuary at station 1) were covered with sand and thus inaccessible. Consequently, this station could not be sampled at this time. Temperature, pH, conductivity, salinity and dissolved oxygen were measured in situ with electronic probes (PCE-PHD 1). Turbidity was detected with a portable turbidimeter (PCE-TUM 20).

Water samples for microbiological analyses were collected at each sampling station at 30 cm below the surface using 500-mL sterile bottles. Additionally, seston fractions were sampled by horizontal tow net (Nitex mesh) at three stations in Río Colorado (RC1, RC3 and RC7) and at four in Río Negro (RN1, RN3, RN7 and RN9). The fractions selected were >200 μm for the mesozooplankton, between 200 and 60 μm, and between 60 and 20 μm for the microplankton and <20 μm for the nanoplankton. Each fraction was kept in 250-mL sterile plastic cups and the filtered volume was calculated using a mechanical flow meter (Hydrobios). Water samples for organic and inorganic nutrient analyses were collected with clean 5-L plastic bottles. To avoid thermal and photodamage, all samples were transported in insulated plastic boxes and processed under laboratory conditions within 6 h.

2.3. Microbiological studies

Duplicates of variable volumes of water (1–50 mL) were filtered through sterile nitrocellulose filters (Gamafil, 0.45-μm pore size). Each filter was placed individually in a petri dish filled with Endo agar (Merck) and incubated overnight at 44 °C, and faecal coliforms were counted the following day. Water for terrestrial heterotrophic bacteria counts was diluted with Locke's solution at different concentrations, and the dilutions were directly spread in plate-counting agar (Britania) at 30 °C. Duplicates of water and homogenised seston fractions ($n = 189$) for culturable *Vibrio* counts were filtered (0.1–50 mL) through sterile nitrocellulose filters. Filters were placed individually on thiosulphate citrate bile salts sucrose agar (Britania) and incubated overnight at 37 °C. Presumptive *Vibrio* colonies were enumerated, and at least 20 of these colonies were transferred to alkaline peptone water (Britania) and incubated as described above. Presumptive colonies ($n = 45$) were separately isolated and incubated overnight at 37 °C. Template DNA from fresh bacterial culture was extracted by a simple boiling method (Hoshino et al., 1998). Briefly, 50 μL of alkaline peptone water were added to 450 μL of 1× Tris-EDTA buffer (Sigma), heated to 100 °C for 10 min in a heat block and centrifuged at 10,500*g* and 4 °C for 3 min. The supernatant was collected and stored at −20 °C for future use.

Vibrio cholerae, V. parahaemolyticus and V. vulnificus were detected by a highly sensitive and specific multiplex Polymerase Chain Reaction (PCR) after the method of Neogi et al. (2010). The primers of this assay (GeneDesign, Inc.) were based on the *toxR* gene for *V. cholerae* and *V. parahaemolyticus* and the *vvhA* gene for *V. vulnificus* (Table 1). Positive controls were DNA templates from the reference strains *V. cholerae* O1 N 16961, *V. vulnificus* IFO 15675 and *V. parahaemolyticus* NBRC 12711. PCR was performed in a 20-μL reaction mixture for each

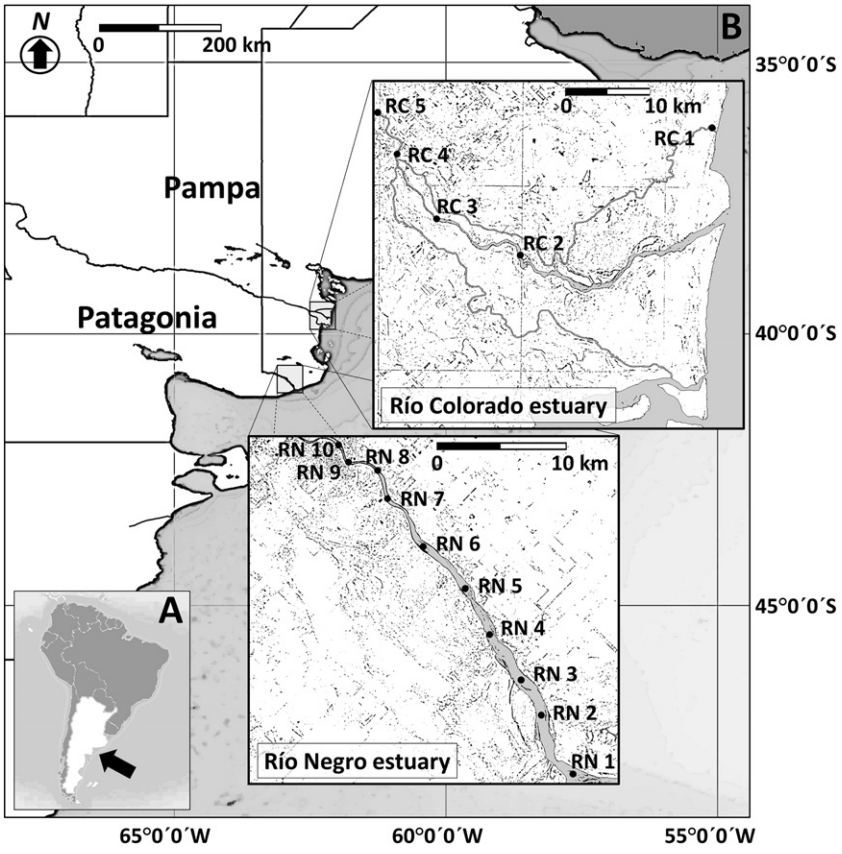

Fig. 1. Location of the Río Colorado and Río Negro estuaries and sampling stations (RN or RC) in Argentinian Patagonia.

Table 1
Primers used for *Vibrio* species and serogroups identification and presence of cholera toxin gene of *Vibrio cholerae*.

Primer	Target gene	Sequence (5′-3′)	Concentration (μM)	References
VC toxR 403F	*V. cholerae*,	GAAGCTGCTCATGACATC	0.50	Neogi et al., 2010
VC toxR 678R	*toxR*	AAGATCAGGGTGGTTATTC		
vvhA 870F	*V. vulnificus*,	ACTCAACTATCGTGCACG	0.30	Neogi et al., 2010
vvhA 1236R	*vvhA*	ACACTGTTCGACTGTGAG		
VP toxR 325F	*V. parahaemolyticus*,	TGTACTGTTGAACGCCTAA	0.15	Neogi et al., 2010
VP toxR 828R	*toxR*	CACGTTCTCATACGAGTG		
O139 F2	*V. cholerae* O139,	AGCCTCTTTATTACGGGTGG	0.27	Hoshino et al., 1998
O139 R2	*rfb*	GTCAAACCCGATCGTAAAGG		
O1F2-1	*V. cholerae* O1,	GTTTCACTGAACAGATGGG	0.50	Hoshino et al., 1998
O1R2-2	*rfb*	GGTCATCTGTAAGTACAAC		
VCT1	cholera toxin,	ACAGAGTGAGTACTTTGACC	0.17	Hoshino et al., 1998
VCT2	*ctxA*	ATACCATCCATATATTTGGGAG		

tube containing 0.5 U of rTaq polymerase (Invitrogen), 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM of dNTP (PB-L), and a variable concentration of each primer set (Table 1). PCR conditions were an initial denaturation of 3 min at 94 °C, followed by 30 cycles of denaturation for 45 s at 94 °C, annealing for 30 s at 55 °C and extension for 60 s at 72 °C, and a final extension step for 8 min at 72 °C in a PCR thermal cycler (IVEMA T-18). PCR products were separated on a 2% agarose gel in TAE [40 mM Tris-acetate (pH 8), 1 mM EDTA] at 85 mV for 1.25 h in an electrophoresis system (Enduro). Bands were visualised under UV light after staining with SYBR Green (Sigma). Images were captured with a slider imager equipped with an LED transilluminator (Maestrogen). The serogroups of *V. cholerae* O1 and O 139 and the cholera toxin were studied according to Hoshino et al. (1998). The primers used were from specific regions of the *rtf* cluster for the two serogroups and from the *ctxa* gene for the toxin (Table 1).

2.4. Pigments and nutrient analyses

Water samples were filtered (Whatmann GF/F) under laboratory conditions and filters for chlorophyll *a* and phaeopigment analyses were placed in buffered acetone (90%) and kept overnight at 4 °C. The pigment concentrations were quantified spectrophotometrically. Filters for particulate organic matter were dried overnight at 50 °C and preserved in an exsiccator for analysis of particulate organic carbon and nitrogen. Additional filtrates were kept frozen at −20 °C in 100 mL PE bottles for subsequent inorganic nutrient determinations. Ammonium, nitrate, nitrite, silicate and phosphate were measured according to standard methods (Kattner and Becker, 1991). Dissolved inorganic nitrogen was estimated as the summation of ammonium, nitrate and nitrite. Filtered water samples for dissolved organic carbon were adjusted to pH = 2 with H_3PO_4 and kept at −20 °C in precombusted glass ampoules. Dissolved organic carbon was determined by high temperature catalytic oxidation with a Shimadzu TOC-V_{CPN} analyser. System performance was verified using consensus reference water (Hansell Lab.). Filters for particulate organic carbon and nitrogen were acidified with 0.1 N HCl to remove inorganic carbon, dried at 50 °C for 12 h, placed in tin capsules, and completely oxidised in the elemental analyser (EURO EA) by flash combustion at 1000 °C under pure O_2. Acetanilide (Hekatech) was used as an internal standard.

2.5. Data analysis

All statistical tests were considered significant at $p < 0.05$. Differences in the mean biogeochemical and hydrological parameters between the estuaries were evaluated using the non-parametric Mann-Whitney test. Spearman correlations (r_s) were performed because of the non-normal distributions and non-linear relations of most of the data. Both analyses were calculated using Statistica 8.0. Parametric linear regressions among some variables were performed with Xact 7.21d. Differences between estuaries and months were evaluated using permutational multivariate analysis of variance (PERMANOVA), which is distribution-free. PERMANOVA was based on Euclidean distances calculated from log-transformed data. In the case of significant differences, pairwise comparisons and similarity percentage (SIMPER) were subsequently run to identify the main variables contributing to dissimilarity. To evaluate relationships between environmental traits and bacterial distribution, data were assessed using distance-based linear models and ordinated with distance-based redundancy analysis. PERMANOVA, SIMPER, distance-based linear models and distance-based redundancy analysis were performed in PRIMER v6 and PERMANOVA.

3. Results and discussion

3.1. Vibrio species and genes

Vibrio cholerae and *V. parahaemolyticus* were detected in 65% and 29% of the samples, respectively, using the multiplex PCR (Supplementary material). Each sample was considered from the pool of colonies. *Vibrio vulnificus* was identified in only three samples associated with microplankton in the Río Negro estuary. To our knowledge, this is the first report of *V. cholerae* with at least one gene related to virulence (*toxR*) in the water courses of Argentinian Patagonia and at latitudes higher than 39°S in coastal regions of the Southwestern Atlantic. Furthermore, this work is the first to mention the *V. parahaemolyticus* and *V. vulnificus* species with virulence or pathogenic genes (*toxR* and *vvhA*) in water and plankton samples from coastal regions of Argentina.

Several genes determine the virulence of *Vibrio* strains; the presence of these toxigenic or virulence genes in the environment signify a risk. The *toxR* gene encodes a trans-membrane protein that controls the transcription of toxin genes and other important proteins involved in pathogenesis and virulence, whereas *vvhA* is responsible for cytolytic activities (Neogi et al., 2010; Krebs and Taylor, 2011; Wetz et al., 2014; He et al., 2015). The presence of *toxR* is generally detected in >99% of *V. cholerae* isolates in Argentina (Bidinost et al., 2004; González Fraga et al., 2007). This finding suggests that this gene is also required for metabolic activities in the environment and is not only related to pathogenesis (Rivera et al., 2001). The presence of *vvhA* is directly involved in the pathogenesis of *V. vulnificus* and is of greater relevance for human health.

3.2. Vibrio cholerae serogroups, cholera toxin gene and culturable state

Vibrio cholerae O1, *V. cholerae*, *Vibrio cholerae* O139 and *ctxA* gene encoding for cholera toxin were not detected in any sample from either estuary. However, these results do not indicate their total absence in the studied systems. Toxigenic strains of *V. cholerae* are infrequently isolated from surface waters by culture methods (Alam et al., 2006). *Vibrio cholerae* O1 in a viable, but non-culturable state, has been reported in the Rio de la Plata estuary and the rivers of Tucumán (Binsztein et al., 2004; Seeligmann et al., 2008). A viable non-culturable state is a dormant phase used by *V. cholerae* to survive unfavourable environmental

conditions; a culturable form can be reverted to under favourable settings or in the host (Li et al., 2014). According to Neogi et al. (2011), this dormant state has a much reduced metabolism and consequently a weaker influence on aquatic biogeochemical processes.

Generally, *Vibrio* incubated in selective media without decreasing temperature is able to grow for several days until reaching a viable, but non-culturable, state (Krebs and Taylor, 2011). Although culturable bacteria may represent up to 3% of the total population in estuarine regions (Amann et al., 1995), culturable *Vibrio* usually exhibits a similar distribution as non-culturable forms (Lara et al., 2011; Neogi et al., 2011). Culturable bacteria are widely used as ecological indicators for water safety because of their reliability and lower-cost. These last two advantages of working with culturable bacteria are crucial for *Vibrio* research in developing countries, which are the most affected by severe *Vibrio* outbreaks but with limited resources for research.

The presence of non-O1 and non-O139 *V. cholerae* also has important implications on human health. Several outbreaks of diarrhoea caused by non-O1 and non-O139 strains have been reported worldwide, even in the north of Argentina (González Fraga et al., 2007). These strains produce several virulence and pathogenic factors such as toxins, proteases and haemolysins (Ottaviani et al., 2009; Ceccarelli et al., 2015), which considerably increase the secretory response of intestinal tissue. Moreover, most environmental strains can acquire virulence or pathogenic genes through horizontal transfer (Faruque et al., 1998; Neogi et al., 2010; Khouadja et al., 2014), and environmental factors can trigger the expression of these genes (Lara et al., 2009; Khandeparker et al., 2015). There is evidence that *V. cholerae* O139 arose by horizontal gene transfer between a non-O1 and O1 strain (Bik et al., 1995). Therefore, it is of great relevance to survey the entire population, rather than only pathogenic forms, as well as their relationships with environmental factors.

3.3. Culturable Vibrio and seston fractions

Approximately half (47%) of the presumptive colonies, which were isolated individually, were positive through PCR identification for at least one of the studied *Vibrio* species. Taking into account the filtered volume and the fractions of seston, 91.5% of the total culturable *Vibrio* counts were registered in the nanoplankton (<20 μm) fraction. In the microplankton fractions, the mean relative abundances were: 6.2% for the 20 to 60 μm fraction and 1.9% for the 60 to 200 μm fraction. This value was the lowest in mesozooplankton (>200 μm, 0.4%). This trend was very similar to other oceanic and tropical estuarine systems, with >98% of bacteria detected in nanoplankton (Lara et al., 2011; Neogi et al., 2011). Nevertheless, the role of zooplankton in *Vibrio* ecology is relevant.

A single copepod of the mesozooplankton may contain an infective dose for cholera (Covazzi Harriague et al., 2008). The chitin in this crustacean can favour the horizontal transfer of genes. *Vibrio cholerae* can acquire new genetic material by natural transformation during growth on chitin (Meibom et al., 2005) and this new genetic material may signify the emergence of new variants that are better adapted to the environment or more pathogenic to humans. Zooplankton and the organisms or organic aggregates in nanoplankton are of great relevance to the study of *Vibrio* ecology. Furthermore, pathogenic *V. vulnificus* was only detected within the microplankton fraction, and this association with plankton or aggregates within this fraction may favour the persistence of pathogens in the environment.

3.4. Hydrological and biogeochemical drivers of Vibrio and impact of sewage

The positive cases of *V. parahaemolyticus* were generally, but not exclusively, associated with higher salinities, while *V. cholerae* followed the opposite trend (Fig. 2). *Vibrio parahaemolyticus* is a halophilic bacteria related to higher salinities (Grimes, 1991; López-Hernández et al., 2015). Culturable *Vibrio* counts, salinity, pH, turbidity, silicate, particulate organic carbon, and the carbon/nitrogen ratio of the particulates were significantly higher in the Rio Colorado than in the Río Negro estuary (Table 2) when considering only the mean values and not the monthly variation or sample location. In contrast, the Rio Negro estuary was characterised by greater faecal coliforms, heterotrophic bacteria, ammonium, nitrate, phosphate, dissolved organic carbon and phaeopigments. Thus, the Rio Colorado estuary reflects salinisation impacts, whereas the Río Negro estuary indicates some eutrophication problems. Consequently, these different states may contribute to dissimilarities in bacterial distribution.

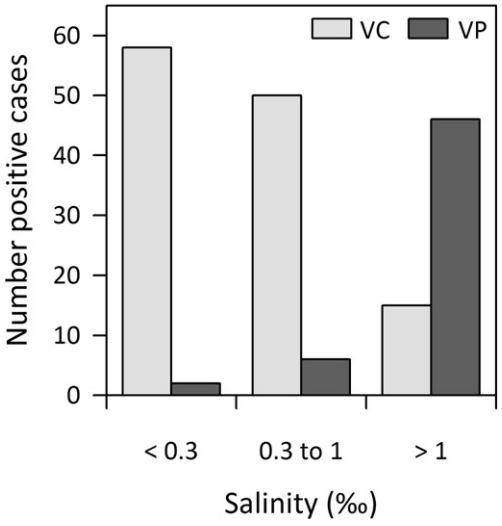

Fig. 2. Salinity relationship to the number of positive cases of *Vibrio cholerae* (VC0) and *Vibrio parahaemolyticus* (VP).

No significant differences were found between the estuaries in temperature, particulate organic nitrogen or chlorophyll *a* after the Mann-Whitney test. The size effect or extreme values in the data set analysis of Table 2 should be carefully analysed. For example, the maximum values of ammonium, faecal coliforms and heterotrophic bacteria coincided with the sewage discharge event and affect the significance of the test. Furthermore, PERMANOVA revealed significant differences between the estuaries (pseudo-$F = 10.41$, $p_{perm} < 0.001$) and the considered months (pseudo-$F = 4.05$, $p_{perm} < 0.001$). There was also an interaction between estuaries and months (pseudo-$F = 1.99$, $p_{perm} = 0.026$). When comparing estuaries within months by pair-wise tests (Table 3) of PERMANOVA, non- significant differences were detected in January and March.

After SIMPER analysis (Table 3), the main dissimilarities at both estuaries between November and December were explained by coliforms, cultivable *Vibrio* counts, salinity and heterotrophic bacteria. In February, the month with the higher influence of sewage discharge, ammonium, coliforms and heterotrophic bacteria were the main factors contributing to the dissimilarities. Considering the tested months within estuaries, November (late spring) was in almost all cases separated from the other months at Río Colorado and Río Negro. Conversely, the warmer months (January, February and March) were not significantly differentiated in the Río Colorado estuary. This trend was very similar in the Río Negro estuary, but in some cases incorporated the month of December. After SIMPER analysis in the month within estuary sections, the primary variables contributing to their distance were principally coliforms and heterotrophic bacteria, and secondary variables were culturable *Vibrio* counts, salinity and ammonium.

However, SIMPER is sensitive to abundance within the variables, and some effects of water temperature are proposed for the dissimilarities of November with the warmer months. There was no significant correlative evidence between temperature and culturable *Vibrio* counts

Table 2
Biogeochemical and hydrological characteristics from November 2013 to March 2014 in the Rio Negro and Río Colorado estuaries. Statistical differences after Mann-Whitney test.

	Rio Negro estuary				Rio Colorado estuary				
Characteristics	Mean		SD	(min–max)	Mean		SD	(min–max)	p
Bacteria									
Vibrio culturable (CFU 100 mL^{-1})	500	±	850	(25–4600)	1020	±	1370	(50–5800)	0.001
Faecal coliforms (CFU 100 mL^{-1})	1600	±	6300	(4–40,000)	81	±	109	(n.d.–440)	0.031
Heterotrophic bacteria (CFU mL^{-1})	10,000	±	50,000	(30–350,000)	1060	±	1820	(60–7830)	0.002
Hydrological characteristics									
Temperature (°C)	22	±	2.9	(17–26)	23	±	1.9	(18–26)	0.339
Salinity	3.7	±	8.1	(0.1–28)	3.8	±	9.3	(0.7–33)	<0.001
pH	8.3	±	0.2	(8.0–8.6)	8.5	±	0.3	(8.1–9.9)	0.001
Turbidity (NTU)	20	±	21	(5.4–130)	31	±	20	(11 − 120)	<0.001
Inorganic and organic nutrients									
Ammonium (μM)	3.4	±	8.4	(0.1–54)	0.6	±	0.3	(0.2–1.2)	<0.001
Nitrate (μM)	5.4	±	4.1	(0.1–22)	4.4	±	6.2	(0.3–29)	<0.001
Phosphate (μM)	0.8	±	0.8	(0.1–4.8)	0.3	±	0.3	(n.d.–1.0)	<0.001
Silicate (μM)	113	±	37	(20–195)	132	±	61	(13 − 132)	0.044
Particulate organic carbon (μM)	73	±	55	(17–260)	102	±	37	(39–191)	<0.001
Particulate organic nitrogen (μM)	14	±	11	(2.8–54)	13	±	4.2	(6.0–21)	0.486
Carbon/nitrogen ratio particulates	5.2	±	0.9	(4.0–6.9)	8.2	±	1.6	(4.4–12)	<0.001
Dissolved organic carbon (μM)	91	±	20	(51–153)	86	±	36	(33–260)	0.015
Pigments									
Chlorophyll *a* (μg L^{-1})	6.8	±	5.9	(1.8–32)	3.8	±	1.5	(1.3–8.0)	0.056
Phaeopigments (μg L^{-1})	1.3	±	1.8	(n.d.–7.2)	1.0	±	2.1	(n.d.–8.3)	0.019

SD: standard deviation, min: minimum, max: maximum, CFU: colony-forming-unit, n.d.: not detected.

(Table 4) and temperature were only weakly correlated to faecal coliforms at Río Colorado ($r_s = 0.48$, $p = 0.003$) and heterotrophic bacteria at Río Negro ($r_s = 0.35$, $p = 0.01$). Water temperature is considered a strong predictor of the abundance and distribution of total *Vibrio* (Neogi et al., 2011; Johnson et al., 2012; Baker-Austin et al., 2013). This work was focused on the warmer months, when human populations were more exposed to estuarine bacteria due to recreational activities. The colder station usually had higher *Vibrio* counts because of the influence of marine water. For these reasons, there were no significant relationship between this environmental factor and *Vibrio*.

The several-fold increase in culturable *Vibrio* counts at stations of higher marine influence showed the marked effect of salinity on their distribution (Fig. 3). Salinity was strongly correlated with culturable *Vibrio* counts considering the total number of samples at both estuaries. This relationship was moderate for Río Negro but not significant for Río Colorado (Table 4). Salinity, together with pH, ammonium, silicate, and

Table 3
Pair-wise tests comparing differences between estuaries (Río Colorado and Río Negro) and months (November to March) after permutational multivariate analysis of the variance (PERMANOVA.). Similarity percentages (SIMPER) are given to each significant difference at $p_{perm} > 0.05$.

Comparisons	d. f.	T	p_{perm}	SIMPER (Contribution %)
Estuaries within months				
November	14	2.61	0.004	Coli (58.9), HB (11.3), CVC (6.4), Sal (6.0)
December	15	2.81	0.001	Coli (51.6), CVC (11.2), Sal (7.6), HB (6.0)
January	15	1.17	0.262	
February	15	1.77	0.044	HB (31.2), Coli (29.7), NH_4^+ (8.7), Sal (8.6)
March	15	1.19	0.215	
Months within estuaries				
Río Colorado estuary				
Nov, Dec	11	1.69	0.019	Coli (31.7), HB (22,7), CVC (10,9), Phae (10.3)
Nov, Jan	11	2.56	0.004	Coli (59.9), HB (11,3), CVC (7,7), NO_3^- (4.9)
Nov, Feb	11	3.25	0.002	Coli (56.8), HB (15.2), CVC (11.9), Phae (5.0)
Nov, Mar	11	2.45	0.002	Coli (35.8), HB (32.1), CVC (10.0), Phae (6.7)
Dec, Jan	12	1.82	0.045	Coli (55.4), CVC (11.8), Sal (10.8), Sil (6.4)
Dec, Feb	12	2.29	0.007	Coli (57.2), CVC (15.0), Sal (9.9), Sil (5.1)
Dec, Mar	12	1.33	0.171	
Jan, Feb	12	0.88	0.427	
Jan, Mar	12	1.51	0.080	
Feb, Mar	12	1.54	0.096	
Río Negro estuary				
Nov, Dec	18	1.67	0.040	Coli (36.7), Sal (10.5), CVC (10.0), HB (7.8)
Nov, Jan	18	1.22	0.201	
Nov, Feb	18	1.70	0.036	Coli (36.7), HB (15.1), CVC(12.9), Sal (11.6)
Nov, Mar	18	1.85	0.028	HB (22.6), Coli(19.3), Sal (18.0), CVC (15.7)
Dec, Jan	18	0.91	0.480	
Dec, Feb	18	1.49	0.088	
Dec, Mar	18	1.78	0.027	Coli (26.5), HB (19.7), Sal(12.9), CVC(10.6)
Jan, Feb	18	1.01	0.344	
Jan, Mar	18	1.65	0.045	
Feb, Mar	18	2.313	0.007	HB (32.4), Coli (25.1), CVC(13.1), Sal (10.1)

d.f: degree of freedom of the denominator, Coli: faecal coliforms, HB: heterotrophic bacteria, CVC: culturable *Vibrio* counts, Sal: salinity, NH_4^+: Ammonium, NO_3^-: Nitrate, Phae: phaeopigments, Sil: silicates.

Table 4
Spearman rank correlations (r_s) of culturable *Vibrio* counts with hydrological and biogeochemical variables in Rio Colorado estuary ($n = 34$), Río Negro estuary ($n = 50$) and total number of samples ($n = 84$).

Variables	Culturable *Vibrio* counts					
	Rio Colorado		Río Negro		Total	
	r_s	p	r_s	p	r_s	p
Hydrological						
Temperature	−0.30	0.081	−0.12	0.404	−0.15	0.184
Salinity	−0.01	0.968	0.56	<0.001	0.62	<0.001
pH	−0.41	0.016	−0.63	<0.001	−0.33	0.002
Turbidity	0.10	0.565	0.26	0.072	0.29	0.008
Biogeochemical						
Ammonium	−0,17	0.350	−0,03	0.847	−0.25	0.021
Nitrate	0.44	0.009	0.24	0.101	0.14	0.199
Phosphate	0.37	0.032	0.12	0.416	−0.07	0.506
Silicate	−0.47	0.004	−0.27	0.059	−0.25	0.023
Particulate organic carbon	0.03	0.886	0.37	0.009	0.39	<0.001
Particulate organic nitrogen	0.13	0.449	0.36	0.011	0.27	0.012
Carbon/nitrogen ratio part.	−0.02	0.905	0.09	0.519	0.26	0.017
Dissolved organic carbon	0.30	0.091	0.30	0.034	0.15	0.168
Chlorophyll *a*	0.30	0.085	0.35	0.013	0.23	0.036
Phaeopigments	−0.12	0.483	0.11	0.466	−0,10	0.368

the carbon/nitrogen ratio of the suspended organic particulates, explain the bacterial distribution with a R^2 of 0.49 according to distance-based linear models. This finding is one of the overall best solutions if we consider that all environmental variables explain the bacterial distribution with a R^2 of 0.55.

The samples with higher marine influence and those from the Río Colorado estuary are ordinated at the negative side of the first axis (32.5% of the variation) of the distance-based redundancy analysis, principally due to salinity (Fig. 4A). This environmental factor also contributed to major differences after SIMPER. Most *Vibrio* have a minimum salinity requirement for growth and persistence (Froelich et al., 2013), as salinity generally stimulates their abundance and distribution (Castañeda Chávez et al., 2005; Lara et al., 2009, 2011; Wetz et al., 2014). The higher salinity in the Río Colorado estuary, near irrigation works and at the stations of higher marine influence in both estuaries, favours *Vibrio* abundance, particularly for *V. parahaemolyticus*.

The estuarine pH is a major factor explaining bacterial distribution after distance-based linear models: higher pH in the Río Colorado coincided with significantly higher *Vibrio* counts after the Mann-Whitney test. *Vibrio* species usually grow better in alkaline media and are associated with an elevated pH in the environment (Costa et al., 2010). Furthermore, it has been suggested that after the earthquake in Haiti, the consequent increased alkalinity of its rivers was a factor contributing

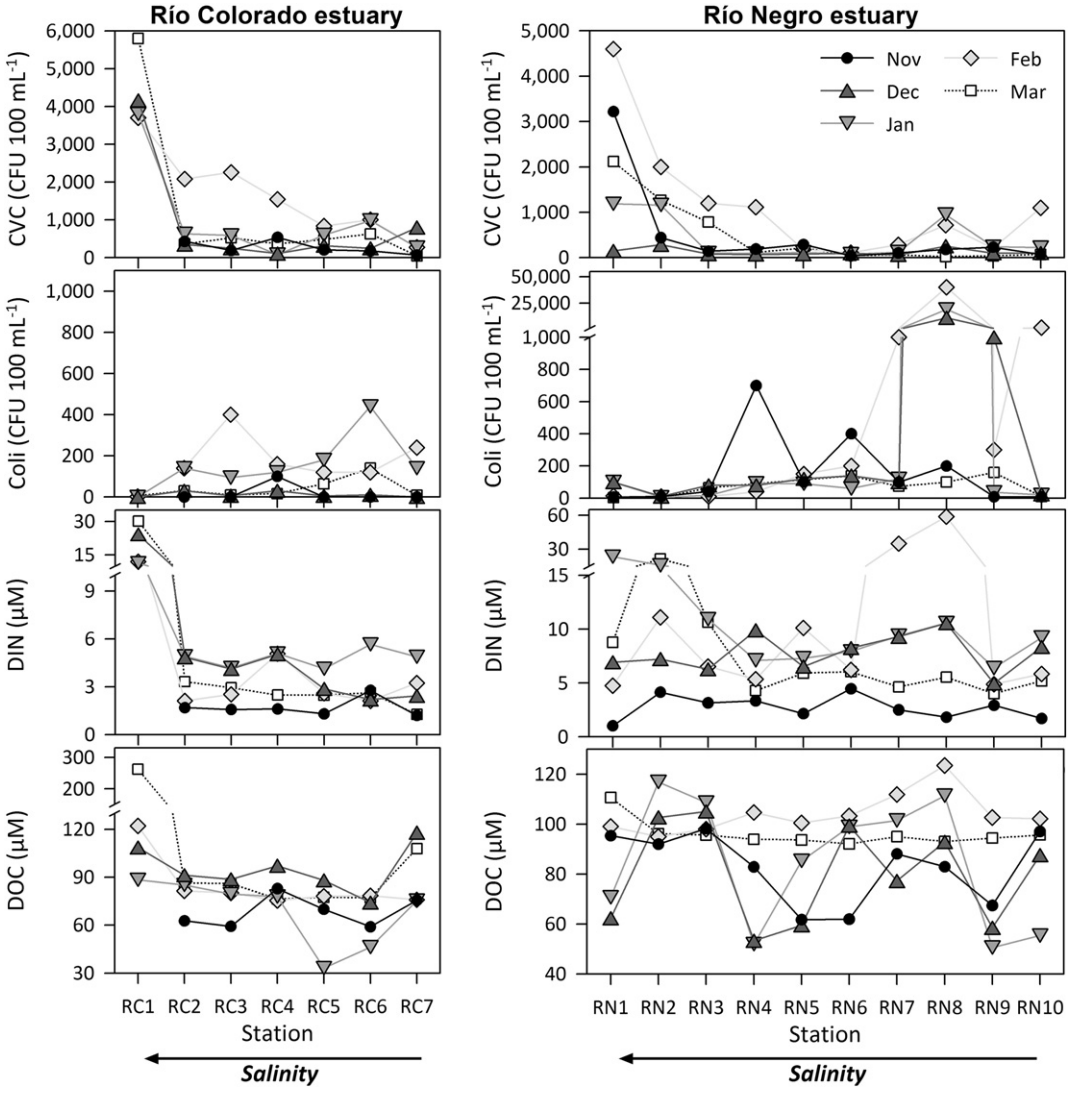

Fig. 3. Monthly and spatial distributions of culturable *Vibrio* counts (CVC), faecal coliforms (Coli), dissolved inorganic nitrogen (DIN) and dissolved organic carbon (DOC) in the Río Colorado and the Río Negro estuaries.

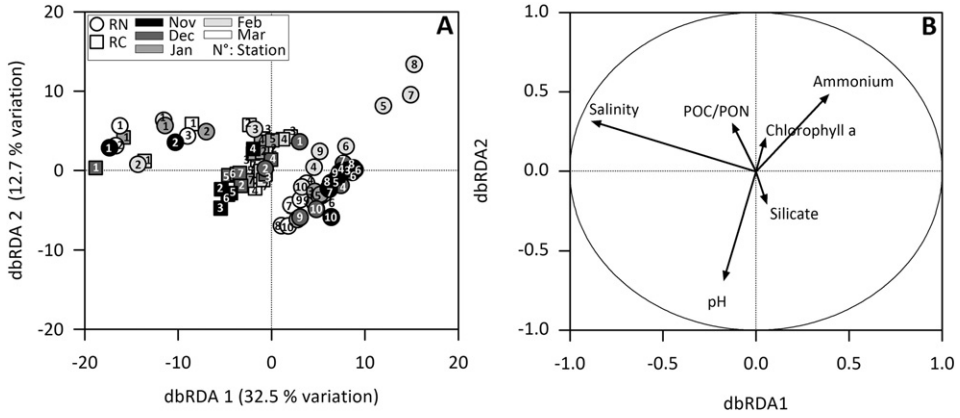

Fig. 4. Distance-based redundancy analysis (dbRDA) ordinating estuaries (figures), months (colour) and station (numbers). POC/PON: particulate organic carbon and nitrogen ratio.

to *Vibrio* outbreaks (Levy, 2015). The irrigation works in the Río Colorado and their consequences for pH may improve conditions for *Vibrio* growth. However, the correlative evidence indicates a significant negative relationship between pH and culturable *Vibrio* counts (Table 4). The marine stations were several tenth of a pH unit lower than the riverine stations. This phenomenon can be explained by the alkaline nature of some Patagonian rivers (Gaiero et al., 2002) and by microbial degradation that increase CO_2 concentrations at the mouth of the estuary. In this case, correlative evidence may not represent causality, and further research is needed to understand the effect of pH on *Vibrio* distribution.

The release of untreated sewage at station 8 of the Río Negro estuary (RN8) is evidenced, particularly in January and February, by higher faecal coliforms, dissolved inorganic nitrogen (mostly ammonium) and dissolved organic carbon (Fig. 3). This phenomenon in the Río Negro estuary is also supported by weak correlations of ammonium with heterotrophic bacteria ($r_s = 0.43$, $p = 0.002$) and faecal coliforms ($r_s = 0.33$, $p = 0.002$). The Spearman correlation is valuable as a non-parametric test but is not greatly influenced by extreme values outside of the rank. Ammonium was major factor explaining the bacterial distribution after distance-based linear models as well as dissimilarities between months and estuaries after SIMPER. This inorganic nutrient is very likely derived from the reductive activity of bacteria on organic matter from wastewater under anoxic conditions. Moreover, it was the main factor at the positive side of the first axis of the ordination (Fig. 4), where sewage samples are located at the positive extreme.

The higher eutrophic conditions of the Río Negro estuary are also suggested in this distance-based redundancy ordination. Ammonium is the dominant feature in sewage outfalls and may enhance the liberation of the green-house gas N_2O in estuarine zones (Ahad et al., 2006). Nitrogen is the principal limiting nutrient in estuaries and coasts (Nixon, 1995; de Jonge et al., 2002) and its abundance favours eutrophication, which enhances plankton blooms and bacterial growth. Waterborne bacterial disease outbreaks have been directly associated with faecal pollution of aquatic resources (Khandeparker et al., 2015; Teklehaimanot et al., 2015; Henry et al., 2016). Cultivable *Vibrio* counts increased slightly at the sewage discharge, compared to the extreme values of coliforms and heterotrophic bacteria (Fig. 3). Despite this slight trend, its faecal origin is of great concern, and the direct discharge of untreated wastewater favours the occurrence of *V. cholerae* (Castañeda Chávez et al., 2005). Furthermore, *V. cholerae* was detected in heavy sewage-contaminated environments of Peru before a cholera outbreak occurred in the community (Gil et al., 2004).

Some effects of the dynamics between dissolved organic carbon and *Vibrio* are inferred in the trends at both estuaries, not only at the sewage discharge but also at the stations with higher salinity (Fig. 3). Dissolved organic carbon was weakly correlated with culturable *Vibrio* counts only in the Rio Negro estuary (Table 4). Dissolved organic matter is the main nutritional source for aquatic bacteria (Jiao et al., 2010) and *Gammaproteobacteria* are associated with elevated dissolved organic carbon (Amaral et al., 2016). Dissolved organic matter has a considerable effect on the regulation of *Vibrio* populations and these bacteria possess a wide variety of enzymes for organic matter degradation, such as mucinases, proteases, lipases, and laminarinases (Neogi et al., 2011 and references therein). We infer that some of the higher dissolved organic carbon values near the marine regions at both estuaries are related to wetland outwelling favouring bacterial abundance in regions of intermediate salinity. Moreover, freshwater organisms suffer a considerable osmotic shock in saline waters, which may facilitate the liberation of dissolved organic nutrients. Microbial degradation may also increase the CO_2 concentration in water and decrease the pH.

The peaks of culturable *Vibrio* counts at station 1 in the Río Colorado estuary (RC1) and at some saline stations in the Río Negro estuary (Fig. 3) coincided with higher values of dissolved inorganic nitrogen (mostly nitrate). At this estuary, cultivable *Vibrio* counts were moderately correlated with nitrate and weakly correlated with phosphate (Table 4). Both inorganic nutrients are relevant for the ecology of microorganisms and plankton as well as eutrophication processes in estuaries and coasts. Phosphate has a direct influence on *Vibrio* metabolism and ecology (Jahid et al., 2006; Chimetto Tonon et al., 2015). Some studies report negative relationships between nitrate and the growth of some *Vibrio* species (Rehnstam-Holm et al., 2010; Khandeparker et al., 2015). In this study, nitrate may indicate co-occurrence with cultivable *Vibrio* counts or represent a substrate for further denitrification, as several species of *Vibrio* have the capability to reduce nitrate (Lara et al., 2011). Although silicate was a major factor explaining bacterial distribution after distance-based linear models and was significantly correlated with culturable *Vibrio* counts, we only infer an effect on diatom abundance, and indirectly on bacteria.

The regressions between particulate organic carbon and chlorophyll *a* were only significant at the Río Negro estuary, whereas the regressions between particulate organic carbon and nitrogen were significant at both estuaries (Fig. 5). The slope of the last regression and its relation to the Redfield ratio indicates a higher terrigenous input in the Río Colorado estuary and this slope, together with the particulate organic carbon relationship to chlorophyll *a*, an autochthonous input (e.g., phytoplankton) in the Río Negro estuary. The carbon/nitrogen ratio of the particulates was also a major factor explaining bacterial distribution after distance-based linear models. Cultivable *Vibrio* counts were weakly but significantly correlated with particulate organic carbon, nitrogen and chlorophyll *a* in the Río Negro estuary and the total number of samples at both estuaries (Table 4). Phytoplankton blooms collaborate in the persistence and spread of *Vibrio cholerae* (e.g., Epstein, 1993).

Particulate organic matter represents an important source of carbon and nitrogen for aquatic bacteria. The quantity of particulate organic matter and sediment resuspension dramatically affects the quantity of

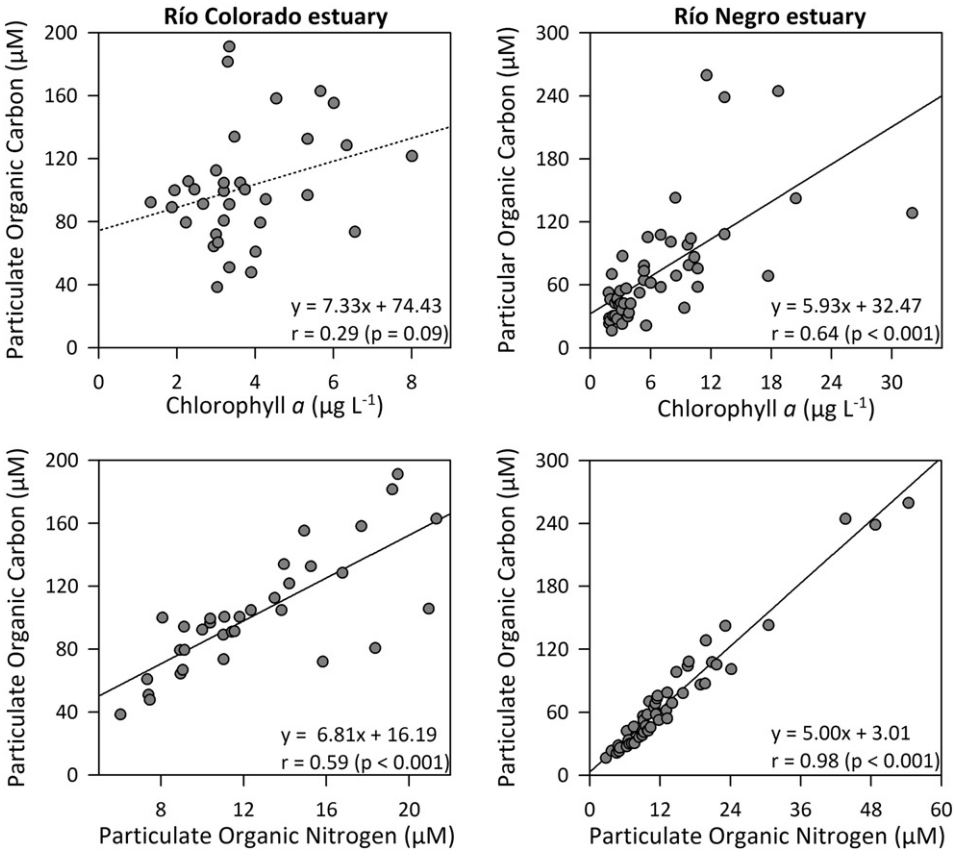

Fig. 5. Linear regressions between particulate organic carbon (POC) and nitrogen (PON) and particulate organic carbon and chlorophyll *a* in Río Colorado and Río Negro estuaries.

Vibrio spp. in estuaries (Lara et al., 2009, 2011; Batabyal et al., 2014). Particulate organic carbon was correlated with turbidity at the Río Colorado ($r_s = 0.50$, $p = 0.002$) and Río Negro ($r_s = 0.79$, $p < 0.001$) estuaries. Turbidity was a good indicator of particulate organic carbon and may suggest sediment suspension. Further studies will help to understand the role of bentho-pelagic coupling on *Vibrio* dynamics. Particulate organic carbon was correlated with salinity in the Río Negro estuary ($r_s = 0.72$, $p < 0.001$) The higher values of particulate organic carbon at the marine stations of the Río Negro estuary and the higher carbon/nitrogen ratio of organic particulates in the Río Colorado estuary also favour the growth and distribution of *Vibrio*.

3.5. Changing baselines

Global change, the synergic interaction of climate change with anthropogenic impacts, is likely to increase the magnitude and frequency of floods, droughts and coastal storms, to increase sea-level and water temperature, to alter biogeochemical processes, to modify current and winds patterns and to lead to increased human use of water resources in Patagonian estuaries (Kopprio et al., 2015). As reviewed by Kopprio and co-workers, possible future changes include deoxygenation in sediment and the water column with release of nutrients and green-house gases, benthos degradation, plankton blooms, elevated concentrations of organic matter, agricultural runoff, ecological changes in nekton and higher growth and activity of bacteria. This uncertain but prospective changing baseline is expected to favour the growth and development of pathogenic and non-pathogenic strains of *Vibrio*. Moreover, recent climate-driven changes have been related to *Vibrio* outbreaks in the Americas, Europe and Middle East (Baker-Austin et al., 2013; Levy, 2015) and climate change is extending the range of tropical infectious diseases to intermediate latitudes (El-Fadel et al., 2012; Wu et al., 2016).

4. Conclusions

The detected genes and species of *Vibrio* represent a risk for human and ecosystem health, particularly *vvhA* for *V. vulnificus*. The negative results for *V. cholerae* O1, *V. cholerae* O139 and cholera toxin in culturable bacteria may not indicate their total absence in the environment. Nanoplankton was the principal seston fraction for *Vibrio* distribution, though zooplankton have important implications for *Vibrio* ecology. Ammonium concentration and salinity were important factors in explaining bacterial abundance and distribution. Organic nutrients represent an important nutritional source for aquatic bacteria and influence the dynamic of culturable *Vibrio*. The salinisation of the Rio Colorado estuary and the higher eutrophic conditions of the Río Negro estuary favour *Vibrio* growth and distribution.

The failure of the sewage treatment plants signifies a severe risk to the ecosystem and human health. Adaptive efforts should be focused on the correct treatment of sewage effluent and adequate development and management of irrigation works to avoid the salinisation and eutrophication of estuaries and rivers. The development of long-term monitoring programs for drinking and recreational water is urgently needed in Argentinian Patagonia. The resilience of aquatic ecosystems to global change can be achieved by reducing their current stressors and improving water quality to an optimal range to buffer future impacts. The development of educational programs about the risk of the expansion of water-borne diseases and hygienic practices are also of great relevance for climate-change adaptation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.11.045.

Funding

This work was granted by FONCYT (PICT 1241) and IB-MINCYT (ARG14WTZ-020). G. Kopprio was supported by a re-invitation

program (9153678) of the German Academic Exchange Service (DAAD) and a Leibniz-DAAD fellowship (57191174).

Acknowledgments

We thank J. E. Garzón Cardona, S. Priotto and M. Pistonesi for sampling support and W. Melo for study-site images. We are also grateful to the Prefectura Naval Argentina for exceptional logistics in the Río Negro estuary and to CORFO for valuable information about the Río Colorado estuary.

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