Estuarine, Coastal and Shelf Science xxx (2017) 1–9

Contents lists available at ScienceDirect

Estuarine, Coastal and Shelf Science

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

The impact of sewage effluents on the fecundity and survival of *Eurytemora americana* in a eutrophic estuary of Argentina

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ARTICLE INFO

Article history:
Received 30 December 2016
Received in revised form
23 July 2017
Accepted 18 August 2017
Available online xxx

Keywords:
Copepods
Environmental pollution
Females fecundity
Argentina

ABSTRACT

The present study shows the results of laboratory experiments addressing the effects of different water qualities on *E. americana*'s fecundity and survival. The study was carried out with cultured females, incubated under different water qualities and controlled conditions during ten days (7 ± 2 °C, 14 h light, salinity of 32 ± 2, *Tetraselmis* sp. and *Nannochloropsis* sp. as food). Four treatments were established: P = subsurface water from sewage plume of Bahía Blanca city, D = the dissolved phase of P, I = bottom water in the sewage discharge point of Bahía Blanca city, and C = water from the low-impacted area of Monte Hermoso, used as a control treatment. Egg production, number of nauplii, number of faecal pellets, survival and fertility-state of females were evaluated. The differences and relationships among copepod factors, environmental variables and pollutants levels (cadmium, lead, copper, zinc, chrome and ammonium-phosphate dissolved), were analysed among treatment using non-parametric multivariate analysis. The copepod factors showed a negative association with pollutants levels and the turbidity. No mortality was observed in C, P and D treatments, whereas 100% mortality was observed in treatment I. Females from C, showed the highest egg production (22.6 ± 6.5 egg/female.clutch), as well as gonads regeneration and a second egg laying. Egg production, number of nauplli and faecal pellets were similar for P and D treatments, but significantly lower than those registered in C. In P treatment, only 40% of females showed regeneration of their gonads for a second egg laying (12.4 ± 2.9 egg/female.clutch) and in D treatment, no female regenerated their gonads for a second egg laying (12.5 ± 3 egg/female.clutch). The results indicate that bioavailable contaminants from dissolved phase of sewage effluent reduce the fertility in *Eurytemora americana*, while the bottom water at the sewage discharge site is undoubtedly lethal for this species.

1. Introduction

Contaminants associated with urban and industrial effluents, such as hydrocarbon derivatives, organochlorines, endocrine disrupting chemicals and heavy metals (Kusk and Wollenberger, 2007; Flint et al., 2012) affect the reproduction of copepods and the viability of their subitaneous and resting eggs (Apostolopoulou et al., 1979; Lotufo, 1998; Lindley et al., 1999; Suderman and Marcus, 2002; Hack et al., 2008). Most polycyclic aromatic hydrocarbons (PAHs) and fuel oil entering aquatic systems deposit in sediments and in storage lipids of the benthic biota, because of their high hydrophobicity and their molecular tensioactive properties (Lotufo, 1998). Bioassays with copepods, polycyclic aromatic hydrocarbons and oil derivatives have shown lethal (Barata et al., 2005) and sub-lethal effects, like bioaccumulation of lipids in eggs (Lotufo, 1998), a decrease in eggs viability and naupliar survival (Suderman and Marcus, 2002) and decreased feeding efficiency (Avila et al., 2010; Jensen and Carroll, 2010).

Amongst heavy metals, Cd is one of the most harmful pollutants in the aquatic environment (Wright and Welbourn., 1994). Studies on resting eggs that evaluate the potential effect of Cu, Pb and Cd metals on the hatching success of *Acartia pacifica* revealed a decrease in hatching success as metal concentration and exposure time increased (Jiang et al., 2007). In addition, these authors also found a significant decrease in egg hatching when mixed treatments were applied, and concluded that Cd was the most toxic

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http://dx.doi.org/10.1016/j.ecss.2017.08.034
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metal for *A. pacifica*. On the other hand, studies involving the intertidal harpacticoid copepod *Tigriopus japonicus* from the Western Pacific coasts demonstrated a significant decrease in the number of nauplii incubated at10 μg $L^{-1.}$ Cd (Raisuddin et al., 2007). *T. japonicus* is considered a model species and its survival, development and reproduction responses could provide effective physiological indicators to monitor marine metal pollution and to assess population responses (Emadeldeen et al., 2011).

The calanoid *Eurytemora americana* (Williams, 1906), has been reported in the cold and temperate latitudes of North America (Williams, 1906; Heron, 1964; Kos, 1977), in northern Europe as invader species (Brylinski, 2009; Noël, 2011) and the northeast Pacific (Kos, 1977; Borutzky et al., 1991). This species was recently reported in the northern part of Korea, with a dense population in the estuary of Jeoncheon Stream (Moon et al., 2016). It has also been reported in the southern hemisphere in the Atlantic Ocean, specifically in Bahía Blanca estuary, Ushuaia Bay and Encerrada Bay (Argentina), as invader species (Hoffmeyer et al., 2009; Biancalana and Torres, 2011; Biancalana et al., 2012a, b). The first report of *E. americana* in the Bahía Blanca estuary (BBE) was in the spring months of 1983 (Hoffmeyer, 1994) and was introduced into this estuary by ballast water translocation inside commercial ships coming from the Northern Hemisphere. This water is discharged into the main channel before entering the commercial port of Bahía Blanca city (Hoffmeyer, 1994). Since its first report, this invading copepod has successfully established in the BBE and shows high abundances within the mesozooplanktonic fraction from winter to spring (Hoffmeyer et al., 2008, 2009). This species manifests two markedly distinct types of reproductive behaviour, depending on the prevailing environmental conditions of the BBE (Berasategui et al., 2009, 2012). After spring, it disappears from the pelagic zone and recruits in subsequent years from the diapause egg bank, located in the inner zone of the BBE (Berasategui et al., 2013). During its planktonic period, *E. americana* is considered an important trophodynamic link (Hoffmeyer et al., 2008), since it is a frequent prey for planktivorous fish in the BBE (López-Cazorla et al., 2011). This species is generally associated with high values of organic matter and is commonly found in disturbed environments (Biancalana and Torres, 2011; Biancalana et al., 2012a,b). However, increasing human activities and elevated pollution of the BBE (Botté et al., 2010, 2007; Arias et al., 2009, 2010; Fernández-Severini et al., 2009, 2011; Marcovecchio et al., 2008) arouse the question about the long-term survival of its population. The effect of a sewage effluent (SW-Vieja channel) on the planktonic community structure and on the environmental quality of the BBE has been approached by several authors (Barría de Cao et al., 2003; Biancalana et al., 2012b; Dutto et al., 2012; Lopez Abbate et al., 2015, 2016). All these studies agreed on the registration of lower abundance, diversity and differences in species composition between planktonic assemblages in heavily impacted vs. low impacted sites within the estuary. However, no studies so far have experimentally evaluated the biological responses of dominant copepods in the BBE to the waters directly affected by the sewage effluents.

Long-term copepod population viability depends on successful reproduction, i.e., successful copulation, high viable egg production and survival at different larval stages (Mauchline, 1998). Bioavailable contaminants, either dissolved or in particulate form in the sewage effluent to the BBE (i.e., SW-Vieja channel), could adversely affect the reproduction of copepods in areas close to the effluent discharges, and affect the survival of these organisms in the long-term.

The aim of the present work was to experimentally evaluate the effect of different water qualities on survival, feeding (as faecal pellets production) and reproductive success (egg production, number of nauplii and fertility) of female *E. americana.* The outcome of this study could contribute to a better understanding of the anthropogenic impact on the biological communities of BBE, as baseline of pollution bioindicators for the BBE, as well as a reference for other polluted estuaries.

2. Materials and methods

2.1. Study area

The general study area is located in the southwest coast of Buenos Aires Province, Argentina (38° 45′-30°40′ S; 61° 45′-62° 30′ W). Inside of this area, two sampling sites with different degrees of anthropic disturbance were established: Site I: the sewage discharge of Bahia Blanca city at Vieja channel (SW), in the inner zone of Bahía Blanca estuary (BBE), and Site II: Monte Hermoso (MH) coastal zone with very low anthropic disturbance (Fig. 1).

BBE is a temperate estuary (water temperature range 5–27 °C), which is characterized by a semidiurnal tidal regime, with tidal flats and shallow channels (Perillo et al., 2001). The combined effects of NW-N winds and tidal currents induce the resuspension of fine sediments, resulting in a highly turbid, vertically homogeneous water column (Perillo and Piccolo, 1999). The estuary has a small freshwater input from Sauce Chico River and Napostá Grande Stream (1.72 m^3 s^{-1} and 1.05 m^3 s^{-1} annual means, respectively) and presents hypersaline marginal areas during the warm season (Freije et al., 2008). Associated to the BBE, there are diverse industrial facilities, including oil refineries, thermoplastics, polyethylene, agrochemicals and petrochemical production plants. BBE also hosts one of the most important ports in southern Argentina, with specialized terminals for the loading and unloading of grains, fuels, gases and other petrochemical products, which implies intense port activity and operation of vessels with draft up to 45 feet. All these features require regular dredging of navigation channels. The inner zone of BBE receives large quantities of raw urban sewage (SW-Vieja channel) from the nearly 300,000 inhabitants of Bahía Blanca city, which adds to the untreated industrial effluent and run-off water from nearly 4200 km^2 of cultivated land. In summary, this coastal marine system receives contaminant influxes from industrial and urban wastewaters, from harbour activities and agriculture run-off, plus aerial fallouts of atmospheric pollutants (Andrade, 2000). Contamination of inner BBE waters due to a complex mix of pollutants is well established. Heavy metals, organochlorides and polycyclic aromatic hydrocarbon (PHAs) have been repeatedly reported in sediments and water samples from the inner zone of BBE (i.e., Botté et al., 2010, 2007; Arias et al., 2009, 2010; Fernández-Severini et al., 2011, 2013; in press; Marcovecchio et al., 2016).

Monte Hermoso is a small town (6495 inhabitants) located in the southwest Buenos Aires Province, nearly 120 km from Bahía Blanca city. The main economic activities at MH are artisanal fisheries (Carroza et al., 2009) and tourism during summertime (Rojas et al., 2014). The coast of MH is characterized by open sandy beaches of gentle slope supported by extensive sand dunes with minimum urban development (Fig. 1) (Delgado et al., 2012). This is a highly diverse ecosystem (Menédez et al., 2016), influenced by the plume of the BBE, but with an overall low degree of anthropic alteration.

2.2. Sampling procedures and methods

Water samples for experimental incubations were taken from SW and MH (Fig. 1) during December 2014. These samples were collected manualy from a boat with polyethylene-terephthalate (PET) bottles (10–20 L), dipping the bottle into the water. The

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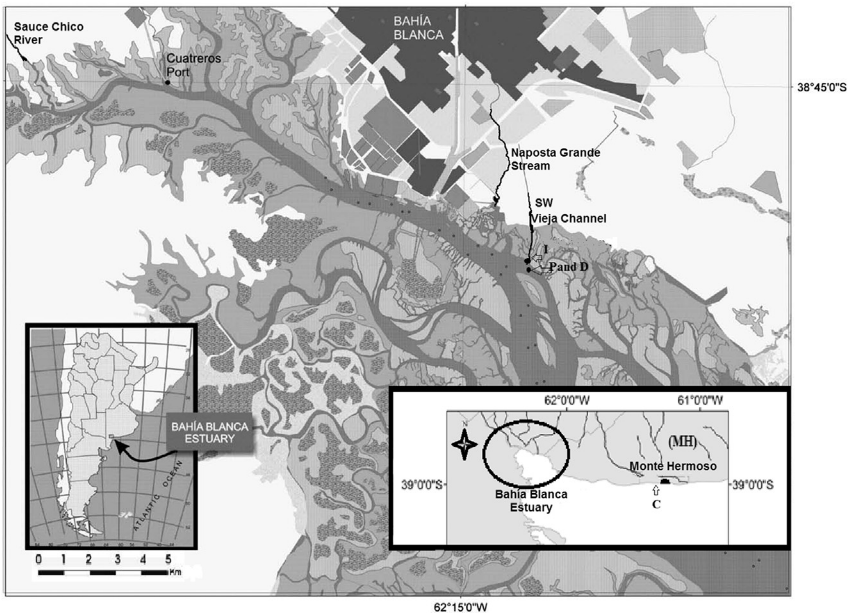

Fig. 1. Map of Bahía Blanca estuary (BBE) and coast of Monte Hermoso. SW = Vieja channel: channel that receives non-treated sewage from the Bahía Blanca City (300000 inhabitants).

water of SW was taken during low tide and three fractions were considered: (P): surface water (0.5 m depth) from the sewage plume, about 100 m from the actual discharge point. The depth at the sampling site P was of 1.5–2 m. (D): the dissolved phase of P; and (I): bottom water from the pelagic-benthic interphase taken from 0.5 m above the sediments bed, at the discharge point. Surface water (<0.5 m depth) from MH was collected at a nearshore site 10–20 m from the coast, in the same way as mentioned above and used as a control treatment (C). Additional surface samples of MH water (n = 4) were collected manually at < 0.5 m depth with 1.5 L PET bottles to determine the concentration of particulate heavy metals (cadmium-Cd, copper-Cu, lead-Pb, zinc-Zn and chrome-Cr) and dissolved nutrients (ammonium and phosphate). Salinity (SAL), PH, dissolved oxygen (OD) and water turbidity (TUR) were measured in each collection site using a Horiba® multisensor probe.

The data concentration of organochlorines and PHAs (surface sediments), heavy metals (dissolved, particulate and surface sediments), and concentration of ammonium and phosphate dissolved in SW, were taken from studies of Marcovecchio et al. (2014). Chemical determination of nutrients and contaminants was carried out in the Marine chemistry laboratory of IADO, following internationally validated methods (Koirtyohann and Wen, 1973; Treguer and Le Corre, 1975; Grasshoff, 1976; McDonald and McLaughlin, 1982; UNEP/IAEA, 1982; US EPA, 1995, 2000; APHA, 1998; El-Moselhy and Gabal, 2004; Botté et al., 2007, 2010).

2.3. Laboratory procedures

To avoid the introduction of predominant planktonic organisms during December in the BBE, like *Acartia tonsa* nauplii (>80 μm) (Sabatini, 1989; Berasategui et al., 2016), water of P and I was filtered through a 60 μm sieve. Likewise, MH water used as control treatment C was reserved in a dark chamber under 6 °C for 24 h and was later filtered by sieves of 200, 20 and 1 μm. This process was carried out to minimize the natural seston contribution (phyto-microzooplankton and detritus), and at the same time, to reduce the trophic incorporation of possible contaminants (Fernandez Severini et al. in press). For treatment D, 5 L of P water were filtered through a 0.45 μm (MILLIPORE filters) and only the dissolved fraction (water <0.45 μm) was used in the D experiments.

Experimental copepods were obtained from a laboratory stock culture kept under controlled conditions of temperature (7 ± 2 °C), photoperiod (14 L) and salinity (30 ± 3), simulating conditions in the natural environment (Berasategui et al., 2009). This culture was initiated by isolating ovigerous females during the period of population growth (July–August 2014) in the BBE (Berasategui et al., 2009, 2012). These copepods were maintained in water collected from MH area (≤1 μm) and were fed with a mix of *Tetraselmis* and *Nanocloropsis* (≥20,000 cells/ml, according to Schipp et al., 1999).

For the experiments, one male and one female with empty oviducts were placed in each experimental device (Erlenmeyers of 250 ml filled with C, I, P or D, water; five replicates per treatment, n = 20). Incubations lasted for ten days under the same temperature, salinity and photoperiod conditions than in the stock culture. Daily observations were not made to minimize the stress manipulation that may affect females' reproductive responses (Sibly et al., 2000). Duration of experimental incubations corresponds to the estimated time taken for *E. americana* to form ovigerous sacks of subitaneous eggs (Berasategui et al., 2009, 2012). Preliminary trials showed that natural seston as the only food was not enough to ensure copepods survival for the experimental period. Therefore, a daily ration of the algal mix *Tetraselmis* sp. - *Nanocloropsis* sp. (≥20,000 cells/ml) was supplied. To avoid dilution of the experimental media, the algal mix was previously concentrated by centrifugation (2500 rpm, for 10 min). At the end of the experimental period, adults were retained using 200 μm sieves in order to evaluate in vivo their vital state and gonadal maturation level (F). The remainder of the sample was fixed with 4% formalin for subsequent analyses. Each fixed sample was washed with seawater on a 60 μm sieve to remove formalin excess, and the number of nauplii (N), and faecal pellets (Pe, as a feeding activity index) were counted in each replicate bottle. Considering that faecal pellet length of calanoid copepods like *Acartia* spp. and *Eurytemora affinis* range from 51 to 203 μm, depending on the type of food ingested (Hansen et al., 1996; Viitasalo et al., 1999), it is assumed that the values of Pe found in our works may be underestimated. The egg production

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(EP, as number of egg/clutch. female) was estimated taking into account the number of unhatched eggs and nauplii (if any).

2.4. Statistical analyses

To analyse the differences among treatments C, I, P and D, taking into account all the copepod factors as biological variables (N, Pe and EP), a non-parametric multivariate analysis of variance test was applied (PERMANOVA-PAIR WISE). In addition, in order to analyse the possible relationships among copepod factors (F, N, Pe and EP), environmental variables (SAL, PH, OD,TUR) and pollutants levels in the treatment (P, D, I) and control, a Principal Components Analysis (PCA) was conducted with a Spearman's rank correlations matrix (standardized data). For this analysis, average values of particulate and dissolved heavy metals (Cd, Pb, Cu, Zn, Cr) from the period 2013–2014 (Marcovecchio et al., 2014) were taken into account for treatments P and D. The values of these heavy metals in sediment were also taken into account, particularly for I treatment. The Hg, PHAs and organochlorines values could not be taken into account in our analysis due to lack of data in some treatments. The values of ammonium (Ammon. D) and phosphate (Phos. D) dissolved in all treatments and the control, were also taken into account for the analysis.

3. Results

The values of the physicochemical variables measured *in situ* in the sewage effluent were similar between I and P, except for turbidity, which reached 32.61 (ntu) for P and 121 (ntu) for I. The Ph values were slightly alkaline in both sites, being 7.95 for P and 7.88 for I, and the values of salinity ranged between 31 and 32. Lower values of dissolved oxygen (OD) were recorded in both sites, being lower in I (4.9 mg/L) than in P (5.5. mg/L). The values of the physicochemical variables recorded in MH were: salinity 33.7, Ph 7.97, turbidity 42.5 (ntu) and OD of 8.96 mg/L. The results of the pollutants measured in SW and in MH were presented in Table 1.

Taking into account the results on survival and gonadal maturation-fertility (F), four categories were established (see illustrative table of the Fig. 2). Mortality was 100% for treatment I. However all females survived in treatments C, P and D (Fig. 2 a,b,c,d). In C treatment 40% of females showed gonadal regeneration and production of a second egg clutch, while the rest of the females (60%) presented incipient regeneration of the gonads for a second egg laying (Fig. 2e). In P treatment, only 40% of females showed regeneration of the gonads for a second egg laying and in D treatment, no female regenerated their gonads for a second egg laying (Fig. 2e). In order to avoid underestimation of EP, only the eggs produced corresponding to the first clutch were taken into account, since incomplete clutch and filled oviducts were observed in the second egg laying. The vital copepod factors Pe, N and EP in treatment I, were considered equal to zero, due to the lethal effect on females. Females from control incubations (C), showed high production of faecal pellets (79.4 ± 25.44), eggs (22.6 ± 6.5 egg/female.clutch) and nauplii (Fig. 3). Instead, females of P and D treatments showed lower egg laying (12.5 ± 2.9 and 12.4 ± 2.8 egg/female.clutch) and faecal pellets production (6.2 ± 2.38 and 10.2 ± 3.49 respectively). In fact, the values of N, Pe and EP, in P and D treatments were significantly lower than those recorded in C, and no significant differences were found between P and D treatments (Table 2).

The results of the PCA showed that the first two components, PC1 and PC2 axis explained 90% of the variation in the data set (Fig. 4). The general trend of the variables was defined mainly by the first axis (PC1 60.9%) in four groups coincident with the four treatments. The variables Pe, N, EP, OD and treatment C showed a positive association with PC1, in contrast Tur, Cu, Ammon.D and treatment I, showed a negative association with PC1. Treatment P and Zn showed a negative association with PC2 axis and the rest of the variables did not show a clear association with these two first axis (axes, plural). On the other hand, the variables Pe, F, EP and N were positively correlated with OD; N and F showed the same trend with SAL (Table 3). Pe, N and EP were negatively correlated with Cu and Zn, and EP and F showed the same trend with TUR. Surprisingly, significant positive correlations were found between Cd and F.

4. Discussion

Water from the benthopelagic interphase (I) was undoubtedly the most toxic for *E. americana*, inducing100% mortality within the 10-days incubation period. Results of PCA analysis suggest a positive association between treatment I and turbidity, dissolved ammonia, dissolved phosphates and most of the metals studied, especially with Cu. This pattern would indicate that these variables were determinants of the high mortality of females. Therefore, this lethal response could be attributed to three non-mutually exclusive causes. First, a higher concentration of contaminants associated to the suspended sediments; second, the higher load of fine cohesive sediments near the benthic-pelagic interface; and third high eutrophication due to high concentration of organic matter and nutrients.

The high turbidity found in I water indicates resuspension of bottom sediments and this, in turn, implies an increase in bioavailability of contaminants associated with the BBE sediments like Cu, Cr, Pb, and Zn (Table 1), generating a higher risk for organisms in water column (Grecco et al., 2011). In fact, the negative

Table 1
Water characterization from the sewage effluent from Bahía Blanca (SW-BBE) and Monte Hermoso (MH). Annual mean - values of heavy metals, organochlorines, polycyclic aromatic hydrocarbon (PAHs) and nutrients values recorded in sewage effluent (SW), were taken from historic report (period 2013–2014) of Marcovecchio et al. (2001)4. Undetectable values (n.d) and suspended particulate matter (SPM).

Quality of experimental water		SW-BBE			MH	
		Disolved	Surface-Sediments	SPM(μg.g^{-1})	SPM(μg.g^{-1})	Disolved
Heavy metals	Cd	(0.50–2.73 μg.L^{-1})	(0.04–0.12 μg g^{-1})	0.39	0.43	_
	Pb	(0.25–14.75 μg.L^{-1})	(18.87–30.24 μg g^{-1})	7.83	n.d	_
	Cu	(1.17–3.86 μg.L^{-1})	(18.87–30.24 μg g^{-1})	24.73	7.40	_
	Zn	(0.00–19.9 μg.L^{-1})	(30.54–42.08 μg g^{-1})	63.61	24.92	_
	Cr	(4.1–15.4 μg.L^{-1})	(7.33–11.28 μg g^{-1})	11.70	6.32	_
	Hg	(n.d.-0.93 μg.L^{-1})	(n.d-0.061 μg g^{-1})	_	_	_
Nutrients	ammonium	(3.94–36.02 μmol.L^{-1})	_	_	_	(0.0–1.90 μg.L-1)
	phosphate	(0.82–1.41 μmol.L^{-1})	_	_	_	(0.0–0.5 μg.L-1)
Organochlorines			(0.00053 μg g^{-1})	_	_	
PAHs		_	(10–900 ppb)	_	_	

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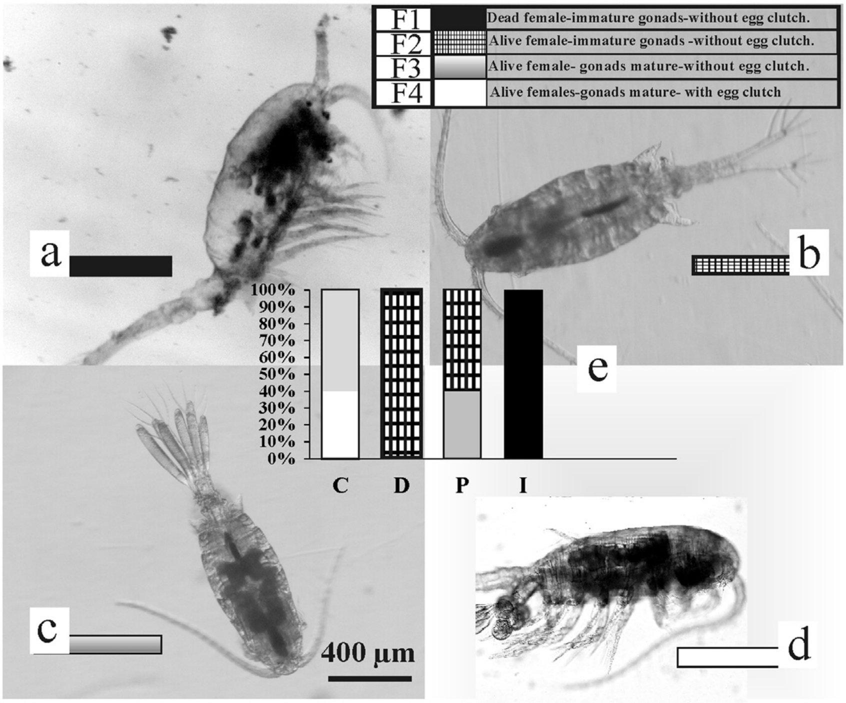

Fig. 2. Survival, female's fecundity and gonadal maturation, F categories (F1, 2, 3 and 4). 2a) Dead females with immature gonads without egg clutch. 2b) Alive females with immature gonads without egg clutch. 2c) Alive females with mature gonads, without egg clutch. 2d) Alive females with mature gonads, with egg clutch. 2e) Survival and gonadal stage of females (F), incubated under different qualities of water expressed in %, taking into account the total number of females subjected in each treatment. C = suitable water for the organisms culture. P = water ≤60 μm taken from 100 m of the sewage discharge area. D = water ≤0.45 μm of P. I water ≤60 μm from interphase pelagic-benthic in the sewage discharge area.

effect of turbidity on the reproduction responses of *E. americana* was corroborated in our study, since EP (egg production) and F (vital state and gonadal maturation level) variables were negatively correlated with turbidity (TUR). The possible negative influence of other pollutants such as PHAs, organochlorines and mercury which are also present in the sediments of BBE (Botté et al., 2007) cannot be ruled out as contributing to responses found in treatment I (Lotufo, 1998; Suderman and Marcus, 2002; Kusk and Wollenberger, 2007; Francois-Gaël et al., 2013). Current results are overall consistent with previous studies on similar topics. For example, in New Zealand, Hack et al. (2008) evaluated the survival and reproduction of the benthic harpacticoid *Robertsonia propinqua* in sediments from polluted and non-polluted areas. They found no significant differences in survival but reproductive output was reduced after exposure times of 24 d to lower mean contaminant concentrations (Pb 4.76 μg g^{-1}, Cu 2.76 μg g^{-1}, Zn 29.6 μg g^{-1} and PAHs 0.0175 μg g^{-1}) than those registered in BBE sediments (Table 1). In another study, Oliveira et al. (2014) evaluated survival of the harparcticoid *Tisbebi miniensis* exposed to extremely polluted mangrove sediments from Recife, Brazil (84 ± 68–570 ± 55 μg g^{-1} Zn and 68 ± 4–93.5 ± 2 μg g^{-1}Cr, higher than sediments of BBE, Table 1), and reported a maximum mortality of 60% ovigerous females after 7 days.

The higher load of fine cohesive sediments in I treatment would also generate lower feeding efficiency making it difficult for *E. americana* to survive. In this sense, it has been reported that egg production rates of *A. tonsa*, became reduced only at the highest sediment concentration of 1000 ppm (White and Dagg, 1989). Results of Gasparini et al. (1999) reported that suspended particulate matter (SPM) concentration could influence egg production rate of *Eurytemora affinis* through prey uptake limitation. Moreover, studies with *Paracalanus* sp. reported lower feeding efficiency and low survival rate of adult females at high levels of suspended sediments (Kang, 2012). Our results also suggest an association between I treatment and the concentration of ammonium and phosphates. This trend is to be expected since water corresponding to treatment I was extracted from the point of sewage discharge and contained higher concentrations of these two nutrients. Previous studies in SW - BBE, reported that the nutrient pool and high particulate organic matter (2740.04 ± 1153.48 μg L^{-1}) from this wastewater contributed with a high eutrophication (López Abbate et al., 2015, 2016). Heavy eutrophication may lead to hypoxia and anoxia in near bottom water (Diaz and Rosenberg, 2008; López Abbate et al., 2015). It is well established that hypoxia-anoxia negatively affect the reproduction and survival of copepods, as well as the viability of their eggs (Lutz et al., 1992; Katajisto, 2004; Marcus et al., 2004; Grego et al., 2014). These facts lead us to consider that hypoxia conditions in treatment I (<4–5 mg/L DO), could also contribute to the death and the negative effect on the female's fecundity (see, Fig. 2a). Present results for the BBE water-sediment interface (I treatment) also suggest a potential hazard for resting eggs in the bottom sediments (Marcus et al., 1994; Katajisto, 2004), which may compromise long-term recruitment of *E. americanana* (Berasategui et al., 2012) and other species in the studied area. In future studies it would be important to elucidate whether current or realistic future contamination levels are also deleterious for the copepod egg bank of the EEB, in order to assess the long-term vulnerability of local population to pollution.

Sub-lethal effects were evident in the reproduction and feeding responses of *E. americana* females incubated with surface water collected in the plume of the sewage discharge (P and D treatments). The water of both treatments belong to the same sampling site, P treatment was integrated by dissolved contaminants and suspended particulate matter from the natural environment and D

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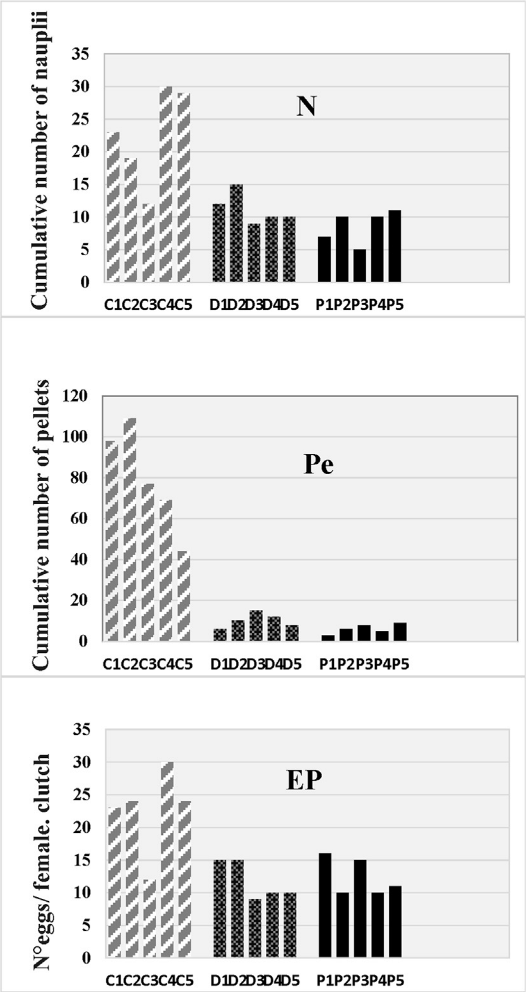

Fig. 3. Results of copepod factors as biological variables. Faecal pellets (Pe), nauplii(N) and eggs produced (EP) in incubations under control conditions (C) and sewage treatments (P- D). C = suitable water for the organisms culture. P = water≤ 60 μm taken from 100 m of the discharge area. D = water ≤0.45 μm of P.

Table 2
Results of PERMANOVA-PAIR WISE TESTS. * Significantly different (P < 0.01), Non-significantly different (ns). Treatments: P (subsurface water from sewage plume of Bahía Blanca city), D (the dissolved phase of P), I (bottom water in the sewage discharge point of Bahía Blanca city), and C (water from the low-impacted area of Monte Hermoso, used as a control treatment).

PERMANOVA-PAIR WISE TESTS	t	P(perm)	P(MC)
C-P	4.95	**0.0086***	0.0002
C-D	5.77	**0.0092***	0.0002
P-D	1.74	0.0665 ns	0.0727

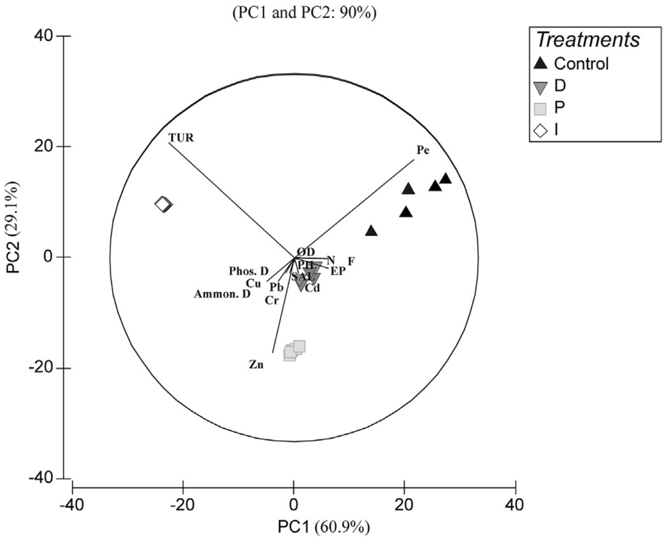

Fig. 4. PCA plots of environmental and copepod factors as biological variables, treatments and contaminants. Environmental variables: Salinity (SAL), PH, dissolved oxygen (OD) and water turbidity (TUR). Biological variables: vital state and gonadal maturation level of females (F), number of nauplii (N), faecal pellets (Pe) and egg production (EP). Contaminats: particulate and dissolved heavy metals as cadmium (Cd), lead (Pb), copper (Cu) zinc (Zn) chrome (Cr). Dissolved ammonium (Ammon. D) and dissolved phosphate (Phos.D). Treatments: subsurface water extracted into sewage plume to 100 m of the discharge point (P), the dissolved phase of P (D), bottom water from the pelagic-benthic interphase (I), water of MH as control treatment (C).

was only integrated by the dissolved phase. Egg production (EP), production of naplii (N) and faecal pellets production (Pe) showed similar patterns in P and D treatments and no significant differences were found between both treatments. Moreover, the PCA and Spearman results show that EP, N and Pe respond negatively to particulate metals and dissolved nutrients. Taking to account that, P and D treatments show similar patterns in reproduction and feeding responses and that both treatment had in common the dissolved phase from the natural environment, maybe the toxic compounds that produced the most negative sub-lethal effects on *E. americana* could be present in the dissolved phase. This theory requires further research into the future, since there were others dissolved contaminants in the BBE that have not been taken into account in our study (Marcovecchio et al., 2008). Nevertheless this theory would be unexpected, since it has been reported that sub-lethal effects in copepods reproduction result from trophic incorporation of hydrophobic organic contaminants or heavy metals associated to the SPM (suspended particulate matter) in natural environment (Hook and Fisher, 2001a,b; Chang and Reinfelder, 2002; Marcus, 2004; Cailleaud et al., 2007). In fact, recent studies in BBE suggested that in general, the SPM (seston >0.45 μm) had higher concentrations of metals than the microplankton (20–200 μm) (Fernadez Severini et al., in press). On the other hand, studies performed with *Acartia hudsonica* and *A. tonsa* investigated the effect of the exposure route on metal accumulation, and reported that dissolved metals resulted in metal deposition in the exoskeleton, whereas exposure to metals via food intake resulted in metal deposition in internal tissues (Hook and Fisher, 2001a). These studies also reported that vitellogenesis was affected by an enrichment of metal concentrations in internal tissues, which occurs primarily after exposure to dietary metals. The same trend has been observed with other pollutants, in fact studies made with *Calanus helgolandicus* and naphthalene dissolved in seawater reported that BAF fornaphthalene ingested through the diet was much larger than the bioaccumulation by dissolved state in seawater (Corner et al., 1976). Moreover, the bioaccumulation of contaminants by microalgae, commonly used to feed copepods, is relatively rapid <24 h (Hook and Fisher, 2001a,b; Calbet et al., 2007). Indeed, it was reported that *Tetraselmis suecica* algae, responded by storing heavy metals in their osmiophilic vesicles (lipid composition), after 5 days exposure to dissolved Cu or Ag (Ballan-Dufrançais et al., 1991). Those findings and our results (low eggs, low nauplii and faecal pellets production) suggest, therefore, that the negative effects on *E. americana* reproduction found under P and D treatments could be due to the dietary incorporation of contaminants originally in dissolved phase and the selective

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Table 3
Spearman's correlation matrix corresponding to the PCA. Significant correlation (light grey< 0.05; dark grey p < 0.01; n = 20). Copepod factors as biological variables: faecal pellets (Pe), number of nauplii (N), egg production (EP) and vital state and gonadal maturation level of females (F). Environmental variables: Salinity (SAL), PH, dissolved oxygen (OD) and water turbidity (TUR). Heavy metals: (Cd Pb Cu Zn Cr). Dissolved nutrients: dissolved phosphate (PD) and dissolved ammonium (AmD).

Variable	Pe	N	EP	F	TUR	SAL	PH	OD	Cd	Pb	Cu	Zn	Cr	PD
Pe														
N	**0.858**													
EP	**0.743**	**0.895**												
F	**0.644**	**0.648**	**0.653**											
TUR	-0.424	-0.329	**-0.455**	**-0.449**										
SAL	0.229	0.411	0.33	**0.496**	-0.062									
PH	-0.012	0.175	0.133	0.338	-0.085	**0.924**								
OD	**0.819**	**0.855**	**0.731**	**0.756**	-0.233	**0.645**	0.362							
Cd	0.501	0.608	0.534	**0.647**	-0.256	**0.938**	**0.845**	**0.772**						
Pb	-0.391	-0.276	-0.188	0.026	-0.355	**0.453**	**0.723**	-0.195	0.364					
Cu	**-0.58**	**-0.642**	**-0.504**	-0.275	-0.34	-0.371	-0.202	**-0.581**	**-0.491**	0.412				
Zn	**-0.776**	**-0.712**	**-0.592**	-0.341	-0.146	0.013	0.296	**-0.616**	-0.179	**0.8**	**0.808**			
Cr	-0.226	-0.016	-0.034	0.109	0.044	**0.778**	**0.946**	0.11	**0.668**	**0.8**	-0.149	0.426		
PD	-0.225	-0.022	-0.038	0.096	0.04	**0.776**	**0.946**	0.106	**0.667**	**0.798**	-0.151	0.423	**0.998**	
AmD	-0.226	-0.016	-0.034	0.109	0.044	**0.778**	**0.946**	0.11	**0.668**	**0.8**	-0.149	0.426	**1**	**0.998**

predation of this copepod (Hoffmeyer and Prado-Figueroa, 1997). Incorporation of dissolved forms could have taken place through the food supplied daily during the experiment (mix of *Tetraselmis* and *Nanocloropsis*). This could also explain the differential behaviour of F observed between P and D treatments (see Fig. 2e). In treatment D, no gonadal maturation was observed in the females, whereas in the treatment P gonadal maturation was observed. This would indicate that the water of the treatment D exerted a stronger negative effect on the gonadal maturation than that of treatment P, which had less influence of contaminants in dissolved phase. The high levels of organic matter (López Abbate et al., 2016) and natural SPM in the P treatment is one factor that may have contributed to decrease the availability of dissolved contaminants by adsorption (Bejarano et al., 2005).

An association between Cd and treatment D was suggested by the PCA, which would point to the influence of this metal on the low production of eggs, nauplii and faecal pellets (EP, N and Pe) in D treatment. However, the interpretation of the effects of Cd remains unclear since a significant positive correlation between Cd and F (vital state and gonadal maturation) was also found. Amongst heavy metals, Cd is considered as one of the most harmful pollutants in the aquatic environment (Wright and Welbourn, 1994) and produces negative effects on the reproduction and survival of copepods (Jiang et al., 2007; Raisuddin et al., 2007). We do not find clear explanations for the unusual pattern of Cd effects found in our study. Heavy metals Cu and Zn showed a negative correlation with Pe, N and EP, suggesting a negative influence on the reproductive responses of *E. americana*. Heavy metals assimilation bioassays made with *Acartia tonsa* and *Acartia hudsonica* revealed that the concentrations of metal (34 for Hg, 37 for Ag, 64 for Cd, and 363 for Zn nmol g^{-1} dry wt, or 23.72 μg g^{-1} dry wt) in phytoplankton, caused measurable toxic effects on egg production (Fisher and Hook, 2002). These authors also reported assimilation efficiencies of 14% for Hg, 15% for Ag, 62% for Cd, 55% for Zn, and between 15 and 30% for Mn, being the Zn, one of the metals with less toxicity among those studied. Concentrations of Zn in SPM in our study (30.54–42.08 μg g^{-1}) were higher than the toxic concentration threshold reported by Fisher and Hook (2002). Reagarding Cu, Chang and Reinfelder (2002) suggested that herbivorous marine zooplankton accumulate that metal mainly by trophic transfer, but that dissolved uptake could be important in contaminated waters.

Control water from Monte Hermoso allowed for proper feeding and reproduction of *E. americana*, with higher performances compared to treatments P, D, and I. Better water quality in control water (lower general contaminant levels, higher oxygen) is consistent with observed biological responses.

In conclusion, the copepod *E. americana* was found to be a good bioindicator species, since it was very sensitive to the effects of the polluted water from sewage discharge. In fact, the effects documented in this study may explain, at least in part, the low abundances recorded in SW stations during the population pulse in relation to other areas within BBE estuary (Dutto et al., 2012). In surface waters, the dissolved fraction had an overriding effect on the feeding and reproductive performance. Water from the pelagic-benthic interface had a lethal outcome on adult females and may imply a risk for the viability of benthic eggs. Taken together lethal and sublethal effects may constitute a threat for the long-term recruitment and population viability of this species in the inner BBE.

Acknowledgements

We wish to thank the Instituto Argentino de Oceanografía (IADO) and CONICET-PIP0630 for making this research possible. We would also like to thank Dr. Melo W. for the preparation of the Bahía Blanca estuary map. We are also grateful to anonymous reviewers for their valuable contribution and suggestions.

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Please cite this article in press as: Berasategui, A.A., et al., The impact of sewage effluents on the fecundity and survival of *Eurytemora americana* in a eutrophic estuary of Argentina, Estuarine, Coastal and Shelf Science (2017), http://dx.doi.org/10.1016/j.ecss.2017.08.034