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Contribution to the Themed Section: ‘The Role of Zooplankton in Marine Biogeochemical Cycles: From Fine Scale to Global Theories’

Zooplankton recolonization of the inner estuary of Bilbao: influence of pollution abatement, climate and non-indigenous species

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Variations in mesozooplankton abundance and community structure in response to water quality improvement and to variations in hydro-climatic conditions were studied in the inner estuary of Bilbao from 1998 to 2011. A process of recolonization was observed with a marked increase in copepod abundance and a smaller increase in the abundances of appendicularians, meroplanktonic bivalves and gastropods, at the expense of the decrease in groups of gelatinous predators. Within the copepods, an initial phase in this recolonization occurred through an increase in neritic copepods. However, in a second phase, the non-indigenous species *Acartia tonsa* and *Oithona davisae* increased and became dominant. In the last 2 years, a third non-indigenous species *Pseudodiaptomus marinus* was observed in lower densities, and *Calanipeda aquaedulcis* abundance increased becoming co-dominant with *A. tonsa*. All non-indigenous copepod species had summer/autumn peaks and were characteristic of brackish conditions, which suggests that unsaturated ecological niches in brackish waters were important for the settlement of non-indigenous species. Pollution abatement allowed for zooplankton recolonization, but variations in community structure, both at the broad taxonomic group and at the copepod species level, were greatly influenced by hydro-climatic factors, temperature being the single best explanatory variable of zooplankton variations.

KEYWORDS: zooplankton; estuary; pollution; climate; non-indigenous species

INTRODUCTION

Estuaries receive much of the waste discharged by mankind into aquatic environments, and they are among the most polluted marine systems (McLusky and Elliott, 2004). Some of these polluted estuaries have undergone restoration/rehabilitation processes (Borja *et al.*, 2010). However, while many studies have been conducted to investigate changes in biotic communities in response to sediment and water quality degradation (González-Oreja and Sáiz-Salinas, 1998; Courrat *et al.*, 2009), the biotic responses to rehabilitation/restoration are less well documented (Mialet *et al.*, 2011). Zooplankton play a key role in the carbon transfer from primary producers to higher trophic levels, but effects of both estuarine pollution enhancement and abatement have been less studied in zooplankton than in other pelagic and benthic fauna (Uriarte and Villate, 2004; Mialet *et al.*, 2011). For European estuaries, the fact that zooplankton are not included in the Water Framework Directive has contributed to this limitation (Mialet *et al.*, 2011). However, being ectotherms with short generation times, zooplankton have the potential capacity to respond fast to environmental changes through phenotypic plasticity or evolutionary adaptation, and therefore, they are considered excellent sentinels of biotic responses to environmental change (Goberville *et al.*, 2014; Rice *et al.*, 2014).

In rehabilitation/restoration processes, recolonization of perturbed estuarine areas occurs, and this provides increased opportunities for the settlement of non-indigenous species too (Winder *et al.*, 2011). In estuaries, non-indigenous species appear to occupy preferentially brackish waters where ecological niches are naturally unsaturated (Wolff, 1999; Paavola *et al.*, 2005). In addition to responding to anthropogenically mediated environmental changes, estuarine zooplankton abundance and community structure are affected by climatic and related hydrological changes, mainly by warming and variations in freshwater discharge (Kimmel and Roman, 2004; Winder *et al.*, 2011; Rice *et al.*, 2014). Anthropogenic and hydroclimatic variables can act synergistically to facilitate the establishment of non-indigenous species (Winder *et al.*, 2011).

The estuary of Bilbao was one of the most polluted estuaries in the Iberian Peninsula up until the late 20th century, but management plans conducive to pollution abatement started to be implemented in 1979, and the estuary has since entered a progressive rehabilitation phase (Borja *et al.*, 2010). Significant steps in the recovery of benthic macrofauna (Borja *et al.*, 2006) and fish communities (Uriarte and Borja, 2009) have been observed. However, for the zooplankton community, only initial signs of recovery have been reported (Albaina *et al.*, 2009), and a rigorous assessment using data from a longer time-series is still lacking.

The aims of the present work were to describe changes in the abundance and community structure of mesozooplankton, both at the level of broad taxonomic groups and at the level of copepod species, during a 14-year period of recolonization of the inner estuary of Bilbao, and to assess the effect of pollution abatement, hydroclimatic factors and invasion by non-indigenous species.

METHOD

Study area

The estuary of Bilbao is a small and shallow system located on the southeastern Bay of Biscay, within 43°23'N to 43°14'N and 3°07'W to 2°55'W (Fig. 1). The main river discharging at the head of the estuary is the Ibaizabal-Nerbioi with a mean flow of $\sim 21 \text{ m}^3 \text{ s}^{-1}$. It is a meso-macrotidal estuary where, except for short periods of high river discharge, euhaline waters (salinity > 30) dominate. Waters of lower salinities are mainly restricted to the thin layer above the halocline, and they are represented by too low volumes (Fig. 1) to maintain stable mesozooplankton communities of their own (Uriarte *et al.*, 2014). The inner estuary, where the present study has been carried out, is strongly stratified, and salinities below the halocline are generally around 30 (Iriarte *et al.*, 2010; Villate *et al.*, 2013; Uriarte *et al.*, 2014).

This system became a heavily polluted estuary with very low levels of biodiversity (González-Oreja and Sáiz-Salinas, 1998), but in the last three decades or so, it has undergone a rehabilitation process due to a substantial industrial decline along its banks and to the implementation of a Comprehensive Plan for the Sanitation of the Metropolitan Area of Bilbao since 1979, which has resulted in a significant decrease in heavy metal concentrations in the sediments (in 2008 sediments from intermediate estuarine areas were classified only as moderately toxic and inner and outer estuarine areas as slightly toxic, whereas in the late 1990s they were classified as heavily polluted) and in an increase in the levels of dissolved oxygen (e.g. from 1998 to 2008 in the intermediate estuary zone dissolved oxygen increased from mean annual values around 40% to mean annual values around 80%) and biodiversity (e.g. there were azoic soft bottom macrobenthic areas in 1989; by 2008 communities with > 25 species and an AMBI index (*AZTI Marine Biotic Index*) of 3 were observed) (Borja *et al.*, 2006; Uriarte and Borja, 2009; Fernández-Ortiz de Vallejuelo *et al.*, 2010; Villate *et al.*, 2013).

Data collection

A time-series of environmental and zooplankton data from the inner estuary of Bilbao was built for the period

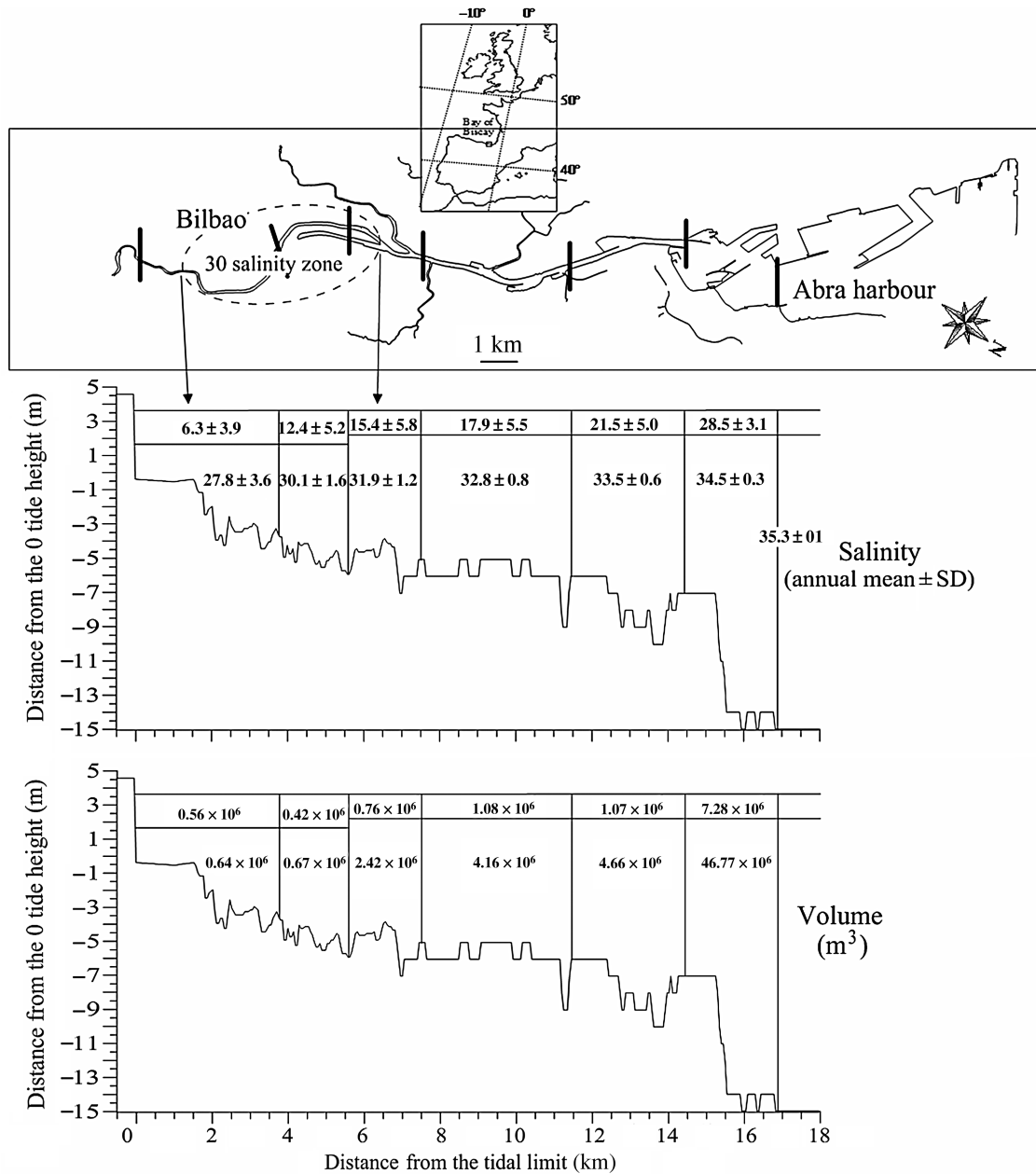


Fig. 1. Map of the estuary of Bilbao, showing the location of the sampling zone and spatial distribution of salinity (11-year annual mean and standard deviation) and water volumes for a tide height of 3.6 m, according to Uriarte *et al.* (Uriarte *et al.*, 2014).

1998–2011. Data were obtained from monthly sampling (usually during the last week of the month) conducted at high tide during neap tides at the 30 salinity zone (Fig. 1), using a Lagrangian type of sampling strategy (see Kimmerer *et al.*, 1998; Modéran *et al.*, 2010). Zooplankton were collected from below the halocline (around 3–5 m) by 2–3 min horizontal tows using a 200 µm mesh size net (mouth diameter 0.25 m) equipped with a Digital Flowmeter. These zooplankton samples were preserved in 4% buffered formalin and identified and counted

under a stereomicroscope. Identification was made to the lowest possible taxonomic level. Vertical profiles of water temperature, salinity and dissolved oxygen were measured *in situ* using WTW Water Quality Meters, but only data from the depth of zooplankton sampling are presented in this study. Water samples were collected also at the depth of zooplankton sampling using a Niskin bottle for measuring chlorophyll *a*, which was analysed spectrophotometrically in triplicate samples according to the monochromatic method with acidification (Jeffrey and

Mantoura, 1997). Secchi disk depths (SDDs) were also recorded.

Additionally, hydro-meteorological data were obtained from various sources. Monthly mean data of rainfall and river flow for the Nerbioi-Ibaizabal river measured at the Abusu station (past the confluence point of the two main streams Nerbioi and Ibaizabal) were provided by the Provincial Council of Bizkaia. For air temperature, ERA-Interim reanalysis data were accessed through the ECMWF data server as monthly means of daily means of 2 m temperatures from an area of [43.283°N, 43.408°N] × [3.088°W, 2.963°W] using a grid of 0.125° × 0.125°. Regional climate indices relevant in the North Atlantic, such as the North Atlantic Oscillation (NAO) index, the East Atlantic (EA) index and the Atlantic Multidecadal Oscillation (AMO) index (unsmoothed), were obtained as monthly mean values from the NOAA (National Oceanic and Atmospheric Administration) Climate Prediction Center (<http://www.cpc.ncep.noaa.gov/>) and Earth System Research Laboratory (<http://www.esrl.noaa.gov/psd/data/climate/indices>) webpages.

Data analysis

Occasional missing values (6%) were filled using the mean value of the previous and following month values. Prior to statistical treatment, zooplankton abundance data were $\log(x + 1)$ transformed. For the purpose of the present study, zooplankton data analysis was carried out at two different levels: (i) the herein termed “group” level, where we distinguished the following holoplankton taxa: Copepoda, Cladocera, Appendicularia, Chaetognatha and Siphonophora; and the following meroplankton taxa: Cirripedia, Decapoda, Gastropoda, Bivalvia, Polychaeta, Pisces and Medusae (which occasionally included holoplanktonic forms); (ii) the herein termed “species” level, which includes the following species or genera (in one case a cluster of genera) within the most abundant group, i.e. the Copepoda: *Acartia tonsa*, *Acartia clausi*, *Acartia margalefi*, *Acartia discaudata*, *Calanipeda aquaedulcis*, *Oithona davisae*, *Oithona* spp. (which includes the neritic species *O. similis*, *O. nana* and *O. plumifera*), *Oncaea* spp. (mostly *O. media*, and the former *O. subtilis* today renamed *Monothula subtilis*), *Pseudodiaptomus marinus*, *Euterpina acutifrons*, *Centropages* sp. (with *C. typicus* as the only species reported for adult individuals), *Temora* spp. (which includes *T. stylifera* and *T. longicornis*), *Calanus* sp. (mostly *C. helgolandicus*), and the herein termed PCPCalanus [which includes the dominant genus *Paracalanus* (mostly *P. parvus*), and the successively less abundant *Clausocalanus* (which includes several unidentified species), *Pseudocalanus* (only *P. elongatus*) and *Ctenocalanus* (only *C. vanus*)]. To visualize the temporal

variability, the sequences of the corresponding monthly data and their moving averages (12 months) were plotted. Spearman rank correlation analyses between zooplankton abundance and time were performed to test for inter-annual trends.

A multivariate ordination method was used to examine the relationship between zooplankton taxa composition and environmental variables using CANOCO software package version 4.5 (Ter Braak and Šmilauer, 2002). First, detrended correspondence analysis (DCA) was performed for the taxa distribution to determine whether a unimodal model such as the canonical correspondence analysis (CCA) or a linear model such as the redundancy analysis (RDA) best fits the data sets. The longest gradient lengths were 1.72 and 3.45 for groups and species, respectively. Since CCA is recommended when values are >4 and RDA when they are <3, with both types of ordination methods working reasonably well in the range between 3 and 4 (Ter Braak and Šmilauer, 2002), we opted for performing RDA analyses for both groups and species. The RDA is a multivariate technique that is frequently used in studies dealing with temporal and spatial changes in zooplankton community composition in relation to environmental variables (Gislason *et al.*, 2009; Mialet *et al.*, 2011). To establish the order of importance of the explanatory variables, the conditional effects, which show the increase in the total percentage of explained variance after including a new variable during a forward selection, were computed. The statistical significance was tested with Monte Carlo permutation tests (999 unrestricted permutations, $P < 0.05$).

RESULTS

Water environment and hydro-climatic factors

The temporal variation of hydro-climatic (air temperature, precipitation and streamflow) and water environment factors (water temperature, dissolved oxygen, SDD and chlorophyll *a*) is shown in Fig. 2.

Air temperature and water temperature showed similar patterns of variation during the study period. Summer maxima were highest in 2003 and 2006 and lowest summer maxima in 2002. Spring and summer water temperature tended to increase during the study period, but no such increase was observed in winter and autumn. Precipitation and streamflow also showed similar patterns of year-to-year variation, but they showed no significant temporal trends.

Dissolved oxygen (DOS) varied between 0.3 and 93.2% and, overall, showed an improvement during the study period. However, it was not a steady increase. There

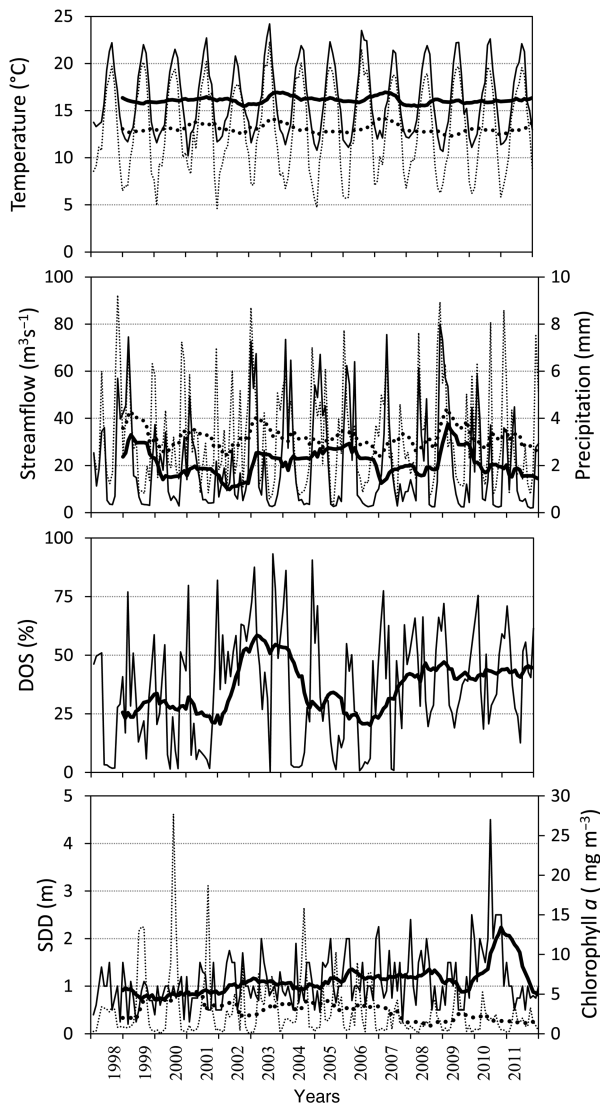


Fig. 2. Monthly time-series of water (solid) and air (dotted) temperature, streamflow (solid), precipitation (dotted), DOS, SDD (solid) and chlorophyll *a* concentration (dotted) of the 30 salinity sites of the estuary of Bilbao from 1998 to 2011. Thicker lines are moving averages.

was an initial increase from 2001 to 2002, but this was followed by a decrease from 2004 to 2006. From then onwards DOS increased again, and during the last 4 years of the series, mean annual values tended to stabilize. Hypoxia was observed most of the years during the summer, but in the last 4 years of the series, the level of hypoxia (ca. 25%) was weaker than in most of the previous years, when below halocline waters became nearly anoxic during part of the summer.

SDDs varied between 0.2 and 4.5 m and showed a general trend of increase until 2010. The latter was a year with particularly high values of SDD, but 2011 did

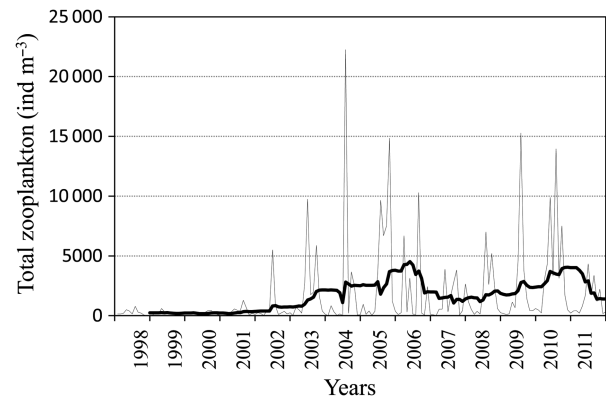


Fig. 3. Monthly time-series from 1998 to 2011 of total zooplankton density. Thicker lines are moving averages.

not follow the increasing trend. Chlorophyll *a* concentration varied from near 0 to 27.7 mg m⁻³ and, in contrast to SDD, showed a general tendency of decrease during the study period.

Zooplankton

Annual mean abundance of total zooplankton was low during the period 1998–2001 and increased about an order of magnitude from 2003 onwards (Fig. 3). The temporal variation of zooplankton abundance, however, showed differences between groups (Fig. 4). While Copepoda, Appendicularia and meroplanktonic molluscs (Bivalvia and Gastropoda) showed increasing trends, gelatinous predators such as Medusae, Siphonophora and Chaetognatha showed decreasing trends during the study period. Cladocera and meroplanktonic Decapoda, Cirripedia, Polychaeta and Pisces showed no statistically significant trends. Within the copepods, the temporal variations showed three distinct periods (Fig. 5). From 1998 to 2002, indigenous neritic species such as *A. clausi*, PCPCalanus and *Oithona* spp. dominated the copepod assemblage. From 2003 to 2009, a replacement in the dominance of indigenous neritic species by the newly arriving non-indigenous brackish species *A. tonsa* and *O. davisae* occurred. In the period 2010–11, two events are worth underscoring, the large increase in abundance of *C. aquaedulcis*, which became co-dominant together with *A. tonsa*, and the appearance for the first time in 2010 of the non-indigenous *P. marinus*. Even within the first period (1998–2002), we can highlight the period from late 2001 to 2002, when PCPCalanus became more abundant than *A. clausi* and when *A. margalefi* and *A. discaudata* showed distinctly high abundances. *A. tonsa*, *O. davisae*, *C. aquaedulcis* and *P. marinus* showed trends of increase, whereas *Temora* spp., *Centropages* sp., *A. clausi*, *E. acutifrons*, *Oithona* spp., PCPCalanus, *A. discaudata*, *Oncaea* spp. and *Calanus*

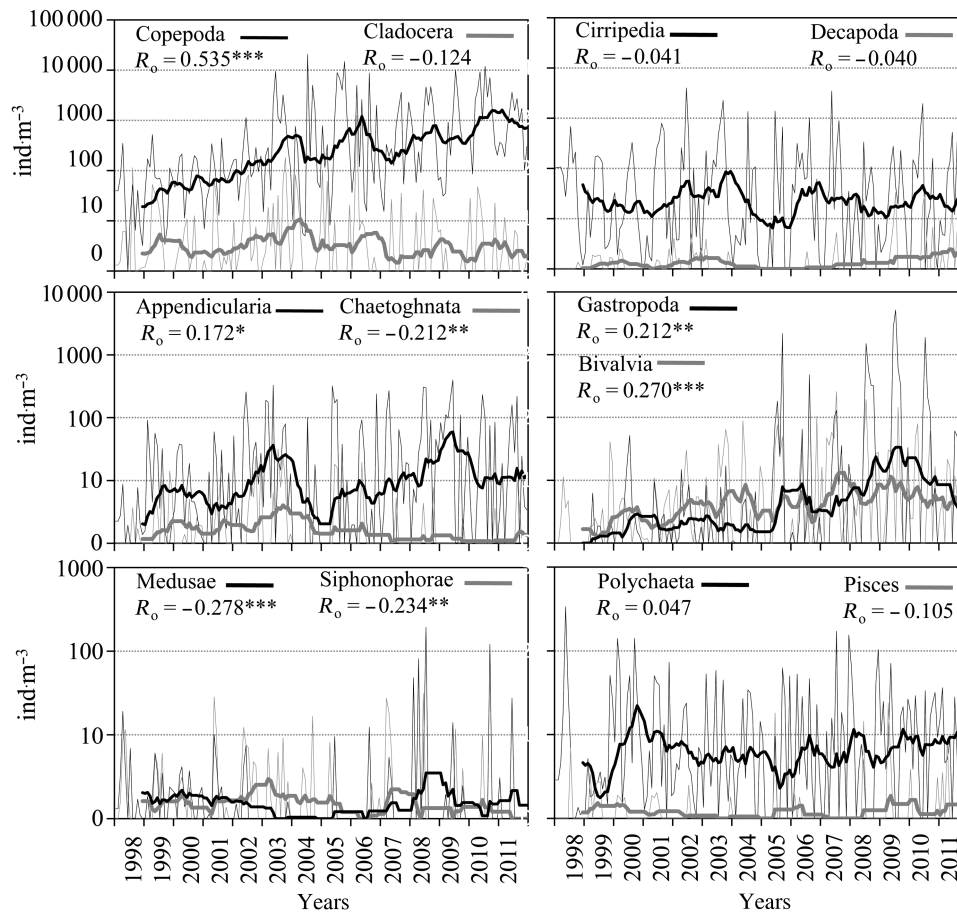


Fig. 4. Monthly time-series from 1998 to 2011 of zooplankton group densities ($\log x + 1$). Top left panel: Copepoda (black) and Cladocera (grey); mid left panel: Appendicularia (black) and Chaetognatha (grey); bottom left panel: Medusae (black) and Siphonophorae (grey); top right panel: Cirripedia (black) and Decapoda (grey); mid right panel: Gastropoda (black) and Bivalvia (grey); bottom right panel: Polychaeta (black) and Pisces (grey). Thicker lines are moving averages. R_o is the Spearman's rank correlation coefficient (zooplankton density vs. time). (***) $P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$.

sp. showed trends of decrease during the study period. *Acartia margalefi* showed no statistically significant trend of variation. At the group level, year-to-year variations occurred more smoothly, whereas at the level of copepod species variations occurred more abruptly because of the arrival of non-indigenous species in specific years (Figs 4 and 5).

From the year-to-year evolution of the percentage contribution of the different taxa to total zooplankton (Fig. 6), the following points can be made. Initially (year 1998) cirripedes were dominant (45.7% of the total zooplankton abundance), followed by copepods (30.2% of the total zooplankton abundance). With the introduction and settlement of non-indigenous copepods and the increase of *C. aquaedulcis*, copepods became more and more dominant during the second half of the time-series (56.5 to 82.9% of the total zooplankton abundance) and cirripedes decreased their relative abundance (4.7–20.8% of the total zooplankton). The per cent contribution of

polychaetes also declined from the first half to the second half of the series. Within the copepod species (Fig. 6), until the settlement of *A. tonsa* in 2003, *A. clausi* was dominant followed by PCPCalanus and *Oithona* spp. From 2004, the other non-indigenous copepod species, *O. davisae* became the second most abundant species until 2010, when *C. aquaedulcis* started to rank second in abundance after *A. tonsa*.

The relationship between zooplankton community structure and water environment and hydro-climatic factors was examined by means of RDA where all the environmental variables were used as continuous explanatory variables. Results are shown in Fig. 7 and Tables I and II for zooplankton groups and in Fig. 8 and Tables I and II for copepod species. For zooplankton groups, all the water environment and hydro-climatic factors considered explained 23.1% of the variation in the taxa data. From the variance explained by the environmental factors, the first two axes explained 81.3% of the taxa–environment relationship.

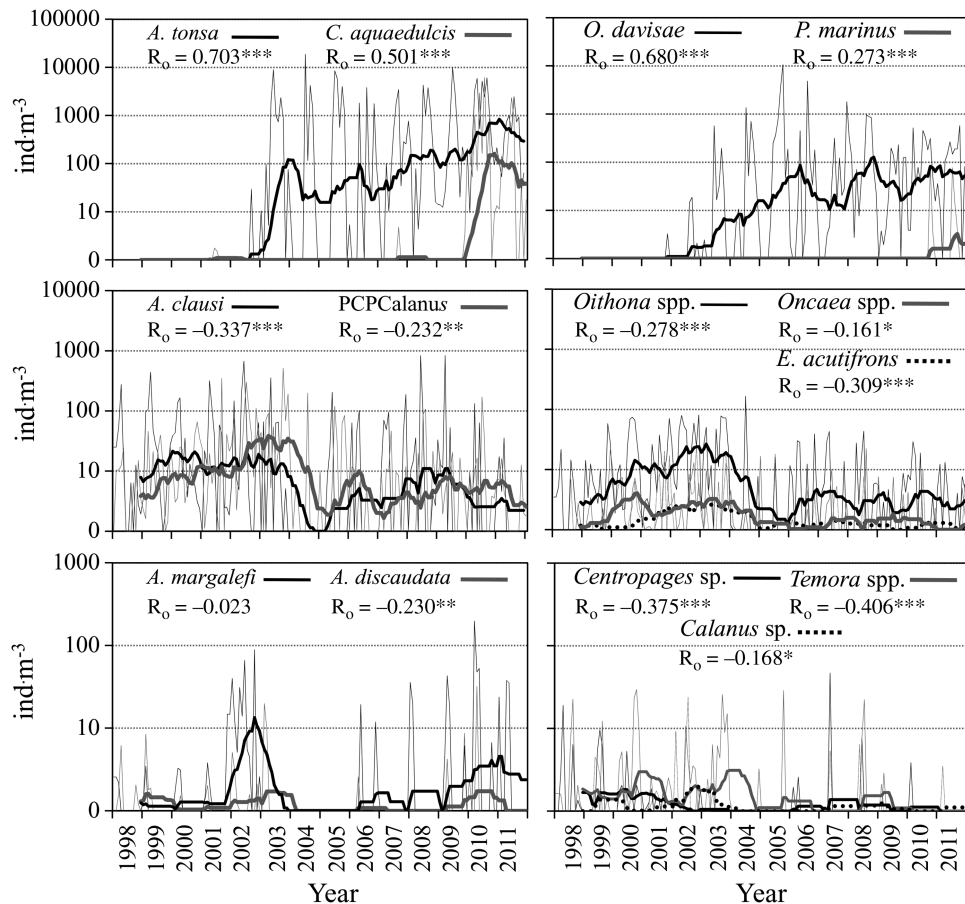


Fig. 5. Monthly time-series from 1998 to 2011 of copepod densities ($\log x + 1$). Top left panel: *Acartia tonsa* (black), and *Calanipeda aquaedulcis* (grey); mid left panel: *Acartia clausi* (black) and PCPCalanus (grey); bottom left panel: *Acartia margalefi* (black) and *Acartia discaudata* (grey); top right panel: *Oithona davisae* (black) and *Pseudodiaptomus marinus* spp. (grey); mid right panel: *Oithona* spp. (black), *Oncaea* spp. (grey) and *Euterpina acutifrons* (dashed); bottom right panel: *Centropages* sp. (black), *Temora* spp. (grey) and *Calanus* sp. (dashed). Thicker lines are moving averages. R_0 is the Spearman's rank correlation coefficient (zooplankton density vs. time). (***) $P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$.

The single best explanatory variable for the variations in zooplankton groups was water temperature, followed by DOS, air temperature and SDD, while the rest of variables considered did not add significantly to the percentage variance explained. Bivalvia and Copepoda were mainly correlated with temperature (positively) and streamflow (negatively). Gastropoda, Medusae, Chaetognatha and Cirripedia showed high correlation among them and were mainly correlated with chlorophyll *a* and SDD. Cladocera were mainly correlated with DOS.

For copepod species, all the water environment and hydro-climatic factors explained 23.3% of the variations in the taxa data. From this 23.3%, the first two axes explained 86.1% of the taxa–environment relationship. The single best explanatory variable for the copepod species variations was also water temperature, in this case followed by SDD, DOS, streamflow, chlorophyll *a*, AMO index and EA index, while the rest of the variables were not selected by the model. *Acartia discaudata* and *A. clausi*

showed positive correlation with precipitation and NAO index, whereas *A. tonsa*, *O. davisae*, *C. aquaedulcis* and *P. marinus* showed positive correlation with temperature and SDD and negative correlation with streamflow. *Euterpina acutifrons*, PCPCalanus and *Oithona* spp. were correlated with chlorophyll *a*, EA index and DOS.

Given that temperature and streamflow, which are variables with strong seasonal patterns, were important explanatory variables accounting for the main mode of variability, correlation analyses were performed between the sample scores (in this case sample scores that are linear combinations of the environmental values were used) and water temperature, air temperature and streamflow by seasons to test whether the relationships between these factors and zooplankton taxa (both groups and species) were due not only to their seasonal variations, but also to their year-to-year variations. At the zooplankton groups level, positive correlations with water temperature were observed in all seasons (winter, $P < 0.05$;

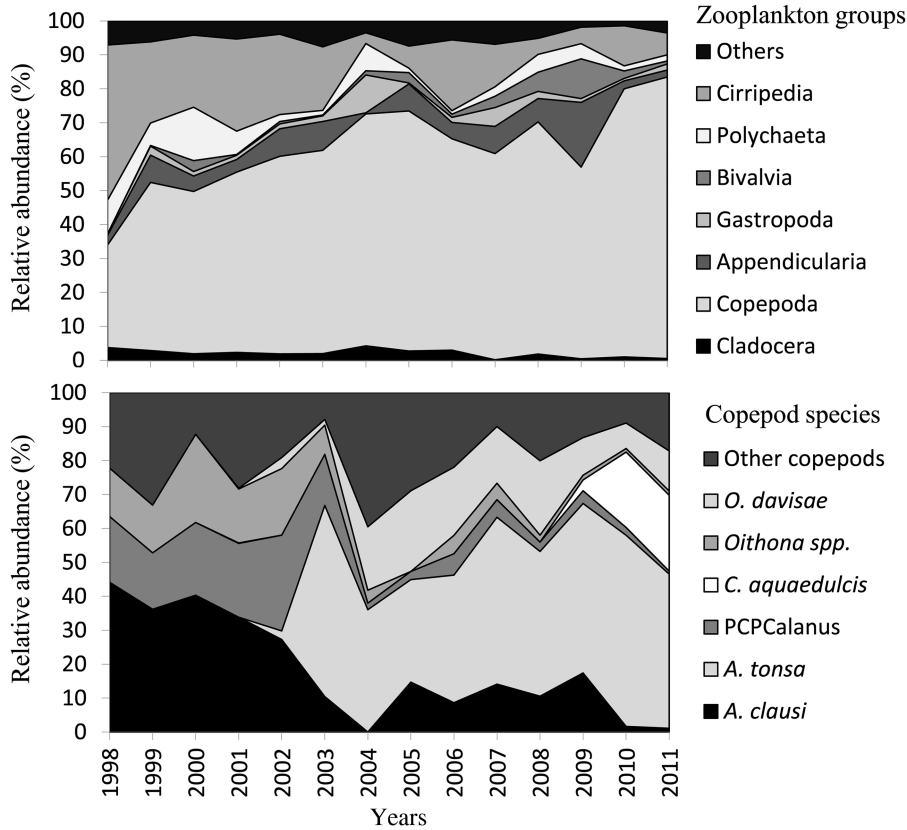


Fig. 6. Temporal variations of the relative abundances (%) of zooplankton groups (top) and copepod species (bottom) from 1998 to 2011.

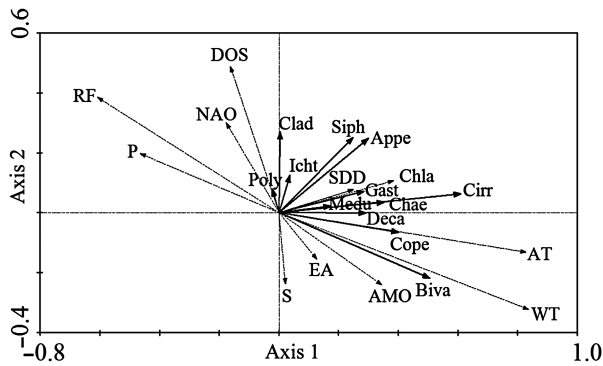


Fig. 7. RDA ordination plots of zooplankton groups and environmental variables. AT, air temperature; WT, water temperature; S, salinity; DOS, dissolved oxygen saturation; Chla, Chlorophyll *a* concentration; SDD, Secchi disk depth; P, precipitation; RF, streamflow; NAO, North Atlantic Oscillation index; AMO, Atlantic Multidecadal Oscillation index; EA, East Atlantic pattern; Cope, Copepoda; Clad, Cladocera; Appe, Appendicularia; Chaec, Chaetognatha; Medu, Medusae; Siph, Siphonophorae; Cirr, Cirripedia; Deca, Decapoda; Gast, Gastropoda; Biva, Bivalvia; Poly, Ploychaeta; Icht, Pisces.

Table I: Conditional effects for the zooplankton groups and copepod species data sets

Zooplankton groups				Copepod species			
Variables	λ	<i>P</i>	<i>F</i>	Variables	λ	<i>P</i>	<i>F</i>
WT	0.12	0.002	22.09	WT	0.08	0.002	14.00
DOS	0.03	0.002	7.11	SDD	0.04	0.002	7.14
AT	0.02	0.032	2.74	DOS	0.03	0.002	6.67
SDD	0.01	0.010	2.74	RF	0.02	0.016	4.11
AMO	0.01	0.068	1.90	Chla	0.02	0.024	3.47
RF	0.01	0.056	1.91	AMO	0.01	0.034	2.76
Chla	0.01	0.100	1.66	EA	0.01	0.044	2.50
S	0.01	0.132	1.49	S	0.01	0.296	1.12
NAO	0.00	0.164	1.39	P	0.01	0.368	1.00
EA	0.01	0.348	1.07	NAO	0.00	0.480	0.78
P	0.00	0.890	0.51	AT	0.00	0.408	0.89

Significant *P*-values in bold.

AT, air temperature; WT, water temperature; S, salinity; DOS, dissolved oxygen saturation; Chla, chlorophyll *a* concentration; SDD, Secchi disk depth; P, precipitation; RF, streamflow; NAO, North Atlantic Oscillation index; AMO, Atlantic Multidecadal Oscillation index; EA, East Atlantic pattern.

spring, $P < 0.001$; summer, $P < 0.05$; autumn, $P < 0.001$) and with air temperature in winter ($P < 0.05$), spring ($P < 0.001$) and autumn ($P < 0.001$). In addition, negative correlations with streamflow in spring ($P < 0.01$), summer

($P < 0.05$) and autumn ($P < 0.001$) were obtained. At the copepod species level, positive correlations with water temperature were obtained in spring ($P < 0.001$), summer ($P < 0.05$) and autumn ($P < 0.05$), and in

Table II: Summary statistics for the first four axes of RDA of zooplankton groups and copepod species data

	Axis 1	Axis 2	Axis 3	Axis 4	Total variance
Zooplankton groups					1
Eigenvalues	0.161	0.026	0.016	0.011	
Species–environment correlations	0.690	0.454	0.352	0.372	
Cumulative percentage variance					
of species data	16.1	18.8	20.3	21.4	
of species–environment relations	69.9	81.3	88.1	92.8	
Sum of all eigenvalues					1
Sum of all canonical eigenvalues					0.231
Copepod species					
Eigenvalues	0.165	0.036	0.016	0.006	1
Species–environment correlations	0.579	0.470	0.440	0.328	
Cumulative percentage variance					
of species data	16.5	20.1	21.7	22.3	
of species–environment relations	70.8	86.1	93.1	95.6	
Sum of all eigenvalues					1
Sum of all canonical eigenvalues					0.233

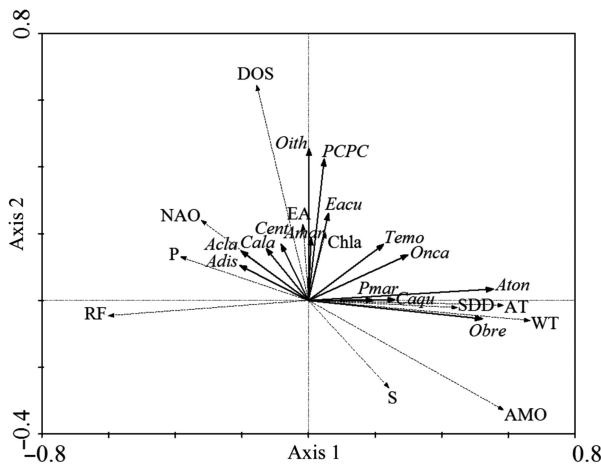


Fig. 8. RDA ordination plots of copepod species and environmental variables. Environmental variable abbreviations as in Fig. 7; Acla, *Acartia clausi*; Aton, *Acartia tonsa*; Adis, *Acartia discaudata*; Amar, *Acartia margalefi*; Cala, *Calanus* spp.; PCPC, *Pseudocalanus* sp., *Clausocalanus* spp. and *P-calanus*; Cent, *Centropages* sp.; Temo, *Temora* spp.; Pmar, *Pseudodiaptomus marinus*; Caqu, *Calanipeda aquaedulcis*; Oith, *Oithona* spp.; Odav, *Oithona davisae*; Onca, *Oncaea* spp.; Eacu, *Euterpina acutifrons*.

spring ($P < 0.01$) and autumn ($P < 0.05$), correlations were significantly positive with air temperature too. Additionally, negative correlations with streamflow were observed in winter ($P < 0.001$), summer ($P < 0.01$) and autumn ($P < 0.001$).

DISCUSSION

The increase in total zooplankton abundance in the low salinity zone of the estuary of Bilbao observed from 2001 onwards coincided with the initiation of a substantial improvement in the levels of DOS. This improvement in the oxygenation of the water column was linked to the decrease in sewage-derived pollution, particularly when

secondary treatment begun to be used in the Galindo wastewater treatment plant in 2001 (García-Barcina *et al.*, 2006; Villate *et al.*, 2013), which, together with the industrial decline of the area, has caused an overall improvement in water and sediment quality parameters (García-Barcina *et al.*, 2006; Fernández-Ortiz de Vallejuelo *et al.*, 2010). The degradation level attained was strong, and for instance in 1995–96, a depleted benthic faunal stage still persisted along intertidal mudflats where anoxic conditions prevailed (González-Oreja and Saiz-Salinas, 1998). Increases in abundance and diversity of benthic and fish communities in the last decade or so have been linked to this amelioration in the health status of the estuary (Borja *et al.*, 2006; Uriarte and Borja, 2009). There is some basis in the literature to support the view that zooplankton also respond to water quality degradation and improvement (Patrioti, 1984; Vecchione, 1989; Uriarte and Villate, 2004; Mialet *et al.*, 2011; Biancalana *et al.*, 2012), and a likely explanation of the total zooplankton abundance increase in the estuary of Bilbao is pollution abatement, particularly if we consider the fact that in the 1980s, when the inner estuary was very strongly polluted, this estuarine reach was practically devoid of zooplankton (Fernando Villate, personal communication). Initial signs of recovery in the zooplankton of the estuary of Bilbao were reported by Albaina *et al.* (Albaina *et al.*, 2009) from the comparison of mesozooplankton communities of the periods 1997–99 and 1999–2001. However, on the one hand, the increase in DOS levels in this inner estuarine site during the period analysed in the present work (1998–2011) did not occur in a steady manner with time, because in addition to sewage pollution abatement, hydro-climatic conditions, mainly water column stratification, streamflow and temperature, are very influential (Villate *et al.*, 2013). On the other hand, total zooplankton abundance did not

appear to respond linearly to DOS levels, and this may partly be explained by the fact that marine biota respond to multiple environmental drivers which cause non-linear changes in the performance of organisms (Boyd and Brown, 2015), the fact that different taxa of a community can respond differently to pollution abatement and improvement and also by the fact that communities may be modified by the occurrence of new species during processes of environmental change.

This was particularly evident in our case, where the zooplankton group that showed the largest increase in abundance over the entire series in the inner reach of the estuary of Bilbao was the Copepoda, as a result of the increase in large numbers of the non-indigenous species *A. tonsa* and *O. davisae*. The more moderate temporal increase in total copepod abundance with water quality improvement before the occurrence of non-indigenous species is consistent with the increase in abundance observed along spatial gradients of decreasing sewage-derived pollution in this estuary (Uriarte and Villate, 2004) and elsewhere (Vecchione, 1989). Thus, the process of recolonization of the inner estuary by copepods occurred in different ways and stages for different species. Firstly, neritic copepod species such as PCPCalanus and *Oithona* spp. started to penetrate further into the estuary, and their abundance increased in quite a gradual way, but then the arrival and development of the brackish non-indigenous populations of *A. tonsa* and *O. davisae*, and later of *C. aquadulcis*, were mainly responsible for the increase in copepods since 2003. *Acartia tonsa* is a calanoid copepod likely originated in West Atlantic and IndoPacific waters that were first found in European waters in the early 20th century (see Brylinsky, 1981) and which were not observed in the inner estuary of Bilbao until 2002. By 2003, it had colonized this inner estuary becoming the dominant copepod and displacing the indigenous neritic congener *A. clausi* to the outer estuary (Aravena et al., 2009). The segregation of *A. clausi* and *A. tonsa* spatially in higher and lower salinity waters is a feature common to many estuaries (Chinnery and Williams, 2004; Azeiteiro et al., 2005). In the estuary of Bilbao, these two congeners appear seasonally segregated too, as observed in other estuaries (Gaudy et al., 2000).

The next non-indigenous copepod species that successfully settled in the inner estuary was *O. davisae*, a cyclopid species native to the Japan and China Seas (Mihneva and Stefanova, 2013). *Oithona davisae*, sometimes initially misidentified as *Oithona brevicornis* (Temnykh and Nishida, 2012), has colonized a number of American and European estuaries and bays in the last decades (Ferrari and Orsi, 1984; Hirakawa, 1988; Altukhov et al., 2014). These two non-indigenous species, *A. tonsa* and *O. davisae*, have been found to be able to inhabit low

oxygen waters (Roman et al., 1993; Itoh et al., 2011), although severe hypoxia can cause lethal effects at least in *A. tonsa* (Marcus et al., 2004).

The progression towards higher oxygen levels in the inner reach of the estuary was quite irregular, with significant ups and downs, weak hypoxia still being a common feature during part of the summer. We hypothesize that the erratic trajectory in the oxygenation recovery and the weak hypoxia still remaining during part of the summer in the inner reach of the estuary of Bilbao may have favoured the settlement of species such as *A. tonsa* and, maybe even *O. davisae*, with a higher tolerance to some degree of hypoxia. Aravena et al. (Aravena et al., 2009) showed that the settlement and dominance of *A. tonsa* in the intermediate and inner estuary of Bilbao, to the detriment of the indigenous congener *A. clausi*, were favoured by the following: the more negative effect of low DOS on *A. clausi* than on *A. tonsa*, the positive effect of temperature on *A. tonsa* and the competitive advantage of the brackish *A. tonsa* over the neritic *A. clausi* in lower salinity waters. The present results also showed the positive correlation of *A. tonsa* abundance with temperature, as observed in other estuaries (Pastorinho et al., 2003; Kimmel and Roman, 2004). We further showed that the abundance of *O. davisae*, which peaks in summer/autumn, was also correlated with temperature. Interestingly, this temperature effect was not explainable solely because of the seasonality of these two species, but it was also due to the year-to-year variations in temperature. These positive correlations with temperature agree with the description of *A. tonsa* as thermophilic (Gubanova et al., 2014) or, more frequently, as eurythermal (Gaudy et al., 2000), but with a capacity to take advantage of increased temperatures and other environmental changes associated with temperature increases when colonizing new systems, such as the marinization of estuaries and/or decreases in DOS (David et al., 2007; Aravena et al., 2009) and of *O. davisae* as thermophilic (Uye and Sano, 1995). Additionally, both these two species showed negative correlation with streamflow in the inner estuary of Bilbao. Extended drought periods have been claimed to facilitate the establishment of non-indigenous zooplankton species in estuaries, because this condition favours increased inoculum frequency, thus providing colonization opportunities (Winder et al., 2011). In our series, the driest period was from 2001 to 2002, just when the first records of *A. tonsa* and *O. davisae* were registered. Therefore, it seems that in the estuary of Bilbao, as in other estuaries (Winder et al., 2011), climate factors can act synergistically with anthropogenic influences to facilitate the establishment of non-indigenous species.

In a later stage, a third non-indigenous brackish copepod species, *P. marinus*, native to the Northwest Pacific (Srinui et al., 2013), irrupted in the estuary of

Bilbao. This species seems, as in the estuary of Bilbao, to have only recently (first recorded in 2007 in the Adriatic Sea, De Olazabal and Tirelli, 2011) arrived to the Mediterranean (De Olazabal and Tirelli, 2011; Sabia *et al.*, 2014) and the North Sea (Brylinski *et al.*, 2012). *Pseudodiaptomus marinus* peaks were observed in summer/autumn in the inner estuary of Bilbao, the abundance of this species showing a positive correlation with temperature. This finding is consistent with the seasonality of this species in Japanese bays (Liang and Uye, 1997; Tachibana *et al.*, 2013) and the fact that its reproduction rate is limited by temperature (Liang and Uye, 1997). *Pseudodiaptomus marinus* was only observed during the last 2 years of the time-series in the estuary of Bilbao, and the maximum abundance attained was only of 80 ind m⁻³, far below that of *A. tonsa* (18 866 ind m⁻³). It is not known whether the population has definitely settled in this inner reach of the estuary and whether it will grow further in the future. That is to say, the other two non-indigenous copepods, i.e. *A. tonsa* and *O. davisae*, have shown a pattern of quite persistent (for nearly a decade) dominance, but it is too early to know whether *P. marinus* is still in a lag phase and will attain larger densities in the coming years, and if so, whether it will show a persistent dominance or a boom-and-bust type of cycle as observed for some other non-indigenous species elsewhere (Simberloff and Gibbons, 2004; Dexter *et al.*, 2015).

All these three colonizer copepods are brackish species. It has already been pointed out that many non-indigenous species that colonize estuaries tend to occur in brackish waters (Nehring, 2006). Accordingly, brackish seas are subject to intense invasions of non-indigenous species (Paavola *et al.*, 2005). It seems that the natural species minimum in brackish waters with unsaturated ecological niches is of great importance to the establishment of non-indigenous species (Wolff, 1999). The low salinity habitats of the inner estuary of Bilbao were characterized by the absence of indigenous brackish species when pollution was high. It is likely that, as the health status of the estuary improved, ecological niches became available in these habitats. These were then occupied by brackish species that are potentially more competitive than neritic species in this lower salinity environment.

In addition, all these three non-indigenous copepods are summer/autumn peaking species. Observations in other estuaries have already shown that non-indigenous zooplankton species are more dominant in summer and autumn (Bollens *et al.*, 2012, 2014), although the specific reasons for this are unclear.

Another copepod species contributing significantly to the increase in the abundance of total copepods in the estuary of Bilbao in the last period of the time-series was *C. aquaedulcis*. Despite it being often considered as a

marine species, it has been observed from freshwater to salt lake systems, being an extremely euryhaline osmoconformer (Svetlichny *et al.*, 2012). This species is commonly found in the upper reaches of other nearby Basque coast estuaries such as Plentzia (Villate and Orive, 1981) and Urdaibai (Villate, 1989), but it was first observed in the estuary of Bilbao in 2001 (Albaina *et al.*, 2009). Present results showed a very large increase in abundance during the last 2 years of the time-series, 2010–11, linked primarily to an increase in water transparency, and under conditions of lowered chlorophyll *a* concentrations and of certain stability in the improved levels of DOS. This is consistent with findings in Mediterranean coastal wetlands where *C. aquaedulcis* is dominant in confined and relatively more oligotrophic conditions (Boix *et al.*, 2005). It is considered as eurythermal, but usually with a preference for cooler temperatures (Frisch *et al.*, 2006 and references therein). Accordingly, in the estuary of Bilbao, it was found that it could attain high abundances already by March, although peak abundances could be attained in July.

Regarding climate factors, regional climatic indices such as AMO and EA were also selected as explanatory variables accounting for the variability of copepod species in our study. The AMO index is defined as the de-trended mean of North Atlantic (0–60°N) sea surface temperature anomalies (Enfield *et al.*, 2001) and, accordingly, in the estuary of Bilbao AMO appeared to be positively related with water temperature. Long-term changes in marine plankton ecosystems have been found to be correlated with sea surface temperature and AMO (Goberville *et al.*, 2014; Harris *et al.*, 2015) and to a lesser extent with EA (Goberville *et al.*, 2014) in the North Sea and Northeast Atlantic. Similarly, in the Middle Atlantic Bight, several zooplankton taxa were positively correlated to surface water temperature and the AMO index (Kane, 2011). Ecosystem responses to other modes of climate variability have also been reported for the North Atlantic. Regarding estuarine zooplankton, first records of non-indigenous copepods such as *A. tonsa* have been suggested to be related to the NAO index in the Gironde estuary, high positive NAO index values being related to dry conditions and, therefore, a higher penetration of marine waters in the estuary (David *et al.*, 2007).

The other holoplankton group that, in addition to Copepoda, significantly increased in abundance during the study period was Appendicularia. This group showed low correlation with DOS. In accordance to this, *Oikopleura*, which is the dominant genus in the estuary of Bilbao, has been classified as sewage pollution tolerant (EPOPEM, 1979; Champalbert and Patrìti, 1982). In the inner estuary of Bilbao, it appeared to be better correlated with water transparency than with DOS, so its increase could be to

some extent related to the progressive decrease in suspended particulate materials during the study period, although a more in-depth study is required to confirm this.

The two other zooplankton groups that showed trends of increase in abundance were meroplanktonic Gastropoda and Bivalvia. In previous studies, spatial variations in abundance along the inner-to-outer estuary gradient of pollution showed that gastropod larvae were sensitive to pollution in the estuary of Bilbao, decreased abundances being linked to lowered DOS content, although no such sensitivity was detected in bivalve larvae (Uriarte and Villate, 2004; Intxausti et al., 2012). Similarly, gastropod larvae have been found to decrease near sewage outfalls in other coastal waters too (Patri, 1984). Studies conducted for soft bottom macrozoobenthic communities revealed that biomass ranged over six orders of magnitude from the lowest polluted to the highest polluted intertidal mudflat area in the mid-1990s in the estuary of Bilbao, and biomass highly correlated with the oxygen content of the water (González-Oreja and Saiz-Salinas, 1998). Present trends of increase of meroplanktonic bivalves and gastropods agree with the progressive recolonization of the benthic inner reaches observed by Borja et al. (Borja et al., 2006), which initially (at least up until 2003) took place through the establishment of species from the outer reaches (*Scrobicularia*–*Cerastoderma* community, sensu Borja et al., 2004). Similarly, the increasing settlement of non-indigenous soft bottom species in the intermediate and inner reaches of the estuary (Zorita et al., 2013) might have contributed to the increase in abundance of gastropod and bivalve meroplankton too. In fact, bivalve larvae abundance, as in the case of the three non-indigenous copepod species, was positively linked to temperature and negatively linked to streamflow, high temperature and low streamflow being environmental conditions that seem to favour the settlement of non-indigenous species in some estuaries (Winder et al., 2011; Bollens et al., 2012).

The abundance of Polychaeta, which in the estuary of Bilbao were mainly composed of spionids, remained relatively stable along the years, but their percentage contribution to total zooplankton decreased with pollution abatement. This is consistent with the high tolerance to pollution and low oxygen conditions of spionid larvae (Sardá and Martín, 1993; Frieztzsche and von Oertzen, 1995), which are often found to be relatively more abundant than other taxa in polluted estuarine areas (Uriarte and Villate, 2004; Biancalana et al., 2012).

Overall, it can be said that pollution abatement has allowed zooplankton recolonization of the inner estuary of Bilbao, but the fact that temporal variations in zooplankton abundance, both at group and at copepod species level, were explained mainly by variations in

climatic factors, particularly temperature, shows the great influence of climatic factors in modulating zooplankton abundance and community structure in the inner estuary of Bilbao.

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