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Health status of the Bilbao estuary: A review of data from a multidisciplinary approach☆

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A B S T R A C T

Estuarine and marine ecosystems are subject to different sources of stress including changes in environmental physicochemical variables (nutrients, oxygen availability, temperature, salinity, pH) or exposure to a large cocktail of environmental pollutants. In the estuary of Bilbao, despite the improvement in environmental quality due to replacement of most polluting industries and the progressive implementation of an integrated sewage treatment plan, chronic pollution by metals and hydrocarbons still remains, together with eutrophication and pollution by emerging contaminants. The Unit of Formation and Research "Protection of Ecosystem Health" was created in 2012 through the strategic aggregation of three consolidated research groups: Cell Biology in Environmental Toxicology (CBET), Phytoplankton of estuaries and coastal areas (FITEAC) and Research on marine and estuarine pelagic environment and planktonic communities (MarEsPlank). The three groups have a long tradition in studying the health of marine and estuarine ecosystems from different perspectives, focusing in the Bay of Biscay and especially in the estuary of Bilbao. In this work we review data on the health status of the Bilbao estuary using early warning tools at the cell and tissue levels, phytoplankton assemblages and zooplankton ecology. In spite of the seasonal and interannual variability recorded for some parameters, a general recovery trend was observed for the health status in the estuary of Bilbao, with sporadic critical events such as the Prestige oil spill.

1. Introduction

Estuarine and marine ecosystems are subject to multiple sources of stress including changes in environmental physicochemical variables (nutrients, oxygen availability, temperature, salinity or pH) or exposure to a large cocktail of environmental pollutants. All these factors can influence ecosystem structure and functioning, affecting diversity and abundance of species or trophic interactions among ecosystem components. Environmental pollutants comprise conventional or historical contaminants such as metals, polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs), and new emerging contaminants, for which there is a lack of published health standards. Endocrine disruptors are a relevant category of emerging contaminants because they can interact with the endocrine system of living organisms and produce effects on their development, reproduction and immune function (Porte et al., 2006; WHO/UNEP, 2013). Among these compounds we can find pharmaceuticals and personal care products, drugs, hormones, steroids, polychlorinated naphthalenes (PCNs) and alkanes (PCAs), perfluorochemicals, alkylphenolic surfactants, synthetic musks and phthalate ester plasticizers. On the other hand, due to the development of nanotechnology and the increasing use of products

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containing nanomaterials, the presence of these compounds is growing, especially in the marine environment, considered as the ultimate sink for contaminants, and their potential impact on marine wildlife is of concern (Corsi et al., 2014). Nano and microplastics have also become a widespread pollutant in aquatic ecosystems across the globe. Moreover, global warming and ocean acidification may pose additional threats for estuarine and marine ecosystems. Interactive effects of temperature and environmental pollutants have been described, with impairment of energy metabolism playing a key role in the synergistic effects of these stressors (Sokolova and Lannig, 2008).

The description above highlights the need to assess the health of estuarine and marine ecosystems with the aim of preventing their degradation and of allowing their sustainable development, thus preserving them for present and future generations. Quality assessment of estuarine and marine environments is driven by regulation. The EU *Water Framework Directive (2000/60/EC)* deals with the compliance of environmental quality standards (EQSs), established for chemical substances at European level, in the water basins, from inland to transitional and coastal waters. The EU *Marine Strategy Framework Directive (2008/56/EC)* covers the water of the first 200 miles and aims to promote the sustainable use of the seas and to conserve marine ecosystems. The final goal is to achieve a "good quality status" of European seas by 2021. An important challenge is to understand and define what good quality status means in terms of ecosystem integrity and functioning, sustainable use of marine resources and maintenance of ecosystems services. It is widely accepted that assessing ecosystem quality or health status will require integration of physical, chemical and biological aspects. Establishing criteria and methods to determine EQSs and good quality status is a priority objective in order to achieve harmonized monitoring procedures combining chemical and biological effect assessment methods.

As to the biological aspects, the influence of pollutants and other environmental variables can be assessed at different levels of biological organization, from molecules to ecosystems. Responses measured at the molecular, biochemical or cellular level are known as biomarkers, which indicate that the organism has been exposed to toxic chemicals and the magnitude of the organism's response to the pollutant (McCarthy and Shugart, 1990). Biomarker responses are rapid and can be reversible, as shown in transplant experiments (Orbea and Cajaraville, 2006; Lekube et al., 2014). The biomarker concept is based on the fact that the first interaction of pollutants with biological systems will occur at the molecular and cellular level (receptor activation, differential regulation of gene transcription, induction of proteins or enzyme activities, alterations in cellular organelles). These changes will then give rise to alterations at the histological and individual level and finally can lead to alterations in populations, communities and ecosystems. Changes at the lower biological organization levels occur earlier and are useful to have a mechanistic understanding on the impact of pollutants or environmental variables whereas changes at higher organization levels occur at longer time scales and are more ecologically relevant (Wilson, 1988; McCarthy and Shugart, 1990; Cajaraville et al., 2000; Adams, 2005).

The Unit of Formation and Research "Protection of Ecosystem Health" was created in 2012 through the strategic aggregation of three consolidated research groups of the University of the Basque Country: Cell Biology in Environmental Toxicology (CBET), Phytoplankton of estuaries and coastal areas (FITEAC) and Research on marine and estuarine pelagic environment and planktonic communities (MarEsPlank). The CBET research group is specialized in early warning tools at the molecular, cellular and tissue levels (Cajaraville et al., 2000, 2003, 2006; Marigómez et al., 2006, 2013), whereas FITEAC and MarEsPlank groups work at the community and ecosystem levels (Orive et al., 2004; Uriarte and Villate, 2004, 2005; Seoane et al., 2005, 2006; Albaina et al., 2009; Villate et al., 2013). The collaborative research of the three groups together provides a multidisciplinary perspective for the diagnosis and evaluation of ecosystem health by integrating responses of ecosystems at different levels of biological complexity, together with physical and chemical variables.

Although different estuaries and coastal areas of the Bay of Biscay have been investigated by the three groups, the Bilbao estuary, due to its larger size and for its recovery process from a very polluted condition, is being the subject of much research and can be taken as a case study. Thus, in this work we review data on the health status of the Bilbao estuary using early warning biomarkers at the cell and tissue levels, phytoplankton assemblages and zooplankton ecology. Further work is in progress to gain new insight into the long-term trends in the health status of the estuary of Bilbao by integrating responses at different biological complexity levels.

2. The estuary of Bilbao

The estuary of Bilbao (Fig. 1) is a small macro-mesotidal system ($\approx$23 km long from the tidal limit to the shore line) located in the inner Bay of Biscay (43°23′- 43°14′N, 3°07′-2°55′W) on the coast of the Basque Country. The original morphology of the system has been strongly modified by land reclamation, dredging and channelization, and today two clearly different zones can be distinguished: the narrow channelized zone that extends 15.2 km from the tidal limit in the innermost part to the Abra harbour, and the harbour itself which is a funnel-shaped wider embayment occupied by port facilities. The channelized zone varies between 25 and 270 m in width, and between 0.5 m mean depth at the head and >10 m at the border with the Abra harbour. In the latter, in turn, there is an inner zone with a maximum width of 1.8 km and a maximum depth of 15 m and an outer zone with a mean width of 3.8 km and a maximum depth of 32 m.

The system is subject to a semidiurnal tidal regime, the tidal amplitude ranging from 4.6 m (spring tides) to 1.2 m (neap tides) in the Abra harbour. The main input of freshwater to the estuary occurs at the head of the main channel from the joint flow of the Ibaizabal and Nerbioi rivers that represents on average 66% of the total input of freshwater, whereas the third main tributary (the Kadagua river) discharges into the middle estuary and contributes 27% of the total (Uriarte et al., 2014). River flow is on average relatively low in comparison to the basin volume and the tidal prism, and as a result, most of the estuary is usually euhaline (salinity > 30). The channelized zone is highly stratified in its inner half all year round, but stratification weakens gradually towards the Abra harbour (Iriarte et al., 2010), where salinity values approach those of the surrounding shelf waters (35.0–35.8). Under unusually high river flow conditions, however, the entire channelized zone may be filled by freshwater at low tide, and the strongest salinity gradient moves to the Abra harbour.

A large history of human activity in the estuary and watershed has been responsible for the dramatic changes that have occurred in the environmental properties of the estuarine waters and sediments, mainly during the last two centuries (Belzunce et al., 2004; Cearreta et al., 2004). In the mid 20th century the estuary became a highly polluted system that received large amounts of industrial wastes and domestic sewage, which enhanced the accumulation of a large mixture of toxicants and the organic enrichment of the system (Belzunce et al., 2001). As a result, extensive areas developed hypoxic or even anoxic conditions and faunal communities were largely wiped out from the channelized zone (González-Oreja and Sáiz-Salinas, 1998). However, the significant industrial decline

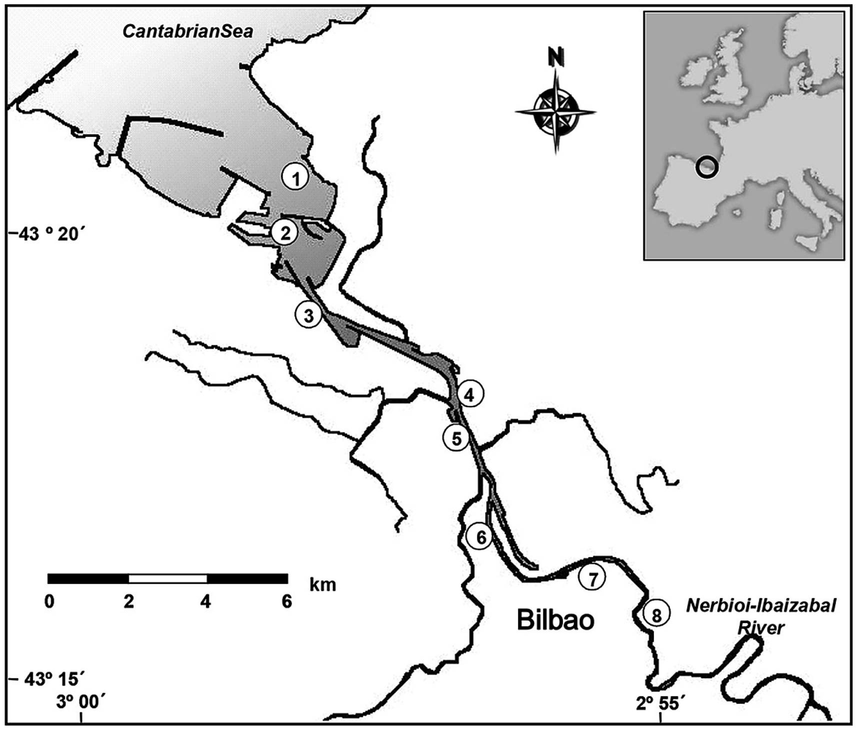

Fig. 1. Map showing the location of the estuary of Bilbao and sampling locations: 1, outer Abra; 2, inner Abra; 3, Biscay Bridge (Getxo); 4, Axpe (Erandio); 5, Rontegi (Barakaldo); 6, Zorroza; 7, Deusto; 8, Arriaga (Abando).

in the area in the last decades, and the implementation of a sewerage scheme in the late 1980s have caused a general improvement of the water quality and a significant recovery of the biological communities of the estuary, although biotic differences associated to pollution gradients along the estuary were still observed in the last years of the 20th century and the early 21st century (Uriarte and Villate, 2004, 2005; Albaina et al., 2009; Borja et al., 2006, 2010; Intxausti et al., 2012). A feature of the recent biotic re-colonization of the most degraded habitats has been the occurrence of non-native species, which in some cases have become dominant (Aravena et al., 2009a; Zorita et al., 2013).

Overall, chronic pollution by metals and hydrocarbons still remains in sediments and biota of the estuary of Bilbao (Prieto et al., 2008; Bartolomé et al., 2010; Puy-Azurmendi et al., 2013). Further, accidental chemical and oil spills have affected the area, such as the oil spill caused by the sink of the Prestige tanker in front of Galician coast in 2002 (Marigómez et al., 2006; Orbea et al., 2006; Bartolomé et al., 2007; Diez et al., 2009). Moreover, emerging pollutants such as endocrine disruptors are increasingly reported in different estuaries of the Bay of Biscay, including the estuary of Bilbao (Rodriguez et al., 2009; Bartolomé et al., 2010; Puy-Azurmendi et al., 2013; Bizarro et al., 2014). Main sources of pollution in the area are urban, industrial and agricultural wastes and run-off, atmospheric deposition, dredging, harbour activities and maritime traffic. In addition, other stress sources such as global climate change have recently been reported to affect the area (Diez et al., 2012). The importance of understanding the interactions between climate variability and other anthropogenic drivers has been recently highlighted (Brown et al., 2011).

3. The early warning biomarker approach

Biomarkers constitute sensitive early warning tools for measuring biological effects caused by environmental stress, including exposure to pollutants (McCarthy and Shugart, 1990; Cajaraville et al., 2000; Viarengo et al., 2007). Cell and tissue level biomarkers have been applied to assess the health status of the estuary of Bilbao since the 1990s. Biomarkers have been measured in mussels *Mytilus galloprovincialis*, used worldwide as bioindicator and sentinel species for estuarine and marine pollution biomonitoring (Goldberg, 1975; Cajaraville et al., 2000). Mussels are ubiquitous sessile filter-feeding bivalve molluscs that accumulate contaminants above levels found in the environment and exhibit a wide range of responses to contaminant exposure. Mussels were collected monthly over 1 year (September 1991–1992) at four sites of the outer estuary of Bilbao (Zierbena, Santurtzi, Arrigunaga and Galea) with different degrees of pollution. Changes in digestive cell lysosomal size and numbers were measured as general biomarker of health status (Marigómez et al., 1996) and peroxisome proliferation as biomarker of exposure to organic pollutants (Orbea et al., 1999), together with tissue levels of metals (Soto et al., 1995) and PAHs (Orbea et al., 1999). In all seasons, biomarker responses were in accordance with pollution levels in the Bilbao estuary, mussels from Galea showing significantly smaller and more numerous lysosomes than those from other sites or from the nearby less polluted estuary of Plentzia (Marigómez et al., 1996). Also, except in summer, mussels from Galea showed a higher degree of peroxisome proliferation compared to those from Plentzia (Orbea et al., 1999). Studied biomarkers varied according to a seasonal pattern at all sites, possibly related to seasonal changes in temperature, food availability and reproductive cycle (Marigómez et al., 1996; Orbea et al., 1999). Interestingly, seasonal variability was much more marked in the less polluted site of Plentzia compared to Galea, which indicates that mussels adapted to a chronically polluted site may show a limited capacity to respond to further environmental changes (Orbea et al., 1999). In September 1994, significant differences in *in vitro* endocytic-phagocytic and oxyradical producing activities of hemocytes were evidenced between mussels from

Arrigunaga at the outer part of the Bilbao estuary and those from Plentzia (Cajaraville et al., 1996).

In 2003, monitoring efforts were extended to 17 localities all along the northern coast of the Iberian peninsula, from Galicia to the eastern part of the Basque Country, in order to assess the consequences of the oil spill provoked by the accident of the Prestige tanker (Cajaraville et al., 2006; Marigómez et al., 2006; Orbea et al., 2006; Garmendia et al., 2011a,b,c; Ortiz-Zarragoitia et al., 2011). The accident, occurred in November 2002 in front of the Galician coast, caused the leakage of more than 60.000 t of heavy fuel oil that affected >1.000 Km of coastline along the Bay of Biscay. The biological effects of the Prestige oil spill on the outer part of the estuary of Bilbao (Arrigunaga) were measured by selected cell and tissue-level biomarkers and histopathological alterations together with reproduction-related effects (Table 1) in sentinel mussels over 3 years (2003–2006). Later on, monitoring has continued regularly up today in selected localities of the Bay of Biscay with the goal of identifying long-term trends in relation with pollutants, especially emerging pollutants such as endocrine disruptors (Puy-Azurmendi et al., 2010). Reference and critical values for the selected biomarkers in mussels from the study area at different seasons of the year have been published by Marigómez et al. (2006).

The most remarkable effects of the Prestige oil spill were alterations in peroxisomal β-oxidation, lysosomal membrane destabilization and enlargement, changes in cell type composition, severe alteration in the general condition of the digestive gland tissue and atrophy of the digestive alveoli (Orbea et al., 2006; Garmendia et al., 2011a,b). Similarly, mussels showed disturbed gametogenesis with high oocyte atresia, small-sized follicles and abnormal Vtg-like protein levels in 2003–2004 (Ortiz-Zarragoitia et al., 2011). Thereafter, the synchronization of gametogenesis was recovered and most severe gonad pathologies disappeared. Similarly, other biomarkers reflected a recovery process of mussels health two years after the Prestige oil spill (Cajaraville et al., 2006). Thus, some biomarkers such as Vv_L, Vv_{BAS} and general histological integrity of the digestive gland returned to reference values by 2004–2005 (Fig. 2), but others as LP values and MLR/MET were not fully recovered and continued warning at the end of the study in April 2006 (Fig. 2) (Garmendia et al., 2011a,b). Similar results were obtained in mussels from Mundaka, in the nearby estuary of Urdaibai (Fig. 2), except for values of AOX activity, that were significantly lower in mussels from the outer estuary of Bilbao than in mussels from Urdaibai. Overall, it seems that some secondary effects of the oil spill persisted in the estuary of Bilbao 4 years after the oil spill, even though the PAH tissue levels (mainly naphthalene) in mussels decreased drastically after February 2004, indicating cessation of the direct impact of the Prestige oil spill.

The early warning biomarker approach using sentinel mussels can also be applied in active biomonitoring strategies (Salazar and Salazar, 1997; Orbea and Cajaraville, 2006; Zorita et al., 2006; Viarengo et al., 2007; De los Ríos et al., 2012, 2013; Marigómez et al., 2013; Lekube et al., 2014). The use of caged or transplanted reference mussels offers the possibility of comparing responses in a controlled population, reducing the genetic variability and adaptation responses occurring when assessing native mussels. Furthermore, this methodology allows the study of pollution effects in sites where no native mussels or other bivalves are available and make it easier the comparison of responses among different regions and works. Three caging studies using mussels have been completed in the estuary of Bilbao. Two in the outer part of the estuary (Orbea and Cajaraville, 2006; Marigómez et al., 2013; Lekube et al., 2014), mainly impacted by maritime traffic and pollutants transported by the river flood, and a third one performed in the middle part of the estuary to assess the impact of the local sewage treatment plant (De los Ríos et al., 2013).

In the first transplant study performed in November 1998, mussels obtained from the UNESCO Biosphere Reserve of Urdaibai were caged for 3 weeks in the marina of Arriluze, at the outer Bilbao estuary. High levels of PAHs and PCBs were measured in mussels caged in Arriluze, as well as in native mussels, in agreement with measured peroxisome proliferation (induction of AOX and catalase activities and high peroxisomal volume density) (Orbea and Cajaraville, 2006). Mussels caged in Arriluze showed signs of disturbed health such as attenuation of antioxidant defense mechanisms (inhibition of superoxide dismutase and glutathione peroxidase enzyme activities) (Orbea and Cajaraville, 2006), changes in cell type composition of digestive gland and disruption

Table 1
Summary of cell and tissue level biomarkers and individual biometric/allometric parameters studied and their biological significance.

Biomarker	Measured parameter	Significance	Reference
Peroxisome proliferation	Acyl-CoA oxidase (AOX) activity	Exposure to organic pollutants	Cajaraville et al. (2003)
Lysosomal Membrane Stability	Labilization Period (LP)	General health status	Marigómez et al. (2006), Garmendia et al. (2011a)
Lysosomal Structural Changes	Lysosomal Volume density (Vv_L), Surface density (Sv_L), Surface to Volume ratio (S/V_L), Numerical density (Nv_L)	General health status	Marigómez et al. (2006), Garmendia et al. (2011a)
Structural Changes in digestive alveoli	Mean epithelial thickness (MET), Mean diverticular radius (MDR), Mean luminal radius (MLR), MET/MDR, MLR/MET	General health status	Marigómez et al. (2006), Garmendia et al. (2011b)
Cell Type Composition of the digestive gland epithelium	Volume density of basophilic cells (Vv_{BAS})	General health status	Marigómez et al. (2006), Garmendia et al. (2011b)
Disease prevalence and intensity	Prevalence and infection intensity of parasitic burden and inflammatory responses	Individual disease condition and immunological status	Kim et al. (2006), Garmendia et al. (2011b)
Gamete development	Gamete developmental stages, Gonad Index (GI), Vitellogenin (Vtg)-like proteins as alkali-labile phosphate levels	Disturbances in gamete development and reproduction	Kim et al. (2006), Ortiz-Zarragoitia et al. (2011)
Biometric/allometric parameters	Flesh weight (FW), Shell weight (SW), Flesh Condition Index (FCI), Shell width/shell height, (W/H), Shell width/shell length (W/L), Shell length/shell height (L/H)	Growth alterations	Soto et al. (2000)

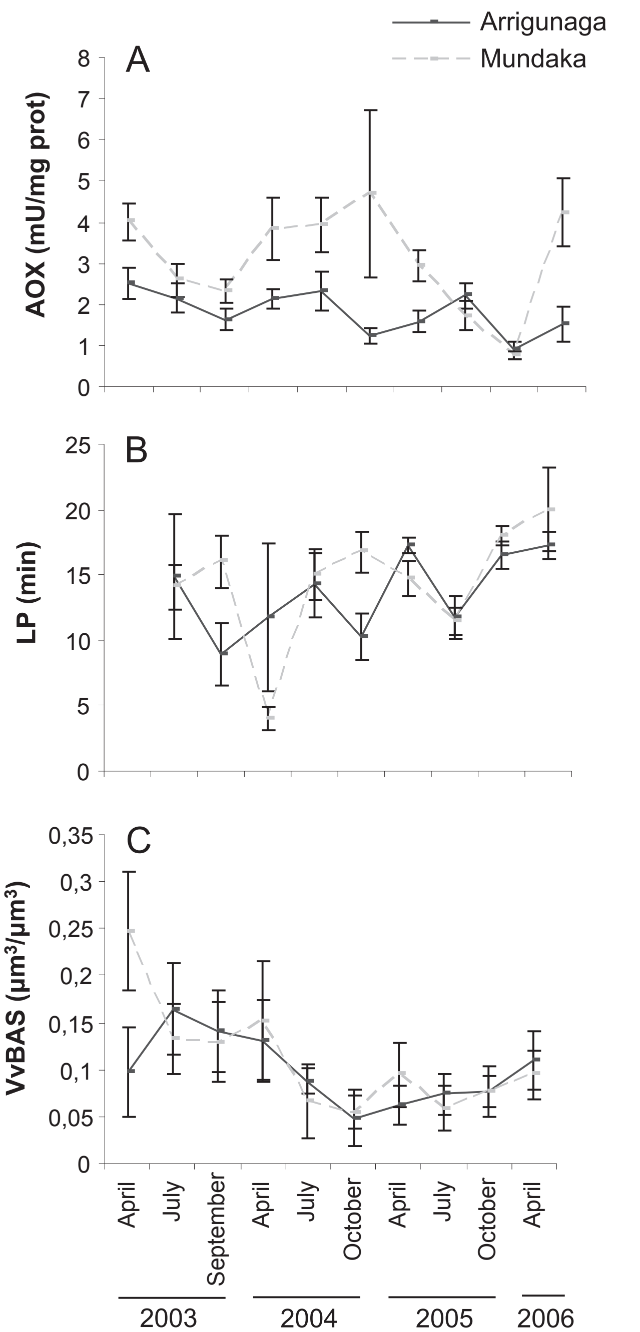

Fig. 2. Temporal trends of mussel biomarker responses in mussels from Arrigunaga, outer estuary of Bilbao, along the period 2003–2006, in comparison to mussels from Mundaka, estuary of Urdaibai. A) Acyl-CoA oxidase activity (AOX) in mU/mg protein, B) Lysosomal membrane labilization period (LP) in minutes, and C) Volume density of basophilic cells (Vv_{BAS}) in $\mu m^3/\mu m^3$. Data are adapted from Garmendia et al. (2011a,b,c).

of the lysosomal system in digestive cells (very low lysosomal membrane stability and enlarged lysosomal size) (Lekube et al., 2014), demonstrating the poor environmental quality of the outer part of the Bilbao estuary. Almost ten years later, in May 2007, a second transplant experiment was performed in the Arriluze marina, but using farmed mussels, deployed for 3 weeks (Marigómez et al., 2013). Mussels caged in Arriluze showed a significant bioaccumulation of PAHs (enriched in high molecular weight PAHs), PCBs and phthalates. Contaminant levels were much higher than those measured in mussels caged in the locality of Gorliz, Plentzia estuary, considered as low to moderately polluted site, but lower than those described previously in the first transplant experiment (Orbea and Cajaraville, 2006). Accordingly, biomarker studies demonstrated that mussels caged in Arriluze showed marked genotoxic effects (high frequency of micronuclei in hemocytes), elevated lipid peroxidation (high MDA and lipofuscin levels in digestive cells) and endocrine disruption effects (high levels of Vtg-like proteins in male gonads). Indeed reduced health status, reflected by very low levels of lysosomal membrane LP and high Vv_{BAS}, was demonstrated, together with reduced survival fitness and condition factor. Thus, although contaminant levels decreased when compared with the 1998 transplant study (Orbea and Cajaraville, 2006), the condition of mussels was still impacted by pollution (Marigómez et al., 2013).

A third transplant study was carried out in October 2009, in which farm mussels were caged for 3 weeks in Sestao, downstream the sewage treatment plant of Galindo, located in the middle part of the Bilbao estuary (De los Ríos et al., 2013). The sewage treatment plant collects all urban wastewaters of the metropolitan area of Bilbao and is a potential point source of pollutants to the estuary. Mussels caged in Sestao showed transitory effects on health condition (reduced values of lysosomal membrane LP) that were recovered after 21 days of experiment, being similar to those measured in mussels caged in the Plentzia estuary. However, high levels of intralysosomal metal accumulation and inhibition of peroxisomal AOX activity were observed at the end of the study. Accordingly, high bioaccumulation levels of Cd and Pb were detected in soft tissues of caged mussels. As in the second caging study performed in the Arriluze marina (Marigómez et al., 2013), endocrine disruption effects were detected in caged male mussels, which showed up-regulated gonad Vtg transcription levels. The same effect was evidenced in mussels caged in other places of the Bay of Biscay affected by treated (Virgen del Mar) and untreated (Peñarrubia) marine outfall discharges containing endocrine disrupting chemicals (De los Ríos et al., 2013).

4. Phytoplankton of estuaries and coastal areas

Due to the close relationships existing between dominant taxa and environmental conditions, phytoplankton assemblages are one of the biological entities considered as bioindicators of water quality in several International Directives including *Water Framework Directive (WFD, 2000/60/EC), Bathing Water Directive (BWS, 2006/7/EC), Marine Strategy Framework Directive (MSFD, 2008/56/EC), Shellfish Water Quality Directive (SWQD, 2006/113/EC) and Convention on the management of ships ballast waters (072-2006/DCG)*. When blooming, phytoplankton can be harmful not only by containing toxins, which is the case of certain species, but also by promoting oxygen depletion, and physical damage to filter-feeding animals, whose filtering structures can be clogged by the organics secreted by the microalgae (Hallegraeff, 2003). Blooms of microalgae are a natural phenomenon necessary to fuel marine food webs. However, when dominated by one or a few species (low diversity blooms), they can be harmful for aquatic animals and human health. Eutrophication is known to contribute to the frequency of apparition of blooms and to the shift for harmful species (de Jonge et al., 2002; Verity, 2010).

Phytoplankton blooms are a common feature in nutrient replete estuaries in summer coinciding with an increase in the residence time of the water in conjunction with higher irradiance and temperature (Paerl, 1996). These also occur in the Bilbao estuary, as found in the phytoplankton monitoring performed since 2000 in eight permanent stations located along the longitudinal axis of the estuary (Orive et al., 2004; Seoane et al., 2005, 2006; Laza-Martínez et al., 2007; Seoane et al., 2009). More details on the sampling

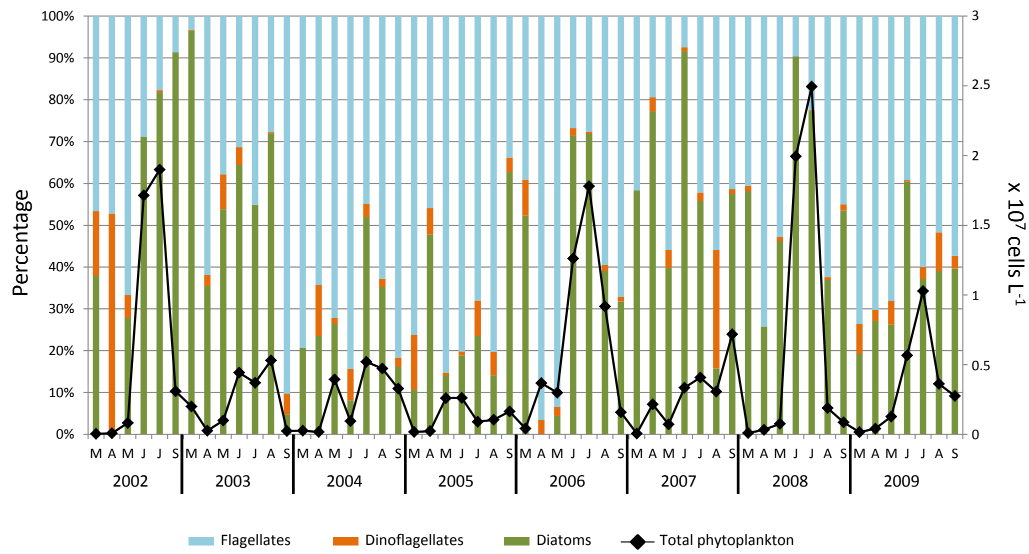

Fig. 3. Percentage of the three main groups of phytoplankton (diatoms, dinoflagellates and flagellates) and total amount of phytoplankton ($\times 10^7$ cells L^{-1}) in the outer estuary of Bilbao during the period 2002–2009, from March to September. Data can be found in the web page of the Bilbao Bizkaia Water Consortium (www.consorciodeaguas.com).

strategy, which includes the analysis of several physical and chemical parameters in addition to the phytoplankton, are given in García-Barcina et al. (2006) and the results obtained can be found in the web page of the Bilbao Bizkaia Water Consortium (www.consorciodeaguas.com). As illustrated in Fig. 3 for the outer estuary (Abra of Bilbao), a clear seasonal pattern of phytoplankton abundance, in terms of cells number, was observed along the estuary, with maxima in summer. Diatoms were the dominant taxon during the sampling period, particularly during the phytoplankton maxima, whereas flagellates dominated in spring. Dinoflagellates contributed with less than 20% to the total community. The routine monitoring was performed with the Utermöhl method (Hasle, 1978), which, although useful for some purposes, does not allow to distinguish among cryptic species or to identify very small or fragile species. For this reason, to gain insight into the taxonomic composition of the phytoplankton, which is of much concern to detect harmful species or species to be used for different applications such as biodiesel production, in addition to the routine monitoring of phytoplankton abundance and composition, live samples from the estuary have been regularly analyzed. Selected strains including harmful and/or blooming taxa have been the subject of much research work by means of microscopical as well as molecular methods after growing them in culture. Molecular analyses have been regularly performed by sequencing the small subunit (SSU), the large subunit (LSU) or the internal transcribed spacers (ITS) of the rDNA. When necessary, the secondary structure of the ITS2 was also derived to gain insight into the species delimitations within determined taxa (Orive et al., 2010, 2013).

Although phytoplankton assemblages displace along the estuary with the tide, most taxa are characteristic of a particular area. Some representative species present in the estuary are shown in Fig. 4. The outer estuary, the most extensive, contained mostly marine species, which in summer can be displaced by the tide into the estuary due to the decreased river discharge. Among harmful diatoms, ten out of about 40 described species of *Pseudo-nitzschia* have been identified in the estuary, including two new: *P. abrensis* and *P. plurisecta* (Orive et al., 2010, 2013). Other potentially harmful diatoms present in the estuary were those of the genus *Chaetoceros*, which can cause physical damage to filter-feeding animals when blooming (Seoane et al., 2005). However, most harmful species found in the estuary were flagellates, some of them ichtyotoxic, such as the genera *Chrysochromulina, Heterosigma, Karlodinium,* and *Prymnesium* (Seoane et al., 2009; Laza-Martinez et al., 2007; de Salas et al., 2008); or harmful for human health as *Alexandrium* or *Dinophysis* (Seoane and Orive, 2012). From all these taxa, most abundant ones to date have been *Pseudo-nitzschia* spp., *Chaetoceros* spp., *Heterosigma akashiwo* and *Chrysochromulina* spp., but this does not preclude that other potentially harmful taxa had bloomed any time. Several cryptophyte species dominated by the genus *Teleaulax* have been isolated from the outer estuary (Laza-Martinez et al., 2012). Although non-toxic, they are obligate preys for ciliates consumed by the diarrheic shellfish poisoning (DSP) dinoflagellate *Dinophysis*, which is of much concern in terms of water quality.

The middle estuary is a transitional zone which harbours autochthonous populations in addition to those reaching from the outer or the innermost estuary. In this estuarine reach, small centric diatoms and flagellates such as the cryptophytes *Hemiselmis* spp. and the haptophyte *Isochrisis galbana* reached high concentrations in late spring and summer (Seoane et al., 2005).

The most highlighting feature in the inner estuary was the presence of large amounts of small centric diatoms, most of them solitary forms dominated by the genera *Cyclotella*-like and *Thalassiosira*-like (Hevia-Orube et al., 2015). Their presence coincided with the minimum oxygen concentrations in the estuary and the highest values of chlorophyll *a*. Another characteristic species of this section was the cryptophyte *Urgorri complanatus*, which forms red blooms in summer and can also contribute to the oxygen depletion (Laza-Martinez, 2012). The potentially ichtyotoxic *Pfiesteria* –like dinoflagellates usually follow the cryptophytes blooms.

Several of the identified species are ichtyotoxic, among which, the most abundant was the raphydophycean *H. akashiwo*. The outer estuary of Bilbao has many artificial semiconfined areas with soft sediment which can be a source of microalgae cyst, which can give rise to a bloom if resuspended cysts of a particular species find favourable conditions in the water column. Such was the case of *H. akashiwo*, which is known to form cysts and that, although it was

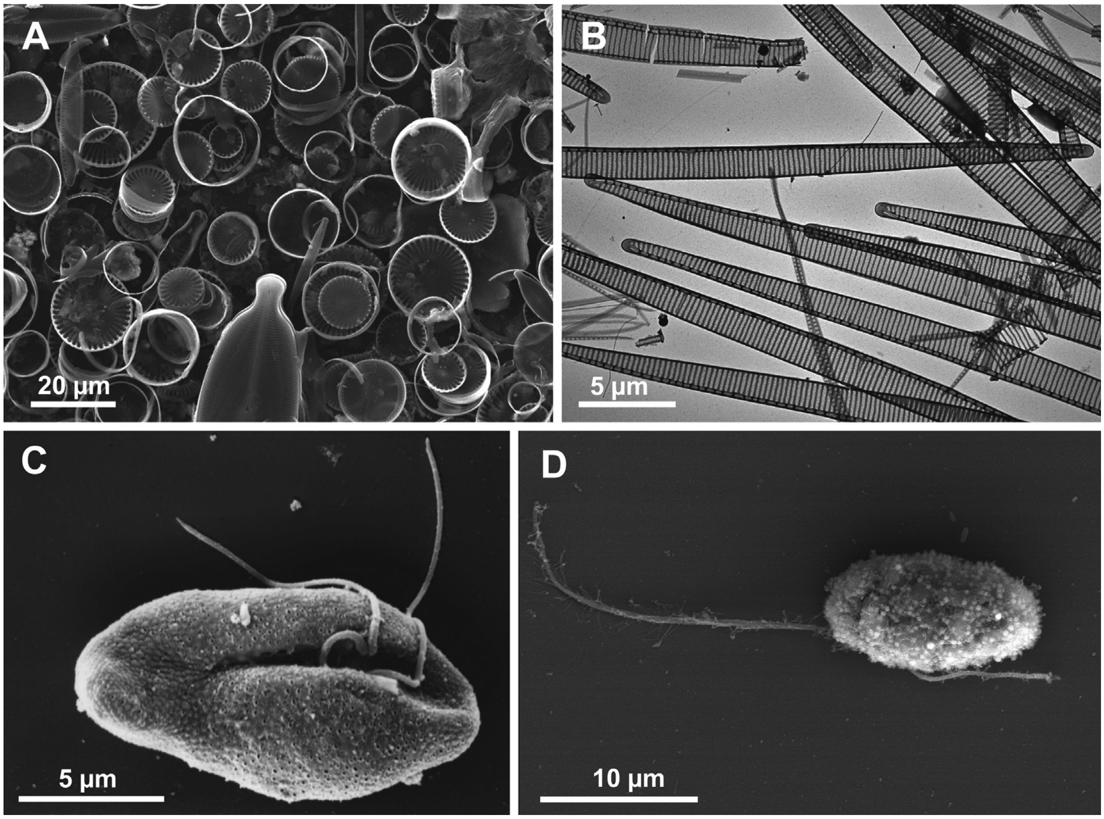

Fig. 4. Electron micrographs of bloom forming and toxic species of phytoplankton isolated from the Bilbao estuary. A, natural assemblage of blooming thalassiosiroid diatoms. B, the potentially toxic diatom *Pseudo-nitzschia arenysensis*. C, the red-tide forming flagellate *Urgorri complanatus* (Cryptophyceae). D, the red-tide forming and toxic flagellate *Heterosigma akashiwo* (Raphidophyceae). These species are described in A) Hevia-Orube et al. (2015); B) Orive et al. (2010, 2013); C) Laza-Martínez (2012) and D) Laza-Martínez et al. (2007).

distributed in the outer and middle estuary, was observed to bloom in the Arriluze marina under specific environmental conditions involving high irradiance levels during calm waters (Laza-Martinez et al., 2007). The blooming of this species is thus predictable to a certain degree. This and the other ichtyotoxic flagellates are known to be mixotrophs, which explains to a great extent their success in eutrophized areas.

Although the estuary of Bilbao is not a shellfish harvesting area and has not aquaculture facilities, the identified microalgae are cosmopolitan and consequently could be shared by closest estuaries such as those of Plentzia, Urdaibai or Txingudi, which are shellfish harvesting areas. Besides, in the Basque Country there are projects for coastal aquaculture and the knowledge on the presence of potential harmful microalgae is of paramount importance. Species noxious for human health include the diatoms of the genus *Pseudonitzschia* (Orive et al., 2010, 2013), one of whose new species, *P. plurisecta*, has proven to be highly toxic (Fernandes et al., 2014). The toxigenic dinoflagellate genus *Dinophysis* can be noxious even at relatively low concentrations. It was observed to bloom on the coast (Seoane and Orive, 2012) and it was very frequent in the estuary in the same way as its feeding-related species of the genus *Teleaulax* (Laza-Martinez et al., 2012).

5. The dissolved oxygen criteria in estuaries and coastal areas

In aquatic environments dissolved oxygen (DO) is a key factor with effects from the individual to the ecosystem level. Oxygen depletion (hypoxia/anoxia) has a well documented negative effect on individual survival, population growth, biodiversity, energy and matter pathways, and ecosystem function and services (Rabalais et al., 2002), while hyperoxic conditions may also have detrimental effects on the organisms by promoting oxidative stress (e.g. Finelli et al., 2006; Vosloo et al., 2013). In addition, DO can provide an indication of the trophic status of the system. The alteration of the natural levels of DO in coastal waters and estuaries has paralleled the increasing settlement of human populations in coastal areas, and the resulting anthropogenic enrichment of coastal waters and estuaries with nutrients and organic compounds (Rabalais et al., 2010). The latter are the main cause of the unbalanced DO dynamics that result in hypoxic/anoxic conditions due to the depletion of oxygen in the microbial decomposition of excessive amounts of organic matter, frequently combined or alternated with hyperoxic conditions by the enhancement of photosynthetic production in nutrient-rich waters. For these reasons the dissolved oxygen criteria is now used for the assessment of the health of estuarine habitats, and the suitability of such habitats for different species inhabiting estuaries (Hanmer et al., 2003).

In the plankton-ecosystem monitoring initiated in the estuary of Bilbao in 1998, DO profiles were obtained monthly along the estuary at the sites of 35, 34, 33 and 30 salinity waters. The evolution of DO at the different depths from 1998 to 2008 (Fig. 5) reflects the improvement of environmental conditions in the estuary of Bilbao associated to the integrated sewage treatment scheme developed by the Bilbao-Bizkaia Water Consortium, but also shows axial and depth-dependent differences in the DO levels and dynamics, in relation to the constraints derived from the basin morphology and estuarine circulation. The most noticeable improvement has been observed in surface waters, which showed hypoxic events (<30% oxygen saturation) along the entire channelized estuary in the warm/dry season until 1999. Since 2000 DO levels showed a clear

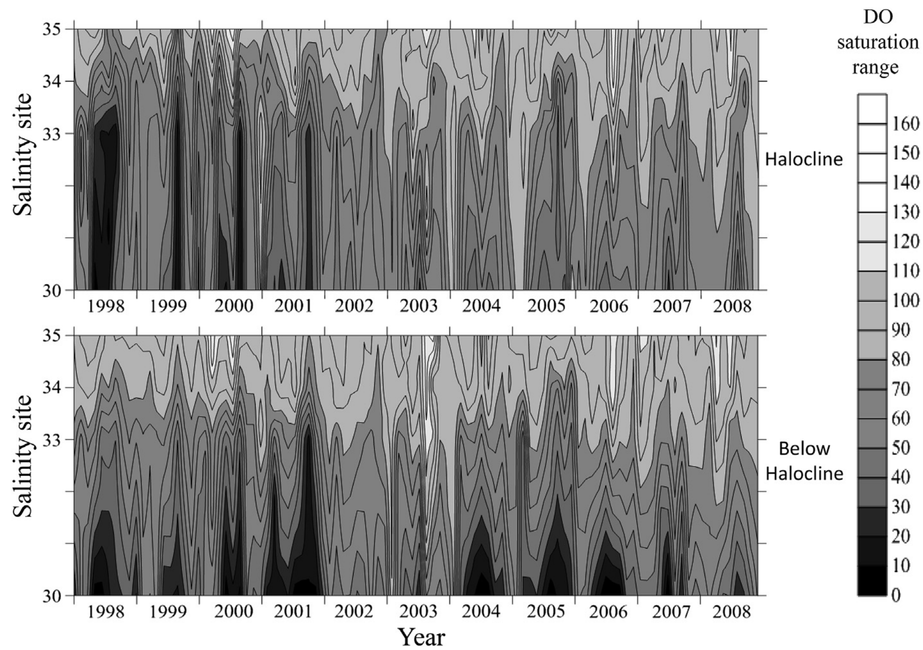

Fig. 5. Axial and temporal variations of dissolved oxygen saturation (%) in halocline and below halocline waters from the site of 35 salinity to the site of 30 salinity in the estuary of Bilbao during the 1998–2008 period. Data are adapted from Villate et al. (2013).

increasing trend. The gradual improvement from 1998 to 2008 was also observed in the vertical gradient or halocline layers of the middle estuary, and was mainly accounted for by the abatement of sewage pollution during this period (Villate et al., 2013). However, the increase of DO in waters below the halocline has been more moderate and fluctuating than in the layer above. In this zone, hydro-climatic factors driving changes in river discharge and water column stratification rather than sewage pollution abatement were found to be the main responsible for the inter-annual DO dynamics (Villate et al., 2013). In the outer estuary, generally with normoxic conditions in the entire water column and a weak increasing trend of DO throughout the study period, the main factor explaining DO variations, both at seasonal and inter-annual scales, was chlorophyll *a* concentration (Iriarte et al., 2010; Villate et al., 2013), this denoting the major contribution of photosynthetic oxygen production to DO dynamics, and the role of eutrophication enhanced phytoplankton growth as responsible for very high-oversaturation DO events. Such events were also observed in the channelized zone, where they may be of greater intensity than in the outer zone but restricted to the thin layer above and within the halocline, whereas in the outer estuary it usually extends to a depth of several meters. The finding that the major factors controlling DO are river discharge and stratification in the inner estuary and phytoplankton biomass in the outer estuary, which are, in turn, related to rainfall and temperature respectively, suggests that in future not only local anthropogenic perturbations, but also climate change may be a major driver of long-term DO dynamics in the estuary of Bilbao.

6. Zooplankton series: a tool to assess environmental changes and ecosystem health

A good understanding of changes occurring in ecosystems, and the causes of such changes, which can be useful for forecasting purposes, can only be achieved by analyzing long-term data sets that integrate the different time-scales of variability. This leads us to acknowledge the need to carry out long-term environmental and plankton monitoring programs in marine systems to obtain more robust evidences of the effects of phenomena such as climate change (Richardson, 2008; Richardson et al., 2009), but also of the effects of local human pressure in coastal and estuarine systems and of their synergic effects. Among the advantages of zooplankton as bioindicator of environmental changes in marine systems are: the rapid turnover of the community, which makes zooplankton a quick response indicator to water quality perturbation (Gibson et al., 2000), the high sensitivity of zooplanktonic organisms to temperature, which strongly affects physiological processes and population growth (Mauchline, 1998), the tight coupling of zooplankton population dynamics and climate (Hays et al., 2005), and the non-linear response of communities to environmental factors that can amplify subtle environmental signals (Taylor et al., 2002).

Early analyses of the first 6 years of the zooplankton series in the estuary of Bilbao revealed an evolution of the zooplankton community attributable to the evolution of water quality at the different salinity sites within the system. The comparison of zooplankton differences between the estuary of Bilbao and the estuary of Urdaibai (used as control) from the 1997–1999 period to the 1999–2001 period (Albaina et al., 2009) showed that the zooplankton of the estuary of Bilbao reflected the environmental recovery of this estuary, mainly in response to the improvement in DO levels. Main evidences were: the reduction in abundance differences of most holoplanktonic taxa between estuaries, the finding of the largest reduction of differences in mid salinity waters, where the greatest improvement of DO conditions occurred, and the fact that the reduction of differences was mainly accounted for by the increase of the relative abundance of pollution-sensitive taxa in the estuary of Bilbao, while they remained stable in the estuary of Urdaibai.

The analysis of the *Acartia* congeneric assemblage in the estuary of Bilbao during the period 1998–2005 (Aravena et al., 2009a) revealed that two scarce species, such as *Acartia margalefi* and *Acartia discaudata* were valuable indicators of year-to-year unusual hydroclimatic changes in this estuary. Both species were found to be only quantitatively relevant in 2002, when they increased in abundance and expanded their seasonal distribution. The year 2002 was climatically peculiar in comparison with previous and

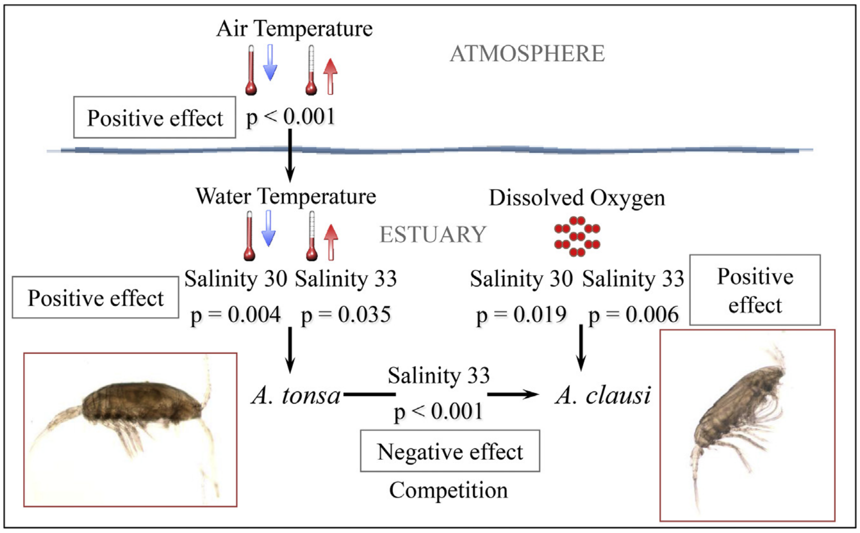

Fig. 6. Diagram showing the links between significant ($p < 0.05$) causal relationships among air temperature, water temperature, dissolved oxygen saturation, abundance of *Acartia tonsa* and abundance of *Acartia clausi* in the estuary of Bilbao during the 1998–2005 period, as inferred by using Transfer Function models. Figure adapted from Aravena et al. (2009a,b).

later years, because it was the period of the time-series with the lowest autumn–winter rainfall and the coldest subsequent summer. In the case of *A. margalefi* it also increased its spatial distribution landwards, this coinciding with the highest levels of DO in the inner estuary.

Results of the zooplankton monitoring in the estuary of Bilbao also showed the irruption of the invasive copepod *Acartia tonsa* in this estuary, where this species was found for the first time in 2001 and became dominant in waters of less than 34 salinity since 2003, ousting *Acartia clausi,* the dominant congeneric species, from waters of around 30 salinity of the inner estuary (Aravena et al., 2009a). *A. tonsa,* although native of the American and Indo-Pacific area, has been found in many European estuaries since the middle 20th century (Bakker and Pauw, 1975) and it was reported for the first time in an estuary of the Bay of Biscay (the Gironde estuary) in 1983 (David et al., 2007). Ship ballast water exchange has been suggested as the most plausible introduction vector of brackish water species such as *A. tonsa* (Paavola et al., 2005). The late appearance (2001) of this species in the estuary of Bilbao, in spite of the large history and intensity of commercial shipping in this estuary, may be due to the unsuitable environmental conditions of previous years. The transfer function (TF) models used by Aravena et al., (2009a) to infer causal relationships between *Acartia* congenerics revealed a negative effect of *A. tonsa* on *A. clausi* in the intermediate salinity (33) waters, where these species showed the highest spatial overlap. In addition, the results of these TF models indicated that the decrease of *A. clausi* abundance in waters of less than 34 salinity was related to the decrease of DO levels, and the increase of *A. tonsa* to the increase of temperature (Fig. 6). This suggests that the balance of the competition between both species would be tipped in favour of *A. tonsa* in a future scenario of general warming. In fact, a general warming is expected to cause also a reduction in DO levels in the inner estuary of Bilbao, according to the negative relationship between temperature and DO observed in this inner zone, likely due to the enhancement of oxygen consumption processes with increasing temperature (Iriarte et al., 2010).

7. Conclusions

As reviewed in previous sections, data on contaminant bioaccumulation and biomarkers in mussels, on phytoplankton and zooplankton communities, and on physicochemical variables have been recorded since 90's in the estuary of Bilbao. Integrating different biological complexity levels could provide new insights for the assessment of the long-term trends in the health status of the estuary of Bilbao. Taking together, results reviewed herein indicate that, in spite of the seasonal and interannual variability recorded for some parameters, a general recovery trend was observed for the health status in the estuary of Bilbao, with sporadic critical events such as, for instance, regular dredging activities and the Prestige oil spill that affected the region between 2002 and 2005, at least. The comparison of responses at different biological complexity levels allows a comprehensive and integrated description of the Bilbao estuary recovery process and the understanding of seasonal dependent fluctuations on selected variables, that could serve as baseline for future monitoring studies.

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References

Adams, S.M., 2005. Using multiple response bioindicators to assess the health of estuarine ecosystems: an operational framework. In: Bortone, S.A. (Ed.), Estuarine Indicators. CRC Marine Science Series. CRC Press, Boca Raton, Florida, pp. 5–18.

Albaina, A., Villate, F., Uriarte, I., 2009. Zooplankton communities in two contrasting Basque estuaries (1999-2001): reporting changes associated with ecosystem

health. J. Plankton Res. 31, 739–752.
Aravena, G., Villate, F., Uriarte, I., Iriarte, A., Ibáñez, B., 2009a. Response of *Acartia* populations to environmental variability and effects of invasive congenerics in the estuary of Bilbao, Bay of Biscay. Estuar. Coast. Shelf Sci. 83, 621–628.
Aravena, G., Villate, F., Iriarte, A., Uriarte, I., Ibáñez, B., 2009b. Influence of the North Atlantic Oscillation (NAO) on climatic factors and estuarine water temperature on the Basque coast (Bay of Biscay): comparative analysis of three seasonal NAO indices. Cont. Shelf Res. 29, 750–758.
Bakker, C., de Pauw, N., 1975. Comparison of plankton assemblages of identical salinity ranges in estuarine tidal, and stagnant environments. II. Zooplankton. Neth. J. Sea Res. 9, 145–165.
Bartolomé, L., Deusto, M., Etxebarria, N., Navarro, P., Usobiaga, A., Zuloaga, O., 2007. Chemical fingerprinting of petroleum biomarkers in biota samples using retention-time locking chromatography and multivariate analysis. J. Chromatogr. A 1157, 369–375.
Bartolomé, L., Etxebarria, N., Martinez-Arkarazo, I., Raposo, J.C., Usobiaga, A., Zuloaga, O., Ringeard, D., Ortiz-Zarragoitia, M., Cajaraville, M.P., 2010. Distribution of organic microcontaminants, butyltins, and metals in mussels from the Estuary of Bilbao. Arch. Environ. Contam. Toxicol. 59, 244–254.
Belzunce, M.J., Solaun, O., Franco, J., Valencia, V., Borja, A., 2001. Accumulation of organic matter, heavy metals and organic compounds in the surface sediments along the Nervión estuary (Northern Spain). Mar. Pollut. Bull. 42, 1407–1411.
Belzunce, M.J., Solaun, O., Valencia, V., Pérez, V., 2004. Contaminants in estuarine and coastal waters. In: Borja, A., Collins, M. (Eds.), Oceanography and Marine Environment of the Basque Country. Elsevier Oceanography Series 70. Elsevier, Amsterdam, pp. 233–251.
Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajaraville, M.P., Ortiz-Zarragoitia, M., 2014. Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay). Mar. Environ. Res. 96, 19–28.
Borja, A., Muxika, I., Franco, J., 2006. Long-term recovery of soft-bottom benthos following urban and industrial sewage treatment in the Nervión estuary (southern Bay of Biscay). Mar. Ecol. Prog. Ser. 313, 43–55.
Borja, A., Dauer, D.M., Elliott, M., 2010. Medium- and long-term recovery of estuarine and coastal ecosystems: patterns, rates and restoration effectiveness. Estuaries Coasts 33, 1249–1260.
Brown, C.J., Schoeman, D.S., Sydeman, W.J., Brander, K., Buckley, L.B., Burrows, M., Duarte, C.M., Moore, P.J., Pandolfi, J.M., Poloczanska, E., Venables, W., Richardson, A.J., 2011. Quantitative approaches in climate change ecology. Glob. Change Biol. 17, 3697–3713.
Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295–311.
Cajaraville, M.P., Cancio, I., Ibabe, A., Orbea, A., 2003. Peroxisome proliferation as a biomarker in environmental pollution assessment. Microsc. Res. Tech. 61, 191–202.
Cajaraville, M.P., Garmendia, L., Orbea, A., Werding, R., Gómez-Mendikute, A., Izagirre, U., Soto, M., Marigómez, I., 2006. Signs of recovery of mussels health two years after the Prestige oil spill. Mar. Environ. Res. 62, 337–341.
Cajaraville, M.P., Olabarrieta, I., Marigómez, I., 1996. *In vitro* activities in mussel hemocytes as biomarkers of environmental quality: a case study in the Abra estuary (Biscay bay). Ecotox. Env. Saf. 35, 253–260.
Cearreta, A., Irabien, M.J., Pascual, A., 2004. Human activities along the Basque coast during the last two centuries: geological perspective of recent anthropogenic impact on the coast and its environmental consequences. In: Borja, A., Collins, M. (Eds.), Oceanography and Marine Environment of the Basque Country: Elsevier Oceanography Series, 70, pp. 27–50.
Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., Pinsino, A., Libralato, G., Marcomini, A., Sabbioni, E., Matranga, V., 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8 (10), 9694–9709.
David, V., Sautour, B., Chardy, P., 2007. Successful colonization of the calanoid copepod *Acartia tonsa* in the oligo-mesohaline area of the Gironde estuary (SW France) – natural or anthropogenic forcing. Estuar. Coast. Shelf Sci. 71, 429–442.
De Jonge, V.N., Elliott, M., Orive, E., 2002. Causes, historical development, effects and future challenges of a common environmental problem: eutrophication. Hydrobiologia 475/476, 1–19.
De los Ríos, A., Juanes, J.A., Ortiz-Zarragoitia, M., López de Alda, M., Barceló, D., Cajaraville, M.P., 2012. Assessment of the effects of a marine urban outfall discharge on caged mussels using chemical and biomarker analysis. Mar. Pollut. Bull. 64, 563–573.
De los Ríos, A., Pérez, L., Ortiz-Zarragoitia, M., Serrano, T., Barbero, M.C., Echavarri-Erasun, B., Juanes, J.A., Orbea, A., Cajaraville, M.P., 2013. Assessing the effects of treated and untreated urban discharges to estuarine and coastal waters applying selected biomarkers on caged mussels. Mar. Pollut. Bull. 77, 251–265.
De Salas, M.F., Laza-Martínez, A., Hallegraeff, G.M., 2008. Novel unarmored dinoflagellates from the toxigenic family Kareniaceae (Gymnodiniales): five new species of *Karlodinium* and one *Takayama* from the australian sector of the Southern Ocean. J. Phycol. 44, 241–257.
Diez, I., Muguerza, N., Santolaria, A., Ganzedo, U., Gorostiaga, J.M., 2012. Seaweed assemblage changes in the eastern Cantabrian Sea and their potential relationship to climate change. Estuar. Coast. Shelf Sci. 99, 108–120.
Diez, I., Secilla, A., Santolaria, A., Gorostiaga, J.M., 2009. Ecological monitoring of intertidal phytobenthic communities of the Basque Coast (N. Spain) following the Prestige oil spill. Environ. Monit. Assess. 159, 555–575.
Fernandes, L.F., Hubbard, K.A., Richlen, M.L., Smith, J., Bates, S.S., Ehrman, J., Leger, C., Mafra, L.L., Kulis, D., Quilliam, M., 2014. Diversity and toxicity of *Pseudo-nitzschia Peragallo* in the Gulf of Maine, Northwestern Atlantic ocean. Deep Sea Res. Part II- Trop. Stud. Oceanogr. 103, 139–162.
Finelli, Ch M., Helmuth, B.S.T., Pentcheff, N.D., Wethey, D.S., 2006. Water flow influences oxygen transport and photosynthetic efficiency in corals. Coral Reefs 25, 47–57.
García-Barcina, J.M., González-Oreja, J.A., De la Sota, A., 2006. Assessing the improvement of the Bilbao estuary water quality in response to pollution abatement measures. Water Res. 40, 951–960.
Garmendia, L., Izagirre, U., Cajaraville, M.P., Marigómez, I., 2011a. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and the Bay of Biscay: lysosomal responses. J. Environ. Monit. 13, 901–914.
Garmendia, L., Soto, M., Vicario, U., Kim, Y., Cajaraville, M.P., Marigómez, I., 2011b. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Bay of Biscay: tissue-level biomarkers and histopathology. J. Environ. Monit. 13, 915–932.
Garmendia, L., Soto, M., Ortiz-Zarragoitia, M., Orbea, A., Cajaraville, M.P., Marigómez, I., 2011c. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Biscay Bay: correlation and multivariate analysis. J. Environ. Monit. 13, 933–942.
Gibson, G.R., Bowman, M.L., Gerritsen, J., Snyder, B.D., 2000. Estuarine and Coastal Marine Waters: Bioassessment and Biocriteria Technical Guidance. EPA 822-B-00–024. U.S. Environmental Protection Agency, Office of Water, Washington, DC.
Goldberg, E.D., 1975. The mussel watch. Mar. Pollut. Bull. 6, 111.
González-Oreja, J.A., Sáiz-Salinas, J.I., 1998. Exploring the relationship between abiotic variables and benthic community structure in a polluted estuary. Water Res. 32, 3799–3807.
Hallegraeff, G.M., 2003. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, Paris, pp. 25–50.
Hanmer, R.W., Capacasa, J.M., Grubbs, G.H., 2003. Ambient Water Quality Criteria for Dissolved Oxygen, Water Clarity and Chlorophyll a for the Chesapeake Bay and its Tidal Tributaries. EPA 903-R-03–002. Chesapeake Bay Program, Annapolis, Maryland.
Hasle, G.R., 1978. The inverted-microscope method. In: Sournia, A. (Ed.), Phytoplankton Manual. UNESCO, Paris, pp. 88–96.
Hays, G.C., Richardson, A.J., Robinson, C., 2005. Climate change and plankton. Trends Ecol. Evol. 20, 337–344.
Hevia-Orube, J., Orive, E., David, H., Diez, A., Laza-Martínez, A., Miguel, I., Seoane, S., 2015. Molecular and morphological analyses of solitary forms of brackish Thalassiosiroid diatoms (Coscinodiscophyceae) with emphasis on their phenotypic plasticity. Eur. J. Phycol. (in press).
Intxausti, L., Villate, F., Uriarte, I., Iriarte, A., Ameztoy, I., 2012. Size-related response of zooplankton to hydroclimatic variability and water-quality in an organically polluted estuary of the Basque coast (Bay of Biscay). J. Mar. Syst. 94, 87–96.
Iriarte, A., Aravena, G., Villate, F., Uriarte, I., Ibañez, B., Llope, M., Stenseth, N., 2010. Dissolved oxygen in contrasting estuaries of the Bay of Biscay: effects of temperature, river discharge and chlorophyll *a*. Mar. Ecol. Prog. Ser. 418, 57–71.
Kim, Y., Ashton-Alcox, K.A., Powell, E.N., 2006. Gonadal analysis. In: Histological Techniques for Marine Bivalve Molluscs. Silver Spring, pp. 1–10. NOAA Technical Memorandum NOS NCCOS 27.
Laza-Martínez, A., 2012. *Urgorri complanatus* gen. et sp. nov. (Cryptophyceae), a red tide-forming species in brackish waters. J. Phycol. 48, 423–435.
Laza-Martínez, A., Seoane, S., Zapata, M., Orive, E., 2007. Phytoplankton pigment patterns in a temperate estuary: from unialgal cultures to natural assemblages. J. Plankton Res. 29, 913–929.
Laza-Martínez, A., Arluzea, J., Miguel, I., Orive, E., 2012. Morphological and molecular characterization of *Teleaulax gracilis* sp. nov. and *T. minuta* sp. nov. (Cryptophyceae). Phycologia 51, 649–661.
Lekube, X., Izagirre, U., Soto, M., Marigómez, I., 2014. Lysosomal and tissue-level biomarkers in mussels cross-transplanted among four estuaries with different pollution levels. Sci. Total Environ. 472, 36–48.
Marigómez, I., Orbea, A., Olabarrieta, I., Etxeberria, M., Cajaraville, M.P., 1996. Structural changes in the digestive lysosomal system of sentinel mussels as biomarkers of environmental stress in mussel-watch programmes. Comp. Biochem. Physiol. 113C, 291–297.
Marigómez, I., Soto, M., Cancio, I., Orbea, A., Garmendia, L., Cajaraville, M.P., 2006. Cell and tissue biomarkers in mussel, and histopathology in hake and anchovy from Bay of Biscay after the Prestige oil spill (Monitoring campaign 2003). Mar. Pollut. Bull. 53, 287–304.
Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., Cajaraville, M.P., 2013. Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. Aquat. Toxicol. 136–137, 32–48.
Mauchline, J., 1998. The biology of calanoid copepods. Adv. Mar. Biol. 33, 710.
McCarthy, J.F., Shugart, L.R., 1990. Biological markers of environmental contamination. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, FL, pp. 3–14.

Orbea, A., Cajaraville, M.P., 2006. Peroxisome proliferation and antioxidant enzymes in transplanted mussels of four Basque estuaries with different levels of polycyclic aromatic hydrocarbon and polychlorinated biphenyl pollution. Environ. Toxicol. Chem. 25, 1616–1626.
Orbea, A., Garmendia, L., Marigómez, I., Cajaraville, M.P., 2006. Effects of the Prestige oil spill on cellular biomarkers in intertidal mussels-results of first year of studies. Mar. Ecol. Prog. Ser. 306, 177–189.
Orbea, A., Marigómez, I., Fernández, C., Tarazona, J.V., Cancio, I., Cajaraville, M.P., 1999. Structure of peroxisomes and activity of the marker enzyme catalase in digestive epithelial cells in relation to PAH content of mussels from two Basque estuaries (Bay of Biscay): seasonal and site-specific variations. Arch. Environ. Contam. Toxicol. 36, 158–166.
Orive, E., Franco, J., Madariaga, I., Revilla, M., 2004. Bacterioplankton and phytoplankton communities. In: Borja, A., Collins, M. (Eds.), Oceanography and Marine Environment of the Basque Country. Elsevier, pp. 367–393.
Orive, E., Laza-Martinez, A., Seoane, S., Alonso, A., Andrade, R., Miguel, I., 2010. Diversity of *Pseudo-nitzschia* in the southeastern Bay of Biscay. Diatom Res. 25, 125–145.
Orive, E., Pérez- Aicua, L., David, H., García-Etxebarria, K., Laza-Martinez, A., Seoane, S., Miguel, I., 2013. The genus *Pseudo-nitzschia* (Bacillariophyceae) in a temperate estuary with description of two new species: *Pseudo-nitzschia plurisecta* sp. nov. and *Pseudo-nitzschia abrensis* sp. nov. J. Phycol. 49, 1192–1206.
Ortiz-Zarragoitia, M., Garmendia, L., Barbero, M.C., Serrano, T., Marigómez, I., Cajaraville, M.P., 2011. Effects of the fuel oil spilled by the *Prestige* tanker on reproduction parameters of wild mussel populations. J. Environ. Monit. 13, 84–94.
Paavola, M., Olenin, S., Leppäkoski, E., 2005. Are invasive species most successful in habitats of low native species richness across European brackish water seas. Estuar. Coast. Shelf Sci. 64, 738–750.
Paerl, H., 1996. A comparison of cyanobacterial bloom dynamics in freshwater, estuarine and marine environments. Phycologia 35, 25–35.
Porte, C., Janer, G., Lorusso, L.C., Cajaraville, M.P., Ortiz-Zarragoitia, M., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. Comp. Biochem. Physiol. 143C, 303–315.
Prieto, A., Zuloaga, O., Usobiaga, A., Bartolomé, L., Etxebarria, N., Cipriain, E., Alonso, A., 2008. Levels and spatial distribution of inorganic and organic contaminants in sediments along the Bilbao estuary. Mar. Pollut. Bull. 56, 2094–2099.
Puy-Azurmendi, E., Ortiz-Zarragoitia, M., Kuster, M., Martínez, E., Guillamón, M., Domínguez, C., Serrano, T., Barbero, M.C., López de Alda, M., Bayona, J.M., Barceló, D., Cajaraville, M.P., 2010. An integrated study of endocrine disruptors in sediments and reproduction-related parameters in bivalve molluscs from the Biosphere's Reserve of Urdaibai (Bay of Biscay). Mar. Environ. Res. 69, 63–66.
Puy-Azurmendi, E., Ortiz-Zarragoitia, M., Vilagrasa, M., Kuster, M., Aragón, P., Atienza, J., Puchades, R., Maquieira, A., Domínguez, C., López de Alda, M., Fernandes, D., Porte, C., Bayona, J.M., Barceló, D., Cajaraville, M.P., 2013. Endocrine disruption in thicklip grey mullet (*Chelon labrosus*) from the Urdaibai Biosphere Reserve (Bay of Biscay, Southwestern Europe). Sci. Total Environ. 443, 233–244.
Rabalais, N.N., Turner, R.E., Wiseman Jr., W.J., 2002. Gulf of Mexico hypoxia, A.K.A. "The dead zone". Annu. Rev. Ecol. Syst. 33, 235–263.
Rabalais, N.N., Díaz, R.J., Levin, L.A., Turner, R.E., Gilbert, D., Zhang, J., 2010. Dynamics and distribution of natural and human-caused hypoxia. Biogeosciences 7, 585–619.
Richardson, A.J., 2008. In hot water: zooplankton and climate change. ICES J. Mar. Sci. 65, 279–295.
Richardson, A.J., McKinnon, D., Swadling, K.M., 2009. Zooplankton. In: Poloczanska, E.S., Hobday, A.J., Richardson, A.J. (Eds.), A Marine Climate Change Impacts and Adaptation Report Card for Australia 2009. NCCARF Publication, ISBN 978-1-921609-03-9, 05/09.
Rodriguez, J.G., Tueros, I., Borja, A., Franco, J., Garcia-Alonso, J.I., Garmendia, J.M., Muxika, I., Sariego, C., Valencia, V., 2009. Butyltin compounds, sterility and imposex assessment in *Nassarius reticulatus* (Linnaeus, 1758), prior to the 2008 European ban on TBT antifouling paints, within Basque ports and along coastal areas. Cont. Shelf Res. 29, 1165–1173.
Salazar, M.H., Salazar, M.S., 1997. Using caged bivalves to characterize exposure and effects associated with pulp and paper mill effluents. Water Sci. Technol. 35, 213–220.
Seoane, S., Laza, A., Urrutxurtu, I., Orive, E., 2005. Phytoplankton assemblages and their dominant pigments in the Nervion River estuary. Hydrobiologia 549, 1–13.
Seoane, S., Laza, A., Orive, E., 2006. Monitoring phytoplankton assemblages in estuarine waters: the application of pigment analysis and microscopy to size-fractionated samples. Estuar. Coast. Shelf Sci. 67, 343–354.
Seoane, S., Eikrem, W., Arlucea, J., Orive, E., 2009. Haptophytes of the Nervión river estuary (Northern Spain). Bot. Mar. 52, 47–59.
Seoane, S., Orive, E., 2012. First report of *Dinophysis acuminata* bloom in Basque waters, Northern Spain. Harmful Algae News 46, 9.
Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201.
Soto, M., Ireland, M.P., Marigómez, I., 2000. Changes in mussel biometry on exposure to metals: implications in estimation of metal bioavailability in 'Mussel-Watch' programmes. Sci. Total Environ. 247, 175–187.
Soto, M., Kortabitarte, M., Marigómez, I., 1995. Bioavailable heavy metals in estuarine waters as assessed by metal/shell-weight indices in sentinel mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 125, 127–136.
Taylor, A.H., Allen, J.I., Clark, P.A., 2002. Extraction of a weak climatic signal by an ecosystem. Nature 416, 629–632.
Uriarte, I., Villate, F., 2004. Effects of pollution on zooplankton abundance and distribution in two estuaries of the Basque coast (Bay of Biscay). Mar. Pollut. Bull. 49, 220–228.
Uriarte, I., Villate, F., 2005. Differences in the abundance and distribution of copepods in two estuaries of the Basque coast (Bay of Biscay) in relation to pollution. J. Plankton Res. 27, 863–874.
Uriarte, I., Villate, F., Iriarte, A., Duque, J., Ameztoy, I., 2014. Seasonal and axial variations of net water circulation and turnover in the estuary of Bilbao. Estuarine. Coast. Shelf Sci. 150, 312–324.
Verity, P.G., 2010. Expansion of potentially harmful taxa in a Georgia Estuary (USA). Harmful Algae 144–152.
Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. 146C, 281–300.
Villate, F., Iriarte, A., Uriarte, I., Intxausti, L., de la Sota, A., 2013. Dissolved oxygen in the rehabilitation phase of an estuary: influence of sewage pollution abatement and hydro-climatic factors. Mar. Pollut. Bull. 70, 234–246.
Vosloo, D., van Rensburg, L., Vosloo, A., 2013. Oxidative stress in abalone: the role of temperature, oxygen and L-proline supplementation. Aquaculture 416–417, 265–271.
WHO/UNEP, 2013. In: Bergman, A., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T. (Eds.), State-of-the-Science of Endocrine Disrupting Chemicals 2012. WHO Press, Geneva, Switzerland.
Wilson, J.G., 1988. The Biology of Estuarine Management. Croom Helm, London.
Zorita, I., Ortiz-Zarragoitia, M., Soto, M., Cajaraville, M.P., 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): an integrated biochemical, histochemical and histological study. Aquat. Toxicol. 78, 109–116.
Zorita, I., Solaun, O., Borja, A., Franco, J., Muxika, I., Pascual, M., 2013. Spatial distribution and temporal trends of soft-bottom marine benthic alien species collected during the period 1989–2008 in the Nervión estuary (southeastern Bay of Biscay). J. Sea Res. 83, 104–110.