Impact of non-indigenous planktonic copepod species on the estuaries of the Basque coast

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PhD Thesis

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LIST OF PUBLICATIONS

Part of the present PhD has been presented in five international conferences or meetings and also in international journals:

Communications

- Z. Barroeta, A. Iriarte, I. Uriarte, F. Villate. Updates on the distribution and impact of *Pseudodiaptomus marinus* in Basque estuaries (Bay of Biscay). EUROBUS, Peniche, Portugal, 2019.
- Z. Barroeta, I. Uriarte, F. Villate, A. Iriarte. Impact of non-indigenous copepod species in the copepod community of two contrasting estuaries of the Bay of Biscay. EMBS 54, Dublin, Ireland. 2019.
- Z. Barroeta, I. Uriarte, F. Villate, A. Iriarte. Euskal kostako estuarioetan bertakoa ez den zooplanktonaren eraginaren analisia. Ikergazte 2019, Baiona, France.
- Z. Barroeta, T. García, F. Villate, I. Uriarte, A. Iriarte. Monitoring the non-indigenous *Pseudodiaptomus marinus* in two estuaries of the Bay of Biscay. Aquainvad-ED, Dublin, Ireland. 2018.
- A. Iriarte, I. Uriarte, Z. Barroeta, F. Villate. The occurrence of *Pseudodiaptomus marinus* in the estuaries of the Basque coast (Bay of Biscay). EUROBUS, Naples, Italia, 2018.

Articles

- Barroeta Z, Villate F, Uriarte I, Iriarte A (2020) Differences in the colonization success and impact of non-indigenous and other expanding copepod species on the zooplankton of two contrasting estuaries of the Bay of Biscay. Biol Invasions 22:3239–3267. doi: 10.1007/s10530-020-02320-7
- Barroeta Z, Villate F, Uriarte I, Iriarte A (2021) The importance of previous conditions in zooplankton community structure and diversity changes caused by the occurrence of non-indigenous copepod species: a case study in two contrasting estuaries of the Bay of Biscay. Estuaries and Coasts (Under revision).

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RESUMEN

Las invasiones biológicas son uno de los principales agentes de pérdida de biodiversidad y de presión sobre los ecosistemas, teniendo un gran impacto tanto ecológico como económico (MEA (Millennium Ecosystem Assessment) 2005). Las especies no nativas (NIS, por sus siglas en inglés "non-indigenous species") pueden llegar a ser invasoras y desplazar a las nativas, causando pérdida de genotipos nativos, modificación de hábitats, cambios en la estructura de las comunidades, modificación de las propiedades de las redes tróficas y alteración de los procesos de los ecosistemas, lo que deterioraría la provisión de servicios del ecosistema con sus consecuentes efectos negativos sobre la salud humana y la economía (Vila et al. 2010). En el caso de ecosistemas marinos, y en un mundo globalizado como el actual, el rápido incremento del comercio, los viajes y el transporte en las últimas décadas ha acelerado las invasiones biológicas marinas. Esto ha sido más evidente en estuarios por ser más vulnerables (Frisch et al. 2006), gracias al transporte marítimo (aguas de sentina), la construcción de canales de navegación, la acuacultura y la acuariofilia (Katsanevakis et al. 2014).

El Convenio sobre la Diversidad Biológica (CBD 2000) incide en la necesidad de incrementar los estudios sobre el impacto de las NIS en la diversidad biológica, los hábitats y los ecosistemas, y de recopilar y divulgar toda la información disponible para su implementación en acciones de prevención y mitigación. En el caso de la Unión Europea, la Directiva marco sobre la estrategia marina (MSFD, por sus siglas en inglés) cataloga a las NIS marinas como la mayor amenaza para la biodiversidad europea y la salud de los ecosistemas. Entre las NIS señaladas se encuentra el copépodo calanoide *Acartia tonsa* (Katsanevakis et al. 2014), que ha colonizado nuevas áreas costeras y estuáricas (Frisch et al. 2006; Brugnano et al. 2011) por introducción antrópica y/o propia propagación, debido a su enorme capacidad para superar barreras geográficas y, sobre todo, a sus estadios de resistencia (Belmonte y Potenza 2001).

En los estuarios de la costa vasca, se identificó por primera vez en 2001 en el estuario de Bilbao, llegando a ser con el tiempo el calanoide dominante en la zona interna del estuario (Aravena et al. 2009). Su presencia también se detectó en el estuario de Urdaibai. Pero no es el único copépodo planctónico NIS observado en los estuarios de la costa vasca, ya que se ha registrado la consecutiva llegada de otros dos copépodos NIS, *Oithona davisae* y *Pseudodiaptomus marinus*, confirmando la paulatina expansión de estas especies a diferentes sistemas (Jha et al. 2013; Gubanova et al. 2014; Cornils y Wend-Heckmann 2015; Sabia et al. 2015). Además, en el año 2010 se detectó la presencia de otras especies en expansión (denominadas en esta tesis OES por sus siglas en inglés, "other expanding copepods"), *Calanipeda aquaedulcis* y *Acartia bifilosa*, en el estuario de Bilbao.

A pesar de su importancia, y aunque sí existe una amplia bibliografía sobre la biología y ecofisiología de *A. tonsa* (p. ej.: Cervetto et al. 1999; Gaudy et al. 2000; Svetlichny y Hubareva 2014), los estudios sobre las otras dos especies son mucho más escasos (Svetlichny et al. 2019, 2021), al igual que los centrados en las causas y efectos de su introducción en los ecosistemas (Katsanevakis et al. 2014; Sabia et al. 2015). En este contexto, se desconocía el posible efecto de las NIS y las OES sobre la biodiversidad, la abundancia y distribución del zooplancton nativo y la adecuación de sus nichos ecológicos en los estuarios de Bilbao y de Urdaibai, así como su posible expansión a otros estuarios de la costa vasca. Por ello, la propuesta de esta tesis doctoral fue estudiar dichos aspectos con el fin de evaluar la influencia de los copépodos planctónicos no nativos en los estuarios vascos y contribuir a un mayor conocimiento general del impacto de las especies invasoras en la biodiversidad y servicios de los ecosistemas.

Para realizar el presente estudio se utilizaron principalmente datos procedentes de las series temporales de zooplancton y factores ambientales básicos, correspondientes al programa de monitorización que desde 1997 se viene realizando a lo largo del gradiente salino de los estuarios de Bilbao (regiones salinas 35, 34, 33, 30 y 10) y de Urdaibai (regiones salinas de 35, 33, 30 y 26). Concretamente, se utilizaron los datos correspondientes al periodo 1998-2015. Para analizar el efecto temporal de la colonización de estas NIS, el periodo de estudio se dividió en tres: periodo 1 (antes de la colonización de *A. tonsa* y *O. davisae*) desde el año 1998 al 2002, periodo 2 (después de la colonización de *A. tonsa* y *O. davisae* pero antes de la colonización de *P. marinus* en el estuario de Bilbao) desde el año 2003 al 2009 y el periodo 3 (después de la colonización de *P. marinus* en el estuario de Bilbao) desde el año 2010 hasta el 2015.

El primer capítulo de esta tesis tuvo como objetivo analizar los cambios de población de las NIS después de la colonización de los estuarios de Bilbao y Urdaibai durante el periodo 1998-2015 y evaluar y comparar el impacto de estas NIS en términos de su relevancia cuantitativa y efecto sobre la abundancia y fenología de copépodos totales, zooplancton total, especies congenéricas y otros componentes relevantes del zooplancton en las diferentes zonas de salinidad de ambos estuarios. Asimismo, analizar la dinámica e impacto de las OES en el estuario de Bilbao. Para ello, se analizaron los cambios interanuales de densidad y de abundancia relativa en los diferentes componentes de la comunidad de zooplancton y se calculó la contribución porcentual al zooplancton total de los mismos para cada zona de salinidad de ambos estuarios durante todo el período de estudio. Por último, se analizó mediante modelos aditivos generalizados (GAM), la contribución de los diferentes taxones a los cambios interanuales en densidad del zooplancton total y copépodos totales. La contribución de los diferentes taxones a la dinámica de copépodos y zooplancton dentro de cada estuario permitió concluir que la sustitución en la dominancia de especies salobres por neríticas a medida que aumenta la salinidad a lo largo del eje longitudinal fue similar en ambos estuarios, siendo las especies salobres las que mostraron la mayor contribución a salinidad 33 e inferiores y especies neríticas a salinidades superiores a 33. La sustitución del copépodo nerítico *A. clausi* por el salobre NIS *A. tonsa* como especie dominante en las salinidades interiores de la ría de Bilbao es común a otras rías y zonas costeras colonizadas por *A. tonsa* (Gaudy et al. 2000; Chinnery and Williams 2004; Azeiteiro et al. 2005; Sei et al. 2006). En el estuario de Urdaibai, sin embargo, la contribución de la especie autóctona salobre *A. bifilosa* fue siempre mayor que la de *A. tonsa* en aguas de salinidad 33 y menor, y la contribución de *A. tonsa* disminuyó más drásticamente al aumentar la salinidad que la de *A. bifilosa*. Esto sugiere que la colonización y el aumento de *A. bifilosa* en los últimos años del período de estudio en la ría de Bilbao podría conducir a un reajuste en la contribución de estas dos especies congéneres en un futuro próximo en esta ría. Asimismo, las NIS y los OES aumentaron considerablemente las densidades del zooplancton y copépodos totales en la parte interna de la ría de Bilbao, llegando a obtener valores similares a las de la zona externa. Sin embargo, este aumento no se vio en el caso del estuario de Urdaibai.

El segundo capítulo de esta tesis tuvo como objetivo describir, comparar y explicar los cambios en la estructura y diversidad de las comunidades de zooplancton en los estuarios de Bilbao y Urdaibai y determinar el papel de las NIS y OES en dichos cambios, así como la influencia de las condiciones ecológicas dispares en estos estuarios. Los cambios en la estructura de las comunidades se evaluaron mediante análisis multivariante de abundancia de taxones y los cambios en la diversidad mediante el uso de diferentes descriptores de diversidad alfa, beta y gamma. Los cambios más evidentes se produjeron en el interior del estuario de Bilbao, donde una comunidad salobre abundante y menos diversa, dominada por los NIS, *Acartia tonsa* y *Oithona davisae*, sucedió a una comunidad de origen nerítico de escasa abundancia y más diversa. El posterior establecimiento de OES, principalmente de *Calanipeda aquadulcis*, supuso nuevos cambios en la estructura de la comunidad y un aumento progresivo de la diversidad. El patrón estacional de diversidad en el estuario interior y la diversidad beta en el estuario también se vieron afectados significativamente por la llegada de NIS y OES. Los resultados pusieron de manifiesto que los cambios observados en la estructura y diversidad del zooplancton dependieron de las condiciones ambientales y biológicas previas a la llegada de las NIS y de propiedades inherentes a cada uno de los sistemas. La disponibilidad de hábitats salobres insaturados y una mayor estabilidad hidrodinámica favorecieron la colonización del sistema por las nuevas especies llegadas al estuario de Bilbao, mientras que la preexistencia de una comunidad salobre bien desarrollada y la menor estabilidad hidrodinámica limitaron el éxito colonizador de dichas especies en el estuario de Urdaibai.

El tercer capítulo tuvo como objetivo caracterizar los nichos ecológicos de NIS y OES dentro de la comunidad de zooplancton a escala regional, representada por los dos estuarios juntos, y determinar sus subnichos espaciales y los de los otros taxones analizados para cada sistema, así como evaluar las diferentes respuestas espaciales y temporales de los diferentes componentes del zooplancton desde que las nuevas especies colonizaron ambos estuarios. Para ello, se utilizaron análisis OMI (“Outlying Mean Index “) y WITOMI (“Within Outlying Mean Index) y se calculó la métrica D de superposición entre especies. Los resultados obtenidos de dichos análisis mostraron que las nuevas especies que llegaron a ambos estuarios colonizaron solo hábitats salobres, pero tuvieron nichos reales más amplios en el estuario de Bilbao, debido a la ausencia de especies salobres dominantes a su llegada, que en el estuario de Urdaibai, donde su nicho fue limitado por interacciones bióticas y factores inherentes al sistema. La colonización de los habitas salobres del estuario de Urdaibai por copépodos portadores de huevos se vería negativamente afectada por la inestabilidad hidrológica. Tras el establecimiento de las especies colonizadoras, en el estuario de Bilbao se observaron cambios espacio-temporales en los nichos de otras especies, que no se observaron en el de Urdaibai y se atribuyeron a los mayores cambios en las condiciones bióticas y abióticas del estuario de Bilbao durante el periodo de estudio.

El cuarto capítulo se trata de un estudio preliminar que tuvo como objetivo detectar la presencia de las NIS *A. tonsa*, *P. marinus* y *O. davisae* en otros estuarios de la costa vasca y compararlo con lo observado en los estuarios de Bilbao y Urdaibai. lo que permitió además profundizar en el conocimiento de los factores que condicionan el éxito de la colonización en diferentes sistemas. Para ello, en el año 2019 se realizaron muestreos adicionales en los estuarios de Plentzia, Oria y Bidasoa en la época de verano, coincidiendo con el periodo de mayor abundancia de dichas NIS. Los resultados indicaron que la colonización de los estuarios de la costa vasca por parte de *A. tonsa, P. marinus* y *O. davisae* ha sido muy desigual en termino de presencia y abundancia. *A. tonsa* fue también encontrado en el estuario de Plencia, mientras que *O. davisae* fue encontrado en los tres estuarios y *P. marinus* en ninguno de ellos. Previamente se realizó la hipótesis que las NIS colonizaron en primer lugar la ría de Bilbao a través de aguas de sentina, y posteriormente se expandieron al resto de estuarios. La disparidad en las colonizaciones observadas en los resultados, sin embargo, pusieron en duda esta hipótesis arrojando más dudas que respuestas y sugiriendo realizar un estudio molecular en el futuro para poder entender mejor las vías de entrada y dispersión de estas NIS. A pesar de ello, los resultados recalcaron la importancia de tener en cuenta los puertos comerciales, las características propias de la especie y las características del nuevo hábitat a la hora de determinar el éxito de colonización de las NIS.

THESIS STRUCTURE

The present work is organized in three main parts:

The first part comprises a general introduction where relevant aspects of the zooplankton, estuaries, biological invasions, the study species and the state of the art are introduced. It is accompanied by the general objective of the present work, as well as, the definition of four specific objectives. This part ends with the description of the study area and the general methodology, including data acquisition and data pretreatment, which are common for the first three chapters of the second part, and therefore, in order not to repeat the information, only once is reported.

The second main part is composed of four chapters which individually cover each one of the four specific objectives. Each chapter starts with a small introduction including the aims and it is accompanied by the method part in which the specific data analysis used to obtain those aims are reported. The chapters end with the results and a discussion of those results.

Finally, the third part is devoted to perform a general discussion, report the general conclusions and used references of this work.

GENERAL INTRODUCTION

1. Study matters

1.1. The zooplankton

Plankton are the miscellaneous assemblage of small organisms that inhabit aquatic systems but do not hold sufficient locomotion abilities to bear against water masses currents and turbulence. The term was coined by Hensen (1887) from the Greek adjective πλαγκτός (planktos), meaning errant, wanderer, and drifter. Among its constituents, there are representatives of all kingdoms, including, as could not be otherwise, the animals (zoon (ζῷον) in Greek). Therefore, zooplankton are an assemblage of phagotrophic organisms (metazoans and also protozoans) drifting in water habitats. However, almost all species have developed some mechanism to move, at least, to change their vertical position within the water column, through flagella and cilia (protozoans), peristaltic contractions (medusae and salps), lobes and wings (pterodopods), tails (appendicularians), special swimming appendages (polychaetes and crustaceans) or contracting the longitudinal muscles (chaetognaths) (Lenz 2000).

According to the life cycle, two main groups are differentiated: 1) the holo(zoo)plankton, constituted by those planktonic species spending their whole life in the pelagic realm (water column), and 2) the mero(zoo)plankton whose species spending only part of their life (larval stage) in the pelagic zone, switching to either nekton (swimming capacity) or benthic (on the substrate) during the juvenile/adult stage. However, the life cycle type is not common to all the organisms of the taxonomic categories. In spite of that, holoplanktonic organisms are represented in foraminifera, amphipods, euphausiids, copepods, siphonophores, ctenophores, pteropods, chaetognaths, and appendicularians, whereas meroplanktonic organisms are represented in polychaetes, molluscs, echinoderms, bryozoans, and decapod switching to a benthic existence and cephalopods and fish switching to a nektic existence. Despite its overall small size, plankton organisms show a wide range of size variation, from 2 µm to >200 mm, and therefore size is used to classify zooplankton too (Lenz 2000):

- Nanozooplankton (2-20 µm): mainly heterotrophic nanoflagelates.
- Microzooplankton (20-200 µm): mainly ciliate protozoans and small metazoan larval stages.
- Mesozooplankton (0.2-20 mm): small hydromedusae, appendicularians, doliolids, crustacean plankton and meroplankton larvae among others. This is the most relevant size fraction among the scientific literature, because is the most sampled in monitoring programs and time series (Riccardi 2010).
- Macrozooplankton (2-20 cm): large hydromedusae, mysids and salps, for example.
- Megazooplankton (20-200 cm): large jellyfish, tunicates and chain-forming salps.

Despite zooplankton is found in all oceans, brackish waters and fresh waters over the world, the species and diversity of them is restricted not only by salinity, but also by three main factors, which are water depth, trophic status of the area and temperature regime (Lenz 2000).

Talking about diversity, zooplankton is a highly diverse group with approximately 36000 species, which creates huge combinations of zooplankton communities (Lenz 2000). That diversity gives the opportunity to have a zooplankton community that survive at extremely different environmental conditions and be important in all the water masses around the world. Indeed, in the marine environment zooplankton is of vital importance because they are the main link between primary producers and higher trophic levels. As their position and significance in the food web is very important, they had a great ecological role. They are considered as the secondary producers, occupying the second and to some extent third level in the food web. In the pelagic realm, the food web is controlled by bottom-up (resource-driven control) control, because the supply of nutrient determine the amount of primary production, but the pivotal role of zooplankton grazing in controlling phytoplankton growth has been getting attention in the last years, determining their importance in the top-down (predator control) control of the food web (Pinheiro-Silva et al. 2020). Likewise, they are able to control and avoid algal blooms (Ger et al. 2019). Apart from their importance in the food webs, zooplankton communities are so diverse that they perform a variety of ecosystem functions, as nutrient recycling. For instance, they are very important in microbial communities, because the regeneration of nitrogen through excretion by zooplankton helps support bacterial and phytoplankton production. Microbes colonize zooplankton faecal pellets and carcasses, making them rich sources of organic carbon for detrital feeders. These zooplankton products slowly rain down on the dark seabed, sustaining diverse benthic communities of sponges, echinoderms, anemones, crabs and fish (Ruhl and Smith 2004). Moreover, zooplankton play an important role in biological pump, because much of the CO_2 that is fixed by phytoplankton is eaten by zooplankton and finally sink in the seabed, locking up in the sediments and removing from the carbon cycle (Richardson 2008). In addition, most zooplankton species are short-lived (less than a year), therefore very sensitive to environmental changes. In fact, some evidence suggests that plankton are more sensitive indicators of change than even environmental variables themselves, because the non-linear responses of plankton communities can amplify subtle environmental signals (Taylor et al. 2002). Therefore, those characteristics make zooplankton a good sentinel to study environmental changes, such as climate change (Ji et al. 2010), habitat degradation (Lougheed and Chow-Fraser 2002), pollution (Webber et al. 2005), biological invasions (Bollens et al. 2002), or even, water masses characteristics (Eisner et al. 2013).

Among the zooplankton, has to be pointed out the group of copepods. Certainly, small planktonic marine copepods (overall, less than 1 mm in length) are the most abundant metazoans on Earth (Sommer and Stibor 2002) representing the 90-97 % of the total biomass of marine zooplankton (Bradford-Grieve 2002), and being also very important in freshwaters (Boxshall and Defaye 2008). Among small copepods they are included adults, copepodites and nauplii of calanoid genera such as *Paracalanus, Pseudocalanus, Acartia* and *Clausocalanus;* cyclopoid genera such as *Oithona*, *Oncaea* and *Corycaeus*; and planktonic harpacticoids of the genus *Microsetella* Copepods are present in any aquatic environment, from deep-ocean trenches to mountain lakes and occupy a wide temperature range, from polar waters to hydrothermal vents (Walter and Boxshall 2020). These small copepods are the link of food webs, preying upon phytoplankton and being prey items for larval fish and other zooplanktivorous consumers (Turner 2004). Likewise, copepods link the viscous and inertial realms, characterized by low and high Reynolds numbers respectively (Naganuma 1996). Apart from that, copepods are important as bioindicators, for example for climate change (Richardson 2008) or ocean acidification (Lewis et al. 2013), and as model animals for ecotoxicological studies or environmental genomics (Raisuddin et al. 2007).

1.2. Estuaries

Fairbridge (1980) defined the estuary as "an inlet of the sea reaching into a river valley as far as the upper limit of tidal use, usually being divisible into three sectors: 1) a marine or lower estuary, in free connections with the open sea; 2) a middle estuary subject to strong salt and freshwater mixing; 3) an upper or fluvial estuary, characterized by freshwater but subject to strong tidal action". The salinity of seawater (marine waters) is approximately 35 and the one of freshwater is always less than 0.5, thus the salinity range of estuarine waters is 0.5-35 and it is named brackish. Consequently, the salinity of the estuarine waters are extremely variable. It is a dynamic ecosystem, because seawater that normally enters through the open sea according to the twice-daily rhythm of the tides, at least, in those places where tides are, is diluted with freshwater flowing into the estuary from rivers (McLusky and Elliott 2004), creating water masses along the estuary of different salinity, which are usually called salinity zones. According to the Venice system (1959) six distinct zones are recognized: limnetic or freshwater (salinity < 0.5); oligohaline (0.5-5); mesohaline (5-18); polyhaline (18-30); euhaline (30-40) and hyperhaline (> 40). The pattern of dilution is different in each estuary depending on the volume of freshwater, the range of tidal amplitude, and the extent of evaporation from the water within the estuary, which is used to classify estuaries in three types: positive, negative or neutral (McLusky and Elliott 2004). The most typical in temperate areas are the positive estuaries, where the evaporation from the surface of the estuary is less than the volume of freshwater entering the estuary, as a consequence, on the bottom we found the incoming saltwater, a gradual vertical mixing and an outgoing stream of fresher surface water. In negative estuaries the opposite situation is found and is typical from tropical areas. In the neutral estuaries, the evaporation equals the freshwater input provoking a static salinity regime, but these type of estuaries are scarce. Within the positive estuaries four main types are recognized depending on the tidal amplitude and volume of freshwater flow (Dyer 1997): salt wedge or highly stratified, fjord, partially mixed and homogenous. In the salt wedge or highly stratified estuaries, the freshwater flows seawards on the surface of the inflowing saltwater; the fjords are similar to the highly stratified with the exception that at the mouth of fjords the inflow of tidal water is more restricted. When the tidal inflow is greater or similar than the freshwater inflow a partially mixed estuary develops and when the estuary is very wide an homogenous estuary, where the Coriolis force will cause a horizontal separation of the flow circulation being across from left to right (in the north hemisphere) rather than vertically as in the other types (McLusky and Elliott 2004). Apart from that, estuaries could be classified depending on the tidal range, in fact, tidal amplitude is not only different in each region of the world but also at local level according to the neap and spring tide cycle. Based on the tidal range four types of estuaries could be found, which are microtidal (< 2 m of tidal range), mesotidal (2-4 m), macrotidal (4-6 m) and hypertidal (> 6 m). Moreover, the tidal amplitude could influence the mudflats and vegetation of an estuary (Kennish 1986).

The river flow, tidal range and sediment distributions in estuaries are continually changing and consequently estuaries are never really “steady-state” systems. Thanks to that, estuaries are important ecologically and for human life. Ecologically, it holds many species during their entire life cycle or during the part of the migration and it is the spawning place and nursery area for several fish species (Ketchum 1983). For humans, estuaries have great uses, for example, provides fish and shellfish, aggregates for building and water for abstraction; land is claimed from the wetlands for agriculture, industry, urban areas, construction of ports and bridges; wastes are discharged and water is use for navigation porpoises. Such activities could change the structure of the estuary or add materials to the system and any disruption could cause a knock-on effect to the whole system. In spite of that, estuaries remains as one of the most resilient habitats on earth (McLusky and Elliott 2004).

As it has been mentioned above, variations in salinity occur vertically, horizontally and with time, which pose the most extreme stress on organisms living within the estuary. Therefore, organisms that live in the estuary could be classified by their tolerance to salinity (McLusky and Elliott 2004): a) oligohaline organisms; organisms living in rivers and other freshwaters that persists at salinities of up to 5, b) true estuarine organism; organisms with marine affinities that live in the central parts of estuaries, commonly at salinities of 5-18. Most of them could live in the sea but are apparently absent from the sea probably due to competition from other organisms. And c) marine species; organisms living in the sea that are able to persist lower salinities and enter to the estuary.

The total quantity of true estuarine species is relatively low compared to the one of oligohaline or marine species. Consequently, the numbers of species of all groups living within estuaries is combined in a single diagram, known as Remane´s diagram (Fig. 1), it can be seen that estuaries are characterized by having fewer species than adjacent aquatic environments have (McLusky and Elliott 2004).

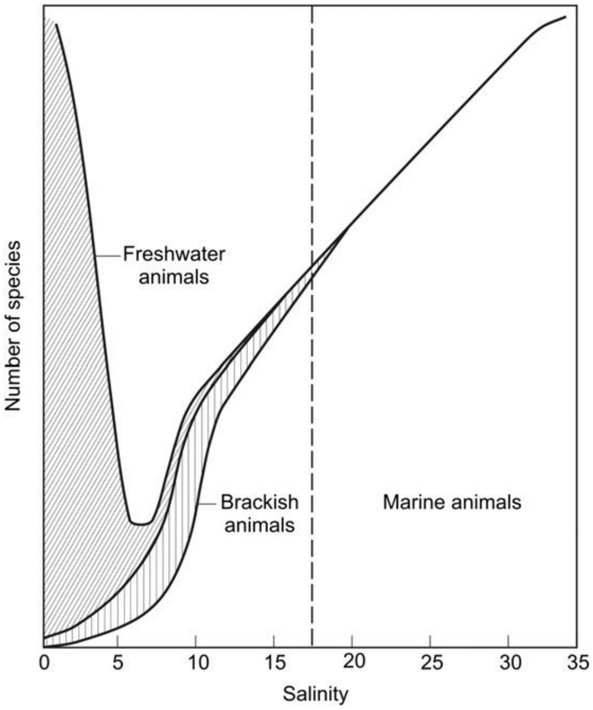

Fig. 1: Remane´s diagram. Source: Whitfield et al. 2012.

1.3. Estuarine zooplankton

Particularly, the (meso)zooplankton of an estuary have been divided by Bousfield et al. (1975) into three groups based of their origins and relationships to salinity:

- Marine coastal species which enter the estuary from the sea (neritic species): two subgroups are included in this group the ones restricted to the deeper more seaward parts of the estuary their distribution being extended by the salt wedge, and the ones that their distribution extends the coastal water but that they reach their population maxima within the estuary.

- Estuarine endemic species which live only in the brackish water of the upper estuary (brackish species).

- Fresh-water species which extend into brackish water in the upper estuary (freshwater species).

Therefore, freshwater species are sporadic species in the estuary. Besides, some neritic and estuarine species are able to maintain relatively high stocks all year round by their ability to graze on non-algal diets, but the majority have seasonal peaks of abundance and show a great decreased or disappear from the estuary till the next year. Therefore, the seasonality of the zooplankton is a key point in the community (Ketchum 1983). Moreover, turbidity and currents are the most limiting environmental factors for the survival of estuarine zooplankton; turbidity because limits the phytoplankton production and currents (particularly in small estuaries) due to the "wash out" effect, namely, high river flow can carry them out to the sea. To avoid this last effect, zooplankton of the estuaries migrate vertically with the different phases of the tide, in fact, maintaining near to the bottom helps not to be washed out from the estuary (McLusky and Elliott 2004).

The zooplankton of estuaries over the world is dominated by copepods, indeed, they are the most numerous organisms and the greatest contributors to zooplankton biomass as it has been explained before. They are classified by their origin and relationship with salinity, like the zooplankton in general (Ketchum 1983). Consequently, in the outer part of the estuary, we usually find a variety of species that are also found in the nearby coastal ocean, for example in the estuaries located in the Northeastern Atlantic coast *Paracalanus* spp., *Clausocalanus* spp, *Acartia clausi, Centropages* spp, *Calanus* spp. among others (Azeiteiro et al. 1999; Chaalali et al. 2013; Fanjul et al. 2017). However, the middle and inner zone of the estuary, which are more affected by salinity reduction, the calanoid genera *Acartia* and *Eurytemora* are the principal contributors (Ketchum 1983), in the Eastern Atlantic estuaries specially *Acartia bifilosa* and *Eurytemora affinis* (Villate et al. 2004; David et al. 2007).

2. Biological invasions

Elton (1958) defined biological invasion as the spread of an organism or species into an area formerly free of it. Several terms have been coined to refer to these species, e.g., alien, allochthonous, exotic, non-native species, but recently the term Non-Indigenous Species (NIS) has taken strength (Uttieri et al. 2020). Once the NIS arrived to the new habitat, they could colonize the new place, start reproducing and creating a permanent population. When the fact of maintaining that population affect the native community, the food web or the environment in any way, is when that NIS become an invasive species (IUCN 2020a). Around the world, the most known hazardous invasive species are: the pampas grass (*Cortaderia selloana*) spreading from Argentina to all around the world in the 20th century, mainly as an ornamental plant (Domènech et al. 2005), the “Killer Algae” (*Caulerpa taxifolia*) invasions in the Mediterranean, California and Australia coasts, and the zebra mussel (*Dreissena polymorpha*) in the worldwide freshwaters (GloBallast Partnership 2020), among others. The intensity of the consequences of this invader species in the new habitat is called invasibility, having species that do not damage in any way or species that become very hazardous. In this sense, the capacity of invasibility of a given NIS is not still clear, being related to some characteristics of the species itself and/or of the colonized environments. On one hand, the colonization success of NIS might vary depending of its capacities to face the biotic and abiotic constraints within the host ecosystem, in other words, the fitness, the minimum generation time, rate of population expansion, competitiveness and the self-modification ability to survive in the environment (Chan and Briski 2017). On the other hand, communities with high species diversity clearly tend to support ecosystem properties that are less variable through time as compared with simple communities (Schindler et al. 2015). Therefore, changes in species richness and composition are most important in facilitating invasions, and this may be the result of increased vulnerability to invasion in a post-disturbance (natural or man-made) community dominated by poor competitors (Kneitel and Perrault 2006). In addition to the importance of low species richness in the establishment of NIS, Marraffini and Geller (2015) concluded that the interaction with other factors (e.g. resource availability) also contributes to the invasibility. Therefore, the pre-invasion conditions of the receiving habitat could greatly influence the consequences of NIS establishment on the native community.

2.1. Impact of invasions

Biological invasion phenomenon is one of the most important ecological disturbances (Mollot et al. 2017), and warnings about the serious threats posed by NIS that become invasive to biodiversity (Vitousek et al. 1997; Strayer 2012; Simberloff and Vitule 2014), and even to economic (Perrings 2011) and human welfare (Bol 2011) have been notorious, because bringing a given species into a new habitat is not neutral to the environment, interacting with resident species or abiotic parameters or energy and matter fluxes. Consequently, there is a growing concern about the potential impacts at several levels (Thomaz et al. 2015).

At ecology level, NIS can affect the recipient community in highly variable ways: competing with native species for food or space, causing the displacement or the eradication of the native species as in the case of the invasion of the American crab in European waters (Nehring 2011); preying upon native species as the ctenophore *Mnemiopsis leidyi*, which has caused the dramatic decline of the population of anchovy (*Engraulis encrasicolus*) in the Black Sea (Oguz et al. 2008); altering habitat as the eucalyptus tree on the soil quality (Cook et al. 2016); altering environmental conditions as the clarity of the water by the zebra mussel (Macisaac 1996); altering the food web and the overall ecosystem, e.g. the introduction of the zooplanktivorous fishes *Rutilus rutilus* and

Alburnus alburnus into Sau Reservoir (Northeast Spain) that caused a trophic cascade effect (Ordóñez et al. 2010). All these impacts end to damage the biodiversity, ecosystem functioning and ecosystem stability. Apart from that, impact on human welfare have to be pointed out. Non-indigenous bacteria and viruses which attack humans rapidly spread through the world due to the globalization. A clear example is the pandemic of Covid-19 that we are suffering nowadays, with until now more than 100 million cases and 2.1 million of deaths worldwide (World Health Organization 2021). Pathogens related invasions not only caused impact on human health, but also in economy by increased pathogen monitoring, testing and diagnostic and treatment costs, and loss of social productivity due to illness and even death in affected persons.

Moreover, biological invasions` ecological impacts also caused direct economic costs due to several effects: invasive pests that cause crops plague in agriculture; alteration of the forests flora in forestry; reductions in fisheries production (including collapse of the fishery); impacts on aquaculture (including closure of fish-farms), especially from introduced harmful algal blooms; physical impacts on coastal infrastructure, facilities and industry, especially by fouling species; impacts or even closure of recreational and tourism beaches and other coastal amenity sites due to invasive species. Furthermore, we could not forget the economic costs by indirect ecological impacts and biodiversity loss, which are not counted usually because is not easy to calculate, or the costs of responding to the problem, including research and development, monitoring, education, communication, regulation, compliance, management mitigation and control costs. The sum of all this problems is huge, in the United States for example, the cost of biological invasions to the economy is estimated at over $100 billion annually (Vila et al. 2010; Perrings 2011).

Taking into account the concerning situation about the NIS, the Convention on Biological Diversity (CBD 2000) has recognized the need to compile and spread the information about them, which threaten the ecosystems, habitats, or native species, in order to use them in a context of prevention and mitigation actions. Moreover, it issued a call to increase the number of researches on the impact of NIS on biological diversity.

2.2. Invasion pathways

It is true that organism dispersion is a natural process and of vital importance for the distribution of life on earth, for the biodiversity and for evolution. The natural dispersal is usually slow and is limited by geographical barriers. However, humans overcome all biogeographical barriers and nowadays inhabit all parts of the world, connecting them by roads, ships or plane and spreading with them domesticated animals, crop plants, pets, pathogens and parasites in relatively short time. Consequently, we distribute hundreds and thousands of species intentionally and unintentionally worldwide (Nentwig 2002). The last decade's globalization and increasing trade have fostered the introduction and establishment of NIS beyond their natural distributional range in all ecosystems (Thomaz et al. 2015). The exact values of the amount of invasive species worldwide is unknown, but an estimation has been done per continent, indicating up to 10000 species of plant, up to 300 vertebrates, more than 5000 invertebrates and many thousands of microorganisms (Nentwig 2002). In the terrestrial habitats the main pathway is transport in vehicles, some of them unintentional and other intentional, such as, as part of human nutrition (the domesticated animals or species for hunting), as biological control agents or as ornamental animals and plants.

Although more studies have been carried out in terrestrial habitats than in aquatic ones (Jeschke et al. 2012; Lowry et al. 2012), the impact of human activities, in general, and of invasions, in particular, are likely greater in aquatic ecosystems than in terrestrial ecosystems (Thomaz et al. 2015). Since 1970, marine traffic has increased at an average rate of 2.1 % per year, surpassing 10 billion tons of cargo in 2015 (UNCTAD 2017). This is more evident in estuaries and connected habitats where human impact has caused a decrease of > 90% of formerly important species, destroyed > 65% of the main habitats, degraded water quality and increased species spreading (Lotze et al. 2006). Indeed, the increase in commercial shipping, channel construction for sailing, aquaculture, life seafood, bait and use of aquariums (Hulme et al. 2008; Katsanevakis et al. 2013) has made estuaries one of the most vulnerable ecosystems to the arrival of new species (Frisch et al. 2006) due to the unavoidable presence of a huge variety of organisms especially by ballast water (Bax et al. 2003; Bailey 2015). In addition, as it has been previously mentioned, salinity is a limiting factor and lowered species richness occur at intermediate salinity brackish habitats, these zones becoming subject to invasion by NIS (Paavola et al. 2005). Apart from estuaries, in the United States and Canada, a considerable transport related to ballast water was documented to impact on lakes and rivers (Bailey 2015).

Ballast water is the best-studied shipping vector, partly due to the relative ease with which samples could be collected, and the certainty that aquatic organisms associated with ballast water are discharged from the vessel when ballast tanks are emptied (Endresen et al. 2004). The mechanism of the introduction by ballast water is the following (Davidson et al. 2017): at the source port, when a commercial ship puts on its load, in order to maintain the stability of the ship it has to fill the tanks with the surrounding water. Together with the water, its pelagic organisms are transported to the tanks. The ship then moved toward the destination port, while the organisms could keep alive inside. Modern international voyages span just a few days or weeks, affording entrained organisms a unique opportunity for transport to biogeographically remote locations which would otherwise be rarely accessible. When the ship arrived to the destination port and discharge the load, it has to pour the tanks in order to maintain the stability again. Therefore, the organisms that where inside the tanks are poured to the destination waters too. Once the organism has been discharged could widespread through currents on the coast or could be introduced into the estuary through active movements or tide movements and colonize also the estuary or nearby coast. It has been estimated that merchant shipping around the world transfers annually approximately 3-5 billion tons of ballast. With this transfer, it is estimated that around 7000 to 10000 different marine microbes, plant and animal species are transported worldwide each day (Küçük 2019). The invasive sea creatures transported in this way have become a serious problem for the marine environment, becoming the four largest global threats to the oceans (Nunes et al. 2014). Regarding that, the International Maritime Organization (IMO) has made an International Convention for the Control and Management of Ships´ Ballast Water and Sediments (the Ballast Water Management Convention or BWMC) (IMO 2004). With this treaty all vessels must carry a ballast water management plan, a ballast water record book and an international ballast water management certificate.

2.3. Zooplankton invasions

Since just one cubic metre of ballast water may contain up to 50,000 zooplankton specimens (Nentwig 2002) and/or 10 million phytoplankton cells, and the majority of marine species include a planktonic phase in their life cycle, there are literally thousands of different marine species that may be carried in ships' ballast water – basically anything that is small enough to pass through a ships' ballast water intake ports and pumps (GloBallast Partnership 2020). This includes bacteria and other microbes, small invertebrates and the eggs, cysts and larvae of various species, including most fish, although not all of these will survive in the ballast tank because it is a hostile environment with considerable disturbance, lack of food and light. Indeed, abundance and species diversity of plankton decreases with the length of the confinement of the organisms in the tanks. Some organisms are harder and survived longer in time. In fact, crustaceans seem to be generally harder than very delicate organisms such as siphonophores, appendicularians and chaetognathes, which survived only a few days; polychaete, bivalve and echinoderm larvae occupying an intermediate position (Gollasch et al. 2000).

Therefore, NIS of zooplankton has an unprecedented capacity and opportunity for dispersal across large geographical distances (Geburzi and McCarthy 2018). A huge increase in the occurrence of NIS zooplankton in aquatic habitats, and particularly in estuaries, has been documented during the last decades (Bollens et al. 2002; Dexter and Bollens 2020; Dexter et al. 2020). Indeed, NIS zooplankton are now reported from water masses over all the world, and in many cases associated with large ecological and economic impacts (Walsh et al. 2016). The increasing number of publications that pertain to NIS zooplankton shows the awareness of this issue among the scientific community, so for instance, only in Europe 170 publications concerning invasive zooplankton species were published between 2000 and 2018 (Dexter and Bollens 2020). But the spreading of NIS is not only a priority for scientists but also for manager and legislators (Galil et al. 2018), as reflected, for instance, in the regulation on the prevention and management of the introduction and spread of invasive alien species across the EU (European Union 2014). Consequently, monitoring programs for early detection, identification of spreading pathways, and study on the factors that facilitate their colonization of new areas would be very helpful for controlling the expansion of NIS zooplankton. Among zooplankton, copepods are the most representative group, are one of the most hardest species in hostile environments and show great colonizing ability on both small and long-range geographical scale (Lee 2016).

3. The colonizers *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae*

Examples of worldwide copepod colonizers in coastal and transitional systems are the calanoids *Acartia* (*Acanthacartia*) *tonsa* Dana, 1849 and *Pseudodiaptomus marinus* Sato, 1913 and the cyclopoid *Oithona davisae* Ferrari F.D. and Orsi, 1984. The success of these species to survive during transportation in ships and to establish and colonize their new recipient environments is, mainly, related to low sensitivity to salinity changes (euryhaline) and, despite their thermophilic character, to the ability to cope with the cold season (Svetlichny et al. 2019, 2021). It is well known that *A. tonsa* is able to survive adverse conditions by producing resting eggs (Holm et al. 2018), whilst *P. marinus* show an apparent ability to survive in a torpid state (Svetlichny et al. 2019) and *O. davisae* overcomes cold season as quasi-diapausing fertilized females (Svetlichny et al. 2016).

The cryptogenic copepod *A. tonsa* (Fig. 2), prior to its widespread in coastal and brackish subtropical and temperate waters of all the world, only occurred in the Indo-Pacific region, but the exact origin of the species, however, remains unknown (VLIZ Alien Species Consortium 2011). The first record was dated around 1900 on the east coast of North America (Jensen 2010), and has since extended its distribution over all Indo-Pacific and Atlantic coasts (Garmew et al. 1994; Mauchline et al. 1998; Chen and Hare 2011; Gomes et al. 2018; Plough et al. 2018; Dexter et al. 2020; Figueroa et al. 2020). Likewise, the first published record of this species in Europe was in Caen in 1925 (Remy 1927), but, a re-examination of older plankton samples determined that it was certainly in the now disappeared large shallow and brackish bay Zuiderzee in 1916 but no earlier (Redeke 1934), likely transported through ballast water (Brylinski 1981). Since first observation, it was rapidly identified in northern European coasts and estuaries; in Ringkøbing Fjord in 1921 (Jespersen 1933), in the Gulf of Riga in 1924 and in the Gulf of Gdansk in 1925 (Segerstråle 1957), in the estuary of the Weser in 1930 (Klie 1933), in the Gulf of Finland (Smirnov 1935) and in Swedish Baltic coast (Jensen 2010) in 1934, and in the British coastal waters of Southampton in 1954 (Conover 1957). The likely colonization of southern water bodies was later; the polyhaline area of the Gironde estuary in the 1970s (Castel 1981), whilst the oligo-mesohaline area in 1983 (David et al. 2007), the estuaries Ría de Aveiro (Morgado 1997) and Mondego (Azeiteiro et al. 1999) around 1990, and the estuary of Bilbao in 2001 and the estuary of Urdaibai in 2003 (Barroeta et al. 2020). According to Jensen (2010), this species is present in the Black Sea from 1976, in the Caspian Sea from 1982 and in the Mediterranean Sea from 1985, where it still is spreading (Camatti et al. 2019). At present, *A. tonsa* is one of the commonest copepods in different coastal and estuarine water bodies of Europe (e. g., Azeiteiro et al. 2005; Leandro et al. 2006; David et al. 2007; Tiselius et al. 2008; Uriarte et al. 2016).

Fig. 2: From left to right: *Acartia tonsa* nauplius, female and male (author: Ziortza Barroeta).

On the other hand, *P. marinus* (Fig. 3) was firstly described from the coast of Hokkaido in 1913 and is considered to be native to the Northwestern Pacific Ocean (Walter 1987), although likely shows a natural presence in the eastern sector of the Indo-Malayan region (Grindely and Grice 1969). It has, however, a long invasion history from 1950, in fact, it is the most worldwide spread species among its numerous congenerics (Sabia et al. 2015). Likely introduced through ballast water in Hawaii in 1964 (Jones 1966), it has spread along the western coast of North America in Puget Sound (Lawrence and Cordell 2010), Mission and San Francisco bays (Fleminger and Hendrix Kramer 1988; Orsi and Walter 1991) and Baja California (Jimenez-Perez and Castro-Longoria 2006), as well as, in Australia in 1963 (Greenwood 1976) where, in 2005, it was included in the ranking of Australian marine pests under medium priority level (Hayes et al. 2005). In Europe, the expansion is seemingly more recent, according to Gubanova et al. 2020 and S Besiktepe (pers. comm.), the first appearance was reported in the Gulf of Izmir (Aegean Sea). Although the first published record was in Rimini in 2007 (De Olazabal and Tirelli 2011), since then, it is being detected in the shelf and coastal/estuarine waters of the Mediterranean and Atlantic: in Lake Faro in 2008 (Sabia et al. 2014), in the Tuscany coast in 2008 (Sabia et al. 2015), in the harbour of Monfalcone (Gulf of Trieste) in 2009 (De Olazabal and Tirelli 2011), in the Köprüçay estuary in 2009 (Erdoğan and Ertan 2014), in the harbour of Calais and along the coast off Gravelines in 2010 (Brylinski et al. 2012), in Berre Lagoon in 2010 (Delpy et al. 2012), in the estuaries of Bilbao and Urdaibai in 2010 and 2011, respectively (Barroeta et al. 2020), in the Gironde estuary in 2011 (Dessier et al. 2017), in the Mondego estuary in 2011 (Uttieri et al. 2020), in the German Bight and in the southern North in 2011 (Jha et al. 2013), in the Gulf of Naples in 2013 (Mahadik and Mazzocchi, unpublished data), in the Belgian part of the North Sea in 2015 (Deschutter et al. 2018), in the Guadalquivir estuary in 2016 (Reyes-Martinez and González-Gordillo 2019), in Sevastopol Bay in 2016 (Garbazey et al. 2016) and very recently in the Berlengas Archipelago (Sonia Cotrim, personal comm.). Nevertheless, the occurrence at the different sites ranges from punctual observations at very low abundances to reach stable settlement (Uttieri et al. 2020) and even to become one of the more abundant species (Gubanova et al. 2020). Likewise, and as a consequence of its rapid spread through Europe, in 2018 scientists from nine European countries established an ICES working group entitled “Towards a EURopean OBservatory of the non-indigenous calanoid copepod *Pseudodiaptomus marinUS*” (EUROBUS WG) in order to increase the ecological, physiological, genetic and biogeographical knowledge on this species (Uttieri et al. 2020). Furthermore, very recently it has been included in the list of IAS of European Union concern, which focuses on the priority species that are likely to arrive, establish, spread, and have an impact on native biodiversity and associated ecosystem services over the next decade in Europe (Tsiamis et al. 2020).

Fig. 3: From left to right: *Pseudodiaptomus marinus* female (eggs carrying) and male (author: Ziortza Barroeta).

Finally, the cyclopoid *O. davisae* (Fig. 4) was firstly described in the Sacramento-San Joaquin delta estuary, to where it likely arrived in ballast waters as early as 1963 (Ferrari and Orsi 1984), although it is considered native to the coastal waters of East Asia, mostly around Japan (Nishida 1985) and China (Razouls et al. 2020). Progressively it spread along the California coast (Ferrari and Orsi 1984; Ambler et al. 1985; Fairey et al. 2002; Bollens et al. 2011) and it has also colonized Puget Sound, on the Northern coast of America (Cordell et al. 2015). In the Southeast Pacific, in Chile, was also recorded, but only in a single sample during a survey, suggesting that might result from a single ballast water discharge, likely from a Japanese ship (Hirakawa 1988). In Europe, apparently the first catches were in 2000 in the harbour of Barcelona for cultivation experiment purposes (Saiz et al. 2003). In 2001 appeared in the estuaries of Bilbao and Urdaibai (Barroeta et al. 2020). In 2001 (Zagorodnyaya 2002) and 2005 (Gubanova and Altukhov 2007) was misidentified as *O. brevicornis* in the Black Sea, but afterwards, continuous observations were made in several areas of this region: in Sevastopol Bay in 2005 (Altukhov et al. 2014), in Varna Bay (Mihneva and Stefanova 2013) and in the Sinop coast (Üstün et al. 2019) in 2009 and in Sürmene Bay in 2010 and in the Anatolian coast in 2013 (Yildiz et al. 2016). Similarly, in the Mediterranean sea: in Marmara Sea and Golden Horn estuary in 2008 (Isinibilir et al. 2016), in the Lakes Faro and Ganzirri (Zagami et al. 2018) and in the Venice Port (Vidjak et al. 2019) in 2014, whilst in the Southeastern Aegean Sea the first record was in 2015 (Terbıyık Kurt and Beşiktepe 2019) in the North Aegean Sea was in 2018 (Dragičević et al. 2019). Likewise, specimens from the Wadden Sea in the North Sea were identified by both morphological and genetic methods in 2010 and 2015 (Cornils and Wend-Heckmann 2015). Nevertheless, the full extent range is uncertain, due to it frequently is misidentified as other congenerics (Uye and Sano 1995; Temnykh and Nishida 2012). Similarly, and although in many sites it achieves high abundance (e.g. Gubanova and Altukhov 2007; Cordell et al. 2015; Uriarte et al. 2016; Dexter et al. 2020), its small size and the use of coarse nets can underestimate its real densities which can be more than 100 times higher (unpublished data).

Fig. 4: From left to right: *Oithona davisae* female and male (author: Ziortza Barroeta).

3.1. Impact on native zooplankton community

Despite their worldwide expansion, the vast majority of the publications about those NIS are limited to the study of their occurrence and means of introduction in new areas (David et al. 2007) and only a few of them aim to analyse the effect of the species in the ecosystem (Katsanevakis et al. 2014) or the evolution in long term of the invasions, maybe due to the low time since invasion in some places or no previous information about the communities. Katsanevakis et al. 2014 identified non-indigenous marine species that have a high impact on ecosystem services and biodiversity in European seas, classified the mechanisms of impact, commented on the methods applied for assessing the impact and the related inferential strength, and reported gaps in available information. It has to point out, that they also study the positive effects that cause NIS, which is often forgotten, as there is often a perception bias against NIS. However, they also concluded that evidence for most of the reported impacts was weak, as it was based on expert judgement or dubious correlations, while only 13 % of the reported impacts were inferred via manipulative or natural experiments.

Among the listed species, the calanoid copepod *Acartia tonsa* can be found, which according to the mentioned research, could affects the following aspects of an environment:

- Food (Positive effect): could be a prey for pelagic fish or use to produce live feed for aquacultured species as turbot reared in the Black Sea (Sørensen et al. 2007).
- Water purification and biological regulation (Positive effect): can serve as a biological control of algal blooms (Leppäkoski et al. 2002), purifying the water from harmful algae.
- Multiple-species impacts (Positive or negative effect): could compete with native copepods, especially congenerics, and may dominate zooplanktonic communities (David et al. 2007).
- Entire ecosystem processes/wider ecosystem functioning (Positive or negative effect): may modify food webs and trophic flows within invaded ecosystems through severe predation or competition for resources.

Concerning the zooplankton community, this NIS or another copepod NIS could affect especially the part of "Multiple-species impacts". If NIS become very abundant or dominate the zooplankton community, this may generate several affections: changes in species density or even total zooplankton density, changes in species or total zooplankton phenology, alterations of the species diversity, displacement of native species niches and/or expansion to near habitats (range-expansion).

3.1.1. Changes in density

When a NIS colonized a new habitat and starts increasing its population if the resources are limited, the first consequence that could be observed in native community is changes in native species densities. NIS may be more resistant to adverse environmental conditions than native ones (Katsanevakis et al. 2014), being more competitive and obtaining the needed resources. In this circumstances, native species density will decrease because is not able to grow and reproduce at the same time as the NIS. An example of this was observed in the Black Sea, when *O. davisae* occurred and started increasing its density, *O. nana* density started to decrease because as both species are congeneric, they compete for the same resources (Gubanova et al. 2019). The higher adaptive potential of *O. davisae* in this situation compared to that of *O. nana* (Isinibilir et al. 2016) caused the decrease in density of the last one. Apart from that, they could also affect to the total zooplankton density, decreasing it due to the competition or increasing it by their contribution; reflecting not only negative but also positive effect. In the first chapter of this thesis, the impact on total zooplankton and total copepod densities of the NIS (*A. tonsa*, *P. marinus* and *O. davisae*) in the estuaries of Bilbao and Urdaibai will be investigated; together with the impact on native species that are their congeneric.

3.1.2. Phenological changes

Phenology is the study of periodic events in biological life cycles, such as migration, flowering or breeding of many plant and animal species, and how these are influenced by seasonal and interannual variations in climate or habitat factors (Walther et al. 2002). Plants and animals exhibit seasonal patterns in their activities because there is a clear seasonality in the suitability of their environment: there is often only a limited period in the year when conditions are favourable enough to successfully reproduce or grow (Fig. 5A). If reproduction or growth takes place outside this window of favourable conditions, there are often large fitness consequences. Therefore, the activity that is the most demanding for an organism should take place at the time of optimal conditions (Visser and Both 2005). If those optimal conditions change in time due to changes at environmental variables, prey phenology, predators phenology or other factors, the phenology of a species is shifting at a different rate from that of the species that make-up its ecological conditions, this leading to a mismatch in phenology (Figs. 5B and C) (Stenseth and Mysterud 2002). An example of this we can found in the marine pelagic community due to climate change. Large differences in the extent of the shifts of the phenology of diatoms and dinoflagellates (primary producers), copepods, other holoplankton groups and meroplankton have been observed in the last four decades (Edwards and Richardson 2004). Diatoms, as their phenology depends on photoperiod or light intensity rather than temperature, have not shifted but the dinoflagellates have by 23 days. The copepod and other holoplankton groups advanced by 10 days their maximum density peak while meroplankton 27 days. Consequently, a mismatch between the successive trophic levels and a change in the synchrony in the timing of primary, secondary and tertiary producers was observed (Edwards and Richardson 2004).

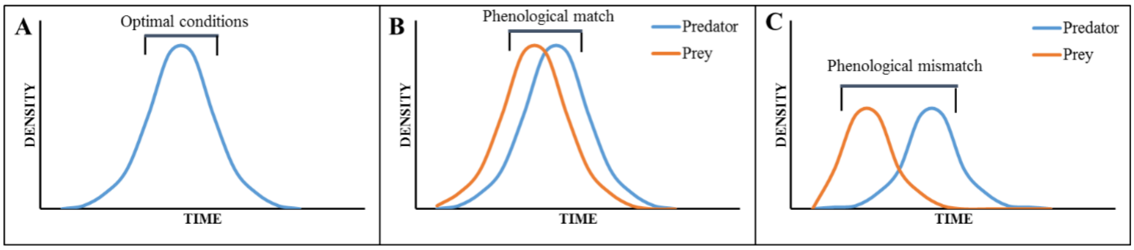

Fig. 5: A) An example of a seasonal pattern in a species density. B) A predator species phenology and the one of its prey, note that both overlap in time. C) A predator species phenology and the one of its prey, note that both do not overlap in time.

Apart from climate change, the occurrence of a new species in the habitat of the same scale at the food web could create between species a competition for the same resources, especially at optimal conditions. If the NIS is more efficient exploiting those resources, the native species is forced to change its habits and be more efficient in less favourable conditions, creating a shift in its phenology. An example of this is the successful colonization of the middle part of the estuary of Gironde (France) by *A. tonsa* led to a shift in the phenology of the native *A. bifilosa.* As both species are congeneric and had similar optimum conditions, they compete directly for the resources and as *A. tonsa* is more competitive it changed the maximum density of *A. bifilosa,* coming one month earlier in time (David et al. 2007). Regarding this fact, the possible consequences on total zooplankton, total copepod and native species phenology by the occurrences of *A. tonsa, P. marinus* and *O. davisae* on the estuaries of Bilbao and Urdaibai have been studied in the first chapter of this thesis.

3.1.3. Diversity changes

After habitat degradation, biological invasions are the main cause of the decrease in total biodiversity, by causing native species extinctions (Bax et al. 2003). Several researchers are intending to calculate the extinction rate that will occurred in the next decades, taking into account present extinction rate and the species that are threatened with extinction according to the red list of the IUCN (IUCN 2020b). These estimates reveal an exceptionally rapid loss of biodiversity over the last few centuries, indicating that a sixth mass extinction is already under way (Ceballos et al. 2015). However, things could be different at local scale. When a NIS became established in a new habitat, it could affect species diversity in two ways. On one hand, it can decrease diversity if it cause extinctions of native species. On the other hand, it can increase diversity by becoming established and contributing to the total number of species present in a region (Sax and Gaines 2003). Nevertheless, the number of species in an area or the species richness is not the only metric that ecologist often use to measure diversity at local scale, because they also included concepts of species evenness (Sax and Gaines 2003). Evenness is a metric that bases in the species abundances, because species abundance could differ largely in a habitat. Thus, if species of an assemblage are equally abundant, then it has high evenness, but if in an assemblage, a species is very abundant and the rest very scarce, then it has low evenness. As there are endless ways of emphasizing different aspects of the species abundance relationship (richness or evenness), there are a huge number of diversity indices that could be used in order to note if the diversity is changing (Magurran 2004).

As NIS could become very abundant, dominating the invaded habitat and decreasing native species abundance, is of highly importance to take into account not only the species richness, but also the evenness in researches where NIS impact on diversity will be studied. Indeed a decrease in the number of species or evenness could altered higher trophic communities or even the whole ecosystem. An ecosystem with low diversity had more probabilities to collapse and fail to overcome from an environmental hazard (Zhou et al. 2010). A clear example of a decrease in diversity due to the introduction of a NIS we could found in the Uruguayan Solís Grande estuary, again with *A. tonsa*, where the evenness and diversity of copepods decreased during summer due to the increase in abundance of *A. tonsa* (Gómez-Erache et al. 2000). Therefore, the impact of NIS (*A. tonsa, P. marinus* and *O. davisae*) on species diversity and community structure changes of the estuaries of Bilbao and Urdaibai has been studied in the second chapter of this thesis.

3.1.4. Ecological niche displacement

The concept of ecological niche has been defined in several ways, but the one given by Hutchinson has been the most influential. Hutchinson (1957) defined the ecological niche as a series of independent environmental variables with simple ranges of suitable conditions defining an “n-dimensional hyperspace” within which the species can survive and reproduce. That definition changed the view of previous authors that the ecological niche is a property of a species and not a property of the environment (Colwell and Rangel 2009). Hutchinson also distinguish two niches: the fundamental niche and the realized niche. The fundamental niche represents the response of all species physiological processes to the effects of environmental factors in the absence of biological interactions. In contrast, the realized niche represents the part of the fundamental niche actually occupied by the species including factors that increase or tight niche breadth. Among the factors that Hutchinson called bionomic and that might distinguish fundamental from realized niches in niche space are living food resources, consumable abiotic resources, competitors, natural enemies and mutualistic partners (Hutchinson 1957). Moreover, Hutchinson viewed the species realized niches reflected in geographical space, in other words, points or regions of the n-dimensional niche space can be projected onto the geographical space guided by the n environmental layers of the map (Colwell and Rangel 2009). This property allow scientist to make questions about the interface between the ecological requirements and broad scale patters of species distributions, helping to explore diversity gradients, the assembly of ecological communities, species invasiveness and responses to global climate change (Pearman et al. 2008).

After occasional introduction, a NIS must either find a niche that is not occupied or compete for an occupied one (Di Castri et al. 1990). When a NIS colonized a new habitat that is not empty, its niche can overlap with native species ones, altering the interactions of the native species by competition for the same resources. Consequently, native species´ realized niche displacement or alteration in their niche breadth could happened. Those changes could destabilised the whole community by causing food web shifts. This must have to take into account, because may happen that a NIS do not altered the diversity or the density of the native species but that it altered their realized niches, changing the interactions and functionalities of the native community. In the Gulf of Finland (Baltic Sea), for example, two *Eurytemora* species (native *E. affinis* and invasive *E. carolleeae*) coexist in the same area. Although, invasive *E. carolleeae* is usually second to *E. affinis* in terms of density, the larger body size and different reproductive traits of *E. carolleeae* confer a potential for it to displace native *E. affinis* species (Sukhikh et al. 2019). Therefore, in the third chapter of this thesis the impact of NIS (*A. tonsa*, *P. marinus* and *O. davisae*) on the native species ecological niches of the estuaries of Bilbao and Urdaibai has been investigated. Moreover, the environmental variables that constrain NIS niches have been determined to assess future risk invasions in nearby habitats.

3.1.5. Range-expansion

After the colonization of a new habitat a NIS is able to range-expansion, which occurs when a population expands into space that was unoccupied by the species. This term usually is called secondary invasion and it used to be via natural dispersal or anthropogenic vectors (Wasson et al. 2001). The NIS *A. tonsa, P. marinus and O. davisae* have a great ability to disperse naturally due to the above-mentioned characteristics: their skill to tolerate sharp salinity changes, high levels of energy metabolism and locomotion activity, the production of resting eggs, in the case of *A. tonsa* (Holm et al. 2018), the ability of *P. marinus* to survive in a torpid state (Svetlichny et al. 2019) and the ability of *O. davisae* to overcome cold season as quasi-diapausing fertilized females (Svetlichny et al. 2016). Thanks to these abilities, among others, *A. tonsa*, for instance, was able to colonize the Marmara Sea by individuals from the Black Sea penetrating through the Bosporus (Hubareva et al. 2008). Likewise, *O. davisae* and *P. marinus* have spread through the coast of the Black Sea because they are able to survive the adverse cold winter–spring environmental conditions of the Black Sea in overwintering stage and does not encounter a strong grazing/predation pressure (Altukhov et al. 2014; Svetlichny et al. 2019).

An anthropogenic vector for secondary transport of NIS that has great influence is the intracoastal ballast water transport (Wasson et al. 2001) and has been supported by several researches. Simkanin et al. (2009) examined intracoastal ballast water transfer along with NIS presence in receiving ports and suggested that intracoastal transport may be a significant vector for the secondary spread of NIS. Cordell et al. (2009) analysed zooplankton in ships arriving in Puget Sound and found that intracoastal transport poses a greater invasion risk, based on the presence of high-risk coastal taxa, than transoceanic transport. Indeed, intracoastal vessels often have shorter voyages, in which organism survivorship is greater (Dibacco et al. 2012).

In the fourth chapter of this thesis, a preliminary study of the range-expansion of the NIS *A. tonsa*, *P. marinus* and *O. davisae* is reported. The research is focused on the presence of those species in several estuaries of the Basque coast, which has allowed us to make an approximation of which variables could have helped the establishment of a population in each estuary in order to take into account for future research.

3.2. Invasions in the estuaries of Bilbao and Urdaibai

Monitoring programs are the best tools to study the impact of NIS in new habitats. In this sense, thanks to two existing monitoring programs in the estuaries of Bilbao and Urdaibai (located in the Bay of Biscay), data on the zooplankton community previous and after the occurrence of the above-mentioned NIS copepods *A. tonsa*, *P. marinus* and *O. davisae* are available. These systems are located in the same biogeographical region but they have different morphological, physical-chemical characteristics and anthropogenic effects. All that makes those monitoring programs a good case study to analyse the impact that *A. tonsa, P. marinus* and *O. davisae* could have in long term in the native zooplankton community.

A. tonsa was identified for the first time in 2001 in the estuary of Bilbao, where it has been the dominant calanoid of the zooplankton in the inner estuary since then, altering the distribution of the native copepod *Acartia clausi* (Aravena et al. 2009). Two years later, it was also observed in the estuary of Urdaibai (Aravena et al. 2009) causing changes in the seasonal pattern of the native species *Acartia bifilosa* (Villate et al. 2018). In the year 2001, *O. davisae* also occurred in the estuaries of Bilbao and Urdaibai, simultaneously, becoming very abundant in the case of the estuary of Bilbao (Uriarte et al. 2016). Afterwards, in the year 2010 *P. marinus* occurred in the estuary of Bilbao (Uriarte et al. 2016). Moreover, in the year 2010 other expanding copepods (OES), such as *A. bifilosa* and *Calanipeda aquaedulcis,* were firstly recorded in the estuary of Bilbao. These species absent in previous studies on this estuary but indigenous in the nearby estuaries of Urdaibai and Plentzia (Villate and Orive 1981; Villate 1989). Before this thesis, the occurrences of those NIS were reported and the impact that they cause on native zooplankton had been reported only in the case of *A. tonsa* and for species of the same family (Acartiidae). Therefore, the impact of those NIS on the total zooplankton and copepod community in a long term is studied in this thesis.

OBJECTIVES

General objective:

To analyse the colonization process by non-indigenous species (NIS) and other expanding species (OES) of planktonic copepods, and the impact of such colonising species on native zooplankton communities, in estuaries of the Basque coast in relation to the natural and anthropogenic differences between estuaries, in order to assess the invasive character of the studied colonizing copepods and the sensitivity of the studied estuaries to biological invasions.

Specific objectives:

1. To describe the process of occurrence and establishment of the NIS *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae* and the OES *Acartia bifilosa* and *Calanipeda aquaedulcis* in the estuaries of Bilbao and Urdaibai during the 1998-2015 period, and to assess and compare their impact in terms of quantitative relevance and effect on the abundance and phenology of total copepods, total zooplankton, congeneric species and other relevant components of the zooplankton in different salinity zones of both estuaries (Chapter 1).

2. To describe comparatively the changes in the structure and diversity of zooplankton communities at different salinities of the estuaries of Bilbao and Urdaibai from 1998 to 2015, and determine the role of the NIS *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae,* and the OES *Acartia bifilosa* and *Calanipeda aquaedulcis* in such changes (Chapter 2).

3. To determine and compare the environmental niche of the NIS *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae,* and the OES *Acartia bifilosa* and *Calanipeda aquaedulcis* in the estuaries of Bilbao and Urdaibai, and assess the effect of the introduction of these species on the realized niche of zooplankton taxa in both systems (Chapter 3).

4. To verify the presence of the NIS *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae* in the estuaries of Plentzia, Oria and Bidasoa, and compare their situations with that observed in the estuaries of Bilbao and Urdaibai to have an overall picture of the expansion of these NIS in the estuaries of the Basque coast and explore the mechanisms of arrival and colonizing success in our study area (Chapter 4).

STUDY AREA

The bulk of the research exposed in this Thesis has been carried out in the estuaries of Bilbao and Urdaibai located on the Basque coast, southeastern Bay of Biscay (Bilbao 43º 23' N, 03º 07' W; Urdaibai 43º 22' N, 02º 43' W) (Fig. 6). Therefore, a common and general description of both systems will be detailed in this part. On the other hand, in Chapter 4 the estuaries of Plentzia, Oria, and Bidasoa were also included, but their description will be addressed in that chapter. All the river systems that make up the estuaries of the Basque coast are characterized by their short course and high slope, a consequence of the short distance between the Cantabrian Mountains, where they originate, and the coast (Villate et al. 1989). Moreover, the area is characterized by a temperate-oceanic climate with moderate winters and warm summers and a variable distribution of precipitation and temperature (Usabiaga et al. 2004). The tidal is semidiurnal, with amplitudes less than a meter in neap tides to more than 4.5 meters in spring tide (Villate et al. 1989).

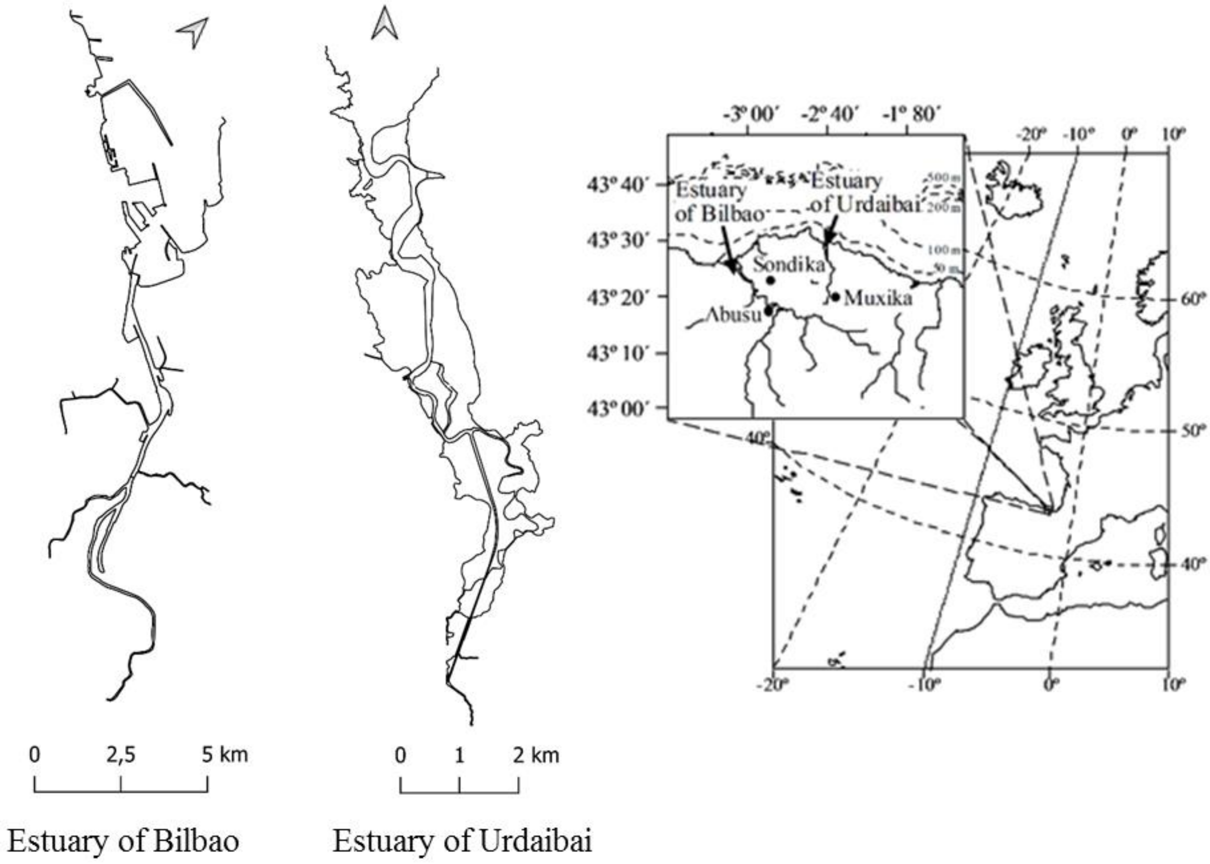

Fig. 6: Schematic map and location of the estuaries of Bilbao and Urdaibai.

1. The estuary of Bilbao

The estuary of Bilbao is a relatively short (23 km) and shallow (0.5 m deep at the inner part and 32 m deep at the outer coastline site) mesomacrotidal system of around 24 km^2, that crosses urban and industrial areas (Fig. 7). The two main rivers flowing into Bilbao estuary head are Ibaizabal and Nerbioi with a mean fluvial of 35.56 m^3/s (Monge-Ganuzas et al. 2019), although some small tributaries flow into the middle part of the estuary (21 m^3/s). The basin area is the largest of the estuaries of the Basque coast, being around 1798.8 km^2 (Borja et al. 2004). As it is a big estuary, the volume of the estuary is around 200 x 10^6 m^3, the average tidal prism is $77 \cdot 10^6$ m^3 and tidal flushing is relatively low, therefore 2 months are needed to infill the mean volume of the estuary (Valencia et al. 2004). Maximum water residence time is around 29 days, being lower in the channelized upper and middle reaches than in the outer Abra embayment, and it is also much lower in above halocline layers (0.1-1.6 days) than in below halocline layers (0.3-11.6 days) for most of the estuary length (Uriarte et al. 2014). In waters below the halocline, salinity decreases from the outer to the inner estuary, presenting an axial decreasing gradient in salinity. Moreover, the inner part is strongly stratified with salinity below the halocline around 30, whereas the outer part is partially mixed (Intxausti et al. 2012). Apart from that, subtidal sediments are muddy sands along the entire estuary, with highest mud and organic matter contents in the intermediate zone and lowest in the outer parts (Cotano and Villate 2006).

In the 19th century, the development of the mining industry surrounding the city of Bilbao (Miribilla and Triano), favored the metallurgical industry to settle on the banks of the estuary. The steel industry supported a whole constellation of companies dedicated to the transformation of the metal and a flourishing shipyards (Euskalduna Company), together with chemical companies that manufacture explosives and textile companies. This industrialization led to the increase of immigration and of the urban areas. At the end of the century, a tenth part of the iron in the world was obtained in Bizkaia. In 1902, a large part of the steel companies was clustered in Altos Hornos de Vizcaya, S.A, and the beginning of the age of large steel factories located basin of the estuary and in the vicinity of the iron ore deposits (Arranz 2012). Industrial discards and the out-of-control raw sewage discharges, led to very high heavy metal concentrations and a permanent hypoxia/anoxia situation in the estuary (Villate et al. 2013; Irabien et al. 2018) affecting all the system, becoming azoic. However, the implementation, in 1979, of a comprehensive plan for the sanitation of the metropolitan area of Bilbao, and the decline of the heavy industry in the 80s, have promoted a rehabilitation process of the environmental conditions of the estuary, that still going on. Heavy metal concentration has decreased (Fdez-Ortiz de Vallejuelo et al. 2010) and dissolved oxygen has increased considerably from mean annual values around 40 % to 80 % (Villate et al. 2013). The improvement in environmental conditions has allowed the species to (re)colonize the estuary and increase the diversity, recovering the macrobenthic community (Borja et al. 2006) and the pelagic community (Uriarte et al. 2016). Nevertheless, organic matter and inorganic nutrient inputs are still high, rendering the system eutrophic (García-Barcina et al. 2006).

Nowadays, the ecological status of the inner estuary has been described as bad, that of the intermediate estuary as poor and that of the outer estuary as moderate (Franco et al. 2004). In fact, it is a totally man-modified estuary, the land reclamation has been very strong and the whole estuary is fully channelized; in fact, today subtidal zones occupy almost 100 % of the estuarine surface area (Cearreta et al. 2004). Moreover, an intense dredging had been done to increase estuary depth in the past. Nonetheless, nowadays they continue dredging in the recently opened channel of Deustu due to an urbanization project that are carrying out in the island of Zorrotzaurre.

Apart from that, the port facilities located in the outer part of the estuary (Abra harbour) are one of the most important marine transport and logistics centers in the European Atlantic Arc. According to their annual balance of 2019 (Bilbao Port 2020) the total traffic was around $35.5 \cdot 10^6$ movements, specially associated to merchant services. Those cargo boats (the ones that introduced species through ballast water) origin are worldwide.

Fig. 7: From left to right, up and down: Images of the inner part, middle parts and outer part of the estuary of Bilbao (author: Ziortza Barroeta).

2. The estuary of Urdaibai

The mesomacrotidal estuary of Urdaibai is shorter (12.5 km long), less extensive (7.7 km^2) and shallower (3 m depth on average) than the nearby estuary of Bilbao, and therefore contains a much lower volume of water (3.3×10^6 m^3) (Borja et al. 2004). In contrast to the estuary of Bilbao, subtidal zones cover around 30 % of the surface area, and extensive intertidal flats and sandy beaches in the outer reaches and salt marshes and reed beds in the middle and inner reaches can be found. However, the inner part is an artificial channel of 15 m wide (Villate et al. 1989) (Fig. 8). The main river flowing into this estuary is Oka, although it also has two small tributaries that are Mape and Golako, with a mean fluvial of 3.6 m^3/s (Monge-Ganuzas et al. 2019) and the basin area is about 183.21 km^2 (Borja et al. 2004). Moreover, it is a system with low water residence time (10 days to infill the mean volume of the estuary) (Valencia et al. 2004), with an average of tidal prism of 4858300 m^3 (Villate et al. 1989). Furthermore, it is characterized by a strong horizontal salinity gradient, indeed, the outer part is dominated by high tidal fluxing and well-mixed marine water at high tide, whilst the middle-inner part is partially stratified (Villate et al. 2017), salinities below the halocline in the inner part being generally around 26. Furthermore, subtidal sediments in the outer zone are exposed sands, in the intermediate and inner zones are muddy sands, with lower organic matter contents in the outer zone and higher ones in the intermediate and inner zones (Cotano and Villate 2006).

The estuary of Urdaibai and its watershed were designated a Biosphere Reserve in 1984, because not only the 12 kilometers of sandy areas that form the estuary at its mouth constitute the most important wetland area in the Basque Country, but also, it becomes a resting and wintering place for many species of rare migratory birds in the Iberian Peninsula such as the gray heron (*Ardea cinerea*), the cormorant (*Phalacrocorax* sp.), the tern (*Sternidae* sp.), the spoonbill (*Platalea leucorodia*) and a large number of waders, along with fish, amphibians and reptiles. In addition, this landscape and ecological diversity is completed by prehistoric sites such as Santimamiñe or the necropolis of the Roman occupation in Forua (Eusko Jaurlaritza/Gobierno Vasco 2020). Nevertheless, in the upper reaches the estuary receives inputs or inorganic nutrients and organic matter from a small sewage treatment plant located in the inner part (Cotano and Villate 2006), the ecological status in this area being described as poor (Franco et al. 2004), whereas in the outer zone, the ecological status is good because as tidal flushing is high, waters of salinities > 34 are flushed out of the estuary with each tidal cycle (Villate et al. 1989). Nevertheless, there are other human activities, for example in the middle of the estuary there is a small shipyard (Astilleros Murueta) whose boats are finished in the nearby estuary of Bilbao. Likewise, in the outer part of the estuary they are several ports located, two of them are small ports basically with recreational boats (the ports of Mundaka and Busturia) and the third one (the port of Bermeo) is relatively a big port, located in an strategic navigational point of the Gulf of Biscay and one of the main fishery ports of the Basque coast. It has also a small dock for recreational boats (Euskal Kirol Portuak 2020).

Fig. 8: From left to right, up and down: Images of the inner part, middle parts and outer part of the estuary of Urdaibai (author: Ziortza Barroeta).

GENERAL METHODS

As the data used for the Chapter one, two and three were gathered from the same monitoring program, and in order to avoid unnecessary duplication, the data acquisition, and pretreatment have been extracted from every chapter and detailed below. For the specific methodologies, and also for further details on the data acquisition of Chapter four, read the method section of the corresponding chapter.

1. Data acquisition

Biological and environmental data used in Chapters one, two and three belong to the 1998-2015 period of the ongoing monitoring program of the zooplankton community that is being carried out in the estuaries of Bilbao and Urdaibai since 1998. Samplings were conducted monthly at high tide at the sites of salinities of 30, 33, 34 and 35 in the estuary of Bilbao (B30, B33, B34 and B35) and salinities of 26, 30, 33 and 35 in the estuary of Urdaibai (U26, U30, U33 and U35) (Fig. 9). At each site temperature, salinity and dissolved oxygen vertical profiles were obtained every 0.5 m using a multi-parameter water quality meter. The Secchi disk depth was also measured. Water collected with a Niskin bottle at mid depth below the halocline was filtered for spectrophotometric chlorophyll *a* determination, values being calculated as in Lorenzen (1967). Additionally, salinity stratification index was calculated as the maximum difference in salinity between consecutive depths (Villate et al. 2013).

Zooplankton samples were also collected below the halocline by 2-3 min horizontal tows using a 200 µm mesh size net equipped with a Mechanical Flow Meter. Contamination by zooplankton located above the halocline was expected to be negligible due to the rapid deployment and retrieval of the net at the beginning and the end of the sampling time, the thinness of the above halocline water layer and the scarcity of mesozooplankton there due to the shorter flushing time (Uriarte et al. 2014). Zooplankton samples were preserved in 4 % buffered formalin. The qualitative and quantitative analyses of zooplankton samples were performed by diluting the sample to a known volume (10-1000 ml), in order to obtain a suitable density of individuals, and by extracting enough aliquots for the identification and counting of individuals until at least 100 individuals of the most abundant taxon and 30 individuals of the second and third most abundant taxa, under an inverted stereomicroscope. Identification was made to the lowest possible taxonomic level, which was the species level for most copepods and main holoplankton groups, but the class or family level for most meroplankton groups.

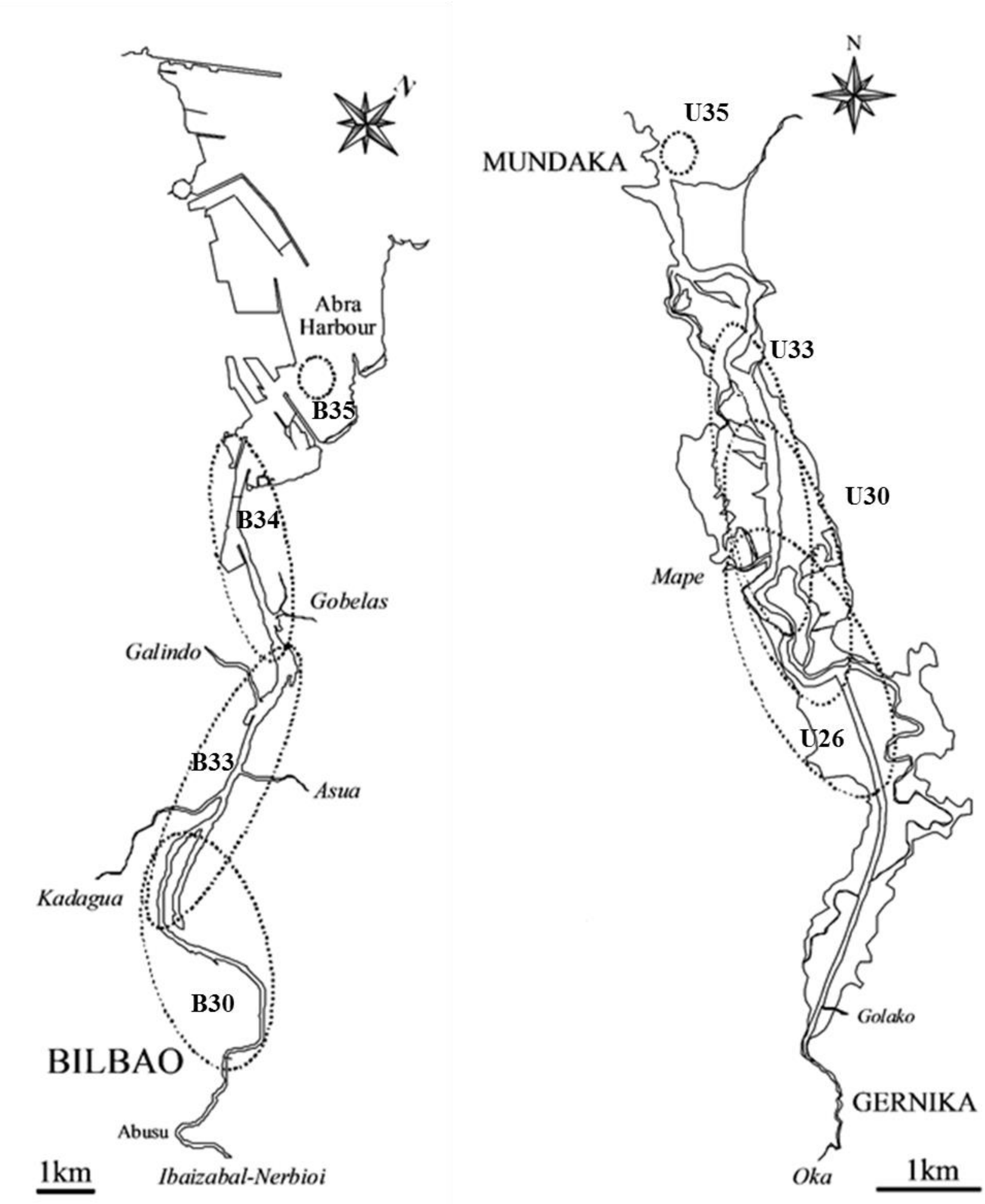

Fig. 9: Maps of the estuaries of Bilbao and Urdaibai showing the spatial range of the sampling salinity zones (26, 30, 33, 34 and 35).

2. Data pretreatment

Sporadic missing values (less than 8-11 % per variable) of both environmental and zooplankton variables in the time series were filled with the mean of the preceding and following month values. However, in the data series of the estuary of Urdaibai more than two consecutive values for U26 chlorophyll *a* concentration were missing between 1998 and 2000 and so, in these years, the annual mean values were not estimated for this variable.

To describe the colonization process, the study period was split into three periods corresponding to three different steps in the establishment of the species: a first period that spans from 1998 to 2002 (before the establishment of *A. tonsa* and *O. davisae* populations), a second period that goes from 2003 to 2009 (since the occurrence of *A. tonsa* and *O. davisae* in large numbers and before the occurrence of *P. marinus*) and a third period that extends from 2010 to 2015 (since the occurrence of *P. marinus* and characterized by the marked increase of *C. aquaedulcis*, a species previously detected occasionally at negligible densities).

CHAPTER 1: Differences in the colonization success and impact of non-indigenous and other expanding copepod species on the zooplankton of two contrasting estuaries of the Bay of Biscay

The colonization success of non-indigenous species (NIS) of copepods that arrived in the estuaries of Bilbao and Urdaibai (Bay of Biscay) and other expanding species (OES) of copepods that arrived in the former estuary during the 1998-2015 period, as well as the impact of such species on the density and seasonal patterns of congeneric species, total copepods and total zooplankton have been analysed. The estuary of Bilbao was successfully colonized by the NIS *Acartia tonsa* and *Oithona davisae* (since 2003) and by the NIS *Pseudodiaptomus marinus* and the OES *Calanipeda aquaedulcis* (since 2010), coinciding with the progressive improvement of environmental conditions in the rehabilitation process of this estuary. The three NIS species also arrived in the estuary of Urdaibai during the same time period, but only *A. tonsa* became abundant, likely due to inherent natural constraints for the other two species. *A. tonsa* in both estuaries, and *O. davisae* and *C. aquaedulcis* in the estuary of Bilbao became influential in the density, composition and dynamics of total copepods and zooplankton in water-masses of 33 or lower salinity. The highest impact was found in the inner estuary of Bilbao, where total copepods and zooplankton reached densities comparable to those of the outer estuary and copepod maxima moved from spring to summer due to the contribution of the colonizing copepod species. The congeneric species most affected by the NIS were the neritic species *Acartia clausi* and *Oithona nana* in the estuary of Bilbao, and the brackish species *Acartia bifilosa* in the estuary of Urdaibai.

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1.1 Introduction

Estuaries have been especially common sites of invasions, accruing from tens to hundreds of NIS per estuary that represent most major taxonomic and trophic groups (Ruiz et al. 1997). However, within the zooplankton, the most abundant metazoans on earth, estuarine NIS impacts have been little studied (Bollens et al. 2002). Monitoring of zooplankton in two Basque coast (southeastern Bay of Biscay) estuaries since 1998 has revealed the introduction of several NIS of copepods of Indo-pacific origin, i.e. *Acartia tonsa*, *Oithona davisae* and *Pseudodiaptomus marinus* and the appearance of other expanding species (OES) of copepods, i.e. *Calanipeda aquaedulcis* and *Acartia bifilosa* (Aravena et al. 2009; Uriarte et al. 2016; Villate et al. 2018). *A. tonsa* was observed for the first time in the year 2001 in the estuary of Bilbao, where it became the dominant calanoid copepod of the inner estuary zooplankton (Uriarte et al. 2016), displacing the distribution of the indigenous *A. clausi* seawards (Aravena et al. 2009). This species was first recorded in the estuary of Urdaibai in 2003, causing changes in the seasonal pattern of the indigenous *A. bifilosa* (Villate et al. 2018). *O. davisae* and *P. marinus* were detected in the estuary of Bilbao in 2001 and 2010, respectively (Uriarte et al. 2016), thus extending the recent spreading of these two species in European waters (Jha et al. 2013; Gubanova et al. 2014; Cornils and Wend-Heckmann 2015; Sabia et al. 2015).

In the estuaries of Bilbao and Urdaibai, the impact of *O. davisae* and *P. marinus* has not been studied yet. In the case of *A. tonsa* the impact on the indigenous species of the same family (Acartiidae) has been analysed (Villate et al. 2018), but there is no information about its impact on the whole copepodian and zooplanktonic assemblages over a relatively long time period. Moreover, no studies on the impact of other expanding copepods, i.e. *C. aquaedulcis*, in these estuaries have been carried out yet.

The aims of the present work were to analyse the population changes of the NIS *A. tonsa, O. davisae* and *P. marinus* after colonizing the estuaries of Bilbao and Urdaibai during the 1998-2015 period, and to assess and compare the impact of these NIS in terms of their quantitative relevance and effect on the abundance and phenology of total copepods, total zooplankton, congeneric species and other relevant components of the zooplankton in different salinity zones of both estuaries. In the estuary of Bilbao, the dynamics and impact of the OES *C. aquaedulcis* and *A. bifilosa,* which arrived in this system during the study period, were also analysed.

1.2 Method

1.2.1 Data analyses

For the purpose of this study, the copepods *Paracalanus* spp., *Clausocalanus* spp., *Pseudocalanus elongatus*, *Ctenocalanus vanus* together with the unidentified copepodites of these species were grouped in a single copepod assemblage under the term "PCPC-calanus", *Paracalanus parvus* being much more abundant than the other species of this group (Fanjul et al. 2017). To summarize environmental variability, the seasonal (winter, spring, summer, autumn) means of water temperature, salinity stratification, percent saturation of dissolved oxygen, Secchi disk depth and chlorophyll *a* were calculated for each period at each salinity zone of both estuaries.

To describe the temporal and spatial dynamics of target species, interannual variations in the density of the NIS *A. tonsa*, *O. davisae* and *P. marinus*, the OES *A. bifilosa* and *C. aquaedulcis*, and the congeneric species of the NIS (*A. clausi, A. margalefi, A. discaudata, P. grani, O. nana, O. similis* and *O. plumifera*) at each salinity site of both estuaries were depicted. Additionally, the interannual changes of density and relative abundance of total NIS of copepods, total OES of copepods, total copepods excluding NIS and OES and total non-copepod zooplankton were also represented. To analyse phenological changes, the monthly means of the densities of NIS, OES, total zooplankton, non-copepod zooplankton, total copepods and copepods excluding NIS and OES for each study period at each salinity site of both estuaries were calculated and their seasonal variability depicted. Density data were log (x+1) transformed to better show density differences between sites.

The percentage contribution to total zooplankton of the main individual taxa, total NIS of copepods, total OES of copepods, total copepods excluding NIS and OES and non-copepod zooplankton was calculated at each salinity site of both estuaries for the entire study period.

The contribution of the different taxa (including NIS, OES and the other zooplankton categories distinguished in this study) to the observed interannual changes in total zooplankton and copepod densities was analysed by means of generalized additive models (GAM) (Wood 2004), using the mgcv package of statistical R software. Prior to this, we tested that the data met the assumptions of the analysis and calculated monthly anomalies (the difference between each value and the mean value for each month for the period under study divided by the standard deviation) in order to avoid the "seasonal effect" which is common in plankton time series.

1.3 Results

1.3.1 Environmental scenario

As shown in Table 1, water temperature was lowest in spring during the last period (2010-2015) and in autumn during the first period (1998-2002), and highest in summer during the intermediate period (2003-2009) at all salinity sites of both estuaries. However, in winter it was highest during the first period in the estuary of Bilbao but during the last period highest values were recorded in the estuary of Urdaibai at all salinity sites. 2003 and 2006 were the warmest years. Salinity stratification was usually much higher in the estuary of Bilbao and increased with decreasing salinity in both estuaries. Throughout the study period it increased from the first to the last period at the 30, 33 and 34 salinity sites of the estuary of Bilbao, whilst no clear pattern was observed at the highest salinity site of Bilbao and in the estuary of Urdaibai. In both systems, the dissolved oxygen saturation (%) at the sampling depth increased with salinity, but values were lower in the estuary of Bilbao where dissolved oxygen did not reach normoxic conditions (60-100%) at the 30 salinity site until the last period. Highest values were recorded during this last period at all sites, with the exception of the 26 salinity site of Urdaibai. Secchi disk depth also increased landwards along the axial salinity gradient and, in most cases, highest values were recorded during the last period. Chlorophyll-*a* concentration showed a general decreasing pattern with salinity that was stronger in the estuary of Urdaibai than in the estuary of Bilbao, and most marked in summer. Chlorophyll *a* maxima occurred in summer, except for the spring maximum at the 35 salinity site of Urdaibai, and in all cases summer chlorophyll *a* decreased from the first to the last period.

Table 1. Mean values of water temperature (ºC), salinity stratification, dissolved oxygen saturation (%), Secchi disk depth (m) and chlorophyll *a* concentration (µg/l) at each salinity site (Sal) of the estuaries of Bilbao (30, 33, 34 and 35) and Urdaibai (26, 30, 33 and 35) in winter (W), spring (Sp), summer (S) and autumn (A), during the periods 1998-2002, 2003-2009 and 2010-2015. The highest values between periods in bold, and the lowest ones in italics.

	Sal	Period	Water temperature (°C)				Salinity stratification				Dissolved oxygen saturation (%)				Secchi disk depth (m)				Chlorophyll *a* (µg/l)			
			W	Sp	S	A	W	Sp	S	A	W	Sp	S	A	W	Sp	S	A	W	Sp	S	A
Estuary of Bilbao	30	1998-2002	**12.25**	**14.75**	*20.63*	*16.03*	*13.88*	*10.81*	*9.33*	*9.40*	*41.91*	*31.75*	*16.03*	39.41	*0.74*	*1.05*	*0.96*	*0.87*	*0.50*	**2.96**	**8.42**	1.61
		2003-2009	*12.00*	14.42	**21.30**	16.84	16.08	16.68	12.81	15.87	59.06	37.96	17.51	*34.85*	**1.15**	1.26	1.02	**1.13**	**0.80**	2.90	5.01	**2.20**
		2010-2015	12.03	*14.25*	20.71	**17.11**	**17.76**	**18.84**	**14.29**	**18.90**	**68.27**	**53.24**	**24.64**	**46.16**	0.96	**1.36**	**1.25**	1.00	0.79	*1.80*	*3.83*	*1.49*
	33	1998-2002	**12.55**	**14.50**	*20.30*	*15.99*	*9.81*	*8.90*	*5.78*	*7.35*	*69.77*	*70.11*	*57.16*	*61.65*	1.19	*1.27*	*1.14*	*1.09*	0.61	**2.60**	**7.91**	**1.25**
		2003-2009	12.46	14.44	**20.85**	16.73	11.45	13.11	6.44	9.40	80.57	78.00	84.46	71.74	**1.29**	1.38	1.54	**1.53**	**0.70**	*1.98*	4.72	1.24
		2010-2015	*12.44*	*14.19*	20.37	**17.00**	**16.28**	**15.20**	**7.73**	**10.10**	**86.79**	**88.48**	**87.04**	**77.83**	*1.12*	**1.62**	**1.67**	1.43	*0.58*	2.45	*3.21*	*1.13*
	34	1998-2002	**12.60**	14.34	*20.18*	*15.98*	*7.57*	*4.33*	*1.91*	*5.01*	*87.73*	*94.32*	*87.24*	*78.86*	1.63	*1.53*	2.04	*1.53*	0.50	2.65	**6.31**	1.01
		2003-2009	12.57	**14.46**	**20.63**	16.72	9.96	8.14	2.83	6.56	88.82	94.58	103.84	84.76	**1.68**	2.03	*1.98*	**2.08**	*0.48*	*2.35*	5.31	**1.16**
		2010-2015	*12.54*	*14.12*	*19.98*	**16.84**	**13.47**	**9.95**	**4.08**	**7.44**	**95.06**	**105.65**	**103.37**	**90.30**	*1.20*	**2.19**	**2.71**	1.86	**0.52**	**2.92**	*3.32*	*0.98*
	35	1998-2002	**12.86**	14.25	20.21	*16.06*	*3.98*	*1.95*	*0.77*	*2.44*	*94.94*	102.79	*101.50*	*89.73*	**3.53**	4.11	*3.29*	*2.95*	**1.05**	**3.38**	**5.97**	*0.76*
		2003-2009	12.66	**14.44**	**20.49**	16.67	**8.03**	3.85	**1.12**	**3.28**	95.87	*101.09*	109.04	100.67	*2.68*	*3.72*	3.40	3.54	0.85	2.37	3.75	**1.87**
		2010-2015	12.66	*14.08*	*19.77*	**16.91**	4.98	**4.29**	1.04	2.92	**105.09**	**111.03**	**114.36**	**102.33**	3.18	**5.92**	**5.82**	**4.26**	0.62	*2.17*	*1.82*	0.78
Estuary of Urdaibai	26	1998-2002	11.05	15.83	22.37	*15.13*	5.24	**3.51**	2.06	*3.83*	*85.10*	*83.60*	60.43	*68.94*	1.32	*1.21*	*1.26*	**1.30**	*0.57*	**7.27**	**10.58**	2.34
		2003-2009	*10.98*	**16.32**	**23.25**	**16.01**	**7.24**	3.23	**3.01**	**4.28**	86.05	88.15	*60.37*	**71.94**	*1.29*	1.40	1.32	1.08	0.85	6.98	6.14	**2.60**
		2010-2015	**11.54**	*15.64*	*22.07*	15.98	*4.27*	*2.80*	*1.78*	4.11	**91.96**	**91.99**	**60.88**	69.25	**1.42**	**1.67**	**1.41**	*1.02*	0.85	*4.12*	*4.26*	*1.92*
	30	1998-2002	11.43	15.53	*22.11*	*15.25*	*3.31*	**3.02**	1.06	2.87	*89.46*	*89.13*	*73.19*	79.47	1.63	*1.53*	1.60	*1.51*	1.09	2.93	**6.73**	*1.34*
		2003-2009	*11.29*	**15.94**	**22.96**	16.18	**5.61**	3.00	*0.65*	*2.27*	90.57	91.49	77.52	*77.82*	*1.52*	1.76	*1.53*	1.54	**0.82**	**5.10**	5.05	**1.78**
		2010-2015	**11.72**	*15.35*	21.72	16.18	4.24	*2.41*	**1.44**	**3.75**	**94.54**	**97.51**	**79.15**	**83.38**	**1.91**	**1.92**	**1.74**	**1.59**	*0.67*	*2.60*	*4.13*	1.44
	33	1998-2002	11.90	15.30	21.69	*15.52*	*2.97*	**2.93**	0.54	2.84	*91.90*	97.70	*86.36*	88.10	**2.11**	*1.86*	*1.91*	*1.71*	0.84	1.97	*3.15*	**1.23**
		2003-2009	*11.64*	**15.49**	**22.35**	**16.37**	**3.94**	*1.99*	*0.57*	*1.08*	94.12	*94.48*	90.28	*87.93*	2.02	2.44	2.02	2.06	**0.87**	**2.60**	3.86	1.13
		2010-2015	**11.98**	*15.03*	*21.21*	16.32	3.57	2.76	**1.04**	**2.92**	**98.78**	**104.62**	**95.45**	**93.92**	*1.94*	**2.47**	**2.03**	**2.13**	*0.61*	*1.73*	**4.08**	*1.01*
	35	1998-2002	12.45	**14.98**	21.08	*16.11*	*0.26*	0.36	0.06	0.25	*95.49*	104.94	*99.29*	*94.97*	**3.27**	3.35	*2.50*	3.00	0.51	**1.50**	**0.76**	**0.93**
		2003-2009	*12.38*	14.85	**21.35**	16.85	**0.51**	*0.34*	0.06	*0.09*	97.50	*97.88*	102.51	96.81	2.87	**4.22**	**3.26**	*2.74*	**0.83**	1.45	*0.58*	*0.67*
		2010-2015	**12.62**	*14.43*	*20.32*	**16.86**	0.27	**0.40**	**0.36**	**0.31**	**103.60**	**112.56**	**108.39**	**104.53**	*2.45*	---	3.00	**3.30**	*0.45*	*0.97*	0.69	0.73

1.3.2 Zooplankton composition

Zooplankton taxa used in this study and their contribution to total zooplankton at each salinity site in both estuaries for the 1998-2015 period are shown in Table 2. In the estuary of Bilbao, in addition to bivalve larvae, only the NIS of copepods (*Acartia tonsa*, *Oithona davisae* and *Pseudodiaptomus marinus*) and OES of copepods (*Calanipeda aquaedulcis* and *Acartia bifilosa*) had the highest contribution to total zooplankton at the innermost salinity site (B30), whilst most copepod species and other holoplankton and meroplankton taxa had the highest contribution at the outermost salinity site (B35). The copepods *Acartia discaudata* and *Acartia margalefi*, cirripede larvae and appendicularians were the only taxa that showed highest contributions at the intermediate salinity sites of 33 and 34. In the estuary of Urdaibai only the NIS *A. tonsa* and gastropod larvae reached the highest contribution at the innermost salinity site (U26), and the NIS *O. davisae* and the native *A. bifilosa* were the only taxa with peak contributions at U30. The number of species and groups that showed their highest contribution at U33 was higher than at U30 and even higher at U35.

In both estuaries total NIS showed the highest contribution within the estuary at the innermost site (B30 and U26), total copepods excluding NIS and OES at the outermost site (B35 and U35) and non-copepod zooplankton at an intermediate salinity (B34 and U33). However, NIS of copepods dominated the zooplankton community for the entire study period only at the lowest salinity site of the estuary of Bilbao (B30). In this estuary, non-copepod zooplankton dominated at intermediate salinities, mainly due to the high contribution of cirripede larvae, and total copepods excluding NIS and OES contributed most at B35, mainly due to the high contribution of *Acartia clausi* and PCPC-calanus. In the estuary of Urdaibai, total copepods excluding NIS contributed most at all salinity sites, mainly due to the high contribution of *A. bifilosa* at salinities ≤33 and to *A. clausi* and PCPC-calanus at U35.

Table 2. Mean contribution (%) of selected taxa to total zooplankton density at each salinity zone of the estuaries of Bilbao (30, 33, 34 and 35) and Urdaibai (26, 30, 33 and 35) during the study period (1998-2015). The highest contributions between salinity sites of each estuary in bold.

			Estuary of Bilbao				Estuary of Urdaibai			
		Taxa	30	33	34	35	26	30	33	35
Copepods	NIS	*Acartia tonsa*	**37.51**	29.51	5.30	0.81	**26.86**	7.61	2.57	0.01
		Oithona davisae	**12.47**	4.08	2.52	0.06	0.02	**0.18**	0.02	0.00
		Pseudodiaptomus marinus	**0.38**	0.21	0.05	0.00	0.00	0.00	0.00	0.00
	OES	*Calanipeda aquaedulcis*	**12.49**	0.25	0.03	0.00	**0.01**	**0.01**	0.00	0.00
		Acartia bifilosa	**2.41**	0.30	0.30	0.00	36.77	**49.45**	22.19	0.12
		Acartia clausi	1.87	7.53	20.51	**24.63**	0.58	2.00	9.27	**35.71**
		Acartia discaudata	0.02	0.09	**0.29**	0.04	0.00	0.00	**0.04**	0.01
		Acartia margalefi	0.20	**0.33**	0.17	0.02	0.00	0.01	**0.13**	0.00
		Paracartia granii	0.00	0.00	0.00	0.00	0.03	0.21	**0.79**	0.00
		Oithona nana	0.27	0.80	2.04	**2.45**	0.23	0.54	1.54	**1.86**
		Oithona similis	0.29	1.12	1.78	**1.84**	0.68	2.77	**5.20**	3.67
		Oithona plumifera	0.02	0.09	0.18	**0.32**	0.01	0.04	0.24	**0.37**
		PCPC-calanus	1.10	4.16	7.32	**13.79**	0.71	2.62	8.45	**17.58**
		Temora longicornis	0.03	0.05	0.14	**0.35**	0.04	0.15	**0.46**	0.29
		Temora stylifera	0.04	0.22	0.53	**1.16**	0.05	0.17	0.58	**0.69**
		Euterpina acutifrons	0.11	0.33	0.89	**1.14**	0.21	0.90	**2.33**	1.90
		Ditrichocorycaeus anglicus	0.01	0.02	0.07	**0.14**	0.01	0.05	0.17	**0.23**
		Oncaea media	0.04	0.12	0.42	**1.07**	0.14	0.36	1.53	**2.37**
		Podon sp.	0.03	0.12	0.72	**1.16**	0.04	0.06	0.11	**0.48**
		Evadne nordmanni	0.07	1.32	2.34	**3.10**	0.13	0.46	1.45	**1.72**
		Evadne spinifera	0.00	0.05	0.14	**0.45**	0.01	0.02	0.22	**0.39**
		Appendicularians	2.01	4.08	**5.26**	4.68	0.20	1.09	2.24	**2.39**
		Doliolids	0.01	0.03	0.33	**1.13**	0.01	0.01	0.08	**0.52**
		Chaetognaths	0.08	0.31	0.36	**0.56**	0.05	0.11	0.25	**0.30**
		Siphonophores	0.05	0.42	0.85	**1.25**	0.04	0.17	0.50	**0.51**
		Bivalve larvae	**12.11**	1.98	2.00	1.34	0.12	0.42	1.25	**1.82**
		Gastropod larvae	0.86	1.08	1.59	**1.93**	**16.42**	10.64	9.93	7.56
		Cirripede larvae	10.41	**36.65**	34.66	24.38	12.29	11.58	**14.08**	9.14
		Decapod larvae	0.05	0.46	0.62	**0.72**	0.14	0.43	**0.68**	0.28
		Ictioplankton	0.04	0.10	0.25	**0.38**	0.02	0.03	0.07	**0.08**
		Medusae	0.20	0.12	0.30	**0.43**	0.13	0.24	**0.50**	0.29
		Echinoderm larvae	0.00	0.02	0.07	**0.12**	0.00	0.02	0.03	**0.05**
		Total NIS	**50.36**	33.80	7.87	0.87	**26.88**	7.79	2.60	0.01
		Total OES	**14.90**	0.54	0.33	0.00	---	---	---	---
		Copepods excluding NIS & OES	7.06	16.38	36.02	**50.05**	41.46	64.23	60.40	**68.39**
		Non-copepod zooplankton	27.68	49.28	**55.78**	49.08	31.66	27.98	**37.00**	31.59

1.3.3 Interannual variations and between-period differences in zooplankton

NIS and OES of copepods

As shown in Figure 1, the density of *A. tonsa* increased abruptly in 2003, the first year of the period 2003-2009, and thereafter it showed relatively high densities in both estuaries at sites of ≤33 salinity. Since 2003 the interannual dynamics of *A. tonsa* density have been quite different in the two estuaries under study and showed higher fluctuations in the estuary of Urdaibai, where its density decreased throughout the 2003-2009 period and was lower than in the estuary of Bilbao during the period 2010-2015. The density of this species decreased clearly with salinity in both estuaries, but at the same salinity, densities were always higher in the estuary of Bilbao. *O. davisae* was first observed in 2001 in the estuary of Bilbao and from 2002 onwards it increased in density more smoothly than *A. tonsa*, reaching highest values during the periods 2003-2009 at B30 and 2010-2015 at B33 and B34. However, in the estuary of Urdaibai the density of *O. davisae* was very low and during some of the years no specimens were found at all. Like *A. tonsa*, *O. davisae* showed a decrease in density with salinity and it was only observed occasionally at B35, but never at U35. *P. marinus* showed a clear pattern of increase in density since its first occurrence in 2010 at all salinity sites of the estuary of Bilbao, except at B35, where it was never found. However, in the estuary of Urdaibai this species was only recorded twice (November 2011 and October 2012) in very low densities. In the estuary of Urdaibai, *A. bifilosa* was always present and very abundant from U33 to U26, where it showed lower fluctuations and a higher mean density in the last period (2010-2015). However, it was first recorded in the estuary of Bilbao in 2007 and it increased in density from 2003-2009 to 2010-2015. *C. aquaedulcis* was also found throughout the entire period of study at all salinity sites of the estuary of Urdaibai but in low densities. In the estuary of Bilbao, it was only occasionally observed until 2009 but from 2010 onwards this species became one of the most abundant copepods at B30. *C. aquaedulcis* showed the strongest (among the NIS and OES under study) pattern of decrease in density as salinity increased in the estuary of Bilbao.

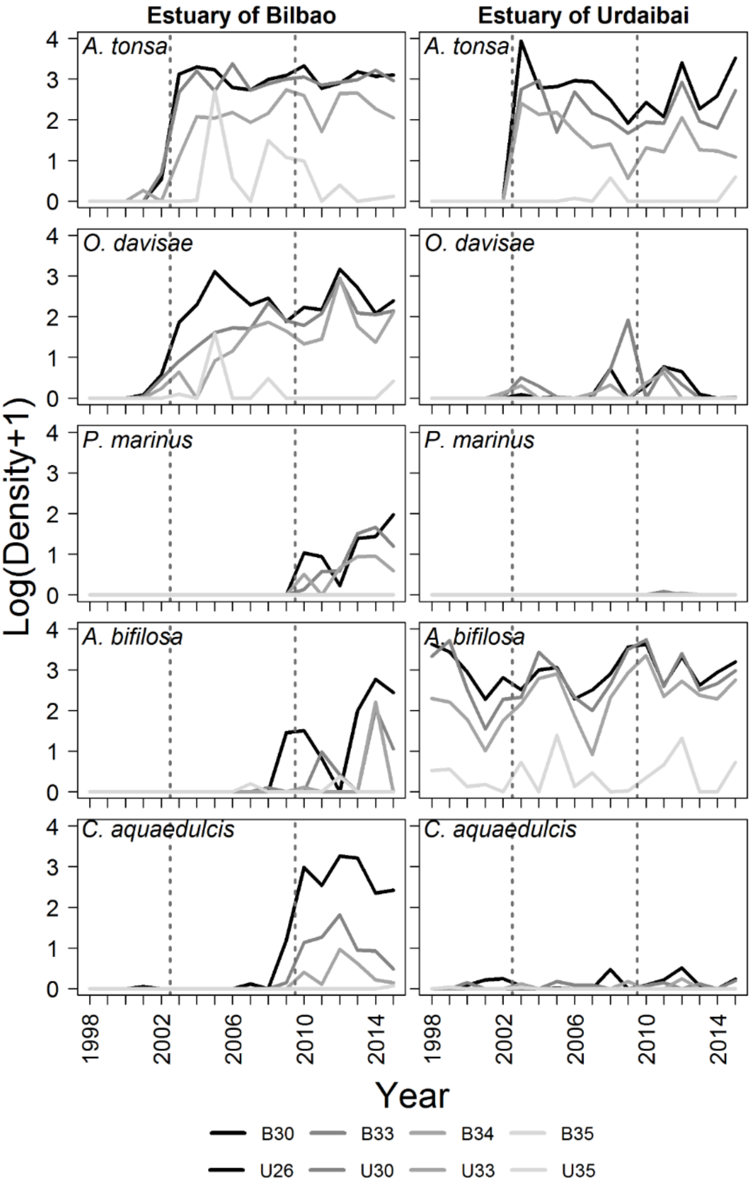

Fig. 1 Interannual variations of mean annual densities (log density+1) of *Acartia tonsa, Oithona davisae, Pseudodiaptomus marinus, Acartia bifilosa* and *Calanipeda aquaedulcis* at the different salinity sites of the estuaries of Bilbao (B30, B33, B34 and B35) and Urdaibai (U26, U30, U33 and U35) from 1998 to 2015. Dashed lines separate the 1998-2002, 2003-2009 and 2010-2015 periods.

The highest increase of NIS at all salinities of both estuaries occurred from the period 1998-2002 to the period 2003-2009, except at B34 of the estuary of Bilbao, where it was between the period 2003-2009 and the period 2010-2015 (Table 3). The increase was highest and of similar magnitude (> 1000 ind m^{-3} on average) at B30, B33 and U26 and negligible (0.4 ind m^{-3} on average) at U35. From 2003-2009 to 2010-2015 NIS showed a moderate increase in the estuary of Bilbao but a decrease at the estuary of Urdaibai at low and intermediate salinity sites. The increase of OES from 2003-2009 to 2010-2014 in the estuary of Bilbao was of two or three orders of magnitude higher at B30 (> 1000 ind m^{-3} on average) than at the other salinity sites.

Table 3. Mean densities (individuals m^{-3}) of NIS, OES, total copepods, total zooplankton, total copepods excluding NIS and OES and non-copepod zooplankton at each salinity zone (Sal) of the estuaries of Bilbao (30, 33, 34 and 35) and Urdaibai (26, 30, 33 and 35) during the periods 1998-2002, 2003-2009 and 2010-2015. The density differences (Diff) between consecutive periods are also shown. In bold highest values of both parameters at each salinity zone.

	Sal	Period	NIS		OES		Total copepods		Total zooplankton		Copepods excluding NIS & OES		Non-copepod zooplankton	
			Density	Diff.	Density	Diff.	Density	Diff.	Density	Diff.	Density	Diff.	Density	Diff.
Estuary of Bilbao	30	1998-2002	1.1		0.0		124.3		349.6		123.2		225.3	
		2003-2009	1573.9	**1572.9**	6.1	6.1	1777.2	**1652.9**	2293.2	**1943.6**	**197.2**	**74.0**	516.0	290.7
		2010-2015	**1714.5**	140.6	**1043.9**	**1037.9**	**2924.0**	1146.8	**4086.3**	1793.0	165.5	-31.7	**1162. 3**	**646.2**
	33	1998-2002	1.2		0.0		484.6		1018.1		483.4		533.5	
		2003-2009	1108.5	**1107.3**	0.1	0.1	**1636.7**	**1152.1**	**3274.2**	**2256.0**	**528.1**	44.7	**1637.5**	**1103.9**
		2010-2015	**1279.9**	171.4	**41.1**	**41.0**	1549.3	-87.4	2946.7	-327.4	228.3	**-299.8**	1397.4	-240.0
	34	1998-2002	0.3		0.0		1281.9		2402.3		**1281.6**		1120.4	
		2003-2009	194.4	194.1	0.0	0.0	1148.1	-133.9	**3203.6**	**801.3**	953.6	**-328.0**	**2055.5**	**935.1**
		2010-2015	**470.1**	**275.7**	**29.3**	**29.3**	**1508.3**	**360.2**	3115.2	-88.3	1009.0	55.3	1607.0	-448.6
	35	1998-2002	0.0		0.0		1577.6		2914.4		1577.6		1336.8	
		2003-2009	**86.1**	**86.1**	0.1	0.1	1577.6	0.0	3631.5	717.1	1491.5	-86.1	2053.9	**717.1**
		2010-2015	2.0	-84.1	**0.3**	**0.2**	**2860.1**	**1282.5**	**5147.3**	**1515.8**	**2857.8**	**1366.5**	**2287.2**	233.3
Estuary of Urdaibai	26	1998-2002	0.0		---		1931.6		2734.4		**1931.6**		802.8	
		2003-2009	**1716.9**	**1716.9**	---	---	**2932.7**	**1001.0**	**4517.7**	**1783.3**	1215.7	**-715.9**	**1585.0**	**782.3**
		2010-2015	1124.5	-592.4	---	---	2920.3	-12.4	4085.2	-432.5	1795.8	580.0	1165.0	-420.1
	30	1998-2002	0.01		---		1934.0		2674.5		1934.0		740.5	
		2003-2009	**341.9**	**341.9**	---	---	1851.1	-83.0	2767.0	92.5	1509.2	-424.8	**915.9**	**175.4**
		2010-2015	280.2	-61.7	---	---	**2506.5**	**655.4**	**3259.3**	**492.3**	**2226.3**	**717.1**	752.9	-163.0
	33	1998-2002	0.1		---		797.3		1175.5		797.2		378.2	
		2003-2009	**91.1**	**91.0**	---	---	1098.0	300.7	**2039.9**	**864.4**	1006.9	209.7	**941.9**	**563.7**
		2010-2015	32.5	-58.6	---	---	**1422.5**	**324.5**	1986.2	-53.7	**1390.0**	**383.1**	563.7	-378.2
	35	1998-2002	0.0		---		1424.4		1904.3		1424.4		479.9	
		2003-2009	0.4	**0.4**	---	---	1617.2	192.8	2837.6	933.3	1616.8	192.4	**1220.4**	**740.5**
		2010-2015	**0.5**	0.1	---	---	**3066.0**	**1448.7**	**4078.0**	**1240.4**	**3065.5**	**1448.7**	1012.0	-208.3

Copepods and Zooplankton

The densities of total zooplankton and copepods showed similar year-to-year fluctuations over the study period in each of the estuaries, but fluctuations were more marked in the estuary of Urdaibai than in the estuary of Bilbao, and at the innermost salinity site in each estuary (B30 and U26) (Fig. 2). As shown in Table 3, both copepods and zooplankton increased progressively from the first period (1998-2002) to the last one (2010-2015) at B30, B35, U30 and U35, and both showed the highest densities in the intermediate period at B33 and U26. However, zooplankton density increased from the first to the last period and copepod density was highest in the intermediate period at B34 and U33. In the estuary of Bilbao, both copepods and zooplankton showed the highest increases between the three consecutive periods at B30. The increases were of the same magnitude (> 1000 ind m^{-3} on average), although they were slightly higher from 1998-2002 to 2003-2009 than from 2003-2009 to 2010-2015. These consecutive increases were mainly related to the increase of NIS from 1998-2002 to 2003-2009 and to the increase of OES from 2003-2009 to 2010-2015, respectively. In the case of zooplankton, the increases were related to the increase of non-copepod zooplankton, mainly of bivalve larvae (Table 2).

At B33, zooplankton and copepods showed increases in density similar to those at B30 from 1998-2002 to 2003-2009 associated to the increase of NIS and, in the case of zooplankton, also to the increase of non-copepod zooplankton, mainly due to cirripede larvae (Table 2). However, at this B33 site both decreased from 2003-2009 to 2010-2015 due to the decrease of non-copepod zooplankton and total copepods excluding NIS and OES that counteracted the slight increment of NIS and OES. At B34, total copepods and zooplankton also decreased in the second and third periods, respectively, since the increases of NIS or NIS and OES were not enough to make up for the decrease of total copepods excluding NIS and OES in 2003-2009 and the decrease of non-copepod zooplankton in 2010-2015. In the estuary of Urdaibai, the NIS contributed most to the high increase of copepod and zooplankton densities at U26 from 1998-2002 to 2003-2009 that occurred in spite of the decrease in density of copepods excluding NIS & OES. The later decrease of NIS from 2003-2009 to 2010-2015 at this site was compensated with the increase of other copepods. At U30, the increase of NIS from the first to the second period was counteracted by the decrease of copepods excluding NIS & OES; at U33, the increase of NIS during the same time span contributed to the increase of copepods and zooplankton to a lesser extent than copepods excluding NIS and non-copepod zooplankton. This was mainly due to the contribution of *A. bifilosa*, gastropod larvae, and cirripede larvae (Table 2). The decrease of NIS at U33 in the last period had little effect on the quantitative changes of copepods and zooplankton. At the highest salinity site of both estuaries (B35 and U35), the density of copepods and zooplankton increased from 2003-2009 to 2010-2015 in the same magnitude (> 1000 ind m^{-3} on average) as at the lowest salinity sites, but at these outermost sites it was mostly due to the increase of copepods excluding NIS & OES, since NIS & OES contribution was negligible.

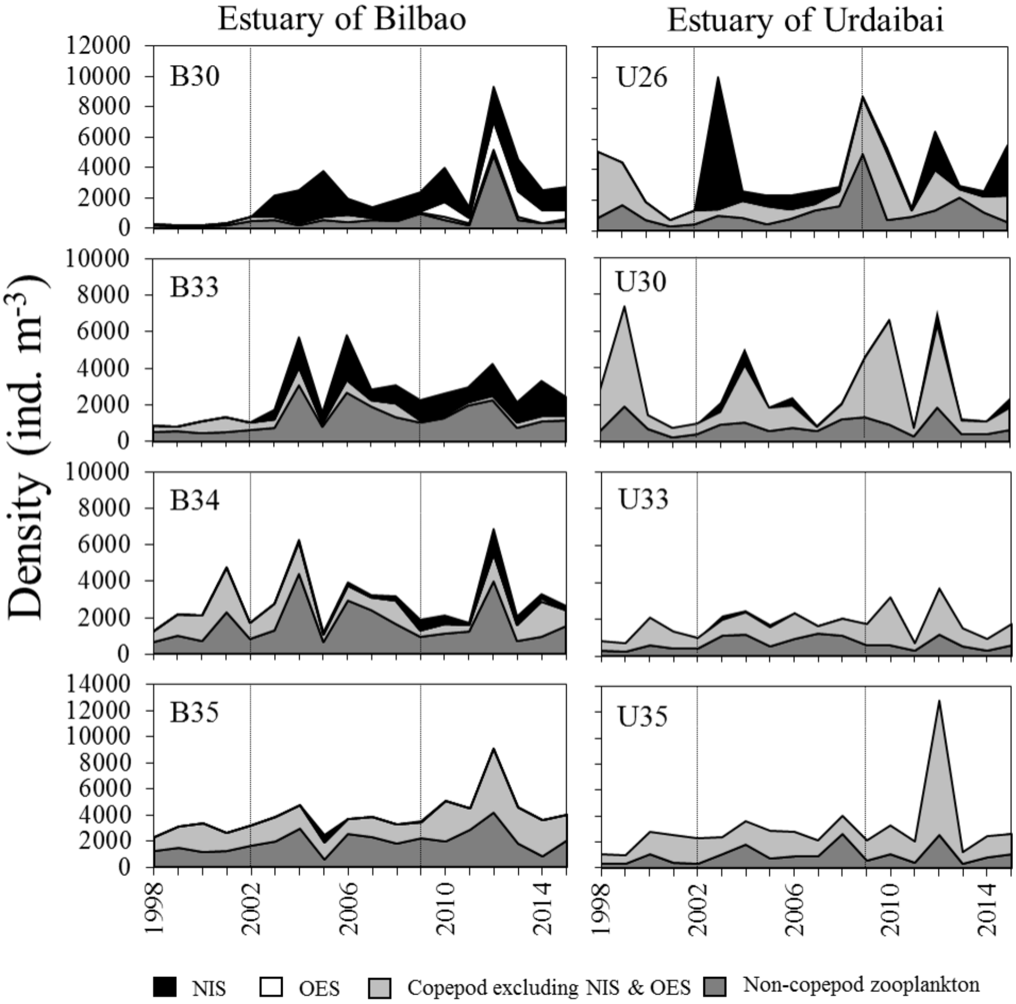

Fig. 2 Interannual variations of mean annual densities of copepod NIS (*Acartia tonsa, Oithona davisae* and *Pseudodiaptomus marinus*) and OES (*Acartia bifilosa* and *Calanipeda aquaedulcis*, only at the estuary of Bilbao), copepod excluding NIS and OES and non-copepod zooplankton at the different salinity sites of the estuaries of Bilbao (B30, B33, B34 and B35) and Urdaibai (U26, U30, U33 and U35) from 1998 to 2015. Dashed lines separate the 1998-2002, 2003-2009 and 2010-2015 periods.

Figure 3 shows that in the first period (1998- 2002) non-copepod zooplankton contributed most to total zooplankton at low salinity sites (B30 and B33) in the estuary of Bilbao (around 55-60%) and copepods excluding NIS and OES did so at high salinity sites (B34 and B35) in the estuary of Bilbao (around 55%) and at all salinity sites of the estuary of Urdaibai (around 70%). After their establishment in the estuary of Bilbao, NIS dominated the zooplankton at B30, but with decreasing contributions from 2003-2009 (55-90%) to 2010-2015 (30-60%) due to the establishment of OES of copepods that reached large densities in the last period (15-40%). At B33, however, the contribution of the NIS increased from the second (25-40%) to the third (30-55%) period. At both sites (B30 and B33), the negative effect of both NIS and OES was higher on the contribution of copepods excluding NIS and OES than on the contribution of non-copepod zooplankton. In the estuary of Urdaibai, the contribution of the copepod NIS to zooplankton fluctuated from more than 80% to less than 5% at the lowest salinity site (U26). The yearly mean contribution of NIS to zooplankton decreased strongly with increasing salinity. At B34, B35 and U30 NIS only exceeded once the level of 20% contribution during the study period, remaining lower than 10% in all cases at U33 and at 0% at U35.

NIS and OES made a negligible or null contribution to the annual mean density of copepods (Fig. 3) at all salinity sites of the two estuaries in the first period (1998-2002), but since 2003 the contribution was highest at the low salinity sites of the estuary of Bilbao. There, NIS dominated with values that decreased from around 85 % in 2003-2009 to around 65% in 2010-2015 at B30, due to the increase of OES (around 30%) in the last period, but increased from around 65% to 80% at B33. In contrast, in the estuary of Urdaibai, after the establishment of the NIS, their contribution fluctuated largely (from 5% to 95%) between years at the lowest salinity site (U26). In both estuaries, as observed for the contribution to total zooplankton, the contribution of the NIS to copepod density showed a marked decrease with increasing salinity, and only occasionally exceeded the value of 50% at B34, of 40% at U30 and of 20% at U33 and B35.

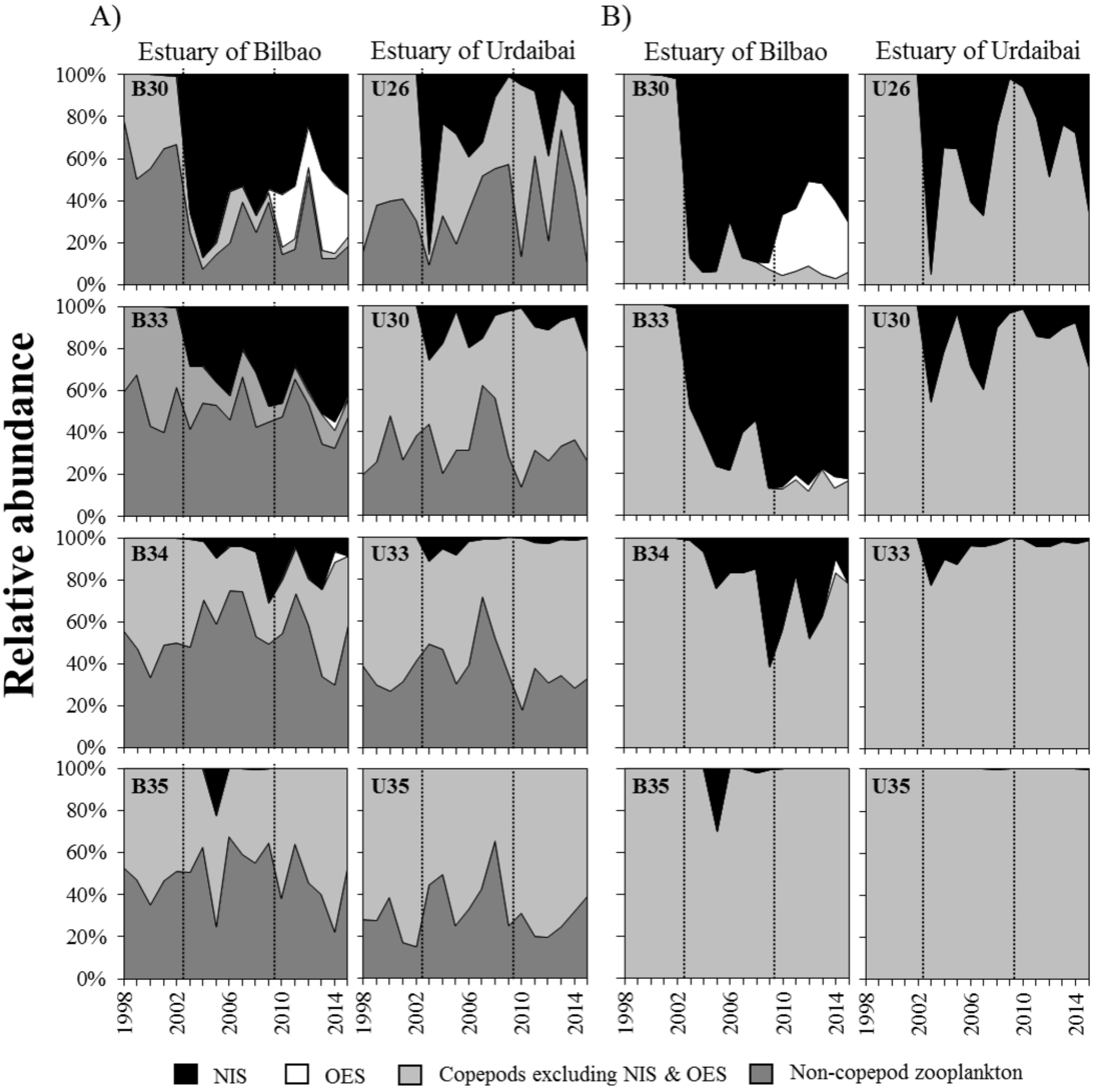

Fig. 3 Interannual variations of A) the contribution (%) of copepod NIS (*Acartia tonsa, Oithona davisae* and *Pseudodiaptomus marinus*) and OES (*Acartia bifilosa* and *Calanipeda aquaedulicis,* only at the estuary of Bilbao), copepods excluding NIS & OES and non-copepod zooplankton to total zooplankton abundance, and B) the contribution (%) of copepod NIS (*Acartia tonsa, Oithona davisae* and *Pseudodiaptomus marinus*) and OES (*Acartia bifilosa* and *Calanipeda aquaedulcis,* only at the estuary of Bilbao) and copepods excluding NIS & OES to the total copepods abundance at the different salinity sites of the estuaries of Bilbao (B30, B33, B34 and B35) and Urdaibai (U26, U30, U33 and U35) from 1998 to 2015. Dashed lines separate the 1998-2002, 2003-2009 and 2010-2015 periods.

Congeneric assemblages of Acartia and Oithona

In the *Acartia* congeneric assemblage (Figs. 4 and 5A), *A. clausi* was the dominant *Acartia* species at all salinity sites of the estuary of Bilbao in the period 1998-2002, although its density showed a strong decrease from B34 to B30. However, when *A. tonsa* arrived in this estuary, it became clearly dominant at B30 (usually > 90%) and B33 (usually > 80%), where it reached densities of similar magnitude to those of *A. clausi* at B34 and B35. The arrival of *A. bifilosa* also caused a slight decrease in the contribution of *A. clausi* to the assemblage at B30. *A. clausi* remained as the dominant *Acartia* species at B34 and B35, although at B34 its contribution to total *Acartia* density decreased progressively from the initial period (1998-2002) to the last one (2010-2015). In contrast, in the estuary of Urdaibai, *A. bifilosa* dominated the *Acartia* assemblage at U26 and U30 and *A. clausi* at U33 and U35 before the arrival of *A. tonsa*. After that, the latter species became dominant sometimes at U26, but generally its contribution to the *Acartia* assemblage decreased from 2003-2009 to 2010-2015 both at U26 and U30. *A. bifilosa* and *A. clausi* alternated being the numerically dominant species of the assemblage at U33, although *A. tonsa* was occasionally dominant in 2003. The overall contribution of *A. bifilosa* increased from the first period (1998-2002) to the last one (2010-2015), whereas *A. clausi* was always dominant at U35.

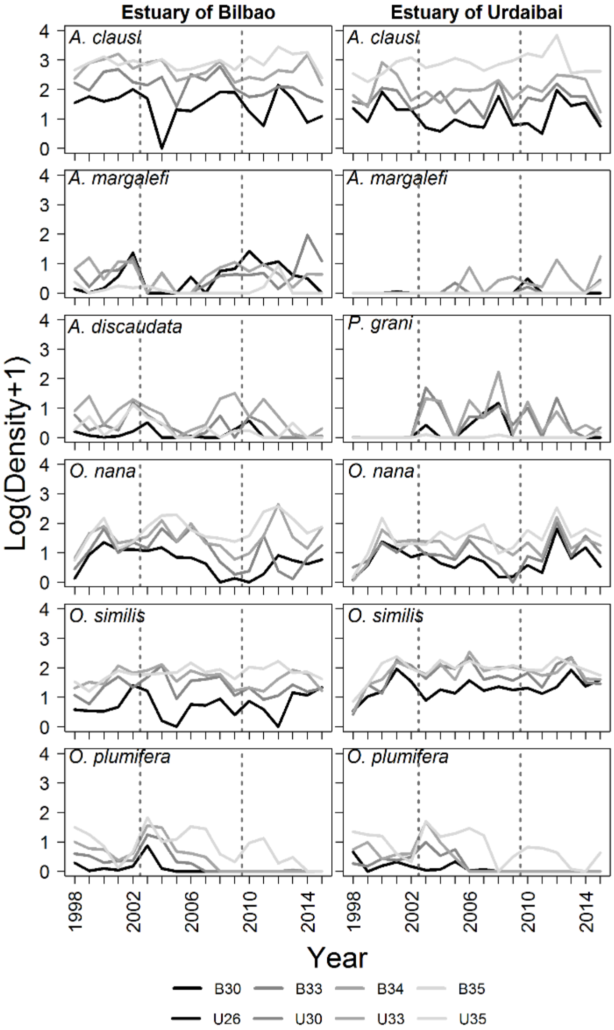

Fig. 4 Interannual variations of mean annual densities (log density+1) of *Acartia clausi*, *Acartia margalefi*, *Acartia discaudata*, *Paracartia grani*, *Oithona nana*, *Oithona similis* and *Oithona plumifera* at the different salinity sites of the estuaries of Bilbao (B30, B33, B34 and B35) and Urdaibai (U26, U30, U33 and U35) from 1998 to 2015. Dashed lines separate the 1998-2002, 2003-2009 and 2010-2015 periods.

In the *Oithona* congeneric assemblage (Figs. 4 and 5B), either *O. nana* or *O. similis* were the dominant species in the estuary of Bilbao until the increase of *O. davisae* density in 2003. Since then, *O. davisae* completely dominated the assemblage at B30 (usually > 90%) and it became progressively dominant at B33 and to a lesser extent at B34, whilst the contribution of *O. nana* at B30, B33 and B34 and of *O. similis* at B30 and B33 decreased, although both of these species maintained or increased their densities at those salinity sites from 2003-2009 to 2010-2015. However, *O. davisae* did not affect the pattern of shared dominance by *O. nana* and *O. similis* at B35, and the most frequent dominance of *O. similis* at any of the salinity sites of the estuary of Urdaibai.

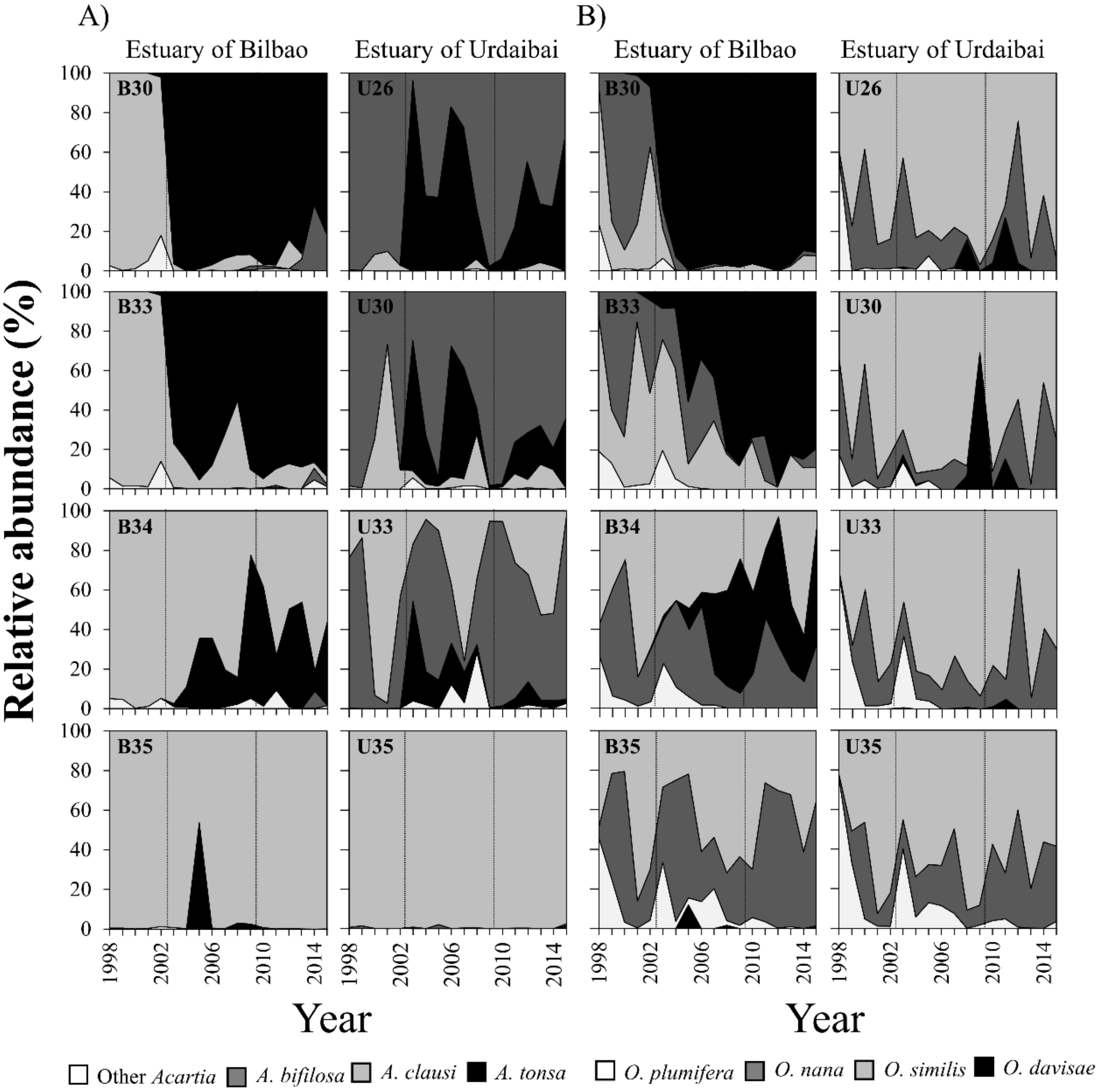

Fig. 5 Interannual variations of the relative abundance (%) of A) *A. bifilosa, A. clausi, A. tonsa* and other *Acartia* species (*A. discaudata, P. grani* and *A. margalefi*) in the *Acartia* genus assemblage and B) *O. nana, O. similis, O. plumifera* and *O. davisae* in the *Oithona* genus assemblage at the different salinity sites of the estuaries of Bilbao (B30, B33, B34 and B35) and Urdaibai (U26, U30, U33 and U35) from 1998 to 2015. Dashed lines separate the 1998-2002, 2003-2009 and 2010-2015 periods.

1.3.4 Phenological changes

Copepod NIS and OES

As shown in Figures 6 and 7, *A. tonsa* peaked in July-August and had a more marked seasonality in the estuary of Urdaibai than in the estuary of Bilbao. It also showed a wider seasonal distribution in the period 2010-2015 than in the period 2003-2009 at the low salinity sites of both estuaries (B30, B33, U26 and U33) due mainly to the increase of spring densities from 2003-2009 to 2010-2015. In the case of *O. davisae*, the timing of its annual maxima changed from late summer-autumn (usually October) at B30 and B33 in the period 2003-2009 to summer (July) in the period 2010-2015 at B30, B33 and B34. *P. marinus* was never found from February to April; it peaked in June-July and was more abundant in the second half of the year. *A. bifilosa* changed the timing of its annual maximum from summer (July) in the period 1998-2002 to spring (usually May) in the period 2003-2009 and period 2010-2015 at the salinity sites of 26, 30 and 33 of the estuary of Urdaibai. In the estuary of Bilbao, after its settlement (period 2010-2015), this species peaked in May, and was never found from July to October. Similarly, in the estuary of Bilbao *C. aquaedulcis* was more abundant in the first half of the year but showed peaks from February to June depending on the salinity site. In the estuary of Urdaibai, this species was scarce throughout the entire period of study and did not show any clear seasonal pattern.

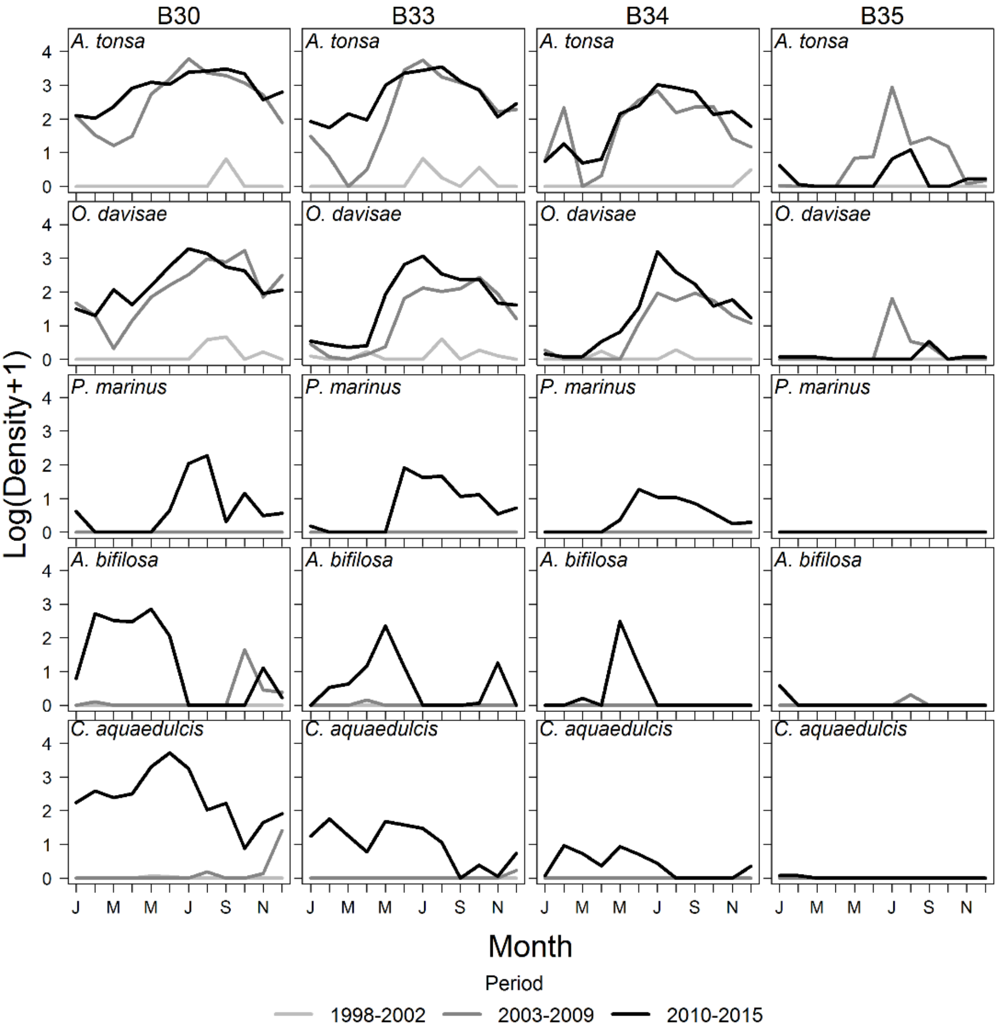

Fig. 6 Month-to-month variations (log density+1) of *Acartia tonsa*, *Oithona davisae*, *Pseudodiaptomus marinus*, *Acartia bifilosa* and *Calanipeda aquaedulcis* during the periods 1998-2002, 2003-2009 and 2010-2015 at the different salinity sites of the estuary of Bilbao (B30, B33, B34 and B35).

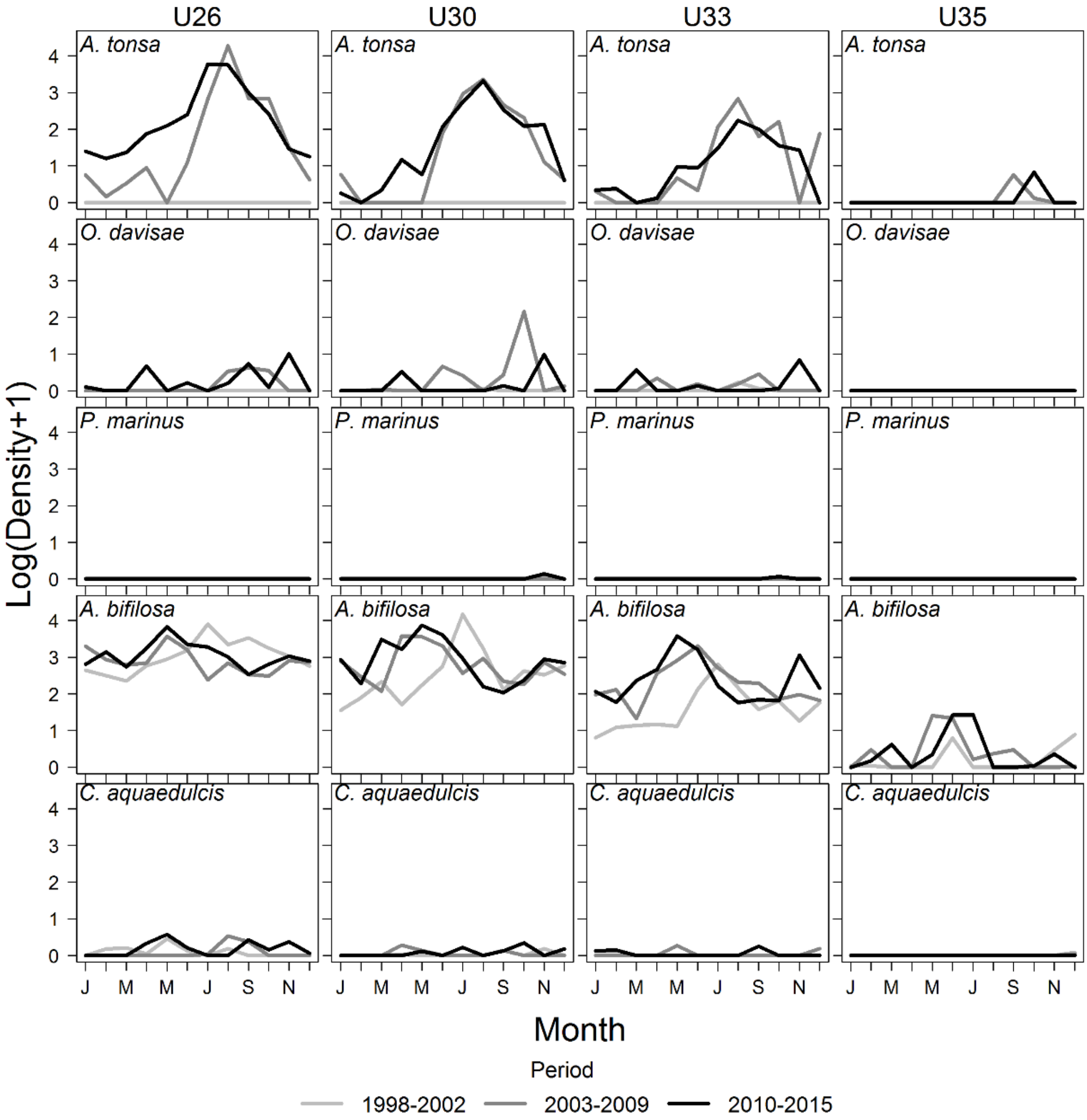

Fig. 7 Month-to-month variations (log density+1) of *Acartia tonsa*, *Oithona davisae*, *Pseudodiaptomus marinus*, *Acartia bifilosa* and *Calanipeda aquaedulcis* during the periods 1998-2002, 2003-2009 and 2010-2015 at the different salinity sites of the estuary of Urdaibai (U26, U30, U33 and U35).

Copepods and zooplankton

As shown in Fig. 8, at B30 total copepod and zooplankton densities increased abruptly from the period 1998-2002 to the periods 2003-2009 and 2010-2015 in the second half of the year, and progressively from one period to the next in the first half of the year. B30 was also the only salinity site where the seasonal pattern of the density of copepods excluding NIS and OES differed largely from that of total copepods in 2003-2009 and 2010-2015, since the timing of the peak in density changed from spring (April) in the period 1998-2002 to summer (June-July) in the two next periods. At B33, the increase in density of zooplankton and copepods from 1998-2002 to the two next periods in the second half of the year was smaller than at B30, and no relevant changes were observed in the first half of the year. At B34 and B35, no marked differences in the seasonal patterns between periods were observed in zooplankton and copepod density. In the estuary of Urdaibai (Fig. 9), the seasonal pattern of zooplankton and copepod density, both including and excluding the NIS and OES, did not change between periods at U35. The timing of their maximum density changed from spring to summer at U33 and from summer to spring at U30. At U26, however, zooplankton and copepod density maxima were in summer (July-August) in all three periods, but in copepods excluding NIS and OES the timing changed from summer (July) in 1998-2002 to spring (May) in 2010-2015.

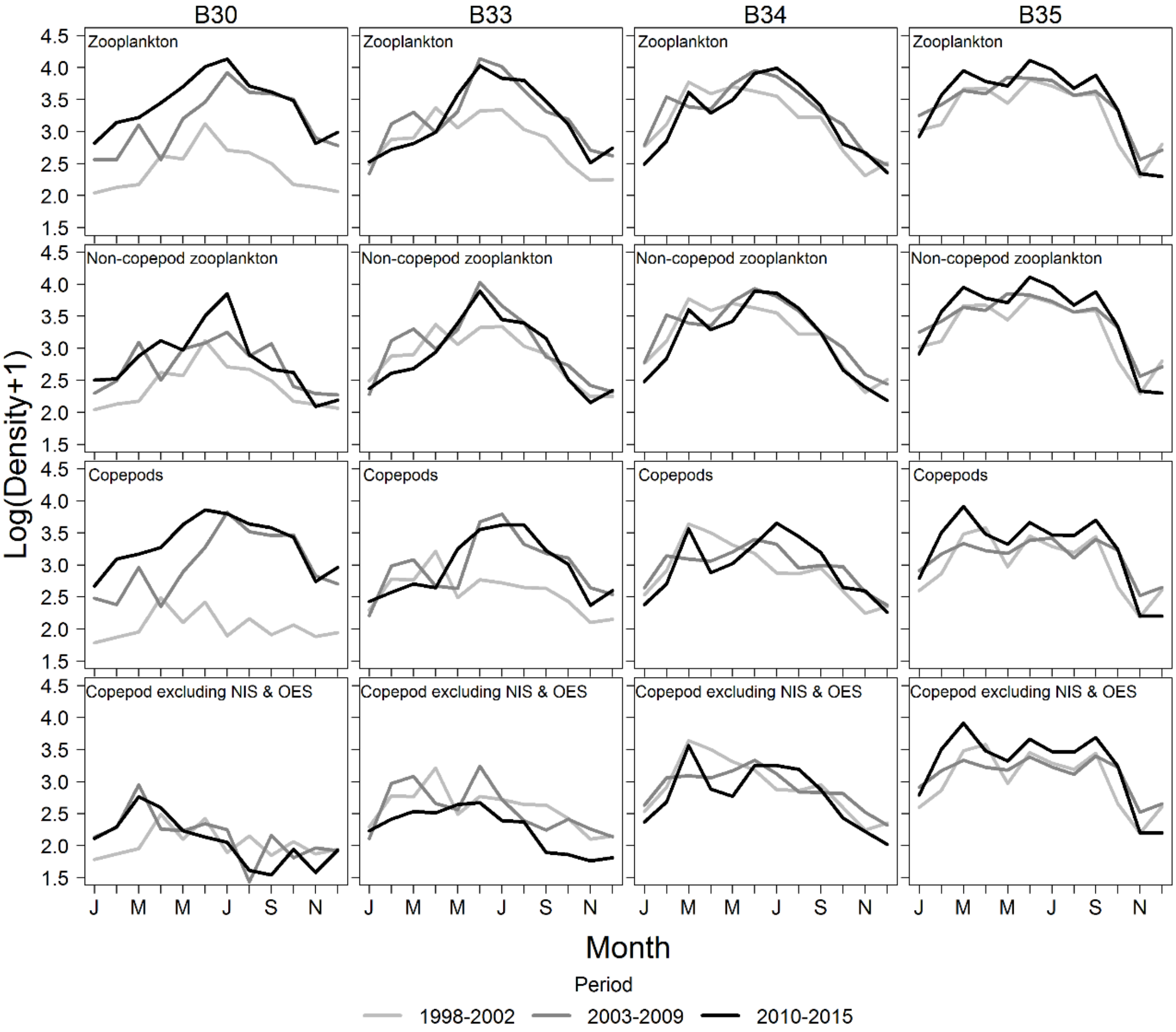

Fig. 8 Month-to-month variations (log density+1) of zooplankton, non-copepod zooplankton, copepods and copepods excluding NIS & OES during the periods 1998-2002, 2003-2009 and 2010-2015 at the different salinity sites of the estuary of Bilbao (B30, B33, B34 and B35).

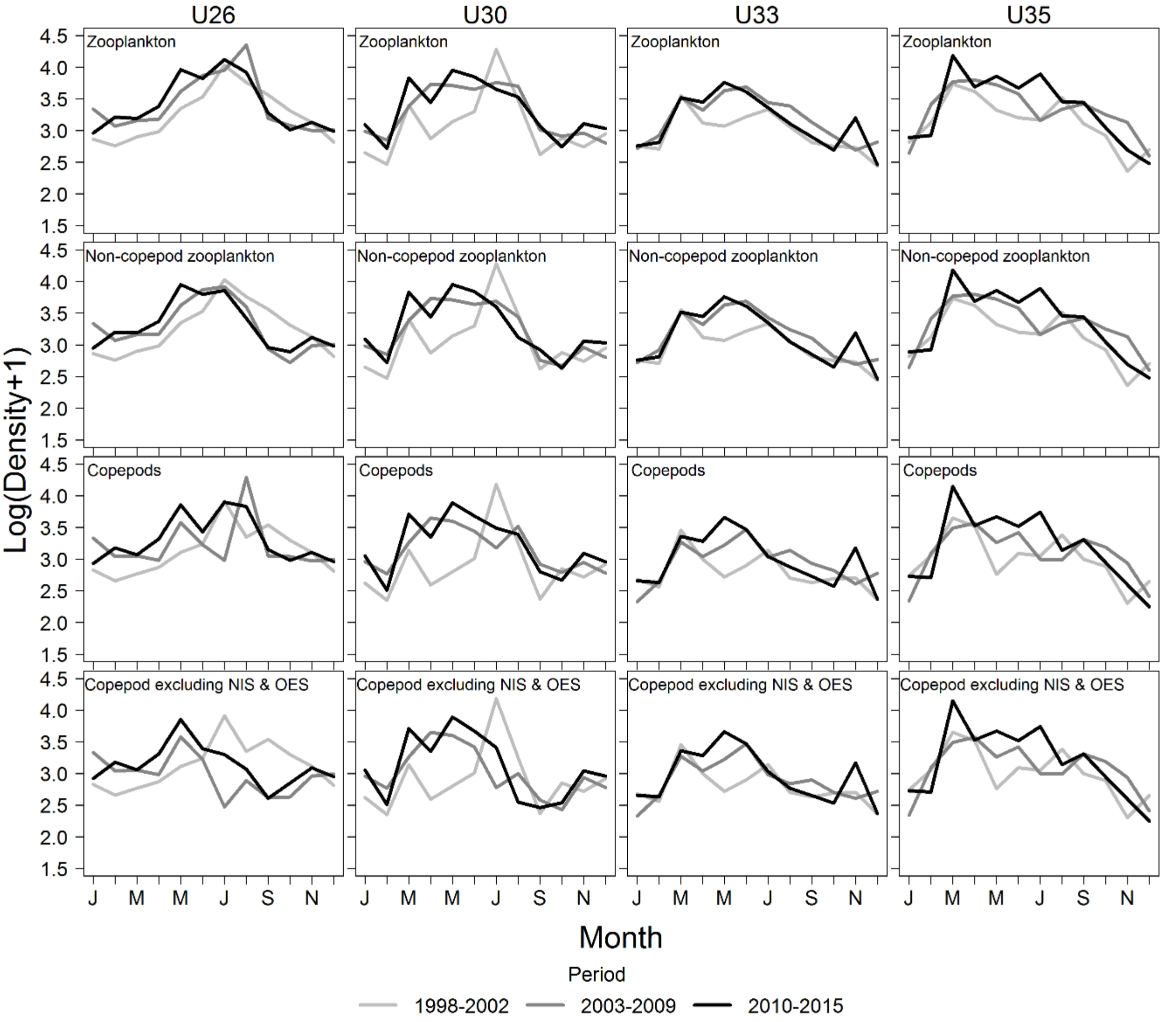

Fig. 9 Month-to-month variations (log density+1) of zooplankton, non-copepod zooplankton, copepods and copepods excluding NIS & OES during the periods 1998-2002, 2003-2009 and 2010-2015 at the different salinity sites of the estuary of Urdaibai (U26, U30, U33 and U35).

1.3.5 Contribution of NIS, OES and other taxa to copepod and zooplankton density variations

GAM analyses results (Tables 4 and 5) showed the contribution of different taxa to total zooplankton and copepod density variations along the study period. In the estuary of Bilbao, *A. tonsa* and *C. aquaedulcis* were the species that contributed most to total zooplankton variations at B30. *A. tonsa* together with cirripede larvae and total copepods excluding NIS & OES made the highest contribution at B33 and the latter two taxa groups at B34 and B35. In contrast, in the estuary of Urdaibai, the taxa with the highest contributions to total zooplankton variations were copepods excluding NIS & OES together with *A. tonsa* at U26, *A. tonsa*, cirripede larvae and gastropod larvae at U30, and cirripede larvae at U33 and U35. Variations in copepod density in the estuary of Bilbao were mostly explained by *A. tonsa*, *O. davisae* and *C. aquaedulcis* at B30, by *A. tonsa* and *A. clausi* at B33, and by *A. clausi* and PCPC-calanus at B34 and B35. In the estuary of Urdaibai, *A. bifilosa* and *A. tonsa* were the taxa with highest contribution (with similar influence) to the variations in copepod density at U26 and U30. *A. bifilosa* influenced most, but followed by PCPC-calanus and *A. clausi* at U33. However, *A. clausi* and PCPC-calanus became the main taxa responsible for copepod density variations at U35. The contribution of both *O. davisae* and *A. tonsa* to explain copepod and zooplankton dynamics decreased with increasing salinity in both estuaries, but it was always much lower in the estuary of Urdaibai.

Table 4. Results of GAM analyses of total zooplankton and total copepod abundances from each salinity zone of the estuary of Bilbao, using different taxa abundances as predictor variables. Only significant smooths are reported and the p-values and F values for predictor variables are given The R^2 value and the percentage deviance explained (D.e. (%)) for each model are also shown. NIS and OES in bold.

30			**33**			**34**			**35**		
Zooplankton											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
A. tonsa	32.495	<2E-16	***A. tonsa***	39.326	<2E-16	Copepods excluding NIS & OES	512.821	<2E-16	Copepods excluding NIS & OES	618.107	<2E-16
C. aquaedulcis	10.738	1.79E-13	Cirripede larvae	22.665	<2E-16	Cirripede larvae	14.891	<2E-16	Cirripede larvae	22.146	<2E-16
Copepods excluding NIS & OES	8.95	6.40E-11	Copepods excluding NIS & OES	22.357	<2E-16	***A. tonsa***	10.601	1.38E-12	Appendicularians	3.894	0.00015
O. davisae	8.027	5.00E-10	***O. davisae***	4.023	0.00014	Appendicularians	17.064	5.48E-05	Cladocerans	4.890	0.00861
Cirripede larvae	13.237	2.87E-09	Polychaete larvae	3.710	0.00077	***O. davisae***	12.994	0.00040	Bivalve larvae	2.596	0.00968
Chaetognatha	4.467	0.00013	Appendicularians	3.026	0.00743	Siphonophores	5.333	0.02205	Fish eggs and larvae	3.591	0.02166
Bivalve larvae	12.217	0.00062									
A. bifilosa	7.571	0.00665									
Polychaete larvae	2.464	0.01383									
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.905	93.4		0.899	92.6		0.898	91.5		0.894	91.0	
Copepods											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
A. tonsa	189.712	<2E-16	***A. tonsa***	36.465	<2E-16	*A. clausi*	64.873	<2e-16	*A. clausi*	203.35	<2E-16
O. davisae	9.586	3.78E-12	*A. clausi*	8.788	5.07E-11	PCPC-calanus	22.446	3.77E-14	PCPC-calanus	129.588	<2E-16
C. aquaedulcis	10.761	1.64E-08	PCPC-calanus	7.78	2.46E-09	***O. davisae***	52.808	7.88E-12	*O. nana*	5.846	0.00067
PCPC-calanus	9.114	0.00288	*A. margalefi*	5.053	4.29E-05	***A. tonsa***	9.549	2.49E-11	*T. sylifera*	6.462	0.00149
			O. davisae	6.833	0.00044	*O. plumifera*	6.429	5.31E-05	*O. similis*	4.050	0.04554
			E. acutifrons	5.172	0.02418	*P. marinus*	3.669	0.0005			
						O. nana	8.575	0.0038			
						E. acutifrons	6.309	0.0129			
						O. similis	5.405	0.0212			
						A. discaudata	4.289	0.0398			
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.798	82.4		0.807	84.8		0.893	91.3		0.895	90.7	

Table 5. Results of GAM analyses of total zooplankton and total copepod abundances from each salinity zone of the estuary of Urdaibai, using different taxa abundances as predictor variables. Only significant smooths are reported and the p-values and F values for predictor variables are given. The R^2 value and the percentage deviance explained (D.e. (%)) for each model are also shown. NIS and OES in bold.

26			**30**			**33**			**35**		
Zooplankton											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Copepods excluding NIS & OES	265.336	<2E-16	Copepods excluding NIS & OES	173.883	<2E-16	Cirripede larvae	194.684	<2E-16	Copepodsexcluding NIS & OES	124.08	<2E-16
A. tonsa	25.735	<2E-16	***A. tonsa***	30.946	<2E-16	Copepods excluding NIS & OES	122.022	<2E-16	Cirripede larvae	8.5	2.83E-09
Gastropod larvae	12.762	2.32E-16	Cirripede larvae	29.821	<2E-16	Gastropod larvae	53.284	7.31E-12	Gastropod larvae	13.464	1.32E-08
Cirripede larvae	12.384	5.86E-12	Gastropod larvae	21.3	<2E-16	***A. tonsa***	18.002	3.60E-05	Cladocerans	20.106	1.29E-05
Decapod larvae	3.057	0.00271	Hydromedusae	5.284	2.00E-05	Fish eggs and larvae	3.88	0.00025	Siphonophores	2.344	0.01100
O. davisae	2.891	0.00393	Chaetognaths	3.818	2.56E-04	Hydromedusae	3.796	0.00030	Chaetognaths	3.069	0.02770
Polychaete larvae	2.772	0.01037	***O. davisae***	2.606	0.03737						
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.888	91.9		0.965	97.6		0.958	96.7		0.927	94.1	
Copepods											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
A. bifilosa	218.402	<2E-16	*A. bifilosa*	65.229	<2E-16	*A. bifilosa*	54.291	<2E-16	*A. clausi*	424.806	<2E-16
A. tonsa	43.199	<2E-16	***A. tonsa***	17.959	<2E-16	PCPC-calanus	21.306	1.17E-10	PCPC-calanus	28.179	<2E-16
T. longicornis	5.32	3.22E-06	*O. similis*	8.522	1.10E-08	*A. clausi*	11.132	3.03E-07	*E. acutifrons*	6.437	2.38E-07
O. davisae	2.129	0.03440	*A. clausi*	14.402	0.00020	***A. tonsa***	6.743	0.00055	*O. nana*	27.946	3.62E-07
			E. acutifrons	2.505	0.03899	*O. similis*	3.789	0.00085	*O. similis*	5.552	3.22E-06
						O. media	2.477	0.02356	*O. media*	3.348	0.00147
									T. stylifera	8.111	0.00494
									O. plumifera	7.027	0.00878
									D. anglicus	3.028	0.00936
									T. longicornis	2.834	0.03037
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.82	85.3		0.801	83.8		0.689	73.8		0.943	95.5	

1.4 Discussion

The results of this study brought to light that the arrival of different copepod NIS (*A. tonsa, O. davisae* and *P. marinus*) to the estuaries of Bilbao and Urdaibai affected in different ways and degrees of intensity the density, relative composition and seasonal distribution of total zooplankton, copepods and congeneric species in both systems. Similarly, the success and influence of *A. bifilosa* and *C. aquaedulcis* after settling in the estuary of Bilbao differed largely from that observed in the estuary of Urdaibai, where both species were present since the beginning of the monitoring programme.

1.4.1 Differences in colonization success of NIS and OES

The copepod NIS densities observed in the two estuaries under study allow us to conclude that *A. tonsa, O. davisae* and *P. marinus* settled successfully in the estuary of Bilbao, whereas only *A. tonsa* was successful in the estuary of Urdaibai. Previous studies already reported the success of *A. tonsa* in colonizing the inner estuary of Bilbao (Aravena et al. 2009; Uriarte et al. 2016) and its brackish behaviour evidenced by the decrease in density from 33 salinity to 35 salinity sites in both estuaries (Villate et al. 2018). This species, however, is also present in brackish open coastal waters in the Black Sea (Altukhov et al. 2014) and it was reported to penetrate into the Golden Horn estuary from the Marmara Sea (Isinibilir et al. 2016). *O. davisae* was found in lower densities than *A. tonsa,* but the real numerical importance of this small cyclopoid has been undoubtedly underestimated, owing to the use of 200 µm plankton nets, as shown for other *Oithona* species of similar size (Villate 1991; Pansera et al. 2014). In the upper San Francisco estuary, the average abundance of *O. davisae* was two orders of magnitude higher than that of the brackish *Acartia* species, and the average autumn maximum exceeded 150000 ind. m^{-3} when a 73 µm mesh plankton net was used (Bollens et al. 2014). These are densities one order of magnitude higher than the maximum values estimated for the estuary of Bilbao. *O. davisae* also showed a brackish behaviour in both estuaries, being found at the highest salinity site only occasionally in the estuary of Bilbao. *P. marinus*, which is larger than the former two NIS species, showed much lower densities than those of *A. tonsa* and *O. davisae,* in agreement with observations in other coastal systems. In the region of origin, in Fukuyama Harbour, *P. marinus* is also much less abundant than other dominant copepods such as *O. davisae* (Liang and Uye 1997). In Lake Faro (Mediterranean), *P. marinus* became the fourth most abundant copepod species, but representing on average only 0.5 % of the total density of a copepod assemblage dominated by Acartiidae and Oithonidae (Sabia et al. 2014), as in the estuary of Bilbao. Notwithstanding, due to the epibenthic nature of *P. marinus,* its density could be underestimated in routine zooplankton samples (De Olazabal and Tirelli 2011; Brylinski et al. 2012).

The seasonal population growth of *A. tonsa* was more constrained in the estuary of Urdaibai than in the estuary of Bilbao. This may be attributed to the fact that the former estuary already had a well-established brackish zooplankton community before the arrival of NIS, which was lacking in the latter estuary (Uriarte et al. 2016; Villate et al. 2018). However, to explain why *A. tonsa* achieved high population densities in both estuaries, while *O. davisae* and *P. marinus* densities were very low in the estuary of Urdaibai, both inherent system hydromorphological features and NIS capabilities need to be taken into account. According to Zagami et al. (2018), optimum conditions for *O. davisae* colonization success are isolated eutrophic environments and limited water exchange, and the estuary of Urdaibai has lower water residence time than the estuary of Bilbao (Valencia et al. 2004), where the two-layered circulation allows a greater stability of below halocline water masses along the estuary (Uriarte et al. 2014). In addition, in the shallower estuary of Urdaibai wind and tide induced water turbulence is higher and during the tidal cycle the brackish zooplankton assemblage moves from the inner part at high tide to the outer part at low tide and may be flushed out of the estuary during extreme flood events (Villate 1997; Uriarte et al. 1998). These unfavourable conditions may be better counteracted by *Acartia* species than by *O. davisae*, because *Acartia* produces resting (diapause) eggs viable in sediments that allow to increase or recover planktonic populations (Katajisto et al. 1998), but no such type of eggs are produced by *O. davisae* (Uye and Sano 1995). In fact, in the estuary of Urdaibai, resting eggs of an *Acartia* species (*A. bifilosa*) have been found in high densities in the sediments (Uriarte and Villate 2006). Moreover, laboratory experiments show that *O. davisae* is more sensitive to turbulence than *A. tonsa* due to its ambush behaviour (Saiz et al. 2003). Likewise, differences between the estuaries of Bilbao and Urdaibai in *P. marinus* presence may be linked to the lack of resting egg production and the negative effects that intense turbulence and transport rate may have on this copepod (Sabia et al. 2015). The length of salinity intrusion zone, with independence of the estuary size, was found to be an important factor accounting for the establishment of other invasive *Pseudodiaptomus* species when several North American estuaries were compared (Cordell and Morrison 1996). This suggests that the high salt wedge that usually penetrates up to the inner estuary of Bilbao during the dry season (Uriarte et al. 2014) favoured the establishment of *P. marinus* in this system. Conversely, the lack or weakness of saline intrusions in the shallower and more mixed estuary of Urdaibai would hinder the establishment of such type of NIS of copepod. Our results in the estuary of Urdaibai, confirm that after their arrival in a new area not all the NIS succeed in establishing and they may not become a threat to the native congeneric species, as reported for the brackish NIS *Eurytemora carolleeae* in the Gulf of Riga (Astra et al. 2018).

Differences between the two estuaries under study in the density of *C. aquaedulcis* may also be attributable to the aforementioned environmental constraints in the estuary of Urdaibai and the achievement of better water quality conditions in the hydrologically more stable estuary of Bilbao in recent years (Uriarte et al. 2016). In small shallow estuaries of the Basque coast, *C. aquaedulcis* was observed since the first studies on zooplankton in the early 1980s, but always in low density (Villate and Orive 1981; Villate 1990). This species is found from limnetic to hyperhaline habitats, evidencing its tolerance to a wide range of salinities, but it only produces subitaneous eggs (Svetlichny et al. 2012a, Svetlichny et al. 2012b). Although the improvement in oxygen conditions during the rehabilitation process of this estuary may have been critical for the colonization of other fauna (Borja et al. 2006), this may not be the case for *C. aquaedulcis*, since it does not seem to be greatly affected by the level of oxygen in water (Svetlichny et al. 2012b). A response to the general trophic change of the system evidenced by the decrease of chlorophyll *a*, organic loads and turbidity (Uriarte et al. 2016) seems a more plausible explanation. In agreement with this, *C. aquaedulcis* has been found to be dominant in oligotrophic environments and better adapted than *A. tonsa* to oligotrophic conditions (Boix et al. 2005; Brugnano et al. 2011).

1.4.2 Interannual variations of NIS densities

The density of *A. tonsa* only increased throughout the 3 periods under study at the innermost site of the estuary of Bilbao, while it decreased from 2003-2009 to 2010-2015 in the estuary of Urdaibai and at middle and outer sites of the estuary of Bilbao. The interannual fluctuations of this species and its high density in 2003 in both estuaries were found to be related to temperature, *A. tonsa* being favoured in hot years (Aravena et al. 2009; Villate et al. 2018). In addition, the sudden rise of this species in 2003, after its absence or negligible presence in 2002, was preceded by the driest autumn-winter and occurred during the warmest spring-summer of the study period in both estuaries (Iriarte et al. 2016). This would agree with the hypothesis that extreme climatic events synergistically with hydrological changes, with the occurrence of drought periods in particular, can facilitate the establishment of invasive species in estuaries (Winder et al. 2011). The decrease of this species density from 2003-2009 to 2010-2015 in the inner estuary of Urdaibai agreed with the decrease of spring-summer temperature in the last period. No such decrease of density was found in the estuary of Bilbao, where differences of spring-summer temperatures between periods were not so evident. Inner estuary water temperature is less fluctuating and less influenced by air temperature in the estuary of Bilbao than in the estuary of Urdaibai because the former is deeper and more stratified, this preventing the warming of waters below the halocline during the warm season (Iriarte et al. 2016). The continuous increase of *A. tonsa* in the inner estuary of Bilbao might be linked to the co-occurring increase in water column stratification, since a hydrologically more stable environment benefits the production of *A. tonsa* (Azeiteiro et al. 2005). In contrast to *A. tonsa*, which irrupted with its highest densities in both estuaries during the warmest year (2003) of the series, the population of *O. davisae* increased more progressively in the estuary of Bilbao during the study period. Similar contrasting histories (sudden *vs* progressive occurrence) in the establishment of introduced copepod species has been observed in the San Francisco estuary, where *O. davisae* and other Oithonid species (*Limnoithona tetraspina*) showed progressive increases from its first occurrence, whilst calanoid species of the genus *Acartiella*, *Pseudodiaptomus* and *Sinocalanus* showed highest densities in their first occurrences (Winder et al. 2011). Interannual dynamics of *O. davisae* in the estuary of Bilbao suggest it was less dependent on temperature and more dependent on stratification than *A. tonsa*. This is supported by the fact that the 2005 peak and the high densities of the period 2011-2013 occurred in years of intermediate values of temperature but highest salinity stratification.

1.4.3 Phenology of NIS and OES

The seasonal pattern of *A. tonsa* density shows a maximum in summer, as in other north European estuaries (Baretta and Malschaert 1988; David et al. 2007) and in the lagoon of Venice in the Mediterranean (Camatti et al. 2019). It usually peaked in July in the period 2003-2009 in the estuary of Bilbao, but in August during the periods 2003-2009 and 2010-2015 in the estuary of Urdaibai. The more seasonally restricted development of *A. tonsa* population in the estuary of Urdaibai has been attributed to the interaction with the dominant indigenous species *A. bifilosa* (Villate et al. 2018). The latter species peaks earlier in the year than *A. tonsa*, this likely being related to the fact that highest egg production rates of *A. bifilosa* occur at submaximal temperatures of around 20°C in the estuary of Urdaibai (Uriarte et al. 1998) while *A. tonsa* achieves highest reproductive success at higher temperatures (Leandro et al. 2006).

The seasonal pattern of *O. davisae* was more skewed towards autumn than that of *A. tonsa* in the estuary of Bilbao, where both species showed well-established populations and exhibited high densities along the summer-autumn period. In its region of origin, but also in areas colonized by them, the seasonal pattern of *O. davisae* is characterized by maxima in early summer or autumn (Uye and Sano 1995; Yildiz et al. 2016). However, in Lakes Faro and Ganzirri coastal ecosystems in the Mediterranean (Zagami et al. 2018) and in San Francisco Bay (Bollens et al. 2011) its density starts to increase in late spring, remains high in summer and decreases in autumn. In the estuary of Bilbao *O. davisae* remained in the winter zooplankton community, in contrast to what has been observed in colder areas like the coastal waters of the Black Sea (Seregin and Popova 2016), probably because water temperature is rarely lower than 10 ºC in the estuary of Bilbao.

The seasonal patterns of *P. marinus* in the estuaries of Bilbao and Urdaibai were similar to those observed in other areas colonized by this species such as a southern California embayment, Belgian harbours and North Sea coast and ports (Fleminger and Hendrix Kramer 1988; Deschutter et al. 2018), with highest densities from June to September, a decrease in autumn and absence in some winter-spring months. (Sato 1913) first described *P. marinus* from embayments along the west coast of Japan as a perennial species (Uye et al. 1982). In Fukuyama Harbour, this species is also perennial, with a large density peak in June but very scarce in winter due to the low reproductive rate induced by low temperatures (Liang and Uye 1997). Because *P. marinus* does not produce resting eggs (Sabia et al. 2014), we hypothesize that the population within the estuary is annually recovered by migration from a perennial coastal population. What maybe happen is that the population is flushed out the estuary of Bilbao in the period of highest river flow (winter-early spring) and later penetrates in the estuary again in spring favoured by the increase of the net upward bottom circulation from spring to summer (Uriarte et al. 2014).

In most salinities, the seasonal pattern of *A. tonsa* remained rather similar in the two estuaries under study since its settlement, but in the innermost site of the estuary of Bilbao the growing season of this species clearly expanded towards spring and autumn in the last period, and showed higher densities all year round. This occurred in spite of the temperature decrease in spring and the successful development of the spring peaking *C. aquaedulcis* in that period. *O. davisae* also increased in density from 2003-2009 to 2010-2015 in spring, however, its seasonal pattern showed higher differences between periods than that of *A. tonsa*, and the annual maximum moved from autumn in the period 2003-2009 to summer in the period 2010-2015. This indicates that the seasonal dynamics of these two species did not respond in the same way to environmental changes over time, and suggests that *O. davisae* modifies its seasonal pattern more easily than *A. tonsa* in response to the environmental factors that shape their annual cycle. The seasonal distribution of *A. tonsa* also expanded towards spring in the period 2010-2015 in the inner estuary of Urdaibai. However, no clear relationship with environmental factors could be established in any of the cases. The coarse comparison of changes in environmental variables and species densities between periods may mask relationships that could be revealed by testing them at shorter time scales. Year-to year or month-to month comparisons should be conducted as a next step to determine adequately the effect of environmental factors on the species dynamics.

As it was also observed in the upper San Francisco Estuary (Bollens et al. 2014), NIS were particularly dominant in the summer and autumn, whilst the OES *C. aquaedulcis* and *A. bifilosa* that arrived later in the inner estuary of Bilbao were more abundant in the first half of the year and had an earlier seasonal timing than in the estuary of Urdaibai. This suggests that competition between the earlier arriving NIS and the later arriving OES in the recovered brackish community of the inner estuary of Bilbao may have occurred. Studies in other areas where *A. tonsa* and *O. davisae* are also abundant have also shown the temporal segregation of these two species. For instance, Frisch et al. (2006) reported highest abundances of the NIS *A. tonsa* in spring and autumn and of *C. aquaedulcis* in summer in artificial brackish ponds of the Doñana Natural Park (Spain).

1.4.4 Copepod NIS and OES effects on total zooplankton, copepods and congeneric species

Results showed that zooplankton and copepod densities shifted twice during the study period at the low salinity sites of the estuary of Bilbao, primarily due to the contribution of the NIS *A. tonsa* and *O. davisae* in the first shift and to the contribution of the OES *C. aquaedulcis*, and to a lesser extent of *A. bifilosa*, in the second one. The observed increase of the density of zooplankton and copepods of one order of magnitude (from hundreds of individuals per cubic meter to thousands of individuals per cubic meter) must have had a positive effect on the recolonization of this estuary by fish during the rehabilitation process and on the improvement of the health status of fish communities that has been observed in the inner estuary since 2004 (Uriarte and Borja 2009), by increasing food availability for fish larvae and zooplanktophagous adults. No shifts of such magnitude in zooplankton and copepod densities were observed in the inner estuary of Urdaibai, where the brackish indigenous species *A. bifilosa* was already occupying the brackish habitat and likely limited the population growth (spatial and seasonal expansion) of the newly established congeneric NIS *A. tonsa*. In Urdaibai, the introduction of *A. tonsa* did not cause the disappearance or dramatic decrease of *A. bifilosa*, but spatial and seasonal segregation of these two species occurred, as in other European estuaries (Baretta and Malschaert 1988; Soetaert and Van Rijswijk 1993; David et al. 2007). The unsuccessful settlement of *O. davisae* in the estuary of Urdaibai made this species irrelevant to the changes in total density of zooplankton and copepods throughout the study period in this system. The successful settlement of the newly arrived NIS and OES in the estuary of Bilbao led to the restoration of the brackish copepod community, lacking in the past as a result of pollution (Villate et al. 2004), and furnished the inner estuary with copepod and zooplankton densities similar to those shown by the neritic community in the outer estuary. However, the brackish community established in the estuary of Bilbao dominated by *A. tonsa*, *O. davisae* and *C. aquaedulcis*, differed from that of the nearby estuary of Urdaibai dominated by *A. bifilosa* and *A. tonsa*, and added the estuary of Bilbao to the increasing group of coastal and estuarine systems dominated by *A. tonsa* (Cervetto et al. 1999; Cubbage et al. 1999; Leandro et al. 2006; Sørensen et al. 2007; Tiselius et al. 2008) and *O. davisae* (Uye 1994; Altukhov et al. 2014). However, in the estuary of Bilbao, the above-mentioned species of copepods were not the only taxa contributing to the increase of zooplankton density in the inner estuary. Bivalve, cirripede and gastropod larvae showed noticeable increases in density during the period of study associated to the rehabilitation process of the estuary of Bilbao. Parallel to the water quality improvement, a new layer of sediments covered the polluted and azoic deposits of the estuary of Bilbao (Irabien et al. 2018), and this allowed the penetration of benthic fauna further and further inside the estuary (Borja et al. 2006).

The development of NIS populations not only resulted in an increase in the density of total copepods and zooplankton, but also in compositional and phenological changes, which were most evident in the inner estuary of Bilbao. Compositional changes may affect the suitability of available prey to consumers, since some zooplanktophagous fishes have been found to select for native zooplankton over NIS (Adams et al. 2015), and differences in size and motility between native species and NIS may result in selective predation. Regarding *Acartia* and *Oithona* congenerics from our estuaries, there are differences in size among them that could make some of them be more or less attractive to predators, since *A. tonsa* is larger than the dominant native copepod *A. bifilosa*, with which it shows some spatial and temporal overlap, and *O. davisae* is the smallest of the three native congeneric species inhabiting the estuaries of Bilbao and Urdaibai. The strongest compositional changes due to the copepod NIS occurred in the inner estuary of Bilbao. NIS were quantitatively irrelevant until 2002 in both estuaries at all salinities, but since 2003 they constituted regularly more that 50% of the zooplankton and more than 85% of the copepods (together with the OES since 2010) at the innermost salinity site of the estuary of Bilbao, at the same time that *A. tonsa* and *O. davisae* constituted regularly more than the 90% of the *Acartia* and *Oithona* assemblages, respectively. In addition, the strong dominance of two NIS originated in the Pacific region in the zooplankton of the inner estuary of Bilbao during the 2003-2009 period entailed the establishment of a brackish community more similar to those typical of Pacific estuaries as compared to typical estuarine communities along the European coasts. Shifts in estuarine zooplankton communities to communities with characteristics typical of other bioregions as a result of NIS colonization have been reported in other estuarine systems too (Cordell et al. 2008). However, the marked contribution of the OES that arrived later to the brackish community of the estuary of Bilbao made this community to change to features more similar to those of other nearby estuaries dominated by native species. This also suggests that competition driven changes in NIS and OES will define the future characteristics of the brackish zooplankton community in this system.

Zooplankton phenological changes are also claimed to have significant consequences in the dynamics of consumers, according to the match-mismatch hypothesis (Cushing 1990). In the inner estuary of Bilbao, the initial spring maximum of copepods due to neritic species was replaced by the summer maximum due to the NIS, and in the inner estuary of Urdaibai, the maximum density of total zooplankton and copepods occurred earlier after the settlement of the NIS. This last phenological change was mainly due to the change in the seasonality of the dominant native species *A. bifilosa*, likely influenced by potential competition effects of the new species *A. tonsa* (Villate et al. 2018 and the present work). Nevertheless, the seasonal and spatial segregation of these two congeneric species in the estuary of Urdaibai seems to be less marked than those reported in other estuaries like Ems (Germany) and the Solent-Southampton Water estuarine system (south coast of England), where *A. bifilosa* is a typical winter-spring species peaking in April-May and with wide spatial distribution, while *A. tonsa* is restricted to the inner part and peaks in September (Baretta and Malschaert 1988; Castro-Longoria 2003). Likewise, in the Gironde estuary (France), the introduction of *A. tonsa* changed the seasonal pattern of *A. bifilosa* without affecting their long-term population stability (David et al. 2007).

The increase of *A. tonsa* density from 2003-2009 to 2010-2015 was not accompanied by a decrease of *A. clausi* density at the 30 salinity site of the estuary of Bilbao. Furthermore, *A. tonsa* density decreased between those two periods at the 33 salinity site. This suggests that the seaward displacement of *A. clausi* by *A. tonsa* from the period 1998-2002 to the period 2003-2005, reported by Aravena et al. (2009), has not been enhanced by the increase of *A. tonsa* density in the inner estuary during the last years. The assessment of the impact of *A. tonsa* on *A. margalefi* and *A. discaudata* in the estuary of Bilbao is difficult because of their low density and sparse distribution (Villate et al. 2018). In the lagoon of Venice, however, Camatti et al. (2019) noticed that the initially abundant *A. margalefi* was not completely excluded but declined in abundance after the establishment of *A. tonsa* in the lagoon.

The impact of *O. davisae* on congeneric species has seldom been investigated, but studies in the Black Sea and Lakes Faro and Ganzirri (Mediterranean Sea) showed that *O. davisae* displaced the indigenous *O. nana* (Isinibilir et al. 2016; Zagami et al. 2018). A similar effect has been observed in the inner estuary of Bilbao, where the settlement of *O. davisae* heavily reduced the presence of *O. nana*. A strong competition between *O. nana* and *O. davisae* is expected, because of their similar size, behaviour and seasonal overlap. Experimental studies concluded that a higher salinity tolerance, a lower body density, a lower sinking speed and a higher swimming speed confer *O. davisae* higher adaptive potential in comparison to *O. nana* (Isinibilir et al. 2016). No evidence of impacts on *O. similis* were detected in the present study, likely due to the low seasonal overlap of this spring peaking species with *O. davisae* (Villate 1991). *O. plumifera* reached highest densities in the second part of the year (Villate 1991) and overlapped seasonally with *O. davisae*, but it showed decreases in density in high salinity waters of both estuaries, where *O. davisae* was scarce or absent, this denoting the main influence of environmental factors common to both estuaries.

The contribution of different taxa to the dynamics of copepods and zooplankton within each estuary allowed us to conclude that the replacement in the dominance of brackish by neritic copepods as salinity increases along the longitudinal axis was similar in both estuaries, with brackish species showing the highest contribution at 33 and lower salinities and neritic species at salinities above 33. The replacement of the neritic *A. clausi* by the brackish NIS *A. tonsa* as the dominant species at the inner salinities of the estuary of Bilbao is common to other estuaries and coastal areas colonized by *A. tonsa* (Gaudy et al. 2000; Chinnery and Williams 2004; Azeiteiro et al. 2005; Sei et al. 2006). In the estuary of Urdaibai, however, the contribution of the indigenous brackish species *A. bifilosa* was always higher than that of *A. tonsa* in waters of 33 and lower salinity, and the contribution of *A. tonsa* decreased more drastically with increasing salinity than that of *A. bifilosa*. This suggests that the colonization and increase of *A. bifilosa* in the last years of the study period in the estuary of Bilbao might lead to a readjustment in the contribution of these two congeneric species in the near future in this estuary.

CHAPTER 2: Impact of colonizer copepods on zooplankton structure and diversity in contrasting estuaries: dependence on pre-arrival conditions

The impact of non-indigenous species (NIS) and other expanding species (OES) of copepods on the structure and diversity of zooplankton communities was analysed for a period of 18 years (1998-2015) in the contrasting estuaries of Bilbao and Urdaibai (Basque coast, Bay of Biscay). Changes in the structure of communities were assessed by using multivariate analyses of taxa abundances and changes in diversity by using different descriptors of alpha, beta and gamma diversity. The most evident changes occurred at the inner reaches of the estuary of Bilbao, where an abundant and less diverse brackish community, dominated by the NIS, Acartia tonsa and Oithona davisae, succeeded a low abundance and more diverse community of neritic origin. The later establishment of OES, mainly of Calanipeda aquadulcis, accounted for further changes in the structure of the community and a progressive increase in diversity. The seasonal pattern of diversity at the inner estuary and the beta diversity in the estuary were also significantly affected by the arrival of NIS and OES. This study revealed that the observed changes in zooplankton structure and diversity were clearly dependent on the initial environmental and biological conditions and the inherent properties of each of the estuarine systems. The availability of unsaturated brackish habitats and higher hydrodynamical stability favoured the colonization of the by new arriving species in the estuary of Bilbao, while the pre-existence of a well-developed brackish community and the lower hydrodynamical stability limited the colonizing success and the consequent impact in the estuary of Urdaibai.

2.1 Introduction

Non-indigenous species (NIS) can affect the recipient community in highly variable ways; from changes in the density, even local extinction of native species, to changes in the structure and functionality (Thomsen et al. 2014; Gallardo et al. 2015; Chan and Briski 2017), which can influence ecosystem stability overtime because species respond differentially to temporal environmental variations (Mccann 2000; Schindler et al. 2015). Likewise, the consequences of multiple invasions within a community should also be addressed (Olden and Poff 2003). One of the main tools to study the changes in long term monitoring's of large numbers of species and ecosystems are diversity indexes (Magurran et al. 2010; Vackár et al. 2012). There are different diversity types depending on the focus: alpha diversity which reflects the variability in a small area of homogeneous habitat, gamma diversity which indicates variability at the regional level (or within a system with different habitats) and beta diversity which gives us the turnover of taxa from one habitat to another (Ricklefs 2010). These indices are easy to calculate and they are not only relevant for making science but also to inform policy, because diversity is something our societies care about (Aslaksen et al. 2015). There are many diversity indexes, some that measure richness, others that measure evenness and others that measure both richness and evenness, although only a few of them are commonly used (Magurran 2004). In studies related to changes in diversity due to NIS introductions, Species richness, Shannon index and Pielou´s evenness are the most frequently used diversity indexes. Species richness gives information about species numbers, Pielou´s evenness measures the dominance level in the community and the Shannon index accounts for both (Magurran 2004). Therefore, after the introduction of NIS in an ecosystem a decrease in species richness will indicate local extinction of native species populations (Albins 2015); changes in evenness may indicate that some local species have become less dominant, both denoting changes in community structure.

Although global scale diversity trends are easier to detect under the current situation of global biodiversity loss, local scale diversity trends are more complex (Richirt et al. 2019), because they may be strongly influenced by local ecological context (Elahi et al. 2015). The biotic resistance hypothesis of Elton (Elton 1958) predicts that more diverse communities will have greater resistance to invasions. Therefore, changes in species richness and composition are most important in facilitating invasions, and this may be the result of increased vulnerability to invasion in a post-disturbance (natural or man-made) community dominated by poor competitors (Kneitel and Perrault 2006). This has also been observed in transitional systems, where salinity is a limiting factor and lowered species richness occur at intermediate salinity brackish habitats, these zones becoming subject to invasion by NIS (Paavola et al. 2005). In addition to the importance of low species richness in the establishment of NIS, Marraffini and Geller (2015) concluded that the interaction with other factors (e.g. resource availability) also contributes to the invasibility. Therefore, low diversity, suitable environmental conditions for invaders and availability of vectors are the ideal combination for brackish water invasions. Therefore, the pre-invasion conditions of the receiving habitat could greatly influence the consequences of NIS establishment on the native community, including diversity changes, but more studies are needed to better evaluate community structure and diversity trajectories at very fine scales at which ecologists often work (Elahi et al. 2015).

However, many of the NIS effects on the ecosystems they colonize are not easy to observe because they may take a long time to reveal, and therefore the full impact on biodiversity may not be apparent in the short term (Gallardo et al. 2015). As for marine systems, zooplankton-monitoring programs are an essential tool to improve our understanding and management of effects related to NIS. These programs have recently started to focus on the variations in community structure and functional diversity based on indicators (Chiba et al. 2018). Accordingly, we made use of the monitoring program of the zooplankton community carried out since 1998 in two contrasting estuaries of the Bay of Biscay, i.e. the estuaries of Bilbao and Urdaibai, to analyze variations in community structure and diversity induced by NIS copepod occurrences. The present work aims to describe, compare and explain the changes in the structure and diversity of zooplankton communities in the estuaries of Bilbao and Urdaibai and determine the role of the NIS and OES in such changes, and the influence of the contrasting ecological conditions in these estuaries. The estuary of Bilbao, which was still highly polluted at the beginning of the study period, has experienced more marked environmental changes than the estuary of Urdaibai, due to a rehabilitation process that favoured the biotic recolonization of the upper brackish zone (Uriarte et al. 2016). Therefore, this also offered a good opportunity to analyse the effect of local pre-invasion conditions on the changes in diversity and community structure driven by colonizer species in estuaries.

2.2 Method

2.2.1 Data analyses

Before calculating diversity indexes, rare copepod species were excluded densities as done in other studies (Richirt et al. 2019; David et al. 2020), retaining only species with a presence >0.01% over the period 1998-2015 (calculations taking into account presence in data pooled for the two estuaries). Zooplankton data were grouped and analyzed at two levels: (i) the zooplankton group level that included 19 groups and (ii) the copepod species level that included 25 taxa (Table 1).

For the multivariate ordination analyses taxa densities were log transformed (log x +1). In order to detect changes on diversity indexes due to NIS, the study period was divided into the three periods described in the General method section of the first part of this thesis.

Multivariate ordination methods were used to model the variability in the taxonomic structure of zooplankton communities at each estuary using the software Canoco v 4.55. A PCA of the zooplankton taxa densities of each estuary was conducted using months and salinity sites as covariables, and the position of each taxa in the two first axes was depicted in order to visualize the contribution of taxa to the main temporal and spatial changes in the structure of communities. Inter-annual density variations of the taxa that contributed most were also plotted. Moreover, separate PCA analyses were performed for each salinity site at both estuaries (B30, B33, B34 and B35 in the estuary of Bilbao; U26, U30, U33 and U35 in Urdaibai) and year scores of the first two axes for each salinity site were depicted, in order to show year-to-year variation patterns in zooplankton community structure and taxa scores were examined to determine which species influenced most community structure pattern.

The diversity of zooplankton groups and copepod species was analysed by the calculation of alpha, beta and gamma diversity indices using Primer 6 software. Species richness (S), Shannon index (H´, log e) and Pielou`s evenness (J) indices were calculated at a monthly scale for each salinity site of each estuary to estimate alpha diversity, and pooled for all salinity sites of each estuary to estimate gamma diversity. To determine beta diversity, the Whittaker index was calculated at a monthly scale in each estuary. Inter-annual variations and seasonal patterns of zooplankton groups and copepod species diversity during each period in each salinity site and estuary under study were depicted. Spearman rank correlations of the diversity indexes seasonal patterns between periods were tested. In this case, the correlations do not serve to observe if seasonal patterns between periods are related, but rather they are useful to explain which have not similar patterns (no significant positive correlation) or which have the opposite pattern (significant negative correlation). Prior to statistical comparisons, the normality of the data was tested by Kolmogorov-Smirnov test. All those tests were performed using statistical RStudio software (RStudio Team 2020). The contribution of the different taxa to the observed inter-annual changes in zooplankton groups and copepod species alpha and gamma diversity were tested using generalized additive models (GAM) (Wood 2004), which were performed using the gam function from the mgcv R package (RStudio Team 2020). In this case, in order to eliminate the seasonal effect, which is common in plankton time series (Ribera D´Alcalà et al. 2004; Benedetti et al. 2019), monthly anomalies were used, which were calculated as the difference between each value and the mean value for each month for the period under study divided by the standard deviation.

Table 1. List of taxa used in the analyses.

		Taxa		Taxa
Copepods	NIS	*Acartia tonsa*	Zooplankton groups	Appendicularians
		Oithona davisae		Doliolids
		Pseudodiaptomus marinus		Chaetognaths
	OES	*Calanipeda aquaedulcis*		Siphonophores
		Acartia bifilosa		Bivalve larvae
		Acartia clausi		Gastropod larvae
		Acartia discaudata		Cirripede larvae
		Acartia margalefi		Decapod larvae
		Paracartia granii		Ichthyoplankton
		Oithona nana		Medusae
		Oithona similis		Echinoderm larvae
		Oithona plumifera		Cyphonaute larvae
		PCPC-calanus assemblage (*Paracalanus* sp., *Clausocalanus* sp., *Ctenocalansu* sp. and *Pseudocalanus* sp.)		Polychaeta larvae
		Temora longicornis		Cladocerans
		Temora stylifera		Ostracoda larvae
		Euterpina acutifrons		Isopods
		Ditrichocorycaeus anglicus		Mysids
		Oncaea media		Ascidian larvae
		Calanus sp.		Copepods
		Calocalanus sp.		
		Centrophages sp.		
		Candacia sp.		
		Fresh water copepods		
		Microsetella sp.		
		Harpacticoids		

2.3 Results

2.3.1Community structure

The first component (axis 1) of the PCA for the estuary of Bilbao (41.4% of the total variability) emphasised the major role of the NIS *A. tonsa*, *O. davisae* and *P. marinus* and the OES *C. aquaedulcis* and *A. bifilosa*, together with some meroplankton groups like bivalve, gastropod and cirripede larvae in the zooplankton community changes during the study period, since they all had positive scores, as opposed to a larger assemblage of taxa which showed negative scores (Fig. 1). The plot of year scores along axis 1 (Fig. 2a) revealed similar trends of interannual variation at the B30, B33 and B34 sites, but the magnitude of the change increased with decreasing salinity and was highest at the innermost site (B30). At B34, however, a slight return trend toward the initial community structure was observed in the year 2009. At the outermost salinity region (B35) no clear trend of community change was observed. The taxa that contributed most to the main pattern of interannual change in the zooplankton community of the estuary of Bilbao, i.e. the NIS copepods *A. tonsa* and *O. davisae*, increased markedly their densities from the end of the 1998-2002 period to the beginning of the 2003-2010 period, while the taxon that showed the third highest contribution, the OES copepod *C. aquaedulcis*, did not show a large increase in density until the beginning of the 2010-2015 period (Fig. 2b). The second component (axis 2) of the PCA (15.7% of the total variability) reflected common patterns of variation in most neritic and brackish taxa that were in contrast to those of freshwater copepods (Fig. 1). This pattern of variation in neritic and brackish taxa was similar at all salinity sites (Fig. 2a), and was mainly related to the increase in density from 1998-2002 to 2003-2010 or 2010-2015 of several groups such as appendicularians, bivalve larvae and gastropod larvae (Figs. 1 and 2b).

In the estuary of Urdaibai, the first component (axis 1) of the PCA analysis accounted for a much lower variability of the zooplankton community (23.7% of the total variability) than in the estuary of Bilbao (Fig. 1), and reflected a common pattern of variation of most neritic taxa that was slightly in contrast with the pattern of some brackish copepods, i.e. the NIS *A. tonsa* and the native species *Paracartia grani* and *Acartia discaudata.* The plot of year scores along the axis 1 (Fig. 3a) showed no clear trends of zooplankton change throughout the study period at any salinity, and revealed that the main interannual fluctuations were similar at all salinities. The strongest change in the community structure of the entire estuary was that occurred in 2012 in relation to previous and later years. The taxa that contributed most to such pattern of interannual variability were neritic copepod species (i.e. *Euterpina acutifrons*, *Oithona nana* and PCPC-calanus) with strong interannual variations of density, and coincident peaks of density during the 1998-2002 period and the 2010-2015 period where showed the maxima in 2012 (Figs. 1 and 3b). The second component (axis 2) (17.3% of the total variability) was mainly accounted by the density variations of the brackish NIS copepod *A. tonsa,* the brackish native copepod *A. bifilosa* and the larvae of gastropod and polychaeta, which opposed to those of some neritic holoplankters, i.e. shiphonophores, doliolids, *Temora stylifera* and *Oithona plumifera* (Fig. 1). Overall, the community showed a progressive change from 1998-2002 to 2010-2015 with similar variations over the study period at all salinities (Fig. 3a), but among the taxa that most contributed to this pattern of zooplankton variability only *A. tonsa* showed strong differences in density between periods because it was not found during in 1998-2002 while appeared in high density since 2003 (Fig. 3b).

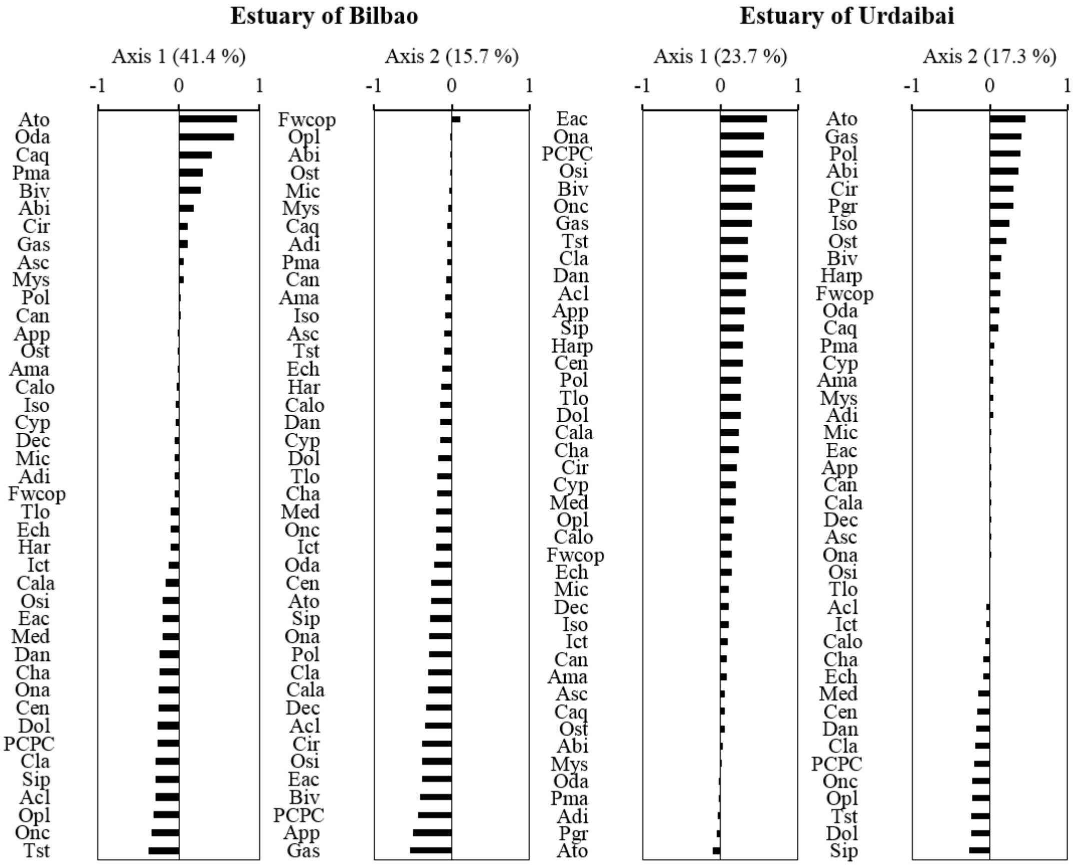

Fig. 1 Taxa scores on the first two axis obtained from PCAs using months and salinity zones as covariables for the estuary of Bilbao and Urdaibai. Abbreviation meaning are explained at Appendix 1.

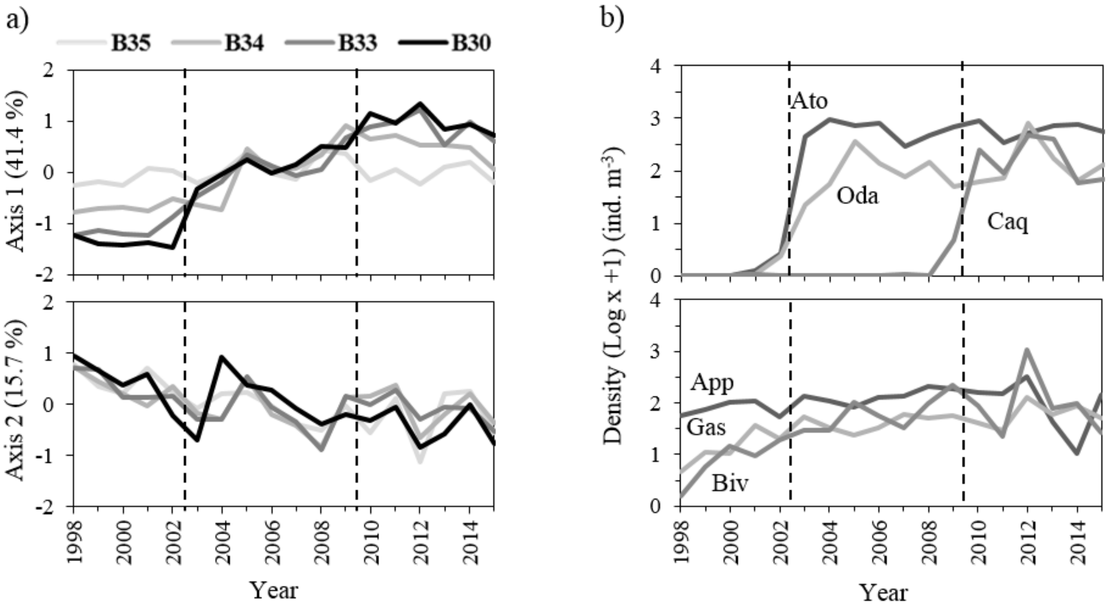

Fig. 2 a) Inter-annual variations of the first two axis for each salinity zone in the estuary of Bilbao. b) Inter-annual variations of the most influencing taxa densities at each axis for the whole estuary of Bilbao.

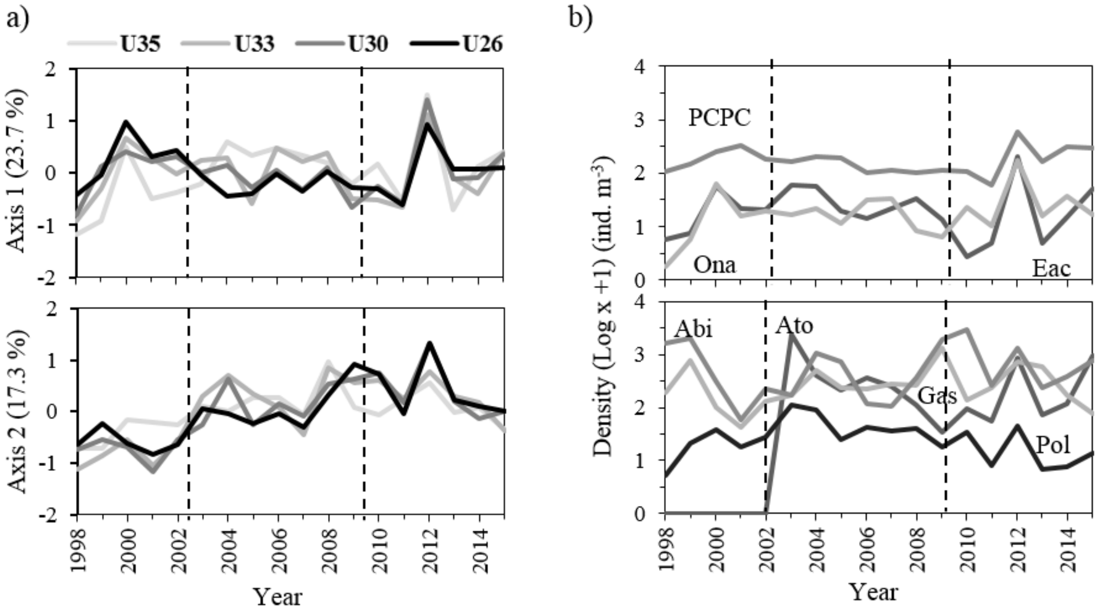

Fig. 3 a) Inter-annual variations of the first two axis for each salinity zone in the estuary of Urdaibai. b) Inter-annual variations of the most influencing taxa densities at each axis for the whole estuary of Urdaibai.

2.3.2 Diversity

Alfa diversity

Between-estuary differences in the spatial and temporal variations of alpha diversity for both zooplankton groups and copepod species were evident during the study period (Fig. 4). In the estuary of Urdaibai, species richness, Pielou's evenness and Shannon diversity of zooplankton groups and copepod species decreased, in general, from high salinity sites (U35 and U33) to the lowest salinity site (U26), and showed no clear trends over the study period but large interannual fluctuations. The main exception to the general decreasing pattern of Pielou's evenness and Shannon diversity with decreasing salinity was found in 2001-2002, with similar high values at all salinities and higher values at the lowest salinity (U26) than at the highest salinity (U35). In contrast with the estuary of Urdaibai, in the estuary of Bilbao, alpha diversity of zooplankton groups and copepod species differed between salinity sites and periods depending on the diversity index, and clearest patterns of change and trends over the study period were observed at the lowest salinity site (B30). Species richness decreased with decreasing salinity but was more similar between salinity sites during the 1998-2002 period than during the next two periods, where the richness of both zooplankton groups and copepod species showed a noticeable decrease at B30. This decrease was followed by a progressive increase in the richness of copepod species throughout the two last periods. Pielou´s evenness showed both for zooplankton groups and copepod species rather similar ranges of values at all salinity sites and throughout the entire study period in the estuary of Bilbao. However, in the last period (2010-2015) the evenness of zooplankton groups decreased at the lowest salinity site (B30) and increased at B34, this increasing between-salinity differences in zooplankton groups' evenness within the estuary. Variations of Shannon diversity for zooplankton groups showed some resemblance to those of evenness, mostly at the innermost site (B30), where both diversity and evenness showed a clear decreasing trend that resulted in highest differences with the other salinity sites during the last period (2010-2015). In contrast, variations of the Shannon diversity for copepod species resembled more those of species richness because, in both, values decreased markedly at the lowest salinity site (B30) from 2002 to 2005 and later they increased progressively.

According to GAM analysis, copepods were the main drivers of interannual changes in zooplankton groups' Shannon diversity and Pielou's evenness at all the salinity sites of both estuaries, while the contribution of other groups was lower and variable among estuaries and salinity sites (Table 2). Appendicularians were found to be the second or third main drivers in most cases in the estuary of Bilbao. In the case of copepod species, however, the main drivers of diversity and evenness differed between estuaries and salinities (Table 3). In the estuary of Bilbao, the main drivers of copepod diversity and evenness at B30 and B33 were the NIS *A. tonsa* and *O. davisae* together with the OES *C. aquaedulcis*, while at B34 and B35 they were neritic species, such as *A. clausi* or *Centropages* sp. Nevertheless, *A. tonsa* was also influential at B34. In the estuary of Urdaibai, *A. bifilosa* was the main driver of the changes in copepod diversity and evenness at all salinity sites, except at U35, where PCPC-calanus and *A. clausi* were the main drivers. The NIS *A. tonsa* also appeared as influential at U26 but in the fourth position.

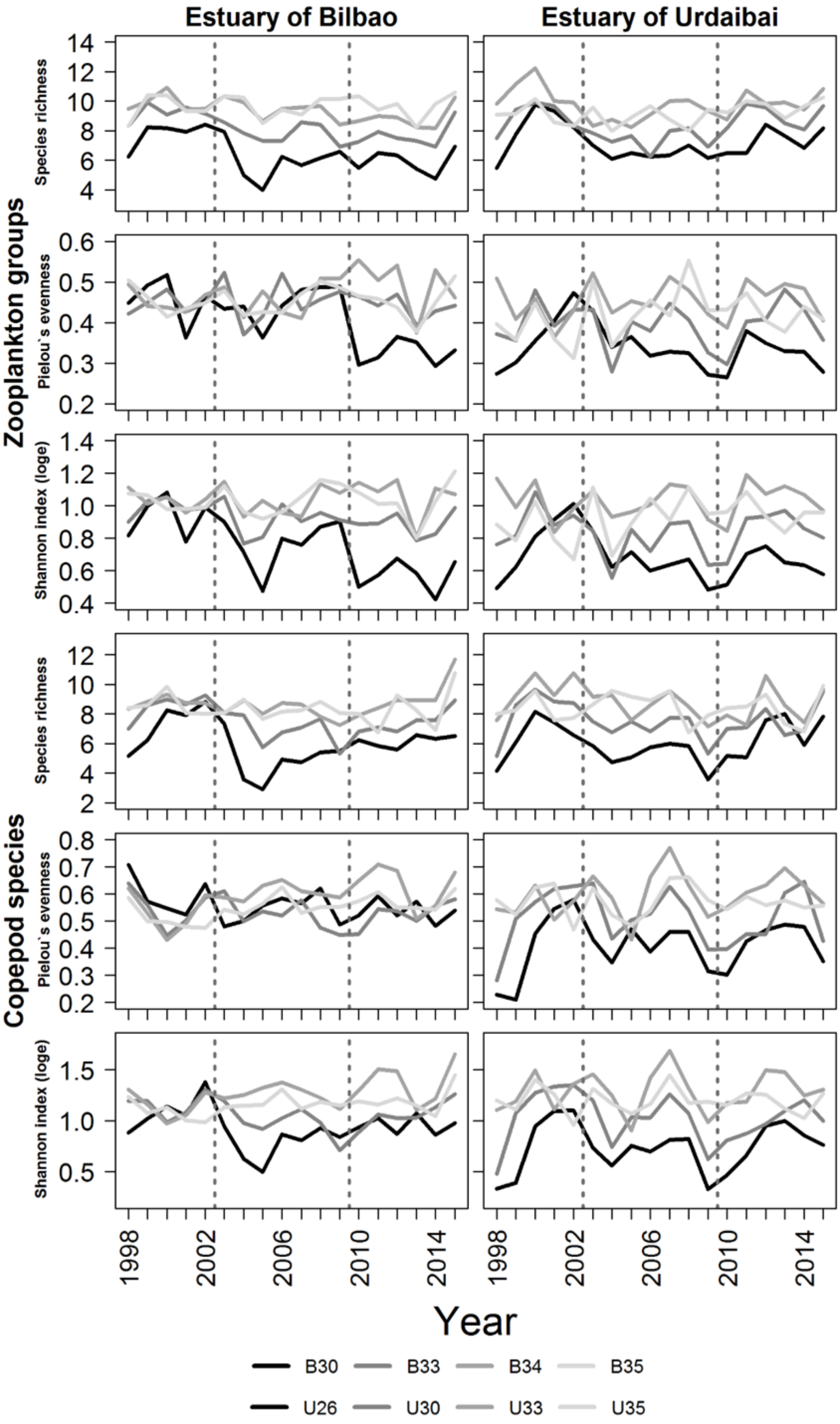

Fig. 4 Inter-annual variations of zooplankton groups and copepod species mean annual alpha diversity indexes (Species richness, Pielou`s evenness and Shannon index (log e)) at different salinity sites of the estuaries of Bilbao (30, 33, 34, 35) and Urdaibai (26, 30, 33, 35) from 1998 to 2015

Table 2. Results of GAM analyses of zooplankton groups' alpha diversity indexes (Pielou`s evenness and Shannon index (log e)) from the salinity sites under study of the estuaries of Bilbao and Urdaibai, using different taxa abundances as predictor variables. Only significant smooths are reported and the p-values and F values for predictor variables, the R square value and the percentage deviance explained for each model are shown. Taxa abbreviations as in Appendix 1. Note that the table continuous in the next page.

B30			**B33**			**B34**			**B35**		
Evenness											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	23.426	2.00E-16	Cop	30.645	2.00E-16	Cop	30.296	3.11E-15	Cop	32.692	2.00E-16
Cla	5.263	0.00283	App	9.853	2.60E-05	App	11.794	8.08E-05	Gas	20.025	1.28E-05
App	8.753	0.00345	Gas	5.566	0.000737	Biv	4.203	0.00114	App	4.376	0.000234
Gas	5.324	0.00416	Cla	6.29	0.012914	Dol	10.861	0.00116	Dol	12.576	0.000487
Biv	7.31	0.00743	Pol	2.317	0.041222	Cir	3.721	0.00142	Biv	4.964	0.001678
						Asc	2.757	0.01633	Cha	9.824	0.001982
									Cla	5.503	0.004309
									Cyp	5.05	0.006384
									Sip	3.904	0.015962
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.439	46.8		0.369	40.6		0.456	52.5		0.539	58.4	
Shannon index											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	27.057	2.00E-16	Cop	43.417	2.00E-16	Cop	40.885	2.00E-16	Cop	39.956	2.00E-16
App	21.913	5.16E-06	App	10.753	1.07E-06	Dol	18.68	2.43E-05	Cha	16.832	5.90E-05
Gas	6.717	0.000759	Pol	5.605	1.54E-06	App	8.685	0.000164	Gas	16.571	6.70E-05
Biv	10.952	0.001106	Ict	8.163	0.00474	Biv	6.943	0.001014	App	7.323	0.000121
Cla	4.988	0.002259	Cla	3.727	0.00575	Cir	2.582	0.018721	Dol	10.667	0.00128
Pol	6.924	0.009159	Ech	2.927	0.00893	Gas	5.271	0.022734	Sip	4.874	0.007235
Dec	4.706	0.010167	Biv	5.737	0.01757	Cla	2.835	0.025951	Biv	3.951	0.007651
Ost	4.168	0.042494	Dec	5.289	0.01839	Asc	2.199	0.044577	Med	6.405	0.012142
			Gas	3.155	0.03428				Cla	3.863	0.014546
									Ict	5.738	0.017518
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.565	59.9		0.577	62.9		0.479	53		0.595	62.8	

U26			U30			U33			U35		
Evenness											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	20.101	2.00E-16	Cop	35.729	2.00E-16	Cop	43.086	2.00E-16	Cop	52.555	2.00E-16
Pol	31.246	6.75E-08	Biv	5.792	0.000745	App	25.207	1.11E-06	Cir	7.973	1.35E-05
Biv	6.578	0.00158	Cir	9.642	0.002172	Gas	9.955	5.54E-05	Gas	6.561	3.75E-05
Iso	5.466	0.02033	Pol	3.523	0.014605	Pol	11.868	0.000694	Biv	15.807	9.69E-05
			Cla	3.401	0.019662	Cir	5.011	0.000725	App	14.849	0.000155
			Ost	2.829	0.037075	Iso	11.667	0.000768	Cla	6.909	0.009227
						Med	8.541	0.003867	Dol	5.996	0.015184
						Biv	7.613	0.006326			
						Dol	6.539	0.011287			
						Cla	4.874	0.028398			
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.467	49.1		0.514	54.9		0.612	64.4		0.583	61.1	
Shannon index											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	25.859	2.00E-16	Cop	34.167	2.00E-16	Cop	58.636	2.00E-16	Cop	52.102	2.00E-16
Pol	22.937	3.17E-06	Biv	12.491	2.93E-08	App	32.65	3.83E-08	App	28.89	2.14E-07
Biv	8.264	0.000178	Cla	13.656	0.000282	Pol	15.652	0.000106	Cir	9.479	2.66E-06
Iso	5.684	0.018035	Cir	7.08	0.008421	Med	9.467	0.000116	Biv	8.879	2.96E-05
Med	3.896	0.013267	Pol	2.623	0.017916	Biv	9.122	0.000307	Sip	4.225	0.000999
Gas	2.714	0.032381	Iso	3.744	0.025689	Cla	12.56	0.000492	Gas	5.268	0.001836
						Gas	6.513	0.001318	Med	6.052	0.014778
						Cir	4.105	0.006162	Ict	5.727	0.017677
						Ost	4.684	0.007667	Iso	2.186	0.021221
						Dol	4.508	0.011675			
						Iso	5.063	0.025551			
						Cha	2.99	0.038603			
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.517	55.1		0.564	59.8		0.693	72.5		0.65	69.5	

Table 3. Results of GAM analyses of copepod species' alpha diversity indexes (Pielou`s evenness and Shannon index (log e)) from the salinity sites under study of the estuaries of Bilbao and Urdaibai, using different taxa abundances as predictor variables. Only significant smooths are reported and the p-values and F values for predictor variables, the R square value and the percentage deviance explained for each model are also shown. NIS and OES in bold. Taxa abbreviations as in Appendix 1. Note that the table continuous in the next page.

B30			**B33**			**B34**			**B35**		
Evenness											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Ato	9.319	0.00256	**Ato**	16.678	3.48E-10	Acl	9.294	1.61E-11	Acl	28.391	1.16E-13
Har	8.316	0.00434	Acl	5.248	3.00E-05	Eac	7.339	0.00731	Cen	25.638	8.80E-07
Onc	6.645	0.01063	PCPC	5.745	0.00055	**Ato**	3.82	0.01681	Osi	6.624	0.000647
Fwcop	2.136	0.03508	Osi	3.266	0.00385	Osi	4.069	0.04497	Ona	3.407	0.008942
			Oda	7.645	0.00622				PCPC	4.944	0.025766
									Onc		0.027244
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.162	20		0.341	39.3		0.269	30.9		0.332	35.9	
Shannon index											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Ato	10.829	6.85E-05	**Ato**	24.999	3.04E-15	Acl	7.963	2.53E-09	Acl	27.425	1.09E-14
Osi	5.239	8.76E-05	Acl	9.494	2.02E-08	Eac	10.315	0.00153	Cen	9.397	8.14E-05
Eac	3.864	0.000333	Osi	24.699	1.42E-06	Mic	8.518	0.00391	Osi	8.078	0.000125
Harp	11.26	0.000951	PCPC	7.948	1.90E-05	Har	5.635	0.01852	Cala	14.817	0.000158
Caq	4.062	0.001502	Ama	4.439	0.000529	Tlo	4.66	0.03204	Onc	14.358	0.000199
Onc	9.573	0.002264	Cen	11.384	0.00089	**Ato**	3.192	0.04047	PCPC	6.428	0.001161
Ama	4.787	0.012451	**Caq**	5.419	0.003252				Eac	3.099	0.005139
Cen	2.881	0.013586	Eac	4.411	0.009802						
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.48	53.8		0.495	54.3		0.308	34.8		0.383	43.1	

U26			**U30**			**U33**			**U35**		
Evenness											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Abi	37.723	2.00E-16	Abi	19.329	2.00E-16	Abi	50.389	1.51E-11	PCPC	8.847	2.21E-08
PCPC	10.121	3.24E-05	Eac	7.615	0.00629	Har	3.409	0.0149	Acl	23.571	2.31E-06
Ona	9.978	0.00182				Calo	3.222	0.0228	Osi	7.943	0.00529
Ato	2.234	0.01525				Cen	3.345	0.038	Cen	5.127	0.00637
Onc	4.428	0.03658				Ama	3.218	0.0405	Eac	5.541	0.0195
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.466	50.4		0.381	40		0.261	29		0.254	28.6	
Shannon index											
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Abi	32.163	2.00E-16	Abi	21.626	2.00E-16	Abi	9.117	1.47E-07	PCPC	10.159	4.27E-10
PCPC	14.095	0.000226	Eac	9.043	1.35E-05	Ona	3.969	0.0142	Acl	13.455	1.74E-07
Acl	4.657	0.002627	Cen	9.518	0.00231	Cen	4.14	0.016	Cen	7.952	0.000389
Ona	6.405	0.01213	Osi	4.246	0.00272	Eac	3.749	0.0221	Osi	12.851	0.000428
Osi	3.088	0.022781				Acl	2.222	0.0398	Eac	6.206	0.002013
Eac	5.017	0.02617				Dan	3.933	0.0487	Cala	3.897	0.007551
Onc	2.995	0.03148							Opl	3.207	0.00998
									Mic	3.713	0.011036
									Ona	4.027	0.019438
									Dan	2.705	0.021264
									Onc	5.278	0.022684
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.543	57.4		0.492	52		0.366	41.5		0.442	51.4	

Gamma and beta diversities

Gamma diversity values and patterns of variation for zooplankton groups and copepod species differed between estuaries depending on the index used (Fig. 5). Regarding zooplankton groups, in general, the richness was higher in the estuary of Urdaibai, whilst Pielou's evenness and Shannon diversity were higher in the estuary of Bilbao. Both evenness and diversity decreased over the study period in the estuary of Bilbao but did not show progressive changes in the estuary of Urdaibai. For copepod species, however, the richness increased more clearly along the study period in the estuary of Bilbao, in such a way that values were in general lower than in the estuary of Urdaibai in the 1998-2002 period and higher than in the estuary of Urdaibai in the 2010-2015 period. Pielou's evenness and Shannon diversity of copepod species showed frequent opposite patterns of variation between estuaries and slightly increasing trends in both estuaries, the highest values being recorded in the estuary of Bilbao during the last period. As shown in Table 4, copepods were the main drivers of gamma diversity changes of zooplankton groups in both estuaries, followed by bivalve larvae in the estuary of Bilbao and polychaete larvae in the estuary of Urdaibai. *A. clausi* and *Oithona similis* were the main species responsible for the changes in copepod evenness and diversity in the estuary of Bilbao, and *A. bifilosa* in the estuary of Urdaibai. The NIS *O. davisae* and *A. tonsa* and the OES *C. aquaedulcis* also influenced, but to a lesser degree, the changes of copepod evenness and diversity in the estuary of Bilbao, while none of the copepod NIS had significant influence in those indices in the estuary of Urdaibai.

The beta diversity represented by the Whittaker index (Fig. 5) increased both for zooplankton groups and for copepod species from the 1998-2002 period to the following periods in both estuaries. However, from 2003-2009 to 2010-2015 both increased in the estuary of Bilbao while they showed a slight decrease in the estuary of Urdaibai.

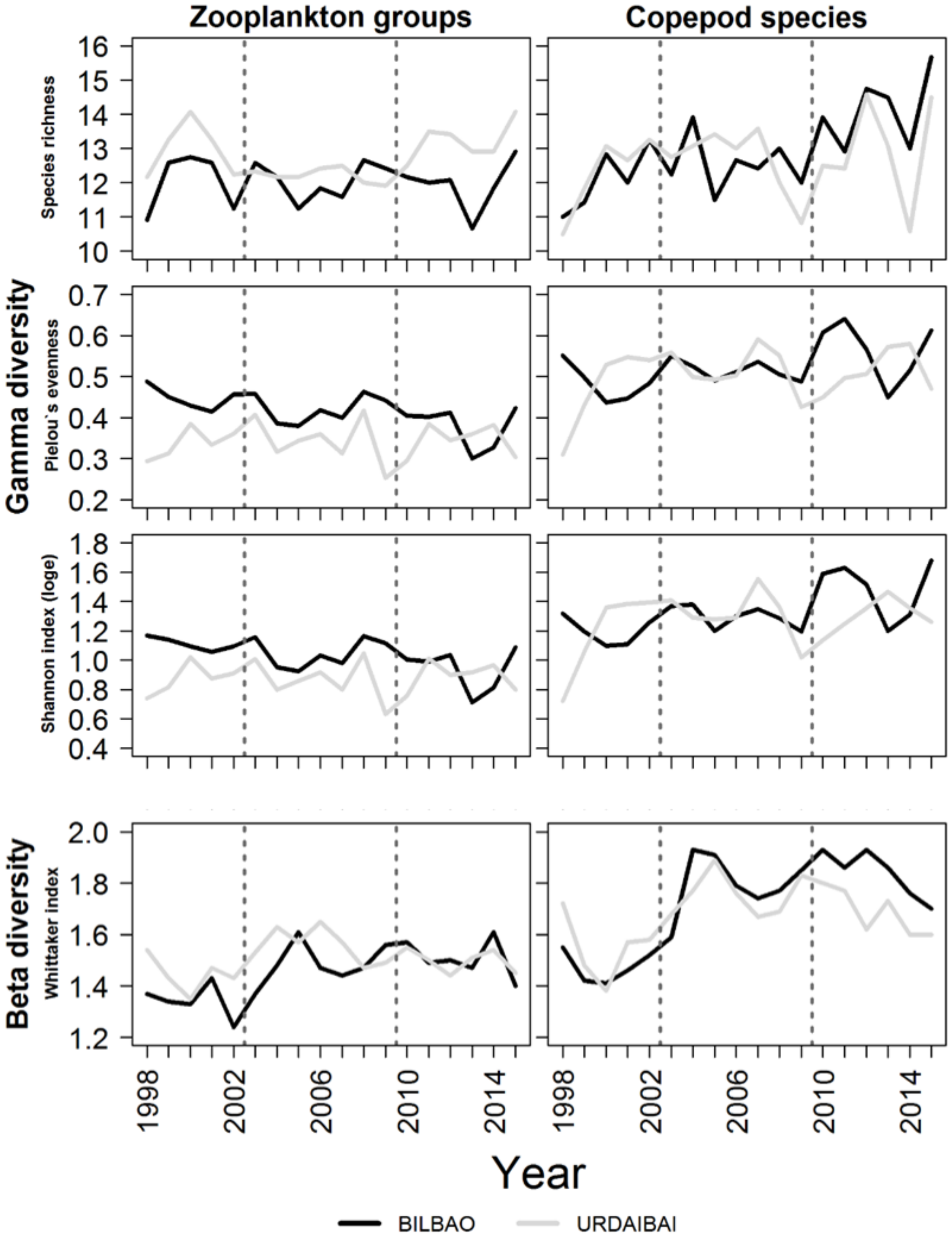

Fig. 5 Inter-annual variations of zooplankton groups and copepod species mean annual gamma diversity indexes (Species richness, Pielou`s evenness and Shannon index (log e)) and beta diversity index (Whittaker index) at the estuaries of Bilbao and Urdaibai from 1998 to 2015.

Table 4. Results of GAM analysis of zooplankton groups' and copepod species' gamma diversity indexes (Pielou`s evenness and Shannon index (log e)) from the estuaries of Bilbao and Urdaibai, using different taxa abundances as predictor variables. Only significant smooths are reported and the p-values and F values for predictor variables, the R square value and the percentage deviance explained for each model are shown. NIS and OES in bold. Taxa abbreviations as in Appendix 1.

BILBAO Zooplankton groups			**BILBAO Copepod species**			**URDAIBAI Zooplankton groups**			**URDAIBAI Copepod species**		
					Evenness						
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	52.663	<2E-16	Acla	33.988	3.19E-14	Cop	39.159	2.00E-16	Abi	61.321	2.00E-16
Biv	25.305	2.80E-07	Osi	20.231	4.45E-06	Pol	24.641	1.47E-06	Osi	3.64	0.00199
Cla	13.129	1.95E-06	**Oda**	20.805	8.83E-06	Biv	8.136	0.000258	Eac	9.224	0.00271
App	8.856	9.23E-06	**Ato**	6.817	1.65E-05	Cla	8.575	0.000338	Onc	8.945	0.00314
Dec	3.958	0.000133	Cen	17.285	4.77E-05	Gas	4.566	0.001066	Dan	7.947	0.00531
Sip	3.879	0.000273	**Caq**	4.408	0.00147	Cir	3.338	0.0035	Har	5.824	0.01673
Iso	8.626	0.003722	Onc	9.983	0.00183	App	7.337	0.007348	Acla	2.434	0.01894
Med	6.385	0.012323	PCPC	2.439	0.01096				Cala	2.636	0.06593
			Pma	4.573	0.03371				Cen	2.128	0.09299
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.703	74.3		0.43	48.7		0.653	68.9		0.476	49.2	
					Shannon index						
Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value	Smooth	F	p-value
Cop	60.966	2.00E-16	Acl	41.181	9.22E-16	Cop	47.152	2.00E-16	Abi	51.577	2.00E-16
Biv	32.856	3.71E-08	Osi	16.632	4.00E-07	Pol	30.965	8.32E-08	Eac	20.457	1.05E-05
Sip	11.805	1.65E-06	Cen	21.736	5.71E-06	Cla	14.24	7.70E-07	Osi	4.668	0.000191
Cla	13.604	2.61E-06	**Ato**	7.68	8.73E-06	Biv	8.158	3.61E-05	Har	7.946	0.002036
App	8.441	0.000115	Onc	18.114	3.21E-05	Cir	3.656	0.00181	Dan	3.76	0.002803
Dec	2.749	0.005419	**Oda**	*10.927*	0.00113	App	8.677	0.00362	Onc	8.096	0.004915
Ict	6.55	0.01128	**Caq**	4.309	0.00127	Gas	3.595	0.01096	Acl	2.54	0.012928
Med	6.244	0.013325	PCPC	3.058	0.00235	Asc	3.605	0.01426	Cala	3.398	0.014793
Pol	3.994	0.015739	Eac	6.928	0.00916	Dec	5.402	0.02116			
Ech	5.671	0.018259	Ama	5.492	0.02011						
Cir	2.137	0.042115	**Abi**	3.963	0.0479						
R^2	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**		**R^2**	**D.e. (%)**	
0.753	79		0.496	55.1		0.697	73.4		0.605	65.3	

Seasonal changes of diversity

Seasonal patterns of alpha diversity in the estuary of Bilbao (Fig. 6) showed strongest changes between periods at low salinities, where seasonal values of species richness and Pielou's and Shannon indices of zooplankton groups and copepod species calculated for the 1998-2002 period correlated negatively with those for one or both of the following periods, or in the case of B30 and B33 sites did not correlate between periods for most diversity indexes. The main seasonal changes between periods were observed for copepod evenness and diversity at the innermost salinity site (B30), with lowest values in winter-spring and highest values in summer-autumn during 1998-2002 but highest values in winter-spring and lowest values in summer-autumn during the next two periods. Summer-early autumn values of copepod evenness and diversity also decreased largely from the first to the two next periods at B33. At the high salinity sites of this estuary (B34 and B35), however, seasonal values of all the alpha diversity descriptors correlated between periods in most cases, and were almost always highest in summer-spring and lowest in winter for zooplankton groups but highest in late autumn or early winter and lowest in spring for copepods.

In the estuary of Urdaibai (Fig. 7) the seasonal patterns of all the alpha diversity indices of zooplankton groups and copepod species showed variations (no significant negative or positive correlation) between periods at all the salinity sites, except for the Shannon diversity of zooplankton groups at the outermost site (U35). At low salinities (U26 and U30), the main differences between periods in the seasonal patterns of the alpha diversity of the zooplankton groups and copepod species were due to the fact that the spring peak of diversity moved earlier in time, around two months, from 1998-2002 to 2010-2015. At U33, the highest values of zooplankton groups' diversity in summer delayed through the study periods, whereas copepod species evenness and Shannon diversity decreased in spring in the third period.

Except for the richness of zooplankton groups in the estuary of Bilbao, the seasonal patterns of gamma diversity of zooplankton groups and copepod species in both estuaries showed changes between the second and the third period in all the salinity zones, and between the first and the second or the third period in some of them (Fig. 8). In the estuary of Bilbao, zooplankton groups' gamma diversity peaked earlier in spring and high values lasted more months in the third period (2010-2015), and the annual peak of copepod species' gamma diversity moved from winter to autumn and summer values increased in the third period. In the estuary of Urdaibai, the highest values of zooplankton groups' gamma diversity moved from spring in the two first periods to summer in the last period, and the values of gamma diversity in copepod species were lower in spring and higher in summer in the last period. The seasonal pattern of beta diversity (Whittaker index) for zooplankton groups and copepod species differed between the three periods in both estuaries (Fig. 8). However, the most obvious variations of the seasonal pattern between periods were observed in copepod species, with the increase of beta diversity values in summer in the estuary of Bilbao and the decrease of beta diversity values in summer in the estuary of Urdaibai in the third period.

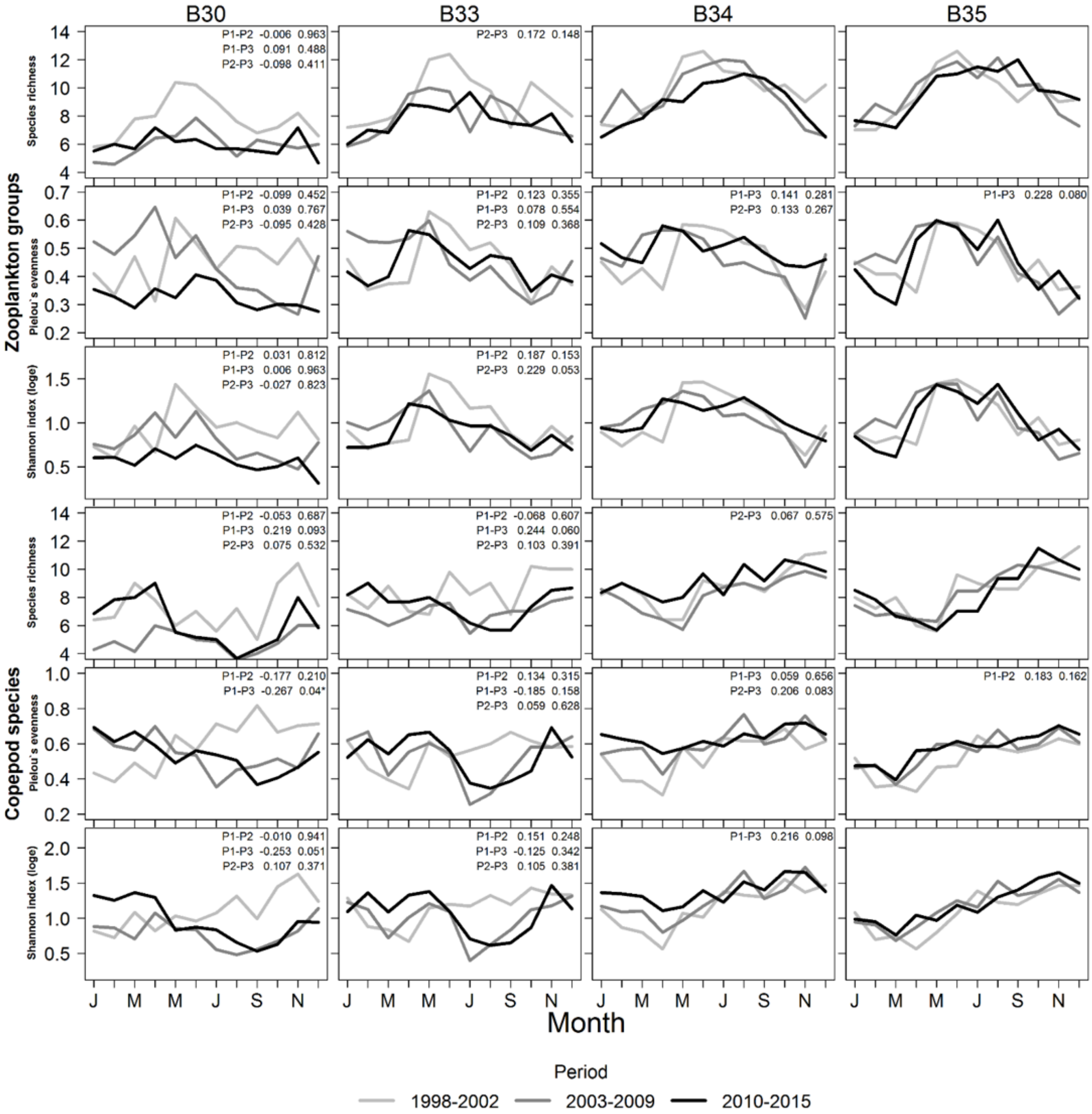

Fig. 6 Seasonal variations of zooplankton groups and copepod species mean annual alpha diversity indexes (Species richness, Pielou`s evenness and Shannon index (log e)) for periods 1 (1998-2002), 2 (2003-2009) and 3 (2010-2015) at the different salinity sites of the estuary of Bilbao (30, 33, 34, 35). Results of Spearman rank correlation test between periods of the alpha diversity indices seasonal pattern at the salinity zones under study are included, indicating in the first column between which periods are the correlations, in the second the coefficient of the correlation and in the third the p-value. Only non-significant p-values and significant p-values with negative correlation are reported.

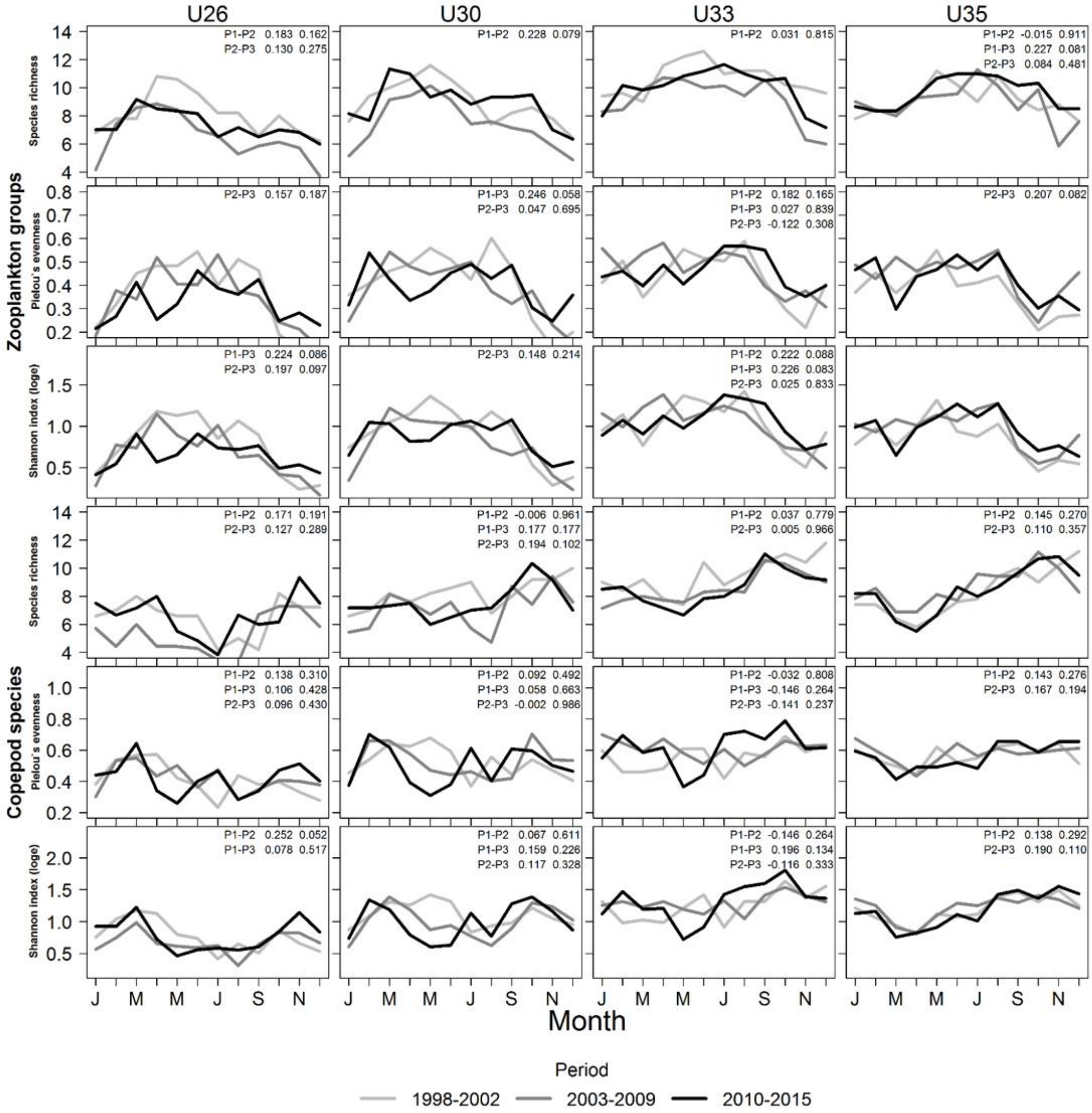

Fig. 7 Seasonal variations of zooplankton groups and copepod species mean monthly alpha diversity indexes (Species richness, Pielou`s evenness and Shannon index (log e)) for periods 1 (1998-2002), 2 (2003-2009) and 3 (2010-2015) at the different salinity sites of the estuary of Urdaibai (26, 30, 33, 35). Results of Spearman rank correlation test between periods of the alpha diversity indices seasonal pattern at the salinity zones under study are included, indicating in the first column between which periods are the correlations, in the second the coefficient of the correlation and in the third the p-value. Only non-significant p-values and significant p-values with negative correlation are reported.

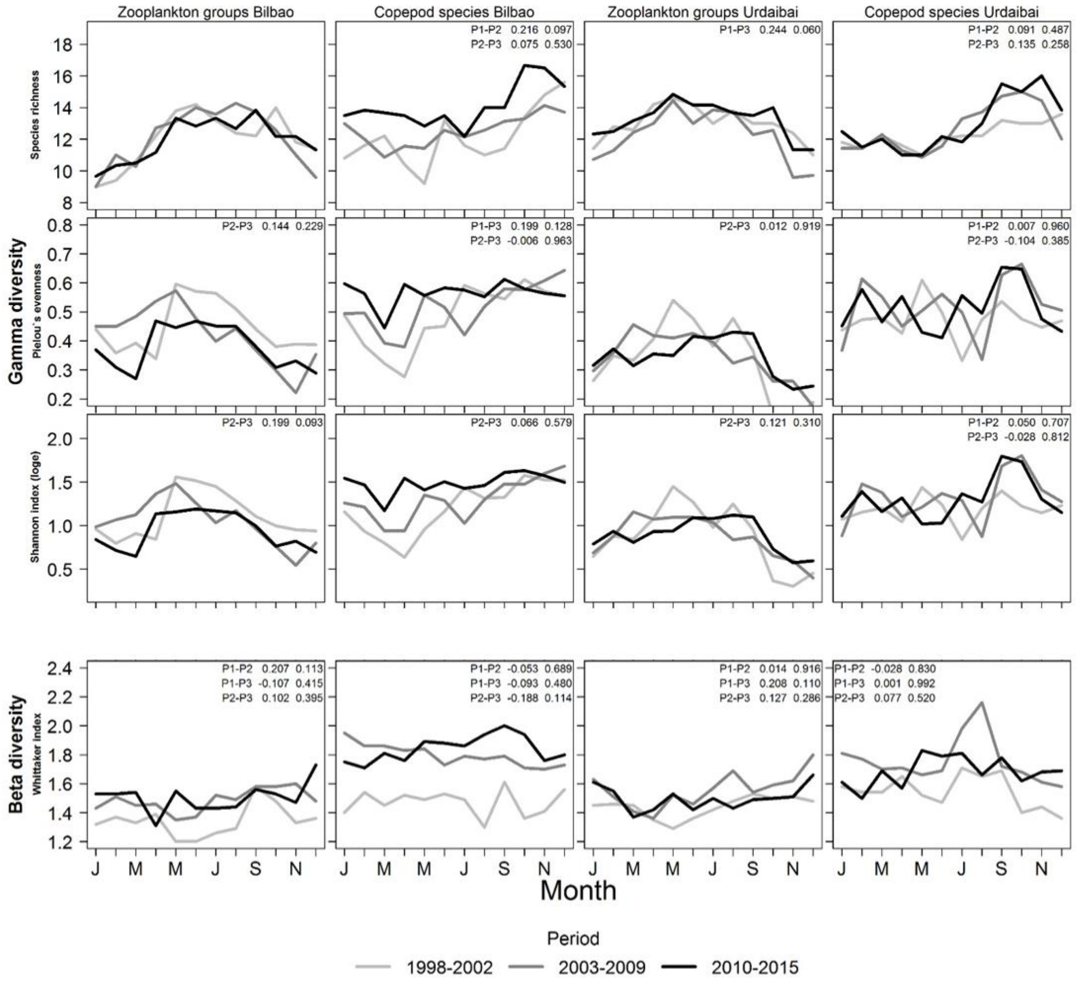

Fig. 8 Seasonal variations of zooplankton groups and copepod species mean monthly gamma diversity indexes (Species richness, Pielou`s evenness and Shannon index (log e)) and beta diversity index (Whittaker index) for periods 1 (1998-2002), 2 (2003-2009) and 3 (2010-2015) at the estuaries of Bilbao and Urdaibai. Results of Spearman rank correlation test between periods of the gamma diversity index seasonal pattern at the estuaries under study are included, indicating in the first column between which periods are the correlations, in the second the coefficient of the correlation and in the third the p-value. Only non-significant p-values and significant p-values with negative correlation are reported.

2.4 Discussion

The NIS and OES of copepods detected during the 18 yr study had a much higher impact on zooplankton community structure and diversity in the estuary of Bilbao than in that of Urdaibai. This impact decreased with increasing salinity in both estuaries, being negligible at the high salinity waters (B35 and U35) located at the lower estuarine zones. This patterns of change in impact on community structure and diversity were in agreement with the patterns of spatial and temporal variations in zooplankton and copepod densities observed in these two estuaries during the same study period (Barroeta et al. 2020). The higher impact in the estuary of Bilbao may be partly attributed to the higher number of new species that arrived in this estuary, which were the NIS *A. tonsa*, *O. davisae* and *P. marinus*, that also arrived in the estuary of Urdaibai, but also the OES *C. aquadulcis* and *A. bifilosa*, which were observed in the estuary of Urdaibai since the beginning of the study period. However, differences in impact may be attributed mainly to the fact that all the NIS and OES of copepods that were detected in the estuary of Bilbao showed a successful colonization, while only *A. tonsa* colonized successfully the estuary of Urdaibai and to a lesser extent than the estuary of Bilbao (Barroeta et al. 2020). The failure in the establishment of abundant and persistent populations of *O. davisae* and *P. marinus* in the estuary of Urdaibai was found to be related to the natural constraints of the system (Barroeta et al. 2020), mixed water column, high marine influence and low water residence time. Consequently, the Urdaibai estuary does not provide the environmental conditions requiered by these species, as reported in the literature (Saiz et al. 2003; Sabia et al. 2015; Zagami et al. 2018). The overall success of NIS and OES and their major role in driving changes of zooplankton structure and diversity in the estuary of Bilbao, and the modest success and impact of *A. tonsa* in the estuary of Urdaibai were found to be a result of the between estuary differences in the initial environmental and biological conditions. The remarkable change in structure and alpha diversity observed in the community of the inner estuary of Bilbao throughout the study period was a consequence of pre-existing empty niches, as observed in other European brackish water systems (Paavola et al. 2005). The estuary of Bilbao was highly polluted before the 1980s and inner brackish habitats were devoid of zooplankton until environmental conditions improved as a results of the rehabilitation process that favoured initially the spread of neritic plankton species towards the inner estuary (Uriarte and Villate 2004; Uriarte et al. 2016) in a process similar to that reported in the Scheldt estuary in Belgium (Mialet et al. 2011). Recovered ecological niches, however, are prone to be invaded by NIS, which show competitive advantage over neritic native species in impaired brackish environments (Aravena et al. 2009; Winder et al. 2011). This supports the hypothesis that the changes in zooplankton community structure and diversity in the estuary of Bilbao were largely a response to the management actions conducive to the improvement of the water quality in the estuary, as shown for example by the increase in dissolved oxygen levels at sites of <34 salinity below the halocline during the study period (Villate et al. 2013). In contrast with the estuary of Bilbao, from the beginning of the study period the inner estuary of Urdaibai housed a well-developed zooplankton brackish community dominated by the copepod *A. bifilosa* with compositional features that agreed well with those described in previous studies carried out in this estuary since the early 1980s (Villate 1990; Uriarte and Villate 2006). In addition, unlike in the estuary of Bilbao, the main changes in the water environment of the estuary of Urdaibai during the study period were attributable to natural hydroclimatic factors rather than to local human interventions on the system (Iriarte et al. 2016).

The effect of the NIS and OES of copepods on the changes in community structure and alpha diversity increased towards the inner estuary in both systems, due to the brackish nature of the arriving species. In the estuary of Bilbao, *C. aquadulcis* had its distribution most restricted to the low salinity upper reaches while *P. marinus* showed the most even distribution along the axial salinity gradient. However, all NIS and OES peaked at salinities of 30-33 and were absent or occurred occasionally in very low numbers in the highest salinity waters of the lower estuary reaches (Barroeta et al. 2020). The NIS *A. tonsa* and *O. davisae* and the OES *C. aquaedulcis* were also the main drivers of changes in alpha diversity at salinities lower than 34. In agreement with this, Villate et al. (2017) established that 33 salinity was the upper salinity boundary of the area in which *A. tonsa* was dominant due to its competitive advantage over the congeneric neritic species *A. clausi* in this estuary. In the estuary of Urdaibai, the main colonizer NIS, *A. tonsa*, occurred in areas upwards from those occupied by the native congeneric *A bifilosa* (Villate et al. 2017), and only showed a significant contribution to the changes of copepod evenness at the innermost salinity zone we studied. The native *Acartia* species, however, appeared as the main drivers of the changes in alpha diversity in all zones with salinities lower than 35. Therefore, the magnitude of the changes induced by NIS and OES with decreasing salinity was much lower in the estuary of Urdaibai than in the estuary of Bilbao.

2.4.1 Changes in zooplankton structure

The changes in the structure of zooplankton communities throughout the study period in the inner estuary of Bilbao clearly depicted the progressive establishment of the copepod NIS *A. tonsa* and *O. davisae*, which dominated the copepod assemblage of the inner estuary since 2003, and of the OES *C. aquadulcis*, which reached high densities at the innermost site since 2010 (Uriarte et al. 2016; Barroeta et al. 2020). The slight return towards earlier conditions in the main mode of variation (PCA, axis 1) of the community in intermediate salinity waters of 34 from the first to the second period suggested a landward increase in the influence of neritic taxa, to the detriment of the seaward advance of brackish taxa. This fact may be related to the changes in water quality during the study period, as we can infer from the evolution of the dissolved oxygen (Villate et al. 2013; Iriarte et al. 2016). Aravena et al. (2009) found that oxygen levels affected the density relationship between the densities of *A. tonsa* and *A.clausi* at intermediate salinities of the estuary of Bilbao, because the density of *A. clausi* increased at higher oxygen levels, whereas *A, tonsa* showed a competitive advantage over *A. clausi* under low oxygen conditions due to its high tolerance to hypoxic conditions (Marcus et al. 2004; Richmond et al. 2006). The general increasing trend of some holoplankton and meroplankton groups with time, mainly appendicularians and larvae of gastropods and bivalves, along the entire estuary evidenced by the second main mode of variation of the zooplankton (PCA, axis 2) corroborated the positive effect of improving environmental conditions on the zooplankton of this system.

In the estuary of Urdaibai, unlike in the estuary of Bilbao, NIS copepods were not involved in the main changes of the zooplankton community structure as shown by their low contribution to the PCA axis 1, and no clear differences in the trends of zooplankton change were identified between salinity habitats. Strong interannual variations mainly driven by neritic copepods such as PCPC-calanus, *O. nana* and *E. acutifrons* were observed. This might be attributable to the stronger hydrodynamics and shallowness of the estuary of Urdaibai which confer to the water environment of this system a higher sensitivity to climate factors (Iriarte et al. 2016), as it has been observed in other estuaries of southern Europe (Vieira et al. 2015). The strong changes in zooplankton observed in 2012 at all the salinity sites in the present study were already reported for the outer marine zone by Fanjul et al. (2017), which they linked to an hydroclimatic event (atypical positive values of the upwelling index for February and March and exceptional changes in specific phytoplankton species related to climate anomalies) that affected also other coastal areas of the Bay of Biscay in 2012 (Díaz et al. 2013; Rodriguez et al. 2015), but which had a smaller response in the zooplankton of the estuary of Bilbao (Fanjul et al. 2017). Brackish copepods had the highest contribution to the second mode of zooplankton variation in the estuary of Urdaibai, being the main contributors together with *A. tonsa*, the native copepod *A. bifilosa* and some meroplankton groups. In the estuary of Urdaibai, unlike in the estuary of Bilbao, before the colonization of *A. tonsa* and *O. davisae,* the estuary had the typical brackish zooplankton community with a dominant species (*A. bifilosa*), functionally similar to *A. tonsa,* that largely constrained the seasonal expansion and the acquisition of quantitative relevance of the NIS of copepods (Villate et al. 2018; Barroeta et al. 2020). As the NIS *A. tonsa* is functionally similar to the indigenous species *A, bifilosa*, no novel impact on zooplankton community that could otherwise cause ecosystem change happened (Doherty-Bone et al. 2019). It is interesting to point out that meroplankton groups like gastropod and polychaete larvae showed a similar level of contribution to that of the dominant brackish copepods to the changes in the zooplankton of the estuary of Urdaibai. In this system, gastropod larvae share salinity habitats with brackish copepods and may become the dominant taxa in the zooplankton assemblage of the inner estuary in summer (Villate et al. 1993; Villate 1997), the season with highest densities of *A. bifilosa* initially, but not after *A. tonsa* was established (Villate et al. 2018; Barroeta et al. 2020).

2.4.2 Changes in diversity

Overall, richness and diversity decreased with decreasing salinity and from the mouth to the head in both estuaries, as predicted by the Remane diagram, and corroborated for zooplankton communities and copepod species in other estuaries (Grindley 1981; Duggan et al. 2008; Whitfield et al. 2012). However, it is interesting to note the homogenization and occasional inversion (higher at low salinity zones than at high salinity zones) of the evenness and diversity patterns of zooplankton groups and copepod species along the salinity gradient of both estuaries, but more marked in the estuary of Urdaibai, observed in 2001-2002. Such event coincided with an unusual change of the seasonal regime of precipitation-river flow, consisting in a strong decrease of freshwater inputs in the autumn-winter of 2001-2002 and an increase in summer of 2002 (Iriarte et al. 2016). This hydroclimatic anomalies contributed during some months to the improvement of environmental conditions in the estuary of Bilbao and allowed the inward advance of copepod species like *Acartia margalefi* and *A. discaudata* (Aravena et al. 2009), this contributing to the increase of species richness in the inner estuary. In the estuary of Urdaibai, the most noticeable effect was the drop in density of the dominant brackish species *A. bifilosa,* which showed the lowest annual densities of the series. River flow changes, in combination with the tidal exchange, have strong effects on the temporal and spatial dynamics and the maintenance of the pelagic populations of *A. bifilosa* within this system (Villate 1997; Uriarte and Villate 2006). The decrease in the density of *A. bifilosa*, and the consequent decrease in dominance resulted in the increase in evenness and diversity in the inner estuary. At the Mondego estuary (Portugal) a clear relationship between river flow variability and changes in zooplankton community structure was observed, dry and rainy years being associated to a prevalence of marine and estuarine species throughout the year, respectively (Primo et al. 2009).

The main change in diversity observed in the estuary of Bilbao during the study period was the decrease of alpha diversity from 2002-2003 to 2004-2005 in the innermost zone after the sudden increase of *A. tonsa* and, to a lesser extent of *O. davisae*. A similar phenomenon was observed in the Eyrac site in Arcachon Bay (France) from the year 2000 to 2014, but in this case, the decrease of diversity was due to the decrease of the neritic and autochthonous *Parapontella brevicornis* and *Ditrichocorycaeus anglicus* and an increase in the dominance of *E. acutifrons* and *Oithona* spp. (Richirt et al. 2019). The later arrival of *P. marinus* and OES (especially *C. aquaedulcis*) to the estuary of Bilbao accounted for the increase of diversity in this period 3, by increasing the species richness. The progressive colonization of the inner estuary by NIS and OES copepods had also an evident effect on the beta diversity, because prior to the occurrence of NIS in large numbers the community was more similar at all salinities due to the dominance of neritic species along the entire estuary, but the recovery of an estuarine community dominated by brackish species increased species heterogeneity within the system. Even if NIS and OES contributed considerably to the increase of gamma diversity in copepods, GAM results showed that gamma diversity was more influenced by neritic species than by brackish species, as it was also found in the estuary of Urdaibai. In some cases, although a modification in species numbers or densities affects the alpha diversity of some sites, gamma diversity stays unchanged, because other sites, maintain the species pool (Bonecker et al. 2013).

In the estuary of Urdaibai, no clear trends in alpha diversity and no remarkable differences in the diversity of the inner estuary after the occurrence of *A. tonsa* in large numbers in 2003 were observed, this denoting that the zooplankton community was resilient to the invasion-induced disruption in terms of diversity level. In fact, the lowest values of zooplankton and copepod diversity at the innermost zone coincided in time with the highest values of density of *A. bifilosa* in 1998-1999 and 2009-2010, this confirming the dominant effect of the native species. Regarding gamma diversity, *A. tonsa* only had a significant contribution to the changes in species richness, but not to changes in the evenness or Shannon diversity. Beta diversity increased in the estuary of Urdaibai after the occurrence of *A. tonsa*, and such increase in heterogeneity was coherent with the observed spatial segregation of the population of *A. bifilosa*, which was found to be displaced seaward after the colonization of the inner estuary by *A. tonsa* (Villate et al. 2018). The seasonal and spatial segregation of these two congeneric species has also been reported in other European estuaries (Baretta and Malschaert 1988; Soetaert and Van Rijswijk 1993; David et al. 2007).

The seasonal patterns of alpha diversity in zooplankton groups and copepod species at the inner estuary of Bilbao were also substantially modified by the progressive colonization of NIS and OES of copepods because these NIS peak in summer-autumn and the OES in spring-summer (Barroeta et al. 2020). Before the occurrence of NIS in large numbers, the seasonal pattern of alpha diversity was similar at all salinities, with low values during the first part of the year (from January to June) and an increase during the second part (from June to December), this being the typical diversity pattern of the neritic community common to the highest salinity zones of both estuaries (Villate et al. 2017). When *A. tonsa* and *O. davisae* became dominant since 2003, the evenness and diversity of copepods decreased during the second part of the year, in a similar way to that reported in summer in the Uruguayan Solís Grande estuary (Gómez-Erache et al. 2000) and Doñana Park (Spain) artificial ponds (Frisch et al. 2006) due to *A. tonsa* increases. When *A. bifilosa* and *C. aquaedulcis* occurred in large numbers from 2010 onwards, the species richness and Shannon diversity of copepods increased in the first part of the year, reflecting a more estuarine-type seasonal pattern of diversity, similar to that found in the inner estuary of Urdaibai (Villate et al. 2017). Copepods' gamma diversity seasonal patterns were the ones that showed highest changes and consisted in a progressive increase in the first half of the year between consecutive periods. The seasonal pattern of copepods' beta diversity showed clear-cut higher values in summer-autumn only during the last period (2010-2015). However, unlike for alpha diversity, changes in the seasonal patterns of gamma and beta diversity in the estuary of Bilbao could not be associated to the observed changes in NIS and OES species, likely due to the higher number and evenness of zooplankton components when the entire estuary is considered instead of particular salinity habitats which are characterized by different dominant taxa.

In the estuary of Urdaibai, the main changes in the seasonal patterns of zooplankton groups' and copepod species' alpha diversity, consisting in the progressive decrease of evenness and diversity in spring in waters of lower than 33 salinity, were associated to changes in the seasonal pattern of *A. bifilosa*. However, an indirect effect of *A. tonsa* may be inferred, since annual highest densities of *A. bifilosa* advanced from summer to late spring after the establishment of the congeneric NIS which had its annual maxima in summer (Villate et al. 2018; Barroeta et al. 2020). In the Westerschelde estuary, *A. tonsa*´s occurrence is limited in time and space, occupying the upstream area in summer, this also affecting *A. bifilosa*´s seasonal pattern and the copepod community diversity pattern (Soetaert and Van Rijswijk 1993). The changes in the seasonal pattern of *A. bifilosa* also appeared associated to the progressive decrease of gamma diversity and the progressive increase of beta diversity in copepods in late spring, coinciding with an increase of gamma diversity and a decrease of beta diversity in early summer.

The zooplankton community of the estuary of Bilbao underwent marked changes in its inner reaches because of the colonization of NIS and OES, while the arrival of the same NIS to the estuary of Urdaibai had not such a predominant role in the interannual variability of the inner zooplankton community. These differences in colonization between estuaries were to a large extent the consequence of the differences in the pre-colonization conditions of zooplankton communities in each estuary; with a well-established brackish community in the inner part differentiated from the neritic community of the outer part in the estuary of Urdaibai, but a lack of dominant brackish species and a dominance of neritic taxa all along the estuary of Bilbao. The successful colonization of the unsaturated low salinity habitats of the estuary of Bilbao by the NIS *A. tonsa* and *O. davisae* since 2003 and by the OES *C. aquaedulcis* since 2010 allowed the establishment of a true brackish community spatially segregated from the neritic one. This altered zooplankton alpha and gamma diversity in two ways: one was the decrease of diversity when *A. tonsa* and *O. davisae* became highly dominant, followed by a later increase associated to the arrival of *A. bifilosa*, *C. aquaedulcis* and *P. marinus*; and the other one was the change of the seasonal pattern of diversity because of the decrease of diversity in the second half of the year due to the dominance of *A. tonsa* and *O. davisae* in summer-autumn, and the increase of diversity in the first half of the year due mainly to the contribution of *C. aquaedulcis.* At the same time, beta diversity increased by the increase of compositional differences between salinity zones, due to the formation of the brackish community dominated mostly by NIS and OES in the inner part. In the estuary of Urdaibai, only the establishment of *A. tonsa* had moderate effects in the structure of the zooplankton community of the inner estuary. Alpha, gamma and beta diversities were presumably affected by *A. tonsa* in an indirect way by assuming that this species was responsible for the observed changes in the seasonal and spatial distribution of *A. bifilosa*, which was really the main driver of community diversity changes in this estuary.

CHAPTER 3: Response of zooplankton communities to the introduction of new copepod species in two Basque estuaries: a niche decomposition approach

The effect of non-indigenous species (NIS) (*A. tonsa*, *P. marinus* and *O. davisae*) and other expanding species (OES) (*A. bifilosa* and *C. aquaedulcis*) on the realized ecological niche of zooplankton taxa in the estuaries of Bilbao and Urdaibai from data obtained from 1998 to 2015 have been analysed. Indeed, the ecological niches of both NIS and OES within the zooplankton community at the regional scale depicted by the two estuaries together has been characterized, their spatial subniches and those of the other analysed taxa for each system have been determined, and our analysis have been decomposed into spatio-temporal subniches in order to assess the different responses of zooplankton communities since NIS and OES colonized both estuaries using the OMI and WITOMI analysis and calculating the D-metric of overlap between species. The results obtained from those analysis determined that NIS and OES colonize principally the inner part of Bilbao estuary, as it lacks of brackish dominant species, while in the estuary of Urdaibai that settlement of NIS was limited by biotic interactions. Moreover, the colonization of the estuary of Urdaibai by egg-carrying copepods may be due to water turbulence. In the estuary of Bilbao, a spatio-temporal change in some neritic and congeneric species niches was observed probably affected by the apparition of NIS and OES in that estuary. In the estuary of Urdaibai no changes were observed probably due to different biotic and abiotic conditions in the estuary.

3.1 Introduction

In general, three main steps are recognised in the invasion process: 1) the introduction of the alien species in the recipient system through different transport pathways; 2) the establishment of a viable and self-sustaining population in the new habitat; and 3) the likely subsequent dispersal of the species outside this ecosystem (Vermeij 1996; Kolar and Lodge 2001; Sakai et al. 2001). Although the majority of policies and directives (i.e. Water Framework Directive, Marine Strategy Framework Directive, Convention on Biological Diversity) are focused on preventing the first step (see Tsiamis et al. 2020 and references therein), it is well known the problems to carry out it (European Environment Agency 2019), and once alien species become established in the marine environment, the severe difficulties to eradicate them (Thresher and Kuris 2004), as a consequence, among others, of their higher capacity to face changing environment (e.g. climate change, human perturbations, ...) than native species (Katsanevakis et al. 2014; Castro et al. 2019). Thus, to help the stakeholders in managing biological invasion, it remains determinant to understand the drivers of the establishment of NIS and to assess their potential effect on their recipient habitat (Woodford et al. 2016).

The colonization success of NIS varies depending of its capacities to face the biotic and abiotic constraints within the recipient ecosystem (Chan and Briski 2017). Competition, exploitation and other biotic interactions may promote (or impede) the spread and establishment of the NIS in the new environment (e.a. Freestone et al. 2013; Alofs and Jackson 2014; Gallardo et al. 2015). It is accepted that species do occupy the same niche in their new range as in their native range (Laeseke et al. 2020). Although the possibility of “niche shift” has widely been debated, this is, changes in the realized niche of a species in relation to the centroid of the niche, the margins, and/or frequency of occupied environmental conditions (Guisan et al. 2014). A wide range of approaches, e.g., ecology, physiology, evolution and genetics, are used to assess the mechanisms and consequences of the establishment of NIS (Geburzi and McCarthy 2018). Among the main goals of invasion biology is to find answers to the questions of why certain species are successful invaders and how invasion processes happen. Recent improvements in mathematical computing allowed relying the species’ response to the environmental conditions within an ecological niche approach (Guisan et al. 2017). Based on observational data it is possible to describe the realized ecological niche theorized by Hutchinson (Colwell and Rangel 2009). Guisan et al. (2014) classified niche studies in two approaches: ordination and ecological niche models. While the first approach is more robust to detect niche changes and quantify niche overlap (Broennimann et al. 2012), the second one tends to provide a better mathematically formalized niche representation. The so called outlying mean index (OMI, Dolédec et al. 2000) and within outlying mean index (WitOMI, Karasiewicz et al. 2017) analyses belong to the ordination framework. They makes up for the drawbacks of old indexes based on resource availability (Levins 1968; Hulbert 1978; Smith and Zaret 1982), densities (Morisita 1959) or relative abundances (Macarthur and Levins 1967; Pianka 1973; Hulbert 1978), giving a greater evenness to the significance of all the sampling units, even in the case of rare species. These methods show more adequately the response to the environmental variations, allowing a better description of the changes in the niches. Thus, it may be a useful tool in cases of environmental alterations, such as the case of the introduction of NIS (Broennimann et al. 2012; Guisan et al. 2014). In addition, those methods are useful for calculating niche metrics such as centroid (i.e. marginality) or niche breadth (i.e. tolerance) (Dolédec et al. 2000; Karasiewicz et al. 2017). By niche decomposition, WitOMI analysis provides new insights to studying ecological niche of NIS at finer scale: the so-called subniche (Karasiewicz et al. 2017). Furthermore, to understand the realization of species ecological niche it may be important to take in account both abiotic and biotic component. Niche overlap between species is a parameter commonly used to assess biological interactions. Among the set of different measurements of niche overlap, the D-metric is one of the most widespread method because of its simplicity (Warren et al. 2008).

The present study analyses the effect of NIS (*A. tonsa*, *P. marinus* and *O. davisae*) and OES (*A. bifilosa* and *C. aquaedulcis*) copepods on the realized ecological niche of zooplankton taxa in the estuaries of Bilbao and Urdaibai from data obtained from 1998 to 2015. The specific aims were (i) to characterize the ecological niches of both NIS and OES within the zooplankton community at the regional scale depicted by the two estuaries together, (ii) to determine their spatial subniches and those of the other analysed taxa for each system to highlight the main discrepancies on the realized subniches between the two estuaries, and (iii) to decompose our analysis into spatio-temporal subniches in order to assess the different responses of zooplankton communities since NIS and OES colonized both estuaries. Finally, in the light of the results obtained we discuss on the potential of invasion and the consequence of the establishment of NIS and OES in the estuaries of Bilbao and Urdaibai.

3.2 Method

3.2.1 Data analyses

Prior to the analyses rare zooplankton species were excluded based on an index mixing species abundances and frequencies (Richirt et al. 2019; David et al. 2020), retaining only taxa with a presence >0.01% over the study period in any of the estuaries. Copepods were studied at species level, whilst, the rest of organisms were grouped into major assemblages. Salinity stratification index was calculated as the maximum difference in salinity between consecutive depths (Villate et al. 2013).

To investigate the ecological niche of the zooplankton species outlying mean index (OMI) and within outlying mean indexes (WitOMI) analyses were performed (Dolédec et al. 2000 ; Karasiewicz et al. 2017). Both multivariate explorative methods allow studying the species (i.e. matrix site-taxon) and environment (i.e. matrix site-environmental variables) relationships, as the commonly used canonical correspondence (CCA) and redundancy analyses (RDA). However, OMI and WitOMI analyses can detect and represent both unimodal and linear responses of every species along the environmental gradient, while CCA (unimodal response) and RDA (linear response) can only catch one of them (Dolédec et al. 2000; Karasiewicz et al. 2017). Those methods have been applied many times in ecology to picture community structure (Hernandez Fariñas et al. 2015; Husson et al. 2017). Thanks to niche decomposition it is possible to detect spatio-temporal niche shifts, which is a determinant index to assess community dynamic in a changing environment (Guisan et al. 2014). The introduction of the notion of niche overlap brings information on the biotic interactions within the community (Broennimann et al. 2012). Although commonly in ecology, the use of ordination analyses such Principal Component Analyses on time series might violate the data indenpendence assumption and thus might led to biased results (Planque and Arneberg 2018). However, the OMI and WitOMI analyses are easy to use and to interpret the outputs, being a good technic to make the first insights into niche dynamics after NIS occurrences.

On the one hand, OMI analysis introduced marginality, tolerance and residual tolerance indexes for every species *j* in its habitat in absolute and relative values (Dolédec et al. 2000). Marginality index (OMI_j) is the distance between the average species habitat (centroid) to the average of the sampling area (origin). Tolerance index (Tol_j) is a measurement of the niche breadth of the species. Residual tolerance ($Rtol_j$) represents the unexplained variance by the analysis. The scores sum of the previous indexes gives the inertia of the explanation of the environmental variables for each species (Dolédec et al. 2000). The input of OMI analysis is a PCA performed on the environmental variables. Convex polygons were used to picture both the available environmental habitat and the realized niche.

On the other hand, WitOMI analysis enables to study species´ niche at a temporal and/or spatial finer scale, as a consequence of the niche decomposition into subsets to create subniches (Karasiewicz et al. 2017). For this purpose, WitOMI analysis combines the OMI properties with the K-select analysis species marginality decomposition (Calenge et al. 2005). For every subset *k* (specific environmental conditions) and species *j*, marginality (WitOMIG_{kj}), tolerance (Tol_{kj}) and residual tolerance ($Rtol_{kj}$) can be calculated in reference to the subset conditions (i.e. the so-called WitOMIG_k analysis) or to the overall conditions (i.e. the so-called WitOMIG analysis). In the present study only the WitOMIG_k analysis was performed.

Zooplankton community niche was studied according the previous steps: (i) first of all, the realized niche of the selected 42 taxa obtained during the study period (1998-2015) at both estuaries jointly was studied by an OMI analysis based on a PCA of the eight monitored environmental variables. The marginality significance ($p<0.005$) for each taxon was assessed by means Monte Carlo permutation test (1000 permutations), under the null hypothesis that each taxon is indifferent from its environment (Dolédec et al. 2000). (ii) Then, the realized subniches for the same taxa than in (i) were analysed from the output of the previous OMI analysis by means a WitOMI analysis. This analysis was carried out under two subsets corresponding to each of the two estuaries. The statistical significance ($p<0.005$) of the marginality for every taxon was assessed in reference to the average environmental conditions of each subset (WitOMIG_k analysis) through a Monte Carlo permutation test (1000 permutations). Finally, (iii) to disentangle the temporal dynamic of colonization of the NIS and OES copepods in each estuary, a WitOMI analysis as in (ii) was performed for 3 periods basing on the steps of colonization by NIS and OES. Therefore, period 1 spans from 1998 to 2002 prior to the establishment of *A. tonsa* and *O. davisae* populations; period 2, from 2003 to 2009 takes place after the occurrence of *A. tonsa* and *O. davisae* in large number, but before the occurrence of *P. marinus*, and finally, period 3, from 2010 to 2015 corresponds to the period initiated with the first occurrences of *P. marinus* and the marked increase of *C. aquaedulcis*, a species previously detected occasionally at negligible abundances, in the estuary of Bilbao. In total six subsets were selected: B.1, B.2, B.3, U.1, U.2, U.3, corresponding to each estuary (B: Bilbao, U: Urdaibai) and time period. Again, the statistical significance ($p<0.005$) of the marginality for each taxon in reference to the average environmental conditions of each subset were tested through a Monte Carlo permutation test (1000 permutations).

Likewise, the niche overlap between NIS copepods and their congenerics and neritic copepods within the same temporal subset has also been calculated for every species that appeared at least five times in the same subset (Broennimann et al. 2012). For this purpose, the ecological niche of every species have been estimated. The subsets environmental conditions represented by the first two axes of the OMI analysis were gridded into a 100 x 100 grid. Then, basing on the species occurrence in every grid in the subset, the species niches was estimated using a kernel density estimation (see Figure 2 in Hernandez Fariñas et al. 2015). Finally, the D-metric has been used to quantify the niche overlap between two species (Warren et al. 2008; Schoener 2013):

$$D_{1,2} = 1 - \frac{1}{2}\sum_{ij}\left|p_{1ij} - p_{2ij}\right|$$

Where p_{1ij} and p_{2ij} are respectively the occupancy of the species p_1 and p_2 in the ij grid. The D-metric ranges between 1 (full overlap between both species) and 0 (no overlap).

All numerical analyses were performed using the R software (Team 2013), with the packages *ade4* (Bougeard and Dray 2018) for OMI and WitOMI analyses and *ecospat* for the niche overlaps calculations (Broennimann et al. 2020).

3.3 Results

3.3.1 Zooplankton community niches in the set of both estuaries

According to the Monte Carlo test, of the selected taxa from the general inventory, only four taxa (*Acartia margalefi*, *Candacia* sp., *Microsetella* sp. and Mysids) were not influenced by environmental variables, and as a consequence, they were not included in the subsequent analyses (Table 1).

The OMI analysis for the zooplankton community of both estuaries jointly explained 96.72 % of the variability (Fig. 1). The first component (OMI1, 55.15% of the variability) underscored seasonal and spatial variations of the zooplankton community associated mainly to opposite variation of water temperature with the other physicochemical variables (salinity, dissolved oxygen and water transparency) (Figs. 1A and 1B, and Table 2). Warm and brackish affinity taxa, such as *A. tonsa*, *P. marinus*, *O. davisae*, *C. aquaedulcis A. bifilosa*, *P. grani*, and bivalve and gastropod larvae had the most positive scores along this axis (Figs. 1C and 1D), which are linked to the hottest months of the year (Fig. 2), whilst late winter-early spring taxa of neritic origin, such as *A. clausi*, *Calanus* sp., *T. longicornis* and *Calocalanus* sp. were plotted to the most negative positions (Figs. 1C and 1D), which are linked to the coldest months (Fig. 2). The second axis (OMI2, 32.28 % of the variability) also reflected seasonal and longitudinal variations, but mainly opposed physicochemical variables (temperature, salinity, dissolved oxygen and water transparency) and hydro-meteorological variables (precipitation, water column stratification and river flow) (Figs. 1A, 2 and Table 2). Here, zooplankton community differences were mainly illustrated by the opposite position of freshwater copepods and *C. aquaedulcis* on the positive end, and neritic taxa of warm affinity (doliolids, *T. stylifera* and chaetognaths) on the negative side of this axis (Fig. 1C). In general, NIS (*A. tonsa*, *P. marinus* and *O. davisae*) and OES (*C. aquaedulcis* and *A. bifilosa*) were related with low salinity and high temperature, and their centroids appeared segregated from the centroids of native copepod species, although, the realized niches (coloured convex polygons) overlapped with most of the copepod’s centroids (Figs. 1C and 1D). Finally, the third component (OMI3, 9.29 % of the variability) mainly separates the lowest salinity zones of two estuaries (Figs. 1B and 1F), in relation to the higher water column stratification and the lower water quality (dissolved oxygen concentration) in the estuary of Bilbao (Fig. 1B and Table 2). The OES copepod *C. aquaedulcis*, the NIS copepods *P. marinus* and *O. davisae* and freshwater copepods were the best related to the high stratification and low dissolved oxygen conditions that characterized the inner estuary of Bilbao (Fig. 1E), in opposition to *A. bifilosa*, which together with some tychoplanktonic forms (benthic harpacticoids and ostracods) was the most distinctive taxon of the inner estuary of Urdaibai (Fig. 1D).

Table 1. Niche parameters of the zooplankton taxa selected by Monte Carlo permutation test. The inertia (I), marginality (OMI), tolerance index (Tol) and residual tolerance index (Rtol) were computed for every taxon at a regional scale (Both estuaries jointly). Inertia (I_K), marginality (WitOMIG$_K$), tolerance (Tol$_K$) and residual tolerance (Rtol$_K$) were calculated for every taxon at a local scale (The estuary of Bilbao and the estuary of Urdaibai subsets). Significant p –values ($p<0.005$) of Monte Carlo permutations (1000) are in bold. Abbreviations of each taxa as in Appendix 1.

	Both estuaries jointly					**Estuary of Bilbao**					**Estuary of Urdaibai**				
Taxa	**I**	**OMI**	**Tol**	**Rtol**	**P**	**I_k**	**WitOMI$_k$**	**Tol$_k$**	**Rtol$_k$**	**P**	**I_k**	**WitOMI$_k$**	**Tol$_k$**	**Rtol$_k$**	**P**
Med	7.62	1.28	1.77	4.58	**0.001**	8.36	1.75	2.21	4.41	**0.001**	5.46	0.87	1.02	3.58	**0.001**
Sip	8.45	2.28	2.02	4.15	**0.001**	8.98	2.93	2.09	3.96	**0.001**	5.00	0.51	0.80	3.69	**0.012**
Cyp	7.09	1.90	1.66	3.53	**0.001**	8.52	3.22	1.95	3.35	**0.001**	4.67	0.91	1.08	2.68	**0.003**
Gas	9.79	2.98	2.07	4.74	**0.001**	9.01	1.51	3.31	4.19	**0.007**	8.27	2.56	1.74	3.96	**0.005**
Biv	9.07	2.88	2.25	3.95	**0.018**	9.67	3.87	1.97	3.83	**0.027**	5.46	0.99	1.02	3.45	0.264
Poly	8.83	0.99	2.89	4.94	**0.001**	7.56	0.14	2.06	5.36	0.287	7.57	1.67	2.44	3.46	**0.001**
Cha	10.83	3.36	1.74	5.73	**0.001**	12.03	4.56	1.89	5.58	**0.001**	6.49	1.92	0.79	3.79	**0.001**
Cla	8.91	2.54	2.32	4.05	**0.001**	9.31	2.99	2.43	3.90	**0.001**	5.94	1.02	1.04	3.89	**0.002**
Ost	6.30	1.40	0.56	4.34	**0.001**	6.41	1.48	0.82	4.12	**0.001**	5.07	0.26	1.82	3.00	0.229
Cir	8.20	1.32	1.32	5.56	**0.001**	8.20	1.76	1.73	4.71	**0.001**	7.34	1.91	1.90	3.54	**0.001**
Abi	7.87	2.26	0.99	4.62	**0.001**	6.46	2.77	1.14	2.55	**0.002**	6.18	0.76	1.43	4.00	0.085
Acl	11.32	3.17	3.15	5.00	**0.001**	11.52	3.23	3.43	4.86	**0.001**	6.90	1.95	0.87	4.07	**0.001**
Ato	9.71	4.33	1.61	3.77	**0.001**	8.05	3.18	1.36	3.51	**0.003**	9.67	7.26	1.00	1.41	**0.001**
Adi	5.59	1.45	0.91	3.23	**0.001**	4.86	0.79	0.76	3.31	0.127	4.15	2.03	0.76	1.36	**0.007**
Pgr	6.75	4.63	0.44	1.68	**0.039**	—	—	—	—	—	5.95	3.83	0.43	1.69	0.093
Cala	14.24	3.06	3.47	7.72	**0.001**	14.28	2.79	3.37	8.12	**0.010**	6.42	1.53	0.92	3.97	**0.027**
Calo	9.24	2.03	1.23	5.98	**0.001**	10.15	2.54	2.06	5.55	**0.001**	6.07	2.59	0.45	3.03	**0.002**
PCPC	9.25	2.30	2.17	4.79	**0.001**	10.41	3.13	2.46	4.82	**0.001**	6.07	1.33	1.54	3.19	**0.001**
Cen	8.74	2.98	1.62	4.14	**0.001**	10.19	3.89	1.86	4.44	**0.001**	5.44	2.12	1.16	2.17	**0.002**
Tlo	9.07	1.75	1.39	5.93	**0.016**	10.48	4.11	2.82	3.55	**0.004**	8.31	4.37	1.74	2.20	**0.004**
Tsty	7.96	3.66	1.23	3.07	**0.001**	9.12	5.11	1.10	2.91	**0.001**	5.64	1.90	1.07	2.67	**0.002**
Pma	10.59	4.93	2.46	3.20	**0.013**	10.86	5.21	2.39	3.26	**0.033**	2.68	1.35	0.14	1.19	0.448
Caq	8.89	5.40	0.37	3.12	**0.001**	8.42	4.95	0.42	3.05	**0.023**	4.75	1.19	1.28	2.28	0.417
Opl	7.02	2.07	1.38	3.56	**0.001**	7.64	3.01	1.55	3.08	**0.001**	6.06	1.60	0.81	3.65	**0.006**
Ona	8.50	2.64	1.86	4.00	**0.001**	9.80	4.03	1.81	3.97	**0.001**	5.69	1.04	1.54	3.11	**0.004**
Osi	7.37	0.73	1.30	5.34	**0.001**	8.99	1.59	2.42	4.99	**0.001**	4.82	0.46	0.82	3.54	**0.002**
Oda	9.57	3.86	2.14	3.57	**0.001**	9.57	3.88	1.92	3.76	**0.002**	1.78	0.35	0.17	1.27	0.600
Fwcop	11.82	5.92	2.62	3.29	**0.002**	10.25	7.14	0.90	2.22	**0.007**	6.35	1.39	2.44	2.52	0.260
Onc	7.11	2.11	1.56	3.44	**0.001**	9.34	5.08	1.43	2.84	**0.001**	4.53	0.71	0.95	2.87	**0.029**
Dan	7.69	2.56	1.47	3.65	**0.001**	8.88	4.11	1.76	3.00	**0.001**	6.13	1.53	1.13	3.47	**0.001**
Eac	7.66	1.47	2.14	4.05	**0.001**	10.14	3.63	2.86	3.66	**0.001**	4.75	0.64	0.99	3.12	**0.012**
Harp	5.98	1.73	0.70	3.55	**0.014**	8.38	0.41	3.99	3.98	0.625	4.48	0.59	0.69	3.21	0.458
Iso	8.44	2.93	1.84	3.67	**0.001**	8.71	0.68	3.08	4.94	**0.013**	6.93	1.77	2.24	2.92	**0.002**
Dec	9.44	2.71	2.37	4.35	**0.001**	10.74	4.09	2.74	3.91	**0.001**	5.46	1.22	1.25	2.99	**0.001**
Ech	9.52	2.81	2.67	4.03	**0.001**	10.08	3.86	2.39	3.82	**0.001**	6.28	1.71	1.03	3.54	**0.005**
App	7.73	1.15	2.10	4.48	**0.001**	7.99	1.32	2.56	4.11	**0.001**	5.55	0.75	1.16	3.64	**0.001**
Dol	9.01	5.69	0.79	2.53	**0.001**	9.68	6.49	0.66	2.53	**0.001**	5.95	2.43	1.34	2.18	**0.018**
Asc	7.42	2.22	2.35	2.86	**0.002**	8.42	3.40	2.34	2.68	**0.008**	3.28	0.20	0.21	2.87	0.914
Ict	8.25	2.43	1.89	3.93	**0.001**	8.52	3.05	1.92	3.55	**0.001**	6.38	0.23	0.75	5.40	0.432

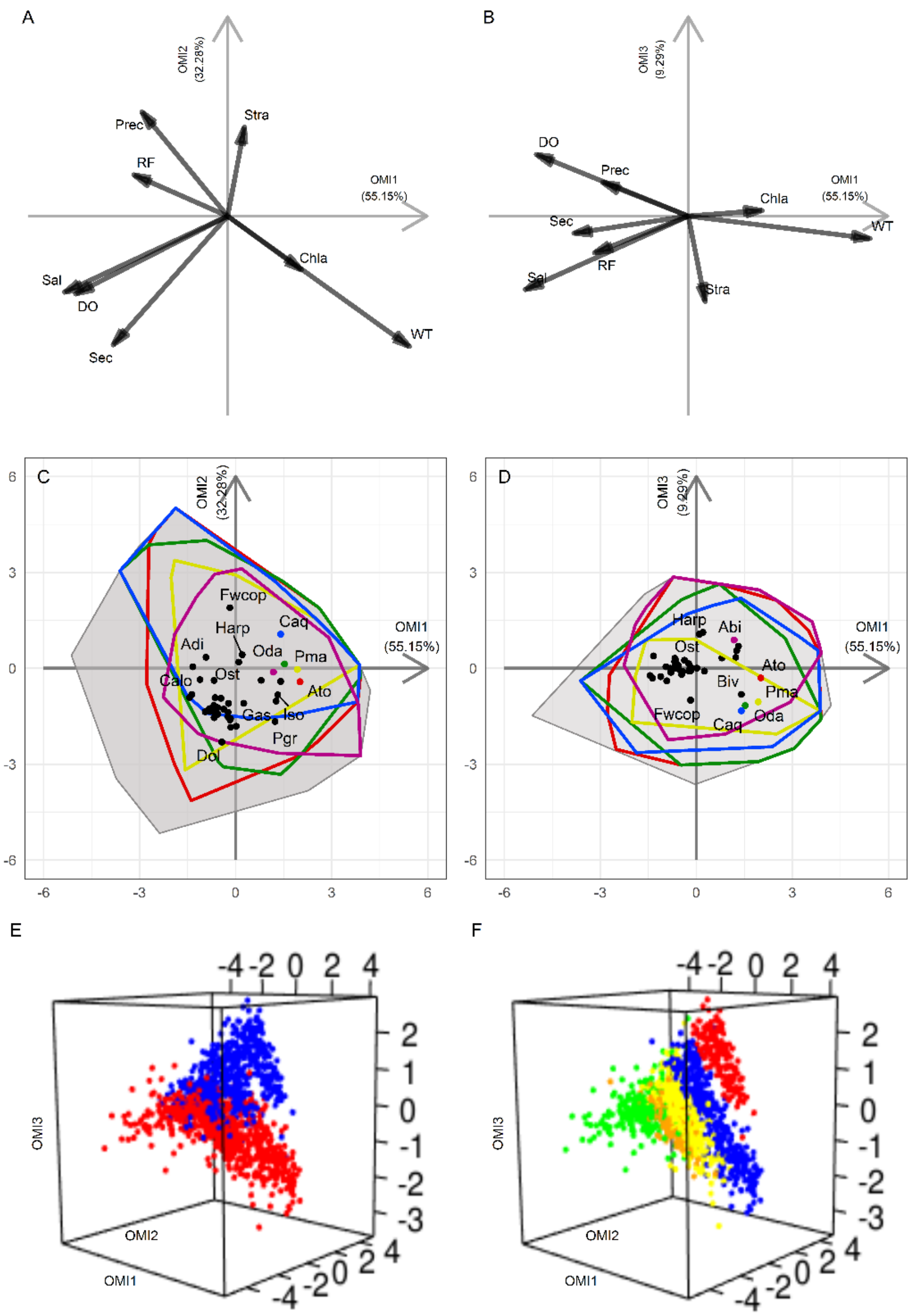

Fig. 1. OMI analysis of the zooplankton community of the estuaries of Bilbao and Urdaibai jointly. (A) Canonical weights of the eight environmental variables on the two first factorial axes, OMI1 and OMI2. (B) Canonical weights of the eight environmental variables on the first and third factorial axes, OMI1 and OMI3. (C) Representation of the statistical significant zooplankton taxa´s realized niches centroids on the first two OMI axes. Coloured points represent NIS and OES. The light grey shaded convex polygon represent the environmental conditions constraint at the regional scale (overall of the two estuaries). (D) Representation of the statistical significant zooplankton taxa´s realized niches centroids on the first and third OMI axes. Coloured points represent NIS and OES. Coloured points represent NIS and OES. The light grey shaded convex polygon represent the environmental conditions constraint at the regional scale (overall of the two estuaries). (E) Representation of every sampling point in the three first factorial axis of the OMI analysis. Red spheres represent point sampled in the estuary of Bilbao, and blue spheres represent point sampled in the estuary of Urdaibai. (F) Representation of every sampling point in the three first factorial axis of the OMI analysis. Red spheres represent point sampled in the 26 salinity zone, blue spheres represent point sampled in the 30 salinity zone, yellow spheres represent point sampled in the 33 salinity zone, orange point represent sampled in the 34 salinity zone and green point represent sampled in the 35 salinity zone. Taxa abbreviations like in Appendix 1.

Table 2. Environmental variables (Precipitation, river flow, water temperature, salinity, stratification, dissolved oxygen, *Secchi* disk and chlorophyll *a*) scores for each of the main three axis of the OMI analysis.

Environmental variables	**Axis 1**	**Axis 2**	**Axis 3**
Precipitation	-0.250	0.398	0.240
River flow	-0.274	0.157	-0.260
Water temperature	0.530	-0.497	-0.159
Salinity	-0.476	-0.290	-0.522
Stratification	0.049	0.340	-0.606
Dissolved oxygen	-0.443	-0.297	0.440
Sechhi disk	-0.334	-0.491	-0.121
Chlorophyll *a*	0.215	-0.205	0.040

Tolerance and marginality of every studied taxon were shown in Figure 3 and Table 1. *Calanus* sp. and *Acartia clausi* showed highest tolerance, whilst *Paracartia grani* and *C. aquaedulcis* were the least tolerant species. Fresh water copepods, doliolids and *C. aquaedulcis,* obtained the highest marginality scores, whereas *Oithona similis* was the less marginal taxon. Overall, the OES copepod *C. aquaedulcis*, together with doliolids and *P. grani*, differed from most of the zooplankton components due to their high marginality and low tolerance. Another OES species (*A. bifilosa*) also showed a relatively low tolerance, but its marginality was similar to most of taxa. The three NIS copepods (*A. tonsa*, *P. marinus* and *O. davisae*), however, mainly differed from the other taxa due to their high marginality. Low values of both tolerance and marginality were found for benthic ostracods and harpacticoids.

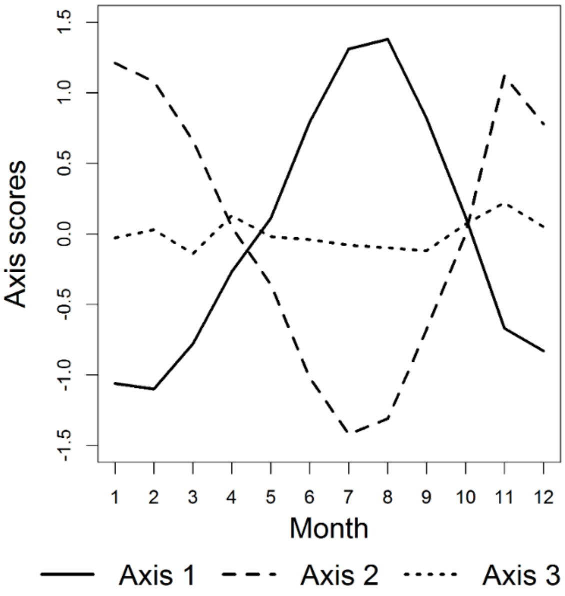

Fig. 2. Month to month variations of the three main axis of the OMI analyses.

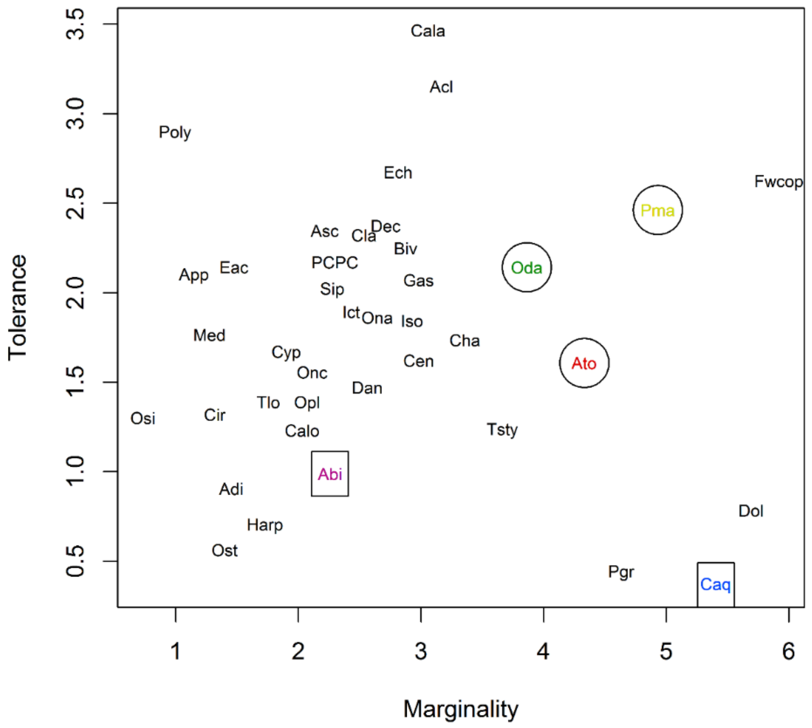

Fig. 3. Bivariate plot of every zooplankton taxon according to their marginality and tolerance. NIS copepods inside a circle and OES copepods inside a square. NIS and OES copepods abbreviations in the same color as in Figure 1. Taxa abbreviations like in Appendix 1.

3.3.2 Between-estuary differences in zooplankton niches

On the 39 selected taxa, the Monte Carlo permutation tests were significant for 26 taxa among the two subsets that were each estuary (Table 1). All NIS and OES copepods were statistically significant in the estuary of Bilbao, whereas in the estuary of Urdaibai subset only the NIS copepod *A. tonsa* was.

In the case of copepod taxa, the tolerance or the marginality of most of them were higher in the estuary of Bilbao than in the estuary of Urdaibai. The most noticeable exceptions were the much higher marginality of *A. tonsa* and the much higher tolerance of freshwater copepods in the estuary of Urdaibai (Fig. 4A). Comparatively, neritic species of warm affinity like *O. nana, T. stylifera and Oncaea* sp. showed lower tolerance and lower marginality in the estuary of Urdaibai, whilst winter-spring neritic species like *T. longicornis* and *Calocalanus* sp. showed higher tolerance and higher marginality in the estuary of Bilbao (Figs. 4B and 4C, and Table 1). Among *Acartia* species, *A. clausi* showed much higher tolerance in the estuary of Bilbao and similar marginality in both estuaries, whilst *A. tonsa* reached higher tolerance in the estuary of Bilbao but showing much higher marginality in the estuary of Urdaibai. For those species in the estuary of Urdaibai, the residual tolerance explained more than 50% of the inertia and was close to 74 % for *O. similis* (Table 1). Non-copepod zooplankton taxa also reached higher tolerance or marginality values in the estuary of Bilbao than in the estuary of Urdaibai (Fig. 4D). As for the percentages of deviance explained by each index, cirripede larvae and doliolids were the only taxa that had higher tolerance in Urdaibai than in Bilbao (Fig. 4E) and cirripede larvae, gastropod larvae and isopods were the only taxa that had higher marginality values in Urdaibai than in Bilbao (Fig. 4F).

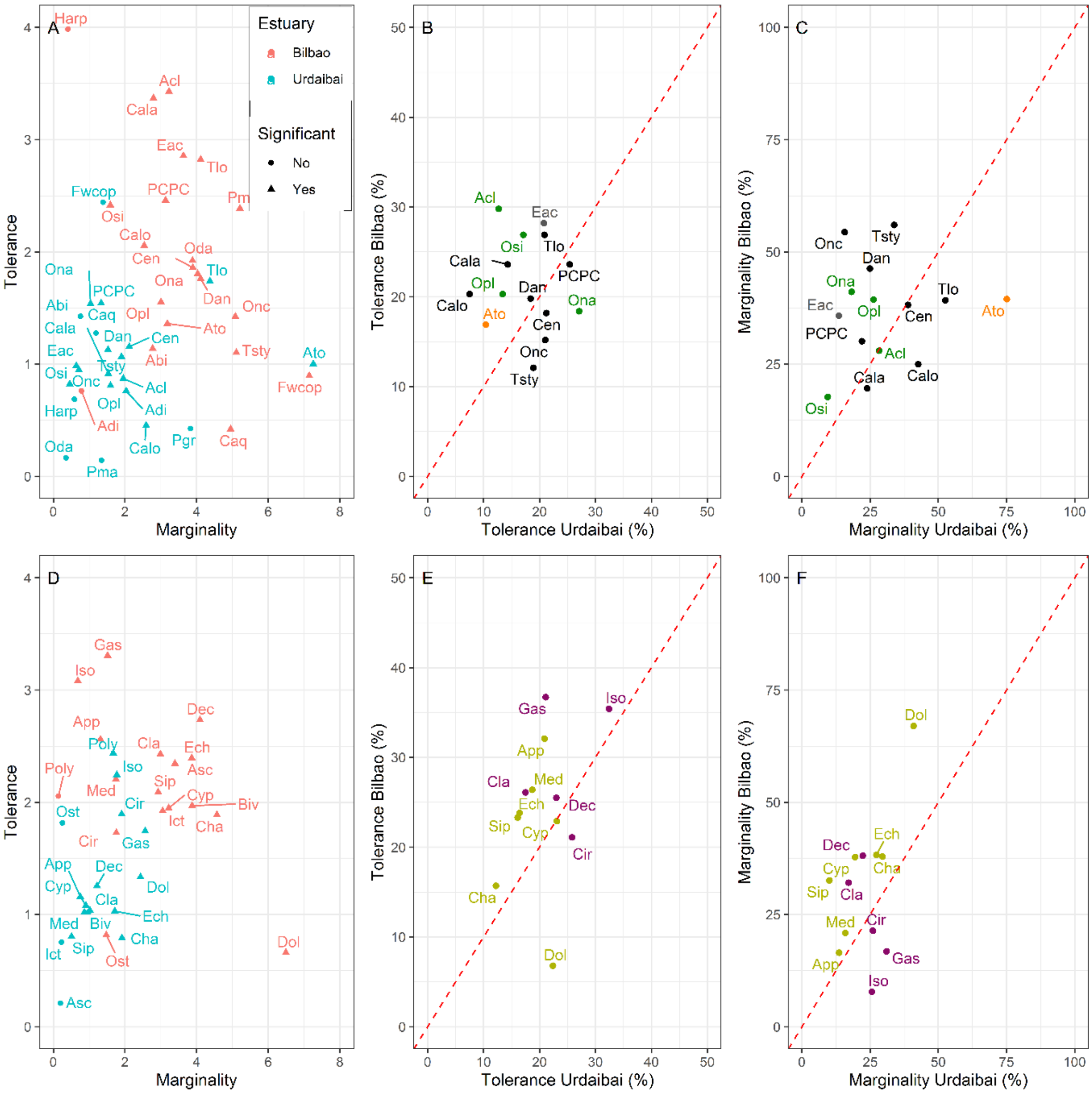

Fig. 4. (A and D) Representation of tolerance (Tol$_k$) and marginality (WitOMIG$_k$) from the suborigin of each subset (i.e. estuary) (the estuary of Bilbao in red and the estuary of Urdaibai in blue). The taxa with statistically significant realized subniches are represented with a triangle. Only the species with statistically significant realized subniches in both estuaries were represented in the next analysis. (B and E) Representation of every significant taxon in both subsets according to their tolerance (Tol$_k$) in percentage in the estuary of Bilbao and Urdaibai subsets. (C) Representation of every significant taxon in both subsets according to their marginality (WitOMIG$_k$) in percentage in the estuary of Bilbao and Urdaibai subset. In green congeneric copepods, in orange NIS, in black neritic copepods, in grey other copepods, in yellow neritic groups and in purple estuarine groups. Upper panels (A, B and C) show copepod species and down panels (D, E and F) non-copepod taxa in order not to overlap the points and have a clear view of the results. Taxa abbreviations like in Appendix 1.

Table 3. Niche parameters of the zooplankton taxa. In the spatio-temporal subsets, inertia (I_K), marginality ($WitOMIG_K$), tolerance (Tol_K) and residual tolerance ($Rtol_K$) were estimates at the local scale ($WitOMIG_K$ analysis, see result section iii) Significant p –values ($p<0.005$) of Monte Carlo permutations (1000) are in bold. Abbreviations of the taxa as in Appendix 1.

Estuaries	**Bilbao**															**Urdaibai**														
Period	**1**					**2**					**3**					**1**					**2**					**3**				
Taxa	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**	**I_k**	**$WitOMI_k$**	**Tol_k**	**$Rtol_k$**	**P**
Med	7.99	1.21	1.50	5.28	**0.031**	7.14	1.86	1.82	3.45	**0.002**	9.79	2.49	2.66	4.64	**0.005**	4.57	0.39	0.74	3.44	0.349	6.32	0.92	0.92	4.48	**0.015**	4.84	1.63	0.92	2.30	**0.005**
Sip	6.40	1.48	1.11	3.80	**0.027**	8.09	2.25	1.50	4.34	**0.001**	12.25	7.34	1.77	3.14	**0.001**	4.82	0.51	1.29	3.02	0.343	5.23	0.61	0.79	3.83	0.078	4.11	1.23	0.64	2.24	**0.025**
Cyp	6.96	2.19	1.26	3.52	**0.024**	6.22	2.41	0.81	3.00	**0.003**	12.12	5.80	3.01	3.32	**0.001**	4.66	1.15	1.08	2.43	0.211	4.45	1.04	0.92	2.48	0.060	5.19	1.69	1.12	2.38	**0.041**
Gas	7.35	1.48	1.71	4.15	0.154	7.02	1.15	2.48	3.38	0.151	10.49	1.93	4.36	4.21	0.113	4.65	2.63	0.71	1.32	**0.043**	10.53	3.01	2.99	4.53	**0.008**	5.66	2.46	1.20	2.00	**0.035**
Biv	13.84	3.42	5.33	5.09	0.077	9.58	3.13	2.48	3.97	0.089	10.73	5.61	1.87	3.25	0.052	5.23	0.53	1.51	3.19	0.547	5.42	1.37	0.87	3.18	0.223	5.58	1.21	1.60	2.78	0.250
Poly	7.04	0.52	1.73	4.79	0.190	7.10	0.16	1.31	5.64	0.509	7.77	0.06	1.21	6.50	0.918	6.11	1.53	1.54	3.04	**0.035**	8.45	2.22	2.77	3.46	**0.001**	5.31	0.53	1.52	3.26	0.185
Cha	6.49	1.94	1.10	3.44	**0.023**	12.48	5.47	2.79	4.22	**0.001**	14.62	9.47	2.67	2.49	**0.001**	6.51	1.05	1.23	4.24	0.137	6.77	1.90	0.74	4.13	**0.003**	5.98	2.75	0.53	2.70	**0.003**
Cla	6.11	2.08	0.98	3.06	**0.010**	6.96	1.88	2.00	3.08	**0.001**	14.98	8.18	3.27	3.53	**0.001**	6.82	1.44	1.25	4.13	**0.032**	5.87	2.78	1.28	1.81	**0.001**	4.28	0.52	0.42	3.35	0.220
Ost	7.28	1.32	1.60	4.37	0.062	7.61	1.46	1.53	4.62	**0.012**	4.92	2.19	0.25	2.48	**0.021**	5.36	0.48	0.98	3.90	0.410	4.65	0.36	1.90	2.39	0.357	5.40	0.42	2.00	2.99	0.360
Cir	10.23	1.61	2.08	6.54	**0.004**	6.00	1.60	1.42	2.98	**0.001**	9.91	2.41	2.38	5.12	**0.001**	8.59	1.22	2.29	5.08	**0.012**	7.88	1.94	1.96	3.99	**0.001**	5.55	2.36	1.36	1.83	**0.001**
Abi	—	—	—	—	—	7.80	6.78	0.52	0.49	**0.001**	6.15	2.47	1.00	2.69	0.064	4.97	2.27	0.83	1.86	0.064	8.04	0.91	1.38	5.75	0.215	5.10	0.48	1.57	3.04	0.537
Acl	7.05	1.85	1.35	3.85	**0.015**	5.73	1.05	0.94	3.75	**0.005**	18.69	9.87	4.05	4.77	**0.001**	7.95	4.36	2.23	1.36	**0.001**	6.74	3.18	1.13	2.43	**0.001**	5.95	2.11	1.45	2.39	**0.002**
Ato	6.16	2.42	2.06	1.68	0.060	8.98	3.74	1.53	3.71	**0.008**	7.65	3.42	1.17	3.06	**0.029**	—	—	—	—	—	10.66	8.09	1.19	1.38	**0.001**	7.43	5.90	0.60	0.94	**0.007**
Adi	4.42	0.85	0.45	3.12	0.513	4.97	1.32	0.97	2.69	0.183	5.33	1.78	0.77	2.78	0.206	3.82	2.45	0.03	1.34	0.155	3.81	2.59	0.55	0.68	**0.036**	5.52	1.83	1.80	1.90	0.169
Pgr	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.95	1.95	0.00	0.00	0.527	6.13	4.13	0.29	1.70	0.131	4.13	2.27	0.86	1.00	0.423
Cala	6.66	3.48	1.00	2.19	0.055	5.46	0.83	1.05	3.59	0.343	25.31	9.10	4.92	11.29	**0.002**	6.22	1.27	2.24	2.71	0.306	6.31	1.91	1.23	3.17	0.100	6.84	2.59	0.34	3.91	0.051
Calo	4.29	1.84	0.42	2.03	0.141	7.53	2.63	2.07	2.83	**0.013**	17.83	6.05	3.51	8.27	**0.008**	3.02	2.02	0.31	0.69	0.165	8.39	3.28	0.62	4.49	**0.008**	8.33	3.76	0.78	3.79	**0.014**
PCPC	8.56	1.68	1.67	5.21	**0.008**	7.08	2.25	1.67	3.16	**0.001**	13.28	5.50	3.16	4.61	**0.001**	4.65	0.36	0.73	3.56	0.195	6.54	1.25	1.34	3.95	**0.007**	6.61	2.92	1.81	1.89	**0.003**
Cen	8.99	3.19	1.31	4.50	**0.019**	6.40	2.25	1.24	2.91	**0.008**	11.94	6.17	1.82	3.95	**0.003**	4.05	0.50	0.89	2.67	0.510	5.94	3.36	0.92	1.66	**0.006**	5.14	1.31	1.15	2.68	0.095
Tlo	5.39	1.16	1.43	2.80	0.441	5.05	1.55	0.89	2.62	0.231	13.45	7.33	2.29	3.84	**0.022**	8.87	6.88	1.14	0.84	**0.019**	7.04	4.26	1.33	1.45	**0.029**	3.26	0.62	0.44	2.20	0.638
Tsty	6.14	3.20	0.57	2.37	**0.036**	8.59	4.79	1.07	2.72	**0.001**	11.87	9.52	1.20	1.14	**0.002**	5.95	2.17	1.49	2.30	0.085	5.38	1.96	0.55	2.87	**0.035**	4.29	1.29	1.09	1.90	0.183
Pma	—	—	—	—	—	—	—	—	—	—	12.34	6.69	2.45	3.21	0.109	—	—	—	—	—	—	—	—	—	—	2.71	1.38	0.10	1.23	0.660
Caq	9.20	8.41	0.02	0.78	**0.044**	8.79	8.37	0.25	0.17	**0.019**	9.12	5.65	0.57	2.90	0.135	4.92	1.81	0.58	2.53	0.537	4.83	1.70	2.23	0.90	0.472	4.52	0.89	0.98	2.66	0.791
Opl	6.91	1.99	1.45	3.46	0.075	7.35	3.23	1.42	2.70	**0.008**	13.87	11.29	1.21	1.37	**0.002**	4.36	0.70	0.93	2.73	0.486	6.36	1.76	0.77	3.83	0.052	5.88	5.35	0.00	0.52	**0.006**
Ona	8.20	1.28	1.18	5.74	0.070	7.45	2.95	1.67	2.83	**0.001**	11.47	7.16	1.73	2.58	**0.001**	5.12	0.24	0.80	4.08	0.757	5.11	0.74	1.21	3.16	0.119	6.14	2.05	1.53	2.57	**0.016**
Osi	6.24	1.24	1.38	3.63	**0.018**	5.79	0.91	1.05	3.83	**0.005**	14.34	4.24	4.29	5.81	**0.001**	3.54	0.60	0.53	2.42	0.092	5.13	0.54	1.01	3.58	**0.039**	4.82	0.83	0.68	3.32	**0.023**
Oda	5.01	1.18	1.29	2.54	0.358	12.38	6.96	2.44	2.98	**0.002**	8.69	4.14	1.11	3.44	**0.045**	2.87	2.87	0.00	0.00	0.080	1.38	0.58	0.13	0.66	0.624	4.21	1.24	0.66	2.31	0.320
Fwcop	7.80	1.96	1.99	3.85	0.308	9.81	7.57	0.53	1.72	**0.012**	13.83	5.75	2.06	6.02	0.088	2.90	0.33	0.34	2.24	0.866	3.98	0.27	0.59	3.13	0.907	9.03	5.32	1.86	1.85	**0.036**
Onc	7.47	3.24	0.96	3.27	**0.007**	7.31	3.91	0.96	2.45	**0.001**	11.03	7.39	1.55	2.08	**0.001**	3.48	0.42	0.63	2.43	0.532	4.75	1.02	0.74	3.00	0.066	5.46	0.94	1.80	2.73	0.124
Dan	8.05	2.81	1.06	4.19	**0.019**	7.70	2.67	1.68	3.35	**0.002**	9.61	5.89	1.83	1.90	**0.003**	5.92	1.37	0.91	3.64	0.102	6.03	1.70	1.04	3.30	**0.019**	6.31	2.33	1.75	2.23	**0.018**
Eac	6.77	1.29	1.39	4.09	0.055	7.32	1.79	1.73	3.81	**0.005**	11.85	5.85	2.96	3.04	**0.002**	6.25	1.21	2.42	2.62	0.056	4.98	1.26	0.89	2.82	**0.026**	3.70	0.66	0.51	2.52	0.156
Harp	7.80	0.24	1.54	6.01	0.860	8.19	0.22	2.61	5.36	0.864	8.44	1.66	3.83	2.95	0.291	5.59	0.65	1.51	3.44	0.530	4.64	0.90	0.58	3.16	0.415	3.62	0.47	0.43	2.71	0.617
Iso	5.57	0.19	0.78	4.60	0.733	6.94	1.22	1.88	3.84	**0.018**	11.73	1.20	3.01	7.52	0.066	5.10	1.04	1.49	2.57	0.124	8.42	2.81	2.58	3.03	**0.004**	4.91	0.75	1.49	2.67	0.148
Dec	8.71	3.12	1.51	4.08	**0.002**	7.05	1.67	2.13	3.25	**0.002**	12.52	5.89	2.74	3.89	**0.001**	5.15	1.41	1.08	2.66	**0.039**	6.03	1.21	1.46	3.37	**0.007**	5.36	1.92	0.94	2.50	**0.006**
Ech	5.61	1.95	1.02	2.64	0.111	7.36	2.89	1.51	2.96	**0.008**	17.33	12.86	2.82	1.64	**0.001**	5.13	1.80	0.62	2.71	0.145	5.78	1.47	1.01	3.30	0.089	7.61	3.34	0.88	3.39	**0.016**
App	7.20	1.23	1.61	4.36	**0.004**	6.27	0.80	2.18	3.29	**0.001**	10.12	2.31	3.02	4.80	**0.001**	6.61	1.57	2.28	2.76	**0.006**	5.10	1.22	1.41	2.48	**0.001**	5.48	1.00	1.23	3.25	**0.004**
Dol	9.26	6.46	0.85	1.96	**0.027**	9.25	6.26	0.56	2.42	**0.009**	10.86	8.54	0.78	1.54	**0.013**	4.14	2.50	0.06	1.58	0.176	7.97	4.69	1.78	1.51	**0.017**	3.71	1.15	0.18	2.37	0.441
Asc	3.34	1.42	0.17	1.76	0.487	7.04	2.56	1.63	2.85	0.109	9.07	4.61	2.30	2.16	**0.066**	7.61	7.61	0.00	0.00	**0.031**	2.15	0.82	0.52	0.81	0.665	6.55	2.92	1.15	2.48	0.138
Ict	5.55	3.06	0.61	1.88	**0.022**	6.45	2.53	1.28	2.64	**0.008**	12.40	4.81	3.45	4.14	**0.003**	3.60	0.28	0.73	2.59	0.766	6.27	0.77	0.78	4.71	0.175	7.52	0.74	1.22	5.56	0.262

3.3.3 Temporal changes of zooplankton niches in each estuary

According the Monte Carlo permutation test only four taxa (cirripede larvae, *A. clausi*, decapod larvae and appendicularians) showed significant values in the six subsets and on the 35 remaining taxa, 25 were significant at least two times in the same estuary (Table 3). In both estuaries, the environmental mean conditions were close to the origin of the OMI components (Fig. 5A and 6A), but in the estuary of Bilbao slight displacement of the centroid over time along the first OMI component was observed (Fig. 5A). The niche decomposition into temporal subsets highlighted niche shifts along the two main OMI components over time for most of the neritic copepod and NIS congeneric taxa in the estuary of Bilbao (Figs. 5B and C). For the other (non-copepods) neritic and estuarine taxa the niche shifts observed in this estuary were less evident (Figs. 5D and E). In the estuary of Urdaibai, for the few significant neritic and congeneric copepods niche shift was not observed, or was clearly slower than in the estuary of Bilbao, except for *A. clausi* which showed a remarkable shift along the second axis from period 1 to period 2 (Figs. 6B and C). As for non-copepods, no shift, or weak shifts were observed in the estuary of Urdaibai (Figs. 6D and E).

The temporal change of the niche overlap between NIS and the other copepod species differed between estuaries (Fig. 7). In the estuary of Bilbao, the niche overlap of *A. tonsa* and *O. davisae* with most of the neritic and congeneric species increased from the period 1 to the period 2, and decreased in the period 3, with the main exception of the overlap of *A. tonsa* with *A. bifilosa*, which clearly increased from the period 2 to the period 3 (Figs. 7A and C). In the estuary of Urdaibai, however, *A. tonsa*´s niche overlap with the most of the taxa increased from the period 2 to the period 3, except with *Calanus* sp. and *P. grani*, (Figs. 7B and D). Similarly, *O. davisae*´s niche overlap increased from the period 2 to the period 3 in relation to congeneric species but was variable depending on the neritic taxa (Figs. 7B and D). As *P. marinus* did not appeared more than 5 times in most of the subsets (i.e. B.1, B.2, B.3, U.2 and U.3), it was not possible to assess the change of its overlap with neritic and congeneric copepods. The species that showed the highest overlap with the NIS *A. tonsa* and *O. davisae* did not changed from the period 2 to the period 3 in any estuary. In the estuary of Bilbao, both NIS showed higher overlap in periods 1 and 2 with PCPC than with any of the respective congeneric species (Figs. 7A and C). In the estuary of Urdaibai, however, both showed the highest overlap in periods 1 and 2 with their congeneric species *A. bifilosa* and *O. nana*, respectively (Figs. 7B and D).

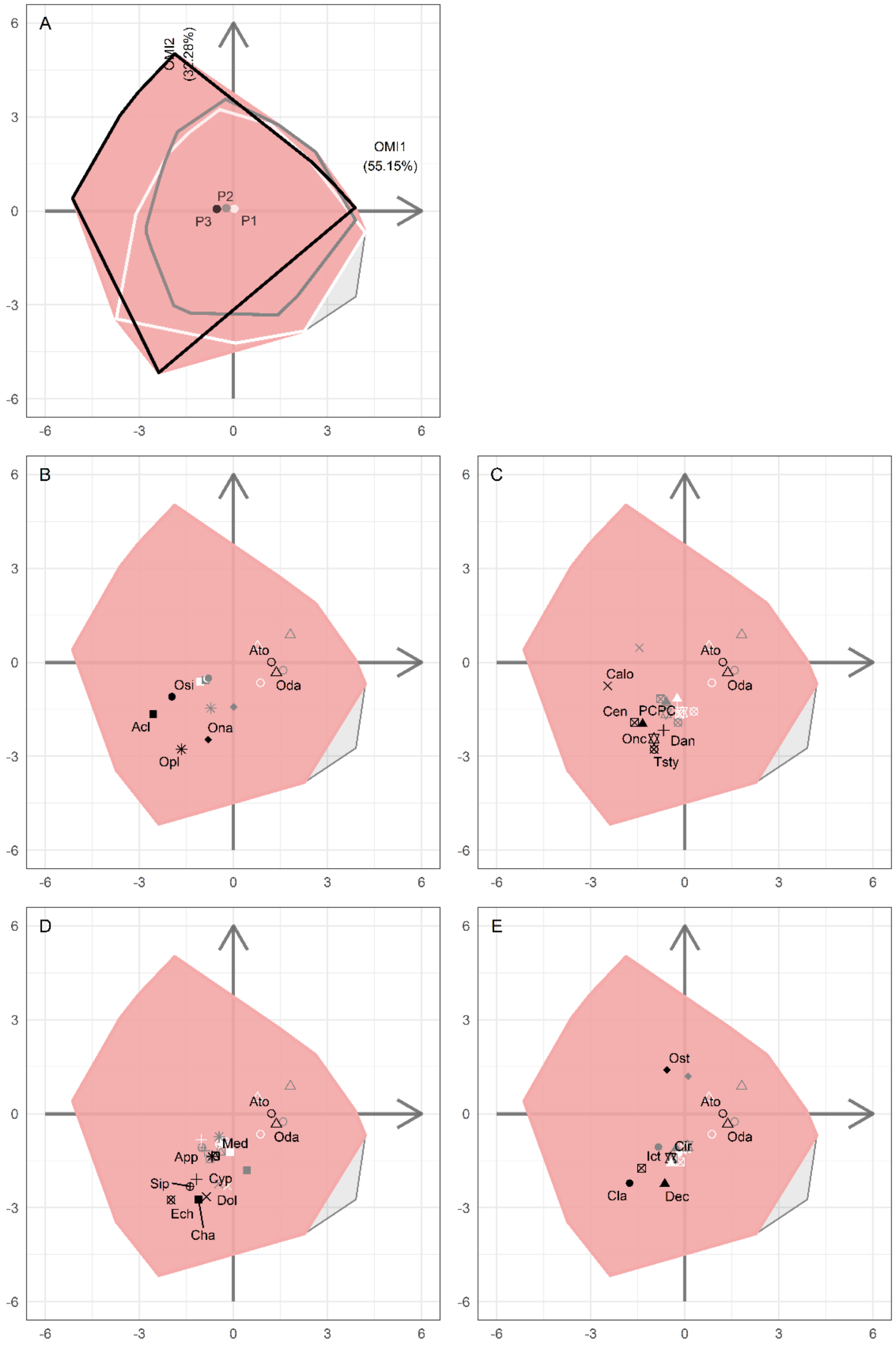

Fig. 5. Representation of the results of the WitOMI analysis of the spatio-temporal subsets in the two first OMI axes. The light grey shaded convex polygon represent the overall environmental conditions constraints at the regional scale, both estuaries jointly. The red couloured shaded convex polygon represent the habitat conditions of the estuary of Bilbao. (A) Representation of the environmenal conditions and the suborigins for each period (Period 1: white polygon and dot; Period 2: grey polygon and dot; Period 3: black polygon and dot). In the estuary of Bilbao the centroid of the NIS and significant congeneric (B), neritic copepod (C), neritic no-copepod (D) and estuarine no-copepod (E) taxa are represented in the two first OMI axes. The shape of the point stand for a same species (see the label in the plot) and the color of the point account for a period (white for period 1,grey period 2, and black period 3). Taxa abbreviations like in Appendix 1.

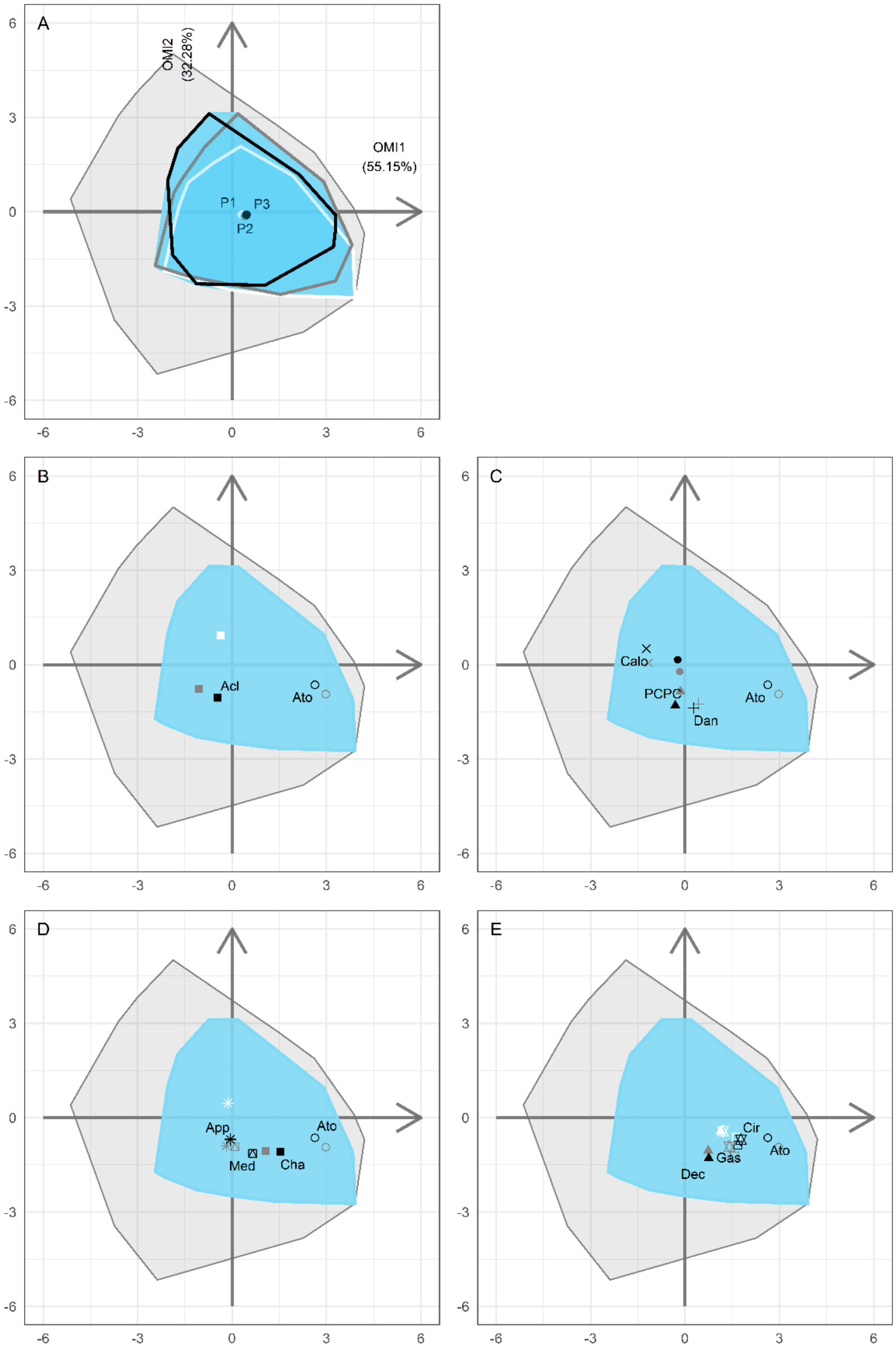

Fig. 6. Representation of the results of the WitOMI analysis of the spatio-temporal subsets in the two first OMI axes. The light grey shaded convex polygon represent the overall environmental conditions constraints at the regional scale, both estuaries jointly. The blue couloured shaded convex polygon represent the habitat conditions of the estuary of Urdaibai. (A) Representation of the environmenal conditions and the suborigins for each period (Period 1: white polygon and dot; Period 2: grey polygon and dot; Period 3: black polygon and dot). In Urdaibai estuary the centroid of the NIS and significant congeneric (B), neritic copepod (C), neritic no-copepod (D) and estuarine no-copepod (E) taxa are represented in the two first OMI axes. The shape of the point stand for a same species (see the label in the plot) and the color of the point account for a period (white for period 1,grey period 2, and black period 3). Taxa abbreviations like in Appendix 1.

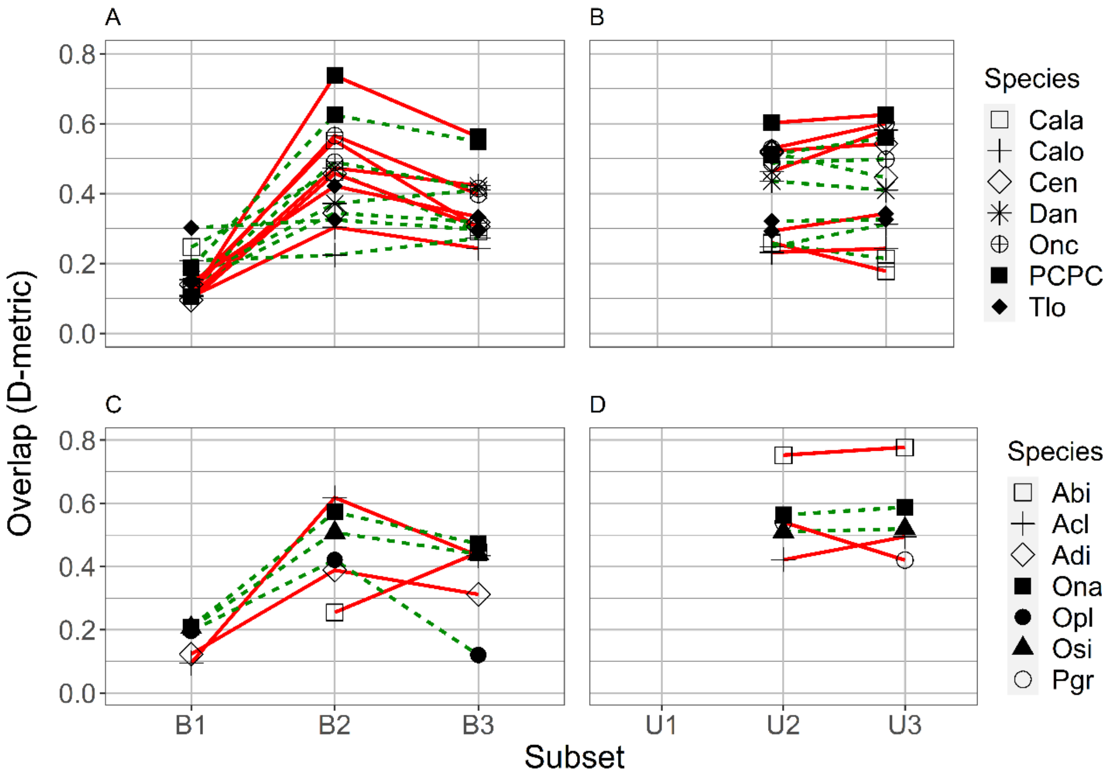

Fig. 7. Bivariate plots of niche overlap changes over time between *A. tonsa* and *O. davisae* with neritic copepod (A and B) and congeneric taxa (C and D) in both estuaries (estuary of Bilbao: A and C; estuary of Urdaibai: B and D). Red line account for overlap regarding to *A. tonsa* niche and green dashed line for *O. davisae* niche. Point shape correspond to the species which its niche overlap with *A. tonsa* or *O. davisae*. Taxa abbreviations like in Appendix 1.

3.4 Discussion

3.4.1 NIS and OES within the regional zooplankton community

Results revealed that a blend of spatial and temporal patterns of water physicochemical features drove the overall environmental segregation of the major zooplankton assemblages that inhabited both estuaries. As in other studies on zooplankton spatio-temporal variability in coastal transitional environments, salinity and temperature showed the higher contribution to model the studied zooplankton communities (Marques et al. 2008; Albaina et al. 2009; Zervoudaki et al. 2009; Oda et al. 2018). To the typical differentiation of estuarine zooplankton freshwater, brackish and marine neritic assemblages, in relation to salinity, the OMI added a clear seasonal differentiation of a neritic winter-spring assemblage and a neritic summer-autumn assemblage as a result of the rotation in the dominance or occurrence of the neritic taxa between the first and the second half of the thermal cycle in the biogeographic region where our estuaries are located (Fanjul et al. 2018).

The components of the brackish assemblage, which included all the NIS and OES that progressively joined the estuarine zooplankton community over the study period (Barroeta et al. 2020), showed weaker temporal segregation in response to thermal preferences than those of the neritic assemblage, but the OES, mainly *C. aquaedulcis*, were found to have less warm-affinity than the NIS. Overall, our field observations agreed with results of laboratory experiments which addressed in characterizing temperature and salinity optimum of *A. tonsa*, *P. marinus*, *O. davisae* and *A. bifilosa* (Lance 1963; Castro-Longoria and Williams 1999; Chaalali et al. 2013; Sabia et al. 2015; Zagami et al. 2018), although a displacement of these species towards higher salinities was observed in our estuaries when compared to larger ones. For instance, the optimal salinity range (between 5 and 10) for reproduction of *C. aquaedulcis* (Svetlichny et al. 2012a) is entirely out of the salinity ranges in which this species has been recorded in the estuaries of Bilbao and Urdaibai. This is attributed to the dominance of marine conditions and small spatial entity of low salinity brackish habitats within these estuaries (Villate 1997; Uriarte et al. 2014) that would force brackish populations to develop at the highest limit of their salinity optimal range.

All the studied NIS and OES are considered to be eurytherm and euryhaline species that can tolerate ranges of salinity and temperature values wider than the commonly monitored in the study area (Lance 1963; Gonzalez 1974; Cervetto et al. 1999; Svetlichny et al. 2012a, 2021; Sabia et al. 2015; Lazareva 2018; Zagami et al. 2018). However, the tolerance of the NIS (i.e. measurement of niche breadth) were closed to the average value of the zooplankton community whilst that of the OES was found to be lower, the lowest tolerance measured in this study being for *C. aquaedulcis*. In contrast, *P. marinus* reached one of the highest tolerance scores. The large discrepancy between *in situ* and laboratory observations might be attributed to the complexity of interacting factors that contribute to the realized niches in natural systems, where the biotic component and hydrodynamic features of the system must be considered when forecasting potential estuarine habitats' colonization by NIS in new ecosystems (Gestoso et al. 2018; Barroeta et al. 2020).

The OMI also evidenced between-estuary differences in the zooplankton assemblages of the inner part, due to the higher presence of freshwater copepods in the estuary of Bilbao and tychoplanktonic forms (benthic harpacticoids and ostracods) in the estuary of Urdaibai, which reveal differing environmental scenarios. Hydrodynamic differences like higher inputs of fresh water that transport limnetic organisms into the estuary in the first one, and higher shallowness and extent of intertidal areas that promotes the incorporation of benthic organisms to the water column by turbulence and tidal washing in the second one (Villate 1997), seem to be the main responsible for such zooplankton compositional differences.

3.4.2 Importance of local conditions in NIS and OES realized niche

WitOMI analysis allowed evaluate at finer scale the community of each estuary highlighting the differences between the estuaries of Bilbao and Urdaibai. On the set of the studied species, only one species showed no significant marginality in both estuaries and twelve species in one of the two estuaries. No significant marginality implies that species might not be influenced by the environmental conditions (Karasiewicz et al. 2017) due to the ubiquity of the species within the studied subset or the no sufficient data availability to evaluate the ecological niche of the species in the subset. In this study, most of the cases of species with no significant marginality (e.g. *P. marinus*, *C. aquaedulcis*, *O. davisae* and *P. grani* in the estuary of Urdaibai, or *Acartia discaudata* in the estuary of Bilbao) can be attributed to the scarcity of observations of the species in the estuary. This also suggest that the presence of such species in the estuary is limited by abiotic and/or biotic factors different from those considered in the present study. In contrast, the no significant marginality of other species, such as the commonly recorded and abundant species *A. bifilosa* in the estuary of Urdaibai, may be attributable to the high adaptability of this species to the different environmental conditions found in the estuary. In addition, the existence of an abundant and active bank of resting eggs of *A. bifilosa* in the sediments of the estuary of Urdaibai (Uriarte and Villate, 2006) guarantees the rapid recovery of pelagic population after any hydrological perturbation tending to remove or reduce drastically them.

The weak water column stability in relation to weak salinity stratification, and the lack of permanent high-salinity marine-water intrusion upward the estuary of Urdaibai, were considered the main responsible for the unsuccessful establishment of *O. davisae*, *P. marinus* and *C. aquaedulcis* in the estuary of Urdaibai (Barroeta et al. 2020), and consequently of the differences of the realized niche of these NIS and OES copepods observed in the present study between the estuaries of Bilbao and Urdaibai. Isolated eutrophic environments and weak hydrodynamics conditions favour *O. davisae* and *P. marinus* establishment (Barry and Levings 2002; Zagami et al. 2018) and explain the successful colonization of the estuary of Bilbao by these two NIS. Although no evidence of *C. aquaedulcis* sensitivity to turbulence was found in the literature, this species could be affected by turbulence in the same way that other species of the family Pseudodiaptomidae, including *P. marinus* (Lee et al. 2011; Sabia et al. 2012; Breckenridge et al. 2015; Jones et al. 2015). Results also suggested preference of *A. bifilosa* by low salinity-stratification conditions and adaptability, as in other estuaries of the northern Europe, where *A. bifilosa* is found in oligo-mesohaline areas (Redeke 1934; David et al. 2007). However, it suggested preference of *A. tonsa* to both mixed and stratified conditions, coinciding with abundances found in another well stratified and mixed estuaries (Price 1986) and other mixed environments, such as the southwestern Atlantic coast, where this species have acquired adaptations as an omnivorous diet, high reproductive rates dependent mainly on temperature, production of resting eggs and a high tolerance to salinity changes in order to survive (Marrari et al. 2004).

Despite the above mentioned adaptability to different hydrodynamics conditions, and the similar range of temperature and salinity in both estuaries (Iriarte et al. 2015), *A. tonsa* showed higher marginality in the estuary of Urdaibai than in the estuary of Bilbao, this denoting that the differences in the realized ecological niche might not be induced by abiotic but mainly by biotic constraints in the estuary of Urdaibai (Guisan et al. 2014). Brackish waters present high level of chlorophyll *a* in both estuaries (Iriarte et al. 2016), essential for *A. tonsa* development in coastal systems (Paffenhöfer and Stearns 1986), but the presence of potential competitors at the arrival of this species differed largely between estuaries. Before the apparition of NIS and OES the zooplankton community of the estuary of Bilbao was lacking of brackish copepod species whilst the congeneric brackish species *A. bifilosa* dominated the zooplankton community of the inner estuary of Urdaibai (Uriarte and Villate 2005; Chapter 2 of this thesis), likely preventing the complete occupation of the brackish niche by *A. tonsa*. In other systems, it has been also hypothesized that biotic resistance exerted by native species could influence the realisation of ecological niche of invaders (Chaalali et al. 2013).

Inherent environmental differences between the estuaries of Bilbao and Urdaibai not only accounted for differences in the realized niches of brackish species but also in the realized niche of the common neritic taxa that inhabits the outer estuary, as it was evidenced by the higher tolerance and lower marginality of warm-affinity neritic taxa (*O. nana*, *T. stylifera*, *Oncaea* sp. and doliolids) in the estuary of Urdaibai, and by the higher tolerance and lower marginality of cold-affinity neritic taxa (*T. longicornis*, *Calocalanus* sp.) in the estuary of Bilbao. The outer estuary of Bilbao is fertilized by the estuarine plume and present mesotrophic conditions and annual peaks of phytoplankton biomass in summer, in contrast with the oligotrophic conditions of the estuary of Urdaibai derived from the decline of phytoplankton biomass in summer, this accounting for the higher density and seasonal spread of summer-autumn taxa in the first one (Iriarte et al. 2015; Fanjul et al. 2017).

3.4.3 Different response of zooplankton communities since the apparition of NIS

Our results highlighted different responses and niche shifts of the zooplankton communities throughout time and between estuaries. In the estuary of Bilbao, between the second (2003-2009) and the third period (2010-2015), a seaward shift of *A. clausi*, *O. similis*, *O. nana and O. plumifera* congeneric species and *Calocalanus* sp., *Centrophages* sp., *Oncaea* sp. and PCPC (among others) native neritic species was inferred from the displacement of their centroids that can be attributed to changes in environmental conditions, or to changes in biotic interactions within the estuary (Pearman et al. 2008). The displacement of the habitat centroid position in the estuary of Bilbao revealed progressive environmental changes that were consistent with the increase of dissolved oxygen concentration in the system during the study period as a result of the rehabilitation plan carried out in this estuary (Villate et al. 2013; Iriarte et al. 2016; Barroeta et al. 2020). However, the displacement of taxa centroids only shared in part the displacement of environmental centroids, this suggesting that biotic interactions were also implied. Water quality improvement in the estuary of Bilbao was associated with a recolonization of the inner part of the estuary by copepods, as in other systems (Mialet et al. 2011), but the effects of biotic interactions was corroborated by the changes in the niche overlap over time.

In this estuary, the niche overlap of *A. tonsa* and *O. davisae* with most of the neritic and congeneric copepods was higher in the period 2, just after the successful colonization of the system by the NIS, than in the period 3, after a long time of cohabitation, and this led us to hypothesize that the centroid displacement (see Fig 1d in Guisan et al. 2014) of neritic and congeneric species was attributable to biotic interactions. Niche shifts of congeneric species since the apparition of *O. davisae* has been also reported in other coastal environments after the NIS established (Isinibilir et al. 2016; Zagami et al. 2018). Biotic interactions between the studied NIS and their congeneric species can lead to a high spatial segregation (Azeiteiro et al. 2005) due to the fact that *A. tonsa*, *O. davisae* or *A. bifilosa* are found to be competitively superior in brackish environments (Gunter 1956, Lance 1963, Calliari et al. 2006) but physiologically limited to compete with their congeneric in high salinity waters (Dahl 1956; Hedgpeth 1957, Gaudy et al. 2000). The effect of the biotic interaction in the decrease of the niche overlap was corroborated by the fact that the changes mainly occurred with the species with highest overlap due to the seasonal coincidence as in the case of *A. tonsa* and *O. davisae* with PCPC, the dominant neritic taxa in the warm period, whilst no change was observed for *O. davisae* with the cold-affinity neritic species *T. longicornis* and *Calocalanus* sp. (Uriarte et al. 2016; Barroeta et al. 2020). In the case of *A. bifilosa*, recently introduced in the estuary of Bilbao (2007), the observed increase in niche overlap with *A. tonsa* from period 1 to period 3 in this system also corroborated that the niche overlap increases during the expanding phase of the new introduced species, since *A. bifilosa* arrived to the estuary of Bilbao when *A. tonsa* was already well established. The continuation of the study in the future might allow us to better understand the competition between both species by using a case study in which the order of arrival of competing species to the system is the opposite to the expected one in natural conditions.

The lack in the estuary of Urdaibai of such niche shifts or changes in niche overlap seems respond to the fact that the estuary of Urdaibai had a well-differentiated neritic and estuarine zooplankton assemblages before the apparition of NIS, with the native brackish species *A. bifilosa* dominating the estuarine community and maintaining neritic species restricted to the high salinity waters of the outer part. In this estuary, *Acartia* species distribution moved seasonally rather than spatially (Villate et al. 2018), and the annual abundance peak of *A. bifilosa* occurred around two month earlier after the establishment of *A. tonsa* in the system (Barroeta et al. 2020).

The use of OMI analysis and WitOMI allowed us to describe the community structure of the zooplankton community in two Basque estuaries and they reacted differently to the NIS settlement. We identified that NIS and OES colonize principally the inner part of Bilbao estuary, as it lacks of brackish dominant species. Such settlement of NIS was limited in Urdaibai estuary by biotic interactions. Additionally, we suggested that the colonization of the estuary of Urdaibai by egg-carrying NIS might be limited due to water turbulence. Finally, since the apparition of NIS and OES in the estuary of Bilbao, it has been observed a displacement seaward of some neritic and congeneric species. In the estuary of Urdaibai no changes were observed probably due to different biotic and abiotic conditions in the estuary.

CHAPTER 4: Occupation of Basque coast estuaries (south-eastern Bay of Biscay) by non-indigenous planktonic copepods in 2019

The colonization of a new system by non-indigenous planktonic species (NIS) may be the result of the transport of individuals, usually by cargo ships ballast water, from their natural area of occurrence or the result of a secondary spread, more likely due transport by recreational boats, or range-expansion from close previously colonized systems as a regional expansion. The NIS copepods *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae* colonized since 2001 with different success two estuaries of the Basque coast monitored from 1997, but there was not information on the regional spread of these NIS to other estuaries of the Basque coast. Therefore, we checked their presence and importance in the nearby estuaries of Plentzia, Oria and Bidasoa in summer 2019, in comparison with that observed during the same period in the monitored estuaries of Bilbao and Urdaibai. Results brought to light that the three NIS did not succeed equally in the colonization of the estuaries of the Basque coast. While *O. davisae* was recorded at all the estuaries but with large differences in abundance, *A. tonsa* was recorded in three systems and *P. marinus* only in the largest and most stratified estuary of Bilbao. Morphological and hydrodynamic differences between estuaries accounted for some of the between-estuary differences in the observed abundance and distribution of NIS species, but did not explained satisfactorily the differences in the regional spread of *A. tonsa* and *O. davisae,* this being a challenge for future studies.

4.1 Introduction

The most probable long-range transfer pathway for aquatic non-indigenous species (NIS) and their introduction in estuaries is ballast water (Geburzi and McCarthy 2018; Dexter et al. 2020). Albeit, it seems unlikely in the case of secondary spread (range-expansion) to other close estuaries, especially, with little or no commercial shipping (Dexter et al. 2020). Therefore, other both abiotic and biotic transport vectors (Christy and Stancyk 1982; Cáceres and Soluk 2002; Frisch et al. 2007; Minchin 2007) can be effective means to facilitate the small-range spread of the NIS. In this sense, regional traffic of smaller crafts, such as recreational boating, can be a noticeable driver of regional dispersion (Clarke Murray et al. 2011; Hänfling et al. 2011; Geburzi and McCarthy 2018). However, to understand invasion processes across regional scales is an arduous task, because the mechanisms and patterns of secondary spread across estuaries remain uncertain largely due to regular zooplankton monitoring programs are carried out at a low number of estuaries (Dexter et al. 2020).

This is the case of the estuaries of Bilbao and Urdaibai, where since 1997 a monitoring program of the zooplankton community has been carried out, which has enabled the detection and the monitoring of the different development of the NIS *Acartia tonsa, Oithona davisae* and *Pseudodiaptomus marinus* (Barroeta et al. 2020). In 2001 *A. tonsa* was firstly recorded in the estuary of Bilbao, quickly becoming the dominant copepod species of the inner part of the estuary (Aravena et al. 2009). In the estuary of Urdaibai, it was first found in 2003, establishing in a very few time period in the inner part of the estuary (Villate et al. 2018). The first occurrence of *P. marinus* in the estuary of Bilbao was recorded in 2010, maintaining a permanent low-abundance population (Barroeta et al. 2020), whilst in the estuary of Urdaibai some unsuccessful attempts to colonize has been registered (unpublished data). *O. davisae* was also found in 2001 in the estuary of Bilbao (Uriarte et al. 2016), becoming, together with *A. tonsa*, the dominant species of the inner part of the estuary. Although it occurred in the estuary of Urdaibai since 2001, it was not able to reach relevant densities (Barroeta et al. 2020). Furthermore, in the estuary of Bilbao, two other expanding species (OES) of copepods arrived in 2010, *Acartia bifilosa* and *Calanipeda aquaedulcis*, the latest dominating the inner estuary in spring (Barroeta et al . 2020). However, currently, there was a complete lack of information from other estuaries of the Basque coast because the studies on their zooplankton communities are concerning the 80-90s (Villate and Orive 1981; San Vicente et al. 1988; Villate 1989, 1991; D´Elbee 1998; Villate et al. 2004). Therefore, this is a preliminary study with the aim of detecting the presence and degree of occupation of the NIS *A. tonsa, P. marinus* and *O. davisae* into different estuaries along the Basque coast (Bay of Biscay). Moreover, the work gave the possibility to get insights into the spreading pathways of theses NIS between neighbour systems, and the role of environmental features and native species in determining the successfulness of colonization.

4.2 Method

4.2.1 Study area

A detailed description of the estuaries of Bilbao and Urdaibai is depicted in the study area section of the first part of this thesis. In this section, however, together with the other estuaries, it is exposed again information of both systems useful for the purpose of this Chapter. The estuaries of Bilbao (43º 23' N, 03º 07' W), Plentzia (43°25' N, 2°57' W), Urdaibai (43º 22' N, 02º 43' W), Oria (43°17' N, 2°07' W) and Bidasoa (43°23' N, 1°46' W) are close systems located within 176 km of the Basque coast, in the south-eastern Bay of Biscay (Fig. 1). Therefore, they belong to a same temperate-oceanic climate type characterized by moderate winters and warm summers. All are relatively short and shallow meso-macrotidal estuaries, showing semidiurnal tides which amplitude range vary from 1 m on neap tides to >4.5 m on spring tides (Villate et al. 1989). Nevertheless, differences in morphological and hydrodynamical features are noticeable (Table 1), as well as in water quality and anthropic impact.

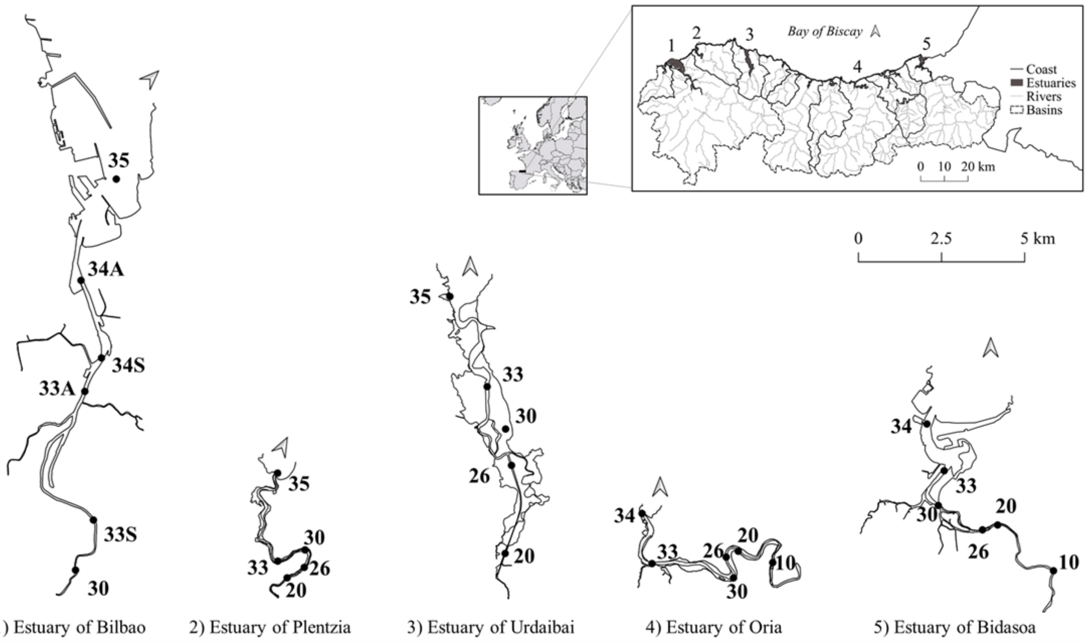

Fig. 1: Map of the estuaries of Bilbao, Plentzia, Urdaibai, Oria and Bidasoa showing their location on the Basque coast of the Bay of Biscay and the sampling salinity points.

Table 1: Main hydro-morphological characteristics of the estuaries of Bilbao, Plentzia, Urdaibai, Oria and Bidasoa.

Main hydro-morphological characteristics of the estuaries											
Estuary	[1]Length (Km)	[2]Area (Km^2)	[4]Basin area (Km^2)	[4]Mean volume (V) (x 10^6 m^3)	[1]Mean fluvial (m^3/s)	[2]Average depth (m)	[2]Mean tidal prism (Ω) (m^3)	[2]Ω/V	[4]% of subtidal area	[4]% of intertidal area	[4]Water column mixing
Bilbao	22.60	[3]1.50	1798.77	402.10	35.56	[4]9.50	[4]77000000	1.09	72	28	Highly stratified
Plentzia	8.53	0.38	172.22	2.20	4.73	3.07	833500	1.20	22	78	Partially mixed
Urdaibai	12.22	1.89	183.21	12.87	3.60	2.59	4858300	1.47	14	86	Partially mixed
Oria	11.35	0.85	881.99	3.13	25.66	3.62	2039800	0.97	16	84	Partially mixed
Bidasoa	15.81	2.30	700.00	45.80	27.19	4.33	5941900	0.84	82	18	Highly stratified

Information sources: [1]Monge-Ganuzas et al. 2019, [2]Villate et al. 1989, [3]Cearreta et al. 2014, [4]Borja et al. 2004

The estuary of Bilbao is the largest, deepest (from 0.5 m at the inner part to 32 m at the outer coastline site) and westernmost of all studied estuaries. The two main rivers flowing into Bilbao estuary head are Ibaizabal and Nerbioi, although some small tributaries flow into the middle part of the estuary. Tidal flushing is relatively low (Valencia et al. 2004) and the estuary varies from partially mixed in the outer part to strongly stratified in the inner (Intxausti et al. 2012). Therefore, the system shows two-layered circulation with much lower residence time in above halocline layers of the channelized upper and middle reaches than in the below halocline layer of outer zone (Abra harbour) (Uriarte et al. 2014). The estuary of Bilbao was one of the most polluted estuaries of Europe in the mid-twentieth century due to the industrialization located in its banks, but since 1980 it has undergone in a rehabilitation process, improving the water quality (Borja et al. 2006; Fdez-Ortiz de Vallejuelo et al. 2010; Villate et al. 2013). Nowadays, it is considered strongly modified and is highly impacted by morphological changes as channelization and dredge associated to urban and industrial land use and intense international maritime traffic, and by chemical pollution (Solaun et al. 2018). Actually, the port facilities located in the outer part of the estuary (Abra harbour) are one of the most important marine transport and logistics centres in the European Atlantic Arc, harbouring cargo ships (and consequently ballast water) from worldwide (Bilbao Port 2020).

The meander-form estuary of Plentzia is the smallest of all, with a maximum depth of 8 m and it flows into a small and semi-enclosed embayment. The main tributary is the Butroi River, at whose basin some industries are settled whose waste effluents result in some pollution (Orbea et al. 2002). During the first half of the 20th century, the estuary of Plentzia was in part channelized and artificial dykes were built for urban occupation and agriculture, impacting directly on the intertidal flats and marshes (Cearreta et al. 2002). Nevertheless, since the second half of the century, natural regeneration of these subsystems has been observed, as a consequence of the decline in agricultural activity (Cearreta et al. 2002), and, now, around 80 % of the estuarine surface is exposed in low tide. In the outer part of the estuary is located a small port harbouring, basically, recreational boats (Euskal Kirol Portuak 2020).

The small estuary of Urdaibai is the most shallow (2-5 m depth in most the estuary) of all. The main river flowing into this estuary is Oka, although it also has two small tributaries that are Mape and Golako. Tidal flushing is high and the estuary is dominated by seawater at high tide, therefore the outer part of the estuary being well mixed and the inner part is partly stratified (Villate et al. 2017). The system shows low water residence time. It is the central axis of the Urdaibai Biosphere Reserve, with extensive intertidal flats and sandy beaches in the outer reaches and salt marshes and reed beds in the middle and inner reaches. An artificial channel meets the main tributaries in the head of the estuary with the natural channel in the mid estuary, and a not very effective small sewage treatment plant (EDAR Gernika) located in the upper artificial channel causes punctual nutrient and chemical pollution (Cotano and Villate 2006; Solaun et al. 2018). Furthermore, in the middle of the estuary is located the Murueta shipyard, where the boat building is started but they, finally, are finished in the nearby estuary of Bilbao. In the mouth of the estuary are located the two small recreational ports of Mundaka and Busturia, and out of the estuary the relatively big port of Bermeo, one of the main fishery ports of the Basque coast, but with a small dock for recreational boats as well (Euskal Kirol Portuak 2020).

The also meander-form estuary of Oria is similar in length and depth (1-6 m depth) to the estuary of Urdaibai, but with lower expanse of intertidal and supratidal areas due in part to channelization and land claim. It has to point out that the middle part of the estuary is shallower than the inner and outer part (Villate et al. 1989). Homonymous with the main river, other small tributaries flow into it. Once a highly polluted system, nowadays the water quality in the whole basin has improved considerably (Provincial Council of Gipuzkoa 2020), as a consequence of the sanitation works and the installation of a sewage treatment plant. The middle-inner part is surrounded, mainly, by cattle and agricultural activity areas, whilst the outer zone shows greater channelization and urbanization. In the outermost part a small mooring for small fishery ships and a small dock for recreational boats are located (Gobierno Vasco 2020).

The estuary of Bidasoa is the easternmost, the second in length and depth (2-6 m depth), and has the biggest mean tidal prism of all studied estuaries (Villate et al. 1989). It takes the same name as the main river flowing into it. Though the channelization of the middle-inner part is very high and the outer part is fully modified, it is characterized by the presence of small isles, channels, and salt marshes in the middle-inner part and of a small bay (Txingudi Bay) in the outer one. However, it shows a good ecological, chemical and global status (Solaun et al. 2018) since low sewage discharge is generated along the basin, showing the best water quality of all estuaries of the western part of the Cantabrian Sea (Provincial Council of Gipuzkoa 2020). Finally, inside the estuary, five recreational ports are located, and moreover, outer the estuary there is one of the most important inshore fishing ports of the Basque coast (Euskal Kirol Portuak 2020).

4.2.2 Data acquisition

In order to assure the likely presence of the abovementioned NIS copepods in the selected estuaries, previous results on the seasonal distribution of these species in the estuaries of Bilbao and Urdaibai (Barroeta et al. 2020) and in other areas (Azeiteiro et al. 2005; Chaalali et al. 2013; Deschutter et al. 2018; Zagami et al. 2018) were taken into account, concluding that the best sampling date to find them was around September. Therefore, we used in the present study the data obtained in the estuaries of Bilbao and Urdaibai on 22 and 23 August 2019, respectively, and 23 and 24 September 2019, respectively, from the ongoing monitoring program of both estuaries, and the data obtained on purpose in the estuaries of Oria, Bidasoa, and Plentzia on 5, 6 and 12 September 2019, respectively. The data of Bilbao and Urdaibai from August and September were used in order to have a reference period within which the data of the estuaries of Oria, Bidasoa and Plentzia were obtained for compare purposes.

All the samplings were conducted at high tide during neap tides period. Samples were collected at selected salinity sites in relation to the salinity distribution peculiarities of each estuary (Fig. 1): 30, 33, 34 and 35 in the estuary of Bilbao; 20 (in Urdaibai only in August), 26, 30, 33, 35 in the estuaries of Urdaibai and Plentzia; and 10, 20, 26, 30, 33 and 34 in the estuaries of Oria and Bidasoa. Both water environmental and zooplankton data were collected and analysed as explained in the general method section of the first part of this thesis. Data of sampling day's air temperature (ºC), precipitation (mm) and river flow (m^3 s^{-1}) and monthly average precipitation (mm) and river flow (m^3 s^{-1}) were gathered in order to have an overall view of the hydro-meteorological conditions. These data were obtained from the Provincial Councils of Bizkaia (estuaries of Bilbao, Plentzia and Urdaibai) and Gipuzkoa (estuaries of Oria and Bidasoa).

4.2.3 Data treatment

Spatial distributions, both longitudinally and vertically, of salinity, percentage of dissolved oxygen and temperature in each estuary during the sampling cruise were depicted by using the software Surfer® 10 (Golden Software, LLC). For every estuary, salinity stratification index, calculated as the maximum difference in salinity between consecutive depths (Villate et al. 2013), was plotted against the relative distance (RD), calculated as the percentage to the total length of the estuary (being 0 m the outermost site of the estuary). Only those species showing a relative abundance >10 % were treated at the species level, while the remaining species were clustered in higher taxonomic levels. The abundance of identified taxa was reported as both density values (ind. m^{-3}) and logarithmic transformed density values (Log (density+1)). Density variations of the main components of the zooplankton community were represented in relation to salinity and relative distance (RD) within each estuary. In order to model the relationship between the zooplankton community and environmental variables two canonical redundancy analysis (RDA) were performed with all estuaries jointly, in the first RDA the environmental variables were added as categorical explanatory variables and estuaries as supplementary variables, and in the second one, salinity was assigned as categorical covariable (thereby removing the effect of salinity). RDAs were performed by means of Canoco v. 4.55 (Ter Braak and Šmilauer 2002). RDAs were performed by Monte Carlo tests with 499 permutations under reduced model (Ter Braak and Šmilauer 2002).

4.3 Results

4.3.1 Environmental scenario

The estuary of Bilbao showed the highest vertical variations of salinity, since euhaline (salinity > 30) water masses reached the upper estuary in depth, below a thin layer of low salinity water that expanded until the mid-zone (Fig. 2). In contrast, the estuary of Oria showed the strongest longitudinal gradient of salinity between the inner and the outer estuary. In the rest of the estuaries, the water masses showed, to a greater or lesser extent, a higher vertical mixing of the water column lengthwise estuaries, related to the tide. The percentage of dissolved oxygen was high in most water masses of all systems (Fig. 2). Overall, values decreased from oversaturation (> 100 %) in the outer part to normoxia (60-100 %) in the middle-inner parts, but in the innermost parts of the estuaries of Urdaibai and Bilbao, and only in depth in the last one, oxygen deficiency (40-60 %) and/or hypoxia conditions (15-40 %), according to the criteria proposed by Hale et al. (2016), were registered. The water temperature showed different spatial patterns depending on the estuary and the sampling date (Fig. 2). Overall, longitudinal gradients were higher in the estuaries of Urdaibai in both months and in the estuary of Bilbao in September, with increasing temperature from the outer to the inner, and in the estuary of Oria, but with increasing temperature from the inner to the outer. In contrast, temperature gradients were higher vertically in the estuary of Bidasoa, with decreasing values from surface to bottom, whilst in the estuary of Bilbao in August and in the estuary of Plentzia vertical variations were weak and the highest temperatures were recorded in the mid zone. As shown in Fig. 3, overall, the greatest salinity stratification was registered in the estuary of Bilbao and the lowest in the estuary of Urdaibai. Likewise, the salinity stratification increased meaningfully towards the innermost part of the estuaries, except in the estuary of Oria with highest stratification in the middle part.

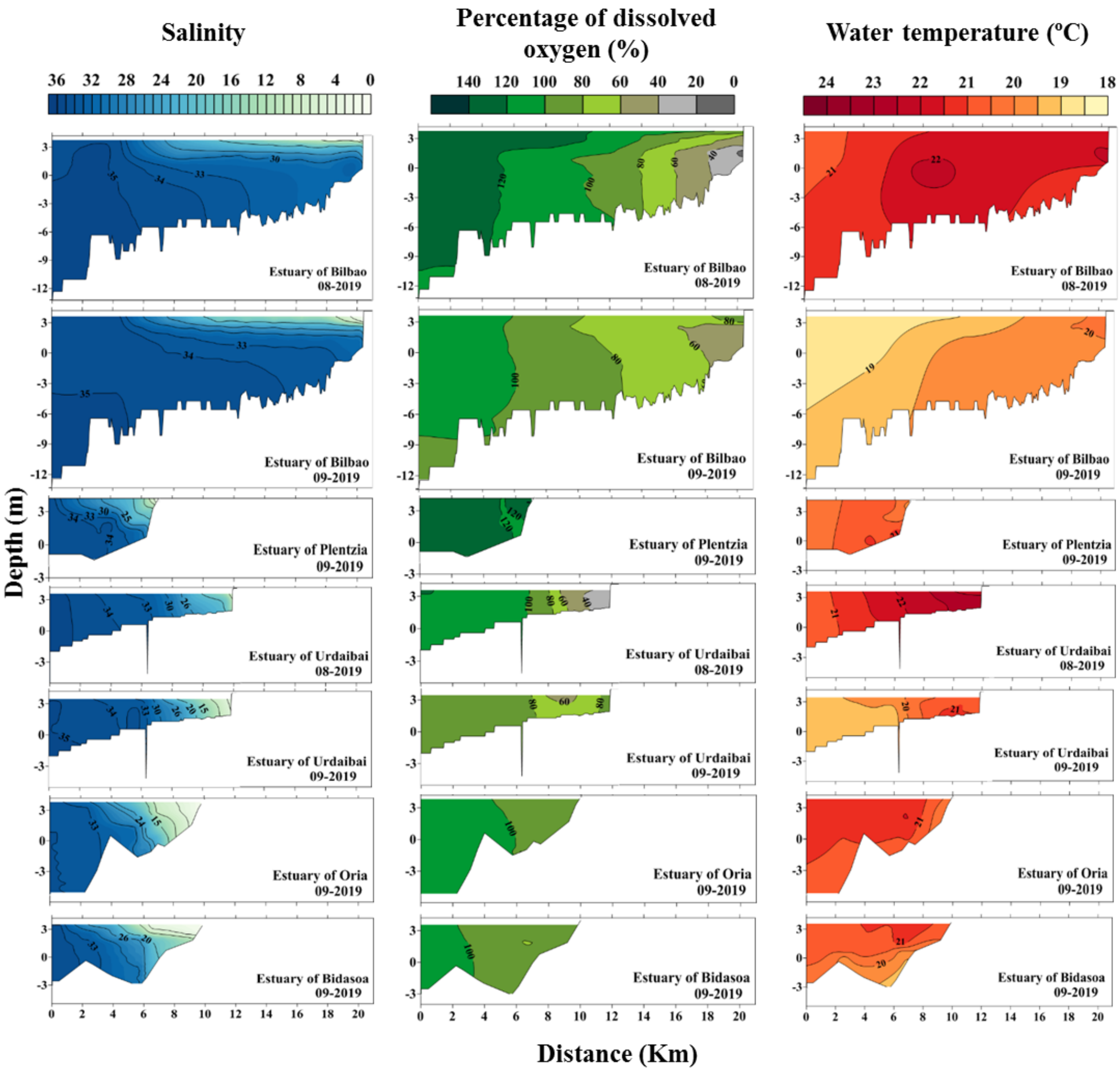

Fig. 2: Spatial variations of salinity, percentage of dissolved oxygen (%) and temperature (ºC) during the sampling cruises in the estuaries of Bilbao and Urdaibai, in both August and September 2019, and in the estuaries of Plentzia, Oria and Bidasoa, in September 2019.

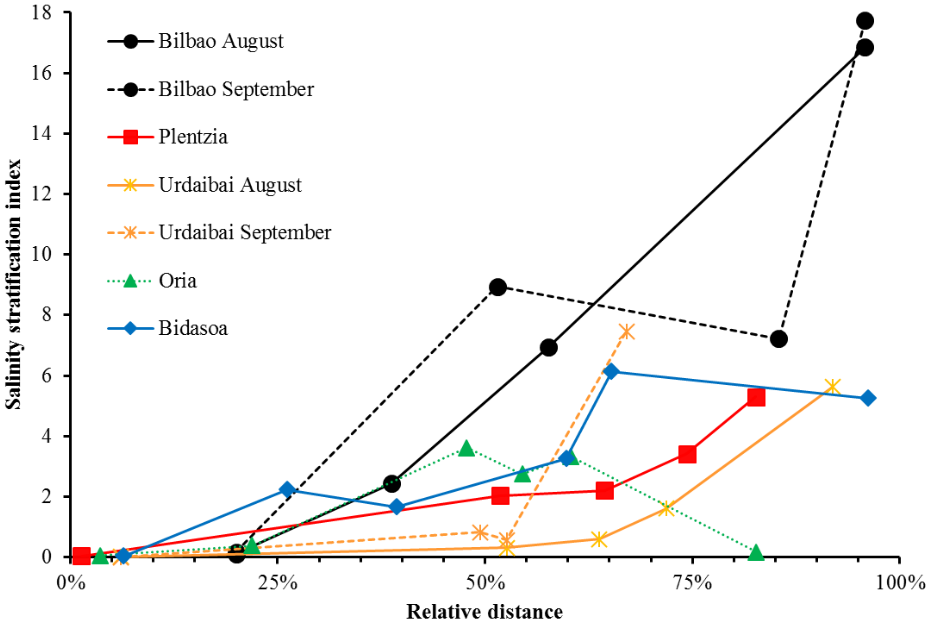

Fig. 3: Variation of the salinity stratification index in relation to the distance from the estuary mouth (in %) during the sampling cruises in the estuaries of Bilbao and Urdaibai, in both August and September 2019, and in the estuaries of Plentzia, Oria and Bidasoa, in September 2019.

4.3.2 NIS occurrence and zooplankton communities in the estuaries of the Basque coast

Densities of the NIS and OES copepods obtained in every estuary are shown in Table 2. *A. tonsa* was recorded in the estuaries of Bilbao, Plentzia and Urdaibai in a meaningful way, with mean densities higher than 1000 ind. m^{-3} in all cases, and even higher than 10000 ind. m^{-3} in the survey of August in the estuary of Urdaibai, but was not registered in the estuaries of Oria and Bidasoa. *P. marinus*, was only recorded in the estuary of Bilbao at low density (< 100 ind. m^{-3}). *O. davisae* was found in all the studied estuaries, but decreasing largely in density from the estuaries of Bilbao and Bidasoa (> 1000 ind. m^{-3}) to the estuaries of Plentzia and Urdaibai (< 10 ind. m^{-3} in average). Similarly, *A. bifilosa* also appeared in all the estuaries, but with lowest densities in the estuary of Bilbao (< 10 ind. m^{-3}), where was not found in September, and highest densities in the estuaries of Oria and Bidasoa, (>1000 ind. m^{-3}). Finally, *C. aquaedulcis* was registered in very low densities (maxima between 2 and 14 ind. m^{-3}) in the estuaries of Bilbao, Plentzia and Oria.

Table 2: Mean, minimum and maximum densities (ind.m^{-3}) of the NIS *Acartia tonsa, Pseudodiaptomus marinus* and *Oithona davisae* and the OES *Acartia bifilosa* and *Calanipeda aquaedulcis* in the estuaries of Bilbao, Plentzia, Urdaibai, Oria and Bidasoa.

Estuary	Date	Metric	*Acartia tonsa*	*Oithona davisae*	*Pseudodiaptomus marinus*	*Acartia bifilosa*	*Calanipeda aquaedulcis*
Bilbao	22/08/2019	Mean	1579.3	243.6	41.0	1.7	0.9
		Minimum	0.0	0.0	0.0	0.0	0.0
		Maximum	3818.6	634.1	110.2	6.8	3.4
Bilbao	24/09/2019	Mean	1740.5	548.6	36.3	0.0	0.0
		Minimum	22.7	39.8	11.4	0.0	0.0
		Maximum	2571.9	1414.0	67.5	0.0	0.0
Plentzia	12/09/2019	Mean	1389.6	9.4	0.0	181.4	2.7
		Minimum	0.0	0.0	0.0	0.0	0.0
		Maximum	3996.3	40.8	0.0	675.9	13.6
Urdaibai	23/08/2019	Mean	5575.2	3.8	0.0	282.1	0.0
		Minimum	0.0	0.0	0.0	0.0	0.0
		Maximum	22168.0	18.9	0.0	1307.3	0.0
Urdaibai	23/09/2019	Mean	627.5	0.2	0.0	87.7	0.0
		Minimum	5.6	0.0	0.0	0.0	0.0
		Maximum	2460.2	0.7	0.0	276.7	0.0
Oria	05/09/2019	Mean	0.0	24.0	0.0	751.2	0.4
		Minimum	0.0	0.0	0.0	0.0	0.0
		Maximum	0.0	96.0	0.0	2645.2	2.6
Bidasoa	06/09/2019	Mean	0.0	582.9	0.0	874.1	0.0
		Minimum	0.0	0.0	0.0	0.0	0.0
		Maximum	0.0	1299.1	0.0	1808.4	0.0

As shown in Figure 4, the neritic assemblage of copepods was dominated by *Paracalanus parvus*, in all the estuaries, followed by *Centropages* sp., *Acartia clausi* or *Oithona nana* depending on the estuary and survey. The brackish assemblage, however, showed clear differences in the dominance of species between estuaries. *A. tonsa* and *O. davisae* dominated in the estuary of Bilbao, *A. tonsa* and *A. bifilosa* in the estuaries of Plentzia and Urdaibai, and *A. bifilosa* and *O. davisae* in the estuaries of Oria and Bidasoa. Differences in the distribution of NIS and the other brackish species with salinity were also evident between estuaries. Thus, the highest densities of these species were obtained in euhaline waters (>30 salinity) in the estuary of Bilbao, polyhaline waters (18-30 salinity) in the estuaries of Plentzia and Urdaibai, and mesohaline-oligohaline waters (<18 salinity) in the estuaries of Oria and Bidasoa. Moreover, the change in dominance from neritic species to brackish species was at different salinities depending on the estuary, being between the salinity sites of 35 and 34 in the estuary of Bilbao, 34 and 33 in the estuary of Plentzia, and 33 and 30 in the estuaries of Oria and Bidasoa. In the estuary of Urdaibai, this replacement in the dominance was found between the salinity sites of 35 and 33 in August but 30 and 26 in September. As for other holoplankton taxa, appendicularians were the most abundant group, except in the estuary of Urdaibai where chaetognaths were the most representative. Cladocera were clearly more abundant in the estuaries of Oria and Bidasoa than in the other estuaries. Overall, all these holoplankton taxa decreased with decreasing salinity. Regarding meroplankton taxa, the most abundant group were cirripedia larvae in all the estuaries, but they increased or decreased with salinity depending on the estuary, and on the survey in the estuary of Bilbao. Gnathiidae increased considerably at lowest salinities, whilst ostracoda showed highest densities at lowest salinity sites in the estuary of Urdaibai and at intermediate salinity in the estuary of Oria.

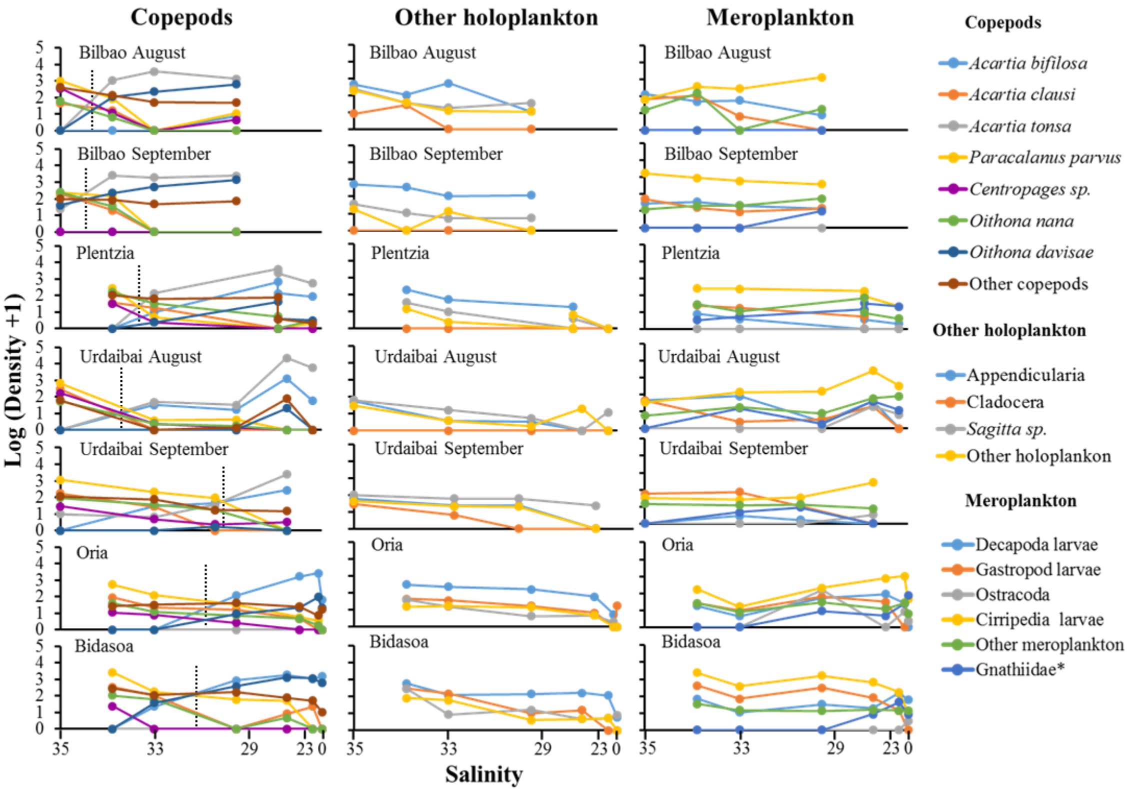

Fig. 4: Variation of density (Log (density +1)) with salinity (at 4/5 scale) of the main copepod taxa, other holoplankton taxa and meroplankton taxa in the estuaries of Bilbao and Urdaibai, in both August and September 2019, and in the estuaries of Plentzia, Oria and Bidasoa, in September 2019. Vertical dotted line in copepods plots represents the change point from neritic to brackish community.

Spatially, the change in the dominance from neritic to brackish copepod species took place at the outer half of the estuaries, except in the estuary of Urdaibai in September, where it occurred upward the mid of the estuary (Fig 5). Overall, the density of the other main holoplankton groups decreased drastically inward the mid of the estuary, except in the estuary of Bilbao, whilst the meroplankton groups showed, in general, higher occupation of the inner half of estuaries and different patterns of variations going into every estuary. Additionally, in contrast with the other estuaries, in the estuary of Bidasoa decapod larvae were the dominant meroplankton group in the innermost site. Interestingly, we found that it was due to the occurrence in large number of zoea larvae of the non-indigenous brachyuran *Rhithropanopeus harrisii*, which showed densities of 142.2 and 59.1 ind.m^{-3} at the salinity sites of 20 and 2, respectively.

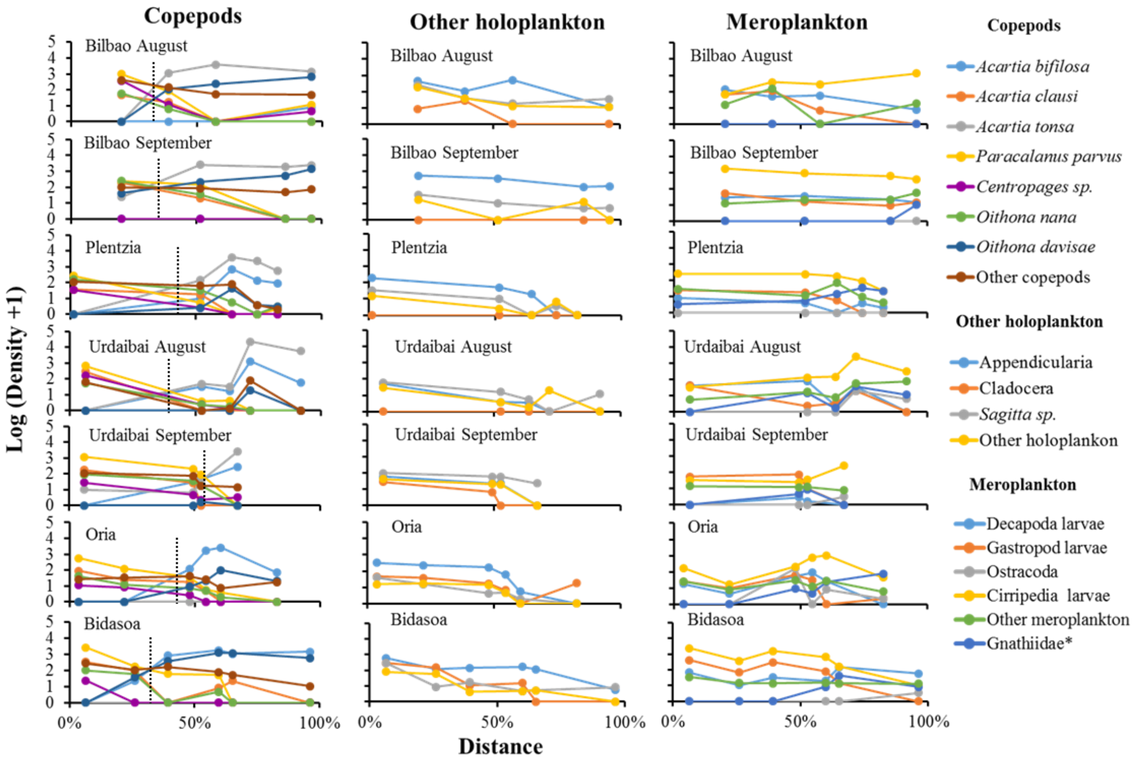

Fig. 5: Variation of density (Log (density +1)) with the distance from the estuary mouth (in %) of the main copepod taxa, other holoplankton taxa and meroplankton taxa in the estuaries of Bilbao and Urdaibai, in both August and September 2019, and in the estuaries of Plentzia, Oria and Bidasoa, in September 2019. Vertical dotted line in copepods plots represented the change point from neritic to brackish community.

4.3.3 Influence of environmental variables on NIS occurrence

The environmental variables selected by the RDA explained more than 90% of the variability in the zooplankton joined set for all the studied estuaries (Fig 6a). The first component (Axis 1; 68.9 % of the total variability) underscored the distribution pattern of zooplankton taxa along the longitudinal gradient of the estuaries, since neritic and brackish taxa positioned oppositely. Among others, *A. clausi*, *P. parvus*, *O. nana*, appendicularians, cladocerans, gastropod larvae and bivalve larvae were plotted on the positive side, whilst *A. tonsa*, *A. bifilosa*, *O. davisae*, *C. aquaedulcis*, and Gnathiidae on the negative side. This pattern was mainly explained by Secchi disk depth, salinity, depth, and percentage of dissolved oxygen, which are higher outward estuary, in opposition to the relative distance from the estuary mouth and salinity stratification index, increasing inward estuary. The second component (Axis 2; 22.7 % of the total variability) emphasized between-estuary differences by separating the estuary of Bilbao from the other estuaries. The estuary of Bilbao was mainly associated to all NIS copepods (*P. marinus*, *A. tonsa* and *O. davisae*) and to cirripide larvae, and to saline stratification and depth. On the opposite side, together with the other estuaries, were plotted brackish taxa such as *A. bifilosa*, isopods Gnathiidae and *R. harrisi* and neritic taxa such as medusae, *Temora stylifera* and *P. avirostris*. Among environmental conditions, dissolved oxygen and temperature were the most opposed to depth and salinity stratification. Once the effect of salinity was removed (Fig 6b), the percentage of zooplankton variability explained by the selected variables diminished slightly (74.6%) but the first component (Axis 1; 53.5 % of the total variability) still pointed out the longitudinal gradient, with a more marked contribution of *A. tonsa*, plotted to the negative side and related to the relative distance from the estuary mouth and salinity stratification index, and the main neritic taxa plotted to the positive side in relation to Secchi disk depth and percentage of dissolved oxygen. In this case, the estuaries of Bilbao, Plentzia and Urdaibai were depicted in the negative side and the estuaries of Oria and Bidasoa in the positive. Similarly, the second axis (21.1 % of the total variability) remarked the differences between the estuary of Bilbao and the estuaries of Urdaibai and Plentzia, depicting in the positive side *P. marinus* and *O. davisae* and cirripede larvae, together with river flow, depth and salinity stratification index, and in the negative side *A. bifilosa*, *Temora stylifera* and medusae, together with the percentage of dissolved oxygen and water temperature.

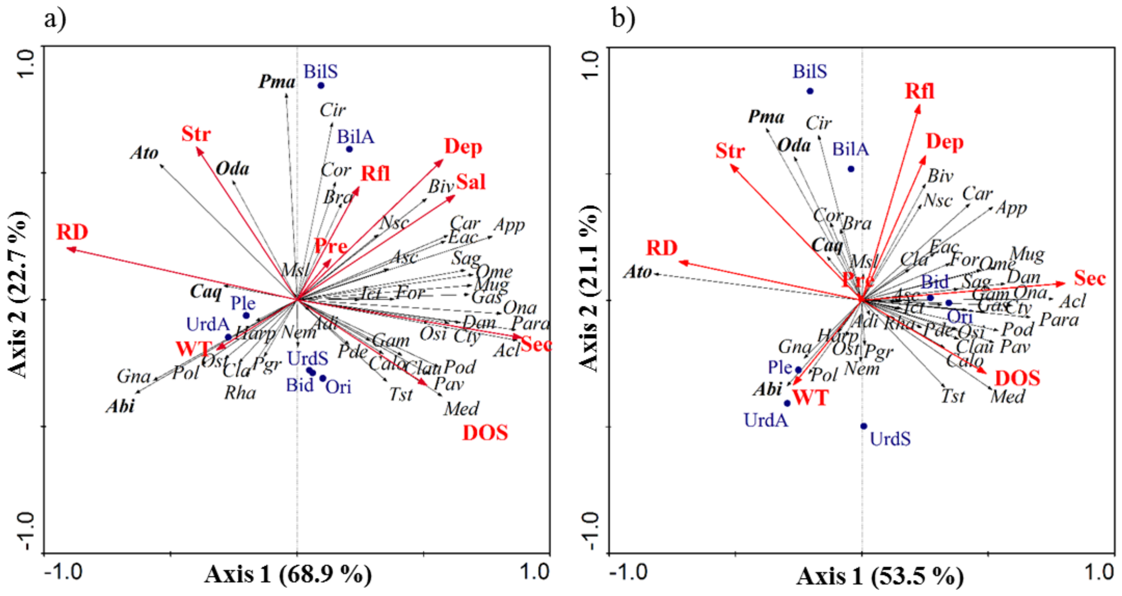

Fig. 6: RDA triplots for the zooplankton community (a) with environmental variables as explanatory variables and estuaries as supplementary variables, and (b) with salinity assigned as covariable in order to eliminate its effect. Taxa are shown by thin arrows, explanatory variables by coloured arrows and estuaries with the coloured dots. NIS (*Acartia tonsa, Oithona davisae, Pseudodiaptomus marinus*) and OES (*Acartia bifilosa, Calanipeda aquaedulcis*) in bold. Abbreviations of environmental variables: Water temperature (WT), Salinity (Sal), Dissolved oxygen (DOS), Stratification index (Str), Secchi disk depth (Sec), Depth (Dep), Proportional distance (RD), Precipitation (Pre) and River flow (Rfl). Abbreviations of taxa as in Appendix 1. Abbreviations of the estuaries: BilS (Bilbao September sampling), BilA (Bilbao August sampling), Ple (Plentzia), UrdS (Urdaibai September sampling), UrdA (Urdaibai August sampling), Ori (Oria) and Bid (Bidasoa).

4.4 Discussion

In studies previous to the year 2000, *A. bifilosa* was reported as the dominant brackish species in the zooplankton of all the estuaries of the Basque coast (Villate et al. 2004), with the exception of the estuary of Bilbao where it did not occurred until 2010 (Barroeta et al. 2020). The monitoring of the zooplankton in the estuaries of Bilbao and Urdaibai since 1997 allowed to detect from 2001 onwards the arrival of the NIS *A. tonsa*, *O. davisae* and *P. marinus* (Uriarte et al. 2016; Villate et al. 2018), which colonized brackish habitats with different success and impact on the zooplankton community of these estuaries (Barroeta et al. 2020). Present results showed that the spread of these NIS to other estuaries of the Basque coast and their colonization success was also different, since *O. davisae* was recorded in the three new estuaries surveyed but in variable abundance (Plentzia, Oria and Bidasoa), *A. tonsa* was only found in the estuary of Plentzia and *P. marinus* was not detected in none of them. Two plausible explanation to the absence of any of these NIS in such estuaries are that they did not arrived or that they arrived, or are periodically arriving, but were not stablished as detectable populations in the date surveyed. This last is supported by the observations in the estuary of Urdaibai, where *O. davisae* and *P. marinus* are recorded occasionally and usually in low number (Barroeta et al. 2020), and the fact that *P. marinus* was not either found in the surveys of the estuary of Urdaibai reported here.

The three NIS were initially detected in the estuary of Bilbao, this suggesting that this estuary could be the entry point of the estuaries of the Basque coast. The most plausible way of introduction was by ballast water, because the estuary of Bilbao holds one of the most important marine transport and logistics centres in the European Atlantic Arc, harbouring cargo ships (and consequently ballast water) from worldwide (Bilbao Port 2020). Likewise, this estuary was the most successfully colonized by all the arriving planktonic copepod NIS, showing the highest receptiveness among the estuaries of the Basque coast analysed until now.

The environmental characteristics that made the estuary of Bilbao a better hosting system for NIS in relation to the other estuaries may be related to its higher size and man-made modifications, which determines present volume and surface/depth ratio, and consequently water circulation and mixing, salinity zonation, tidal exchange and water residence time (Uriarte et al. 2014). Its length, from the coastal line to the inner tidal limit, doubles in most of the cases the length of the other systems, and its depth is clearly over that of the others due to historical dredging interventions to favour navigation (Cearreta et al. 2004). Additionally, in contrast with the other estuaries, almost all intertidal areas were occupied and the entire estuary was turned into a narrow artificial channel up to the Abra embayment. This morphological transformation favoured marine water intrusions in depth and reduced vertical mixing, resulting in a stratified system with two layered circulation and high residence time of inner water masses below the halocline (Uriarte et al. 2014).

The absence of *P. marinus* in the records of all estuaries, excepting the estuary of Bilbao, reinforced the hypothesis of Barroeta et al. (2020) that the presence of this species in the estuary of Bilbao is associated to the high salt wedge that usually penetrates up to the inner estuary during the dry season and the epibenthic behaviour of *P. marinus* (Sabia et al. 2015). Although this species has been occasionally recorded in the estuary of Urdaibai (unpublished data), in 2019 it was not recorded, this suggesting that the unsuitability of this estuary for housing *P. marinus* would be comparable to that of the estuaries of Plentzia, Oria and Bidasoa. *P. marinus* has had a rapid spread through European coasts from the Mediterranean Sea to the North Sea in the last years (Uttieri et al. 2020), similar to that observed for other species of the same genus in other areas, as it is the case of *P. inopinus* (Cordell and Morrison 1996) and *P. forbesi* (Dexter et al. 2020) in the Pacific coast of North America. Interestingly, the success of *Pseudodiaptomus* species colonizing estuaries was also associated to the extent of marine intrusions (Cordell and Morrison 1996). Apart of the estuary of Bilbao, as no such hydrologic conditions take place in the other estuaries of the Basque coast, the occurrence of this species in them will remain improbable although they are located within the wide geographic space occupied by *P. marinus* in last years.

Stratification itself seems also a condition required for the successful colonization of Basque coast estuaries by *O. davisae*, which showed decreasing densities from the highest stratified estuary of Bilbao and the moderately stratified estuaries of Bidasoa and Oria to the best mixed estuaries of Plentzia and Urdaibai. These new results reinforce the hypothesis done in previous chapters of this thesis that directly related the contrasting successfulness in the maintaining of *O. davisae* populations in the estuaries of Bilbao and Urdaibai to stratification levels and fresh water retention. Accordingly, they corroborate that *O. davisae* requires stable waters to ensure its reproduction throughout the year (Zagami et al. 2018), while turbulent mixing may have negative effect on individuals survival by affecting feeding behaviour (Saiz et al. 2003).

Surprisingly, *A. tonsa* which seems to be not affected in such a way by turbulence (Saiz et al. 2003) and shows high adaptability to different environmental conditions including deteriorated ones by abnormal inputs of pollutants and trophic loadings (Camatti et al. 2019), even taking advantage of eutrophic or impacted ecosystems (Brylinski 1981; Bianchi et al. 2003; David et al. 2007; Biancalana et al. 2014; Bandpei et al. 2017), was not found in two of the three new surveyed estuaries. The settle success of this species, however, seems also be enhanced in habitats with poor native zooplankton populations (Baretta and Malschaert 1988) as was supported by our findings in the estuaries of Bilbao, with no native brackish copepods when *A. tonsa* arrived, and Urdaibai, previously inhabited by a well-developed brackish assemblage dominated by the congeneric species *A. bifilosa* (Villate et al. 2018; Barroeta et al. 2020). Therefore, the presence of *A. bifilosa* in high abundance in the estuaries of Bidasoa and Oria might be seen as a handicap in the establishment of *A. tonsa* in these systems but does not explain by itself the lack of this species in them, since *A. tonsa* was found in large number cohabiting with *A. bifilosa* in the estuaries of Urdaibai and Plentzia. In both, *A. bifilosa* was reported historically as the largely abundant and only dominant species of brackish waters prior to the occurrence of *A. tonsa* (Villate and Orive 1981; Villate 1989). If the estuary of Bilbao is considered the source of *A tonsa* to colonize neighbour estuaries the connectivity between estuaries might be argued given the proximity of the estuaries of Plentzia and Urdaibai to the estuary of Bilbao in relation to the distance of the estuaries of Oria and Bidasoa from the former ones.

The absence of great commercial port facilities in the rest of the estuaries hinders NIS transfer from the estuary of Bilbao through ballast water, the most likely means of transferring these species (Gubanova 2000), being most plausible a regional spread from this estuary by means of recreational boating (Hänfling et al. 2011; Geburzi and McCarthy 2018). All the studied estuaries have leisure ports, and in the case of the estuaries of Plentzia and Urdaibai this vector is highly probable due to their proximity to the estuary of Bilbao. Proximity makes also plausible NIS spread from estuary to estuary by range-expansion. However, the same spread mechanisms and constraints should be considered for *O. davisae*, which shares brackish habitat with *A. tonsa* in the estuary of Bilbao but, unlike *A. tonsa*, it was present in all the estuaries. Nevertheless, another source of introduction could be the international commercial port of Bayonne, located in the mouth of the estuary of Adour. The plume of this estuary reach until the estuary of Bidasoa, unlike the plume of the estuary of Bilbao (Fernández-Nóvoa et al. 2019), likely facilitating individuals of NIS range-expansion. Consequently, the concurrent absence of *A. tonsa* and the presence of *O. davisae* in the estuaries of Oria and Bidasoa poses questions rather than clues about the mechanisms implied in the spread of NIS across the estuaries of the Basque coast and remain as a challenging issue for future studies.

The densities of *A. tonsa* recorded in the estuary of Plentzia in the present work suggest that presently its quantitative relevance in summer in this estuary is similar to those registered in the estuaries of Bilbao and Urdaibai, becoming the dominant brackish copepod in the warmest period. Unfortunately, no studies on zooplankton have been carried out in the estuary of Plentzia since the early 80s (Villate and Orive 1981) but knowing the almost time-coincident occurrence and sudden increase of this species in the estuaries of Bilbao and Urdaibai (Barroeta et al. 2020) we hypothesize a likely introduction around 2001-2003 given that the estuary of Plentzia is spatially located between the two former estuaries. In the same way, the similarity of the estuaries of Plentzia and Urdaibai in that concern to environmental features and seasonal records of *A. bifilosa* (Villate and Orive 1981; Villate 1989) lead as to think that the population of *A. tonsa* in the estuary of Plentzia could not persist all along the year as in the estuary of Bilbao but only around summer months as in the estuary of Urdaibai. Similar comparative assumptions might be drawn for *O. davisae*, which was found in the estuary of Plentzia in low densities similar to those of the estuary of Urdaibai, likely in response to the coincident small size and depth, weak stratification and high tidal exchange of two both systems.

Conversely, *O.* davisae showed high densities in the estuary of Bidasoa, similar to those obtained in the estuary of Bilbao, and intermediate densities in the estuary of Orio, in agreement with the degree of stratification and tidal exchange. The estuary of Oria showed lower stratification than the estuaries of Bilbao and Bidasoa, and has relatively low tidal exchange due to the fact that the middle part is shallower than the inner part, this limiting the penetration of the marine front till there (San Vicente et al. 1988). As in the estuary of Plentzia, previous studies on zooplankton in the estuaries of Orio and Bidasoa go back to the last decades of the past century, and only permit to state that *O. davisae*, just like *A. tonsa*, was not recorded in these systems (San Vicente et al. 1988; D´Elbee 1998). The fact that *O. davisae* has colonized these two estuaries located far away from the estuary of Bilbao, whist *A. tonsa* did not, opens the way to the alternative hypothesis of an independent spread from another source different from this estuary, unlike in the case of the estuaries of Plentzia and Urdaibai. This is also compatible with the hypothesis that *A. tonsa* has been unable to arrive to the estuaries of Oria and Bidasoa, since displacement/eradication by native/competitive species after eventual colonization seems unlikely in the light of the cases that inform in the opposite sense (Camatti et al. 2019), with dominances and alterations on the native species (Bianchi et al. 2003; Azeiteiro et al. 2005; Sei et al. 2006; David et al. 2007). Once *A. tonsa* is established, its replacement by other *Acartia* species might require strong environmental changes, such as the reported for Berre lagoon (Mediterranean Sea), where rehabilitation processes resulting in an increase of salinity led to the replacement of *A. tonsa* by *A. clausi* (Delpy and Pagano 2018). Anyway, this case illustrates the replacement of a brackish species by a neritic species, and there is no evidence of such strong environmental change in the estuaries of Oria and Bidasoa, which keep original brackish habitats and communities.

The solution to present uncertainties on the spread sources and ways of *A. tonsa* and *O. davisae* across the Basque coast estuaries would be in future researches using molecular analyses to track the spreading route of these species in the basis of phylogeographic distribution of populations' haplotypes (Albaina et al. 2016).

An additional case of between-estuary differences in the spread of NIS in the estuaries of the Basque coast was the case of the crab *R. harrisii*. We found larvae of this decapod in large number in the plankton of the inner zone of the estuary of Bidasoa, but such larvae were not recorded in the other estuaries. This species was already found in this estuary in 1994-1995 (D´Elbee 1998), whilst it was not mentioned in the list of benthic NIS reported during the period 1989-2008 in the estuary of Bilbao (Zorita et al. 2013). This crab native to the west coast of North America inhabits shallow and low salinity habitats (Eno et al. 1997), spreading through much of continental Europe since 1870 (Cuesta et al. 1991; Minchin and Sheehan 1995; Eno et al. 1997), possibly in ballast water or clinging to hulls (Eno et al. 1997). All this suggest that inherent environmental features of a particular estuary, in this case the estuary of Bidasoa, may allow the stablishment of a given NIS which is unable to colonize neighbour estuaries. For instance, the estuary of Bidasoa, with a semi-enclosed embayment shape, several channels and salt marshes in the mid part and high fresh water retention in the inner part, seems to provide a suitable habitat for this species that could be no found in the rest of the estuaries of the Basque coast. Another example of irregular occurrence of a brackish species among estuaries was the case of *C. aquaedulcis*, which was found only at the estuaries of Bilbao, Plentzia and Oria in few densities. Nevertheless, we could not make clear conclusions about its expansion from this data due to the fact that this species has been also reported in the estuary of Urdaibai in other studies, and it reaches highest densities in spring dropping in abundance in summer (Villate and Orive 1981; Villate 1989; Barroeta et al. 2020).

Results on the overall zooplankton communities revealed between-estuary differences not only in the assemblage of NIS, but also in native components that were also attributable to the most contrasting environmental condition of the estuary of Bilbao against the other estuaries, mostly against the estuaries Urdaibai and Plentzia. After *P. marinus*, cirripede larvae were the main zooplankton category that differentiated the estuary of Bilbao from the other estuaries, and after *A. bifilosa* the isopods Gnathiidae the main taxon that differentiated the others estuaries from the estuary of Bilbao. The higher contribution of cirripede larvae to the total zooplankton of the estuary of Bilbao is attributed to the higher surface of hard natural and artificial substrates, i.e. the suitable habitat for sessile barnacles, in this system (Villate et al. 2017), while the occurrence of isopods Gnathiidae and other temporary zooplankton in the water column is favoured in the other estuaries by the combined effect of shallowness and tides (Villate 1997).

Another interesting finding was that the abundance maxima of brackish species and the transition from neritic species dominance to brackish species dominance, this including the dominance of NIS, were more related to the position within the estuary (relative distance from the mouth) than to salinity. The RDA that included both variables as explanatory corroborated this finding, because the relative distance from the mouth showed the highest contribution, instead of salinity, accounting for the segregation of brackish taxa from neritic ones. This is in agreement with that reported in other transitional systems such as Mission Bay and Senegal River estuary, where spatial variations in zooplankton communities were also related to distance from the mouth (Champalbert et al. 2007; Elliot and Kaufmann 2007). The differences in the salinity of transition from neritic to brackish species observed between the two surveys of the estuary of Urdaibai reinforced the hypothesis of the predominant role of the relative distance from the mouth to explain the replacement in the dominance of neritic species by brackish species within estuaries. This may be related to the effect of tides that determines the position of water masses and inhabiting zooplankton populations within the estuary. Our result show the position at high tide in all cases, but according to Villate (1997) brackish zooplankton populations move from the inner zone, at high tide, to the outer zone, at low tide, over distances of more than one third of the estuary length in the estuary of Urdaibai during spring tides. This suggest that the replacement of brackish species by neritic species, (between the 25 % and the 50 % of the length from the mouth) in the dominance of the zooplankton communities of this meso-macrotidal system would occur in the water masses that just arrive to the mouth of the estuary, because water masses fluxed out of the estuary at ebb tide may be mixed with waters of the surrounding coastal water, this dispersing estuarine population and preventing their return to the estuary at the next flood tide.

GENERAL DISCUSSION

The NIS *A. tonsa* and *O. davisae* occurred in the year 2001 in the estuary of Bilbao and in the year 2003 in the estuary of Urdaibai, since then establishing lasting populations in the inner zone of both estuaries. Later, in 2010, another NIS, *Pseudodiaptomus marinus,* and the OES *A. bifilosa* and *C. aquaedulcis* occurred in the estuary of Bilbao, increasing their abundances also in the inner estuary (Uriarte et al. 2016; Barroeta et al. 2020). The NIS *A. tonsa, P. marinus* and *O. davisae* showed differences in achieving a successful colonization of the estuaries of Bilbao and Urdaibai, because although the three NIS established successfully in the former one, only *A. tonsa* was successful to colonize the estuary of Urdaibai. *A. tonsa* showed a sudden raise of density from 2002 to 2003 in both estuaries, coinciding with the change from a cool year to the warmest one in transitional waters. This corroborated that great environmental changes, such as extreme climatic events and/or hydrological changes, can facilitate the establishment of invasive species in estuaries (Winder et al. 2011). Later population changes of this species were also found to be related to temperature, being favored in hot years as it was reported in previous studies (Aravena et al. 2009; Villate et al. 2018). In contrast, the establishment of *O. davisae* was not so abrupt, in the estuary of Bilbao increasing its abundances more progressively, in agreement with that observed in other colonized estuaries (Winder et al. 2011). Nevertheless, according to Chapter 1 results, this had an increasing trend through the study period, which seemed not to reach yet its maximum in the innermost part. Although *O. davisae* was always found in lower densities than *A. tonsa* in our samples, this does not mean that it had a lower numerical importance than *A tonsa*. The real abundance of this small cyclopoid resulted clearly underestimated in our study due to the use of a 200 µm plankton net, as we have observed (unpublished data) and has been observed for other *Oithona* species of similar size (Villate 1991; Pansera et al. 2014). *P. marinus,* the largest of the three NIS, showed much lower densities than those of *A. tonsa* and *O. davisae*, like in the region of origin, where it is also much less abundant than other dominant copepods such as *O. davisae* (Liang and Uye 1997). But in contrast to the other studied NIS, *P. marinus* has an epibenthic nature and, therefore, its density could also be underestimated in routine zooplankton samplings (De Olazabal and Tirelli 2011; Brylinski et al. 2012). As for the OES, *A. bifilosa* had a progressive increasing trend in the estuary of Bilbao, whilst *C. aquaedulcis* has a more rapid increase in abundance after occurring in 2010.

Spatial and temporal distributions of all the NIS in the estuaries of Bilbao and Urdaibai were clearly shaped by salinity and temperature (Chapter 1 and 3) due to their brackish and warm affinities. However, the observed salinity optimum of *A. tonsa, P. marinus* and *O. davisae,* as well as of the OES *A. bifilosa* and *C. aquaedulcis*, was displaced in our estuaries towards higher salinities (euhaline-polyhaline waters) that those reported for laboratory experiments and larger estuaries (Lance 1963; Castro-Longoria and Williams 1999; Svetlichny et al. 2012a; Chaalali et al. 2013; Sabia et al. 2015; Zagami et al. 2018), likely due to the fact that both estuaries are small sea dominated systems in which mesohaline-oligohaline water masses are not enough entity to allow a successful development of brackish planktonic copepods. Both *A. tonsa* and *O. davisae* showed a drastic decrease in density from 33 to 35 salinity sites in both estuaries (Villate et al. 2018). Nevertheless, through our study period, *A. tonsa* and *O. davisae* expanded to higher salinity waters in the estuary of Bilbao, while in the estuary of Urdaibai *A. tonsa* was more constrained to low salinity waters. *P. marinus* was the NIS with the most even distribution with salinity. In general, the NIS occupied a wider salinity range than the OES *A. bifilosa* and *C. aquaedulcis* in the estuary of Bilbao, whilst in the estuary of Urdaibai *A. tonsa* showed a lower spread toward high salinity waters than the native *A. bifilosa*. *A. tonsa* had a very marked seasonal pattern in both estuaries, peaking clearly in the warmest period (July - August), similarly as in other

European colonized areas (Baretta and Malschaert 1988; David et al. 2007; Camatti et al. 2019). However, during the last period (2010-2015) its seasonal distribution was less constrained to the summer in the estuary of Bilbao, and clearly expanded towards spring and autumn, showing higher densities all year round. The results of Chapter 1 and 3 suggested that *O. davisae* was less dependent on temperature and more dependent on stratification than *A. tonsa*, although it also was a clear warm affinity species. Indeed, the annual peak of *O. davisae* was skewed towards autumn in the estuary of Bilbao in the period 2003-2009 but moved to summer in the period 2010-2015. Although the observed seasonal pattern of this species was similar to that described in its region of origin and other colonized areas (Uye and Sano 1995; Yildiz et al. 2016), in the estuary of Bilbao, *O. davisae* remained in winter months, in contrast to what has been observed in colder areas like the coastal waters of the Black Sea (Seregin and Popova 2016), probably because water temperature is rarely lower than 10 ºC in the estuary of Bilbao. The seasonal pattern of *P. marinus* in the estuary of Bilbao was also similar to those reported in other areas colonized by this species (Fleminger and Hendrix Kramer 1988; Deschutter et al. 2018), with highest densities from June to September, decreasing in autumn and being absent in most winter-spring months. Since *P. marinus* does not produce resting eggs (Sabia et al. 2014), the population within the estuary is likely annually recovered by migration from a perennial coastal population. In this way, in the period of highest river flow (winter-early spring) the population would be flushed out of the estuary and later penetrates into the estuary again in late spring favored by the increase of the net upward bottom circulation from spring to summer (Uriarte et al. 2014).

The differences in the success of colonization of the estuaries of Bilbao and Urdaibai by the NIS, discussed in Chapter 1, were attributed to the combination of inherent differences in system hydromorphological features and NIS capabilities. The estuary of Urdaibai has lower water residence time than the estuary of Bilbao (Valencia et al. 2004), where the two-layered circulation allows a greater stability of below halocline water masses along the estuary (Uriarte et al. 2014). In addition, in the shallower estuary of Urdaibai wind and tides induced water turbulence is higher and during the tidal cycle the brackish zooplankton assemblage moves from the inner part at high tide to the outer part at low tide, and may be flushed out of the estuary during extreme flood events (Villate 1997; Uriarte et al. 1998). These conditions are unfavorable for *P. marinus* and *O. davisae,* whose optimum conditions are stratified waters with limited water exchange and also, for the former species, a large salinity intrusion zone (Cordell and Morrison 1997; Saiz et al. 2003; Sabia et al. 2015; Zagami et al. 2018). *A. tonsa* may better counteract those conditions because, in contrast to *P. marinus* (Sabia et al. 2014) or *O. davisae* (Uye and Sano 1995), this species produces resting eggs that remain long time viable in sediments and allow to increase or recover the planktonic populations (Katajisto et al. 1998). Likewise, the differences in the density and interannual dynamics of the OES *C. aquaedulcis* between the two estuaries may also be related to the aforementioned environmental constraints in the estuary of Urdaibai and the improvement of the water quality conditions in the hydrologically more stable estuary of Bilbao in recent years (Uriarte et al. 2016). WitOMI analysis (Chapter 3) allowed evaluating at finer scale the community of each estuary highlighting the differences between the estuaries of Bilbao and Urdaibai and reinforcing the achieved conclusions in Chapter 1 about the unsuccessful establishment of *O. davisae, P. marinus* and *C. aquaedulcis* in the estuary of Urdaibai.

Moreover, the results shown in Chapter 3 helped to understand the factors behind the higher marginality of *A. tonsa* in the estuary of Urdaibai, denoting that in the estuary of Urdaibai the differences in the realized ecological niche might not be induced by abiotic but mainly by biotic constraints (Guisan et al. 2014). Those results are in agreement with the results observed in

Chapter 2. Before the occurrence of NIS, the indigenous species *A. bifilosa* was already the dominant brackish copepod inhabiting the estuary of Urdaibai (Uriarte et al. 2016; Villate et al. 2018) and likely limited the spatial and temporal expansion of the newly established congeneric NIS *A. tonsa*. In contrast, in the estuary of Bilbao, there was no a zooplankton brackish community at the beginning of the study as a result of the historical pollution of the system (Villate et al. 2004), and brackish waters were initially occupied by neritic species in low number. Therefore, the sequential arrival and establishment of numerically abundant populations of NIS and OES during the study period led to the restoration of the brackish copepod community in this estuary as a result of the ongoing plan of rehabilitation.

The different success of colonization of the three NIS in each estuary determined their impact in native zooplankton community in terms of total zooplankton and copepod abundance, community structure and diversity, and spatial and temporal distribution of native populations (Chapter 1, 2 and 3). At congeneric species level, Aravena et al. (2009) reported the seaward displacement of the neritic species *A. clausi* by *A. tonsa* from the period 1998-2002 to the period 2003-2005, and this study showed that such displacement had not been enhanced by the increase of *A. tonsa* density in the inner estuary during the last years. A similar effect was observed with *O. davisae*, which heavily reduced the presence of the neritic species of similar size and behaviour *O. nana*, likely due to the competitive interaction enhanced by their seasonal overlap. The displacement of *O. nana* by *O. davisae* has been also observed in other invaded areas (Isinibilir et al. 2016; Zagami et al. 2018) and can be attributed to the competitive superiority conferred by the higher salinity tolerance, lower body density, lower sinking speed and a higher swimming speed of *O. davisae* in comparison to *O. nana* (Isinibilir et al. 2016). In the estuary of Urdaibai, the introduction of *A. tonsa* did not cause the disappearance or heavy decrease of *A. bifilosa*, but spatial and temporal segregation of these two species occurred, as in other European estuaries (Baretta and Malschaert 1988; Soetaert and Van Rijswijk 1993; David et al. 2007). *A. tonsa* was restricted to summer months and to the innermost site of the estuary, while the annual peak of *A. bifilosa* moved to two month earlier in time, from summer to spring, and its spatial distribution skewed toward higher salinity.

However, effects on abundance and realized niches were not limited to congeneric species, and niche overlap results of Chapter 3 revealed that *A. tonsa* and *O. davisae* niches overlapped with those of most of the neritic copepods, the overlap being higher initially, just after the successful colonization of the system by the NIS, than after a long time of cohabitation. The highest overlap was not only due to the salinity gradient, but also due to the seasonal coincidence as in the case of *A. tonsa* and *O. davisae* with PCPC, the dominant neritic taxa in the warm period, whilst no change was observed for *O. davisae* with the cold-affinity neritic species *T. longicornis* and *Calocalanus* sp. (Uriarte et al. 2016; Barroeta et al. 2020). In the estuary of Urdaibai, no niche shifts or changes in niche overlap was observed, likely due to the fact that neritic and brackish assemblages already coexist before the apparition of NIS.

Regarding zooplankton community structure, the effect of NIS on the copepod assemblage and the overall zooplankton differed between estuaries and between salinity sites within estuaries as can be seen in Chapter 2. Strongest changes were observed in the inner estuary of Bilbao, where the arrival and sudden increase of *A. tonsa* and *O. davisae* caused a noticeable decrease of alpha diversity, as happen when a species become dominant (Richirt et al. 2019). The later introduction of *P. marinus* and the OES, mainly *C. aquaedulcis*, had the opposite effect and consequently alpha diversity increased again. This progressive incorporation of new species of brackish affinity that became abundant and dominant in the inner estuary had also an evident effect on the beta diversity, because the recovery of a brackish assemblage, clearly segregated from the neritic assemblage, in the estuary of Bilbao increased greatly species heterogeneity within the system. NIS and OES introduction also increased the gamma diversity in copepods, but the diversity of copepods in the entire estuary was always more influenced by neritic species than by brackish species, as it was also found in the estuary of Urdaibai, because the rest of parts of the estuary maintain the species pool (Bonecker et al. 2013). In addition, noticeable changes in the density of some neritic taxa were also observed, indeed, the general increasing trend of appendicularians and larvae of gastropods and bivalves (Chapter 1) corroborated the positive effect of improving environmental conditions on the zooplankton of this system, as we can infer from the changes of dissolved oxygen during the study period (Villate et al. 2013; Iriarte et al. 2016). These results were consistent with the displacement of the habitat centroid position that was observed in Chapter 3. In the estuary of Urdaibai, unlike in the estuary of Bilbao, NIS had a weak impact in the interannual variations of the zooplankton community structure, which were mainly driven by neritic copepods such as PCPC-calanus, *O. nana* and *E. acutifron*. Among brackish copepods, the indigenous species *A. bifilosa* was the main contributor to the variation of zooplankton structure. In this case, as the NIS *A. tonsa* was less abundant than the indigenous species *A. bifilosa*, and shared the niche previously occupied by this species, no significant novel impact on zooplankton community that could otherwise cause ecosystem change happened (Doherty-Bone et al. 2019). It could be said that the zooplankton community of the estuary of Urdaibai was somewhat resilient to the invasion-induced disruption in terms of diversity level, the changes driven by *A. tonsa* being indirect via its impact on *A. bifilosa* dynamics (Villate et al. 2018).

NIS also affected zooplankton and copepod phenology. Main phenological changes took place in the inner estuary of Bilbao, where the initial spring maximum of copepods due to neritic species was replaced by a summer maximum caused by the new dominant NIS (Chapter 1). Once NIS colonized the estuary of Bilbao, the zooplankton of this estuary showed the typical differentiation of estuarine zooplankton in freshwater, brackish and marine neritic assemblages, with the last one clearly differentiate in a winter-spring assemblage and a summer-autumn assemblage (Chapter 3). This was a result of the rotation in the dominance or occurrence of the neritic taxa between the first and the second half of the thermal cycle in the biogeographic region where our estuaries are located (Fanjul et al. 2018). In the estuary of Urdaibai, the main phenological changes in zooplankton and copepod density also occurred in the inner estuary, but were driven by the phenological changes of *A. bifilosa*, likely induced by competitive interaction with the new species *A. tonsa* (Villate et al. 2018). In this case the maximum density of the zooplankton and copepods come earlier. The seasonal patterns of alpha diversity in zooplankton groups and copepod species at the inner estuary of Bilbao were also substantially modified by the establishment of NIS, since lowest values of alpha diversity moved from the first part of the year to the second one with the introduction of *A. tonsa* and *O. davisae*, as it has been reported in other colonized areas (Gómez-Erache et al. 2000; Frisch et al. 2006). Later, when *A. bifilosa* and *C. aquaedulcis* occurred in large numbers, the species richness and Shannon diversity of copepods increased in the first part of the year, then reflecting a more estuarine-type seasonal pattern of diversity, similar to that found in the inner zone of the estuary of Urdaibai (Villate et al. 2017). However, changes in the seasonal patterns of gamma and beta diversity in the estuary of Bilbao could not be associated to the observed changes in NIS and OES species, likely due to the higher number and evenness of zooplankton components when the entire estuary is considered instead of particular salinity habitats, which are characterized by different dominant taxa. In the estuary of Urdaibai, the main changes in the seasonal patterns of zooplankton groups' and copepod species' alpha diversity, gamma diversity and beta diversity were associated to changes in the seasonal pattern of *A. bifilosa*, and consequently to the indirect effect of *A. tonsa*.

The preliminary study of Chapter 4 took as a premise that the studied NIS likely arrived, by means of ballast water, firstly to the estuary of Bilbao, then spreading to the other estuaries of the Basque coast. The estuary of Bilbao had a greatly active commercial port and gathered some environmental characteristics, i.e. high-stratified vertically stable water column in the inner part, eutrophic conditions and initial lack of brackish native copepods that make it a good habitat for NIS colonization. Similarly, the estuary of Bidasoa has an international commercial port and the water column of the inner part is well stratified, likely being an introduction way of NIS by ballast water. The absence of great port facilities in the other estuaries of Basque coast let unlikely the arrival through ballast water, the most likely means of transferring these species (Gubanova 2000), being most plausible a regional spread from the estuary of Bilbao by means of recreational boating (Hänfling et al. 2011; Geburzi and McCarthy 2018) or via natural mechanisms, since individuals of brackish species may easily be fluxed out these small estuaries by the effect of river flood and tidal exchange (Villate 1997; Islam and Tanaka 2007).

However, noticeable differences in the spread of the two main NIS, *A. tonsa* and *O. davisae,* to the other studied estuaries was found. While *O. davisae* was recorded in all them *A. tonsa* was not found in the estuaries of Oria and Bidasoa. This suggested that both species did not have a parallel spreading pathway among the estuaries of the Basque coast, since no inherent constraints for the establishment of *A. tonsa* could be argued by the reasons discussed in Chapter 4. Seasonally, unlike in the estuary of Bilbao, *A. tonsa* was constrained to the summer months in the estuary of Urdaibai (Barroeta et al. 2020), where the population likely recovers through the hatching of resting eggs. This seasonal limitation was related to the previous presence of the native congeneric species *A. bifilosa*. The presence of these both species in the estuary of Plentzia, with similar densities in summer to those found in the estuary of Urdaibai, suggests that the result of the interaction between both species in the estuary might be similar to that observed in the estuary of Urdaibai. This is also supported by the fact that both systems have similar features, i.e. small, shallow, with low stratification levels and high tidal exchange, which also serve to explain the unsuccessful establishment of *O. davisae* in both of them. *O. davisae* was observed, however, in the estuary of Oria and Bidasoa, in moderate densities in the first one and in high densities in the last one. Stratification was again the best explanatory factor accounting for such differences in *O. davisae* abundance, since the estuary of Oria had moderate stratification levels, and relatively low tidal exchange due to morphological features that limit the upward advance of the marine front (San Vicente et al. 1988), whilst the estuary of Bidasoa had higher stratification, lowest tidal exchange and geomorphological features that favor the retention of fresh water within the system. The lack of *A. tonsa* in both estuaries is hypothesized to be likely due to difficulties to arrive rather than to environmental constraints to colonize the estuaries of Oria and Bidasoa, which show good environmental quality (Solaun et al. 2018; Provincial Council of Gipuzkoa 2020) and suitable brackish habitats still occupied by the native species *A. bifilosa,* as in the estuaries of Urdaibai and Plentzia.

GENERAL CONCLUSIONS

Differences in NIS and OES establishment on the estuaries of Bilbao and Urdaibai

- The NIS *A. tonsa*, *P. marinus* and *O. davisae* colonized successfully the estuary of Bilbao, but only *A. tonsa* had success in the estuary of Urdaibai.
- *A. tonsa* and *O. davisae* reached high densities and became dominant in the inner zone of the estuary of Bilbao, and spread seawards until drop suddenly in waters around 35 salinity, whereas in the estuary of Urdaibai *A. tonsa* was more restricted to the innermost site and *O. davisae* occurred very sporadically and in very low density.
- *P. marinus* also showed highest density in the inner estuary of Bilbao, but had a more even distribution with salinity than *A. tonsa,* and *O. davisae.*
- The OES *C. aquaedulcis* and *A. bifilosa* colonized successfully the innermost zone of the estuary of Bilbao, mainly the former one which reached high densities and became dominant occasionally.
- All the NIS showed a thermophilic behaviour and peaked in summer. In the estuary of Bilbao, *A. tonsa* expanded its seasonal distribution through the study period, *O. davisae* showed a seasonal distribution more skewed towards autumn initially but moved towards summer in the last period of the study, and *P. marinus* peaked from June to September depending on the year.
- *C. aquaedulcis* and *A. bifilosa*, however, were not so thermophilic, and once colonized the system both peaked in spring in the estuary of Bilbao.

Differences in estuaries properties affecting the colonization success

- In the estuary of Bilbao, the higher stratification and stability of the water column, the large salinity intrusion zone, the high eutrophication and moderate pollution, and the absence of a brackish community favoured the colonization of all the arriving NIS.
- In contrast, the estuary of Urdaibai showed unfavourable conditions for the establishment of *P. marinus* and *O. davisae* due to the higher turbulence/instability and lower stratification of the water column induced by wind and tides, and the existence of a well-developed native brackish community dominated by *A. bifilosa* likely constrained the spatial and temporal distribution of *A. tonsa* by competence.

Impact of NIS and OES in zooplankton community

- In the estuary of Bilbao NIS, mainly *A. tonsa* and *O. davisae*, and OES, mainly *C. aquaedulcis,* contributed strongly to the increase of copepods and total zooplankton abundance in the inner estuary, which in this way reached densities similar to those of the outer estuary. In contrast, in the estuary of Urdaibai, the quantitative impact of NIS in total zooplankton and copepods was negligible.
- In the estuary of Bilbao, *A. tonsa* replaced the neritic *A. clausi* as the dominant species, and *O. davisae* heavily reduced the presence of *O. nana* in the inner zone. The competitive pressure was higher in the *Oithona* congeneric due to their larger seasonal overlap.
- The niche overlap of *A. tonsa* and *O. davisae* with congeneric species and most of the neritic copepods diminished through the study period, likely due to the relaxation of the competitiveness with the time of cohabitation.
- In the estuary of Urdaibai, the contribution of the indigenous brackish species *A. bifilosa* to the changes of zooplankton abundance was always higher than that of *A. tonsa*, and the contribution of *A. tonsa* decreased more drastically with increasing salinity than that of *A. bifilosa*. However, the movement of the annual maximum of *A. bifilosa* from summer to

spring, two months earlier in time, was attributed to the competitive pressure of *A. tonsa* in summer.

- In the estuary of Bilbao NIS produced phenological changes in the zooplankton and copepods of the inner part, where the initial spring maximum of copepods due to neritic species was replaced by a summer maximum caused by the NIS. In the estuary of Urdaibai the phenological changes in zooplankton and copepods were due to changes in the seasonal pattern of *A. bifilosa*, attributed to the competence of *A. tonsa.*
- The successful colonization of the unsaturated low salinity habitats of the estuary of Bilbao by the NIS *A. tonsa* and *O. davisae* and by the OES *C. aquaedulcis* allowed the establishment of a true brackish community spatially segregated from the neritic one, and consequently a notable increase of beta diversity in the system due to the increase of compositional differences between the inner and the outer estuary.
- In the inner community of the estuary of Bilbao, alpha and gamma diversity decreased strongly when *A. tonsa* and *O. davisae* became highly dominant, but increased again with the arrival of *A. bifilosa*, *C. aquaedulcis* and *P. marinus*.
- The seasonal patterns of alpha and gamma diversity were also modified by both NIS and OES, which caused an increase of diversity in the first half of the year due mainly to the contribution of *C. aquaedulcis*, and a decrease in the second half of the year due to the dominance of *A. tonsa* and *O. davisae* in summer-autumn.
- In the estuary of Urdaibai, alpha, gamma and beta diversities were affected by *A. tonsa* in an indirect way by assuming that this species was responsible for the observed changes in the seasonal and spatial distribution of *A. bifilosa*, which was really the main driver of community diversity changes in this estuary.

The expansion of NIS to other estuaries

- Apart from the estuaries of Bilbao and Urdaibai, *A. tonsa* was found in the estuary of Plentzia but not in the estuaries of Oria and Bidasoa. *O. davisae* was found in all the three new estuaries analysed and *P. marinus* in none of them.
- The preliminary premise that supposed that the studied NIS colonized first the estuary of Bilbao by ballast water and then expanded to the other estuaries was put on doubt after observing the different expansion pattern of *A. tonsa* and *O. davisae* in the other estuaries of the Basque coast, which suggested no parallel dispersal pathways. Molecular approaches are proposed to clarify the routes of NIS dispersion across Basque coast estuaries
- Results on the presence/absence and abundance differences of NIS and native brackish copepods point at the importance of chance of arrival, and both inherent system and species features, in determining the success of colonization.

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APPENDIXES

Appendix 1: List of the abbreviations and its correspondent name used through the thesis.

Holoplankton		Meroplankton	
Abbreviation	Taxa	Abbreviation	Taxa
Abi	*Acartia bifilosa*	Asc	Ascidian larvae
Acl	*Acartia clausi*	Biv	Bivalve larvae
Adi	*Acartia discaudata*	Bra	Brachiura larvae
Ama	*Acartia margalefi*	Car	Caridea larvae
App	Appendicularians	Cir	Cirripede larvae
Ato	*Acartia tonsa*	Cyp	Cyphonaute larvae
Cala	*Calanus* sp.	Dec	Decapod larvae
Calo	*Calocalanus* sp.	Ech	Echinoderm larvae
Can	*Candacia* sp.	Gam	Gammaridae
Caq	*Calanipeda aquaedulcis*	Gas	Gastropod larvae
Cen	*Centrophages* sp.	Gna	Gnathiidae
Cha	Chaetognaths	Har	Harpacticoids
Cla	Cladocerans	Iso	Isopods
Clau	*Clausocalanus* sp.	Mys	Mysids
Cop	Copepods	Nem	Nematoda
Cor	Corynidae	Pol	Polychaeta larvae
Cty	*Centrophages typicus*	Rha	*Rhithropanopeus harrisii*
Dan	*Ditrichocorycaeus anglicus*		
Dol	Doliolids		
Eac	*Euterpina acutifrons*		
For	Foraminifera		
Fwcop	Fresh water copepods		
Ict	Ichthyoplankton		
Med	Medusae		
Mic	*Microsetella* sp.		
Msl	*Mesopodosis slabberi*		
Mug	*Muggiaea* sp.		
Nsc	*Noctiluca scintillans*		
Oda	*Oithona davisae*		
Ona	*Oithona nana*		
Onc	*Oncaea media*		
Opl	*Oithona plumifera*		
Osi	*Oithona similis*		
Ost	Ostracoda larvae		
Para	*Paracalanus* sp.		
Pav	*Penilia avirostris*		
PCPC	PCPC-calanus assemblage		
Pde	*Pirimela denticulata*		
Pgr	*Paracartia granii*		
Pma	*Pseudodiaptomus marinus*		
Pod	*Podon* sp.		
Sag	*Sagitta* sp.		
Sip	Siphonophores		
Tlo	*Temora longicornis*		
Tst	*Temora stylifera*		