Universidade do Algarve

Faculdade de Ciências do Mar e do Ambiente

The life cycle of *Engraulis encrasicolus sensu lato* in the Guadiana estuary: Ecology, Ecohydrology and Biology

Doutoramento em Ecologia, especialidade Ecologia das Populações

Pedro Miguel Coutinho Victorino Borges Morais

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Aos meus pais

“Não faças do pensamento blocos de duro cimento”

(inscrição numa parede dum prédio no Porto)

NOME: Pedro Miguel Coutinho Victorino Borges Morais

FACULDADE: Faculdade de Ciências do Mar e do Ambiente

ORIENTADOR: Maria Alexandra Anica Teodósio Chícharo

CO-ORIENTADOR: Luís Manuel Zambujal Chícharo

DATA: 07/2007

TÍTULO DA TESE: O ciclo de vida de *Engraulis encrasicolus sensu lato* no estuário do Guadiana: Ecologia, Ecohidrologia e Biologia

RESUMO: O estuário do Guadiana é o segundo estuário português mais bem conservado, mas também o segundo mais vulnerável aos impactes antropogénicos. As barragens são uma das principais fontes de impactes na bacia do Guadiana. Uma visão holística é então necessária para a melhor gestão deste ecossistema, a qual tem vindo a ser alcançada com uma abordagem ecohidrológica. Os objectivos principais deste trabalho foram compreender a dinâmica do estuário do Guadiana e da zona costeira adjacente, durante o primeiro ano de enchimento da barragem de Alqueva; estudar o ciclo de vida do biqueirão *Engraulis encrasicolus sensu lato* neste estuário e integrar a informação da abundância e distribuição dos ovos num modelo hidrodinâmico do estuário, para a criação duma ferramenta de gestão ecohidrológica. Durante o enchimento desta barragem, o caudal foi preponderante na definição das alterações abióticas e bióticas na área de estudo. As principais mudanças foram o deslocamento do máximo de turbidez do estuário para montante, a alteração da dinâmica de nutrientes nas secções médias e altas do estuário e o aumento do fornecimento de nutrientes ao estuário para níveis apenas registados durante anos hidrológicos com caudais elevados. Uma descarga não controlada da barragem de Alqueva provocou a advecção dos estados larvares de biqueirão para a zona costeira. A desova deste peixe é feita no interior do estuário por espécimens residentes. Em condições de caudal reduzido ($Q< 3.2 \pm 0.1\ m^3.s^{-1}$), a transição entre o baixo e o médio estuário constitui o local ideal para a desova. A zona de transição entre o médio e o alto estuário é o local de desenvolvimento das larvas e juvenis. Os adultos encontram-se predominantemente a jusante destes locais. Biqueirões adultos foram capturados na zona terminal do estuário e na pluma apenas durante períodos de cheia ($Q> 3000\ m^3.s^{-1}$). O biqueirão presente no estuário do Guadiana era geneticamente idêntico ao da zona costeira adjacente, podendo ser a espécie que foi recentemente descrita- *Engraulis albidus*. As análises isotópicas de carbono e oxigénio aos otólitos indicaram que são duas populações. A concentração de Sr, ao longo dos otólitos de espécimens da população costeira, indicou que esta é formada maioritariamente por indivíduos provenientes de estuários. O modelo ecohidrológico permitiu avaliar os impactos sobre a permanência dos ictioplanctontes no estuário, quando o caudal é gerido de forma a prevenir ou mitigar situações de eutrofização no alto estuário do Guadiana. Dos vários perfis de caudal testados, apenas os de caudais máximos de $20\ m^3.s^{-1}$ e $50\ m^3.s^{-1}$ não comprometem a presença de ictioplanctontes no interior do estuário. Esta ferramenta tem o potencial para ser aplicada noutros ecossistemas e deve ser desenvolvida e melhorada.

PALAVRAS-CHAVE: biqueirão, ciclo de vida, estuário do Guadiana, caudal, barragem de Alqueva, modelação hidrodinâmica.

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TÍTULO DA TESE EM INGLÊS: The life cycle of *Engraulis encrasicolus sensu lato* in the Guadiana estuary: Ecology, Ecohydrology and Biology

ABSTRACT: The Guadiana estuary is the second more ecologically preserved portuguese estuary, but also the second most vulnerable to antropogenic impacts. Dams are one of the main sources of impacts to the Guadiana basin. An ecohydrological approach is needed to efficiently manage this ecosystem. The main objectives of this work were to understand the dynamics of the Guadiana estuary and adjacent coastal area during the filling of the Alqueva dam; to study the life cycle of anchovy *Engraulis encrasicolus sensu lato* in this estuary; and to integrate the information on the abundance and distribution of eggs in a hydrodynamic model of the estuary, in order to create an ecohydrological management tool. During the filling of the dam, the river flow was preponderant in the definition of abiotic and biotic changes. An upstream displacement of the estuarine turbidity maximum region, changes in nutrient stoichiometry in the middle and upper estuary and the increase of nutrient load to the estuary, to levels only reach during years with high inflow, were observed. One uncontrolled discharge from the Alqueva dam caused the advection of anchovy larval stages to the coast. The spawning of anchovy is made by resident specimens. During periods of low inflow ($Q< 3.2 \pm 0.1$ $m^3.s^{-1}$), the transition between the low and middle estuary was the ideal location for spawning. The transition area between the middle and upper estuary was the nursery area for larvae and juveniles, while the adults were predominantly downstream. Adults of anchovy were captured in the terminal area of the estuary and in the plume, only during flood events ($Q> 3000$ $m^3.s^{-1}$). The anchovies from the Guadiana estuary were genetically identical to those from the coastal area, being probably the new anchovy species- *Engraulis albidus*. Carbon and oxygen isotopic analysis to the otoliths revealed that they were two populations. The concentration of Sr, along the otoliths of coastal specimens, showed that the majority had migrated from an estuary. The ecohydrological model allowed evaluating the impacts on the permanence of ichthyoplankton in the estuary, when the river flow is managed to prevent or mitigate eutrophication in the upper Guadiana estuary. Between the tested river discharge scenarios, those with maximum discharges of 20 $m^3.s^{-1}$ and 50 $m^3.s^{-1}$ do not compromise the presence of ichthyoplankton inside the estuary. This tool has the potential to be applied in other ecosystems and must be developed and improved.

KEY-WORDS: anchovy, life cycle, Guadiana estuary, river flow, Alqueva dam, hydrodynamic model.

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FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Escreve na areia aquilo que dás, grava sobre a rocha aquilo que recebes.

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Chapters **Page**

The life cycle of *Engraulis encrasicolus sensu lato* in the Guadiana estuary: Ecology, Ecohydrology and Biology

In Portugal, the construction of the Alqueva dam, at 150 km from the Guadiana river mouth (SE-Portugal/SW-Spain), was theme of political debate for almost 60 years and centre of environmental attention, particularly during the last decade. The scientific studies on the environmental characterization of the Guadiana basin are few and those regarding the estuary are even less, sparse and mainly restricted to the last 15 years. The creation of the University of the Algarve in 1979 was the trigger that slowly increased the scientific knowledge about the Guadiana estuary.

The holistic study of the lower Guadiana basin started 10 years ago with the support of UNESCO, through the International Hydrological Program- IHP-V. This holistic approach is designated as Ecohydrology, a discipline that aims to study the functional inter-relations between hydrology and biota at the catchment scale. The Guadiana estuary is recognised as a pioneer site in the development of this concept in estuaries and adjacent coastal areas. UNESCO granted the University of the Algarve, through Prof. Luís Chícharo and Prof. Alexandra Chícharo, the responsibility to continue to develop this concept in the Guadiana estuary, by continuing to pursue the scientific knowledge of this ecosystem and to promote its communication with the remaining stakeholders. Ultimately, these continuous tasks will be accomplishment with the creation of a demosite in the Guadiana estuary.

It was under this background that this thesis was brought up in March 2002, one month after the beginning of the fill of the Alqueva dam. Previously to this work, one planktivorous fish inhabiting in the estuary, identified as *Engraulis encrasicolus* - European anchovy, was recognised as a potential key species to identify meaningful changes in the catchment of the Guadiana estuary. Hypothetically, these changes could be noticed on the abundance of the larval stages of anchovy and on the composition of the landings of fish captured in the vicinity coastal area. A hypothesis on the anchovy life cycle was proposed previously to this work, however some doubts subsisted. Moreover, a new anchovy species was identified in the south coast of France, *Engraulis albidus* sp. nov. - White anchovy, described to inhabit in estuaries, coastal embayments and coastal areas. These facts raised some doubts about the life cycle of anchovy in the Guadiana estuary and on which anchovy species is present, *E. encrasicolus* or *E. albidus*.

The main objectives of this work were to understand the dynamics of the Guadiana estuary and adjacent coastal area during the filling of the Alqueva dam; to study the life cycle of anchovy *Engraulis encrasicolus sensu lato* in this estuary; and to integrate the information on the abundance and distribution of eggs in a hydrodynamic model of the estuary, in order to create an ecohydrological management tool. These topics are presented in chapters 2 to 4 and summarised in Chapter 5.

The Chapter 1 ("*Impacts caused by damming and watershed development in an Iberian basin (SW-Europe)- focus on the Guadiana estuary*") aims to provide a holistic background of the lower Guadiana basin, highlighting its ecological status and constrains.

After this introductory chapter, this thesis uses anchovy as the cement that holds three distinct, but complementary, topic objectives- "Guadiana estuary", "Ecohydrology" and "Biology and Ecology of anchovy" (Fig. 1).

The Chapter 2 ("River inflow as an ecological structuring agent in the Guadiana estuary and adjacent coastal area (SW-Iberia), during the filling of the Alqueva dam") intends to investigate the changes that occurred in the Guadiana estuary during the first year of the Alqueva dam filling, and in which way it may have affected anchovy larval stages.

FIG. 1 Diagram of anchovy life cycle, the cement that holds the distinct objectives pursued in this thesis.

The Chapter 3 ("*The life cycle of* Engraulis encrasicolus sensu lato *in the Guadiana estuary*") uses a multidisciplinary approach to clarify the life cycle of anchovy in the Guadiana estuary, and aspects regarding its biology and ecology, such as: spawning and nursery areas, migration patterns and which anchovy species is actually present in the Guadiana estuary and in the adjacent coastal area.

The Chapter 4 ("*Anchovy eggs as an assessment tool for estuarine ecohydrological management*") aims to create an ecohydrological tool by merging the abundance and distribution of anchovy eggs in a hydrodynamic model of the Guadiana estuary, in order to manage river flow without compromising the presence of anchovy larval stages inside the estuary.

The last chapter, Chapter 5, summarizes the major achievements of this thesis.

Furthermore, samples collected and analysed for this Ph.D. allowed publishing three research articles in an international peer-reviewed journal, included in the Citation Index:

1) *Inter-annual differences of ichthyofauna structure of the Guadiana estuary and adjacent coastal area (SE Portugal/SW Spain): Before and after Alqueva dam*

construction by Chícharo MA, Chícharo L & Morais P (2006). (Estuarine, Coastal and Shelf Science 70: 39-51);

2) *Ichthyoplankton dynamics in the Guadiana estuary and adjacent coastal area (SE-Portugal/SW-Spain)* by Faria A, Morais P & Chícharo MA (2006) (Estuarine, Coastal and Shelf Science 70: 85-97)
3) *An ecohydrology model of the Guadiana Estuary (South Portugal)* by Wolanski E, Chícharo L, Chícharo MA & Morais P (2006) (Estuarine, Coastal and Shelf Science 70: 132-143).

The first article is also an outcome of project ERIC- “The effect of river inflow changes on the ichthyofauna of Douro, Tejo and Guadiana estuaries and adjacent coastal areas. Ecological and socioeconomic predictions” (FCT/P/MAR/15263/1999). The sampling program of this project allowed collecting the majority of the anchovy specimens used for this Ph.D. thesis. The second article is based on the degree thesis of A. Faria, while the latter is the first Ecohydrological model for the Guadiana estuary.

Chapter 1

Impacts caused by damming and watershed development in an Iberian basin (SW-Europe) - focus on the Guadiana estuary

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Abstract

The Guadiana estuary (Iberian Peninsula, Europe) suffers reduced agriculture, industry and urban pressure, in opposition to other major Iberian estuaries; however this situation is changing. At 150 km from the river mouth it was constructed the Alqueva dam, one of the biggest artificial lakes in Europe that will support 110,000 ha of irrigation areas. Moreover, the estuarine margins are suffering increase human pressure. The risks that the Alqueva dam will pose to the downstream ecosystems, mainly to the estuary and adjacent coastal area, are evaluated. Other issues, as increased human desertification in the Portuguese basin, real estate pressure and the impact of the construction of a public infrastructure in the Portuguese lower estuary are analysed. Enlarged problems of sediment load reduction, water quality deterioration and shifts on the downstream fish populations are expected due to higher river flow control. Irreversible changes on estuarine margins and increased risk of pollution in the estuary will result from the multiple urban pressures. Lack of forest management and increased risk of wildfires, leading to water quality degradation and increased soil erosion, are a result of human desertification in the upper Portuguese basin. Various solutions to tackle these multiple problems have to be considered. Creating the International Natural Park of the Lower Guadiana would not be enough and tourism should not be regarded as the only way to develop such a poor region. An ecohydrological approach to the basin should be implemented, to establish scientific based solutions to help solving conflicts and testing scenarios. Ultimately, the creation of a Portuguese-Spanish Basin Management Council for the lower Guadiana, relating those that are interested in the sustainable development of the Guadiana (local populations and associations, private stakeholders (dam managers, tourism entrepreneurs, farmers), local and national authorities, ENGO’s and scientists) would be essential to produce ecological and sustainable management decisions.

1. Introduction

Estuaries are highly dynamic systems, where complex interactions linking physical, chemical, geological and biological components occur. Linking them are multiple variables such as tidal amplitude, river flow, episodic events (*e.g*. floods), local (*e.g*. rainfall, droughts) and large-scale atmospheric phenomena (*e.g*. North-Atlantic Oscillation (NAO)) and global climate change (Stein et al., 2004; Struyf et al., 2004; Trigo et al., 2004). Estuaries undergo intense anthropogenic impact from urban, agricultural and industrial runoffs along their path, but also from the disturbances that occur in the entire basin (Saiz-Salinas, 1997; Grousset et al., 1999; Cearreta et al., 2000; Suzumura et al., 2004), even if located hundreds of kilometres apart. Indeed, damming is one of such disturbances that cause meaningful deleterious changes to downstream ecosystems (Ly, 1980; Nixon, 2004).

In the Guadiana basin (SW-Iberian Peninsula, Europe) (Fig. 1) the main ecological constrains are damming (Vasconcelos et al. 2007), water abstraction from aquifers and water pollution (Sanz, 1999). Even so, the estuary is one of the best preserved estuaries of the Iberian Peninsula, being ranked as the second Portuguese estuary with less anthropogenic pressure (0.21); however, it is the second most vulnerable according to its natural characteristics (0.71) (Vasconcelos et al., 2007) (Fig. 2). Its ecological status is constantly being pressured. Nowadays, it has to deal *i*) with the impact caused by one of the major European dams, the Alqueva dam, and *ii*) with the creation of 110,000 ha of irrigation areas until 2015 in the surroundings of this dam and *iii*) with the destruction of vast natural areas along the estuarine margins that will be converted to tourist resorts.

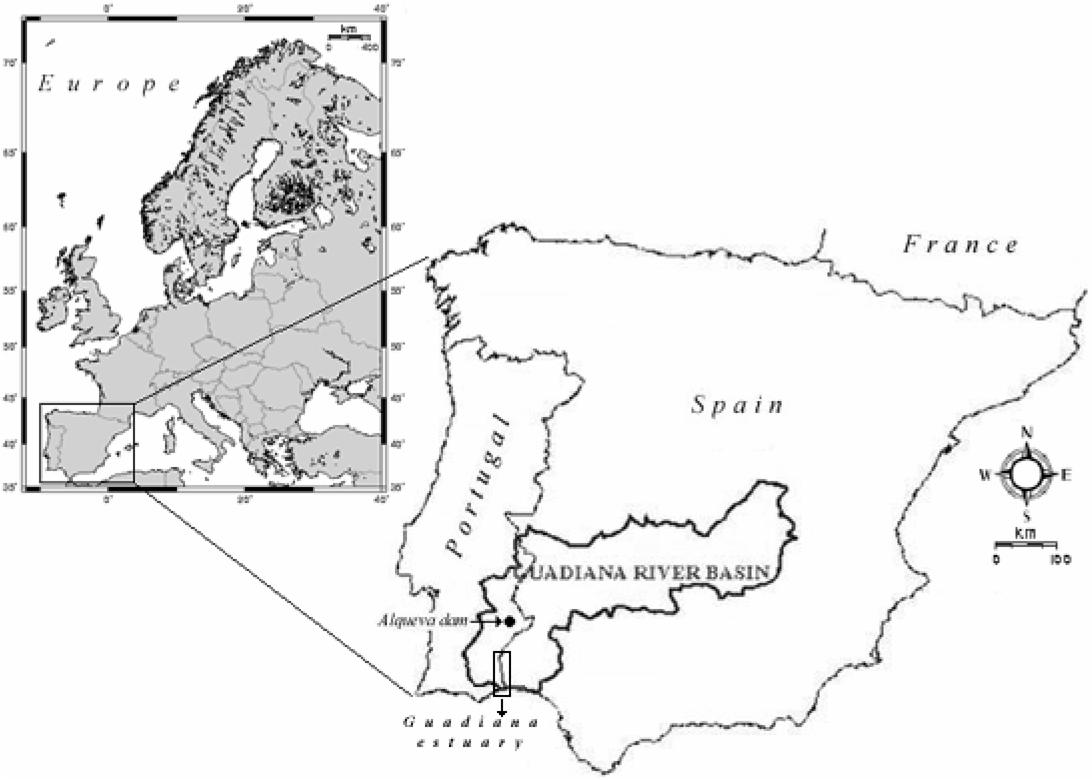

Fig. 1 Geographical location of the Guadiana river basin, Alqueva dam and Guadiana estuary in the Iberian Peninsula (Europe). Map of Europe modified from http://www.aquarius.geomar.de.

One of the solutions to help solving or preventing ecological constrains to the estuary should be based on an ecohydrological approach of the ecosystem. It consists on the study of functional inter-relationships between hydrology and biota at the catchment scale, in order to propose sustainable water management practises (Zalewski et al., 1997). Successful approaches were already achieved in worldwide aquatic ecosystems (Hickley et al., 2004; Wagner-Łotkowska et al., 2004; Trepel & Kieckbusch, 2005). This concept was formally extended to estuaries and coastal areas recently (Wolanski et al., 2004); however it is applied in the Guadiana estuary for the past 10 years (Chícharo et al., 2001; Wolanski et al., 2006).

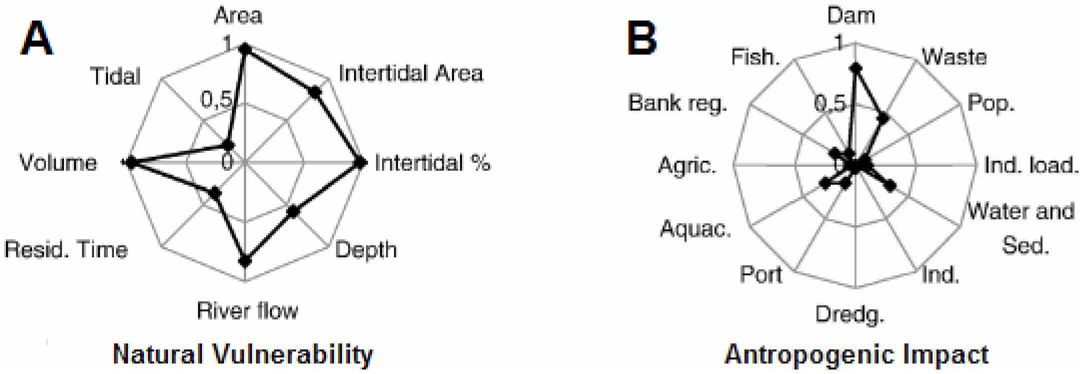

Fig. 2 Natural vulnerability and anthropogenic impact of the Guadiana estuary. Scores range between 0 (low vulnerability and impact) and 1 (high vulnerability and impact). Legend 2B: Dams, Wastewater Treatment, Population, Industrial loads, Water and Sediment Quality, Industry, Dredging, Port activities, Aquaculture, Agriculture, Bank regulation and Fishing. Modified from Vasconcelos et al. (2007).

This study aims to describe the main ecological constrains of the Guadiana basin, focusing on the current threats that the Guadiana estuary and the adjacent coastal area has to deal in the present and future. The impacts caused by damming to these ecosystems are described, namely those related with sediment load reduction, water quantity and water quality decline and with the impact on fish populations. Preventive and mitigation measures are presented.

2. The Guadiana basin (SW-Iberian Peninsula, Europe)

The Guadiana basin is the 4th largest in the Iberian Peninsula and is shared by Portugal and Spain (Fig. 1). The Guadiana river rises in Lagunas de Ruidera (Spain) at 1700 m and drains in the Atlantic Ocean, near the city of Vila Real de Santo António (Portugal). It has an area of 66,889 km^2 (55,364 km^2- Spain; 11,525 km^2- Portugal). The river has a total length of 810 km, 550 km in Spain and 150 km in Portugal; the remaining forms the border between these countries. The estuary has its tidal limit in Mértola, 70 km away from the coast. The last 50 km form the southern border of Portugal and Spain. The estuary is mesotidal and tide amplitudes range from 1.3 to 3.5 m, it occupies an area of 22 km^2 and has an average depth of 6.5 m. Annual average temperature varies between 14 and 18 ºC. Rainfall is very irregular between years; 80% is concentrated during autumn and winter, while summers are very dry, and it ranges between 561 and 600 mm in the Portuguese basin. River flow has a clear variability between and within years, since the majority of the basin is under the influence of Mediterranean climate. During dry years the average annual river flow oscillate between 8 and 63 $m^3.s^{-1}$; in a regular year it averages between 170 and 190 $m^3.s^{-1}$ and between 412 and 463 $m^3.s^{-1}$ in a humid year (Bettencourt et al., 2003).

There are 1.92 million inhabitants in the entire basin, 88% of which in Spain (Euronatura & IIDMA, 2003). Along the estuary, the human presence is sparse, mainly in Spain. In the low estuary there are the two main population agglomerates, Vila Real de Santo António (Portugal) and Ayamonte (Spain), with an approximate resident population of 13,880 and 17,500 inhabitants, respectively. Direct sources of pollution result from the runoffs and the partly treated or untreated sewages of these cities, of other small villages and of two aquaculture enterprises. Diffusive pollution from agriculture, mainly of orange groves in mid estuarine Spanish margins, and from decommissioned mines runoffs in upper estuarine regions also occurs. New tourist resorts are planned for the middle estuary margins and one is almost constructed; therefore they will have to be included in the map of pollution sources. These infrastructures raise huge expectations by the local populations, to improve the local economy, and great concern to regional non-governmental environmental organizations.

There are three ecological constrains in the Guadiana river basin. The first is the aquifers overexploitation in the upper basin, which supports extensive irrigation areas. The second ecological constrain is the diffusive pollution originated in these areas and from industries, uncomissioned mines, untreated sewages, sewage treatment plants and landfills. The aquifer overexploitation in the upper basin is trying to be mitigated with sustainable water use and with water transfer from the Tagus basin; however this led to the introduction of alien fish species in the Guadiana basin (Fornés et al., 2000).

The third main ecological constrain of the Guadiana basin is the intense damming. Initially, dams were essential to maintain water supply during the sporadic, but sometimes long draught periods. The first dams of this basin, and also of the Iberian Peninsula, were built by the Romans in II a.C.- Cornalvo (10 hm^3) and Proserpina (4 hm^3). After, several others were built and until 1956, when the Cijara dam (1670 hm^3) was built, the total amount of water stored in reservoirs was *ca*. 115 hm^3. In 1964, this value increased to *ca*. 3850 hm^3, increasing slightly until 1988, when it raised again to *ca*. 7540 hm^3 and to *ca*. 8575 hm^3 in 1990. Today, after the construction of the Alqueva dam in February 2002, *ca*. 12730 hm^3 of water can be stored in the 1,824 dams of the Guadiana basin. The average total flow of the Guadiana is 4400 hm^3 (Dias & Ferreira, 2001), however the major 86 dams retain *ca*. 150% of the average annual rainfall (UNEP, 2006).

The intense damming of the basin raises great concern on water availability, mainly along the Portuguese-Spanish border (Brandão & Rodrigues, 2000). The amount of water drained to the Portuguese basin decreased *ca*. 60% in the last 30 years and the quality is rather poor. Of the total needs for irrigation and domestic water supply, 81% and 75% are consumed and generated in Spain, respectively. The land use is predominantly rural and irrigation accounts for 93% of water consumption in Spain. Here, non-sustainable agriculture practises cause conflicts between environmental sustainability and socioeconomic interests (Sanz, 1999, Fornés et al., 2000).

The impact caused by the construction of the Alqueva dam in the upper Portuguese basin is now the focus of concern. The Alqueva dam, and the subsidiary Pedrógão dam, will control the river flow before water reaches the estuary, since they are the last in the river main course. The Alqueva dam is located approximately at 150 km from the river mouth (Fig. 1). It forms at its maximum capacity (152 m level) one of the biggest artificial lakes in Europe (Hellsten, 2003), with an area of 250 km^2 (63 km^2 in Spain), a perimeter of approximately 1,000 km, a total capacity of 4,150 hm^3 and an useful capacity of 3,150 hm^3. The objectives of the Alqueva dam construction, and of those that constitute the Alqueva project, were to reinforce the capacity of hydroelectrical production, to develop tourism, to promote the regional employment market, to organize intervention in environmental and patrimony domains, to fight physical desertification and climate change, to modify the agriculture model of south Portugal and to regularize river flow.

3. Damming and shifts on downstream ecosystems

3.1. Water quantity and sediment load

Before intensively damming the Guadiana basin, ore exploitation (late 19th and early 20th centuries) and the "wheat campaign" (*ca*. 1938-1945), which occurred in the upper Portuguese basin, increased the sediment transported to the coast. Afterwards, damming had an opposite effect, causing a decline on the quantity of sediment exported to downstream regions, originating deleterious effects on coastal ecosystems (Fig. 3).

These contrasting situations drastically changed the area of a sand bank in the river mouth- the O'Brill Bank (Dias et al., 2004). The O'Brill Bank reached its maximum area, *ca*. 6 km^2 around 1910, when mining was at its maximum. Mining needed huge amounts of timber, causing deforestation and soil erosion. However, around 1990, after intense basin damming and littoral drift interruption, the O'Brill Bank had an area of 0.7 km^2, only 11.6% of the former maximum area (González et al., 2001).

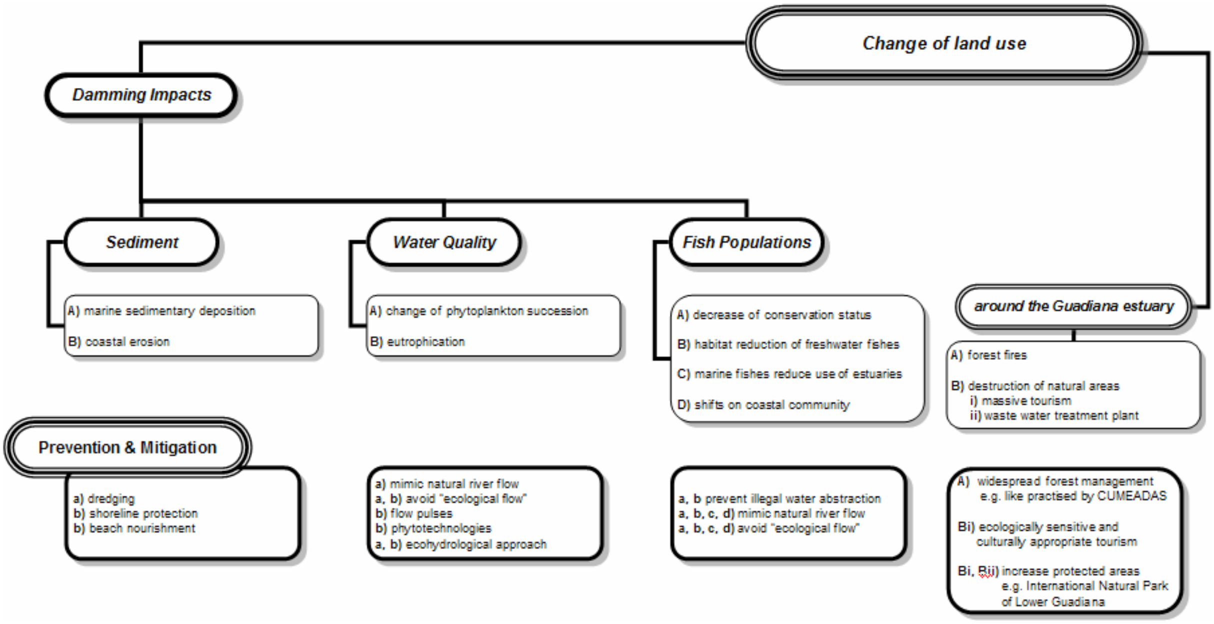

Fig. 3 Main impacts caused by damming and change of land use around the Guadiana estuary and their putative prevention and mitigation measures.

The Guadiana river mouth was highly dynamic until the construction of two jetties (1972-1974) (Bettencourt et al., 2003), that drastically changed the sediment dynamics (Dias et al., 2004). The western jetty interrupted the predominant eastward littoral drift (Lobo et al., 2002), estimated in 180,000 $m^3.year^{-1}$ (González et al., 2001). Nowadays, marine sediment deposition occurs in the river mouth due *a*) to the cumulative effect of river flow reduction, *b*) to the inherent higher flood velocities, *c*) to the predominant W and SW coastal wave regime and *d*) to the hampering effect on flooding caused by intensive damming (LNEC, 2001).

Despite marine sediment accumulation in the river mouth, there is a deficit of supply to the coast. Coastal erosion started in late 1950's in the Spanish coast (Dias & Ferreira, 2001). The sediment is retained in dams and coastal jetties, which interrupts the predominant eastwards littoral drift (Lobo et al., 2002). The Alqueva dam is expected to amplify coastal erosion (Del Río et al., 2002); however its harshness is unknown. Similar situations are known from other Portuguese (Dias et al., 2000; Veloso-Gomes et al., 2002) and worldwide coastal areas (Ly, 1980; Palanques et al., 1990; Barusseau et al., 1998; Chen & Zong, 1998).

The foreseen sea-level raise will intensify coastal erosion and recession of delta systems (Yang et al., 2001) and will cause saltwater penetration into coastal aquifers and estuaries, contaminating urban water supply and affecting agriculture production (Gornitz, 1991). Climate change will also cause a reduction on river discharge, up to 60% (Cunha et al., 2002), aggravating the negative effects of damming in coastal sedimentary dynamics.

3.2. Water quantity and water quality

The water reduction imposed by the Alqueva dam construction, in addition to the impact of all the other dams in the Guadiana basin, will have negative impacts on water quality and consequently on the biological communities of downstream areas (Fig. 3).

Phytoplankton dynamics in the upper Guadiana estuary is being study during the last decade (Rocha et al., 2002; Domingues et al., 2005). During the filling of the Alqueva dam (2002-2003), nutrient and phytoplankton dynamics changed drastically (Domingues et al., 2005). Silica concentration increased significantly, but the diatom spring bloom did not occur. Moreover, cyanobacteria abundance increased, not only during summer, as common, but also in autumn and winter. The higher incidence of cyanobacteria, which is mainly driven by long water residence time and water column stability (Paerl, 1996; Morais et al., 2003), is also associated to water degraded systems, mainly in periods of high water and atmospheric temperatures (Hickel, 1982; Douterelo et al., 2004). Several cyanobacteria species synthesize toxins (Carmichael, 1994; Feuillade, 1992), nuisance to aquatic (Pizzolon, 1996) and terrestrial communities (Rodas & Costas, 1999), strongly affecting the water quality. This would be critical in periods of consecutive years of droughts, which is not unusual in the Guadiana basin (INAG, 2004).

A long draught period was experienced, in southern Portugal, from 1990 to 1993. The draught, in combination with agricultural (high loads of nitrates, phosphates and pesticides), industrial and urban contamination, caused the blooming of an aquatic fern (*Azolla* spp.), in vast areas of the Guadiana river. Local and military authorities were involved in its harvest to minimize aquatic eutrophication after the closing of *Azolla* spp. life cycle. The singularity of this event instigated great concern among local populations, in respect to the ecological status of the Guadiana river (Baioa, 1997).

3.3. Water quantity and fish populations

Water retention in dams influences stream fish populations, estuarine ichthyofauna and coastal communities (Fig. 3). A significant reduction of river flow causes a decrease on the abundance of marine fishes that use the estuary as a spawning ground and of freshwater fishes habitat (the barbells *Barbus comiza*, *B. microcephalus*, *B. sclateri* and *B. steindachneri*) (Chícharo et al., 2006). These species are endemic to Iberian southern freshwater ecosystems and classified as threatened (SNPRCN, 1991). Other brackish and freshwater species, such as allis shad (*Alosa alosa*) and twaite shad (*Alosa fallax*), have a vulnerable status, meaning that they will be in danger if the limiting factors, over-fishing and general habitat destruction, persist (IUCN, 2001).

Five fish species present in the streams of the lower Guadiana are listed in the Annex II of Habitats Directive: lamprey (*Petromyzon marinus*), several cyprinidae (*Rutilus alburnoides*, *Cobitis maroccana* and *Anaecypris hispanica*) and sturgeon (*Acipenser sturio*). *Anaecypris hispanica* is an endemism of the Guadiana streams and is in threat of extinction (Collares-Pereira & Cowx, 2001). *Acipenser sturio* is probably extirpated from this basin; since the early 1980's that no sturgeon is caught (Almaça & Elvira, 2000). The conservation status of

Anaecypris hispanica changed drastically in few years, from abundant to threaten of extinction (Collares-Pereira et al., 1999; Salgueiro et al., 2003). Damming and water abstraction are probably the major cause of change, once it destroys or damages the habitat, interferes with reproduction opportunities, may generate polluted effluents and facilitate the introduction of exotic competitor species (Collares-Pereira et al., 2000).

Damming can also cause coastal fisheries decline. Indeed, river flow was also the factor that better explained the Guadiana coastal fisheries shifts (Erzini, 2005). In the years following significant changes of river flow, the structure of coastal community also shifts. In dry years, the landings of planktivorous fish, such as sardine and anchovy, were significantly lower. Between years of high and low inflow, sardine landings decreased, in average, 69%; while landings of carnivorous fish (*e.g.* white sea-bream, axillary sea-bream, red sea-bream and red-porgy) increased between 112% and 128% (Chícharo et al., 2003). The coupling between river flow and fishery landings and larval recruitment was also reported elsewhere (*e.g.* Ebro River- Spain (Lloret et al., 2004); Gironde estuary- France (Bergeron, 2004), Nile River- Egipt (Nixon, 2003, 2004), Pó River- Italy (Marasović, 1990)).

4. Change of land use around the Guadiana estuary

The Guadiana estuary has low agriculture, industry and urban pressure, in comparison to the most important Iberian estuaries (Tagus, Douro, Guadalquivir and Ebro estuaries) (Solé et al., 2000; Silva et al., 2004; Ferreira et al., 2004; Riba et al., 2004, Vasconcelos et al., 2007). However, this situation is about to change, not only due to the activities that occur in the distant Alqueva dam and upstream areas, but also in the surrounding estuarine areas. Here, the occurrence of forest fires and destruction of vast natural areas are now the main threats of its ecological balance (Fig. 3).

In the upper regions of the Guadiana estuary, intense mining and agriculture have caused water contamination and deterioration (Bettencourt et al., 2003). Nowadays, it can result from the lack of land use and management, a consequence of demographic regression in Portugal and Spain (Euronatura & IIDMA, 2003). The Portuguese Guadiana river basin is one of the poorest regions of the European Union. The increase of local populations' age and the decrease of manpower committed to maintain forests in proper conditions lead to an increase of the probability for the occurrence of forest fires. Wildfires increase the risk of soil nutrient depletion and erosion (Townsend & Douglas, 2000; Shakesby et al., 2003); consequently, water quality decrease (Rab, 1996; Townsend & Douglas, 2004), mainly in streams, small rivers and water reservoirs. Even so, wildfires negative impacts might be buffered in major rivers (Cornish & Binns, 1987), in this case, by the estuary.

In the middle estuary, the construction of an enormous tourist resort in Spain (north of Ayamonte), and of others that are approved for the Portuguese margin (near Almada de Ouro and Castro Marim), will destroy vast natural areas and dramatically change others. The construction of the Spanish tourist resort is being finished (Figs. 4, 5). It will host 20,000 inhabitants, lodged in 6,000 houses and in 3 luxury hotels, and equipped with 7 shopping centres, 2 golf courts and 1 marina, which undoubtedly represents a tremendous change on land and estuary use. It is located in a site without previous human pressure, near the northern limit of the Natural Reserve of Castro Marim and Vila Real de Santo António. Moreover, it is in the vicinity of a preponderant estuarine area for the development of several resident and migratory fish species, such as eels (*Anguilla anguilla*), meagres (*Argyrosomus regius*), temperate bases (*Dicentrarchus* sp.), gilt head sea breams (*Sparus aurata*) and common soles (*Solea vulgaris*). These infrastructures will also increase the threat of pollution, as well as its dissemination and bioaccumulation in an estuary classified as low polluted (Ruiz, 2001).

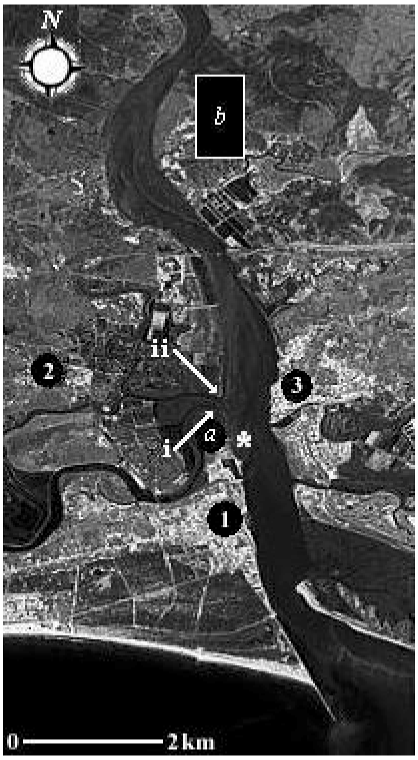

Fig. 4 Aerial photograph of the lower section of the Guadiana estuary.

Legend: 1- Vila Real de Santo António; 2- Castro Marim; 3- Ayamonte; a- Rato saltmarsh; b- tourist resort; i- Carrasqueira creek; ii- Lezíria creek; *- possible location for water sewage treatment outflow.

Photograph modified from http://mapmachine.nationalgeographic.com

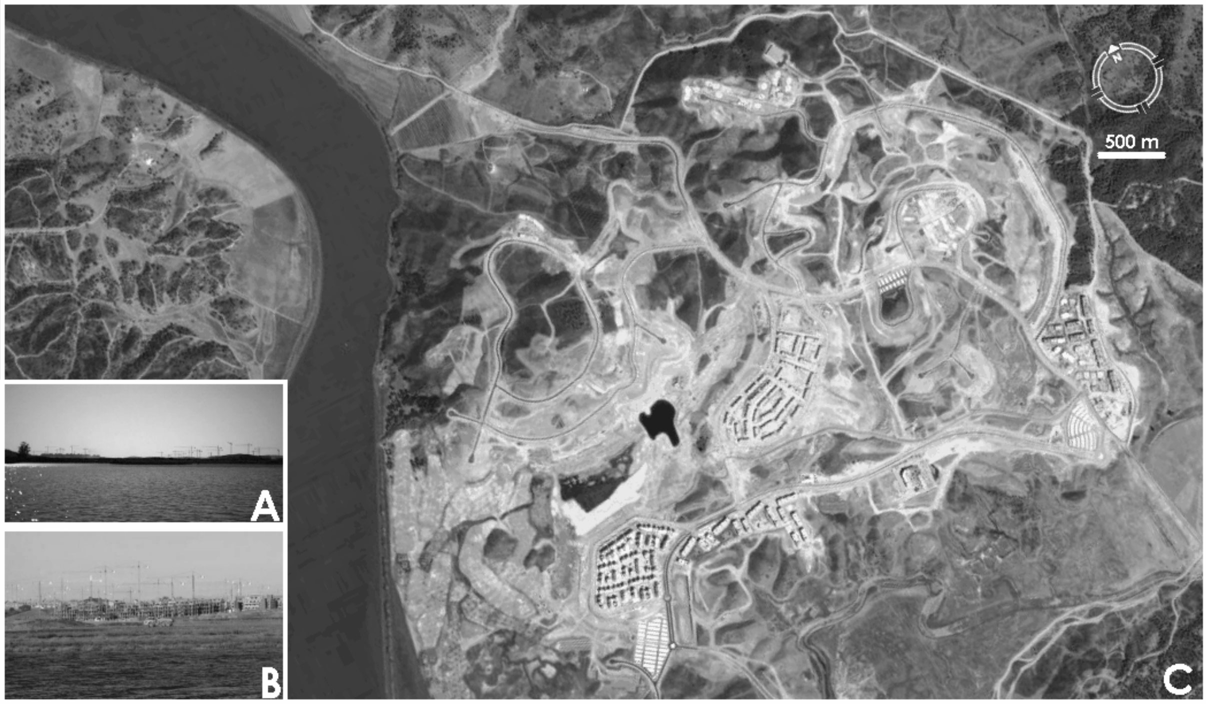

Fig. 5 Photographs of a tourist resort that is being constructed in the Spanish middle Guadiana estuary.

Photograph 4C modified from Google Earth.

In the low estuary, a sewage treatment plant, with tertiary treatment, will be built north of Vila Real de Santo António (Portugal) to fulfill the requirements of the residual water discharge legislation (Fig. 4). This station will treat sewages that are currently discharged untreated to the environment and substitute several sewage treatment plants with inappropriate functioning (PROCESL, 2001). This is, undoubtedly, an important environmental achievement, since it will reduce the amount of organic matter, nutrients and microbial pathogens to the environment; however it will be located in Rato salt marsh. This salt marsh is at 6 km from the estuary mouth and at the entrance of two creeks, "Esteiro da Carrasqueira" and "Esteiro da Lezíria", in the Natural Reserve of Castro Marim and Vila Real de Santo António, an area of high terrestrial and estuarine biological diversity. This Natural Reserve is classified as a Zone of Special Protection (PTZPE0018), a humid area of international importance (RAMSAR site- 7PT010), an area of special protection for birds (directive 79/409/EEC), a region under the jurisdiction of Habitats Directive (92/43/EEC) and inscribed in the Natura 2000 network (PTCON0013). The construction of the sewage treatment plant implies the destruction of 12 ha- 2% of the salt marsh. This area is a protected habitat by the Annex I of Habitats Directive (habitats 1410, 1420 and 1430) and the impact on flora is meaningful and irreversible. Rato salt marsh has the most important area of primary salt marsh of the natural reserve and the more diverse bird population (Dias, 1999), being extremely important for several nidifying birds, winter migrants and in obligatory passage (SPEA, 2002). This salt marsh is vital for several steppe species (*Burhinus oedicnemus*, *Melanocorypha calandra*, *Tetrax tetrax* and *Calandrella rufescens*), the nesting place for birds of prey (*Circus aeruginosus* and *Circus pygargus*) (Leitão, 2002, 2003). The species *C. rufescens* has in this area the only location to nidify in Portugal (Leitão, 2003). The intertidal areas adjacent to the sewage treatment plant are feeding grounds for nine limnic species (*Arenaria interpres*, *Calidris alpina*, *Calidris ferruginea*, *Charadrius alexandrinus*, *Charadrius hiaticula*, *Limosa limosa*, *Pluvialis squatarola, Sterna albifrons* and *Tringa tetanus*); with some included in the Annex I of Bird Directive (Dias, 1999; Dias et al., 2003).

The sewage treatment plant outflow will impact a vital estuarine area for the reproduction and growth of ichthyofauna, during their larval (Faria et al., 2006), juvenile and adult life stages (Bexiga, 2002; Chícharo et al., 2006), including some species with high economical value species (*e.g. Diplodus* spp., *Dicentrarchus* spp., *Solea senegalensis*, *Sparus aurata*, *Spondyliosoma cantharus*). The sewage outflow will be located 250 m off "Esteiro da Carrasqueira" creek (Fig. 4), supposedly to protect these creeks of singular ecological sensibility, of great importance to aquatic life and to salt production. This way, the pollutants released in the estuary would be transported far from the creeks during the flood (PROCESL, 2001). Nevertheless, this estuarine area and the areas upstream are extremely important for ichthyofauna. Food chain contamination and bioaccumulation (Polprasert, 1982) should have been considered, since sewage treatment plants can be a major point source of heavy metal contamination on aquatic biota and ecosystems (Al-Jundi, 2000). Therefore, redirecting the sewage treatment outflow to the estuary does not eliminate the threats to creeks' biota.

5. Prevention and mitigation

The preventive and mitigation measures proposed by the scientific community can only be effectively achieved if the knowledge is clearly transmitted to the non-scientific community interested in the sustainable development of the Guadiana. The creation of a Portuguese-Spanish Basin Management Council for the lower Guadiana would be essential to produce ecological and sustainable management decisions for this estuary. A connection with local and national media would be crucial to raise the aware of populations to the local environmental issues (Fig. 6).

The problems caused by the reduction of sediment transported to the coast can only be solved with expensive engineering works, namely beach nourishment and ultimately shoreline protection. The hampering of floods results in the deposition of marine sediments in the river mouth, which can only be solved with dredging (Fig. 3).

In basins where river flow is greatly controlled by dams, the natural river flow is drastically altered. In these places, there is a time lag between maximum rainfall and maximum river discharge. This delay can sometimes compromise the recruitment of estuarine fish species; this way mimicking natural river flow regime could help preventing some negative impacts on fish populations (Loneragan & Bunn, 1999) and shifts on the natural patterns of other biological communities. It is in this sense that ecological flow should be avoided for long periods (Fig. 3).

Dam managers can control or mitigate eutrophication phenomena in the downstream areas of the Alqueva dam by setting flow pulses. With this procedure, secondary production would be promoted and primary producers' biomass controlled. This is only achieved when the magnitude and periodicity of hydraulic flushing and nutrient loading are large (Maier et al., 2001; Roelke et al., 2003). Nevertheless, the intensity and length of hydraulic flushing have to be site specific and should have into account the impact on estuarine fish populations. The conservation status of freshwater fishes, as well as native bivalves, is threatened by the illegal water abstraction from streams, mainly during summer. Therefore, such practise should continue to be prevented by the local authorities (Fig. 3).

The questions regarding ecosystem balance can only be answered with robust ecosystem modelling, achieved after a long lasting ecohydrological approach to the ecosystem. Such questions could be *i*) "Will the Guadiana coastal fisheries decrease sharply after the construction of the Alqueva dam?", *ii*) "Which will be the impact of the 110,000 ha of the

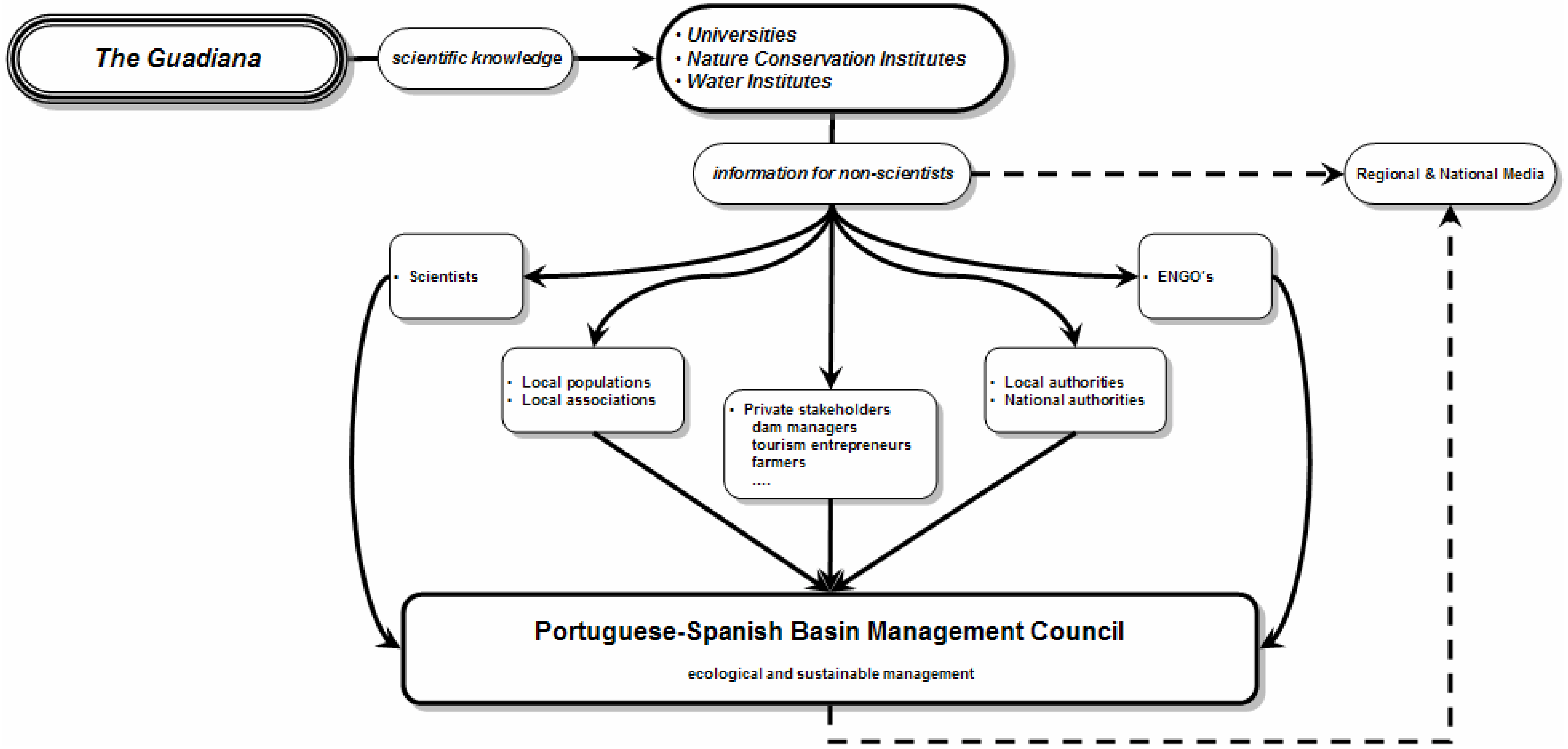

Fig. 6 Diagram on the flow trough of processed scientific knowledge to the several partners that should contribute to the decisions of a Portuguese-Spanish basin management council for the lower Guadiana.

Alqueva irrigation complex on the water quality of aquifers and in all the downstream areas, mainly during periods of reduced river flow?” and *iii*) “Will the nutrients used in the Alqueva irrigation complex trigger an increment in coastal productivity and inherently on coastal fisheries?”. One of the major benefits of an ecohydrological approach is that it allows weighting the benefits and risks of the multiple water uses (*e.g.* damming, irrigation, human consumption, electrical production).

If phytotechnology solutions would be implemented in the Alqueva irrigation complex it would be possible *i*) to augment resilience and capacity of natural systems to absorb impacts, *ii*) to prevent and control pollutant release and environment degradation, or even *iii*) to remediate and restore degraded ecosystems and contaminated sites (Zalewski & Wagner-Lotkowska, 2004). This is possible because riparian wetlands, and also saltmarshes, help to regulate hydrology, sedimentation, nutrient status and sequestrate pollutants.

The occurrence of forest fires is part of a complex socio-economical problem. The preventive measures of forest management should be broadly implemented: *a*) to protect the forest by implementing a global management plan, *b*) to prevent and fight forest fires and to recover such areas, *c*) to develop and promote sustainable agriculture practices in an area that now faces severe problems of desertification, *d*) to recover ancient but effective agriculture procedures, *e*) to develop rural tourism and *f*) to promote environmental education among the local populations (CUMEADAS, personal communication, 2005).

Finally, the destruction of vast natural areas around the Guadiana estuary is of great concern, because some of these areas are inscribed in Natura 2000 network (site PTCON0036). The local ENGO’s propose the creation of a Biosphere Reserve or the International Natural Park of the Lower Guadiana. This measure would resist/stop the development of massive tourism projects in the area, as it occurs in the littoral areas of southern Portugal and Spain, and would serve to compensate the loss of biological diversity forced by the construction of the Alqueva dam.

It is difficult to make a compromise between effective nature conservation and economical improvement of such a poor region. Positive impacts in local economy should be achieved with sustainable and rational growth of human activities, including tourism. Tourism is regarded as the solution to achieve economic development in southern Portugal. However, the achievement of economically viable tourism in this region has to be ecologically sensitive and culturally appropriate (Wall, 1997). Therefore, nature protection and valorisation of the high natural patrimony that characterize the lower Guadiana would not be neglected.

6. Conclusions

The conflicts and constrains on the use of Guadiana water and land exist along its entire path, posing multiple management difficulties. Achieving a social development of the lower Guadiana basin, with sustainable environmental decisions, is a tremendous endeavour. It has to compulsory rely on solid scientific input, on the commitment of basin managers, on the determination of local and regional authorities, on the active involvement of ENGO's, on the awareness of populations and will of private entrepreneurs, which could be set by a Portuguese-Spanish basin management council.

The creation of the International Natural Park of the Lower Guadiana would not resolve the conflict "nature protection" *versus* "water demand and dam impacts", even though it would be needed to avoid the destruction of vast natural areas of great ecological relevance. But it could allow achieving the sustainable development of such a poor region with environmental sensitive tourism projects.

A huge step-forward for a balanced basin management would result from the implementation of a multidisciplinary study of the Guadiana basin. The scientific based proposals would help solving conflicts and testing management scenarios, but for that long lasting monitoring programs and an ecohydrological approach are compulsory.

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Chapter 2

River inflow as an ecological structuring agent in the Guadiana estuary (SW-Iberia) during the filling of the Alqueva dam

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Abstract

During the unique period of filling of the Alqueva dam, this study aimed to *i*) determine which factors control abiotic and biotic variability along the Guadiana estuary and in the adjacent coastal area, *ii*) determine the influence of river inflow on abiotic and biotic water parameters and on egg abundance and distribution in anchovy (*Engraulis encrasicolus sensu lato*, Linnaeus 1758), and *iii)* detect the putative influences of the filling of the Alqueva reservoir on the Guadiana estuary. River inflow was the most decisive factor in variability and in drastic changes of abiotic and biotic parameters along the estuary and in the adjacent coastal area. Continuous river inflows of approximately 50 $m^3.s^{-1}$ were enough to give a nutrient stoichiometry that was similar along the estuary. In contrast, long periods of low inflow led to a marked abiotic and biotic gradient. Six days prior to the April 2002 sampling, a sudden increase of river inflow caused a reduction of 99.99% in the maximum abundance of anchovy eggs. Possibly, anchovy eggs were exported out of the estuary. The filling of the Alqueva dam probably caused marked changes in the estuary. The estuarine turbidity maximum zone moved to the upper estuary, to at least 38 km from the river mouth, differing 8 to 16 km from previous records. In the upper and middle estuary, the nutrient stoichiometry dynamics was more N limited than P limited during the whole year, and Si limitation was only frequent on the coast. Previously, the upper estuary evolved from potential P limitation during winter, to Si limitation during spring to mid summer, and to N limitation from mid summer to autumn. The flooding of vast areas possibly caused an increase in DSi minimum and maximum concentrations; in fact, this value was slightly smaller than the maximum observed during a year of high inflow. In conclusion, after the filling of the Alqueva dam it is advisable that dam managers mimic, as much as possible, the natural river flow, in order to minimize the impact on downstream ecosystems.

1. Introduction

Estuaries and the vicinity coastal areas are highly dynamic ecosystems, mainly in temperate regions where marked biological (Coull, 1999), physical (Bodineau et al., 1998), chemical (Cabeçadas et al., 1999) and geological (Sherwood & Creager, 1990) seasonal changes occur; in addition to those that happen as a result of innate characteristics of tidal ecosystems (Gianesella et al., 2000). Estuaries are the interface between land and ocean and river inflow is one of the major structuring factors of abiotic parameters and biota of this environment and of the adjacent coastal area (Cabeçadas et al., 1999; Snow et al., 2000), occasionally more than tides (Ande & Xisan, 1989). Therefore, it affects primary and secondary productivities (Canuel, 2001; Nixon, 2003), since river inflow is crucial in setting nutrient concentration and stoichiometry (Grange et al., 2000; Nixon, 2003). Ultimately, it will affect estuarine dynamics and coastal fisheries landings (Loneragan & Bunn, 1999; Whitfield & Harrison, 2003; Erzini, 2005), with an inherent economical impact.

River inflow has a marked variability within and between years in temperate estuaries under the influence of a Mediterranean climate, such as the Guadiana estuary (SW Iberia, Europe) (CEDEX, 2006; INAG, 2006) (Fig. 1). The numerous dams that exist in the Guadiana basin control largely the river flow (Euronatura & IIDMA 2003). This will increase river inflow variability and shifts on the natural flow regime (Brandão & Rodrigues, 2000). The Alqueva dam was the last to be built; its floodgates were closed on February 8th 2002, hence river flow regulation increased from 75% to 81% (Rocha et al., 2002). Many studies draw attention to the putative changes in the Guadiana estuary due to the increased regularization of river flow after the dam construction (Brandão & Rodrigues, 2000; Chícharo et al., 2001a,b; Rocha et al., 2002; Lopes, 2004; Domingues et al., 2005; Erzini, 2005). Prior to its construction, the location of the ETM was evaluated (Chícharo & Chícharo, 2000;

Chícharo et al., 2001a) and modelled (Lopes, 2004). Phytoplankton dynamics and the link with nutrient variability were established (Rocha et al. 2002; Domingues et al., 2005). Additionally, it was found a positive relationship between river inflow with the abundance of the larval stages of the most abundant planktivorous fish in the Guadiana estuary- anchovy (*Engraulis encrasicolus sensu lato* (Linnaeus, 1758) (Chícharo et al., 2001b; Chícharo et al., 2006). Thus, for the unique period of the Alqueva dam filling, this study aimed to *i*) assess which factors cause abiotic and biotic variability along the Guadiana estuary and in the adjacent coastal area, *ii*) determine the influence of river inflow on abiotic and biotic water parameters and on the abundance and distribution of anchovy eggs and *iii)* detect the putative influences of the filling of the Alqueva reservoir on the estuary.

2. Material and Methods

2.1. Study site

This study took place in the Guadiana estuary and in its adjacent coastal area, which is on the southern border of Portugal with Spain (Fig. 1a). The estuary is approximately 70 km long, with an area of 22 km^2 and an average depth of 6.5 m. It is a mesotidal estuary and tide amplitudes range from 1.3 to 3.5 m. The estuary is partially stratified when average river flow (~150 m^3.s^{-1}) and tidal prism (~3 x10^7 m^3) are observed (Michel, 1980). River flow has a striking variability between and within years, since the majority of the basin is under the influence of a climate with Mediterranean characteristics. The annual average temperature varies from 14 to 18 ºC. Rainfall is very irregular within the year, ~80% occurs during autumn and winter and summers are very dry. The annual average rainfall fluctuates between 561 and 600 mm in the Portuguese basin. Climate variability imposes a similar trend to river flow;

thus, the average river inflows are as follows: dry years, 8–63 $m^3.s^{-1}$; average years, 170–190 $m^3.s^{-1}$; humid years, 412–463 $m^3.s^{-1}$ (Bettencourt et al., 2003).

Intense regularization of river flow has occurred in the Guadiana basin since the mid 1950s (Brandão & Rodrigues, 2000). The Alqueva dam was the last to be built, it is at 150 km from the river mouth, creating one of the biggest artificial lakes in Europe (Hellsten, 2003). This reservoir has a maximum area of 250 km^2, a perimeter of more than 1000 km and a total capacity of 4,150 hm^3 (INAG, 2006).

2.2. Sampling strategy and methodology

Eulerian sampling was carried out during new moon spring tides at low and high tides, from March 2002 to February 2003 at 9 sampling stations: 7 stations inside the estuary and 2 in the coastal area. Station 1 was off Praia de Santo António, outside the direct influence of the estuarine outflow, and station 2 was positioned in the area where the river plume is formed. Station 9 was the uppermost station, situated in the high estuary in front of Alcoutim (Portugal) and Sanlucar de Guadiana (Spain), at 38 km from the river mouth (Fig. 1b). Sampling was performed from a boat equipped with an 80 hp engine, except in February 2003, when a boat equipped with a 30 hp engine was used because of technical problems. Therefore, high tide sampling in January and February 2003 was not performed.

In each station, vertical profiles of temperature and salinity in the water column were recorded with a YSI 6600 probe. Sub-superficial zooplankton trawls were made with a 250 μm mesh net, equipped with a flowmeter, and the collected samples were immediately preserved in buffered formaldehyde (4% final concentration). At the same depth as the zooplankton trawls, water samples were collected for the analysis of dissolved inorganic macronutrients, seston, suspended organic matter, chlorophyll *a* and phaeopigments. These samples were preserved cooled until processed in the laboratory.

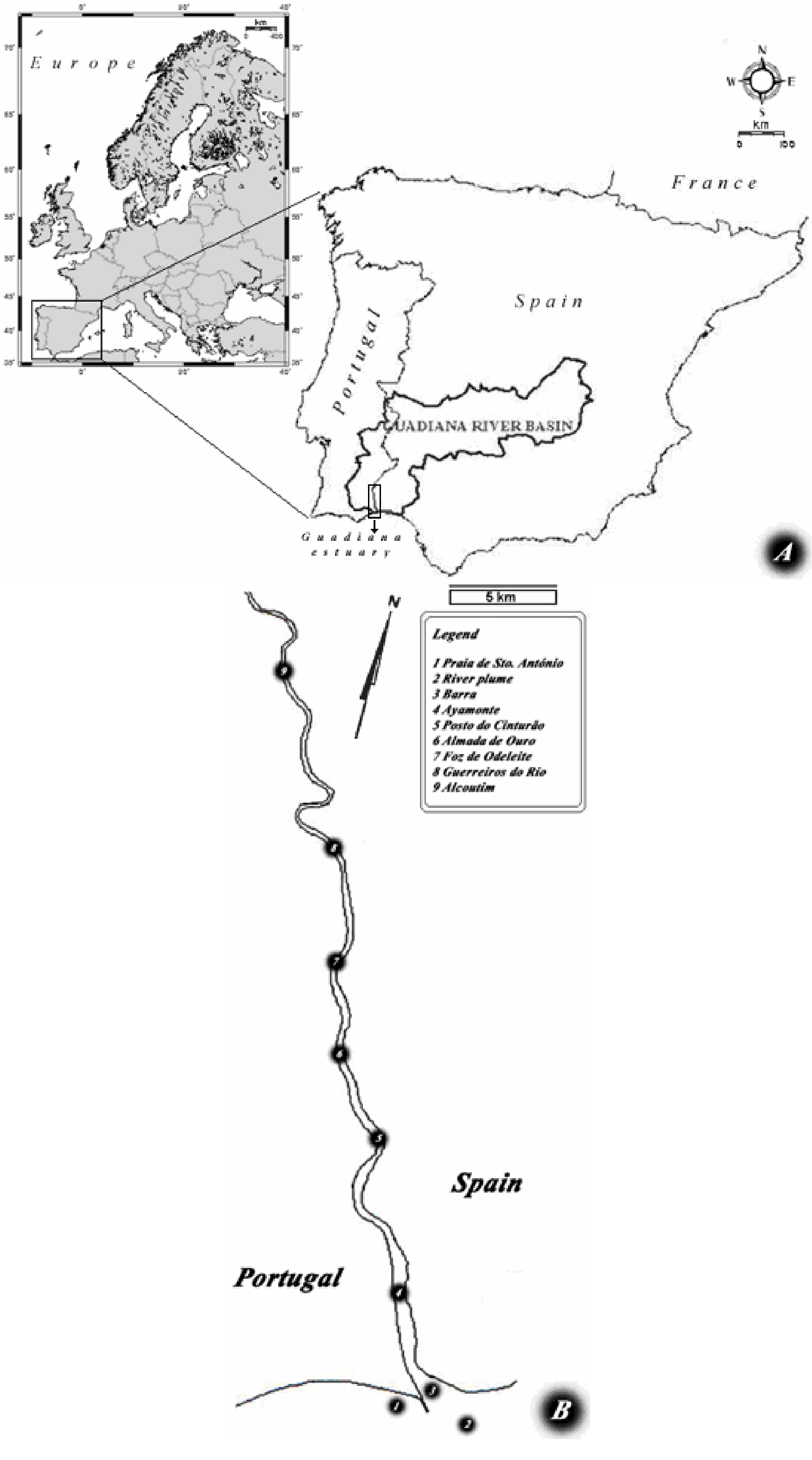


Fig. 1 Geographical context of the Guadiana estuary in the Iberian Peninsula (Europe) (A) and location of sampling stations along the estuary and in the adjacent coastal area (B).
Map of Europe modified from http://www.aquarius.geomar.de.

2.3. Laboratory analyses

To determine dissolved inorganic macronutrients (ammonium, nitrate, nitrite, orthophosphate and silicate), water samples were filtered through 0.45 µm pore size cellulose acetate filters (MSI) and preserved frozen (Kirkwood, 1996) until spectrophotometric analyses (Grasshoff et al, 1983). Water samples to be analysed for seston and suspended organic matter were filtered through 0.7 µm pore size filters (Whatman GF/F); then the filters were washed with distilled water (three times the filtered sample volume), dried at 60 ºC and finally burned at 450 ºC (Greenberg et al., 1992). Water samples for analysis of chlorophyll *a* and phaeopigments were filtered through 0.7 µm pore filters (Whatman GF/F), without exceeding 100 mm Hg of filtration pressure; then they were kept frozen (-20 ºC) until fluorimetric analyses (Knap et al., 1996).

Anchovy eggs were sorted from the zooplankton samples and their abundance was determined.

2.4. Data analysis

Data on the Guadiana river inflow, rainfall and N and P loading was made available by the Portuguese National Water Institute (INAG, 2006). River inflow and N and P loadings to the estuary were measured in Pulo do Lobo hydrometric station (code: 27L/01), situated at 15 km above the tidal limit of the estuary and where *ca*. 90% of water going to the estuary passes (Ribeiro et al., 1988). Monthly average river inflows and cumulative rainfall were calculated for the periods elapsed between samplings. N loading results from the summation of ammonium, nitrate and nitrite, while P loading corresponds to total phosphorus data. Rainfall was determined at Martim Longo meteorological station, the closest to Pulo do Lobo.

Surface plots of temperature, salinity and seston were made, using kriging (linear variogram model) as the gridding method.

A one-way ANOSIM and a non-parametric multidimensional scaling (MDS) analysis were performed to evaluate spatial and temporal evolution patterns of nutrients, seston, organic matter, chlorophyll *a* and phaeopigments in the water in relation to eight established factors: *Month* (month when sampling occurred), *Season* (*spring*- April, May and June 2002; *summer*- July, August and September 2002; *autumn*- October, November and December 2002; *winter*- March 2002, January and February 2003), *Station* (from sampling station 1 to 9), *Studied Area* (*coastal area*- st. 1 and 2; *low estuary*- st. 3 and 4; *middle estuary*- st. 5, 6 and 7; *upper estuary*- st. 8 and 9), *Tide* (*low tide* and *high tide*), *Inflow* (*low*- < 8 $m^3.s^{-1}$; *moderate/low*- 19.0 $m^3.s^{-1}$, *moderate*- between 47.6 and 56.0 $m^3.s^{-1}$; *high*- 105.3 $m^3.s^{-1}$), *Rainfall* (*undetermined*- March 2002; *low*- < 8 mm; *moderate*- between 31.4 and 77.6 mm; *high*- > 133.1 mm) and *Coastal upwelling* (*present and persistent*; *absent*; *undetermined*). The similarity matrix constructed to perform these analyses was made after log (x+1) data transformation and setting Euclidean distance as the measure of similarity. The non-parametric ANOSIM, which employs R statistics, was used to examine the existence of meaningful differences between the established groups within each factor. R values close to 0 indicate that there are small differences in the evolution pattern of the analysed parameters, in opposition to R values near 1 (Clarke & Warwick, 2001). Both analyses used Primer 5 software (Primer-E Ltd.).

Degree of coastal upwelling was inferred by analysing NOAA 17 satellite images from the 7 days prior to each sampling. Those images have a dimension of 760 × 1100 pixels and a resolution of 8 bits $pixel^{-1}$ (NERC & PML, 2004).

Dissolved inorganic nitrogen (DIN) was calculated as the sum of ammonium, nitrate and nitrite. Dissolved reactive phosphorus (DRP) and dissolved silica (DSi) correspond to orthophosphate and silicate concentrations. The concentration of DIN, DRP and DSi will here be analysed along the studied period in stations 1, 4, 6 and 9, which can be considered as characteristic of the four areas studied. N:P and Si:N molar ratios were calculated and plotted on an *XY* logarithmic graph (Rocha et al., 2002).

To evaluate the effect of intra-annual changes of river inflow on several water parameters, a comparison between three distinct months was done. Selection criteria regard the one-way ANOSIM results, plus data on river inflow and anchovy eggs. Such evaluation relies on analyses of the shifts of temperature and salinity profiles in the water column, as well as on changes in nutrient stoichiometry along the estuary in each of these 3 months.

3. Results

3.1. Global analysis

Daily average river inflow varied from 1.5 ± 0.0 $m^3.s^{-1}$ (July 3rd 2002) to 656.3 ± 405.0 $m^3.s^{-1}$ (April 9th 2002), with an annual average river flow of 24.0 ± 61.3 $m^3.s^{-1}$ (Fig. 2A). Usually, river inflow was lower than the average historical river inflow. Drastic changes in river inflow were observed, the main one being 6 days prior to the April sampling, when there was a 44.8 fold increase in only 2 days (Fig. 2A). If those 6 days are omitted in calculating the annual average river inflow, then this parameter drops by 20.4%. The highest average river inflow determined between samplings was 105.3 ± 179.8 $m^3.s^{-1}$ (March 28th to April 15th). Moderate inflows were observed from February 27th to March 27th (47.6 ± 55.8 $m^3.s^{-1}$), December 4th to January 5th (56.0 ± 16.1 $m^3.s^{-1}$) and January 6th to February 4th (48.0 ± 12.1 $m^3.s^{-1}$). The lowest inflows, lower than 7.7 ± 0.6 $m^3.s^{-1}$, were registered between May 14th and December 4th (Fig. 2B).

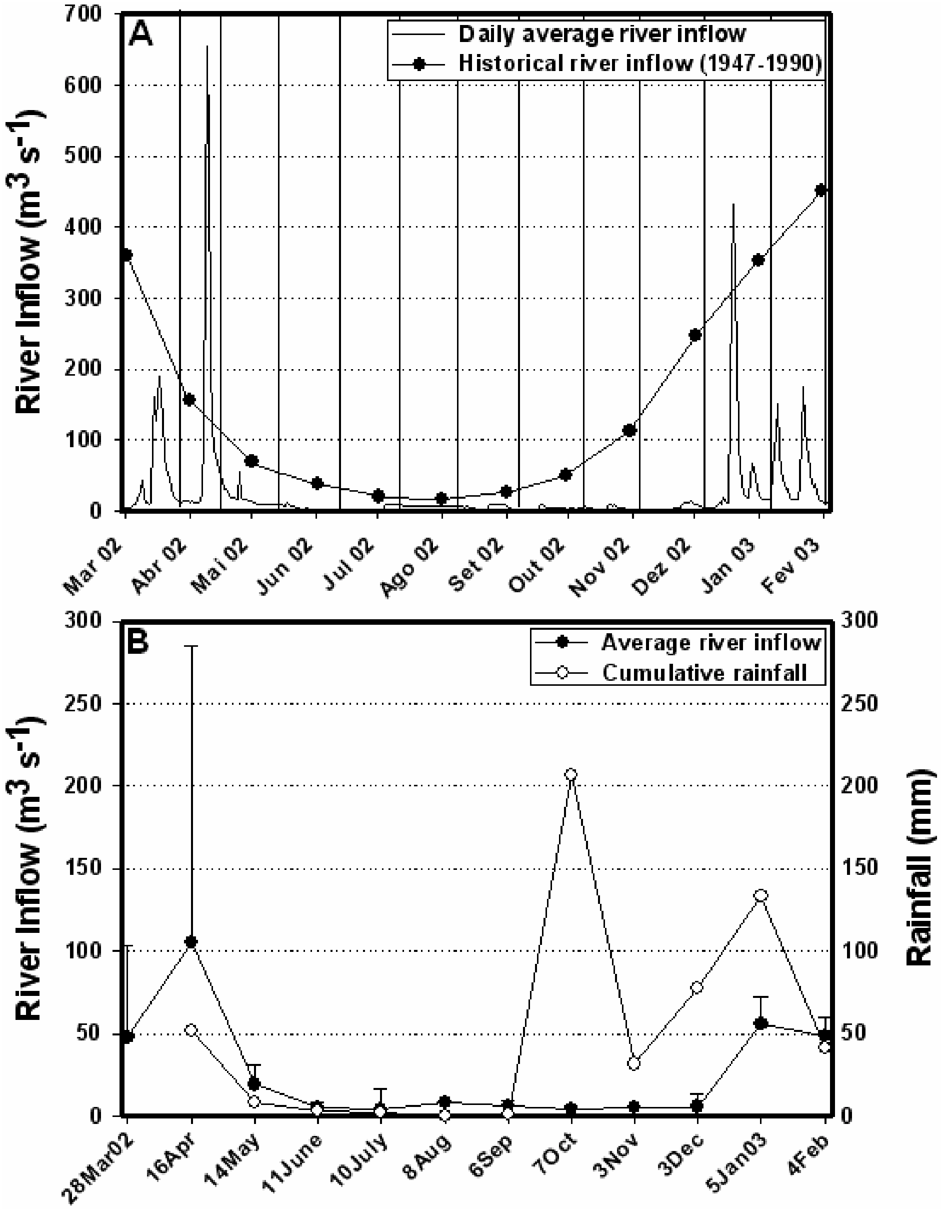


Fig. 2 A- Daily average Guadiana river inflow and Guadiana historical river inflow monthly average (1947-1999) registered at Pulo do Lobo hydrometric station. Vertical lines correspond to the days of sampling.
B- Average river inflow (±SD) and cumulative rainfall registered between the different sampling moments at Pulo do Lobo hydrometric station and Martim Longo meteorological station, respectively.

The evolution patterns of monthly average river inflow and cumulative rainfall did not always coincide, namely when the maximum cumulative rainfall was registered (206.2 mm from September 7th to October 7th). A similar pattern between these parameters was observed from March 28th to April 15th; nevertheless, the observed cumulative rainfall was inconsistent with the high river inflow. October cumulative rainfall was 4 times higher than in April (a similar period was considered), whereas monthly average river inflow remained low and constant (4.1 ± 1.8 $m^3.s^{-1}$) (Fig. 2B).

Surface temperature varied from 11.6 to 26.7 ºC, showing the usual seasonal evolution. Higher temperatures were generally registered in the upper estuary and decreased towards the coast, yet practically disappeared (March and April 2002) or inverted (December 2002 to February 2003) during the colder months (Fig. 3A). Surface salinity decreased towards upstream stations, varying from 0.09 to 36.5. Periods of higher river inflow (Fig. 2) coincided with weaker intrusion of saline water, namely during April 2002 and from December 2002 to February 2003 (Fig. 3B).

Seston tended to decrease towards the coastal area, varying from 3.0 $mg.L^{-1}$ (st. 1, September 2002) to 132.0 $mg.L^{-1}$ (st. 9, August 2002). Maximum seston concentration occurred in the uppermost station during low inflow periods, but during more intense river inflow it was located between stations 6 (January 2003) and 8 (March 2002 and February 2003) (Fig. 3C).

DIN (Fig. 4B), DRP (Fig. 4C) and DSi (Fig. 4D) concentrations showed the same pattern. Their average concentrations were higher during low tide (data not shown), generally decreasing towards the coast. Maximum concentrations were concomitant with higher river inflow (Fig. 2) and N and P loadings (Fig. 4A). Maximum N loadings reached 10,046 t in January 2003, while P loading was maximum in February 2003 with a supply of 77 t to the estuary. Maximum DIN, DRP and DSi concentrations were 101.2 µM (Jan. 2003, st. 9), 3.9 µM (Feb. 2003, st. 9) and 179.0 µM (Feb. 2003, st. 9), respectively. Chlorophyll *a* concentration was maximum in June and July 2002 (12.3 $\mu g.L^{-1}$) (Fig. 4E), coinciding with minimum DIN and DRP concentration. Later, chlorophyll *a* concentration decreased sharply, while DIN and DRP concentrations increased, without correspondence with increased N and

P loading. DSi concentration also increased after reaching its minimum in August 2002, although there was no increase in river inflow.

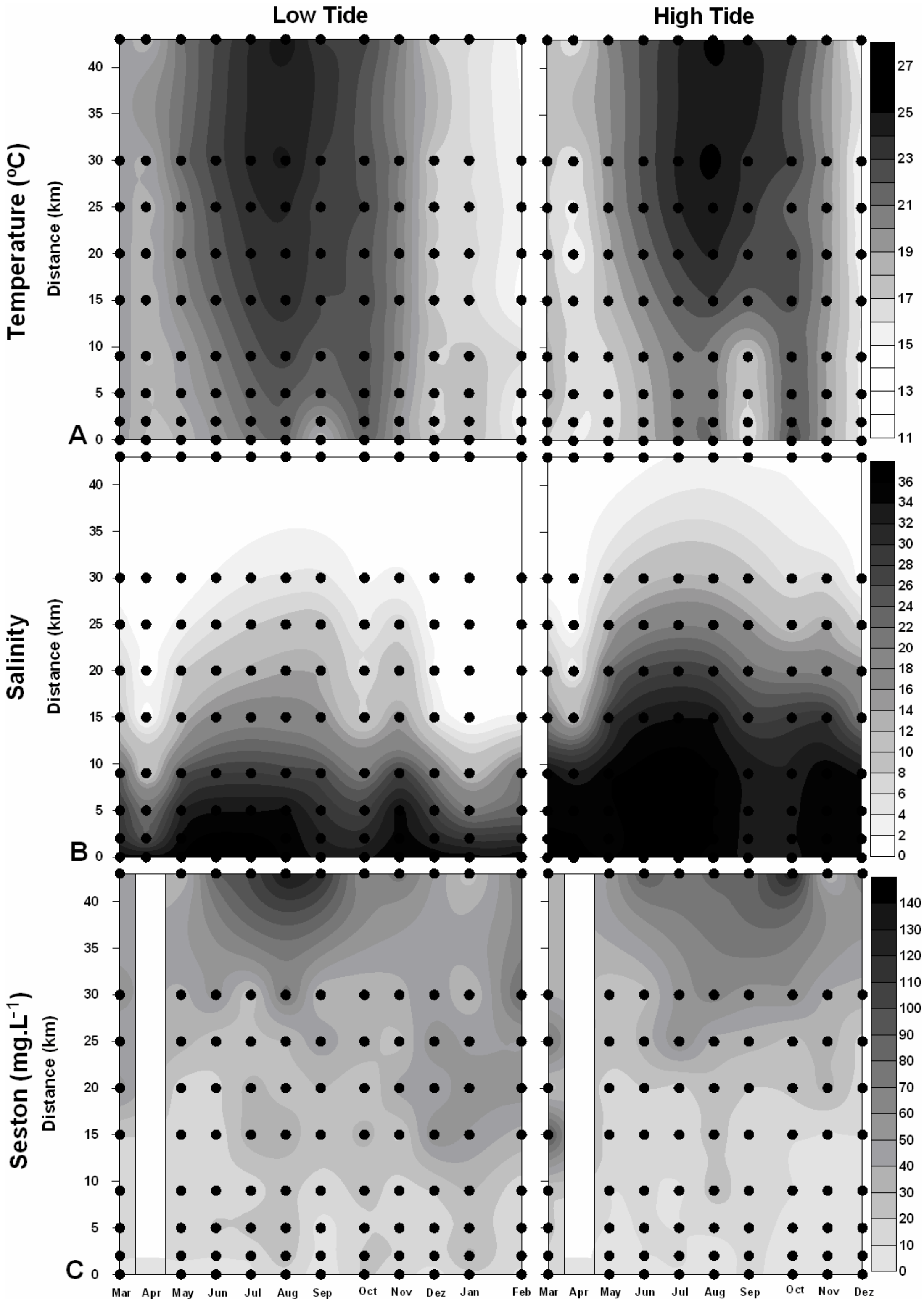


Fig. 3 Spatial and temporal evolution of surface temperature, salinity and seston in the Guadiana estuary and adjacent coastal area, at low and high tides, from March 2002 to February 2003.

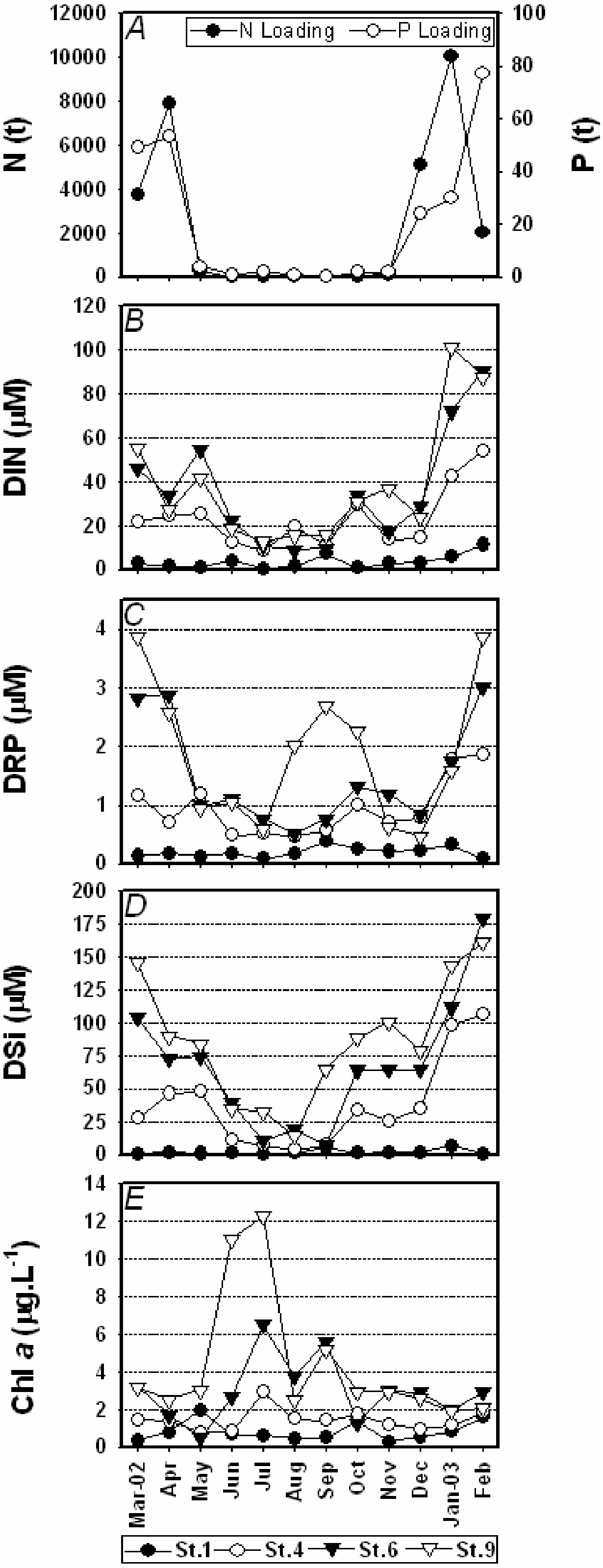


Fig. 4 Temporal evolution of N and P loadings at Pulo do Lobo (A) and DIN, DRP, DSi and Chl *a* concentrations in stations 1, 4, 6 and 9 during low tide (B-E), from March 2002 to February 2003.

Seasonal evolution of nutrient stoichiometry reveals that the Guadiana estuary tends to be P limited. However, during summer, N is potentially more limiting in the high and middle estuary and Si more limiting in the low estuary (Figs 5A-C). Seasonally, the coastal area (st. 2 and st. 1) was more heterogeneous, being potentially N limited during spring and autumn, Si limited during summer and P limited during winter (Fig. 5D).

Studied area and *Station* were the factors that best explained the differences of abiotic and biotic parameters, with global R values of 0.460 (p= 0.1) and 0.431 (p= 0.1), respectively. The similarity between studied areas and sampling stations decreased with increasing distance; minimum similarities were observed between stations *1* and *9* (R=1.000; p= 0.1) and between *coastal area* and *upper estuary* (R= 0.960; p=01). *Inflow*, *Month*, *Season* and *Rainfall* had global R values between 0.170 (p= 0.1) and 0.114 (p=0.1) (Table I). The biggest differences in the *Inflow* factor were observed between the periods of *low* and *moderate inflow* (R= 0.252; p= 0.1) and *high inflow* (R= 0.194; p= 0.1). Seasonally, *winter* and *summer* were the most distinct periods (R= 0.426; p= 0.1), in contrast with *spring vs. autumn* (R= 0.009; p= 20.7) and *spring vs. winter* (R= 0.074; p= 1.4). Therefore, August and February were the most distinct months (R= 0.747; p= 0.1). August showed the greatest difference from the remaining sampling months, the least difference was with September (R= 0.167; p= 0.3). With regard to *Rainfall*, the periods of *low vs. high rainfall* (R= 0.142; p= 0.1) and *moderate rainfall* (R= 0.125; p= 0.1) show the highest differences. *Tide* (R= 0.054; p= 0.2) and *Coastal Upwelling* (R= -0.040; p= 87.6) had the lowest global R values (Fig. 6).

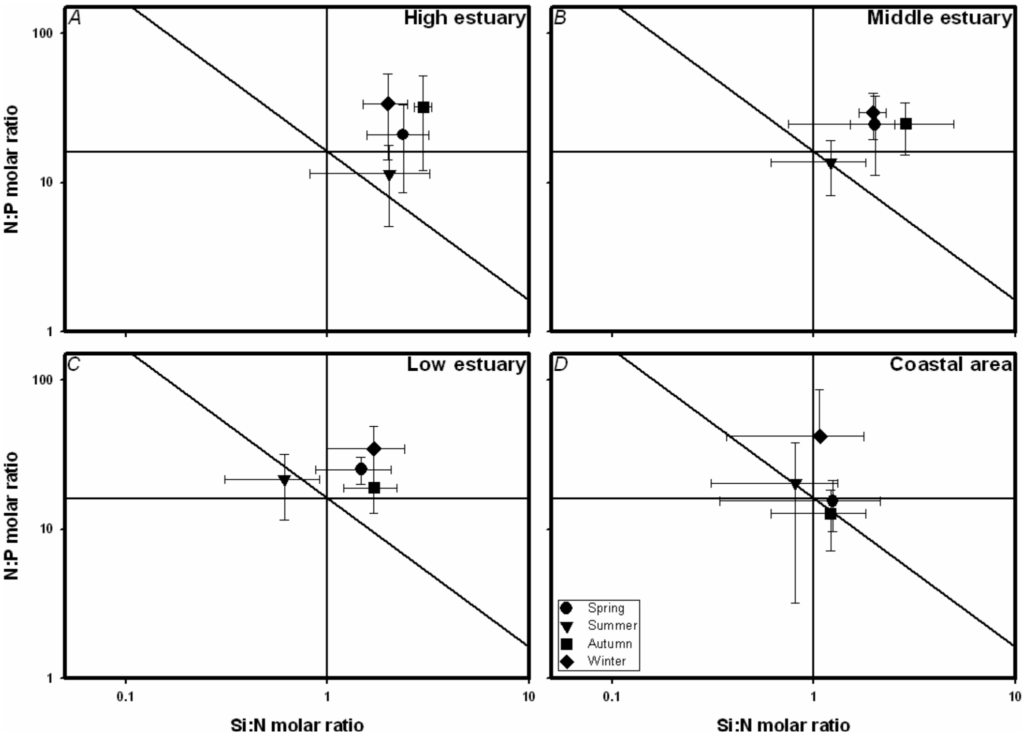
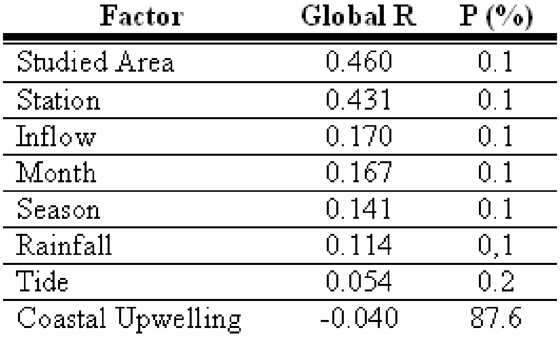


Fig. 5 Nutrient stoichiometry, during low tide, along the Guadiana estuary and in the adjacent coastal area from March 2002 to February 2003.

Table I Global R values for each factor obtained in the non-parametric ANOSIM analysis.



Factor	Global R	P (%)
Studied Area	0.460	0.1
Station	0.431	0.1
Inflow	0.170	0.1
Month	0.167	0.1
Season	0.141	0.1
Rainfall	0.114	0,1
Tide	0.054	0.2
Coastal Upwelling	-0.040	87.6

Anchovy eggs were distributed mainly through the lower and middle estuary and were collected from March to November 2002. Maximum abundance was registered in June 2002, with 2106 eggs.100 m^{-3} (station 5; high tide), and abundance steadily decreased until November 2002. From March to April 2002, maximum abundance decreased by 99.99%, down to 0.4 eggs.100 m^{-3} (Fig. 7).

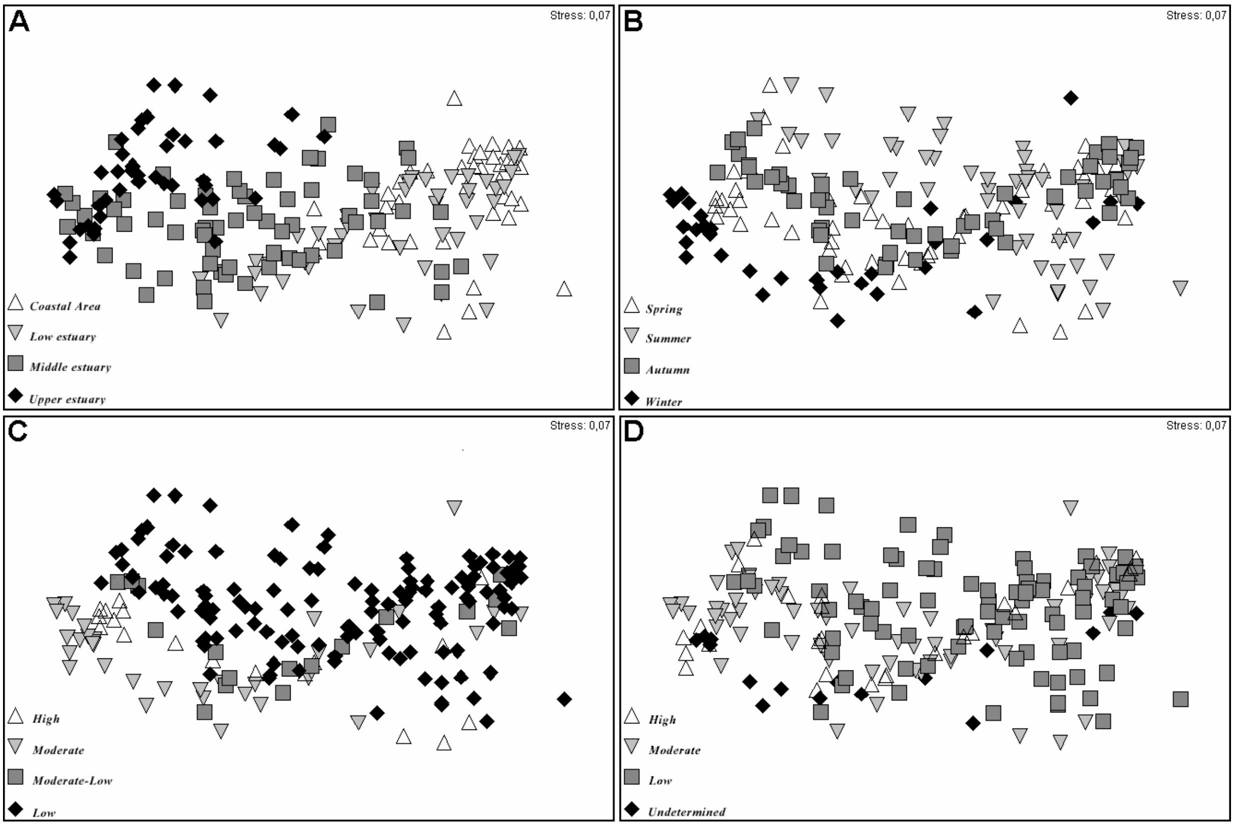


Fig. 6 MDS plots of the factors *Studied Area* (A), *Season* (B), *Inflow* (C) and *Rainfall* (D).

3.2. Comparison of three periods with distinct river inflow characteristics

The samplings of April 2002, August 2002 and February 2003 were selected for this insight analysis. August 2002 and February 2003 were the months with lowest similarity (R= 0.747; p= 0.1). The highest river inflows were registered in April 2002, with the maximum daily average river inflow (656.3 ± 405.0 $m^3.s^{-1}$) occurred 6 days before sampling (Fig. 2). Moreover, in April 2002 the maximum abundance of anchovy eggs decreased by 99.99% (Fig. 7). In these 3 months, there were striking differences in temperature and salinity vertical profiles (annex III), seston (see previous section), nutrient concentration (see previous section) and stoichiometry (Fig. 8).

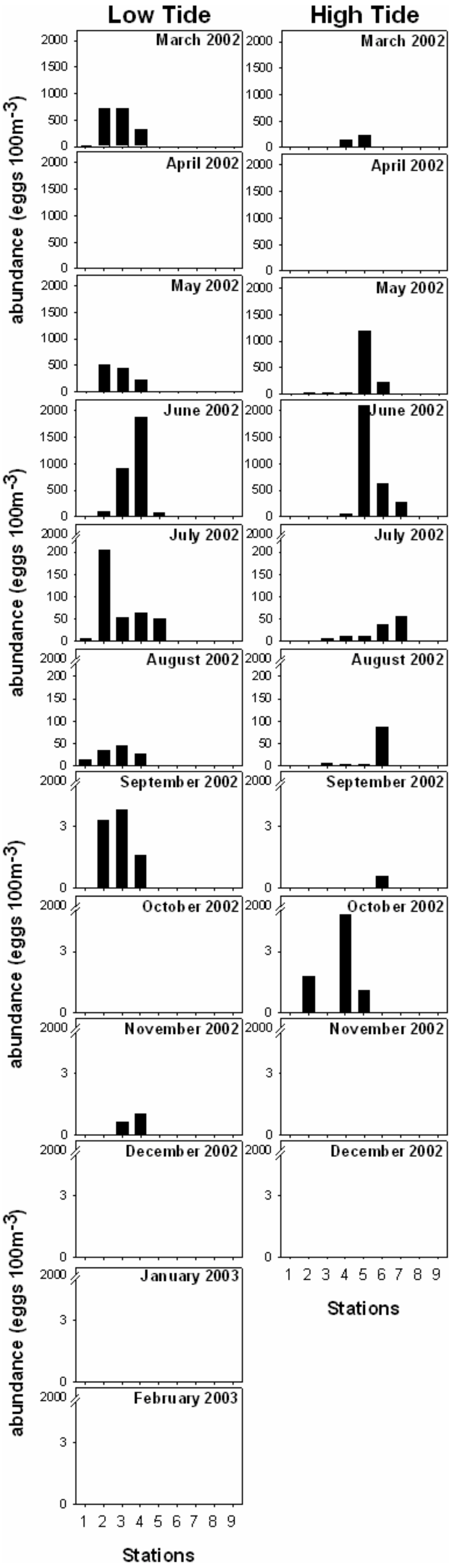


Fig. 7 Spatial and temporal evolution of abundance of anchovy eggs at low (A) and high (B) tides.

In August 2002 there was no vertical stratification of temperature and salinity through the water column, but a sharp gradient was observed from the coast to the upper estuary. Higher temperatures were registered in the upper estuary, decreasing towards the coast. The greatest difference between the highest and lowest temperature was 6.1 ºC. In April 2002 and February 2003, maximum and minimum temperature differences were lower, 2.0 ºC and 2.5 ºC, respectively. Despite the reduced differences in water temperature in February 2003, warmer temperatures were registered at the coast, decreasing slightly towards the upper estuary.

During low tide in April 2002 and February 2003, sharp stratification of salinity down the water column was observed from stations 2 to 4 and from stations 3 to 5, respectively. In the high tide of April 2002, only station 5 was stratified. In these months, the isohaline 0-2 reached station 5, whereas in August 2002 it remained close to station 9 (Fig. 3B, annex III).

Nutrient concentration was higher during periods of higher inflow (April 2002 and February 2003) (Figs. 4B-D), decreasing sharply during periods of low inflow (August 2002) (Figs. 4B-D). In April and August 2002, estuarine waters tended to be potentially N limited (Fig. 8Ai), mainly in the middle and/or upper studied areas (Figs. 8A, B). In August 2002, nutrient stoichiometry showed a marked gradient along the studied areas (Fig. 8B), similar to that observed for surface temperature and salinity (Figs 3A-B). In contrast, a greater homogeneity was observed in April 2002 (Fig. 8A), and even greater in February 2003 (Fig. 8C), when it was preceded by a continuous period of high and moderate river inflow (Fig. 2). In these months, all estuarine stations and station 2 had similar potential P limitation (Si:N= 1.9 ± 1.4; N:P= 31.9 ± 25.0), in clear contrast to station 1, which was silica limited (Si:N= 0.06; N:P= 131.6) (Fig. 8Ci). Coastal waters were usually silica limited, imposing this characteristic in downstream stations during periods of low inflow (August 2002) and at the high tide (Fig. 8Bii). Intrusion of coastal water in to the estuary led to the influence of tides in changing nutrient stoichiometry from stations 1 to 4 in April 2002 (Fig. 8A). In August 2002, tidal influence markedly biased nutrient stoichiometry as far as the middle estuary (Fig. 8B).

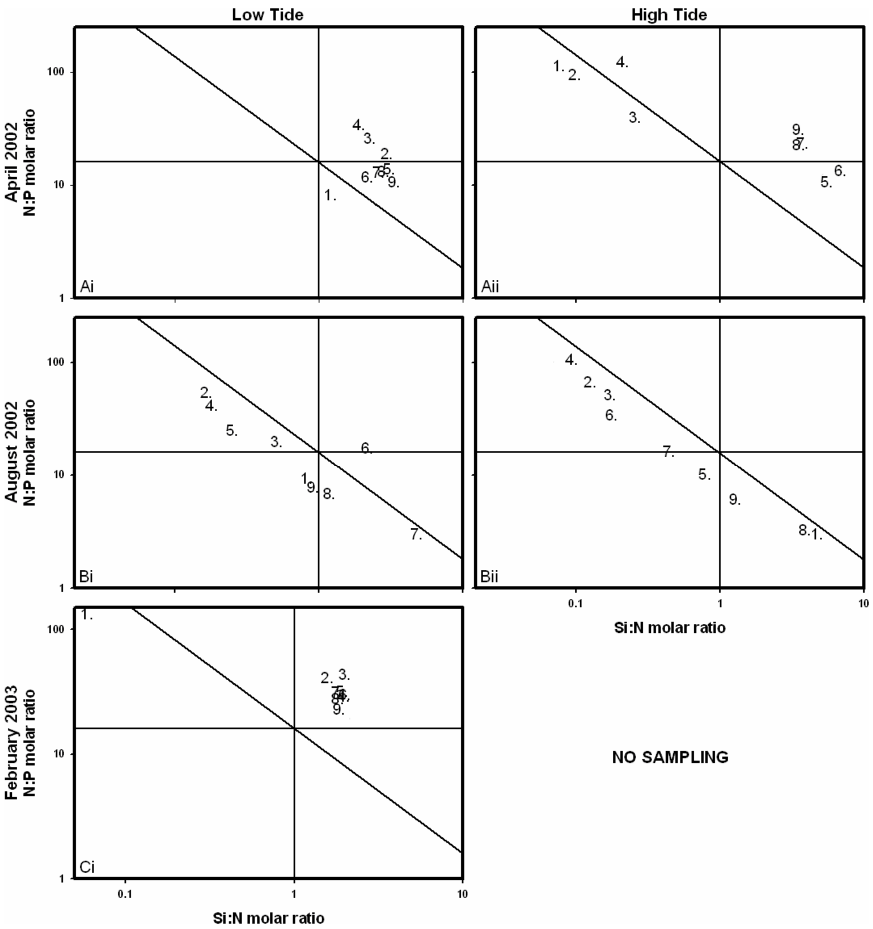


Fig. 8 Si:N:P ratios in the Guadiana estuary and adjacent coastal area, from sampling stations 1 to 9, at low and high tides, during April 2002, August 2002 and February 2003.

4. Discussion

4.1. Which factors control abiotic and biotic variability along the Guadiana estuary?

Distance to river mouth was the factor that set the striking differences of abiotic water parameters and biota along the Guadiana estuary, as previously observed elsewhere (Blaber et al., 1997; Huang et al., 2003; Scharler & Baird, 2003); yet it is seasonality that imposes their dynamics.

In dammed basins, mainly in regions where water is scarce and crucial to supply reservoir needs, there is a time lag between rainfall and an effect on river inflow. Coastal upwelling can be important for quite large periods in some estuarine ecosystems (Pérez et al., 2000), while in others, it only has a significant effect during reduced river inflows (Taylor, 1992).

A prolonged upwelling event (late August to early September 2002) started near Cape São Vicente (SW-Portugal) on the west coast of Portugal, and extended eastwards along the southern coast as far as the Guadiana estuary. Since this was a single event and its influence was restricted to the lower estuary, this explains its low overall significance. Rainfall had also a restricted importance, except in January and February 2003. Finally, seasonality was overlapped by river inflow from mid April to mid December 2002, once river inflow had become fairly constant, below 10 $m^3.s^{-1}$ in 90% of the days (INAG, 2006). Thus, river inflow was the decisive factor influencing the dynamics of the studied area.

4.2. Influences of Guadiana river inflow on abiotic and biotic water parameters

The influence of river inflow on water temperature was significant from March to April 2002, when it decreased 2 ºC. The remaining temperature fluctuations were influenced by tide and seasonality.

Horizontal and vertical salinity profiles clearly reflect the influence of river inflow variability and allow an evaluation of the relationship between rainfall and water release/retention on dams. In April 2002, a fall in salinity coincided with the extraordinary increase of river inflow, caused by the release of water, when it reached the lower gates of the Alqueva dam. On the other hand, in October 2002 a slight fall in salinity was probably a result of local rainfall events. Most likely, intense retention of water on dams occurred up to December 2002.

During the two most disparate samplings, August 2002 and February 2003, nutrient stoichiometry seemed also to reveal the impact of distinct river inflow; however, the seasonality effect over nutrient dynamics should not be neglected (Ragueneau et al., 2002). The constant high and moderate inflows that preceded the February 2003 sampling forced an intense estuarine homogenization as far as 12 km (st. 5) from the river mouth. Thus, all estuarine stations and the one under direct influence of estuarine outflow shared similar stoichiometry, with potential P limitation. The effect of river inflow was so striking that the only station outside the direct and indirect estuarine outflow, even with a river inflow of 1000 $m^3.s^{-1}$ (Cunha et al., 2000), exhibited strong silica limitation. Conversely, in August 2002, the prolonged reduced river inflow caused a marked stoichiometry gradient. In this month, the majority of the estuary was potentially Si limited, reflecting the decreasing terrestrial input (Rocha et al., 2002; Domingues et al., 2005) and stronger coastal influence. In fact, between August 2002 and February 2003, after some periods of high and moderate rainfall, DSi concentration increased 13.8 times, from an overall average of 8.4 to 115.2 µM. Finally, it would be expected that the outstanding increase in river inflow prior to the April sampling would result in a stoichiometry pattern similar to the one determined in February 2003, or even more homogenised. In fact, similar ratios were observed during the low tide, but not as much as in February 2003, probably because constant high or moderate inflows might be more important in stoichiometry homogenization than a single enormous output a few days before sampling.

The contrasting inflows observed in April (105.3 ± 179.8 $m^3.s^{-1}$) and August 2002 (7.7 ± 0.6 $m^3.s^{-1}$) changed the tidal influence on estuarine stoichiometry along 18 km. This is also supported by the marked change in the positions of the salt wedge and ETM, commonly used to detect changes in river flow (Kurup et al., 1998; Nagy et al., 2002).

Lopes (2004) modelled sediment transport, determining that, for a river flow of 20 $m^3.s^{-1}$, the ETM should lie between 22 and 30 km from the river mouth. In fact, field data generally support this (Chícharo and Chícharo, 2000). However, during the extended periods of low inflow during the filling of the Alqueva dam, the ETM was positioned more upstream, at least around station 9, 38 km from the river mouth. Chícharo et al. (2001a) consider that stratification of the water column and location and strength of the ETM may significantly influence the retention of zooplankton in the Guadiana estuary. Effects of changes in river inflow on chlorophyll *a* concentration were not perceptible in this study. However, on a shorter time interval between sampling, Sobrino et al. (2004) found that the exceptional river inflow observed in early April 2002 was accompanied by 58% reduction of chlorophyll *a* content in the area between Mértola and Foz de Odeleite. Indeed, intense hydraulic flushing is one of the decisive factors in the dynamics of planktonic ecosystems, with the potential to enhance biodiversity, control eutrophication and prevent harmful algal blooms (Roelke et al., 2003). However, the magnitude and periodicity of flushing must be controlled and the potential impacts on adjacent ecosystems ought to be evaluated first.

4.3. River inflow and anchovy eggs

Temperature is one of the most important triggers that dictate fish spawning periods (Holmes & Henderson, 1990; Palomera, 1992). For anchovy, a planktivorous fish with pelagic eggs and a broad reproductive period (Millán, 1999; Plounevez & Champalbert, 1999), no larval stages have been captured in waters with temperatures below 13 ºC (Demir, 1965; 1991; Ré, 1984; Chícharo & Teodósio, 1991a; Ribeiro, 1991; Motos et al., 1996). This is likely to relate to their presence in the estuary only in late March 2002, when the average temperature was 18 ºC.

Since anchovy had already started its broad and continuous spawning by late March 2002, it was surprising to observe that in mid April 2002 the maximum abundance of anchovy eggs had decreased by 99.99%. Biological and hydrological hypotheses, or both, might be postulated to interpret this finding. Biologically, if populations of the prey of adult anchovies had collapsed, then it might have caused ovarian atresia (Abaunza et al., 2003). With regard to hydrological causes, the sudden increase of river inflow 6 days prior to sampling might have led to the advection of anchovy eggs out of the estuary. The currently available data are only relevant to this second hypothesis, and further analysis is required to corroborate the first hypothesis.

The intense river inflow resulted in a decrease in the successful development of anchovy larval stages, as a result of either increased osmotic stress or increased probability of predation in the coastal area, which can cause a reduction of the subsequent year’s recruits (Whitfield, 1994).

For other species that have restricted reproductive periods, sudden shifts in river inflow might be vital for recruiting success (Whitfield, 1994; Loneragan & Bunn, 1999). This sudden shift in river inflow, and the decrease in the average annual river inflow by 20.4% if the 6 days prior to April 2002 sampling are omitted, clearly support the theory that it is preferable to mimic the natural inflow regime than to discharge similar annual amounts of water (Loneragan & Bunn, 1999) or to compensate the reduced inflows.

The remaining dynamics of anchovy eggs was not linked with any other aspect of variability in river inflow. Its abundance peaked in June, in accordance with previous observations that recorded maximum spawning in June and July in the Guadiana estuary (Chícharo & Teodósio, 1991a). The abundance of anchovy eggs started to decrease from July 2002 onwards, probably as a result of decreasing intensity of anchovy spawning. However, it might also be associated with intense predation by the enormous population of jellyfish

(Morais**,** unpublished data), which are important regulators of zooplanktonic communities (Schneider & Behrends, 1994).

The maximum abundance of anchovy eggs and larvae decreased 4.7 and 14.5 times in comparison with the maximu registered in 1988, a year of moderate inflow (Chícharo & Teodósio, 1991a - February-July 1988). Moreover, Faria et al. (2006), in work performed simultaneously with this study, made an inter-annual comparison between river inflow and ichthyoplankton abundance, reporting that the abundance of gobies (*Pomatochistus* spp.) increased in comparison to anchovy larvae (Chícharo & Teodósio, 1991b). Faria et al. (2006) associated the abundance of these species with their life cycle strategies and with the enhanced productivity effect of river inflow (Bergeron, 2004). Then, 2002 could be a less productive year than 1988. Several works positively relate the intensity of river inflow with abundance of estuarine and coastal fish and crustaceans (Loneragan & Bunn, 1999; Quiñones & Montes, 2001; Nixon, 2003), some stating that, if in appropriate quantities and at suitable times, stronger river inflow may be favourable to fisheries, either by enhanced production mechanisms (Bergeron, 2004) or by promoting organism concentration and catchability (Loneragan & Bunn, 1999).

4.4. Influence of the filling of the Alqueva reservoir on the Guadiana estuary

Prior to construction of the Alqueva dam, nutrient stoichiometry in the upper Guadiana estuary evolved from potential P limitation during winter, to Si limitation during spring to mid summer and to N limitation from mid summer to autumn. For the period after the construction of the dam, it was foreseen an increased dominance of cyanobacteria in the upper estuary (Rocha et al., 2002; Domingues et al., 2005). According to the available data, Rocha et al. (2002) stated that low Si-loading and low N:P ratios, plus high water temperatures, constitute the main triggering features for the occurrence of cyanobacteria blooms in the upper estuary. However, Domingues (unpublished data) later found that even with high Si concentrations, high Si:N and low N:P, cyanobacteria dominate the phytoplankton community, and deduced that the transition to a cyanobacteria-dominated community has to rely on other factors than those that were considered. The concern regarding the occurrence of cyanobacteria blooms is prompted by the presence of several potentially toxic species (*e.g. Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Oscillatoria* spp. and *Anabaena* spp.; Caetano et al. (2001)). Such blooms are frequently related to the death of aquatic and terrestrial animals and to problems of public health (Vasconcelos, 1999).

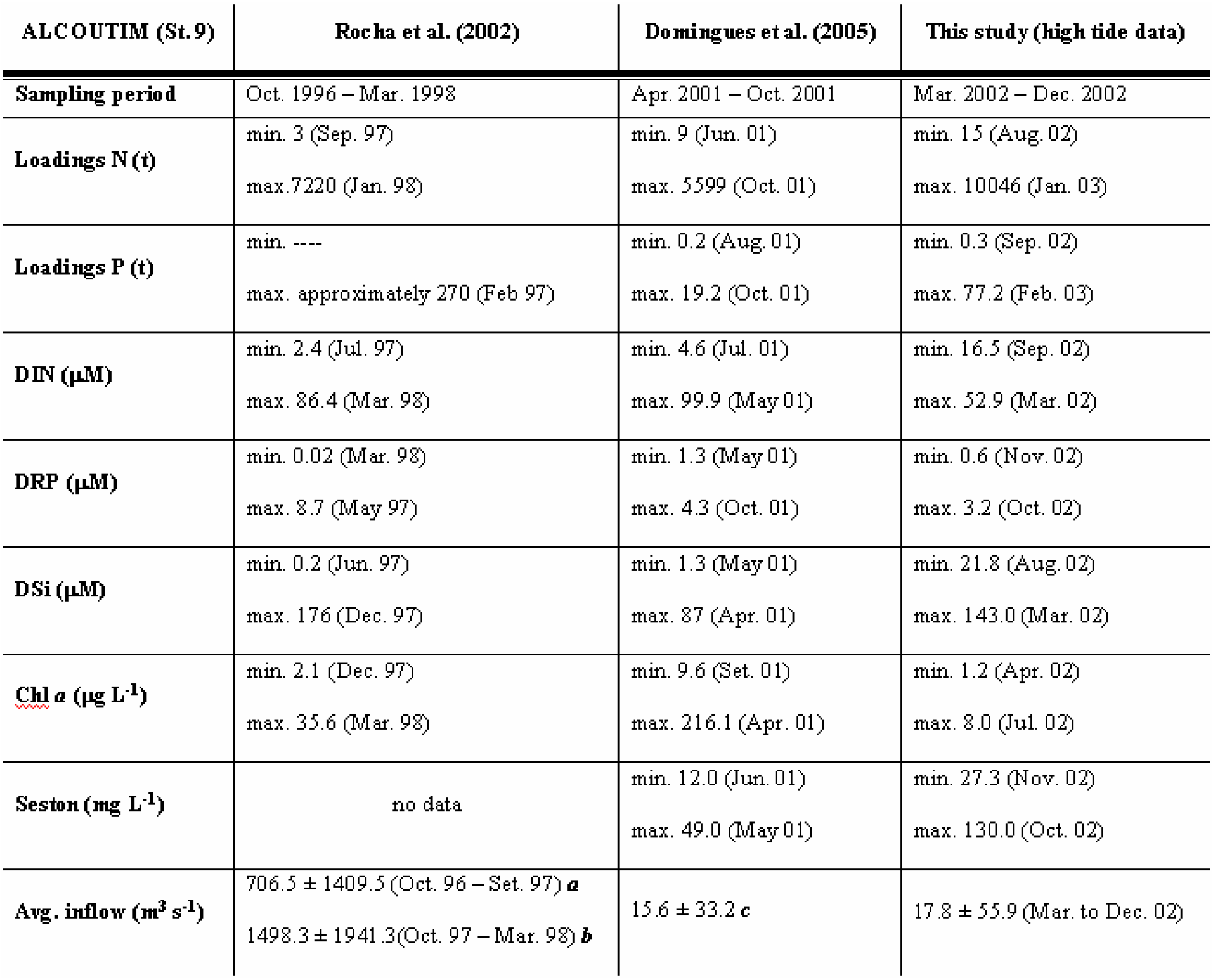
During the first year of the filling of the reservoir (March 2002 to February 2003), the above-mentioned seasonal pattern in the evolution of the nutrient stoichiometry was not observed. Throughout this period, the high and middle estuary tended to be more P limited and potential Si limitation was frequent only in the coastal area. It was expected that the concentration of DSi would decrease after the construction of the Alqueva dam; however, during reservoir filling the converse has happened. The lowest concentration (21.8 μM) increased 70.5 times and the maximum was only 19% lower (143.0 μM), when compared with a year of higher river flow (Table II) (Rocha et al., 2002). Increased soil displacement and the inundation of vast terrestrial areas are the putative reasons for such an occurrence, increasing the minimum DSi concentration and setting the maximum concentration similar to that recorded in a year of high river inflow (Domingues et al., in press).

Maximum and minimum DIN and DRP concentrations coincided with their maximum and minimum loadings. The lowest concentration of DIN continued to be concomitant with a cyanobacteria bloom (Rocha et al., 2002; Domingues et al., 2005; Domingues, unpublished data), precisely in summer when the upper estuary was more nitrogen limited. DRP concentration peaked from August to October, like that of DIN, even though there was no significant increase in their loading. Remineralization is likely to have occurred, since this reaction is temperature dependent and the source of organic matter (Humborg et al., 2000) might have been the collapse of a cyanobacteria bloom (Domingues, unpublished data).

This change in nutrient stoichiometry and concentration (Table II) implies shifts in phytoplankton dynamics. Rocha et al. (2002) and Domingues et al (2005) observed a spring bloom of diatoms, corresponding to maximum chlorophyll *a* concentrations of 35.6 and 216.1 $\mu g.L^{-1}$ (Table II). However, during reservoir filling, the common spring bloom of diatoms did not occur and cyanobacteria bloomed from summer to winter (Domingues, unpublished data). The maximum chlorophyll *a* concentration in the present study at high tide (with the same tidal moment as in the studies mentioned), was 8.0 $\mu g.L^{-1}$ (12.3 $\mu g.L^{-1}$ at low tide) and was determined in July 2002, when cyanobacteria were blooming. Microcystin concentration had increased 5.8 times, up to 1010 $ng.L^{-1}$ (study period from March to May 2002 - Sobrino et al., 2004), when compared with 1999, a year with similar average river inflow (25.2 ± 79.6 $m^3.s^{-1}$) (study period from March to October 1999 - Caetano et al., 2001).

From March 2002 to February 2003, the typical food web, diatom → zooplankton → fish (DZF), was replaced by a non-DZF food web, which was based on cyanobacteria from summer to winter (Domingues, unpublished data). A recurrence of non-DZF food webs might be more frequent after the development of the Alqueva irrigation plan, which will affect 110,000 ha. This is a real threat to aquatic ecosystems and can not be neglected. There is a worldwide trend in aquatic ecosystems for increased P and Si limitation and a higher incidence of noxious planktonic blooms, generally an outcome of increased eutrophication due to higher nitrate loading (Turner et al., 2003). The ingredients to promote increased degradation of water quality in the Guadiana basin may be a reality in the near future. Thus, “How to prevent such a trend in the Guadiana basin?” is a question that should concern all, from local populations, to scientists and policy makers. A broad ecohydrological approach to management might be a potential solution to minimize the threats to the Guadiana basin, which has up to now been one of the more preserved Portuguese river basins (Vasconcelos et al. 2007).

Table II Comparison of minimum and maximum values of N and P loadings, DIN, DRP, DSi, chlorophyll *a* and seston concentrations determined in Alcoutim (St. 9) by Rocha et al. (2002), Domingues et al. (2005) and Morais et al. (this study). To allow more accurate comparisons, only high tide data were assessed. This study of average river inflow corresponds to the period from late February (previous month to first sampling) to December 2002 sampling. Legend- No daily average river flow for the following periods: ***a*** 30 Nov., 28 Feb.-2 Mar.; 7-17 Mar.; 12-19 May; 1 Jun.-14 Jul.; 23 Jul.-18 Aug.; 24 Aug.-30 Sep.; ***b*** 3-7 Nov. 97 and 31 Mar. 98; ***c*** 1-25 Apr.



ALCOUTIM (St. 9)	**Rocha et al. (2002)**	**Domingues et al. (2005)**	**This study (high tide data)**
Sampling period	Oct. 1996 – Mar. 1998	Apr. 2001 – Oct. 2001	Mar. 2002 – Dec. 2002
Loadings N (t)	min. 3 (Sep. 97) max.7220 (Jan. 98)	min. 9 (Jun. 01) max. 5599 (Oct. 01)	min. 15 (Aug. 02) max. 10046 (Jan. 03)
Loadings P (t)	min. ---- max. approximately 270 (Feb 97)	min. 0.2 (Aug. 01) max. 19.2 (Oct. 01)	min. 0.3 (Sep. 02) max. 77.2 (Feb. 03)
DIN (µM)	min. 2.4 (Jul. 97) max. 86.4 (Mar. 98)	min. 4.6 (Jul. 01) max. 99.9 (May 01)	min. 16.5 (Sep. 02) max. 52.9 (Mar. 02)
DRP (µM)	min. 0.02 (Mar. 98) max. 8.7 (May 97)	min. 1.3 (May 01) max. 4.3 (Oct. 01)	min. 0.6 (Nov. 02) max. 3.2 (Oct. 02)
DSi (µM)	min. 0.2 (Jun. 97) max. 176 (Dec. 97)	min. 1.3 (May 01) max. 87 (Apr. 01)	min. 21.8 (Aug. 02) max. 143.0 (Mar. 02)
Chl *a* (µg L^{-1})	min. 2.1 (Dec. 97) max. 35.6 (Mar. 98)	min. 9.6 (Set. 01) max. 216.1 (Apr. 01)	min. 1.2 (Apr. 02) max. 8.0 (Jul. 02)
Seston (mg L^{-1})	no data	min. 12.0 (Jun. 01) max. 49.0 (May 01)	min. 27.3 (Nov. 02) max. 130.0 (Oct. 02)
Avg. inflow ($m^3\ s^{-1}$)	706.5 ± 1409.5 (Oct. 96 – Set. 97) ***a*** 1498.3 ± 1941.3(Oct. 97 – Mar. 98) ***b***	15.6 ± 33.2 ***c***	17.8 ± 55.9 (Mar. to Dec. 02)

5. Conclusions

River inflow was the most important factor in determining abiotic and biotic variability in the Guadiana estuary and in the adjacent coastal area, during the unique period of the filling of the Alqueva dam. The seasonality effect was overlaid by long periods of low inflow (mid April to early December 2002).

The shifts in river inflow were fundamental in drastic changes to abiotic and biotic water parameters and to abundance and distribution of anchovy eggs. Continuous river inflows of approximately 50 $m^3.s^{-1}$ were enough to produce a similar nutrient stoichiometry along the Guadiana estuary, whereas long periods of low inflow ended in a marked abiotic and biotic gradient.

River inflow management can be a useful tool to resolve downstream ecological constraints, but bad management practises may increase such constraints. The assessment of anchovy eggs seems to be an excellent indicator of the effects of river inflow.

The filling of the Alqueva dam was probably responsible for causing marked changes in the estuary. The ETM zone moved to the upper estuary, to at least 38 km from the river mouth. The nutrient stoichiometry dynamics also changed in the high and middle estuary, where it was more N limited than P limited during the whole year, and Si limitation was only frequent on the coast. The flooding of vast areas was probably responsible for increasing DSi minimum and maximum concentrations, which was slightly smaller than the maximum observed during a year of high inflow.

After the filling of the Alqueva dam, it is advisable that dam managers mimic the natural river flow as much as possible, in order to minimize the impact on downstream ecosystems. Moreover, dam managers should not be restricted to upstream of the dam wall, but their management should begin in the distant coastal area.

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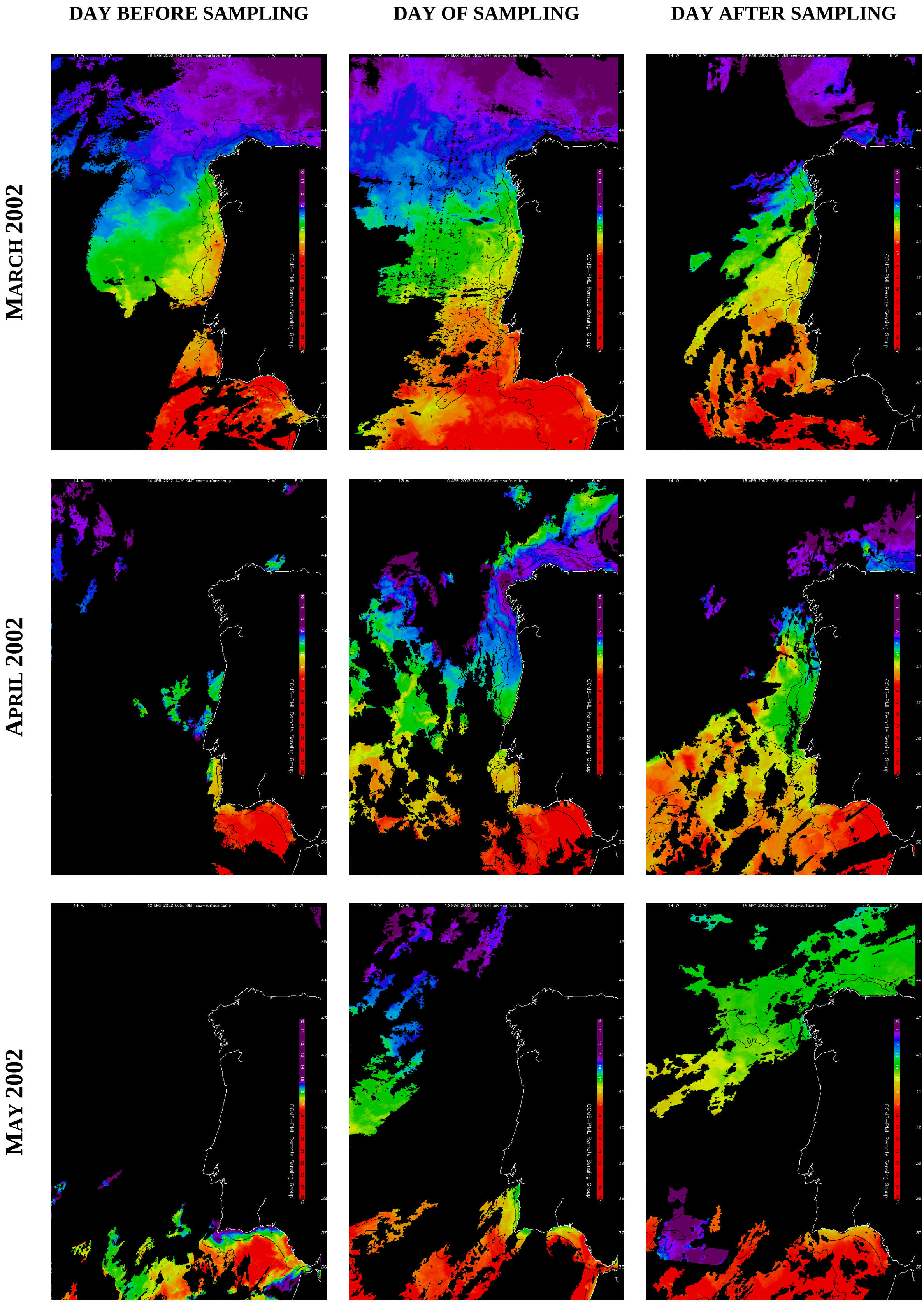
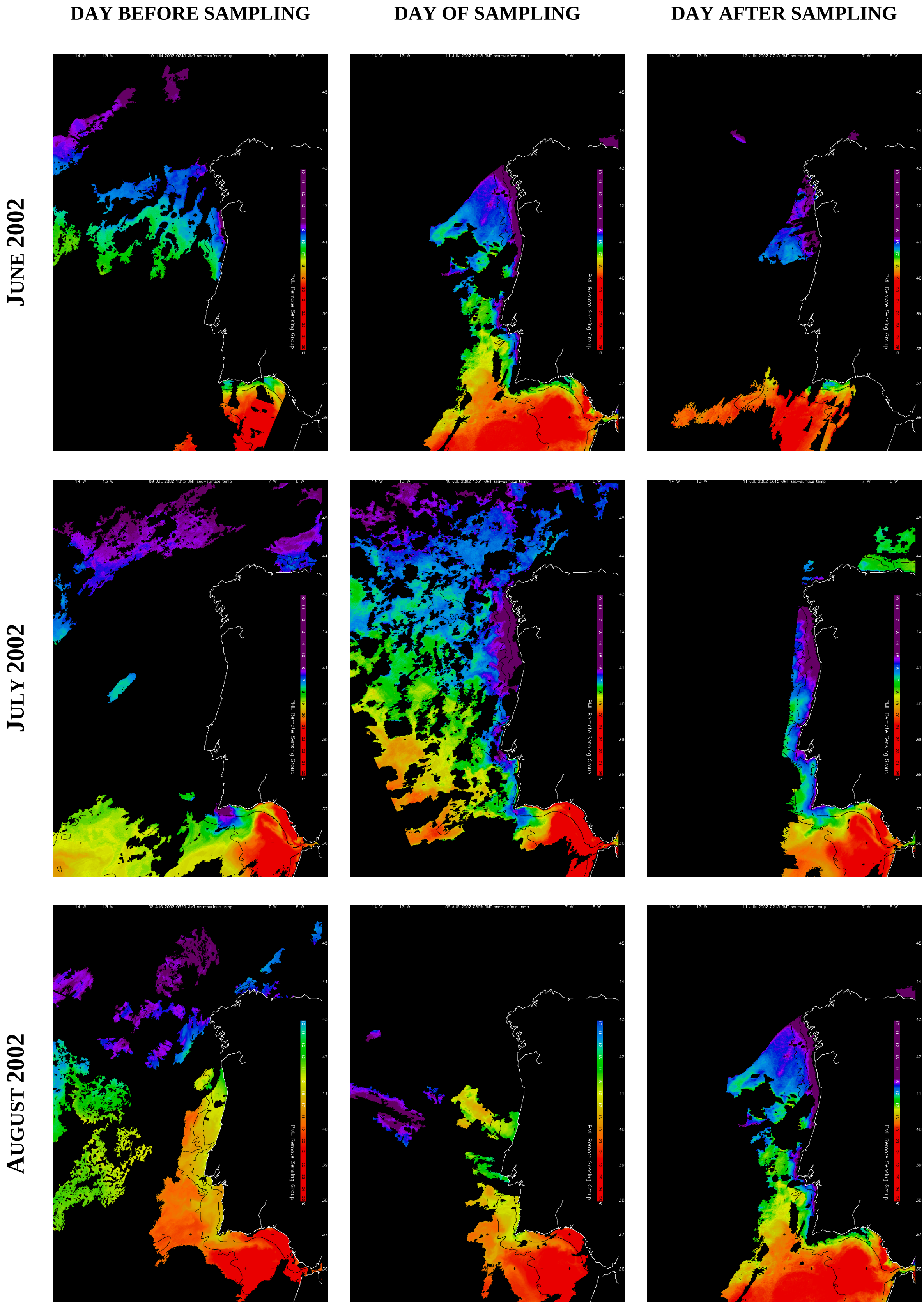
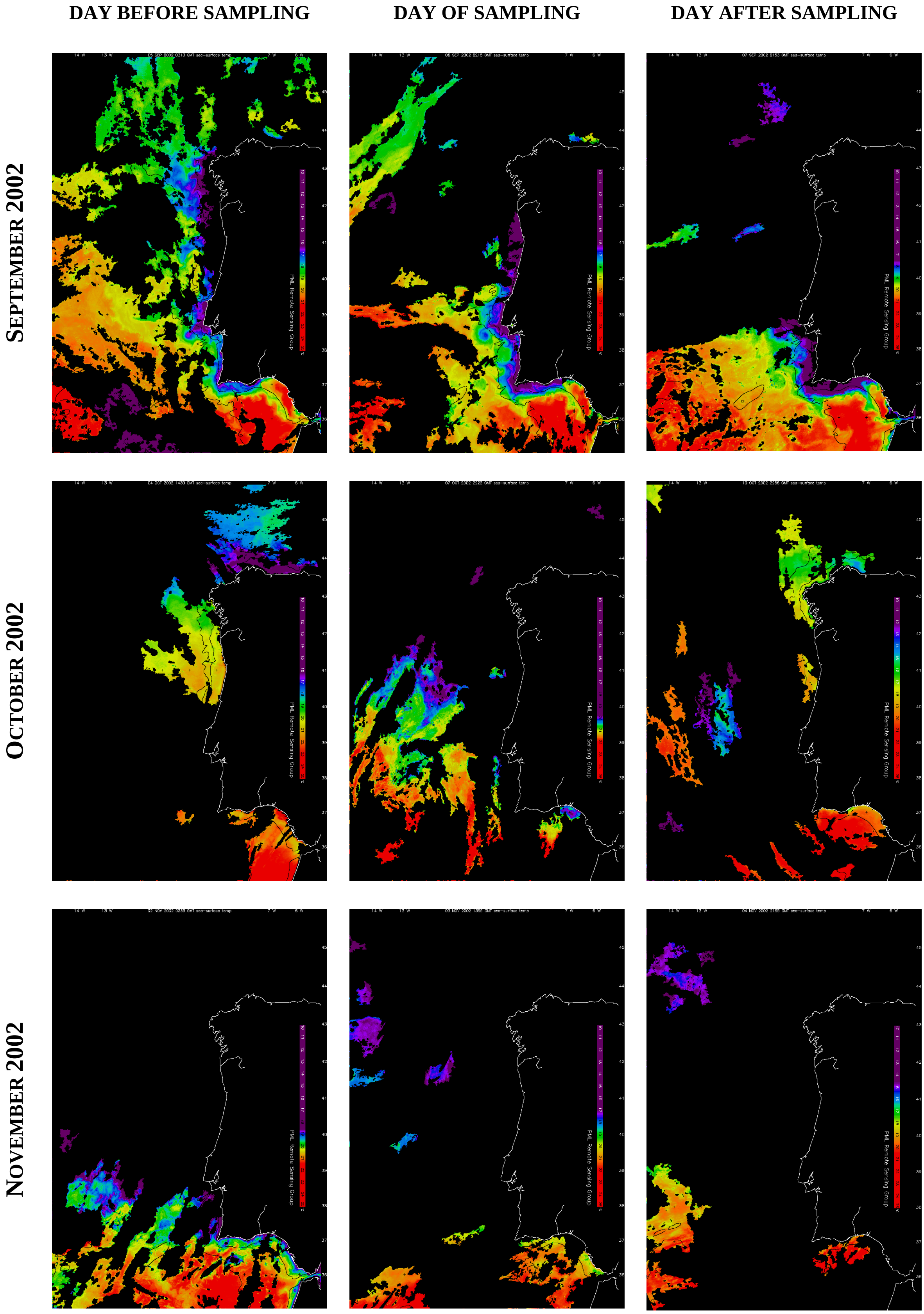


Annex I Sea surface temperature obtained with NOAA 17 satellite. For each month of sampling (March 2002 to February 2003), it is shown the photographs from the day before, during and after sampling.



Annex I (continuation) Sea surface temperature obtained with NOAA 17 satellite. For each month of sampling (March 2002 to February 2003), it is shown the photographs from the day before, during and after sampling.



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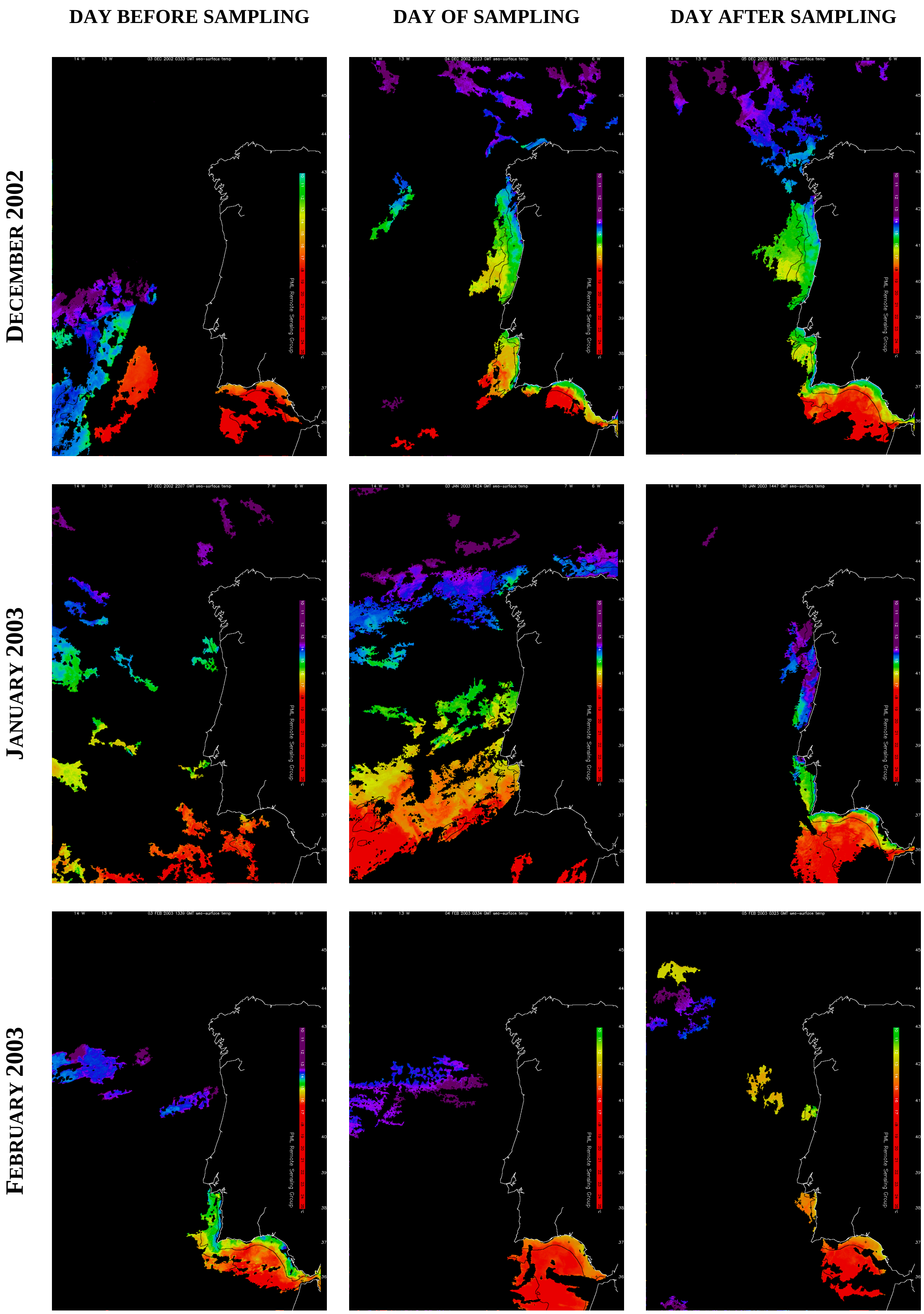

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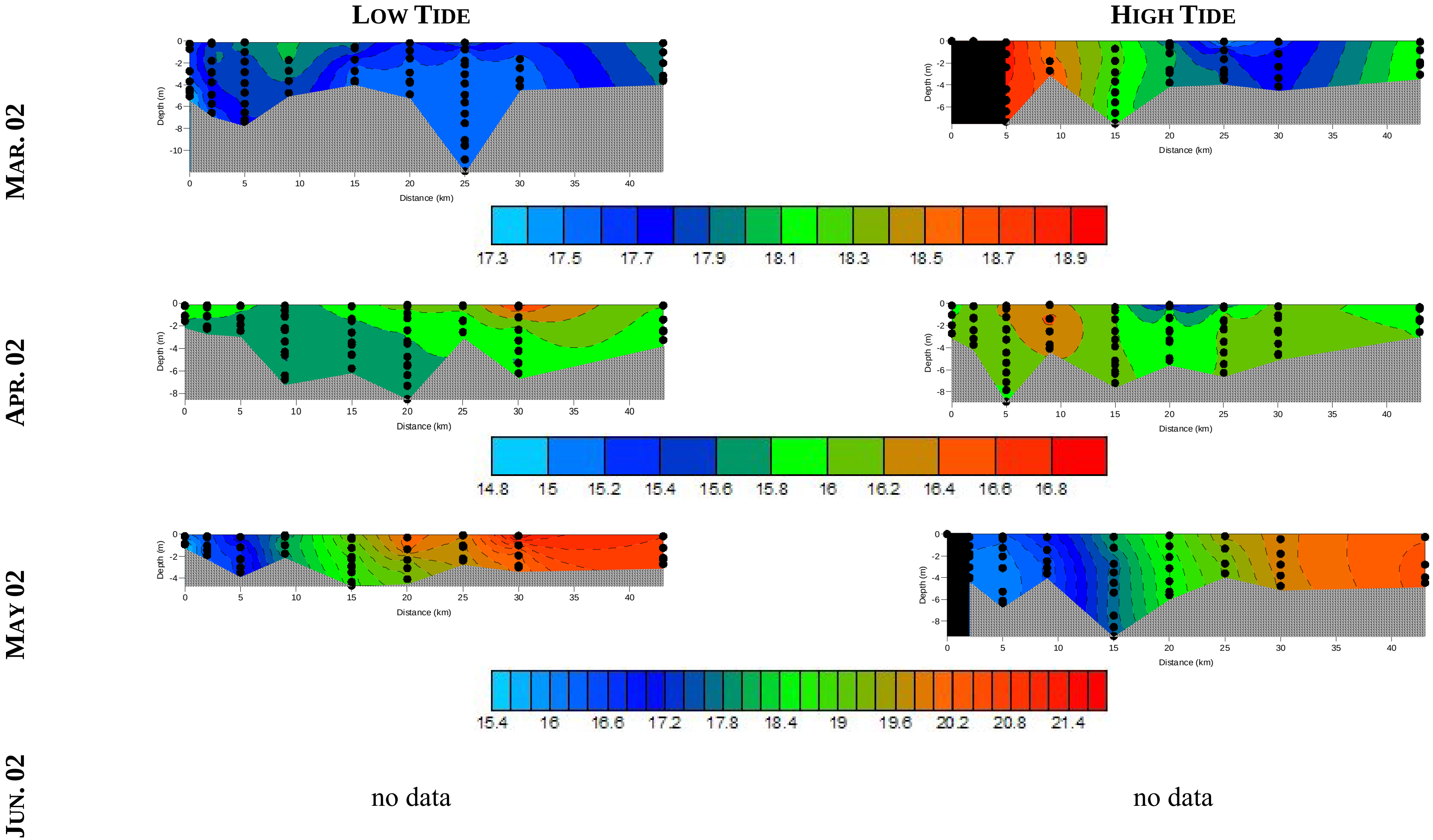
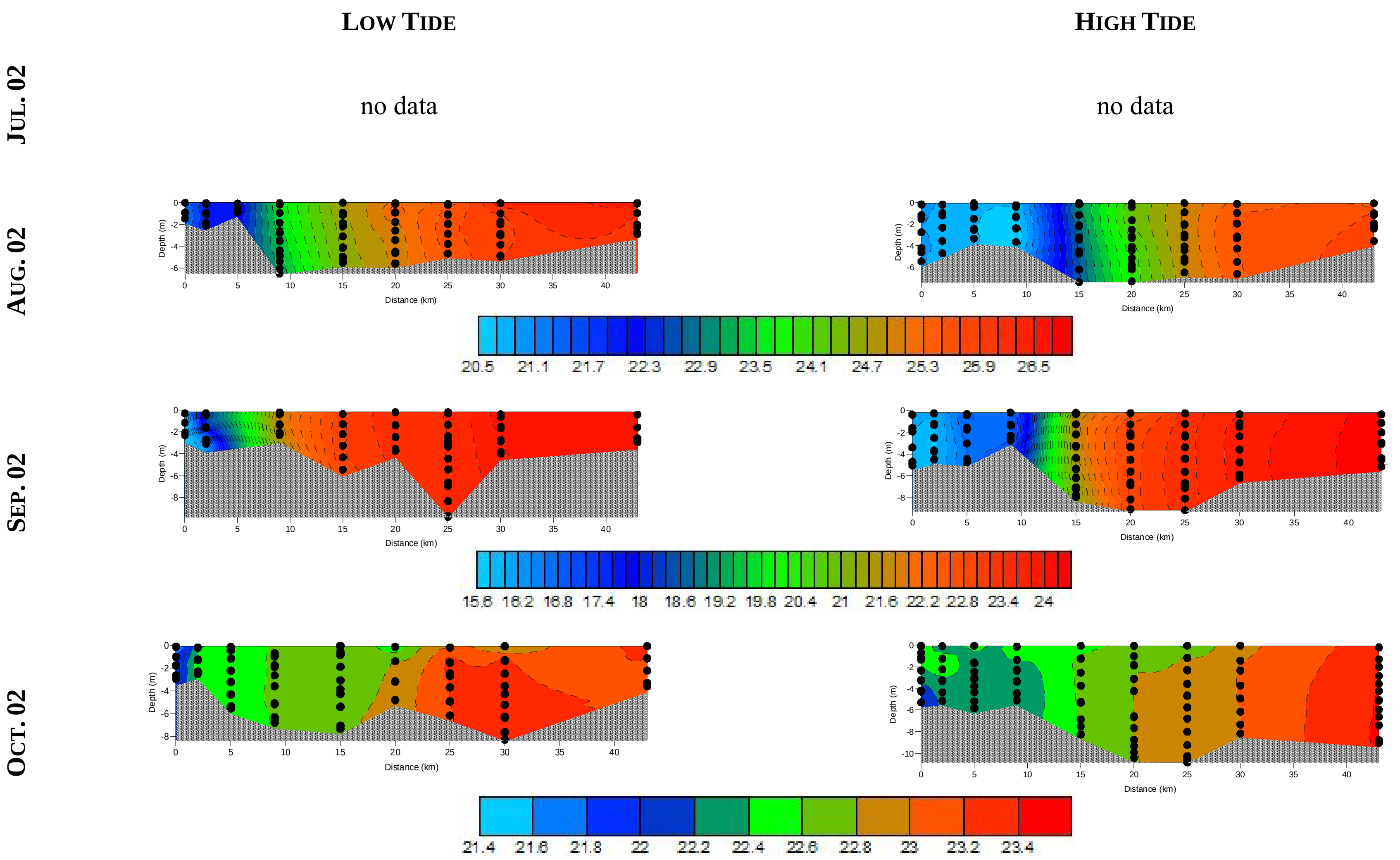
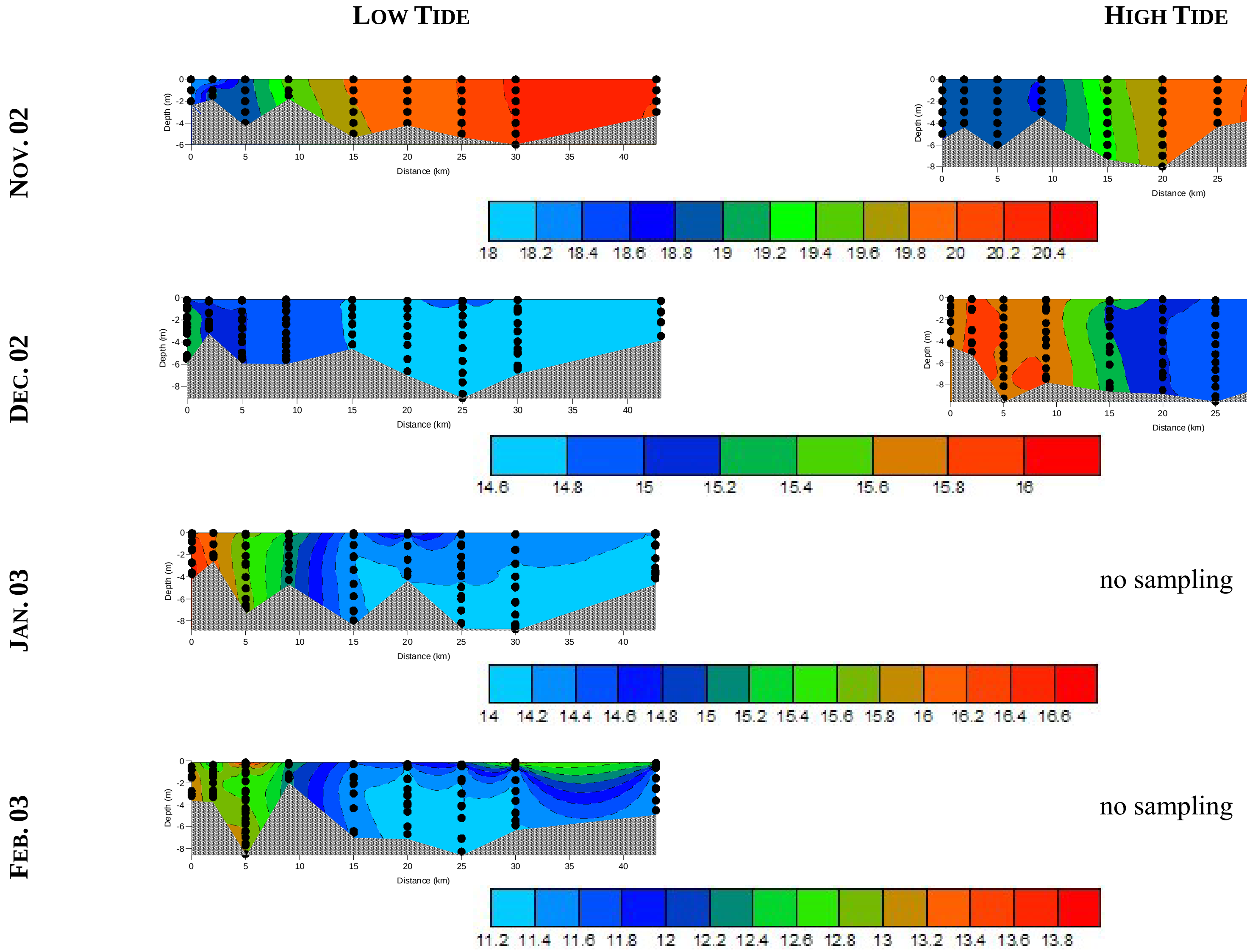


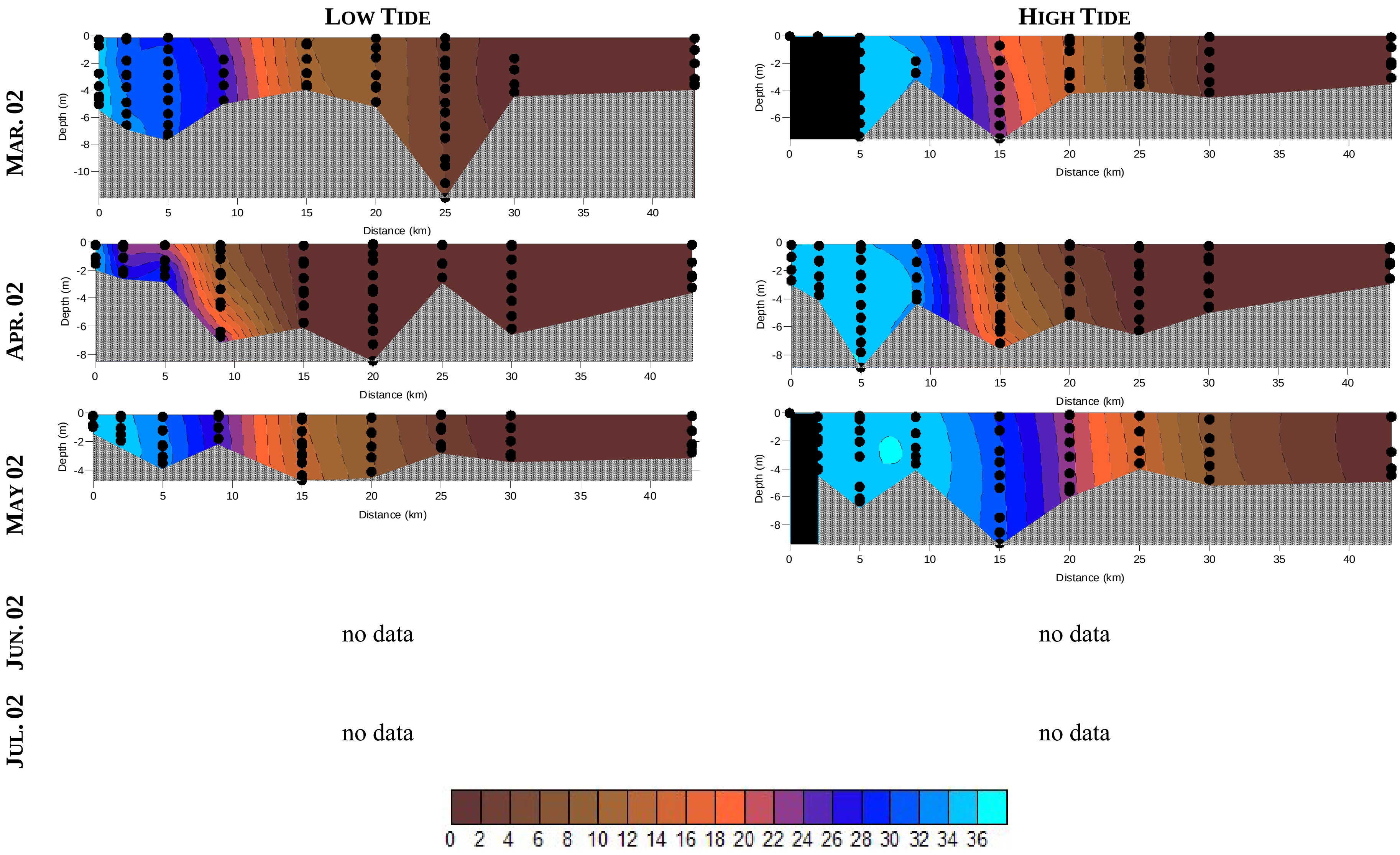
Annex II Vertical profiles of temperature (ºC) from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



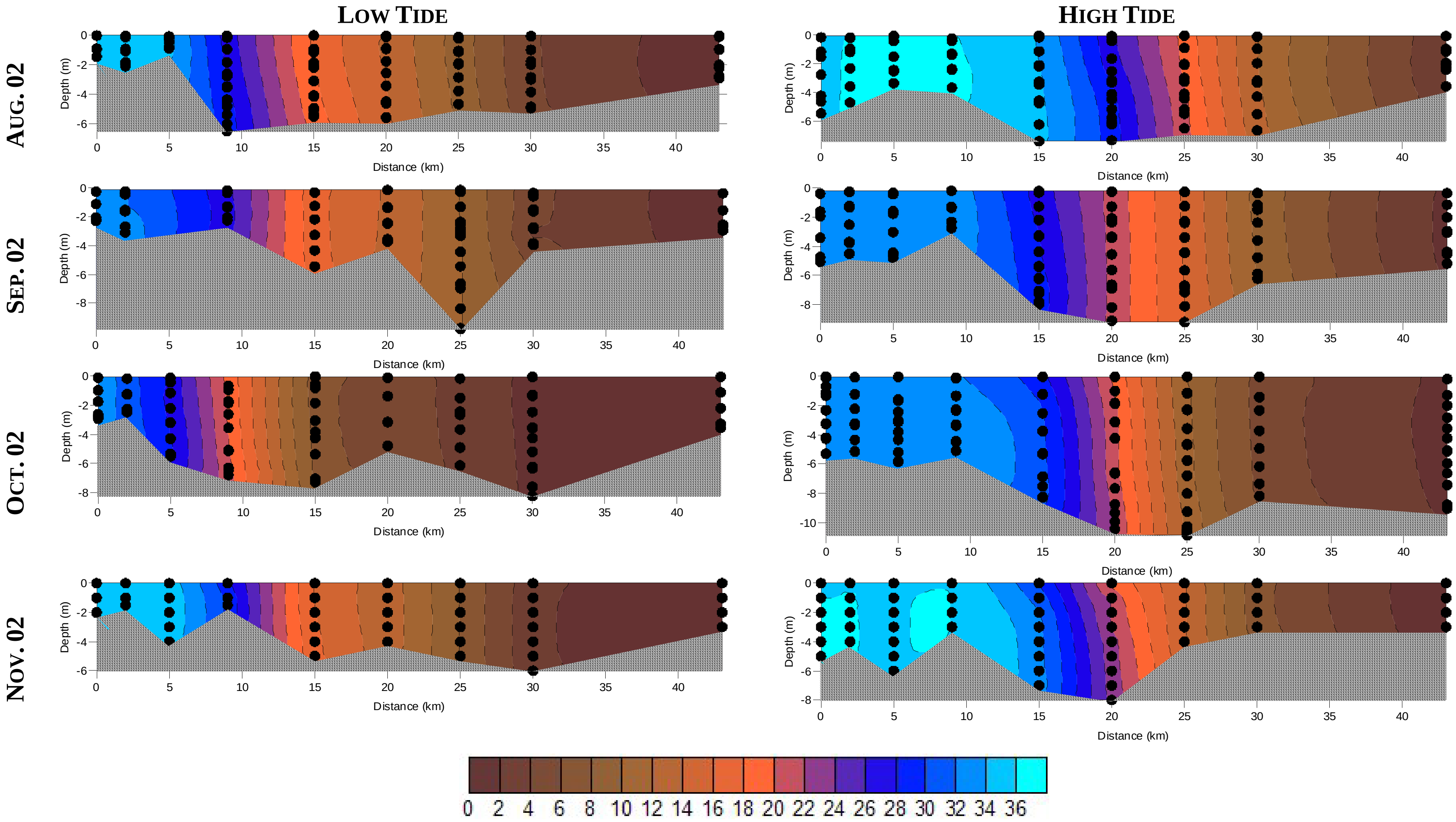
Annex II (continuation) Vertical profiles of temperature (ºC) from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



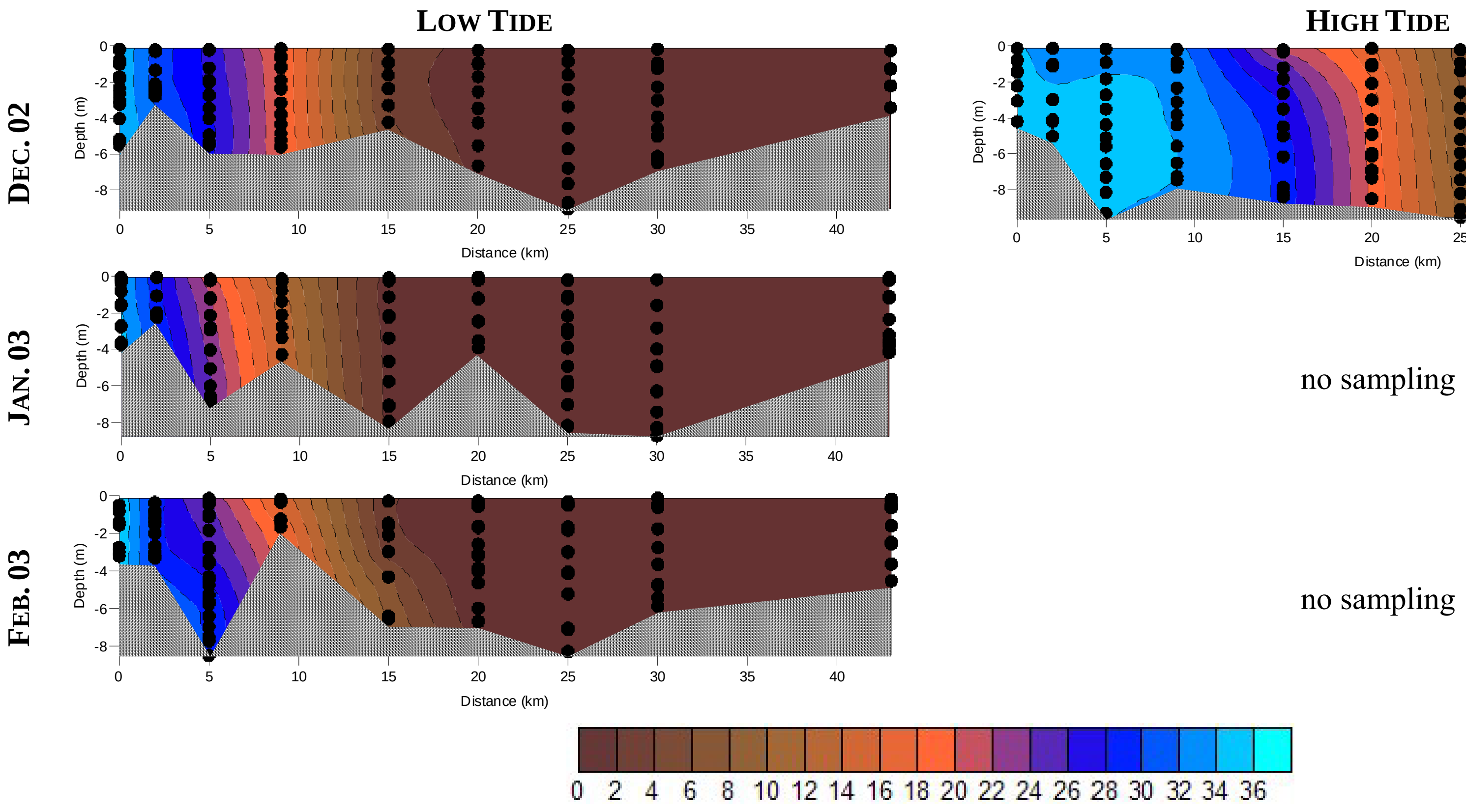
Annex II (continuation) Vertical profiles of temperature (ºC) from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



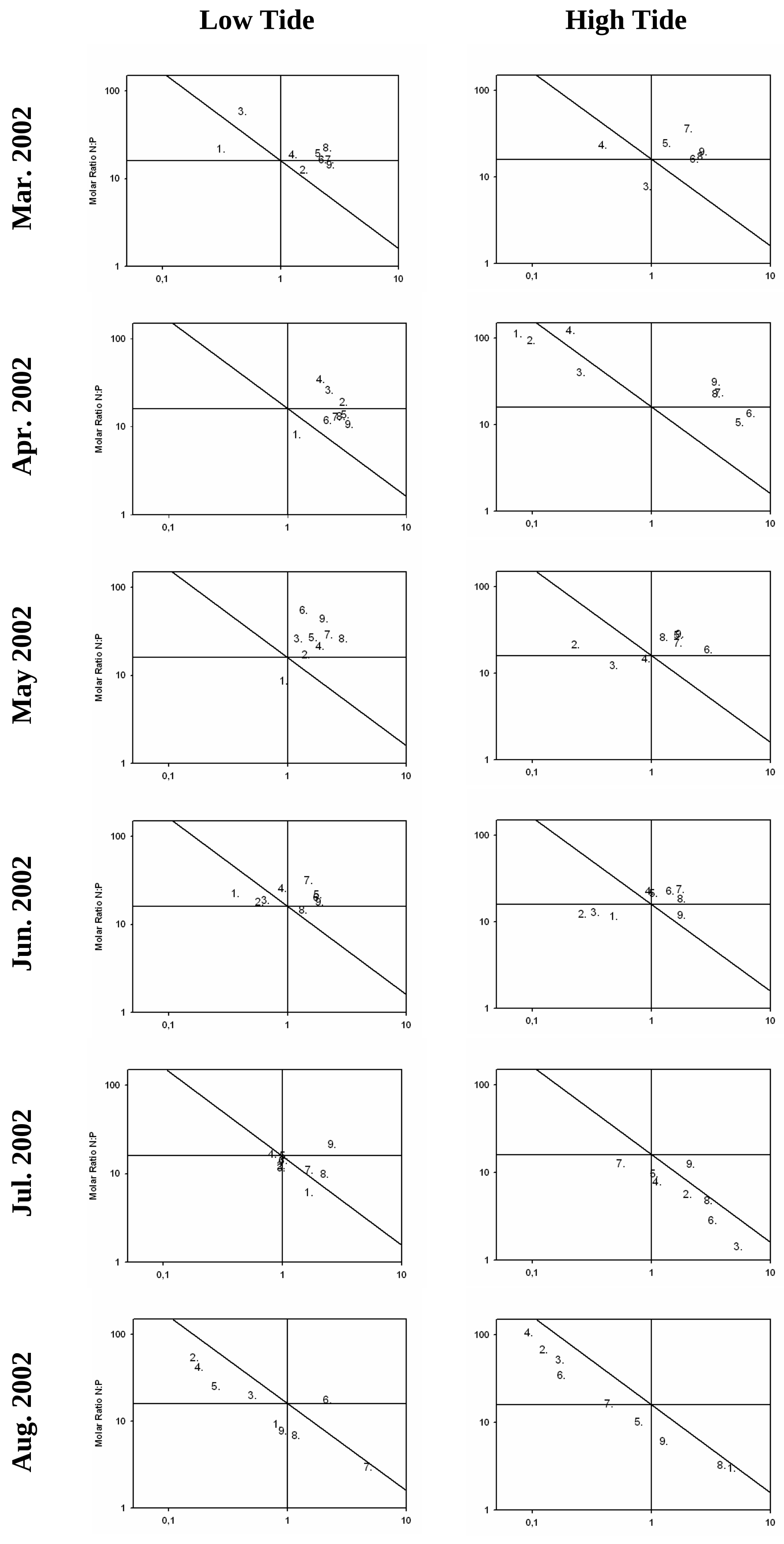
Annex III Vertical profiles of salinity from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



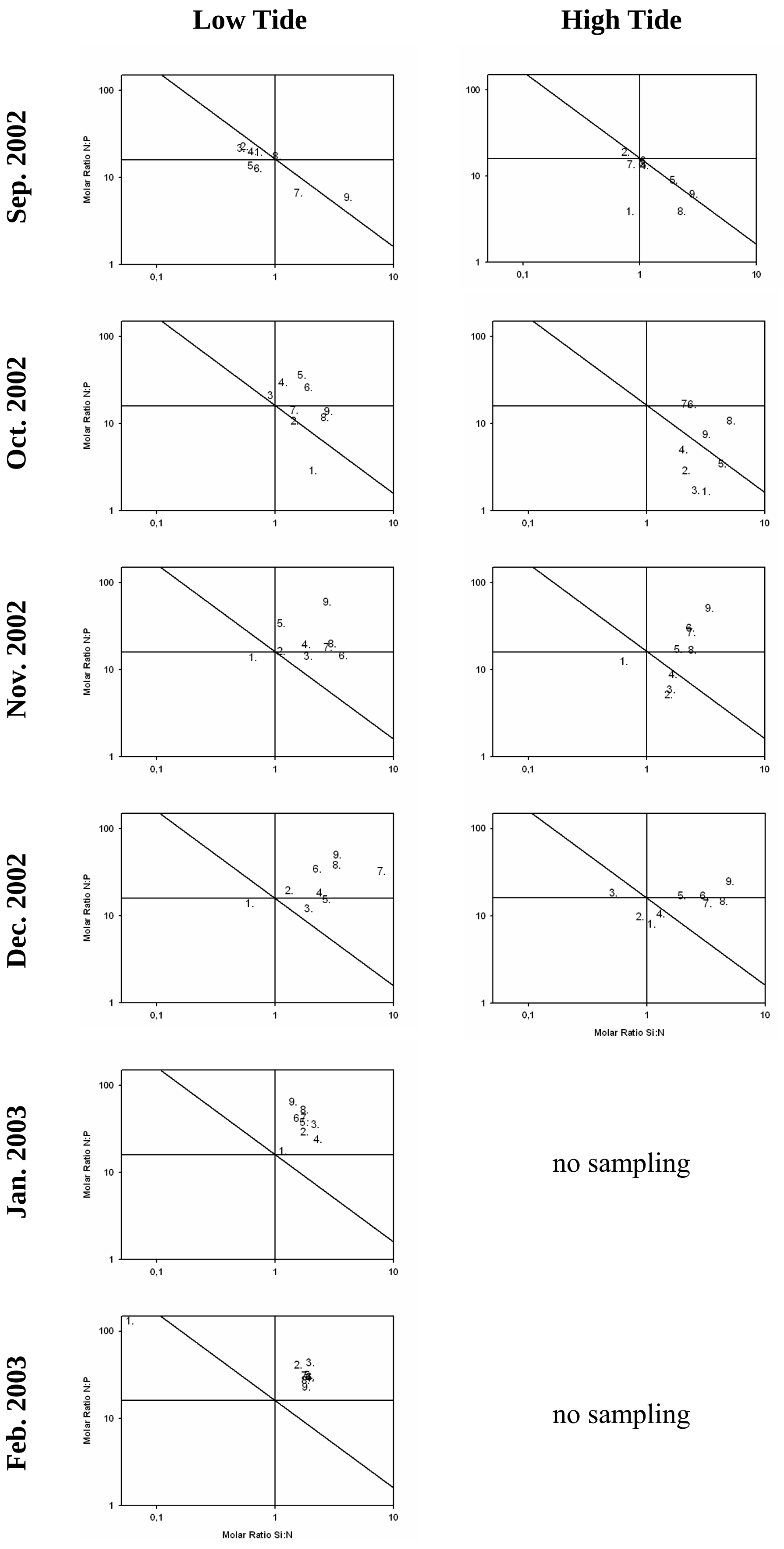
Annex III (continuation) Vertical profiles of salinity from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



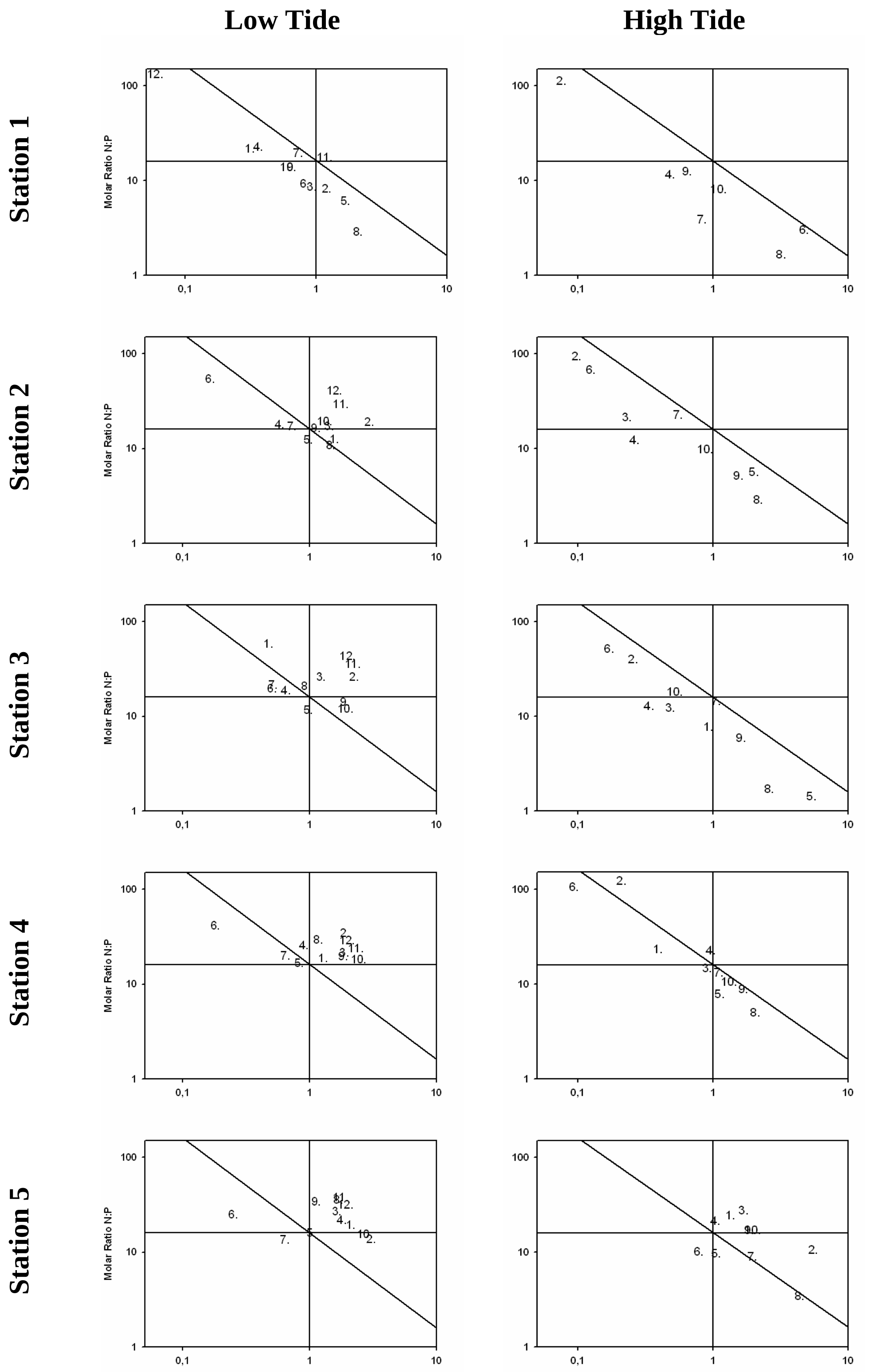
Annex III (continuation) Vertical profiles of salinity from the coastal area of the Guadiana estuary till Alcoutim, from March 2002 to February 2003.



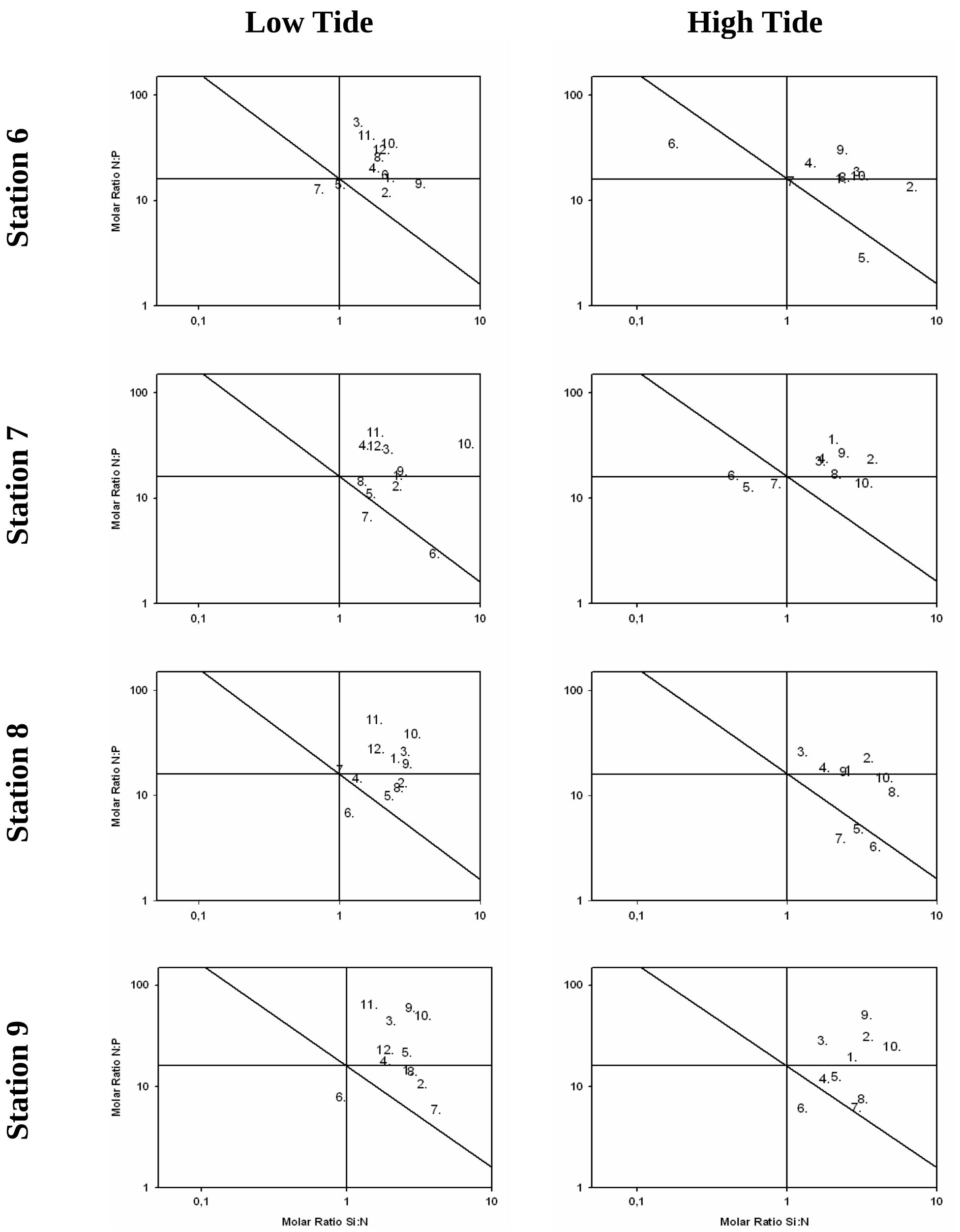
Annex IV Nutrient stoichiometry along the Guadiana estuary from March 2002 till February 2003 at the low and high tide. In each graph, the numbers correspond to the number of the sampling station.



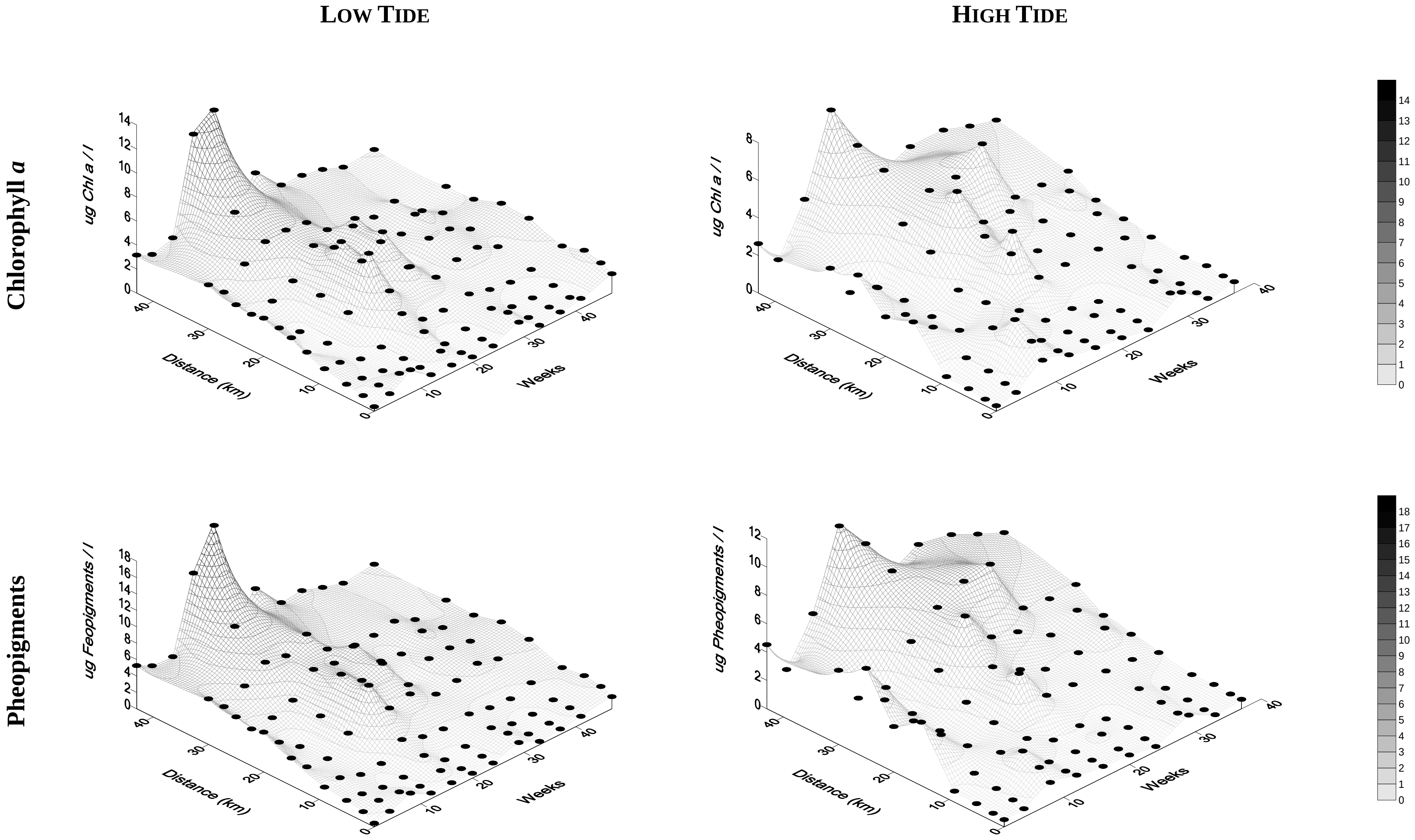
Annex IV (continuation) Nutrient stoichiometry along the Guadiana estuary from March 2002 till February 2003 at the low and high tide. In each graph, the numbers correspond to the number of the sampling station.



Annex V Nutrient stoichiometry from March 2002 till February 2003 in each sampling station, at the low and high tide. Legend: 1- March 2002, 2- April 2002, 3- May 2002, 4- June 2002, 5- July 2002, 6- August 2002, 7- September 2002, 8- October 2002, 9- November 2002, 10- December 2002, 11- January 03, 12- February 2003.



Annex V (continuation) Nutrient stoichiometry from March 2002 till February 2003 in each sampling station, at the low and high tide. Legend: 1- March 2002, 2- April 2002, 3- May 2002, 4- June 2002, 5- July 2002, 6- August 2002, 7- September 2002, 8- October 2002, 9- November 2002, 10- December 2002, 11- January 03, 12- February 2003.



Annex VI Chlorophyll *a* (µg.L^{-1}) and Phaeopigments concentration (µg.L^{-1}) from the coastal area adjacent to the Guadiana estuary till Alcoutim, from March 2002 till February 2003, at the low and high tides.

Chapter 3

The life cycle of **Engraulis encrasicolus sensu lato** *in the Guadiana estuary*

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Abstract

Until recently it was thought that *Engraulis encrasicolus* was the anchovy species present in the Guadiana estuary. The description of a new estuarine anchovy species, *Engraulis albidus*, led to question about which species is present in this estuary, *E. encrasicolus or E. albidus*? Moreover, a multidisciplinary approach was made to investigate the life cycle of anchovy in the Guadiana estuary. The presence of *E. albidus* was not confirmed for the Guadiana estuary and for the adjacent coastal area. However, the majority of the analysed specimens are genetically distinct from *E. encrasicolus*. Thus, the anchovy present in the Guadiana estuary has to be classified as *Engraulis encrasicolus sensu lato*. The genetic difference between the anchovy collected in the Guadiana estuary and in the adjacent coastal area is not significant. Data from oxygen and carbon isotopes on the otoliths revealed significant differences between sites, suggesting that they are two populations. The spawning of anchovy occurs inside the Guadiana estuary, by resident specimens. Periods of low river flow, *ca.* 3.2 ± 0.1 $m^3.s^{-1}$, sets the transition between the low and mid estuary as the spawning ground, which is probably the ideal spawning area. The transition between the middle and upper estuary is the nursery area. The distribution of anchovy juveniles and adults is intrinsically linked with river flow. Anchovies were collected at the entrance of the estuary and plume region, only during a flood event in January 2001 ($Q > 3000\ m^3.s^{-1}$). A differential distribution of juveniles and adults was observed during Summer ($Q < 8m^3.s^{-1}$), when juveniles were mainly in the middle and upper estuary, while adults were from the middle estuary to the upper part of the low estuary. The concentration of Fe and Zn along the otoliths was not suitable to conjecture on the migration patterns of anchovy. The analyses of Sr concentration allowed identifying two distinct patterns for the estuarine specimens: *i*) no migration along estuarine salinity gradients *ii*), migration to high salinity estuarine regions,

after spending the beginning of the life cycle in those with low salinity. Anchovies captured in the coast have probably born in an estuarine environment, then progressively migrated along an estuary to the coast.

1. Introduction

1.1. The family Engraulidae and the genus Engraulis

The family Engraulidae (Kingdom Animalia; Phylum Chordata; Class Actinopterygii; Order Clupeiformes) comprises 143 species of 16 genera (*Amazonsprattus*, *Anchoa*, *Anchovia*, *Anchiovella*, *Cetengraulis*, *Coilia*, *Encrasicholina*, *Engraulis*, *Jurengraulis*, *Lycengraulis*, *Lycothrissa*, *Papuengraulis*, *Pterengraulis*, *Setipinna*, *Stolephorus* and *Thryssa*). The common name for the majority of the species is anchovy. This family has species distributed along the Atlantic, Indian and Pacific oceans and in their adjacent seas. There are also species inhabiting in brackish and freshwater ecosystems (Froese & Pauly, 2006).

Among the Engraulidae, the genus *Engraulis* Cuvier 1817 contributes the most to global fisheries landings (FAO, 2007). Therefore, *Engraulis* spp. (*E. anchoita* Hubbs & Marini, 1935 - Argentine anchovy; *E. australis* (White, 1790) - Australian anchovy; *E. encrasicolus* (Linnaeus, 1758) - European anchovy; *E. eurystole* (Swain & Meek, 1885) - Silver anchovy; *E. japonicus* Temminck & Schlegel, 1846 - Japanese anchovy; *E. mordax* Girard, 1854 - Californian anchovy; *E. ringens* Jenyns, 1842 - Peruvian anchovy) have meaningful economical (Bariche et al., 2006) and ecological impacts (Zhao et al., 2003; Crawford et al., 2006; García-Godos & Goya, 2006; Thayer & Sydeman, 2007) due to the enormous populations that exist in many coastal regions of the world (Fig. 1) (FAO, 2007). Engraulidae landings averaged 12.2 x 10^6 ton (± 1.7 x 10^6 ton) in the period 2001-2005, representing, on average, 11.5 ± 1.3 % of global fish landings. However, it was during the 1960's that this family contributed the most to world fish landings, averaging 22.3 ± 2.2 % (Fig. 2) (FAO, 2007). Anchovies are sold fresh, dried, smoked and as canned fish for human consumption; it is also used to produce fish flour and fish bait.

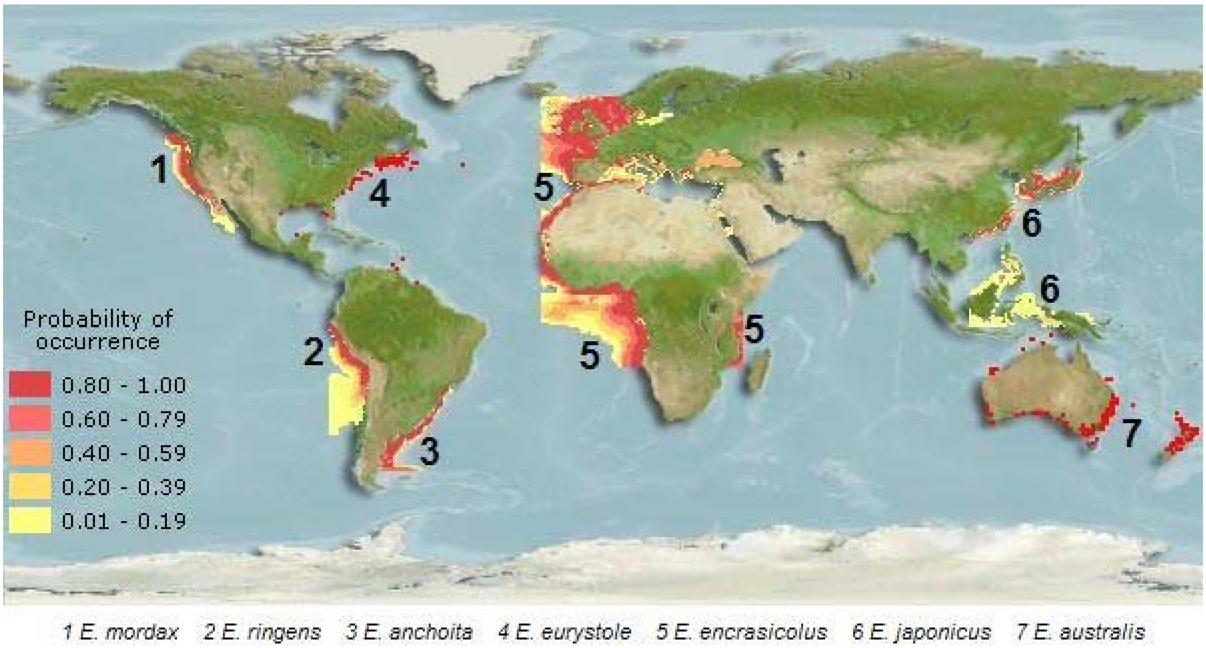


Fig. 1 Geographical distribution of 7 species of the genus *Engraulis*. Modified from Froese & Pauly (2006).

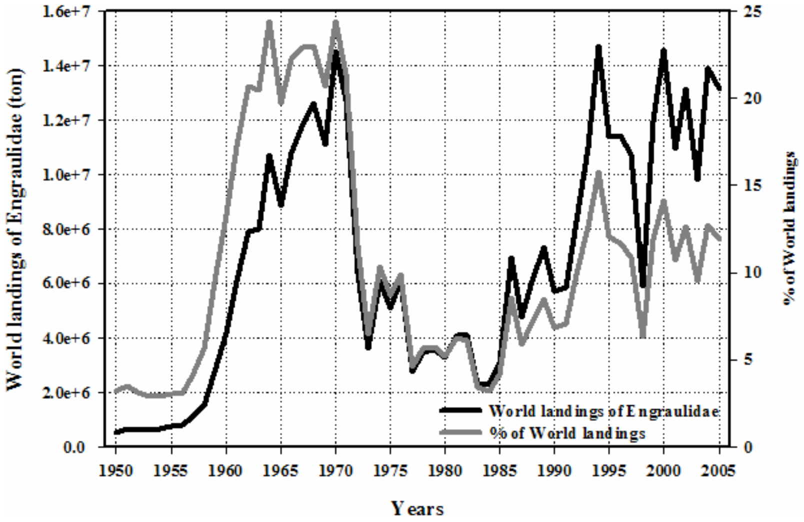


Fig. 2 World landings (ton) of Engraulidae and its relative contribution (%). Data source: FAO (2007).

1.2. The species Engraulis encrasicolus *(Linnaeus, 1758)*

The species *Engraulis encrasicolus* (Linnaeus, 1758) (European anchovy - Table I, Fig. 3) is the one of its genus with widest distribution. It is a pelagic species and its habitat was described as extending from coastal areas to lagoons, estuaries and lakes. In coastal waters it is mainly captured in the mesopelagic realm. *E. encrasicolus* is present from the NE-Atlantic (Bergen- Norway) to W-Indian Ocean (Mauritius, Seychelles and in the upwelling area from Northern Mozambique to Southern Somalia); also all of Mediterranean, Black, Azov and Baltic seas. There are stray individuals in Suez Canal and Gulf of Suez; also recorded from St. Helena. The populations present in the SW and E-African coasts are those once reported as *Engraulis capensis* (Grant & Bowen, 1998).

Table I Taxonomic hierarchy of *Engraulis encrasicolus* (Linnaeus, 1758) - European anchovy.

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Osteichthyes
Class	Actinopterygii
Subclass	Neopterygii
Infraclass	Teleostei
Superorder	Clupeomorpha
Order	Clupeiformes
Suborder	Clupeoidei
Family	Engraulidae
Subfamily	Engraulinae
Genus	*Engraulis* Cuvier, 1817
Species	*Engraulis encrasicolus* (Linnaeus, 1758) - European anchovy

It was set that *E. encrasicolus* is a euryhaline species, tolerating salinities ranging from 5 to 41. It is a dioic species with external fertilization and without parental care. It is a partial spawner, *i.e.* the spawning period is broad (usually 6 to 9 months), having oocytes in various stages of development in the ovaries. Spawning is usually maxima at the beginning of the warmer months. The eggs are pelagic and have an ellipsoid shape. They have a slight positive buoyancy (Blaxter, 1969) and since the development is temperature dependent (Lo, 1985), hatching time vary between 24h to 67h. Larvae hatch with *ca*. 3.5 mm and approximately at day 20 (~10 mm) the gas bladder is completely formed, allowing larvae to actively regulate their position in the water column (Ré, 1986). The metamorphosis occurs when larvae reach 35-40 mm, approximately at 60 days (Ré, 1996). The life span is generally 2 years, but it can reach 4 years. The recorded maximum length was included in the size class [18-19[cm. As adults, the swimming mode is carangiform, *i.e*. by movements of body and/or caudal fin. Anchovies are planktivorous, employing filter- and raptorial-feeding modes on phytoplankton and zooplankton (meso- and macro-), which comprises the majority of their diet (Demir, 1965; Plounevez & Champalbert, 2000).

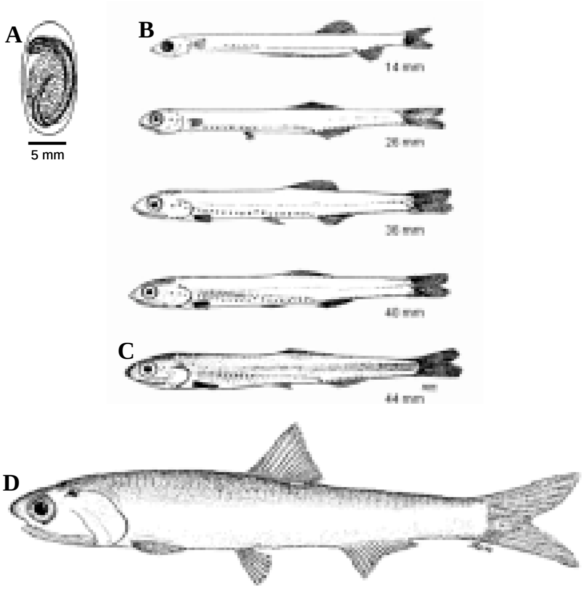


Fig. 3 Illustration of *Engraulis encrasicolus*: egg (A), larvae (B), juvenile (C) and adult (D).
Images from: A- Moser & Ahlstrom (1985); B and C- Arias & Drake (1990); D- Froese & Pauly (2006).

The migratory pattern of *E. encrasicolus* varies according to the regions where the populations are found. However, the pattern usually described for this species states that in March, but mainly from April to June, it occurs in coastal superficial layers (Whitehead et al., 1984). During summer it would approach coastal shallower waters, even penetrating into lagoons and estuaries. Spawning areas have been identified in these locations (Ré, 1984, Ribeiro, 1991, Ré, 1996; Faria et al., 2006). From September to January, they would move to deeper (generally *ca*. 100-150 m) and warmer coastal waters (Whitehead et al., 1984). Nevertheless, circadian vertical migratory patterns were also related with a protective strategy to avoid fish eating birds; thus, they migrate to deeper waters during the day and to the surface during the night (Nikolski, 1963 *in* Demir, 1965).

Table II Chronologically ordered list of synonyms of *Engraulis encrasicolus*.
Adapted from Demir (1965), FAO (2006) and Froese & Pauly (2006).

Synonyms	Author	Local	Status
Clupea encrasicolus	Linnaeus, 1758	"European Ocean"	original combination
Clupea encrasicholus	Lacépède, 1798-1803	?	synonym
Clupea engraticolus	Asso, 1801	?	synonym
Clupea encrassicholus	Delaroche, 1809	?	synonym
Clupea engrasicholus	Ramis, 1814	?	synonym
Engraulis encrasicholus	Cuvier, 1817	?	synonym
Engraulis amara	Risso, 1827	Nice (France)	synonym
Encrasicholus encrasicholus	Fleming, 1828	?	synonym
Engraulis meletta	Cuvier, 1829	Mediterranean Sea	synonym
Engraulis vulgaris	Nilsson, 1832	Sweden	synonym
Engraulis argyrophanus	Valenciennes, 1848	Equatorial Atlantic off W-Africa	synonym
Engraulis encrassicholus	Greals, 1855	?	synonym
Engraulis encrasicholus antipodum	Günther, 1868	Australia, New Zealand, Tasmania	synonym of *E. australis*
Clupea encracicolus	Poggi, 1881	?	synonym
Engraulis capensis	Gilchrist, 1913	Southern African Coasts	synonym
Engraulis encrasicholus atlanticus	Pusanov & Zeeb, 1926	Atlantic Ocean	synonym
Engraulis encrasicholus maeoticus	Pusanov, 1926	Azov Sea	synonym
Engraulis encrasicolus mediterraneus	Pusanov & Zeeb, 1926	Mediterranean Sea	synonym
Engraulis encrassicholus atlantica	de Buen, 1926	Gulf of Cadiz (Spain) and Larache (Morocco)	synonym
Engraulis encrasicolus mediterraneus pontica	Pusanov, 1926	Sevastopol (Ukraine)	synonym
Engraulis encrasicolus aquitanius	Aleksandrov, 1927	Bay of Biscay and probably near shores of Portugal.	synonym
Engraulis encrasicholus atlanticus	Aleksandrov, 1927	North Atlantic Ocean	synonym
Engraulis encrasicolus adriaticus	Aleksandrov, 1927	Ionian and Adriatic Seas, E-Mediterranean Sea	synonym
Engraulis encrasicolus mediterraneus	Aleksandrov, 1927	E-Mediterranean Sea	synonym
Engraulis encrasicholus ponticus	Aleksandrov, 1927	Adriatic and W-Black Sea	synonym
Engraulis encrasicholus ponticus occidentalis	Majorowa, 1934	W-Black Sea	synonym
Engraulis encrasicholus ponticus orientalis	Majorowa, 1934	E-Black Sea	synonym
Engraulis encrasicolus symaetensis	Dulzetto, 1938	Catania – E-Sicily	synonym
Engraulis encrasicolus russoi	Dulzetto, 1947	Messina – NE-Sicily	synonym
Engraulis russoi	Dulzetto, 1947	Messina – NE-Sicily	synonym
Engraulis russoi oliveri	Scuderi, 1957	Lago Verdi (brackish marine pond) NE-Sicily	synonym
Anchoviella guineensis	Rossignol & Blache, 1961	Gabon, Congo, Ivory Coast	synonym
Anchoa guineensis	(Rossignol & Blache, 1961)	21°30' to 16°45'N	synonym
Engraulis guineensis	(Rossignol & Blache, 1961)	Senegal to Pointe Noire (Congo)	synonym

It was established that *E. encrasicolus* is the representative of Engraulidae in the NE-Atlantic. However, since its description as *Clupea encrasicolus* Linnaeus, 1758 (original combination), seven populations of *E. encrasicolus* were described as new anchovy species: *E. amara*, *E. meletta*, *E. vulgaris*, *E. argyrophanus*, *E. capensis*, *E. russoi*, *Anchoviella guineensis* (synonyms *Anchoa guineensis*, *E. guineensis*). Twelve subspecies and three races were also defined. Nowadays, they are all considered synonyms of *E. encrasicolus* (Table II). The reason for the differentiation of the northern populations of *E. encrasicolus* in different species, subspecies and races is now better understood. Unlike the majority of small pelagic oceanic fishes, *E. encrasicolus* has a high phylogeographic structure, which was revealed by modern genetic analyses (Magoulas et al., 1996, 2006). Moreover, high morphological differentiation was observed between genetically homogeneous populations (Tudela, 1999; Tudela et al., 1999).

Magoulas et al. (1996, 2006) revealed the existence of two clades in samples collected from the Bay of Biscay down to Senegal and along the Mediterranean till the Black sea, using RFLP analysis of mtDNA. A mosaic pattern is observed with abrupt changes between some areas. Clade A has the highest frequencies in the Black Sea and slightly lower in the Aegean Sea and from N-Portugal to Senegal. Conversely, clade B has the highest frequencies in the Adriatic Sea, overcoming clade A in the Ionian Sea, N-Mediterranean Sea and in the Bay of Biscay (Fig. 4). Four main geographical groups where identified for the studied area (Atlantic, central Mediterranean, Aegean Sea and Black Sea), with high level of genetic differentiation among populations (F_{ST}= 0.148, p< 0.0001) (Magoulas et al., 2006). The coexistence of these two phylads, over a large and ecologically diverse area, refutes the suggested existence of many species and races of anchovy (Table II) (Magoulas et al., 1996).

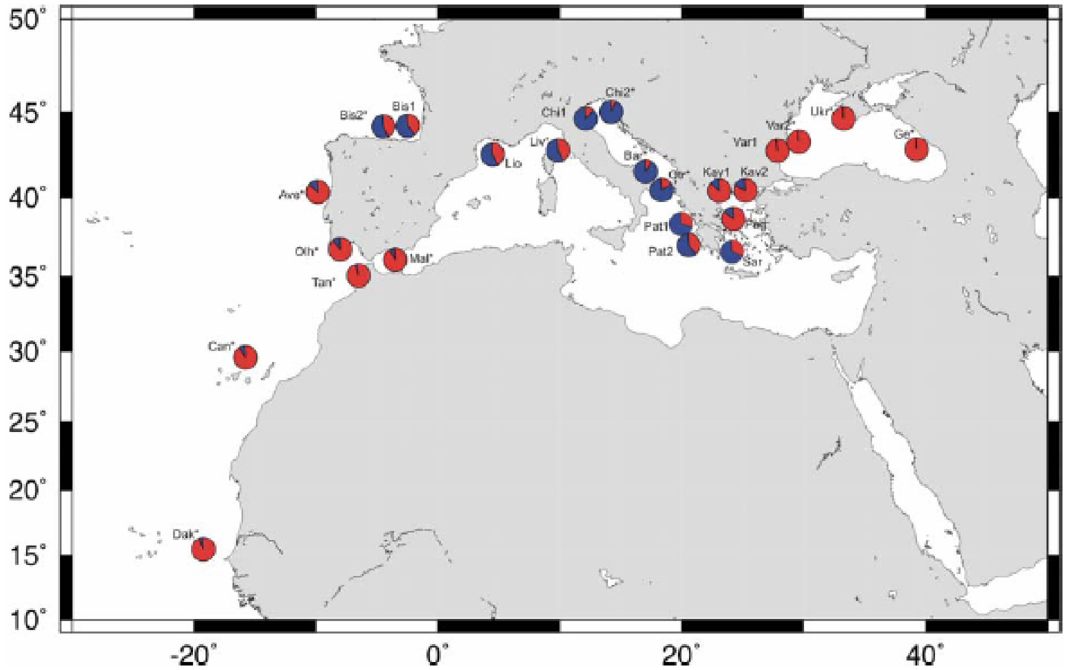


Fig. 4 *Engraulis encrasicolus* clade frequencies for clades A (red) and B (blue), from the Bay of Biscay to Senegal and along the Mediterranean Sea till the Black Sea (from Magoulas et al., 2006).

It was established that estuaries, lagoons and local embayments are habitats that may provide special opportunities for genetic divergence among fishes (Watts & Johnson, 2004). This same hypothesis is being suggested for *E. encrasicolus* for the last three decades. In the Gulf of Gascoigne, coastal and oceanic populations of anchovy were reported as different on the basis of morphological and biological analyses (Gueraul & Avrilla, 1978 *in* Ré, 1984). Furthermore, allozyme-frequency differences revealed the same division in the Gulf of Lion (Pasteur & Berrebi, 1985 *in* Borsa, 2002). Later, Borsa (2002) speculated that the divergence among coastal and oceanic *E. encrasicolus* occurs because there are at least two cryptic species (Group I and Group II), both present in the Gulf of Lion and in the North Adriatic Sea. The existence of cryptic species is common among marine invertebrates, but not among marine fishes (Thorpe et al., 2000 and references therein). The Group I inhabit the inshore areas of both regions, while Group II is in the oceanic domain of the Bay of Biscay, W-Mediterranean Sea, central and S-Adriatic Sea and Ionian Sea. Inshore and oceanic populations have greater genetic difference within a region ($\hat{G}_{ST}$= 0.035-0.067), than broadscale geographical variation for each of both forms ($\hat{G}_{ST}$= 0.005-0.006). Morphometric, allozymic and mtDNA analyses led to hypothesize on the existence of a third species in the N-Aegean Sea and Sicily. Afterwards, Borsa et al. (2004) only proved the existence of a new anchovy species, the White anchovy- *Engraulis albidus* sp. nov., by analysing nuclear DNA. *E. albidus* was set to inhabit in the inshore, coastal lagoons and estuaries, while *E. encrasicolus* would be restricted to the open-sea. The genetic difference between these forms, apart 50-70 km, was higher ($\hat{\theta}$ =0.397-0.586) than between oceanic populations distant thousands of kilometers ($\hat{\theta}$= −0.006-0.042). The designation *Engraulis encrasicolus sensu lato* should be used when the classification of anchovy is uncertain.

1.3. Engraulis encrasicolus sensu lato *in the Guadiana estuary*

The dynamics of *Engraulis encrasicolus sensu lato*, and of other Clupeoids, along coastal regions has been strongly linked with river inflow (Motos et al., 1996). The river plumes that are formed might propitiate optimal conditions for larval fish to develop and may explain recruitment variability, once river flow exports nutrients to coastal areas and it may enhance water column stability and biological production (García & Palomera, 1996; Motos et al., 1996; Plounevez & Champalbert, 1999; Bergeron, 2004).

The coastal area around the Guadiana estuary (SW-Portugal/SE-Spain), *i.e.* the Bay of Cadiz, is considered an important spawning and nursery area of anchovy (Millán, 1999; Bellido et al., 2000) and supports an important fishery (Uriarte et al., 1996). The Guadiana estuary was also identified as a fundamental spawning and nursery area for anchovy (Chícharo, 1988; Faria et al. 2006), which is the most abundant planktivorous fish in this estuary (Chícharo et al., 2006). However, a comprehensive knowledge on the biology and ecology of anchovy is scarce in the Guadiana, when compared to other Portuguese estuaries, namely Mondego (Ribeiro, 1991), Tagus (Ré, 1984; Monteiro, 2002) and Mira (Ferreira & Ré, 1993; Ré, 1996; Esteves et al., 2000a, 2000b). The first hypothesis about the life cycle of anchovy (*E. encrasicolus*) in the Guadiana estuary was proposed by Chícharo et al. (2001). This hypothesis stated that anchovy would spawn in the lower estuary or in the adjacent coastal area (Fig. 5A). Eggs and recently hatched larvae would be found in these areas (Fig. 5B) and, as larvae would get older, they would migrate upstream, up to the estuarine turbidity maximum (ETM) region (Fig. 5C). Finally, juveniles and/or recruits would migrate downstream, to the coastal area, to integrate the harvested stock (Fig. 5D).

Moreover, it was suggested that moderate river flow (1 x 10^6 $m^3.month^{-1}$) could attract more adults from the coastal area to the low estuary and to the coastal area where the plume is formed; thus, the abundance of anchovy larval stages would increase. Higher river flow (3.5 x 10^6 $m^3.month^{-1}$) could attract even more anchovy adults to the estuarine plume and to the low estuary; however advection would send larval stages away from the spawning and nursery areas inside the estuary (Chícharo et al., 2001).

1.4. Tools to study fish's life histories and historical demography

The research of fish's life histories and historical demography should use a multidisciplinary approach to study these aspects in different time scales perspectives (Waldman, 1999).

1.4.1. The mitochondrial DNA

The broadest time scale that can be assessed in the study of fish life histories is through

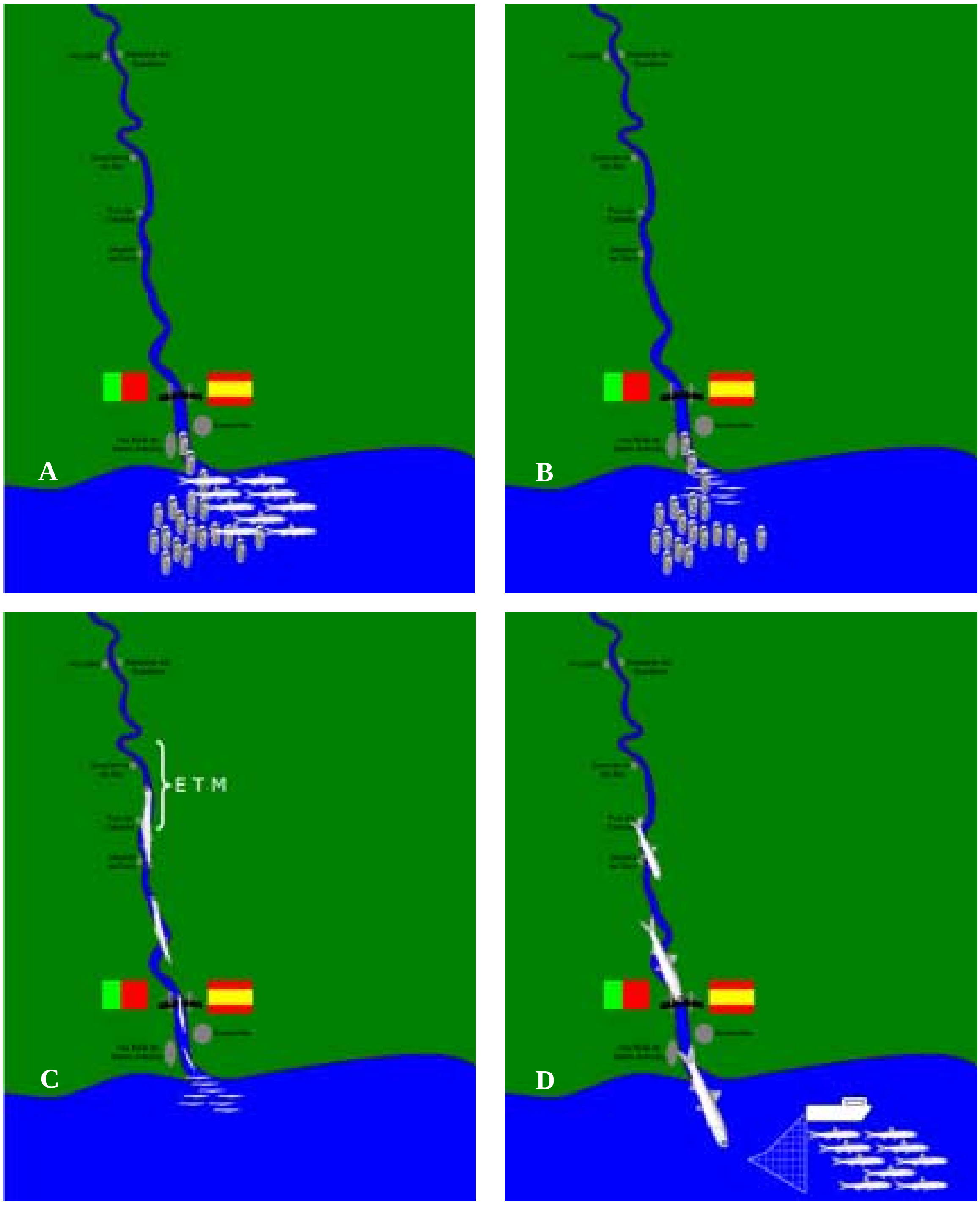


Fig.5 Anchovy life history in the Guadiana estuary and adjacent coastal area, according to Chícharo et al. (2001).

the analysis of genetic markers. One of the most powerful tools in population genetics studies is the mitochondrial DNA (mtDNA) (Castro et al., 1998).

The mtDNA of multicelular organisms is highly conservative in size, function and organization. In several vertebrates it was observed that mtDNA does not have huge variations in the position of the genes along the molecule (Pereira, 2000). The mtDNA is small and simple, in opposition to nuclear DNA, consisting of a small double strand circular molecule, with sequences that occur only once in the genome. The mtDNA contains 2 genes that are involved in transcription of DNA into proteins (ribosomal RNA genes), 22 genes that code for transfer RNA and 13 genes concerned with the biochemical process of oxidative phosphorylation (NADH dehydrogenase subunits 1 to 6 (ND1, ND2, ND3, ND4, ND5, ND6), cytochrome c oxidase subunits I to III (COX1, COX2, COX3), ATP synthase F0 subunits 6 and 8 (ATP6, ATP8) and cytochrome *b* (CYTB)). The mtDNA of vertebrates has also one non-coding control region (displacement loop or d-loop), where the replication and transcription of the molecule occurs (Fig. 6).

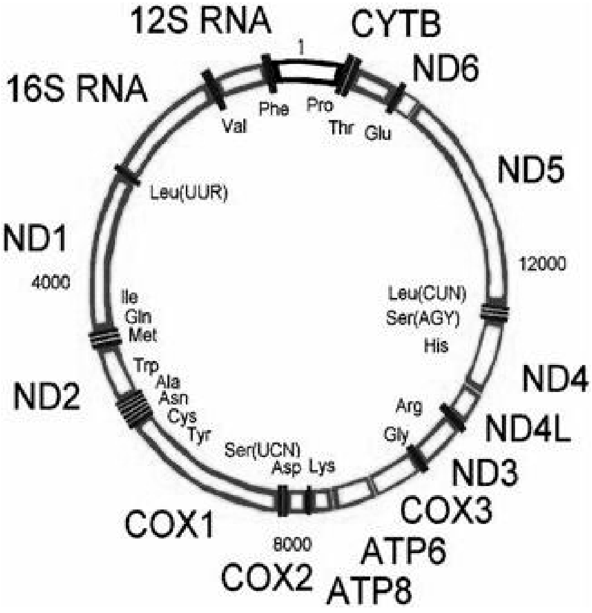


Figure 6 Simplified representation of the mtDNA**.** The nucleotide base 1 is at the 12 o'clock position. The numbering of the nucleotide bases goes in an anti-clockwise direction. The coding areas for the genes are shown in red, with their corresponding labels outside. The tRNA's are shown in blue with the three-letter abbreviations for the amino acids inside. Adapted from Mitomap (2007).

The mtDNA is generally inherited from the female progenitor; therefore its evolution occurs more straightforward and discernable than if it would have been inherited from both progenitors. However, some species can inherit the mtDNA from the male progenitor (Obata et al., 2006; Sherengul et al., 2006), as it occurs for *E. encrasicolus* (Magoulas & Zouros, 1993). The mutation rate of mtDNA is 10 times higher than nuclear DNA (Wallace, 1994 *in* Miyazono et al., 2002), *ca.* 2% per million years (Magoulas et al., 1996), which is caused by lack of protective histones, inefficient DNA repair systems and to continuous exposure to the mutagenic effects of oxygen radicals released during the oxidative phosphorylation process (Sharma et al., 2005). The analyses of mtDNA is used to differentiate species (Castilho et al., 2007) and analyse speciation (Sato et al., 2003), to support conservation programs (Saillant et al., 2004; López et al., 2007), to investigate phylogeography (Scoles et al., 1998; Salgueiro et al., 2007) and historical demography (Saillant et al., 2004; Francisco et al., 2006).

1.4.2. Fish otoliths

The entire life cycle of fishes is commonly assessed by analysing their sagittal otoliths. These are one of the three pairs of otoliths that fishes have in their membranous labyrinth or inner ear, just behind the brain. The sagittal otoliths are located in the sacculus and the other two pairs, the lapillus and asteriscus, are located in the utriculus and lagena, respectively (Fig. 7). The sagittal otoliths are formed by calcium carbonate ($CaCO_3$) crystals, mainly in the form of aragonite, but also as vaterite and calcite, in a non-collagenous organic matrix. In all teleost fishes, otoliths are involved in hearing, balance and depth resolution, but are also important for sensitivities to gravity and linear acceleration (Campana, 1999; Begg et al., 2005).

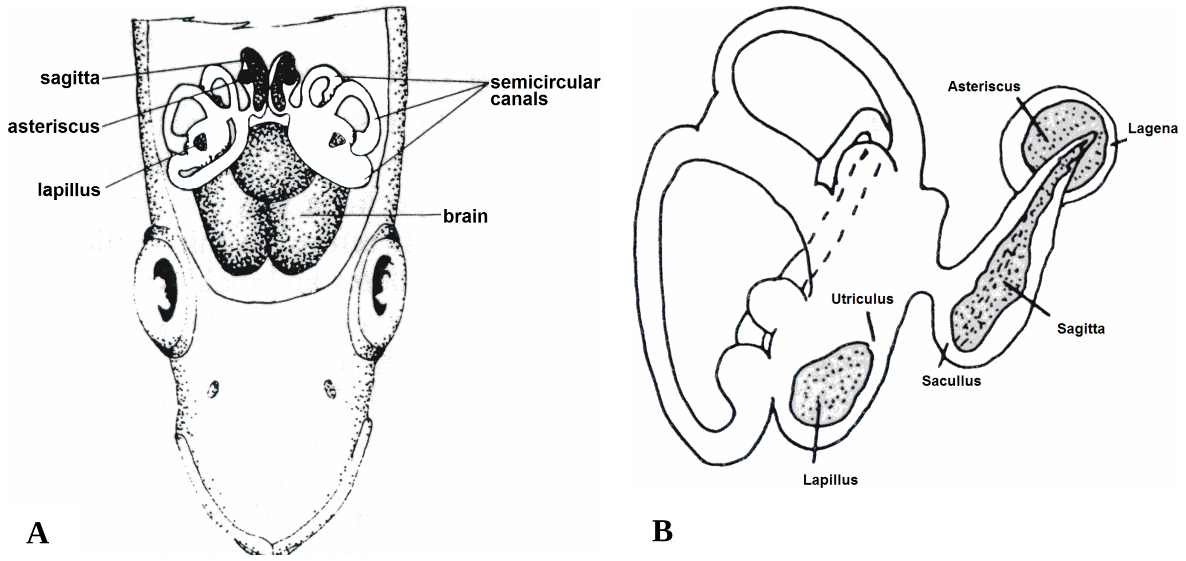


Fig. 7 Anatomy of the vestibular apparatus in a teleost fish. A- Dorsal view from the head, where the top was removed. B- Otoliths location within the right labyrinth system. Modified from Stevenson & Campana, 1992.

The otoliths are metabolically inert; *i.e*. there is no resorption once the $CaCO_3$ mineral is deposited within the otolith. Some chemical elements are incorporated into the otoliths proportionally to their concentration in the ambient water, in function of fish metabolism or dependent of their food items (Campana, 1999). The absorption pathways of inorganic elements are through branchial uptake or intestine assimilation into the plasma and then to the crystallizing otolith (Campana, 1999). These characteristics turn otoliths particularly suitable to delineate stocks, to infer migration patterns and to reconstruct environmental history (Campana, 2005), other than just estimate fish's age and growth and discriminate species.

The microchemical analyses of otoliths are undoubtedly a major achievement in fisheries biology, once it can substitute in many species the traditional capture-recapture methods. For example, the concentration of strontium (Sr) along otoliths is mostly used to track migration patterns along salinity gradients (Edmonds et al., 1999; Elfman et al., 1999, 2000; Zlokovitz et al., 2003). The general assumption is that the concentration of Sr is substantially higher in the seawater than in estuaries or freshwater ecosystems (Secor & Rooker, 2000).

Other examples are the stable isotope ratios of oxygen ($^{18}O/^{16}O= \delta^{18}O$) and carbon ($^{13}C/^{12}C= \delta^{13}C$). The $\delta^{18}O$ detects differences on water temperature and rainfall between sampling locations, since these isotopes are deposited in the otolith in equilibrium, or close to equilibrium, with ambient water. The locations with higher temperature and evaporation rate are more depleted in ^{16}O than in ^{18}O, while locations influenced by rainfall are enriched in ^{16}O water. On the other hand, the $\delta^{13}C$ are not deposited in equilibrium with ambient sea water, instead the observed disequilibria have been attributed to metabolic effects associated with age, to changes in habitat, with habitat depth and to nutrient sources (Schwarcz et al., 1988; Nelson et al., 1989; Edmonds & Fletcher, 1997; Edmonds et al., 1999; Stephenson et al., 2001; Sherwood & Rose, 2003).

1.4.3. Fish monitoring programs

In the study of fish life histories, shortest and discontinuous time scale analyses, such as on daily and monthly bases, allow to complement, to support and to enlighten the hypothesis suggested by data from genetic and sagittal otoliths microchemical analyses.

The monitoring programs of fish larval stages in an estuary allow, for example, to: a) determine spawning and nursery sites (Ré, 1984); b) infer larvae migration strategies (Raynie & Shaw, 1994; Forward Jr. et al., 1996, 1998); c) calculate larval stages mortality (Bassista & Hartman, 2005); d) evaluate the influence of natural and managed river flow on the advection of larval stages (Potter & Hyndes, 1999; Quinn et al., 1999; Kanandjembo et al. 2001; Strydom, 2002; North et al., 2005); e) evaluate ecosystem changes by detecting pronounced shifts on the abundance of larval stages (Chícharo et al., 2001, 2006; Faria et al., 2006).

Moreover, the study of fish larvae, juveniles and adults populations allow corroborating or rejecting hypothesis on fish’s migration patterns raised by otoliths microchemical analyses.

These studies often consist in analysing the differential use of an estuary by larvae, juveniles and adults (Ribeiro, 1991), in determining life history parameters that might allow to outline populations (Sinovcic, 2004) and in setting locations that should be preserved due to their relevance to fish populations (Valesini et al., 1997; Gillanders, 2005; Sá et al., 2006).

1.5. Objectives

The mentioned constrains on the knowledge of anchovy in the Guadiana estuary and its ecological relevancy in this estuary, lead to formulate the following main questions:

1. Which anchovy species is present in the Guadiana estuary and in the adjacent coastal area, *E. encrasicolus* or *E. albidus*?
2. Which are anchovy spawning and nursery areas in the Guadiana estuary and how it reallocates with different sets of river inflow?
3. Which anchovy specimens spawn in the estuary, those that hypothetically migrate from a coastal population or those that already inhabit the estuary?
4. What is the distribution pattern of anchovy juveniles and adults along the Guadiana estuary and in the adjacent coastal area?
5. Are Carbon and Oxygen isotope ratios of juveniles and adult anchovy otoliths useful proxies to distinguish between two populations/species of anchovy (Guadiana estuary *vs*. adjacent coastal area) and complement genetic information?
6. What is/are the migratory pattern(s) of two anchovy populations/species of anchovy as revealed by Fe, Sr and Zn concentrations along the otoliths?

2. Material and Methods

2.1. Genetic analyses

In order to determine which anchovy species, *E. encrasicolus* or *E. albidus*, is present in the Guadiana estuary and adjacent coastal area, the genes cytochrome *b* (cyt *b*) and displacement loop (d-loop) of mitochondrial DNA (mtDNA) were analysed. Adult specimens were collected in front of Ayamonte (37º12'45" N, 7º24'50" W, January 22nd 2004) and in the coastal area off Praia Verde- Portugal (approximately 37º9'39" N, 7º28'38" W, June 11th 2004), which are *ca.* 15 km apart (Fig. 8). Anchovies from Guadiana estuary were collected with an otter trawl, while those from the coastal area were bought to local fishermen.

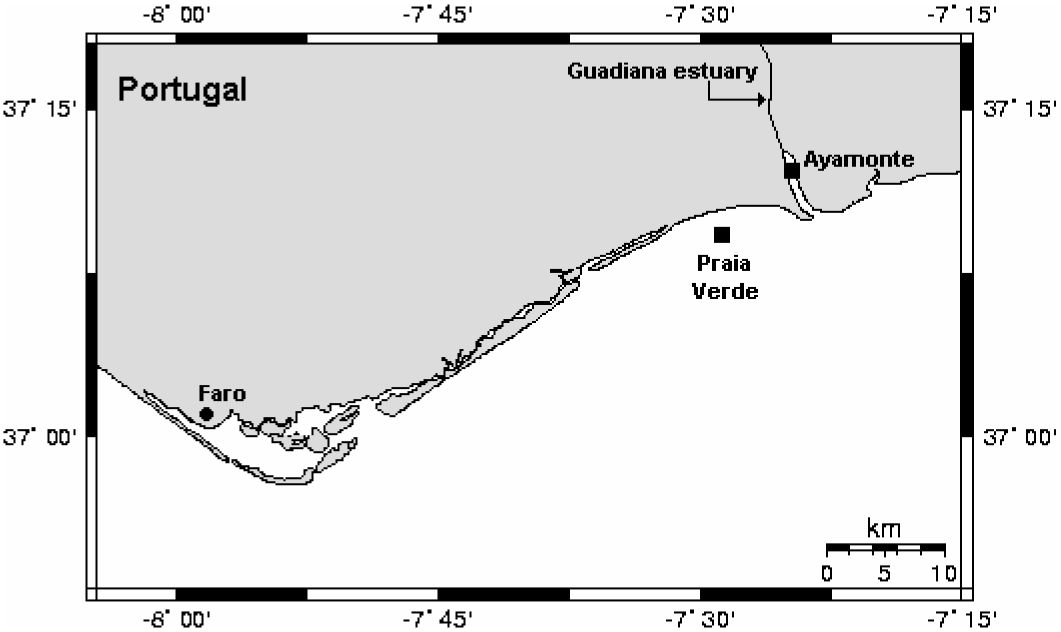


Fig. 8 Location of sampling stations for the analyses of anchovy mtDNA.

Anchovy muscle samples were removed from the anterior region to the dorsal fin and stored in ethanol 96%. DNA extraction was made with *ChargeSwitch® gDNA Mini Tissue Kit*

(Invitrogen, USA- CS11204) using the manufacturer´s procedure (Version A). The primers cyt *b*-F (5’-CCT ACT CAA GAT CGC TAA CGA-3’) and cyt *b*-R (5’-CAC TGC ACT AAT TCA GTG CC-3’) were used for cyt *b* analysis, while for d-loop analysis the primers used were Dloop-F (5’-CTA CCT CCA ACT CCC AAA GC-3’) and Dloop-R (5’-ATA GTG GGG TAT CTA ATC CCA GTT-3’). The extraction product was sent to Macrogen (Seoul, Korea) for polymerase chain reaction (PCR) and sequencing. The PCR amplifications were carried out in a MJ Research PTC-225 Peltier Thermal Cycler (GMI, USA) and was performed using the following profile: an initial denaturing step of 3’ at 95 ºC; 35 cycles of denaturing at 95 ºC for 1’, annealing at 52 ºC for cyt *b* and at 54 ºC for d-loop for 1’, and extending at 72 ºC for 1’; a final extending step of 10 min at 72 ºC. Sequencing reactions were performed in a *MJ Research PTC-225 Peltier Thermal Cycler* using an *ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits* (Applied Biosystems, USA), with *AmpliTaq DNA polymerase (FS enzyme)* (Applied Biosystems, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the mentioned primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an *ABI PRISM® 3730xl DNA Analyzer* (96 capillary type) (Applied Biosystems).

The cyt *b* and d-loop sequences were aligned using MEGA 3.1 (Kumar et al., 2004). Since the gene d-loop was sequenced in both directions, a multiple sequence alignment was done in “Multalin Interface Page” (http://bioinfo.genopole-toulouse.prd.fr/multalin) (Corpet, 1988). The alignments were confirmed by examining the sequences chromatograms in BioEdit 7.0.5.3 (Hall, 1999).

Several measures of DNA polymorphism were calculated with DnaSP 4.10.9 (Rozas et al., 2003), namely: number of polymorphic (S), conservative and parsimony informative sites;

number of haplotypes (*h*); haplotype diversity (*Hd*); nucleotide diversity (π); average nucleotide differences (*Kt*); number of mutations (Eta). These estimates where made for each group of samples and for the bulk of both samples. The parameters *h*, *Hd*, π and *Eta* were also calculated for each codon position of the cyt *b* sequences.

Average sequence differences within and among samples were calculated from pairwise sequence differences obtained by MEGA 3.1 (Kumar et al., 2004). The insertions and deletions were treated as the fifth state and no mutations were weighted for sequence difference estimation.

The genetic differentiation, within and between sampling locations was calculated with DnaSP 4.10.9 (Rozas et al., 2003). The alignment gaps were considered as the fifth state and the permutation test was performed by setting 1000 replicates. Two distinct classes of test statistics, Hudson's haplotype (*Hst*) and sequence-based statistical tests (*Kst**, *Z**), were applied (Hudson et al., 1992). The haplotype statistic (*Hst*) is based on haplotype frequencies in the samples and does not matter whether two haplotypes differ by one or hundreds of nucleotides. The sequence based statistics (*Kst**, *Z**) use the information on the number of nucleotide differences between haplotype sequences. This class of statistics is a more appropriate method when the haplotype diversity is very high and the sample sizes are small.

The base (T, C, A, G) frequencies were calculated for each fragment of mtDNA cyt *b* and d-loop genes. The global base content and in each codon position was calculated. The difference between samples was evaluated with a t-test. However, if the assumptions of this test, data normality and equal variances, are not achieved then data has to be transformed. Ultimately, if data transformation fails to achieve t-test assumptions then the Mann-Whitney test (U-test) is performed (Sokal & Rohlf, 1995).

An haplotype network was constructed by using Network 4.2.0.1 (Fluxus Technology Ltd.- http://www.fluxus-technology.com). The median joining algorithm was implemented

(Bandelt et al.,1999) and maximum parsimony was set as a post-processing option, this way superfluous links and median vectors are purged from network (Polzin & Daneschmand, 2003).

A phylogenetic tree was constructed with the cyt *b* sequences determined in this study and with those available in GenBank (2007) for others *Engraulis* spp. No phylogenetic tree was inferred with d-loop sequences because there were no sequences of *E. encrasicolus* in GenBank (2007), the species that was intended to be compared with that/those captured for this study.

The evolutionary model was determined with FINDMODEL (2007), a web implementation of Modeltest (Posada & Crandal, 1998). The Tamura-Nei model was the chosen evolutionary model (Tamura & Nei, 1993), which corrects for multiple hits, taking into account the differences in substitution rate between nucleotides and the inequality of nucleotide frequencies. It distinguishes between transitional substitution rates between purines and transversional substitution rates between pyrimidines. It also assumes equality of substitution rates among sites. The phylogenetic tree was implemented with the neighbor-joining method (Saitou & Nei, 1987) and the reliability of nodes on trees was estimated using bootstrap analyses (Felsenstein, 1985) with 1000 replicates, in MEGA 3.1 (Kumar et al., 2004).

2.2. Anchovy larval stages

Eulerian sampling was carried out during new moon spring tides at low and high tides, from March 2002 to February 2003 at 9 sampling stations: 7 stations inside the estuary and 2 in the coastal area. Station 1 was off Praia de Santo António, outside the direct influence of the estuarine outflow, and station 2 was positioned in the area where the river plume is formed. Station 9 was the uppermost station, situated in the high estuary in front of Alcoutim (Portugal) and Sanlucar de Guadiana (Spain), at 38 km from the river mouth (Fig. 9). Sampling was performed from a boat equipped with an 80 hp engine, except in February 2003, when a boat equipped with a 30 hp engine was used because of technical problems. The high tide sampling in January and February 2003 was not performed.

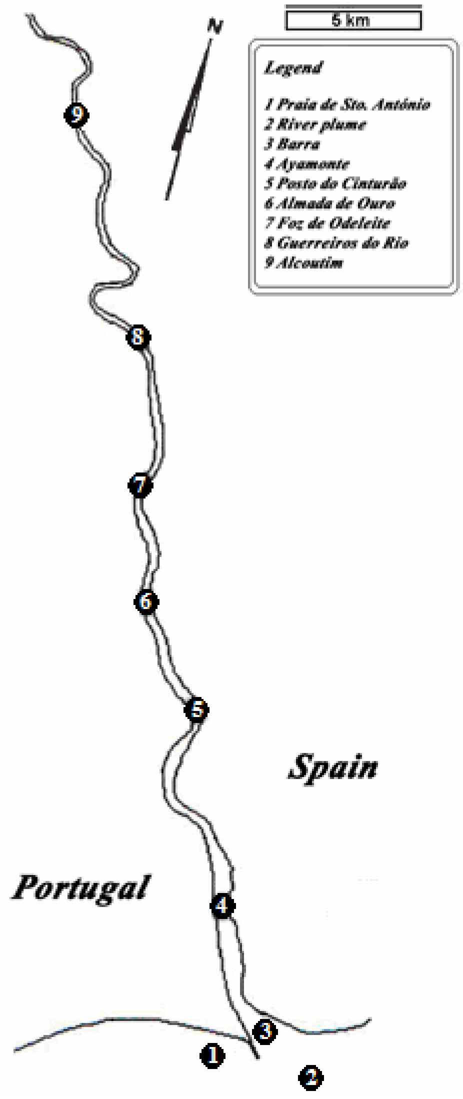


Fig. 9 Location of sampling stations along the Guadiana estuary and in the adjacent coastal area.

In each station, water column profiles of temperature and salinity were recorded with an YSI 6600 probe. Sub-superficial zooplankton trawls were made at each station at an average speed of 2 knots (Harris et al., 2000). The collecting device has 0.9 m in length and 0.4 m in diameter, it is made with a 250 µm net mesh and equipped with a flowmeter (General

Oceanics). The collected samples were immediately preserved with buffered formaldehyde (4% final concentration). Trawling time varied along the studied area, 10 minutes at the coastal area and decreasing towards the upper estuary; where, during the high abundance periods of siphomedusae, trawling did not exceed 3 minutes. The reduction of trawling time had to be performed to avoid clothing, decreasing filtering efficiency and to diminish the extrusion of the collected organisms (Harris et al., 2000). At the laboratory, anchovy eggs and larvae were sorted from the zooplankton samples and their abundance was determined. Development stages were assigned to each egg, according to the 11 stages defined by Moser & Ahlstrom (1985) for *Engraulis mordax*. The average time elapsed since spawning was calculated with Lo's (1985) stage-to-age model, using the parameters defined for *Engraulis encrasicolus* by Motos (1994). Larvae standard length was measured and corrected for the shrinking effect caused by trawling and preservation (Theilacker, 1980). A length-to-age model was fitted to infer the age of larvae, considering a daily growth of 0.51 mm.day^{-1} (Ribeiro, 1991).

Freshwater inflow data, obtained from the Instituto Nacional da Água (INAG, 2004), were measured at the Pulo do Lobo hydrometric station (37º48'N, 7º38´W), which is located a few kilometers above the last point of tidal influence (Mértola) and from the uppermost sampling station (Alcoutim), in order to relate this parameter with the variation of the position of the spawning and nursery areas.

A one-way analysis of variance, followed by an a posteriori Tukey HSD test, were used to assess spatial differences of anchovy larval standard length, along the studied area, in the month of their widest distribution at the low and high tides. A confidence interval of 95% was set and the power of the analysis was assessed with $\alpha = 0.05$. If data normality ($P < 0.010$) and equal variance ($P < 0.010$) were not observed, then the Kruskal-Wallis One Way Analysis of

Variance on Ranks was used, followed by an a posteriori test, the Dunn's Method (Sokal & Rohlf, 1995).

2.3. Anchovy adults

The temporal and spatial distribution of juveniles and adults of anchovy, along the Guadiana estuary and in the adjacent coastal area, were assessed in 9 sampling stations, every two months, from January 2001 to September 2002. These stations correspond to the same that were assessed to study anchovy larval stages (Fig. 9). Sampling was carried out during intermediate ebb tides with an otter trawl. This is a conical-shaped fishing gear, with a stretched mouth of 3 m and an overall length of 25 m, equipped with two otter boards weighing 12 kg each. The net was constructed with two panes. The outer pane, made from 30-mm stretched mesh net, protected the inner pane, which was made from 10-mm stretched mesh net. The trawling process consisted of throwing the cod end of the net into the water and rapidly lowering the rest of the net, without allowing it to rest on the bottom and always maintaining the mouth open. When the otter boards hit the bottom, the trawl time and distance were recorded using an Eagle Explorer GPS. Once the trawling procedure finished, the net was rapidly retrieved and manually hauled from the water. Trawls were performed in a boat that was equipped with an 80 hp engine and, depending on the site location and on the abundance of fish and cnidarians, trawl durations varied between 5 and 35 min.

A factorial analysis was impossible to be performed, once data is disconnected. Therefore, one-way analysis of variance (ANOVA), followed by an a posteriori Tukey HSD test, were used to assess spatial and temporal differences of fish total length. A confidence interval of 95% was set and the power of the analysis was assessed with $\alpha = 0.05$. If data normality ($P < 0.010$) and equal variance ($P < 0.010$) were not observed, then the Kruskal-

Wallis One Way Analysis of Variance on Ranks was used, followed by an a posteriori test, the Dunn's Method (Sokal & Rohlf, 1995). Three factors were defined and analysed, years (2001 and 2002, data from November 2001 was excluded), seasons (January and March- Winter; May- Spring; July and September- Summer; November- Autumn) and sampling area (stations 1 and 2- coastal area; stations 3 and 4- low estuary; station 5 to 7- middle estuary; station 8 and 9- upper estuary). By grouping months in seasons and sampling stations in sampling areas, the robustness of the analyses increases, due to the low number of specimens in some sampling stations and months. The month with widest distribution of anchovy was also analysed separately.

Additional samples of anchovy juveniles and adults were collected, every month, from March 2002 to February 2003. This sampling was made two-three days before, or after, the zooplankton sampling campaign. All specimens were measured (total length, TL, mm) and weighted (total weight, TW, g). The sex was assigned and the gonadosomatic index (GSI= gonad weight x (gonad-free body weight)$^{-1}$ x 100) determined. The chi-square test was used to check for significant differences in the ratios of males and females in each month. (Sokal & Rohlf, 1995).

2.4. Otolith microchemistry analysis

2.4.1. Isotopic analysis

The Carbon (C) and Oxygen (O) isotopic ratios of anchovy otoliths were determined from the adult specimens captured in front of Ayamonte (Guadiana estuary) and off Praia Verde (coastal area) (Fig. 8), from the same individuals used for the genetic analysis mentioned in chapter 2.1.

The two sagittae otoliths were removed, weighed, cleaned with a nylon brush and distilled water, rinsed in distilled water, air dried, powdered and deproteinated with hydrogen peroxide. The carbonate was decomposed to carbon dioxide (CO_2) with 100% phosphoric acid and the $^{18}O/^{16}O$ and $^{13}C/^{12}C$ ratios of the resultant CO_2 measured by standard mass spectrometric techniques at the CSIRO Division of Water Resources (Perth, Australia). Resulting values are reported in the standard notation relative to the PDB-1 standard (Epstein et al., 1953).

Adjustments to $\delta^{13}C$ and $\delta^{18}O$ measurements should be made if a significant correlation is determined with otolith weight (Stephenson et al., 2001). The relationships between these variables with otolith weight were investigated by using the parametric Pearson Product Moment Correlation Pearson or the nonparametric Spearman Rank Order Correlation, depending if the assumptions of data normally and equal variance are registered or not, respectively (Sokal & Rohlf, 1995). Since there were no significant correlations between variables, no adjustments were made to $\delta^{13}C$ and $\delta^{18}O$ measurements.

The difference between samples was investigated with a t-test. However, if the assumptions of this test, data normality and equal variances, are not achieved then data has to be transformed. Ultimately, if data transformation fails to achieve t-test assumptions then the Mann-Whitney test (U-test) should be performed (Sokal & Rohlf, 1995).

The correlation between $\delta^{13}C$ and $\delta^{18}O$ with total fish length and total fish weight was determined by using Pearson or Spearman correlations. The criteria for using these analyses are explained above.

2.4.2. Fe, Sr and Zinc analysis

The concentration of Iron (Fe), Strontium (Sr) and Zinc (Zn) were determined in the left otoliths of juveniles and adult anchovies captured in front of Ayamonte in the Guadiana estuary and in the adjacent coastal area off Praia Verde (Chapter 2.1; Fig. 8).

The sagittae otoliths were extracted, cleaned and embedded with epoxy resin in disc probe mounts (Ø 25 mm). The embedded otoliths were then ground by hand through the sagittal plane with 600, 1200 and 2400 silicon carbide abrasive paper to expose the otolith core. Then, they were polished with 6, 3 and 1 µm diamond pastes and, finally, with alumina solution (1:20). The surface of the otolith must be highly polished to prevent diffraction of the x-rays and subsequent analytical error. This quality control was performed in a metallographic microscope (Meiji ML7100). After polishing, the embedded otoliths were cut from this initial disc. Then, to significantly diminish the time of analysis, six embedded otoliths were re-embedded with epoxy resin in a disc probe mount (Ø 25 mm). Prior to analysis, the discs were cleaned with absolute ethanol in an ultrasonic bath for 5 min, rinsed with deionised water and carbon coated. The measurement of Fe, Sr and Zn concentration were made along a cross section of the otoliths in the µ-PIXE (Proton Induced X-ray Emission) microprobe of the Guelph PIXE Group (Department of Physics, University of Guelph, Canada). The proton beam entered the otolith surface at a 45° angle. Beam energy was 3 MeV, diameter was 5-10 µm and current was approximately 5 nA. The excitation volume was approximately 30 µm in depth. X-ray intensity was obtained by rastering the proton beam from the core to the post-rostrum; thus, incorporating the entire life of the specimens. Further details on proton microprobe procedures are given by Halden et al. (1996) and Campbell et al. (1999).

The difference in the concentration of Sr in the core ([0-100[μm) and in the last 100 μm of the post-rostrum of otoliths from samples collected in the Guadiana estuary and adjacent coastal area was investigated with a t-test. However, if the assumptions of this test, data normality and equal variances, are not achieved then data has to be transformed. Ultimately, if data transformation fails to achieve t-test assumptions then the Mann-Whitney test (U-test) should be performed (Sokal & Rohlf, 1995).

The existence of positive or negative significant correlations between Sr concentration and distance to core was determined, for all analysed otoliths, with the parametric Pearson Product Moment Correlation. However, if the assumptions of data normally and equal variance are not registered, then the nonparametric Spearman Rank Order Correlation is used (Sokal & Rohlf, 1995).

A cluster analysis was performed to the measurements of Sr concentration, which were grouped in distance classes to otoliths' core. The joining or tree clustering was the chosen cluster analysis method, which groups together the variables into successively larger clusters. The single linkage was the chosen amalgamation rule, in which the distance between two clusters is determined by the distance of the two closest objects in the different clusters. The joining clustering uses the dissimilarities or distances between variables to form the clusters and the Euclidean distance was the one that was chosen. The greatest advantage of using the Euclidean distance is that the distances between any two objects is not affected by the presence of outliers (StatSoft, Inc., 2004).

3. Results

3.1. Anchovy genetic analyses

3.1.1. The gene Cyt-B

Table III Nucleotide sequence of a 390 bp fragment of the mtDNA Cyt-B from individual E1 collected in the Guadiana estuary. Underline letters indicate the variable sites in 23 haplotypes.

1	ATTTCAGTCT	**11**	GATGAAATTT	**21**	TGGATCCCTT	**31**	TTAGGACTAT	**41**	GCTTGGCCAC
51	ACAAATCCTT	**61**	ACAGGACTTT	**71**	TCCTAGCCAT	**81**	ACACTACACC	**91**	TCAGACATCG
101	CTACCGCTTT	**111**	CTCGTCAGTG	**121**	GCCCACATTT	**131**	GTCGAGACGT	**141**	AAATTACGGG
151	TGACTTATCC	**161**	GAAATATGCA	**171**	TGCGAACGGA	**181**	GCCTCATTCT	**191**	TTTTCATCTG
201	CATTTATGCA	**211**	CACATTGCTC	**221**	GGGGACTGTA	**231**	CTACGGTTCT	**241**	TATCTTTACA
251	TAGAAACTTG	**261**	AAACATCGGA	**271**	GTAGTACTAC	**281**	TTCTTTTAGT	**291**	TATGATGACT
301	GCCTTCGTTG	**311**	GGTACGTACT	**321**	ACCCTGAGGA	**331**	CAAATGTCCT	**341**	TCTGAGGGGC
351	AACTGTCATT	**361**	ACTAACCTTA	**371**	TGTCTGCAGT	**381**	TCCTTACGTG		

Table IV Number of variable, conservative and parsimony informative sites, number of haplotypes, haplotypes and nucleotide diversity with standard deviation (s.d.) for samples from the Guadiana estuary and adjacent coastal area. Data for the analyses of both locations is also shown.

mtDNA Cyt-B	**Guadiana estuary n= 22**	**Coastal area n= 20**	**Both locations n= 42**
Variable sites (bp)	16	21	26
Conservative sites (bp)	374	369	364
Parsimony informative sites (bp)	4 (150, 168, 228, 315)	7 (150, 168, 228, 315)	13 (150, 156, 168, 186, 92, 204, 222, 228, 252, 315, 348, 360, 390)
H	11	15	23
***Hd* ± s.d.**	$0.849 \pm 6.4 \times 10^{-2}$	$0.958 \pm 3.3 \times 10^{-2}$	$0.912 \pm 3.1 \times 10^{-2}$
π± s.d.	$6.53 \times 10^{-3} \pm 1.34 \times 10^{-3}$	$8.0 \times 10^{-3} \pm 1.72 \times 10^{-3}$	$7.27 \times 10^{-3} \pm 1.08 \times 10^{-3}$
Kt	2.545	3.121	2.834
Mutation (Eta)s	16	22	27

The aligned mtDNA sequence data consisted of part of the Cyt-B containing 390 base pairs (bp) (Table III). A total of 26 variable positions and 23 haplotypes were found in the 42 individuals from both locations. The number of variable sites and haplotypes from individuals from the Guadiana estuary was 16 and 11, respectively; which is lower than those from the coastal area, 21 and 15, respectively (Table IV).

The global frequency of variable sites in this fragment of the mtDNA Cyt-B was 6.7% (26/390 bp); 4.1% (16/390 bp) for samples from the Guadiana estuary and 5.4% (21/390 bp) for samples from the coastal area (Table IV).

A total of 4 parsimony informative sites were found for samples from the Guadiana estuary and 7 for samples from the coastal area. When considering both locations, the number of parsimony informative sites determined was 13 (Table IV).

The haplotype diversity (Hd) was 0.849 in the Guadiana estuary, which is lower than that determined for samples from the coastal area of 0.958. The nucleotide diversity (π) was also lower in the Guadiana estuary than in the coastal area, 6.53×10^{-3} and 8.0×10^{-3}, respectively. The average number of nucleotide differences (Kt) was lower in samples from the Guadiana estuary (2.545), than in those from the coastal area (3.121) (Table IV).

Table V Number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity and number of mutations (Eta) in each codon position.

Cyt-B	1st codon	2nd codon	3rd codon
h	3	1	22
***Hd* ± s.d.**	0. 094 ± 0.061	0	0.902 ± 0.032
π ± s.d.	$0.0007 \pm 4.8 \times 10^{-4}$	0	$0.021 \pm 3.2 \times 10^{-3}$
Eta	2	0	25

The gene Cyt-B, from samples collected in the Guadiana estuary and in the adjacent coastal area, is richer in the base T, 32.4 ± 0.2 % and 32.3 ± 0.2 %, respectively. In the 1st codon position, the content of the base A is higher (31.3 ± 0.7 % in the Guadiana estuary; 31.0 ± 0.6 % in the coastal area), but in the other two positions T content is also higher. The difference in base content frequency is not significant between sampling locations, either globally or for each codon position (U-test: p= 0.109 to p= 0.990) (Tables V & VI).

The average sequence divergences within samples from the Guadiana estuary was 2.54 ± 0.81 %, while for those from the coast was 3.12 ± 0.75 %. The overall difference between samples from both sites was 2.86 ± 0.75 %.

The several genetic differentiation estimates reveal no significant differences between sites: Hst= 0.012 (p=0.099), Kst^{*}= 0.005 (p= 0.235), Z^{*}= 5.778 (p= 0.118). The results for gene flow estimates were as follow: Gst= 0.012, γst= 0.030, Fst= 0.010.

It were defined 23 haplotypes, of which 18 (42.9%) correspond to exclusive haplotypes, 7 in the estuarine samples and 11 in the coast. The most abundant haplotype was h4 (n= 10; 23.8%), which predominate in the estuary (80%); then it was h7 (n=8, 19.0%), with equal distribution between samples, and then haplotypes h3 (100% in the estuary), h9 (50% for each site) and h21 (100% in the coast), accounting each 4.8% (n=2). The haplotypes h6 (estuary, ind. E9) and h13 (coast, ind. C3) were the most dissimilar when compared to the remaining haplotypes (Figs. 10, 11).

Table VI Base (T, C, A and G) frequencies (in percent) for the 390 bp fragment of mtDNA Cyt-B, considering the entire fragment or each codon position, for each individual captured in the Guadiana estuary or in the adjacent coastal area. The average values for each location and for the combination of both locations are shown.

Sample	**T**	**C**	**A**	**G**	**Total**	**T-1**	**C-1**	**A-1**	**G-1**	**Pos #1**	**T-2**	**C-2**	**A-2**	**G-2**	**Pos #2**	**T-3**	**C-3**	**A-3**	**G-3**	**Pos #3**
E1	32.3	24.4	24.1	19.2	390	27.7	30.8	30.0	11.5	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E2	32.3	24.4	24.1	19.2	390	27.7	30.8	30.0	11.5	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E3	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E4	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E5	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E6	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E7	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E8	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E9	31.8	24.6	25.4	18.2	390	26.2	31.5	33.8	8.5	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E10	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E11	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E12	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E13	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E14	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E15	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E16	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E17	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E18	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E19	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E20	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E21	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
E22	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
Average	32.4	24.3	24.5	18.8	390	27.9	30.5	31.3	10.3	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
St. dev.	0.2	0.2	0.2	0.2	0.0	0.5	0.4	0.7	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C1	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C2	32.1	24.6	24.4	19.0	390	26.9	31.5	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C3	31.3	25.1	24.6	19.0	390	24.6	33.1	31.5	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C4	32.3	24.4	23.8	19.5	390	27.7	30.8	29.2	12.3	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C5	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C6	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C7	32.1	24.9	24.4	18.7	390	26.9	32.3	30.8	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C8	32.6	24.1	24.4	19.0	390	27.7	30.8	30.8	10.8	130	30.0	17.7	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C9	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C10	32.3	24.4	24.4	19.0	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C12	32.6	24.1	24.4	19.0	390	28.5	30.0	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C13	32.1	24.6	24.6	18.7	390	26.9	31.5	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C14	32.1	24.6	24.1	19.2	390	26.9	31.5	30.0	11.5	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C15	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C16	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C17	32.6	24.1	24.6	18.7	390	28.5	30.0	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C18	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C19	32.6	24.1	24.4	19.0	390	28.5	30.0	30.8	10.8	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
C20	32.3	24.4	24.1	19.2	390	27.7	30.8	30.8	10.8	130	29.2	18.5	23.1	29.2	130	40.0	23.8	18.5	17.7	130
C21	32.3	24.4	24.6	18.7	390	27.7	30.8	31.5	10.0	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
Avgerage	32.3	24.4	24.4	18.9	390	27.5	30.9	31.0	10.6	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
St. dev.	0.3	0.3	0.2	0.2	0.0	0.9	0.8	0.6	0.6	0.0	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Global avg.	32.3	24.4	24.5	18.9	390	27.7	30.7	31.1	10.4	130	29.2	18.5	23.8	28.5	130	40.0	23.8	18.5	17.7	130
Global st. dev.	0.2	0.2	0.2	0.2	0.0	0.7	0.6	0.7	0.6	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0

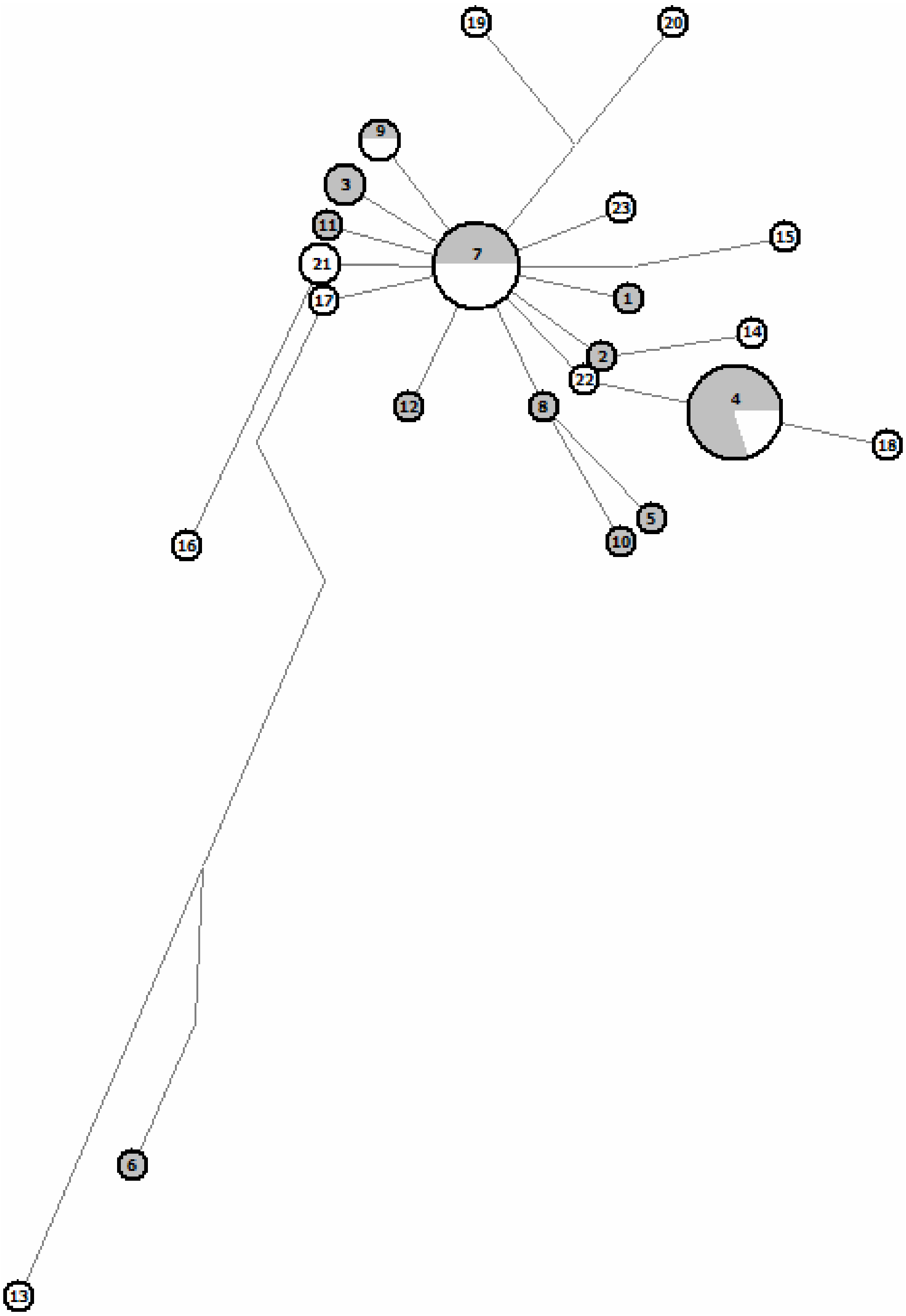


Fig. 10 MtDNA haplotypes network for the gene Cyt-b, from samples collected in the Guadiana estuary (grey) and in the coastal area off Praia Verde (white). The number of each haplotype is inside the circle. The frequency of each haplotype is proportional to the circle size, for example h1= 1, h3= 2, h4= 10 and h7= 8.

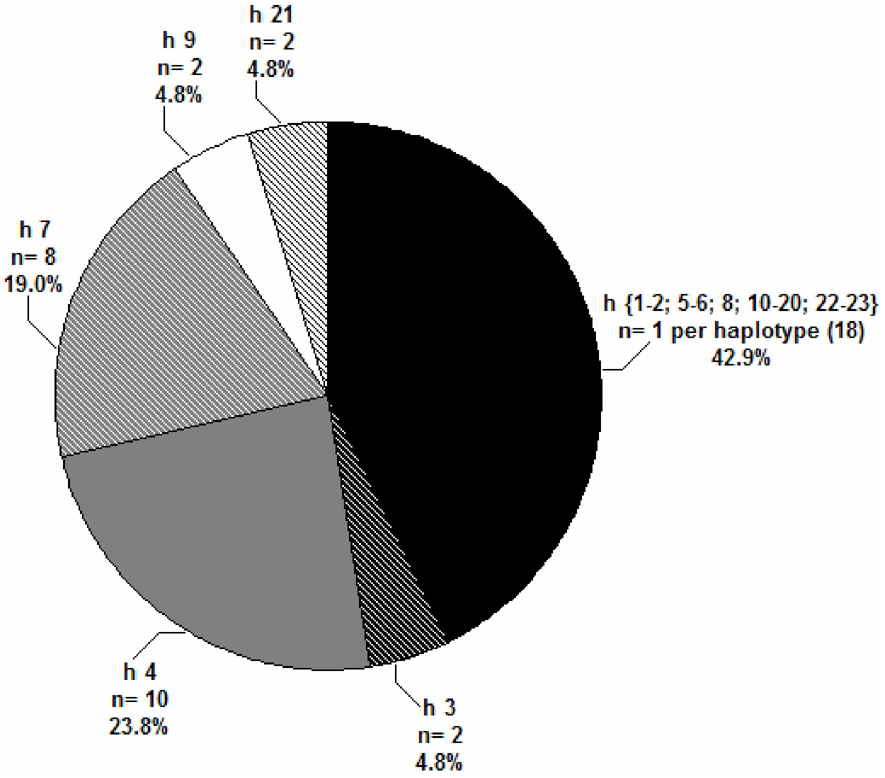


Fig.11 Haplotypes frequency for the Cyt-b gene from samples collected in the Guadiana estuary and in the coastal area off Praia Verde. Haplotypes with one individual were grouped.

In the phylogenetic tree it can be observed that the individuals E9 (h6) and C3 (h13) were the only, from those collected in this study, that were grouped among the majority of the *E. encrasicolus* sequences available in GenBank. However, there are 6 sequences of *E. encrasicolus*, corresponding to 40% of the sequences available in Genbank , which were grouped with the samples collected in this study. The remaining analysed taxa (*Engraulis anchoita*, *Engraulis japonicus*, *Engraulis mordax*, *Engraulis ringens*) are clearly separated from each other (Fig. 12).

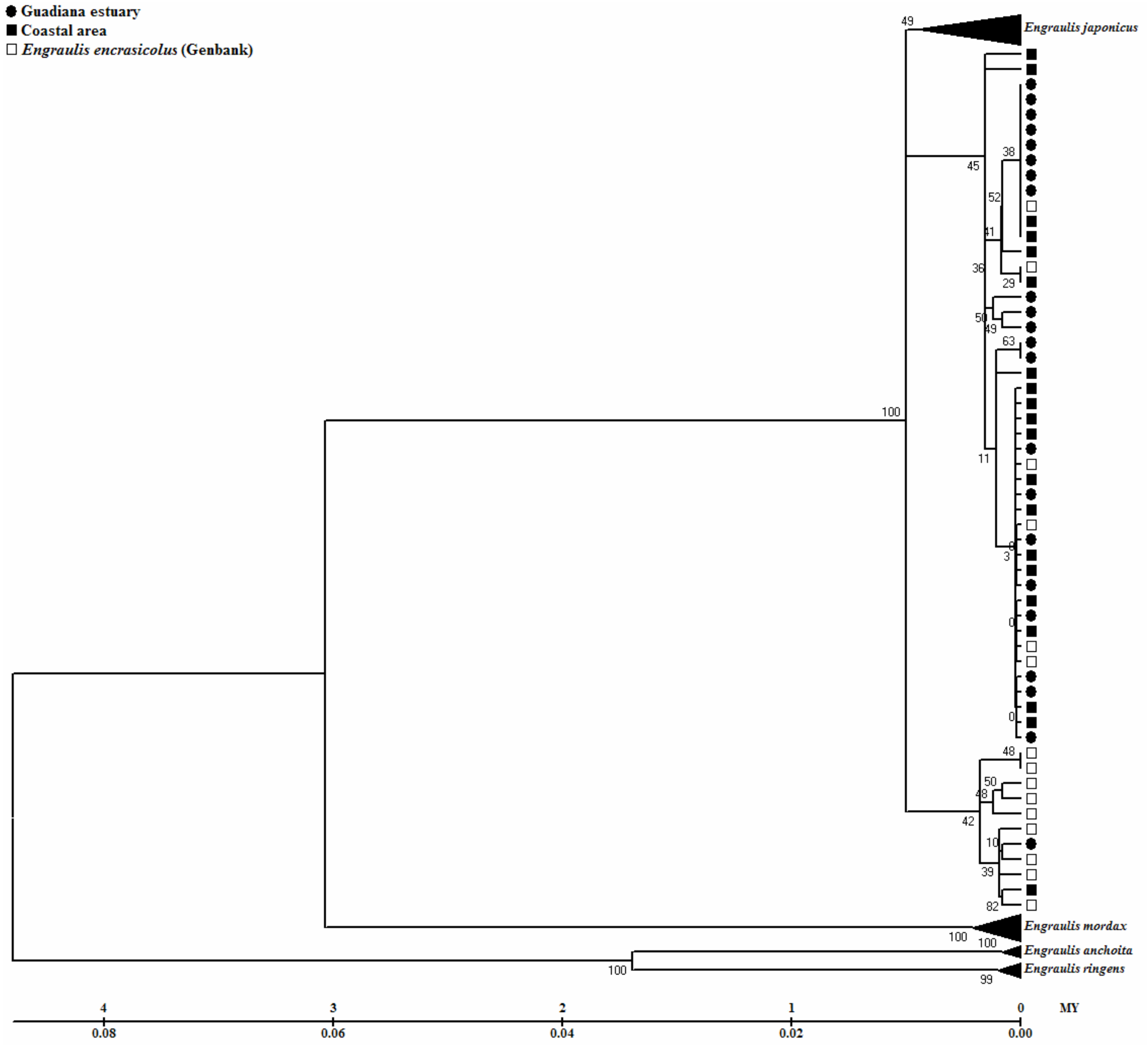


Fig. 12 Phylogenetic tree based on cyt *b* sequences with 319bp, from those determined in this study and available in GenBank (2007). The bootstrap percentage is indicated at the nodes of the trees. The evolutionary time scale (in million years- MY) and the frequency of sequence divergence are shown.

3.1.2. The gene d-loop

Table VII Nucleotide sequence of a 548 bp fragment of the mtDNA d-loop from individual E9 collected in the Guadiana estuary. Underline letters indicate the variable sites in 37 haplotypes.

```
  1 CTAGCATTCT  11 AAAGTTAAAC  21 TACCCTCTGG  31 TAATGCGGCC  41 GCCGCGCCC-
 51 –AAGTAGTGC  61 TT-GATGCCC  71 TTAGGCAGTT  81 CAAGCACTGG  91 TTCATGACTG
101 CGCAGAGCAT 111 TCATGGACAT 121 ATATGTATTA 131 TTTTACATAT 141 ATTATGGTGT
151 TAACCCATAT 161 ATGCATAATA 171 TTACATACAC 181 TATGGTGTTA 191 ATGCATACTA
201 TGTATAACTT 211 TACACTACTT 221 ATGCATGAGT 231 AAATACCTTA 241 AGGTATAATA
251 TACTGAATCT 261 GAGGACATAG 271 AAACATTATC 281 AACATAAATA 291 TAACTAAACC
301 CAACCAAGTA 311 ACAATAAAAC 321 TTAGACAGAC 331 ATAAACTGCA 341 AACAGAATAC
351 TCACGAAGAA 361 CTCCAACGCA 371 GCTGAGTAAT 381 AGAGTGATCC 391 CCATAACTCT
401 GTTTAACCAT 411 TTTCTATGCG 421 TTCCCCAACA 431 TTACTCGATA 441 ACTCACTTAA
451 TTAATGTAGT 461 AAAGTCCCAC 471 CATCGGTTGC 481 ATCCTAATGT 491 GGATCATGAA
501 TGATGGTCAG 511 GTCCATTTAT 521 CGTGGGGGTC 531 GCACAGAATG 541 AATTATTT
```

The aligned mtDNA sequence data consisted of part of the d-loop containing 548 bp (Table VII). A total of 54 variable positions and 37 haplotypes were found in the 43 individuals from both locations. Of those variable positions, two individuals (E9- haplotype 7, C3- haplotype 21) have a G (guanine) and a C (cytosine) deletion at positions 50 and 51, respectively; other individual (E13, haplotype 11) has a T (timidine) insertion at position 63. The number of variable sites and haplotypes from individuals from the Guadiana estuary was 39 and 18, respectively; which is lower than those from the coastal area, 43 and 20, respectively (Table VIII).

The global frequency of variable sites in this fragment of the mtDNA d-loop was 9.9% (54/548 bp); 7.1% (39/548 bp) for samples from the Guadiana estuary and 7.8% (43/548 bp) for samples from the coastal area (Table VIII).

A total of 17 parsimony informative sites were found for samples from the Guadiana estuary and from the coastal area. When considering both locations, the number of parsimony informative sites determined was 35 (Table VIII).

Table VIII Number of polymorphic (S), conservative and parsimony informative sites, number of haplotypes (*h*), Haplotypes diversity (*Hd*) and nucleotide diversity (π) with respective standard deviation (s.d.) and average number of nucleotide differences for samples from the Guadiana estuary and adjacent coastal area.

mtDNA d-loop	**Guadiana estuary n= 22**	**Coastal area n=21**	**Both locations n= 43**
Polymorphic sites (bp)	39	43	54
Conservative sites (bp)	508	504	493
Parsimony informative sites (bp)	17 (68, 89, 124, 127, 142, 154, 198, 261, 270, 272, 282, 295, 355, 368, 438, 443, 476)	17 (54, 89, 104, 142, 154, 164, 193, 198, 270, 271, 273, 295, 304, 355, 368, 443, 476)	35 (35, 49, 54, 68, 89, 104, 142, 154, 164, 180, 190, 193, 198, 219, 224, 227, 261, 270, 271, 272, 273, 274, 282, 294, 295, 296, 304, 355, 368, 384, 386, 438, 443)
H	18	20	37
***Hd* ± s.d.**	0.970 ± 2.8 x 10^{-2}	0.995 ± 1.6 x 10^{-2}	0.987 ± 1.1 x 10^{-2}
π± s.d.	1.26 x 10^{-2} ± 3.3 x 10^{-3}	1.36 x 10^{-2} ± 2.8 x 10^{-3}	1.32 x 10^{-2} ± 2.2 x 10^{-3}
Kt	6.879	7.395	7.190
Eta	43	51	63

The haplotype diversity (*Hd*) was 0.970 in the Guadiana estuary, which is lower than that determined for samples from the coastal area (0.995). The nucleotide diversity (π) was also lower in the Guadiana estuary (1.26 x 10^{-2}) than in the coastal area (1.36 x 10^{-2}). When considering samples from both locations, the *Hd* and π has intermediate values, 0.987 and 1.32 x 10^{-2}, respectively. The average number of nucleotide differences (Kt) was 7.418 (Table VIII).

In both populations, the gene d-loop is richer in the base A, globally (34.8 ± 0.3 %) and in each codon position (maximum: 38.9 ± 0.6 % for the Guadiana estuary and 38.8 ± 0.4 % for the coastal area). The difference in base content frequency is not significant between sampling locations, either globally or for each codon position (U-test: p= 0.100 to p= 0.836) (Table IX).

Table IX Base (T, C, A and G) frequencies (in percent) for the 390 bp fragment of mtDNA d-loop, considering the entire fragment or each codon position, for each individual captured in the Guadiana estuary or in the adjacent coastal area. The average and standard deviation for each location and for the combination of both locations are shown.

Sample	**T**	**C**	**A**	**G**	**Total**	**T-1**	**C-1**	**A-1**	**G-1**	**Pos #1**	**T-2**	**C-2**	**A-2**	**G-2**	**Pos #2**	**T-3**	**C-3**	**A-3**	**G-3**	**Pos #3**
E1	30.2	20.3	34.9	14.6	547	32.8	19.1	35.0	13.1	183	27.3	17.5	39.3	15.8	183	30.4	24.3	30.4	14.9	181
E2	29.4	20.7	34.4	15.5	547	32.2	19.1	35.5	13.1	183	26.8	18.0	37.7	17.5	183	29.3	24.9	29.8	16.0	181
E3	30.2	20.1	34.7	15.0	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	29.8	15.5	181
E4	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E5	30.0	20.1	35.3	14.6	547	32.2	19.1	35.5	13.1	183	27.3	16.9	39.9	15.8	183	30.4	24.3	30.4	14.9	181
E6	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E7	30.0	20.3	34.9	14.8	547	32.8	18.6	35.5	13.1	183	27.3	17.5	39.9	15.3	183	29.8	24.9	29.3	16.0	181
E8	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E9	29.0	20.9	33.6	16.5	545	32.2	20.2	33.9	13.7	183	26.4	17.0	37.9	18.7	182	28.3	25.6	28.9	17.2	180
E10	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E11	30.0	20.3	34.6	15.2	547	32.2	19.1	35.0	13.7	183	27.3	17.5	38.8	16.4	183	30.4	24.3	29.8	15.5	181
E12	30.0	20.3	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.3	17.5	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E13	29.9	20.4	34.7	15.0	548	32.2	19.1	35.5	13.1	183	27.9	16.9	38.3	16.9	183	29.7	25.3	30.2	14.8	182
E14	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
E15	30.0	20.3	35.1	14.6	547	32.2	19.1	35.5	13.1	183	27.3	17.5	39.3	15.8	183	30.4	24.3	30.4	14.9	181
E16	30.0	20.3	34.9	14.8	547	32.8	18.6	35.5	13.1	183	27.3	17.5	38.8	16.4	183	29.8	24.9	30.4	14.9	181
E17	30.0	20.3	35.1	14.6	547	32.2	19.1	35.5	13.1	183	27.3	17.5	39.3	15.8	183	30.4	24.3	30.4	14.9	181
E18	30.0	20.3	35.1	14.6	547	32.2	19.1	35.5	13.1	183	27.3	17.5	39.3	15.8	183	30.4	24.3	30.4	14.9	181
E19	30.0	20.3	34.7	15.0	547	32.8	18.6	35.0	13.7	183	27.3	17.5	39.3	15.8	183	29.8	24.9	29.8	15.5	181
E20	29.6	20.7	34.6	15.2	547	32.2	19.1	35.5	13.1	183	27.3	17.5	37.7	17.5	183	29.3	25.4	30.4	14.9	181
E21	30.0	20.3	34.9	14.8	547	32.8	18.6	35.5	13.1	183	27.3	17.5	39.9	15.3	183	29.8	24.9	29.3	16.0	181
E22	29.8	20.5	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.3	17.5	38.8	16.4	183	29.8	24.9	30.4	14.9	181
Avgerage	30.0	20.3	34.8	14.9	547.0	32.3	19.1	35.4	13.2	183.0	27.4	17.3	38.9	16.4	183.0	30.0	24.6	30.1	15.2	181.0
St. dev.	0.3	0.2	0.3	0.4	0.5	0.3	0.3	0.4	0.2	0.0	0.4	0.3	0.6	0.8	0.2	0.5	0.4	0.5	0.6	0.3
C1	30.2	20.5	34.2	15.2	547	32.8	19.1	34.4	13.7	183	27.3	18.0	38.3	16.4	183	30.4	24.3	29.8	15.5	181
C2	30.2	20.1	35.1	14.6	547	32.2	19.1	35.5	13.1	183	27.9	16.9	39.3	15.8	183	30.4	24.3	30.4	14.9	181
C3	29.7	20.4	34.3	15.6	545	33.3	19.1	35.0	12.6	183	26.4	17.6	37.9	18.1	182	29.4	24.4	30.0	16.1	180
C4	29.6	20.7	34.9	14.8	547	31.7	19.7	35.0	13.7	183	26.8	18.0	39.3	15.8	183	30.4	24.3	30.4	14.9	181
C5	30.2	20.1	34.7	15.0	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.3	16.9	183	30.4	24.3	30.4	14.9	181
C6	30.0	20.5	34.9	14.6	547	31.7	19.7	35.5	13.1	183	27.9	17.5	38.8	15.8	183	30.4	24.3	30.4	14.9	181
C7	30.3	19.9	34.9	14.8	547	33.3	18.0	35.5	13.1	183	27.3	17.5	38.8	16.4	183	30.4	24.3	30.4	14.9	181
C8	29.6	20.7	34.9	14.8	547	31.7	19.7	35.5	13.1	183	27.3	17.5	39.3	15.8	183	29.8	24.9	29.8	15.5	181
C9	29.8	20.5	34.4	15.4	547	31.7	19.7	34.4	14.2	183	27.3	17.5	38.8	16.4	183	30.4	24.3	29.8	15.5	181
C10	30.0	20.3	34.7	15.0	547	32.2	19.1	35.5	13.1	183	27.3	17.5	38.3	16.9	183	30.4	24.3	30.4	14.9	181
C11	30.2	20.1	35.1	14.6	547	32.8	18.6	35.5	13.1	183	27.3	17.5	39.3	15.8	183	30.4	24.3	30.4	14.9	181
C12	30.0	20.3	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.3	17.5	38.8	16.4	183	30.4	24.3	30.4	14.9	181
C13	30.0	20.3	34.4	15.4	547	32.2	19.1	33.9	14.8	183	27.9	16.9	39.3	15.8	183	29.8	24.9	29.8	15.5	181
C14	30.0	20.3	34.6	15.2	547	32.2	19.1	35.0	13.7	183	27.9	16.9	38.8	16.4	183	29.8	24.9	29.8	15.5	181
C15	30.2	20.3	34.6	15.0	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.3	16.9	183	30.4	24.9	29.8	14.9	181
C16	30.2	20.1	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.9	16.9	38.8	16.4	183	30.4	24.3	30.4	14.9	181
C17	30.0	20.3	34.9	14.8	547	32.2	19.1	35.5	13.1	183	27.3	17.5	38.8	16.4	183	30.4	24.3	30.4	14.9	181
C18	30.2	20.5	34.2	15.2	547	32.2	19.1	35.0	13.7	183	28.4	16.9	38.3	16.4	183	29.8	25.4	29.3	15.5	181
C19	30.0	20.3	34.7	15.0	547	32.2	19.1	35.0	13.7	183	27.3	17.5	38.8	16.4	183	30.4	24.3	30.4	14.9	181
C20	29.8	20.3	34.7	15.2	547	32.2	19.1	35.0	13.7	183	27.3	17.5	38.8	16.4	183	29.8	24.3	30.4	15.5	181
C21	29.8	20.3	34.9	15.0	547	32.2	19.1	35.0	13.7	183	27.3	17.5	38.8	16.4	183	29.8	24.3	30.9	14.9	181
Avgerage	30.0	20.3	34.7	15.0	546.9	32.3	19.1	35.2	13.4	183.0	27.5	17.4	38.8	16.4	183.0	30.2	24.5	30.2	15.2	181.0
St. dev.	0.2	0.2	0.3	0.3	0.4	0.4	0.4	0.5	0.5	0.0	0.5	0.4	0.4	0.5	0.2	0.3	0.3	0.4	0.4	0.2
Global avg.	30.0	20.3	34.8	15.0	546.9	32.3	19.1	35.3	13.3	183	27.5	17.3	38.8	16.4	183	30.1	24.5	30.1	15.2	181
Global st. dev.	0.3	0.2	0.3	0.4	0.5	0.4	0.3	0.4	0.4	0.0	0.4	0.3	0.5	0.7	0.2	0.5	0.4	0.4	0.5	0.3

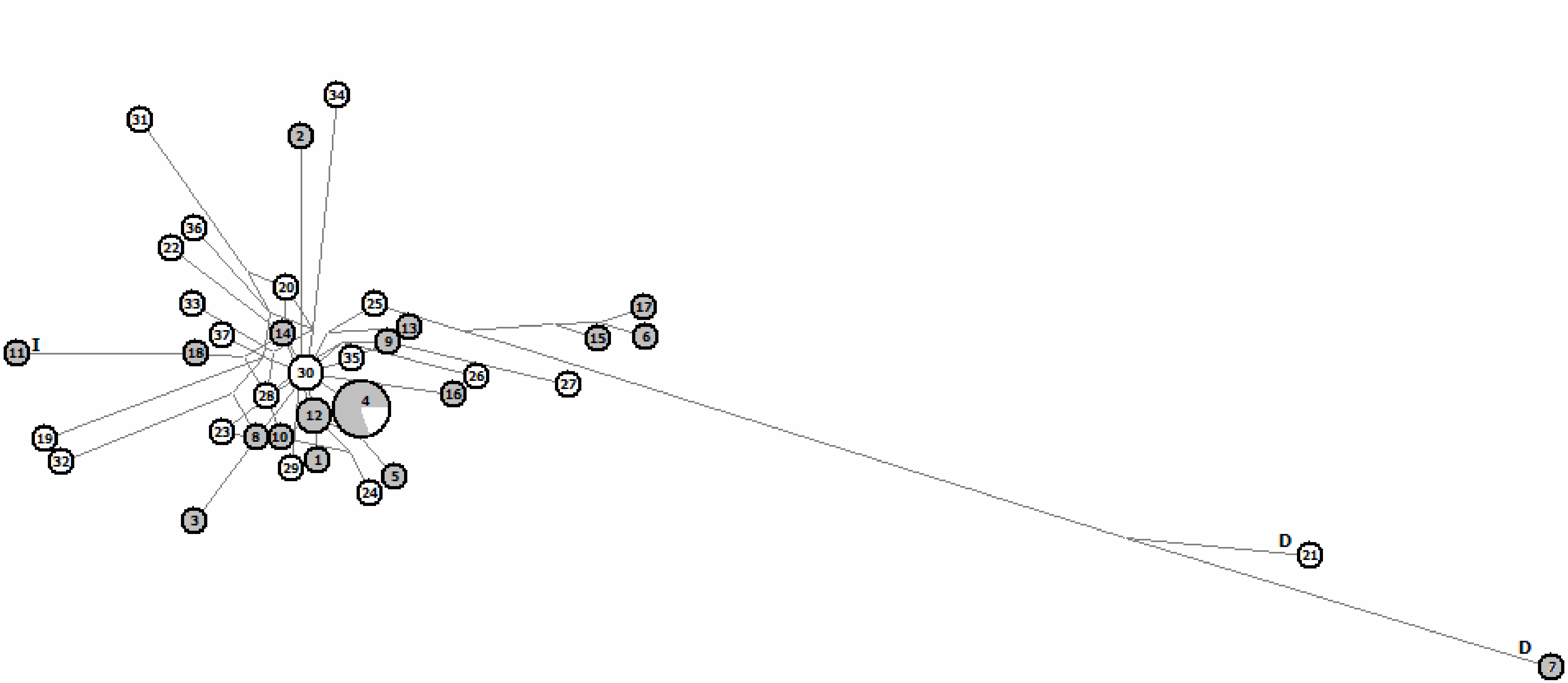


Fig. 13 MtDNA haplotypes network for the gene d-loop, from samples collected in the Guadiana estuary (grey) and in the coastal area off Praia Verde (white). The number of each haplotype is inside the circle. The frequency of each haplotype is proportional to the circle size, for example h1= 1, h4= 5 and h12= 2. The letters I and D correspond to the presence of an insertion and deletions in the respective haplotype.

The average sequence divergences within samples from the Guadiana estuary was 6.88 ± 1.19 %, while for those from the coast was 7.40 ± 1.28 %. The overall difference between samples from both sites was 7.25 ± 1.17 %.

The several genetic differentiation estimates reveal no significant differences between sites: Hs= 0.982, Hst= 0.012 (p=0.099), Kst^*= 0.005 (p= 0.235), Z^*= 5.778 (p= 0.118). The results for gene flow estimates were as follow: Gst= 0.005, γst= 0.031, Fst= 0.014.

It were defined 43 haplotypes, of which 34 (79.1%) correspond to exclusive haplotypes, 16 in the estuarine samples and 18 in the coast. The most abundant haplotype was h4 (n= 5 11.6%), which predominate in the estuary (80%); then it was h12 (n=2, 4.7%) and h30 (n= 2, 4.7%), both exclusive to the estuary and to the coast, respectively. The haplotype h11 has one insertion and the two most dissimilar haplotypes, h7 (estuary) and h21 (coast), both have two deletions (Figs. 12, 13).

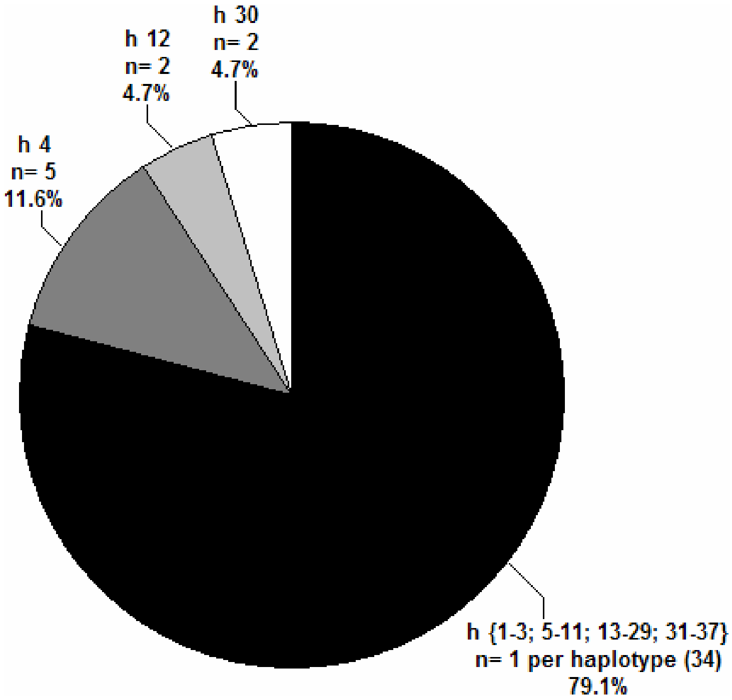


Fig. 14 Haplotypes frequency for the d-loop gene from samples collected in the Guadiana estuary and in the coastal area off Praia Verde. Haplotypes with one individual were grouped.

3.2. Anchovy larval stages

3.2.1. Abiotic setup

The average daily river inflow varied from 1.5 ± 0.0 $m^3.s^{-1}$ (July 3rd 2002) to 656.3 ± 405.0 $m^3.s^{-1}$ (April 9th 2002). The average annual river flow was 24.0 ± 61.3 $m^3.s^{-1}$. River inflow was lower than the average historical river inflow, except for two temporary occasions in April and December 2002. Six days prior to the April sampling, river inflow increased 44.8 times in only 2 days (Fig. 15).

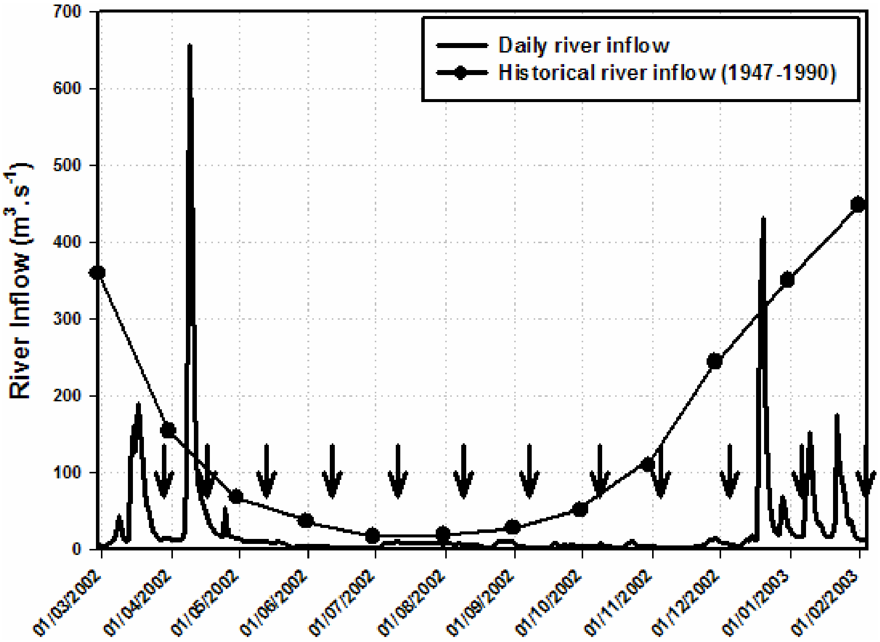


Fig. 15 Daily average Guadiana river inflow and historical monthly average river inflow (1947-1999) registered at Pulo do Lobo hydrometric station. Arrows on the graph correspond to the days of sampling.

Surface temperature varied from 11.6 to 26.7 ºC, presenting a standard seasonal evolution (Fig. 16). Surface salinity increased towards downstream stations, varying from 0.09 (station 9) to36.5 (station 1) (Fig. 17).

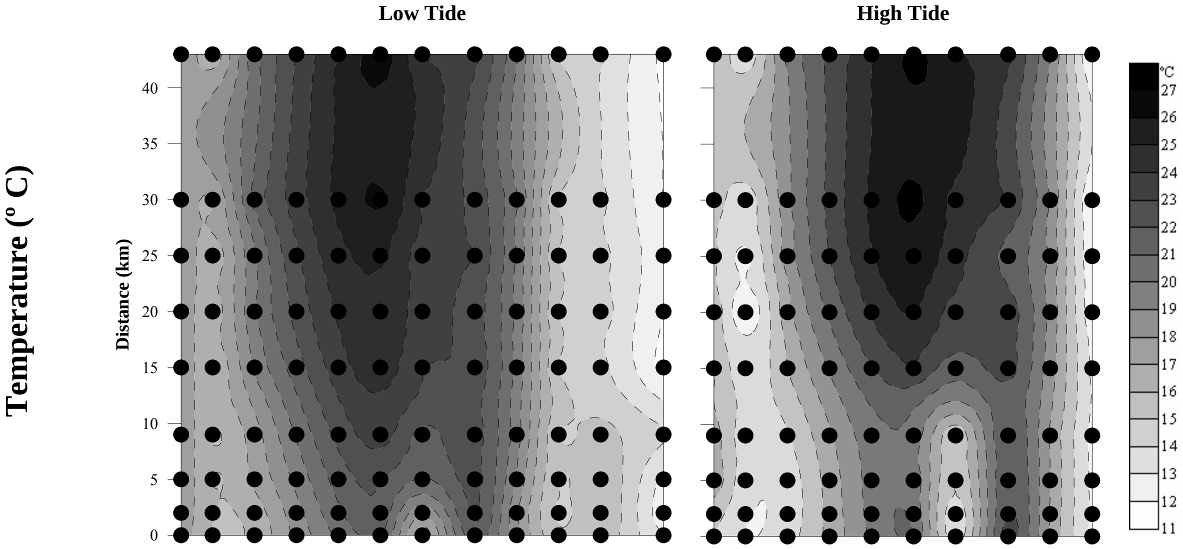


Fig. 16 Spatial and temporal evolution of surface temperature in the Guadiana estuary and adjacent coastal area, at low (A) and high tides (B), from March 2002 till February 2003.

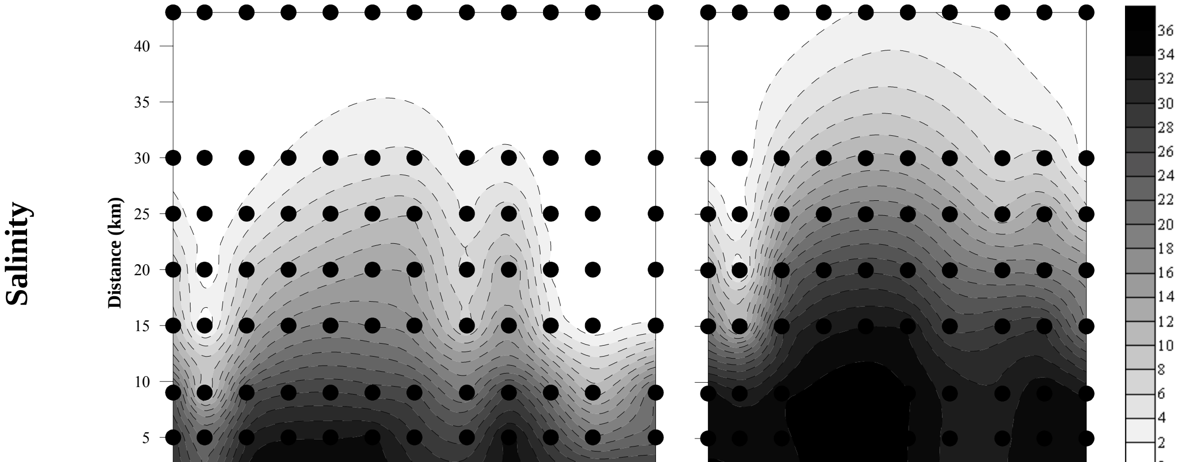
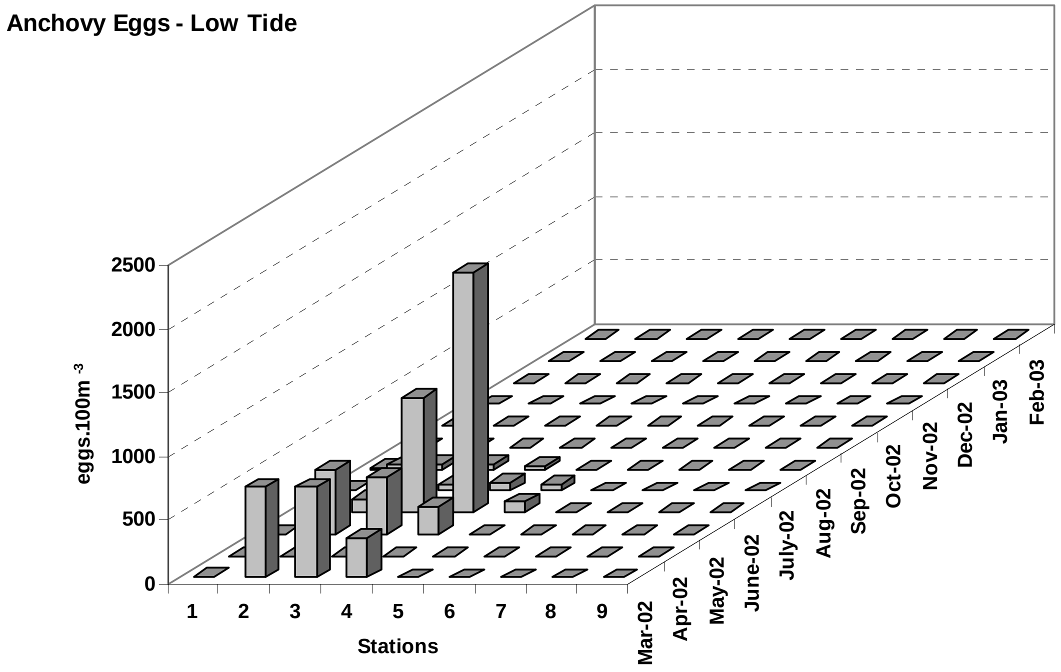


Fig. 17 Spatial and temporal evolution of surface salinity in the Guadiana estuary and adjacent coastal area, at low (A) and high tides (B), from March 2002 till February 2003.

3.2.2. Anchovy larval stages - distribution and abundance

Anchovy eggs and larvae were collected from March to November 2002, in every sampling station (Figs. 18, 19). The temperature range, in which eggs and larvae were collected, varied from 15.9 ºC to 24.7 ºC and from 16.1 ºC to 23.5 ºC, respectively (Figs. 16). Anchovy eggs and larvae were distributed along a salinity gradient that varied from 0.2 to 36.4 and from 0.4 to 36.4, respectively (Fig. 17). Anchovy eggs were captured more abundantly at an average salinity of 29.1 ± 5.5 (Fig. 20), while larvae were predominantly collected at a lower average salinity, 22.6 ± 12.0 (Fig. 21). During the three months of maximum abundance of eggs, the average river flow, in the week previous to sampling, was 30.8 ± 17.6 $m^3.s^{-1}$ in March, 10.5 ± 0.5 $m^3.s^{-1}$ in May and 3.2 ± 0.1 $m^3.s^{-1}$ in June 2002. (Fig. 15).

In June 2002, the maximum abundance of anchovy eggs was 2106 $eggs.100m^{-3}$ (Fig. 18) and of larvae was 218 $larvae.100m^{-3}$ (Fig. 19). The maximum abundance of eggs was registered in the upper part of the low estuary, during the low tide, and in the low part of the middle estuary, at the high tide. The highest abundance of larvae were recorded in the middle estuary, at both tides. Two major decreases in anchovy larval stages abundance occurred in April and in July 2002. In April 2002 the abundance of eggs decreased 99.99%. In July 2002, eggs and larvae abundances decreased 91.3% and 86.6%, respectively (Figs. 18, 19). The average temperature along the studied area, in the June 2002, the month with highest abundance of anchovy larval stages, was 20.1 ± 1.9 ºC (Fig. 16). In this month, the eggs and larvae were more abundant at an average salinity of 29.1 ± 5.5 and 22.6 ± 12.0, respectively (Fig. 21).



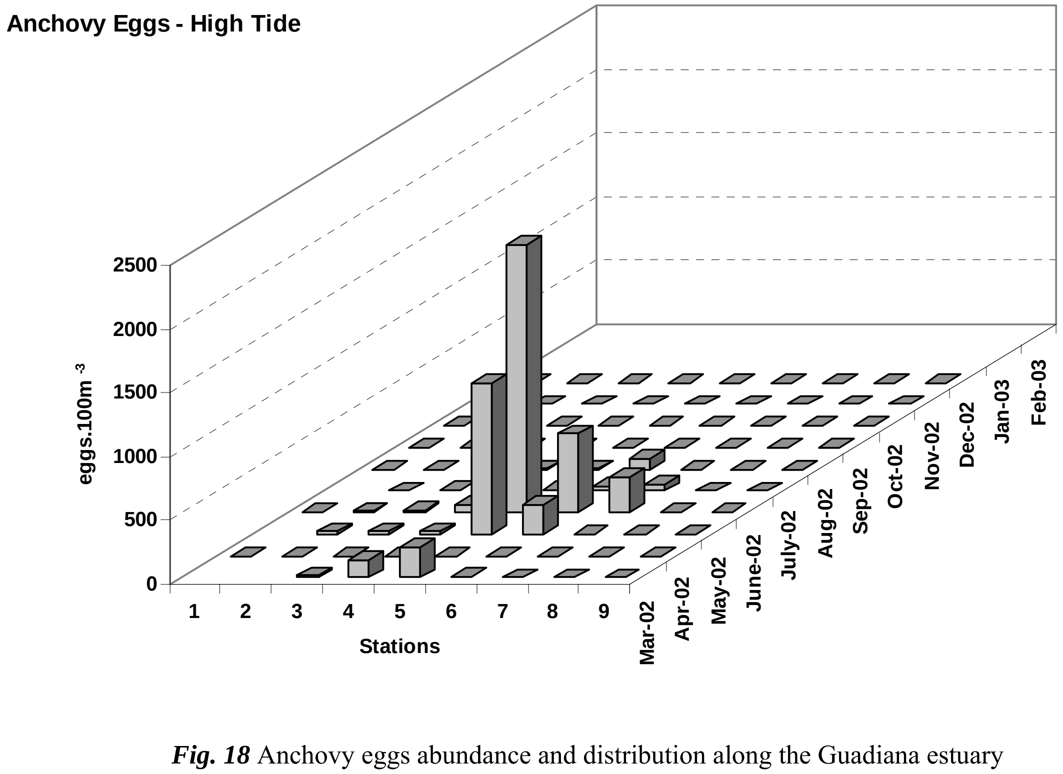
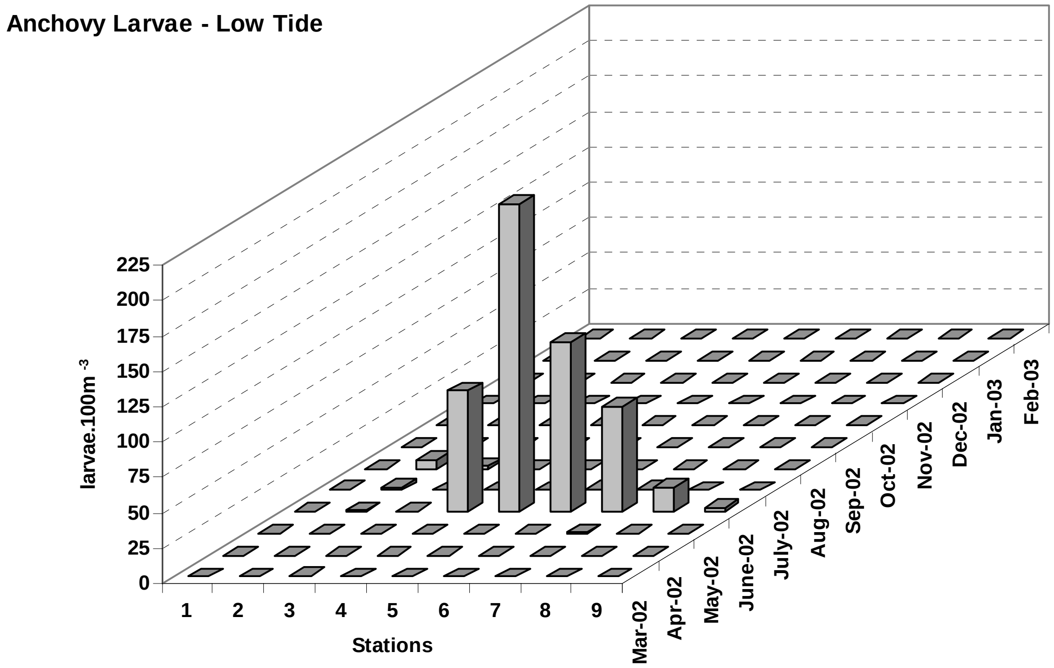


Fig. 18 Anchovy eggs abundance and distribution along the Guadiana estuary and adjacent coastal area, at low and high tides, from March 2002 to February 2003.



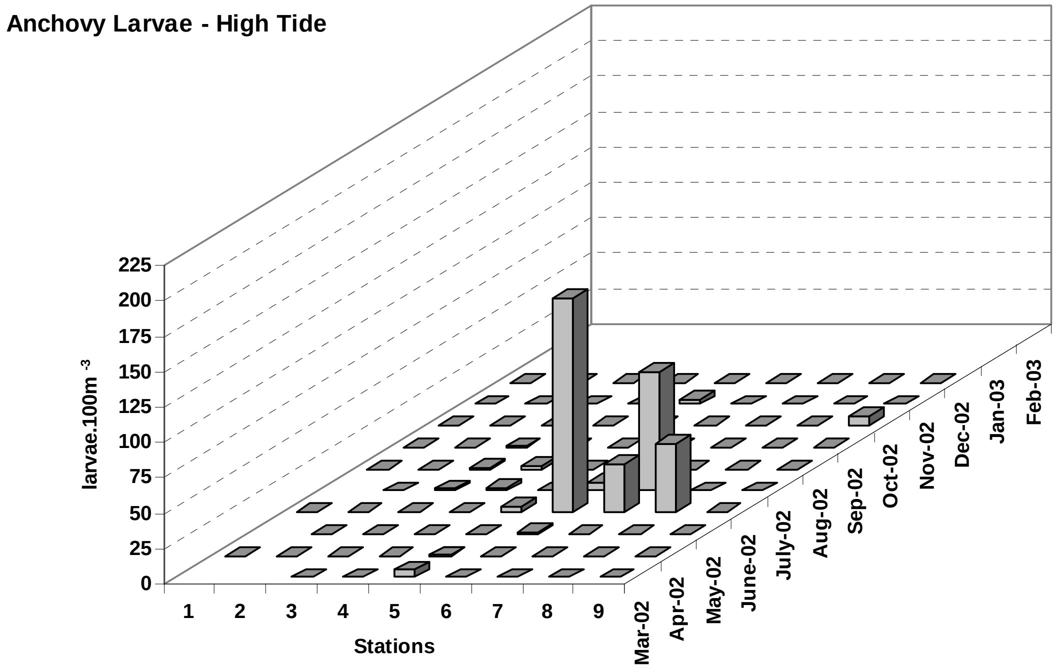


Fig. 19 Anchovy larvae abundance and distribution along the Guadiana estuary and adjacent coastal area, at low and high tides, from March 2002 to February 2003.

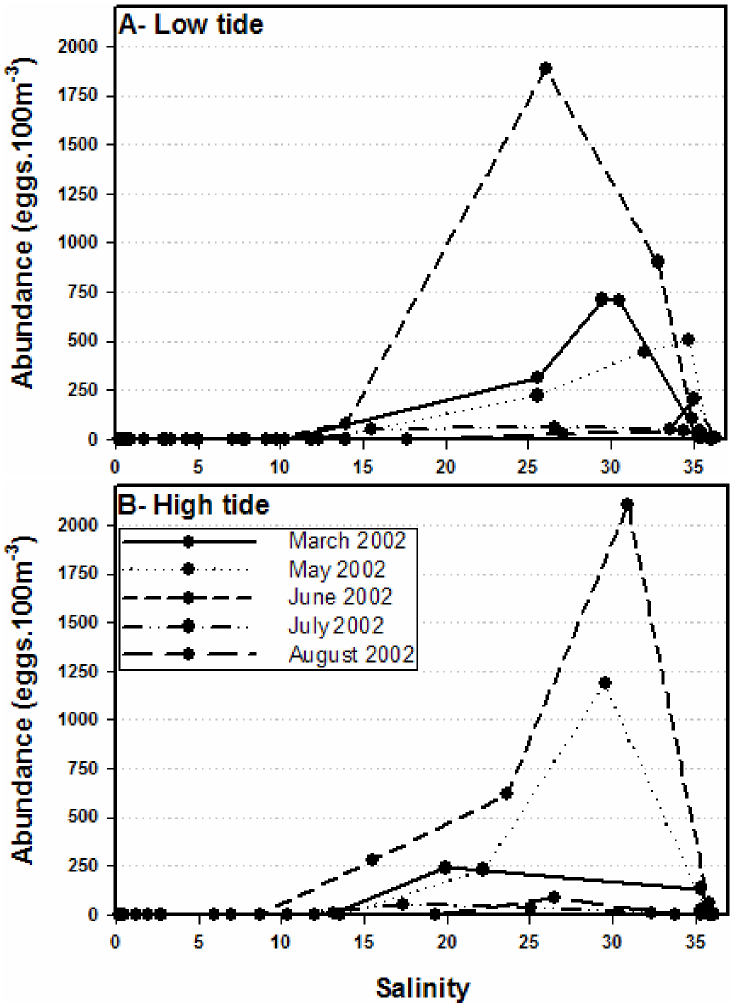


Fig. 20 Abundance of anchovy eggs along the salinity gradient measured in the Guadiana estuary and adjacent coastal area, during the months of higher egg abundance.

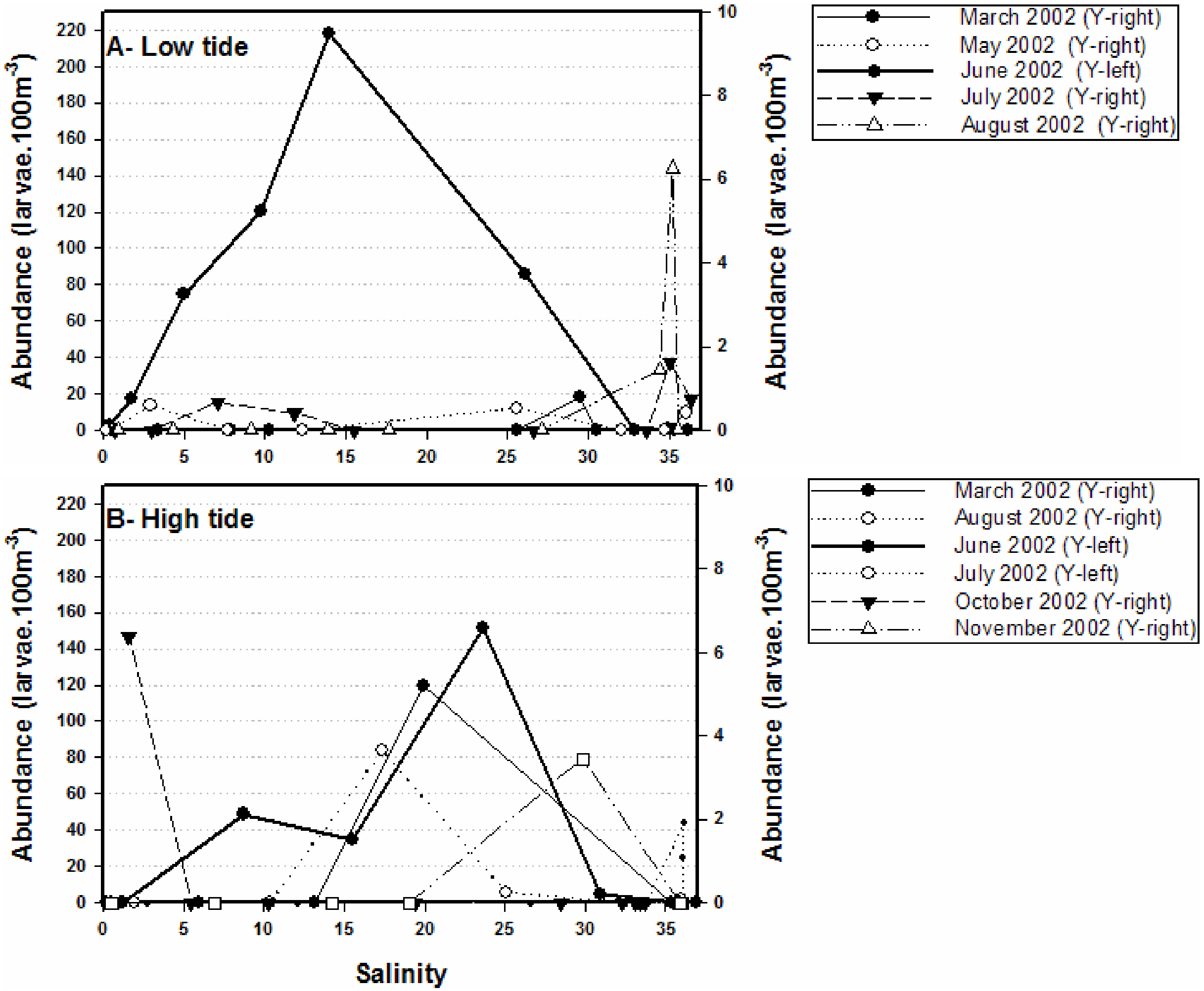


Fig. 21 Abundance of anchovy larvae along the salinity gradient measured in the Guadiana estuary and adjacent coastal area, during the months of higher larvae abundance.

3.2.3. Anchovy spawning and hatching grounds

Anchovy eggs in early development, stages I and II, occurred predominantly at the high tide, in the areas where abundance was maximum. Thus, at the high tide of March (Q_{7days}= 30.8 ± 17.6 $m^3.s^{-1}$) and May 2002 (Q_{7days}= 10.5 ± 0.5 $m^3.s^{-1}$), maximum abundances were observed at station 5, where 64% (153 eggs.100 m^{-3}) and 82% (972 eggs.100 m^{-3}) of the eggs were in stage II, respectively. However, in June 2002, when river inflow was minimum (Q_{7days} = 3.2 ± 0.1 $m^3.s^{-1}$), the eggs collected at the high tide at stages I and II were mainly in station 7, at the upstream limit of anchovy egg distribution. These stages represented 73% of the collected eggs (203 eggs.100 m^{-3}), while in the station of maximum abundance (station 5) they account for 4% (84 eggs.100 m^{-3}) (Figs. 22 to 24).

Anchovy eggs at late development, stages X and XI, are the smallest fraction of the collected eggs. In March 2002, no eggs in these stages were collected. In May 2002, the latter egg stages were caught in stations 2 (2.3% - 12 eggs.100m^{-3}) and 3 (2.5% - 0.5 eggs.100m^{-3}), at the low and high tide, respectively. In June 2002, eggs in late development were caught in upstream areas, up to stations 4 (HT - 3.8% - 2.4 eggs.100m^{-3}) and 5 (HT - 5.4% - 112.8 eggs.100m^{-3}) (Figs. 22 to 24).

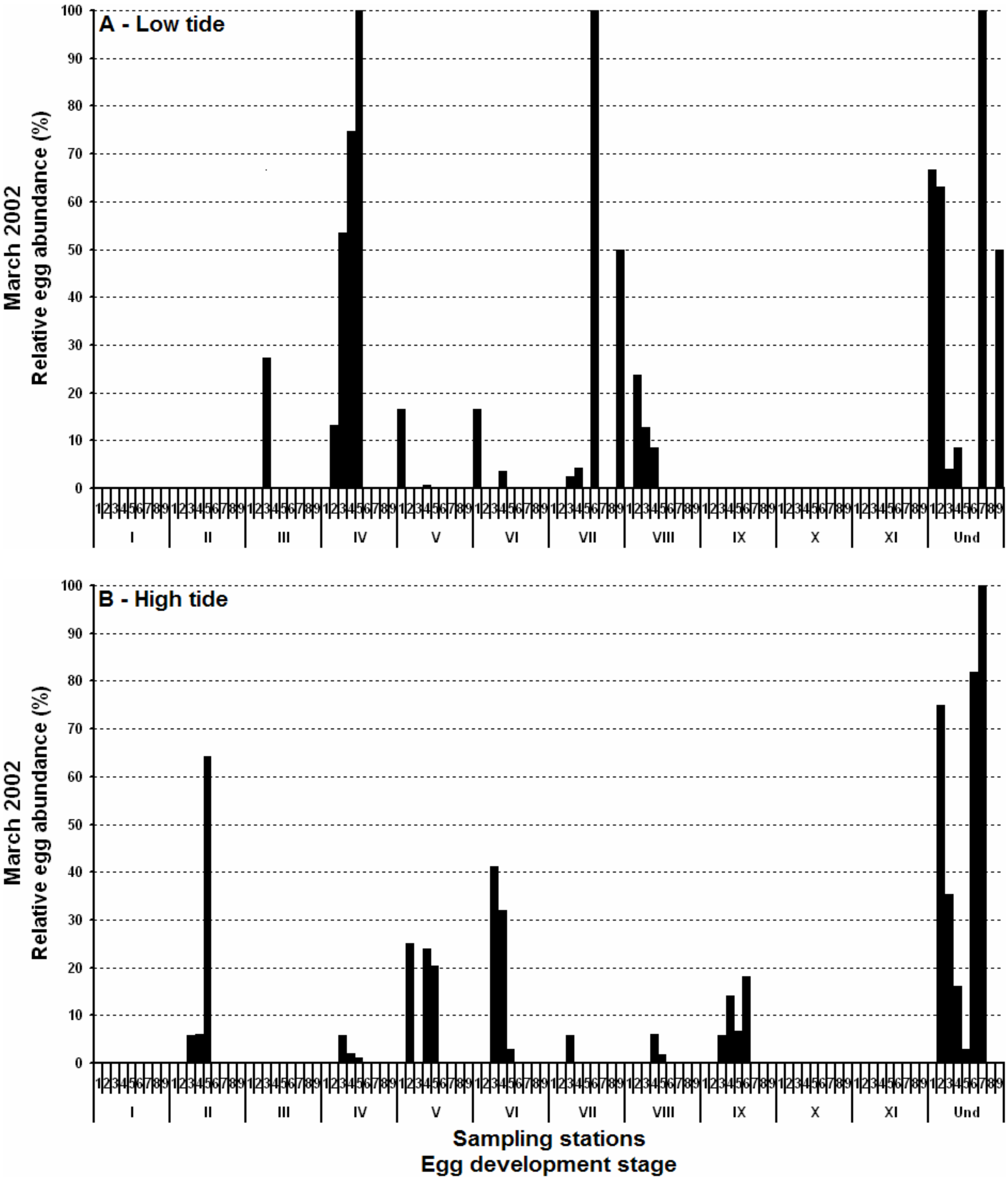


Fig. 22 Frequency of anchovy eggs development stages (Roman numerals) distributed by sampling stations (Arabic numerals), at the low (A) and high (B) tides of March 2002.

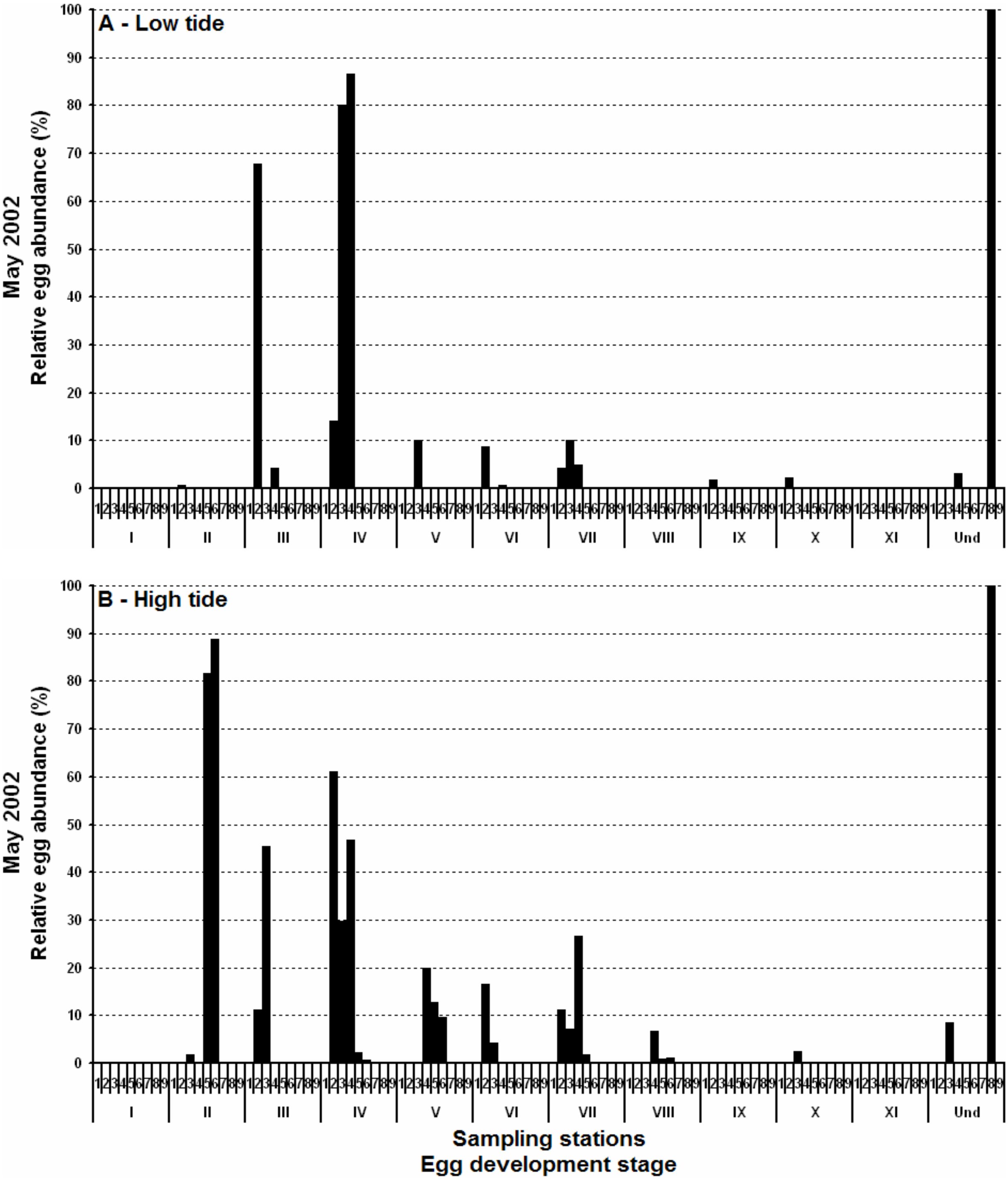


Fig. 23 Frequency of anchovy eggs development stages (Roman numerals) distributed by sampling stations (Arabic numerals), at the low (A) and high (B) tides of May 2002.

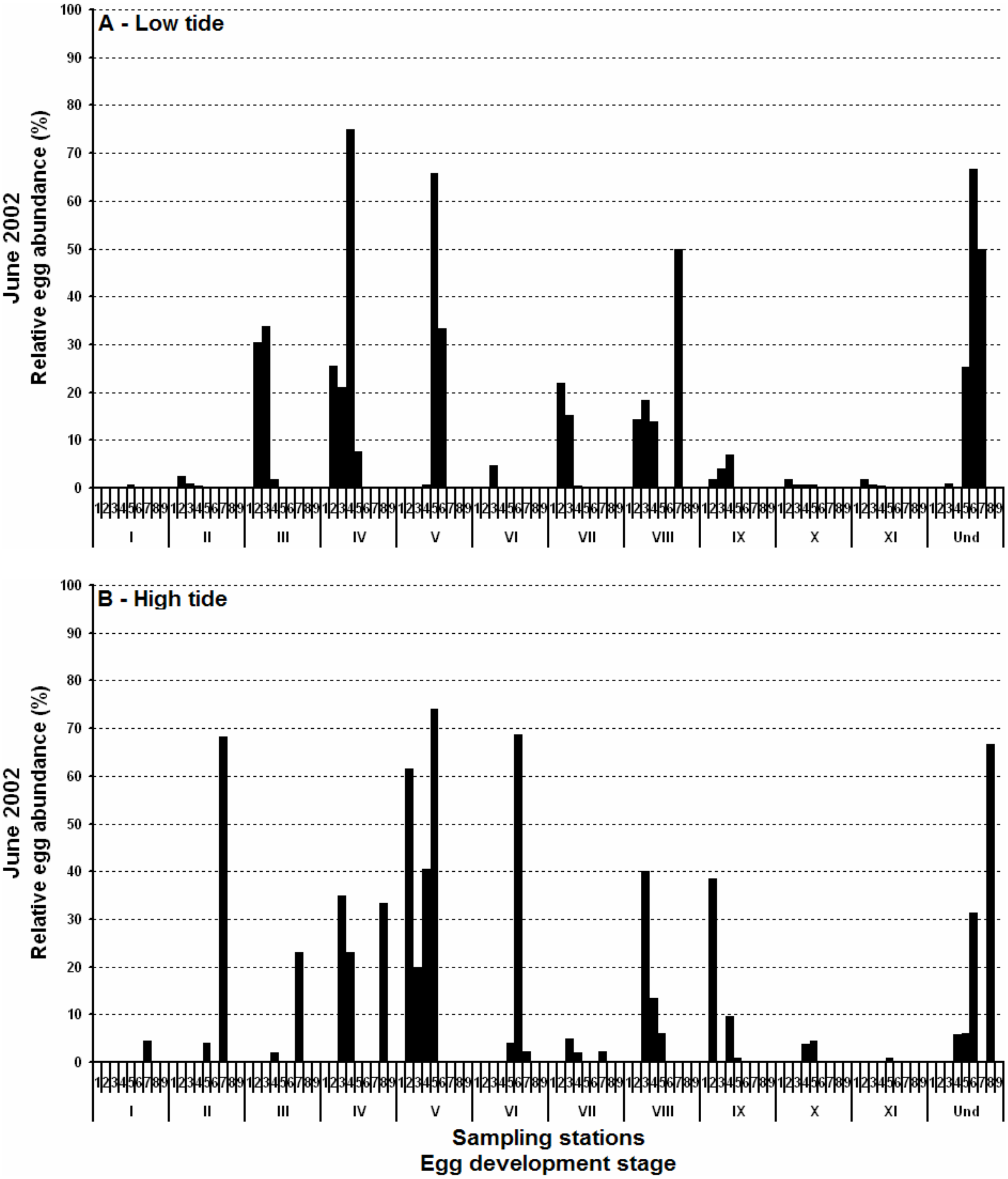


Fig. 24 Frequency of anchovy eggs development stages (Roman numerals) distributed by sampling stations (Arabic numerals), at the low (A) and high (B) tides of June 2002.

Table X Kruskal-Wallis One Way Analysis of Variance on Ranks made for the age of anchovy larvae captured in June 2002 in the Guadiana estuary at low tide. Multiple comparisons between stations are shown.

June 2002 – Low tide

Groups (stations)	**N**	**Average**	**25%**	**75%**
4	30	0.670	0.370	0.810
5	33	1.550	1.372	3.325
6	38	2.040	1.330	5.930
7	33	5.110	3.565	6.938
8	23	6.340	4.288	7.768
9	4	9.890	5.555	11.375

H= 96.638 with 5 degrees of freedom (P≤ 0.001)

Dunn's method

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
9 vs 4	115.367	4.649	yes
9 vs 5	68.788	2.787	no
9 vs 6	52.763	2.153	don't test
9 vs 7	21.848	0.885	don't test
9 vs 8	10.304	0.408	don't test
8 vs 4	105.062	8.131	yes
8 vs 5	58.484	4.618	don't test
8 vs 6	42.459	3.447	don't test
8 vs 7	11.544	0.912	don't test
7 vs 4	93.518	7.952	yes
7 vs 5	46.939	4.090	don't test
7 vs 6	30.915	2.787	don't test
6 vs 4	62.604	5.498	yes
6 vs 5	16.025	1.445	don't test
5 vs 4	46.579	3.961	yes

Table XI Kruskal-Wallis One Way Analysis of Variance on Ranks made for the age of anchovy larvae captured in June 2002 in the Guadiana estuary at high tide. Multiple comparisons between stations are shown

June 2002 – High tide

Groups (stations)	**N**	**Average**	**25%**	**75%**
5	5	1.8	1.3	1.9
6	144	1.4	1.2	1.9
7	31	1.9	1.5	3.4
8	71	3.9	2.5	5.6

H= 87.710 with 3 degrees of freedom (P≤ 0.001)

Dunn's method

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
8 vs 6	97.978	9.306	yes
8 vs 5	85.487	2.545	no
8 vs 7	56.468	3.613	don't test
7 vs 6	41.510	2.888	yes
7 vs 5	29.019	0.829	don't test
5 vs 6	12.490	0.378	no

The size range of the collected larvae varied from 3.4 mm to 26.3 mm, corresponding to recently hatched larvae and to larvae with 42 days old, respectively. A consistent analysis on the distribution pattern of anchovy larvae is only possible in June 2002. In this month, significant differences on the age and size of larvae were found between sampling stations, at both tides (P≤ 0.001). Younger larvae were collected in downstream areas, progressively increasing its age and size towards the upper estuary. The lowest average age was 7.2 ± 0.5 days in station 4 (low tide) and the maximum was 15.1 ± 4.7 days in station 9 (low tide) (Fig. 25, Table X & XI).

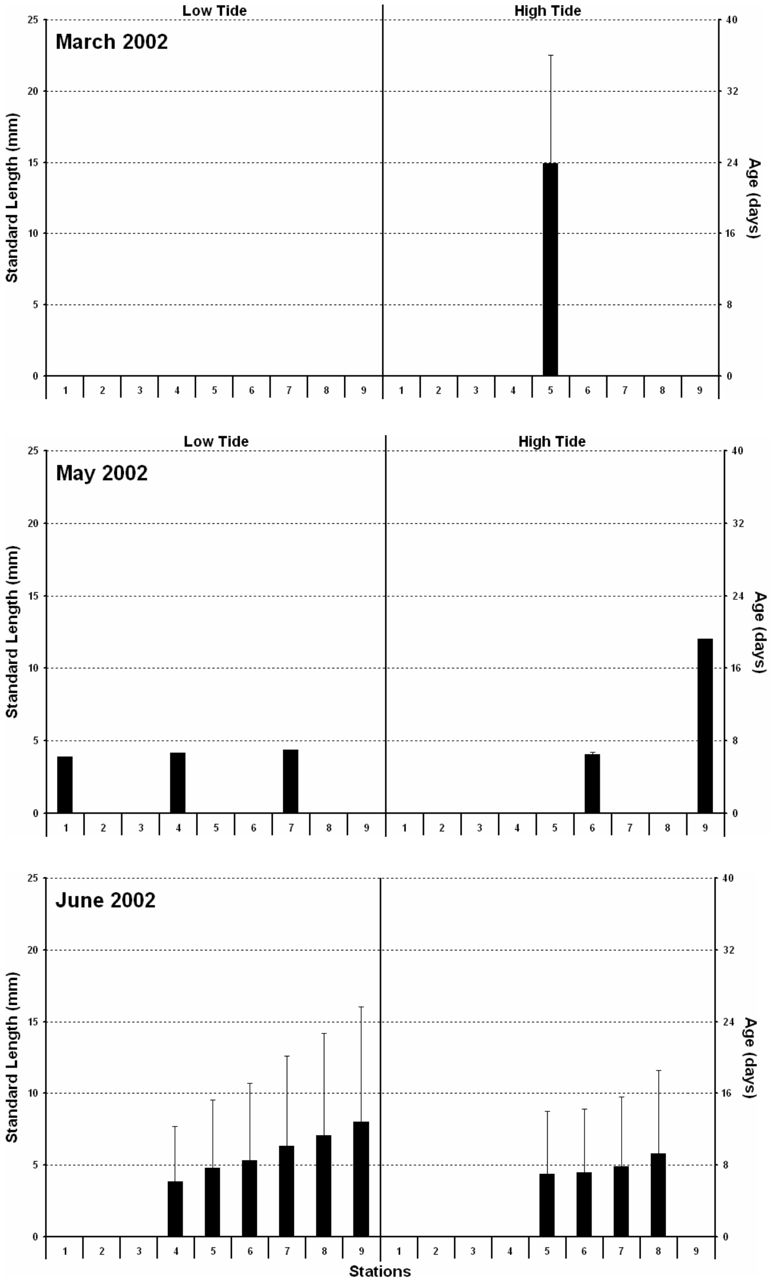


Fig. 25 Standard length (mm) and age (days) of anchovy larvae captured along the Guadianaestuary and in the adjacent coastal area, in March 2002 and from May to November 2002, at the low and high tides.

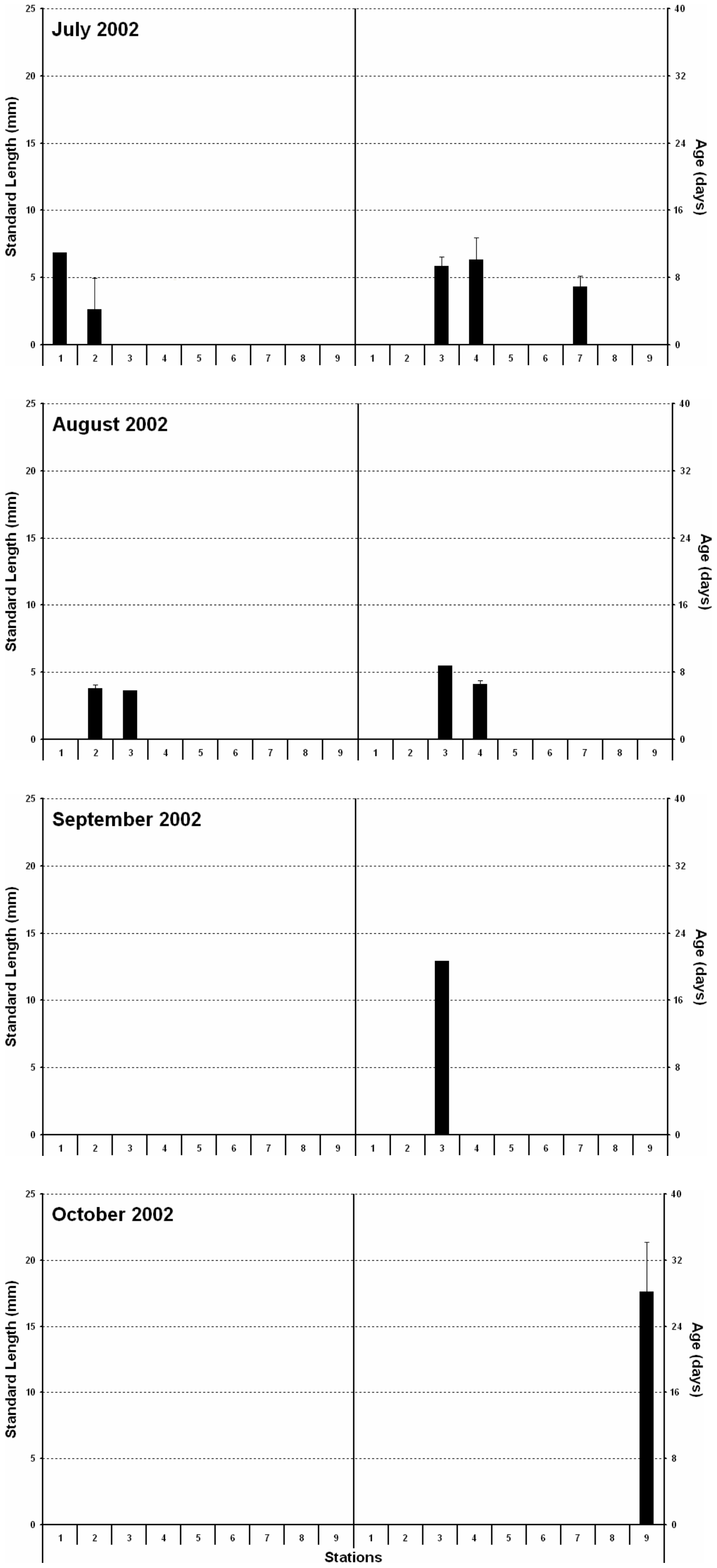


Fig. 25 (cont.) Standard length (mm) and age (days) of anchovy larvae captured along the Guadianaestuary and in the adjacent coastal area, in March 2002 and from May to November 2002, at the low and high tides.

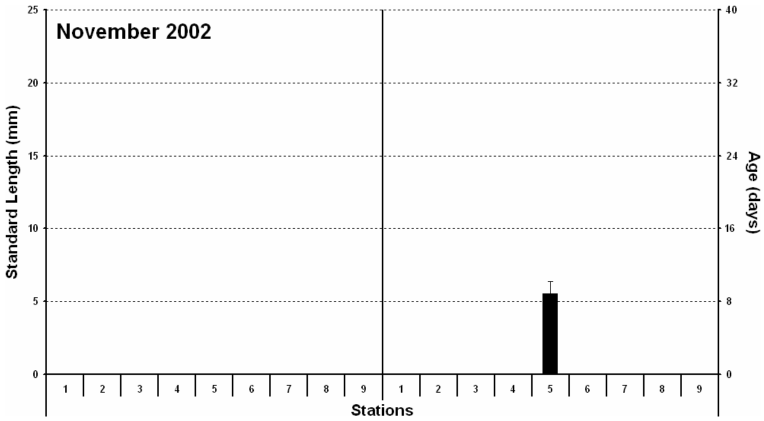


Fig. 25 (cont.) Standard length (mm) and age (days) of anchovy larvae captured along the Guadianaestuary and in the adjacent coastal area, in March 2002 and from May to November 2002, at the low and high tides.

3.3. Anchovy juveniles and adults

The Guadiana river flow varied markedly from January 2001 to September 2002. The highest river flow, 3257 $m^3.s^{-1}$, was registered in early February 2001. Long periods of reduced river flow were determined during Summer of 2001 and late Spring and Summer of 2002. In January 2001 intense river flows were observed, over 3000 $m^3.s^{-1}$, but could not be registered in the hydrometric station due to flooding (Fig. 26).

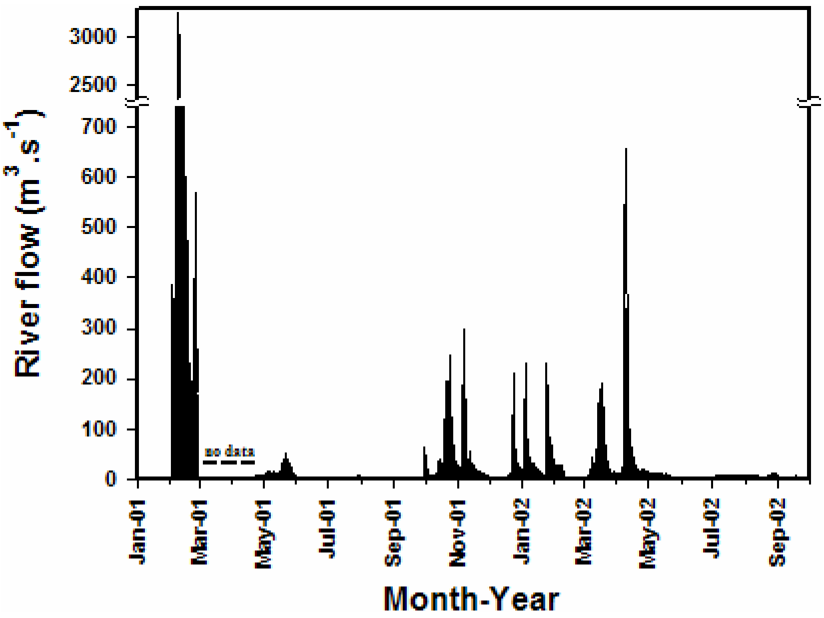


Fig. 26 Guadiana river flow from January 2001 to September 2002, at Pulo do Lobo hydrometric station. Data source: INAG (2004).

Table XII. Presence (black squares)-absence (white squares) table of the juveniles and adults of anchovy along the Guadiana estuary and in the adjacent coastal area from January 2001 to September 2002.

	Jan-01	Mar-01	May-01	Jul-01	Sep-01	Nov-01	Jan-02	Mar-02	May-02	Jul-02	Sep-02
st.9	□	□	□	□	□	□	□	□	□	□	■
st.8	□	□	□	□	□	□	□	□	□	□	■
st.7	□	□	■	■	■	■	□	□	■	■	■
st.6	□	□	□	■	■	■	□	□	■	□	■
st.5	□	□	■	■	■	■	■	■	■	■	■
st.4	□	■	■	□	□	■	□	□	■	■	■
st.3	■	□	□	□	□	■	□	□	□	□	□
st.2	■	□	□	□	□	□	□	□	□	□	□
st.1	□	□	□	□	□	□	□	□	□	□	□

The juveniles and adults of anchovy were captured from stations 2 to 9. Their distribution was less dispersed during Winter (January) and early Spring (March). In September 2002, it was observed the highest dispersion of anchovies in the Guadiana estuary, being captured from stations 4 to 9 (Table XII). The maximum abundance was 45.1 individual.Km^{-2}, registered in station 3 in November 2001, (Fig. 27A), which corresponded to a biomass of 295.8 g.Km^{-2} (Fig. 27B).

The average length of anchovy juveniles and adults in 2001 (9.8 ± 1.5 cm) was not significantly different from 2002 (9.8 ± 1.8 cm) (P= 0.752). This analysis do not include data from November 2001 (Table XIII).

It was possible to observe a significant gradient ($P \leq 0.001$) of anchovy sizes, along the estuary, during Summer. Significant differences in the size of anchovies were found between "low estuary *vs*. middle estuary" and "low estuary *vs*. high estuary". Bigger anchovies were captured in the low estuary (10.8 ± 1.1 cm), while smaller were captured in the upper estuary (8.0 ± 1.9 cm) (Table XIV).

Within each sampling area, the size of anchovies varied significantly along seasons in the low and middle estuary ($P \leq 0.001$). In the low estuary, significant differences were observed between Summer and Autumn. The size of anchovies averaged 10.8 ± 1.1 cm during Summer, while during Autumn they averaged 9.8 ± 1.1 cm (Table XV).

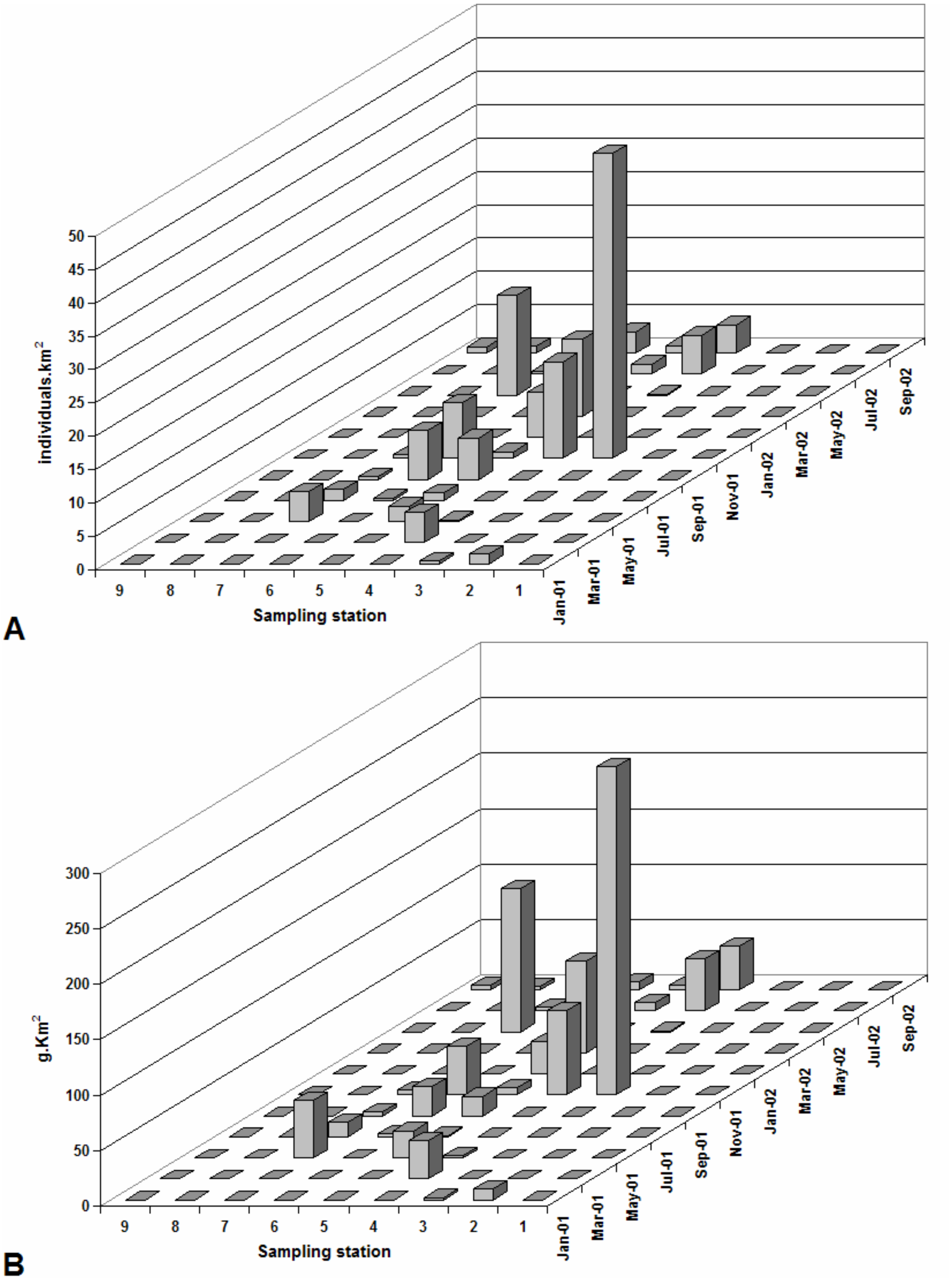


Fig. 27 Density (individuals.Km^{-2}) (A) and biomass (g.Km^{-2}) (B) of anchovy juveniles and adults along the Guadiana estuary and in the adjacent coastal area, from January 2001 to September 2002.

Table XII. Kruskal-Wallis One Way Analysis of Variance on Ranks made for the total length of anchovy juveniles and adults in 2001 and 2002. Data from November 2001 was not considered.

2001 *vs* 2002

Group	N	Average	25%	75%
2001	87	10.1	8.6	11.0
2002	327	10.3	8.7	11.1
H= 0.0996 with 1 degrees of freedom (P= 0.752)				

Table XIV Kruskal-Wallis One Way Analysis of Variance on Ranks made for the total length of anchovy juveniles and adults captured in each season and for the groups coastal area (CA), low estuary (LE), middle estuary (ME) and upper estuary (UE). Multiple comparisons between sampling areas are shown for the seasons were significant differences were found.

Spring

Group	N	Average	25%	75%
Low estuary	2	10.4	10.0	10.7
Middle estuary	73	11.0	10.3	11.5
H= 1.181 with 1 degrees of freedom (P= 0.277)				

Summer

Group	N	Average	25%	75%
Low estuary	77	11.0	10.4	11.5
Middle estuary	93	7.9	6.7	10.4
Upper estuary	8	7.4	6.8	9.2
H= 55.661 with 2 degrees of freedom ($P \leq 0.001$)				

Dunn's method

Comparison	Diff of Ranks	Q	P<0.05
LE *vs.* UE	66.485	3.473	Yes
LE *vs.* ME	57.293	7.216	Yes
ME *vs.* UE	9.192	0.484	No

Autumn

Group	N	Average	25%	75%
Low estuary	177	9.7	9.1	10.6
Middle estuary	67	10.1	9.4	11.0
H= 2.855 with 1 degrees of freedom (P= 0.091)				

Winter

Group	N	Average	25%	75%
Low estuary	11	10.0	9.8	10.2
Middle estuary	14	10.1	9.7	10.5
Upper estuary	136	9.9	8.7	10.7
H= 1.293 with 2 degrees of freedom (P= 0.524)				

Table XV Kruskal-Wallis One Way Analysis of Variance on Ranks made for the total length of anchovy juveniles and adults captured in each sampled area and for the groups Spring (Sp), Summer (Su), Autumn (Au) and Winter (W). Multiple comparisons between seasons are shown for the areas where significant differences were found.

Coastal area

Group	**N**	**Median**	**25%**	**75%**
Winter	11	10.0	9.8	10.2

Low estuary

Group	**N**	**Average**	**25%**	**75%**
Spring	2	10.4	10.0	10.7
Summer	77	11.0	10.4	11.5
Autumn	178	9.7	9.1	10.6
Winter	14	10.1	9.7	10.5

H= 46,536 with 3 degrees of freedom (P≤ 0,001)

Dunn’s method			
Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Sp *vs*. Su	30,390	0,541	don’t test
Sp. *vs*. Au	42,365	0,760	no
Sp. *vs*. W	15,643	0,264	don’t test
Su. *Vs*. Au	72,755	6,806	yes
Su. *Vs*. W	46,032	2,022	no
Au *vs*. W	26,722	1,228	don’t test

Middle estuary

Group	**N**	**Average**	**25%**	**75%**
Spring	**73**	**11,0**	**10,3**	**11,5**
Summer	**93**	**7,9**	**6,7**	**10,4**
Autumn	**67**	**10,1**	**9,4**	**11,0**
Winter	**136**	**9,9**	**8,7**	**10,7**

H= 72,194 with 3 degrees of freedom (P≤ 0,001)

Dunn’s method			
Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Sp *vs*. Su	140,013	8,395	yes
Sp. *vs*. Au	78,815	5,093	yes
Sp. *vs*. W	59,899	3,319	yes
Su. *vs*. Au	80,115	4,687	yes
Su. *vs*. W	18,916	1,188	no
Au *vs*. W	61,198	4,264	yes

Upper estuary

Group	**N**	**Median**	**25%**	**75%**
Summer	8	7.5	6.8	9.2

Table XVI One Way Analysis of Variance made for the total length of anchovy juveniles and adults captured in September 2002 along the Guadiana estuary. The significance of multiple comparisons between sampling stations are shown.

September 2002

Group (Station)	N	Average	25%	75%
4	62	10.9	10.6	11.6
5	6	8.8	7.50	9.9
6	30	6.9	6.0	7.1
7	10	9.2	8.1	10.5
8	7	8.3	7.0	9.9
9	1	5.9	-	-

F= 41.57 with 3 degrees of freedom ($P< 0.001$)

Tukey HSD Test (P values)

	Station 4	Station 5	Station 6	Station 7	Station 8
Station 5	0.010				
Station 6	<0.001	0.043			
Station 7	0.009	0.993	<0.001		
Station 8	<0.001	0.990	0.189	0.795	
Station 9	0.009	0.413	0.981	0.235	0.606

In September 2002, the month when maximum dispersal of anchovy juveniles and adults was observed, a significant decreasing gradient ($P\leq 0.001$) of anchovy sizes was observed towards the uppermost sampling stations. Significant differences in the size of anchovies were found between station 4 (10.9 ± 1.1 cm) and the remaining, as well as between stations 5 (8.8 ± 1.9 cm) and 6 (6.9 ± 1.6 cm) and between stations 6 and 7 (9.2 ± 1.7 cm) (Table XVI).

From March 2002 to February 2003 a total of 783 juveniles and adults of anchovy were captured in the Guadiana estuary. The size ranged from 4.1 cm (February 2002) to 13.8 cm (March 2002). The lowest average size was determined in October 2002 (7.7 ± 1.5 cm) and the highest in June 2002 (11.5 ± 0.7 cm). In June 2002, the size class [11-12[cm contributed with 58.8% of the individuals. In October 2002, the size classes that were predominantly collected were [6-7[cm (37.7%) and [7-8[cm (34.8%). The widest size range was registered in February 2003, from 4.1 cm to 12.8 cm, and with a variance (S^2) of 6.2. The narrowest size range was determined in July 2002, from 9.6 cm to 12.3 cm, and with a S^2 of 0.4 (Fig. 28, Table XVII).

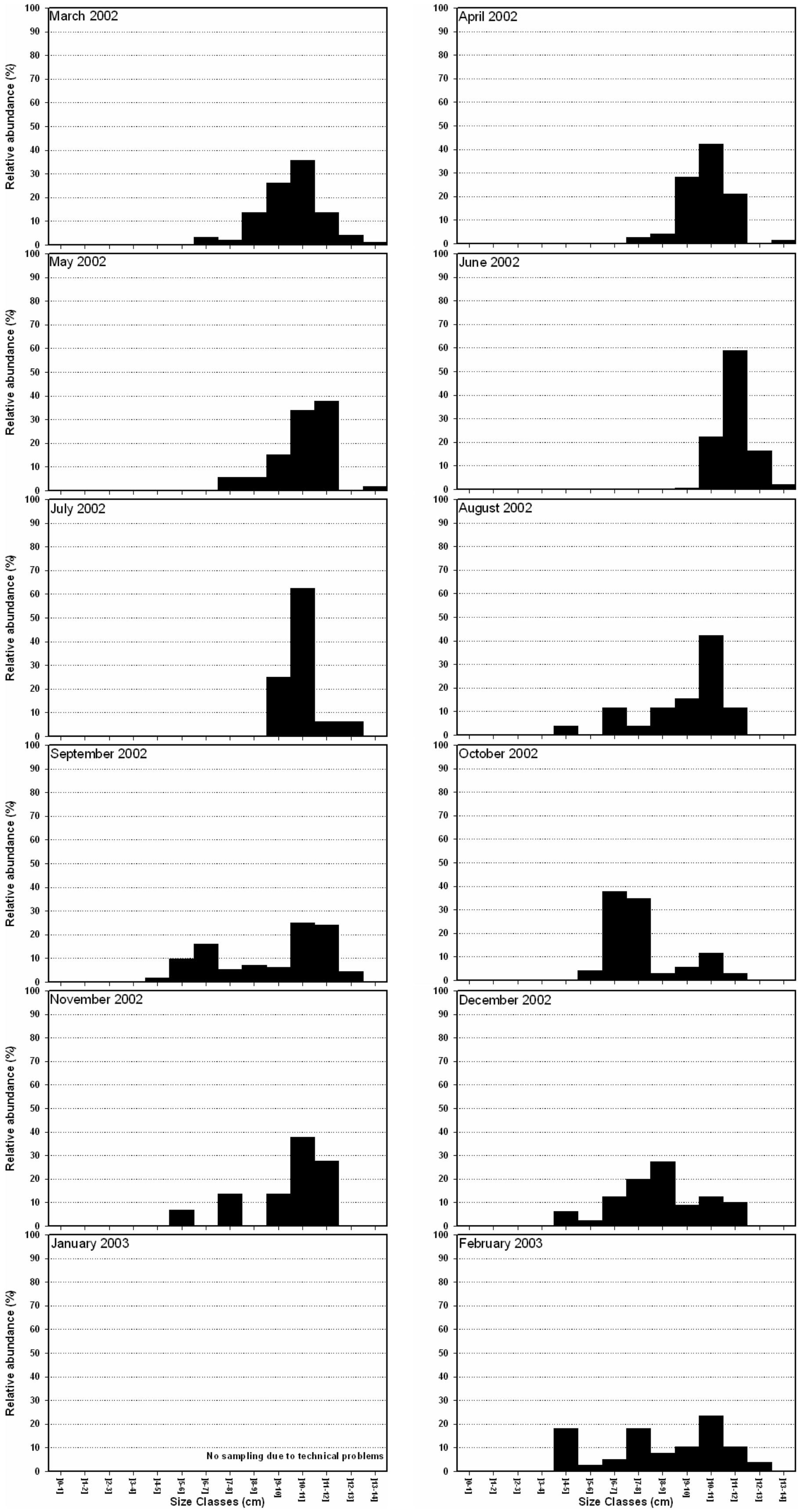


Fig. 28 Relative frequency of anchovy size classes in the Guadiana estuary from March 2002 to February 2003.

Table XVII Descriptive statistics (number of specimens- N; sample average- $\bar{x}$; variance: S^2; standard deviation: st. dev.; minimum size: min.; maximum size: max.; 25th Perc: 25th percentile; 75th perc: 75th percentile) for the anchovy juveniles and adults captured in the Guadiana estuary from March 2002 to February 2003.

	Total length (cm)							
	N	$\bar{x}$	S^2	**st. dev.**	**min.**	**max.**	**25th Perc.**	**75th Perc.**
March 2002	95	10.0	1.5	1.2	6.1	13.8	9.3	10.7
April 2002	71	10.4	0.9	0.9	7.8	13.6	9.9	11.0
May 2002	53	10.6	1.4	1.2	7.7	13.0	10.0	11.5
June 2002	155	11.5	0.4	0.6	10.0	13.5	11.1	11.8
July 2002	16	10.5	0.5	0.7	9.6	12.3	10.1	10.8
August 2002	26	9.5	3.1	1.8	4.7	12.0	8.6	10.7
September 2002	112	9.3	5.4	2.3	4.6	12.9	6.7	11.2
October 2002	69	7.7	2.2	1.5	5.5	11.2	6.8	7.9
November 2002	29	9.8	3.2	1.8	5.3	11.9	9.2	11.1
December 2002	80	8.4	3.2	1.8	4.5	12.0	7.5	9.6
January 2003	-	-	-	-	-	-	6.7	10.6
February 2003	77	8.5	6.2	2.5	4.1	12.8	9.3	10.7

The sex ratio between males and females was significantly different in May (P = 0,031) and June 2002 (P = <0,001). The highest frequency of undetermined individuals, 21%, was registered in September 2002 (Fig. 29).

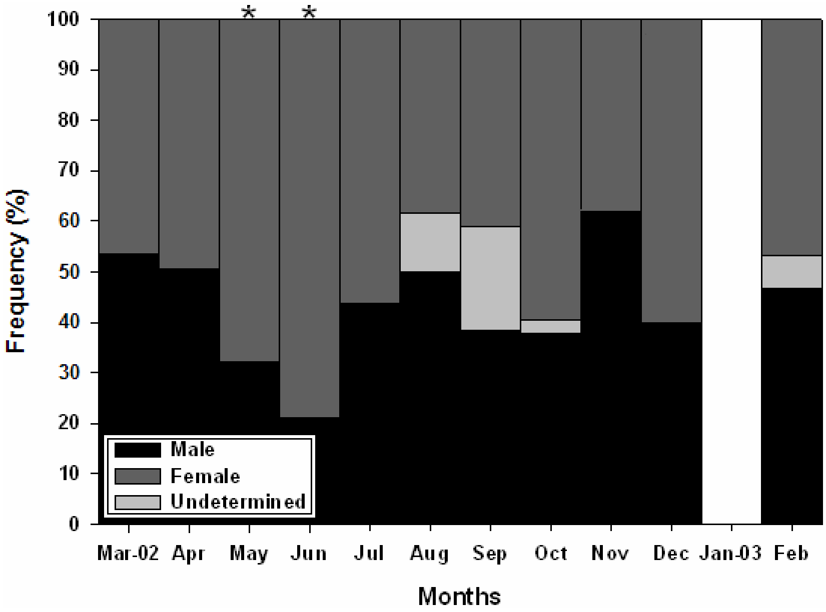


Fig. 29 Frequency of males, females and undetermined specimens of anchovy captured in the Guadiana estuary from March 2002 to February 2003. Significantly different ratios are marked with an asterisk (*).

The maximum average of GSI was determined in June 2002, both for males (9.7 ±1.8 %) and females (8.0 ± 1.8 %) (Fig. 30). A highly significant correlation was found between maximum egg abundance and the average GSI of anchovy males (n= 10; R= 0.827; P< 0.001) and females (n= 10; R= 0.845; P< 0.001), captured in the Guadiana estuary.

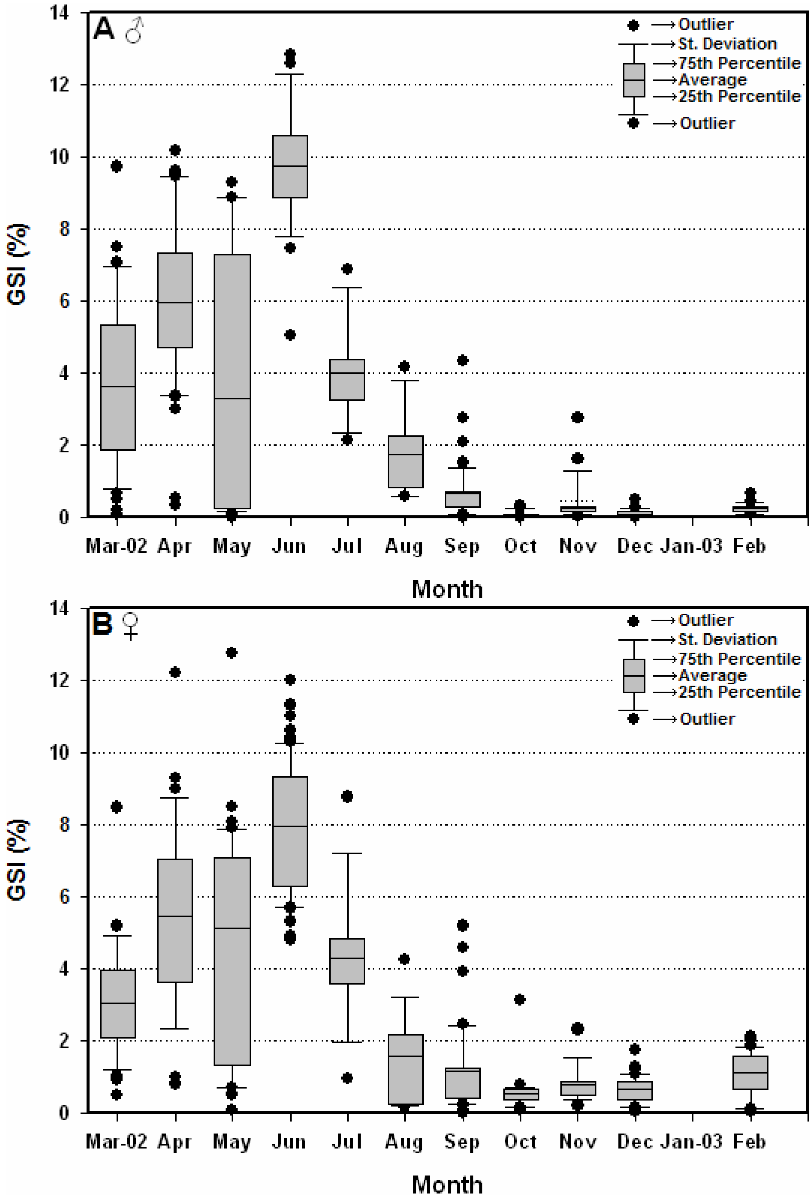


Fig. 30 Evolution of the gonadosomatic index (GSI %) of anchovy males and females in the Guadiana estuary, from March 2002 to February 2003.

3.4. Carbonate $\delta^{13}C$ and $\delta^{18}O$ of anchovy otoliths

The otolith carbonate $\delta^{13}C$ ranged from -8.6‰ to -3.2‰ in the Guadiana estuary (average: -6.4 ± 1.7 ‰), while in the coastal area ranged from -3.7‰ to -2.1‰ (average: -3.0 ± 0.5 ‰). The $\delta^{18}O$ varied between -1.8‰ and 1.0‰ in the Guadiana estuary (average: -0.9 ± 0.9 ‰) and between 0.2‰ and 1.5‰ in the coastal area (average: 0.9 ± 0.3 ‰) (Fig. 31). The average otolith carbonate $\delta^{13}C$ and $\delta^{18}O$ are significantly lower (U-test: $p \leq 0,001$, $\alpha = 0.05$) in the Guadiana estuary.

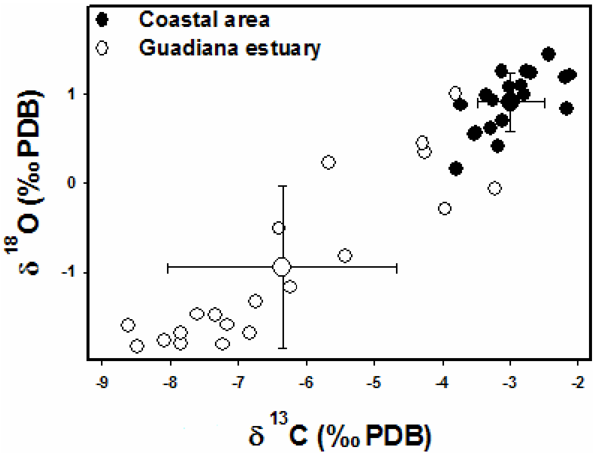


Fig. 31 Anchovy otolith carbonate values of $\delta^{13}C$ (‰ PDB) and $\delta^{18}O$ (‰ PDB) at the Guadiana estuary and coastal area. Average and standard deviation are shown for each site.

Table XVIII Pearson (r) and Spearman (R) correlation coefficients and significance (p) between the otolith carbonate of $\delta^{13}C$ and $\delta^{18}O$ with total fish length and total fish weight at the Guadiana estuary and coastal area.

	Guadiana estuary		**Coastal area**	
	total length	**total weight**	**total size**	**total weight**
$\delta^{13}C$	r= -0.080 p= 0.758	r= 0.025 p= 0.918	r= 0.173 p= 0.465	r= 0.183 p= 0.440
$\delta^{18}O$	R= -0.150 p= 0.521	R = -0.181 p= 0.440	r= 0.187 p= 0.430	r= 0.233 p= 0.323

The average length and weight of specimens captured in the Guadiana estuary was 8.7 ± 1.1 cm and 4.2 ± 1.4 g, respectively. These values are significantly lower (t-test: $p \leq 0{,}001$, $\alpha = 0.05$) than those of specimens analysed in the coastal area (13.0 ± 0.6 cm and 15.2 ± 2.3 g). The otolith carbonate values of $\delta^{13}C$ and $\delta^{18}O$ from both sampling sites was not significantly correlated either with total fish length or total fish weight (Table XVIII). For both sites, the dispersion of $\delta^{13}C$ and $\delta^{18}O$ *vs*. total fish length and total fish weight are plotted in Fig. 32.

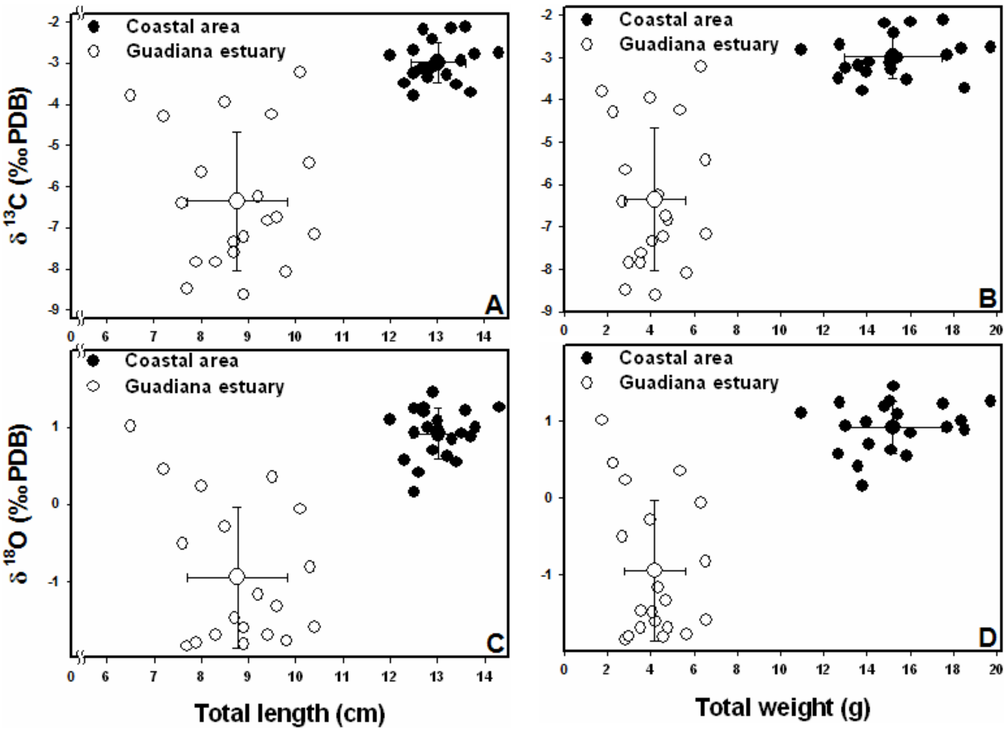


Fig. 32 Anchovy otolith carbonate values of $\delta^{13}C$ (A, B) and $\delta^{18}O$ (C, D) from specimens captured in the Guadiana estuary and coastal area *versus* total fish length (A, C) and weight (B, D). Average and standard deviation are shown for each site.

3.5. Concentration of Fe, Sr and Zn along anchovy otoliths

The analyses of Fe and Zn concentrations along anchovy otoliths were not successful, either because concentration was below detection limit (Fe) or due to lack of discernable pattern (Zn) (Fig. 33).

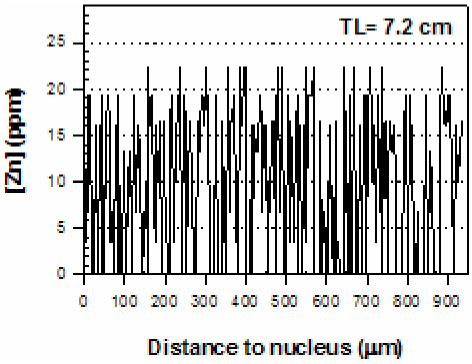


Fig. 33 Variation of Zn concentration (ppm) along an anchovy otolith.

The concentration of Sr, in the otoliths collected from specimens captured in the Guadiana estuary, varied from 500 ppm to 2170 ppm (Fig. 34A), while those collected in the adjacent coastal area ranged from 385 ppm to 2720 ppm (Fig. 34B). The concentration of Sr decreases significantly from core to post-rostrum for specimens collected both in the Guadiana estuary (R= -0.106, p= 0.000) (Fig. 34A) and in the adjacent coastal area (R= -0.587, p= 0.000) (Fig. 34B). The adjustment of the 1st order regression curve is lower for the Guadiana estuary (R^2= 0.041), than that calculated for the adjacent coastal area (R^2= 0.587) (Fig. 34).

The average concentration of Sr in the core of otoliths from specimens captured in the Guadiana estuary was 1255 ± 350 ppm ($N_{specimens}$= 16; N_{obs}= 416), which is significantly lower (U-test: p< 0.001) than for those captured in the coastal area of 1535 ± 290 ppm ($N_{specimens}$= 15; N_{obs}= 393) (Fig. 35A). The average concentration of Sr in the last 100 µm of the post-rostrum of specimens captured in the Guadiana estuary was 1085 ± 200 ppm ($N_{specimens}$= 16; N_{obs}= 416), which is significantly higher (U-test: p< 0.001) than for those captured in the coastal area of 810 ± 200 ppm ($N_{specimens}$= 15; N_{obs}= 393) (Fig. 35B). There is also a significant difference (U-test, p< 0.001) between the measurements made in the core and in the last 100 µm of the post-rostrum of specimens from both sites.

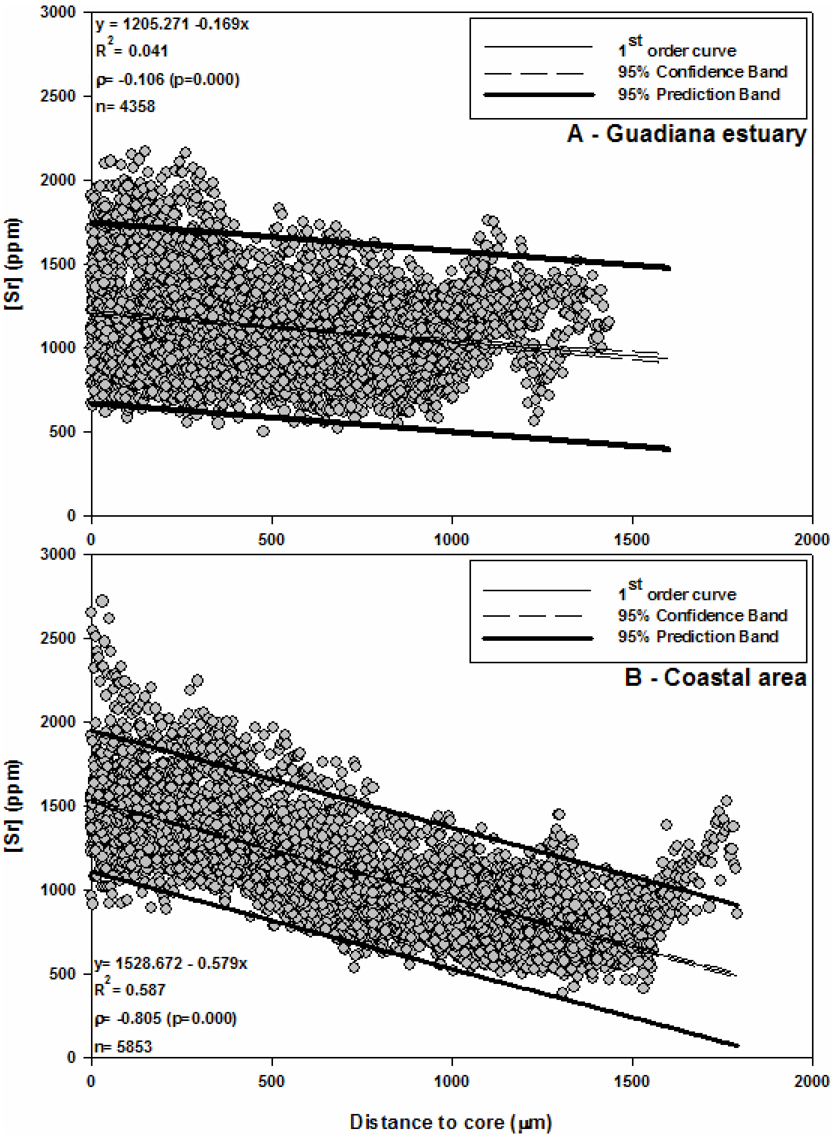


Fig.34 Concentration of Sr *vs.* distance to core for the measurements made in all the specimens collected at the Guadiana estuary (A) and coastal area (B).

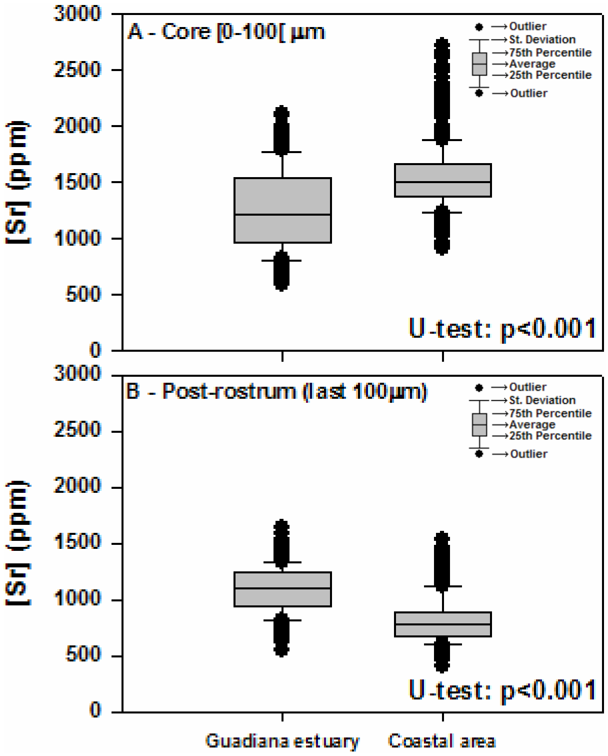


Fig. 35 Concentration of Sr in the core (0-100 µm) (A) and in the post-rostrum (last 100 µm) (B) of specimens captured at the Guadiana estuary and coastal area. The average Sr concentration, the 25th and 75th percentiles, standard deviation and outliers are shown.

Table XIX Pearson and Spearman correlation coefficients between Sr concentration and distance to core, in otolith samples collected from specimens captured in the Guadiana estuary.

Guadiana estuary	**Pearson Correlation (r)** **Spearman correlation (R)**	**p**	**n**
A	R= -0.539	0.000	369
B	R= 0.333	0.000	351
C	R= -0.636	0.000	321
D	R= 0.007	0.903	315
E	R= 0.178	0.002	291
F	R= 0.135	0.022	288
G	R= -0.679	< 0.001	281
H	R= -0.747	0.000	279
I	r= 0.201	0.001	268
J	R= -0.535	0.000	260
K	R= -0.234	0.000	241
L	r= -0.443	< 0.001	240
M	R= -0.761	0.000	240
N	R= 0.068	0.294	240
O	r= -0.036	0.617	196
P	R= -0.561	0.000	178

Table XX Spearman correlation coefficients between Sr concentration and distance to core, in otolith samples collected from specimens captured in the coastal area off Praia Verde.

Coastal area	**Spearman correlation (R)**	**p**	**n**
A	R= -0.547	0.000	460
B	R= -0.861	0.000	423
C	R= -0.880	0.000	405
D	R= -0.935	0.000	399
E	R= -0.925	0.000	398
F	R= -0.903	0.000	391
G	R= -0.630	0.000	368
H	R= -0.928	0.000	367
I	R= -0.699	0.000	359
J	R= -0.914	0.000	358
K	R= -0.935	0.000	346
L	R= -0.871	0.000	343
M	R= -0.865	0.000	174
N	R= -0.840	0.000	304
O	R= -0.923	0.000	336

In the Guadiana estuary, the correlation between Sr concentration and distance to otolith core was not significant for 3 specimens (D, N and O); significant negative correlations were determined for specimens {A, C, G, H, J, K, L, M, P}, while significant positive correlations were determined for specimens {B, E, F, I} (Table XIX, Fig. 36). The specimens captured in the coastal area had highly significant (p= 0.000) and negative correlations between Sr concentration and distance to core (Table XX, Fig. 37).

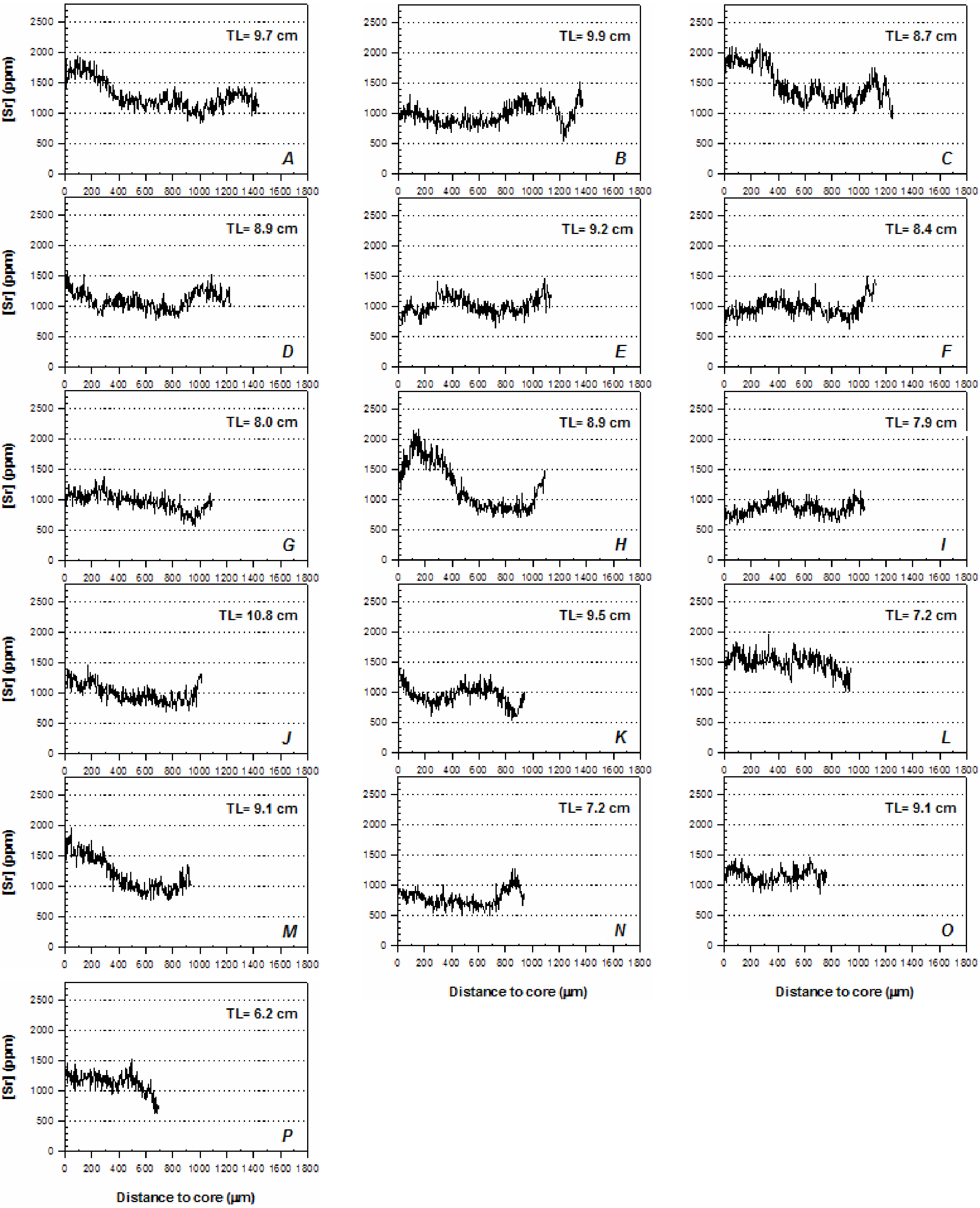


Fig. 36 Concentration of Sr along the otoliths of anchovy captured at the Guadiana estuary.

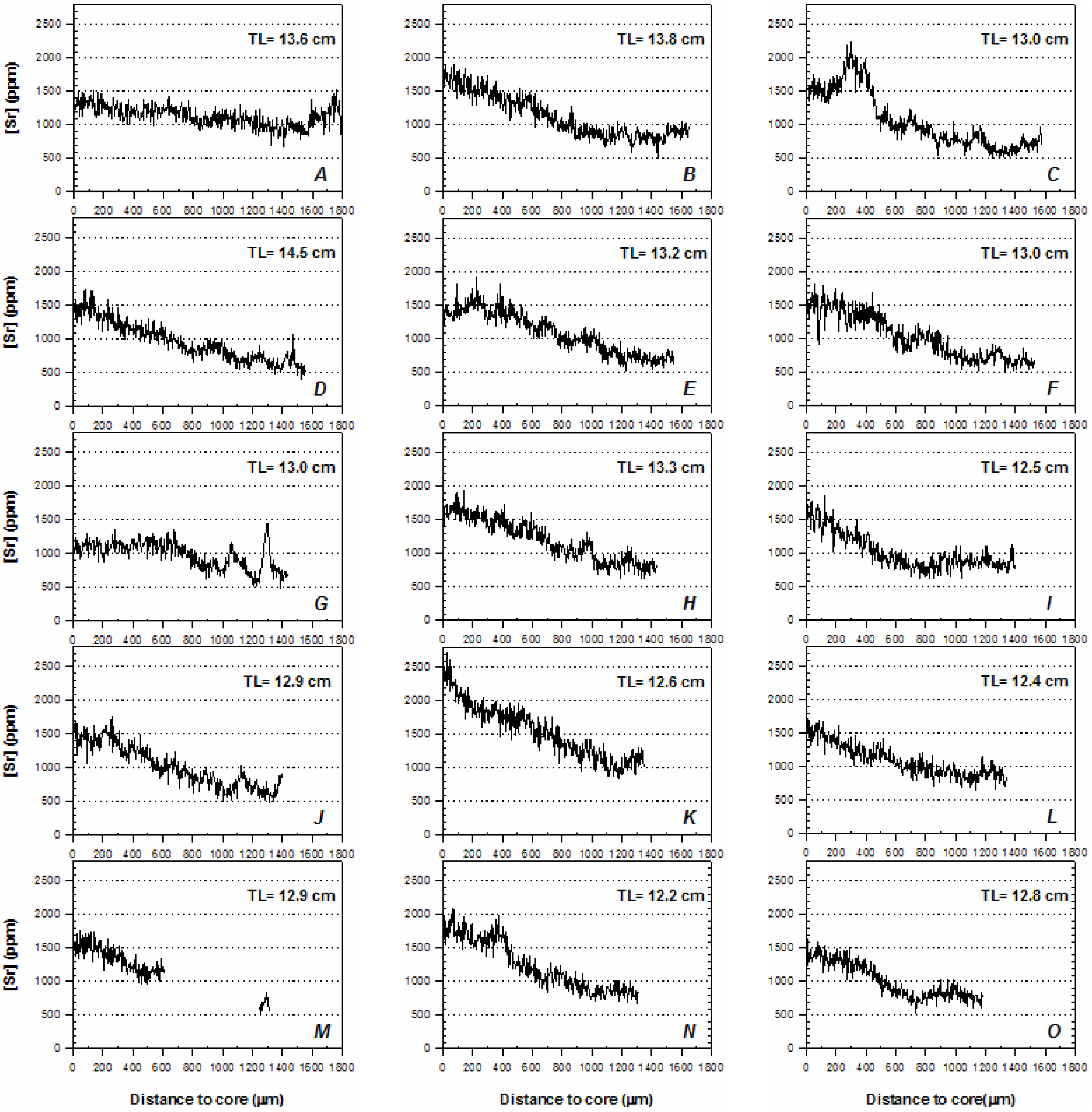


Fig. 37 Concentration of Sr along the otoliths of anchovy captured at the coastal area.

Table XXI Descriptive statistics for each distance class (µm) of Sr concentration along anchovy otoliths from the Guadiana estuary.

Dist. classes (µm)	**[0-100[**	**[100-200[**	**[200-300[**	**[300-400[**	**[400-500[**	**[500-600[**	**[600-700[**	**[700-800[**	**[800-900[**
avg. ([Sr] ppm)	1255	1235	1195	1160	1090	1045	1030	1000	985
st. dev.	350	365	350	280	195	220	230	220	200
min. ([Sr] ppm)	585	625	555	550	500	555	515	580	550
max. ([Sr] ppm)	2110	2170	2160	2040	1650	1830	1730	1665	1555
25th percentile	970	945	930	980	955	905	880	850	845
75th percentile	1525	1555	1475	1295	1215	1145	1135	1085	1110
$N_{specimens}$	16	16	16	16	16	16	16	15	14
$N_{measurements}$	416	416	400	416	400	416	414	366	364

Dist. classes (µm)	**[900-1000[**	**[1000-1100[**	**[1100-1200[**	**[1200-1300[**	**[1300-1400[**	**[1400-1500[**
avg. ([Sr] ppm)	1010	1155	1230	1115	1230	1160
st. dev.	185	185	175	245	150	105
min. ([Sr] ppm)	580	695	880	560	850	1000
max. ([Sr] ppm)	1460	1660	1755	1445	1520	1340
25th percentile	870	1020	1110	900	1140	1120
75th percentile	1155	1280	1325	1335	1315	1220
$N_{specimens}$	14	10	6	4	2	1
$N_{measurements}$	287	220	119	70	44	10

Table XXII Descriptive statistics for each distance class (µm) of Sr concentration along anchovy otoliths from the coastal area.

Dist. classes (µm)	**[0-100[**	**[100-200[**	**[200-300[**	**[300-400[**	**[400-500[**	**[500-600[**	**[600-700[**	**[700-800[**	**[800-900[**
avg. ([Sr] ppm)	1535	1485	1440	1380	1280	1185	1100	1025	945
st. dev.	290	230	220	245	200	215	200	190	160
min. ([Sr] ppm)	915	890	880	930	835	765	650	540	555
max. ([Sr] ppm)	2720	2205	2245	2050	1960	1960	1760	1760	1435
25th percentile	1370	1340	1275	1200	1140	1045	965	905	835
75th percentile	1655	1610	1575	1510	1375	1285	1210	11265	1045
$N_{specimens}$	15	15	15	15	15	15	15	14	14
$N_{measurements}$	416	416	400	416	400	416	393	375	390

Dist. classes (µm)	**[900-1000[**	**[1000-1100[**	**[1100-1200[**	**[1200-1300[**	**[1300-1400[**	**[1400-1500[**	**[1500-1600[**	**[1600-1700[**	**[1700-1800[**
avg. ([Sr] ppm)	920	855	825	820	765	730	790	1050	1255
st. dev.	160	160	145	165	170	130	165	130	165
min. ([Sr] ppm)	555	515	500	505	385	465	415	820	855
max. ([Sr] ppm)	1435	1365	1300	1445	1445	1130	1380	1265	1525
25th percentile	810	755	725	695	640	640	660	955	1165
75th percentile	1010	940	915	915	860	795	905	1150	1370
$N_{specimens}$	14	14	14	14	14	8	6	2	1
$N_{measurements}$	375	390	386	363	315	192	125	39	24

The concentration of Sr along anchovy otoliths was grouped in distance classes to core, as shown in Fig. 38A for Guadiana estuary samples and in Fig. 38B for coastal area samples. Results are summarized in Tables XXI and XXII.

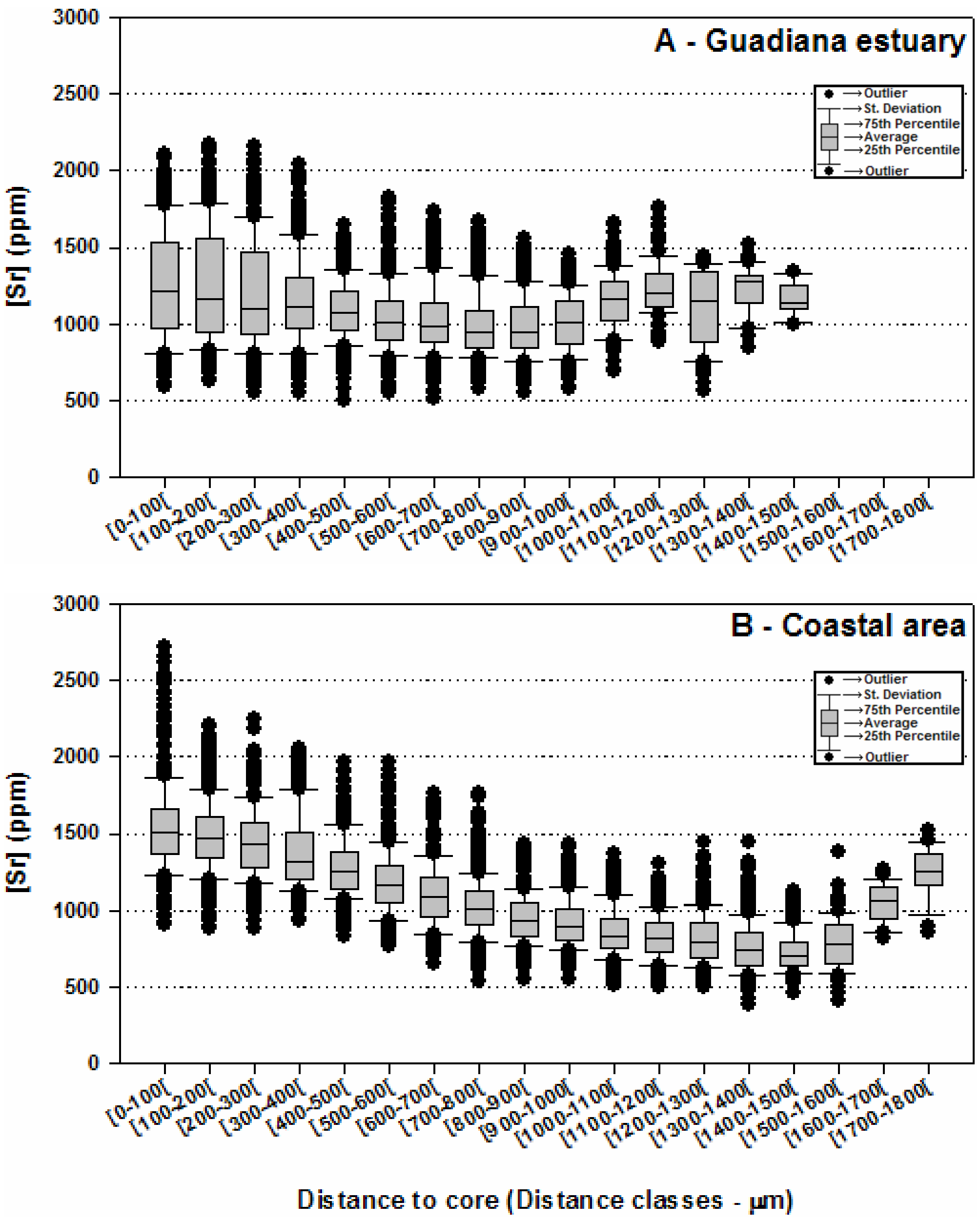


Fig. 38 Concentration of Sr in each distance classes determined for otolith samples collected in the Guadiana estuary (A) and in the coastal area (B). The average Sr concentration, the 25th and 75th percentiles, standard deviation and outliers are shown.

The average Euclidean distance among distance size classes calculated for samples collected in the Guadiana estuary was 2000 ± 600 (n= 105) (Fig. 39A), which is significantly lower (U-test: p≤ 0.001) than that determined for the coastal area of 2755 ± 1605 (n= 153) (Fig. 39B).

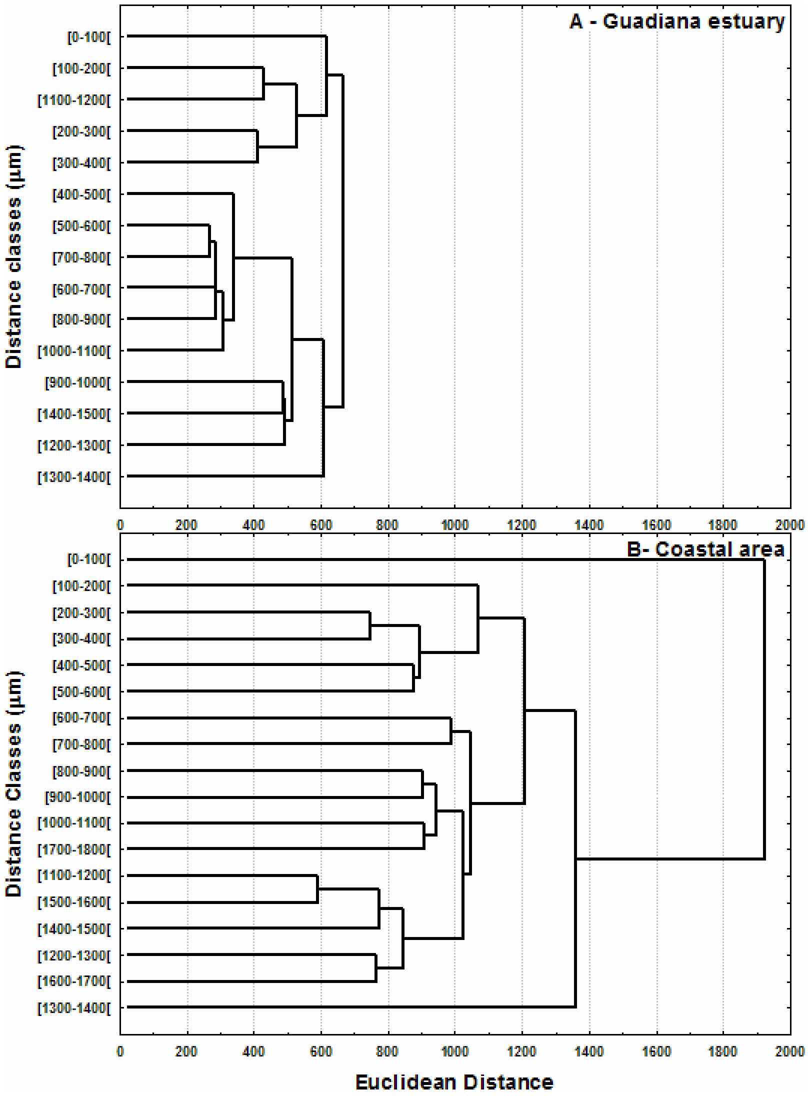


Fig. 39 Euclidean distances between distance classes (µm) of Sr concentration along anchovy otoliths from the Guadiana estuary (A) and coastal area (B).

The cluster analysis defined 3 distance class groups for samples collected in the Guadiana estuary: *G-1*) [0-100[µm to [300-400[µm and [1100-1200[µm; *G-2*) [400-500[µm to [800-900[µm, [1000-1100[µm; *G-3*) [900-1000[µm, [1200-1300[µm to [1400-1500[µm (Fig.

39A). The average Euclidean distance within group was 705 ± 235 (n= 10) for *G-1*, 495 ± 180 (n= 21) for *G-2* and 525 ± 55 (n= 3) for *G-3* (Fig. 39A).

The cluster analysis defined 4 distance class groups for samples collected in the coastal area: *CA-1*) [0-100[µm; *CA-2*) [100-200[µm to [500-600[µm; *CA-3*) [600-700[µm to [1000-1100[µm and [1700-1800[µm; *CA-4*) [1100-1200[µm to [1600-1700[µm (Fig. 39B). The average Euclidean distance within group was 1115 ± 310 (n= 10) for *CA-2*, 1275 ± 380 (n= 15) for *CA-3* and 1210 ± 440 (n= 15) for *CA-4*. The average Euclidean distance between CA-1 and the remaining distance classes was 5040 ± 1825 (n= 17) (Fig. 39B).

4. Discussion

4.1. Which anchovy is in the Guadiana estuary, Engraulis encrasicolus *or* Engraulis albidus?

The results obtained in this study have not provided straightforward evidence of which anchovy species, *E. encrasicolus* or *E. albidus*, inhabits in the Guadiana estuary and in the adjacent coastal area. The genetic similarity between populations and the inexistence of mtDNA sequences (cyt *b* and d-loop), of *E. albidus* in GenBank, disabled a clear and concise answer to this objective. Therefore, the specimens from the Guadiana estuary and from the adjacent coastal area will be described as *E. encrasicolus sensu lato*, until robust results are available, demonstrating the need for further insights.

The visual identification of specimens and, phylogeny trees point towards the presence of *E. albidus* in the Guadiana estuary and adjacent coastal area. This hypothesis goes against that of Magoulas et al. (2006), which stated that *E. albidus* represents locally adapted populations to coastal environments in clade-B dominated regions, since the south coast of Portugal is a clade A dominated region, as well as the surrounding coastal areas: West Portugal, Canary

Islands, Morocco and Alboran Sea (Fig. 4). Grant et al. (2005) affirmed that if *E. albidus* is a valid species then there are two distinct hypotheses that might explain its existence: 1) *E. albidus* reflect a back dispersal of *Engraulis eurystole* from the western Atlantic Ocean, or 2) *E. albidus* have recently diverged from *E. encrasicolus*, perhaps resulting from a post-glacial divergence. Up to now there are no available sequences of *E. eurystole* to check for the first hypothesis. This study, although not conclusive, points also towards a recent divergence between *E. albidus* (putatively those collected in this study) and *E. encrasicolus* (those available in GenBank) of *ca.* 0.5 MY (BP) (Fig. 12).

With the phylogeny tree obtained with cyt *b* sequences, the majority of specimens collected in this study, except specimens E9 (haplotype h6) and C3 (haplotype h13) were grouped together. There are some *E. encrasicolus* specimens mingled among those analysed in this study, which might indicate that those specimens identified as *E. encrasicolus* are *E. albidus*, as suggested by Borsa (2002) in a comprehensive review on previous published data. The haplotypes h6 and h13 are mingled among the majority of *E. encrasicolus* samples; perhaps these haplotypes correspond to *E. encrasicolus* specimens, which were among the hypothetical *E. albidus* populations of the Guadiana estuary and adjacent coastal area. However, Borsa et al. (2004) would discourage this hypothesis, since they established that these anchovy species are ecologically specialised. They would also discourage the existence of hybrid specimens, because they would be clearly in disadvantage; although these authors state that the reproductive isolation may be partial. The existence of hybrids is strongly discouraged due to persistent genetic differences, at neutral genetic markers, between coastal and oceanic anchovy (Borsa el al., 2004).

The nucleotide and haplotype diversity is reduced in the Guadiana estuary and in the adjacent coastal area, despite the high incidence of exclusive haplotypes, 42.9% for cyt *b* and 79.1% for d-loop. For both genes, there are more exclusive haplotypes in the coast than in the

Guadiana estuary, suggesting that the coast is a sink of genes more than a source of genes into the estuaries that exist around the Gulf of Cadiz (Guadiana, Piedras, Tinto-Odiel, Guadalete, Guadalquivir) and along the South Portuguese coast (Ria Formosa and Ria de Alvor Lagoons and Arade estuary). However, this hypothesis might just be an artefact resulting from the low number of analysed specimens in each location; but ontogenic and environmental factors might reinforce the suggested hypothesis, namely:1) Sr concentration along anchovy otoliths suggests a predominant migration from an estuary to the coast (chapter 4.5); 2) an important fraction (> 50%) of the anchovy eggs present inside the estuary are exported to the coast, during periods of moderate river flow (*ca.* 50 $m^3.s^{-1}$), and there is no evidence that this eggs are not viable to hatch and succeed in the coastal environment; 3) during river floods (*e.g.* January 2001, Q> 3000 $m^3.s^{-1}$) the population of Guadiana anchovy moves only towards the low estuary and to the estuarine plume, re-entering to the estuary as river flow diminishes. Probably, the estuarine connectivity and genetic homogenization of anchovy populations, among the estuaries around the Gulf of Cadiz and along the coastal ecosystems of South Portugal, is more intense only during periods of extreme river flow (Watts & Johnson, 2004).

Ultimately, the existence of *E. albidus* in coastal areas is a fact, at least in the areas where Borsa et al. (2004) collected their samples. When the presence of *E. albidus* is confirmed for the coastal area adjacent to the Guadiana estuary and elsewhere, then it will be mandatory to determine the implication of the co-existence of *E. albidus* and *E. encrasicolus* in anchovy stock management.

4.2. Anchovy larval stages in the Guadiana estuary

Anchovy larval stages are among the most abundant species in many Portuguese estuaries (Ré, 1984; Chícharo, 1988; Ribeiro, 1991; Ré, 1996; Faria et al., 2006), except in those of the northern part of the country, namely Lima (Ramos et al., 2006) and Minho estuaries (R. Sousa, personal communication). One of the most pronounced differences, between this study and that of Chícharo (1988), was the decrease of maximum abundance of anchovy larval stages abundance. In this study, 4.7 and 14.5 times fewer eggs and larvae were captured, respectively. It might be possible that 2002 could have been a less productive year than 1988. Several works positively relate the intensity of river inflow with abundance of estuarine and coastal fish and also crustaceans (Loneragan & Bunn, 1999; Quiñones & Montes, 2001; Nixon, 2003), some stating that, in appropriate quantities and at suitable times, stronger river inflow may be favourable to fisheries, either by enhanced production mechanisms (Bergeron, 2004) or by promoting organism concentration and catchability (Loneragan & Bunn, 1999).

The spawning areas of fishes are essential in allowing the retention of larval stages within an estuary (Ré, 1991). An ideal spawning area should combine appropriate physical, chemical and biological characteristics. The Guadiana low estuary is not the ideal spawning area, due to its low residence time, even during periods of low inflow- as low as 6 days for 10 $m^3.s^{-1}$ (Oliveira et al., 2006). When the abundance of larval stages was highest, June 2002, the spawning area was located in the transition between the low and middle estuary. Here, the pronounced *S* shape morphology of the estuary enables the formation of secondary flows, thus the retention of particles (Martins et al., 2001). In this month, the average salinity was 29.1 ± 5.5; yet, it is not absolutely indicative of the ideal spawning area, once maximum abundances of anchovy eggs were registered in multiple sets of salinity. The maximum abundances have been registered along a broad salinity range, from 26 to 35 in this study, from 18 to 25 also in the Guadiana estuary in 1988 (Chícharo, 1988), from 18 to 25 in the Mira estuary (Ré, 1996) and from 16 to 25 in the Mondego estuary (Ribeiro, 1991). The availability of food for the adults and larvae seems also to be important (Ribeiro, 1991); indeed, maximum abundances of larval stages also coincided with sites with high zooplankton biomass (Morais, unpublished data). Anchovy eggs in early development stages (I to III) were mainly captured during late afternoon and early morning, because anchovy spawns mainly at dusk and during the first hours of darkness (Ré, 1984; Ribeiro, 1991; Ré, 1996). The presence of eggs in late development stages (IX to XI) in stations 2 (coastal area) and 3 (low estuary) is probably consequence of advection.

The identification of spawning and nursery areas is fundamental for the evaluation of impacts caused by river flow management, for instance to mitigate eutrophication in upper estuarine regions (consult "An assessment tool for estuarine ecohydrological management" for further details). Therefore, as river inflow decreased from March to June 2002, the spawning area shifted upstream. During the low tide, from March (Q_{7days}= 30.8 ± 17.6 $m^3.s^{-1}$) to June 2002 (Q_{7days}= 3.2 ± 0.1 $m^3.s^{-1}$), the spawning area shifted from the plume area and low estuary to the upper part of the low estuary, a maximum displacement of 8 km. In the same period, but during the high tide, the spawning area shifted to more upstream areas of the middle estuary. Uncontrolled river discharge in April 2002 was responsible for a 99.99% decrease of anchovy eggs in the Guadiana estuary, despite the 1.8 times increase of female GSI. The decrease of anchovy larval stages in July 2002, 91.3% for the eggs and 86.6% for the larvae, is result of the 54% decrease of spawning intensity and probably also of predation by jellyfishes. Jellyfishes are important zooplanktonic community regulators (Schneider & Behrends, 1994; Rilling & Houde, 1999), which increased 528%, from 6.0 ± 11.9 jellyfish.100m^{-3} (June 2002) to 37.8 ± 75.6 jellyfish.100m^{-3} (July 2002), being mainly located in the middle and upper estuary (Morais, unpublished data).

In June 2002, recently hatched larvae of anchovy were collected near the spawning area, but displaced to the upstream station in both tides. The time elapsed from spawning to hatching, 49.0 ± 3.8 h (Morais, unpublished data), is probably enough to exhibit this difference, because the length of the ebb is longer than the flood (LNEC, 1998). The shift on the nursery area, due to changes on the river flow, cannot be objectively assessed, due to insufficient data obtained during the majority of the reproductive period of anchovy.

The retention and migrations of anchovy larvae to the upper estuary might be a result of active or passive retention mechanisms (Hare et al., 2005). Examples of active retention mechanisms are *a*) selective tidal stream transport and residual bottom inflow, which might be synchronized with vertical migrations of larvae with formed gas bladder (Ré, 1984, 1996) and *b*) lateral migrations to the margins, where the river flow is lower or to take advantage of the residual up-estuary flow near the margins of the low estuary (Oliveira et al., 2006). Examples of passive retention mechanisms are tidal and wind advection; although wind has little effect in the advection of inert particles in narrow estuaries (Braunschweig et al., 2003), like the Guadiana. The relative importance of these mechanisms differ among species and changes with larval development (Hare et al., 2005). For example, the presence of the sardine *Sardina pilchardus* (Clupeidae) inside estuaries was considered restricted to the terminal areas (Ré, 1984; Chícharo, 1988; Ribeiro, 1991); however, the capture of larvae with 32.9 ± 2.0 mm, at 15 km from the Guadiana river mouth and near the bottom, are probably explained by active retention mechanisms and selective tidal stream transport, other than by passive retention mechanisms due to high river flow (Morais et al., 2003). Indeed, Oliveira et al. (2006) found that organisms with vertical migration capabilities can remain inside the Guadiana estuary under strong river flows, by taking advantage of the phase lag along the vertical profiles of velocity under stratified conditions. In Chesapeake Bay (USA), the net up-estuary flux, of the Atlantic menhaden *Brevoortia tyrannus* (Clupeidae) larvae, was dominated by residual bottom inflow and wind forcing (Hare et al., 2005). Other estuarine anchovy, *Anchoa mitchilli*, perform up-estuary migration by tracking the diurnal migrations of their main preys (Copepoda: *Acartia tonsa*) (North & Houde, 2004 and references therein). Microchemical analyses to the otoliths of *A. mitchilli* also revealed the up-estuary migration (Kimura et al., 2000), as well as in the anchovy otoliths analysed in this study (see chapter 4.5).

In the vicinities of estuaries that surround the Gulf of Cadiz, it were registered several spawning areas of anchovy (Uriarte et al., 1996). Advection of anchovy larval stages into the estuary did not occur or, if it did, it was very insignificant. It is the estuarine population of anchovy that spawns in the Guadiana estuary. The highly significant positive correlation between maximum egg abundance and the GSI of estuarine anchovy supports this finding. The distribution pattern of anchovy eggs in the estuary is clearly that of an estuarine spawning species. The major difference on the distribution of eggs, of estuarine (anchovy) and coastal spawning species (sardine), is that the eggs of the latter are mostly present in the low estuary during the high tide and almost absent during the low tide (Faria et al., 2006).

4.3. Distribution of anchovy adults and juveniles

River inflow was the most important factor in determining abiotic and biotic variability in the Guadiana estuary and in the adjacent coastal area (Erzini, 2005; Chícharo et al., 2006; Faria et al., 2006). The influence of river flow on the distribution of anchovy juveniles and adults along the Guadiana estuary is also meaningful.

Migration of anchovy adults from the coast to spawn in the low estuary, or in the vicinities of the estuary, is refuted based on the size of specimens. The biggest anchovy collected, in a two year survey in the Guadiana estuary and the adjacent coastal area, had 13.8 cm (n= 692); while those captured by fishermen in the South coast of Portugal and Gulf of Cadiz were bigger (Fuzeta: 18.0 cm, n= 34; Isla Cristina: 17.6 cm, n= 40; Praia Verde: 14.5 cm, n= 50). The migration patterns inferred by microchemical analyses of the otoliths also support this finding (chapters 4.4. and 4.5).

Ribeiro (1991) stated that anchovy adults use the Mondego estuary mainly during the reproductive period, probably performing daily migrations to the estuarine area of reproduction. In opposition, the anchovy adults of the Guadiana estuary were predominant along the year, except in October 2002, and no daily migrations occur, as suggest by the concentration of Sr along the otoliths (see chapter 4.5). Ribeiro (1991) also declares that anchovy might form two separate shoals, one predominantly of males and other of females. In this study, significantly different sex ratios were only observed in May and June 2002, but the sampling size is not robust to validate or reject this hypothesis.

During this study, juveniles and adults of anchovy were collected along the entire estuary and in the plume region. During Spring, when river flows varied between 8 $m^3.s^{-1}$ and 20 $m^3.s^{-1}$, anchovy adults were dispersed from the upper part of the low estuary to the middle estuary, but mainly in the upper part of the middle estuary.

In Summer, when river flows were lower than 8 $m^3.s^{-1}$, anchovies were dispersed from the upper part of the low estuary to the upper estuary, but mainly in middle estuary or in the upper part of the low estuary, which coincides with the location of the spawning area. A differential distribution of juveniles and adults was observed along the estuary. The juveniles where in the location where the older larvae where collected, the upper estuary, at least up to 40 km from the river mouth. Progressively bigger anchovies were collected downstream.

In Autumn (November 2001), when river flow was *ca.* 300 $m^3.s^{-1}$, anchovies, either juveniles or adults, were dispersed along the low and middle estuary, but mainly in the low estuary.

In Winter, when several peaks of river flow occur, anchovies were less dispersed along the estuary than during the preceding seasons. The presence of anchovies at the entrance of the estuary and in the plume region is rare and restricted to periods of high inflow, as during a flood event in January 2001, when river flow should have been higher than $Q> 3000\ m^3.s^{-1}$.

The life cycle of anchovy in the Guadiana estuary is similar to that proposed to *A. mitchilli* in the Chesapeake Bay (Fig. 40, Jung & Houde, 2000). The spawning area is located downstream to the nursery ground. The late stage larvae, young of the year and recruits are also in the upper regions of the bay during Summer and Autumn; while during Winter they migrate downstream. They question the existence of adult immigration from open ocean into the Chesapeake Bay. On the contrary, in the Guadiana estuary it is proposed the occurrence of emigration into the adjacent coastal area (chapter 4.5).

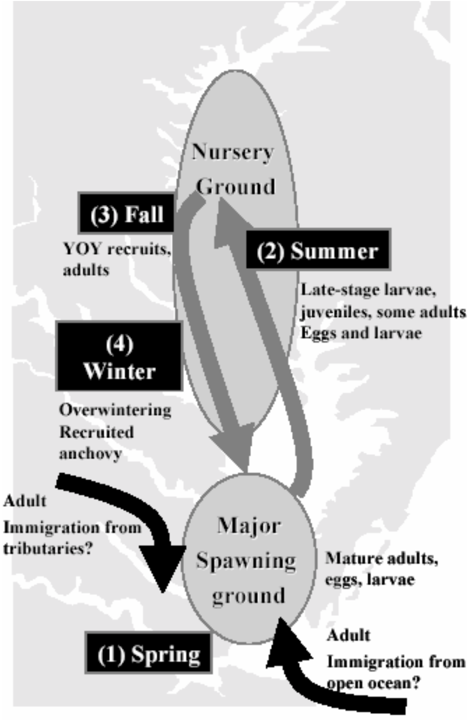


Fig. 40 Hypothetical representation of bay anchovy (*Anchoa mitchilli*) ontogenic migration in Chesapeake Bay and possible immigrations of adults from tributaries and open ocean. From Jung & Houde, 2000.

4.4. Carbonate $\delta^{13}C$ and $\delta^{18}O$ of anchovy otoliths

The information on the $\delta^{13}C$ and $\delta^{18}O$ composition of anchovy otoliths revealed significant differences between the anchovy populations of the Guadiana estuary and coastal area, which were imperceptible to the genetic analyses. This finding supports the suggestions of several authors (Babaluk et al., 2002; Morris Jr. et al., 2003; Bergenius et al., 2005; Veinott & Porter, 2005; Correia et al., 2006), which advised on the necessity of complementing genetic analyses with the microchemical analysis of otoliths, and vice-versa, in order to infer population/stock distinction. According to Stephenson et al. (2001), distinct oxygen and carbon isotopic signatures, as those found in this study, do not imply that specimens are genetically different, once larval dispersion and migration of adults between sites might occur. Moreover, they state that such differences can suggest that the analysed specimens occupied a different habitat for a long period of their lives and that they do not mix entirely. Indeed, the genetic analyses proved the genetic homogeneity between individuals from these sites (chapter 4.1.) and larval advection and adult migration from the Guadiana estuary to the coast might occur. The advection of larval stages is likely to occur more intensively during peaks of river inflow, either with natural or anthropogenic origins (chapter 4.2.). The migration of anchovy adults from the Guadiana estuary to the coast, or from other estuaries in its vicinities, is also very probable, as it will be shown in chapter 4.5.

The differences on the $\delta^{18}O$ of anchovy otoliths between sites might be attributed to differences on water temperature. The lowest average $\delta^{18}O$ was determined in the anchovy from the Guadiana estuary, where the annual average water temperature, 19.4 ± 3.5 ºC was significantly higher (t-test, d.f.= 171, P= 0.005) than in the coast, 17.7 ± 2.6 ºC. This trend is commonly observed elsewhere for other fish species (Nelson et al., 1989; Edmonds et al., 1997; Edmonds et al., 1999; Stephenson et al., 2001).

Various factors influence the deposition of $\delta^{13}C$ on fish otoliths, such as a) habitat depth, b) metabolism and fish age and c) food (Nelson et al., 1989; Edmonds et al., 1997; Schwarcz et al., 1998; Stephenson et al., 2001; Sherwood & Rose, 2003).

a) The influence of habitat depth on the deposition of $\delta^{13}C$ on the otoliths of anchovy is impossible to be objectively assessed, since there is no record of the depth where coastal anchovy was captured. Nevertheless, the coastal area has higher depths than the Guadiana estuary (average depth- 6.5 m) (Bettencourt et al., 2003). Significantly lower $\delta^{13}C$ values were measured in the shallower location- the Guadiana estuary, as it is commonly referred to occur (Schwarcz et al. 1998). The $\delta^{13}C$ in the otoliths from anchovy collected in the Guadiana estuary was -6.4 ± 1.7 ‰, while in the coastal area it was -3.0 ± 0.5 ‰. Iacumin et al. (1992 in Sherwood & Rose, 2003) refer an intermediate value of $\delta^{13}C$ -4.3‰ for *Engraulis encrasicolus* that was captured at a maximum depth of 400m.

b) The age of anchovy does not appear to have significantly influenced the $\delta^{13}C$ deposited in the otoliths of specimens from both locations, as it can be deduced by the inexistence of significant relationships between $\delta^{13}C$ and total fish length for both groups of samples.

c) According to Santos (unpublished data), there is an increasing gradient of $\delta^{13}C$ along the Guadiana estuary, from fluvial regions to areas of more marine influence. Therefore, it could be possible that the trophic characteristics of the estuarine and coastal environments, where anchovy samples were caught, explain the differences of $\delta^{13}C$ in their otoliths, since significant lower values were registered for estuarine samples. The $\delta^{13}C$ gradient along the Guadiana estuary is detected in the taxonomic groups/organisms of several trophic compartments, such as: 1) freshwater phytoplankton [-29‰, -27‰] and marine phytoplankton [-24‰, -19‰]; 2) upper and middle estuarine zooplankton: -26.5 ± 2.7 ‰; low estuarine zooplankton: -21.0 ± 2.6 ‰; 3) the average values for *Mytilus galloprovincialis* were -21.6‰ in Foz de Odeleite (st. 6) and -17.8‰ in Esteiro da Carrasqueira (st. 3); 4) the average values for *Crangon crangon* were -20.9‰ in Foz de Odeleite (st. 6) and -18.0‰ in Esteiro da Carrasqueira (st. 3); 5) the average values for *Pomatoschistus microps* were -21.3‰ in Foz de Odeleite (st. 6) and -17.6‰ in Esteiro da Carrasqueira (st. 3); 6) the average values for *E. encrasicolus sensu lato* were -19.8‰ in Foz de Odeleite (st. 6) and -19.5‰ in Esteiro da Carrasqueira (st. 3).

Five individuals from the Guadiana estuary had $\delta^{13}C$ and $\delta^{18}O$ signatures very similar to those from the coastal area. This may suggest the migration of these specimens from the coast to the estuary. However, as confirmed by Sr analyses, it is more likely that these specimens, juveniles and adults, have not migrated from the coast. The widest range of $\delta^{13}C$ and $\delta^{18}O$ might just reveal the extreme variability of estuarine ecosystems, when compared to coastal environments. The impact of the Guadiana outflow, and of other major rivers, in the coastal area is spatial and time limited, except during intense river flows (Cravo et al., 2006). Furthermore, the occurrence of upwelling events are scarce and its intensity is weak (NERC & PML, 2004). In future studies, the combination of stable isotopes analysis in the tissues and in otoliths would be extremely useful to clarify the existence, or not, of recent migrations between ecosystems (Herzka, 2005).

4.5. Concentration of Fe, Sr and Zn along anchovy otoliths

The measurements of Fe and Zn concentrations along the otoliths of anchovy were unable to detect the migration patterns of this species, although they are commonly used to investigate stock discrimination (Edmonds et al., 1989, 1991). The concentration of Fe was undetectable and Zn measurements were not discernable. However, Fe had detectable concentrations in the otoliths of *Engraulis japonicus* (Zenitani et al., 2003), and Zn revealed usefulness in complementing the information set by Sr concentration along the otoliths of anadromous and non-anadromous Artic char *Salvelinus alpinus* (Halden et al., 2000) and in providing a mean of estimating food uptake history (Campbell et al., 1999). Nevertheless, these elements might be useful to study the life history of freshwater fishes inhabiting near decommissioned mines, that exist along the Guadiana lower basin (Barriga, 1990), hence detecting mine tailings contamination (Saquet et al., 2002).

The isotopic analyses of the otoliths showed results that are consistent with the trends described in the literature (Schwarcz et al., 1988; Nelson et al., 1989; Edmonds & Fletcher, 1997; Edmonds et al., 1999; Stephenson et al., 2001; Sherwood & Rose, 2003), but this was not the case for the analysis of Sr concentration along otoliths. In general, the concentrations of Sr reflect salinity changes, being substantially higher in the seawater than in estuaries or freshwater ecosystems (Campbell et al., 1999; Secor & Rooker, 2000, Campbell et al., 2002). However the opposite pattern was found with the concentration of Sr in the last 100 µm of the post-rostrum being significantly lower in the samples from the coast than in those from the Guadiana estuary. Truly, Kraus & Secor (2004) and Elsdon & Gillanders (2005) suggested that local geology and ground waters can influence ambient concentration of Sr, setting higher concentrations in freshwater or estuarine ecosystems than in the ocean.

The Guadiana river flows through two geological formations which characteristics might modify the common pattern of Sr concentration along salinity gradients:

i) the Iberian Pyrite Belt (IPB)- an area of Devonian-Carboniferous volcanic and sedimentary rocks containing massive polymetallic sulfide deposits. The IBP forms an arcuate belt, about 250 km long and up to 60 km wide, trending westwards from near Seville in Spain to west-northwest in South Portugal. Both the eastward and westward extents of the belt are covered by Tertiary sedimentary rocks (Fig. 41). The IPB is possibly the largest and most important volcanogenic massive sulfide metallogenic province in the world. There is an impressive amount of metals, in concentrations that range from small lenses with thousands of tons to giant bodies with hundreds of million tons, in such a relatively small area, representing an outstanding global geochemical anomaly of Bi, Cd, Co, Cu, Fe, Hg, In, Pb, S, Sn, Sr and Zn, ranging from tens to hundreds of ppm (Barriga, 1990; Barriga et al., 1997; Carvalho et al., 1999).

ii) the limestone that form some aquifers in the lower Guadiana basin. The limestone is a sedimentary rock composed of calcium carbonate and is enriched in Sr, up to concentrations of *ca.* 1000 $mg.kg^{-1}$. Sr is easily mobilised during weathering, especially in oxidising acid environments. In acid soils, Sr is highly leached and coastal areas have an apparent enrichment in surface soil (Salminen, 2005). The soils surrounding the Guadiana estuary probably have such suitable conditions, because the soil pH ranges from 5.6 to 6.5 (Instituto do Ambiente, 2007). The influence of limestone can be detected in the platform sediments off the Guadiana estuary, which have high levels of Ca contents (max. 11.1%) when compared to other sites. Moreover, it was off Guadiana estuary that were determined the highest concentrations of Sr in the sediments of the continental platform (*ca.* 200-360 $mg.kg^{-1}$), when compared to those of the continental platform off the Tagus (*ca.* 139-313 $mg.kg^{-1}$) and Douro (*ca.* 130-227 $mg.kg^{-1}$) estuaries and off Galician Rias (*ca.* 155-286 $mg.kg^{-1}$). Despite the increase of Ca availability in the coast off Guadiana estuary, the Sr:Ca ratio is still higher in the Guadiana (280), than in Tagus (220) and Douro (110) (Fig. 42) (Araújo, unpublished data).

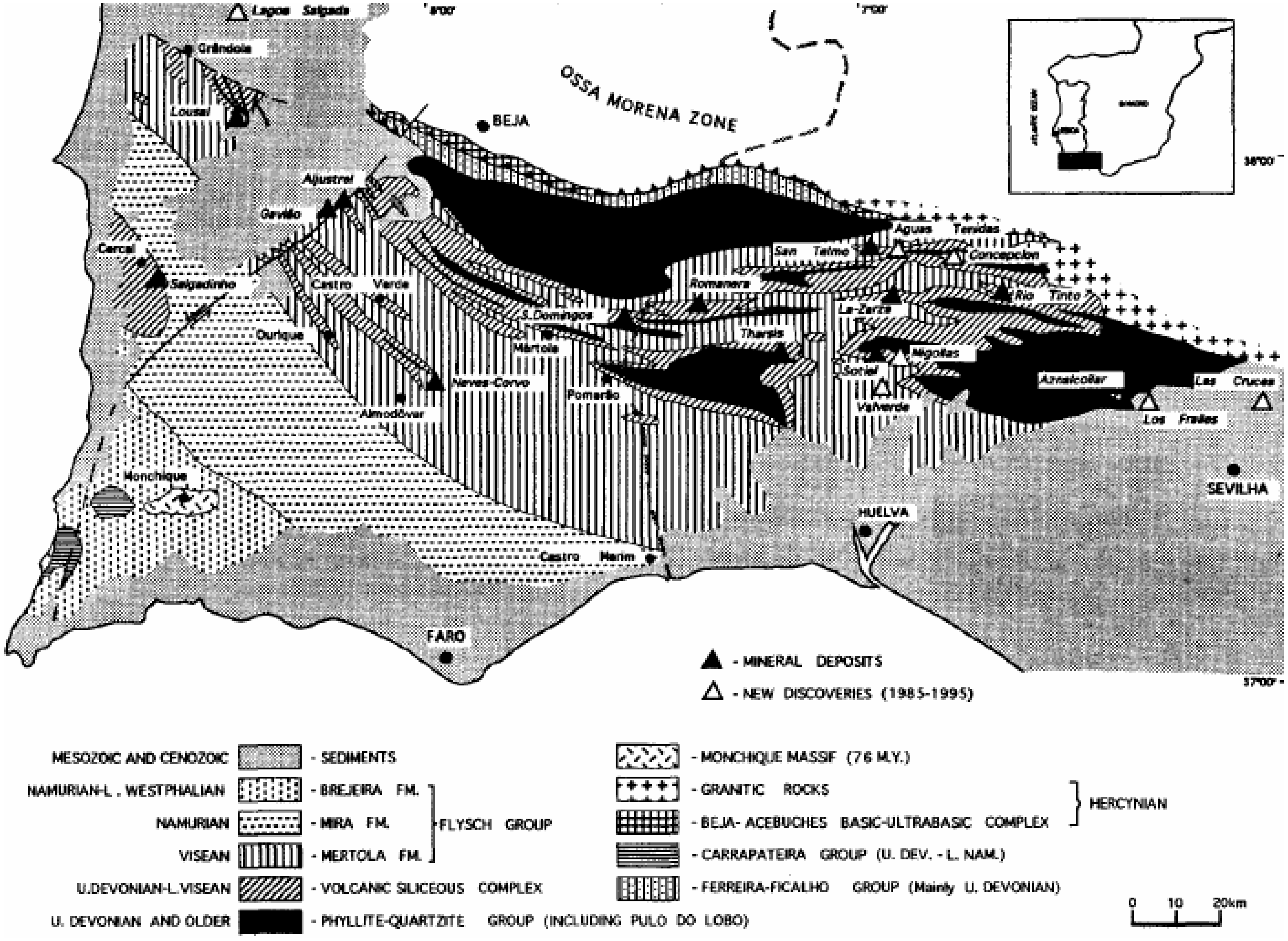


Fig. 41 Location and general geology of the Iberian pyrite belt. Adapted from Carvalho et al. (1999).

A comprehensive geochemical survey in the entire United States of America showed that 12% of the analysed streams and rivers had higher Sr concentration than adjacent coastal regions (Kraus & Secor, 2004). Unfortunately, data on Sr concentration in the Guadiana river basin and from others in the Algarve (S-Portugal) and in Andaluzia (SW-Spain) are not available.

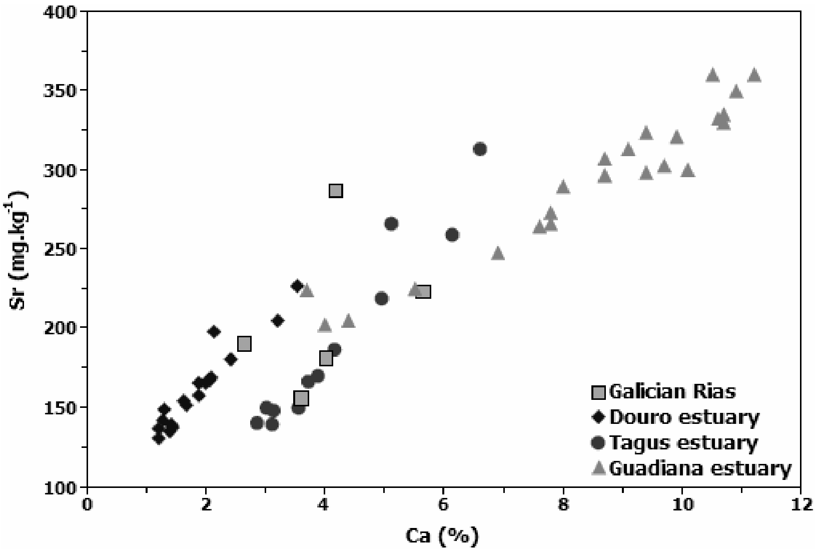


Fig. 42 Concentration of Sr (mg.kg^{-1}) vs. Ca (%) content in the platform sediments off Galician Rias, and Douro, Tagus and Guadiana estuaries (Araújo, unpublished data).

Therefore, it seems feasible that the concentration of Sr in the lower Guadiana basin is higher than that in the coastal area. For instance, two main reasons might support this hypothesis: a) local geological formations are enriched in Sr; b) the differences on the patterns of Sr concentration along the analysed anchovy otoliths from both site are only logical and coherent, with other data on anchovy life cycle, if we consider that the concentration of Sr is higher in the estuary than in the coast. Therefore, the following analyses are made based on this assumption.

The concentration of Sr on the otoliths of anchovy captured in the coast has a highly significant negative correlation with distance to core. This fact suggests that these specimens have progressively migrated from an area enriched in Sr, the Guadiana estuary or an estuarine system in its surroundings (Carreras, Piedras, Odiel-Tinto, Guadalete or Guadalquivir estuaries), to a less enriched one, the coastal area. The concentration of Sr can be relatively constant along wide oceanic areas (Kraus & Secor, 2004); because of this we assumed that 73.3% of the specimens captured in the coast (B, C, D, E, F, H, I, J, L, N, O) (Fig. 37) ([Sr]< 1000 ppm) have spent between 25% (specimen C) to 44% (specimen B) of their life cycle in the coast.

There are two specimens with particular patterns of Sr concentration along the otoliths. The pattern obtained for specimen G suggests that it may have entered into an estuary (*ca.* 1250-1350 μm) after probably spending its entire life in the coast. The most complex migration pattern was registered for specimen C, which suggests that it was born inside an estuary (*ca.* 1500 pmm Sr); then migrated to an oligohaline estuarine region (*ca.* 150-300 μm; max. 2400 ppm Sr), afterwards it suddenly migrated to a lower estuarine area (400-500 μm; 2000 to 1200 ppm Sr) and then progressively moved to the coast. The ontogenic migration to the upper estuary is confirmed by the distribution of anchovy larvae in the Guadiana estuary (see Fig. 25 and chapter 4.2) and the specimen H captured in the Guadiana estuary also has a similar ontogenic migration at the initial stage of its life cycle (*ca.* 0-150 μm) (Fig. 38). Kimura et al. (2000) also confirmed, for another anchovy species- *Anchoa mitchilli*, a late larval stage and juvenile ontogenic migration to oligohaline regions in the Chesapeake Bay with Sr analyses to the otoliths.

For the specimens collected in the Guadiana estuary, we will consider that a core concentration of Sr around 1000 ppm will correspond to the low estuary, since data from the last 100 μm of the post-rostrum, thus including the time of capture in the low estuary, averaged 1085 ± 200 ppm. Therefore, we assume that two groups of specimens born in the estuary, those with core concentrations above 1500 ppm (α) and others with core concentrations circa 1000 ppm (β). This distinction can be also observed by the shifting of the spawning area with river inflow (chapter 4.2).

α) This group include the specimens A, C, D, H, K, L and M that probably born in the middle estuary, except specimen C that might have born in an upper region. Specimens A and H performed a clear migration to an upper estuarine region, probably up to the upper estuary. Specimens D, K and M passed to a region with lower Sr concentration, probably the low estuary, while specimen L remain in the same location almost until the time is was collected.

β) This group include the specimens B, E, F, G, I, J, N, O and P, which were probably born in the low estuary and spend almost the entire life in the same location or accompanying a water mass with similar characteristics, as corroborated by the adult distribution of anchovy along the estuary (chapter 4.3). There are some exceptions: specimen B might have migrated into the coast and returned to the estuary (*ca.* 1100 to 1300 μm); specimen N might have been transported off the estuary and then entered in it (> 700 μm).

For 56.25% of the specimens captured in the Guadiana estuary (specimens B, E, F, G, H, J, K, M and N), there is a substantial increase of Sr concentration at capture time. Usually, the deposition of chemical elements into the otoliths diminishes with age due to a reduction of fish metabolism, so this hypothesis is less likely to explain this increase of Sr. However, it can be just a sign of the onset of a migration (Campbell et al., 1999; Elfman et al., 1999, 2000). However, Morris Jr. et al. (2003) established a connection between Sr concentrations in otoliths with a flooding event in Roanoke River (North Carolina, USA). If this connection could be established in the Guadiana estuary, it would be a useful tool to investigate former shifts in Guadiana river flow by analysing fossil otoliths. With current data, this connection cannot be established, however, from late November to mid December 2003, two major increases of river flow were registered in Pulo do Lobo (Fig. 43) that might have increased the availability of Sr in the estuary (INAG, 2004).

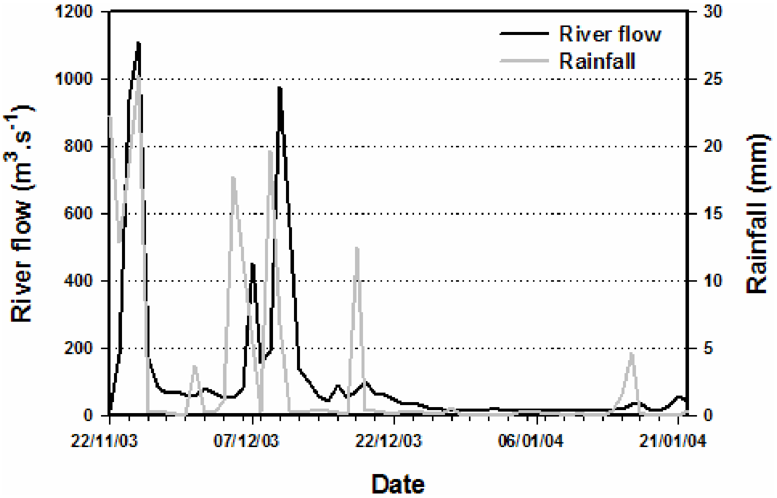


Fig. 43 Guadiana river flow (Pulo do Lobo hydrometric station) and rainfall (Alcoutim meteorological station), measured during the 2 months preceding the sampling of anchovy in the low estuary, for Sr analyses along otoliths. Data source: INAG, 2004.

Hence, in a future study of anchovy otoliths in the Guadiana estuary or elsewhere, the analysis of the concentration of microchemical elements in the water should be done simultaneously to otolith collection. The analysis of other elements (*e.g.* Ba, Ca, Mn, Mg) and analytical techniques (LA-ICPMS, SB-ICPMS) should be tested.

5. Conclusions

The presence of the new anchovy species *Engraulis albidus* was not confirmed for the Guadiana estuary and for the adjacent coastal area off Praia Verde. However, the majority of these specimens are distinct from *E. encrasicolus*, whose sequences are available in GenBank. The anchovy present in the Guadiana estuary remains classified as *Engraulis encrasicolus sensu lato*; therefore, this subject demands further investigation.

The genetic differentiation between the anchovy collected in the Guadiana estuary and in the adjacent coastal area is not significant. However, data from oxygen and carbon isotopes on the otoliths revealed significant differences between sites, suggesting that they are two populations.

The spawning of anchovy occurs inside the Guadiana estuary, by resident specimens. Periods of low river flow, *ca.* 3.2 ± 0.1 $m^3.s^{-1}$, sets the transition region of the low and mid estuary as the spawning ground. This is probably the ideal spawning area, since the estuarine morphology favours the retention of inert particles in this area. Higher river inflow, Q_{7days}= 30.8 ± 17.6 $m^3.s^{-1}$, sets the low estuary as the spawning area. An uncontrolled discharge from the Alqueva dam, in April 2002 ($Q_{daily\ max.}$= 656.3 ± 405.0), has compromised the development of anchovy larval stages inside the estuary.

The maximum abundance of recently hatched anchovy larvae is near the spawning area. Increasingly elderly larvae were more abundantly collected towards the upper estuary. To reach such location, the use of active migration strategies must have been employed. The transition between the middle and upper estuary is the nursery area of anchovy.

The distribution of anchovy juveniles and adults is also intrinsically linked with river flow. Anchovies were collected at the entrance of the estuary and the plume region, only during a flood event in January 2001 (Q> 3000 $m^3.s^{-1}$). Massive migration of anchovy adults from the coast to spawn in the low estuary, or in its vicinities, does not occur; as revealed by the size of specimens and by the migration patterns inferred by microchemical analyses of the otoliths. A differential distribution of juveniles and adults along the estuary was observed during Summer (Q< $8m^3.s^{-1}$), when juveniles were mainly in the middle and upper estuary, while adults were from the middle estuary to the upper part of the low estuary.

The concentration of Fe and Zn along the otoliths was not suitable to conjecture on the migration patterns of anchovy. However, the analyses of Sr concentration allowed to identify two distinct patterns: *i*) there are specimens that do not migrate along estuarine salinity gradients, *ii*), there are specimens that make a differential use of the estuary along their lives, occupying lower salinity regions during the beginning of the life cycle and, afterwards, they migrate to regions of higher salinity. The patterns inferred for specimens captured in the coast suggest that they probably born in an estuarine environment, progressively migrating along an estuary to the coast.

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Annex I. List, in alphabetic order, of the 143 species of the family Engraulidae (Froese & Pauly, 2006).

Scientific Name	Author	English common name
Amazonsprattus scintilla	Roberts, 1984	Rio Negro pygmy anchovy
Anchoa analis	(Miller, 1945)	Longfin Pacific anchovy
Anchoa argenteus	Schultz, 1949	
Anchoa argentivittata	(Regan, 1904)	Regan's anchovy
Anchoa belizensis	(Thomerson & Greenfield, 1975)	Belize anchovy
Anchoa cayorum	(Fowler, 1906)	Key anchovy
Anchoa chamensis	Hildebrand, 1943	Chame Point anchovy
Anchoa choerostoma	(Goode, 1874)	Bermuda anchovy
Anchoa colonensis	Hildebrand, 1943	Narrow-striped anchovy
Anchoa compressa	(Girard, 1858)	Deep body anchovy
Anchoa cubana	(Poey, 1868)	Cuban anchovy
Anchoa curta	(Jordan & Gilbert, 1882)	Short anchovy
Anchoa delicatissima	(Girard, 1854)	Slough anchovy
Anchoa eigenmannia	(Meek & Hildebrand, 1923)	Eigenmann's anchovy
Anchoa exigua	(Jordan & Gilbert, 1882)	Slender anchovy
Anchoa filifera	(Fowler, 1915)	Longfinger anchovy
Anchoa helleri	(Hubbs, 1921)	Heller's anchovy
Anchoa hepsetus	(Linnaeus, 1758)	Broad-striped anchovy
Anchoa ischana	(Jordan & Gilbert, 1882)	Gulf of California slender anchovy
Anchoa januaria	(Steindachner, 1879)	Rio anchovy
Anchoa lamprotaenia	Hildebrand, 1943	Big-eye anchovy
Anchoa lucida	(Jordan & Gilbert, 1882)	Bright anchovy
Anchoa lyolepis	(Evermann & Marsh, 1900)	Shortfinger anchovy
Anchoa marinii	Hildebrand, 1943	Marini's anchovy
Anchoa mitchilli	(Valenciennes, 1848)	Bay anchovy
Anchoa mundeola	(Gilbert & Pierson, 1898)	False Panama anchovy
Anchoa mundeoloides	(Breder, 1928)	Northern Gulf anchovy
Anchoa nasus	(Kner & Steindachner, 1867)	Longnose anchovy
Anchoa panamensis	(Steindachner, 1877)	Panama anchovy
Anchoa parva	(Meek & Hildebrand, 1923)	Little anchovy
Anchoa pectoralis	Hildebrand, 1943	Bigfin anchovy
Anchoa scofieldi	(Jordan & Culver, 1895)	Scofield's anchovy
Anchoa spinifer	(Valenciennes, 1848)	Spicule anchovy
Anchoa starksi	(Gilbert & Pierson, 1898)	Starks's anchovy
Anchoa tricolor	(Spix & Agassiz, 1829)	Piquitinga anchovy
Anchoa trinitatis	(Fowler, 1915)	Trinidad anchovy
Anchoa walkeri	Baldwin & Chang, 1970	Walker's anchovy
Anchovia clupeoides	(Swainson, 1839)	Zabaleta anchovy
Anchovia macrolepidota	(Kner, 1863)	Bigscale anchovy
Anchovia surinamensis	(Bleeker, 1866)	Surinam anchovy
Anchoviella alleni	(Myers, 1940)	Allen's anchovy
Anchoviella balboae	(Jordan & Seale, 1926)	Balboa anchovy
Anchoviella blackburni	Hildebrand, 1943	Blackburns's anchovy
Anchoviella brevirostris	(Günther, 1868)	Snubnose anchovy
Anchoviella carrikeri	Fowler, 1940	Carriker's anchovy
Anchoviella cayennensis	(Puyo, 1946)	Cayenne anchovy
Anchoviella elongata	(Meek & Hildebrand, 1923)	Elongate anchovy
Anchoviella guianensis	(Eigenmann, 1912)	Guyana anchovy
Anchoviella jamesi	(Jordan & Seale, 1926)	James's anchovy
Anchoviella lepidentostole	(Fowler, 1911)	Broadband anchovy
Anchoviella manamensis	Cervigón, 1982	Manamo anchovy
Anchoviella nattereri	(Steindachner, 1879)	Natterer's anchovy

Anchoviella perezi	Cervigón, 1987	
Anchoviella perfasciata	(Poey, 1860)	Poey's anchovy
Anchoviella vaillanti	(Steindachner, 1908)	Vaillant's anchovy
Cetengraulis edentulus	(Cuvier, 1829)	Atlantic anchoveta
Cetengraulis mysticetus	(Günther, 1867)	Pacific anchoveta
Coilia borneensis	Bleeker, 1852	Bornean grenadier anchovy
Coilia brachygnathus	Kreyenberg & Pappenheim, 1908	Yangtse grenadier anchovy
Coilia coomansi	Hardenberg, 1934	Cooman's grenadier anchovy
Coilia dussumieri	Valenciennes, 1848	Goldspotted grenadier anchovy
Coilia grayii	Richardson, 1845	Gray's grenadier anchovy
Coilia lindmani	Bleeker, 1858	Lindman's grenadier anchovy
Coilia macrognathos	Bleeker, 1852	Longjaw grenadier anchovy
Coilia mystus	(Linnaeus, 1758)	Osbeck's grenadier anchovy
Coilia nasus	Temminck & Schlegel, 1846	Japanese grenadier anchovy
Coilia neglecta	Whitehead, 1967	Neglected grenadier anchovy
Coilia ramcarati	(Hamilton, 1822)	Ramcarat grenadier anchovy
Coilia rebentischii	Bleeker, 1858	Many-fingered grenadier anchovy
Coilia reynaldi	Valenciennes, 1848	Reynald's grenadier anchovy
Encrasicholina devisi	(Whitley, 1940)	Devis' anchovy
Encrasicholina heteroloba	(Rüppell, 1837)	Shorthead anchovy
Encrasicholina oligobranchus	(Wongratana, 1983)	Philippine anchovy
Encrasicholina punctifer	Fowler, 1938	Buccaneer anchovy
Encrasicholina purpurea	(Fowler, 1900)	Nehu
Engraulis albidus	Borsa et al. 2004	White anchovy
Engraulis anchoita	Hubbs & Marini, 1935	Argentine anchoita
Engraulis australis	(White, 1790)	Australian anchovy
Engraulis encrasicolus	(Linnaeus, 1758)	European anchovy
Engraulis eurystole	(Swain & Meek, 1885)	Silver anchovy
Engraulis japonicus	Temminck & Schlegel, 1846	Japanese anchovy
Engraulis mordax	Girard, 1854	Californian anchovy
Engraulis ringens	Jenyns, 1842	Anchoveta
Jurengraulis juruensis	(Boulenger, 1898)	Jurua anchovy
Lycengraulis batesii	(Günther, 1868)	Bates' sabretooth anchovy
Lycengraulis grossidens	(Agassiz, 1829)	Atlantic sabretooth anchovy
Lycengraulis limnichthys	Schultz, 1949	
Lycengraulis poeyi	(Kner, 1863)	Pacific sabretooth anchovy
Lycothrissa crocodilus	(Bleeker, 1851)	Sabretoothed thryssa
Papuengraulis micropinna	Munro, 1964	Littlefin anchovy
Pseudosetipinna haizhouensis	Peng & Zhao, 1988	
Pterengraulis atherinoides	(Linnaeus, 1766)	Wingfin anchovy
Setipinna breviceps	(Cantor, 1849)	Shorthead hairfin anchovy
Setipinna brevifilis	(Valenciennes, 1848)	Short-hairfin anchovy
Setipinna melanochir	(Bleeker, 1849)	Dusky-hairfin anchovy
Setipinna paxtoni	Wongratana, 1987	Humpback hairfin anchovy
Setipinna phasa	(Hamilton, 1822)	Gangetic hairfin anchovy
Setipinna taty	(Valenciennes, 1848)	Scaly hairfin anchovy
Setipinna tenuifilis	(Valenciennes, 1848)	Common hairfin anchovy
Setipinna wheeleri	Wongratana, 1983	Burma hairfin anchovy
Stolephorus advenus	Wongratana, 1987	False Indian anchovy
Stolephorus andhraensis	Babu Rao, 1966	Andhra anchovy
Stolephorus apiensis	(Jordan & Seale, 1906)	Samoan anchovy
Stolephorus baganensis	Hardenberg, 1933	Bagan anchovy
Stolephorus brachycephalus	Wongratana, 1983	Broadhead anchovy
Stolephorus carpentariae	(De Vis, 1882)	Gulf of Carpenteria anchovy
Stolephorus chinensis	(Günther, 1880)	China anchovy

Stolephorus commersonnii	Lacepède, 1803	Commerson's anchovy
Stolephorus dubiosus	Wongratana, 1983	Thai anchovy
Stolephorus holodon	(Boulenger, 1900)	Natal anchovy
Stolephorus indicus	(van Hasselt, 1823)	Indian anchovy
Stolephorus insularis	Hardenberg, 1933	Hardenberg's anchovy
Stolephorus multibranchus	Wongratana, 1987	Caroline anchovy
Stolephorus nelsoni	Wongratana, 1987	Nelson's anchovy
Stolephorus pacificus	Baldwin, 1984	Pacific anchovy
Stolephorus ronquilloi	Wongratana, 1983	Ronquillo's anchovy
Stolephorus shantungensis	(Li, 1978)	
Stolephorus tri	(Bleeker, 1852)	Spined anchovy
Stolephorus waitei	Jordan & Seale, 1926	Spotty-face anchovy
Thryssa adelae	(Rutter, 1897)	Swatow thryssa
Thryssa aestuaria	(Ogilby, 1910)	Estuarine thryssa
Thryssa baelama	(Forsskål, 1775)	Baelama anchovy
Thryssa brevicauda	Roberts, 1978	Short-tail thryssa
Thryssa chefuensis	(Günther, 1874)	Chefoo thryssa
Thryssa dayi	Wongratana, 1983	Day's thryssa
Thryssa dussumieri	(Valenciennes, 1848)	Dussumier's thryssa
Thryssa encrasicholoides	(Bleeker, 1852)	False baelama anchovy
Thryssa gautamiensis	Babu Rao, 1971	Gautama thryssa
Thryssa hamiltonii	(Gray, 1835)	Hamilton's thryssa
Thryssa kammalensis	(Bleeker, 1849)	Kammal thryssa
Thryssa kammalensoides	Wongratana, 1983	Godavari thryssa
Thryssa malabarica	(Bloch, 1795)	Malabar thryssa
Thryssa marasriae	Wongratana, 1987	Marasri's thryssa
Thryssa mystax	(Bloch & Schneider, 1801)	Moustached thryssa
Thryssa polybranchialis	Wongratana, 1983	Humphead thryssa
Thryssa purava	(Hamilton, 1822)	Oblique-jaw thryssa
Thryssa rastrosa	Roberts, 1978	Fly river thryssa
Thryssa scratchleyi	(Ramsay & Ogilby, 1886)	New Guinea thryssa
Thryssa setirostris	(Broussonet, 1782)	Longjaw thryssa
Thryssa spinidens	(Jordan & Seale, 1925)	Bengal thryssa
Thryssa stenosoma	Wongratana, 1983	Slender thryssa
Thryssa vitrirostris	(Gilchrist & Thompson, 1908)	Orangemouth anchovy
Thryssa whiteheadi	Wongratana, 1983	Whitehead's thryssa

Chapter 4

Anchovy eggs as an assessment tool for estuarine ecohydrological management

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Abstract

Managing water discharge in dams may enhance the advenction of fish larval stages from their spawning and nursery areas in the estuary, when trying to solve eutrophication phenomenon in estuaries. The abundance and distribution of anchovy eggs and larvae were assessed in the Guadiana estuary and adjacent coastal area. Seven river discharge scenarios were tested using the *MOHID Water Modelling System*, at 2 tidal situations, to determine the residence time of the upper, middle and lower estuary, the sections where anchovy eggs were collected. The abundance of anchovy eggs were also merged as tracers in MOHID lagrangian model, to select the discharge scenarios that do not compromise the presence of anchovy eggs and of non-competent larvae inside the estuary. Data on anchovy eggs development stages and larvae age were used to validate the lagrangian model and to infer the spawning and nursery areas of anchovy. MOHID hydrodynamic model allowed determining that the lower section of the middle estuary was the anchovy spawning area during the period of maximum spawning, June 2002. It also reproduced the general distribution pattern of younger anchovy larvae, setting the upper and lower sections of the middle and upper estuary, respectively, as the nursery ground. It was decisive in establishing the river discharge scenarios that can be applied, to solve or mitigate eutrophication in the upper estuary, without compromising the presence of anchovy larval stages within the estuary. Scenarios B (Q_{max}= 20 $m^3.s^{-1}$) and C2 (Q_{max}= 50 $m^3.s^{-1}$ sudden end) should be applied during neap tides, to maximize the chances of fish larval stages to remain inside the estuary. The choice between scenarios will depend on the harshness of the eutrophication, on the effectiveness of an inexistent monitoring program of the Guadiana estuary and on plankton response experiments to flushing and increased nutrient loading. This approach resulted in an easy-to-use management tool for Guadiana managers and may serve as an example to other estuarine managers.

1. Introduction

A new approach to achieve sustainable water management is being attained with the implementation of UNESCO IHP-V (1996-2001) and IHP-VI (2002-2007) programmes. They established the concepts and principles of Ecohydrology, briefly defined as the study of functional inter-relations between hydrology and biota at the catchment scale (Zalewski et al., 1997), which has been applied in many worldwide freshwater ecosystems (Boruah & Biswas, 2002; Porporato et al., 2002; Trepel & Kluge, 2002; de Cabo et al., 2003; Luz & Loucks, 2003). However, the formal extension to estuaries and adjacent coastal areas occurred recently (Wolanski et al., 2004). In the Guadiana estuary (southeast Portugal), it is pursued for the last decade (Chícharo et al. 2001b), resulting in an ecohydrological model of the ecosystem (Wolanski et al., 2006). Although presenting good adjustments and reliable predictive scenarios, further ecohydrological approaches are needed.

In February 2002, the construction of the Alqueva dam was concluded at 150 km from the river mouth. This reservoir is one of the biggest in Europe, with a maximum area of 250 km^2, a perimeter of more than 1000 km and a total capacity of 4,150 hm^3 (INAG, 2005). A proper water management is undoubtedly a challenging task to accomplish in this reservoir, due to the multiple uses that water will have, *e.g.* hydroelectric production and river flow regularization.

The management of river discharges might be a useful tool to solve downstream ecological constrains (Chícharo et al., 2006a), such as eutrophication, but bad management practises may increase them. A proper management could be obtained through the application of the flow pulse concept, an expansion of the flood pulse concept (Tockner et al., 2000; Junk & Wantzen, 2004); both developed for freshwater ecosystems. The flood pulse concept considers that rivers and their fringing floodplains are integrated components of a single dynamic system, linked by strong interactions between hydrological and ecological processes. The flow pulse concept added that the interaction between temperature and flow plays a major role in structuring habitat conditions and biotic communities (Tockner et al. 2000). In fact, it is possible to set a deterministic plankton dynamics only when the magnitude and periodicity of hydraulic flushing and nutrient loading are large. Thus, biodiversity may be enhanced, eutrophication controlled and harmful algal blooms prevented (Roelke et al., 2003). The application of this concept to estuaries should be considered. In the Guadiana estuary it would help to mitigate or prevent the problems caused by toxic cyanobacteria blooms that often occur in the upper estuary (Caetano et al., 2001; Sobrino et al., 2004).

The intensity and length of hydraulic flushing is site specific. Modelling flushing scenarios, estimating estuarine water residence time or even primary productivity is a partial "biological-blind" approach that should be avoided, while applying the flow pulse concept to estuaries. Estuaries are the spawning and nursery areas of many coastal and estuarine fishes (Potter et al., 1990; Blaber et al., 1995). Therefore, an inadequate high flow pulse can increase the advection of fish eggs off the estuary, compromising fish recruitment, mainly of those with reduced reproductive periods and non-competent larvae at the hatching time (Strydom et al., 2002), such as Engraulidae.

Anchovy (Pisces: Engraulidae- *Engraulis encrasicolus sensu lato*) is the most abundant planktivorous fish in the Guadiana estuary (Chícharo et al., 2006b; Faria et al., 2006), has a central position in its trophic web (Canário, 2001) and was designated as a potential key species in detecting ecosystem changes (Chícharo et al. 2001a, 2003). Anchovy eggs can be an excellent indicator of river flow impacts. Moreover, the wide reproductive period (March to November) (Faria et al., 2006), and the easy identification of their pelagic eggs, turn it an excellent natural lagrangian tracer. Fish eggs could be used to validate lagrangian models. In return, these models can allow the definition of spawning (Page et al., 1999) and nursery areas

(Parada et al., 2003) and infer on larval transport mechanisms (Allain et al., 2003). The hydrodynamic model MOHID *Water Modelling System* is one of the many models used to model many aquatic ecosystems, either oceanic (Coelho et al. 2002), coastal (Cancino & Neves, 1999; Salgueiro, 2002) or estuarine environments (Gómez-Gesteira et al., 2000; Braunschweig, 2003; Martins et al., 2001; Trancoso et al., 2005), including the Guadiana estuary (Cunha, 1998; Lopes, 2004).

The transport and distribution of fish eggs depends on the flow (Faria et al., 2006). In the Guadiana estuary, the flow is very complex, mainly due to the interaction with bathymetry (Lopes, 2004). The release of particles in a 2D Guadiana estuary model as passive tracers might be a good approach. Maximum time period of lagrangian simulations was 10 days, long before larvae become competent (Ré, 1986).

Thus, comprehending anchovy early-larval stages dynamics needs, but also complements, the knowledge on flow dynamics. Therefore, the abundance and distribution of anchovy eggs and larvae were assessed in the Guadiana estuary and adjacent coastal area. Considering the flood pulse concept and using *MOHID*, 7 river discharge scenarios were tested to determine the residence time of the upper, middle and lower estuary, the sections where anchovy eggs were collected. Moreover, the abundance of anchovy eggs were merged as tracers in MOHID lagrangian model, to select the discharge scenarios that do not compromise the presence of anchovy eggs and of non-competent larvae inside the Guadiana estuary. Finally, data on anchovy eggs development stages and larvae age were used to validate the lagrangian model and also to infer the spawning and nursery areas of anchovy.

2. Material and Methods

2.1. Study site

This study took place in the Guadiana estuary and in its adjacent coastal area, which is on the southern border of Portugal with Spain (Fig. 1). The estuary is approximately 76 km long, with an area of 22 km^2 and an average depth of 6.5 m. It is a mesotidal estuary and tide amplitudes range from 1.3 to 3.5 m. The estuary is partially stratified when average river flow (~150 m^3.s^{-1}) and tidal prism (~3 x10^7 m^3) are observed (Michel, 1980). River flow has a striking variability between and within years, since the majority of the basin is under the influence of a climate with Mediterranean characteristics. The annual average temperature varies from 14 to 18 ºC. Rainfall is very irregular within the year, 80% occurs during autumn and winter, while summers are very dry. The annual average rainfall fluctuates between 561

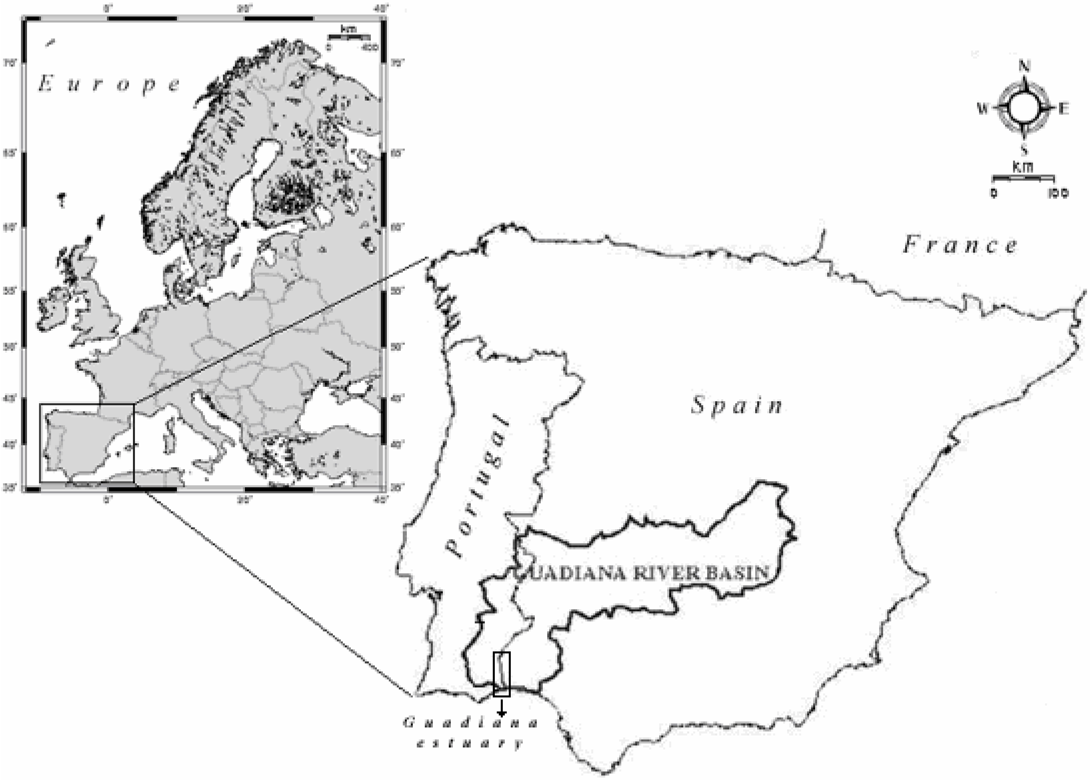


Fig. 1 Location of the Guadiana estuary in the Iberian Peninsula (Europe).

and 600 mm in the Portuguese basin, however higher variations are expected between years. Climate variability imposes a similar trend to river flow; thus, the average river flows are as follows: dry years, 8–63 $m^3.s^{-1}$; average years, 170–190 $m^3.s^{-1}$; humid years, 412–463 $m^3.s^{-1}$ (Bettencourt et al., 2003).

2.2. Relevant aspects of anchovy larval stages

Anchovy eggs have slight positive buoyancy (Blaxter, 1969) and development is temperature dependent (Lo, 1985). In the Guadiana estuary, the time elapsed from spawning to hatching might vary between 24h (August 2002) to 67h (May 2002) (unpublished data).

Anchovy larvae hatch from pelagic eggs, less developed than those hatching from benthonic eggs (Dando, 1984). Recently hatched anchovy larvae (~3.5 mm) tend to have neutral floatability, due to its huge yolk sac. The drag caused by the yolk sac turns movement highly energetic expensive. At this stage, larvae just perform regular short bursts to adjust their position and to search for microhabitats with oxygen saturation greater than 60%, since oxygen is exchanged by cutaneous diffusion until the gills are developed. When smaller than 5 mm, anchovy larvae can only progress through active swimming, because viscosity and drag will force them to stop when they beat-and-glide. As the yolk sac is consumed, they tend to have negative floatability. The yolk sac is fully absorbed at day 5 (~5 mm) and intermittent motion becomes the more efficient way of swimming (Weihs, 1980a,b). The development of the dorsal fin and of the gas bladder occurs at days 11 and 13, respectively. The gas bladder is completely formed at day 20 (10 mm), allowing fish to regulate its position easily. Consequently, they become able to control their position efficiently, performing diel rhythms of vertical migration (Ré, 1986).

2.3. Data on anchovy larval stages

Eulerian sampling campaigns were performed during new moon spring tides at low and high tides, from March 2002 till February 2003 in 9 sampling stations, 7 inside the estuary and 2 in the coastal area. Sampling started at the beginning of the flood in station 1 and continued upstream, towards station 9. Station 1 was re-sampled at the beginning of the ebb; the remaining stations were sampled afterwards (Fig. 2). In each station, water column vertical profiles of temperature and salinity were recorded with an YSI 6600 probe. Sub-superficial zooplankton trawls were made with a 250 µm net mesh, equipped with a flowmeter (General Oceanics). Samples were immediately preserved with buffered formaldehyde (4% final concentration) until processing.

On the laboratory, anchovy eggs and larvae were sorted from the zooplankton samples to determine their abundance. Development stages were assigned to each egg, according to the 11 stages defined by Moser & Ahlstrom (1985) for *Engraulis mordax*. The average time elapsed since spawning was calculated with Lo's (1985) stage-to-age model, using the parameters defined for *Engraulis encrasicolus* by Motos (1994). The percentage of each egg stage along the estuary will allow detecting preferential spawning areas and analysing the effect of advection.

To determine eggs mortality rate, the staged eggs collected in the different sampling stations were gathered in two groups, the low and high tide groups. In each group the presence of daily cohorts was investigated, by checking the correspondence between egg stage and average time since spawning. The decrease between each daily cohort was determined and the final monthly value results from the average of both tides. Confidence on the analysis relies on a high number of collected eggs and on a broad distribution of the eggs among the different stages.

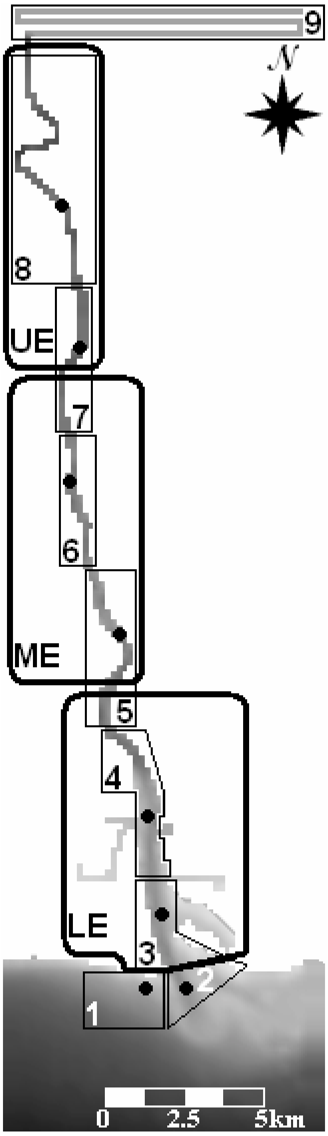


Fig. 2 Representation of the grid used in MOHID hydrodynamic model to simulate the hydrodynamic processes that occur in the Guadiana estuary. Legend: *Black dots*- discharge points and sampling stations; *Thick lines*- release and monitor boxes used to estimate residence time; *Thin lines*- monitor boxes used to infer anchovy spawning and nursery areas and to assess the impact of each river discharge scenario.

Larvae standard length was measured and corrected for the shrinking effect caused by trawling and preservation (Theilacker, 1980). A length-to-age model was fitted to infer the age of larvae, considering a daily growth of 0.51 mm.day^{-1} (Ribeiro, 1991).

2.4. What is MOHID?

MOHID is a 3D water modelling system (www.mohid.com), with modular numerical models, programmed in object oriented ANSI FORTRAN 95, and a set of graphical user interfaces programmed in VB.NET. It comprises models for hydrodynamics, lagrangian transport, water quality and sediment. This approach allows the adoption of an integrated modelling, not only of processes, but also of different scales and systems (Leitão, 2002). The velocity fields are computed in the hydrodynamic module, using a 3D formulation with hydrostatic and Boussinesq approximations. The equations are solved using the finite volume method with *Alternate Direction Implicit* discrimination. The horizontal grid is the orthogonal Arakawa C type staggered grid. At the open boundaries, both radiative conditions and imposed values can be set (Coelho et al., 2002). Tide gauge elevations at the open boundary and river discharge of the Guadiana River (5 $m^3.s^{-1}$, average discharge between the samplings of April and June 2002) were imposed explicitly to the hydrodynamic model. In tidal flats a moving boundary condition is implemented enabling the drying and flooding of cells as a function of the water level. The bottom stress was employed implicitly using a quadratic law and the Backhaus scheme (Backhaus, 1983). No meteorological forcing data was imposed.

The lagrangian module was used to construct a dependency matrix to know the fate of water masses inside the estuary. Lagrangian tracers are born at origins and those with equal origin have the same properties and parameters for random walk. Tracers carry explicit information of its origin, relating their position in any instant of time with their origin. Residence time and water or particles history can then be analysed. There are three ways to define origins in space: *Point Origin* (emits tracers at a given point), *Box Origin* (emits tracers over a given area) and *Accident Origin* (emit tracers in a circular form around a point). Origins can emit tracers during a period of time - *Continuous Origin,* or at one instant in time-

Instantaneous Origin. Mean velocity is the major factor responsible for particle movement (MOHID, 2005).

2.5. Setup of numerical simulations

MOHID is a freeware program (www.mohid.com) and version 4.5 was used. The grid defined to model the hydrological processes in the Guadiana estuary begins at 5 km from the estuary mouth, ending few kilometres downstream of Alcoutim (station 9), but holding the area where anchovy eggs were collected. The domain of calculus is composed by 205x53 cells with a spatial step of 180x180m (Fig. 2). The bathymetry was crafted on this grid using the Portuguese Hydrographic Institute surveys. One sigma layer was used for the vertical discrimination; therefore the model behaves as a 2D depth integrated model. This configuration was used because the estuary is shallow and vertical stratification is only important during extreme discharge periods in the estuary mouth. Field measurements of hydrodynamic parameters (elevation, velocity and direction) and water properties (salinity and cohesive sediments) were used in calibration (Lopes, 2004). River discharge was determined at 16 kilometres above the tidal limit of the estuary, in Pulo do Lobo hydrometric station (code: 27L/01) (INAG, 2005), where *ca*. 90% of the water going to the estuary passes (Ribeiro et al., 1988).

After stabilizing hydrodynamic and salt transport, using a constant river discharge of 5 $m^3.s^{-1}$, it were prepared several simulations. Distinct estuarine sections and river discharge scenarios were defined for the 3 numerical simulations.

The methodology proposed by Braunschweig et al. (2003) was used to estimate residence times. It states that the water located inside the estuary at high waters is divided into water masses and transported in the lagrangian model as passive tracers. Emission boxes and points were used to mark the water masses; in order to identify their origin. Using monitor boxes allows determining the fraction of water coming from each location in the place of interest.

Seven river discharge scenarios were tested in sections 2.4.2.1. and 2.4.2.2. Scenario A has a constant discharge of 5 $m^3.s^{-1}$. The remaining 6 scenarios have maximum river discharges of 20 $m^3.s^{-1}$ (scenario B), 50 $m^3.s^{-1}$ (scenarios C1 and C2), 100 $m^3.s^{-1}$ (scenario D), 250 $m^3.s^{-1}$ (scenario E) and 500 $m^3.s^{-1}$ (scenario F). Except for scenario C2, the discharge increases steadily during 4 days until reaching the maximum discharge, remains constant for 2 days and then decreases progressively until reaching 5 $m^3.s^{-1}$ at day 10. The difference between scenarios C1 and C2 exists in the last 4 days, once scenario C2 has an abrupt decrease from 50 $m^3.s^{-1}$ to 5 $m^3.s^{-1}$ at day 6. Simulations were made in the month when maximum abundance of anchovy eggs was registered, June 2002. The maximum time period of lagrangian simulations was 10 days, long before larvae can actively regulate their position in the water column (Ré, 1986). The details of each simulation are summarized in figure 3.

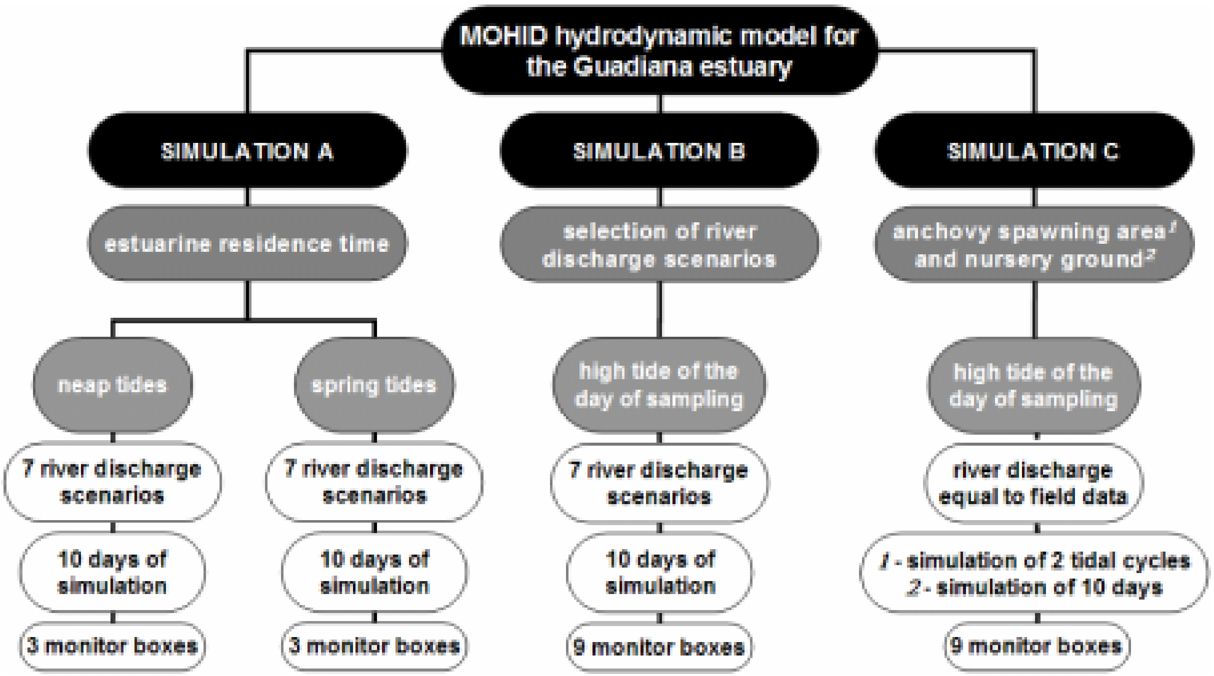


Fig. 3 Summary of the setup used to determine estuarine residence time (Simulation A), to select the river discharge scenarios that do not compromise the existence of fish larval stages inside the Guadiana estuary (Simulation B) and to infer anchovy spawning and nursery areas (Simulation C).

2.5.1. Estimating estuarine residence time (Simulation A)

The 7 river discharge scenarios were tested in two distinct tidal situations, during the weaker neap tides and strongest spring tides of late May and early June 2002, respectively. It was set 3 release and monitor boxes, corresponding to the upper, middle and lower estuary (Fig. 2). This subdivision was made according to the hidrology, sedimentology and biology of the estuary (Morales, 1993; Chícharo et al. 2001; Faria et al., 2006). The number of particles released in each box was proportional to its volume; thus, 1140, 1110 and 1610 particles were released in the upper, middle and low estuary, respectively. The time span of each simulation was 10 days (Figs. 3 and 4). The effect of tides on the number of particles remaining in the estuary, for each river discharge scenario, was evaluated with a t-test. If the Kolmogorov-Smirnov test (with Lilliefors' correction) (p= 0.05) reveals that data has not a normal distribution, then it was applied the Mann-Whitney rank sum test (p= 0.05).

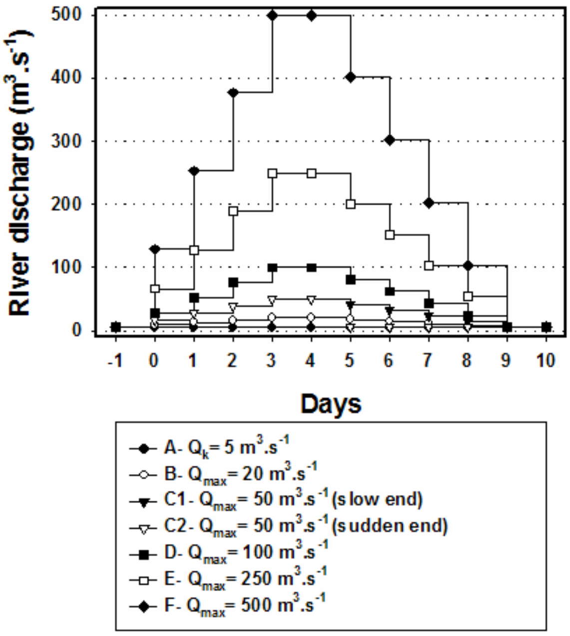


Fig. 4 River discharge scenarios applied in MOHID hydrodynamic model for the Guadiana estuary.

2.5.2. Selection of river discharge scenarios (Simulation B)

The 7 river discharge scenarios were tested to select those that do not compromise the presence of anchovy larval stages inside the Guadiana estuary. The number of particles released in each releasing point was proportional to the abundance of anchovy eggs registered during the field campaign of June 2002 high tide. Thus, 1, 111, 175, 614, 21064, 6202, 2782 and 13 lagrangian tracers were released in releasing points 1 to 8, respectively. These releasing points correspond to a sampling station. Simulation started at the high tide of the day of sampling and lasted for 10 days. The number of particles was calculated in 9 monitor boxes, at the high tide of days 5 and 10. Boxes boundaries were set at the half way between stations (boxes 3 to 8) or comprising a particular area (boxes 1, 2 and 9). No particles were released in box 9, but there number was monitored, although it does not correspond to the contour of the estuary (Fig. 2). The median was calculated to compare the various results, once it is less sensitive to extreme scores than the mean, thus a better measure of highly skewed distributions (Sokal & Rohlf, 1995).

2.5.3. Inferring anchovy spawning and nursery areas (Simulation C)

The reliability of this transport model can be evaluated by comparing the advection of particles with data regarding the abundance and distribution of eggs and larvae, the development stage of eggs and age of larvae. This allows confirming and explaining the spawning and nursery locations. Thus, lagrangian tracers were released in 8 releasing points, corresponding to the sampling stations where anchovy eggs were collected- stations 1 to 8. The number of particles released in each box was proportional to the abundance of anchovy eggs, registered during the field campaign of June 2002 high tide. The number of lagrangian tracers used in this simulation was equal to the one used in *Simulation B.* A constant river discharge of 5 $m^3.s^{-1}$ was set. To determine anchovy spawning and nursery areas, lagrangian simulations lasted for 2 tidal cycles and 10 days, respectively. Number and origin of particles were tracked in 9 monitor boxes (Figs. 2 and 3).

3. Results

3.1. Anchovy eggs and larvae

In June 2002, maximum egg abundance was registered in station 4 at the low tide (1883 eggs.$100m^{-3}$) and in station 5 at the high tide (2106 eggs.$100m^{-3}$). At the low tide, anchovy eggs were sampled from stations 2 to 7, but predominantly in the low estuary (stations 3 and 4). At the high tide, the distribution ranged from stations 2 to 8, but they were mainly collected in the middle estuary (stations 5 to 7) (Fig. 5A).

Younger egg stages (stages I to II) were more abundant in station 7 (high tide)- 190 eggs.$100m^{-3}$ (68%) in stage II. Eggs at stages IV and V were more abundant in station 4 (low tide)- 1410 eggs.$100m^{-3}$ (75%), and in station 5 (high tide)- 1559 eggs.$100m^{-3}$ (74%), respectively. The oldest egg stages (IX to XI) were more abundant in stations 4 and 5 and were collected from stations 2 to 5. At the low tide, 131 eggs.$100m^{-3}$ at stage IX were collected in station 4 (7.0%); while at the high tide, 96 eggs.$100m^{-3}$ (4.6%) were collected in station 5 at stage X (Figs 5A and 6).

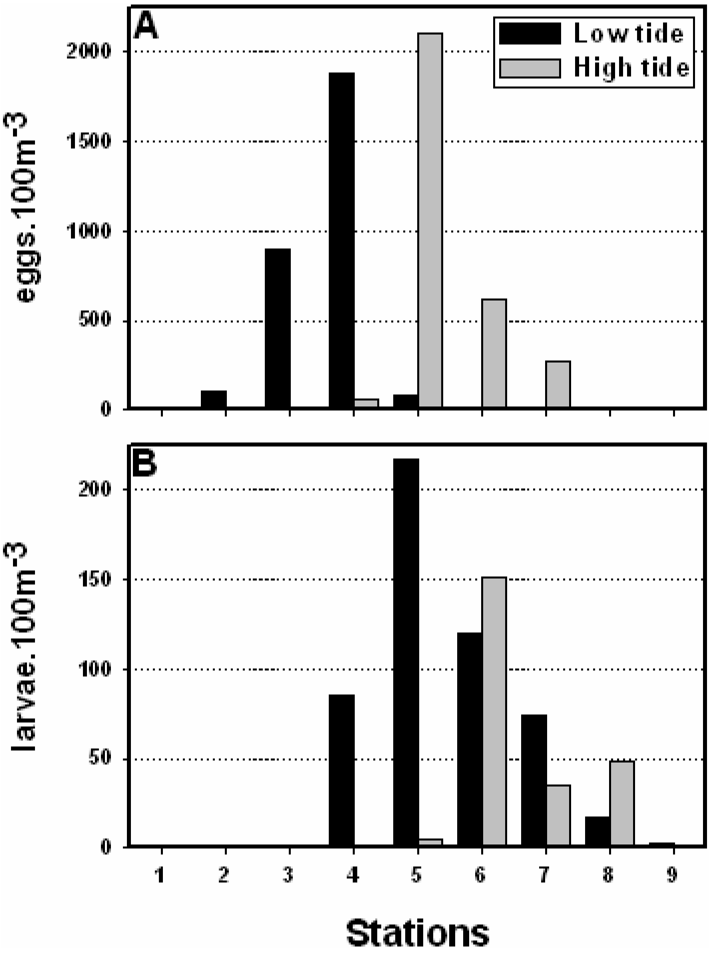


Fig. 5 Abundance of anchovy eggs (A) and larvae (B), in each sampling station, at the low and high tide of June 2002.

The average time length (h) of each stage was as follow: stage I- 1.2 ± 0.0 h; stage II 5.1 ± 0.7 h; stage III 10.7 ± 1.5 h; stage IV 16.5 ± 2.7 h; stage V 22.6 ± 3.9 h; stage VI 25.9 ± 3.2 h; stage VII 35.1 ± 4.9 h; stage VIII 39.4 ± 5.6 h; stage IX 45.3 ± 4.0 h; stage X 47.6 ± 3.9 h; stage XI 49.0 ± 3.8 h. Therefore, it was possible to identify 2 complete cohorts at the low and high tide. At the low tide, cohort 1 gathered stages III to V, while cohort 2 was represented by stages VII to IX. At the high tide, cohort 1 had eggs in stages IV to VI, while cohort 2 had eggs in stages VIII to X. The average egg mortality was 59.0 ± 12.4 % (Fig. 7).

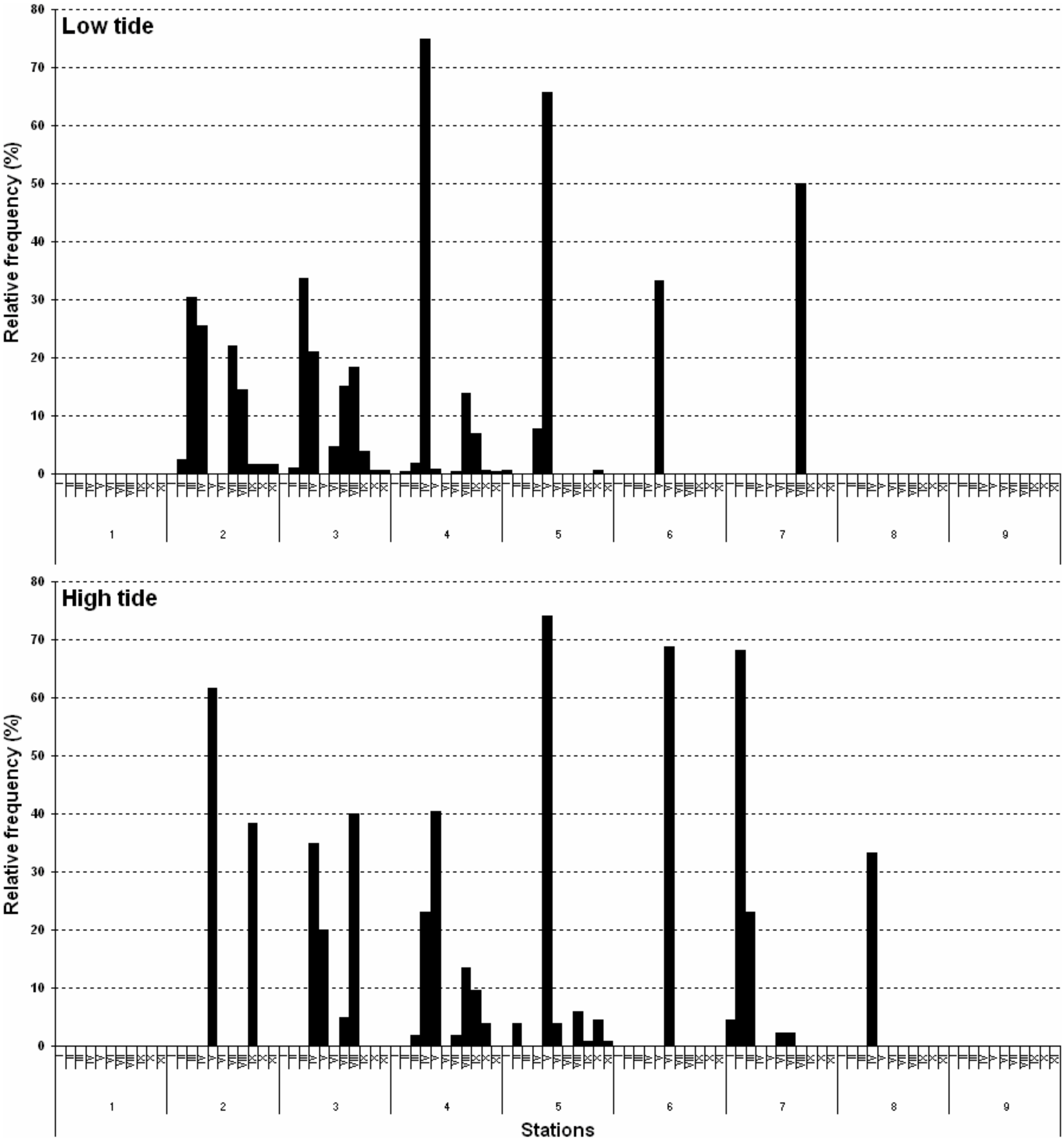


Fig. 6 Relative frequency of anchovy egg stages (Roman numerals)
in each sampling station (Arabic numerals), at the low and high tide of June 2002.

Maximum larvae abundance was registered in station 5 during the low tide (218 larvae.100m^{-3}) and in station 6 during the high tide (151 larvae.100m^{-3}). Anchovy larvae were collected from stations 4 to 9, but predominantly in the middle estuary at both tides (Fig. 5). Younger larvae are collected in the middle estuary (8.2 ± 0.5 days), while older were collected in the upper estuary, with 15.1 ± 4.7 days (station 9, low tide) (Fig. 8).

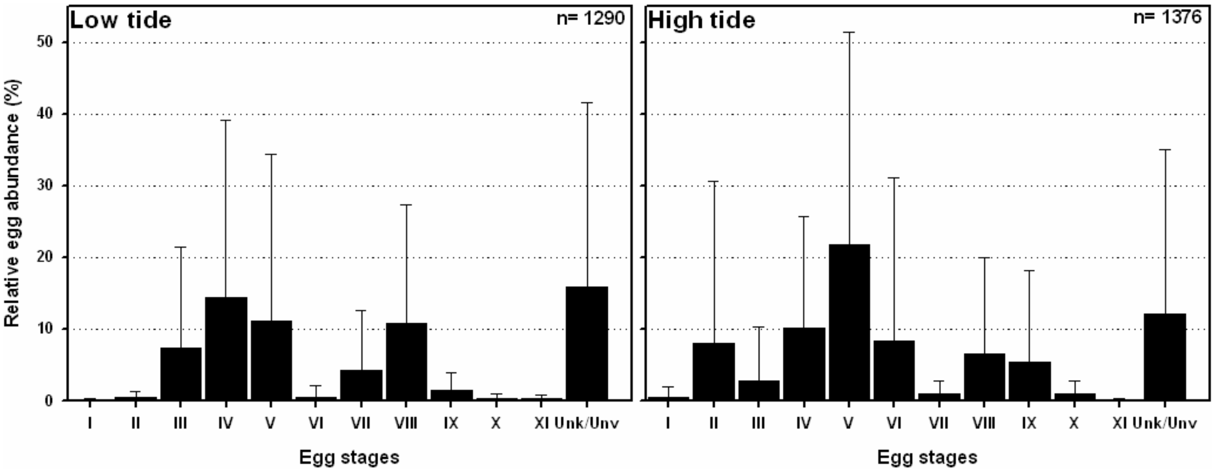


Fig. 7 Relative egg abundance in each stage at the low and high tide of June 2002.

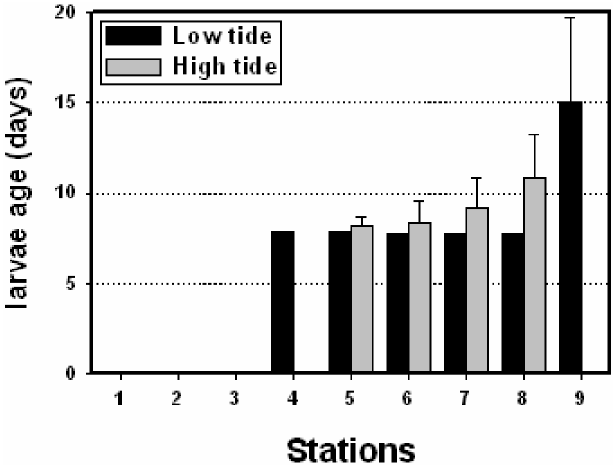
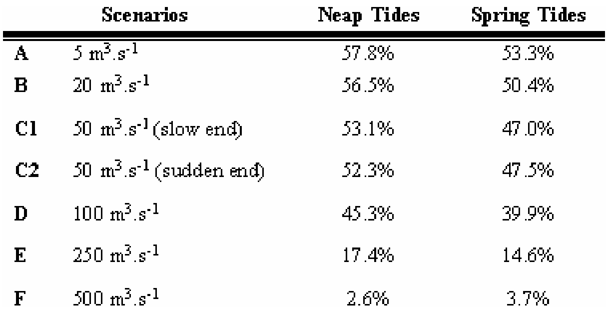


Fig. 8 Average (± standard deviation) age of anchovy larvae, in each sampling station, at the low and high tide of June 2002.

3.2. Estimating estuarine residence time (Simulation A)

The median percentages of particles inside the estuary for each scenario and tide, along 10 days, are summarized in table I. Estuarine residence time is significantly lower (n= 21600; P≤ 0.001) during spring tides, except for scenario F (Q_{max}= 500 $m^3.s^{-1}$). Hereafter, the analysed scenarios correspond only to this tide. Thus, in scenario A (Q_k= 5 $m^3.s^{-1}$), 53.3% of

Table I Median percentage of particles in the estuary for each tidal condition, along the 10 days of simulation.



	Scenarios	Neap Tides	Spring Tides
A	5 $m^3.s^{-1}$	57.8%	53.3%
B	20 $m^3.s^{-1}$	56.5%	50.4%
C1	50 $m^3.s^{-1}$ (slow end)	53.1%	47.0%
C2	50 $m^3.s^{-1}$ (sudden end)	52.3%	47.5%
D	100 $m^3.s^{-1}$	45.3%	39.9%
E	250 $m^3.s^{-1}$	17.4%	14.6%
F	500 $m^3.s^{-1}$	2.6%	3.7%

the particles remain in the estuary after 10 days. This value decrease as maximum river discharge increases, down to 3.7% in scenario F (Q_{max}= 500 $m^3.s^{-1}$), which has a water residence time of 6.2 days. There is a significant difference (n= 10810; P≤ 0.001) between scenarios C1 (Q_{max}= 50 $m^3.s^{-1}$ slow end) and C2 (Q_{max}= 50 $m^3.s^{-1}$ sudden end), for the period when river discharge pattern start to differ. The median values were 39.2% (scenario C1) and 39.7% (scenario C2) (Fig. 9). For scenarios D, E and F, the particles remainning inside the estuary after 10 days correspond only to 28.7%, 5.8% and 1.5% of those remaining in the estuary for scenario A, respectively (Fig. 9).For scenario A, water residence time in the low

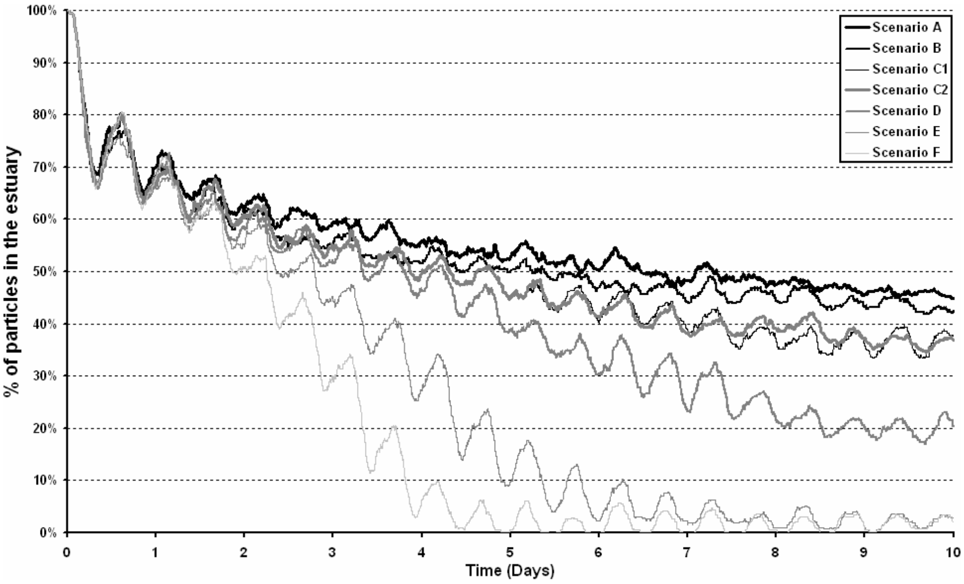


Fig. 9. Percentage of particles remaining in the estuary for every discharge scenarios.

estuary was 9.5 days, decreasing to 1.6 days in scenario F (Fig. 10). Also for scenario F, the middle and upper estuary residence times; are 3.8 and 4.7 days, respectively (Fig. 10).

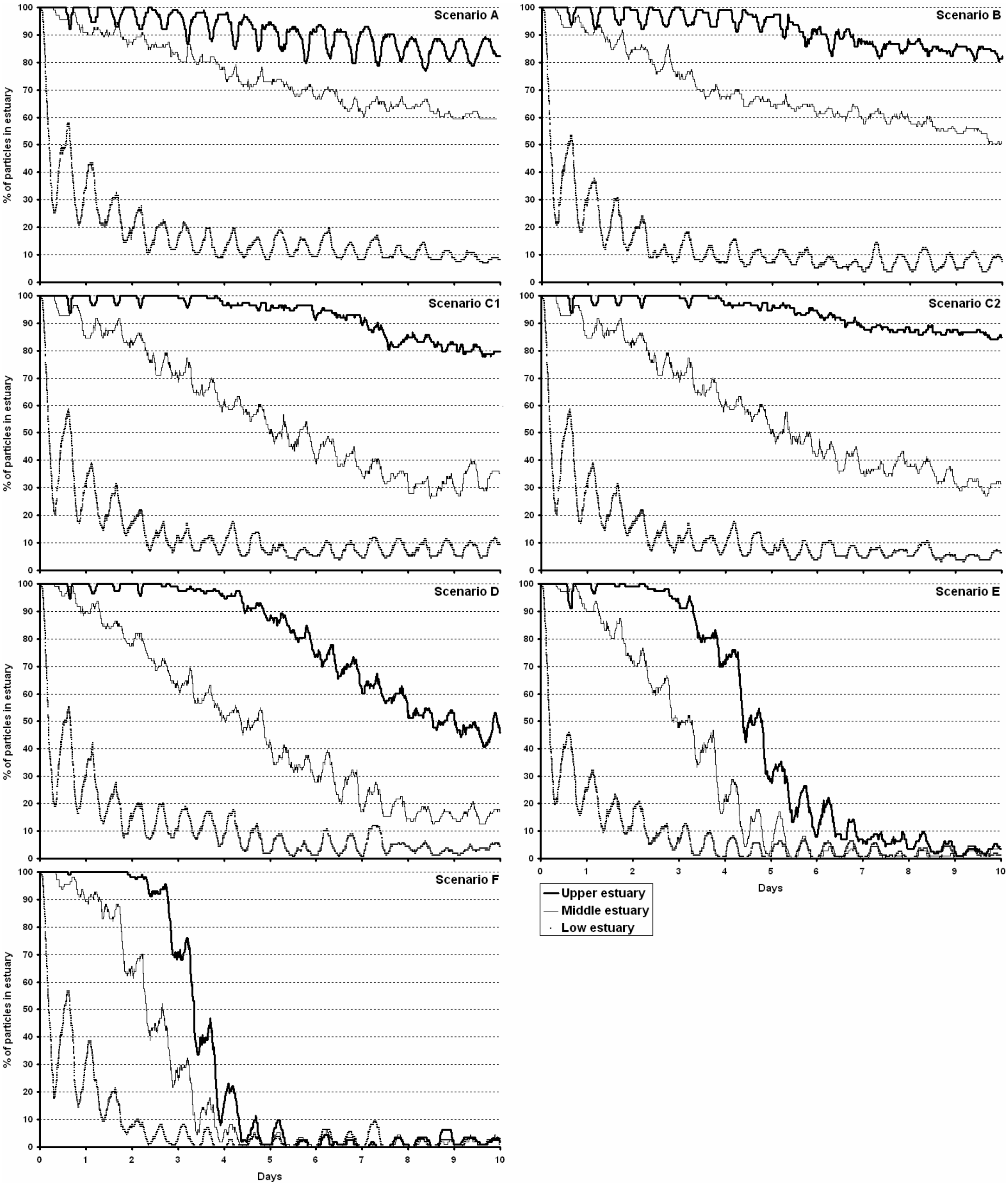


Fig. 10 Percentage of particles in each estuarine section along the 10 days of simulation, for each discharge scenario (Simulation A).

3.3. Selection of river discharge scenarios (Simulation B)

For scenario A (Q_{k}= 5 m^{3}.s^{-1}), 76.2% and 63.2% of the particles released in each station remain in the estuary after 5 and 10 days, respectively. Particles remain largely in the middle estuary. The distribution pattern observed for scenarios B ($Q_{max=}$ 20 m^{3}.s^{-1}), C1 (Q_{max}= 50 m^{3}.s^{-1} slow end) and C2 (Q_{max}= 50 m^{3}.s^{-1} sudden end) are similar with scenario A, despite the reduction of particles inside the estuary. For scenarios C1 and C2, 67.8% of particles are inside the estuary after 5 days, decreasing to 42.6% and 47.6% after 10 days, respectively. In scenario D ($Q_{max=}$ 100 m^{3}.s^{-1}), 22.5% of particles are in the estuary after 10 days, but their distribution pattern is more even than for the previous scenarios. For scenarios E ($Q_{max=}$ 250

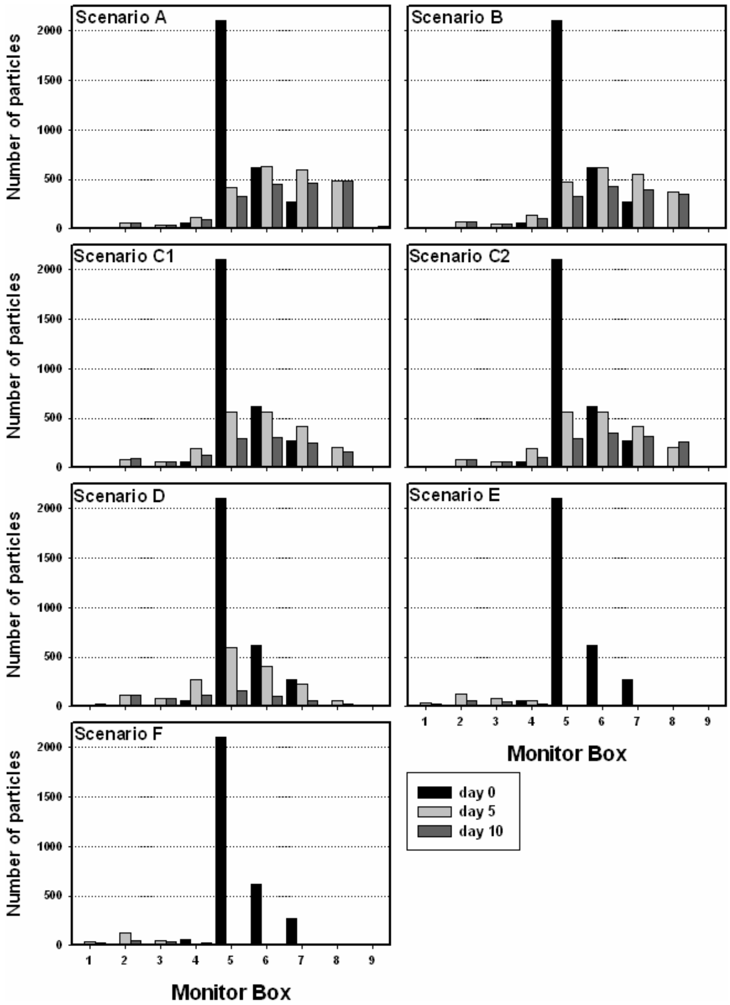


Fig. 11 Number of particles, in each monitor box, at the high tide of days 0, 5 and 10, for each river discharge scenario (Simulation B).

$m^3.s^{-1}$) and F ($Q_{max=}$ 500 $m^3.s^{-1}$), the particles remaining in the estuary after 10 days are only present in the low estuary- 5.0% and 4.8% respectively (Fig. 11).

3.4. Inferring anchovy spawning area and nursery ground (Simulation C)

Particles were released in each releasing point proportionally to the abundance of anchovy eggs. Thus, after 2 tidal cycles, the particles released in all stations produced a similar distribution to the one observed in the estuary (Figs. 5A, 12). Station 5, *i.e.* the lower section of the middle estuary, was the one that produced the most similar distribution pattern (Fig. 13). After 10 days of simulation at Q_k= 5 $m^3.s^{-1}$ (scenario A), 63.2% of the released particles remain inside the estuary (Fig. 11). The distribution of particles along the estuary is more even at day 10 than at day 0, being observed a marked increase of particles in station 7 (166%) and 8 (37762%), *i.e.* in the upper and lower sections of the middle and upper estuary, respectively (Fig. 13). The highest differences were as follow. In June 2002, the eggs captured in station 3, during the low tide, correspond to 4.8% of the eggs captured in station 4, the one with highest abundance. The simulation output showed a higher number of particles in monitor box 3- 14.2%. In monitor box 5, it was observed the same. It was accounted 84.0%

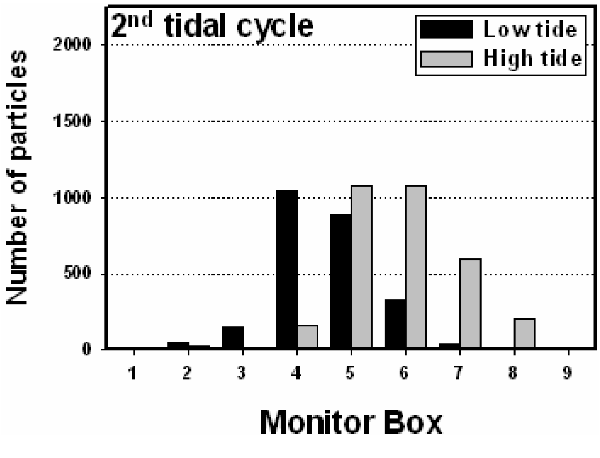


Fig. 12 Number of particles in each monitor box after 2 tidal cycles, for Q_k= 5 $m^3.s^{-1}$. Particles were released from each monitor box at the same proportion to the one observed in June 2002 high tide (Simulation C).

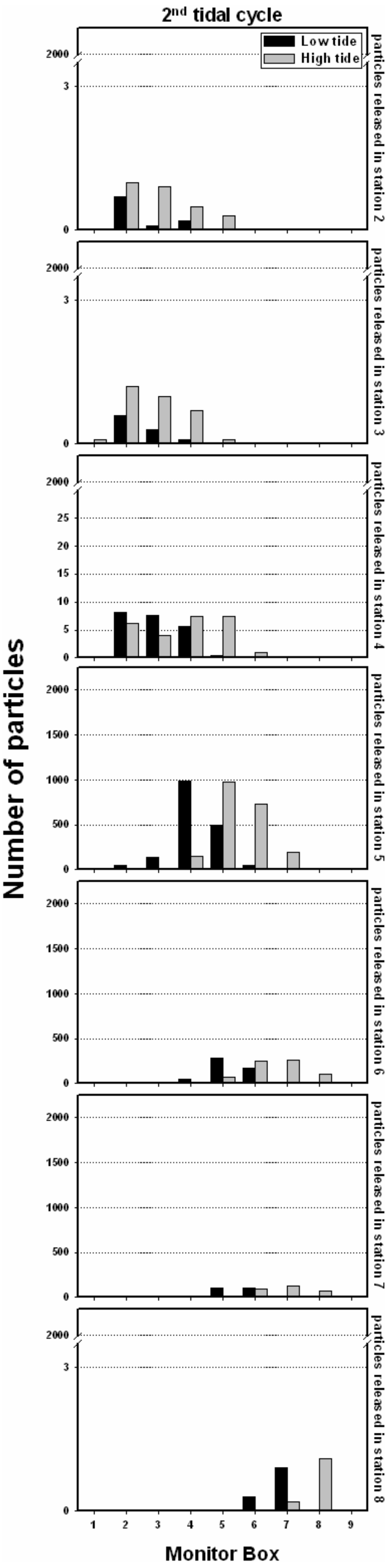
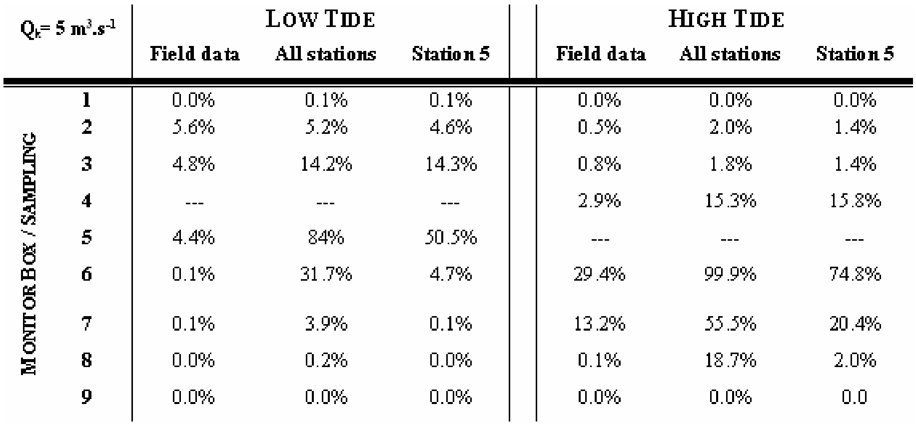


Fig. 13 Tracking the advection and number of the particles released in each monitor box after 2 tidal cycles with a Q_k= 5 $m^3.s^{-1}$. Particles were released from each monitor boxat the same proportion to the one observed in June 2002 high tide (Simulation C).

more particles in this box than when comparing station 4 and 5, only a 4.4% difference. During the high tide, the most striking difference was observed when comparing the output model for monitor box 6 and station 6. In station 6, the collected eggs corresponded to 29.4% of the eggs captured in station 5. However, the particles registered in monitor box 6 correspond to 99.9% of the particles present in monitor box 5 (Table II).

Table II Relative abundance of lagrangian particles (all station) in each monitor box compared with the one with the highest abundance, after 2 tidal cycles and with Q_k= 5 $m^3.s^{-1}$. Field data concerning anchovy egg abundance in June 2002 is also shown.



Q_k= 5 $m^3.s^{-1}$		LOW TIDE			HIGH TIDE		
		Field data	**All stations**	**Station 5**	**Field data**	**All stations**	**Station 5**
MONITOR BOX / SAMPLING	**1**	0.0%	0.1%	0.1%	0.0%	0.0%	0.0%
	2	5.6%	5.2%	4.6%	0.5%	2.0%	1.4%
	3	4.8%	14.2%	14.3%	0.8%	1.8%	1.4%
	4	---	---	---	2.9%	15.3%	15.8%
	5	4.4%	84%	50.5%	---	---	---
	6	0.1%	31.7%	4.7%	29.4%	99.9%	74.8%
	7	0.1%	3.9%	0.1%	13.2%	55.5%	20.4%
	8	0.0%	0.2%	0.0%	0.1%	18.7%	2.0%
	9	0.0%	0.0%	0.0%	0.0%	0.0%	0.0

4. Discussion

4.1. Residence time and anchovy spawning and nursery areas

In estuaries, suitable spawning areas must have circulation patterns and residence times that allow fish larval stages to fulfill their need for exogenous food and to develop their capability to actively control their distribution (Page et al., 1999; Brown et al., 2005). Based on our results, the spawning area of anchovy in the Guadiana estuary seems to be located in the transition between the low and middle estuary, somewhere between stations 4 and 5, or even a little upstream. Probably, during the lowest neap tide periods, spawning would have been limited to the middle estuary. Less intense tidal transport and advection would support this hypothesis. Additionally, the particles released in station 5 were those that better reproduced the distribution pattern of anchovy eggs in the estuary (Fig. 13). After 10 days of simulation at $Q_k= 5\ m^3.s^{-1}$ (scenario A, spring tide), 60% of the released particles remain in the estuary. Spawning in the low estuary is less appropriate because it has a very low residence time, 9.5 days (Fig. 10), thus the majority of anchovy larvae would be flushed off the estuary before they turn competent. However, advection from the spawning area, located in upper estuarine areas, possibly explains the presence of late egg stages in the low estuary during the low tide (Fig. 6). Stage I eggs were found in upstream stations during late afternoon, since anchovy spawns preferentially at dusk and in the following hours (Ré, 1984; Ribeiro et al., 1996).

The transition between the low and middle estuary has pronounced *S* shape morphology, enabling the formation of secondary flows and, thus, retention of particles (Martins et al., 2002) (Fig. 14). This supports the marked difference between the number of particles remaining in the low and middle estuary, after 5 and 10 days at $Q_k= 5\ m^3.s^{-1}$. There is also a residual trend of particles transport towards upstream, which occurs because the length of the flood is slightly longer than the ebb, mainly in periods of low river flow (Fortunato et al., 2002; Pinto et al., 2003). The distribution pattern obtained with a $Q_k= 5\ m^3.s^{-1}$ after 10 days of simulation (Fig. 11) is comparable with the one observed for anchovy larvae (Fig. 6B), which had an average age of 8 days (Fig. 8), still without active swimming abilities (Ré, 1986). However, a more analogous pattern could have been obtained if anchovy egg mortality (59.0 ± 12.4 %) and larvae mortality along the estuary would have been integrated in MOHID. Anchovy larvae were mainly collected in the middle estuary (Fig. 5B), the location with higher zooplankton biomass, 1.4 ± 0.5 g $DW.100m^{-3}$ (except for station 9 at the high tide- 7.7 g $DW.100m^{-3}$) (Morais, unpublished data). Older anchovy larvae are found in the upper estuary (Fig. 8), but transport strategies, other than passive mechanisms have to be considered (Kimura et al., 2000; Hare et al. 2005).

Examples of active retention mechanisms are a) selective tidal stream transport and residual bottom inflow, which might be synchronized with vertical migrations of larvae with formed gas bladder (Ré, 1984) and b) lateral migrations to the margins, where the river flow is lower or to take advantage of the residual up-estuary flow near the margins, which occurs in the low Guadiana estuary (Oliveira et al., 2006), which even allow to counter-act strong river inflows. Examples of passive retention mechanisms are tidal and wind advection; although wind has little effect in the advection of inert particles in narrow estuaries (Braunschweig et al., 2003), like the Guadiana. The relative importance of these mechanisms differs among species and changes with larval development (Hare et al., 2005). In Chesapeake Bay (USA), the net up-estuary migration of the Atlantic menhaden *Brevoortia tyrannus* (Clupeidae) larvae was dominated by residual bottom inflow and wind forcing (Hare et al., 2005). An estuarine anchovy, *Anchoa mitchilli* (Clupeidae), perform up-estuary migration by tracking the diurnal migrations of their main preys (Copepoda: *Acartia tonsa*) (North & Houde, 2004 and references therein).

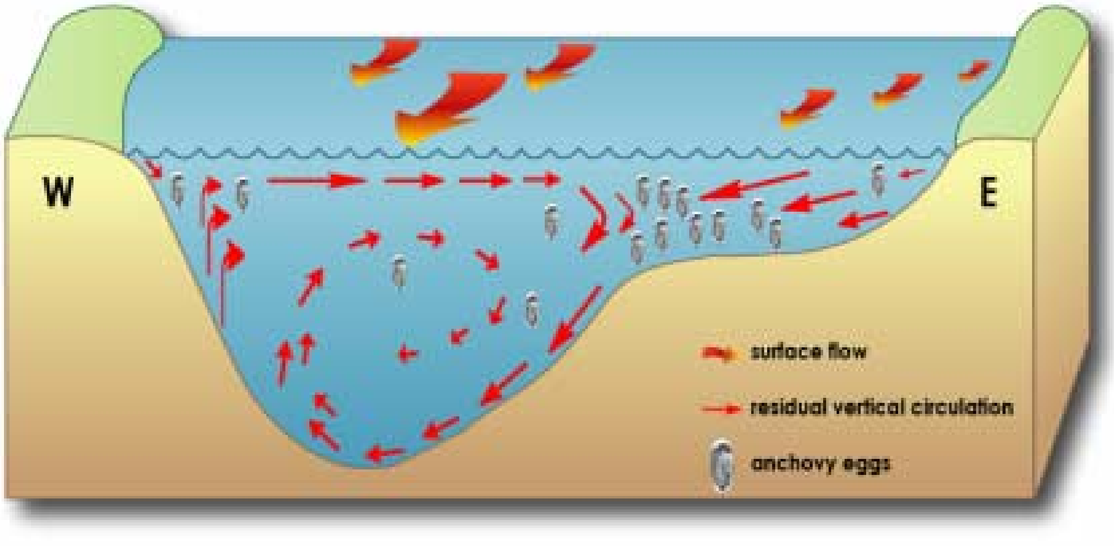


Fig. 14 Hypothetical representation of residual vertical circulation in the transition between the low and middle Guadiana estuary.

4.2. Residence time and selection of river discharge scenarios

Managing the Guadiana water resources is undoubtedly a difficult task to accomplish, mainly in the Alqueva reservoir. Ecosystem services to humans are broad and management based on ecosystem services requires a full understanding of the complexity in which these services benefit humans (Farber et al., 2006). Thus, trying to set river discharge scenarios that would not jeopardize the presence of fish larval stages inside the Guadiana estuary, in order to solve or mitigate eutrophication in the upper estuary, is just one of the many aspects that need evaluation. Another vital issue that must be addressed is the use of river discharges to minimize the colonization success of non-native aquatic species, namely of the Asiatic clam *Corbicula fluminea* (Müller). Ecosystem perturbation is the best discourage factor to their colonization success, however setting increased discharges to prevent post-larvae to bury in the sediment might coincide with anchovy maximum spawning intensity (Doherty et al., 1987).

In this paper, 7 river discharge scenarios were tested in two distinct tidal situations, during the lowest neap tides and highest spring tides of June 2002. Since there is a significant difference between them, the application of one of the chosen scenarios should occur during neap tides periods. Thus, chances of fish larval stages to remain inside the estuary are maximized.

Assuming that anchovy spawning occurred in the middle estuary in June 2002; then, scenarios D (Q_{max}= 100 $m^3.s^{-1}$), E (Q_{max}= 250 $m^3.s^{-1}$) and F (Q_{max}= 500 $m^3.s^{-1}$) clearly compromise the presence of larval stages inside the estuary. The application of these scenarios would flush the larvae to the coast before they turn fully competent, which would occur around day 20 after they hatch (Ré, 1986).

Depending on the magnitude of the eutrophication in the upper estuary, scenarios B (Q_{max}= 20 $m^3.s^{-1}$), C1 (Q_{max}= 50 $m^3.s^{-1}$ slow end) or C2 (Q_{max}= 50 $m^3.s^{-1}$ sudden end) would be those that hypothetically would be applied during the neap tide. A definitive choice between one of these scenarios has to rely in their simulation in controlled plankton experiments. When deciding between scenarios C1 and C2, it is important to recall that scenario C2 is more conservative, *i.e.* the number of particles remaining inside the estuary is greater. Moreover, scenario C2 requires 30% less water, which is vital in a region with reduced water resources and highly variable supply.

5. Conclusions

MOHID hydrodynamic model for the Guadiana estuary was useful in enlightening the spawning and nursery areas of anchovy, *i.e.* the lower section of the middle estuary was the location of spawning in June 2002. It was able to replicate the general distribution pattern of younger anchovy larvae, setting the middle estuary and the lower part of the upper estuary (stations 5 to 8) as the nursery ground. MOHID was decisive to choose the river discharge scenarios that can be applied to solve or mitigate eutrophication problems in the upper estuary. Scenarios B (Q_{max}= 20 $m^3.s^{-1}$) and C2 (Q_{max}= 50 $m^3.s^{-1}$ sudden end) should be applied during neap tides to maximize the chances of fish larval stages to remain in the estuary. The choice between scenarios will depend on the harshness of the eutrophication and on the effectiveness of an inexistent monitoring program. Plankton response experiments to flushing and increased nutrient loading have to be accomplished, to achieve a broader ecohydrological approach to the Guadiana basin. Above all, this work produced an easy-to-use management tool for Guadiana managers, which can serve as an example to other estuarine sites around the globe.

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Chapter 5

Synthesis

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1. The present study revealed that during the unique period of filling of the Alqueva dam major changes occurred in the Guadiana estuary. The estuarine turbidity maximum zone moved to the upper estuary, to at least 38 km from the river mouth, differing 8 to 16 km from previous records. In the upper and middle estuary, the nutrient stoichiometry dynamics was more N limited than P limited during the whole year, and Si limitation was only frequent on the coast. Previously, the upper estuary evolved from potential P limitation during winter, to Si limitation during spring to mid summer, and to N limitation from mid summer to autumn. The flooding of vast areas possibly caused an increase in DSi minimum and maximum concentrations; which was slightly smaller than the maximum observed during a year of high inflow.
2. A reduction of 99.99% in the maximum abundance of anchovy eggs occurred six days prior to the April 2002 sampling. The eggs where flushed to the coast because of an uncontrolled river discharge from the Alqueva dam. Therefore, it is advisable that dam managers mimic, as much as possible, the natural river flow, in order to minimize the impact on downstream ecosystems.
3. Up to now it was not possible to confirm the presence of the new anchovy species, *Engraulis albidus*, in the Guadiana estuary. Therefore anchovies from the Guadiana estuary and adjacent coastal area were classified as *E. encrasicolus sensu lato*. Although, the analysed specimens were genetically distinct from *E. encrasicolus*.
4. Data from oxygen and carbon isotopes on the otoliths complemented the information revealed by genetic analyse, because it suggests the existence of two populations of anchovy, apart only 15 km.
5. The spawning of anchovy occurs inside the Guadiana estuary, by resident specimens. Periods of low river flow, *ca.* 3.2 ± 0.1 $m^3.s^{-1}$, sets the transition between the low and mid estuary as the spawning ground, which is probably the ideal spawning area. The

transition between the middle and upper estuary is the nursery area. The distribution of anchovy juveniles and adults is intrinsically linked with river flow. Anchovies were collected at the entrance of the estuary and plume region, only during a flood event in January 2001 (Q> 3000 $m^3.s^{-1}$). A differential distribution of juveniles and adults was observed during Summer (Q< 8 $m^3.s^{-1}$), when juveniles were mainly in the middle and upper estuary, while adults were from the middle estuary to the upper part of the low estuary.

6. The concentration of Sr along the otoliths allowed identifying two distinct patterns for the estuarine specimens: *i*) no migration along estuarine salinity gradients *ii*), migration to high salinity estuarine regions, after spending the beginning of the life cycle in those with low salinity. The majority of anchovies captured in the coast have born in an estuarine environment; then they progressively migrated along an estuary to the coast.
7. This study merged the abundance of anchovy eggs in a hydrodynamic model of the Guadiana estuary, to provide dam managers a tool that evaluates the impact caused by river discharge, as a mean to solve or mitigate eutrophication in the upper estuary, on the presence of anchovy larval stages in their spawning and nursery areas. The tested scenarios that do not compromise the presence of anchovy larval stages within the estuary were scenarios B (Q_{max}= 20 $m^3.s^{-1}$) and C2 (Q_{max}= 50 $m^3.s^{-1}$ sudden end). These scenarios should be applied during neap tides, to maximize the chances of fish larval stages to remain inside the estuary. Finally, it provided an easy-to-use management tool for Guadiana managers and may serve as an example to other estuarine managers.
8. This study revealed unique data about the Guadiana estuary during the filling of the Alqueva dam, clarified the life cycle of anchovy in this estuary and provided dam

managers a modeling tool that allow understanding the impact of dam discharges on downstream populations of larval fishes. Aspects on the genetics of anchovy bear further insights and the developed management tool has the potential to be applied in other ecosystems and must be further developed and improved.