



Article

# Preliminary Insight into Winter Native Fish Assemblages in Guadiana Estuary Salt Marshes Coping with Environmental Variability and Non-Indigenous Fish Introduction

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**Abstract:** This work aims to undertake a preliminary characterization of winter fish assemblages in the salt marsh areas of Guadiana lower estuary (South-East Portugal) and discusses the potential risks of habitat dominance by a non-indigenous species (NIS). To this effect, six field campaigns were carried out in four sampling sites during winter season targeting the collection of fish species. A total of 48 samples were collected. Individuals from seven different taxa (marine and estuarine) were collected, although the assemblage was dominated by two estuarine species—the native *Pomatoschistus* sp. (goby) and the NIS *Fundulus heteroclitus* (mummichog). Goby was the most abundant taxa in the majority of salt marsh habitats, except for one specific, marsh pool, where extreme environmental conditions were registered, namely high temperature and salinity. Such conditions may have boosted the intrusion of mummichog in this area. This species is well adapted to a wide range of abiotic factors enabling them to colonize habitats where no predators inhabit. Impacts of mummichog introduction in the Guadiana salt marsh area are still unpredictable since this is the first time they have been recorded in such high density. Nevertheless, in scenarios of increased anthropogenic pressure and, consequently, habitat degradation, there is a potential risk of mummichog spreading to other habitats and therefore competing for space and food resources with native species.

**Keywords:** *Pomatoschistus* sp.; *Fundulus heteroclitus*; temperature; salinity; anthropogenic pressures; habitat degradation

## 1. Introduction

Worldwide, estuarine areas and their associated salt marsh habitats are described as highly productive and valuable aquatic ecosystems [1]. Due to high levels of primary production, large reserves of organic matter and habitat diversity, these areas are considered biochemical hotspots that offer optimal conditions for numerous birds and aquatic species [2]. These systems provide potential advantages for the growth and survival of young fish, namely high prey availability and refuge from

predators [1] and, consequently, support the offshore stocks of economically valuable species [3,4]. The Guadiana estuary is no exception to this general observation since it provides an exceptionally suitable environment for fish spawning, breeding, feeding, and growth not only for estuarine but also economically important marine species, such as sardine *Sardina pilchardus*, seabream *Diplodus* sp., and sole *Solea* spp. [5,6]. In particular, the sardine is a small pelagic fish of great socio-economic importance for Portugal. Fluctuations in their productivity may cause serious issues for fisheries management and policies in the region [7]. Since winter is the main spawning season of sardine in the study area [8], sampling campaigns for this preliminary study were carried out between January and February. Tidal shifts in fish assemblages have been reported by several authors [9–11] and, therefore, fish were sampled in both tidal stages.

Over the years, estuarine and coastal areas became increasingly affected by anthropogenic activities such as urbanization, industrialization, and tourism [12]. Also, these ecosystems are facing impacts of climatic changes [13]. Such anthropogenic and natural pressures exert a great influence on several environmental factors including temperature, salinity, and macronutrients concentration which in turn will disturb fish. They respond to environmental changes by changing their numbers and/or distributional range, in particular, larvae and juvenile fish which are highly susceptible to environmental fluctuations [14].

The completion of the Alqueva dam located 150 km from the Guadiana River mouth promoted a reduction in freshwater inflow leading to the degradation of salt marsh vegetation in the lower Guadiana estuary [15]. Moreover, climate change scenarios predict an increase in temperature, length, and frequency of dry periods for this region [13]. Predicted lower precipitation will potentiate retention of river waters and sediments by upstream dams and consequently the degradation of water quality and lack of sediment for plant accretion on salt marsh areas of the lower Guadiana estuary.

The resulting degradation observed in salt marsh habitats per se are a threat to associated fish species, but also increase the risk of invasion by exotic opportunistic species [16]. Invasions are also facilitated by altered environmental conditions. Environmental changes encourage the establishment of non-indigenous species (NIS) which may become potential competitors for native species [17]. In the Guadiana estuary, a community shift has already been documented, including plankton and fish [15,18,19]. In addition, the first occurrences of several marine invasive species have also been recorded, with potentially detrimental effects on native biota [13]. In 2008, the invasive *Blackfordia virginica* (cnidarian) and *Palaemon macrodactylus* (caridean shrimp) were first observed within the Guadiana estuary [17]. For both species, there is the potential competition for space and resources with native fish such as *Sardina pilchardus*, *Engraulis encrasicolus*, *Pomatoschistus* sp., *Solea* sp., *Diplodus* sp., *Syngnathus* sp. Although the sources of introduction for *B. virginica* and *P. macrodactylus* to the Guadiana Estuary remain unknown, previous research has shown that reduction of variability of river flow in estuaries has facilitated the establishment of NIS species [20].

Mummichog is a native species and a dominant faunal component of salt marshes in the Atlantic coast of the United States and Canada [21]. It is described as an opportunistic species due to its high tolerance to extreme and highly variable environmental conditions, namely high salinity and a wide range of temperature [22] which makes mummichog a potential candidate as an invasive species. It has reportedly been introduced to Hawaii, and from there to the Philippines, in the early 20th century [23]. Later, in the 1970s, mummichog arrived in the southwestern Iberian Peninsula apparently due to cross-contamination of ballast water of ships coming from the United States [24]. Occurrences of the species remain poorly documented in the Guadiana [5,6]. There are doubts as to the outcomes of introductions but for now there are no records of native species disappearance where mummichog has been introduced [25].

The objectives of this work are: (a) to characterize winter fish communities in salt marsh areas of Guadiana estuary and their occurrence variability according to tide regime; (b) to investigate the effect of environmental factors on fish species distribution; and (c) to discuss the potential risks of habitat dominance by mummichog, an NIS species.

## 2. Results

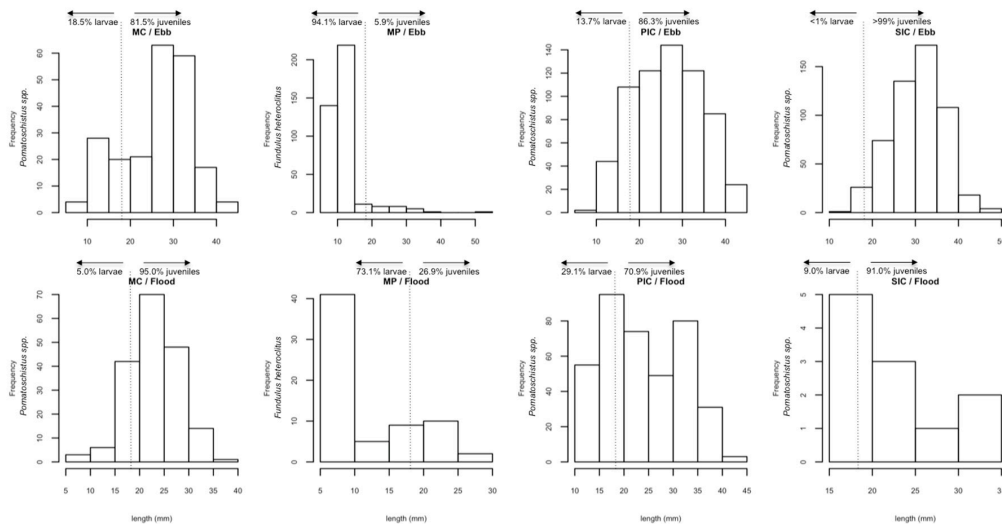
### 2.1. Composition of Fish Communities

A total of 2550 individuals from seven different families were collected from Guadiana estuary salt marshes. Among these, four were estuarine residents (goby, *Pomatoschistus* sp.; mummichog, *Fundulus heteroclitus*; sand smelt, *Atherina* sp.; and pipefish, *Syngnathus* sp.). One species was marine seasonal (sardine, *Sardina pilchardus*), the other two were marine juveniles (seabream, *Diplodus* sp. and sole, *Solea* spp.). Catches were primarily dominated by the estuarine resident goby and secondarily by mummichog, which collectively made up more than 99% of the total catch.

Marine species were caught at larval stages, except the sole that was also caught in the juvenile stage. The estuarine resident pipefish was caught at the juvenile stage, goby at both larvae and juvenile stage and mummichog at all stages (from egg to adult). The majority of the captured gobies were juveniles (87%) and only 13% were larvae. Mummichog catches were dominated by larvae (91%) with only 9% of juveniles (Table 1, Figure 1).

**Table 1.** List of captured fish species during the study period in the lower Guadiana estuary salt marsh areas. N: total number; F (%): frequency; % of larvae; % of juveniles; Mean size (mm); Size range (mm); Spatial occupancy: MC “main channel”, PIC “principal intertidal creek”, SIC “secondary intertidal creek” and MP “marsh pool”.

Species	N	F (%)	Larvae (%)	Juveniles (%)	Mean Size (mm)	Size Range (mm)	Spatial Occupancy
<i>Atherina</i> sp.	1	0.04	-	100.0	36.0	-	MC
<i>Diplodus</i> sp.	1	0.04	100.0	-	16.0	-	PIC
<i>Fundulus heteroclitus</i>	479	18.93	90.6	9.4	12.1	1.6–52.0	MP
<i>Pomatoschistus</i> sp.	2043	80.75	13.0	87.0	27.1	7.0–47.0	MC, PIC, SIC, MP
<i>Sardina pilchardus</i>	2	0.08	100.0	-	27.0	26.0–28.0	MC, SIC
<i>Solea</i> spp.	3	0.12	66.7	33.3	13.2	6.5–26.0	MC
<i>Syngnathus</i> sp.	1	0.04	-	100.0	92.0	-	MP

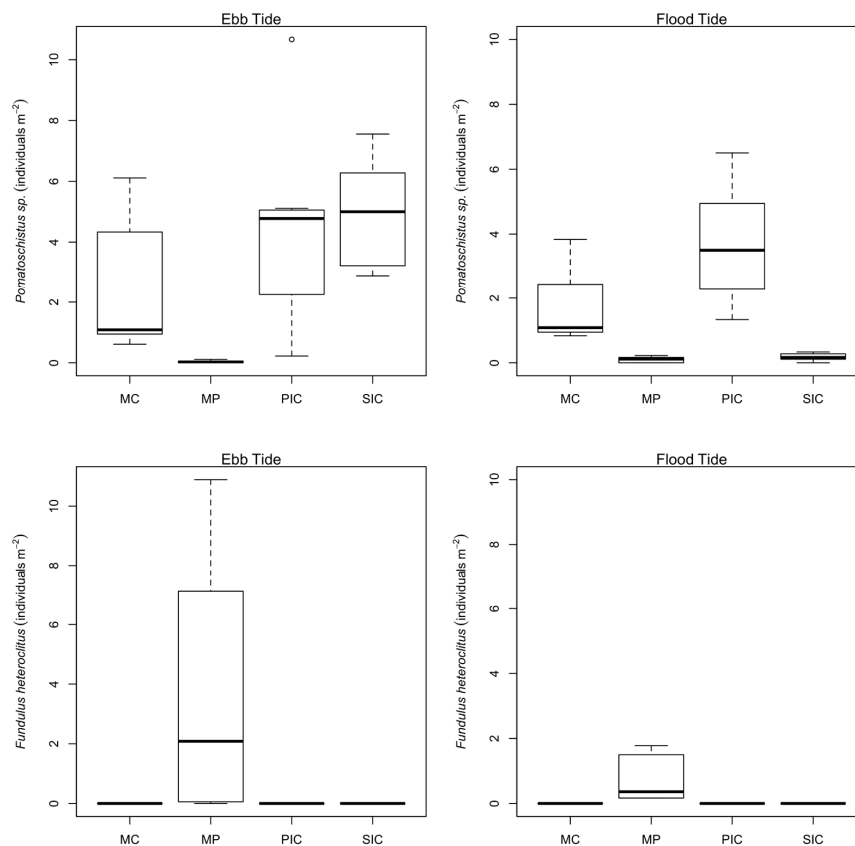


**Figure 1.** Size and frequency distributions of the two most abundant species per site and tide. Dotted vertical line indicates length at metamorphosis of each species (*Pomatoschistus* sp.: 17 mm; *Fundulus heteroclitus*: 18 mm).

### 2.2. Fish Distribution among Habitats

Overall, fish density was always higher during ebb tide except for Marsh Pool (MP) habitat. Principal Intertidal Creek (PIC) and Secondary Intertidal Creek (SIC) were the habitats with higher

densities during the ebb tide. However, fish density in SIC abruptly dropped during the flood (Figure 2) and this habitat presented the lowest percentage of fish larvae (Figure 1). The Analysis of Variance (ANOVA) performed with density data indicated that goby distribution varied significantly between habitats nested with the tide (two-way ANOVA: habitat × tide  $p < 0.001$ , degrees of freedom (df) = 3, Tables 2 and 3, Figure 2). The largest differences were found between MP and the remaining habitats. Main Channel (MC), PIC, and SIC were heavily used by fish during ebb tide. MC and PIC were the most and SIC the least used habitats during the flood. Gobies were present in all habitats while mummichog was exclusively observed in MP (Figure 2) justifying the extremely low habitat overlap between the two species in both tide stages (Pianka’s measure of niche overlap ( $O_{pf}$ ) = 0.0036 in the ebb tide and  $O_{pf} = 0.0107$  in the flood). Moreover, marine species were never observed in MP habitat (Table 1). Sampling week did not affect the density of goby recorded in each habitat (two-way ANOVA: habitat × week,  $p > 0.05$  for week factor (df = 1) and habitat × week interaction (df = 3)).



**Figure 2.** Box plots of fish density (no. individuals·m<sup>-2</sup>) of *Pomatoschistus* sp. and *Fundulus heteroclitus* per site and tide captured in the lower Guadiana estuary salt marsh area. The box includes observations from 25th to the 75th percentile, the horizontal line within the box represents the median value. Lines outside the box represent the 10th and 90th percentiles.

**Table 2.** Results of two-way factorial Analysis of Variance (ANOVA) performed on the fish density (no. individuals·m<sup>-2</sup>, squared root transformed) of the most abundant species (*Pomatoschistus* sp.) using habitat and tide as fixed factors. Level of significance: \*  $p < 0.001$ .

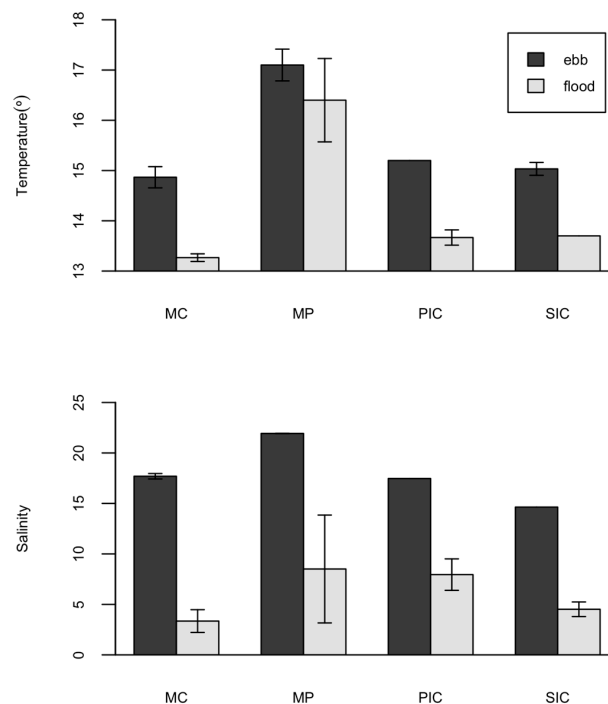
	Degrees of Freedom (df)	Mean Square	F Ratio
Habitat	3	7.333	40.59 *
Tide	1	4.098	22.68 *
Habitat × Tide	3	2.243	12.42 *
Error	40	0.181	

**Table 3.** Results of Tukey (HSD) test for habitat comparisons in each tide state performed on the fish density (no. individuals·m<sup>-2</sup>, squared root transformed) of the most abundant species (*Pomatoschistus* sp.). Levels of significance: ns, not significant; \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ .

Tide	Habitat	$p$ Value
Ebb	MC vs. PIC	0.009 **
	MP vs. PIC	0.000 ***
	SIC vs. PIC	0.999 ns
	MP vs. MC	0.000 ***
	SIC vs. MC	0.041 *
	SIC vs. MP	0.000 ***
Flood	MC vs. PIC	0.236 ns
	MP vs. PIC	0.000 ***
	SIC vs. PIC	0.000 ***
	MP vs. MC	0.005 **
	SIC vs. MC	0.020 *
	SIC vs. MP	0.999 ns

### 2.3. Environmental Parameters

Maximum water temperature was recorded in the MP habitat during the ebb (17.1 °C) and minimum in the PIC during the flood (13.3 °C). The temperature ranges within MC, PIC, and SIC were very similar. The patterns of temperature variation among sites were analogous in both tidal stages with a slight decrease in temperature observed during the flood (Figure 3). The relationship between environmental factors and the density of the two dominant species (goby and mummichog) were explored using the Spearman's rank correlation. A more complex analysis—Generalized Additive Models (GAM)—was not possible due to the lack of robustness of collected data. Residuals normal distribution assumption was violated even after data transformation. There was a negative correlation between temperature and goby larvae density, and a positive correlation between temperature and mummichog (larvae and juveniles) density (Table 4).

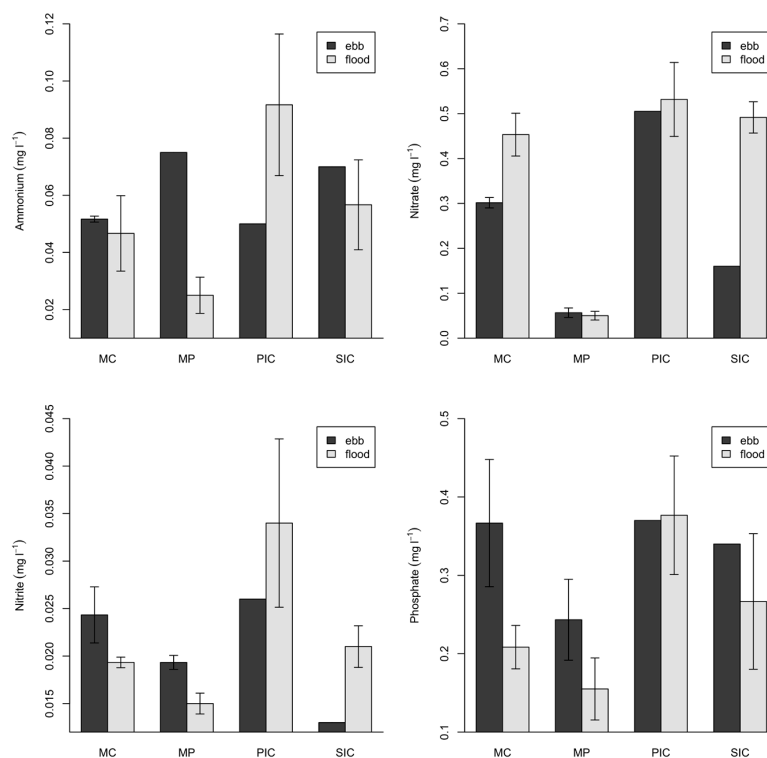


**Figure 3.** Spatial and tidal variation of temperature and salinity measured at each sampling site during the study period in the lower Guadiana estuary salt marsh areas.

**Table 4.** Results of Spearman correlation indices ( $\rho$ ) between larvae and juvenile densities of the two most abundant species and the environmental variables. Level of significance \*  $p < 0.05$ .

	Density of <i>Pomatoschistus</i> sp.		Density of <i>Fundulus heteroclitus</i>	
	Juveniles	Larvae	Juveniles	Larvae
Temperature (°)	−0.244	−0.332 *	0.564 *	0.517 *
Salinity	−0.105	−0.143	0.060	0.211
Dissolved Oxygen (mg·L <sup>−1</sup> )	−0.208	−0.133	−0.084	−0.137
Cha (mg·L <sup>−1</sup> )	0.109	0.214	0.160	0.031
Ammonium (mg·L <sup>−1</sup> )	0.017	−0.103	0.048	−0.131
Nitrate (mg·L <sup>−1</sup> )	0.467 *	0.645 *	−0.508 *	−0.703 *
Nitrite (mg·L <sup>−1</sup> )	0.190	0.493 *	−0.185	−0.213
Phosphate (mg·L <sup>−1</sup> )	0.489 *	0.361 **	−0.310 *	−0.250

Salinity ranged between 3 and 22 with significantly higher values during ebb. A peak of salinity, as for temperature, was also registered in the MP habitat in both tides (Figure 3). There was no correlation between salinity and fish density (Table 4). Dissolved oxygen (DO) concentration ranged between 5.6 and 9.3 mg·L<sup>−1</sup> with higher values during the flood. The highest values (2.53 mg·L<sup>−1</sup>) of chlorophyll *a* (*Cha*) were registered in the MC habitat during ebb tide and the lowest values (1.10 mg·L<sup>−1</sup>) in SIC also during ebb tide. There was no correlation between fish density and both DO or *Cha*. Maximum of all nutrients concentration occurred in the PIC and minimum in the MP habitat except for nitrite (Figure 4). Nitrate concentration was extremely low in the MP during ebb and flood (0.065 ± 0.025 and 0.050 ± 0.022 mg·L<sup>−1</sup>, respectively). Goby juvenile's density was positively correlated with nitrate and phosphate and goby larvae with nitrite, nitrate, and phosphate. There was a negative correlation between mummichog (larvae and juvenile) density and nitrate. Mummichog juvenile density was negatively correlated also with phosphate (Table 4).

**Figure 4.** Spatial and tidal variation of macronutrients concentration (ammonium, nitrite, nitrate and phosphate) measured at each sampling site during the study period in the lower Guadiana estuary salt marsh areas.

### 3. Discussion

For the first time, the occurrence of mummichog in such high densities is reported in South Portugal and their potential risk for native species discussed. The present study was performed during winter months and thus, fish density and diversity registered herein were relatively low, as expected for this season and region [5,6] and in other salt marsh areas of Portugal [11], North Europe [26,27] and United States [28,29]. All the seven fish genera captured had been previously observed in the study area [5,6] although mummichog in very low densities [5]. Goby and mummichog, two estuarine residents, the first being native and the latter NIS, were the two dominant species. Overall, the degree of habitat overlap between these two species was very low in both tidal stages, as shown by the low values of Pianka's index. This indicates that the two species occupy different habitats with the NIS mummichog being exclusively present in an isolated habitat (MP). Gobies are among the most abundant genera all over European salt marshes [6,27,30], and are particularly successful in temperate estuarine environments [31]. There are records of mummichog in southwest Spain [22]; however, occurrences of mummichog in Portugal are poorly documented. Veiga recorded their presence between 2001 and 2002 in Guadiana salt marsh subtidal creeks but as low as one individual in the total catch [5].

In the salt marshes, marine species were mainly caught at larval stages, while estuarine residents occurred at both larval and juvenile stages. These results indicate that marine species use salt marshes mainly as a nursery area while estuarine species depend on salt marshes during their entire life cycle [32].

Tides affected goby density in MC, PIC and SIC habitats in a similar way, with higher densities registered during ebb. The authors of [33] developed a hydrodynamic model for Guadiana estuary. Results indicate water surface velocities of  $5.3 \text{ cms}^{-1}$  (SIC) and  $12.9 \text{ cms}^{-1}$  (MC) during the ebb and  $8.8 \text{ cms}^{-1}$  (SIC) and  $15.8 \text{ cms}^{-1}$  (MC) during the flood. Lower velocities in the ebb may facilitate fish spread throughout the sampled salt marsh habitats especially for the youngest. In fact, changes in fish density according to the tidal stages occur due to the movement of fish from permanently inundated areas towards the inundated intertidal areas with the flood tide [11]. Differences in fish assemblages may also be influenced by marsh plain availability in the proximity to each sampling site. However, this does not seem to be the case if marsh plain area and vegetation type is very similar among habitats. It is worth mentioning that the presence of estuarine fish larvae in all the sampled sites indicate an active habitat selection capacity from an early life stage. Our data do not support the same assumption for marine species due to the small number of marine larvae caught. Nevertheless, previous studies performed in some of the fish genera caught during this investigation reported larvae swimming speeds (Table 5) in the range of simulated current velocities by [33]. It should be noted that although the model developed by [33] was calibrated with data recorded after the construction of the Alqueva dam, it is not guaranteed that similar velocities occurred during samplings. Results show that despite inter- and subtidal salt marsh creeks not being continuously available habitats, they play a major role in both estuarine and marine species, and for both larvae and juvenile fish.

**Table 5.** Critical swimming speeds ( $U_{crit}$ ) of three marine fish larvae species.

Species	$U_{crit}$ Range ( $\text{cms}^{-1}$ )	Size Range (mm)	Reference
<i>Atherina presbyter</i>	3.6–18.7	6.6–21.0	[34]
<i>Solea senegalensis</i>	0.0–5.0	3.5–7.5	[35]
<i>Sardina pilchardus</i>	1.6–9.5	7.9–23.4	[36]

MP was the most dissimilar habitat, both in terms of fish composition and environmental parameters, presenting the highest temperatures and salinities and the lowest macronutrients concentration, namely nitrates and phosphates. Extreme abiotic conditions registered were caused by the lack of water renewal since the MP is only partially flushed during high tides. Mummichog inhabits a wide range of salinities but prefers the most saline sites, usually above 25 [22,25,37]. They present a

great euryhaline range, covering 0 to 128 [38]. Altogether, with their wide thermic acclimation range, this species is able to colonize new habitats with great success [25]. MP was also the habitat with the lowest concentration of macronutrients, in particular phosphates and nitrates. According to [39], phosphate and nitrate concentrations registered at MP indicate a low impacted area, in comparison to the other three habitats (SIC, PIC, MC) that presented typical values of moderate to high eutrophic sites. Nutrient enrichment is known to stimulate primary production causing a bottom-up enrichment of the food web, fostering increased fish biomass and body size [39]. Particularly, nitrogen and phosphorus enrichment stimulates benthic algae [40], which in turn stimulates infauna and epibenthic invertebrates [41]. Benthic algae, infauna and epibenthic invertebrates all serve as food resources for most estuarine dependent fish species [39]. However, a nutrient over-enrichment can have deleterious consequences, namely a decrease in dissolved oxygen leading to a reduction in fish growth rates [39]. Fish such as gobies, that make use of eutrophic environments, are not likely to stay long enough to experience the negative effect of hypoxia on their growth. As observed by [42], they invade the salt marshes through tidal creeks, forage there for up to a few hours and swim back at the ebb. Such habitats are available for a limited time dependent on tides. Instead, for short periods, they colonize salt marsh creeks and main channel edge and benefit from the high availability of food which in turn are probably influenced by the high concentration of macronutrients [41]. Accounting for the lower concentration of macronutrients observed in MP, also a lower stimulation of primary production and consequently less food availability may be expected for this habitat which might thus explain the lower occurrence of gobies.

Gobies are described as opportunistic carnivores feeding on prey according to its availability. Most important prey items in their diet are polychaetes, mysids, isopods and decapods [31]. Mummichog is also an opportunistic species but omnivore. Their diet is mainly based on amphipods, isopods, and snails [39]. There is some overlap on feeding preferences of both species but mummichog is highly flexible, easily adapting to a more herbivory diet (plant tissues) in case of animal prey reduction [39].

Mummichog growth is quite fast, being able to reproduce within the first year of life. Their eggs are resistant to desiccation, their development is fast and thus, the post-hatched larvae start with great advantages due to their size [37]. Such characteristics provide this fish species an opportunistic life-history strategy effectively adapting to habitats with extreme environmental conditions as observed in MP. Although goby is described as a widespread species, relatively tolerant to fluctuations in environmental conditions [43], the establishment of mummichog in MP suggests that this habitat was not attractive to goby as the remain three sampled habitats [22]. In fact, the majority of the studies observed that mummichog is occupying extreme habitats (empty niches) not previously used by native fish species [25,37,44] as it seems to be the current scenario in Guadiana salt marshes. This accounts for the low degree of habitat overlap (Pianka's index) between gobies and mummichog. However, a species with such an expansion capacity, along with its productivity, must have a great influence on the local fish populations.

At least 35 NIS fish species have been introduced into the Iberian Peninsula in the last century and, although not all of them prospered, most are now widespread in this area especially linked to degraded environments [45]. Extensive urban development has occurred in the Guadiana River basin over the past century: the consequent reduction in river flow contributed to decreases in water quality [46]. The presence of mummichog does not necessarily imply that a successful invasion has occurred. We did, however, find specimens over a wide range of sizes (1.6–52.0 mm) and development stages that imply local reproduction. As such, mummichog must be classified as an NIS species in Guadiana salt marsh area, i.e., a species introduced outside its natural distribution that might survive and subsequently reproduce. Not all NIS species turn into invasive species, defined as species with the potential to cause native species extinction, modify ecosystem processes and act as disease vectors [47]. However, some species out of their natural habitats lose their natural predators or control



agents. As a result, they are able to increase to levels which are potentially detrimental to the native environment [47].

Mummichog establishment was recorded in an isolated and low attractive area for native species due to extreme environmental conditions registered. However, similar to what is happening in areas relatively close in South Spain, Guadiana Estuary salt marsh habitats are facing the threat of an expansion of this NIS species to close areas of great value for native species. More sampling effort at reference and impact sites in the study area will be needed to verify the potential effects of mummichog on native communities. Various measures are being taken to improve management of water bodies in the Guadiana river basin under the Water Framework Directive in conjunction with the new European Marine Strategy Framework Directive. The new strategic plan which came into force in 2016 and will be in action until 2021 provides for several measures that will potentially mitigate the risk of mummichog expansion, among them: (a) reduce or eliminate discharges of pollutants; (b) define and implement ecological flows; and (c) increase monitoring and supervising plans [48]. The authors recommend that future monitoring studies should be carried out in the study area to evaluate the effectiveness of the new measures implemented in control not only of this but other NIS species.

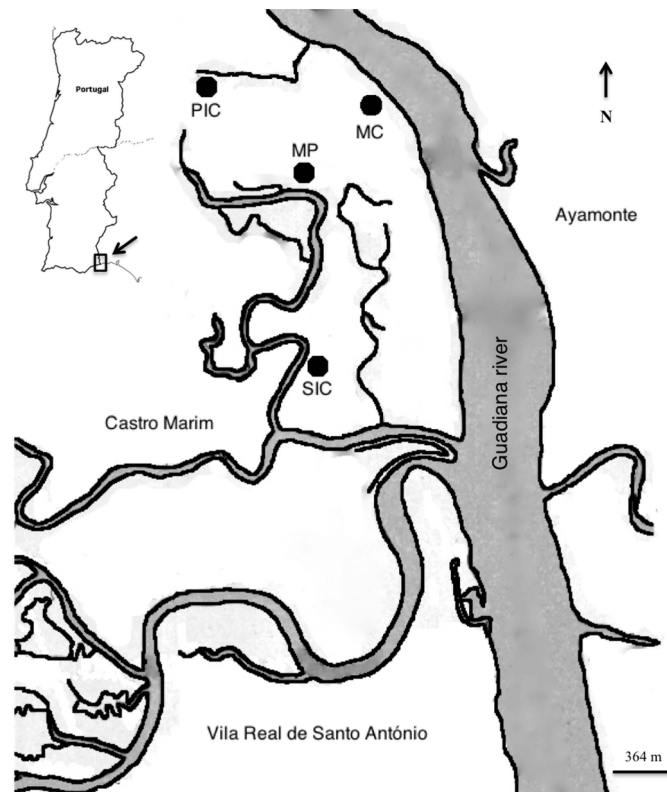
## 4. Materials and Methods

### 4.1. Sampling and Field Methodology

Larval and juvenile fishes were collected between January and February of 2013 at four sites in the Natural Reserve of Castro Marim and Vila Real de Santo António (South-East Portugal) salt marsh area. The first sampling site, Main Channel (MC, 37°13'59" N, 7°25'8" W), was set on the edge of the main course of the Guadiana estuary; the second, Primary Intertidal Creek (PIC, 37°14'2" N, 7°25'23" W), a small creek directly connected to the main channel; the third, Secondary Intertidal Creek (SIC, 37°13'16" N, 7°25'52" W), also a small creek with secondary connection to the main channel; and the fourth, Marsh Pool (MP, 37°13'52" N, 7°25'44" W), an area permanently inundated due to the partially obstruction of water flow by a small dyke (Figure 5). A preliminary survey was performed in order to evaluate the adequacy of sampling points and methodologies to the characteristics of the habitats. Accessibility and representativeness were the main factors considered for the selection of sampling sites.

A total of six diurnal field campaigns were carried out weekly—three at the end of ebb and the other three at the end of flood tides. Samples were collected weekly at each of the four sites. In each week at each site, two samples were collected at the end of ebb or flood tide. This gave a total of 48 samples ( $n = 12$  samples per site;  $n = 24$  samples per tide;  $n = 6$  samples per site  $\times$  tide). Fishes were captured with a seine net (1 mm net size, 5 m length and 0.5 m height). Capture procedure consisted of encircling an area of approximately 18 m<sup>2</sup> near the edge of the main channel, intertidal creeks and marsh pool. One of the operators stood on a fixed point holding one of the extremities of the net, while a second operator dragged the net manually in a circle, meeting the first operator at the fixed point. When the two operators meet each other, the net was pulled close to the shore. The bottom of the seine net was equipped with weights in order to guarantee that it remained in permanent and direct contact with the bottom. Each seine took approximately 10 min and was performed twice at each point per sampling week. Collected samples were immediately preserved in buffered formaldehyde solution (4% final concentration) for further analysis. Animal handling was performed following the European Directive 2010/63/EU of European Parliament and of the Council of European Union on the protection of animals used for scientific purposes. Additionally, physical-chemical parameters (temperature, salinity, dissolved oxygen, and pH) were recorded at each station with a YSI Model 85 multiparameter system probe (YSI Incorporated, Yellow Springs, OH, USA) immersed approximately 10–20 cm from the water surface. Finally, for each point, water samples were collected from the surface for the analysis of dissolved inorganic macronutrients (ammonium, NH<sub>4</sub><sup>+</sup>; nitrate, NO<sub>3</sub><sup>-</sup>; nitrite, NO<sub>2</sub><sup>-</sup>; and

phosphate,  $\text{PO}_4^{3-}$ ) and chlorophyll *a*. Samples were immediately stored in 330 mL containers in the dark and at low temperature, until further processing.



**Figure 5.** Geographical context of the Natural Reserve of Castro Marim and Vila Real de Santo António in Portugal and sampling sites localization in the Natural Reserve salt marsh area.

#### 4.2. Laboratory Analysis

In the laboratory, preserved marine juvenile fishes were measured for total length (precision  $\pm 1.0$  mm) and identified according to [49,50]. Fish larvae were measured for total length (precision  $\pm 0.1$  mm) and identified according to [51,52] under a Leica S8 APO stereomicroscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). Identified fish species were classified into ecological guilds, according to their biology and behavior reported in the literature (Table 6).

**Table 6.** Ecological guild definition of captured fish species during the study period in the lower Guadiana estuary salt marsh areas

Ecological Guild	Definition	Reference
Estuarine resident	Spend their entire lives in the estuary	[53]
Marine seasonal	Have regular seasonal visits to the estuary, mainly as adults	[53]
Marine juvenile	Use the estuary as nursery ground, usually spawning and spending much of their adult life at sea with seasonal visits to the estuary	[53]

Chlorophyll *a* concentration was determined by filtering water samples through  $0.7 \mu\text{m}$  pore Whatman GF/F filters (GE Healthcare Life Sciences, Buckinghamshire, UK) without exceeding vacuum pressures of 100 mmHg. The filters were kept frozen until fluorimetric analysis using the Fluorimeter 10 AU (Turner Designs, Sunnyvale, CA, USA). Finally, dissolved inorganic macronutrients concentrations were determined according to a spectrophotometric method using Spectroquant cell

test photometric kits (Merck Millipore, Darmstadt, Germany) and the Spectroquant Nova 60 photometer (Merck Millipore).

### 4.3. Data Analysis

Two-way factorial Analysis of Variance was used to test for differences in fish densities of the most abundant species (*Pomatoschistus* sp.) among sampling sites and tide stages. Both independent variables were considered as fixed factors. Fish density data was transformed to square root in order to reduce the heteroscedasticity of the data. Tukey's HSD test was used for post hoc comparisons when significant differences were detected ( $p < 0.01$ ). The Spearman correlation coefficient was used to explore patterns of association among the environmental variables and the density of larvae and juveniles of the two most abundant species (*Pomatoschistus* sp. and *Fundulus heteroclitus*). The habitat niche overlap between *Pomatoschistus* sp. and *Fundulus heteroclitus* was evaluated through Pianka's index [54], and applied to the mean density (no. individuals·m<sup>-2</sup>) of each species in each habitat sampled. Pianka's formula is  $O_{pf} = \frac{\sum P_{ip} P_{if}}{\sqrt{\sum P_{ip}^2 \sum P_{if}^2}}$ , where  $O_{pf}$  is Pianka's measure of niche overlap between *Pomatoschistus* sp. and *Fundulus heteroclitus*;  $P_{ip}$  is the proportion of the resource  $i$  out of the total resources used by *Pomatoschistus* sp.;  $P_{if}$  is the proportion of the resource  $i$  out of the total resources used by *Fundulus heteroclitus*;  $i$  could range from 1 to  $n$ , where  $n$  is the total number of habitats considered (in our case  $n = 4$ , the number of sampled habitats). The value of index  $O$  could range from 0 (no overlap) to 1 (full overlap). All statistics were applied using the open source software R version 2.15.1 [55]. Spaa package [56] was applied for Pianka's index calculation.

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