

## Microplastics in wild mussels (*Mytilus* spp.) from the north coast of Spain

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**Summary:** Microplastic content (MPs) in mussels (*Mytilus* spp.) from two areas of the north coast of Spain was measured for the first time. Additionally, a comparison of microplastic levels observed in mussels digested with nitric acid and with potassium hydroxide was carried out. The average microplastic concentration in mussels digested with nitric acid was significantly lower than that observed in mussels digested with potassium hydroxide ( $p < 0.05$ ). The average concentration of microplastics in mussels from the Cantabrian Sea ( $2.55 \pm 2.80$  MPs  $g^{-1}$  WW) was slightly higher than that in mussels from the Ria of Vigo ( $1.59 \pm 1.28$  MPs  $g^{-1}$  WW). Both in the Ria of Vigo and in the Cantabrian Sea the observed pattern of pollution was fitted to the one expected. Consequently, mussels have been confirmed as suitable sentinel organisms for microplastic pollution.

**Keywords:** biota samples; NW Spain; seafood; biomonitoring; bivalve molluscs; microplastics.

### Microplásticos en mejillones silvestres (*Mytilus* spp.) de la costa del norte de España

**Resumen:** Se midió por primera vez el contenido en microplásticos (MPs) presente en mejillones (*Mytilus* spp.) procedentes de dos áreas de la costa del norte de España. Además, se llevó a cabo una comparación de los niveles de microplásticos observados en mejillones digeridos con ácido nítrico y con hidróxido de potasio. La concentración promedio de microplásticos que se observó en los mejillones digeridos con ácido nítrico fue significativamente inferior a la observada en los mejillones digeridos con hidróxido de potasio ( $p < 0.05$ ). La concentración promedio de microplásticos presente en los mejillones del mar Cantábrico ( $2.55 \pm 2.80$  MPs  $g^{-1}$  de peso fresco) fue ligeramente superior a la observada en los mejillones de la Ría de Vigo ( $1.59 \pm 1.28$  MPs  $g^{-1}$  de peso fresco). Tanto en la Ría de Vigo como en el mar Cantábrico el patrón de contaminación observado se ajustó al esperado. Por tanto, se ha confirmado que los mejillones son organismos centinela adecuados para el seguimiento de la contaminación por microplásticos.

**Palabras clave:** muestras biológicas; noroeste de España; alimentos marinos; biomonitorización; moluscos bivalvos; microplásticos.

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## INTRODUCTION

Plastic pollution has become an environmental problem of growing importance because of the release of large quantities of this material into the marine environment (e.g. Jambeck et al. 2015). Due to their physical properties (malleability, tenacity, resistance, etc.) and low cost, plastics are used in a wide range of activities and products (Andrady and Neal 2009).

Microplastics are plastic particles with a length lower than 5 mm (Arthur et al. 2009). According to their origin, microplastics are classified as primary (when they have been designed for use with a microscopic size) or secondary (when they are derived from degradation of larger plastic items; Auta et al. 2017).

Microplastic presence has been reported in virtually all oceanic areas, including those that are very remote from zones with human presence (Ivar do Sul

and Costa 2014). A number of studies have shown that microplastics tend to concentrate in convergence zones of oceanic subtropical gyres (see e.g. Law et al. 2010, Eriksen et al. 2013).

The presence of microplastics has been reported in a wide variety of marine organisms, among them zooplankton (Desforges et al. 2015), sea cucumbers (Graham and Thompson 2009), molluscs (Karlsson et al. 2017), lugworms (Van Cauwenberghe et al. 2015), fishes (Foekema et al. 2013), seabirds (Amélineau et al. 2016) and cetaceans (Lusher et al. 2015). The small size of microplastics is a key factor for their availability to marine organisms (Wright et al. 2013). It has been reported that microplastics can induce mechanical damages to organisms that ingest them, such as digestive tract blockage and ulcer formation (Wright et al. 2013). Moreover, they can adsorb persistent organic pollutants to their surface, potentially intensifying their biomagnification through the trophic chain (Ziccardi et al. 2016).

Mussels are intertidal bivalve molluscs naturally present on rocky shores (Little and Kitching 1996). Moreover, they are cultured for human consumption (Labarta and Corbacho 2002). Because of their filter-feeding habits, the existence of a wide knowledge about their biology, their key role in the ecosystem, their cosmopolitan distribution and their sessile lifestyle, mussels are extensively used as sentinel organisms in marine pollution monitoring programmes (Beyer et al. 2017). Additionally, because of their filter-feeding habits, mussels are more prone to microplastic ingestion than species with other feeding modes, and their shell minimizes the risk of procedural contamination of soft

tissues during sampling and laboratory processing (Li et al. 2019). As they are widely consumed as food by human beings, the study of the accumulation of pollutants in mussels also has great relevance in food safety studies (Catarino et al. 2018). Microplastic ingestion by mussels has been reported in laboratory studies (see e.g. Browne et al. 2008, von Moos et al. 2012).

The present study pursued two main aims. The first aim was to evaluate the spatial pattern of microplastic pollution along the north Spanish coast. As far as we know, this is the first study to assess microplastic levels in wild mussels (*Mytilus* spp.) from the north coast of Spain. Two areas were selected: the Ria of Vigo, to verify that microplastic levels in mussels enable discrimination among very nearby zones (i.e. separated by few kilometres); and the coast of the Cantabrian Sea, to verify the variability among remote zones (separated by hundreds of kilometres). The second aim was to determine whether digestive treatment with HNO<sub>3</sub> induces an underestimation of microplastic levels observed in mussels in comparison with alkaline digestion. To this end, we compared microplastic concentrations observed in mussels digested with HNO<sub>3</sub> (acid digestion) and with KOH (alkaline digestion).

## MATERIALS AND METHODS

### Study areas

The Spanish coast of the Cantabrian Sea (Fig. 1A) is characterized by the alternation of cliffs, beaches, and estuaries (Díez et al. 2000). In the same area, there are major urban and industrial centres that hold trad-

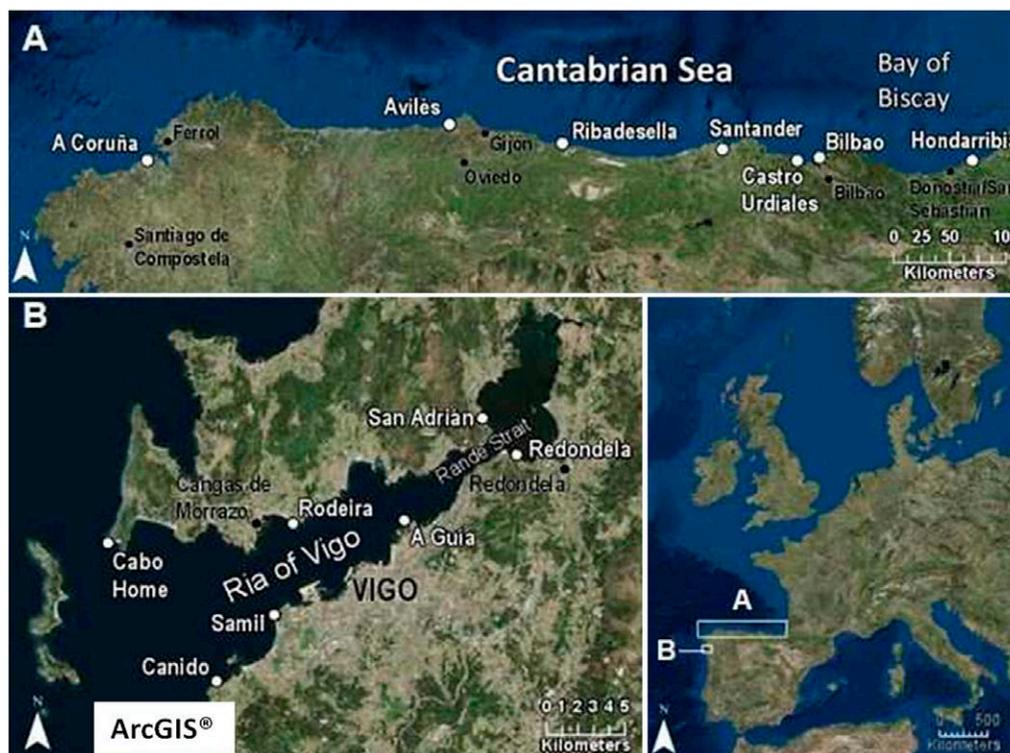


Fig. 1. – Location of sampling stations along the coast of the Cantabrian Sea (A) and the Ria of Vigo (B). Maps were created using ArcGIS® software. ArcGIS® is the intellectual property of Esri and is used herein under licence. Copyright © Esri.

Table 1. – Brief description of sampling stations.

| Geographical region | Sampling station | Coordinates  |               | Description  |
|---------------------|------------------|--------------|---------------|--|
|                     |                  | Latitude (N) | Longitude (W) |  |
| Ria of Vigo         | Canido           | 42°11.486    | 8°48.614      | Exposed and moderately urbanized zone. Small fishing port.           |
|                     | Samil            | 42°13.177    | 8°46.604      | Beach close to Vigo. Moderately urbanized zone.                      |
|                     | A Guía           | 42°15.604    | 8°42.133      | Industrialized zone on the outskirts of the city of Vigo.            |
|                     | Redondela        | 42°17.287    | 8°38.265      | Close to the town of Redondela. Adjacent to the Rande Strait.        |
|                     | San Adrián       | 42°18.227    | 8°39.467      | Presence of a marina. Adjacent to the Rande Strait.                  |
|                     | Rodeira          | 42°15.493    | 8°45.932      | Moderately exposed beach and close to the town of Cangas de Morrazo. |
| Cantabrian Sea      | Cabo Home        | 42°15.007    | 8°52.333      | Exposed zone far from urban and industrial areas.                    |
|                     | A Coruña         | 43°22.178    | 8°23.160      | Station placed inside the city of A Coruña.                          |
|                     | Avilés           | 43°34.759    | 5°58.180      | Moderately urbanized beach close to the city of Avilés.              |
|                     | Ribadesella      | 43°28.051    | 5°3.742       | Station close to the town of Ribadesella.                            |
|                     | Santander        | 43°25.937    | 3°47.476      | Highly industrialized and urbanized port zone. Estuarine site.       |
|                     | Castro Urdiales  | 43°21.868    | 3°11.663      | Quite urbanized beach. Close to the town of Castro Urdiales.         |
|                     | Bilbao           | 43°22.917    | 3°0.885       | Beach close to the city of Bilbao. Exposed zone.                     |
|                     | Hondarribia      | 43°22.119    | 1°47.474      | Inside urban station. Estuarine zone. Close to an airport.           |

ing ports. Upwelling processes which take place on the Cantabrian coast lead to great fishing activity in this zone, with major fisheries such as sardine, mackerel and anchovy.

The Ria of Vigo (Fig. 1B) is an ecosystem subjected to a strong human impact (Fernández et al. 2016). Its surroundings are highly urbanized, with a total population of ~428000 inhabitants. The urban pressure is especially great in the central zone of the south seashore, where the city of Vigo is situated (~295000 inhabitants). Around the Ria of Vigo there is a great presence of industry, which stimulates the traffic of goods through the Port of Vigo, which is also one of the main fishing ports in the world. Urban and industrial activities coexist with culture of molluscs (mainly mussels) in the inner part of the Ria. In spite of the generalized impact, some areas of the Ria of Vigo have remained little affected and therefore possess a high environmental value.

### Biological material

Mussels were collected at 14 sampling stations located in the Ria of Vigo and the Spanish coast of the Cantabrian Sea (Fig. 1) throughout autumn 2016 in the framework of the Spanish Marine Environmental Monitoring programme carried out by the IEO (Instituto Español de Oceanografía). No distinction was made during sampling between both mussel species present along the north coast of Spain (*Mytilus edulis* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819). Consequently, the studied mussels were covered by the denomination *Mytilus* spp.

Table 1 provides a brief description of the sampling stations. For this study, only mussels with a shell length of between 35 and 65 mm were selected. The shells of mussels were discarded and the soft tissues were placed in aluminium vessels and preserved at 20°C. Prior to each digestion, the sample was defrosted in a refrigerator (~3°C) for a minimum time of 3 hours.

### Digestion

For extraction of microplastics, the mussels were digested with 65% HNO<sub>3</sub> (acid digestion) and with 10% KOH (alkaline digestion). The digestive proce-

dures performed with both digestive agents are described below.

### Acid digestion

The digestive procedure was carried out according to Claessens et al. (2013). Three replicates with mussel soft tissue were carried out for each station. Each mussel was weighed and individually treated. Then 10 mL of 65% HNO<sub>3</sub> per gram of mussel soft tissue were added. Because of the destructive effect on microplastics caused by excessively high temperatures (Munno et al. 2018), the digestive procedure was carried out at room temperature for 24 hours (instead of carrying out a digestion at 100°C for 2 hours, as proposed by Claessens et al. 2013). The beakers were not stirred.

### Alkaline digestion

The digestive procedure was carried out as described by Foekema et al. (2013). Three replicates with mussel soft tissue per station were carried out. Each mussel was weighed and individually treated. Then 20 mL of 10% KOH per gram of mussel soft tissue were added. Soft tissues of mussels were digested at room temperature for 21 days. The beakers were submitted to vigorous manual stirring on a daily basis.

### Filtration

Once the digestive procedure had finished, the resulting extract of each replicate was diluted in water at room temperature in the proportion 1:10 (v/v). It was then filtered with a Millipore vacuum pump through a glass-fibre filter (GF/C Whatman) with a 0.7 µm pore diameter. Once the filtration procedure was finished, each filter was individually preserved in a Petri dish until further analysis.

### Identification and count

Microplastics were identified and counted with a Leica Zeiss AxioCam ERc 5s stereomicroscope. The procedure used for identifying suspected particles as plastic were 1) absence of cellular or organic structures, 2) having a constant thickness along their length,

3) having a uniform colour, and 4) consistency when poked with a punch (Hidalgo-Ruz et al. 2012).

An image of each microplastic was taken by means of ZEN software. Observed microplastic particles were classified according to their morphology, colour and length, as suggested by Galgani et al. (2013).

### Measures for prevention of procedural and airborne contamination

To avoid contamination of samples, several measures were taken, such as carrying out the digestive procedure in a laminar flow cabinet, avoiding the use of plastic (e.g. nitrile gloves, glass beakers and metal spatulas), and covering the samples with clock glass (acid digestion) or aluminium foil (alkaline digestion and filtration procedure).

Moreover, in order to estimate contamination, in each experiment a procedural blank (a replicate without mussel tissue) and a control blank (a Petri dish with a GF/C filter open in the laboratory) were carried out in parallel to three replicates. The procedural blanks contained the same volume of digestive material as that which would be used to digest 1 g of mussel tissue and comprised the whole analytical method, while the control blanks only took into account airborne contamination during the count. The microplastic quantity observed in each individual mussel (hereinafter, MPs/individual) was corrected by excluding from counting those items that were similar in morphotype and colour to the ones present in the procedural blanks (similar to Digka et al. 2018).

### Statistical analysis

In the statistical analysis concentration of microplastics (microplastics per gram of wet weight, hereinafter, MPs g<sup>-1</sup> WW) was used as variable. Normality of data was tested by means of the Shapiro-Wilk normality test. Homogeneity of variances was tested by means of the Bartlett test. Because of the lack of normality of data, the existence of significant differences between digestive treatments and between geographical regions was tested by means of the Mann-Whitney test (equivalent to the Wilcoxon rank sum test). The existence of significant differences among sampling stations was tested by means of the Kruskal-Wallis test. Pairwise comparisons were carried out by means of the Conover test (a non-parametric equivalent to the Bonferroni test). The existence of significant differences in the proportion in which each microplastic type was present in the two digestive treatments and the two study regions was tested by means of a chi-squared test. All statistical analysis was carried out using R software (R Core Team 2017).

## RESULTS

### Size of mussels and blank correction

The wet weight of the studied mussels ranged between 0.59 and 4.22 g, with an average of 1.64±0.82 g.

In the mussel replicates, observed microplastic quantity per filter ranged between 0 and 14 MPs filter<sup>-1</sup>, with an average of 3.05±2.67 MPs filter<sup>-1</sup>.

In the procedural blanks, contamination ranged between 0 and 19 MPs filter<sup>-1</sup>, with an average of 4.29±4.68 MPs filter<sup>-1</sup> in the digestive treatment with KOH and 1.93±1.77 MPs filter<sup>-1</sup> in the one with HNO<sub>3</sub>. Both in the procedural blanks of the digestive treatment with KOH and in those of the one with HNO<sub>3</sub> a high proportion of fibres was observed (68% and 63% of total microplastics, respectively), while fragments were the second most abundant morphotype (24% and 30%, respectively). Regarding the colour, blue microplastics were the most abundant both in the alkaline and acid procedural blanks (37% and 30%, respectively), followed by yellow (15%) in the alkaline procedural blanks and by white (19%) in the acid procedural blanks. In the control blanks, the number of microplastics observed in each blank ranged between 1 and 11 MPs filter<sup>-1</sup>, with an average of 4.79±4.06 MPs filter<sup>-1</sup>. The most abundant morphotype were fibres (78%), followed by fragments (18%). The most frequent colour was blue (40%), followed by yellow (22%).

### Comparison of digestive treatments: acid vs base

Average concentrations of microplastics observed in mussels digested with each digestive agent are shown in Figure 2. The average concentration of microplastics in mussels digested with HNO<sub>3</sub> was 1.22±1.42 MPs g<sup>-1</sup> WW. In mussels digested with KOH, the average concentration of microplastics was 2.07±2.21 MPs g<sup>-1</sup> WW. The microplastic concentration observed in mussels digested with HNO<sub>3</sub> was significantly lower than that observed in those digested with KOH (p<0.05).

The proportion of fibres in the digestive treatment with HNO<sub>3</sub> (52%) was higher than the one observed in the digestive treatment with KOH (44%). However, pellets were present in a greater proportion in the digestive treatment with KOH (23%) than in the digestive treatment with HNO<sub>3</sub> (4%). The differences in the proportions in which each morphotype was present in the two digestive treatments were significant (p<0.05).

Regarding microplastic colour, no yellow microplastics were observed in the digestive treatment with KOH, while green and orange microplastics (tending-

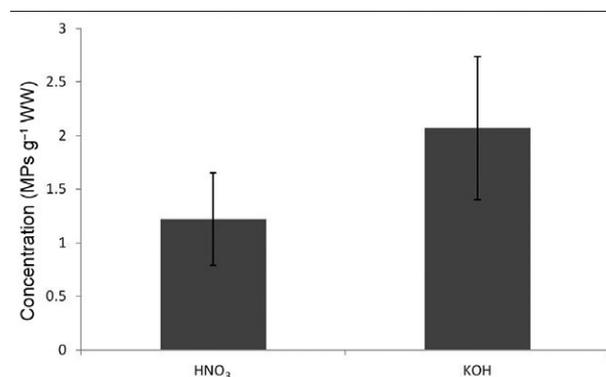


Fig. 2. – Average concentration of microplastics (MPs g<sup>-1</sup> WW) observed in mussels digested with HNO<sub>3</sub> and with KOH.

to-yellow colours) accounted for 5% and 2% of total microplastics, respectively. In the digestive treatment with HNO<sub>3</sub>, yellow microplastics were present in low proportion (1%), similar to that for green and orange microplastics (3% and 1%). A higher proportion of black microplastics was seen in the digestive treatment with HNO<sub>3</sub> (48%) than in the digestive treatment with KOH (11%). The differences between the two digestive treatments with regard to the proportion in which microplastics of each colour were present were significant ( $p < 0.05$ ).

Similar proportions of microplastics in each length range were seen in the two digestive treatments, the most frequent length range of microplastics being 200-500 µm both in the one with KOH and the one with HNO<sub>3</sub> (38% and 31% of observed microplastics, respectively). The highest length range (1000-5000 µm) and the lowest one were present in a lower proportion in the digestive treatment with HNO<sub>3</sub> than in the one with KOH. However, the differences between the two digestive treatments with regard to the proportion in which each length range was found were not significant ( $p > 0.05$ ).

**Geographical pattern of pollution**

To analyse the geographical pattern of pollution, only the results obtained from the digestive treatment with KOH were used. Microplastics were observed in 88% of the studied mussels. Microplastic quantity individually observed ranged between 0 and 10 MPs ind.<sup>-1</sup>, with an average value of  $2.19 \pm 1.57$  MPs ind.<sup>-1</sup> in the Ria of Vigo and  $2.81 \pm 2.80$  MPs ind.<sup>-1</sup> in the Cantabrian Sea. The microplastic concentration observed in each mussel ranged between 0 and 8.90 MPs g<sup>-1</sup> WW. The average concentration of microplastics in the Ria of Vigo was  $1.59 \pm 1.28$  MPs g<sup>-1</sup> WW, while in the Cantabrian Sea it was  $2.55 \pm 2.80$  MPs g<sup>-1</sup> WW (Fig. 3). The difference between the two regions with regard to microplastic concentration was not significant ( $p > 0.05$ ).

The variation of microplastic concentration among the sampling stations of the Ria of Vigo is shown in Figure 4A, while the variation among stations of the Cantabrian Sea is shown in Figure 4B. In the Ria of Vigo, the highest average concentration of microplastics was observed at A Guía, with  $2.48 \pm 1.00$  MPs g<sup>-1</sup> WW,

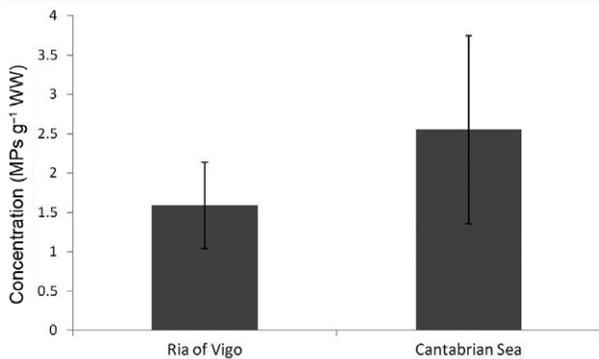


Fig. 3. – Average concentration of microplastics (MPs g<sup>-1</sup> WW) observed in mussels from the Ria of Vigo and the Cantabrian Sea coast.

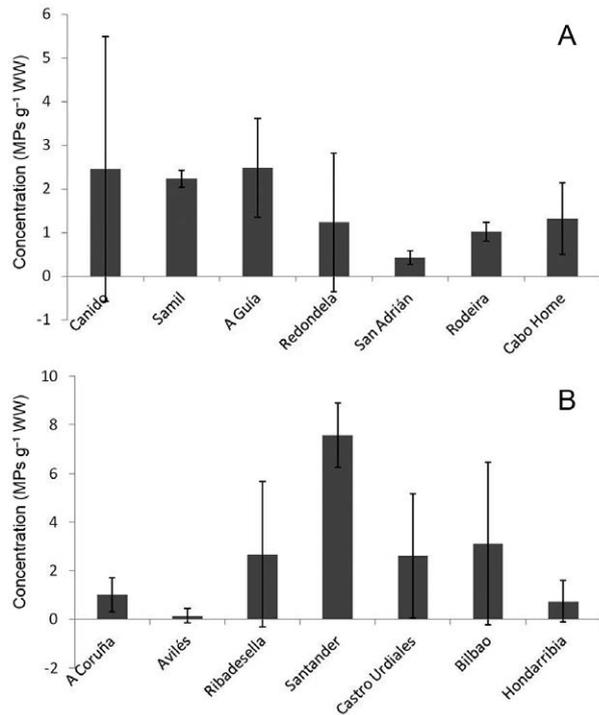


Fig. 4. – Average concentration of microplastics (MPs g<sup>-1</sup> WW) observed in mussels from the stations of the Ria of Vigo (A) and the Cantabrian Sea coast (B).

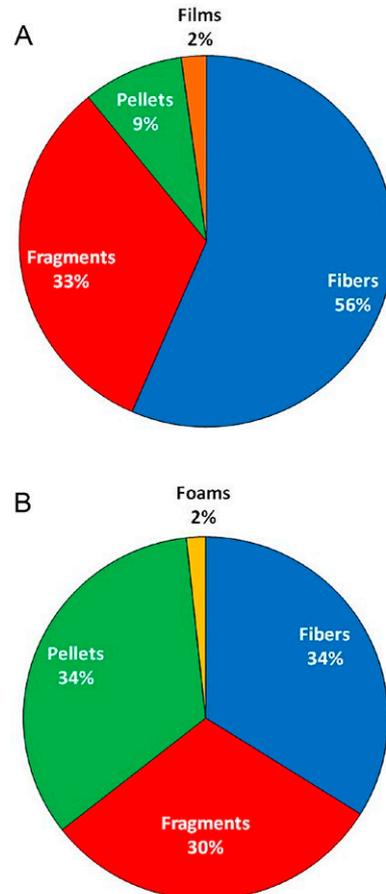


Fig. 5. – Proportion in which each morphotype was observed in the Ria of Vigo (A) and the Cantabrian Sea (B).

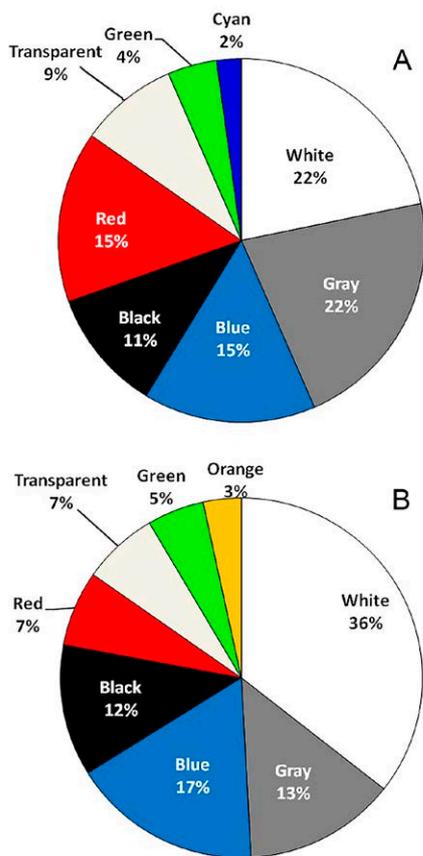


Fig. 6. – Proportion in which microplastics of each colour were observed in the Ria of Vigo (A) and the Cantabrian Sea (B).

while the lowest was observed at San Adrián, with  $0.42 \pm 0.14$  MPs  $g^{-1}$  WW. No significant differences among stations were observed in the Ria of Vigo ( $p > 0.05$ ). In the Cantabrian Sea, the highest average concentration of microplastics was observed at Santander, with  $7.57 \pm 1.16$  MPs  $g^{-1}$  WW, while the lowest concentration was observed at Avilés, with  $0.15 \pm 0.16$  MPs  $g^{-1}$  WW. In the Cantabrian Sea, significant differences were only found between these two stations ( $p < 0.05$ ).

#### Physical characteristics of microplastics

As in the previous case, to analyse the physical characteristics of observed microplastics in each region, only the results from mussels digested with KOH were used. In the Ria of Vigo the most common morphotype (Fig. 5A) were fibres (56% of observed microplastics), followed by fragments (33%), and pellets (9%). In the Cantabrian Sea (Fig. 5B), fibres and pellets were the two main morphotypes (34% each one), followed by fragments (30%).

Regarding the colour of observed microplastics (Fig. 6), the most frequent ones in the Ria of Vigo were both white and grey (accounting for 22% of total microplastics each), followed by blue and red (15% each). In the Cantabrian Sea, white was also the most frequent microplastic colour (36%), followed by blue (17%), and grey (13%).

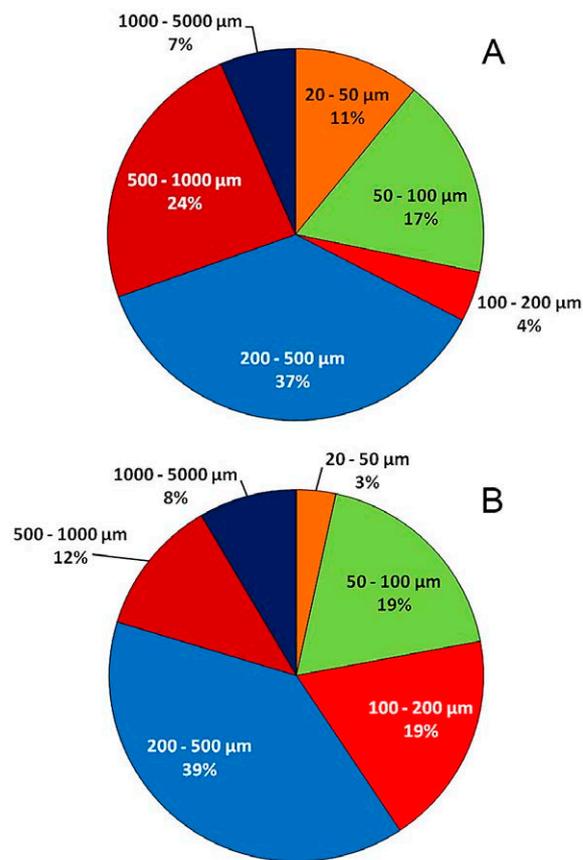


Fig. 7. – Proportion in which microplastics of each length range were observed in the Ria of Vigo (A) and the Cantabrian Sea (B).

The proportions in which microplastics of each length range were observed in mussels from the two regions are shown in Figure 7. The most abundant length range of extracted microplastics in both the Ria of Vigo and the Cantabrian Sea was 200-500  $\mu m$  (37% and 39%, respectively). The second most frequent length range was 500-1000  $\mu m$  in the Ria of Vigo (24%) and 100-200  $\mu m$  in the Cantabrian Sea (19%).

#### DISCUSSION

##### Blank contamination

The microplastic quantity observed in the procedural blanks ( $4.29 \pm 4.68$  MPs  $filter^{-1}$  for the digestive treatment with KOH and  $1.93 \pm 1.77$  MPs  $filter^{-1}$  for the one with  $HNO_3$ ) was lower than the that observed in the procedural blanks by Catarino et al. (2018), who observed  $6.5 \pm 0.95$  particles  $filter^{-1}$ , and higher than that observed in other studies, in which 1.02 particles per filter (Brâte et al. 2018), 1 particle  $filter^{-1}$  (Karlsson et al. 2017) and  $0.67 \pm 0.82$  particles  $filter^{-1}$  (Li et al. 2016) were observed. Therefore, blank contamination is in the range of that observed in previous studies.

##### Comparison of digestive treatments

In the present study a lower concentration of microplastics was observed in mussels digested with  $HNO_3$

than in those digested with KOH (Fig. 2). In previous studies it has been reported that HNO<sub>3</sub> exerts a destructive effect on microplastics when they are exposed to it in the laboratory, either directly (Claessens et al. 2013, Dehaut et al. 2016) or spiked in biological samples (Karami et al. 2017). Consequently, it has been suggested that the use of HNO<sub>3</sub> as a digestive agent induces an underestimation of the microplastic concentration observed in biota samples. The results obtained in the present study confirm this hypothesis.

In a study on recovering microplastics previously spiked in mussel tissue, it was observed that fibres were more sensitive to the destructive effect of HNO<sub>3</sub> than other morphotypes such as pellets due to their higher surface/volume ratio (Claessens et al. 2013). Contrarily to expected, the proportion of fibres in mussels digested with HNO<sub>3</sub> was higher than that in mussels digested with KOH, and the opposite situation happened in the case of pellets. Additionally to their higher surface/volume ratio, Claessens et al. (2013) attributed the greater destructive effect of HNO<sub>3</sub> on fibres observed in their study to the polymer plastic type that composed the tested morphotypes (mainly nylon in the case of fibres and polypropylene in that of pellets), coming to consider it a most probable reason. As in the present study analytical determination of plastic type was not carried out, it is not possible to draw conclusions about the reason why the proportions of fibres and pellets differed from the expected ones.

It has also been observed in the laboratory that HNO<sub>3</sub> induces a yellowing effect in microplastic colour (Claessens et al. 2013). No yellow microplastics were seen in the digestive treatment with KOH, but they were observed in the one with HNO<sub>3</sub>, where they accounted for 1% of total microplastics. By contrast, green and orange

microplastics were present in a higher proportion in the alkaline treatment (5% and 2%, respectively) than in the acid treatment (3% and 1%, respectively). Unexpectedly, a very high proportion of black microplastics (48%) was observed in the digestive treatment with HNO<sub>3</sub>. Since in the acid procedural blanks black microplastics were only 4% of total microplastics, this finding cannot be attributed to contamination of samples.

### Comparison of microplastic levels with other geographical areas

The average microplastic concentration observed in the Cantabrian Sea (2.55±2.80 MPs g<sup>-1</sup> WW) was higher than that observed in the Ria of Vigo (1.59±1.28 MPs g<sup>-1</sup> WW), as shown in Figure 3. In a previous study carried out on the north coast of Spain it was reported that average levels of pollutants in mussels were higher in the Cantabrian Sea than in the northwestern Spanish coast, where the Ria of Vigo is placed (Bellas et al. 2014). Even so, the difference between the two regions observed in the present study was not significant. The lower intensity of upwelling processes in the Cantabrian Sea than on the western Galician coast (Díez et al. 2000), where they tend to induce the exit of surface waters from the Rias and their replacement by deep-waters from the open ocean, could explain why microplastic levels were higher in the Cantabrian Sea than in the Ria of Vigo.

A review of microplastic levels (concentration and individual quantity) reported in geographical areas worldwide is provided in Table 2. Average microplastic concentrations observed in both the Ria of Vigo and the Cantabrian Sea are in the range of those observed in the United Kingdom, both on the west coast of Scot-

Table 2. – Microplastic concentrations reported in wild mussels from geographical areas worldwide.

| Geographical area               | Specific location                    | Digestive material   | Concentration (MPs g <sup>-1</sup> WW)                      | Quantity (MPs ind. <sup>-1</sup> ) | Reference  |
|---------------------------------|--------------------------------------|--|---|------------------------------------|--|
| North coast of Spain            | Ria of Vigo                          | 10% KOH  | 1.59±1.28   | 2.19±1.57                          | Present study  |
|                                 | Cantabrian Sea                       | 10% KOH  | 2.55±2.80   | 2.81±2.80                          | Present study  |
| Norway                          | All Norwegian coast                  | 10% KOH  | 0.97  | 1.5                                | Bråte et al. (2018)                                      |
| Finland                         | Island close to the SW Finnish coast | Enzymes  | 0.26±1.3  | 0.04±0.19                          | Railo et al. (2018)                                      |
| United Kingdom                  | West coast of Scotland               | Corolase 7089  | 3±0.9   | 3.2±0.52                           | Catarino et al. (2018)                                   |
|                                 | Coast of England and Wales           | 30% H <sub>2</sub> O <sub>2</sub>                                      | 0.7 – 2.9   | 1.1–6.4                            | Li et al. (2018)   |
| Southern coast of the North Sea | Netherlands                          | Proteinase K<br>65% HNO <sub>3</sub> + sonication                      | 37 (dry weight)<br>19–105 (dry weight)                      |                                    | Karlsson et al. (2017)<br>Leslie et al. (2017)           |
|                                 | Belgian coast                        | 65% HNO <sub>3</sub><br>HNO <sub>3</sub> :HClO <sub>4</sub>            | 0.2±0.3<br>0.26 (exposed areas)<br>– 0.51 (sheltered areas) |                                    | Van Cauwenberghe et al. (2015)<br>De Witte et al. (2014) |
| French Atlantic coast           | Area around the Loire Estuary        | 10% KOH  | 0.23±0.20   | 0.60±0.56                          | Phuong et al. (2018)                                     |
| Portugal                        | Tagus Estuary                        | 65% HNO <sub>3</sub><br>HNO <sub>3</sub> :HClO <sub>4</sub>            | 0.34±0.33<br>0.08±0.09                                      |                                    | Vandermeersch et al. (2015)                              |
| Mediterranean Sea               | Ebro Delta (Spain)                   | 65% HNO <sub>3</sub><br>HNO <sub>3</sub> :HClO <sub>4</sub>            | 0.15±0.33<br>0.11±0.12                                      |                                    |  |
|                                 | Po Estuary (Italy)                   | 65% HNO <sub>3</sub><br>HNO <sub>3</sub> :HClO <sub>4</sub>            | 0.05±0.11<br>0.16±0.11                                      |                                    |  |
|                                 | Greek coast of the Ionian Sea        | 30% H <sub>2</sub> O <sub>2</sub>                                      |   | 1.9±0.2                            | Digka et al. (2018)                                      |
| China                           | Whole Chinese coast                  | 30% H <sub>2</sub> O <sub>2</sub><br>30% H <sub>2</sub> O <sub>2</sub> | 2.7<br>1.52–5.36  | 1.5–7.6<br>0.77–8.22               | Li et al. (2016)<br>Qu et al. (2018)                     |

land (Catarino et al. 2018) and along the coasts of England and Wales (Li et al. 2018). They are also similar to those observed along the Chinese coast (Li et al. 2016, Qu et al. 2018). Comparison with studies carried out in the Netherlands is harder, because their results are presented as a function of dry weight. Nevertheless, the concentrations reported there seem higher than the ones from both the Ria of Vigo and the Cantabrian Sea (Karlsson et al. 2017, Leslie et al. 2017). In contrast, lower levels than in the present study were reported on the French Atlantic coast (Phuong et al. 2018), along the coast of Norway (Bråte et al. 2018), and in the southwest of Finland (Railo et al. 2018). Microplastic concentrations from both the Ria of Vigo and the Cantabrian Sea are higher than the ones observed on the Belgian coast (De Witte et al. 2014, Van Cauwenberghe et al. 2015), in some parts of the Mediterranean Sea (in the Ebro estuary and in the Po estuary) and in the Tagus Estuary, in Portugal (Vandermeersch et al. 2015). In the case of these studies, the low levels observed can be attributed, at least partially, to the use of acid compounds as digestive agents, which induces an underestimation in microplastic levels, as reported in the present study.

As in other studies, a high variability in microplastic concentrations among individuals was observed, which is attributable to the effect of biological factors influencing the individual accumulation of pollutants, such as body size, sex and lipid content (Newman 2010). In order to minimize the effect of natural variability in biological factors, OSPAR (2012) recommends analysing a pool of 20 individuals by replicate. Thus, the inter-individual variability observed in this study could be avoided in future studies by analysing a higher number of individuals.

Regarding microplastic quantity per individual (see Table 2), the ones observed in this study ( $2.19 \pm 1.57$  MPs ind.<sup>-1</sup> in the Ria of Vigo and  $2.81 \pm 2.80$  MPs ind.<sup>-1</sup> in the Cantabrian Sea) were lower than those observed on the west coast of Scotland (Catarino et al. 2018) and higher than those observed on the Greek coast of the Ionian Sea (Digka et al. 2018), on the Norwegian coast (Bråte et al. 2018) and in the southwest of Finland (Railo et al. 2018). Comparison with other results is more complicated because the above studies inferred microplastic quantity per individual from that observed in a pool of various mussels. Microplastic quantity per individual observed both in the Ria of Vigo and the Cantabrian Sea coast is also in the range observed on the coast of China (Li et al. 2016, Qu et al. 2018). Quantities in the range of this study were also reported on the coast of England and Wales (Li et al. 2018). However, the ones observed in our study are higher than those reported on the French Atlantic coast (Phuong et al. 2018).

## The geographical pattern of pollution

### *The Ria of Vigo*

Overall, microplastic concentrations were higher at stations on the southern shore of the Ria than at

the ones on the northern shore (Fig. 4A), although the observed differences among stations were not significant in this region. Higher microplastic levels were expected on the southern shore because the city of Vigo and its port are located there. Therefore, the overall pattern of microplastic pollution observed in this region is fitted to the one expected.

The highest concentration was reported in A Guía, which is attributable to the high urban and industrial pressure on the surroundings of this station. Likewise, high levels observed in Canido are attributable to the presence of human population, tourism, the activity of the fishing port and the proximity to a sewage treatment plant. Also, the levels observed at Samil can be attributed to the presence of human population and tourism. Despite being away from urban or industrial zones, Cabo Home showed a medium level of pollution. The most likely explanation for this finding is that microplastics arrive at this area from the inner part of the Ria pulled by outer currents, which tend to deviate to the right owing to the Coriolis effect (Rosón et al. 2008). Both Redondela and Rodeira showed moderate levels of microplastic pollution that could be attributable to their closeness to small towns. The lowest microplastic levels were observed in San Adrián, and can be attributed to the existence of strong tidal currents throughout the Rande Strait (up to  $75 \text{ cm s}^{-1}$ ; Rosón et al. 2008).

### *The Cantabrian Sea coast*

Microplastic concentrations in the Cantabrian Sea were higher in the central zone of this region (Fig. 4B). This zone was also shown to be the most polluted in routine monitoring studies (Bellas et al. 2014). In the present study, significant differences were only found between Santander and Avilés, where the highest and the lowest microplastic concentrations of the Cantabrian Sea were observed, respectively.

The highest microplastic concentration was observed in Santander, which is attributable to the port and industrial activities carried out in the area around this station. High microplastic concentrations have also been observed in Bilbao, and are attributable to the closeness of the city. Likewise, the high microplastic concentration observed in Ribadesella and Castro Urdiales can be attributed to the closeness of both stations to towns. The medium levels observed in A Coruña can be attributed to the location of this station inside an urban area, and the moderate levels observed in Hondarribia are probably due to the closeness to a town. The lowest level was observed in Avilés, which is attributable to the low degree of urbanization in the area around this station. However, this result is surprising since the concentrations of pollutants reported in Avilés for a majority of pollutants in routine environmental monitoring studies are among the highest of the Cantabrian coast (see e.g. Bellas et al. 2014, Besada et al. 2014).

## Physical characteristics of microplastics

### *Morphotype*

Fibres were the most common morphotype in mussels from both the Ria of Vigo and the Cantabrian Sea, together with pellets in the latter (Fig. 5). Fibres were also the most common morphotype in mussels from the majority of studied geographical areas worldwide, such as the Belgian coast (De Witte et al. 2014), the Netherlands (Karlsson et al. 2017), the coasts of England and Wales (Li et al. 2018), Norway (Bråte et al. 2018) and China (Li et al. 2016, Qu et al. 2018). The high abundance of fibres in mussels in comparison with other morphotypes has been attributed to a slower egestion of fibres, therefore resulting in a higher long-term accumulation (Li et al. 2019). The proportion of fibres in mussels from the Ria of Vigo (56%) was higher than that in mussels from the Cantabrian Sea (34%). Urban areas have been identified as an important source of microplastic fibres entering the marine environment through effluent of wastewater treatment plants, and fishing elements and plastic equipment abandoned in the environment or stored outdoors are also an important source (Gago et al. 2018). Consequently, the higher proportion of fibres in the Ria of Vigo than in the Cantabrian Sea could be attributed both to release of urban wastewater through treatment plants and to fishing and aquaculture activities carried out in the ria.

Fragments were the second most abundant morphotype in mussels from the Ria of Vigo (33%), and a similar proportion was found in the Cantabrian Sea (30%). Fragments have been identified as the main morphotype on the French Atlantic coast (Phuong et al. 2018) and on the Greek coast of the Ionian Sea, where their high abundance was attributed to breakage in the marine environment of plastics reaching the sea (Digka et al. 2018).

Pellets were the third most common morphotype in mussels from the Ria of Vigo (9%) and, together with fibres, the main one in those from the Cantabrian Sea (34%). The abundance of pellets in both the Ria of Vigo and the Cantabrian Sea can be attributed to the presence of urban areas and industrial activities. However, it has been suggested that mussel's pearls may be misidentified as plastic pellets (Bråte et al. 2018), so caution is needed in order to explain the high proportion of pellets observed in the Cantabrian Sea.

### *Colour*

White was the most frequent microplastic colour in the Cantabrian Sea and, together with grey, also in the Ria of Vigo (see Fig. 6). Grey accounted for 13% of microplastics in the Cantabrian Sea. Brilliant grey was the dominant colour among microplastics in mussels from the French Atlantic coast (Phuong et al. 2018). Blue accounted for a similar proportion of total microplastics in both study regions (15% in the Ria of Vigo and 17% in the Cantabrian Sea). Blue was also the most common colour of microplastics on the Greek coast of the Ionian Sea (Digka et al. 2018). The high

abundance of blue microplastics is attributable to the extensive use of blue as a colour of synthetic clothes worldwide (Gago et al. 2018) and, in the case of the Ria of Vigo, to the use of blue plastic elements as equipment in mussel farming (Digka et al. 2018).

### *Length*

In both mussels from the Ria of Vigo and the Cantabrian Sea the most common length range was between 200 and 500  $\mu\text{m}$  (Fig. 7). Microplastics of this size accounted for 37% in the Ria of Vigo and 39% in the Cantabrian Sea. A higher proportion of long microplastics (500-1000  $\mu\text{m}$  and 1000-5000  $\mu\text{m}$ ) was observed in the Ria of Vigo than in the Cantabrian Sea. Similar microplastic sizes have been reported in some previous studies: average microplastic length in mussels from the Netherlands was 200  $\mu\text{m}$  (Karlsson et al. 2017), and in mussels from the Greek coast of the Ionian Sea the most common length range was between 100 and 500  $\mu\text{m}$  (Digka et al. 2018). A higher length range has been reported in other geographical areas: the most common size range in microplastics in mussels from the Belgian coast was between 1000 and 1500  $\mu\text{m}$  (De Witte et al. 2014), and on the Norwegian coast average length of microplastics was 770  $\mu\text{m}$  (Bråte et al. 2018). By contrast, in mussels from the French Atlantic coast, 52% of observed microplastics had a length between 50 and 100  $\mu\text{m}$  (Phuong et al. 2018).

It is worth mentioning that a number of microplastics observed in this study had a length higher than 1000  $\mu\text{m}$  (7% in the Ria of Vigo and 8% in the Cantabrian Sea). As in a previous study it was suggested that mussels are unable to ingest particles higher than 1000  $\mu\text{m}$  (Beecham 2008), microplastics ingested by mussels are not expected to exceed that length. A possible explanation for the presence of microplastics of such high length in mussels is that they have not been ingested but have rather adhered to the body surface.

## CONCLUSIONS

In the present study it has been confirmed that the use of acid compounds such as  $\text{HNO}_3$  as digestive agents leads to an underestimation in microplastic concentrations observed in biota samples in comparison with  $\text{KOH}$ . However, no selective effect of  $\text{HNO}_3$  on morphotype, colour or size has been confirmed. The use of  $\text{HNO}_3$  should be avoided in a future standardized protocol of microplastic extraction.

Slightly higher microplastic levels observed in the Cantabrian Sea ( $2.55 \pm 2.80$  MPs  $\text{g}^{-1}$  WW) than in the Ria of Vigo ( $1.59 \pm 1.28$  MPs  $\text{g}^{-1}$  WW) could be attributable to the lower effect of upwelling in the Cantabrian Sea, although the difference between the two regions with respect to microplastic concentrations was not significant. In the Ria of Vigo, higher microplastic levels were observed on the southern shore, fitting the expected pattern of pollution, though the differences among stations were not significant. In the Cantabrian Sea, the highest levels were observed in the central zone, which is consistent with previous studies. These

results support the use of mussels as sentinel organisms of microplastic pollution.

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