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BARCODING AS A TOOL FOR EARLY DETECTION OF HITHHIKING INVASIVE SPECIES ON FLOATING MARINE DEBRIS IN THE CANTABRIAN COAST

Barcoding como herramienta genética para la detección temprana de especies invasoras transportadas por basura marina en la costa Cantábrica

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Barcoding as a tool for early detection of hitchhiking invasive species on floating marine debris in the Cantabrian coast. Marta Gómez Agenjo. University of Oviedo. Department of Functional Biology. Genetics Area. C/.Julián Clavería, s/n 33006 Oviedo Asturias SPAIN

ABSTRACT

Floating devices, such as plastic bottles and fishing gear, are additional hard surfaces able to provide new habitat for several organisms. This process helps species' spreading process in non-native areas, hazarding the ecosystem dynamics and populations of endemic individuals. Due to the lack of data from the Cantabrian coast about the increasing of alien species occurrence, an exhaustive sampling process was performed along 22 beaches. Bottles and fishing gear found on the shore with attached organisms, were collected per beach. Barcoding techniques were used to identify the specimens to species level. From the 17 identified species, 3 were reportedly invasive. Although there were differences among bottles and fishing gear, cosmopolitan species were the most numerous found organisms. Moreover, in both items, alien specimens were recorded. The principal aim of this exploratory study was to create social awareness about the relationship between marine debris and the enhancement of invasive species spreading.

Keywords: Plastic, fishing gear, alien species, Cantabrian coast, barcoding

1. INTRODUCTION

Marine debris added to other problems, such as ocean acidification, global warming and biodiversity loss, has been acknowledged as one of the major concerns of our time (Sutherland et al., 2010). In addition, it has been documented that 8 million tons of plastic were spilled into the ocean in 2010 (Laura Parker, 2015). Apart from the aesthetic problem (Mouat et al., 2010), this waste has severe consequences for sea life development and human health (Derraik, 2002; Gregory, 2009).

Man-made debris types and quantity varies around the world, but it is obvious that plastic items represent the higher percentage of the total amount. Bearing in mind that plastic degradation takes about 100 to 1000 years, plastics suffer from fragmentation, resulting from the effects of sunlight, oxidation and wave action. When these fragments are smaller than 5 millimeters in diameter, they are denominated microplastics, (Arthur et al., 2009; Barnes et al., 2009) and the quantity of this tiny plastic pieces, nowadays worldwide found, is predicted to increase in the marine environment (Goldstein et al., 2012; Thompson et al., 2009). Furthermore, plastic is no longer a unique material. In 1988, the Society of Plastic Industry (SPI) established the Resin Identification Code (The Plastic Industry Trade Association, 2015). According to that code plastic can be based on several distinct resins with features, *Table 1*. Substances like antimicrobials bisphenol A (BPA), flame retardants, monomers and oligomers are potential toxic chemicals that are integrated when manufacturing plastics, and can be released into marine ecosystems (Lithner et al., 2011). Particularly, phthalates and flame retardants negatively affect mammals, fish and mollusks (Oehlmann et al., 2009; Teuten et al., 2009). Moreover, plastic toxic compounds threat human health through plastic toys, deli containers or food and drink packaging.

However, plastics are not at the top on the list from the point of view of marine debris size. Accumulation of Abandoned, Lost or Otherwise Discarded Fishing Gear (ALDFG), had considerably spread over the last 50 years (Macfadyen et al., 2009). Referring, not only to ropes, but also to fishing nets, this items are gaining popularity in terms of concerning. Those ALDFG made of natural materials such hemp, cotton or straw, takes about 3 to 14 months to completely degrade in the water column. Unfortunately, there is a growing trend of replacing those traditional fishing gears by stronger and cheaper modern materials that last very much longer in the ocean. It has been demonstrated that ALDFG made of plastic, suppose a hazard not only for human health, but also for fish stock and marine environment (Macfadyen et al., 2009).In addition, these fishing gear items, especially abandoned nets, can stay in the ocean for years and travel long distances (Kaiser et al., 1996). Animals can get trapped therein, a phenomenon known as ghost fishing. Moreover, since many animals cannot distinguish between trash and preys, thereby, most of them end up dying of suffocation.

Symbol	Name	Used for	Recycles?	Leach?	Toxicity
1 PETE	Polyethylene Terephthalate	Bottling beverages	YES	NO	Use with caution
2 HDPE	High Density Polyethylene	Milk jugs, cosmetics	YES	NO	Safe
3 PVC	Polyvinyl chloride	Plastic wraps, toys, spray bottles	NO	YES	Avoid
4 LDPE	Low Density Polyethylene	Shopping bags, plastic wraps, baby bottles, reusable plastics	YES	NO	Safe
5 PP	Polypropylene	Baby bottles, yogurt, deli containers, reusable plastics	YES	NO	Safe
6 PS	Polystyrene	Plastic cutlery, egg containers	NO	YES	Avoid
7 OTHER	Bisphenol A and all other plastics	Food packaging	NO	MAYBE	Use with caution

Table 1. R.I.C., plastic name, uses, recycling, leached and toxicity

Besides the aspects cited above, there is another matter of concern about debris presence in the oceans, the ability of providing a new habitat for species. As long as this waste is highly composed by solid items, this implies the addition of new surfaces for organisms' colonization

(Harrison et al., 2011; Wahl, 1989; Ye and Andrady, 1991). This floating debris has become the main ocean transport system for several species (Jokiel, 1990; Thresher and Brothers, 1985). Hence, oceanic debris represents an important vector for species dispersal, affecting the relative abundance of such organisms outside their native distribution. In order of abundance, the most common organisms living on marine trash are bryozoans, barnacles, polychaete worms, hydroids and molluscs. Such as goose barnacles belonging to *Lepas* family, (*Lepas anatifera* and *Lepas pectinata),* species with a cosmopolitan distribution, are the most numerous found animals attached to marine debris (Barnes, 2002). Problems rise when the carried species start proliferating in non-native areas, causing severe consequences for both environmental and economic aspects (Colautti and Macssa, 2004). Alien species settlement due to the ecosystem alteration is enhanced by the effects of the climate change. Moreover, the increase of biological invasions promotes introgression and hybridization phenomena, challenging the biodiversity conservation (Simberloff, 2005; Simberloff et al., 2013). In the new habitat, the introduced non-native individuals compete for the natural resources leading to a population decrease of endemic species or even to their extinction. As long as the definition of an alien species involves those species who have been introduced, intentionally or not, by humans to a new habitat (ISSG, 2015); this affirmation agrees with the cited features of biota inhabiting on marine litter.

Despite the magnitude of the problem, data about organisms carried by marine litter are relatively scarce and concentrate on a few regions of the world. One of the gaps is the north Iberian coast. Hence, owing to the lack of data from the Asturias' coast and the increasing occurrence of alien organisms therein (Devloo-Delva et al., 2016), this exploratory study will focus on plastic bottles and fishing gear-attached macro-biota. Molecular techniques will be employed to identify the different organisms and for detecting the invasive species. Thereby, this investigation will join the still modest group of studies pointing the increasing relationship among biological invasions and floating debris in the ocean.

2. OBJECTIVES

1. Determine the invasive species adhered to plastic bottles and fishing gear, along the coast of Asturias. These items were chosen according to their most probable origin, land littering for plastic bottles and ocean littering for fishing gear (likely thrown or lost from boats or ships)
2. Compare the frequency of these two types of items

3. Another objective is to find out if there is any kind of item preference by hitchhiker species
4. Obtain DNA Barcodes from hitchhiker species, for species confirmation and building on a genetic database of coastal organisms carried by marine litter.
5. Finally, identifying all found organisms and differentiating among invasive and non-invasive species employing molecular techniques and comparing with international databases such as ISSG list (ISSG - Invasive Species Special Group, 2016) and DAISIE online database (DAISIE - Delivering Alien Invasive Species Inventories for Europe, 2016).

3. MATERIAL AND METHODS

3.1 Study area and sampling design

The location chosen for the study was the coast of Asturias, north Spain, within the Cantabrian Sea. A total number of 22 beaches (*Table 2*) were selected in order to obtain representative coverage of the coast between the borders with the Autonomic Communities of Galicia and Cantabria. The items were collected during the low tides in twelve different days, starting on February 8, 2016, ending on 16 March, 2016.

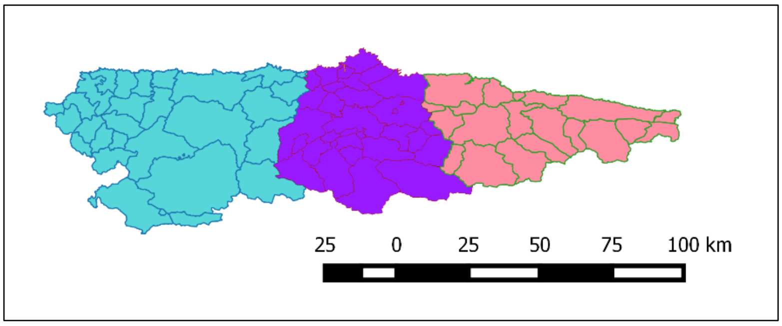

Figure 1. Map of Asturias and differentiated zones

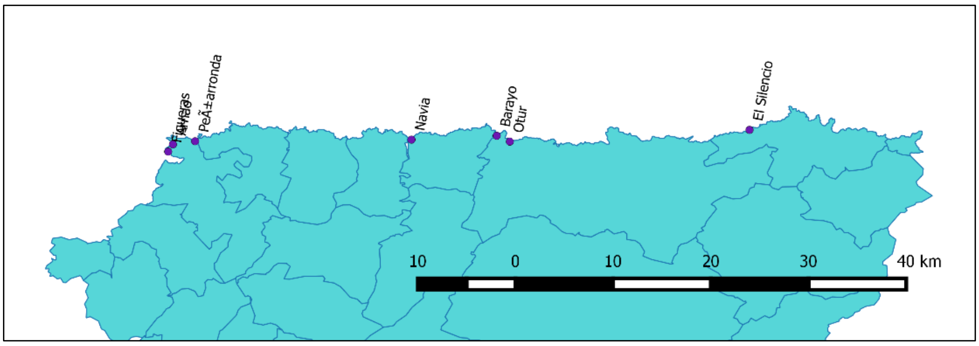

Figure 2. Western zone beaches: Figueras, Arnao, Peñarronda, Navia, Barayo, Otur, El Silencio

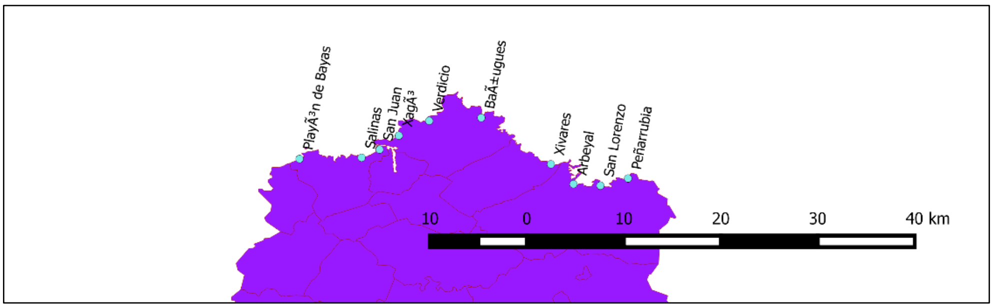

Figure 3. Central zone beaches: Bayas, Salinas, San Juan, Zelán, Xagó, Verdicio, Bañugues, Xivares, Arbeyal, San Lorenzo, Peñarrubia

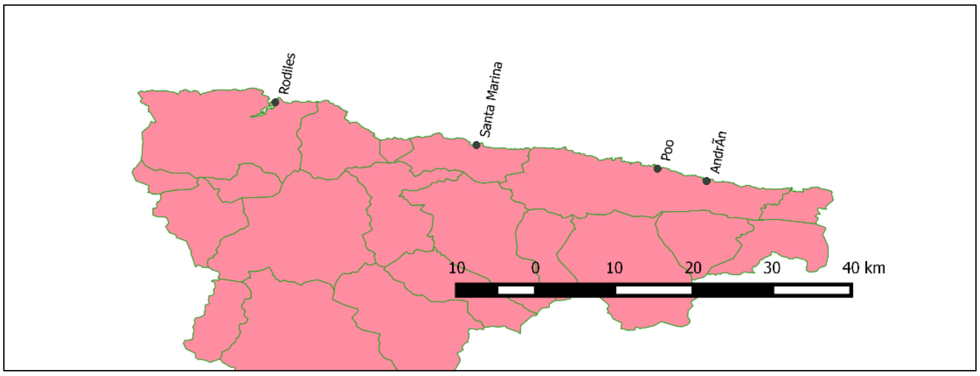

Figure 4.. Eastern zone beaches: Rodiles, Santa Marina, Poo and Andrín

Table 2. Table of target beaches

Name of the beach	*Coordinates*	*Substratum type*
Figueras	43°32′2.47″N 7°1′19.87"W	Rocks and sand
Arnao	43°32′55″N 7°01′17"W	Sand
Peñarronda	43°33′06″N 6°59′24"W	Sand
Navia	43°33′19″N 6°43′20"W	Sand
Barayo	43°33′42″N 6°36′55"W	Sand
Otur	43°33′08″N 6°35′51″W	Sand
El Silencio	43° 33′ 59″N 6° 17′ 45″W	Pebbles and rocks
Playón de Bayas	43°34′31″N 6° 2′ 39″W	Sand
Salinas	43°34′39″N 5°57′44″W	Sand
San Juan de Nieva	43°35′14″N 5°56′21″W	Sand
Zeluán	43°35'23"N 5°54'50"W	Sand
Xagó	43°36′20″N 5°54′53″W	Sand
Verdicio	43°37′35″N 5°52′36″W	Sand
Bañugues	43°37′42″N 5°48′37″W	Sand

Xivares	43°34'13.0"N 5°43'14.2"W	Sand
Arbeyal	43°32′36″N 5°41′37″W	Sand
San Lorenzo	43°32′26″N 5°39′18″W	Sand
Peñarrubia	43°33′02″N 5°37′35″W	Pebbles and rocks
Rodiles	43°31′57″N 5°22′57″W	Sand
Santa Marina	43°27′55″N 5°04′12″W	Sand
Poo	43°25'47.8"N 4°47'09.0"W	Sand
Andrín	43°24′35″N 4°42′00″W	Sand

The objective was to quantify the biota transported by each type of material in different beaches. Hence sampling was focused on collecting sufficient items of each type of material to be representative from each beach. Depending on the size of the beach, two distinct sampling protocols were applied:

- < 1.5 km length: An exhaustive sampling all over the area, from the vegetation line to the waterline. This was the case of Figueras, Arnao, Peñarronda, Navia, Barayo, Otur, El Silencio, San Juan de Nieva, Zeluán, Verdicio, Bañugues Xivares, Arbeyal, Peñarrubia, Rodiles, Santa Marina, Poo and Andrín beaches.
- > 1.5 km length: This was the case of Bayas, Salinas, Xagó and San Lorenzo beaches. In these beaches, a transect of five meters above and five meter below the last waterline was surveyed. Only the items found inside the transect were collected for the research.

All the plastic bottles were counted and weighted in each beach. For those with attached organisms additional data were taken, such as number or plastic type codes. Ropes and nets with adhered fauna were classified as organic (e.g. straw, esparto) or inorganic (plastic).

All attached specimens were collected, preserved in ethanol 70% and taken to the laboratory for further analysis. A maximum of 15 individuals per item were analyzed.

3.2 Species identification

3.2.1 Species morphological identification

An initial morphological identification was performed for all samples to species category, especially for those that did not have tissue available for the DNA extraction such as barnacles and other animals with only exoskeleton remains. The taxonomic guides employed for classification was (Lindner, 1978).

3.2.2 Species molecular identification

Several individuals, minimum of 1 and maximum of 15 individuals, from each species identified *de visu*, were genetically analyzed to confirm unambiguously the species assignation from DNA.

3.2.2.1 DNA extraction

Small pieces of muscle tissues of approximately 2 mm^3 were taken from each specimen, cut into very small pieces, and mixed with 500 µl of a 10% Chelex solution previously warmed at 55°C. Then 7.5 µl of K-proteinase were added to this mix for the DNA digestion. This enzyme degrades cell membranes and releases the DNA. Samples were placed in a preheated oven at 55ºC for 90 minutes, vortexing them every 10 minutes. Then, samples were heated to 100°C for 20 minutes to deactivate the enzyme. DNA aqueous solutions were stored in the fridge at 4ºC for posterior genetic analyses. The E.Z.N.A. Mollusc DNA Kit was employed for small specimens with high content of muccopolysacharids. The kit uses chloroform as a main agent to remove them and purify the extraction. The kit was employed according to manufacturer's directions.

3.2.3 COI sequencing

DNA amplification for all samples was performed with Geller primers, jgLCO1490 and jgHCO2198 (Geller et al., 2013) (*Table 3)* a version of Folmer primers (Folmer et al., 1994). These modified primers were created with degenerated nucleotide sites, thus they are able to amplify COI gene fragments from a wider group of marine invertebrates belonging to very different taxa because the primers' specificity is lower (Geller et al., 2013).

PCR mixtures contained 1x Taq buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs, 1 µM primer jgLCO1490, 1 µM primer jgHCO2198, 0.03 µ/µM Taq polymerase (Promega), 0.2 mg/ml BSA and 4 µl of isolated DNA; summing up a final volume of 40 µl. Minor modifications were applied from the PCR amplification protocol proposed by (Geller et al., 2013): Initial denaturation at 95°C for 5 min, then 40 cycles of 1 min denaturation at 95°C with a 1 min annealing temperature of 48°C, and a 1 min extension phase at 72°C. The final extension was realized a this same temperature for 5 min. PCR products were resolved on 2% agarose gels, stained with SimplySafe, a safe nucleic acid stain employed instead of ethidium bromide.

3.2.4 18S rDNA

Amplification of the gene 18S rDNA (Medlin et al., 1988) (*Table 3*) was done to identify polychaete specimens. Final volume of 40 µl of PCR product, containing: 1x Taq buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs, 1 µM primer 18S-EukF, 1 µM primer 18S-EukR, 0.03 µ/ µM Taq polymerase (Promega), 0.2 mg/ml BSA and 4 µl of isolated DNA. PCR conditions were an initial denaturation at 95ºC for 5 min, then 35 cycles of denaturation at 95ºC for 1 min, 1 min at 55ºC as annealing temperature, extension at 70ºC for 1 min; and a final extension of 10 min at 72ºC. PCR products were resolved on 2% agarose gels, stained with SimplySafe, a safe nucleic acid stain used instead of ethidium bromide.

Table 3. Primers used for PCR

Primer	Sequence (5'-3')	Target loci	Reference
jgLCO1490	TITCIACIAAYCAYAARGAYATTGG	COI, mtDNA	(Geller et al., 2013)
jgHCO2198	TAIACYTCIGGRTGICCRAARAAYCA		
18S-EukF	WAYCTGGTTGATCCTGCAGT	18 rDNA	(Medlin et al., 1988)
18S- EukR	TGATCCTTCYGCAGGTTCACCTAC		

3.2 Barcoding species assignation

The total volume of the PCR product obtained per sample was sent to the DNA sequencing company Macrogen (Macrogen, 2016) based in Korea, Europe, USA and Japan. Result sequences from Macrogen were review and edited with Bioedit software. Lately, sequences were contrasted with Bold Systems (BOLDsystems, 2015) and BLAST in NBCI (NCBI, 2015), both online public databases. Species identification was accepted when the maximum score was at least 97% nucleotide identity. World Register of Marine Species (WoRMS - World Register of Marine Species, 2015) was employed to corroborate the taxonomic nomenclature of all identified species.

3.3 Phylogenetic trees

Also known as 'Dendrograms', phylogenetic trees are graphic branching diagrams that show the evolutionary relationships of a group of individuals. For this research, these diagrams were built for invasive species samples in order to confirm, not only, their species status using a second method after BLAST/BOLD, but also to try to infer their geographic origin. Reference sequences were taken from the public database GenBank (National Center of Biotechnology Information, 2015). Whenever possible, sequences obtained from different countries were employed. Then, with BioEdit software, sequences' alignments were created by comparing the downloaded sequences with those belonging to collected specimens. Alignments were analyzed with DnaSP software package in order to confirm sequences had not the same haplotypes in order to appreciate differences in the tree distribution Final step was to construct the evolutionary tree using MEGA7 software using the statistical method of Neighbour-Joining and Maximum Likelihood model. Number of bootstrap replications was 1000.

4. RESULTS

4.1 Stranded marine debris on the shoreline

A total amount of 777 bottles were counted and weighted (*Table 4*). Only 8 bottles, a 1.02% from the total percentage, carried fauna attached and were thus collected for the study. The material of these bottles were Polyethylene Terephthalate (PET) and High Density Polyethylene (HDPE), resins 1 and 2 from the R.I.C. (*Table 1*). Both of them are used for beverage and cosmetic packaging, reportedly safe, even PET items (International Life Sciences Institute, 2000). Plastic resin was not preferred for fouling (attached) fauna.

Table 4. List of sampled beaches from West to East showing bottle data: Number of found bottles per beach, total weight, number of taken bottles for the study, type of R.I.C. and current status of the found species (NIS for non-indigenous species, N for native, C for Cosmopolitan)

Beach	*Nº of bottles*	*Weight (kg)*	*Taken bottles (attached fauna)*	*R.I.C.*	*Found species*
Figueras	10	0.276	1	HDPE (2)	NIS, N
Arnao	8	0.255	0	-	-
Peñarronda	20	0.638	2	Both PET (1)	C
Navia	27	0.694	0	-	-
Barayo	4	0.131	0	-	-
Otur	0	0	0	-	-
El Silencio	0	0	0	-	-
Playón de Bayas	89	2.952	0	-	-
Salinas	3	0.141	0	-	-
San Juan de Nieva	43	1.109	0	-	-
Zeluán	64	2.638	1	PET (1)	NIS
Xagó	40	0.966	0	-	-
Verdicio	24	0.833	1	PET (1)	C

Bañugues	17	0.458	0	-	-
Xivares	18	0.519	0	-	-
Arbeyal	4	0.125	1	PET (1)	N
San Lorenzo	0	0	0	-	-
Peñarrubia	5	0.429	1	PET (1)	C
Rodiles	10	0.341	1	HDPE (2)	C
Santa Marina	242	5.078	0	-	-
Poo	7	0.108	0	-	-
Andrín	42	1.227	0	-	-

Plastic bottles were quantified and weighted and data has been recorded in *Table 4*. Highest values correspond to Santa Marina beach (Ribadesella), a touristic place where 242 plastic bottles were found, 5.078 kg in total. It has a total length of 1200 m and sampling procedure was performed all over its surface. Most part of the items were found retained in wood remains. According to Asturias local holiday calendar of 2015 (Principado de Asturias, 2015) , carnival day was February 9. In 2016, the Carnival was on February 9, 12 and 13. Owing to major part of beaches are cleaned for three different periods (before Easter holidays, May and summer season), and that this beach was sampled on February 24; debris has been accumulating and the amount probably has increased due to the carnival. No bottles with attached fauna were found there.

Playón de Bayas and Zeluán beach were the second ones of the list with 2.638 kg and 2.952 kg respectively. Beaches were completely different. In the first case, a 3 km beach where 89 bottles were counted along the waterline nearby mounts of debris. Zeluán beach has a maximum length of 300 m and 64 bottles were noted down, most of them located in the upper part of the area. This means that their presumably origin was terrestrial instead of marine. From the total amount of found bottles, only one was taken for this exploratory study.

No bottles were found in Otur, El Silencio or San Lorenzo beaches. In the case of Otur, this beach was sampled days before Easter holidays, thereby, as it was mentioned before, the beach was clean. San Lorenzo beach is located in a highly urbanized area, thereby, such as Salinas beach, it is cleaned almost every day. El Silencio beach is the opposite of this last one. It is located in an isolated area and only small pieces of plastic, probably from bottles, were found here. The pebble/rocky substratum type combined with the wave action, could be the reason of bottles' fragmentation.

Beaches where bottles were collected for the study and all bottle data can be seen in *Table 5*. The percentage of bottles carrying macro-biota was calculated dividing the number of taken bottles by the total number of quantified bottles in each beach. These percentages showed that the numbers of bottles with attached fauna were relatively small compared to those without adhered biota per beach. The last column shows the species status: Cosmopolitan, Invasive or Not Invasive (C, I or NI).

Table 5. List of sampled beaches from West to East showing bottle data: Number of found bottles per beach, number of taken bottles for the study, percentage of bottles carrying biota and current status of the found species (Invasive or Cosmopolitan)

Beach	***Nº of total bottles***	***Taken bottles***	***% of bottles carrying biota***	***Species status***
Figueras (FIG)	10	1	10%	I and NI
Peñarronda (PN)	20	2	10%	C
Zeluán (ZLN)	64	1	1.56%	I
Verdicio (VER)	24	1	4.16%	C
Arbeyal (ARB)	4	1	25%	NI
Peñarrubia (PNR)	5	1	20%	C
Rodiles (ROD)	10	1	10%	C

For fishing gear (ALDFG), at first instance it was considered to weight all the ropes and nets washed ashore but in order to get accurate results, items should be completely dry, otherwise, weight values will be bigger than real ones. As long as this was not possible, measuring the surface of the items collected for the study was tried, but they were highly entangled and the results were not reliable. Therefore, only numbers were given to those items to differentiate them when collecting the attached organisms (see below). All the found fishing gear found in this study was made of inorganic fibers.

4.2. Genetic Barcoding

A total number of 191 specimens were genetically analyzed, from which 58 were identified to species level over 97% nucleotide identity. From the 58 sequences, 3 of them were detected as bacteria, therefore, 55 eukaryote individuals were rightly identified. These sequences of COI gene will be submitted to GenBank, where they will be available.

Reasons of non-identifying 133 individuals are exposed below:

- No tissue available for DNA extraction was found on 40 individuals. As long as it was possible it was tried to morphologically identify them.

- PCR failure. In 48 cases, no bands were visible in the agarose gel. Inhibitors present in the tissue pieces for DNA extraction might have interfered with PCR mixtures.
- Unreliable species identification. DNA sequences from 44 individuals did not reach 97% nucleotide identity with any reference from databases. Although they were edited with Bioedit software, it was not possible to obtain a reliable species assignation.
- Weak PCR amplification. For one individual bands in agarose were not enough clear and were not sent out for sequencing.

Detailed data are in Supplementary *Table 1a and 1b*. The species Barcoded in this study (*Table 6*) corresponded to 9 different orders:

- **Order Alcyonacea:** *Paragorgia arborea*, not invasive species belonging to *Paragorgiidae* family.
- **Order Decapoda:** *Pachygrapsus marmoratus* (*Grapsidae*) and *Polybius henslowii* (*Portunidae*), both of them not invasive species.
- **Order Lepadiformes:** *Lepas anatifera* and *Lepas pectinata*, both belong to *Lepadidae* family. These two species have a cosmopolitan distribution, that is the reason why they are the most numerous individuals.
- **Order Mytilida:** *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*, all of them from *Mytilidae* family. This last one is a not indigenous species.
- **Order Neogastropoda:** *Nassarius reticulatus* from *Nassaridae* family. Not invasive species.
- **Order Ostreida:** *Crassostrea gigas, Ostreidae* family, reportedly invasive species.
- **Order Phyllodocida:** *Eumida bahusiensis* from *Phyllodocidae* family. This is a not indigenous species.
- **Order Sabellida:** *Neodexiospira nipponica* from *Serpulidae* family, reportedly not indigenous species.
- **Order Sessilia:** *Balanus modestus (Balanidae)*, *Chthamalus stellatus (Chthamlidae)*, *Amphibalanus amphitrite (Balanidae)* and *Austrominius modestus (Austrobalanidae).* From these species, the two last ones were reportedly invasive according to ISSG list and DAISIE online databases. In the case of *Neocasta laevigata (Archaeobalanidae)*

Table 6. List of species genetically identified from over 97% nucleotide identity; currently recognized status (Invasive, Not invasive or Cosmopolitan); item where species were attached (bottle, rope or both); number of individuals Barcoded in this study (N).

Species	***Status***	***Item***	***N***
Amphibalanus amphitrite	Invasive	bottle	1
Austrominius modestus	Invasive	both	7
Balanus perforatus	Not invasive	rope	1
Chthamalus stellatus	Not invasive	bottle	3
Crassostrea gigas	Invasive	both	2
Eumida bahusiensis	Not indigenous	rope	1
Lepas anatifera	Cosmopolitan	both	25
Lepas pectinata	Cosmopolitan	both	4
Mytilus edulis	Not invasive	both	2
Mytilus galloprovincialis	Not invasive	rope	1
Mytilus trossulus	Not indigenous	rope	1
Nassarius reticulatus	Not invasive	rope	1
Neocasta laevigata	Not indigenous	rope	1
Neodexiospira nipponica	Not indigenous	bottle	1
Pachygrapsus marmoratus	Not invasive	rope	1
Paragorgia arborea	Not invasive	rope	1
Polybius henslowii	Not invasive	rope	1

Focusing on non-indigenous species (*Table 7),* the 15 individuals found in this study were concentrated in a few objects found from three beaches (Figueras, Xagó, Zeluán). Eight were attached to bottles and seven to ropes.

Table 7. List of individuals from NIS genetically identified over 97% nucleotide identity in this study. Beach where they were detected; items they were attached to ("r" means "rope" and "b" means "bottle"); number of individuals attached to each item.

NIS species	***Location (Beach)***	***Item***	***Nº of individuals***
Amphibalanus amphitrite	Zeluán	b.1	1
Austrominius modestus	Zeluán	r.1; b.1	3; 4
Crassostrea gigas	Figueras	b.1	1

	Zeluán	r.1	1
Eumida bahusiensis	Xagó	r.3	1
Mytilus trossulus	Figueras	b.1	1
	Zeluán	r.1	1
Neocasta laevigata	Xagó	r.3	1
Neodexiospira nipponica	Figueras	b.1	1

More abundant species attached to bottles were the cosmopolitan gooseneck barnacles: *Lepas anatifera* and *Lepas pectinata.* Invasive specimens were *Amphibalanus amphitrite* (Indian and Pacific Ocean), *Austrominius modestus* (Australia) and *Crassostrea gigas* (Pacific Ocean).Not indigenous species individuals of *Neodexiospira nipponica* (Japan) were found too. Native species were individuals of *Mytilus edulis.*

All the fishing gear items found in this study were made of inorganic materials. Such as in the case of the bottles, the cosmopolitan species *Lepas anatifera* and *Lepas pectinata*, were the dominant organisms. In common with bottles, alien species were*: Austrominius modestus* and *Crassostrea gigas*, non-indigenous species of *Mytilus trossulus* (North Atlantic, North Pacific and Baltic Sea); *Eumida bahusiensis* (North Sea) and *Neocasta laevigata* (Australia) individuals were only detected in ropes. *Mytilus edulis* specimens were also found on ropes.

A phylogenetic approach was tried to infer, if possible, the origin of the NIS found in this study. For *Amphibalanus amphitrite* and *Neodexiospira nipponica* only one valid sequence from GenBank was available. No voucher sequences of 18S marker were available in the case of *Neocasta laevigata* species.

The *Figure 5* shows the evolutionary tree for *Austrominius modestus* species reconstructed from sequences of 11 base pairs. The sequences of this study correspond to individuals taken from two different items found in Zeluán beach; 4 from a bottle (ZLNb1_1, ZLNb1_3, ZLNb1_4 and ZLNb1_5) and the rest (ZLNr1_13, ZLNr1_14 and ZLNr1_15) from a rope. Relatively low bootstrapping support (numbers appearing next to the nodes) was found for most branches, with a maximum of 68 for the cluster containing ZLNb1_3 and ZLNr1_14. Only references from Germany were available in GenBank, therefore further origin inference was not possible for this species.

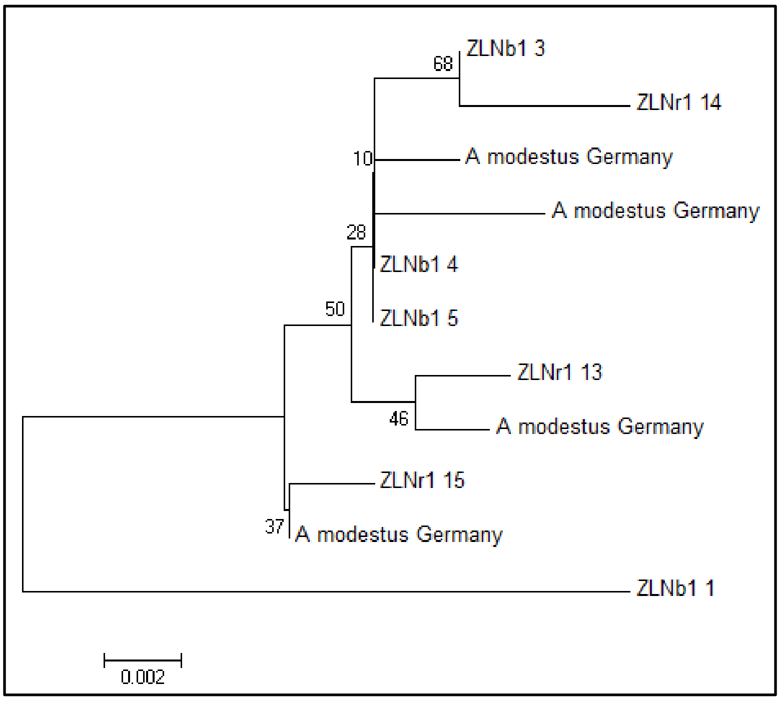

Figure 5. Phylogenetic tree of seven Austrominius modestus specimens. Samples were attached to a plastic bottle and to a rope found in Zeluán beach

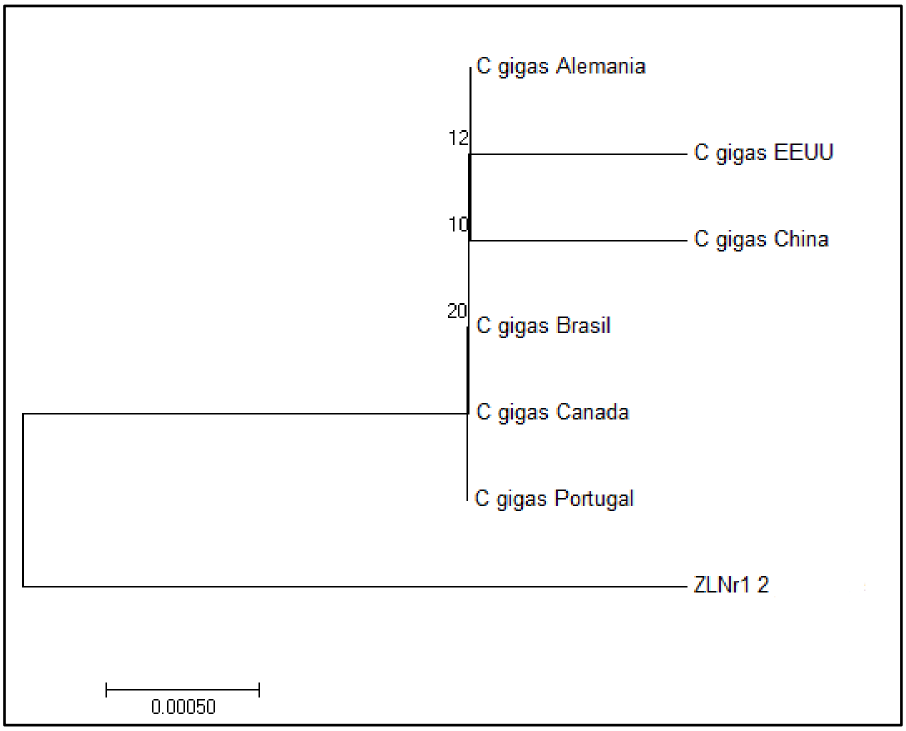

Figure 6. Phylogenetic tree of one Crassostrea gigas specimen. Sample was found attached to a rope found in Zeluán beach.

Figure above (*Figure 6)* represents the phylogeny of an individual of *Crassostrea gigas* that was found adhered to ashore fishing gear in Zeluán beach. Sequences from different countries were available for constructing the tree with 6 base pairs. Although bootstrapping values were low, it can be seen that all these sequences cluster together, whereas the individual of the study was out of this group. Thus these data are not enough data to infer the origin of the *Crassostrea gigas* specimen from the study.

When the sequence obtained from one putative *Eumida bahusiensis* specimen was contrasted with online databases, two reference species were retrieved with 100% nucleotide identity: *Eumida bahusiensis* and *Eumida sanguinea*. The first one is a polychaete native from the North Sea (Bergstrom, 1914), and the second one appears in the northeast part of the Atlantic Sea, including Mediterranean and Cantabrian Sea from Spain (Örsted, 1843) . The *Eumida* genus tree displayed in *Figure 7* based on 5 base pairs did not solve this conflict. In the upper part *Eumida sanguinea* and *Eumida bahusiensis* clustered in distinct groups with a high bootstrapping support. The individual found attached to a rope in Zeluán beach clustered, also with 100% support, with two sequences of different species. The conflict was therefore not solved from this phylogenetic approach.

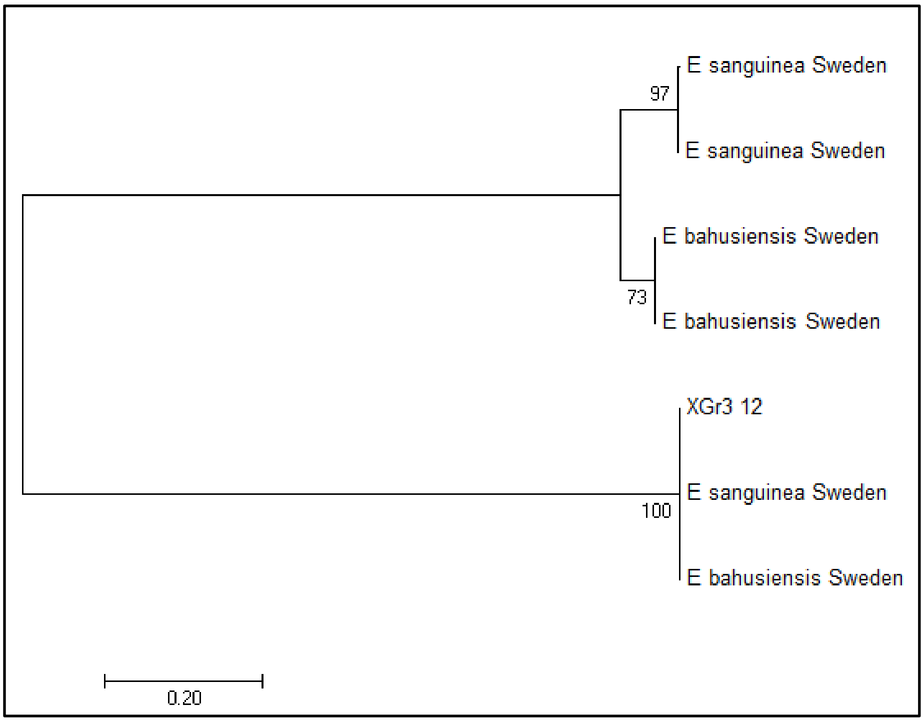

Figure 7. Phylogenetic tree of one Eumida genus. Sample XGr3 12 was found attached to a rope found in Zeluán beach.

Since there were difficulties when identifying some different members of *Mytilus* genus, the evolutionary tree could give clues about the most likely species assignation with those problematic specimens. Sequences of the native *Mytilus galloprovincialis* and *Mytilus edulis* species from varied origins were added to the dendrogram. From BLAST/BOLD, individual ZLNr1_7 was identified as *Mytilus trossulus*, but in the figure it appears grouped with sequences of *Mytilus edulis* with a 62% bootstrapping support. The same occurred with ZLNr1_6 classified as *Mytilus edulis* from barcoding techniques, but clustered with *Mytilus trossulus* with a 65% support value. Consistent assignation was given to ZLNr1_8, as *Mytilus galloprovincialis*.

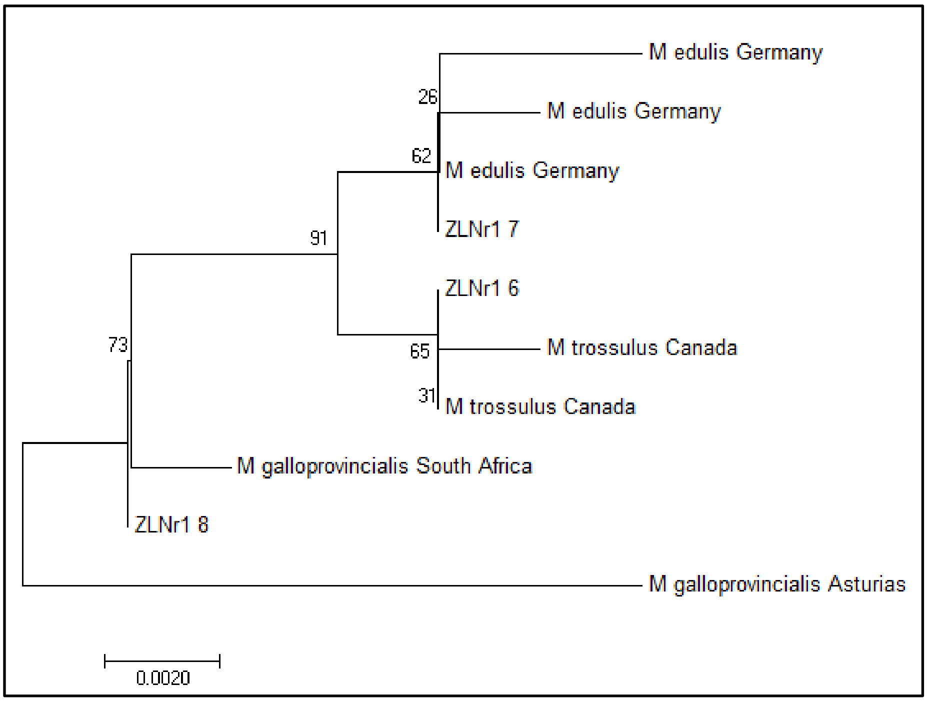

Figure 8. Phylogenetic tree of Mytilus genus. One sample per species was employed, all of them attached to different ropes, found in Zeluán beach.

5. DISCUSSION

Either floating in the water column or stranded along the shoreline, marine debris has a common human origin, but two possible different sources: land and ocean. The majority of plastic bottles are probably from land, whereas ropes and nets were likely thrown from boats or ships ending up in the ocean. Then, these items are carried by the currents until they reach the coast and get ashore. Some of these objects can be colonized by rafting organisms that use them as a transport vector that enhances their own spread. In this exploratory study, cosmopolitan species were the most abundant organisms recorded in both types of items. Known as stalked barnacles too, it has been suggested that these crustaceans may have a role as foundation species when colonizing a new surface (Dayton, 1972). These individuals support the settlement of another hitchhiking species, such as barnacles or mussels, by giving extra morphological complexity (Astudillo et al., 2009). This fact is supposed to enhance positive species interactions like mutualism, which will allow to afford a huge organisms' biodiversity (Bertness and Callaway, 1994; Bruno, 2012). Nevertheless, in collected bottles, specimens from *Lepas* family were the only attached fauna which does not agree with the literature. A possible explanation could be that this items were not enough time in the water column for other species to establish. Moreover, in most of the bottles and ropes with *Lepas sp.* adhered to them, the colonization did not exceed 10 individuals per item (Supplementary *Table 1a and 1b*), leaving large empty spaces. Perhaps long floating time and dense object coverage by these foundation species are needed for the assemblage of other species.

Regarding the invasive species detected in this study, *Austrominius modestus* and *Crassostrea gigas* were found on both types of items (*Table 7). Amphibalanus amphitrite* individuals were also morphologically identified also in fishing gear (although molecular techniques were only able to identified the individuals to genus level). This species as well as *Austrominius modestus* (Barnes and Milner, 2005) have been suggested to be usual hitchhikers of plastic surfaces which promotes their worldwide spreading process (Winston et al., 1997).

For *Mytilidae* family, morphologic characteristics do not seem to be sufficient to reliably identify species within the genus *Mytilus* due to their plasticity. Their shell shape is conditioned by the surrounding environment (Inoue et al., 1995), which makes it hard to differentiate individuals without molecular techniques. Moreover, they hybridize commonly and hybrids occur in the study region (Crego-Prieto et al., 2014). For these reasons, *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus* are grouped together as "*Mytilus complex".* Results obtained from sequencing and those acquired after sequence editing with BioEdit software, were not consistently in many cases, assigning more than one possible species in some cases. A different molecular marker or gene (not mitochondrial, that does not allow to distinguish hybrids) is necessary to identify those species. Thus, the only certain fact from our exploratory study is that *Mytilus sp* individuals were found in both types of items, bottles and fishing gear.

For *Crassostrea gigas*, individuals have been reported to settle over mussel's bed due to their gregarious behavior (Fey et al., 2010; Gerhard, 2001; Wolff and Reise, 2002). These specimens were found together in fishing gear from Zeluán and on a bottle from Figueras. Moreover, during sampling it was observed that mussels were growing over big plastic bags, and oysters, probably *Crassostrea gigas* were settled between them, which supports this behavior. Taking into account all the findings and comments above, item preferences of hitchhiker species cannot be established in this study. The comparison of the available literature about the selected objects of study gives the impression that there is lack of data for relating marine debris with rafting species, especially for fishing gear.

On the other hand, although origin traceability of NIS could not be estimated here, the results obtained in this investigation may serve to suggest that a revision would be needed in some groups. The individual from *Eumida* genus classified *de visu* as *Eumida bahusinesis* also could be *Eumida sanguinea*, since both of them have 100% nucleotide identity for the gene here employed, a point that can be also appreciated in *Figure 7. E. bahusiensis* is an NIS worm original from the North Sea, while *E. sanguinea* is a native species. Both species should be deeply investigated in order to find accurate methods for distinguishing between them, otherwise NIS will pass unnoticed and could represent a potential threat for indigenous biota. Something similar happened when trying to classify the individuals of *Mytilus* genus (

Figure 8). Members belonging to *Mytilus* complex were not clearly identify, thereby another barcoding methods or different genetic markers should be considered. According to Rawson et al. (1996), the molecular marker Glu 5', a nuclear locus, could be used for genetic discerning between *M. edulis*, *M. galloprovincialis* and *M. trossulus*, as explained in (Crego-Prieto et al., 2014).

Barcoding approach for early detection of invasive species is a useful tool from the point of view of biosecurity, but its implementation should be improved with more Barcodes and different genes. This is especially important for *Eumida*, a genus that could benefit from a taxonomic review supported by molecular techniques. Due to the lack of performed investigations about this genus, a taxonomic joint study involving morphology and genetics would be useful for future research about these species.

Moreover, voucher sequences for *Neocasta laevigata* and *Neodexiospira nipponica* would be also needed. Databases were consulted in June 2016, and the sequences obtained in this study will be incorporated there very soon. In the future, it would be desirable to include more reference sequences in order to improve the utility of barcoding techniques. Nevertheless, there is a need to broaden the available literature about the potential relationship among non-indigenous organisms and marine debris.

Summing up, in this exploratory study has been demonstrated that both, plastic bottles and fishing gear, are susceptible items of being colonized by different rafting organisms. What it is more, despite the fact that they are wave-action high exposed objects, 3 reportedly alien species as *Austrominius modestus*, *Amphibalanus amphitrite* or *Crassostrea gigas* individuals, were found in this investigation. This affirmation, added to the reality that marine debris is filling the ocean faster than we think, littering our shores and damaging the ecosystem; reinforces the conviction that several organisms are able to use these items as a dispersal mechanism. Such species proliferating in non-endemic places, put in hazard the native biota, changing the habitat dynamics and its development. That is the reason why biological invasions are known as the world second cause of biodiversity loss.

6. CONCLUSIONS

This exploratory study, summed with previous literature, proves that biological invasions are reinforced by the presence of human manufactured objects in the sea. For the first time in North Iberia the issue of marine litter as carrier vector of invasive species has been tackled: invasive barnacles from Australia, *Austrominius modestus*; Pacific oysters *Crassostrea gigas*; the barnacle *Amphibalanus amphitrite*, and other 52 species were found attached to plastic bottles and fishing gear, in particular ropes. More efforts for controlling litter disposal are needed in this region, especially for plastic bottles that are likely produced in land.

In general lines, even if humans try to keep debris out of the sea, there are many factors that we cannot control such as natural disasters, earthquakes and tsunamis, which can bring debris back to the sea, ruining our efforts. Solution is evident, there is need to reduce the amount of plastic production and raise the awareness about how marine debris is not only an aesthetic issue, but also one of the first responsible of habitat and biodiversity loss.

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ADDENDA

Table 1a. Supplementary table of identified individuals using Chelex for DNA extraction. Columns indicate were the item was collected, the type of item ("R" means "rope" and "B" means "bottle"), number of samples per item, type of recorded organism, DNA extraction and PCR performances, band results in agarose gel and if samples were sequenced or not. Next columns show morphologic identification approach, barcoding species result, and percentage of nucleotid identity assigned by Macrogen. After receiving these results, sequences below 97% nucleotid identity were edited with BioEdit software and new sequences were BLAST/BOLD contrasted resulting in new values and/or new species. Finally, species currently recognized status (Invasive, Not invasive, Not Indigenous (NIS) or Cosmopolitan) and origin of Invasive and NIS individuals.

BEACH	ITEM	SAMPLES	ANIMAL	DNA EXTRACTION	PCR	BANDS	SEQUENCING	MORPHOLOGIC ID	BARCODING	% MG	BLAST	BOLD	STATUS	ORIGIN
ZELUAN	R.1	1	clam	yes	yes	no	no	*Cerastoderma edule?*	X	X	X	X	not invasive	X
		2	oyster	yes	yes	yes	yes	*Ostrea edulis?*	***Crassostrea gigas***	98	99%	X	invasive	Japan
		4	crab	yes	yes	yes	yes	*?*	***Pachygrapsus marmoratus***	99	100%	X	not invasive	X
		5	crab	yes	yes	yes	yes	*?*	***Galathea raventosae***	79	75% (Thysanoessa inermis)	X	NIS	West Pacific
		6	mussel	yes	yes	yes	yes	*Dreissena burgensis?*	***Mytilus trossulus***	98	99% (Mytilus edulis)	98.82%	not invasive	X
		7	mussel	yes	yes	yes	yes	*Mytilus galloprovincialis*	***Mytilus trossulus***	99	99%	X	NIS	North Pacific, North Atlantic and Baltic Sea
		8	mussel	yes	yes	yes	yes	*Dreissena burgensis?*	***Mytilus trossulus***	97	99% (Mytilus galloprovincialis)	X	not invasive	X
		9	striped barnacle	yes	yes	yes	yes	*balanus amphitrite?*	***Amphibalanus amphitrite***	96	99% Balanidae sp.	99.33%	invasive	Indian or Pacific ocean
		10	striped barnacle	yes	yes	no	no	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		11	striped barnacle	yes	yes	no	no	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		12	striped barnacle	yes	yes	no	no	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		13	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	99%	X	invasive	Australia
		14	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	99%	X	invasive	Australia
		15	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	99%	X	invasive	Australia
	B.1	1	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	98	99%	X	invasive	Australia
		2	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	X	X	X	X	X	X
		3	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	98%	X	invasive	Australia
		4	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	99%	X	invasive	Australia
		5	starry barnacle	yes	yes	yes	yes	*Elminius modestus*	***Austrominius modestus***	99	100%	X	invasive	Australia
		6	striped barnacle	yes	yes	yes	yes	*balanus amphitrite?*	***Amphibalanus amphitrite***	93	97% (Balanidae sp. & A. amphitrite)	97.49%	invasive	Indian or Pacific ocean
		7	striped barnacle	yes	yes	yes	yes	*balanus amphitrite?*	***Amphibalanus amphitrite***	93	93%	X	invasive	Indian or Pacific ocean
		8	striped barnacle	yes	yes	yes	yes	*balanus amphitrite?*	***Amphibalanus amphitrite***	93	92%	X	invasive	Indian or Pacific ocean
		9	striped barnacle	yes	yes	yes	no	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
XAGÓ	R.1	1	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		2	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		3	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		4	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		5	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		6	starry barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean

XAGÓ	R.2	1	striped barnacle	yes	yes	no	no	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		2	striped barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		3	striped barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		4	striped barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		5	striped barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
		6	striped barnacle	no tissue	X	X	X	*balanus amphitrite?*	X	X	X	X	invasive	Indian or Pacific ocean
	R.3	1	oyster	yes	yes	no	no	*Crassostrea gigas?*	X	X	X	X	invasive	Japan
		2	oyster	yes	yes	yes	yes	*Crassostrea gigas?*	***seudoalteromonas haloplankti***	79	75% (Shewanella violacea)	X	X	X
		3	oyster	yes	yes	no	no	*Crassostrea gigas?*	X	X	X	X	invasive	Japan
	R.5	1	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	96	99%	X	cosmopolitan	X
		2	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	95	98%	97.63%	cosmopolitan	X
		3	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	88	88%	X	cosmopolitan	X
		4	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	99	99%	99.42%	cosmopolitan	X
		5	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	95	96%	X	cosmopolitan	X
		6	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	92	93%	X	cosmopolitan	X
		7	goose barnacle	yes	yes	no	no	*lepas anatifera?*	X	X	X	X	cosmopolitan	X
		8	goose barnacle	yes	yes	no	no	*lepas anatifera?*	X	X	X	X	cosmopolitan	X
		9	goose barnacle	yes	yes	yes	yes	*lepas anatifera*	***Lepas anatifera***	97	98%	97.76%	cosmopolitan	X
		10	goose barnacle	yes	yes	yes	yes	*lepas anatifera*	***Lepas anatifera***	100	100%	100%	cosmopolitan	X
		11	goose barnacle	yes	yes	yes	yes	*lepas anatifera*	***Lepas anatifera***	100	99%	99.16%	cosmopolitan	X
		12	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	99	99%	100%	cosmopolitan	X
		13	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	96	97%	X	cosmopolitan	X
		14	goose barnacle	yes	yes	yes	yes	*lepas anatifera*	***Lepas anatifera***	98	98%	98.42%	cosmopolitan	X
		15	goose barnacle	yes	yes	yes	yes	*lepas anatifera?*	***Lepas anatifera***	98	99%	X	cosmopolitan	X
VERDICIO	B.1	1	goose barnacle	yes	yes	yes	yes	*Lepas pectinata?*	***Lepas pectinata***	93	98%	X	cosmopolitan	X
		2	goose barnacle	yes	yes	yes	yes	*Lepas pectinata?*	***Lepas pectinata***	96	96%	X	cosmopolitan	X
		3	goose barnacle	yes	yes	yes	yes	*Lepas pectinata?*	***Lepas pectinata***	99	99%	X	cosmopolitan	X
PEÑARRUBIA	B.1	1	goose barnacle	yes	yes	no	no	*Lepas pectinata?*	X	X	X	X	cosmopolitan	X
		2	goose barnacle	yes	yes	no	no	*Lepas pectinata?*	X	X	X	X	cosmopolitan	X
ARBEYAL	B.1	3	mussel	yes	yes	yes	yes	*?*	***Mytilus edulis***	97	98%	97.07% (galloprovincialis	not invasive	x
RODILES	B.1	1	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		2	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		3	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		4	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		5	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		6	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		7	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X
		8	goose barnacle	no tissue	X	X	X	*lepas pectinata?*	X	X	X	X	cosmopolitan	X

RODILES	B.1	9	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		10	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		11	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		12	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		13	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		14	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
		15	goose barnacle	no tissue	X	X	X	lepas pectinata?	X	X	X	X	cosmopolitan	X
PEÑARRONDA	R.1	1	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	99	99%	X	cosmopolitan	X
		2	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	98	98%	X	cosmopolitan	X
		3	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	98	99%	X	cosmopolitan	X
		4	goose barnacle	yes	yes	yes	yes	Lepas pectinata?	Lepas pectinata	99	99%	X	cosmopolitan	X
		5	goose barnacle	yes	yes	yes	yes	Lepas pectinata?	Lepas pectinata	99	99%	X	cosmopolitan	X
	R.2	1	crab	yes	yes	yes	yes	Planes minutus	Gecarcoidea natalis????	87	87% (Eriocheir leptognathus)	X	NIS	Christmas Island, Indian Ocean; leptognatus: China
		2	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	97	99%	X	cosmopolitan	X
		3	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	99	99%	X	cosmopolitan	X
		4	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	97	98%	X	cosmopolitan	X
		5	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	98	98%	X	cosmopolitan	X
	B.1	1	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	98	98%	X	cosmopolitan	X
		2	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudomonas sp.	93	94% (Pseudomonas antartica)	X	X	X
		3	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudoalteromonas sp.	83	84%	X	X	X
		4	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudoalteromonas sp.	85	86%	X	X	X
		5	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudoalteromonas sp.	85	86%	X	X	X
		6	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudoalteromonas sp.	85	86%	X	X	X
		7	goose barnacle	yes	yes	yes	yes	lepas pectinata?	Pseudoalteromonas sp.	86	85% (Echiura sp.)	X	X	X
	B.2	1	goose barnacle	yes	yes	yes	yes	lepas anatifera	Lepas anatifera	96	97%	97.46%	cosmopolitan	X
		2	goose barnacle	yes	yes	yes	yes	lepas anatifera	Lepas anatifera	100	100%	99.56%	cosmopolitan	X
		3	goose barnacle	yes	yes	yes(dil)	yes	lepas anatifera	Lepas anatifera	97	99%	98.93%	cosmopolitan	X
		4	goose barnacle	yes	yes	yes(dil)	yes	lepas anatifera	Lepas anatifera	98	99%	100%	cosmopolitan	X
		5	goose barnacle	yes	yes	yes(dil)	yes	lepas anatifera	Lepas anatifera	99	98%	97.88%	cosmopolitan	X
		7	goose barnacle	yes	yes	no	no	lepas anatifera?	X	X	X	X	cosmopolitan	X
		8	goose barnacle	yes	yes	no	no	lepas anatifera?	X	X	X	X	cosmopolitan	X
		9	goose barnacle	yes	yes	yes (dil)	yes	lepas anatifera?	Lepas anatifera	99	100%	100%	cosmopolitan	X
		10	goose barnacle	yes	yes	yes	yes	lepas anatifera	Lepas anatifera	87	86%	X	cosmopolitan	X
		11	goose barnacle	yes	yes	yes	yes	lepas anatifera	Lepas anatifera	94	95%	X	cosmopolitan	X
		13	goose barnacle	yes	yes	no	no	lepas anatifera?	X	X	X	X	cosmopolitan	X
		14	goose barnacle	yes	yes	no	no	lepas anatifera?	X	X	X	X	cosmopolitan	X
		15	goose barnacle	yes	yes	yes	yes	lepas anatifera?	Lepas anatifera	99	98%	X	cosmopolitan	X

FIGUERAS	**R.1**	**1**	striped barnacle	yes	yes	yes	yes	*balanus amphitrite?*	***Balanus perforatus***	**100**	**100%**	**X**	not invasive	**X**
	B.1	**1**	mussel	yes	yes	yes	yes	*Mytilus edulis?*	***Mytilus galloprovincialis***	**85**	**83% (Mytilus trossulus)**	**X**	not invasive	**X**
		2	mussel	yes	yes	yes	yes	*Mytilus edulis?*	***Mytilus trossulus***	**98**	**100% (Mytilus edulis& Mytilus sp.)**	**98.87% (Mytilus sp)**	not invasive	**X**
		4	oyster	yes	yes	yes	yes	*Crassostrea gigas?*	***Crassostrea gigas***	**99**	**100%**	**X**	invasive	Japan
BAYAS	**R.1**	**1**	goose barnacle	**X**	**X**	**X**	**X**	*lepas anatifera?*	**X**	**X**	**X**	**X**	cosmopolitan	**X**
	R.2	**1**	black crab	yes	yes	yes	yes	*?*	***Polybius henslowii***	**100**	**100%**	**X**	not invasive	**X**
		2	red crab	yes	yes	yes	yes	*?*	***Pseudoalteromonas translucida***	**84**	**X**	**X**	**X**	**X**

Table 1b. Supplementary table of identified individuals using E.Z.N.A. Mollusc DNA Kit for DNA extraction. Columns indicate were the item was collected, the type of item ("R" means "rope" and "B" means "bottle"), number of samples per item, type of recorded organism, DNA extraction and PCR performances, band results in agarose gel and if samples were sequenced or not. Next columns show morphologic identification approach, barcoding species result, and percentage of nucleotid identity assigned by Macrogen. After receiving these results, sequences below 97% nucleotid identity were edited with BioEdit software and new sequences were BLAST/BOLD contrasted resulting in new values and/or new species. Finally, species currently recognized status (Invasive, Not invasive, Not Indigenous (NIS) or Cosmopolitan) and origin of Invasive and NIS individuals.

BEACH	ITEM	SAMPLE	ANIMAL	DNA EXTRACTION	PCR	BANDS	SEQUENCING	MORPHOLOGIC ID	BARCODING	%	BLAST	BOLD	STATUS	ORIGIN
NAVIA	R.1	1	briozoo	yes	yes	no	no	*Bugula neritina*	X	X	X	X	X	X
		2	worm	yes	yes	yes	yes	*Pomatoceros triqueter*	***Cecidomyiidae sp.***	85	81%	X	X	X
		3	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		4	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		5	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Neoacasta laevigata***	83	88%(Balanus sp.)	X	NIS	X
		6	snail	yes	yes	yes	yes	?	***Nassarius reticulatus***	98	100%	X	not invasive	X
		7	terebelid	no tissue	X	X	X	?	X	X	X	X	X	X
		8	terebelid	no tissue	X	X	X	?	X	X	X	X	X	X
		9	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
XAGÓ	R.3	1	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Wanella sp.***	83	96% (Galkinia sp.)	X	not invasive	X
		2	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Oncholaimidae sp.***	92	95%	X	not invasive	X
		3	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Wanella sp.***	96	98% (Neocasta laevigata)	X	NIS	Australia
		4	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Calyptronema sp***	87	89%	X	not invasive	X
		5	pink worm	yes	yes	yes(18S)	yes	?	***Uncultured eukaryote***	88	92%	X	X	X
		6	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		7	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Syllis hyalina***	87	86%	X	not invasive	X
		8	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		9	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	X	X	X	X	X	X
		10	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Tetraclita squamosa***	78	76% (Psammoneis sp)	X	not invasive	X
		11	pink barnacle	yes	yes	yes	yes	*Megabalanus coccopoma*	***Cantellius hoegi***	89	89%	X	??	???
		12	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Eumida bahusiensis***	98%	100% (E. bahusiensis & E. sanguinea)	X	NIS (bahusiensis	North Sea
		13	pink barnacle	yes	yes	yes	yes	*Megabalanus coccopoma*	***Balanus balanus (Semibalanus cariosus)***	87	88% (Balanidae sp.)	X	NIS	Artic Ocean
		14	pink barnacle	yes	yes	yes	yes	*Megabalanus coccopoma*	X	X	X	X	NIS	California, South America, Equador
		15	worm (no tube)	yes	yes	yes(18S)	yes	?	***Wanella milleporae***	96	99% (Wanella milleporae)	X	NIS	Australia
	R.6	1	clam	yes	yes	no	no	?	X	X	X	X	X	X
		2	clam	yes	yes	yes	yes	?	X	X	X	X	X	X
		3	oyster	yes	yes	no	no	*Crassostrea gigas*	X	X	X	X	invasive	Japan
		4	briozoo	yes	yes	no	no	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		5	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		6	worm	no tissue	X	X	X	?	X	X	X	X	X	X

XAGÓ	R.7	1	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Paragorgia arborea***	97	97%	X	not invasive	X
		2	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		3	worm	yes	yes	no	no	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		4	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		5	briozoo (pink)	yes	yes	no	no	?	X	X	X	X	X	X
		6	worm	yes	yes	no	no	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		7	pink worm	yes	yes	yes	yes	?	X	X	X	X	X	X
		8	clam	yes	yes	no	no	?	X	X	X	X	X	X
		9	clam	yes	yes	no	no	?	X	X	X	X	X	X
		10	oyster	yes	yes	no	no	?	X	X	X	X	X	X
		11	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		12	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		13	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		14	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		15	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
	R.8	1	briozoo (pink)	yes	yes	no	no	?	X	X	X	X	X	X
		2	briozoo (pink)	yes	yes	no	no	?	X	X	X	X	X	X
	R.9	1	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Uncultured Colpodellidae***	85	*94% (Margolisiella islandica)*	X	not invasive	X
	R.10	1	briozoo (red)	yes	yes	no	no	?	X	X	X	X	X	X
		2	briozoo (red)	yes	yes	yes	yes	?	***Shewanella baltica***	94	97%	X	NIS	Baltic Sea, Black Sea
RODILES	R.1	1	worm	yes	yes	yes	yes	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		2	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		3	briozoo (white)	yes	yes	yes	yes	/	X	X	X	X	X	
		4	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
	R.2	1	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		2	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
BAYAS	R.1	1	oyster	yes	yes	yes	yes	*Crassostrea gigas*	X	X	X	X	invasive	Japan
		2	sponge	yes	yes	no	no	?	X	X	X	X	X	X
		3	sponge	yes	yes	no	no	?	X	X	X	X	X	X
		4	oyster	yes	yes	yes	yes	*Crassostrea gigas*	X	X	X	X	invasive	Japan
FIGUERAS	B.1	1	spiral worms	yes	yes	yes(18S)	yes	*Spirorbis spirorbis*	***Neodexiospira nipponica***	93	99%	X	NIS	Japan
ARBEYAL	B.1	1	barnacle (small)	yes	yes	yes	yes	?	***Chthamalus stellatus***	96	98%	X	not invasive	X
		2	barnacle (small)	yes	yes	yes	yes	?	***Chthamalus stellatus***	97	97%	X	not invasive	X
		3	barnacle (mix)	yes	yes	yes	yes	?	***Chthamalus stellatus***	98	98%	X	not invasive	X
		4	worm	yes	yes	yes(18S)	yes	*Pomatoceros triqueter*	***Pomatoceros triqueter***	91	95% (Pomatoceros lamarkii)	X	not invasive	X
		5	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X

BAÑUGUES	R.1	1	briozoo (orange)	yes	yes	no	no	?	X	X	X	X	X	X
		2	briozoo (white)	yes	yes	no	no	?	X	X	X	X	X	X
		3	briozoo (pink)	yes	yes	no	no	?	X	X	X	X	X	X
		4	briozoo (white)	yes	yes	no	no	?	X	X	X	X	X	X
		5	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		6	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		7	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		8	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
	R.2	1	briozoo (pink)	yes	yes	no	no	?	X	X	X	X	X	X
		2	briozoo	yes	yes	no	no	?	X	X	X	X	X	X
		3	worm	yes	yes	no	no	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		4	terebelid	yes	yes	yes(18S)	yes	?	***Sabellaria cementarium***	**87**	**89% (Sabellaria intoshi)**	X	not invasive	X
		5	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X
		6	worm	no tissue	X	X	X	*Pomatoceros triqueter*	X	X	X	X	not invasive	X