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The use of ecological studies, genetic tools, and citizen science to assess the biological invasion status of the green seaweed *Codium fragile* subsp. *fragile* (Suringar) Hariot 1890 in the southern area of the Bay of Biscay (Cantabrian Sea).

El uso de estudios ecológicos, herramientas genéticas y ciencia ciudadana en el estudio y evaluación de las invasiones biológicas de la macroalga verde *Codium fragile* subsp. *fragile* (Suringar) Hariot 1890 en la zona sur del Golfo de Vizcaya (mar Cantábrico).

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Roberta Skukan

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: El uso de estudios ecológicos, herramientas genéticas y ciencia ciudadana en el estudio y evaluación de las invasiones biológicas de la macroalga verde <i>Codium fragile</i> subsp. <i>fragile</i> (Suringar) Hariot 1890 en la zona sur del Golfo de Vizcaya (mar Cantábrico).	Inglés: The use of ecological studies, genetic tools, and citizen science to assess the biological invasion status of the green seaweed <i>Codium fragile</i> subsp. <i>fragile</i> (Suringar) Hariot 1890 in the southern area of the Bay of Biscay (Cantabrian Sea).

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RESUMEN (en español)

Codium fragile subsp. *fragile* Suringar (Hariot) 1890 está catalogado como uno de los 100 peores invasores de Europa. Cinco especies de *Codium* se registran en la fauna intermareal marina a lo largo de la costa cantábrica, como nativas (*C. tomentosum*, *C. decorticatum*, *C. vermilara* y *C. adhaerens*) y subespecie *C. fragile* subsp. *atlanticum*. Creamos una base de datos de registros anteriores sobre la distribución de especies exóticas y nativas porque puede proporcionar un punto de partida para una mejor comprensión de la distribución espacial de algas nativas e invasoras en la costa norte de España, actualizando el estado actual de la *C. fragile* para el futuro propósito de vigilancia. La identificación de las especies es importante, con el código de barras de ADN, para el monitoreo y la gestión, especialmente en el caso de las macroalgas marinas, donde la identificación morfológica suele ser ambigua. No se ha llevado a cabo un monitoreo intensivo de los estudios sobre las identificaciones genéticas de las especies de *Codium* y la diversidad genética a lo largo de la costa cantábrica. En España, las actividades de ciencia ciudadana dirigidas a los niños como los principales participantes en las encuestas ambientales marinas son bastante escasas. El juego educativo '*Encontrar algas invasoras*', desarrollado durante este estudio, se incluyó en un proyecto de ciencia ciudadana marina que vinculó a estudiantes de secundaria con investigadores de la Universidad de Oviedo.

Recopilamos y analizamos una base de datos de investigaciones previas sobre algas marinas por investigadores de la Universidad de Oviedo (Asturias, 2002-2003) y nuestro estudio (Asturias, 2016-2017). Los ecosistemas costeros se encuentran entre las áreas más invadidas del mundo. Nuestros resultados sugieren que los factores ambientales podrían influir en los conjuntos de algas marinas en una escala espacial diferente. Detectamos diferencias significativas en la variabilidad espacial entre las playas y entre los sitios dentro de las playas observados tanto para especies exóticas como para de especies nativas. Las algas nativas eran dominantes en todos los lugares de muestreo. La propagación de *C. fragile* no mostró ningún aumento ni disminución en los 15 años. Esto indica que las comunidades de algas nativas todavía están bien establecidas a lo largo de la costa de Asturias.

También evaluamos la probabilidad de detección de *C. fragile* y su distribución espacial



se compara con las especies nativas de *Codium* en el mar Cantábrico. *Codium* nativo spp. fueron dominantes en el este (19.2% de *C. fragile* y 80.7% de *Codium* spp. nativo) y en la zona oeste (12% de *C. fragile* y 88% de *Codium* spp. nativo). Se encontraron diferencias significativas en la proporción de *C. fragile* a ambos lados del cabo Peñas, lo que indica que la combinación de factores ambientales, las playas menos expuestas hacia la parte oriental del mar Cantábrico y las temperaturas más altas, podrían proporcionar un hábitat más favorable para su establecimiento. La probabilidad de detección de invasores no se vio influenciada por las escalas espaciales en nuestro diseño jerárquico, lo que infiere que el muestreo aleatorio de playas para 100 plantas debe usarse como un umbral de distribución en los futuros monitoreos para la evaluación de la proliferación de *C. fragile*.

Actualizamos los conocimientos sobre la presencia de especies de *Codium*, mediante estrategias de código de barras basadas en marcadores moleculares de plástidos y nuevos protocolos de identificación genética para materiales de herbarium. Se identificaron tres especies de *Codium*: *C. fragile* subsp. *fragile*, *C. tomentosum* y *C. vermilara* utilizando las secuencias *tufA* y *rbcL*. Se encontraron niveles bajas de variación genética poblacional para las especies de *Codium* nativas / invasoras examinadas, lo que sugiere mecanismos sexuales y de propagación similares para todas las especies. El marcador FCO*tufA* de nuevo diseño fue útil para el código de barras de ADN de muestras recientes y de herbario. Se encontraron dos haplotipos diferentes de *C. fragile* dentro del clado invasor, incluido un nuevo haplotipo para una muestra de rango nativo, Corea del Sur.

Se diseñó un juego para enseñar a los niños a reconocer las algas invasoras y animarlas a participar en actividades de ciencia marina ciudadana. El juego se probó por primera vez en un estudio piloto y luego se llevó a cabo en el Colegio Público de Salinas (Asturias). El juego consistió en una explicación de las especies invasoras y se evaluó con pruebas. Los resultados mostraron un impacto positivo en las ganancias de conocimiento y diferencias significativas en la conciencia ambiental marina y las acciones de conservación.

RESUMEN (en Inglés)

Codium fragile subsp. *fragile* Suringar (Hariot) 1890 is listed as one of the top 100 worst invaders in Europe. Five *Codium* species are recorded in marine intertidal fauna along the Cantabrian coast, as native (*C. tomentosum*, *C. decorticatum*, *C. vermilara*, and *C. adhaerens*) and one subspecies *C. fragile* subsp. *atlanticum*. Here, creating a database of previous records on the distribution of nonindigenous and native species can provide a starting point for a better understanding of native and invasive seaweed spatial distribution in the North Coast of Spain, updating the present status of the invasive *C. fragile* for future surveillance purpose. Accurate species identification, with DNA barcoding, is important for monitoring and management decision, especially in the case of invasive marine macroalgae, where morphological identification is often ambiguous.

We gathered and analyzed a database of previous research on native and invasive seaweeds by researchers from University of Oviedo (province Asturias, 2002) and our study (province Asturias, 2016-2017). Coastal ecosystems are among the most invaded areas in the world. Our results suggest that environmental factors could influence seaweed



assemblages on a different spatial scale. We detected significant differences in spatial variability among beaches and among sites within beaches observed for both nonindigenous and native species. However, native seaweeds were still dominant in all sampling locations. The spread of *C. fragile* did not show any increase nor decrease in the 15 years. This indicates that native seaweed communities are still well established along the coast of Asturias.

As well, we assessed the detection probability of *C. fragile* and its spatial distribution is compared to native *Codium* spp. in the Cantabrian Sea. Native *Codium* spp. were dominant in the east (19.2% of *C. fragile* and 80.7% of native *Codium* spp.) and the west zone (12% of *C. fragile* and 88% of native *Codium* spp.). Significant differences in the proportion of *C. fragile* reported from both sides of Cape Peñas were confirmed, indicating that mix of environmental factors, less exposed beaches toward the eastern part of the Cantabrian Sea and higher temperatures, could provide solid habitat for its establishment. Detection probability of invader was not influenced by spatial scales in our hierarchical design inferring that random sampling of beaches for 100 plants should be used as a distributional threshold in the future monitorings for the assessment of *C. fragile* proliferation.

We updated current knowledge about the presence of native and invasive *Codium* species, using barcoding strategies based on previous plastid molecular markers and new genetic identification protocols for herbarium material. Three different *Codium* species: *C. fragile* subsp. *fragile*, *C. tomentosum*, and *C. vermilara* were identified using *tufA* and *rbcL* sequences. Low levels of genetic variation for native/invasive *Codium* species analyzed was detected suggesting a similar sexual and propagation mechanisms for all the species. The newly designed FCO*tufA* marker was useful for barcoding herbarium and fresh samples. Two different *C. fragile* haplotypes within the invasive clade, including one new haplotype for a South Korean sample.

A game was designed to teach children how to recognize invasive seaweed and to encourage them to participate in marine citizen science activities. The game was first tested in a pilot study, and then it was carried out in Salinas High School (Asturias, Spain). Game-based training consisted of an explanation of invasive species and was evaluated with tests before and after the activity. Results showed a positive impact on knowledge gains and significant differences in marine environmental awareness and conservation actions.

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GENERAL INTRODUCTION

INVASIVE SEaweEDS

Algae are simple nonvascular photosynthetic organisms yet widely adopted and distributed, in almost every habitat around the world (Cocquyt, 2008). Between taxonomical groups, different species vary in size (from microalgae to giant kelps) and have very diverse morphological traits that often disenable algae identification (Bold and Wynne, 1985; Saunders, 2005). Seaweeds are macroalgae that grow attached to a hard substrate in the marine environment (Diaz-Pulido and McCook, 2008). As important drivers of ecosystem functioning (Egan et al., 2012; Wernberg and Straub, 2016), seaweed communities have a significant functional (as primary producers) and integral (habitat forming) part in marine ecosystems (Mineur et al., 2015). They prevent nutrients loading, anchor sediment (Duarte, 1995) and provide breeding, feeding and hiding ground for many marine organisms (fishes, invertebrates) (Ferrer, 2002; Bailey and Owen, 2014). Thus, displacement or retreat in a seaweed assemblage can have a direct or indirect influence on other marine flora and fauna communities (Schaffelke and Hewitt, 2007). In the light of that, biodiversity and ecosystems interactions concept needs to be perceived for minimizing negative shifts in ecosystem functioning (Elmqvist et al., 2010; Science for Environment Policy, 2015). Apart from global warming and habitat exploitation, an Invasive Alien Species (IAS) pose a serious threat to ecosystems worldwide (Doelle, 2007), causing detriment shifts in the natural balance (Elmqvist et al., 2010; Science for Environment Policy, 2015) and inflict loss of biodiversity (McNeely et al., 2001; Molnar et al., 2008). The term IAS refers to a species introduced intentionally or unintentionally outside of their native (donor) area, displaying negative impacts on the level of biodiversity, ecosystem functioning and sustainable community development of invaded (recipient) area (Olenin et al., 2010; Wilson et al., 2009). IUCN (GISP, 2008) states that the degradation of habitat is affecting biodiversity in a proportional level as a biological invasion. Although introduced, not all species have to become invasive and are called Nonindigenous Species (NIS) (Olenin et al., 2010). Environmental conditions in recipient area (e.g. climate, water chemistry, currents) influence nonindigenous marine species (NIMS) establishment and define the last step in the efficacy of some species becoming

invasive (Gallardo and Aldridge, 2013a). International Maritime Organization (IMO), specialized UN agency with responsibility for the marine safety, security of shipping and the prevention of marine pollution by ship, states that biological invasion is growing and presents one of the greatest threats to the ecological and the economic well-being of the planet. Around 7000 coastal and marine species are traveling through ballast water in tanks every day undetected, with a high risk of becoming invasive (WWF, 2009). Seaweeds are recognized as a major constituent of NIMS (Schaffelke et al., 2006), with continuous raising number of global estimates for introduced macroalgae from 163 (Ribera Siguan, 2002) to 277 (Williams and Smith, 2007). In ALIENS, a project about invasive seaweed distribution along the coastline of six European countries, 15 taxa were recorded in 90 l of ballast water (Guala et al., 2003). EU biodiversity strategy to 2020 draws attention on IAS, encouraging the Commission and Member State to establish a cost-effective and environmentally desirable measure on prevention, monitoring, eradication, and management on invasive alien plant and animals. *Regulation EU No 1143/2014* notes that contribution of the scientific community is important due to their constant effort invested in stressing the problems raised by IAS and implementation of a risk assessment (assessment of potential pathways, the likelihood of introduction, establishment, and propagation of IAS in biogeographical regions and accompanying climate change). Thus, it is necessary to place legislative measures targeting prevention (before IAS occur) and management (when IAS occur and spread in the recipient area). Comprehension about consequences of invasive seaweeds is considered to be one of the least investigated compared to other nonindigenous taxa (Schaffelke and Hewitt, 2007) and more scientific research calls for their attention (Inderjit et al., 2006). Introduced intentionally (for aquaculture purpose) or unintentionally (common for many seaweeds, ballast water or more important biofouling - on a surface of hull vessel, large ships, yacht, smaller crafts, the shell of oysters etc) (Johnson and Chapman, 2007; GISP, 2008), invasive seaweeds can have a negative consequences on native flora and fauna inside the area they are occupying (Schaffelke and Hewitt, 2007) because of the competition for the living space and food resources. Regardless of prevention efforts, IAS do occur outside of their native range and accordingly, it is important to detect them early (Kaiser and Burnett, 2010). Urgent need calls for the development of risk assessments, to identify consequences of marine biological invasions (Hewitt and Campbell, 2005). NIS are usually problematic and costly to eradicate when their populations grow out of control and when they spread to a large spatial magnitude (Hyder et al., 2008). Thus, management

decisions should be dynamic and incorporate the fact that if control is effective, the environment should change; as the environment changes, so too should control efforts (Frei and Scär, 2002; Hyder et al., 2008).

CODIUM FRAGILE SUBSP. *FRAGILE* (SURINGAR) HARIOT (1890)

The genus *Codium* belongs to a group of green siphonous macroalgae (order Bryopsidales), counting roughly 150 species around the globe (Goff, 1992; Verbruggen et al., 2007). The principal axis (thallus) comes in a variety of shapes; erect with cylindrical or flat-like branches to spherical and ground-hugging mat thallus, without branches (Maggs and Kelly, 2007; Verbruggen et al., 2007). The common feature of all known *Codium* species is an interior structure of thallus: multinucleate cells called siphons, intricate in the central part (medulla) and narrow wrapped siphons (utricule) in the cortex (Borden and Stein, 1969; Verbruggen et al., 2015). They are mostly marine inhabitants, attached to rock substratum from the mid-littoral to sublittoral (up to 200 m depth) (Burrows, 1991; Pedroche et al., 2002). Some species can be found in estuaries (Huisman et al., 2015). *Codium fragile* subsp. *fragile* (Suringar) Hariot (1890) (hereafter *Codium fragile*) is a widely distributed species inside genus, listed as one of the top 100 worst invaders in Europe (Galil, 2006) among all invasive seaweeds (Nyberg and Wallentinus, 2005) (Fig. 1.). As an example, the Gulf of St. Lawrence (Canada) served as a case study for estimation of *C. fragile* establishment and spreading. Using quantitative risk assessment tool, output data revealed that *C. fragile* is already well established in the Gulf of St. Lawrence and is expected to spread considerably. Overall, the literature review and expert survey suggest that *C. fragile* will have a considerable effect on the ecosystem but less on economic and social sectors (Drouin and McKindsey, 2007). Originally, from Japan (Silva, 1955; Trowbridge, 1995; Provan et al., 2005), it inhabits all continents, besides Antarctica (Goff et al., 1992).

C. fragile first detection in Europe dates from 1900, on the coast of Holland (described by Silva, 1955). In the following 120 years, it was recorded along Atlantic coast of Europe (1939 in British Isle (Devon) (Silva, 1955); 1946 in Norway (Silva, 1955); 1966 in Spain (Pérez-Cirera et al., 1989)), Mediterranean (1946 in France (Verlaque, 1994); 1974 in Sicily (Furnari, 1974); 1992 in Greece (Tsiamis and Panayotidis, 2007)) and Adriatic Sea (2015 in southernmost limit of Italian coast (Bellissimo et al., 2018)).



Figure 1. *Codium fragile* subsp. *fragile* Suringar (Hariot) (1890)

C. fragile is asexual and reproduce by parthenogenesis (development from unfertilized or fertilized gametes) (Bridgwood, 2010). Five *Codium* species are recorded in marine intertidal fauna along the Cantabrian coast (Gorostiaga et al., 2004; Cremandes et al., 2005; Cires Rodríguez and Moliner, 2010), considering four species as native (*Codium tomentosum*, *Codium decorticatum*, *Codium vermilara*, and *Codium adhaerens*) and one subspecies *Codium fragile* subsp. *atlanticum* (Maggs and Kelly, 2007), often misidentified as *Codium fragile* due to their cryptic nature, as invasive.

MONITORING AND CONTROL OF INVASIVE SEAWEEDS

In the past, miscellaneous outcomes occurred from physical and mechanical removal attempts from established populations of invasive seaweeds. Manual removal of *Codium fragile* did not seem effective because of its reproductive traits and mechanical removal can reduce the population density temporarily but that is an expensive process and the population is quickly renewed. There is no other attempt of *C. fragile* removal since its physiological traits would, most probably, made it ineffectual (GISD, 2014). In eradication measure of brown seaweed *Sargassum muticum* in Southern California, putty knives were used to scrap a holdfast but in 9 months the population was fully recovered

(Smith, 2015). In New Zealand, mechanical removal of brown seaweed *Undaria pinnatifida* over a long period was successful in reducing recruitment of an isolated population but unsuccessful at locations where repeated introductions occurred (Hunt et al. 2009; Forrest and Hopkins 2013). However, in San Diego, California, rapid response to early detection of green seaweed *Caulerpa taxifolia* resulted in successful eradication by using traps and chlorine treatments (Andreson, 2005). In New Zealand, the invasive kelp *Undaria pinnatifida* was removed from the sunken boat by heat-treatments and established population was never found (Wotton et al., 2004). Cases, as previously mentioned *U. pinnatifida* and *C. taxifolia*, are rare (Johnson & Chapman, 2007) so the following question can be raised: what can be done when there are historical records and obvious evidence of IAS presence?

CBD (Convention on Biology Diversity, 2010) states that prevention of the occurrence is the most cost-effective strategy for NIS management. The expansion rate of NIMS is often much higher than that of terrestrial IAS (Grosholz, 1996). This makes that introductions to coastal areas are likely to have a greater influence on native communities in marine than in terrestrial habitats (Altman and Whitlatch, 2007). Here, systematic monitoring (also called survey or surveillance) provide important information for controlling NIS spread. Monitoring is a systematic long-term continuous collection of the abiotic and biotic data about population distribution, size and impact (Zampoukas et al., 2012; Lehtiniemi et al., 2015). They serve as a base ground for establishing management measures when tackling the dynamics of IAS. Habitat disturbance may create patches of free space or increased resources, which creates opportunities for newly arriving species to establish (Hobbs, 1989; Stohlgren et al., 1999; Alpert et al. 2000; Davis et al., 2000; Clark and Johnston 2005; Belote et al., 2008). Noteworthy, native species richness and disturbance are considered the two most important factors influencing the susceptibility to invasion, of both marine and terrestrial ecosystems (e.g. Olyarnik et al., 2009). Invasibility is the susceptibility of an ecosystem to the establishment and spread of introduced species (Alpert et al., 2000); it is often expressed as the number of NIS in an area. Strong competition effects lead to a negative diversity-invasibility relationship in homogenous ('biotic resistance hypothesis', Elton, 1958) whereas a heterogeneous environment favors co-existence of native and non-indigenous species. The latter results in a higher overall species richness in places with favorable environmental conditions ('the rich get richer', Stohlgren et al., 2003). Herein, monitoring of native species can be

beneficial. While conducting monitoring, researchers could use the opportunity to check whether there is any NIMS present (Lehtiniemi et al., 2015) before they display invasive characteristics.

Many times, the presence of NIS is less obvious due to their limited abundance and/or low impacts on native communities (Yorke and Metaxas, 2011). At this point, the optimal detection strategy of target species depends mainly on 'detectability' (ease of detection) and the biological relations of each distinct species (Mehta et al., 2011). It is calculated as probability (p) variable and used for the estimation of the probability in the detection of rare species, as well as IAS. It is not yet quite clear how spatial heterogeneity affects the detection probability of IAS (Hauser and McCharty et al., 2009). Therefore, monitorings accompanied with additional biological and environmental parameters to discern distributional patterns have also proved meaningful. This scientific-based approach can reveal the small spatial scale preferences of invaders, such as *C. fragile*, which colonize and grow better on the sheltered sections of artificial structures compared to exposed sections (Bulleri et al. 2006). The ability of monitoring to disclose relevant processes can frequently be scale dependent. Small-scale monitoring deduce the invasion history at particular sites but large-scale monitoring can show regional patterns of range, dispersal and habitat preference (Gust and Inglis 2006).

The complex structure of coastal zones and a broad range of substrate in the south area of Bay of Biscay (the Cantabrian Sea, North Coast of Spain) provides living ground for a diverse flora and fauna communities (Sánchez and Olaso, 2004), such as seaweed assemblages (Borja et al., 2004). The Cantabrian Sea is a mountainous and coastal region, with a coastline stretching around 800km, in the warm-temperate area of the Atlantic Ocean (Álvarez et al., 1988). Various ecological studies along the coast of Cantabria have indicated the influence of longitudinal position (following east-west coastline) on macroalgal composition and abundance (Anadón and Niell, 1981; Arrontes, 1993). The underlying reason for this could be the geographical position of the coast or anthropogenic influence (García et al., 2011). Furthermore, other studies have shown that increasing sea surface temperature (SST) is a major cause for spatial changes in native benthic communities in the Cantabrian Sea (Voerman et al., 2013; Fernández, 2016). Thus, for seaweed-dominated systems, precise prediction tools should be developed and enable tracking ecological effects of climate change for conservation and management purposes

(Harley et al., 2012). It is supposed that IAS will benefit from direct impacts of climate change (Low, 2008) and manifest negative impacts for native species, hence they need to be closely monitored (Occhipinti-Ambrogi, 2007).

THE UTILIZATION OF GENETIC TOOLS IN INVASION BIOLOGY OF SEAWEEDS

The application of molecular methods is becoming more relevant since they can contribute to invasion risk assessments (Geller et al., 2015) and rapid detection of NIMS (Montes et al., 2016). Nowadays molecular techniques (such as DNA barcoding) demonstrate great importance for marine biodiversity assessment and conservation, especially in the risk assessment and control of NIS (Trivedi et al., 2016). DNA barcoding is a molecular method that enables species recognition from a short sequence of a well-marked gene region (Purty and Chatterjee, 2016). It reduces the time usually needed to distinguish species where morphological plasticity hampers proper identification (Conklin et al., 2009) and has proved to be highly effective in brown (McDewitt and Saunders, 2009), red (Saunders, 2005) and green (Saunders and Kucera, 2010) seaweeds. Around 100 *Codium* spp. reported by Goff et al. (1992), increased up to around 150 species (Verbruggen et al., 2007), and with respect to accessible molecular tools (Oliveira et al., 2012). Ten subspecies of *C. fragile* were identified worldwide, with the help of *tufA* and UCP6 plastid markers (Maggs and Kelly, 2007; Armitage and Sjøtun, 2016). As well, the first record of *C. fragile* in Western Australia was supported using UCP6 plastid marker (McDonald et al., 2015). Invasive red seaweed *Gracilaria vermiculophylla* was screened for the first time in the waters of British Columbia during routine marine flora surveys with mitochondrial markers (Saunders, 2009). Manghisi et al. (2010) emphasize that DNA barcoding is a very beneficial tool for rapid detection of invasive seaweeds by confirming the presence of red seaweed *Agardhiella subulata* in Italy for the first time, which would be impossible just from morphological identification due to the immature stage of the specimen.

The donor area and route of IAS are overflowing in a geographical and gene structure of introduced species, therefore, they need to be acknowledged for effective management strategies (Boothe et al., 2007). By using microsatellite and plastid marker, Provan et al., (2004) state that there was most probably only one introduction event of *C. fragile* from donor region (due to a high degree of geographical structuring of haplotype distribution

in NW Atlantic coast of Europe and the Mediterranean Sea) as a result of maritime traffic. Phylogenetic and phylogeography studies can help to detect donor of NIMS and trends in biological invasions (Provan et al., 2005). Hence, this knowledge can be provided from population genetic studies accompanied by molecular markers as nuclear, mitochondrial, chloroplast DNA, for effective management strategies. The marine environment is one of the most interesting environments to investigate invasive species because it enables their dispersion capacity, diversity of life-history traits type and frequency of introduction vectors (Rius et al., 2015). The cryptic invasion, that some seaweeds, like in the case of *C. fragile* and its subspecies *atlanticum*, are prone to, is a subject of pertinent relevance because it could lead to unserviceable risk assessments and misassumptions about the ecological feature and consequences of invasive species (Geller et al. 2009).

CITIZEN SCIENCE

Citizen science (CS) comes from the participation of citizens in scientific research projects (Bonney et al., 2009). CS has recently emerged a tool to involve people but now in a new definition as a citizen scientist (Science Communication Unit, 2013). Today, CS projects involve a broad range of research subjects regarding the preservation of marine and terrestrial environments e.g. IAS monitoring (Scyphers et al., 2015). The appearances and propagation of NIMS frequently go undetected, as only a few people are skilled to perceive underwater events, including e.g. marine scientists and SCUBA divers (Poursanidis and Zenetos, 2013). Furthermore, especially in the last years, NIS have received more attention due to the rising interest and focus on protection and prevention measures accompanied by increasing ecological awareness (e.g. from the public) (Davis et al., 2018; de Sá Dechoum et al., 2018). Every volunteer must be conscious about the detrimental consequence of biological invasions and their contribution cannot be neglected, especially where scientific activities are confined due to available human resources (Thiel et al., 2014). Often scientists from a different branch of occupation interact and this nexus is like a reversible reaction: both sides will eventually benefit from knowledge exchange. Citizen scientists are actively involved in a marine conservation project, thus any kind CS activity must have a sociological aspect that enables the knowledge trade between scientist and common citizens. Training authorities in sampling methods that are easy to approach, and public consciousness can enable a rapidity of IAS

identification (Roux & Wicczorek, 2008). For unintentional introductions, as it is in a case of most invasive seaweed, quarantine end-points (prevention of the introduction and spread of NIMS) and public education are probably the most effective model for risk assessment (Galil, 2006). Raising public awareness about the invasive seaweed occurrence is important and aided by sailors, fishers, and divers (Meinesz, 2007). For example, the U.S. Environmental Protection Agency and the Hawai'i Department of Land and Natural Resources made easy educational gadgets (waterproof cards for boaters, divers and anglers) for Hawaii IAS monitoring (Smith et al., 2002). Meinesz (2007) noted that around 2000 pressed articles related to *C. taxifolia* invasion, which have been published by media, are obtained due to an informed public. In addition, testing of ballast water treatment methodologies and ballast water sampling may require co-operation with other stakeholders (involved in the industry) (Ojaveer et al., 2015). Raising public awareness should cover and be accessible to all age groups, for example, school students. The enrollment in a big-scale sampling of marine litter along the coast of Chile resulted in 96% (<5000) of the school students telling that they would like to participate in future CS activities (Eastman et al., 2014). Noteworthy, school programs can benefit from scientific inputs because of participation in the CS projects, if participants are conscious about the point of the game and purpose of their involvement (Hofstein & Rosenfeld, 1996). Learning can stimulate knowledge gain by collaborative participation through games and citizens can benefit, especially from group activities (Ernst and Colthorpe, 2007). Games provide many of the essential affordances that are needed for learning and present a possibility to use the interests of children as a method to educate them in a situated manner for the sort of skills increasingly needful for thriving in a globalized world (Foreman et al., 2004; Barab et al., 2007). Salinas (North Coast of Spain, region Asturias) is a parish under the council of Castrillon. The population of Salinas counts around 4500 inhabitants that live quite near the coast. The younger population is presented in a minor percentage of the total population and there is only one school (Salinas High School), with an average number of 15 students per classroom (Fig. 2.). During summer, many events (festivals) take place in Salinas, with educational programmes for children ('*Aula del Mar*'). Many of the children are actively involved in marine activities, especially swimming and surfing, some even in SCUBA diving.



Figure 2. Researchers from the University of Oviedo giving a class about invasive seaweeds in the Cantabrian Sea in Salinas High School (Asturias)

The accessibility of references for citizen science activities in Spain, focusing children as the target-group involved in an environmental monitoring's or surveys are is on the promising sunrise as scientific staff becomes conscious that younger generations must be enlightened by long-term nature conservation and its constant threats.

GENERAL AIMS OF THE THESIS

The general aim of this doctoral thesis is to study the application of ecological, genetics and public outreach approach for the assessment of current and future control of well-established invasive green seaweed *Codium fragile* subsp. *fragile* (Suringar) Hariot 1890 in the South Bay of Biscay (the Cantabrian Sea, North Coast of Spain). To achieve this general purpose, this study was divided into four chapters, with the following objectives:

1. To combine studies from other projects and our datasets of nonindigenous and native seaweed communities and assess the susceptibility to invasion proliferation along the coast of Asturias for prioritizing management measures, focusing on *C. fragile* subsp. *fragile*.
2. To describe the distribution pattern of *Codium* spp. and detection probability of *Codium fragile* subsp. *fragile* on different spatial scales using DNA barcoding, environmental variables and data simulations which could serve as a groundwork for developing future monitoring strategies.
3. To update current knowledge about the presence and genetic diversity of native *Codium* spp. and *Codium fragile* subsp. *fragile* using barcoding strategies based on plastid molecular markers. In addition, we aim to design new genetic identification protocols using smaller fragments from the *tufA* genetic marker for effective identification of the invasive *C. fragile* species on this type of seaweed materials.
4. To motivate and test the knowledge increase of the general public, particularly younger generations, about negative impacts of invasive seaweeds using educational game-based approach.

CHAPTER 1.

HISTORICAL EVIDENCE SUGGEST THAT THE PRESENCE OF NONINDIGENOUS SPECIES DID NOT ALTER WELL ESTABLISHED NATIVE SEAWEED COMMUNITIES ALONG THE COAST OF ASTURIAS, SOUTH AREA OF THE BAY OF BISCAY

INTRODUCTION

Competitive dominant IAS can affect the abundances of less competitive native species communities (Rodriguez, 2006) perpetuating unwanted ecological and economic consequences for ecosystem services (Pejchar and Mooney, 2009). Successful invasions are numerous but can be classified at two levels. First through competition; inter- and intraspecific interactions in recipient habitats might change; this is indicated by a high abundance of the invaders, the resultant monopolization of space and reduce abundance and biomass of native species (Schaffelke and Hewitt, 2007). Second by means of predation, these changes in community structure and composition may affect higher trophic levels by altered food-web dynamics, toxicity and/or habitat modification (Schaffelke and Hewitt, 2007). Notwithstanding the increased documentation on the negative effects imposed by introduced species, it needs to be highlighted that not all NIS cause major problems. Most introductions pass by unnoticed, as they are merely an addition to the local species richness (cf. ‘tens rule’ by Williamson and Fitter 1996, Reise et al. 1999). Nevertheless, recent insights into the importance of alternative stable states and tipping points exposes the importance of thresholds in invasion biology (CBD, 2010). In this sense, biological invasions are not always the cause (*drivers*) of ecosystem degradation but often the consequence (*passengers*) of the ecosystem’s response to global change (MacDougall and Turkington, 2005). Reports of the negative impact of invasive seaweeds are increasing worldwide (Dijkstra et al., 2017) thus if suitable prevention and management measures are not implemented, their number will increase in coming years (Ashton et al., 2012). Herein, long-term monitoring provides guidelines on how to tackle with potentially harmful species (Lee et al., 2008), in alliance with researchers at universities, institutes and research centers (Iriarte et al., 2005). Native species richness

was long thought to be negatively correlated with the success of introduced species. The reasoning behind this is formulated in Elton's biotic resistance hypothesis (Elton, 1958), which states that species-rich ecosystems are more resistant to invaders because the available resources are used more efficiently, leaving fewer free niches for potential invaders. Nowadays, the biotic resistance hypothesis is being questioned as many observational and experimental studies report contradicting outcomes for the correlation between native species richness and invasibility (Stachowicz and Byrnes, 2006). Most observational studies covering a larger spatial scale (landscape scale) find a positive correlation (e.g. Lonsdale 1999) whereas small scale (<1 m², neighborhood scale *sensu* Levine, 2000) observational surveys and experimental studies mostly find a negative correlation or no correlation at all (e.g. Stachowicz et al., 1999; Levine, 2000; Fargione et al., 2003). Here, invasibility could provide deeper knowledge about the relationship between NIS and native communities, reflecting the stability of a certain ecosystem. At small spatial scales, native communities could provide a buffer against the spread of newly introduced species by a (nearly) complete utilization of the limiting resources (Post and Pimm, 1983; Law and Morton, 1996). Alternatively, a more positive relationship can be expected at larger spatial scales as mild environmental conditions, high availability of resources and/or habitat heterogeneity may support high numbers of both non-indigenous and native species. Davies et al. (2005) found a similar pattern, studying terrestrial plants, of a negative relationship between NIS and native diversity at small spatial scales with a positive relationship at larger spatial scales. For seaweed communities on rocky shores, free space is often a limiting resource (Dayton, 1971; Altman and Whitlatch, 2007) and thus disturbance effects, which create free patches, allow colonizers, including NIS, to establish. This opportunistic behavior was previously recorded in other invasive seaweeds along the coast of Europe: *Caulerpa racemosa* in Italy (Piazzi et al., 2001a; Ceccherelli and Campo, 2002), *Caulerpa taxifolia* in France and Italy (Boudouresque et al., 1992; Piazzi et al. 2003), *Fucus evanescens* in Sweden (Wikström and Kautsky, 2004), *Sargassum muticum* in Italy and Denmark (Curiel et al., 1998; Stæhr et al., 2000), *Womersleyella setacea* in Italy (Piazzi and Cinelli, 2001).

For the last two decades, *C. fragile* has been of scientific and public concern due to its invasive tendency. The impacts of this invader are recorded in many published articles; negative influence on commercial bivalve (Throwbridge, 1998) and cultivation of red algae (Neill et al., 2006), impacts on ecosystem service (Vilà et al., 2010), indirect

detrimental influence on nursery ground for fishes and decapods (Levin et al., 2002) and the retreat of sea urchins (Scheibling and Anthony, 2001). *C. fragile* is considered an opportunistic species, occupying free habitat because of the retreatment of native communities, e.g. it forms stiff meadows that constrain re-colonization by native seaweeds and kelps along Nova Scotia (Scheibling and Gagnon, 2006) and Gulf of Main (Levine et al., 2002). However, more argument and field studies are necessary to define the role of species richness in community resistance versus *C. fragile* invasion (Chavanich et al., 2006)

For this work, we created and analyzed a database of previous research on native and invasive seaweeds along the central coast of northern Spain, from 'ALIENS' project gathered by researchers from University of Oviedo (province Asturias, 2002) and spatial data of *Codium* spp. distribution from Cires Rodríguez and Rico Ordás (2007) (province Asturias, 2002-2003) and our study (province Asturias, 2016-2017). If temporal data were available, they were included in the analysis. We investigated the native and NIS species richness in all localities from 'ALIENS' project. As well, we explored the changes in the proportion of *Codium fragile* at two spatial scales (i.e. beaches and sites) between Cires Rodríguez and Rico Ordás (2007) and our study. Two spatial scales (beach and site) were used as a reference to assess population variability of native and NIS communities from 'ALIENS' project as well as from Cires Rodríguez and Rico Ordás (2007) and our work. This approach can provide a starting point for a better understanding of native and invasive seaweed spatial distribution in the North Coast of Spain, updating the present status of the invasive *C. fragile* for future surveillance purpose.

MATERIALS AND METHODS

GENERAL DESCRIPTION OF ALL STUDIES

The study area is situated in the Northeast Atlantic Ocean along the northern coastline of Spain (province of Cantabria, Asturias, and Galicia). The North Coast of Spain is characterized by powerful swells generated over the Atlantic. The upper intertidal zone is dominated by invertebrates whilst the mid- and low intertidal zones are dominated by seaweeds. No substantial differences in topography, tidal regime and weather conditions

appear along the coast of Asturias (Arrontes and Anadón, 1990). The maximal tidal range in the sampled area is 4.5 m. The sampling localities were distributed along 233 km along the Cantabrian coastline. The survey was done in the form of a parallel transect, following the shoreline. In each study, random sampling was conducted, in a form of hierarchical sampling design. Each beach was divided into three sites, that were randomly selected. Sampling was carried out in the low intertidal when a natural slope was present or in the upper subtidal zone. The time and location of all sampled beaches are indicated in Table 1. All statistical analysis was done in PAST software ver. 3.20 (Hammer et al., 2001).

'ALIENS' PROJECT

The data from 'ALIENS' project were based on visual inspection of native and invasive seaweeds (binary data). Conspicuous seaweeds were defined as large algae (>5 cm) attached to the primary substratum (no epiphytes) *or* small (<5 cm) algae growing in dense patches with a primary cover of at least 25 cm². At each locality a hierarchical sampling design was implemented: three sites of ±15 m shore stretch were randomly selected and within each site, three 50×50 cm quadrats were randomly placed. The non-indigenous seaweeds were denoted using (1) a checklist for non-indigenous seaweeds of the Atlantic shores from the ALIENS (Algal Introductions to European Shores) project compiled by Boudouresque and Verlaque (unpublished) based on a checklist published by Wallentinus (2002) and (2) the online database DAISIE (European Invasive Alien Species Gateway, 2012).

The differences in the number of native and non-indigenous richness between beaches and site localities were investigated using one way ANOVA. To meet the assumptions of ANOVA and avoid homoscedasticity, data were square root transformed (Shapiro-Wilk's test ($p>0.05$)). The correlation between the native and non-indigenous species richness was analyzed using simple linear regressions (SLR), at two spatial scale-beaches and sites inside beaches.

A SAMPLING OF *CODIUM* SPP. ALONG THE ASTURIAN COASTLINE

The differences in a population variability of invasive *C. fragile* has been assessed on a two different spatial scale: beaches and sites. Beaches were randomly selected and

divided into three sites, 30×30 m shore stretch. Historical data could provide a vivid answer about positive and negative outcomes between receipt community and invasive species (Trowbridge, 2001). The available data about the distribution of *Codium* spp. from the Asturian Coast of the Cantabrian Sea by Cires Rodríguez and Rico Ordás (2007) enable us to compare relative abundances of *Codium* spp. from the past (2003-2005) in comparison with this study. The relationship between *C. fragile* and time of sampling (presented in years) was analyzed using simple linear regressions (SLR), at two spatial scale-beaches and sites inside beaches with Cires Rodríguez and Rico Ordás (2007) and our data.

RESULTS

NATIVE COMMUNITY STRUCTURE AND NIS RICHNESS IN 'ALIENS' SURVEY

Across all localities from 'ALIEN' project, 139 native taxa were identified, including 100 Rhodophyta, 20 Ochrophyta, and 19 Chlorophyta. The most common species were: *Ulva rigida* C. Agardh 1823 (present in all sites), *Mesophyllum lichenoides* (J. Ellis) Me.Lemoine 1928 (27 out of 33 sites), and *Corallina elongata* J. Ellis and Solander 1786 (24 out of 33 sites).

In total 8 NIS were found in the 33 sites from all the localities, namely: *Anotrichium furcellatum* (J. Agardh) Baldock 1976, *Antithamnionella ternifolia* (J.D. Hooker and Harvey) Lyle 1922, *Asparagopsis armata* Harvey 1855, *Codium fragile* (Suringar) Hariot 1889, *Colpomenia peregrina* (Sauvageau 1927), *Neosiphonia harveyi* (J.W. Bailey) M.S. Kim, H.G. Choi, Guiry and G.W. Saunders 2001, *Sargassum muticum* (Yendo) Fensholt 1955 and *Undaria pinnatifida* (Harvey) Suringar 1873. Both locality and site influenced seaweeds assemblages (ANOVA; $F=209.4$; $p<0.001$ and $F=338.5$; $p<0.001$, respectively) indicating spatial variability in the occurrence of NIS and native communities. The accumulative NIS richness per locality was on average 2.73 ± 1.35 and ranged from 1 in Viavelez and Percebera to 5 in Cudillero.

The negative correlation between native and non-indigenous species richness was detected at beach level ($p=0.07$; $R^2=0.306$) (Fig. 1). A same negative correlation was

detected between sites, only with significant values ($p=0.006$; $R^2=0.213$) (Fig. 2), respectively. The R^2 values (coefficient of determination) ranged from 0.213 to 0.306, meaning that between 21.3% and 30.6% of the variation in NIS richness could be explained by the native species richness. There was no tendency to an increase in slope when moving from sites to beaches.

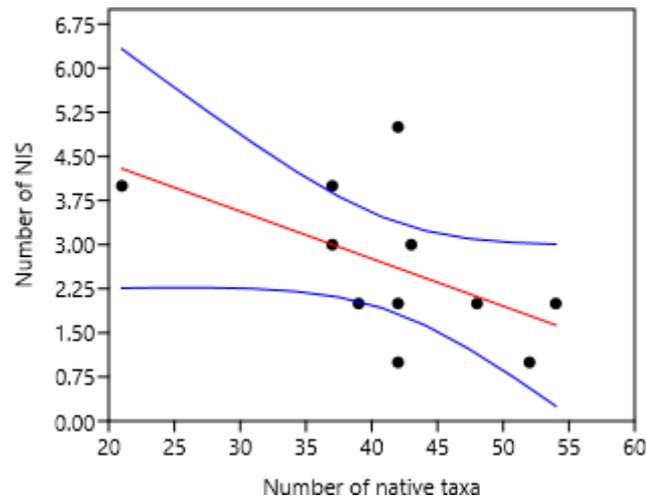


Figure 1. SLR's between the number of NIS and the number of native taxa at the scale of the beach. Red line represents the linear regression model and blue lines are the 95% confidence intervals on the prediction.

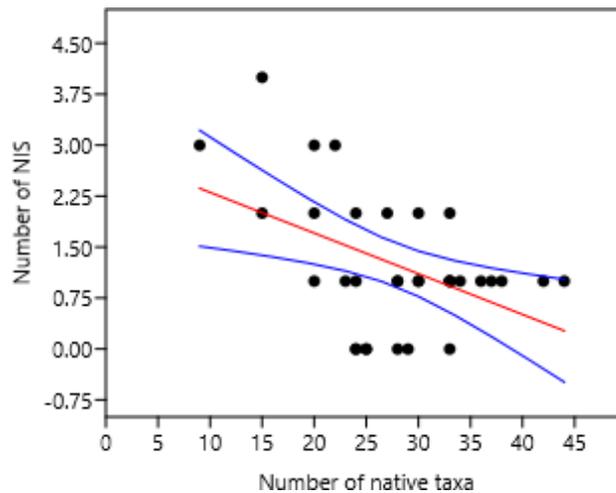


Figure 2. SLR's between the number of NIS and the number of native taxa at the scale of the site. Red line represents the linear regression model and blue lines are the 95% confidence intervals on the prediction.

CODIUM SPP. DISTRIBUTION FROM PRESENT AND CIRES RODRÍGUEZ AND RICO ORDÁS (2007) STUDY

The available data about the distribution of *Codium* spp. from the Asturian coast of Cantabrian Sea by Cires Rodríguez and Rico Ordás (2007) enable us to compare relative abundances of *Codium* spp. from previous (2003-2005) and this study (2016-2017) along the coast of Asturias, showing that until now there is not an evident increase in *C. fragile* (Fig. 3).

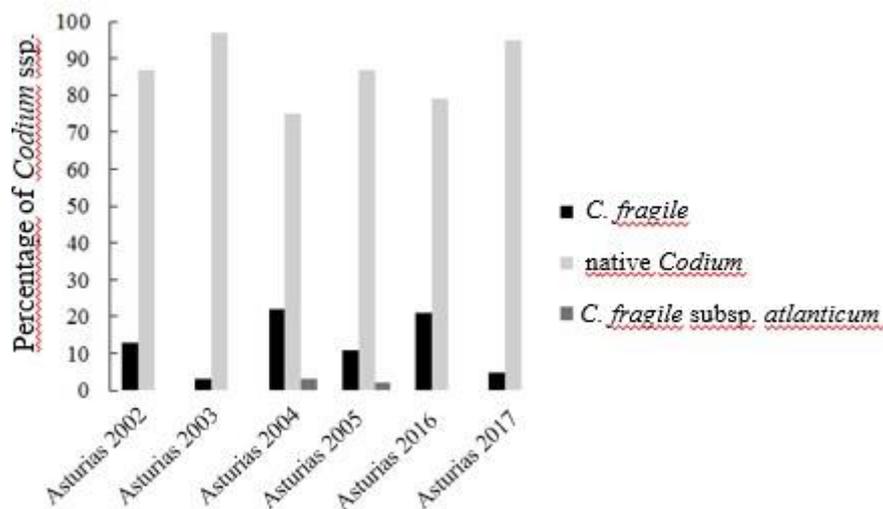


Figure 3. The relative abundance of *Codium* spp. in Asturias, from 2003-2005 (Cires Rodríguez and Rico Ordás, 2007) and 2016-2017 (this study)

The results of simple linear regression indicate there are no significant differences on the scale of beach and site between sampling period (presented in years) from 2002 until 2017 ($p=0.776$, $R^2=0.003$; $p=0.511$, $R^2=0.009$) (Fig.3; Fig.4). The R^2 values (coefficient of determination) ranged from 0.003 to 0.009, meaning that between 0.3% and 0.9% of the variation in *C. fragile* could be explained during those sampling periods. The slope in Fig. 4. indicates there is a slight increase in the proportion of *C. fragile* during years among beaches, however with no significance. The slope in Fig. 5. slightly diminished, which could suggest a decrease of *C. fragile* proportion among sites but again no significant values were detected.

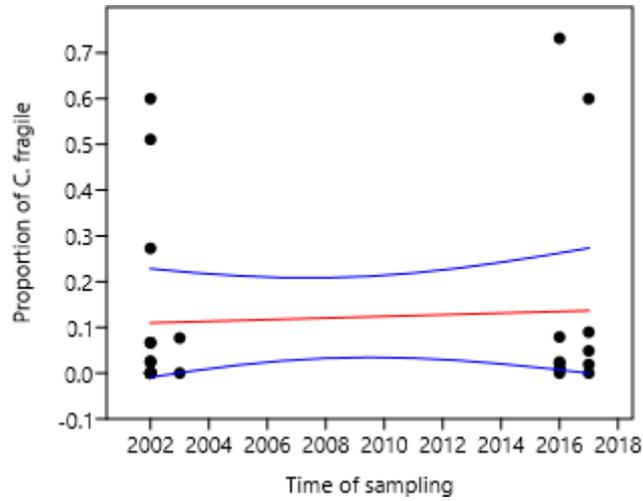


Figure 4. SLR's between the number of proportions of *C. fragile* and the time of sampling (in years) on a beach level. Red line represents the linear regression model and blue lines are the 95% confidence intervals on the prediction.

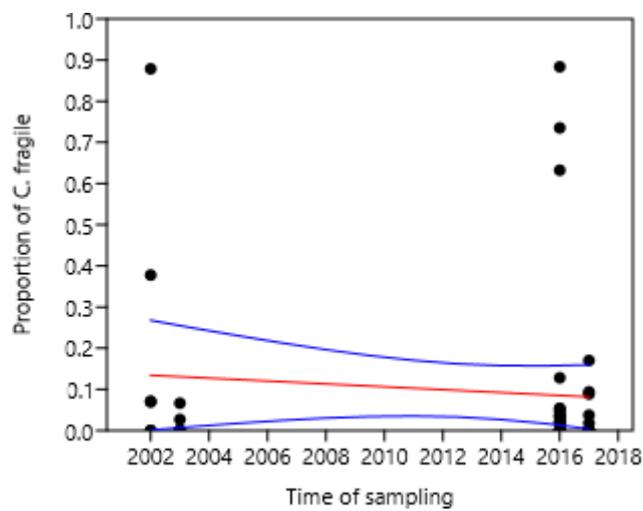


Figure 5. SLR's between the number of proportions of *C. fragile* and the time of sampling (in years) on a site level. Red line represents the linear regression model and blue lines are the 95% confidence intervals on the prediction.

DISCUSSION

Eight NIS (*Anotrichium furcellatum*, *Antithamnionella ternifolia*, *Asparagopsis armata*, *Codium fragile*, *Colpomenia peregrina*, *Neosiphonia harveyi*, *Sargassum muticum*, and *Undaria pinnatifida*) and 139 native seaweed taxa were identified during the sampling along the northern Spanish coast in 'ALIEN' project (2002). All NIS was known to the area prior to 2002. Overall, 5.4% (8 out of 147) of the identified seaweed taxa were non-indigenous. This value is in line with previous studies by Klein *et al.* (2005, the French part of the Mediterranean), Ribera and Boudouresque (1995, Mediterranean) and Mineur *et al.* (2008, Irish sea and the Channel).

Biodiversity loss may have serious effects, not only with regard to a possible decline in the resilience against introduced species (Farrell and Fletcher, 2006) but also concerning the productivity and stability of the ecosystem as a whole (Hooper *et al.*, 2005). We detected significant differences in spatial variability among beaches and among sites within beaches observed for both NIS and native species richness in 'ALIENS' project as well as negative correlation, emphasizing that the native species are well established. This strongly suggests that although spatial heterogeneity, spatial scale, and productivity are all known as important factors influencing the susceptibility of ecosystems to invasion (Davies *et al.*, 2005, 2007), environmental factors and coastal topography could influence seaweed assemblages on a different spatial scale. Substratum availability can influence seaweed species composition and performance (e.g., Lubchenco, 1983; Airoldi, 2000). For example, Morelissen *et al.* (2006) showed that, regardless of disturbance in native seaweeds communities around beaches, the recruitment of invasive *U. pinnatifida* would be a result of available resources of light and space. In addition, the low number of NIS collected in 'ALIENS' project might have lowered the statistical power of the analysis, increasing the probability of a type II error. The significant negative correlation found for native and NIS species richness on a site level supports previously mentioned the idea that native communities could resist the spread of NIS on a small spatial scale, by utilization of the limiting resources (Post and Pimm, 1983; Law and Morton, 1996). Furthermore, the correlation on a beach and site level is not showing any significant decrease or increase in the distribution of *C. fragile* from this and Cires Rodríguez and Rico Ordás (2007) work, meaning that the slope at both levels shows the position that is more horizontal. This proposes that the progression of *C. fragile* is equally maintained

for 15 years. In a prior study, which in particular examined the coexistence of *C. tomentosum* and *C. fragile* along the North Coast of Spain, authors found that native *C. tomentosum* was not outcompeted by the presence of *C. fragile* (Rojo et al., 2014; García et al, 2017). Relative abundances of *Codium* spp. from Cires Rodríguez and Rico Ordás (2007) and this study (2016-2017) along the coast of Asturias, also supports previous results.

The disturbed localities (e.g. harbors) tend to be characterized by a very homogeneous substrate (concrete) whereas the pristine localities have a heterogeneous rocky surface. Homogeneous habitats hold few possibilities for utilizing different resources and therefore competition effects are stronger. The magnitude of the negative effects of seaweed on native community stability tends to augment with human impact levels (Tamburello et al., 2015). Lower native species richness encountered in disturbed (harbors) areas in 'ALIEN' project (Cudillero, Gijón harbor, Tapia) could indicate a loss of biodiversity under the influence of anthropogenic disturbance. Sampling Gijón harbor in 2017, we detected higher numbers of invasive *C. fragile*, compared to native *Codium* spp. Gijón is one of Spain's leading harbors regarding international trade, fishing, and recreational shipping. The surrounding area is an important industrial and urbanized area with a long history of human alterations to the coastal system. Even though this was not the aim of this study, the difference in direction of the diversity-invasibility relationship might be explained by the difference in biotic characteristics between disturbed and pristine localities.

The strong temperature gradient along the Iberian Atlantic coasts forms the southern or northern distribution limit of many organisms, especially in macroalgae (e.g. Lima et al. 2007 and references therein). The role for the behavior of native species in mitigating the effects of habitat-forming invasive species is important, as shown by Wright et al. (2010), where the *C. taxifolia* propagation was determined by available space. Here, an experimental approach would be a valuable addition to future studies to directly link causality and outcome of increasing temperature and NIS spread. Implementing monitoring strategies for controlling the establishment of nonindigenous seaweeds should be, considered a priority for preserving biodiversity. On the other hand, control of invaders at disturbed sites (harbors) should be warranted to diminish their role as propagule sources. The problems, related to invasive seaweeds, are likely to increase in

the future as coastal ecosystems are under ever-increasing pressure due to growing coastal populations in combination with increased trade and industry (MEA, 2005; Altman and Whitlatch, 2007).

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Table 1. List of the sampling localities from our study (2016-2017) and 'ALIEN' project

Sampling location	Region	Latitude	Longitude	Time of sampling
Cangas de Foz	Galicia	43.63044	-7.33456	March 2017
Peizas	Galicia	43.58879	-7.28119	March 2017
Tapia de Casariego	Asturias	43.57023	-6.94391	December 2016
Tapia de Casariego	Asturias	43.57023	-6.94391	April 2017
Cadavedo	Asturias	43.54464	-6.38827	February 2016
Concha de Artedo	Asturias	43.56762	-6.19198	February 2016
Viodo	Asturias	43.64034	-5.84165	February 2016
Gijon Harbour	Asturias	43.55446	-5.69971	June 2016
La Vega	Asturias	43.47485	-5.1346	February 2016
La Isla	Asturias	43.48035	-5.22534	February 2016
Vidiago	Asturias	43.40156	-4.65133	February 2016
Vidiago	Asturias	43.40156	-4.65133	December 2016
Liñera	Cantabria	43.37792	-4.38220	April 2017
San V. de la Barquera	Cantabria	43.38131	-4.39706	April 2017
Viavélez	Asturias	43.56300	-6.83337	July 2002
Cudillero	Asturias	43.34431	-6.85737	July 2002
Lastres	Asturias	43.30571	-5.15191	July 2002
Percebera	Asturias	43.33257	-6.24301	July 2002
Campiello	Asturias	43.34155	-6.17122	July 2002
Novellana	Asturias	43.34451	-6.16472	July 2002
Torbás	Asturias	43.33235	-6.47224	July 2002

CHAPTER 2.

HIERARCHICAL SAMPLING TO UPDATE THE CURRENT DISTRIBUTION PATTERNS AND DETECTION PROBABILITY OF INVASIVE *CODIUM FRAGILE* SUBSP. *FRAGILE* (SURINGAR) HARIOT 1890 IN THE SOUTH AREA OF BAY OF BISCAY, NORTHERN SPAIN

INTRODUCTION

The marine environment represents a natural resource with vital importance for human communities. Currently, it is under serious threats. Invasive seaweeds have a negative repercussion on native flora and fauna inside the newly colonized area (Katsanevakis et al., 2014). For example, it has been reported that the green seaweed *C. fragile* (also referred as invader further in the text) has an opportunistic behavior that resulted in a retreat of native kelp communities (Scheibling and Gagnon, 2006); or that the native fauna community has been negatively affected as a results of native algae extruded by the brown seaweed *Sargassum muticum* (Salvaterra et al., 2013) or finally, that the chemical components of *Caulerpa racemosa* caused negative performance of sea urchins in the Mediterranean Sea (Tomas et al., 2010).

Eradication methods of invasive aquatic taxa appear to be quite limiting compared to terrestrial ones, especially in an open-water body from the marine environment, due to the common lack of geographical boundaries and physical processes therein (Simberloff, 2014; Rius et al., 2015; Xiong et al. 2016). Invasive seaweeds have various reproductive mechanisms such as vegetative (*Caulerpa* sp.), parthenogenetic (*Codium fragile*), sexual, with high fecundity rate (*Asparagopsis armata*) (Andreakis and Schaffelke, 2012) or by self-fertilization (*Sargassum muticum*) (Joseffson and Jansson, 2016) that facilitate their propagation. Management decisions are based on the prediction of future outcomes that at last depend on present and future environmental conditions (known as environmental stochasticity) (Hyder et al., 2008), that are under the strong influence of climate change and direct human transformation of landscapes (Peterson, 2003). Climate change seems

also to determine onward colonization and growth of *C. fragile* in some areas (e.g.: the northeast coast of Canada where it was not recorded previously (Gagnon et al., 2015)).

Molecular studies confirm species diversity in the genus *Codium* (Oliveira-Carvalho et al., 2012; Verbruggen and Costa, 2015) as well as multi-introduction events (Canada; Kusakina et al. 2006) and two possible introduction events in Europe (Provan et al., 2005).

In the past 25 years, sea surface temperature (SST) in the Northeast Atlantic has increased 0.3-0.8°C (Taboada González and Anadón, 2012; Fig. 1. from Voerman et al., 2013).

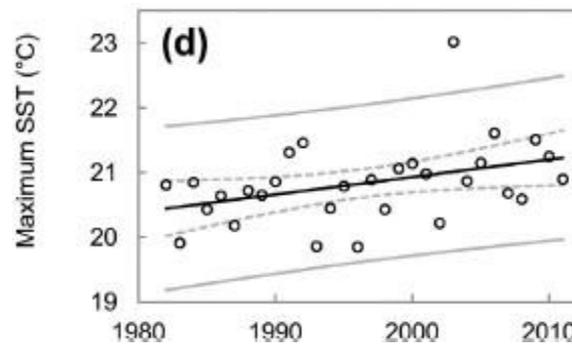


Figure 1. Maximum SST from 1982-2012, calculated per year (from Voerman et al., 2013)

Cape Peñas is the northernmost point of the region Asturias and is considered to be a transitional point in a seaweed composition along the Cantabrian Coast (Anadón and Fernández, 1986). In a context of increasing temperatures changes, seaweed communities also exhibit changes in the distribution along latitudinal gradient in the North Coast of Spain; a displacement of cold-temperate seaweed assemblages (fucooids, kelps) (toward west point of Galicia) by warm-temperate seaweed assemblages from the east (Basque Country) (*Cystoseira* sp., *B. bifurcata* and coralline algae) (Fernández, 2016).

Surveillance (monitoring) programs have been advised for marine areas to obtain valuable feedback and to warrant the application of useful management tools (risk assessment) for prediction of invasion and succeeding consequences (Hewitt and Campbell, 2007). The probability of detecting an established IAS depends on surveillance effort or resources expended; the higher the effort, the chances of non-detection decrease (Hauser and McCharty, 2009). The following question unfolds: which is the minimum quantity of individuals that assures a good estimate of some assemblage (Fatela and

Taborda, 2002) or IAS presence. Phleger (1960), in the study on Foraminifera, suggests that the counting of more than 300 individuals does not increase significantly the trustworthiness of the study. In general, Patterson and Fishbein (1989) discuss that 500-1000 individuals need to be collected to compensate up to 5% chances for the assessment of certain community while several thousand individuals need to be collected to compensate 1% chances for a plausible assessment of the communities. In the case of already invaded areas, a database about spatial and habitat distribution of target IAS should be made providing guidelines for areas that are in the risk of invasion or advising mitigation measures against the continuous spread of IAS (Otero et al., 2013; Smith et al., 2002). *C. fragile* can settle in a wide range of habitats (various types of hard substrate, semi-exposed/covered beaches, sea/estuary) and attach to artificial floating objects where its spread will depend on a water temperature, wind pattern, recipient community and waves exposure (Drouin et al., 2007, Gagnon et al., 2015).

Most of the studies in the Cantabrian Coast have focused on the changes in the native seaweed assemblages on a various spatial scales (Fernández, 2011; Lamela Silvarrey et al., 2012; Voerman et al., 2013; Fernández, 2016) and indeed the information about the distribution of invasive species are rather scarce except for the case of the well-known *Sargassum muticum* invasion in Spain (Andrew and Viejo, 1998; Sánchez and Fernández, 2005; Incera et al., 2011). To our knowledge, three studies about *Codium* spp. molecular and morphological identification, distribution and reproductive traits were carried out in the north coast of Spain (Cires Rodríguez and Rico Ordás, 2007; Rojo et al., 2014; Garcia et al., 2018). All *Codium* species seem to coexist although with lower abundances from 2003-2016, and a greater proportion of *C. fragile* have been reported towards the east of the Cantabrian coast, in upper levels on the shore, where conditions are more stressful (Cires Rodríguez and Rico Ordás 2007; Rojo et al., 2014). In this study, we aimed to update to assess the difference in the spatial pattern of distribution and abundances of *C. fragile* following the east-west longitudinal axis from both sides of Cape Peñas. Furthermore, we evaluated the correlation between environmental variables on the current *Codium* spp. Distribution, as well as the detection probability of *C. fragile* inside different spatial hierarchical levels

MATERIALS AND METHODS

STUDY AREA AND SAMPLING METHOD

The spatial distribution of invasive *C. fragile* and native *Codium* species was investigated in this study, following the Cantabrian coastline (located in the North Coast of Spain), from west (Cangas de Foz (43°37'49" N; 7°20'15" W), Galicia) to the east (San Vicente de la Barquera (43°39'41" N; 3°39'33" W), Cantabria). *Codium* species inhabit intertidal and subtidal zones, attached on rocky substrates, thus our sampling was focused on rock-forming beaches, 0.5-2 m above Lowest Astronomical Tide (LAT) in the low eulitoral. Tides in the Cantabrian Sea are semidiurnal and range from 1.0 m to 4.5 m (Flor and Flor-Blanco, 2006). Overall, 12 localities were sampled between February 2016 and April 2017: February 2016 (Cadavedo, Concha de Artedo, Viedo, La Isla, La Vega, Vidiago), June 2016 (Gijón harbour), December 2016 (Tapia de Casariego and Vidiago), March 2017 (Peizas, Cangas de Foz) and April 2017 (Tapia de Casariego, Liñera, San Vicente de la Barquera) (Fig. 2.).

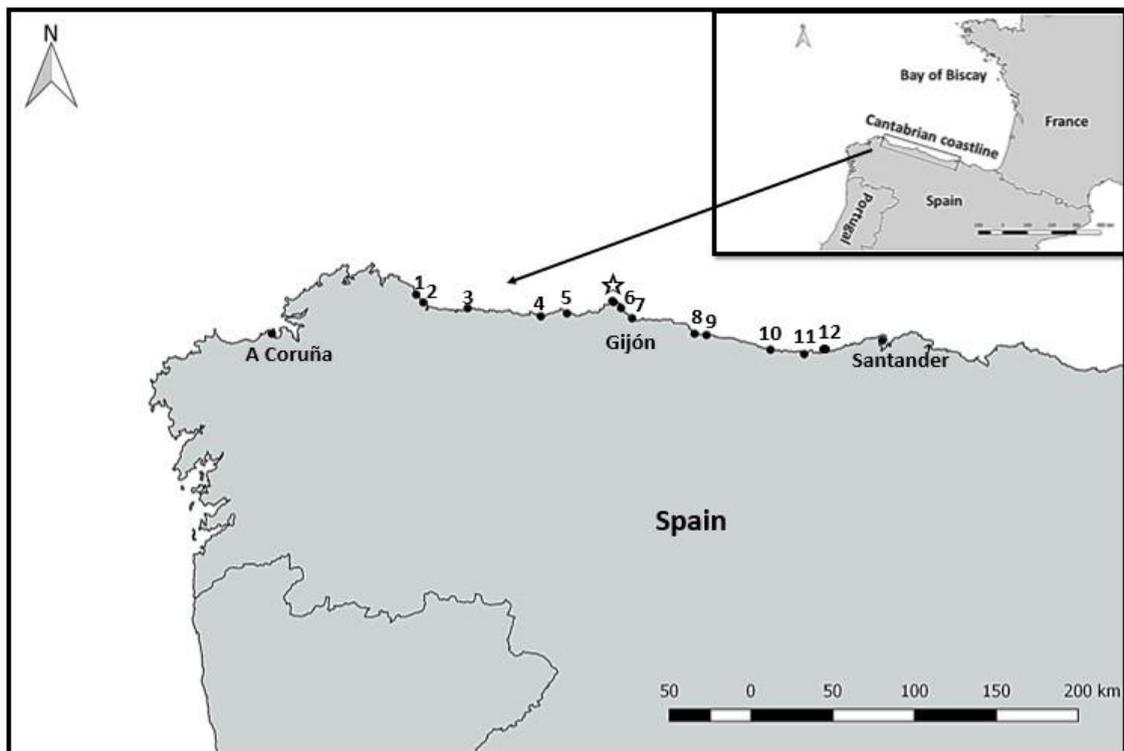


Figure 2. Localities from the Bay of Biscay included in a sampling of *Codium* spp. in this study: 1. Peizas 2. Cangas de Foz 3. Tapia de Casariego 4. Cadavedo 5. Concha de Artedo 6. Viedo 7. Gijón harbor 8. La Isla 9. La Vega 10. Vidiago 11. Liñera 12. San Vicente de la Barquera.

The differences in a population variability of invasive *C. fragile* has been assessed on a four different spatial scale: 2 zones were selected westward and eastward from Cape Peñas (henceforth east and west zone) including beaches, sites, and plots. Hierarchical designs have become useful tools, since scales at which variability is higher can be identified through the nested distribution of samples (Underwood, 1997) thus it was used in this study. At each locality a hierarchical sampling design was implemented: six beaches per zone were randomly chosen. Three sites of 30×30 m shore stretch were randomly selected inside beach and within each site, three 2×2 m quadrats were randomly placed. The survey was done in the form of a parallel transect, following the shoreline.

SPECIES IDENTIFICATION BASED ON MORPHOLOGICAL IDENTIFICATION AND DNA BARCODING

Samples were preserved in 96% ethanol for morphological identification and stored in silica-gel for molecular work (Verbruggen et al., 2015). All collected material was checked under a microscope and assigned morphologically as described in Maggs and Kelly (2007). Sharp ending on the top of utricule, mucron, was used as a morphological trait to differentiate native (non-mucronate form) from invasive *Codium* species (mucronate form) (Silva, 1955). Species ID confirmations were conducted using barcoding genetic procedures on a set of 20 representative samples from each beach/morphotype. We extracted genomic DNA from approximately 30-35 mg dried thalli ground in liquid nitrogen until a fine powder was obtained and afterward used for DNA extraction with GeneMATRIX Plant and Fungi Purification Kit (EURx Cat. No. E3595, Roboklon GmbH, Berlin, Germany; GeneMATRIX purification Kit). DNA barcoding was conducted, using the *tufA* elongation plastid factor gene since it lacks intronic regions and enables distinct delineation between green algae (Saunders and Kucera, 2010). The gene was amplified using published primers: the forward *tufA* primer *tufGF4* (5' GGNGCNGCNCAAATGGAYGG 3') from Saunders and Kucera (2010) and the reverse primer *tufA R* (5' CCTTCNCGAATMGCRAAWCGC 3') from Fama et al. (2002). PCR procedures were performed in a total volume of 20 µl with 0.5 µl of DNA extracts: 1x of PCR Buffer, 2.5 mM MgCl₂, 0.5 mM dNTPs, 0.2 µM of F and R primers and 1U of Taq Polymerase. All samples were diluted with 1:100 prior to PCR processing. PCR cycling was optimized according to Fama et al. (2002), including initial denaturation

(95°C for 5 min), followed by 40 cycles of denaturation (95°C for 1 min), primer annealing (45°C for 1 min), extension (72°C for 2 min) and final extension (72°C for 5 min). PCR products were sent to MACROGEN (Amsterdam, Netherlands) for purification and sequencing using the forward and reverse primers. The obtained sequences were manually checked and edited using the freeware BIOEDIT (Hall, 1999). Alignments were made using CLUSTALW (Thompson et al., 1994). The different sequences found in this study were submitted to GenBank. After alignment and corrections, species identification was carried out through blast attempts against GenBank databases. Species identifications were accepted if they showed more than 98% of similarity with the reference sequences available in Genbank.

DATA ANALYSIS

Standard statistical tests for assessing the distribution of *C. fragile* (invader) at both sides of Cape Peñas could not be used due to a large number of zeros in the data set. Thus, abundance was evaluated using a simple randomization test (Manly, 2007). Five beaches to the west (with 3, 13, 6, 9 and 3 plants of *C. fragile* per beach) and five to the east of Cape Peñas (with 0, 4, 1, 0 and 0 plants per locality) were randomly allocated to any of the two sides. There were only three cases in which the number of invaders eastward is smaller or equal to the observed numbers. These three ordinations are (order ignored): [3 1 0 0 0], [3 1 0 0 0] and [4 1 0 0 0].

PRINCIPAL COMPONENT ANALYSIS (PCA)

Principal component analysis (PCA) was conducted using PAST software ver. 3.20 (Hammer et al., 2001) to evaluate the correlation between environmental variables of sampled locations and port distances from those locations, as they are areas with strong anthropogenic impact, and their effect on the distribution of *C. fragile*. Gagnon et al. (2015) have argued that wind effect can influence the propagule dispersion of *C. fragile* significantly on a local scale, and have assumed that fragments could travel up to 4 km/year. This measure was taken into the account when calculating the distance of beaches from nearby Gijón harbor, as ship fouling is one of the vectors for the

introduction of *C. fragile* (Carlton and Scanlon, 1985). Most of the beaches in the Cantabrian Sea are wave-exposed, except the ones that are rather protected from northwest waves by Cape Peñas, with a temporal height of 1.7 m (Flor et al., 2014). Thus, the orientation of beaches in regard to Cape Peñas was defined (west or east from Cape Peñas) and wave-exposure was calculated based on an average fetch length, the distance between nearest land and the coast. The exposure of the coast increases proportional with the extent of the fetch in a certain direction, thus higher the fetch more energy will allocate on the surface of the water, resulting in more open shapeliness of the beach (Seers, 2018). The Cantabrian shoreline is formatted from clastic rocks in the east (toward Galicia), mixed sediment in the central part (around Cape Peñas, sand and quartz sediment) and calcareous rocks to the west (toward Cantabria) (Flor et al., 2014). Based on the work of Flor Blanco (2006) and Flor et al. (2014) environmental description for sampled locations in this study was compound: Cangas de Foz and Playa de Peizas are beaches with the clastic rock formation. Although location Tapia de Casariego is less than 1 km away from the local harbor, the original appearance of the beach was not influenced by human impact and firm clastic sediment is still dominant. Cadavedo and Concha de Artedo are as well beaches with clastic sediment. Viodo and Aramar, centered around Cape Peñas, are beaches with sand-quartz sediment. Following east, most of the beaches are constituted of gravel-calcareous sediment (apart from La Isla and de Vega that are mostly calcareous-forming with a smaller portion of sand, thus we assigned them as mixed beaches). Beside salt water, *C. fragile* can survive in brackish waters (Wolff, 2005) thus the presence/absence of river estuary in sampling points was taken into consideration. The following variables were used for PCA analysis (Table 2.): orientation of the beach from Cape Peñas, presence of river estuary (yes/no), approximate distance from Gijón harbor, beach sediment type and exposure of beach to waves (fetch length). Wave exposure was calculated using fetchR package, in RStudio ver. 1.1.414 (Seers, 2018). A biplot of variable loading vectors and site scores on the principal components was done to make the main features of the data easier to see (Ter Braak, 1983). Variable vectors inside biplot with an angle close to 0° have a high positive correlation, angles close to 90° show no correlation and angles toward 180° indicate a high negative correlation. Longer vectors show positions of more significance (Santos, 1993). PCA analysis was performed with PAST software ver. 3.20 (Hammer et al., 2001).

DETERMINISTIC AND STOCHASTIC SIMULATION MODELS

We used field data to quantify the probability of failure to detect the presence of *C. fragile* on different hierarchical levels. Both simulation models were evaluated using the proportion of invaders observed in the field. It is implicitly assumed that invaders are identified with no lapse.

For the deterministic simulations, we assume that the invader is randomly distributed within the homogenous environment (in our case beach) and that the probability of failing to detect an invader declines exponentially with the search effort. The probability of no detection after evaluating a sample of size N is given by $(1-p)^N$. Variation in the probability of failure with increasing sample sizes was evaluated for a few proportions: 0.001, 0.01, 0.1 and 0.035. The latter is close to the average proportion of invaders in localities, in which the invader was found (excluding Gijón harbor).

THE STOCHASTIC SIMULATION INCLUDES THREE SCENARIOS:

- 1) The first scenario includes simple random-area based sampling of both *C. fragile* and native *Codium* species from the whole beach. Sites within beach and plots within sites are ignored. Each individual is evaluated using binomial probabilities, with the proportion 0.035 of invaders. The probability that any given population is detected (i.e., at least one trial is successful at detecting the population) can be estimated as a binomial distribution (Niell et al., 2014). This method is reliable if *C. fragile* is not overcome excessively the number of native *Codium* species and they are well mixed. Scenarios 2 and 3 below would give equivalent results to scenario 1 only if no aggregation of *C. fragile* exists and it is well mixed with the native plants.
- 2) The second scenario assumes some heterogeneity at small spatial scales. Some plots may have invaders and some not. Nine plots were evaluated for each beach. Sampling simulated random allocation of plots in the beach. Plots were evaluated for the presence of invaders using the proportions observed in the field: from beaches with invader presence, 22 plots had the invader and 50 lacked it. Thus, plots were evaluated for the presence of invaders by sampling a binomial distribution with $p=0.306$. For plots with invaders, the identity of the plants was

determined using the proportions of *C. fragile* in plots with invaders. That proportion is 0.1161.

- 3) The third scenario assumes heterogeneity at two spatial scales: sites and plots. The invader may be present in some areas of a given beach but not in all of them. In those areas with presence, the invader may also show a heterogeneous distribution. The simulation is now a three steps process. At each beach the presence of *C. fragile* is assumed, three sites are evaluated for the presence of the invader by using the frequency of sites with *C. fragile* in beaches (16 sites out 24, $p = 0.667$). For sites where *C. fragile* is detected, three plots are evaluated for the presence of the invader by using the proportion of plots with *C. fragile* in sites (22 out 48, $p = 0.458$). Then the identity of the plants is evaluated using the frequency of *C. fragile* in plots with the presence of *C. fragile* (0.1161 from Scenario 2).

In all three stochastic scenarios the probability of detection is evaluated for different sample sizes: scenario 1 for the beach (18-1500 plant); scenario 2 and 3 for plots (2 to 175 plants). The procedure is repeated 5000 times for each scenario. For an individual replicate, the invader is detected if at least one invader plant is “found”. The probability of detection is readily obtained as the proportion of replicates with positive detection.

RESULTS

DISTRIBUTION OF *CODIUM* SPP. AND GENETIC ASSIGNMENT

A total of 1,952 *Codium* spp. individuals were collected and examined morphologically in the years 2016 and 2017. After morphological analyses, we identified three different species from the analyzed samples: *C. fragile*, *C. tomentosum* and *C. vermilara*. This was confirmed (100% of correspondence) after obtaining three different *tufA* haplotypes blasting with 100% of similarity with the species mentioned above (Genbank IDs: voucher FCO-Alg. 2287 for *C. vermilara*, FCO-Alg. 2230 for *C. fragile* and FCO-Alg. 2239 for *C. tomentosum*). The species *C. decorticatum* or *C. fragile* subsp. *atlanticum*, although previously morphologically described in the Bay of Biscay, were not recorded during this study.

Samples collected in February 2016, March 2017 and April of 2017 were dominated by native *Codium* spp. (2.8% of *C. fragile* and 97.2% of native *Codium* spp.). Native *Codium*

spp. were dominant in the east (19.2% of *C. fragile* and 80.7% of native *Codium* spp.), as well as in the west zone (12% of *C. fragile* and 88% of native *Codium* spp.) of the Bay of Biscay. Higher proportion of *C. fragile* was detected in June 2016, in the port of Gijón (73.9%, respectively), as well as in December 2016 material collection from Tapia de Casariego and Vidiago (i.e. 52.2% of *C. fragile* and 47.7% of native *Codium* spp.) (Figure 3, Table 1.).

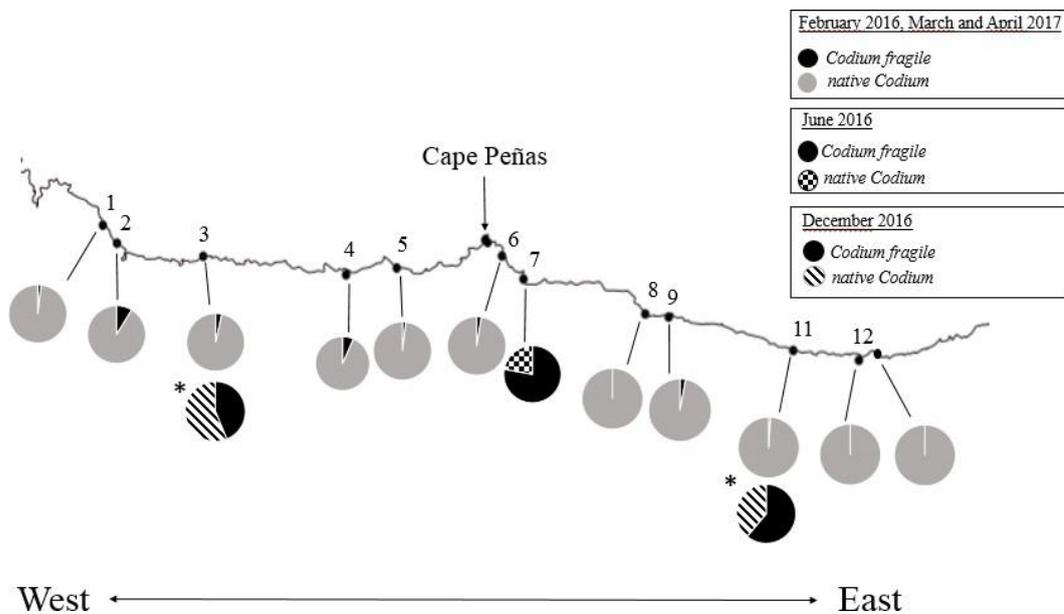


Figure 3. The *Codium* spp. distribution found in this study by localities from the Bay of Biscay. 1. Peizas 2. Cangas de Foz 3. Tapia de Casariego 4. Cadavedo 5. Concha de Artedo 6. Viodo 7. Gijon Harbour 8. De la Isla 9. De la Vega 10. Vidiago 11. Liñera 12. San Vicente de la Barquera *This locations were omitted from simulation models

PCA AND DISTRIBUTION OF *C. FRAGILE* AT BOTH SIDE OF CAPE PEÑAS

The PCA analysis revealed a biplot chart with three principal components explaining 87.24% of the total variation in variables used in this study. The first principal component represented 53.85% of the total variance and includes fetch wind; second principal component represented 19.74% of the total variance and mainly consisted of the approximate distance of beaches from port of Gijón while third principal component represented 13.65% of total variance and covers beach substratum type and the presence of estuary (Figure 4).

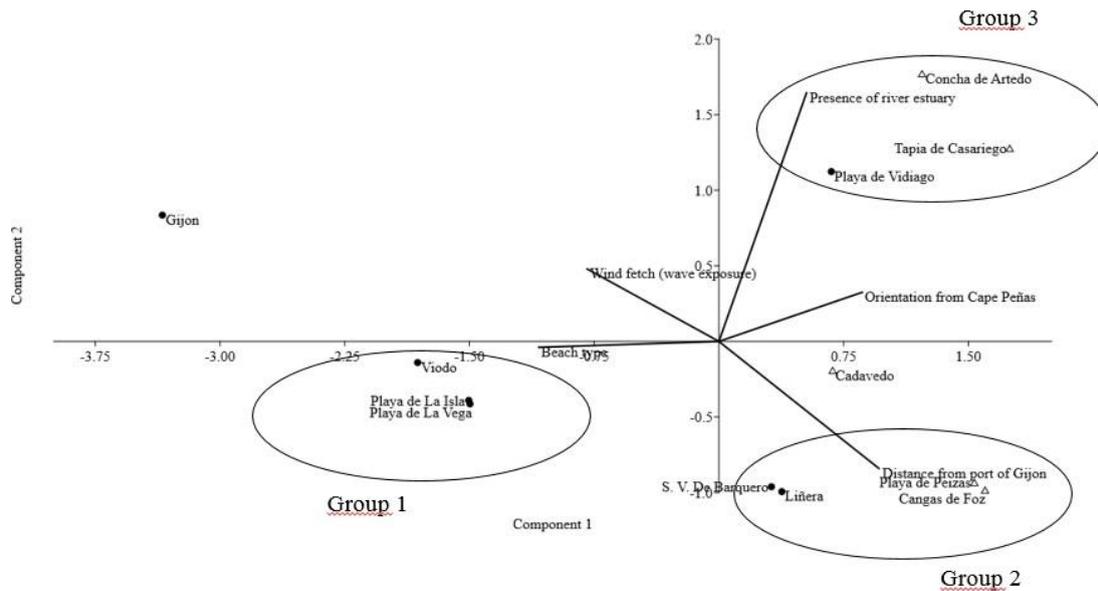


Figure 4. Biplot chart of the variables representing the relationship between the environmental factors, distance from the Gijón harbour and distribution of invasive *Codium fragile* (LONG-longitude, Wind Fetch (wave exposure)-fetch wind, Distance from port of Gijón-approximate distance from the port of Gijón, Orientation from Cape Peñas-orientation in regard to Cape Peñas, Beach type-beach substratum type and Presence of river estuary - presence of the estuary)

PCA figure shows clear division of three groups: group 1 includes beaches east from Cape Peñas that have resembling values of fetch wind (less exposed than the beaches on the western side from Cape Peñas). Beach substratum type does not seem to correlate with the establishment of *C. fragile*. The group 2 includes beaches, both from west and east side of Cape Peñas that are very distant from Gijón harbor and fewer abundances of *C. fragile* could be expected since these locations are under the minor influence of traffic activity. The group 3 includes beaches where estuaries are present however, it the abundances of *C. fragile* did not seem higher or lower compared to other locations. Gijón harbor was not joined in any of the groups and was not explained with variables used in PCA. It is a location where *C. fragile* was collected in the highest quantity, compared to other locations that are not under strong anthropogenic influence. There is a defined separation between beaches of all three groups of samples, suggesting that local parameters could be responsible for differences in the spatial distribution of *C. fragile* from both sides of Cape Peñas. Those findings are supported with results from a simple randomization test where for a level of significance of 0.05, the observed abundance of *C. fragile* is significantly different to both sides of cape Peñas.

DETERMINISTIC AND STOCHASTIC SIMULATION

We used field data to quantify the probability of failure to detect (no detection) the presence of *C. fragile* (invader). A plot with the number of plants collected versus the probability of no detection and associated proportion (p) of the invader under different scenarios from deterministic and is given in Fig. 5. This figure may be used to compute the minimum sample size needed to detect an invader that occurs with a proportion p on different hierarchical levels (excluding zones). The probability of detection is readily obtained as the proportion of replicates with positive detection.

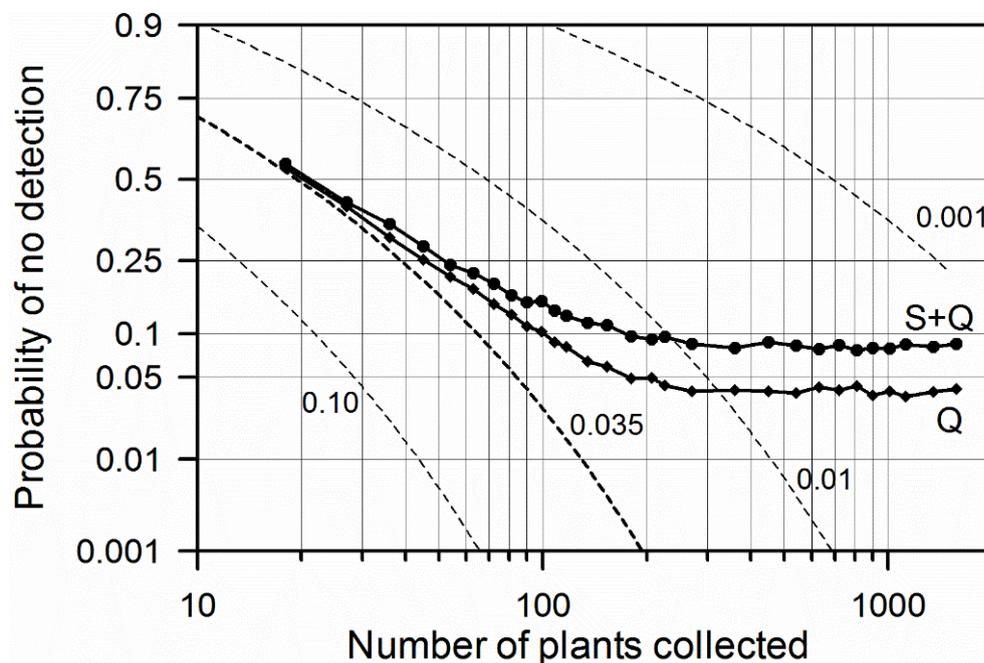


Figure 5. Variation of the probability of no detection of the invader *C. fragile* with increasing sample sizes under different scenarios. Discontinuous lines are for deterministic simulations. Numbers are the proportion of the invader in the localities. The simulation for $p = 0.035$ mimics data with the average proportion observed in the Cantabrian Sea. Continuous lines are for stochastic simulations and assume heterogeneous distribution of the invader in relation to the native. S+Q considers three sites per locality and three quadrats per site. The line marked with Q is for simulations considering nine randomly selected quadrats per locality, ignoring sites. Stochastic simulations ignoring both sites and quadrats were done but not presented. The probability of no detection followed exactly the discontinuous line for $p = 0.035$ and is omitted for clarity.

The deterministic simulation for $p = 0.035$ imitates data with the average proportion of invader observed in sampled beaches from the North Coast of Spain (Fig. 5). In general,

the stochastic simulation obviously shows that the probability of failure in the detection of *C. fragile* for given proportions in this study (0.1, 0.035, 0.01 and 0.001 from deterministic simulation) increases with samples size per beach. The application of stochastic simulation models shows that invader in which $p \geq 0.035$ is represented in samples with at least 100 counted individuals. In fact, counting 100 specimens for a $p \geq 0.035$ implies between 1 and 5% of the failure in the detection of *C. fragile*, if the beach is randomly sampled. For example, counting 20 plants for $p \geq 0.1$ reduces the probability of not detecting invader to 1% or less. Since in this study, the *C. fragile* was very patchy distributed with the average proportion of $p=0.035$, we suggest that counting 100-150 individuals is a very satisfactory procedure for detecting invader with more than 10% of probability. Furthermore, stochastic simulations assume heterogeneous distribution of the invader in relation to the native. Taking into the account frequency of plots inside beach (Q) where invader was present and the proportion of the invader was calculated ($p=0.1161$), and the frequency of plots inside sites and sites inside beaches (S+Q), Fig. 5. also indicates that with increasing samples size per Q and S+Q the probability of invader to remain undetected is quite similar. For a $p=0.116$, that counts around 20 plants, the probability of not detecting invader is 50% and is the same value for Q and S+Q. Even with the sample size for the invader (for example 1000 plants), the chances to detect *C. fragile* would be less than 7% for Q and S+Q. This further suggests that hierarchical level sampling does not assure higher detection probability, in the case of *C. fragile* as target species.

DISCUSSION

This study focused on describing the spatial distribution pattern of *Codium* species, as the groundwork for the future monitoring of rocky beaches along the Cantabrian coast. The predominant *Codium* species were native ones, as similarly observed in previous work in the coastline of northern Spain (Rojo et al., 2014; García et al., 2018). In a prior study, which in particular examined the coexistence of *C. tomentosum* and *C. fragile* of the British Isles (Guernsey Shore and western Ireland), authors found that native *C. tomentosum* was not outcompeted by the presence of *C. fragile* (Trowbridge, 2001; Trowbridge et al., 2004).

Decrease of *C. fragile* on Atlantic shore of Europe is detected since more than 15 years ago (Trowbridge, 2001) and a possible reason points on shifts in climate pattern and presence of specialist herbivores or pathogens (Trowbridge et al., 2004). From personal observation, a higher proportion of *C. fragile* was noticed in the summer months (July-September) of sampling, thus future studies should focus on temporal changes of *Codium* spp. in the Cantabrian Sea. Cires Rodríguez and Rico Ordás (2007) remark that there are differences in the abundance of *Codium* spp., with respect to native *C. tomentosum* present in the spring and *C. fragile* in the summer. Similar differences in the proportion of *Codium* spp., with invader being more frequent toward the east coast of Cantabrian Sea, especially in July, were detected (Rojo et al., 2014; Garcia et al., 2018). The underlying reason for this temporal pattern may be explained by the maturation of *C. fragile* reproductive organs in the early summer and maximum length during late autumn and winter (Churchill and Moeller, 1972; Cotton, 1912; Burrows, 1991).

From a larger-spatial scale point of view, temperature could influence seaweed communities more than wave activity, as is in the Cantabrian Sea, where cold temperate algae exhibit long-term displacement by warm ones while on a small-spatial scales, seaweed composition could be significantly affected by wave activity (Ramos et al., 2017). As a result of this work, significant differences were revealed in the distribution of *C. fragile*, between east and west zone of the coast of Asturias, suggesting that temperature may be one of the responsible factors for the spatial pattern of the invader as previously suggested by Rojo et al. (2014) and Garcia et al. (2018). The beach exposure to waves is one of the important physical factors that can influence relative abundance of species on different shores, especially intertidal communities (Ballantine, 1961; McQuaid and Branch, 1984) and seaweeds assemblages along the Cantabrian coast are, among rest, under continuous effect of waves (García et al., 2011). Among substratum type, habitat availability and temperature, wave exposure is a pertinent factor for *C. fragile* habitat selection (Drouin et al., 2007) contrary to the case of invasive eg. *Sargassum muticum*, which show no decrease in abundance on exposed beaches (Andrew and Viejo, 1998). Beaches that are less exposed to wave actions could perpetuate the establishment of *C. fragile*, as its survival probability will presumably be higher in a semi-exposed bays or shallow lagoons than uncovered cliffs (Drouin et al., 2007, Gagnon et al., 2015). Our PCA showed that on a local scale, beaches on the east side of Cape Peñas could provide habitat for *C. fragile* establishment as they are less exposed. This could support the

previous finding, of retreatment in the distribution of cold temperate seaweeds toward the west coast of the Cantabrian Sea. Aside from the fact that *C. fragile* prefers less exposed beaches, a higher proportion of the invader toward the eastern part of the Cantabrian Coast coast, with records of higher temperature, could perpetuate its establishment. Interactions between environmental conditions could favor and potentiate further spread of IAS (Rodil et al., 2008). Native *Codium vermilara* and *Codium tomentosum* are often found in the lower intertidal levels (Rojo et al., 2014; Trowbridge, 2001) thus it is possible that they are not under the strong influence of waves, unlike invader that is found in the upper levels, where conditions are more aggravating.

The anthropogenic impact affects the seaweed composition in the Cantabrian Sea (García et al., 2011). Majority proportion of *C. fragile* individuals were detected in economically important port in Spain, “El Mussel” harbor in Gijón, Asturias. Due to human alteration, initial physical appearance of the port changed moderately: sediment is rather rocky, with patches of sand covering bottom and reduced wave activity (Flor and Flor-Blanco, 2006) which may provide a ground floor for *C. fragile* establishment, as well as for previously recorded invasive red seaweed *G. imbricata* in “El Mussel” (Montes et al., 2016). In the case of *C. fragile*, propagation could be facilitated by shipping activities among ports along the Cantabrian coast, as was also spotted in the Greece harbors (Tsiamis and Panayotidis, 2007). For instance, region Asturias has two state and 24 regional ports (Flor and Flor-Blanco, 2006) where human-mediated dispersal could elevate the spread of *C. fragile* (Begin and Scheibling, 2003) to other pristine areas.

Hierarchical sampling is an advisable prerequisite for avoiding inaccurate interpretation of the population variability, especially on a small scale (Morrissey et al., 1992), where interaction between biological and physical processes influence population variability, with emphasize on the benthic assemblages (Fraschetti et al., 2005). In the assessment of seaweed communities, the small spatial scale can contribute significantly to the entire variation of the community (Smale et al., 2011). Our analysis suggests that random sampling on lower levels (plots and sites) do not guarantee higher detection probability of the invader, even if the invader is present in same or similar proportions. A possible explanation could be that environmental conditions are not distributional drivers of *C. fragile* on a smaller spatial scale in the Cantabrian Sea. Thus future studies should focus on the interactions between *C. fragile* and other native seaweed assemblages that are

sharing the same habitat and natural resources since it seems that even on a smaller scale, *C. fragile* is not influencing the distribution of native *Codium* spp. With the average proportion of *C. fragile* (0.035) obtained from the scale of the beach, the chances of not detecting the invader are between 1 and 5% for 100 individuals. Probability of detection within the context of a given study does not depend only on the presence of target species; a smaller proportion of *C. fragile* would require more sampling effort meaning more cost-demanding resources. Our results demonstrate that the simple application of our analysis has considerable potential as a management tool.

Our sampling covered 231 km from total 2080 km of the Cantabrian coastline providing a knowledge about the distribution of *Codium* spp., which can serve as an insightful tool for monitoring programmes, as seaweed assemblages are an important ecological indicator of the quality status in rocky communities along the Cantabrian coast (Juanes et al., 2008). It is advisable that, at least, state harbors would be included in monitoring programs, with the permission of the Harbour Authority since shipping activity may contribute to the secondary spread of *C. fragile*, as suggested by McDonald et al. (2015). Ecological and molecular approach from this study provides a solid baseline that can be used to direct management priorities, concerning the monitoring of both native and invasive *Codium* spp. in the Cantabrian Sea.

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Table 1. Sampling points in this study with location, latitude and longitude, time of sampling and total number of *C. fragile* and native *Codium ssp.*

Sampling location	Region	Latitude	Longitude	Time of sampling	N (<i>Codium fragile</i>)	N (native <i>Codium</i>)
Cangas de Foz	Galicia	43.63044	-7.33456	March 2017	3	155
Peizas	Galicia	43.58879	-7.28119	March 2017	13	132
Tapia de Casariego	Asturias	43.57023	-6.94391	December 2016	60	75
Tapia de Casariego	Asturias	43.57023	-6.94391	April 2017	6	117
Cadavedo	Asturias	43.54464	-6.38827	February 2016	9	105
Concha de Artedo	Asturias	43.56762	-6.19198	February 2016	3	139
Viodo	Asturias	43.64034	-5.84165	February 2016	1	124
Gijon Harbour	Asturias	43.55446	-5.69971	June 2016	131	48
La Vega	Asturias	43.47485	-5.1346	February 2016	0	145
La Isla	Asturias	43.48035	-5.22534	February 2016	4	160
Vidiago	Asturias	43.40156	-4.65133	February 2016	1	95
Vidiago	Asturias	43.40156	-4.65133	December 2016	81	54
Liñera	Cantabria	43.37792	-4.38220	April 2017	0	143
San V. de la Barquera	Cantabria	43.38131	-4.39706	April 2017	0	148

Table 2. Environmental variables for sampling points, used in PCA analysis

Sampling location	Cape Peñas orientation	Presence estuary	Beach type	Fetch wind
Cangas de Foz	West	No	Clastic	95,17
Peizas	West	No	Clastic	105,04
Tapia de Casariego	West	Yes	Clastic	117,89
Cadavedo	West	No	Clastic	108,89
Concha de Artedo	West	Yes	Clastic	59,35
Viodo	East	No	Mixed	131,36
Gijon Harbour	East	No	Mixed	
La Vega	East	No	Mixed	129,62
La Isla	East	No	Mixed	100,59
Vidiago	East	Yes	Calcareous	133,87
Liñera	East	No	Calcareous	98,01
San V. de la Barquera	East	No	Calcareous	126,36

CHAPTER 3.

DNA BARCODING FOR UNRAVELING SPECIES DELIMITATIONS, DETECTIONS, AND DIVERSITY ON THE *CODIUM* SPECIES FOUND IN THE CANTABRIAN SEA, BAY OF BISCAY

INTRODUCTION

One of the greatest threats to coastal marine ecosystems is the introduction of non-indigenous species (NIS) that may become invasive. Biological invasion processes related with invasive seaweeds are indeed a particular focus of attention since propagules can be easily transported by vessels or be introduced as accompanying biota of species of economic interest (i.e. aquaculture) (Katsanevakis et al., 2014). Moreover, intensive monitoring and morphological analysis alone can be ineffective for invasive seaweed species early identifications leaving cryptic introductions undetected (Saunders, 2009; Montes et al., 2016).

The *Codium fragile* subsp. *fragile* (hereafter *C. fragile*) is one of the worst invasive species around the world. It is maybe for this reason that it is a major research subject for many ecological and genetical studies since harmful impacts on native flora and fauna communities and also on ecosystem services have been reported so far for this species (Trowbridge, 1995; Chapman, 1999 ; Schiebling and Anthony, 2001; Vilà et al., 2010). Moreover, its invasion pathways are also obscured by its cryptic nature (Provan et al., 2005; Provan et al., 2008; Kusakina et al., 2006; Armitage and Sjøtun, 2016).

Four different *Codium* entities (*C. fragile* and one subsp. *atlanticum*, *C. tomentosum*, *C. vermilara*, *C. decorticatum*) have been previously reported in the intertidal zone of the Cantabrian coast mainly using morphological characters as identification keys (Gorostiaga et al., 2004; Cremades et al., 2005; Cires Rodríguez and Moliner, 2010). However, the existence of a broad variety of morphological forms inside genus *Codium* (Pedroche, 2001) has lead authors conclude that taxonomical assignment should be

supported with genetic analysis (Silva, 1955; Verbruggen et al., 2007). DNA barcoding has been shown as an effective tool to distinguish species where morphological identification is ambiguous as it is in the case of macroalgae with a high level of phenotypic plasticity or cryptic nature (Saunders, 2005; Cross et al., 2011). Notably, the representative molecular markers (barcodes genes) should be simultaneously highly variable to discern species (Saunders and Kucera, 2010) and highly conserved so primers (a short sequence of RNA or DNA from which DNA replication can initiate) can be designed (Saunders and Kucera, 2010). The UCP6 (*rpl16-rps3*), *tufA* and *rbcL* (RuBisCO) are predominant chloroplast molecular markers, used so far for tracking the invasion history (with fresh and herbarium samples) and subspecies discernment in genus *Codium* (Provan et al., 2005; Provan et al., 2008; Verbruggen et al., 2007; Lee and Sook, 2015; Verbruggen and Costa, 2015; McDonald et al.; 2015; Verbruggen et al., 2017). The *tufA* elongation plastid factor is a molecule that mediates the entry of an amino-acyl-tRNA into the acceptor site of a ribosome during the elongation of the nascent polypeptide chain in protein synthesis (Lewin, 1997). The *RbcS* gene for the small subunit of the chloroplast photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) have been generally utilized for phylogenetic studies in macroalgae (e.g. Freshwater et al., 1994; McIvor et al., 2002). On the other hand, the UCP6 marker is universal and commonly used for identification of species inside phyla Chlorophyta (Provan et al., 2004).

Molecular methods provide insight on origin, genetic diversity, introduction pathways of IAS (Taylor and Kelly, 2007) and are becoming more relevant to assess invasion risks (e.g. cryptic invasions, evolutionary and ecological consequences inside invaded area) (Estoup and Guillemaud, 2010; Geller et al., 2019). In this, herbarium samples proved to be helpful historical evidence, providing knowledge about misidentified IAS, time and routes of invasion, commonly used in invasion studies of plants before 1990 (Lavoie, 2013). Since the earliest molecular work on kelps Laminariales (Mayes et al., 1992) and first amplification from red algae (Goff and Moon, 1993), various genes (mostly plastid and mitochondrial) were tested on herbarium samples for DNA barcoding, genetic diversity and origin of invasive seaweeds (Provan et al., 2008; Conklin et al., 2009; Montes et al., 2016; Armitage and Sjøtun, 2016; Steen et al., 2017). The quality of DNA from plants weakens during herbarium preparation due to a high level of cellular and

metabolic stress responses (Savolainen et al., 1995). In order to obtain data from such valuable resource, short species-specific and lineage-specific primers can be designed (Booth et al., 2007) as they show higher efficiency in amplification than larger fragments (Lee and Lee, 2018). Provan et al. (2008) use these short DNA fragments (360bp) on herbarium samples and they were able to demonstrate that *C. fragile* was often misidentified in the past, mostly with subsp. *atlanticum* and was introduced to Europe earlier than previously thought (in Ireland by 1845, Scotland by 1891 and England by 1894).

Several studies reported that non-indigenous species introduction to recipient area sometimes reflects the substantial loss of genetic diversity (see Roman and Darling, 2007). In general, seaweeds are referred to as organisms with the lowest level of genetic variation than in any other natural population (Sossa and Lindstrom, 1999). This has been also corroborated for invasive seaweeds eg. introduction event of *Polysiphonia morrowi* in South Atlantic (Geoffroy et al., 2016) and widespread *Caulerpa taxifolia* (Jousson et al., 2000) even when propagule pressure (a measure of the number of individuals of a species released into a region to which they are not native), and wide environmental conditions are among important factors for non-indigenous species to thrive outside of the donor area (Johnston et al., 2008; Pyšek et al., 2010). Moreover, low genetic variation of invasive seaweeds in donor and recipient area have formerly been reported in genetic studies of *C. fragile* (Provan et al., 2005), *Gracilaria vermiculophylla* (Kim et al., 2010) or cases where genetic variation was completely absent - *Sargassum muticum* (Cheang et al., 2010). Provan et al. (2004) strongly suggest that the founder effect (loss of genetic variation when a very small number of individuals from a larger population establishes a new population) could be an explanation for the low level of genetic variation of *C. fragile* in a donor and recipient area. Until now, no evidence of hybridization between native and invasive *Codium* species has been recorded. There are some indications that subsp. *fragile* and *atlanticum* may interbreed but it has to be thoroughly investigated (Kusakina et al., 2006). On the other hand, Verbruggen et al. (2017) had recently suggested two species (native and invasive) for *C. fragile* when using DNA barcodes and morphometric data.

This study aims to update current knowledge about the presence of native and invasive *Codium* species in the Cantabrian Sea, the Bay of Biscay using barcoding strategies based on plastid molecular markers. This also implies to obtain currently absent data about levels of genetic diversity in those natives and invasive *Codium* species. Moreover, since DNA working with herbariums specimens is often troubled, we aim to design new genetic identification protocols using smaller fragments from the *tufA* genetic marker for effective identification of the invasive *C. fragile* species on this type of seaweed materials.

MATERIALS AND METHODS

PLANT MATERIAL AND STORAGE

Codium species were collected 0.5-2 m above LAT (Lowest Astronomical Tides), in the Cantabrian Sea (northern Spain) (from east to west part of the Cantabrian Coast: Vidiago, de Vega, Port of Gijón, Aramar and Tapia de Casariego) (Figure 1). One location from North Coast of Portugal (Viana do Castelo) was added to our samples (Figure 1).

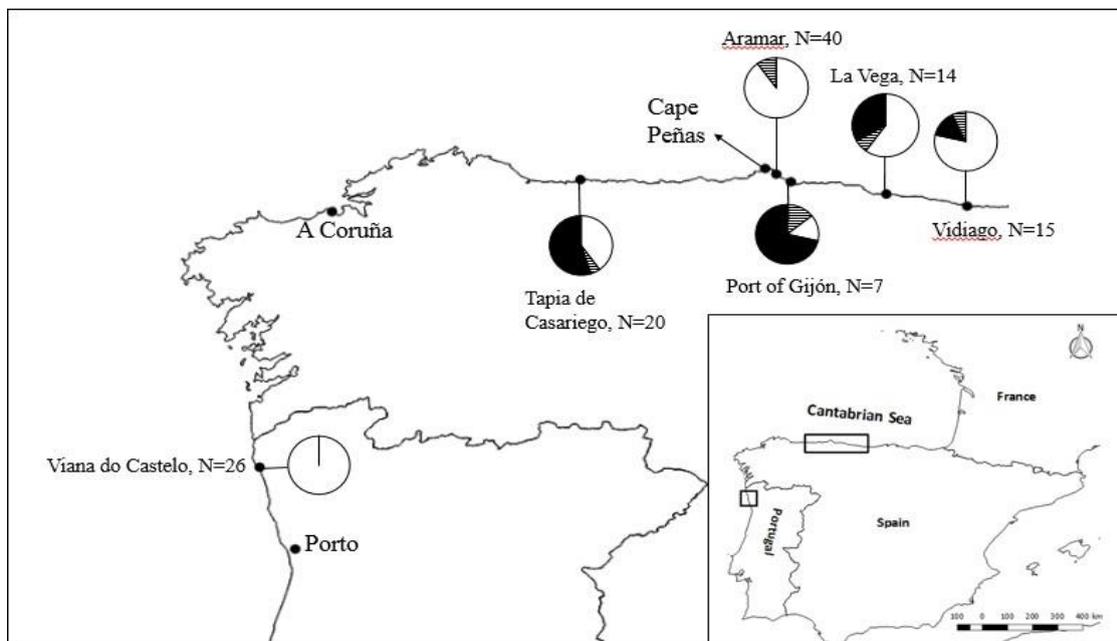


Figure 1. *Codium* sampling locations in the Atlantic (Viana de Castelo, Portugal) and in the Cantabrian Sea (Vidiago, La Vega, Port of Gijón, Aramar, Tapia de Casariego). The sample sizes

by location (Total N=122) and the results of species identification using genetic assignments based on the *tufA* gene are shown (black - *C. fragile*, white - *C. tomentosum*, black stripes - *C. vermilara*).

Thalli were collected randomly in each location (at least 1 m apart). For the purpose of morphological identification, samples were preserved in 96% ethanol. All collected material was checked under the microscope and assigned morphologically as described in Maggs and Kelly (2007). Sharp ending on the top of utricule, mucron, was used as a morphological trait to differentiate native (non-mucronate form) and invasive *Codium* species (mucronate form) (Silva, 1955). For molecular work, tips of thalli were dried in silica-gel (as suggested in Verbruggen et al., 2015).

Codium herbarium and fresh samples were also requested to external labs. Gently we received *C. fragile subsp. fragile* fresh samples from Mokpo and Jindo Geumgap in South Korea (five samples collected in 2017 by Professor Hyul Je Lee) and fresh samples from Portlooe, Plymouth, UK (four samples *C. vermilara*, collector Laura Cappelatti, 2017). Herbarium samples were also received from France (one sample *C. fragile subsp. Fragile*, collector Marc Verlaque, 2002). Professor Christine Maggs gently sent to us six *C. fragile subsp. fragile* herbarium samples from Awaji, Hyogo Prefecture, Japan (2006); Carraroe, Greatmans Bay, Galaway, Ireland (2005, 2006); Toyo, Kochi Prefecture, Japan (2003, 2004); and from the Adriatic Sea, Izola, Gulf of Trieste (). All Herbarium samples were stored at -4°C in the FCO Herbarium of the University of Oviedo, upon the arrival.

DNA EXTRACTION AND PCR AMPLIFICATION

All samples were crushed in a mortar, using liquid nitrogen to obtain a fine powder. The DNA extraction was performed on pulverized samples, using GeneMATRIX Plant and Fungi Purification Kit (EURx Cat. No. E3595, Roboklon GmbH, Berlin, Germany; GeneMATRIX purification Kit) from 20-30 mg of both fresh and herbarium samples. DNA PCR products using two plastid genes: The *tufA* marker, amplified using primers from Saunders and Kucera (2010) (forward *tufGF4* (5' GGNGCNGCNCAAATGGAYGG 3') and from Fama et al. (2002) (reverse *tufGF4* (5'

GGNGCNGCNCAAATGGAYGG 3'); and the *rbcL* marker following Verbruggen et al. (2007). All samples were diluted with 1:100 prior to amplification. For *tufA*, PCR (polymerase chain reaction) procedure was performed in a total volume of 20 µl with 0.5 µl of DNA extracts, 1x of PCR Buffer, 2.5 mM MgCl₂, 0.5 mM dNTPs, 0.2 µM of F and R primers and 1U of Taq Polymerase. We used slightly modified PCR conditions from those reported by Fama et al. (2002): initial denaturation (95°C for 5 min), followed by 40 cycles of denaturation (95°C for 1 min), primer annealing (45°C for 1 min), extension (72°C for 2 min) and final extension (72°C for 5 min). For *rbcL*, PCR procedure was performed in the same volume as for *tufA*, using 1 µl of DNA extracts. We optimized PCR conditions: initial denaturation (94°C for 4 min), followed by 35 cycles of denaturation (94°C for 30s), primer annealing (52°C for 1 min), extension (72°C for 1 min) and final extension (72°C for 7 min). When PCR amplification was not successful, we re-purified samples using the classical phenol/chloroform DNA extraction method. The PCR products were checked on a 2% agarose gel electrophoresis, containing SimplySafe™ (EURx Cat. No. E4600-01). Promega 100 bp DNA Ladder Molecular Weight Marker (Promega Corporation 2800 Woods Hollow Road Madison, WI 53711, USA) was used to check band lengths. PCR products were sent to Macrogen (Amsterdam, Netherlands) for purification and double sequencing using forward and reverse primers.

DESIGN OF SHORT BARCODES IN THE *TUFA* MARKER

FCO*tufA* primers were designed using web interface Primer3Plus (Untergasser et al., 2007) and default parameters. Primer length ranges between 20-25 (bp) and PCR product size up to 200 bp. For this, the *tufA* sequences of *Codium* sp. from public Genbank database and from this study were used for designing forward (FCO*tufA*Fw) and reverse (FCO*tufA*Rv) primers. After the design, we used the same PCR procedure and conditions for the *tufA* marker, as described above.

MOLECULAR ANALYSES

Forward and reverse sequences of *tufA* and *rbcL* were aligned to produce consensus sequences, using freeware BioEdit (Hall, 1999). The consensus sequences were blasted

in the NCBI (Altschul et al., 1997) and Barcode of Life Data System databases. Available *Codium* species sequences from these databases (if a voucher code was available and besides this, if the sequence was previously used in an SCI publication) were downloaded and used as references for obtaining alignments using the MUSCLE algorithm from MEGA ver. 10.0.5 (Kumar et al., 2018). Distinctive haplotypes for both markers were detected using DnaSP v.5 software (Librado and Rozas, 2009). Diversity metrics for each marker were computed using the software. The General Time Reversible model (GTR) with gamma-distributed invariable sites for *tufA*, the Tamura-Nei model (TN93) gamma-distributed invariable sites for *rbcL* and Tamura-3-parameter (T92) with gamma-distributed sites model were used as a model to build phylogenetic trees (ModelTest software available inside MEGA ver. 10.0.5) (Kumar et al., 2018). Sequences of *Bryopsis* (sister-genus of *Codium*) and of *Ostreobium* sp. (a distant relative of Bryopsidales order) were used as outgroups (Verbruggen et al., 2007). We used a Bayesian phylogenetic approach to analyze the data following Verbruggen et al. (2007) and using BEAST ver. 1.7.1 (Drummond and Rambaut, 2007) to generate haplotype trees using the Yule model as the tree prior. This is a simple model of speciation that is generally more appropriate when considering sequences from different species (Drummond and Rambaut, 2007). We made runs of 20 (*tufA*) - 30 million (*rbcL*) generations sampling every 1000 under a molecular clock model of Relaxed Clock: Uncorrelated Log-normal so as to account for lineage-specific rate heterogeneity and using the mentioned above nucleotide substitution models for the markers. In all cases, effective sample sizes (ESS) were above 3000, and a good mix of the chains was observed. Summary statistics and trees were generated using the last ten (50% of burning) million generations. Cluster analysis using the Neighbor-Joining method with 2000 bootstraps was used in MEGA 7 ver. 10.0.5 (Kumar et al., 2018) for the FCO*tufA* marker.

RESULTS

Eighty three new *tufA* sequences were obtained in this work from different sampling locations in the Atlantic (Viana de Castelo), Bay of Biscay and UK (Portlloe, Plymouth) (Fig. 1.). Overall, three different haplotypes corresponding to three different species were identified: *C. fragile* subsp. *Fragile* (Voucher Code: FCO-Alg.2230 - Genbank ID:

MK569393, 100% similarity with the *C. fragile* Genbank record KX855882.1 -Voucher Code: MJB0088); *C. tomentosum* (Voucher Code: FCO-Alg.2239 - Genbank ID: MK569394, 100% similarity with *C. tomentosum* Genbank record KX855760.1 - Voucher Code MEL:G.419) and *C. vermilara* (Voucher Code: FCO-Alg.2287 - Genbank ID: MK569392, 100% similarity with Genbank record KP685880.1 -Voucher Code: KRK002) (Table 1). The Bold database was not informative for the goal of *Codium* spp. identifications using the *tufA* genetic marker (Table 1).

Phylogenetic analysis was conducted after the trimming of sequences using a final alignment of 675 bp and including a total of one-hundred and forty-one *tufA* sequences (83 sequences from this study and 58 from GenBank). Diversity levels found with this analysis revealed $S=299$ as a number of polymorphic/segregating sites and global haplotype and nucleotide diversities of Hd : 0.8031 and $\pi= 0.0829$, respectively. A total of 39 *tufA* distinctive haplotypes were found ($\pi=0.1254$). The Maximum clade credibility tree (the tree with the highest product of the posterior probability of all its nodes) obtaining using the Beast software was perfectly rooted with the external nodes and revealed good support (HPP=0.9999) for the three main clades that have been previously reported for the genus *Codium* even when a few taxa fall apart from the main clades (Fig. 2.). The *C. fragile* species clustering was well supported clearly indicating the invasive (including the FCO-Alg.2230 haplotype) and the *C. fragile* native variants (Fig. 3.). The *C. vermilara* and *C. tomentosum* haplotypes found in this work were clustered with their similar Atlantic *Codium* species in two different groups clades (Fig. 3.).

Table 1. The *Codium* species genetic identification using BLAST procedures on plastidial haplotypes obtained from specimens collected in Asturias (North Coast of Spain) and Viana do Castelo (Portugal).

Place and year of collection	Coordinates	Voucher code	Gen/ GenBank ID	Assignment in GenBank (% of similarity)	Assignment in BOLD system (% of similarity)
<i>Codium vermilara</i>					
Aramar, February 2016	43°60'99"N, 5°78'55"W	FCO- Alg.2287	<i>tufA</i> (MK569392)	KP685880.1 - Voucher Code: KRK002 (100)	No match
<i>Codium fragile</i> subsp. <i>fragile</i>					
Port of Gijón, June 2017	43°55'44"N, 5°69'97"W	FCO- Alg.2230	<i>tufA</i> (MK569393)	KX855882.1 Voucher Code: MJB0088 (100)	No match
<i>Codium tomentosum</i>					
Tapia de Casariego, December 2016	43°57'02"N, 6°94'39"W	FCO- Alg.2239	<i>tufA</i> (MK569394)	KX855760.1 - Voucher Code MEL:G.419 (100)	No match
<i>Codium vermilara</i>					
Aramar, February 2016	43°60'99"N, 5°78'55"W	FCO- Alg.2298	<i>rbcL</i> (MK569395)	<i>Codium vermilara</i> EF108092.1 (100)	<i>Codium vermilara</i> EF108092.1 (100)
<i>Codium fragile</i> subsp. <i>fragile</i>					
Tapia de Casariego, December 2016	43°57'02"N, 6°94'39"W	FCO- Alg.2238	<i>rbcL</i> (MK569396)	KJ909134.1 (100)	No match
Viana de Castelo, March 2016	41°68'78"N, 8°84'66"W	FCO- Alg.2272	<i>rbcL</i> (MK569397)	No match*	No match*

*Assigned to *C. tomentosum* with *tufA* gene (see the text).

Table 2. The *Codium* specimens collected and analyzed in this work (fresh and herbarium seaweeds) using the FCO*tufA* (short fragment of the *tufA* gene) and results of the genetic assignments.

Voucher Code	Material storage	Gene used for species assignment	Species assignment (Genbank ID and %)	Collector	Location (Year)
FCO-Alg. 2355	Fresh	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Hyul Je Lee	Mokpo, South Korea (2017)
FCO-Alg. 2356	Fresh	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Hyul Je Lee	Mokpo, South Korea (2017)
FCO-Alg. 2357	Fresh	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Hyul Je Lee	Jindo Geumgap, South Korea (2017)
FCO-Alg. 2358	Fresh	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Hyul Je Lee	Jindo Geumgap, South Korea (2017)
FCO-Alg.2359	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Christine Maggs	Awaji ,Hyogo Prefecture, Japan (2006)
FCO-Alg.2360	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Christine Maggs	Carraroe, Greatmans Bay, Galaway, Ireland (2006)
FCO-Alg.2361	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Christine Maggs	Carraroe, Greatmans Bay, Galaway, Ireland (2005)
FCO-Alg.2362	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Christine Maggs	Toyo, Kochi Prefecture, Japan (2003)
FCO-Alg.2363	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1 Voucher Code: MJB0088 (100)	Christine Maggs	Toyo, Kochi Prefecture, Japan (2004)
FCO-Alg.2364	Herbarium	FCO <i>tufA</i>	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1	Marc Verlaque	France (2002)

Voucher Code: MJB0088 (100)

FCO-Alg. 2365	Fresh	FCO _{tufA}	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1	Hyul Je Lee	Jindo Geumgap, South Corea (2017)
			Voucher Code: MJB0088 (98,91%)		
FCO-Alg.2367	Herbarium	FCO _{tufA}	<i>Codium fragile</i> subsp. <i>Fragile</i> KX855882.1	Christine Maggs	Adriatic Sea, Izola, Gulf of Trieste
			Voucher Code: MJB0088 (100)		

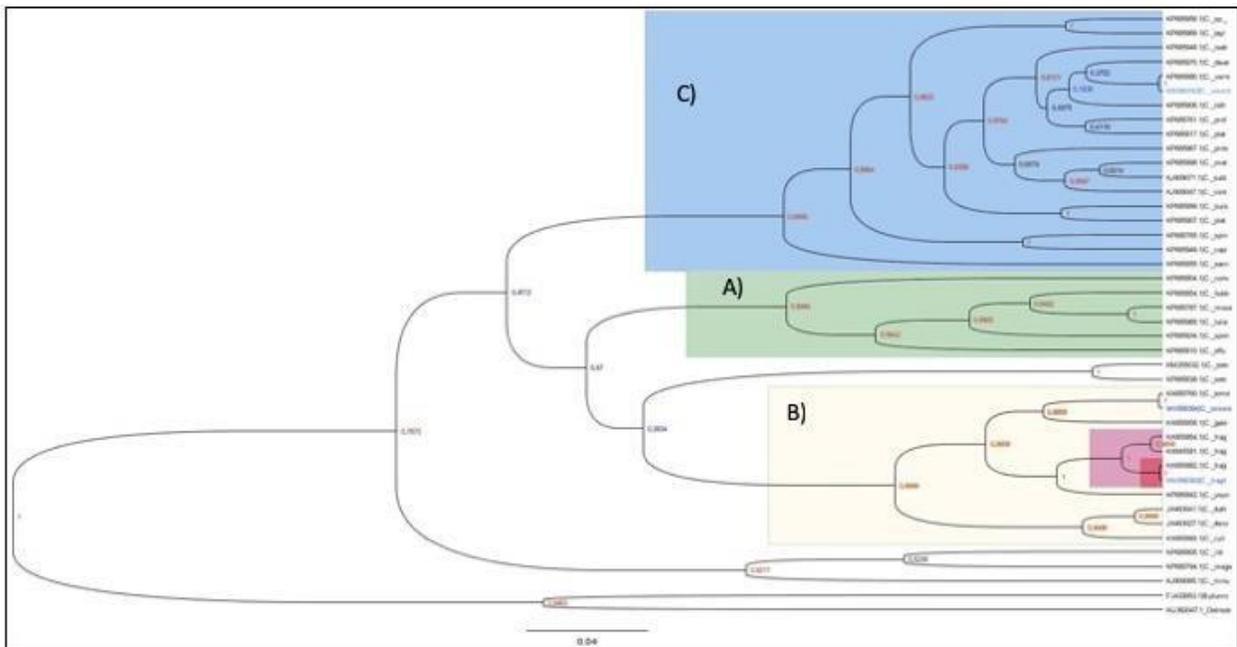


Figure 2. Evolutionary relationships of taxa based on a Bayesian phylogenetic approach of the *tufA* sequences (675 bp) in BEAST ver. 1.7.1 to generated haplotype trees using the Yule model as the tree prior. The evolutionary distances were computed using the GTR +G+I model. The posterior probability (support) for each node is shown and the scale is in the units of the number of base substitutions per site. The *C. fragile* species (invasive and native) is inside a pink square while in red is shown the invasive variants. The Bay of Biscay samples taxa labels in Genbank are in blue. The three previously described clades in Verbruggen et al. (2007) are shown in different colors.

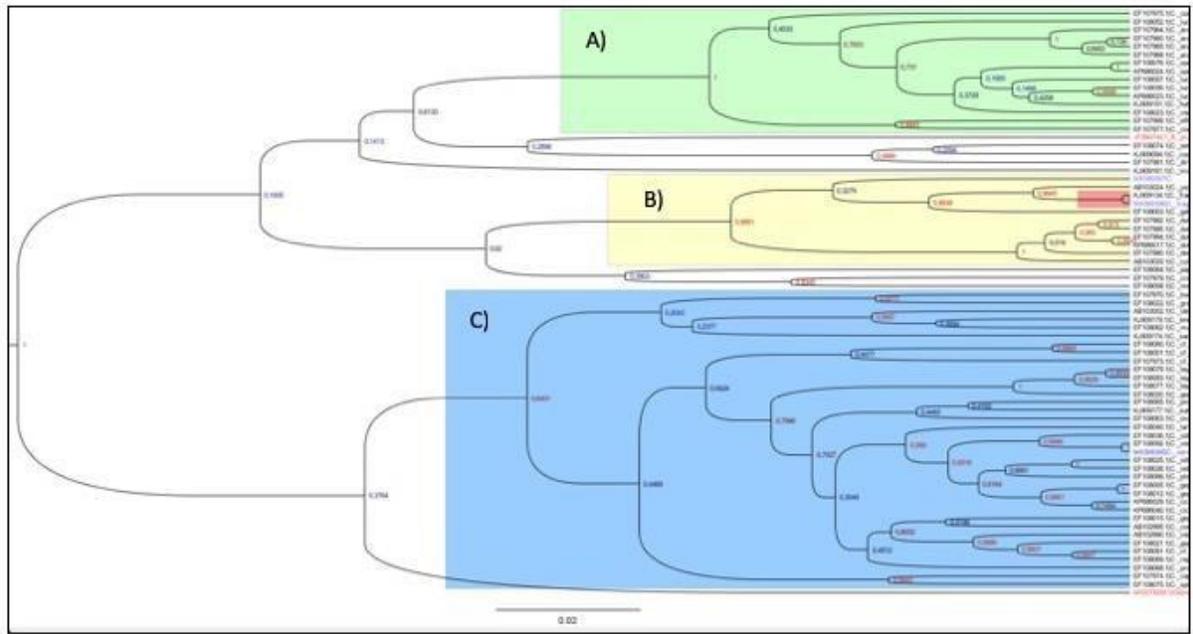


Figure 3. Evolutionary relationships of taxa based on a Bayesian phylogenetic approach of the *rbcL* sequences (601 bp) in BEAST ver. 1.7.1 to generated haplotype trees using the Yule model as the tree prior. The evolutionary distances were computed using the TN93+G+I model. The posterior probability (support) for each node is shown and the scale is in the units of the number of base substitutions per site. The *C. fragile* species clade is inside a red square. The Bay of Biscay samples taxa labels in Genbank are in blue while outgroups taxa labels are in red. The three previously described clades in Verbruggen et al. (2007) are shown in different colors

One-hundred and thirty-two *rbcL* sequences were obtained in this work. Again, three different *rbcL* haplotypes but only two species (see below) were detected using BLAST procedures (>99,83% similarity): *C. fragile* subsp. *Fragile* (Voucher Code: FCO-Alg.2238 - Genbank ID: MK569396) and *C. vermilara* (Voucher Code: FCO-Alg.2298 - Genbank ID: MK569395) (Table 1). There were no *C. tomentosum* sequences amplified with *rbcL* gene available in GenBank and thus not match, at least considering 98% of similarity as an effective cutoff for species identification, was found in Genbank for the sample with the Voucher Code: FCO-Alg.2272 - Genbank ID: MK569397 (Table 1). However, our previous *tufA* analysis indicated that this sample was clearly the species *C. tomentosum* (100% similarity with *C. tomentosum* Genbank record KX855760.1 - Voucher Code MEL: G.419). The Bold database was either no informative for the goal of *Codium* spp. identifications using

the marker *rbcL* (Table 1). Genetic analyses using *rbcL* haplotypes included 132 sequences from this study and 93 from GenBank. A 601bp alignment after trimming revealed lower levels of genetic diversity ($S=194$, $Hd: 0.7850$ and $\pi= 0.0576$) than previously found in the *tufA* gene. A total of 70 distinctive haplotypes were found ($\pi= 0.0777$). The *rbcL* Bayesian phylogenetic analyses showed lesser support for the *Codium* reported clades (0.84-1.00) and chaotic dispersal rooting for the external taxa (*Bryopsis* and *Ostreobium sp.*) or rare clustering for a few taxa (Figure 3). When possible (availability of reference sequences for the species), the Bay of Biscay haplotypes found in this work clustered well with references for the gene under analysis (Fig. 3).

We found serious difficulties while trying to amplify herbarium and fresh Korean samples with longer *tufA* fragment (>600bp). Thus we designed short primers (FCO*tufA*) that facilitate successful amplification of 200bp from those samples. In silico analyses with the software, Primer-blast from Genbank showed that the designed FCO*tufA* marker may also be useful for the identification of herbarium or problematic samples not only when analyzing *C. fragile* species but also for *C. tomentosum*, *C. yezoense*, *C. galeatum*, *C. cylindricum*, *C. duthieae*, *C. decortdatum*, and *C. contractum*. The newly designed FCO*tufA* marker was useful for barcoding herbarium and fresh samples in this work (four *C. fragile* fresh samples from South Korea, three herbarium specimens from Japan, three herbarium specimens from Atlantic coast of Europe (Ireland) and one herbarium specimen from the Adriatic Sea). After sequencing, a final trimmed alignment of 185bp from *tufA* fragments in 29 samples was obtained including these new 11 sequences and reference sequences from *Codium* species where the new tool could be useful. The FCO*tufA* analysis revealed 16 different haplotypes ($S=69$, $Hd: Hd: 0.8050$ and $\pi= 0.0685$). Two *C. fragile* haplotypes within the invasive *C. fragile* clade, including one new haplotype for the FCO-Alg. 2365 sample from South Korea, was found in the newly analyzed samples which could indicate the probable existence of a higher genetic diversity inside the Asian native range for the invasive *C. fragile* sub-species not previously detected here with the *tufA* longer fragment in fresh samples (Table 2, Figure 4). The rest of the *Codium* samples from herbariums share the same haplotype with *C. fragile subsp. fragile* species (Fig. 4.).

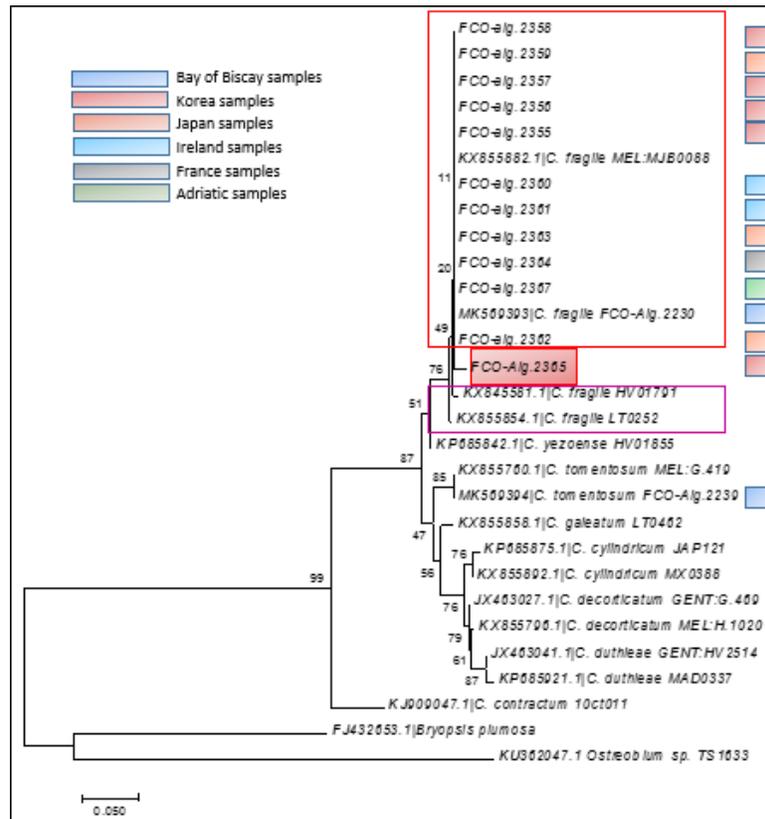


Figure 4. Evolutionary relationships of taxa using the Neighbor-Joining method for PCR bands sequences using the *FCOtufA* marker (185bp/2000 replicates). The evolutionary distances were computed using the Tamura 3-parameter G+I method and are in the units of the number of base substitutions per site. The known two *C. fragile* haplotypes (Invasive and native) are inside red squares and pink squares, respectively, while the new Korean haplotype is pointed out with a red shadow.

DISCUSSION

Molecular tools should refine taxonomical identification of species, especially in the cases of algae (macro and microalgae) with a high level of phenotypic plasticity or a small size (Pečnikar and Buzar, 2014). Accordingly, Rojo et al. (2014) confirmed the presence of *C. fragile* species along the Cantabrian Coast by employing plastid marker UCP6 for DNA barcoding. In this work, we have obtained a confirmation of the presence of the invasive

variant of *C. fragile* and our study further confirms the presence of two native species (*C. tomentosum* and *C. vermilara*) with the support of *tufA* and *rbcL* analyzed genes. In addition, the designed short primers (FCO*tufA*) has facilitated successful amplification of herbarium and fresh samples with different geographical origins and thus the identification of invasive *C. fragile* in this type of plant material.

There have been some records about spatial changes in the seaweed communities along the Cantabrian Coast in the past 20 years (Voerman et al., 2013; Fernández, 2016). Perhaps these changes are associated with increasing temperatures in the Northeast Atlantic (Taboada-González and Anadon, 2012) and the geographical boundaries of cold-temperate macroalgae have moved westward of the Cantabrian Coast. The ecological data about spatial and temporal changes of invasive *C. fragile* and native *Codium* species in the north coast of Spain have been also investigated (Cires Rodríguez and Rico Ordás, 2007; Rojo et al., 2014; Garcia et al., 2018). It seems that native *Codium* species are still thriving, as no displacement was shown due to the presence of *C. fragile*. However, the abundance of *C. fragile* is increasing with temperature, when moving toward the east side of the Cantabrian Coast.

Accurate species identification is important for monitoring and management decision, especially in the case of invasive marine macroalgae (Melton et al., 2016). The *tufA* gene is well-conserved chloroplast region and provides good species-level identification in green macroalgae (Fama et al., 2002). Our results, obtained from *tufA* gene (designed by Fama et al., 2002 and Saunders and Kucera, 2010) and FCO*tufA* support the claim of Verbruggen & Costa (2015); *tufA* gene should be used as a primary marker for clear species distinction in *Codium* species. It is obvious that using concatenated genes (as in Verbruggen et al. (2007)), it is possible to obtain a deeper phylogenetic signal. However, our screening in genetic databases searching by identical voucher codes and both sequences for the *tufA* and *rbcL* genes only reach less than 10 species for the genus *Codium*. In any case, *tufA* marker showed much stronger phylogenetic signal and higher levels of genetic variation than *rbcL* in this study. The small size in FCO*tufA* marker (<200bp) constraints its utility in phylogenetic

studies. However, we suggest that it can be used for rapid identification of invasive *C. fragile*, especially in herbarium or degraded samples.

No intensive monitoring for studies about the *Codium* species genetic identifications and its genetic diversities have been conducted along the Cantabrian Coast so far. The previously described *C. decorticatum* species for the studied area was not recorded among Bay of Biscay samples from this work with either *tufA* nor *rbcL* markers. There are no *tufA* or *rbcL* sequences of subsp. *atlanticum* in Genbank or BOLD databases, however, our samples of subsp. *fragile* were all grouped within subsp. *fragile* cluster from available records in the Genbank database (Fig. 2, 3).

We detected a low level of genetic variation in the invasive *C. fragile* that coincides with previous studies along the European Coast with UCP6 marker (Provan et al., 2005; Provan et al., 2008). Our results, obtained from *tufA* and *rbcL* markers, also indicate a low level of genetic diversity in native *C. tomentosum* and *C. vermilara* since one haplotype is present per species, even for the samples from Portugal and UK. The type of reproductive mechanism could explain this. Even though sexual reproduction has been mentioned in subsp. *fragile* (Prince, 1988) it is a common belief that this species reproduces asexual and it looks that it is the same for native *Codium* species. It is possible that asexuals are more inclined to the low level of genetic variation and high homozygosity in founders population (Roman and Darling, 2007), but also in established native ones, thus low level of genetic diversity within and – among populations can be expected (DeWalt et al., 2004). The asexual reproduction decreases effective population size, causing genetic drift, which results in a reduced genetic variation (Valero et al., 2001). The loss of genetic variation could occur because of a founder effect in cases where introduced genotypes render plastic responses to novel environments or possess phenotypes with broad environmental tolerances (Roman and Darling, 2007; Sakai et al., 2001). It seems that the manifestation of the invasive character is not hampered by the low level of genetic variation (Roman and Darling, 2007) which contribution is still not clear (Bock et al., 2015) and sometimes limited (Rius et al., 2015).

Genetic mechanisms such as epigenetic preadaptation or post-invasive adaptive mutation could explain a role in the invasive success (Puillandre et al., 2008). However, the lack of nuclear markers and knowledge about fitness-related traits between donor and recipient area of *C. fragile* limits those findings. Steen et al. (2017) state that a low level of genetic diversity of *C. fragile* and *S. muticum* should be treated as an exception and not a rule since meta-analysis by Rius et al. (2015) shows that 74% of non-indigenous species demonstrate similar genetic diversity inside the donor and recipient area. The distinction in genetic diversity between donor and recipient areas manifests as a complex interplay of short- and long-term natural processes, as well as an anthropogenic influence (Provan et al., 2013). Genotyping with high-resolution and variable markers like SNP (single nucleotide polymorphism, substitution of a single nucleotide that occurs at a specific position in the genome) or microsatellite (tandem repeats of the two to six nucleotides) would allow the comparison of genetic diversity on a deeper molecular level, as well as how mating systems influence patterns of genetic variation within and between populations (Provan et al., 2013; Candeias et al., 2015).

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CHAPTER 4.

Under revision (Journal for Environmental Education)

FIND INVASIVE SEAWEED: AN OUTDOOR GAME TO ENGAGE CHILDREN IN SCIENCE ACTIVITIES THAT DETECT MARINE BIOLOGICAL INVASION

INTRODUCTION

NIS and, particularly, IAS are among the most dangerous hazards in marine and coastal ecosystems worldwide. These biological pollutants pose a threat to biodiversity and can have several effects on the native community structure (e.g., Chown et al., 2015). The global impact of invasive seaweeds is extensive, affecting coastal communities in most biogeographical regions (Thomsen et al., 2016). A general problem in ecosystems worldwide, marine biological invasions require complex management involving researchers; managers and citizens for successful control and biosecurity (see for example Hershendorfer et al., 2007, Larson et al., 2011). Thus, it is of key importance that the general public is informed about breakthroughs regarding new knowledge and management of NIS and IAS, as well as advances in scientific contributions (Silvertown, 2009). Among other approaches, citizen science can be a participatory strategy that connects the general public with scientific advances in this context.

CITIZEN SCIENCE

Citizen science is a participatory strategy in which citizens and scientists work together in research projects (e.g. Irwin, 1995; Bonney et al., 2014). Recently, the number of citizen science projects augmented exponentially (McKinly et al., 2015; Pocock et al, 2017). Over the period of 2010 to 2015, the number of citizen science projects published in the Web of Science increased from 124 to 402, primarily focusing on biology, environment, and ecology (Kullenberg & Kasparowski, 2016). However, marine citizen science has lagged behind its

terrestrial counterpart and it is fragmented, relative to wider spatial and long-term integration of terrestrial initiatives (Theobald et al., 2015; Garcia-Soto et al., 2017; Science Europe, 2018). Logistic obstacles (expensive boats, equipment in SCUBA diving projects, transportation to the coast), safety issues (especially when involving children) and the fact that most of the humans spend a minor part of their life on the water could be some of the reasons why marine environment poses a challenge when it comes to engaging the general public in such activities (Cigliano et al., 2015). Thus, the need to comprehend how people learn about, become involved with (Ballard et al., 2017), perceive and connect with the sea is essential to successfully engage the general public in citizen science projects related to marine conservation (Vincent, 2011; Fletcher et al., 2012).

EDUCATIONAL GAMES

In that scenario, games are a powerful tool for learning and can engage participants in difficult tasks (Hoffman & Nadelson, 2009), Games appears to work well when implemented in citizen science projects to motivate and encourage participation (e.g., Iacobides et al., 2013), more so when the performance of the task can be quantified in the form of a survey or test that provides an opportunity to learn and correct misapprehensions (Eveleigh et al., 2013). Games also seem to be an effective means to motivate, engage and foster a spirit of conservation in children (Davis 1998, Davis & Elliot, 2003) when there has been a relative absence of approaches stressing environmental perspectives in curriculum theory, policy, and practice (Hart, 1997; Davis, 1998).

The efficacy of gaming as an effective intervention in education can be extrapolated from the growing number of papers published on games, game-based learning and gamification experiences over the last few decades (e.g., Hamari et al., 2014). Educational games help students develop a better understanding of the teaching-learning process in which they are involved (Franco-Mariscal et al., 2015; Huizenga et al., 2017; Martí-Centelles & Rubio-Magnieto, 2014). It has been shown that participants develop new cognitive and operational abilities by teaching and learning specific subjects, and new possibilities emerge with regard to obtaining an improved understanding and reinforcing complex concepts that are difficult to comprehend (Al-Azawi et al., 2016; Miralles et al., 2013).

Under the hypothesis that games could help to foster citizen science and by the same time knowledge about invasive species and marine conservation, an educational game was originally created for children under formal education but is accessible to people of all ages and contexts. It was developed to *i*) teach citizens to identify invasive seaweeds based on taxonomic phenotypic characteristics and *ii*) motivate and encourage the general public, particularly younger generations, to participate in marine citizen science activities. The game was first tested in a pilot study among different audiences and was ultimately included in a citizen science project with high school students. Levels of recruitment, number, and type of NIS/IAS seaweed species detected by children within the area, and pre- and post-tests were used to evaluate the game with regard to its learning and motivation efficacy.

MATERIALS AND METHODS

GAME MATERIALS

- 'Marine invasive seaweed cards' (Fig. 1) containing information about invasive seaweed along the north coast of Spain (Cantabrian Sea, Atlantic Ocean), species that have been introduced to Europe some time ago and display an adverse ecological and economic impact outside of their native range (Williams, 2007). Cards can be modified to any other marine region to include the invasive species therein. 'Marine invasive seaweed cards' had a picture of a real species of seaweed, with its scientific name on the front of the card and a short description of its taxonomic, economic and environmental impact on the reverse. Based on Cires and Moliner (2010) and Peteiro (2014), the game included the following species:
 - *Codium fragile* subsp. *fragile* (Suringar) Hariot 1889 (Scheibling & Gagnon, 2006; DAISIE project),
 - *Undaria pinnatifida* (Harvey) Suringar 1873 (Casas et al., 2004; DAISIE project),
 - *Sargassum muticum* (Yendo) Fesholt 1955 (Britton-Simmons, 2004),

- *Grateloupia imbricata* Holmes 1896 (Ramalhosa et al., 2016; Montes et al, 2016),
 - *Asparagopsis armata* Harvey 1855,
 - *Colpomenia sinuosa* var. *peregrine* Sauvageau 1927 (Cotton, 1908) and
 - *Polysiphonia schneideri* B. Stuercke & D. W. Freshwater 2010.
- Invasive specimens preserved as herbarium material from the University of Oviedo (<https://www.unioviedo.es/bos/Herbario/FCO.htm>) (Fig. 2, Item A).
 - Fresh samples of marine invasive seaweeds preserved in absolute ethanol (Fig. 2, Item B).



Figure 1. Cards showing the seven marine invasive seaweeds occurring in the Cantabrian Sea and used in the ‘*Find invasive seaweed*’ game in Salinas (Asturias, Spain). These cards can be modified to include local invasive species of different geographical areas.

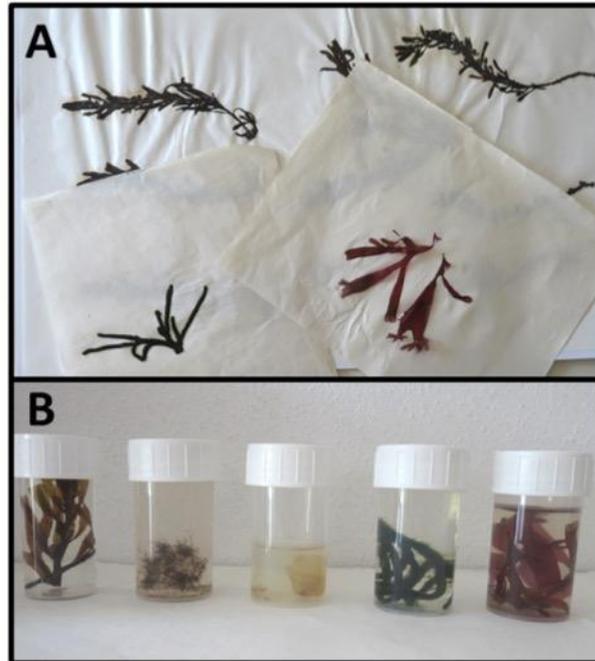


Figure 2. Algae materials used for playing the ‘Find invasive seaweed’ game. A) Herbarium material from the University of Oviedo. B) Fresh samples of marine invasive algae preserved in absolute ethanol.

GAME ACTION

Before starting the game, a secure place (e.g., a playground or fenced area) must be found where samples of Item A and Item B can be hidden. Additionally, a game leader (the person who will be in charge of the game) should supervise the activity. Based on the number of participants in the game, small working teams can be made to stimulate participants to reach the common goal of the game: to collect and identify as many hidden items as possible. However, the game may also be played individually, as some participants may have limitations that preclude them from engaging in group play mode (Iacovides et al., 2013).

The game starts when all items (A and B type) are concealed in the selected area. Then, cards for each species are given to the participants. To obtain better outcomes a brief explanation about each species is also recommended before the game starts. Independent searching is not allowed in the case of team play. All participants start the game at the same time.

When the participants find one item (A or B type), they have to compare it to all of the photos from the 'Marine invasive seaweed cards', to correctly identify the target seaweed. If identification is correct, the game leader asks them a question about the biological, ecological or economic impact of that species. Cards are a helpful accessory to the game because the most important information about each species of seaweed can be found on it. Interaction between team members to answer the question correctly increases team efficiency (Rania et al., 2015). If the team correctly identifies the seaweed species and answers the question, they get one point and can proceed with the game. Even if the identification is incomplete or the question is answered incorrectly, they must continue the game by looking for other hidden items. The game concludes when all the items have been found. If the time allotted for the activity ends and/or participants cannot find all the items, the team with more points wins. In such cases, the points system can help establish a ranking. Additionally, competition principles within the game motivate children to play. As suggested by Ruggiero (2013) and Huizenga et al. (2017), competition in a game can be an important element that contributes to engagement, motivation to learn, and cognitive learning outcomes.

ETHICS STATEMENT

This study adheres to the European Code of Conduct for Research Integrity. Informed consent was obtained from all participants to use the collected data for research publication. While playing, all children were under adult supervision. This scientific game was specifically designed and developed for educational purposes, to implement training and to recruit a volunteer for citizen science. The game has been registered in Spain under the copyright protection number 05/2017/358. To avoid the unlikely possibility of collecting any living specimens or any space possibility of NIS dispersion, all participants were awarded about biosecurity rules. These biosecurity guidelines included clear rules to be followed, such as not taking algae from other beaches, not transferring algae between beaches, keeping all fragments under supervision until they were stored in ethanol, checking shoes for small seaweed fragments before leaving the beach, etc. Specifically, on the beach, only detached specimens found lying on the sand were collected. All collected samples were immediately stored in absolute ethanol and transported to the Department of Organisms and Systems

Biology at the University of Oviedo. All game participants promised not to keep samples or transport samples outside of the study area to avoid the accidental dispersion of putative nonnative fragments.

INITIAL PILOT STUDY

Prior to the case study, we tested 'Find invasive seaweed' game in a pilot study, to avoid potential obstacles and identify possible constraints on the way to achieve the game main objectives and clarify doubts (Hassan et al., 2016). The first game trial was completed during the '*Aula del Mar*' marine environmental education project as part of the activities program during an international surf competition (Surf Music and Friends, August 2016). This event was selected to reinforce the idea of Cigliano et al. (2015) to inform junior audiences about marine systems whenever practicable. All children were under adult supervision. Before playing with the game a short explanation was provided in which the children were familiarized with the invasive seaweed in Asturias and all of the possible environmental and economic issues that could arise following their establishment and dispersion. Later, children gathered in teams and searched for items A and B of invasive seaweed with the help of the cards. The items were hidden randomly in a selected festival area, within a demarcated plot. Since '*Aula del Mar*' was 4 days in duration and it was located only 10 meters from the beach, we suggested to children to bring any detached fragments of possibly invasive seaweed that they could find lying on the beach to be identified, as an approach of a future possible citizen science monitoring project for detecting NIS. *In situ* identification of seaweed, specimens were determined by scientific experts from the University of Oviedo, Department of Organisms and Systems Biology. All seaweed specimens were carefully monitored and preserved in absolute ethanol to avoid any possibility of dispersion and to confirm all detected species by further scientific identification at the University of Oviedo.

THE CASE STUDY

The '*Find invasive seaweed*' game was assessed among children of ages 11-12 (according to Spanish Educational Law it is classified as grade 1 of Educación Secundaria Obligatoria

(ESO); grade KS4 in the UK and grade 8 in the USA) at Salinas High School (Asturias, North Spain), which is located in front of the longest sandy beach in Asturias (Salinas beach; length approx. 2600 m.). This study took place from April to June 2017.

Before starting, all seaweed (items A and B) were hidden inside of a fenced playground. The total time of 'Find invasive seaweed' game-based training lasted approximately 50 minutes and took place during one biology class. First, a pre-test was completed before the game and again, the same test was done at the end of the game, as a post-test. Ten minutes was considered sufficient time to complete the test in both cases. After the pre-test, a short but consistent introduction on the origin of seaweed invasion, the vectors of their introduction and ecological and economic impact were explained to the children, paying special attention to seaweeds invading Asturias coast in the Cantabrian Sea. Explanations were accompanied by pictures, examples and preserved samples. Immediately after the short talk, the game began on the playground. Students worked in different teams to look for items A and B. After finishing the game, everybody came back into the classroom, and the posttest was given. The activity was completed in two different groups (1°ESO-A and 1°ESO-B).

Finally, a modest marine citizen science project for monitoring of invasive seaweed was carried with the students. Two glass containers with absolute ethanol were placed in the biology class for 3 months. Each group (1°ESO-A and 1°ESO-B) had one container and all students were encouraged to bring in detached fragments of potentially invasive seaweed found lying on Salinas beach for final identification. From April to June 2017, students went to the beach on weekends and brought seaweed fragments into class on Mondays. Although students were only allowed to take fragments of dead seaweed they found lying on the closest beach, both teacher and researchers strongly insisted on biosecurity rules to avoid any possibility of dispersion.

TRAINING AND QUESTIONNAIRE

The game-based training was based on a short talk (10-15 minutes) on the problematic nature of marine invasive species, particularly seaweed invading the Asturias Coast, followed by

the game. To assess previous knowledge about invasive algae, a pre-test was administered before the training, and a post-test was also completed immediately after the game to evaluate the knowledge acquired during the activity and the success of the training.

A five-item test was designed in accordance with the objectives of the game to obtain clear responses to simply structured questions (Supplementary Materials Table S1). The first three items of the test assessed current knowledge of seaweed that was included in their school curricula: 1 (Q1): the meaning of invasive species; 2 (Q2): where seaweed live; and 3 (Q3): how seaweed feed. All answers were scored as a 0 (I do not know), 1 (incorrect answer) or 2 (correct answer). Participants awareness of invasion issues was evaluated with the last two items (Q4 and Q5), which were scored using a Likert scale; ranging from 1 to 4, with 1 being the lowest level and 4 the maximum level of awareness; 4 (Q4): awareness of consequences and effects of invasive species; and 5 (Q5): management actions when invasive seaweed is found. An initial trial of the test was administered to 30 children and adults who were not involved in the study to check for inconsistencies and to confirm the understandability of the questions. For each of the items (questions) the correlation between the item and the completed test, once the score of the former was removed from the score of the latter, was assessed using the Corrected Index of homogeneity (IHC) as suggested by Peters and Van Vorhis (1940). Only questions showing $IHC > 0.20$ were used in the final questionnaire.

Differences between ‘correct,’ ‘incorrect’ and ‘don’t know’ answers (acquired knowledge: Q1-Q3) were evaluated using the Pearson chi-square test and comparisons of column proportions were evaluated using Bonferroni corrections (IBM SPSS Statistics 24). Evaluations of Q4 (awareness) and Q5 (management actions) before (pretest) and after (posttest) the game included comparisons in terms of means and distributions, using Levene’s Test to check for homogeneity of variance and paired nonparametric Wilcoxon Signed-Ranks Test (IBM SPSS statistics 24).

RESULTS

INITIAL PILOT STUDY

Twelve children that were 8 to 16 years of age participated in the first pilot game activity. However, in the following days, more children (n=18, original 12 participants in the game-based training plus an additional 6 children) brought in specimens of detached seaweed for scientific evaluation. The original 12 children told their friends to help them, so more children than initially participated in the game helped collect detached seaweed. Thus, engagement in data collection and participation in this marine citizen science approach was surprisingly high, although the initial sample was small. Seaweed collection was successful and samples were preserved in ethanol for species identification. All species were mostly exotic and invasive: *Grateloupia imbricata*, *Colpomenia sinuosa*, and *Codium fragile* subsp. *fragile*.

CITIZEN SCIENCE CASE STUDY

In the final case study, 46 high school children (ages 11-12) participated in the game-based training and marine citizen science project. Test results were similar across the different classes suggesting that any class bias could be dismissed and all data could be pooled for statistical analyses. Answers in response to questions concerning knowledge gained from the curricula (items Q1, Q2, and Q3) revealed an increase in correct answers and a decrease in incorrect and 'don't know' answers after the game-based training (Fig. 3).

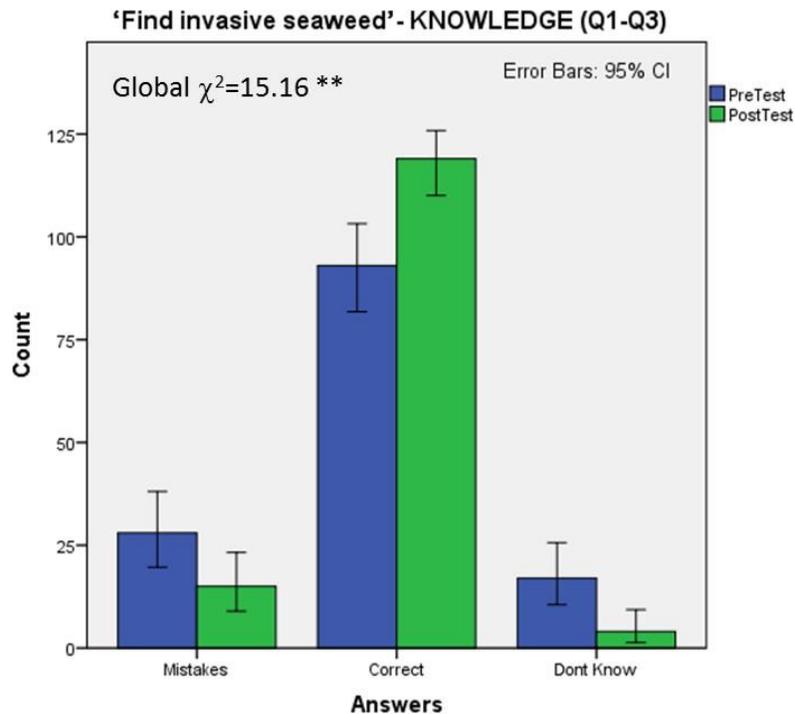


Figure 3. Evaluation of acquired knowledge (Q1, Q2, and Q3 items) about seaweeds in the game-based training with children from a secondary school of Salinas (Asturias, Spain). Answers from Q1, Q2, and Q3 items were counted, added up and presented in the pre- (blue) and post- (green) tests evaluations, its error bars and Pearson test included.

Global significant differences were found between pre- and post-tests (Chi-square=15.167; $p=0.001$) and for each of the answers (comparison of column proportions: p -value (correct)= 0.001; p -value (mistakes)= 0.031; p -value (don't know)= 0.003) (Fig. 3). Individually, significant differences were found for the item Q1, relating to the definition of invasive species, between pre-test and post-test - ($P_{Q1}=0.0187$) and also in items about the relevance and impact of invasive seaweeds Q4 (consciousness; $P_{Q4}=2.66E-06$) and Q5 about management actions ($P_{Q5}=6.345E-11$). Awareness was also positively increased after training (Fig. 4).

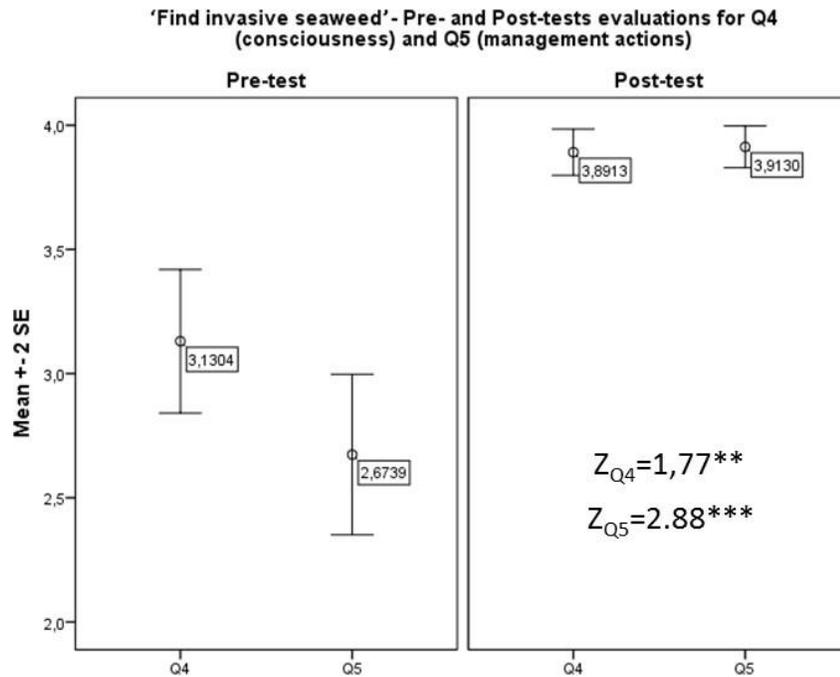


Figure 4. Comparison of Q4 (consciousness) and Q5 (management actions) mean in the pre- and post-tests evaluations when playing the ‘Find invasive seaweed’ game with children from a secondary school of Salinas (Asturias, Spain). Values are shown on a Likert scale where 1 is the lower and 4 the maximum awareness values. Kolmogorov-Smirnov values included.

The Wilcoxon signed-rank test revealed significant differences before and after the activity for awareness ($Z_{Q4} = -4.018$, $p\text{-value} = 0.0001$) and management ($Z_{Q5} = -5.056$, $p\text{-value} = 0.0001$) (Figure 4). Only 52% of participants noted that it is highly important to conserve marine ecosystems for the environment, economy, and society on the pre-test, but this increased to 89.13% on post-test (Supplementary Materials Figure S1). Evaluation of Q5 showed that although at the pretest just 36.9% of the participants knew how to proceed upon finding invasive seaweed, this number increased to 91.3% after the game (Supplementary Materials Figure S2). Thus, after completing the game-based training, children were able to recognize effective individual conservation actions therefore, saying that they would inform experts and authorities about their encounters with invasive seaweeds.

The two groups collected algae samples from Salinas beach during the project time and the students confirmed that they collected only detached fragments lying on the sand. No differences were found between the two containers with regard to the abundance of collected fragments. However, the number of participants in one group was highly different from that in the other (12 students in one group and just 2 in the other). Although they should have only collected invasive species, one native species, *Fucus vesiculosus* L. (Gallardo et al., 2016), was identified in one container. The proportion of native fragments was very low (less than 10% of the total). Moreover, the two containers from the two different groups also had unidentified organic material that was difficult to distinguish from the seaweed samples. Nevertheless, this marine citizen science project made it possible to detect another invasive species in the area: *Asparagopsis armata*.

DISCUSSION

Our living marine legacy is declining fast (McCauley et al., 2015) and the success of biodiversity protection relies on social, cultural, economic and political influences (Hawkins et al., 2016). Indubitably, citizens are an important factor in marine conservation processes. In Spain, citizen science and research activities targeting children as the primary participants in marine environmental surveys are rather scarce. In this project, we developed and evaluated an educational game about invasive seaweed to include it into a marine citizen science project. Using ‘*Find invasive seaweed*’ game-based training, local young citizens were enrolled in a modest but valuable citizen science project that detected four different invasive species around Salinas beach in 2016-2017: *Asparagopsis armata*, *Grateloupia imbricata*, *Colpomenia sinuosa*, and *Codium fragile* ssp. *fragile*. These species were previously reported in the Cantabrian Sea (Cires & Moliner, 2010; Peteiro, 2014), but this was the first report of these four NIS in the area (Salinas, Asturias, North Spain). In general, the cryptic nature of seaweeds sometimes hampers direct visual identification, even for experts, thus the help of taxonomist is always desirable. For example, the detection of cryptic species and lineages inside the genus *Asparagopsis* could easily lead to misidentification of *A. armata* (Zanolla et al., 2014). The fact that some specimens of *A. armata* could be found by students and identified by taxonomist during the project emphasizes how valuable this

activity could be for researchers and the scientific community. This type of research could be beneficial for both scientists and citizens (particularly students), as well as for the marine environment, as this project involves marine conservation principles and procedures. Volunteering with experts (e.g. scientists) at an early age increases motivation toward active participation in citizen science projects later in life (Morrow, 2003). Furthermore, citizen science projects engage participants in science, increasing participants' scientific knowledge and developing positive attitudes toward science (e.g., Eastman et al., 2014; Crall et al., 2013). For all that, the game '*Find invasive seaweed*' and the methodology presented here could be an easy tool to use in schools as a curriculum-based activity to engage and motivate children in marine conservation activities. Before conducting this project, the target students learned about the necessity for environmental protection (e.g. pollution, floating marine plastics, etc.). Thus, the game-based training was focused to raise environmental awareness about the negative impact of invasive species, since they are recognized as a menace for ecosystem functioning (Elmqvist et al., 2003). Public awareness can positively contribute to achieving conservation objectives (Jefferson et al., 2015). Here, we surveyed awareness of invasive species among high school students and the results revealed a significant increase in knowledge and awareness after the game-based training.

Engaging young citizens in structured learning through group interaction with the environment under professional guidance is a comprehensive training system in which the investments could reap longstanding benefits (Hogan, 2002; Davis, 1998; Krasny & Tidball, 2009). By employing emotion, attention, and concentration, citizen science activities augment people knowledge and consciousness about local biodiversity (Jordan et al., 2011). This seems to be true for the "*Find invasive seaweed*" game, which was employed as training material that enabled high school students to learn concepts while playing, working in teams, and carrying out a long-term project (3 months, in this case) with researchers from the University of Oviedo in order to find and identify one of the most dangerous bio-pollutants of marine environment: invasive species. Most citizen science project undertakings have illustrated that recruitment is one of the hardest components (Council & Horvath, 2016). Despite this, the '*Find invasive seaweed*' game enjoyed a successful recruitment rate in our pilot study among children of 8 to 16 years of age (18 participants from an initial group of

12 children; 150%); and a more modest engagement in the case study (14 participants from an initial group of 46; 30%). The differences in recruitment might be explained by the different contexts, as the pilot study was completed during the holidays, and the case study was completed within a formal education program. Moreover, it can be an informal but effective way of learning for students, as well as supporting communication between scientific and educational institutions (Mueller et al., 2012). In any case, conclusions about this recruitment should be taken with caution since the effect of small sampling size (n=64) and/or focus group (since it was conducted in a group of children that were living in a coastal area) could be affecting. For future studies, we recommend using a bigger sampling size and different groups of study.

Games in educational environments should challenge students (Hamari, 2016; Kavak, 2012) while allowing them to observe, explore, and manipulate a wide range of variables and receive immediate feedback on their actions (Lester et al., 2014). In the “*Find invasive seaweed*” game, several learning challenges can be found (from correctly identifying the different species of seaweed to answering specific questions about them), while students can directly manipulate, observe and explore the main target of the learning: invasive seaweed. Usually, educational activities require some intellectual effort, but, at the same time, participants should be able to experience enjoyment and motivation (Khan & Pearce, 2015). On the other hand, most educational games focus on the teaching process of a specific subject/topic (Antunes et al., 2012; Barclay et al., 2011; Franco-Mariscal et al., 2015; Giménez et al., 2011; Gutiérrez, 2014; Kavak, 2012; Lester et al., 2014; Martí-Centelles & Rubio-Magnieto, 2014; Russell, 1999). When playing “*Find invasive seaweed*,” students can learn about marine science by acquiring knowledge about invasive species and the importance of the marine environment. Moreover, there is parallel learning of bioethics, citizenship education and other subjects included in the formal education curricula (Huizenga et al., 2017). It seems that the game dynamics could help develop general competencies, such as working in groups, collaboration, and reflection skills.

Motivation is a broad and complex issue in education (see e.g., Fredricks et al., 2004, Perry et al., 2006). Recent studies have revealed that children seem to be motivated to play games

not only because of the visual appearance of the game design but also because of their interest in science (Iacovides et al., 2013; Curtis, 2015). Indeed, it is difficult to create an attractive educational game for the teaching-learning process, while, at the same time, getting students to pay attention for long durations and perform difficult tasks (Giménez et al., 2011). When developing the “*Find invasive seaweed*” game, the focus was set on developing an attractive game in which students could learn to identify seaweed while playing without losing interest, getting bored or getting frustrated with taxonomic issues. Children have shown positive biases for learning a large amount of new information (Lucas et al., 2014) and that was achieved during this study. It appears that students learned the difficult task of identifying invasive algae while experiencing enjoyment, motivation, and involvement in a citizen science project. Even though, we recommend for the future implication of the “*Find invasive seaweed*” game to interview the same group of children several years later to test long-term engagement and motivation in marine activities. The game was used to do foster two complementary things: citizen science and by the same time knowledge, attitudes, and action towards an environmental issue, invasive species and marine conservation.

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GENERAL DISCUSSION

In conjunction with an ecological and molecular work in this study, we were able to infer deeper understanding into invasion biology of *C. fragile* along the Cantabrian Coast, along with its native congeners, *Codium tomentosum*, and *Codium vermilara*. The dominant *Codium* species in the sampling period of 2016-2017 were native ones. This spatial pattern has been previously recorded in the Cantabrian Sea (Cires Rodríguez and Rico Ordás, 2007; Rojo et al., 2014; García et al., 2017) and along the North European coast (Trowbridge, 2001; Trowbridge et al., 2004). We hypothesize there are three possible explanations for this: opportunism, environmental changes, and temporal variation.

First, *C. fragile* may manifest opportunistic behavior if found in the sites with a lot of bare space or colonized fast in overharvested surfaces (Trowbridge, 1995; Chapman, 1999; Chavanich et al., 2006). Thomsen and McGlathery (2007) suggest that any superior growth of *C. fragile* compared to native macroalgae is possibly constrained to microhabitats where stress (space availability, desiccation, and grazing) is minimal and where bivalve shells facilitate recruitment and long-term perseverance. Space availability influences the expression of invasive character, as previously observed for *S. muticum* in the coast of Asturias (Andrew and Viejo, 1998). Macroalgae are evaluated as an important ecological indicator of the quality status in rocky communities along the Cantabrian Coast (Juanes et al., 2008). Based on the previous records from 'ALIEN' project, it seems that native communities are still thriving over NIS, among which some are invasive. Species-rich ecosystems tend to leave fewer niches available because the resources are used more efficiently (Elton, 1958). However 'ALIEN' project data are based on the presence/absence analysis, whereas temporal and spatial interspecific niche models, combining different type of data (categorical, count, continuous) (Geange et al., 2011), could reveal possible threats of invasion processes.

Second, the environment condition in which a given IAS is introduced has a great effect on its capacity to adapt successfully (Leicht-Young et al., 2007). Large spatial scale and climate-driven changes have lately been perceived in the coastal ecosystems (Aksnes et al. 2009, Iles et al. 2012, Hernández-Fariñas et al. 2014). North Coast of Spain is not an exception and those findings were corroborated by many ecological studies, especially for macroalgae communities. As an example, the retreat of large cold-temperate brown macroalgae toward the western part of the Cantabrian coast (Borja and Gorostiaga, 1990) could be a good indicator for the analysis of climate change effects (Guinda et al, 2012). Major differences in the distribution of macroalgae communities, following east-west latitudinal axis, were first pointed out by Sauvageau (1897) and further confirmed by the increasing amount of studies (Anadón, 1983; Anadón and Niell, 1981). Our simple randomization test demonstrates that these changes are present also on a species level, as we detected significant differences in the distribution of *C. fragile* between both sides of Cape Peñas, that is considered to be the transitional point. Temperature is a limiting factor for the distribution of macroalgae (Van den Hoek, 1984) including light attainability for photosynthetic activities (Lüning, 1981), wave action (Johansson, 2002) and salinity (Sjøtun et al., 2015). These environmental conditions tend to be heterogeneous on a different spatial scale (local, regional and national) (Drouin et al., 2016). Previous studies from the Cantabrian Sea indicate that temperature could be a limiting factor for the distribution of *C. fragile* toward the west side (Galicia) (Rojo et al., 2014; García et al., 2017). This is contrary to well known invasive *U. pinnatifida*, which patchy distribution along the Cantabrian coast seems to be influenced by the chemical potential for primary productivity (Báez et al., 2010). Although temperatures were not used in this study and our sampling was focused more on the central part of North Coast of Spain, our results indicate that invasional pattern of *C. fragile* could follow the trend that was previously confirmed in other macroalgae communities. Surveys should be capable to infer natural processes, substantial for invasion dynamics, as they can be scale-dependent (Johnston et al., 2019). For example, in New Zealand, the field observation indicates that *C. fragile* is less abundant in wave-exposed areas (Trowbridge, 1995). D'Amours and Schiebling (2007) demonstrate that wave dynamics could influence badly the survivorship of adult *C. fragile*. Theriault et al. (2006) encounter *C. fragile* dispersed inside sheltered/exposed areas, although with higher

abundance in sheltered ones. In general terms, description of algal assemblages can be explained with multiple statistical approaches, among rest multivariate analysis, in order to understand which environmental (Díez et al., 2003), or anthropogenic parameters contribute most to species richness. Our results indicate that less exposed beaches toward the eastern part of the Cantabrian Sea, along with increasing temperature, could provide better habitat for the establishment of *C. fragile*, than the ones from the west. Exposure to moving water makes bigger thallus to damage by breaking waves (D'Amours and Schiebling, 2007). This too may help explain why *C. fragile* was present in a bigger proportion in the wave-sheltered beaches. The biggest proportion of *C. fragile* during this study was encountered in Gijón harbor (“El Mussel”). “El Mussel” was sampled as a part of the ongoing project “*The prevention and control of invasion biology in the port of Gijon*”, as first monitoring that includes a genetic tool for early detection of non-indigenous species. Among 20% of non-indigenous seaweeds detected, *C. fragile* was present in an utmost relative abundance. Adverse changes in ecological factors could result in negative impacts on native flora and fauna communities, leaving the possibility for invasive species to exhibit opportunistic behavior (Scheil et al., 2018b). This industrial port makes an example of the area with strong human impact, thus invasive seaweeds, that can tolerate a wide range of environmental conditions and prefer inhabit sheltered areas, could be dominant over native ones (as in the case of *Undaria pinnatifida*, Fletcher and Farrell, 1998).

Third, we noticed that a higher amount of *C. fragile* occurred during late summer sampling, although temporal variability was not included in our analysis. The underlying reason for this temporal pattern may be explained by the maturation of *C. fragile* reproductive organs in the early summer and maximum length during late autumn and winter (Churchill and Moeller, 1972; Cotton, 1912; Burrows, 1991). Strong seasonal trends in frond morphology and chloroplast physiology suggest that *C. fragile* adaptive traits are well prepared for extreme conditions in the summer (when abiotic conditions are more extreme and competition with other algae greater), using nutrient reserves accumulated during early winter for survival (Benson, 1983). A similar pattern was previously shown for *summer-*

annuals algae (Martínez et al., 2012; Sears and Wilce, 1975) and may as well be advantageous for opportunistic IAS to thrive.

MOLECULAR STUDIES OF *C. FRAGILE*

DNA barcoding reduces the misapprehension and time needed to distinguish species where morphological identification is uncertain, as it is in the case of the macroalgae (especially green) or fungi (Cross et al., 2011; Guoying et al., 2014). As molecular makers are facing some shortcomings (intergenic/intronic region within DNA), the utilization of multiple genes should compensate those disadvantages and form a solid ground for species distinction (Pathwardan et al., 2014; Friedheim, 2016). Therefore, two or more markers have been commonly used for DNA barcoding rather than relying on a single marker (Saunders and Kucera, 2010). In terms of applied considerations, DNA sequencing of the plastid and nuclear markers is easy and cost-effective (Stam and Olsen, 2006). The DNA extraction and PCR amplification of *tufA*, *rbcL* and a primer that was designed for herbarium samples in this work (FCO*tufA*) can be accomplished in most laboratories, while the sequencing enables rapid characterization of the desired gene. Service supplier provides access to sequences and the client, with freeware software, can further do analysis. Besides *C. fragile*, we confirmed the presence of two more native *Codium* spp. (*C. tomentosum* and *C. vermilara*) using two plastid markers, *tufA*, and *rbcL*. *tufA* marker showed much strong phylogenetic signal and higher levels of genetic variation than *rbcL* in this study. In general, *tufA* provides easy identification of the species clades for the entire genus *Codium* (Stam and Olsen, 2006; Verbruggen et al., 2017). *RbcL* can be valuable for assessing relationships between red algae at species, genus, family, and other higher taxonomic levels (Freshwater, 1994; Nam et al., 2000) however for phylogeographic studies in green plants it provides a good resolution just at the tribal and family levels. The slow rate of divergence between *Codium* species is a possible reason for these findings (Xiang et al., 1998; Verbruggen et al., 2007; De Oliveira-Carvalho et al., 2012). Guoying et al. (2015) suggest the application of this marker might be prolonged if a more suitable fragment in this gene would be designed. In addition, they indicate that *rbcL* should be used as an accessory marker combined with *tufA*. For example,

using a concatenated tree (*rbcL* and UCP6) Hoffman et al. (2017) were able to detect the presence of new nonindigenous *Codium* spp., *Codium pulvinatum*.

Low level of genetic variation recorded in this study could be explained by the type of asexual reproductive mechanism, both for native and invasive *Codium* spp. Kusakina et al. (2006) suggest that sexual reproduction in *C. fragile* might occur only once in several years (possibly under a stressful environment), and the introduction is too recent to develop any genetic variation. We were already able to detect one more haplotype of *C. fragile* inside its native range Korea, using designed FCO*tufA* primer. We strongly suggest the utilization of *tufA*, or our FCO*tufA* primer, to test again the introduced haplotypes in Europe, as until now only UCP-6 maker was used (Provan et al., 2005). As well, more in-depth molecular markers that are currently absent (like nuclear and mitochondrial) should be developed for future phylogenetic studies of *Codium* spp. Genomic studies to produce a first genome draft for *C. fragile* is currently in progress (Skukan et al., Note from the author). Sequencing and assembly of a first *Codium* genome draft is currently being afforded using three different strategies (short Illumina fragments using HiSeq P150, long PacBio Sequel fragments producing longer reads to help in anchoring the HiSeq P150 assembly and finally a RNA HiSeq sequenced library for annotating the genome). In this way, Pacbio sequences allow a better assembly of the genome and those of Illumina PE150 (with greater sequencing depth) allow the correct identification of simple nucleotide polymorphisms (SNPs). Libraries were sequenced in the genomic center Rapid Genomics (USA). In total, we have obtained data from two smart cells of PacBio and a total of 310,524,647 PE150 reads for Illumina. This will allow to identify new diagnostic molecular tools to study the invasion process.

Until now, only two studies tried to assess deeper insights into *C. fragile* subsp. *atlanticum* presence in Europe, combining morphological and molecular tools (Provan et al., 2008; Armitage and Sjøtun, 2016). Provan et al (2008) discovered that all the specimens thought to be *Codium fragile* ssp. *scandinavicum*, are actually phenotype of *Codium fragile* subsp. *fragile*. In addition, all identified *C. ssp. atlanticum* were misidentified and are actually *C. fragile* subsp. *fragile*. Low genetic variation was again detected. Accordingly, further studies of genus *Codium* should not exclude a genetic approach when facing cryptic invasion and

morphological plasticity cause it could lead to unserviceable prevention measures against the spread of invasive *C. fragile*.

Provan et al. (2008) work include herbarium samples and species-specific primers that provided more knowledge about the arrival of invasive *C. fragile* and taxonomic ascertainment of its cryptic subspecies. The herbarium samples of *C. fragile* were amplified with short length (<200bp) marker designed for our study (FCOtufA) and were able to identify morphologically and assigned correctly this valuable specimens. These findings highlight the importance of designing the primers for species identification, especially in the case herbarium samples that are contemporaneous record of the invasion processes but at the same time exposed to some level of degradation (or are not well preserved).

DETECTION PROBABILITY OF *C. FRAGILE*

Although it presents nuisance for most ecologists, in the case of IAS control, the rate of detection is important in setting up management measures of this increscent environmental issue (Christy et al., 2010). Most of the uncertainties about the designing proper model for distributional estimations are related with the values of information from incidental sightings compared to data from designed field surveys, which is often in IAS studies (Gormley et al., 2011). Here, one of the most important components, when dealing with models that are trying to estimate the presence of target species, is variation in abundances (heterogeneity) since assumption of constant abundance among sites is not common in a real environment (Royle and Nichols, 2003). Existing literature on IAS pays less attention to how different spatial scales in detection probabilities should influence monitoring effort (Hauser and McCharty, 2009). By using deterministic simulation, we were able to set up the distributional threshold of *C. fragile* among beaches ($p=0.035$), employing proportion data from our field study and stochastic simulations to infer conclusions about detection probability of *C. fragile* on a different spatial scale in the central coast of North of Spain. Our results suggest that random sampling on lower spatial levels (plots and sites) do not guarantee higher detection probability of the invader, even if the invader is present in same or similar proportions. Furthermore, randomization test indicates there are significant differences in the distribution

of *C. fragile* among beaches, on both sides of Cape Peñas. A possible explanation could be that environmental conditions are not distributional drivers of *C. fragile* on a smaller spatial scale in the Cantabrian Sea. As shown in 'ALIEN' project, on a spatial scale of sites, a significant negative correlation was found between NIS and native species richness indicating that indigenous seaweeds provide solid resistance to NIS spread. However, this data is collected more than 15 years ago and we emphasize that periodical studies on the interactions between *C. fragile* and other native seaweed assemblages should be conducted, mainly focusing on previous records of the trend of increasing temperature in the Cantabrian Sea.

With the average proportion of *C. fragile* (0.035) obtained from the scale of the beach in this work, the chances of not detecting the invader are between 1 and 5% for 100 individuals. Probability of detection within the context of a given study does not depend only on the presence of target species (meaning only absence-present data); a smaller proportion of *C. fragile* would require more sampling effort meaning more cost-demanding resources. Our results demonstrate that the simple application of our analysis has considerable potential as a management tool and that detection rates are important for surveillance prioritization. Future studies should focus on assessing the clear relationship between the budget and the probability of success, which can be very useful to pest-control agencies and policy makers (Chaco et al., 2010). Furthermore, these studies may include the public media, the establishment of a direct connection (e.g. hotline) to obtain public reports, pedagogic visits to schools, other community organizations, and perhaps reward payment for genuine positive reports (Chaco et al., 2011).

‘FIND INVASIVE SEAWEED’

Marine conservation projects contribute to worldwide knowledge about policy making and management actions and CS should be a component that changes the conservation behavior of citizens toward environmental issues. It is never too late to start, people often say. In this study, we emphasize: it is never too early to start. Youths (e.g. children) are naturally led to exploring nature around them and may even be more sensitive to changes that happen inside

the environment, however not often involved in stakeholders' decisions (adults rarely think of children specifically in relation to water rights) (Mueller et al., 2011). One way to involve children in monitoring programs is to promote partnerships among volunteers, educational centers, summer schools and camps (Kountoupes and Oberhauser, 2008). Our pilot study for '*Find invasive seaweed*' game was tested during the summer festival in Salinas, in a workshop '*Aula de Mar*' for educating children about the importance of marine conservation. This event was good feedback for testing the game concerning thematic issues, like the motivation of children to participate in the game based on a complex biological discipline such as biological invasion. With simple explanation about the potential impact of invasive seaweeds, we were able to involve and motivate children to participate in the game until the end. Next step was to test the game in Salinas High School. Although the sample size of test group in school was small (n=64), we concluded that '*Find invasive seaweed*' could become an effective method for creating a powerful and beneficial exchange of data between scientists and younger generations (junior citizens). Similar 'hide and seek' trail was done in Ireland, with high-school children and it seems that outdoor activities motivate children, even more, to participate in this type of CS projects (Donnelly et al., 2014). Currently, the number of CS has greatly increased (McKinley et al., 2015) and the involvement of citizens in marine and coastal studies can have a substantial impact on marine environments (Cigliano et al., 2016). Salinas is a small parish, located close to the coast and we decided to conduct our pilot and case studies there because marine activities are part of its habitant's daily lives. As well, since the establishment of invasive seaweeds from the game along the whole Cantabrian coast is well known, this modest CS project could be implemented in any summer event or other regional schools. If and when possible, the leader of the game should spend more time with the same group of school children to test long-term engagement and motivation in marine activities. In addition, other community institutions can implement this game in social events, as happened during Salinas festival '*Aula del Mar*'.

GENERAL CONCLUSIONS

1. Native seaweed communities seem to be well established along the coast of Asturias since no major alteration in population variability was detected on a different spatial scale, in comparison with records of nonindigenous and native species richness from European project 'ALIENS'. This suggests that environmental factors and coastal topography still favor native species-rich ecosystems, that are more resistant to invaders.
2. Historical records of *C. fragile* show no significant decrease or increase in its distribution in the past 15 years ago, although native *Codium* spp. are still present in the vast majority. Here, future experimental studies in the field on *C. fragile* invasion would be a valuable addition to define the role of species richness in community resistance and its native congeners.
3. The spatiotemporal shifts in invasion dynamics could lead to variations in the sudden occurrence of *C. fragile* and recuperation of native *Codium* spp. Since we detected significant differences in the distribution of *C. fragile* west and east from Cape Peñas, an equal proportion of more sheltered beaches from both sides of should be taken into account when establishing monitoring programs
4. Using our dataset as an example, we observed that counting 100 plants is enough to detect *C. fragile* on a scale of the beach, a conclusion that may put forward management decisions for tracking its invasion proliferation with future environmental changes.
5. Using DNA barcoding, we were able to detect three species from genus *Codium* that were previously described morphologically along the Cantabrian Coast. The *tufA* gene is an informative molecular marker for the DNA barcoding of species from genus *Codium* and FCO*tufA* marker enables easy amplification and sequencing for rapid recognition of invasive *C. fragile* from fresh and herbarium samples. With the help of *tufA* marker designed for this study, we were able to detect one more haplotype inside the native range of *C. fragile*, Korea.

6. There is no research done on the underlying genetic mechanisms of adaptive traits in seaweeds communities along the Cantabrian Coast. Future studies should focus on developing more variable markers (eg SNP, microsatellite), like in the case of *Codium* species, where the deficiency of this molecular resources confines in-depth insight into low genetic variation patterns, detected in all *Codium* spp. during this work, as that could elucidate the coexistence of congeneric *Codium* species in the Cantabrian Sea.
7. Educational games have shown to be an effective tool for engaging students in difficult tasks while learning. For example, the “*Find invasive seaweed*” game teaches players how to taxonomically identify different invasive species of algae and learn about their impact on marine ecosystems while playing. Students demonstrated a positive learning attitude toward task-related environmental issues about invasive seaweeds and were able to overcome challenges, in both pilot and case study.
8. The game showed a positive outcome and was included in a modest marine citizen science project that linked high school students with researchers from the University of Oviedo. This valuable union made it possible to raise awareness concerning marine conservation among students and to identify four different invasive species of seaweed in the study area. Indeed, the game was used to do foster two complementary things: citizen science and by the same time knowledge, attitudes, and action towards an environmental issue, invasive species and marine conservation.

RESUMEN

Introducción

Durante las últimas dos décadas, el alga verde *C. fragile* ha centrado un creciente interés científico, y público, debido a su éxito como especie invasora. En este contexto, multitud de estudios han intentado profundizar en su caracterización biológica, ecológica y genética con el objetivo de proporcionar pautas sobre cómo abordar el manejo de esta, y otras especies, potencialmente dañinas a los nuevos ecosistemas que conquistan. La creación de una base de datos que incluya registros previos sobre la distribución de las especies exóticas y la riqueza de especies nativas puede proporcionar un punto de partida para una mejor comprensión de la distribución espacial de algas en el norte de España. A la vez, puede ayudar a actualizar el estado actual de *C. fragile* para futuros planes de vigilancia en la región. En general, los conjuntos de algas marinas son un importante indicador ecológico del estado de salud y calidad en comunidades rocosas a lo largo de la costa cantábrica, por lo que la elección de un diseño experimental adecuado es un factor crucial para determinar correctamente su distribución espacial, especialmente en el caso de especies exóticas invasoras. Tres estudios sobre *Codium* spp. se han realizado hasta ahora en la costa norte de España; en ellos se han abordado parcialmente la identificación molecular, morfológica, la distribución y los rasgos reproductivos de las especies. Sin embargo, todavía faltan directrices claras para las decisiones de gestión con respecto a la distribución y manejo de *C. fragile*. Es imprescindible el monitoreo y vigilancia sobre el estado actual de esta especie al estar catalogada entre las peores especies invasoras de Europa. En este trabajo detectamos diferencias significativas en la variabilidad espacial entre las playas, y entre los sitios dentro de las playas, tanto para especies exóticas, como para las especies nativas. Las algas nativas eran dominantes en todos los lugares de muestreo. La propagación de *C. fragile* no mostró ningún aumento, ni disminución, en los últimos 15 años. Esto indica que las comunidades de algas nativas todavía están bien establecidas a lo largo de la costa de Asturias. Por otro lado, la probabilidad de detección de invasores no se vio influenciada por las escalas espaciales en nuestro diseño jerárquico, lo que infiere que el muestreo aleatorio de playas para 100 plantas debe usarse

como un umbral de distribución en los futuros monitoreos para la evaluación de la proliferación de *C. fragile*.

Hasta el momento, tampoco se ha llevado a cabo en el litoral cantábrico, un monitoreo intensivo utilizando identificaciones genéticas de las especies de *Codium*, ni una prospección sobre los niveles de diversidad genética en este género. La introducción de especies invasoras en áreas receptoras casi siempre provoca la pérdida sustancial de la diversidad genética en estas áreas. En general, las algas marinas se cuentan entre los organismos con niveles más bajos de variación genética en comparación con otros organismos de cualquier otra población natural. Las herramientas genéticas pueden ayudar en la identificación precisa de las especies, lo cual es fundamental para la caracterización de los ecosistemas y para el monitoreo y las decisiones de manejo, especialmente en el caso de las macroalgas marinas invasoras, donde la identificación morfológica suele ser ambigua. En este sentido el código de barras de ADN, utilizando genes plastidiales y/o mitocondriales se ha mostrado como una herramienta eficaz para distinguir especies también en el caso de las macroalgas. En este estudio hemos analizado la diversidad genética de *Codium* nativo (*C. tomentosum* y *C. vermilara*) y *C. fragile* del mar Cantábrico, incluidas muestras de herbario de una colección personal de la Universidad de Bournemouth y muestras frescas del área nativa de esta especie invasora (Corea del Sur). Se encontraron niveles bajos de variación genética poblacional para las especies de *Codium* nativas/invasoras examinadas, lo que sugiere mecanismos sexuales y de propagación similares para todas las especies. Desarrollamos un nuevo marcador, FCO_{tufA}, que además nos fue útil para obtener el código de barras de ADN de muestras recientes y de herbario. El trabajo y caracterización genética con este tipo de muestras habitualmente es imposible por problemas de degradación de las muestras y/o conservación incorrecta de los materiales. Sorpresivamente, con este marcador se encontraron dos haplotipos diferentes de *C. fragile* dentro del clado invasor, incluido un nuevo haplotipo para una muestra del rango nativo en Corea del Sur.

Por otro lado, se ha demostrado que cualquier política para la detección temprana y manejo de especies invasoras necesita de una implicación real y efectiva de los ciudadanos. En este sentido el rol de la educación ambiental, desde edades tempranas, parece jugar un papel

esencial. En España, las actividades de ciencia e investigación ciudadana, especialmente aquellas dirigidas a los niños como los principales participantes en las encuestas ambientales marinas, son bastante escasas. En este trabajo se diseñó un juego para enseñar a los niños a reconocer las algas invasoras y animarles a participar en actividades de ciencia marina ciudadana. El objetivo estuvo centrado en crear un juego educativo atractivo para el proceso de enseñanza-aprendizaje, donde los alumnos prestan atención durante largos períodos de tiempo y realizan tareas difíciles. El juego "*Encuentra un alga invasora*" enseña a los jugadores cómo identificar taxonómicamente diferentes especies invasoras de algas y aprender sobre su impacto en los ecosistemas marinos mientras disfrutaban del juego. Los resultados obtenidos mostraron un impacto positivo en las ganancias de conocimiento en los niños y demostraron que se puede mejorar la conciencia ambiental sobre el medio marino y la predisposición a participar en acciones de conservación.

Objetivos

El objetivo general de esta tesis doctoral es aplicar un enfoque ecológico, genético y de ciencia ciudadana para la evaluación del control actual, y futuro, del alga verde invasiva *Codium fragile* subsp. *fragile* (Suringar) Hariot 1890 en el sur de la Bahía de Vizcaya (Mar Cantábrico, costa norte de España). Para lograr este propósito general, este estudio se dividió en cuatro capítulos, con los siguientes objetivos específicos:

1. Compilar y combinar datos previos, y otros obtenidos en este proyecto, sobre estudios de las comunidades de algas nativas y no indígenas en la región y evaluar la susceptibilidad a la proliferación de invasiones a lo largo de la costa de Asturias con el objetivo de priorizar las medidas efectivas de control y gestión en particular para la especie *C. fragile* subsp. *fragile*.
2. Describir el patrón de distribución de *Codium* spp. y la probabilidad de detección de *Codium fragile* subsp. *fragile* en diferentes escalas espaciales a lo largo de la costa cantábrica utilizando códigos de barras de ADN, variables ambientales y simulaciones de datos que podrían servir de base para el desarrollo de futuras estrategias de monitoreo.

3. Actualizar el conocimiento sobre la presencia y diversidad genética de las especies nativas de *Codium* y *Codium fragile* subsp. *Fragile* utilizando estrategias de código de barras basadas en marcadores moleculares plastidiales. Además, diseñar nuevos protocolos de identificación genética utilizando fragmentos más pequeños del marcador genético *tufA* para la identificación efectiva de las especies invasoras de *C. fragile* en distintos tipos de materiales de algas marinas, incluyendo muestras de los herbarios.

4. Desarrollar una estrategia para aumentar el conocimiento de los ciudadanos, especialmente de las generaciones más jóvenes, sobre los impactos negativos en los ecosistemas de las algas marinas invasoras utilizando un enfoque educativo basado en juegos.

Principales Resultados y Discusión

La introducción de especies invasoras y exóticas (NIS) puede tener importantes influencias en las poblaciones nativas, la dinámica de la comunidad y los procesos de los ecosistemas. Aunque la mayoría de los estudios se centran en especies invasoras particulares, los estudios sobre la invasión de los ecosistemas marinos están aumentando, ya que los ecosistemas costeros se encuentran entre las áreas más invadidas del mundo. En este trabajo hemos investigado la riqueza de especies nativas y NIS, centrándonos en la proliferación e invasión de la especie *C. fragile*, a lo largo de la costa asturiana, Golfo de Vizcaya, en el norte de España. Recopilamos y analizamos una base de datos de investigaciones previas sobre algas marinas nativas e invasivas a lo largo del proyecto 'ALIENS' reunido por investigadores de la Universidad de Oviedo (Asturias, 2002), datos espaciales de *Codium* spp. y su distribución realizada por Cires Rodríguez y Rico Ordás (2007) (Asturias, 2002-2003) y datos de nuestro estudio (Asturias, 2016-2017). En todos los estudios se realizó un muestreo jerárquico, ya que las escalas en las que la variabilidad de la población es mayor se pueden identificar a través de la distribución anidada de las muestras. Nuestros resultados sugieren que los factores ambientales y la topografía costera podrían influir en los conjuntos de algas marinas en una escala espacial diferente. Detectamos diferencias significativas en la variabilidad espacial entre las playas, y entre los sitios dentro de las playas, para la riqueza de especies no

indígenas y nativas en el proyecto 'ALIENS'. Sin embargo, las algas nativas aún eran dominantes en todos los lugares de muestreo. Además, la propagación de *C. fragile* no mostró ningún aumento, ni disminución, en los últimos 15 años. Esto indica que las comunidades de algas nativas todavía están bien establecidas a lo largo de la costa de Asturias, ya que no se detectó ninguna alteración importante en la variabilidad de la población. El fuerte gradiente de temperatura a lo largo de la costa norte de España forma el límite de distribución sur, o norte, de muchos organismos, especialmente las macroalgas. Por lo tanto, los estudios futuros deben incluir un enfoque experimental para vincular directamente el impacto de los cambios globales, principalmente los resultados del aumento de la temperatura con la diseminación de especies no indígenas.

Para el establecimiento de las pautas de monitoreo del alga *C. fragile* a lo largo de la costa de Cantabria (Bahía central de Vizcaya), evaluamos la probabilidad de detección de la especie y su distribución espacial en comparación con las especies nativas de *Codium*. Para describir la distribución espacial utilizamos una prueba de aleatorización simple y probamos la influencia de las variables ambientales con un análisis de componentes principales (PCA). Las especies de *Codium* spp. nativas fueron dominantes sobre *C. fragile*. Confirmamos diferencias significativas en las proporciones encontradas de *C. fragile* en el litoral cantábrico, tal y como ha sido reportado en resultados publicados anteriormente, lo que indica que la combinación de factores ambientales, las playas menos expuestas hacia la parte oriental del mar Cantábrico y las temperaturas más altas, podrían proporcionar un hábitat sólido para su establecimiento. La probabilidad de detección de invasores no se vio influenciada por escalas espaciales en nuestro diseño jerárquico, lo que infiere que el muestreo aleatorio de playas para 100 plantas debe usarse como un umbral en los futuros monitoreos para la evaluación de la proliferación de *C. fragile*. Los enfoques ecológicos utilizados aquí proporcionan una base útil que se puede usar para dirigir las prioridades de gestión y manejo y que se deben tener en cuenta al monitorear y gestionar tanto *Codium* spp nativo, como el invasivo, en el Mar Cantábrico.

Los procesos biológicos de invasión relacionados con las algas marinas invasoras son un foco particular de atención en todo el mundo. Hasta ahora, el monitoreo intensivo y los análisis

morfológicos han demostrado ser parcialmente ineficaces para la identificación temprana de especies de algas invasoras. Los análisis de rutina de NIS, combinando métodos moleculares y anatómicos, se han iniciado muy recientemente en las costas del mar Cantábrico del Golfo de Vizcaya. En este trabajo, nuestro objetivo ha sido actualizar el conocimiento actual sobre la presencia de especies de *Codium* spp. nativas e invasivas en esta área mediante estrategias de código de barras basadas en marcadores moleculares de los cloroplastos. Además, dado que trabajar con especímenes de herbarios a menudo es problemático, otro objetivo se centró en diseñar nuevos protocolos de identificación genética utilizando fragmentos más pequeños del marcador genético *tufA* para la identificación efectiva de *C. fragile* en este tipo de materiales de algas marinas. Los especímenes de *Codium* spp. fueron recolectados en seis lugares diferentes en el mar Cantábrico y en Portugal (Viana do Castelo). Los análisis de las secuencias de los genes *tufA* y *rbcL* revelaron tres haplotipos diferentes para cada marcador genético que permiten la identificación genética de tres especies diferentes de *Codium*: *C. fragile* subsp. *fragile*, *C. tomentosum* y *C. vermilara*. Como se esperaba, la especie *C. fragile* detectada en esta región se agrupa, luego de análisis filogenético bayesianos, en el clado con la variante invasiva para esta especie. Por otro lado, no encontramos ninguna diferencia en términos de niveles cuantitativos de variación genética para las especies de *Codium* nativas/invasivas analizadas, lo que sugiere mecanismos sexuales y de propagación similares para todas las especies. El marcador, de nuevo diseño, FCO*tufA* fue útil para obtener datos genéticos de muestras de herbario y muestras frescas. Se encontraron dos haplotipos diferentes de *C. fragile* dentro del clado invasivo, incluido un nuevo haplotipo para una muestra de Corea del Sur, lo que podría indicar la posible existencia de una mayor diversidad genética dentro del rango nativo asiático para el subgrupo invasivo de la especie.

Las especies invasoras amenazan la biodiversidad mundial. El éxito en las estrategias de conservación del medio marino y en la implicación efectiva de los ciudadanos para enfrentar este problema, a largo plazo, puede ser posible a través de la participación de audiencias más jóvenes. En este trabajo, se diseñó un juego para enseñar a los niños a reconocer las algas invasoras y animarles a participar en actividades de ciencia ciudadana marina. El juego se probó por primera vez en un estudio piloto y luego se llevó a cabo en una escuela secundaria de Salinas (Asturias, España). El juego "*Encuentra un alga invasora*", consiste en una

explicación de las especies invasoras, seguido de un juego por equipos para encontrar tarjetas con representaciones de las algas y responder preguntas relacionadas con el tipo de alga y su peligro para el ecosistema. Se utilizaron pruebas Pre y Post juego para evaluar posibles aumentos en el conocimiento y en la motivación de los niños con respecto a la temática. Se motivó a los niños a utilizar sus paseos y actividades por las playas de la región para coleccionar restos de algas depositadas en la arena y enviarlas al equipo investigador. Después de la experiencia, los estudiantes estuvieron enviando posibles restos de especies de algas invasoras durante 3 meses. El análisis de este material permitió identificar cuatro especies invasoras en el área de estudio. Los resultados muestran un impacto positivo en las ganancias de conocimiento en los niños y diferencias significativas en la conciencia ambiental marina, y la disposición a participar en las acciones de conservación, antes y después de la experiencia.

Conclusiones generales

1. Las comunidades de algas nativas parecen estar bien establecidas a lo largo de la costa de Asturias, ya que no se detectó ninguna alteración importante en la variabilidad de la población en diferentes escalas espaciales, en comparación con los registros de riqueza de especies nativas y no indígenas del proyecto europeo 'ALIENS'. Esto sugiere que los factores ambientales y la topografía costera siguen favoreciendo a los ecosistemas nativos ricos en especies, que son más resistentes a los invasores.
2. Aunque las especies nativas de *Codium* siguen siendo mayoritarias, los registros históricos de *C. fragile* no muestran una disminución significativa ni un aumento en su distribución desde hace 15 años. Por ello, futuros estudios experimentales en el campo sobre la invasión de *C. fragile* serían una adición valiosa para definir el papel de la riqueza de especies en la resistencia de la comunidad y de sus congéneres nativos.
3. Los cambios espaciotemporales en la dinámica de invasión podrían llevar a variaciones en la aparición repentina de *C. fragile* y la recuperación de *Codium* spp. nativo. Dado que detectamos diferencias significativas en la distribución de *C. fragile*

al oeste y al este de Cabo Peñas, un programa de seguimiento de cambios en la abundancia de la especie invasora debería incluir un número semejante de playas a ambos lados del cabo Peñas

4. Utilizando nuestro conjunto de datos como ejemplo, observamos que examinar 100 plantas es suficiente para detectar *C. fragile* a la escala de la localidad (playa), una conclusión que puede facilitar decisiones de gestión para el seguimiento de la proliferación de esta especie con futuros cambios ambientales.
5. Utilizando el código de barras del ADN, pudimos detectar tres especies del género *Codium* que anteriormente se describían morfológicamente a lo largo de la costa cantábrica. El gen *tufA* es un marcador molecular informativo para el código de barras del ADN de especies del género *Codium* y el marcador FCO*tufA* permite una fácil amplificación y secuenciación para el reconocimiento rápido de *C. fragile* invasiva de muestras recién recogidas y de herbario. Con la ayuda del marcador *tufA* diseñado para este estudio, pudimos detectar un haplotipo más dentro del rango nativo de *C. fragile* procedente de Corea.
6. No se han realizado investigaciones sobre los mecanismos genéticos subyacentes de los rasgos adaptativos en las comunidades de algas marinas a lo largo de la costa cantábrica. Los estudios futuros deberían centrarse en el desarrollo de más marcadores variables (p. ej., SNP, microsatélite), como en el caso de las especies de *Codium*, donde la deficiencia de estos recursos moleculares limita el conocimiento profundo de los patrones de baja variación genética, detectados en todas las especies de *Codium* durante este trabajo, y podría ayudar a caracterizarla coexistencia de las especies de *Codium* en el Mar Cantábrico.
7. Los juegos educativos han demostrado ser una herramienta efectiva para involucrar a los estudiantes en tareas difíciles mientras aprenden. Por ejemplo, el juego "Encontrar algas invasoras" enseña a los jugadores cómo identificar taxonómicamente diferentes especies de algas invasoras y aprender sobre su impacto en los ecosistemas marinos mientras juegan. Los estudiantes demostraron una actitud de aprendizaje positiva

hacia los problemas ambientales relacionados con las tareas sobre las algas marinas invasoras y pudieron superar los desafíos, tanto en el estudio piloto como en el de caso.

8. El juego tuvo un resultado positivo y se incluyó en un proyecto de ciencia ciudadana marina modesto que vinculó a estudiantes de secundaria con investigadores de la Universidad de Oviedo. Esta unión valiosa hizo posible crear conciencia sobre la conservación marina entre los estudiantes e identificar cuatro especies invasoras de algas en el área de estudio. De hecho, el juego se utilizó para fomentar dos cosas complementarias: la ciencia ciudadana y, al mismo tiempo, el conocimiento, las actitudes y la acción hacia un problema ambiental, las especies invasoras y la conservación marina.

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