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## Research Article

## Investigating the risk of non-indigenous species introduction through ship hulls in Chile

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### Abstract

Ship hull fouling is recognized as an important vector for the introduction of non-indigenous species (NIS), which has been studied globally but no empirical works exist in the Southeast Pacific. The present study examined fouling organisms on the hulls of three ships in one international Chilean port, and compared them with those on settling plates and natural substrates. Also, genetic analyses were performed on the most common NIS recorded in order to explore number and potential origin of the genetic lineages found. The highest number of total taxa and NIS were found on ship hulls, with three species identified as NIS in ship samplings. Settling plates displayed the largest number of taxa, with a large abundance of the invasive tunicate *Ciona robusta*, which also showed high mitochondrial genetic diversity. This study showed that Chilean coasts are subjected to both NIS colonization and propagule pressure through ship hulls. Biosecurity measures should urgently be taken on ship hulls along these coasts.

**Key words:** biofouling, maritime traffic, invasive species, survey, experimental study, molecular barcoding

### Introduction

Non-indigenous species (NIS) are major drivers of marine biodiversity changes, impacting both the economy and human health (Bax et al. 2003; Ojaveer et al. 2018); thus, they are an important biosecurity issue. To increase the effectiveness of management actions, early detection and pre-border actions are encouraged (Simberloff et al. 2013; Ojaveer et al. 2015). In marine systems, international shipping traffic is a main introduction pathway (Molnar et al. 2008), and it is expected that it will continue to sustain NIS introduction at a global level (Seebens et al. 2016; Sardain et al. 2019). As compared to ballast water, which was targeted in the International Ballast Water Convention, biofouling has more recently been identified as

a major introduction vector (eg, Sylvester et al. 2011; Davidson et al. 2018). A sampling carried out on 186 ships in the North Sea found NIS on 96% of the examined hulls (Gollasch 2002). The number of NIS transported on ship hulls has also proven to be influenced by ship routes, with a higher NIS richness in ships travelling through more ecoregions (*sensu* Spalding et al. 2007) (Sylvester et al. 2011). In this context, the GEF-UNDP-IMO launched the GloFouling project providing guidelines to manage and control hull biofouling, notably for commercial vessels, to reduce the probability of NIS introduction and dispersal.

To prevent biofouling and the transport of species on ship hulls, anti-fouling paints have been applied. Currently, the maintenance of anti-fouling coatings is a volunteer practice carried out by ship owners, with the aim of reducing hydrodynamic resistance and fuel consumption (IMO 2004). Fernandes et al. (2016) showed that fuel consumption might be higher with NIS on ship hulls, suggesting that NIS mitigation measures could be worthwhile, even if biofouling prevention measures increase operational costs. However, anti-fouling coatings that are active with water friction do not work appropriately when ships are moored in ports. Furthermore, there are refuge areas where anti-fouling coatings are not effective (eg, friction protected areas) or not applied, such as the sea chest, which favor biofouling growth (Coutts and Taylor 2004; Sylvester and MacIsaac 2010). Additionally, the prohibition of tri-butyl-tin (TBT) further increased the potential for NIS to adhere to ship hulls (Davidson et al. 2008; Piola et al. 2009). In this way, when a ship is docked for loading and unloading or for maintenance, the probability that attached NIS release propagules or that new species settle on its hulls increases (Davidson et al. 2009, 2018; Ruiz et al. 2022).

Chile has a low number of NIS compared to those reported in other Pacific countries (Castilla et al. 2005). For example, 305 marine NIS have been reported in New Zealand (Cranfield et al. 1998), and up to 99 in only one port of Australia (Port Phillip Bay) (Hewitt et al. 2004). In contrast, throughout the entire Chilean coast, which is over 6,435 km long, only 51 NIS have been reported (Castilla and Neill 2009; Villaseñor-Parada et al. 2017). In most cases, the introduction vector(s) is(are) unknown, with the exception of species that were intentionally introduced for aquaculture and some particular taxa, such as the anemone *Anemonia alicemartinae* whose expansion was attributed to maritime activity (Canales-Aguirre et al. 2015; Pinochet et al. 2019). The low number of NIS currently reported in Chile contrasts with a high introduction potential; its extensive coastal area, with multiple ecoregions (and consequently different environmental conditions; Camus 2001; Hormazabal et al. 2004; Hernández et al. 2005; Spalding et al. 2007) in addition to its high number of ports (27 international commercial ports) with intensive international maritime traffic (ca. 8,020 landings in 2018; Directemar 2020) should result in high colonization and propagule

pressure. The number of NIS reported thus far in Chile may have been underestimated due to a lack of dedicated surveys (ie, targeting ports and ships; Leclerc et al. 2018). No studies have examined whether NIS are present on ships' hulls in Chile, and more globally in the South East Pacific. One recent study did however target port areas along 100 km of coastline in the Biobío region of Chile, and documented a total of 78 taxa, mostly sessile fauna (68 taxa). Among them, 26 taxa were NIS or cryptogenic species (Leclerc et al. 2020).

Marinas, docks, harbors, and other artificial hard structures are often built in soft-bottom environments, where hard surfaces are scarce, sparsely distributed, or absent. Changes in substrate composition caused by human activity can dramatically change habitat structure (Reise 2005). In addition, port infrastructure can provide artificial habitat for marine organisms, as well as function as marine corridor, favoring population connectivity (Airoldi et al. 2015). In this way, major modifications of natural environments can benefit invasive species, with artificial structures providing different substrata in terms of form, type and composition which may facilitate NIS establishment (Dafforn et al. 2009; Lagos et al. 2017).

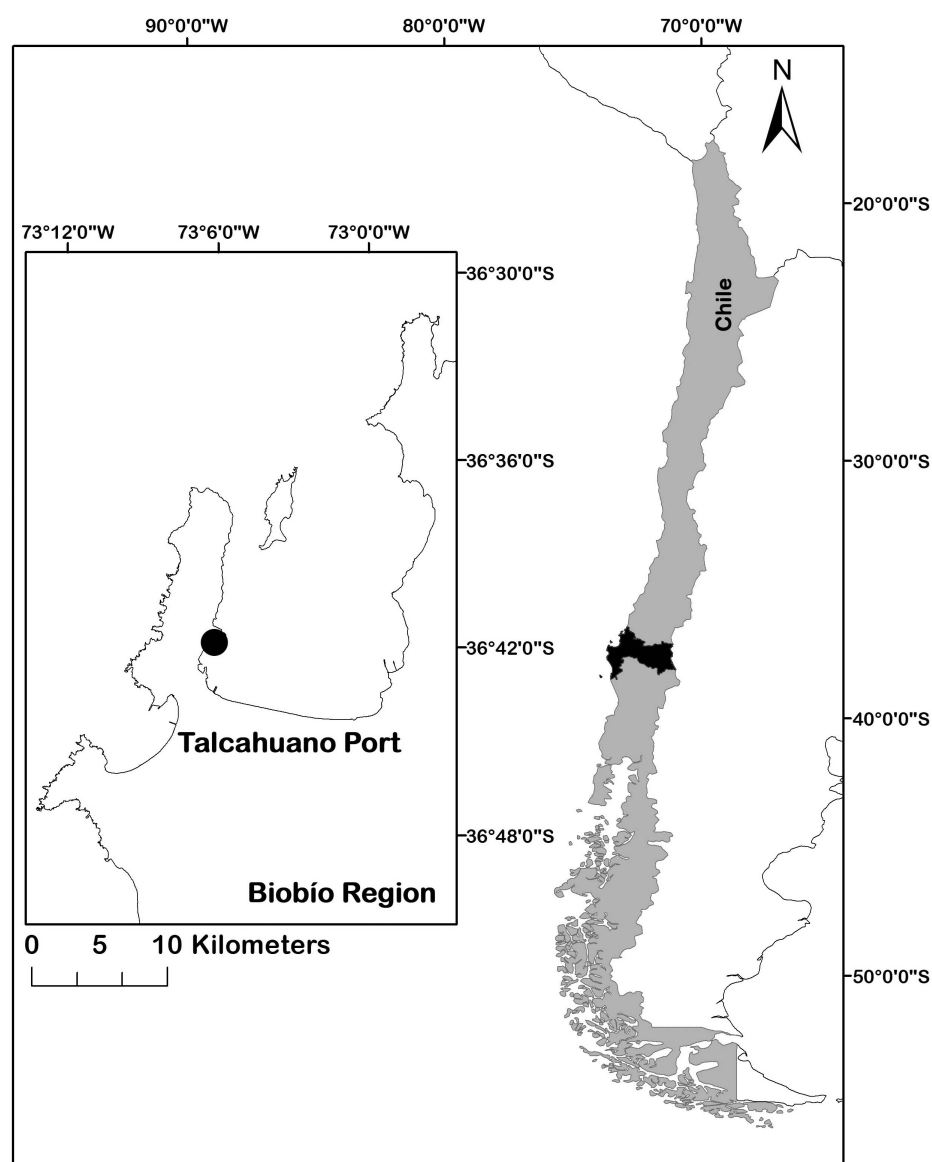
Non-native tunicates are important members of the fouling community associated with artificial substrates and man-made structures in harbors and marinas (Shenkar and Swalla 2011; Cordell et al. 2013). Being efficient fouling species and despite their limited natural dispersal ability, they are successful invaders through aquaculture and maritime transportation (Clarke and Castilla 2000; Coutts and Dodgshun 2007). For example, in the San Francisco Bay, it was found that *Ciona robusta* negatively effect local species richness in the San Francisco Bay (Robinson et al. 2017). In Chile, the invader ascidian *C. robusta* is distributed along the coast occupying mainly infrastructure associated to aquaculture, ports and ship hulls (Castilla and Neill 2009; Turon et al. 2016).

In the present work, using the international port of Talcahuano, located in the Biobío region, Chile, as a reference location, we aimed to: i) examine fouling species on international ships' hulls, ii) compare these data with those obtained in the port area, using settlement plates made of different materials and deployed at different depths, as well as on a nearby natural rocky substrata, and (iii) evaluate genetic diversity of the important invader ascidea *C. robusta* inhabiting this port in order to assess the extent of the propagule pressure.

## Materials and methods

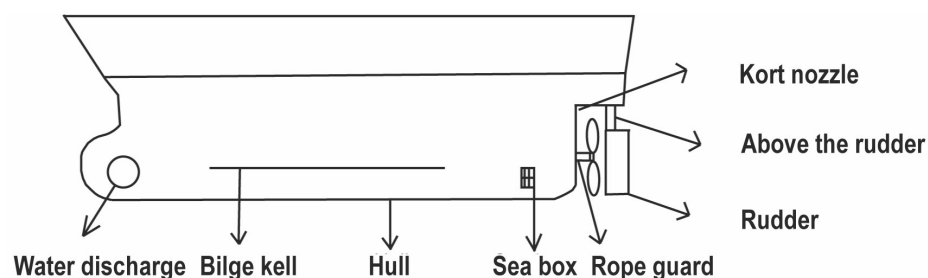
### *Ship hull sampling*

The sampling of sessile taxa found in the biological fouling (biofouling) of the hull was performed in the port of Talcahuano (36°43'S; 73°07'W), in the bay of Concepción, in the Biobío region of Chile (see Figure 1). Only ships



**Figure 1.** The black circle corresponds to the locality where the sampling was carried out in each type of sampling (ships, settlement plates in the port of Talcahuano and natural substrates in the El Manzano pier).

with an international travel route were considered, meaning that these ships had visited at least one non-Chilean locality in the last year. Three ships, two oil tankers and a sail training tall ship operated by the Chilean Navy, and which also differ in size, were sampled between December 2014 and October 2015. This low number is due to the difficulty in obtaining authorization to access these vessels, in relation to security issues and the private business nature of maritime transport. Samplings on the dry-dock were carried out for both exposed and protected areas of the hulls (Figure 2). The exposed areas have greater contact with water friction, and are thus expected to display fewer specimens as compared to protected areas. Protected areas included the sea chest, rope guard, water discharge holes, the top of the rudder and the Kort nozzle (Figure 2). All of the fouling assemblage (sessile taxa; specimens >1 cm) was hand removed within 20 × 20 cm quadrants,



**Figure 2.** Location of the different areas of the vessel from which the samples were obtained. Protected areas: sea box, rope guard, water discharge holes, top of the rudder and the Kort nozzle. Exposed areas: bilge keel, the helm in general and the rudder. Modified from Sylvester and MacIsaac (2010).

with three quadrants per area, and collected in plastic bags to be transported to the laboratory.

### *Sampling on artificial and natural substrates in the port area*

To compare the sessile species found on the ships' hulls with those found on artificial structures in the port, settling plates were deployed in the Talcahuano port. The sampling of port structures was not allowed for security reasons. As the substrate is known to influence settlement (Li et al. 2016; Pinochet et al. 2020), plates were made of three materials commonly used in such experimental studies: polypropylene (Correx), PVC and aluminum. Three  $10 \times 10$  cm plates of each material were suspended on floating structures attached to a floating dock at depths of 50 cm and 1 m, with a total of 18 plates. Settling plates were deployed twice, over a three-month period, from April 16 to June 16, and from June 18 to August 18, the same year (2015). At the end of each deployment period, the plates were retrieved to be examined (top side of the plate) at the laboratory. In addition, sampling was performed on rocky natural substrates, around El Manzano pier adjacent to the Talcahuano port ( $36^{\circ}44'28.4''\text{S}$   $73^{\circ}07'45.8''\text{W}$ ). This pier is located approximately 1 km from the area where the settling plates were deployed. The sampling was carried out with autonomous diving at six points, separated by 5 m. All sessile organisms were collected in  $10 \times 20$  cm quadrants and stored in plastic bags to be transported to the laboratory.

The sampled sessile taxa included macroalgae, ascidians, bivalves, bryozoans and hydrozoans. For further in-depth morphological and/or molecular analyses, the collected organisms were preserved with different protocols. For bivalves, the soft tissue and valves were separated and preserved in 95% ethanol. For ascidians, the anesthetic BZ-20 was used to prevent tissue contraction before their preservation in 95% ethanol. Bryozoans were directly preserved in 95% ethanol. Every specimen was photographed prior to preservation.

### *Taxonomic identification*

Each collected specimen was morphologically identified at the lowest possible taxonomic level using taxonomic keys (Aldea and Valdovinos 2005;



Häussermann and Försterra 2009). Expert taxonomists were also consulted for each group (see Acknowledgments).

In addition, DNA barcoding was used to confirm the identification of animal species. A total of 95 specimens were examined, including bryozoans, hydrozoans, mussels and tunicates. DNA was extracted from ethanol preserved tissue using the NucleoSpin 96 tissue kit (Macherey-Nagel, Duren, Germany) following the manufacturer's protocol. Final DNA elution was carried out in 100 µL of the supplied elution buffer. Sequencing of the Cytochrome c Oxidase subunit I gene (COI) was performed, after amplification with either the universal primers LCO-1490 and HCO-2198 defined by Folmer et al. (1994), or specific primers as detailed in Supplementary material Table S1. Additional sequences for the 18S nuclear ribosomal gene, the 16S mitochondrial ribosomal gene or other taxon-specific informative genes were also obtained for some taxa (see Table S1). PCR products were checked with a 1.5% agarose gel. Purification and Sanger sequencing of PCR products were performed at Eurofins Genomics (Ebersberg, Germany). Sequences were edited using CodonCode Aligner 5.1.4 software (CodonCode Corporation, Dedham, MA, USA). Taxonomic assignment was carried out with BLAST® using the GenBank database ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)), and with BOLD (<http://www.boldsystems.org>). Finally, each specimen identified at the species level was classified as native or non-indigenous for Chile according to literature; the other taxa remained undetermined.

### *Statistical analysis*

The number of taxa were computed for each sampled habitat (ship hulls, plates, and natural substrates), over all taxa and per status (native, non-indigenous, and cryptogenic species). Taxon assemblages in each sampling habitat (ships, settlement plates in the Talcahuano port, and natural substrates at El Manzano pier) was visualized with a non-metric multidimensional scaling analysis (nMDS), using the Bray-Curtis similarity distance, with square-root transformed data. A one-way non-parametric analysis of similarity (ANOSIM) was performed to test for the effect of the habitat on taxonomic richness. Both tests were run using PAST 2.7 software (Hammer et al. 2001). The measurements were normalized to the smallest area (i.e., 10 × 10 cm plates used in the port of Talcahuano) because the sampling areas were different across sampling types (ie, hulls, plates, natural substrates). Species accumulation curves were also computed for each habitat to make sure that each sampling effort was sufficient.

Regarding the sampling carried out on ships, the total richness of taxa in the protected and exposed areas of the hulls was compared, along with its possible relations with the number of ports visited by the ships. An nMDS was performed, using the Bray-Curtis similarity measure (PAST 2.7 program).

For the settlement plates dataset, a substantial number of plates was lost during the second deployment time (because of weather conditions and/or

**Table 1.** Synthesis of the presence of specimens collected from the three sampling sites, namely ships' hulls, settlement plates in the Talcahuano port and the natural substrate at El Manzano pier. Specimens were identified by morphology for algae, and by morphology and/or DNA barcoding for animals (see Table S2). When identified at the species level, the status (native, non-indigenous, cryptogenic) is indicated.

Taxa	Class	Status	Ship			Settlement plates		Natural substrate
			Esmeralda	Tanker (USA)	Tanker (ARG)	Period 1 (06/16/2015)	Period 2 (08/18/2015)	
<i>Asterocarpa humilis</i>	Ascidacea	NIS	0	x <sup>(2)</sup>	0	0	0	0
<i>Ciona robusta</i>	Ascidacea	NIS	(1,2) <sub>x</sub>	(1,2) <sub>x</sub>	0	x	x	0
<i>Bugulina flabellata</i>	Gymnolaemata	NIS	(1,2) <sub>x</sub>	0	0	x	x	x
<i>Ectopleura larynx</i>	Hydrozoa	NIS	0	0	0	0	x	0
<i>Choromytilus chorus</i>	Bivalvia	Native	1,(2) <sub>x</sub>	(1,2) <sub>x</sub>	0	0	0	0
<i>Mytilus chilensis</i>	Bivalvia	Native	(1,2) <sub>x</sub>	(1,2) <sub>x</sub>	(1,2) <sub>x</sub>	0	0	x
<i>Mazzaella laminarioides</i>	Florideophyceae	Native	(1,2) <sub>x</sub>	0	0	x	0	x
Hydrozoans 1	Hydrozoa		0	0	0	x	x	0
Hydrozoans 2	Hydrozoa		0	0	0	0	0	x
<i>Ulva</i> sp.	Ulvophyceae		(2) <sub>x</sub>	(2) <sub>x</sub>	(2) <sub>x</sub>	x	0	x
<i>Rhodomenia</i> spp.	Florideophyceae		(2) <sub>x</sub>	0	0	0	x	0

<sup>(1)</sup> species found in exposed areas of ship hulls.

<sup>(2)</sup> species found in protected areas of ship hulls.

human actions). When testing only for the effect of deployment time on species richness, there were no significant differences between the two periods (ANOVA,  $F_{1,34} = 103.4$ ;  $p = 0.45$ ). The two datasets were thus pooled to conduct a two-way ANOVA with taxa richness as the dependent variable and type of plate material (aluminum, PVC, or polypropylene) and depth (0.5 and 1 m) as the independent variables. A Tukey *a posteriori* test was applied in cases of significant differences. Prior to these analyses, normality assumptions were evaluated using the Shapiro-Wilk test and homoscedasticity of the errors with the Levene test. All of the statistical tests were performed with STATISTICA 8.0 software (Statsoft Inc.; Weiß 2007).

### *Ciona* sp. genetic diversity analysis

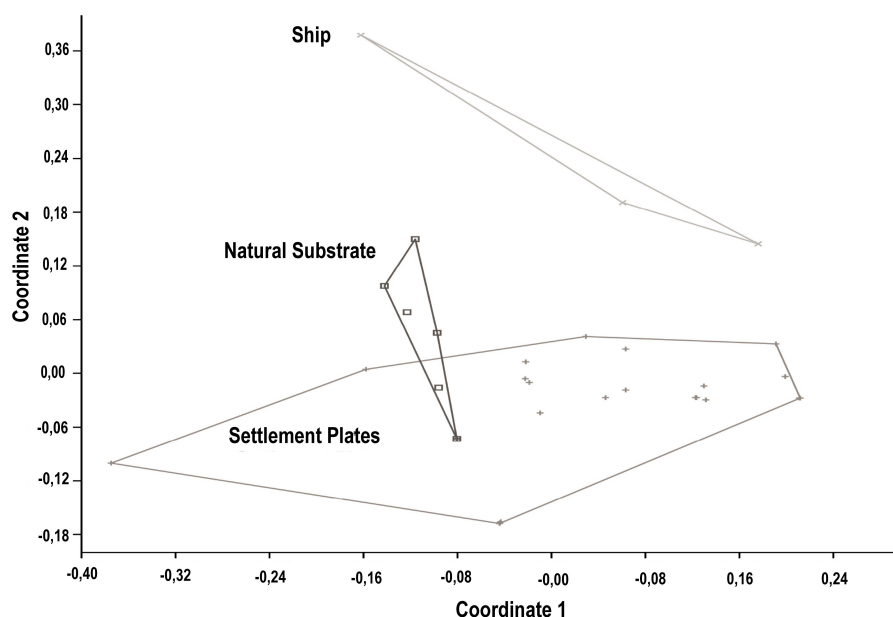
For 36 *Ciona* specimens collected on plates and ship hulls, and analyzed with the COI gene as described above, an alignment of 704 base pairs was obtained. The number of haplotypes and polymorphic sites, as well as the haplotypic and nucleotide diversity, were computed using DnaSP5.0 software (Rozas et al. 2003). Haplotypes obtained were compared with sequences archived in the GenBank database using a BLAST analysis.

## Results

### Taxa identification

Main groups composing the biofouling in the different habitat types were mussels, ascidians, hydrozoans and bryozoans, and macroalgae (Table 1). Specimens were morphologically identified to the species or genus level, except for hydrozoan specimens that were assigned to two distinct morphological groups (hydrozoans 1 and hydrozoans 2), for which DNA barcoding could not be used (it is i.e. amplification failure or poor quality sequences). The





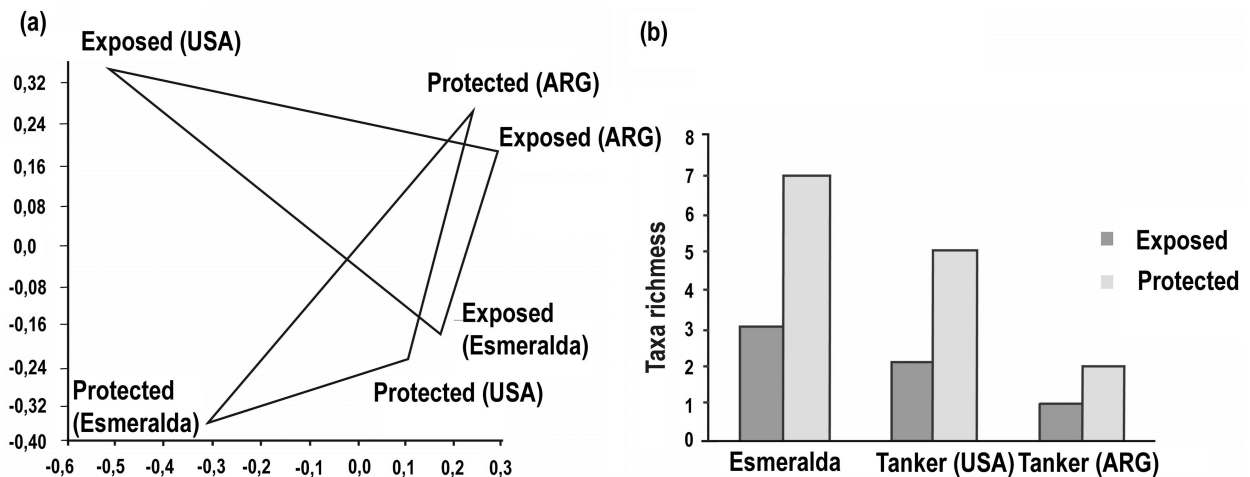
**Figure 3.** Graphical representation using a nMDS based on Bray-Curtis distances, of the taxa assemblages in each sampling type (ships, settlement plates in the Talcahuano port, and natural substrates at El Manzano pier). Stress value is 0.17.

results of the taxonomic assignments made with DNA barcoding are detailed in Table S2. The tunicates were identified as *Ciona robusta* and *Asterocarpa humilis* (previously reported by Pinochet et al. 2017). In addition, the hydrozoa *Ectopleura larynx* and the bryozoan *Bugulina flabellata* were identified. Among mussels, 30 individuals were unambiguously morphologically recognized as *M. chilensis*. For the remaining six individuals, sequences of the genes COI, GLU and EF-bis were assigned to *M. chilensis* (see the Discussion section for a deeper analysis on the genetic identification of this species).

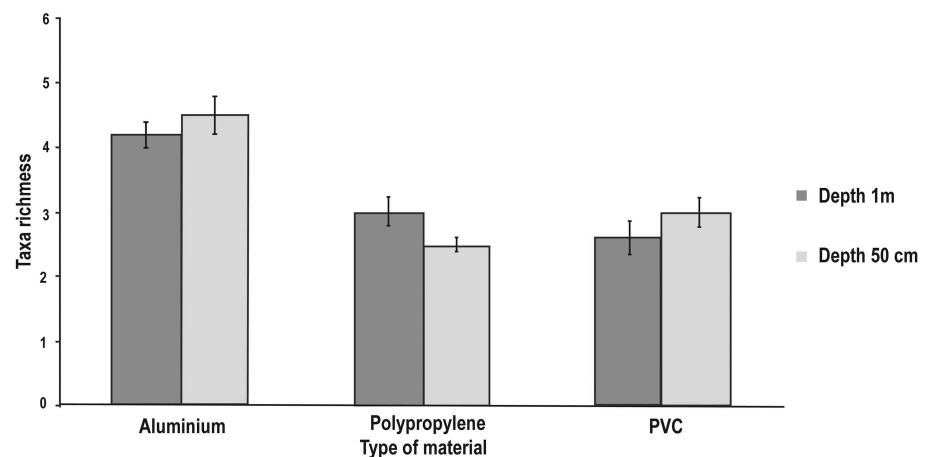
#### *Species number across collection sites*

Specimens were collected from 24 plates deployed in the port of Talcahuano, six quadrants on a natural substrate at El Manzano pier and three ships' hulls docked at the Talcahuano port. Table 1 details the distribution of specimens per taxa for each sampling type. In total, four NIS and three native species were recorded. Within the NIS group, three species (*Asterocarpa humilis*, *Bugulina flabellata* and *Ciona robusta*) were observed on artificial structures and only one on natural substrates (*Bugulina flabellata*; Table 1).

The nMDS clearly showed that the community composition observed on ships was different from that on plates and natural substrates, which were more similar to one another (Figure 3). The ANOSIM performed on standardized measures indicated significant difference in richness among the three sampling types ( $p = 0.0037$ ), with plates showing the highest taxonomic richness. The nMDS also showed a clear difference between protected and exposed areas of ships' hulls (Figure 4a), except for the ARG



**Figure 4.** Taxa distribution on ships' hulls of the three studied vessels (Esmeralda, USA and ARG tankers) (a) nMDS showing the taxa assemblages in protected vs. exposed areas of the ships' hulls. (b) Taxa richness in exposed and protected areas of the hulls of the three studied vessels. (c) Relationship between the number of taxa found on the hulls and the number of ports visited by each ship.



**Figure 5.** Taxa richness recorded in the Talcahuano port on settlement plates made of different types of materials and maintained at different depths for two months total, considering the two periods of time. Values include averages and  $\pm$  one standard error.

tanker, on which only two and one species were observed, for each zone respectively (Figure 4b). For the three studied vessels, protected areas (Figure 4b).

#### *Taxa richness on settlement plates of different materials*

On the 24 plates that were recovered, a total of seven taxa were observed, corresponding to three NIS, three undetermined taxa and one native species (Table 1). The ANOVA showed significant differences in the total richness between plates of different materials ( $F_{2,28} = 12.46$ ;  $p < 0.0001$ ), with most taxa observed on the aluminum collector plates (6 species; Tukey test:  $p < 0.01$ ; Figure 5). No significant differences were detected between depths (Tukey test:  $p > 0.05$ ).

#### *Analysis of genetic diversity of Ciona robusta*

A total of 12 haplotypes was obtained following the sequencing of 36 *Ciona robusta* collected on artificial substrates (N = 26) and ships (N = 10) (Table 2a,

**Table 2.** COI genetic diversity of *Ciona robusta* a) from data obtained in this study, and b) from data obtained in previous studies. Genetic diversity indices are indicated per population, with mean values per population for this and previous studies (Table 2b; Npop indicates the number of populations), and all individuals for a given area (Overall). The number of individuals (Nind), haplotypes (Nhap) and polymorphic sites (S) are indicated with gene diversity (He) and nucleotide diversity ( $\pi \times 10^2$ ) estimates. SEP, Med. Sea, NEA, SEA-IP, NEP and NWP stand for Southern East Pacific, Mediterranean Sea, North East Atlantic, South East Atlantic-Indian Ocean, North East Pacific and North Western Pacific, the latter being the only area where *C. robusta* is thought to be native.

a – This Study							
	Status	Npop	Nind	Nhap	S	He	$\pi$ (10 <sup>2</sup> )
Talcahuano (plates)	NIS		26	9	16	0.871 ± 0.043	0.476 ± 0.046
Esmeralda	NIS		5	5	7	1.000 ± 0.126	0.455 ± 0.089
Tanker (USA)	NIS		5	4	4	0.900 ± 0.161	0.313 ± 0.070
<b>Mean per pop.</b>			12	6	9	0.923 ±0.067	0.415 ± 0.088
<b>Overall</b>		3	36	12	16	0.876 ± 0.038	0.441 ± 0.039

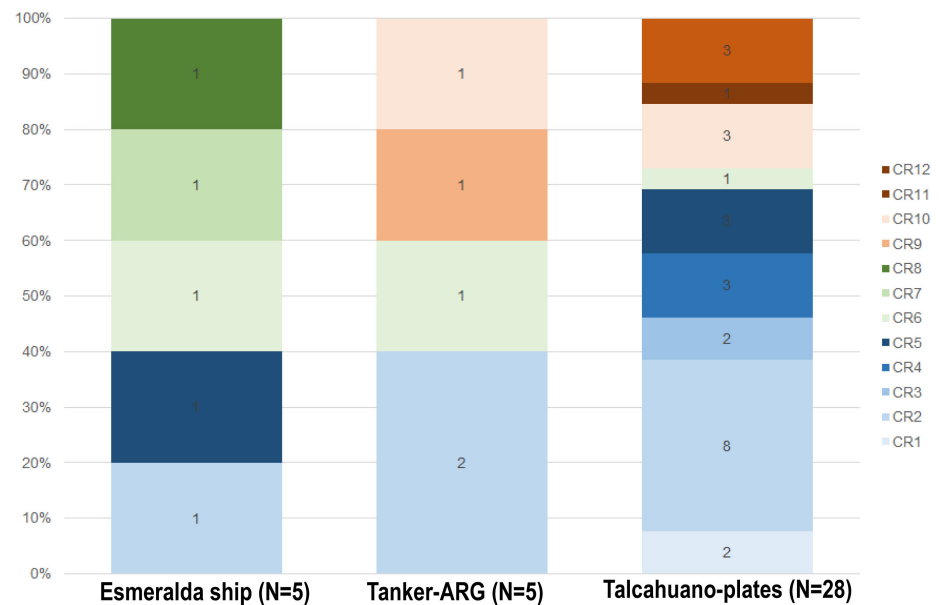
b – Previous studies								
Mean per pop	Status	Npop	Nind	Nhap	S	He	$\pi$ (10 <sup>2</sup> )	Source of the data
SEP (Chile)	NIS	5	14.4	6.4	11.6	0.848 ± 0.103	0.447 ± 0.057	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
Med. Sea (Italy & France)	NIS	2	19.0	3.0	2.5	0.383 ± 0.541	0.083 ± 0.001	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
NEA (France & UK)	NIS	14	21.7	1.6	0.9	0.110 ± 0.173	0.028 ± 0.0491	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
SEA-IP (South Africa)	NIS	7	27.0	5.1	NP	0.426 ± 0.377	0.243 ± 0.222	Table S1 in Rius et al. (2014)
NWP (Japan)	Native	2	31.0	8.0	14.0	0.811 ± 0.048	0.442 ± 0.099	New analyses of raw data used by Bouchemousse et al. (2016) <sup>(1)</sup>
<b>Overall</b>								
SEP (Chile)	NIS		72	17	23.0	0.853 ± 0.026	0.436 ± 0.253	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
NEP (US)	NIS		20	11	NP	0.916 +-0.041	NP	Tables 1 & 2 in Nydam and Harrison (2007)
Med. Sea (Italy & France)	NIS		38	5	5.0	0.451 ± 0.096	0.082 ± 0.074	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
NEA (France & UK)	NIS		304	6	7.0	0.125 ± 0.026	0.032 ± 0.041	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>
SEA-IP (South Africa)	NIS		189	18	NP	0.589 ± NP	0.300 ± NP	Table S1 in Rius et al. (2014)
NWP (Japan)	Native		62	10	17.0	0.815 ± 0.037	0.446 ± 0.258	Raw sequencing data used by Bouchemousse et al. (2016) <sup>(1)</sup>

Bouchemousse et al. (2016) analyzed a dataset made of concatenated sequences obtained with two mitochondrial genes (COI and Cox3-ND1). The supplementary information associated with this paper did not provide the COI dataset alone. The distribution of the haplotype frequencies per population and region, for COI only are thus provided in Table S4.

Figure 6). Most haplotypes were rare, although CR2 was common, with a frequency of 31% across all samples. Comparisons with GenBank data (made in August 2020) showed that one haplotype (CR9, Genbank accession number MZ152218, found on one ship; Figure 6) have not been reported in previous studies (Table S3). Almost as many haplotypes (Nh = 9) as specimens (Nind = 10) were observed on two ships, leading to haplotypic diversity values reaching 0.90 and 1.00 for the USA tanker and the Esmeralda ships, respectively (Table 2a). Three out of these nine haplotypes were found only on the ships (Figure 6). On the plates, the diversity was also very high, with nine haplotypes and an haplotypic diversity of 0.87 (Figure 6, Table 2a). All the other metrics were congruent with the high mitochondrial genetic diversity in the study samples (Table 2a).

## Discussion

The importance of ship hulls as an introduction vector for non-indigenous species (NIS) has been poorly evaluated, especially in Chile. The present study



**Figure 6.** Distribution of the COI haplotypes of *Ciona robusta* per sample, with their number indicated in the barplot. For each sample, the number of specimens is indicated in parentheses. The correspondence between the haplotypes found in this study, and those referenced in GenBank is given in Table S3.

showed the presence of diverse sessile taxa on the hulls of the three evaluated ships, which travel internationally and land on Chilean coasts. The taxa found include three NIS, two of which were also found on natural and artificial substrates in this port, where an additional NIS was also observed. International ships and ports appear to be important contributors to the spread and settlement of NIS in the study area.

#### *Ships' hulls are home to eight taxa, including three non-indigenous species*

A total of eight taxa were reported for the three ships, although the taxa richness greatly varied among them (from two to seven). In particular, only two species were recorded on the ARG tanker. The low number of species on the ARG tanker may be due to the small number of ports visited by this ship (three ports including the Port of Talcahuano), compared to the USA tanker (five ports) and the Escuela Esmeralda Ship (seven taxa).

Among the eight taxa recorded, three NIS were found on two of the evaluated ships (USA tanker, and Esmeralda). One of them, *Asterocarpa humilis* was found exclusively on the hull of the USA tanker. This non-native ascidian was described in New Zealand, and subsequently reported in different coastal regions around the world, including the continental Chilean coast, and in insular areas, such as the Juan Fernández archipelago (Kott 1985; Clarke and Castilla 2000; Bishop et al. 2013; Turon et al. 2016). This finding was previously reported in Pinochet et al. (2017), who also documented the presence of other specimens on a mussel farm in Concepción Bay, where the Talcahuano port is located. Another non-native ascidian, *Ciona robusta*, was observed on the hulls of two of the studied ships. This species, assumed to be native to the Northwest Pacific (Asia),

has been reported as an introduced species in the North and South Atlantic Ocean, the Mediterranean Sea, and the North-East and South-East Pacific Ocean (Bouchemousse et al. 2016). In Chile, *C. robusta* is distributed throughout the country and considered as an invader (Castilla and Neill 2009; Turon et al. 2016). It is important to note that because taxonomic revision has occurred only recently (Gissi et al. 2017) this species had been previously reported as *Ciona intestinalis* in Chile by Castilla and Neill (2009) and Jofré Madariaga et al. (2014). Our report, thus, confirmed the presence of *C. robusta* in Chile (Bouchemousse et al. 2016; Turon et al. 2016), and it is the first to document its presence on international ships. The third NIS is a bryozoan, *Bugulina flabellata*, native to the Northeast Atlantic (Ryland 1960). *Bugulina flabellata* is presumably transported by ships (Gordon and Mawatari 1992; Hayward and Ryland 1998) and has been introduced into various parts of the world, including Australia, New Zealand, and Chile (Castilla et al. 2005). A recent study, based on rapid assessment surveys and the deployment of settlement panels in 2016–2017, reported the presence of this bryozoan in five of six local or international ports in the Biobío region (where the Talcahuano port is located; Leclerc et al. 2018), suggesting that this species is well established in this region. Interestingly, the three NIS identified on the ships' hulls in this study were also detected, following in-water and dry-dock samplings, on the hull of an oceanographic vessel based in the Port of Mar del Plata (Argentina) and navigating through the southwestern Atlantic and Southern Oceans (Meloni et al. 2021). In this study, a greater number of NIS or cryptogenic species (77% of 53 taxa identified at the species level) were observed, though this proportion is similar to that found in our study (60%; 3 NIS out of 5 species). The differences in the number of taxa observed may be due to the sampling strategy and targeted taxa, as Meloni et al. (2021) examined every taxa, including mobile and sessile specimens with a size of > 1 mm. It could also be explained by the sailing route and sailing duration; for instance, the oceanographic vessel was in the water for 754 days straight, as compared to the Esmeralda ship that was cleaned every year.

Contrary to the three NIS cited above, the hydroid *Ectopleura larynx*, the fourth NIS reported in our study, was not found on ships' hulls. It was observed only on settlement plates, suggesting the presence of a cryptic population in the Talcahuano port. This species is cryptogenic in the Northern Hemisphere, with a circumglobal distribution in temperate and cold waters, but it was introduced into Chile. In the Biobío region, it has been reported in only one other port (Coronel port) in 2016–2017, on settlement plates and artificial structures (Leclerc et al. 2018). Considering that our sampling effort was carried out two years before (2014–2015) the aforementioned study, the results obtained so far suggest that this species is slowly expanding. Thus, dedicated surveillance to document the establishment and spread of *E. larynx* is required.

### *Species are unequally distributed on ships' hulls*

We observed substantial differences in taxa richness between the protected and exposed areas of the studied ships' hulls, with ten and three taxa, respectively. In the protected areas, *Bugulina flabellata*, *Ciona robusta* and *Mazzaella laminarioides* were the most commonly observed species. Only *Choromytilus chorus*, *C. robusta* and *Ulva* sp. were found in the exposed areas. The ARG tanker is an exception because the two taxa observed on this vessel (ie, *Mytilus chilensis* and *Ulva* sp.) were found in both exposed and protected areas. Besides the variable effectiveness of the antifouling paint, differential exposure to hydrodynamic flow could account for the variation in the amount and composition of the hull biofouling (Coutts and Taylor 2004; Coutts and Dodgshun 2007). Our data suggest that protected areas are better shelters for species than exposed areas, as several studies have previously shown (Coutts et al. 2003; Sylvester and MacIsaac 2010). These niche areas can considerably increase the risk of NIS transport (Sylvester and MacIsaac 2010; Moser et al. 2017) since they are less effectively targeted by antifouling coatings or in-water cleaning procedures (Tamburri et al. 2020).

NIS are not the only concern. Ships can also transport native species, with varied possible consequences, such as an increase of connectivity between populations otherwise poorly connected by natural dispersal (Lacoursière-Roussel et al. 2012), or the rapid spread of pathogens between distant locations (Ruiz et al. 2000). In this context, the presence of the native mussel *Mytilus chilensis* on the hulls of the three studied ships is noteworthy. This mussel has recently been shown to harbor neoplastic cells that can disseminate across individuals (ie, transmissible cancer) in Chile and Argentina since 1998 (Yonemitsu et al. 2019). A first strain of this transmissible cancer was identified in bay mussels (*M. trossulus*) in the Northern Hemisphere (Metzger et al. 2016), and then a second strain was identified and proved to be shared by both the Chilean mussel (*M. chilensis*) and the European blue mussel (*M. edulis*) (Riquet et al. 2017; Yonemitsu et al. 2019). Yonemitsu et al. (2019) hypothesized that the transmission of this strain across the two species, and thus the two hemispheres and two oceans, is most likely due to the accidental transport of mussels with neoplastic cells through shipping vessels. Although the present data cannot ascertain this hypothesis, the presence of several adult specimens (3 to 5 cm long) of *M. chilensis* on each of the three studied vessels does support this scenario. So far, only farmed populations of *M. chilensis* have been examined; 5–10% of the individuals showed the presence of disseminated neoplasia (Yonemitsu et al. 2019). The mussel populations, developing on ship hulls, require further genetic investigation to determine their origin and test for the presence of neoplastic cells.



*NIS can be particularly pervasive across habitat and substrates*

Species richness was higher on ships ( $N = 8$  taxa) than on natural substrates ( $N = 5$  taxa), but roughly similar to settlement plates ( $N = 7$  taxa) in the Talcahuano port, and the three categories shared a substantial fraction of the recorded species. Plates and natural habitats were, however, more similar to one another, as compared to the ships' hulls. The geographical proximity between the Talcahuano port (plates) and El Manzano pier (natural substrate) could facilitate individual migration between these two habitats, which could explain this similarity. While NIS richness was higher on ships, they were more abundant on plates ( $N = 83\%$  of all specimens) and natural substrates ( $N = 48\%$ ) than on ships ( $N = 33\%$ ), a result largely due to the high number of *C. robusta* and *B. flabellata* specimens settled on plates, and, to a lesser extent, on natural substrates. These two non-native species are widely distributed around the world (Moyano 1996; Nydam and Harrison 2007; Zhan et al. 2010; Rius et al. 2014; Bouchemousse et al. 2016), reflecting their high invasive potential, particularly on artificial substrates.

The colonization potential of the two NIS cited above was illustrated by the settlement plates' experiment carried out in this study. Contrary to expectations of a greater number of species in shallower waters, especially for algae (due to light availability, for instance; Gattuso et al. 2006), no significant differences were observed according to depth (0.5 and 1.0 m) in the settlement plates' experiment. Settlement was, however, significantly higher on collectors made of rough material (aluminum) compared to smooth surfaces (PVC and polypropylene), with aluminum being the material with the highest number of fixed specimens. Rough materials are considered better artificial substrates for the fixation of organisms because they have a more complex surface that promotes the adhesion of spores and post-larval states (Clifford et al. 1992; Saliu and Ovuorie 2007). Nevertheless, the two NIS, *Ciona robusta* and *Bugulina flabellata*, showed no preference in this regard, and settled at both depths and on the three materials. The ubiquitous presence of both NIS in this study is in agreement with other experimental studies carried out in Chile, where these same species were commonly found on PVC settlement plates (e.g., Leclerc et al. 2018).

Along with other taxa, such as hydrozoans, bryozoans and mytilids, ascidians have been affirmed to be major contributors to the species assemblages that colonize human-made habitats (Piola and Johnston 2008; Shenkar and Swalla 2011). This is exemplified by *C. robusta*, one of the most abundant species in the San Francisco Bay, USA (Chang et al. 2018). This also holds true in ports of the Biobío region, Chile, in which the most dominant species were *C. robusta*, *B. neritina* and *Bugulina stolonifera* (Leclerc et al. 2018). Here, two of the three non-indigenous species observed on ships (*C. robusta*, *B. flabellata*) were also found in the Talcahuano port,

or in its vicinity (El Manzano pier), in large abundance, suggesting the presence of a high number of reproducing individuals, a hypothesis also supported by genetic data (see below). This propagule pressure, exerted by locally and sustainably established populations on natural and/or artificial habitats, might further facilitate the colonization of ships' hulls arriving to these ports, along with the subsequent spread, at local or global scales, through maritime traffic.

*High propagule pressure in ports and on ships' hulls: an example with Ciona robusta*

The 36 individuals of *Ciona robusta* that were examined with Sanger sequencing and the COI gene showed high haplotypic diversity, with 12 haplotypes. All, except one rare haplotype (CR9; Figure 6), have been reported in previous genetic studies on *C. robusta* (Table S3). The CR2 haplotype was the most commonly found in our study (in 31% of the individuals), and shared by the three sampling points (Figure 6). This haplotype has been recorded in other regions, such as North America, South Africa, and Europe, where it can be found at high frequencies (e.g., 93% of the individuals introduced in the North East Atlantic (raw data from Bouchemousse et al. 2016). It was, however, not recovered in native populations (Japan) studied by Bouchemousse et al. (2016). The rare occurrence of this haplotype in its native range, but its high occurrence across several regions of introduction suggests similar origins for these introductions (through primary or secondary introductions). The haplotype CR3, observed in two individuals and only on settlement plates, has been previously reported only in South Africa (MF479429, Rius et al. 2014), which could be related to existing maritime traffic from this area to Chilean ports (Directemar 2020).

The high genetic diversity observed in all evaluated individuals was also observed when analyzing each sampling category separately (Table 2a), with gene diversity estimates reaching 0.9 to 1 for the two ships. These numbers are worth comparing to previous data obtained with the same marker (Table 2b). All of the genetic diversity indices computed in our work, for either all individuals or as an average per population, were found to be far higher than those computed in other parts of the introduction range of *C. robusta*, such as South Africa (Rius et al. 2014), the North East Atlantic or the Mediterranean Sea (Bouchemousse et al. 2016) (Table 2b). Nonetheless, these numbers compare to what has been observed in the native range in Japan, and in other introduced Chilean populations spread over more than 1,500 km of coastline (SEP in Table 2b). Similar or higher genetic diversity of introduced populations as compared to native populations is not unusual in marine introduced species (Rius et al. 2015), a pattern suggesting multiple introductions and high propagule loads (Viard et al. 2016). Multiple introductions and propagule pressure can increase establishment success, for instance by increasing the genetic

diversity on which selection may act (ie, selection on standing genetic variation) and/or due to the introduction of new advantageous traits (Rius et al. 2015). The high traffic of ships in the Talcahuano port could not only exert this type of high propagule pressure, but, when accompanied by the sustainable settlement of local populations on the human-made structures in this port, it could, in turn, be the source of rapid colonization of new ships by genetically diversified individuals. Therefore, not only should the introduction of new species be avoided, but a substantial effort should also be made to avoid secondary introductions and reduce the previously established species.

*NIS identification through DNA barcoding: a helpful  
(and sometimes needed) approach*

DNA barcoding confidently identified the four NIS found in our study. This DNA-based approach has been critical to ascertain identification at the species level. This is exemplified by the hydrozoans of the *Ectopleura* genus, which is composed of species such as *E. larynx* and *E. crocea* that can be easily confounded (Schuchert 2010). The use of DNA barcoding has been facilitated by the availability of reliable references for several *Ectopleura* species, including the two species cited above. For NIS, the reliability of species identification is crucial, as both false negatives (ie, a NIS that goes undetected even though it is present) and false positives (ie, identifying a NIS which is actually not present) have important implications in terms of management actions (Sepulveda et al. 2020; Trebitz et al. 2017). The confidence in the species assignment carried out in our study is thanks to the availability of both specific primers that were designed in previous studies and reliable sequences that could be used as references (eg, Bishop et al. (2013) for *A. humilis*, Nydam and Harrison (2007) for the *Ciona* genus; Fehlaue-Ale et al. (2013) for *Bugulina* species). As emphasized by several studies (eg, Comtet et al. 2015; Trebitz et al. 2017), the availability of curated reference databases is a key issue in DNA (meta) barcoding approaches; an issue that deserves a close interaction between taxonomists and molecular biologists. In the case of hydroids, we did however face technical limitations, with poor quality DNA that prevented us from using a DNA barcoding approach, although primers were available (Ender and Schierwater 2003). This failure might be due to the poor quality of the samples (related to tissue fixation), that resulted in specimens' damage during transport and preservation. We were thus unable to provide a species name for the two groups that were identified based on morphology, which may correspond to NIS or cryptogenic species already reported in the study area (Leclerc et al. 2018) or novel for the study area. On the other hand, we are relatively confident that our species list is devoid of false positives, though false negatives cannot be excluded, and further specimens' collection and DNA work are needed to identify the two groups of hydrozoans.

For mussels, six specimens were not possible to identify morphologically. Thus, sequences for COI, GLU and EF-bis were obtained. In the *Mytilus* species complex, a multi-locus approach is highly recommended because of the mosaic composition of *Mytilus* genomes (see for example Larraín et al. 2019). For the COI gene, three distinct haplotypes (GenBank accession numbers MZ152219-21) were found with a 99.8% similarity with COI sequences of *M. chilensis* samples from Chile (Table 1). For the EFbis gene, two distinct haplotypes (accession numbers MZ152222-23) were detected. The closest similarity was found with *M. trossulus* from the Baltic Sea (GenBank EU684165, 66, 67; Faure et al. 2008), but these sequences were longer than ours. The region that we sequenced indeed correspond only to the second half of the sequences obtained by Faure et al. (2008), whereas published sequences for *M. chilensis* from Yonemitsu et al. (2019) (MN546736-41) correspond to the first half, and thus do not overlap with our sequences, preventing a comparison with those *M. chilensis* reference sequences. *M. chilensis* published sequences are very similar to published *M. trossulus* sequences, including the best hit sequences of our haplotypes, and other sequences by Metzger et al. (2016) (KX018532, 37, 39, 40, 42). Therefore, our sequences could be actually also assigned to *M. chilensis* with the EF-bis locus. For the GLU locus, reference sequences from *M. chilensis* samples are absent from GenBank. Following Tarifeño et al. (2012), the *M. chilensis* sequences deposited into GenBank by Santaclara et al. (2006) are indeed *M. galloprovincialis*. The three distinct haplotypes that we found showed best hit in GenBank with *M. edulis* from Northern Europe, but in absence of *M. chilensis* in GenBank. Our study thus provides the first GLU sequences for well-identified *M. chilensis* samples (GenBank accession numbers MZ152224-26). According to the results of these three genes, and morphological observations made on the 30 other *Mytilus* specimens, these six mussel individuals were confidently assigned to *M. chilensis*.

## Conclusion

Non-indigenous species (NIS) are members of the biological fouling on ships' hulls. One of the three NIS (the tunicate *Asterocarpa humilis*) observed on vessels in this study was first reported some few years ago for the Biobío region (Pinochet et al. 2017), whereas the other two species are common for the region, and globally distributed in South America. These NIS co-exist on ships' hulls with native species, such as the mussel *Mytilus chilensis*, which has been shown to host disseminated neoplasia in Chile. Ships' hulls may thus facilitate both novel NIS and disease expansion along receiving coasts, in addition to promoting connectivity between established populations. Three NIS (two found on vessels in this study) were indeed also found in ports of the Biobío region, on artificial (plates) and natural substrates, with commonly large abundances and/or genetic diversities (in

the case of *C. robusta*), which suggests that these NIS are well established. This study documented that ships' hulls can be important contributors to both colonization and propagule pressure of NIS. Transport via ships' hulls is thus a latent risk for marine biodiversity and should be urgently targeted by biosecurity measures. This work is pioneer in Chile because it combines the study of ships, natural and artificial substrates. The collaboration with the Chilean Navy and port authorities was essential to carry out this work. Their involvement is required, not only to pursue and expand similar studies, but also to encourage good practices in maritime ports, in the context of the GloBallast and GloFouling initiatives promoted by the International Maritime Organization (IMO).

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## Data availability statement

Data (number of specimens per sample type and taxa) are provided in Table 1, and the results of the DNA barcoding approach in Table S2. Sequences for the new haplotypes of *M. chilensis* and *C. robusta* were deposited in GenBank (accession no. MZ152218–26).

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## Authors' contribution

FT, FV, JP conceived the idea and designed the study. FV, FT, JP, CD, designed the methodology. JP, FV, CD, FT, AB performed the analyses, prepared the figures and table and interpreted the results. FV, FT, JP, and AB led the writing of the manuscript. FT, FV, JP and AB significantly contributed to the manuscript writing and critical review.

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## Supplementary material

The following supplementary material is available for this article:

**Table S1.** Molecular markers used for the DNA barcoding approach.

**Table S2.** Taxonomic identification through DNA barcoding.

**Table S3.** NCBI Blast analysis of COI haplotypes detected for *Ciona robusta* specimens collected in this study.

**Table S4.** COI haplotype distribution in populations (and regions) studied by Bouchemousse et al. (2016).

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