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A massive ingression of the alien species *Mytilus edulis* L. (Bivalvia: Mollusca) into the Mediterranean Sea following the Costa Concordia cruise-ship disaster

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Abstract

The Costa Concordia cruise-ship disaster occurred just off the coast of Italy on January 13th, 2012, and entailed the largest marine salvage operation in history. The salvage employed vessels from different European harbours, providing an unexpected means for transporting alien species into the Mediterranean. In this work we identified mussel species using fragments length polymorphism of a nuclear locus and report the first evidence of the transport of the blue mussel, *Mytilus edulis* Linnaeus, 1758 (Bivalvia: Mollusca), into the Mediterranean Sea, as a part of the fouling community of the hull of an accommodation barge arrived from a NE Atlantic location in October 2012. Furthermore, we describe the rapid growth of this species, under the ASV Pioneer, until its almost total extinction during the summer of 2013, which left a covering of mussel shells on the underlying *Posidonia oceanica* (Linnaeus) Delile, 1813 meadow. This high mortality rate indicated that *M. edulis* had been exposed to high stress conditions, probably due to different salinity, temperature, and oligotrophic conditions from its place of origin, and there was no spawning event or known settlement on the nearest infralittoral natural habitats. This event reminds us of how the Mediterranean Sea is constantly under alien species pressure, due to human activities.

Keywords: Blue mussel, Non-indigenous marine species, Hull fouling, Mediterranean Sea, Costa Concordia

Introduction

Over the last twenty years the arrival of alien species has been a major concern to the scientific community and policy makers, due to the role of these species as drivers of ecological change. Marine invasions have historical roots, being strongly linked to the development of human settlement and trade. As underlined by Galil (2000), the opening of interoceanic routes in the 16th century, and the Suez Canal in the 19th century, with its consequent increase in shipping, promoted the introduction of alien biota to the Mediterranean Sea. The synergy between environmental factors, geographical history and anthropogenic activities taking place in the Mediterranean Sea as nowhere else, makes this basin one of the most affected by alien species, with 986 reported (Galil, 2000; Zenetos *et al*., 2005, 2011, 2012). Nowadays, the mechanisms of introduction and the pathways of spread of non-indigenous marine species have become clearer: vessel traffic has been identified as a primary source of invasion, either via ballast waters or by hull fouling, and only a secondary role, although still important, has been attributed to aquaculture, fisheries and accidental release by the aquarium industry (Hewitt *et al*., 2009; Katsanevakis *et al*., 2013; Nunes *et al*., 2014). Therefore, it is not difficult to understand why the Mediterranean basin, with all its ports and marinas, shipping lanes, human settlements, coastal tourist facilities, and fishing and aquaculture, is so susceptible to biological invasions.

Hull fouling has recently been recognized as a major force in the global spread of non-indigenous marine species (hereafter NIS), being a complex pathway able to move both sessile and motile species worldwide (Gollasch, 2002; Davidson *et al*., 2009; Hewitt *et al*., 2009; Sylvester *et al*., 2011). The potential for invasive aquatic species being transferred through biofouling has been recognised by the International Maritime Organization (IMO), the Convention on Biological Diversity (CBD), several United Nations Environmental Programme (UNEP) Regional Seas Conventions (e.g., the Barcelona Convention for the Protection of the Mediterranean Sea Against Pollution), the Asia Pacific Economic Cooperation Forum (APEC), and the Secretariat of the Pacific Regional Environmental Programme (SPREP). Although the IMO adopted guidelines to manage ballast waters in 2004 (IMO, 2004), no such proposals were made for the management the hull fouling on commercial vessels and recreational craft until only a few years ago (IMO, 2011;

2012). However, there are still no international regulations to guarantee consistency in the management of ships' hulls in the Mediterranean Sea. This lack is highlighted by the 22nd article of the Mediterranean Action Plan (UNEP/MAP, 2005) which clearly states: "Given the importance of the shipping-mediate introduction of non-indigenous species into the Mediterranean… a regional project be developed to overcome gaps for the Mediterranean countries, and strengthen the capacities of the countries to reduce the transfer of aquatic organisms via ships' ballast water and sediments and hull fouling". Furthermore, the adoption of an IMO convention prohibiting the application of tributyltin (TBT)-based antifouling paints as of January, 2003 could have increased the number of fouled hulls, and consequently, hull-transferred species.

Blue mussels have a worldwide distribution, reflecting their tolerance of salinity and temperature variations (Braby & Somero, 2006). The most common species in Europe are *Mytilus galloprovincialis* Lamarck, 1819 and *Mytilus edulis* Linnaeus, 1758, although *Mytilus trossulus* Gould, 1850 has also been recently reported along north European coasts (Väinölä & Strelkov, 2011). *Mytilus galloprovincialis* is endemic to the Mediterranean and Ibero-Moroccan area, is thought to have evolved from *M. edulis*, is more tolerant of warm temperatures and high salinity conditions, and has been introduced to the Atlantic (South Africa) and Pacific (California and Japan) (Seed, 1992). Although *M. edulis* is mostly found in the Atlantic, it has recently been reported, together with *M. trossulus,* in Ukrainian waters (Black Sea and Sea of Azov, with unintentional introduction by shipping in 2001). It has not been described as an established species, but there is a potential risk of its settlement in the fouling community and consequent hybridization (Alexandrov *et al*., 2007). Hybridization often occurs within the genus *Mytilus*, and the presence of *M. galloprovincialis-edulis* hybrids together with the pure genotypes has been reported in several contact zones (France, SW England and Ireland) (Edwards & Skibinski, 1987; Gosling *et al*., 2008; Hilbish *et al*., 2012). However, Lourenço *et al.* (2015) recently hypothesized that divergent environmental conditions between the two basins (Atlantic Ocean and Mediterranean Sea) block the hybrids at the edge of Mediterranean waters (in fact they are broadly distributed in relatively low numbers in SW Iberia), preventing the ingression so that they have not yet been detected in any Mediterranean study site.

The cruise ship Costa Concordia hit the reefs of Giglio Island (Tuscany, Italy) and partially sank in proximity of Giglio Harbour on 13th January 2012. This maritime disaster, with 32 fatalities, required the biggest wreck removal project ever attempted, which started in May 2012. In order to remove the Costa Concordia wreck five different phases were planned: a holdback system and stabilization, underwater support and portside sponsons, parbuckling, starboard side sponsons, refloating and towing (www.parbucklingproject.com). During the first three phases two huge barges were employed for various purposes, the ASV Pioneer and the Micoperi Trenta (M30). The ASV Pioneer is an accommodation support vessel (100.6 m x 30.4 m x 6.0 m) used for supply, accommodation, diving and remote operated underwater vehicle (ROV) applications. It set sail from Middlesbrough (U.K.) and arrived at Giglio Island on 22nd October 2012, sailing through both Atlantic and Mediterranean waters (Fig. 1). The M30 is a heavy lift/accommodation barge (122.1 m x 27.6 m x 8.2 m) used for heavy lifting, diving and ROV operations. The M30 left Ravenna (Italy) in September 2012, arriving at the island in the same month by circumnavigating the Italian peninsula. We collected the data for this study during the routine surveys conducted to evaluate the environmental situation and to assess the impact of the salvage works.

The aim of the present paper is to report a particular case of the entrance of a non-indigenous mussel species into the Mediterranean Sea, due to unusual events and situations, using both ecological observations and genetic tools. The data we have obtained provided useful information on the risk posed to the basin, and an opportunity to investigate the consequences of the introduction of an alien species, highlighting the urgent need for laws, rather than guidelines, to manage major NIS ingressions.

Materials and Methods

Study site

The study site was the area of Giglio Island north of Giglio Harbour (42°21'37.20'' N, 10°55'22.50'' E). The island has been incorporated in the Tuscan Archipelago National Park (DPR 22/07/1996) due to its notable landscape and natural interest. In the study area the natural landscape and seascape have been deeply affected by man-made structures. The presence of the Pioneer and M30 inside the shipyard area provided additional substrates for sessile species. Routine environmental monitoring revealed a total covering of the ASV Pioneer's hull with mussels and a partial covering of the M30's hull. The ASV Pioneer was moored close to the right side of the bow of the Costa Concordia for one year (from October 2012 to October 2013), in an area of sandy bottom and gentle slope, characterized by a healthy *Posidonia oceanica* (Linnaeus) Delile, 1813 meadow extending from a depth of 10 m down to 36 m. The M30 was moored to the left side of Costa Concordia wreck, in deeper waters (Fig. 1).

Sampling methods and data analyses

Monthly dives were carried out in the area, from early summer 2012 until the end of summer 2014, for environmental monitoring, and it was possible to follow the evolution of the hull fouling community to evalu-

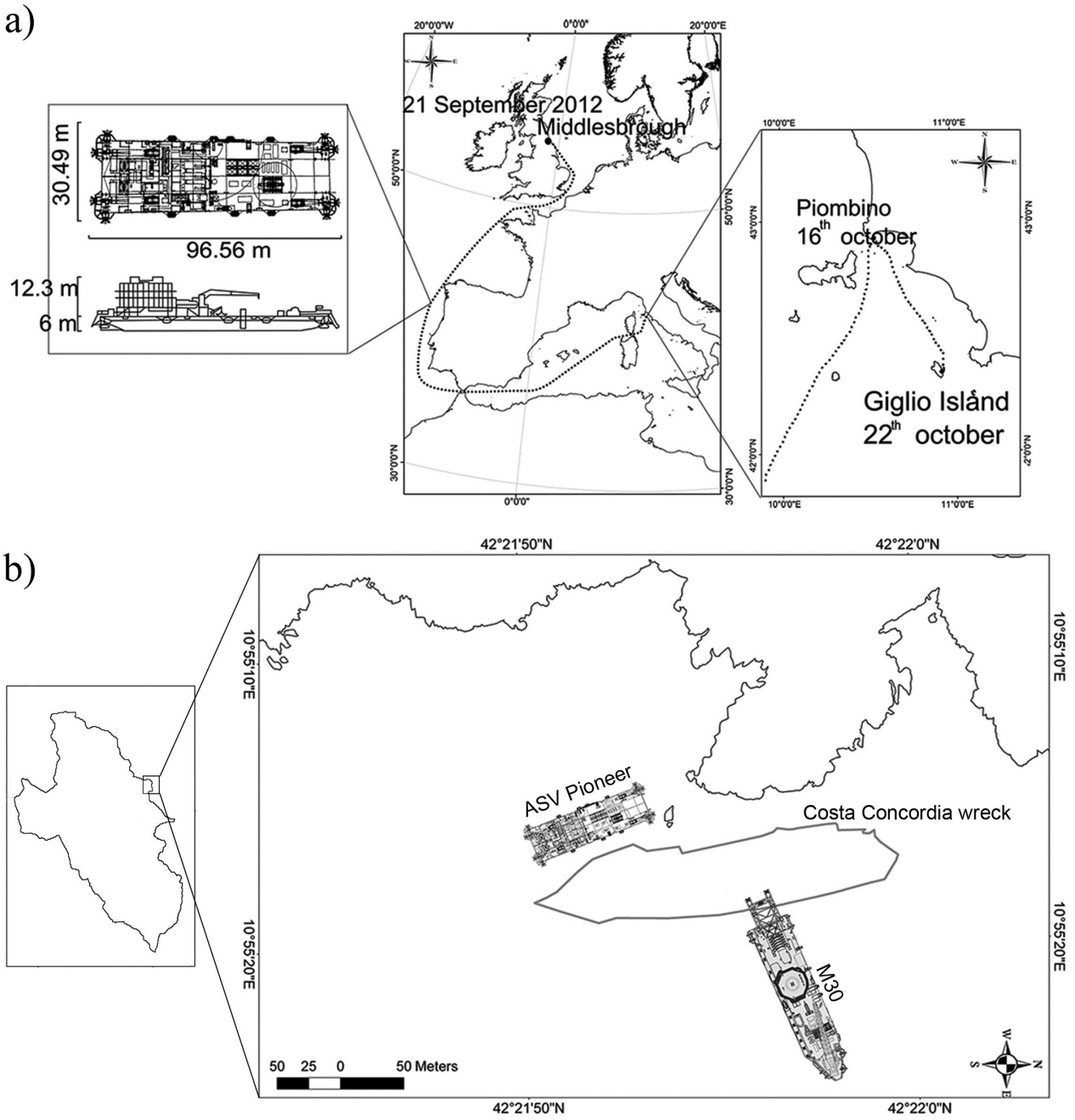

Fig. 1: Map showing the study area. a) ASV Pioneer route; b) Costa Concordia wreck site and vessel displacement.

ate the percentage coverage beneath both vessels. Furthermore, the sea surface temperature (hereafter SST), salinity, chlorophyll α concentration, dissolved oxygen and pH were monitored daily using two conductivity-temperature-depth (CTD) probes (IP010 and MAR-330 IdromarAmbiente CTD probes). Mussels were collected by scuba divers, randomly scraping the hulls of the two aforementioned barges. We sampled a total of 353 mussels from the two barges' hulls for both genetic and length analyses. In order to genetically support the morphological species attribution, we used PCR amplification and amplicon length polymorphism analysis following Rawson *et al*. (1996a). A total of 38 *Mytilus* specimens, 21 from the Pioneer and 17 from the M30, were collected, stored in Ethanol 100° and subjected to Glu-5' PCR assays (Rawson *et al*., 1996a). DNA extraction was performed using the standard phenol-chloroform method (Oliverio & Mariottini, 2001) on a tissue fragment obtained from the foot of the mussels. We used the primer pair JH-5 and JH-54 (Rawson *et al*., 1996a) to amplify the 5' region of the Glu gene, using the original PCR protocol with slight modifications (35 amplification cycles of 94°C for 30'', 54°C for 30'' and 72°C for 1'). Amplified fragments were visualized by running 10 µl of the PCR product on a 1.5% agarose gel stained with ethidium bromide. In *M. edulis* the Glu-5 PCR assay retrieves

a single 350bp in the majority of individuals, but a single 380bp can also be found, as well as both bands (Rawson *et al*., 1996a). Specimens belonging to *M. galloprovincialis* mostly produce two bands, 300bp and 500bp, but occasionally only the 300bp band is obtained. Moreover, an additional 200bp product can be found in some *M. galloprovincialis* specimens, due to the presence of multiple annealing sites for the reverse primer JH-54 on the Glu gene (Rawson *et al*., 1996a).

Shell lengths of 35 specimens collected each month (total 315 specimens) were measured with a digital electronic caliper from the apical umbone to the end of the shell. Then we performed a cubic (or Hermite) spline interpolation in order to fit the growth curve as a typical Von Bertalanffy model could not be applied due to the strong linear relationship of the data. Cubic Hermite spline (often called c-spline) is a spline where each piece is a third-degree polynomial specified in Hermite form: that is, by its values and first derivatives at the end points of the corresponding domain interval. Cubic splines are typically used for interpolation of numerical data specified at given argument values x1, x2,…xn , to obtain a smooth continuous function. The resulting spline will be continuous and will have a continuous first derivative. Subsequently, we computed the monthly growth rate by calculating the first discrete derivative of each spline's node with $\frac{\Delta(f)}{\Delta(t)}$, where f represents the shell length, and t refers to time.

Finally, the constant *routine* surveys of specific Mediterranean key habitats (e.g. the *P. oceanica* meadow) during the course of the project, allowed us to obtain a detailed picture of the dynamics of this community during the various phases of the Costa Concordia's removal. The *P. oceanica* meadow under the ASV Pioneer was mapped annually (August 2012, 2013, 2014) and shoot density measurements were carried out at 10, 15, 20, 25, and 30 m depths in order to monitor and evaluate any impact.

Results

The genetic analyses revealed that the M30 specimens were *M. galloprovincialis* whereas the Pioneer samples were *M. edulis*. The Glu-5' PCR assays returned visible bands on the agarose gel for 24 *Mytilus* samples (63% of the total number of samples) (Fig 2, Table 1), however 33% of the samples collected from the Pioneer 's hull and 41% of the samples collected from the M30 's hull were not successfully amplified. Samples collected from the Pioneer hull consistently yielded an amplicon of 380bp, but in two instances a second product of 500bp was present. Samples collected from the M30 's hull yielded a pattern with two bands, 300bp and 500bp for all samples except one, in which the 500bp was missing; in three samples an additional band of 200bp was also observed. Literature data indicate that in *M. edulis* individuals the Glu-5 PCR assay retrieves a single 350bp in the majority

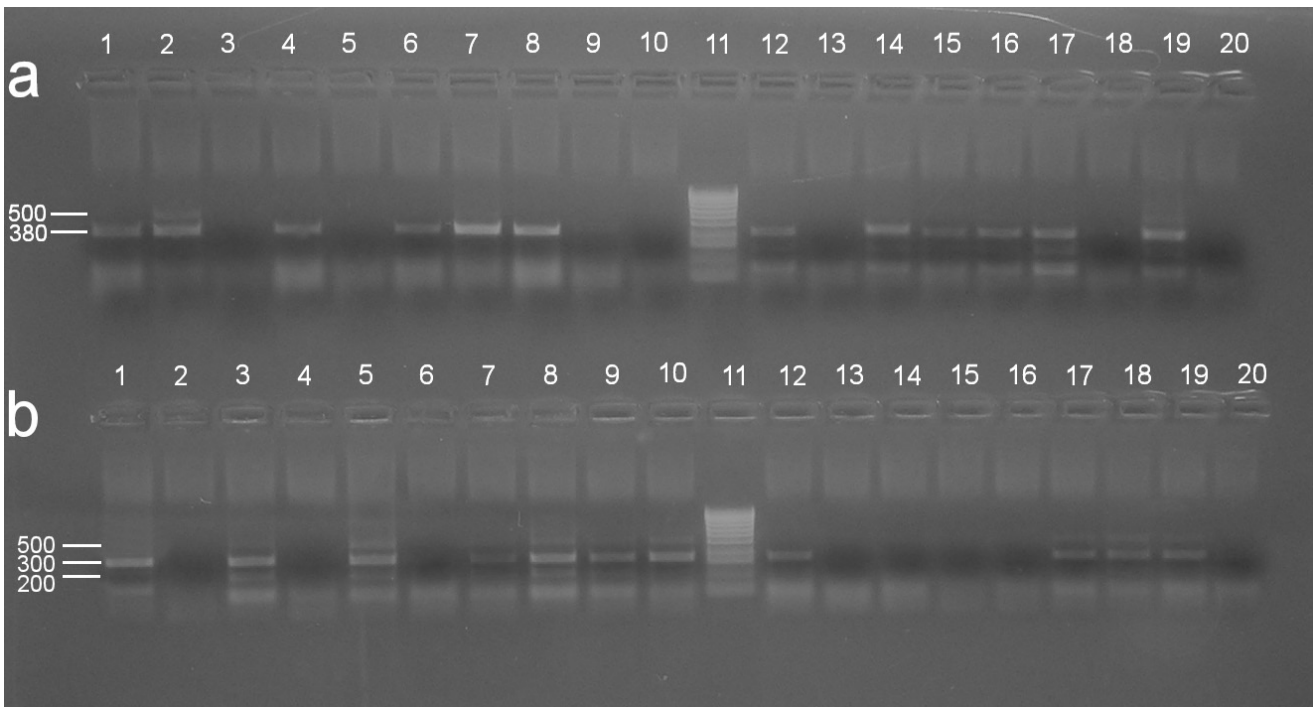

Fig. 2: Agarose gel with PCR products: row a, Pioneer *Mytilus* samples; row b, M30 *Mytilus* samples. Lane 11 on both rows is 100bp PCR Molecular Ruler (200, 300, 380 and 500 base pairs heights are indicated); lanes 10 and 20 (row a) and 20 (row b) are negative controls.

Table 1. Table summarizes results of genetic analyses; specimen ID, source, bp of characteristic bands and species identification have been reported here.

Specimen ID	Source (vessel)	Band(s)	Species identification
M-1454	Pioneer	380	*M. edulis*
M-1455	Pioneer	380-500	*M. edulis*
M-1456	Pioneer	-	-
M-1457	Pioneer	380	*M. edulis*
M-1458	Pioneer	-	*M. edulis*
M-1459	Pioneer	380	*M. edulis*
M-1460	Pioneer	380	*M. edulis*
M-1461	Pioneer	380	*M. edulis*
M-1462	Pioneer	380	*M. edulis*
M-1463	Pioneer	-	-
M-1464	Pioneer	380	*M. edulis*
M-1465	Pioneer	-	*M. edulis*
M-1466	Pioneer	380	*M. edulis*
M-1467	Pioneer	380	*M. edulis*
M-1468	Pioneer	380	*M. edulis*
M-1469	Pioneer	380	*M. edulis*
M-1470	Pioneer	-	-
M-1471	Pioneer	380-500	*M. edulis*
M-1472	Pioneer	-	-
M-1473	Pioneer	380	*M. edulis*
M-1474	Pioneer	-	-
M-1475	M30	300-500	*M. galloprovincialis*
M-1476	M30	-	-
M-1477	M30	300-500	*M. galloprovincialis*
M-1478	M30	-	-
M-1479	M30	300-500	*M. galloprovincialis*
M-1480	M30	300-500	*M. galloprovincialis*
M-1481	M30	300-500	*M. galloprovincialis*
M-1482	M30	300-500	*M. galloprovincialis*
M-1483	M30	300	*M. galloprovincialis*
M-1484	M30	-	-
M-1485	M30	-	-
M-1486	M30	-	-
M-1487	M30	-	-
M-1488	M30	300-500	*M. galloprovincialis*
M-1489	M30	300-500	*M. galloprovincialis*
M-1490	M30	300-500	*M. galloprovincialis*
M-1491	M30	-	-

of cases, but a single 380bp can also be found, as well as both bands (Rawson *et al*. 1996a). Specimens belonging to *M. galloprovincialis* mostly produce two bands, 300bp and 500bp, but occasionally only the 300bp band is obtained. Moreover, an additional 200bp product can be found in some *M. galloprovincialis* specimens and has been explained by the presence of multiple annealing sites for the reverse primer JH-54 on the Glu gene (Rawson *et al*., 1996a). The presence of an accessory 500bp band in some *M. edulis* specimens, which has not been previously reported, could be produced by a slight difference in the nucleotide sequence at the annealing site for the primer JH-54 or be ascribed to a variation in the PCR conditions, as already proposed for *M. galloprovincialis* (Rawson *et al*., 1996a). Instead, *M. edulis* PCR products at 350bp and 380bp are supposedly created by two dis-

tinct and well-differentiated alleles, only one of which is represented in our samples (380bp).

The percentage mussel coverage in relation to the mean SST and chlorophyll α concentration over time is shown in Fig. 3; the degree of coverage and the pattern of hull fouling community evolution clearly differ between the ASV Pioneer and the M30. The former displayed an high percentage coverage of *M. edulis* from October 2012. Although the covered area remained stable during the entire winter and spring seasons, the actual thickness of the layer of mussels increased, reaching a maximum of 10 kg m^{-2} of live mussels at the end of April 2013. Mass mortality then occurred during the early summer of 2013. From the end of June the mussels started dying, reaching a maximum mortality rate at the end of July, and their shells started dropping on the seafloor. By the mid August, 2013 only a few patches of living mussels remained, and these definitively disappeared in September 2013 (Fig. 3 a). Hence, only a few patches of *M. galloprovincialis* were found on the hull of the M30, never exceeding 5% of the total available surface. No changes were observed during the whole year and in relation to SST and chlorophyll α, and no mortality event was reported as is shown in Fig. 3b.

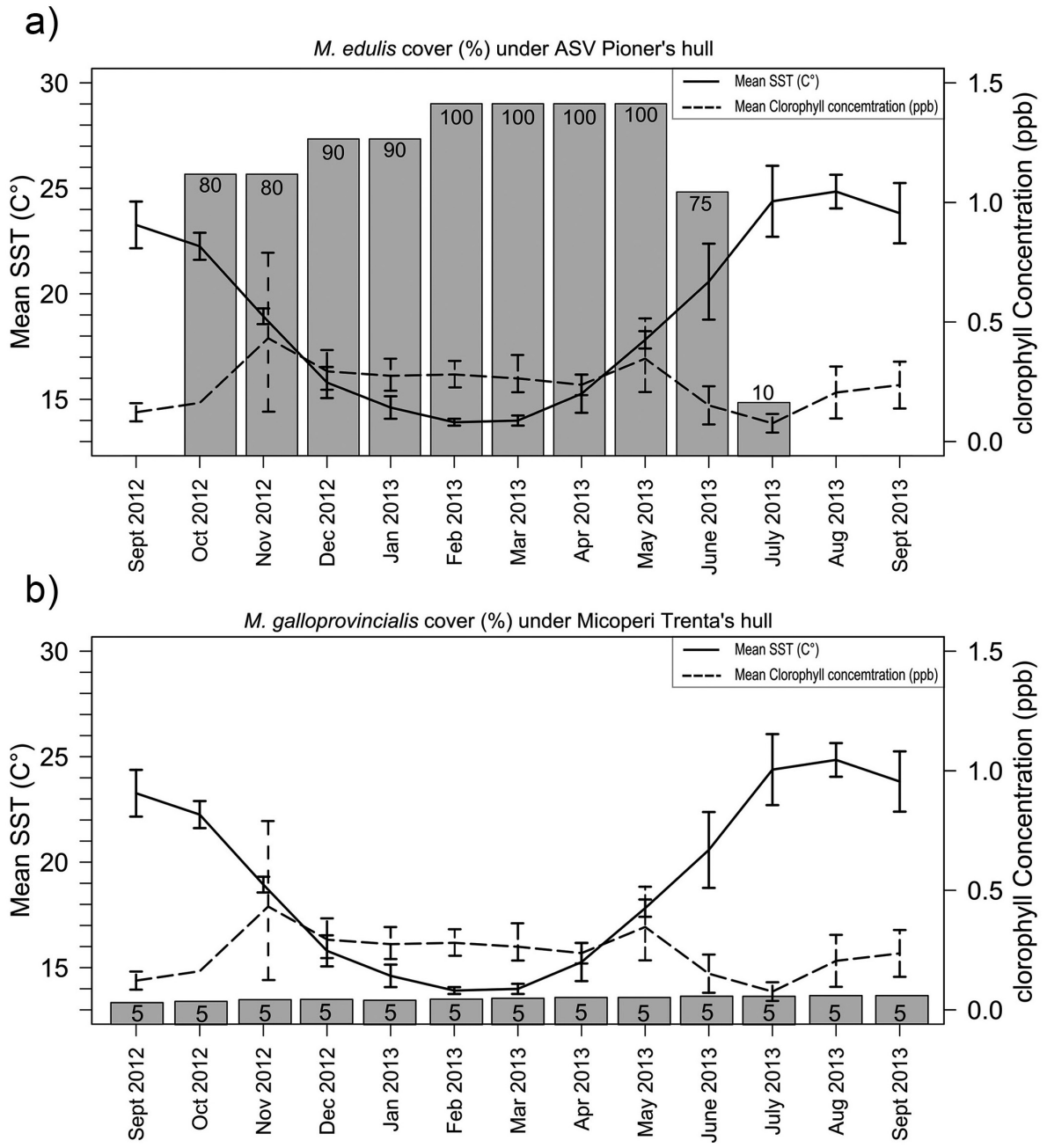

Fig. 3: Mussel cover in relation to mean (±SD) Sea Surface Temperature and Chlorophyll α concentration. a) ASV Pioneer; b) Micoperi 30.

Juvenile *M. edulis* [3.24 ± 1.11 mm, mean length ± standard deviation (SD)] were observed settled on the barge's hull in October 2012; they clearly arrived with the ASV Pioneer from Middlesbrough. From this starting point they followed a linear growth, reaching a maximum length of 52.43 mm at the end of June 2013 as shown by the growth curve (Fig. 4a). The monthly growth rate reached a maximum in October-November and November-December 2012 with a monthly increment in their mean length of 6.21 mm ± 2.8 SD and 10.35 mm ± 0.92 SD, respectively (Fig. 4b). During the following winter months (from January to March 2014) the growth rates stabilized around 4.81 mm ± 0.1 SD per month. The strong growth rate increment that occurred at the end of

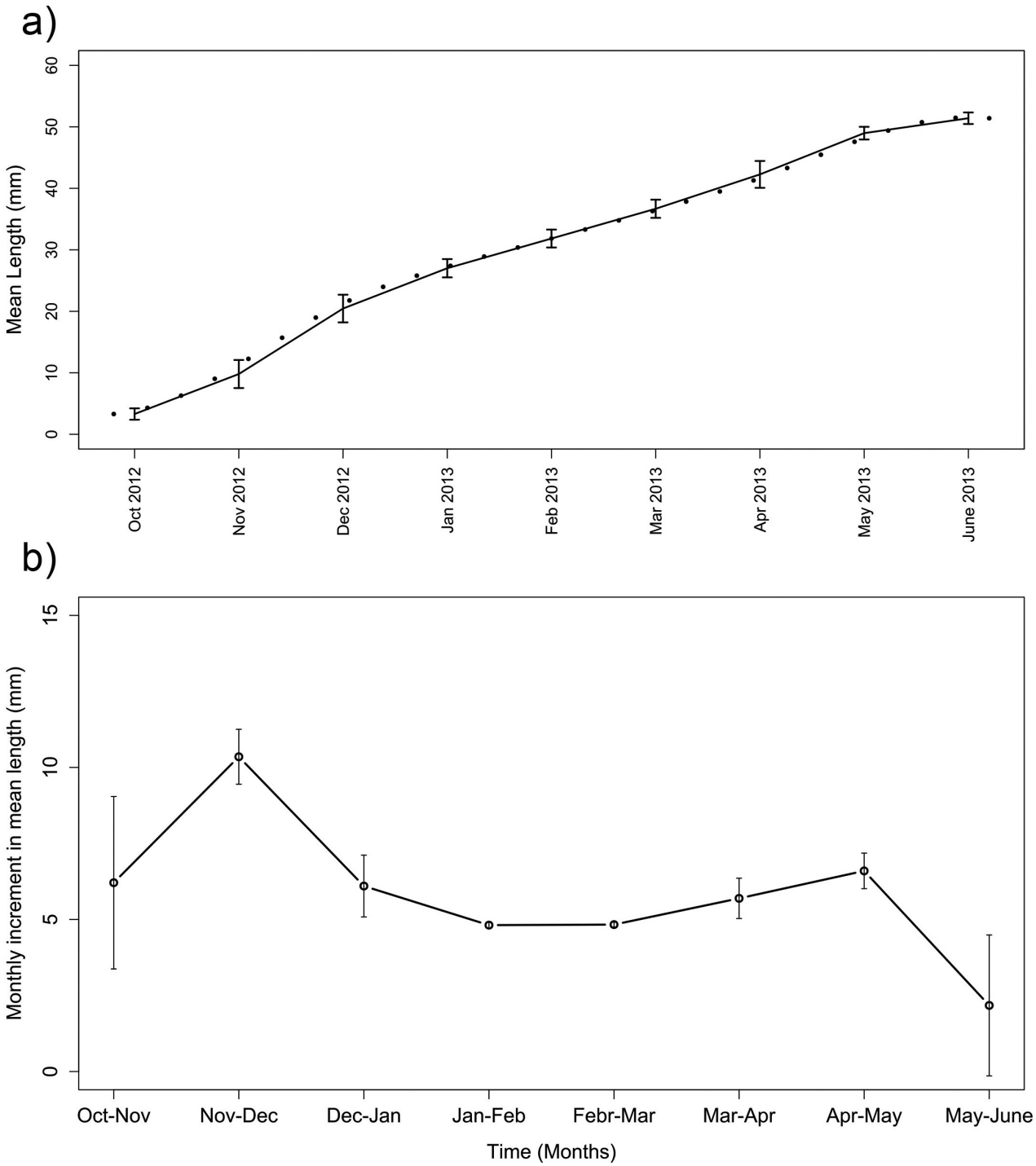

Fig. 4: a) Growth curve of *M. edulis* under the ASV Pioneer's hull. Black dots represent the nodes of the c-spline obtained by combining all size measurements recorded. The error bars represent the standard deviation from the monthly mean length. b) Estimated monthly increment in mean length (mm ± SD) of *M. edulis* from October 2012 to June 2013.

November 2012 could be correlated with the optimum mean temperature for shell increment of 20°C (Almada-Villela *et al*. 1984; Reuter 2004) and with the availability of food resources as signaled by the peak of the chlorophyll α concentration (considered as a proxy for the phytoplanktonic component). This *scenario* could be confirmed by the other pulse in the growth rates (6.5 mm ± 0.6 SD) recorded from April to May 2013, which could be correlated with another chlorophyll peak that occurred in late May 2013 (Fig. 3a). After this weaker peak, the monthly growth rate decrease dramatically reaching 2.1 mm ± 2.3 SD at the end of June 2013. During the following months the mussels stopped growing and began to die, as demonstrated by the reduction in the percentage coverage of the hull of the ASV Pioneer. This mortality event seems to have been strongly linked to the SST; in fact, as soon as the surface temperature rose over 22°C *M. edulis* began to die.

As soon as the Pioneer was moored, baseline observations were carried out; it was noted that there was a non-impacted *P. oceanica* meadow covering the entire sea floor from a depth of 10 m to a depth of 36 m with a shoot density (maximum 237, minimum 120 shoots m^{-2}) decreasing naturally with depth (Fig. 5a). In September 2013, after one year of permanent mooring and parbuckling operations, only a few patches of living *P. oceanica* remained, shoot density had dramatically decreased (max. 60, min. 0 shoots m^{-2}). We attributed this to the impact of the Pioneer's shadow and the dropping of dead mussel shells. As soon as the wreck removal phase was completed (late summer, 2014) a detailed survey allowed us to pinpoint a well defined area where the seabed was totally covered by a thick layer (up to 20 cm) of dead mussel shells (Fig. 5b). The depth range affected by the dead mussel layer ranged from 15 down to 35 m. In this area *P. oceanica* had not survived with the exception of small and isolated patches, displaying very low shoot densities (max. 45, min. 0 shoots m^{-2}). The meadow seemed to have maintained its natural condition only in the shallower portion, where we recorded only a slight reduction in shoot density (Fig. 5c). Fig. 6 shows the *M. edulis* shells fall and the resulting covering of *P. oceanica* meadow.

Discussion

This study is the first report of the entrance of *M. edulis* into the Mediterranean Sea from a well-defined source in the North Atlantic Ocean. Previous findings indicated trade, including import and discard in the field from markets and ponds, and escape from aquarium, as the most plausible vector of introduction for *M. edulis* in Italian waters (Crocetta, 2012).

Bivalve molluscs are considered, together with cirripede crustaceans, to be among the most important group of invasive species introduced by shipping (Zvyagintsev & Mikhailov, 1978; Goallasch, 2002); mussels have frequently featured in references to alien invasive species (Hicks & Tunnell, 1993; Crooks & Khim, 1999).

M. edulis lives in the temperate-cold Atlantic waters of Europe and North America while *M. galloprovincialis* is a warm water inhabitant of the Mediterranean but can also be found in the Atlantic as far north as the coasts of France and the United Kingdom (Gosling, 1984). In

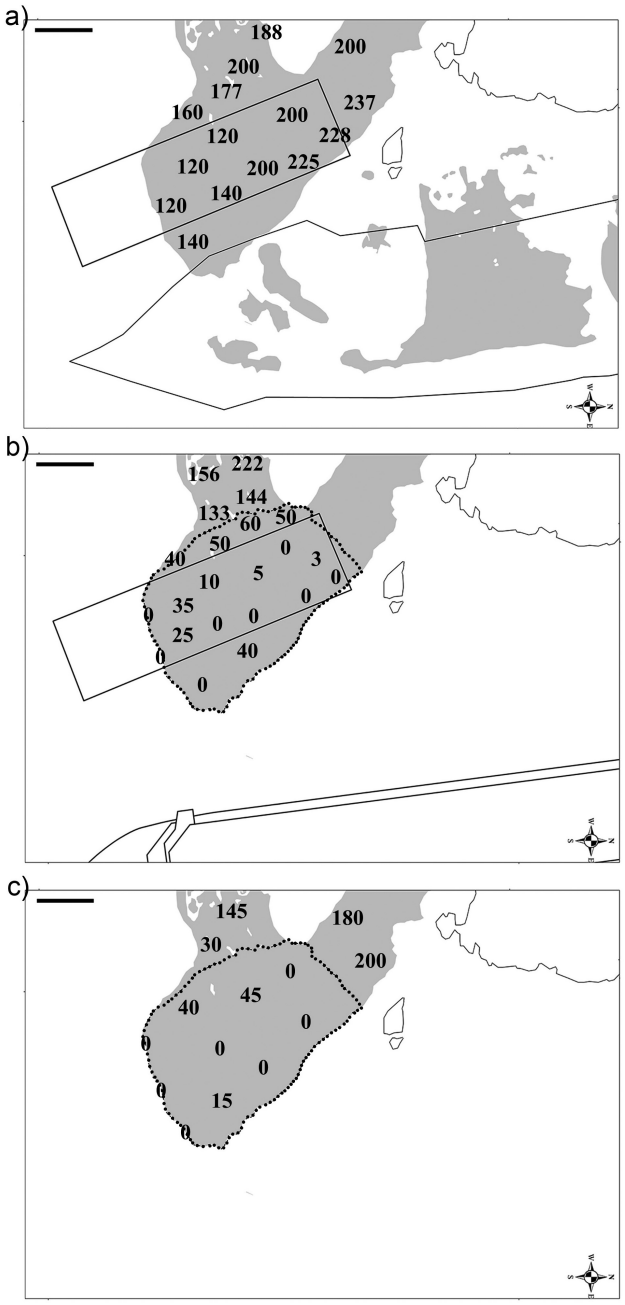

Fig. 5: *P. oceanica* meadow evolution throughout 2012 (a), 2013 (b), 2014 (c); values are reported as number of shoots/m^2. Light grey indicates *P. oceanica* presence, the ASV Pioneer and Costa Concordia's positions are represented by black lines (2012 and 2013), whereas the area involved in the *Mytilus* shells fall is defined by the dotted line. All scale bars equal 30 meters.

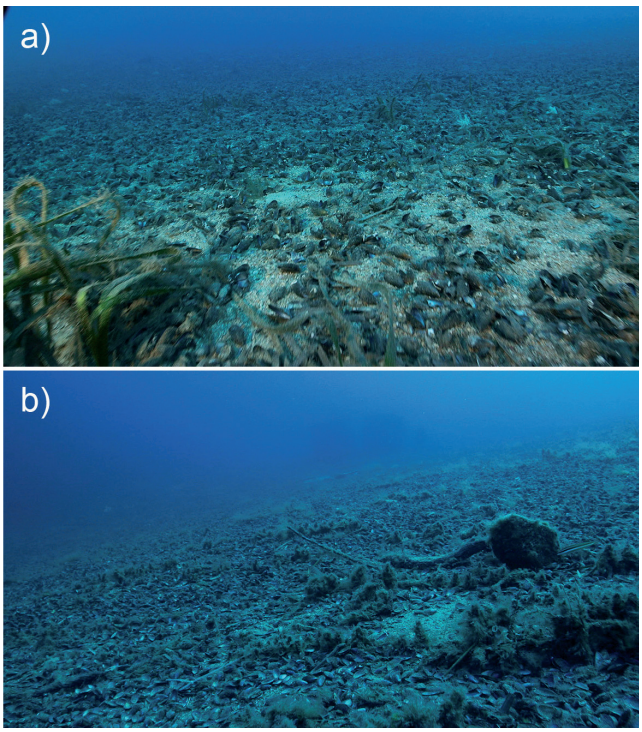

Fig. 6: Underwater photographs at the study site, showing the dead *Mytilus* shells and the resulting massive covering of the *P. oceanica* meadow (a-b).

accordance with these physiological differences the two species occur sympatrically only in some areas of the United Kingdom, Ireland and France, where both hybridization and introgression have been documented, as previously mentioned (Edwards & Skibinski, 1987; Lopez *et al*., 2002; Bierne *et al*., 2003; Gosling *et al*., 2008; Hilbish *et al*., 2012). PCR amplification of diagnostic loci represents a valuable tool to easily and securely identify mussel species (Apte *et al*., 2000), being a morphology-based identification, although perhaps only reliable at a local scale (Freeman *et al*., 1994). Identification is complicated by the high frequency of natural hybridisation that has been observed whenever any two mussel taxa of this complex are sympatric (Gosling, 1992). In fact, the most intensely studied hybrid zones at sea are those of the *M. edulis* species complex, particularly the *M. edulis* × *M. galloprovincialis* hybrid zone in southwestern Europe (Gosling, 1992; Gardner, 1994), and the two *M. edulis* × *M. trossulus* hybrid zones on opposite sides of the North Atlantic (Scandinavia and Canada) (Riginos & Cunningham, 2005). Allozyme markers used in the past for identifying mussel species (Gosling, 1992), although highly successful in distinguishing between species, sometimes prove problematic in hybrid zones with differences often not completely established (Riginos & Cunningham, 2005). Conversely, several other nDNA-based markers, which rely on the detection of length variants or restriction digests of PCR products, are 100% reliable in distinguishing between species (Heath *et al*., 1995; Rawson *et al*., 1996a; Rawson *et al*., 1996b). Here, we used a PCR-based approach that benefits from length polymorphisms at the nuclear locus Glu, encoding the mussel polyphenolic adhesive protein to identify the *Mytilus* species collected from the M30 's and Pioneer's hulls. This protein, produced in the foot gland of the mussel, is crucial for attachment to the substrate (Waite, 1992) and displays a divergent primary sequence in the *Mytilus edulis* complex, which can be visualized as a different banding pattern upon running the PCR products on an agarose gel stained with ethidium bromide (Rawson *et al*., 1996a).

The occurrence of *M. edulis* described here might be included in the category of alien (*sensu* Zenetos *et al*., 2005), that is a species occurring outside its known geographical range and beyond its natural dispersal potential as a result of direct or indirect introduction by humans, but the death of all our mussels and the absence of evidence of reproduction or spawning events during the year of permanence in the Mediterranean sea, did not allow us to report it as either established or invasive. Although there is no proof, the mussel settlement on the Pioneer's hull may have taken place several months before its departure from Middlesbrough (Summer 2012); Bayne (1964) reported a high density of late plantigrade stages in the Menai Straits in late July and August. In accordance with Bayne (1964), Dean & Hurd (1980) observed late summer settlement of *M. edulis* on fouling panels in Delaware. Furthermore, the slow speed of the Pioneer during its voyage and the small plantigrade size allowed the permanence of mussels as hidden passengers.

M. edulis at Giglio Island reached a maximum shell length of 52 mm in nine months and the same growth patterns can be found in other congeneric species (*M. californianus, M. galloprovincialis*) from warmer areas. Coe & Fox (1942) reported an average growth rate of 7 mm per month for *M. californianus* with a total increment in shell length of 80 mm in 11 months. Coe (1945) highlighted how the *M. californiunus* monthly rate of increase during the winter is only about half as great as in summer, and that they reached their maximum size of 78 mm after one year. *M. galloprovincialis* also showed a similar growth in Tyrrhenian coastal waters, reaching 30 mm 5 months after settlement (Ardizzone *et al*., 1996). It must be noted that the *Mytilus* species (*M. edulis* and *M. trossulus*) growing in cold waters could take up to ten years to reach the same lengths, as reported for the Baltic Sea (Kautsky, 1982; Antsulevich *et al*., 1999) and in the Gulf of Alaska (Blanchard & Feder, 2000). In the light of these findings, growth rates may be interpreted as a function of environmental features, reflecting water temperature effects on metabolic activity, more than species linked, as clearly shown from the comparison of our results with other data found in previous studies on the *Mytilus* species (Fig. 7).

The identification of physiological determinants has a pivotal role in understanding of the biogeographical

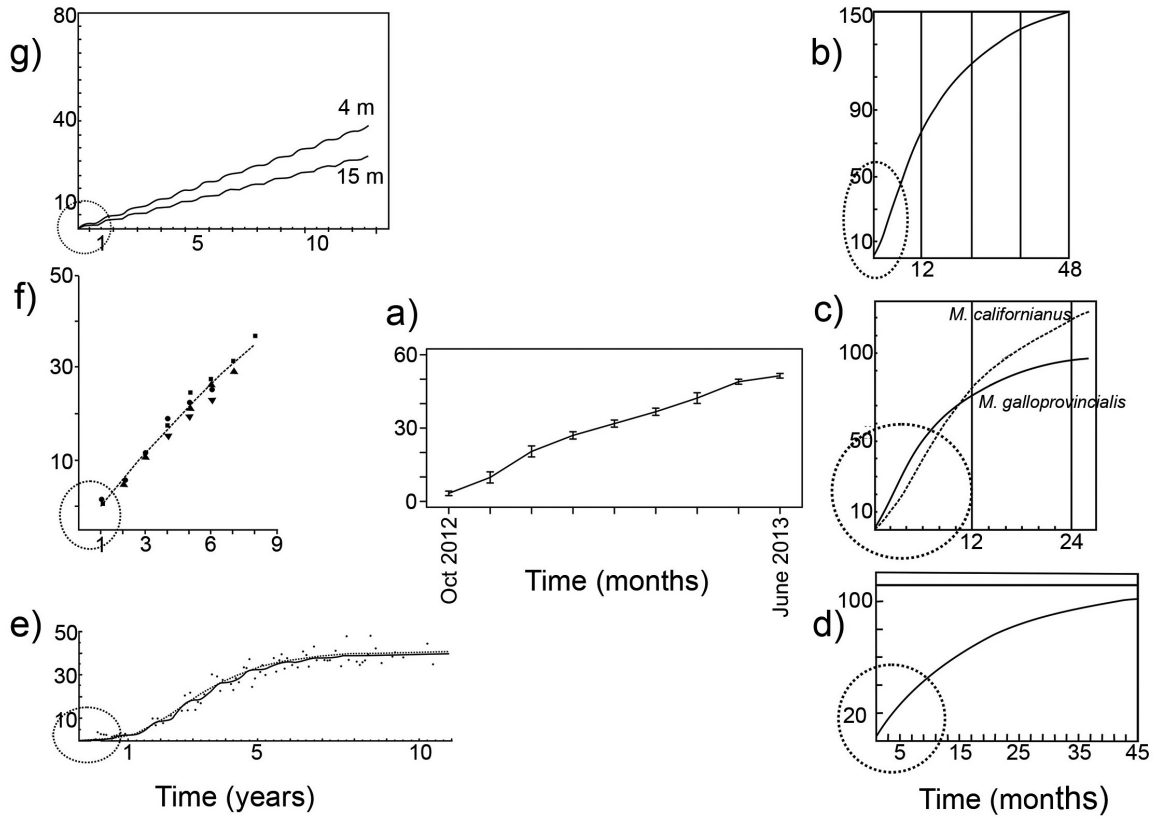

Fig. 7: Graphic comparison of different growth curves on *Mytilus* species from literature data and our study. On the left we report data from cold areas, instead the right graphs represent data from warmer areas. Dotted circles highlight the period of approximately nine months that correspond to the duration of our study. a) present study; b) Coe & Fox, 1942; c) Coe, 1945; d) Ardizzone *et al*. 1996; e) Blanchard & Feder, 2000; f) Antsulevich *et al*. 1999; g) Kautsky, 1982.

patterns of species distribution and the changes in biological communities. Specifically, temperature and salinity, as mentioned above, lead to a specific distribution of mussel species. These parameters are known to influence blue mussels' respiration, heart rate, valve closure, growth and feeding (Schulte, 1975; Lutz & Porter, 1977; Incze *et al*., 1980; Hawkins *et al*., 1991; Braby & Somero, 2006; Riisgård *et al*., 2012), both in field and laboratory studies. The mortality reported in this work might be due to the effect of temperature and salinity-induced stress, which in turn required high energy requirements that could not be supported by the local food supply. In fact, the waters around Giglio Island can be considered as oligotrophic waters and the phytoplanktonic fraction never reaches high concentration values, especially during the summer period.

Considering these findings, the *M. edulis* shells fall in Giglio Island may be considered as an in-water self-cleaning event, caused by the tolerance range of the blue mussel being exceeded. The release of viable propagules by marine organisms could be explained as a response to new environmental cues or prolonged permanence in a recipient region (Apte *et al*., 2000; Floerl & Inglis, 2005). Recently biosecurity risks resulting from self-cleaning in water have been discussed and compared to unmanaged risks. Besides the risk of defouled material settling on the seabed, another risk is represented by propagule release during operations (Hopkins & Forrest, 2008). Unfortunately no evidence or data that could prove larval dispersion or plantigrade settlement has been reported. On the other hand, mussel shell deposition has been well reported and documented. Great attention is often given to competition or displacement phenomena, or to the economic and social impact that took place after a NIS appearance. In this case something rare occurred: the state and health of the underlying *P. oceanica* meadow was affected not only by the shadow of the hull but also the mussel shell deposition. Although we did not find other direct effects of interaction between alien and endemic species (e.g. competition), this episode is a clear example of "habitat disturbance" against an endemic and engineering species such as *P. oceanica*. In fact, to mitigate this NIS intrusion, in the first months of 2015, the Site Remediation Project started removing the mussel shells from the sea bottom, using a purpose-built suction pump to avoid further impacting the remnants of the *P. oceanica*. A total of 50 tonnes of dead mussel shells were removed from the sea bottom, leaving matte and seagrass rhizomes free from the bivalve cover. The damaged *P. oceanica* is being monitored in order to test its eventual recovery capability.

Nowadays the Mediterranean Sea is one of the busiest waterways in the world, occupying an important place in world trade; furthermore its coasts are inhabited by millions of people and affected by economic and tourist activities (IUCN, 2008; REMPEC, 2008; Mounime *et al*., 2014). Both ship transit and maritime activities are expected to rise over the next ten years, by as much as in the last 18 years: from 1997 to 2006 a mean of 3.14 billion deadweight tonnes (hereafter DWT, referring to the carrying capacity of a vessel) per year arrived in Mediterranean ports, whereas an average of 383.3 billion DWT per year transit through Mediterranean Sea (REMPEC, 2008). Oil Tankers, Containerships, other Tanker and Passenger vessels are the categories most involved in Mediterranean traffic; barges may be included in the category "Other vessels" that represents less than 4% of the average DWT in Mediterranean ports from 1997 to 2006, and less than 1% of the average DWT vessels passing through the basin in the same period. Taking into account that the ASV Pioneer could reach a maximum of approximately 6838 DWT, that represents 1.39 10^{-6}% of the total "other vessels" deadweight tonnage passed through Mediterranean Sea in 2006 (REMPEC, 2008), the real scale of the problem becomes clearer. Barges are flat-bottomed vessels involved in several commercial and military operations; as hull fouling is strongly linked to sailing speed, time of permanence in a port (or region), anti-fouling treatments, and propagule and colonization pressure (Sylvester *et al*., 2011), we could hypothesize that this vessel category is more likely affected by hull fouling communities than other types of ships. Effectively, barges clearly differ from other commercial vessels because they tend to remain in a limited area for long periods for work reasons and due to their reduced maneuverability, and their need to be towed, could be considered unmanaged hulls.

Finally, these results must be taken into account seriously, especially in the light of the opening of the enlarged Suez Canal in August 2015 that put the Mediterranean Sea under even greater pressure. No environmental risk assessment was undertaken before the enlargement project; that event and the lack of directives that impose defouling treatments on inter-basin traffic, leave the matter still unsolved and needing urgent attention.

Conclusions

This study, as well as representing a case of alien species ingression, highlights the urgent need for real common management measurements to avoid any impact on Mediterranean biodiversity. In fact, it must be considered that the cascade of events described here, consuming enormous economic resources and time as it did, has been caused by a single ship; then we should presume several implication regarding the introduction of alien species if we take into account the enormous number of ships entering Mediterranean waters each day. The RAC/SPA (Regional Activity Centre for Specially Protected Areas) constantly highlights and updates data on Mediterranean alien biota, but up to now practical management of commercial vessels by Contracting Parties to the Barcelona Convention and other international organizations, such as the IMO, RINA and the FAO, is still missing or insufficient if we are to avoid the unintentional introductions of alien species.

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Conflict of interest

All the authors, as scientists of the "Sapienza" University of Rome and University of Genoa, were commissioned by Titan-Micoperi group to assess environmental risks of operations and monitor the environmental conditions of the areas in order to protect marine habitats during removal operations of the Concordia wreck. Their work was controlled and continuously shared with an italian governmental body (The Osservatorio) who had the public function to verify the performance of the monitoring. This paper was authorized for the publication by Costa Crociere S.p.A.

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