Review Article

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Major ascidian species with negative impacts on bivalve aquaculture: Current knowledge and future research aims

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Abstract: Biofouling constitutes one of the main obstacles in the sector of shellfish farming. Under this perspective, it is of highly importance to critically combine the updated information regarding the invasive potential of ascidian species, together with the factors contributing toward these events. The biological features of each species in relation to the gathering of the main non-indigenous species in the Mediterranean basin represent the first step toward mitigation of negative effects of the phenomenon. Further, there are limited studies investigating the physiological changes of bivalves caused by biofouling while leading to an increase in stress biomarkers. In the present review, the major ascidian species negatively affecting bivalve culture in the Mediterranean Sea are presented, alongside monitoring of ascidians from four Greek mussel farming locations as typical mussel culture cases. Among the main ascidian species, *Styela plicata*, *Clavelina oblonga*, *Ciona robusta*, *Aplidium* sp., *Didemnum* sp., *Botryllus schlosseri*, and *Didemnum drachi* are included, with the last three being the most harmful for this aquaculture sector. Based on the existing literature and research conducted so far, future research directions are proposed, in an effort to effectively control or efficiently manage ascidian biofouling organisms. Overall, perspectives toward the way we manage the biofouling phenomenon, such as the use of ascidian's by-products in feedstuffs, chemical and pharmaceutical industry, or their incorporation in bivalve co-culture and integrated multitrophic aquaculture systems represent promising alternative approaches.

Keywords: aquaculture, bivalves, ascidians, Greece, invasions, *Ciona*, biofouling, *Clavelina*, *Styela*, stress

1 Introduction

Following the continuous growth of the human population, there is an urgent need for sustainable aquaculture development, keeping in mind that it constitutes one of the fastest-growing food production industries [1]. The aquaculture sector represents a commercial activity with a sharp increase during the last years [2]. Aquaculture, apart from seafood production contributes to wild population retention, as it leads to natural catch reduction [3]. Generally, bivalve culture contributes to 60% of the global marine aquaculture production [4]. More specifically, bivalve farming is categorized among the most sustainable aquaculture forms [5]. Within cultured bivalves, oysters, clams, scallops, and mussels are included, with the latter characterized by a total yield of 2 million tons per year, equal to 94% of the total harvest, demonstrating the major role that farming enacts in mussel production [6]. Marine bivalves are rich in proteins and are characterized as high-value aquatic products oriented for human consumption [7]. Bivalve populations, both from natural and cultured stocks, suffer from severe losses due to several pathogens [8,9], biofouling [10], heat waves [11], and urban and agricultural wastes [12].

Among the main threats bivalve aquaculture faces, the biofouling phenomenon refers to the accumulation of undesirable organisms on the underwater surfaces [10]. Biofouling threatens the marine aquaculture industry both from a profitability and a viability point of view, as it is responsible for periodic mortality and removal of fouled organisms [13]. Additionally, biofoulants cause severe

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negative outcomes on yield and growth rates of cultured species [14,15]. Economic losses owing to biofouling are calculated at the levels of 5–10% of the total production value [16]. Further, regarding the market price, the above phenomenon may cause 20 and 30% losses for oysters [17] and scallops [18], respectively. Nevertheless, it needs to be mentioned that losses due to biofouling can significantly fluctuate between aquaculture locations and cultured species, mainly associated with the different practices that farmers use in each condition [19].

Apart from the economic losses, biofouling is also responsible for ecological disturbances with biofoulant organisms characterized by high spread rates and frequent population outbreaks [20]. Considering bivalve aquaculture species, both bivalve shells and artificial substrates provide ideal extended submerged surfaces [21], free of predators, constructing a safe place for fouling organisms to grow [22]. Additionally, fouling organisms are characterized by high invasive potential, increased tolerance, and easy adaptation to new ecosystems. All of these can transform them into the dominant organisms of the new habitat [23]. Furthermore, plenty of biofouling species are competitive toward space and can succeed high biomass and densities in short time periods [24].

One of the most harmful and prolific biofouling communities toward shellfish aquaculture is the ascidian species [25] which grow as independent individuals and occasionally establish aggregations mainly due to their high fecundity [26]. Further, apart from the aggregation they also include colonial forms. Ascidians (Phylum: Chordata, Subphylum: Tunicata) also known as "sea squirts" colonize a plethora of surfaces located underwater and, although their dispersal capacity is low, human activities such as trade via shipping can result in their increased geographic spread [27,28]. Keeping in mind that their geographic expansion is enhanced by human actions, their high invasion ability is accompanied and attributed to many factors including their ability to regenerate from fragments, their short period for reaching sexual maturity, their high growth rate, hermaphroditism, high fecundity, dispersal of pelagic larval phase, their ability to produce metabolites that are often harmful to predators, and extended reproduction period [29,30].

Knowledge regarding the invasive ability of the main ascidian species in the Mediterranean Sea toward bivalve aquaculture, their presence in bivalve farms, as well as the physiological impact of biofouling phenomenon on the fouled organisms is of high importance for the design of effective mitigation strategies. Thus, the scope of the present review can be summarized in four basic components: first, to collect the main factors contributing to ascidian dispersal and their invasion success; second, to gather the main non-indigenous species (NIS) in the Mediterranean region; third, to critically discuss the above topics with the combination of novel data concerning the presence of ascidian species in a typical paradigm of bivalve aquaculture negatively influenced by the presence of those biofoulants, i.e. long line mussel farming from Greece; and lastly, to highlight the research gap concerning the physiological impact of fouling on farmed animals. Keeping in mind that the problem of ascidians still exists, our scope was additionally to propose the future research aims that have to be targeted to mitigate the negative impacts of biofouling in bivalve aquaculture.

2 Ascidians' dispersal and invasion capability

Estimation of the dispersal potential of invasive species is of high importance in an effort to design appropriate management practices [31]. More specifically, to succeed in an in-depth understanding of the invasion potential of invasive species in the field, some of the major fundamental steps concern the investigation of dispersal vectors and distances, propagule supply, interactions between species, diversity of resident communities, disturbance regimes, environmental conditions encountered, and predator–prey relationships [32].

A key feature in the life cycle of benthic marine invertebrates is the limited adult movement, which influences the dispersal of pelagic early life stages [33]. The dispersal of the planktonic propagule, which is a major parameter toward the connection of geographically separated marine populations, together with the spread and the persistence of native populations, lead to the re-establishment in previously introduced areas and the definition of species' range limits [34]. The dispersal capability is influenced by many factors including food resources, pelagic larvae duration, behavior (migration type), predators, and oceanographic conditions such as sea currents [35]. Factors related to human activities, e.g., the influence of the global trade by ascidian growth on the keels of boats and ships, contribute to a great extent in the further geographic spread even at a global scale, as they accelerate the spread rates by carrying them within ballast water; attached to vessel hulls [36,37], aquaculture [38], and recreational and fisheries watercraft [39].

Ascidians have generally aroused interest on account of their high invasive nature, i.e., due to propagule pressure; release of enemies and environmental changes, and their potential environmental impacts [20,40]. They are sessile filter-feeders, categorized among the main biofouling taxa with several cosmopolitan species successfully established worldwide [41,42]. Ascidian species can adapt in a wide range of salinities, temperatures [43], and highly polluted areas [44], whereas they can flourish when the organic load is enriched [36]. Ascidians do not have a long dispersal larva, but they are characterized by a short-lived tadpole larva that usually disperses just several meters or even less, especially regarding colonial species [45]. There are some exceptions where larvae live for many days and move with the aid of currents, e.g., 1–5 days for *Ciona intestinalis* and rafting of fragmented colony parts in colonial species (e.g., *Botrylloides violaceus*) that can contribute to wider natural dispersal [46,47]. Further, it has been reported that ascidian species can spread as they attach to other species such as American lobsters (*Homarus americanus*) and rock crabs (*Cancer irroratus*) [38]. Thus, human-mediated introductions have been recorded in all continents except Antarctica [20]. On the other hand, invasive species originating from Antarctica have invaded southern regions [48].

Hence, there are plenty of vectors contributing to the transport of the non-indigenous ascidian (NIA) species into new habitats helping them overcome the geographical barriers and thus spread in new environments. However, the multiple dispersal means and ways do not entail their invasion success. Consequently, ascidian species constitute representative examples of evaluating to which extent dispersal dynamics and geographical distributions contribute to their establishment and domination in new ecosystems.

Apart from understanding the dispersal potential of NIA species, evaluation of the biotic and abiotic factors that lead to the successful establishment of NIA in natural ecosystems constitutes an essential component for mitigating the negative impacts of ascidians' invasion. As mentioned above, ascidians are tolerant toward a broad range of salinities and temperatures, ranging between 25 and 40‰, and 5–25°C respectively, with some exceptions surviving in even broader ranges including salinities <11‰ and temperatures reaching from −1 to 35°C [20]. Apart from that, ascidians revealed tolerance toward pollution, including heavy metals such as copper, iron, and mercury [49].

Invasive ascidian species usually establish on artificial substrates of harbors and marinas, while there are some cases that later spill over to natural environments as well [50]. It is observed that ascidian species reveal preferences for artificial substrates [51], and therefore, the factors leading to the successful establishment in natural environments have been explored in an effort to explore their spread [52]. In respect to artificial substrates, key factors regarding successful invasion seem to be the absence of predators or failure to recognize introduced prey, diversity of native species, different pollutants, environmental conditions, and propagule pressure [53–56]; but is this the case for the dispersal in natural substrates as well? It was observed that extended sewage-spill events revealed a decrease in native ascidian species while no significant effect had the proximity to international ports [52]. However, this could be achieved by an indirect pathway as it was observed that the proximity of aquaculture to ports is important for propagule pressure [57]. Temperature has been proposed to be the most important driver for areas suitable for invasion [58] and eutrophication has already been connected with ascidians bio-invasion [36]. Seasonal data described in the present study are in agreement with this fact (see Section 3). Furthermore, when native and invasive populations of the same ascidian species (*Herdmania momus*) were compared, higher resilience toward higher temperature range was observed in its new habitat in the invasive population. The above observation indicates the important effect of temperature on the invasive ascidians [59]. It has also been observed that the non-native ascidian *Ascidia sydneiensis* and *Phallusia nigra* populations had higher tolerance toward a large range of salinities and temperatures in comparison to native species [60]. In a previous study, conducted in regions with colder temperatures, it was observed that invasive ascidians fully colonize underwater surfaces and can thrive in the absence of benthic predators, while this is not the case when the structures are fixed with other benthic organisms [61–63].

Apart from the factors contributing to the successful invasion and wide dispersal capacity of ascidian species, it is of main importance to assemble the information regarding the main NIS among the Mediterranean basin to apply a more focused approach toward mitigating the negative effects of the biofouling phenomenon.

3 Major NIA species in the Mediterranean Sea

Apart from the ecological losses, invasive ascidian species possess a serious threat to bivalve aquaculture, marine infrastructures, and fisheries at a global scale through the fouling phenomenon [64]. Many NIA species are considered invasive and constitute a growing global concern [65], with their dispersal enhanced by passive transportation. While biofouling decreases profitability and increases management costs [14], it simultaneously operates as a stepping-stone for the subsequent spread of non-indigenous invasive species along the nearest coastal areas [66].

The Mediterranean Sea is globally considered to be the most invaded sea by NIS [67] and it is recognized as a “hot spot” of biological incursion [64,68]. Plenty of the NIS are transferred into the new environment accidentally by ballast water and tanks, mariculture production [69], ship hitchhikers [70], and through canals [71]. In Greece, there are at least 18 ports with very dynamic operations. Most of the coastal cities in Greece have at least one port receiving cargo and cruise ships [72]. Global regulations aimed to control marine bio-invasions by forcing international ships to manage their ballast water and sediments [73]. More specifically, The International Convention for the Control and Management of Ballast Water and Sediments of Ships was adopted by the International Maritime Organization (IMO), in 2004. To the best of our knowledge, however, there are no regulations concerning the control of ship ballast waters for the presence of invasive species when entering ports, in none of the Mediterranean countries.

Invasive ascidians *Ciona* sp. and *Styela plicata* have been introduced in the Mediterranean Sea and are categorized among the most serious ascidian threats. More specifically, *S. plicata* was first detected in the Mediterranean during the 19th century [74]. Since then, it has been reported in many locations in the Mediterranean Sea such as Israel [75], Italian coasts [76], Aegean and eastern Adriatic Sea [77], and Iberian Peninsula [78]. It is considered a real pest [79] with an impressive ability to survive in many environmental conditions, such as tolerance to moderately wide changes in salinity and temperature [80,81] and high tolerance to water pollution [44]. *C. intestinalis* was the object of many developmental and evolutionary studies [82]. However, its taxonomic position remains unclear, with at least four cryptic but genetically distinct species. More specifically, *C. intestinalis* sp. A (assigned to *Ciona robusta* [83]) is widely distributed in the Mediterranean while sp. B is connected with the “true” intestinalis having a restricted distribution in the Mediterranean. *C. intestinalis* sp. C has not received a formal name yet, and it was detected in only one place of the Mediterranean Sea [84,85].

Didemnum vexillum is referred to have a negative impact on local biodiversity by rapidly overgrowing on both natural and artificial substrates [47]. It is a very harmful species present in Western Mediterranean [86] and Eastern Mediterranean [87] and is characterized by high dispersal potential, invasiveness, and capability of altering benthic communities [88]. A putative new taxon was detected in Roscoff, France, with the preliminary results supporting the existence of a new species *Didemnum pseudovexillum* sp. nov with doubts arising regarding the reports of *D. vexillum.* Although the new species *D. pseudovexillum* sp. nov is characterized as cryptogenic, there is evidence supporting its introduction [89].

Microcosmus squamiger (Michaelsen, 1927) and *Microcosmus exasperatus* (Heller 1878) are categorized among solitary ascidian species with their origin probably located in Australia. *M. squamiger* is invasive in many marine areas globally and has been detected in the Mediterranean Sea since 1995 [44]. After the first detection in Spain, it was also identified in Italy [90]. *M. squamiger* can form dense, monospecific crusts that outcompete native species in shallow water communities, operating as a threat to Mediterranean littoral communities [91]. *M. exasperates* has been detected in several locations in the Mediterranean since the 1960s [92] with the most recent one being in Cyprus [93].

The solitary ascidian species *H. momus* has its native range in Indo-Pacific area and it is supported to have been introduced in the Mediterranean Sea via Suez Canal [94]. Although it was first reported in Egypt, later its presence was demonstrated in Israel, Lebanon [95], Cyprus [96], Turkey [97], and Greece [64], while more recently, it was detected in the central Mediterranean area as well [98]. More importantly, it was observed that the invasive individuals had increased resistance toward higher temperatures in the habitat where they were introduced, implying the ability of the species to further expand its distribution into the Mediterranean [59].

Clavelina oblonga was first and recently recorded in the Mediterranean [99]. However, there is evidence that *C. oblonga* and *Clavelina phlegraea* may be the same species, with its presence in Mediterranean known for over 80 years. A bloom of this species occurred late in the summer of 2011 in Spain causing the loss of almost all mussel juveniles [99]. Thus, a lot of attention must be paid, as *C. oblonga* detected on farmed mussels constitutes a serious stress factor responsible for low productivity and even mortality [100].

The colonial ascidian *Botryllus schlosseri* is categorized among the most aggressive invaders [101], and it is considered a cryptic species, although some research groups have accepted it as native or introduced [102]. There are five genetically divergent clades (A–E) with clade E identified only in European waters so far [103], while the Mediterranean Sea is proposed to be a center of diversity for this species [104]. It has been recorded in the Mediterranean since the 18th century and in some locations, this species represents a very serious problem for shellfish aquaculture [14]. This species has also established populations in the Eastern Mediterranean since the 20th century [68]. Further, in the Mediterranean, another five *Botrylloides* NIS have been reported. These species are the introduced *B. violaceus*, *B. pizoni* which is a synonym with the invasive *B. giganteus* [105,106], the Lessepsian migrants *B. niger* (Herdmann, 1886) and *B. anceps* (Herdmann, 1891), and *B. israelience*, the taxonomic status of which remains uncertain [107].

Polyclinum constellatum is a colonial ascidian recently introduced in the Eastern Mediterranean Sea with the first record reported from Egypt in 2016 [108] and a recent record in Greece [109].

Although their presence is not detected so far, two (*A. sydneiensis* and *Didemnum perlucidum*) and two (*C. intestinalis* and *Corella eumyota*) ascidian species are indicated to have high and intermediate risks, respectively, of introduction and establishment in coastal regions of Mediterranean Sea [101] while *A. sydneiensis* has been reported as "low risk of introduction" in a recent study [110].

Taking into consideration all the above information regarding the NIS existing in the Mediterranean Sea, a more precise approach to the development of managemental practices will be possible, combining also the data concerning their characteristic toward their dispersal and invasive capability. It should be also mentioned that, although numerous ascidians are considered invasive species, their area of origin is not clarified.

4 Ascidian presence in a typical paradigm of bivalve aquaculture, Greek mussel farming

The shellfish farming sector is of high importance to the Greek national economy supporting regional development, and representing the second most important aquaculture sector after fish farming [111]. Greek seas are very susceptible to invasion of NIS operating as a crossroad between the Black and Red Seas and the Western Mediterranean, enhanced by the high levels of maritime traffic and the presence of several human activities. The Aegean Sea circulation is important and has supported bivalve farming development, mainly by seawater purification [112]. The only cultivated bivalve species in the Mediterranean Sea is the mussel *Mytilus galloprovincialis* which is farmed mainly in the coastal regions of Northern Greece. Other bivalve species that have been licensed to be farmed in Greece are *Ostrea edulis*, *Ruditapes decussatus*, *Aequipecten opercularis*, and *Venus verrucosa* [11].

One of the major threats to the bivalve industry is the introduced ascidian species and the biofouling phenomenon that they cause which leads to economic losses [14]. Aquaculture facilities serve as an ideal place for ascidians, as they provide many substrates for attachment, such as ropes, bivalve shells, buoys, and plenty of suspended food. The limited studies with prediction models [101,112] indicate that the main five species with high introduction risk have already been detected in aquaculture facilities [22]. Some of the more aggressive invaders are *A. sydneiensis*, *B. violaceus*, *B. schlosseri*, *C. robusta*, and *D. perlucidum* [100]. The most widely distributed species were *B. schlosseri* and *S. plicata.* The Mediterranean Sea was characterized by the greatest species richness while it was also included in the regions with the highest risk of primary introductions [101,112].

The ascidian species observed in marine areas characterized by high mussel farming activity in Greece (Figure 1) are shown in Table 1. Generally, an intensively seasonal pattern is indicated, probably influenced by climatic conditions. In Greece, i.e., a typical Mediterranean country of temperate latitude in terms of climatic conditions, summer months are characterized by increased water temperatures, while they are accompanied by heat waves leading to mass mortality events of *M. galloprovincialis* [113]. Heat waves are expected to be more common during the next years mainly in response to the ongoing temperature increase due to climate crisis. Concerning spring and autumn periods, increased rainfalls have been recorded, while winter periods are generally characterized by colder temperatures. The ascidian species *S. plicata* is the most common species, whereas *C. oblonga*, *Aplidium* sp., *Ciona robusta*, and *Didemnum* sp. are present in all marine areas during certain periods. *B. schlosseri* is characterized by a lower spread, present in Rodopi and Amvrakikos gulf, while *D. drachi* is detected in three out of four marine areas, namely in Chalastra, Rodopi, and Amvrakikos gulf.

An update list of NIS in Greece was conducted by Zenetos et al. [68], including ascidians. More specifically, *Ascidiella aspersa*, *C. robusta*, *Diplosoma listerianum*, *S. plicata*, and the cryptogenic species *B. schlosseri* and *Clavelina lepadiformis* have established populations as they have been found in Greek waters since the 20th century [67]. More recent records of *P. nigra* and *H. momus* were made on natural and artificial substrates of Rhodes, Chalkidiki, and Porto Heli Bay for the first one, and in Kastellorizo for the second [63,114–116]. Further, *Symplegma brakenhielmi* was also detected [69] while the colonial ascidian *P. constellatum* was found in the two westernmost locations of its distribution range, and one of them was the Greek marina in Heraklion [108]. The rare ascidian species *Ecteinascidia turbinata* was detected along the coasts of Crete Island in 2019 [117]. Even more recently *C. oblonga*, *C. robusta*, *Botryllus*

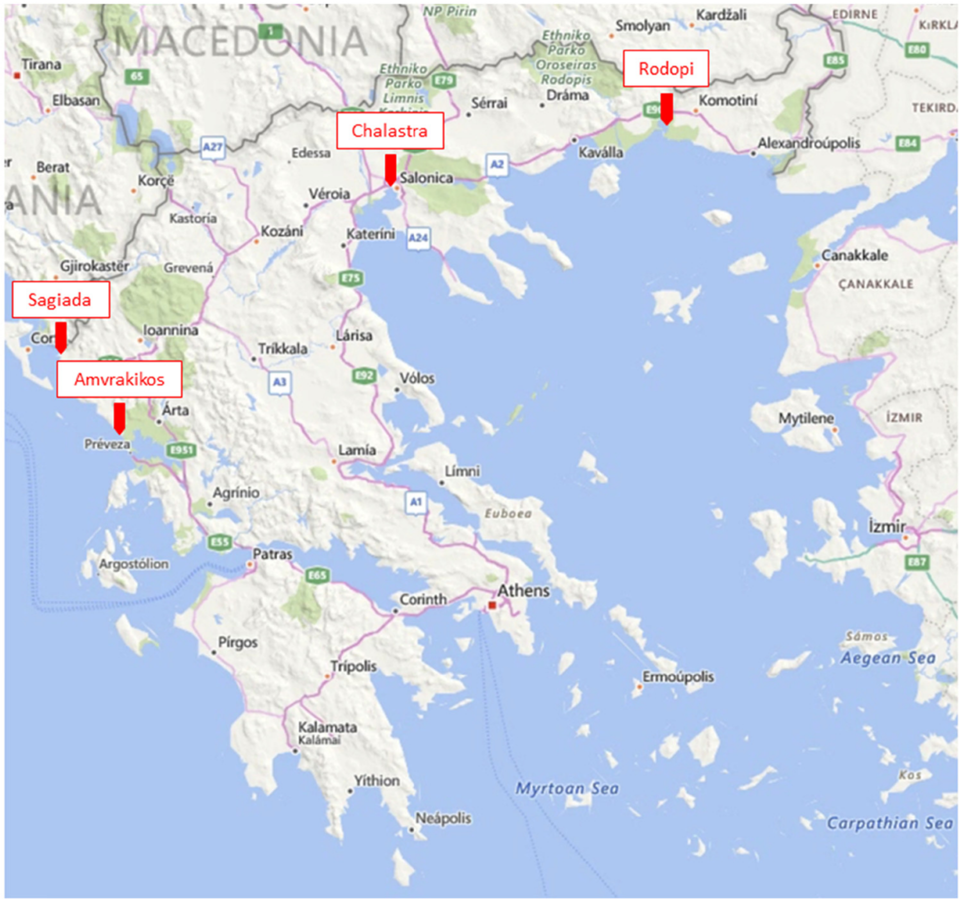

Figure 1: Marine areas in Greece characterized by high levels of mussel farming activity, where high seasonal densities of ascidian presence are observed.

sp., and *S. plicata* were found to be the dominant biofouling species on farmed mussels in Vistonikos Bay [99].

As the bivalve industry is a well-developed sector in Greece, contributing significantly to the national economy, highlighting the major NIS ascidian species already existing, and causing significant losses in the bivalve farms represent the first step toward their confrontation and management. At the same time, the ones that are present in Mediterranean but not detected in Greece yet can operate as an early sign to be prepared for their possible future dispersion.

5 Physiological impact of ascidians and the fouling phenomenon on farmed bivalves

In the plethora of studies discussing the impact of biofouling on farmed organisms (mainly shellfish), the fitness reduction is claimed as a major impact. Nevertheless, there are some studies reporting that ascidians can also have a positive effect when examined in an ecosystem approach, supporting biodiversity and balance among various taxa [118]. The reduced fitness can be translated by weight, growth, welfare, and survival reduction [119–121]. It is proposed that the above parameters are strongly associated with the competition for resources or indirectly via smothering or impede proper valve functioning [122–125], with, however, limited evidence supporting those observations [20,126]. The majority of the conclusions are drawn from observational studies in which some initial parameters among fouled and non-fouled individuals can lead to confusing results. One representative example that can result in an erroneous assumption is that fouling reduces growth rate when slower-growing stock may present higher susceptibility toward settlement of fouling on shells [20].

A solution to the aforementioned misleading conclusions may be the investigation of the cellular physiology of fouled shellfish. Scarce data existed until recently regarding the effect of biofouling on farmed bivalves. Thus, further investigation of biochemical and physiological indexes

Table 1: The ascidian species observed in the four sampling sites

Ascidian species	Sampling site												
	Rodopi					Chalastra				Amvrakikos			Sagiada
	Date												
	2020	2021											
	25 November	12 March	28 April	18 June	01 September	18 November	26 February	26 May	28 June	30 November	20 March	10 September	01 September
Clavelina oblonga	+	+		+	+			+	+	+	+	+	+
Styela plicata	+	+	+	+	+	+	+	+	+	+	+	+	+
Aplidium sp.		+		+			+	+				+	+
Botryllus schlosseri		+										+	
Ciona robusta	+			+	+	+	+	+	+	+	+	+	+
Didemnum drachi						+	+		+				+
Didemnum sp.				+	+				+	+	+	+	+

estimating the overall well-being of an animal will shed light toward the impact caused by ascidian species on the physiology of cultured fouled and non-fouled bivalves.

In this context, two recent studies from our group investigating the role of temperature fluctuations in comparison with the stress caused by ascidians on the antioxidant defense system, the apoptotic and autophagic pathways, and other physiological stress biomarkers of *M. galloprovincialis* enlightened the physiological pathways triggering stress attributed to ascidians presence [99,127]. The majority of the biomarkers examined in these two studies revealed elevated levels of stress in fouled mussels compared to non-fouled. Taking into consideration the fact that ascidians cause hypoxia to fouled mussels and that hypoxic conditions favor the increase of reactive oxygen species (ROS) by multiple cellular systems, increased oxidative stress could be the factor triggering both the *hsp* mRNA levels and the Hsps induction levels in fouled mussels compared to control mussels [99]. The oxidative stress fouled mussels are subjected to, which was evident in these studies both by the downregulated levels of hydroxy-Hif-1α and the increased levels of lipid peroxidation in combination with the enzymatic antioxidant defense system [127]. At the same time, these hypoxic conditions are responsible for the MAPKs' increased phosphorylation, since it is well-documented that oxidative stress plays a key role in MAPK activation [99]. Since it has been shown that Hsps are induced via MAPK signaling pathways [128–131], we propose that hypoxia in the fouled mussels first triggers the MAPKs signaling cascade, by phosphorylation, which in turn activates Hsp cytoprotection.

Hypoxic conditions cause oxidative stress leading to cell death pathways activation of the fouled organisms. Specifically, these results indicate that ascidian fouling drives the observed differences in cell death, rather than the temperature. The level of fouling or the species of ascidians covering the mussels may be the leading factor. Moreover, these hypoxia-sensitive organisms exhibited activation of autophagic processes in order for an energy supply to be provided during prolonged hypoxia [126]. The latter goes hand in hand with the fact that epibiotic pressure due to ascidian fauna also triggered the metabolic machinery in order for fouled mussels to exploit all available energy deposits [99]. It is likely that valve closure due to ascidian fouling restricts both feeding and oxygen access. Starvation ignites a metabolic turnover since it affects the utilization of internal energy reserves [132–135] such as the liver glycogen, which is primarily mobilized for homeostasis of glucose in plasma [136]. It has been shown that food restriction also results in oxidative stress generation in mammals [137–139] and fish [140,141]. Probably, the energy reserves' oxidation through the aerobic pathways can trigger the generation of ROS [142], which can have multifaceted effects on animal physiology. However, we cannot be sure regarding the extent to which each of these parameters (food restriction and oxygen unavailability) contributes, or their synergistic role in the induction of autophagy and the cell death pathway of apoptosis. It should also be noted that the pattern of hydroxy-Hif-1α levels presented herein is similar to the one of apoptosis. This can be attributed to the fact that Hif-1-dependent signaling may play a significant role during hypoxia-induced cell death (Figure 2).

This enhanced physiological stress seems to be season independent and can be attributed to the oxidative stress and/or feed deprivation caused by ascidian biofouling, thus illuminating the biological impact of this phenomenon. Hence, these two studies provide some clear evidence that ascidian species constitute an important stressor parameter for cultured bivalves.

The gap in the information regarding the physiological mechanisms by which fouling organisms exhibit an adverse impact on farmed animals is eventually emphasized, with only a few conducted studies so far investigating the topic.

6 Discussion

In shellfish aquaculture, ascidian species represent a severe obstacle to bivalve productivity, as they compete with the farmed species for food resources adding weight to the infrastructure, leading to a smaller final size and a higher mortality percentage [143]. Knowledge regarding the presence and biological cycles of the fouling ascidian species is very important for their successful management, and even more important in cases where the seasonality of the fouling species synchronizes with the seasonality of the farmed species [144], while an increase in seawater temperature can contribute to organisms' invasions [145] which is often the case in bivalve farming.

We can highlight that enlightening the knowledge concerning the dispersal potential of ascidian species is of main importance and necessary for the design of appropriate management practices. For instance, apart from the already known non-indigenous species that negatively impact Greek farmed bivalves, there are records of NIAs species from Turkish and Italian coasts, as well as from other marine areas within the Mediterranean Sea. Thus, probably, it is a matter of time to spread into neighboring waters as well. By understanding the key factors behind their invasion, appropriate measurements can be implemented. Many of the main NIA species have also been observed in Greek locations; however, some of them remained undetected. Additionally, a lot of attention should be paid to ascidian species that are predicted to spread into the Mediterranean Sea, with some of them being characterized by high risk of introduction and establishment (i.e., *A. sydneiensis*, *B. violaceus*, and *D. perlucidum*).

Apart from the observational impacts of the ascidian species on shellfish aquaculture, i.e., increased weight on the ropes of the long line system or the nets and physical damage [18,146], there is evidence that they cause a decrease in the overall shellfish fitness [99,127]. Until recently, apart from limited information [121], scarce data were available regarding the impact of fouling species on fouled organisms

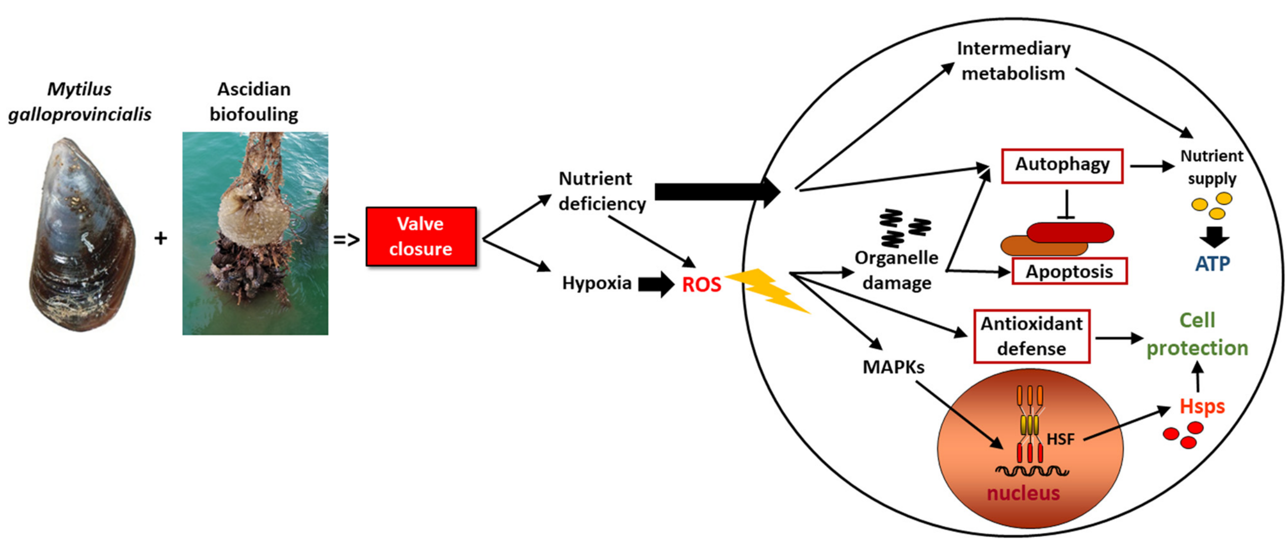

Figure 2: A model of the cellular responses elicited by the effect of ascidian biofouling on *Mytilus galloprovincialis*.

on molecular and cellular levels. Recently, it has been observed that in fouled organisms, cell death is more likely triggered by biofouling-related stress rather than from abiotic stress, such as temperature increase [127], and that ascidian biofouling causes starvation stress, therefore triggering several cell protective and signaling pathways in fouled mussels [99].

Although the molecular and cellular mechanisms of the fouled organisms underlying the biofouling phenomenon are still under investigation, the abiotic parameters with special regard to environmental conditions cannot be overlooked. Climate change can operate as a threat toward shellfish production as it may favor ascidian invasions or their increased densities. More specifically, temperature is among the most important abiotic parameters affecting the distribution of the world's biota [147], while an increase in seawater temperature can contribute to organisms' invasions [148]. As observed from *H. momus* populations, invasive individuals had developed resistance toward higher temperatures in comparison with the native ones [58]. Investigation of epigenetic variations can shed some light on the mechanisms underlying the above adaptations to the introduced environment [149]. More specifically, DNA methylation variations were correlated with environmental factors such as temperature and salinity in the highly invasive ascidian species, *C. robusta* and *D. vexillum*, implying that these changes can be involved in rapid local adaptations [150,151].

As the impact of climate change on the invasion of ascidian species is well recognized, management strategies are of high importance not only for the protection of biodiversity but for the protection of the bivalve farming sector as well [151]. More specifically, the temperature increase that is expected during the next decades is predicted to cause dramatic changes in both ecosystem structure and function at a global scale. The climate crisis can alter the composition of the biota resident in a specific region by enhancing the spread of invasive species. The temperature increase has a strong influence on ascidian dispersion potential [43,48]. More specifically, following the temperature increase, many ascidian species have expanded their distribution range in regions where they were too cold for their successful establishment [57]. Furthermore, their increased metabolic and reproductive rate according to increased temperature can lead to higher population densities [127]. The alterations in the habitat structure can also contribute to ascidians by eliminating predators or by altering sea currents. There are plenty of treatment methods that have been tested in the past. These methods include organic acids and bases application [152], pressure washing [153], washing with freshwater [154], addition of a culture medium [155], application of silicone release coatings [156], air exposure [157], manual removal of ascidians [158], heat exposure [159], and biocontrol [160]. However, the combination of more than one treatment method is probably more effective while the treatment timing was found to play a crucial role toward successful treatment [153,159]. More attention should be paid toward treatment practices, as in some cases the beneficial impact on farm productivity or farmed organism's fitness can be more detrimental to cultured shellfish than biofouling removing [126]. Further, apart from the natural compounds that can be used as antifoulants [161], selective breeding can lead to genetic improvement of broodstock which can be resistant toward biofouling [162], whereas simple eco-friendly methods such as aeration and pressure are still applied with high efficiency [163].

One very promising sector rising is the use of ascidian substances to improve human life quality. A number of studies highlight the useful properties of ascidian bioactive compounds [28,164]. Furthermore, toxins produced from ascidian species have been found to exhibit potential toward drug development [165]. Additionally, several ascidian-derived metabolites exhibit antimicrobial properties [166]. Consequently, apart from being a major threat to the shellfish farming sector, bio-invasive ascidian species can operate as a source of high-added value products due to their previously mentioned properties [167].

7 Conclusions and future research directions

The present study follows the above main pathways to reach some conclusions and to summarize some substantial information: first of all, the determination of the main factors contributing to the successful dispersal and invasion of fouling organisms is of critical importance. Second, the gathering of the main NIS in the Mediterranean Sea in combination with the existing knowledge of biological characteristics is the first step toward the development of proper managemental practices. Finally, the investigation of the physiological factors by which fouling organisms cause stress on farmed animals can pause specific targets for mitigating the phenomenon.

In conclusion, biofouling constitutes a serious problem for shellfish aquaculture. There is evidence that ascidians cause stress in fouled organisms at many different levels of biological organization, from the molecular and cellular levels to the organismal and population ones. In the biofouling equation, an important variable is the environmental conditions, which cause additional stress to the cultured organisms. Further, in line with climate change, the invasive ascidian species introduced in bivalve farming

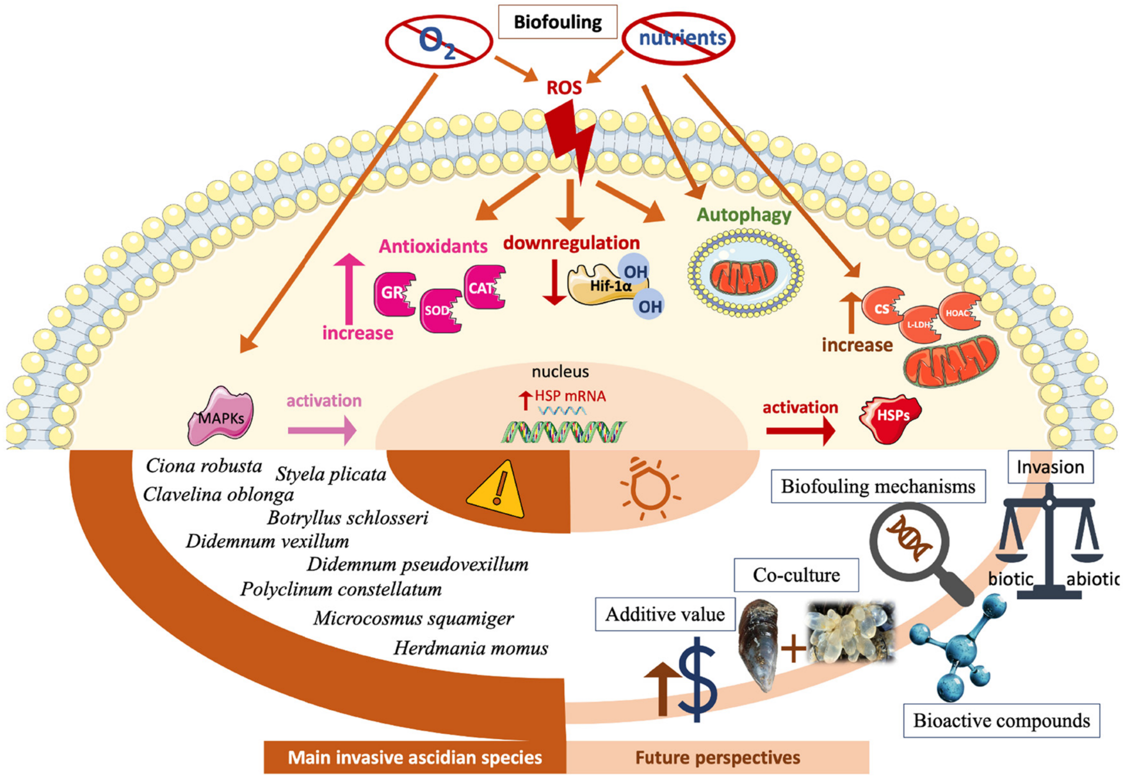

Figure 3: Schematic depiction of the overall stress of ascidian biofouling on *Mytilus galloprovincialis* in combination with the main NIS in Mediterranean and some future perspectives that will provide solutions to management efforts.

areas are increasing. To eradicate the problem, suitable management practices should be implemented, taking into consideration the impact of these practices on cultured bivalves and the balance toward the substantial cost of fouling removal. A rising sector with promising results is the use of bio-invasive ascidians as a source of added value to farmed products due to its bioactive compounds and metabolites that have antimicrobial and antitumoral properties under the scope of drug development (Figure 3).

Despite the plethora of research conducted so far concerning aspects such as species distribution, management strategies, and economic effects on cultured organisms, particular future research goals may provide new perspectives and alternatives in the proper management and control of ascidian biofouling organisms. Based on the aspects analyzed in the present study, we therefore determined and proposed five specific future research goals for biofouling attributed to ascidians.

i. Although the role of shipping and human transportation in the spread of NIA species is well documented, evaluation of the precise biotic and abiotic factors that lead to the successful establishment of non-indigenous species is crucial. These factors should be emphasized in the different geographic locations taking into consideration the specific oceanographic features of each marine area. Further, ascidian species represent an example of which, although extensive knowledge exists, mainly regarding its ecology, adhesion capability, and surface selection, limited information has been implied toward the development of antifouling surfaces [167].

ii. Ascidian species have been indicated as potential carriers of beneficial food and pharmaceutical ingredients. Thus, in light of the “omics” era, future research should investigate qualitatively and quantitatively the major metabolites that are present in the different ascidian taxa. More specifically, many natural products have been isolated from ascidian species, among the most important being cellulose, which can be found almost exclusively in the ascidian’s tunic. Regarding fatty acids, ascidians can be a good source of n-3 polyunsaturated fatty acids, such as EPA and DHA. Additionally, the inner body is protein rich, which amino-acid composition linked to egg albumin. Further, many other compounds with antifungal, antibacterial, anti-inflammatory, and antitumoral properties such as cyclic peptides, alkaloids, polypeptides, sulfated polysaccharides collagens, sterols, and glycosaminoglycans can be used as by-products. The above-proposed ascidian substances as a good alternative in aquafeeds formulation and in applications in fishing bait, health supplement tablets, as well as in the pharmaceutical and chemical industry [168].

iii. Based on the above, polyculture systems with the simultaneous rearing of low-value bivalves and ascidian for production may be a promising perspective that should be targeted in future studies [169]. Particularly, bivalves that are either of lower economic value or characterized by very large populations may serve as the basis for rearing beneficial ascidians. Apart from that, assuming that in some cases, fouling cannot be avoided due to increased cost, the synchronization of husbandry practices along with fouling patterns may be a possible solution [121,126]. Further, an integrated multitrophic aquaculture system consisting of the ascidian *Styela clava* and the sea cucumber *Apostichopus japonicus* with microalgae was developed to mutually benefit both organisms [170].

iv. So far, research has concluded to the fact that biofouling is responsible for the low productivity of bivalve farms. On the contrary, as described in Section 4, low-productivity mussels may attract more ascidians. Hence, keeping in mind that ascidians foul only partially the mussel lines, ascidian species on mussel farms may serve as the means for selection of higher welfare and fitness mussels, which may perform better against various factors such as seawater temperature and oxygen availability.

v. Finally, since our knowledge concerning the precise pathways of the physiological stress caused by ascidians on bivalves is only based on very few recent studies, further research should be conducted, utilizing novel technologies such as transcriptomics and epigenetics toward the estimation of the bivalves' response against biofouling, in both lad and field conditions.

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