Antibacterial activity of marine macroalgae against fish pathogenic *Vibrio* species

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Abstract: In mariculture, diseases of microbial origin can cause significant economic losses worldwide; the evolution of microorganism resistance to antibiotics has resulted in a growing need for new antibacterial compounds that are effective in veterinary medicine and characterized by limited undesirable side effects. Increased attention has recently been turned to seaweeds as a promising source for metabolites with antimicrobial activity. *Vibriosis* is a common disease, caused by bacteria of the genus *Vibrio*, that can result in high mortality in aquaculture. The aim of this study was to identify seaweeds with antibacterial activity against some pathogenic *Vibrio* species, in order to identify a possible alternative to the commonly used antibiotics in aquaculture. Chloroform/methanol lipidal extracts of six seaweed species (*Chaetomorpha linum*, *Cladophora rupestris*, *Gracilaria dura*, *Gracilaria gracilis*, *Gracilariopsis longissima*, *Ulva prolifera*) were tested for their antibacterial activities against six fish pathogenic *Vibrio* species using the disc diffusion method. Different susceptibilities to lipidal algae extracts were observed. All six of the seaweed extracts tested demonstrated inhibition of *Vibrio ordalii*. The best was that from *Gracilariopsis longissima*, showing activity against *Vibrio ordalii*, *Vibrio salmonicida*, *Vibrio alginolyticus* and *Vibrio vulnificus*. The results confirmed the potential use of seaweed extracts as a source of antibacterial compounds or as a health-promoting feed for aquaculture.

Keywords: Antibacterial compounds • Mar Piccolo of Taranto • Mediterranean Sea • Seaweeds • *Vibrio* spp.

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1. Introduction

In aquaculture, diseases of microbial origin cause high mortality rates and lesions on fish skin, with consequent economic losses worldwide [1]. Bacteria, mainly of the genus *Vibrio*, such as *Listonella anguillarum* (formerly *Vibrio anguillarum*), *V. ordalii*, *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. salmonicida*, have been identified as the etiological agents responsible for the most common disease outbreaks in fish and shellfish, called vibriosis [1-3]. Moreover, these microorganisms can accumulate in the reared animal’s flesh and become a serious threat for human health. For example, *Vibrio vulnificus* is considered as one of the worst foodborne pathogen, mainly causing gastroenteritis. It is often present, together with *V. parahaemolyticus*, in the edible *Mytilus galloprovincialis* from the Italian Apulian farms [4,5].

The use of commercial antibiotics for disease treatment produces undesirable side effects, including toxicity to the reared organisms and the release of chemical residues into the environment. These chemical residues can then pose risk to the animal and human health [6]. In addition, it was reported that some *Vibrio* pathogenic strains, including *Vibrio harveyi*, *Vibrio parahaemolyticus* and *Vibrio splendidus*, are resistant to several antibiotics in common use [7]. This has led to a growing need for new antibacterial substances that can be effective in veterinary medicine and characterized by limited undesirable side effects [8].

Marine organisms are a rich source of structurally novel and biologically active metabolites [9,10]. Many of these marine compounds, which exhibit a range
of different activities, have been isolated and some of them have been tested for potential use as new pharmaceuticals [10,11]. For centuries, many seaweeds have been used in folk medicine due to their therapeutic potential for the treatment of a variety of diseases [12]. Several studies have shown that secondary and some primary metabolites from green, brown and red marine algae may be potential bioactive compounds of interest for the pharmacological industry, on account of their in vitro capabilities of inhibiting bacteria, viruses, fungi and other epibionts (e.g. cytostatic, antiviral, antihelmintic, antibacterial, antifungal activity) [12]. The antimicrobial compounds derived from seaweeds consist of diverse groups of chemicals such as macrolides, cyclic peptides, proteins, polyketides, sesquiterpenes, terpenes and fatty acids [13], which have been shown to have antibacterial activity against both Gram-positive and Gram-negative bacteria [14]. These activities are dependent on many factors, such as the species of seaweed, the region of the thallus, the microorganisms, the season and the growth conditions [15-17]. Reports on the inhibition of human pathogens by algal extracts are more numerous than those on the effects against fish pathogenic bacteria, which are also more recent [8,18,19].

In this study we aimed to investigate the antibacterial activities of the lipidic extracts of six marine macroalgae (i.e. Chaetomorpha linum, Cladophora rupestris, Gracilaria dura, Gracilaria gracilis, Gracilariopsis longissima, Ulva prolifera) against a set of well known fish pathogenic Vibrio species such as Vibrio ordalii, Vibrio salmonicida, Vibrio alginolyticus, Vibrio splendidus, Vibrio harveyi and Vibrio vulnificus. These pathogens are widely distributed in a variety of economically important warm and cold water fish and seafood species and are causative of severe financial loss to the aquaculture industry.

2. Experimental Procedures

2.1 Study site and species collection
The six seaweed species (Table 1) were collected in the Mar Piccolo of Taranto: a semi-enclosed Transitional Water System located to the north of Taranto (Southern Italy, Ionian Sea, Mediterranean Sea) (Figure 1). The basin is divided into two smaller inlets named the First and the Second Inlet with a maximum depth of 12 m and 8 m, respectively. Mobile substrates are sandy near the coast and muddy in the central zone; hard substrata are mainly artificial, due to the high anthropogenic pressure.

Seaweeds were collected in 2010 and 2011, during the season of maximum growth of each species, from three sampling stations sharing the same environmental features.

For unattached species, each year three replicates consisting of about 500 g of fresh material were harvested by a rake at a maximum depth of 50 cm; for lower depths SCUBA diving was necessary. For attached

<table>
<thead>
<tr>
<th>Rhodophyta</th>
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<tr>
<td>Gracilaria dura (C. Agardh) J. Agardh</td>
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<tr>
<td>Gracilaria gracilis (Stackhouse) Steentoft, L.M. Irvine et Farnham</td>
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<td>Gracilariopsis longissima (S.G. Gmelin) Steentoft, L.M. Irvine et Farnham</td>
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<th>Chlorophyta</th>
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<tr>
<td>Chaetomorpha linum (O.F. Müller) Kützing</td>
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<td>Cladophora rupestris (Linnaeus) Kützing</td>
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<td>Ulva prolifera O.F. Müller</td>
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Table 1. Species of seaweeds from the Mar Piccolo of Taranto used for extraction and assay tests.

Figure 1. Map of the Mar Piccolo of Taranto. (●) indicates the sampling stations.
species, 3 thalli were collected by SCUBA diving each year for each station. All the collected material was then taken to the laboratory to be processed.

2.2 Studied species
Among the studied species, 3 were Rhodophyta and 3 were Chlorophyta (Table 1). *Gracilaria dura* (Gracilariales, Gracilariaceae) was collected in the unattached seaweed community, at a depth of about 5 m. *Gracilaria gracilis* and *Gracilariopsis longissima* (Gracilariales, Gracilariaceae), both present as unattached thalli dominant in a mixed population, were collected at a depth of about 70 cm. Once in the laboratory, the two species were separated on the basis of morphological characteristics, according to [20].

*Chaetomorpha linum* (Cladophorales, Cladophoraceae) is the dominant species throughout the year in the unattached seaweed populations in the Mar Piccolo, where it forms “mattresses” up to 50 cm thick, densely colonised by a wide variety of invertebrates. It was collected at a depth of about 50 cm. The species was identified according to [21]. *Cladophora rupestris* (Cladophorales, Cladophoraceae), was collected as attached thalli, at a depth of about 1 m. The species was identified according to [21]. *Ulva prolifera* (Ulvales, Ulvaceae) was collected in the attached form in the Second Inlet at a depth of 30 cm, in a zone characterised by the freshwater input of a small river. On the basis of morphological features, the species was identified according to [22].

2.3 Test organisms
The strains of *Vibrio ordalii*, *Vibrio salmonicida*, *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio harveyi* and *Vibrio vulnificus* were isolated and identified from seawater samples of the Mar Piccolo of Taranto as previously reported in [4].

2.4 Preparation of lipidic extracts from macroalgae
Algae samples were cleaned of epiphytes and other marine organisms with a mixture of ethanol and sodium hypochlorite [23] and necrotic parts were removed. The samples were furtherly rinsed with sterile water to remove any associated debris. The freshly cleaned material was air-dried and powdered, then 3 g of each sample was extracted in 150 mL of chloroform/methanol (2:1 at 55-60°C for 24 h) using a soxhlet apparatus. Extraction solvents were evaporated under vacuum at controlled temperature. 5 milligrams of extract was then dissolved in 1 mL of ethanol and assayed for antimicrobial activity by paper disc diffusion method [24].

2.5 Antimicrobial activity
Antimicrobial activity was evaluated using the Kirby Bauer method [25]. Sterile paper discs, 7 mm in diameter (AA; Whatman International Ltd., Maidstone, Kent, UK), were impregnated with 10, 20, 30, 40, 60, 80, 100 µL of each extract (corresponding to 50, 100, 150, 200, 300, 400, 500 µg, respectively) and left to air-dry at room temperature for 4 h. To verify the possibility that antibiotic activity could be affected by residues of the solvents used for extraction (MeOH/CHCl₃, chloroform anhydrous >99% with ethanol as stabilizer, methanol >99.8%; both by Sigma–Aldrich), discs impregnated only with 100 µL of solvent were used as a negative control; the positive control was represented by the vibriostatic agent O/129 at a concentration of 10 µg.

For each assay, autoclaved marine agar plates were seeded with 100 µL of test bacteria suspension (about 10⁶ CFU mL⁻¹) [9,26], using a sterile swab to give a uniform covering. Impregnated discs and controls were laid onto the agar surface and the plates were then incubated for 24 h at 30°C. The clear zone around the discs was evidence of antibacterial activity (Figure 2). The diameter of the microbial growth inhibition was taken as the diameter of the clear zone (measured in millimetres). Each test was prepared in triplicate for every bacterial strain.

2.6. Statistical analysis
One way-ANOVA was performed using the statistical package STATSOFT STATISTICA v. 6.0 (Stat-Soft, Inc., USA), to assess significant differences in the inhibition of bacterial growth produced by each alga against each *Vibrio* species.

![Figure 2. Disc diffusion assay. Gracilaria longissima lipidic extract against Vibrio vulnificus (A = Negative control; B = Disc impregnated with algal extract).](image-url)
3. Results

All the six algal species used in this research demonstrated antibacterial activity (Figure 3). In particular, tests performed against *V. salmonicida*, *V. alginolyticus*, and *V. vulnificus* were able to demonstrate this activity using only 10 µL of each algal extract (corresponding to 50 µg of dry extract). The results obtained using 30 µL of each algal extract are summarized in Figure 3. This figure also shows that none of the extracts tested demonstrated inhibition against *Vibrio splendidus* and *V. harveyi*.

Statistical analysis revealed that *Gracilariopsis longissima* had an inhibition activity against both *V. alginolyticus* and *V. vulnificus* significantly higher (P<0.01) than against the other *Vibrio* species and not significantly different from the positive control. This algal species was also active against *V. ordalii* and *V. salmonicida*, although the clear zone of inhibition was less extensive. *Chaetomorpha linum* showed a inhibition zone against *V. vulnificus* not significantly different from both *G. longissima* and the positive control against this strain. The same diameter of inhibition was also recorded for *V. ordalii* and it was comparable to that produced by the positive control. *Cladophora rupestris* showed a significant lower (P<0.01) bioactivity against *V. vulnificus* in comparison with *G. longissima*, *C. linum* and the positive control. The lipidic extract of this algal species was also capable of inhibiting the growth of *V. salmonicida*, and *V. ordalii*. The extract of *Gracilaria dura* demonstrated activity against *V. ordalii* and *V. alginolyticus* with similar diameters of growth inhibition. Finally, the extract of *Gracilaria gracilis* was active only against *V. salmonicida*, while that of *Ulva prolifera* only against *V. ordalii*.

Statistical analysis revealed that the diameters of bacterial inhibition were all similar apart those produced by *G. longissima* and *C. linum* against *V. vulnificus*, by *G. longissima* against *V. alginolyticus* and by *C. linum* against *V. ordalii*, which were significantly higher (P<0.05).

4. Discussion

The main purpose of this work was to evaluate the capability of different seaweed species from the Mar Piccolo of Taranto to inhibit the growth of some species of fish pathogenic *Vibrio* species with the aim of assessing them as possible alternatives to common antibiotics in aquaculture. This study revealed different levels of bioactivity in the chloroform/methanol extracts from the six seaweeds analysed as well as different susceptibilities of the *Vibrio* strains under investigation. *Gracilaria longissima* extract had the broadest antibacterial spectrum; indeed it was active against four *Vibrio* species, although with different inhibition strength. A previous study [27] also demonstrated that the chloroform/methanol lipidic extract of *G. longissima* from the Mar Piccolo of Taranto had bacteriostatic activity against *Vibrio alginolyticus* and *V. vulnificus*.

*Chaetomorpha linum* was second to *G. longissima* in activity, although its extract was active only against two *Vibrio* species, *i.e.* *V. ordalii* and *V. vulnificus*. Chloroform, methanol and ethanol extract of *C. linum* from an Indian lake demonstrated activity against several Gram-positive and Gram-negative bacteria, but not against *Vibrio cholerae* [28]. No other paper on the potential use as a source of bioactive compounds of this species seems to be present in the literature. The positive results obtained in the present study could, therefore, represent a valid basis for the development of the research on *C. linum*, considering that this is
usually a dominant species in the lagoon unattached communities in several world regions [29,30].

Concerning *Cladophora rupestris*, our results showed that its extract exerted bioactivity against three of the tested *Vibrio* species (i.e. *V. ordalii*, *V. salmonicida*, *V. vulnificus*), although with a lower inhibitory power compared to the foregoing species. The solid extract of *C. rupestris* collected in Spanish waters proved to be active against Gram-positive bacteria [31], while the dichloromethane extract of Atlantic French thalli was active against Gram-negative bacteria [32]. No *Vibrio* species were tested in either study. On the other hand, the crude methanol extract of *Cladophora glomerata* from India showed a considerable activity against several species of fish pathogenic *Vibrio* spp. (i.e. *V. fischeri*, *V. vulnificus*, *V. anguillarum*, *V. parahaemolyticus*) [33]. Moreover, the methanol extract of Indian *Cladophora albidia* had a weak/medium activity against *V. harveyi*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. alcaligenes* [34]. Therefore, the antibacterial activity of the chloroform/methanol extract of *C. rupestris* from the Mar Piccolo of Taranto against three of the six *Vibrio* species tested, could confirm a good perspective for the use of the genus *Cladophora* in these new veterinary medicine applications. These results provide encouragement for continuation of research on species of this genus in order to verify their antibacterial activity against *Vibrio* spp with different extraction methods.

In the present study, *Gracilaria dura* extracts demonstrated activity against two of the *Vibrio* species tested. Finally, the less active extracts were those of *Gracilaria gracilis* and *Ulva prolifera* which were effective only against one *Vibrio* species. It is well established that antimicrobial activity varies among species belonging to the same genus [35,36]. Indeed, concerning the *Gracilaria* genus, there is considerable literature regarding their bioactive content [37 and references therein], but the toxicity levels depend on the seaweed species, the microbe species and the extraction method used. For example, the ethanol extract of *Gracilaria corticata* from India proved to be highly active against *Vibrio cholerae* and *V. parahaemolyticus* but lesser against *Pseudomonas aeruginosa* and *Shigella flexneri* [38]. *Gracilaria fisheri* was studied for its antimicrobial activity by [39], who reported that the ethanol, the hexane, the chloroform and the methanol extract all possessed potent antimicrobial activity against *V. harveyi*, but the ethanol extract resulted the greatest activity with the lowest concentration. In Iberian thalli of *G. dura*, the solid extract obtained by pounding the fresh material, without any solvent, showed a low-medium activity against Gram-positive and Gram-negative bacteria and yeasts, but it was not tested against any *Vibrio* species [31]. Among the extracts of fresh *G. gracilis* from Turkish coast produced using different solvents, the extract obtained with diethyl ether gave the best results against yeasts, Gram-positive and Gram-negative bacteria. Neither in this case were *Vibrio* species tested [40]. Therefore, the presence of bioactivity against *Vibrio* spp. in the thalli of both *G. dura* and *G. gracilis* from the Mar Piccolo of Taranto widens their known antibacterial spectrum.

The extract of *Ulva prolifera* (as *Enteromorpha prolifera*) from Alexandria harbour in Egypt, obtained with methanol and acetone, was shown to be active against several Gram-positive and Gram-negative bacteria, but it was not tested against any *Vibrio* species [41]. The extract of the same species obtained with ethanol 95% in Korea exhibited a good antioxidant activity which made it a good candidate for the production of health-care products [42]. This could stimulate research into other applications for this species, also from the Mar Piccolo of Taranto.

Concerning the *Vibrio* species tested in this study, *V. ordalii* was shown to be the most susceptible to the extracts of all the seaweeds analysed, except for *G. gracilis*. *Vibrio salmonicida*, *V. alginolyticus* and *V. vulnificus* were inhibited by the extract of only some of the seaweeds tested. On the contrary, *V. splendidus* and *V. harveyi* were not inhibited by any seaweed extract from the Mar Piccolo of Taranto.

Since the aim of this paper aim was to identify seaweed extracts active against fish pathogenic *Vibrio* spp., we examined the available literature on this subject. Several species of *Vibrio* fish pathogens were tested worldwide with seaweed extract in order to find new bioactive molecules against fish and crustacean diseases in aquaculture plants. However, scarce information are present to date about seaweed bioactivities specifically against the species of *Vibrio* examined in this study.

*Vibrio alginolyticus* was shown to be inhibited by the methanolic extract of the Rhodophyta *Gracilaria corticata* in India [43] and *Ceramium rubrum* in Germany [44]. This could indicate that methanol is a suitable method for extracting a specific group of substances present in this division. Since the solvents used in our work were able to extract lipids, we suppose that different lipophilic compounds may exhibit antimicrobial activity as also demonstrated from other species of macroalgae [17].

Different strains of *V. harveyi* and *V. vulnificus* were weakly inhibited by the ethanol extract of *Asparagopsis taxiformis* from the Sicily Channel [45]. Ethanol was also used for thalli of *Gracilaria fisheri* from India, the extract of which showed a high inhibition against *V. harveyi* [39]. Since the chlorophorm/methanol extracts of the Mar
Piccolo of Taranto seaweeds were not effective against *V. harveyi*, we suppose that in this case the solvent used for the extraction was not suitable for extracting the antimicrobial compounds.

5. Conclusions

Our results showed that the investigated seaweeds from the Mar Piccolo of Taranto are a potential source of antibacterial compounds which could be used for the prevention and treatment of fish diseases due to *Vibrio* species, either as an adjunct to classical antibiotics or to completely substitute them. In particular, the best candidates are *G. longissima* and *C. linum*, the chloroform/methanol extract of which was active against *V. alginolyticus, V. vulnificus* and *V. ordalii*.

Moreover, all the investigated seaweeds could be a potential source of biologically active compounds: in fact ongoing studies on lipids from some of them have already shown potential for use as an enrichment and/or innovative fodder for fish nourishment in aquaculture plants.

Acknowledgements

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Seaweed antibacterial activity against Vibrio spp.


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