

Dual effect of macroalgal extracts on growth of bacteria in Western Baltic Sea

Dualidad en el efecto de extractos macroalgales del mar Báltico occidental
sobre el crecimiento bacteriano

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Resumen. - Se asume que las características biológicas de las cepas bacterianas utilizadas para test de bioactividad tienen fuerte influencia sobre la susceptibilidad contra compuestos antibacterianos. Por lo tanto, la selección de bacterias para pruebas microbiológicas puede acelerar conclusiones respecto al efecto de extractos y metabolitos macroalgales sobre el crecimiento microbiano. Para probar tal presunción, se analizó la actividad biológica de extractos crudos de 16 macroalgas del fiordo de Kiel (Alemania). Los extractos macroalgales fueron probados frente a un set de 10 microorganismos que involucraron 5 cepas bacterianas de común uso en análisis estándar y 5 cepas de bacterias asociadas a macroalgas. Catorce macroalgas (88%) presentaron actividad antibiótica contra al menos una cepa. Pese a la alta proporción de extractos activos, sólo 3 cepas estándar fueron susceptibles a ellos y la actividad antibiótica fue baja (menos de 80% de inhibición). La mayoría de los extractos inhibió a *Bacillus subtilis*, mientras que no hubo actividad inhibitoria frente a *Erwinia amylovora*, *Escherichia coli* y las cepas macroalga-asociadas. Por el contrario, la totalidad de extractos estimuló el crecimiento de al menos 2 cepas bacterianas. Mientras que la estimulación del crecimiento sobre las cepas de uso estándar fue rara (22,5%) con la excepción del fitopatógeno *Erwinia amylovora* y 2 casos con *Staphylococcus lentus*, fue común frente a las bacterias asociadas a macroalgas (77,5%), especialmente frente a *Bacillus algicola*, *Pseudomonas marincola* y los dos patógenos de algas. El presente estudio demostró que los extractos macroalgales pueden producir diferentes efectos tal como la inhibición o estimulación del crecimiento bacteriano, dependiendo del origen de las bacterias empleadas para los test, ya sea por el uso de un set de cepas estándar o provenientes del ambiente marino, respectivamente.

Palabras clave: Algas marinas, actividad antibacteriana, estimulación del crecimiento bacteriano, fiordo de Kiel

Abstract. - It is assumed that the biological characteristics of the bacterial strains used in bioactivity tests have strong influences on their susceptibility against antibacterial compounds. Therefore, the selection of bacterial test strains may rush conclusions on the effect of macroalgal extracts and metabolites on bacteria. To proof this assumption, we have analysed the biological activities of crude extracts of 16 macroalgae from the coastal waters of Kiel Fjord (Germany), and tested their effect against a panel of 10 microorganisms comprising 5 standard test strains of bacteria and 5 macroalga-associated bacteria. Fourteen macroalgae (88%) displayed antibacterial activity against at least one of the test strains. Despite the high proportion of extracts exhibiting antimicrobial activity, only 3 strains of the standard set were susceptible to macroalgal extracts and the overall activities were low (less than 80% of inhibition). Most of active extracts inhibited *Bacillus subtilis*, while no inhibition effects were found against *Erwinia amylovora*, *Escherichia coli*, and the macroalga-associated bacteria. In contrast, all extracts produced stimulatory growth effects of at least two of the tested bacteria. While growth stimulation of standard set of bacteria was rare (22.5% of total tests) with exception of plant pathogen *Erwinia amylovora* and two cases of *Staphylococcus lentus*, it was common among bacteria associated with macroalgae (77.5%), especially *Bacillus algicola*, *Pseudomonas marincola* and both algal-pathogenic bacteria. This study demonstrates that macroalgal extracts can display different effects, i.e., inhibition or stimulation of bacterial growth depending on the origin of the test strains, which are derived from a standard panel or from the marine environment, respectively.

Key words: Marine algae, antibacterial activities, stimulation of bacterial growth, Kiel fjord

INTRODUCTION

Secondary metabolites mediate numerous biological interactions and play a particular important role in mediating host-microbe associations in the ocean (Lane *et al.* 2010). Recently, it has been addressed that chemical interactions regulate the bacteria-macroalga relationships and may cause specific associations (Goecke *et al.* 2010, Sneed & Pohnert 2011a). Indeed, different species of marine macroalgae growing in the same habitat under the same environmental conditions support different bacterial communities (Lachnit *et al.* 2009, Nylund *et al.* 2010). The biological active compounds (both deterrents and attractants) produced by macroalgae as well as those from the associated bacteria may be involved in shaping these epiphytic bacterial communities (Egan *et al.* 2000, Lachnit *et al.* 2010).

Macroalgal chemistry is rich and diverse, spanning most natural product classes and including functional group characteristics found from no other source (Maschek & Baker 2008). The antibacterial activity of different extracts of macroalgae from almost all groups has been described in many studies around the world (Sridhar & Vidyavathi 1991, Hellio *et al.* 2000, Magallanes *et al.* 2003, Freile-Pelegrin & Morales 2004, Engel *et al.* 2006, Lane *et al.* 2009, Muñoz-Ochoa *et al.* 2010). Although several metabolites with antimicrobial activities have been already characterized from macroalgae, results of different studies on antibacterial activities of crude extracts of one and the same species are equivocal: While some studies reported antibacterial activities others did not (Sandsdalen *et al.* 2003).

Biotic factors such as reproductive state, age of the thallus of the macroalgae, as well as abiotic factors such as seasonality and geographic location have influence on the bioactivity of macroalgal extracts (Hellio *et al.* 2004, Paul & Puglisi 2004, Arunkumar *et al.* 2010). Furthermore there are discrepancies related to the different extraction procedures and to the target microorganisms used in the bioassays (Sridhar & Vidyavathi 1991, Kanagasabhapathy *et al.* 2006). Unfortunately, assessments of the antibacterial activity exhibited by macroalgae in the natural products literature has focused on biomedically-relevant strains, using standard microbial strains from terrestrial origin or of medical relevance (Engel *et al.* 2006, Paul *et al.* 2006, Hughes & Fenical 2011). Bacteria from the marine habitat were rarely included, although bioactivity against these would make possible to draw conclusions in regard to the ecological role of the substances in the macroalga-bacteria interactions

(Jormalainen & Honkanen 2008). It is expected that macroalgae should produce antibacterial compounds against microbial pathogens and that commensal bacteria should be adapted to grow within the algal phycosphere and its metabolites. Hence, the selection of bacteria for a test panel should be relevant in order to evaluate the ecological effects of secondary metabolites due to different responses of the chosen bacteria.

In order to prove if a selection of ecologically relevant bacteria results in different responses in the bioactivity tests as compared to a standard set of bacteria, we studied the effect of extracts from different macroalgae of the Kiel Fjord, Germany, upon bacterial growth.

MATERIALS AND METHODS

SAMPLING OF THE MACROALGAE

Samples of 16 species belonging to 11 families of marine macroalgae were taken from distinct sites in the Kiel Fjord, Western Baltic Sea, Germany (54°25.5'N, 10°12'E) (Table 1). The macroalgae were collected between 1 to 6 m depth. Until processing within 3 h after collection, the samples were stored in the dark at ambient seawater temperature using coolers. In the laboratory, the macroalgae were manually cleaned from sand, epiphytes and animals, and rinsed with sterile and filtered Baltic Sea water to remove associated debris, planktonic and loosely attached microorganisms.

Part of the macroalgae was fixed in 4% formaldehyde for its taxonomic identification. Algae were identified by examination of their thallus architecture and special morphological characters: Fronds, branching, and reproductive structures (Pankow 1971, Maggs & Hommersand 1993). For the filamentous macroalgae histological cuts were performed and observed by light microscopy. The names of the species were used according to Guiry & Guiry (2011). Voucher specimen were deposited in the Herbarium of Museo Nacional de Historia Natural, Santiago, Chile (code SGO).

MACROALGAL EXTRACT PREPARATION

Ten grams of the macroalgae were extracted by immersing them with 200 ml dichloromethane (DCM) at room temperature and shaking them by hand (modified method from Nylund *et al.* 2005). The extracts were centrifuged at 4000 rpm for 10 min and filtered through Whatman 542 filter paper (Freile-Pelegrin & Morales 2004). All extracts were concentrated separately under reduced pressure in

Table 1. List of macroalgae sampled from the Kiel Fjord (Baltic Sea, Germany). Names according to Guiry & Guiry (2011) / Lista de macroalgas del fiordo de Kiel (Mar Báltico, Alemania). Nombres según Guiry & Guiry (2011)

Macroalgal species	Order	Family	Sampling site at Kiel Fjord	Sampling date
Chlorophyta				
<i>Chaetomorpha linum</i> (Müller) Kützinger, 1849	Cladophorales	Cladophoraceae	Strande	21.10.2009
<i>Cladophora rupestris</i> (Linnaeus) Kützinger, 1843	Cladophorales	Cladophoraceae	Strande	21.10.2009
Heterokontophyta				
<i>Fucus serratus</i> Linnaeus, 1753	Fucales	Fucaceae	Stohl	19.01.2010
<i>Fucus vesiculosus</i> Linnaeus, 1753	Fucales	Fucaceae	Bülk	14.07.2009
<i>Halosiphon tomentosus</i> (Lyngbye) Jaasund, 1957	Tilopteridales	Halosiphonaceae	Falkenstein	24.04.2011
<i>Saccharina latissima</i> (Linnaeus) Lane <i>et al.</i> , 2006	Laminariales	Laminariaceae	Tonnenhof	14.07.2009
Rhodophyta				
<i>Bonnemaisonia hamifera</i> (tetrasporoph.) Hariot, 1891	Bonnemaisoniales	Bonnemaisoniaceae	Falkenstein	20.11.2010
<i>Callithamnion corymbosum</i> (Smith) Lyngbye, 1819	Ceramiales	Callithamniaceae	Falkenstein	20.11.2010
<i>Ceramium tenuicorne</i> (Kützinger) Waern, 1952	Ceramiales	Ceramiceae	Strande	24.04.2011
<i>Ceramium virgatum</i> Roth, 1797	Ceramiales	Ceramiceae	Strande	21.10.2009
<i>Dasya baillouviana</i> (Gmelin) Montagne, 1841	Ceramiales	Dasyaceae	Strande	21.10.2009
<i>Delesseria sanguinea</i> (Hudson) Lamouroux, 1813	Ceramiales	Delesseriaceae	Strande	14.07.2009
<i>Dumontia contorta</i> (Gmelin) Ruprecht, 1850	Gigartinales	Dumontiaceae	Strande	01.06.2010
<i>Polysiphonia elongata</i> (Hudson) Sprengel, 1827	Ceramiales	Rhodomelaceae	Strande	21.10.2009
<i>Polysiphonia nigra</i> (Hudson) Batters, 1902	Ceramiales	Rhodomelaceae	Strande	21.10.2009
<i>Rhodomela confervoides</i> Hudson) Silva, 1952	Ceramiales	Rhodomelaceae	Falkenstein	20.11.2010

a Speedvac RVC2-33 (Christ, Germany) until completely dry, weighed and kept at +4°C. For further tests, 1 mg of the solid residue was resuspended in 1 ml methanol.

ANTIMICROBIAL TESTING

The antimicrobial activity of crude extracts of macroalgae was tested against a panel of ten microorganisms comprising standard test strains and macroalga-associated bacteria. The following microorganisms and nutrient media were used:

(i) Five microorganisms usually tested in standard laboratory tests of antibiotic activity (hereafter ‘standard set’): *Erwinia amylovora* DSM 50901 (nutrient medium M1 = 5 g l⁻¹ peptone, 3 g l⁻¹ meat extract in distilled water, pH 7), *Escherichia coli* DSM 498 and *Pseudomonas aeruginosa* DSM 50071 (both nutrient media TSB12 medium = 12 g l⁻¹ Difco tryptic soy broth, 10 g l⁻¹ NaCl, pH 7.2 in distilled water), as Gram-negative strains; and *Bacillus subtilis* DSM 347 and *Staphylococcus lentus* DSM 6672 (both nutrient media: TSB12) as representatives of Gram-positive bacteria. All strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

(ii) Five environmental strains that have been identified to be associated with macroalgae in previous studies (hereafter ‘macroalga-associated set’): iia) three isolates were utilized which were obtained from the surface of Baltic Sea macroalgae *Fucus vesiculosus* and *Delesseria sanguinea*. The sampling procedure for isolating strains of bacteria associated with the macroalgae was performed according to Staufenberger *et al.* (2008). PCR amplifications of 16S rRNA gene and subsequent sequencing were performed at the Institute for Clinical Molecular Biology (University Hospital Schleswig-Holstein, Kiel, Germany). Phylogenetic analysis was performed as described by Heindl *et al.* (2010). 16S rDNA sequences of these strains (isolates AB423f, AB236d and AB251f) were deposited at NCBI under the accession numbers FR775437-FR775439. The isolates affiliated with >99% 16S rRNA gene sequence similarity to other macroalga-associated bacteria the Gram-positive *Bacillus algalicola* KMM 3737^T (AY228462) (grown on TM: 5 g l⁻¹ yeast extract, 1 g l⁻¹ peptone, 30 g l⁻¹ tropic marine sea salt in distilled water), *Paenibacillus lautus* JCM 9073^T (AB073188), and the Gram-negative *Pseudomonas marincola* KMM 3042^T (AB301071) (grown on TSB12); iib) Two Gram-negative bacterial strains identified as

macroalgal pathogens (by Sawabe *et al.* 1998, 2000), *Algicola bacteriolytica* ATCC 700679^T ('red spot disease') and *Pseudoalteromonas elyakovii* ATCC 700519^T ('spot-wounded fronds'), originally isolated in Japan from diseased beds of *Saccharina japonica* (Areschoug) Lane *et al.* (formerly *Laminaria japonica*). Both were purchased from Institute Pasteur CIP (Paris, France), and grown cultivated on nutrient medium TM.

The bioactivity tests were modified according to Schneemann *et al.* (2010). Assay mixtures were prepared by transferring 10 µl aliquots of methanolic solutions of extracts into a sterile 96-well microtiter plate and evaporating the solvent in a vacuum centrifuge. 200 µl overnight cultures of each test strain were diluted to an optical density (OD) of 0.03 determined by spectrophotometry in the corresponding cultivation media (see above). Cultures of the standard set were incubated at +36°C for 5 h under constant shaking at 200 rpm except *E. amylovora* at +28°C. The macroalga-associated strains were cultivated at +28°C for 20 h; and the final OD was

determined. We corrected the natural absorbance of the extract fractions by subtracting initial extract-only blank values from values obtained for treatments according to Lane *et al.* (2009). The tests were performed in 3 replicate treatments. By bacterial set a total of 240 tests were performed (16 macroalgal extracts x 5 bacterial strains x triplicate). The resulting values were compared to those for a positive control (100 µg ml⁻¹ chloramphenicol) and respective negative controls wells: solvent ('no extract') and nutrient medium ('no extract, no solvent') on the same plate.

RESULTS

The antimicrobial assay showed that extracts of 14 from 16 macroalgal species (88% of the total) inhibited at least one of the tested organisms (Fig. 1a, b). Antimicrobial activity was demonstrated in members of the three phylogenetic divisions of macroalgae (Chlorophyta, Heterokontophyta and Rhodophyta, Table 2). Concerning the test organisms, Gram-positive bacteria and in

Table 2. Antibacterial activity and growth stimulation effect of the dichloromethane extracts of macroalga against a standard set of bacteria and a set of macroalga-associated bacteria. MTP: growth stimulation (+) = 20-49%, + = 50-79% ++ = ≥ 80%; 0 = no biological activity; growth inhibition (-) = 20-49%, - = 50-79%, -- = ≥ 80% / Actividad antibacteriana y de estimulación del crecimiento presentada por los diferentes extractos diclorometánicos de las macroalgas frente a un set estándar de cepas y uno de bacterias asociadas a macroalgas. La estimulación en el crecimiento fue designada con (+) = 20-49%, + = 50-79%, ++ = ≥ 80%; 0 = sin actividad biológica; la inhibición del crecimiento bacteriano con (-) = 20-49%, - = 50-79%, -- = ≥ 80%

Macroalga	Standard Set					Macroalga-associated Set				
	<i>B.s.</i>	<i>S.l.</i>	<i>E.a.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>B.a.</i>	<i>P.l.</i>	<i>P.m.</i>	<i>A.b.</i>	<i>P.e.</i>
Chlorophyta										
<i>C. linum</i>	0	(+)	+	0	0	(+)	(+)	+	++	+
<i>C. rupestris</i>	(-)	(+)	(+)	0	0	(+)	0	+	+	(+)
Heterokontophyta										
<i>F. serratus</i>	-	0	(+)	0	0	(+)	0	+	0	0
<i>F. vesiculosus</i>	-	(-)	+	0	0	(+)	0	+	0	+
<i>H. tomentosus</i>	-	0	+	0	0	(+)	(+)	+	(+)	(+)
<i>S. latissima</i>	--	0	(+)	0	0	(+)	0	+	(+)	(+)
Rhodophyta										
<i>B. hamifera</i>	(-)	(-)	+	0	0	(+)	(+)	0	(+)	0
<i>C. corymbosum</i>	-	0	+	0	0	(+)	0	+	(+)	(+)
<i>C. tenuicorne</i>	(-)	0	(+)	0	-	+	0	+	0	(+)
<i>C. virgatum</i>	-	0	(+)	0	0	(+)	0	+	++	(+)
<i>D. baillouiana</i>	-	0	+	0	0	(+)	(+)	(+)	++	++
<i>D. sanguinea</i>	0	0	(+)	0	0	(+)	0	+	++	++
<i>D. contorta</i>	(-)	(-)	+	0	0	(+)	(+)	+	++	+
<i>P. elongata</i>	(-)	0	+	0	0	0	(+)	+	+	0
<i>P. nigra</i>	--	0	(+)	0	0	(+)	0	+	+	+
<i>R. confervoides</i>	-	(-)	(+)	0	0	0	(+)	++	(+)	(+)

Abbreviations / abreviaciones. Bs: *Bacillus subtilis*, Sl: *Staphylococcus lentus*, Ea: *Erwinia amylovora*, Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, Ba: *Bacillus algicola*, Pl: *Paenibacillus lautus*, Pm: *Pseudomonas marincola*, Ab: *Algicola bacteriolytica* y Pe: *Pseudoalteromonas elyakovii*

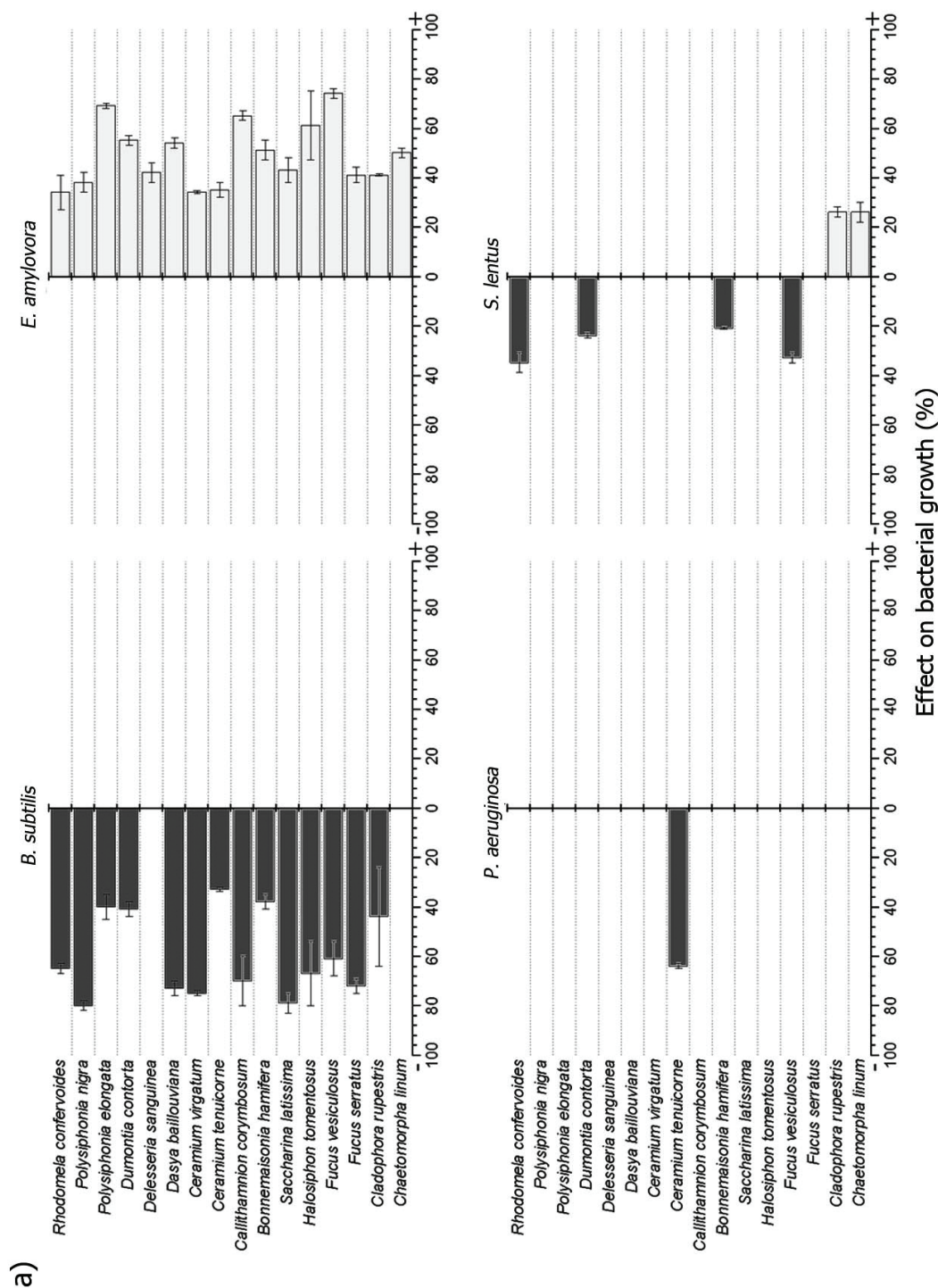
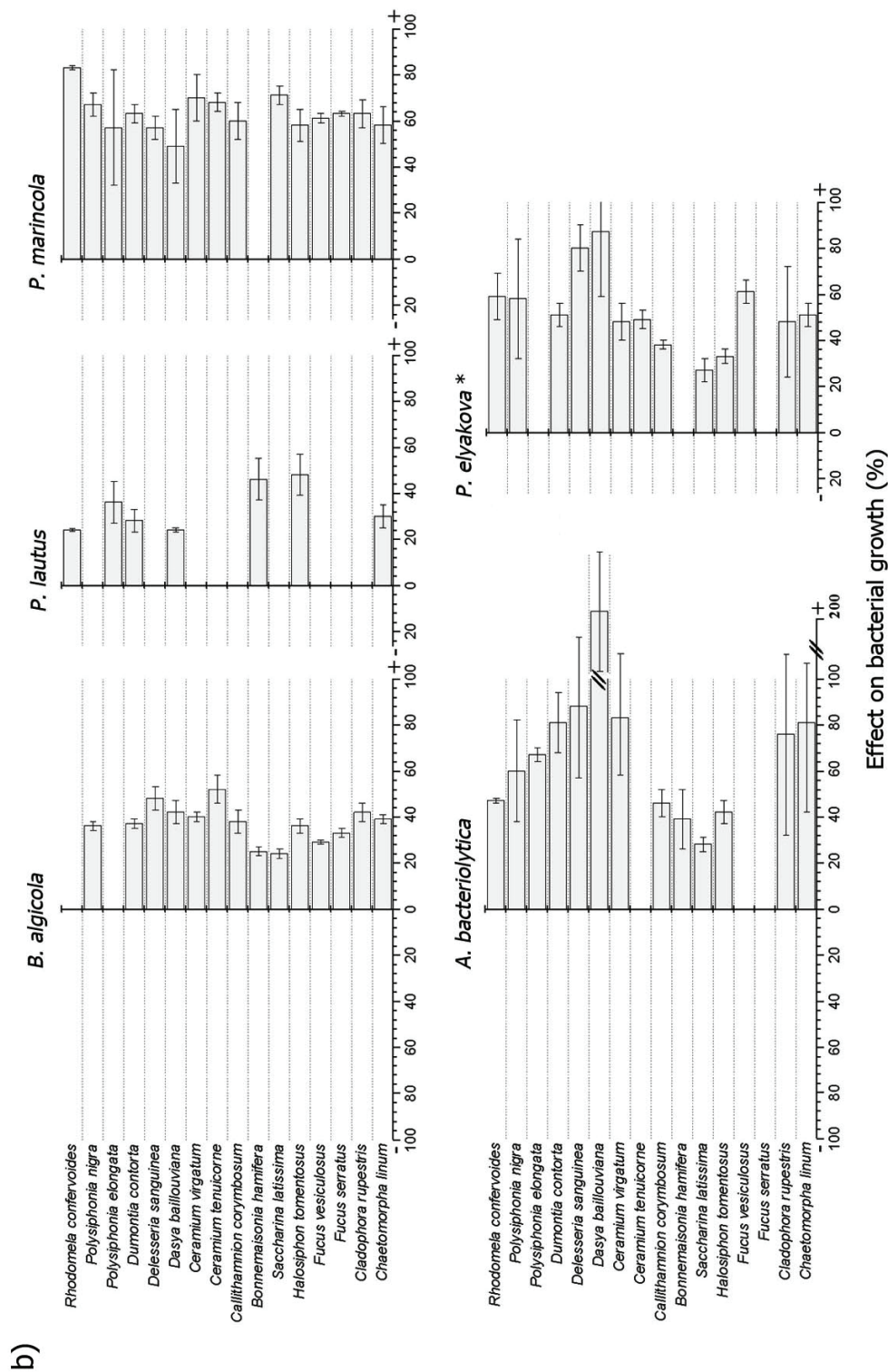


Figure 1. Effect of macroalgal extracts on the growth of 9 microorganisms: a) Standard set of test strains for four of 5 bacteria (*Escherichia coli* is not shown because was not affected by any extract) and b) Macroalga-associated set with 3 surface-associated bacteria and two pathogenic strains (marked with asterisk). Positive values represent enhancement of growth in comparison to the controls (clear bars), and negative values inhibition of growth (dark bars). Average $\pm$ SE (n = 3) values between -20% and +20% were not contemplated / Efecto de los extractos macroalgales sobre el crecimiento de 9 cepas de microorganismos: a) Set estándar para cuatro de las 5 bacterias (*Escherichia coli* no fue contemplada debido a no presentar ninguna variación respecto al control). b) Set macroalga-asociado que incluyó 3 bacterias asociadas a macroalgas locales y 2 cepas patógenas de macroalgas (estas últimas con asterisco). Los valores positivos representan un aumento en el crecimiento (barras claras) en comparación al control negativo, mientras que los valores negativos una inhibición en el crecimiento bacteriano (barras oscuras). Los valores (promedio $\pm$ DS, n = 3) entre -20% y +20% no fueron considerados

Figure 1 Continued / Continuación Figura 1



particular *B. subtilis* were most susceptible (Fig. 2). From the standard set *E. amylovora* and *E. coli* were not inhibited by any extract tested (Table 2, Fig. 2). Also, no extract presented inhibition of the algal pathogens or the other strains of the macroalga-associated set (Fig. 1b). The extracts of *P. nigra* and *S. latissima* presented the strongest inhibitory effect on the bacterial strains with around 80% growth inhibition of *B. subtilis* (Table 2, Fig. 1a). No particular trend in the biological activity was observed by taxonomical macroalgal division (Fig. 3). Although growth inhibitory effects were generally weak (only against 3 bacterial strains and less than 80% of growth inhibition, Fig. 1a), there was a higher proportion of macroalgal species which inhibited the standard test strains (23.8% of the total tests performed) compared to species associated with macroalga (0%, Fig. 2). Only 12% of the algal species did not present any antibiotic activity (Fig. 1, 3).

Interestingly, the present study revealed significant growth stimulation of macroalga-associated bacteria (Table 2, Fig. 1b). The totality of the extracts stimulated the growth of at least two of the tested bacteria (Fig. 3). While growth stimulation of the standard set of bacteria was rare (22.5% of the total tests) - with the exception of plant pathogen *E. amylovora* and just two cases of *S. lentus* (Fig. 2) - it was common among bacteria associated with macroalgae (77.5%), especially *B. algicola* and *P. marincola*. Quite interestingly, also growth of the macroalgal pathogens *A. bacteriolytica* and *P. elyakovii* was stimulated by most of the macroalgal extracts (both 81.3% of the total), and surprisingly none of the macroalgal extracts inhibited these two strains (Figs. 1, 2). The extract of *D. baillouviana* presented the strongest stimulatory growth effects on the bacterial strains with around 200% growth stimulation of *A. bacteriolytica* (Fig. 1b).

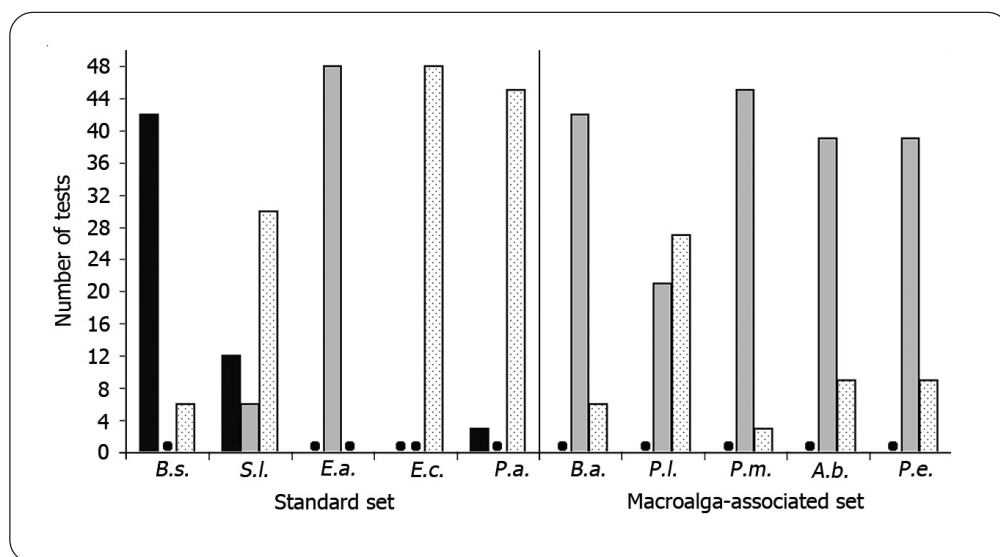


Figure 2. Summary by bacterial strain of the effect on the growth performed with the macroalgal extracts. To the left, standard set and to the right, macroalga-associated set. The bars represent enhancement of growth (grey), inhibition of growth (dark stripes) and no significant variation of growth (points) in comparison to negative controls. Absence of inhibitory/stimulatory activity is designated with a black dot. A total of 48 tests were performed for each strain. See the list of abbreviation in Table 2 / Resumen del efecto en el crecimiento bacteriano según microorganismos producido por los extractos macroalgales investigados. A la izquierda, set estándar y a la derecha, set macroalga-asociado. Las barras representan estimulación del crecimiento (gris), inhibición del crecimiento (negro) y sin efecto significativo (barras con puntos) en comparación a los controles negativos. La ausencia de actividad biológica fue designada con un punto negro. Un total de 48 pruebas fue realizado para cada cepa. Ver lista de abreviaturas en Tabla 2

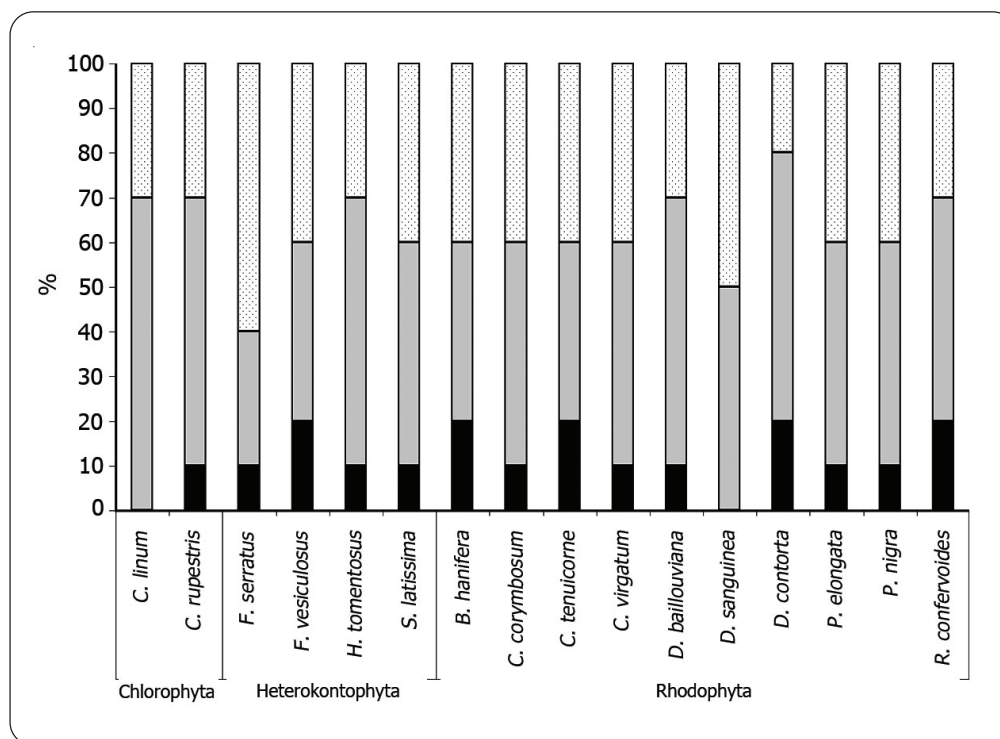


Figure 3. Proportion of growth inhibition (dark stripes), enhancement of growth (grey), and no significant variation of growth (points) of microorganisms exposed to extracts of different macroalgal species / Proporción de la inhibición del crecimiento (barras negras), estimulación del crecimiento (barras grises), y la ausencia de una variación significativa (barras con puntos) en el crecimiento de microorganismos expuestos a los diferentes extractos macroalgales

DISCUSSION

In the majority of prior studies, bacterial growth inhibiting activities of different macroalgal extracts were investigated on human pathogens or standard terrestrial strains (Dubber & Harder 2008). In the present study, we investigated the different effects macroalgal extracts can display over the growth of bacteria especially by testing ecologically relevant microorganisms. Hughes & Fenical (2011) suggested recently that antibacterial activity can only be evaluated in the context of the bacteria strains that are selected. We demonstrated that one macroalgal extract can display different effects on growth of microorganisms especially by testing macroalga-associated bacteria in comparison with a standard set of bacteria.

In the present study, 88% of the macroalgae displayed antibacterial activity against at least one of the test strains. The antibiotic effect was observed only against 3 bacterial

strains from the standard set and less than 80% of inhibition. Extracts of some macroalgal species usually associated with high antimicrobial activity as *Rhodomela confervoides* (Glombitza 1969) and *Bonnemaisonia hamifera* (Nylund *et al.* 2005, Persson *et al.* 2011; Fig. 1a) were active too. *B. subtilis* from the standard set was the most susceptible to the macroalgal extracts (Fig. 2) in accordance with other studies on macroalgae (Freile-Pelegrin & Morales 2004, Dubber & Harder 2008, Sánchez-Saavedra *et al.* 2010).

Just a few studies have investigated the biological activities of macroalgal extracts of the German coasts, and most of them have used standard bacteria. For example, Roos (1957) investigated 27 species of macroalgae of the Kiel Fjord, and tested them against standard strains including *Staphylococcus aureus*, *B. subtilis* and *E. coli*. Most species (82%) were active

against at least one of the tested microorganisms. As in the present study, Roos also found antibacterial activity in *F. serratus*, *F. vesiculosus*, *P. elongata*, *R. confervoides* (as *R. subfusca*), and *S. latissima*. Later, Glombitza (1969) confirmed some of these results during a study with 41 macroalgae from the coastal zone of the Helgoland Island in the German North Sea including *C. rupestris*, *D. contorta* (as *D. incrassata*), *S. latissima*, *F. vesiculosus*, and *R. confervoides*. The author also used standard bacteria in the tests (*i.e.*, *Bacillus cereus*, *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Sarcina lutea*, and *S. aureus*). From the same German island, Duber & Harder (2008) tested macroalgal extracts of *Ceramium rubrum*, *Mastocarpus stellatus* and *Laminaria digitata* against 7 fish pathogenic bacteria and 12 bacteria from marine sediments. Extracts of those 3 macroalgae presented high activity against growth of different strains. In another study, extracts of 4 German North Sea macroalgae (*C. rubrum*, *Hypoglossum hypoglossoides*, *S. latissima*, and *Plocamium cartilagineum*) were tested against 5 fish pathogenic strains, inhibiting all the macroalgae at least one bacterial strain, from which *S. latissima* was active only against *Vibrio anguillarum* (Bansemir *et al.* 2006).

Although these studies clearly showed that macroalgae commonly contain active metabolites with antibacterial properties, it is not known whether the metabolites have an active role within the ecological interactions with their natural enemies (Jormalainen & Honkanen 2008), and/or symbionts. Important variations in the effect of different macroalgal extracts on different marine fouling microorganisms have been shown by Hellio *et al.* (2000, 2001). In a study in the Caribbean Sea, the marine bacterial strains (including *P. elyakovii*) selected were most sensitive to extracts of *Sargassum polyceratum* compared to a standard bacterial strains, suggesting that defence strategies of this brown alga are specific (Thabard *et al.* 2011). Such targeted defence strategies have been described for some algal species before (see Paul & Puglisi 2004).

Different crude extracts tested in the present study have significantly stimulated the growth of bacteria, especially ecologically relevant strains (both surface associated and pathogenic strains, Table 2). One of the extract, of the introduced species *D. baillouviana*, presented the strongest stimulatory effects on the bacterial strains with ~ 200% growth stimulation of the pathogen *A. bacteriolytica* (Fig. 1b). Although several studies have shown stimulation of bacterial growth by algae, unfortunately, they have been based generally on

exudates rather than extracts, and usually of phytoplanktonic sources (see Bell *et al.* 1974, Larsson & Hagström 1979, Brock & Clyne 1984, Murray *et al.* 1986, Coveney & Wetzel 1989). Algal exudates, unknown or partially specified, have been shown to significantly affect the community structure of bacteria in biofilms and in the pelagic zone near the macroalgae (see Dobretsov *et al.* 2006, Lam & Harder 2007, Lachnit *et al.* 2010, Persson *et al.* 2011, Saha *et al.* 2011, Sneed & Pohnert 2011a, b). It is well known that macroalgae release large amounts of organic carbon into the surrounding environment, providing nutrients for microorganisms (Koop *et al.* 1982, Wada *et al.* 2007). Heterotrophic bacteria can directly utilize products excreted by algae as growth substrates (Larsson and Hagström 1979, Brock & Clyne 1984). Excreted compounds may also trigger chemotactic behaviour and stimulate growth (Goecke *et al.* 2010). These compounds are quite selective in their stimulation of bacteria, because different bacteria differ considerably in their ability to respond to these products (Bell *et al.* 1974). The strain-specific preferences for certain substrates and strain specific pro- or antifouling activities of algal metabolites play an important role in establishing ecological associations (Wahl *et al.* 2010). It has been suggested that chemical defences may affect marine communities by promoting some microbes on algal surfaces while deterring others (Lane & Kubanek 2008). However, after more than 20 yrs of research on this topic, there is still no experimental evidence demonstrating if or how host organisms selectively attract and harbour their epibionts (Harder 2009); especially because the studies focussed on inhibitory activities of extracts or metabolites and rarely were concerned with stimulatory effects on growth of the microorganisms.

The bacterial growth stimulatory effect may have different explanations. As mentioned recently, those organic extracts and compounds may rather resemble dead algal material available for microbial degradation (Bengtsson *et al.* 2011). Nevertheless, the possibility of the presence of certain algal substances that specifically stimulate a selected array of species or even strains has also been suggested recently (see Sneed & Pohnert 2011b).

In the present study, we tested activities of macroalgal extracts against two macroalgal pathogens: *A. bacteriolytica* (formerly *Pseudoalteromonas bacteriolytica*) and *P. elyakovii*. All studied macroalgae revealed no inhibitory activities against these pathogens; on the contrary, growth stimulatory activities were in

general displayed (Fig. 1b). The extract of *S. latissima*, which was the only studied brown macroalga that belong to Laminariales, stimulated growth of both macroalgal pathogens (but <30%). Members of this genus were originally affected by those pathogens (Sawabe *et al.* 1998, 2000). Recently, in tropical environments, extensive investigations of different macroalgal extracts have shown high biological activity against *A. bacteriolytica* (Engel *et al.* 2006, Puglisi *et al.* 2007, Lane *et al.* 2010). This indicates highly variable amounts and different composition of active compounds, probably depending on the biotic and abiotic pressures onto the macroalgae. It is accepted that chemical defences are elaborated to a greater extent and are more important in tropical than temperate or cold areas as in the German coast (see Pereira & da Gama 2008). Unfortunately, with the exception of one study using causative agents of the macroalgal ice-ice disease (by Vairappan *et al.* 2010) and *P. elyakovii* (by Thabard *et al.* 2011), bioactivity tests of algal extracts or compounds against other known bacterial pathogens of macroalgae were rarely performed.

As recommended by Engel *et al.* (2006) caution must be exercised about drawing ecological conclusions of the role of secondary metabolites on the observed biological activity. The concentration used in our assays followed standard procedure (see Bansemir *et al.* 2006, Muñoz-Ochoa *et al.* 2010, Sánchez-Saavedra *et al.* 2010, Villarreal-Gómez *et al.* 2010), and crude extracts are usually complex mixtures of compounds. Therefore it is unknown which substances at which concentration exhibited the bioactivity in nature (Persson *et al.* 2011). Despite the inconveniences using crude extracts of whole organism already mentioned by several authors (see Paul *et al.* 2006, Lane *et al.* 2009, Nylund *et al.* 2010), there is no doubt that such experiments provide insights into the potential interactions mediated by algal metabolites, especially by using environmental test strains. We confirmed that macroalgal extracts exert effects of growth inhibition and stimulation according to the nature of the bacterial strains selected. We demonstrated that macroalgal extracts have growth stimulant effects on macroalga-associated bacteria including algal pathogens.

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