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Seasonal antibacterial activity of two red seaweeds, *Palmaria palmata* and *Grateloupia turuturu*, on European abalone pathogen *Vibrio harveyi*

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Abstract – *Vibrio harveyi* is the main pathogen of the European abalone *Haliotis tuberculata*, and recently caused important mortalities at the production sites of this marine gastropod in France. In the present work, the monthly antibacterial activity of two red seaweed species from the French Atlantic coast, the native *Palmaria palmata* and the introduced *Grateloupia turuturu*, were investigated against the abalone pathogen *Vibrio harveyi* strain ORM4. Water-soluble extracts were screened using the microplate method. *Grateloupia turuturu* showed an antibacterial activity with a maximal growth inhibition in spring of around 16%. In contrast, *Palmaria palmata* was inactive, as further growth of the bacteria was observed. Preliminary one-dimensional proton nuclear magnetic-resonance (^{1}H NMR) profiles identified the differences between the two water-soluble extracts.

Keywords: Antibacterial activity / Growth inhibition / Immunity / Water-soluble extracts / *Vibrio harveyi* / *Haliotis tuberculata*

1 Introduction

World aquaculture production of molluscs, with an average in 2011 of 14.4 million tons, is the second fastest growing aquaculture sector in the world (FAO 2013). The intensity of aquaculture activities has led to increasing problems of disease caused by viruses, bacteria and other pathogens (Bondad-Reantaso et al. 2005). *Vibrio* is recognized as one of the most important bacterial genera to consider (Austin and Zhang 2006). For example, studies showed the pathogenicity of *Vibrio harveyi* against salmonids and shrimps (Zhang and Austin 2000; Paillard et al. 2004b; Austin and Zhang 2006), and even mortalities induced by *V. aestuarianus*, *V. splendidus* and *V. tapetis* in the oyster *Crassostrea gigas* and the clam *Venerupis philippinarum* (Lacoste et al. 2001; Gay et al. 2004; Paillard 2004a; Garnier et al. 2007; Paillard et al. 2008). Declines in abalone stocks have thus been associated with the emerging Gram-negative bacterium *Vibrio*, especially *V. harveyi* in relation to the European abalone *Haliotis tuberculata* (Nicolas et al. 2002; Huchette and Clavier 2004). Antibiotics are widely used to prevent and control outbreaks caused by *Vibrio* spp. in aquaculture, especially in shrimp and salmonid farming (Holmström et al. 2003). The development of various solutions against diseases, other than the use of antibiotics, was thus initiated to ensure the profitability of aquaculture, for which the production of safe and healthy products is a priority. For example, using probiotics in aquaculture to limit or inhibit the growth of the pathogenic species clade *Vibrio harveyi* is reported in several studies (Kesarcodi-Watson et al. 2008; Wang et al. 2008; Prado et al. 2010; Jiang et al. 2013). The interest in marine organisms as potential new drugs in aquaculture has increased during recent years (Genovese et al. 2012; Mata et al. 2013). In the past three decades, several studies have shown that seaweeds are one of the richest and most promising sources of bioactive primary and secondary metabolites (Bhakuni and Rawat 2005; Cardozo et al. 2007; Gupta and Abu-Ghannam 2011). Many seaweed species, especially red ones, contain biological compounds that inhibit the growth of pathogenic Gram-positive and Gram-negative

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bacteria (Kumar and Rengasamy 2000; Val et al. 2001; Freile-Pelegrin and Morales 2004; Bansemir et al. 2006; Pang et al. 2006; Plouguerné et al. 2008; Goecke et al. 2010). *Palmaria palmata* and *Grateloupia turuturu* are widespread seaweeds along the French coast (Simon et al. 2001; Le Gall et al. 2004). On the one hand, several studies have shown the preferred consumption of *Palmaria palmata* over other seaweeds, due to its biochemical composition, by gastropods like *Haliotis tuberculata* (Rosen et al. 2000; Demetropoulos and Langdon 2004). On the other hand, the proliferative nature of *G. turuturu* provides the added advantage of being available most of the year in significant quantities (Plouguerné 2006). Moreover, a number of molecules and/or extracts from both species present biological activities, like antibacterial and antiviral (Hudson et al. 1998; Hellio et al. 2000; Bansemir et al. 2006; Pang et al. 2006; Plouguerné et al. 2006; Cox et al. 2010). These two seaweed species may thus be good candidates for sources of bioactive compounds in the context of their use in the culture of the European abalone *Haliotis tuberculata*. The aim of this work was to evaluate the seasonal in vitro activity of aqueous extracts of two marine red seaweeds, *Palmaria palmata* and *Grateloupia turuturu*, against the abalone pathogen *V. harveyi*.

2 Materials and methods

2.1 Seaweed sampling and extraction

Red seaweeds, *Grateloupia turuturu* Yamada 1941 (Rhodophyta, Halymeniaceae) and *Palmaria palmata* (Linnaeus) Weber and Mohr 1805 (Rhodophyta, Palmariaceae), were harvested monthly between May 2011 and April 2012 in the mediolittoral zone at Batz-sur-Mer (Atlantic Coast, France).

Thalli were successively rinsed with seawater, tap water and distilled water before freeze-drying. Freeze-dried samples were then ground in liquid nitrogen and the resulting fine powder was used for water-soluble component extraction for 20 min in a 1/20 ratio (w/v) of phosphate buffer (20 mM; pH 7.1) before being centrifuged at 25 000 g at 4 °C for 20 min then stored at −20 °C until use.

2.2 Biochemical composition of seaweeds

The resulting supernatants were called aqueous crude extracts (CE). Water-soluble proteins in the CE were analyzed following the Bradford method (Bradford 1976). Protein content (in mg g^{-1} dry weight of seaweed) was determined using bovine serum albumin (BSA, Sigma) as a standard (from 0 to 50 mg L^{-1}).

Total water-soluble carbohydrates were analyzed in the CE using the modified colorimetric phenol-sulfuric acid method (Chaplin and Kennedy 1994). Carbohydrate content (in mg g^{-1} dry weight of seaweed) was determined using glucose (Sigma) as a standard (from 0 to 100 mg L^{-1}).

2.3 Bacterial strain

The marine bacterial *V. harveyi* pathogen strain ORM4 obtained from the culture collection of the LEMAR (IUEM-UBO, Brest, France) was used for the experiment. The virulent bacteria studied were isolated from diseased European abalone *Haliotis tuberculata* in Normandy in 1999 (Nicolas et al. 2002).

Before inoculation, bacteria were grown in test tubes with lysogeny broth (LB, Sigma) supplemented with extra NaCl (20 g L^{-1}) (LBS) in a temperature-controlled shaker at 28 °C for 18 h. Each plate well had the same bacterial content of 10^5 colony-forming unit, CFU ml^{-1}.

2.4 Antimicrobial assays

The monthly samples of water-soluble extracts from *P. palmata* and *G. turuturu* were tested for their activity against the growth of the ORM4 strain. Seaweed extracts were incubated with the bacteria in 96-well plates (Falcon®VWR). Two controls were performed for every assay, LBS medium and the water-soluble extracts, to ensure no influence of the microbial growth. Control LBS medium + bacterial strain was used to compare with our extracts. All the extracts and controls were tested on 6 replicates.

From each extract, 10 μl CE + 200 μl sample culture were transferred to wells (6 wells per month/seaweed) of a Bioscreen plate (C BIOSCREEN®MBR) for incubation for 24 h. Experiments were carried out at three temperatures: 16 °C (lowest temperature of growth capacity of the bacteria in seawater), 20 °C (mean temperature of seawater when the abalone stock declined along the French coastline) and 28 °C (high optimal growth temperature of *V. harveyi* – Travers et al. 2009). Bacterial growth was monitored by measuring optical density (OD) at 490 nm every 30 min for 24 h. After incubation, the intensity of growth in the presence of the tested compounds and controls was compared. Growth rate (Eq. (1)) was defined as the variation in population density occurring in different conditions compared to a control, and was calculated for each CE concentration (Bazes et al. 2006). Positive values of equation (1) are seen as inhibition growth while negative values are induced growth. Exponential phase speed variation (EPSV, Eq. (2)) was defined as the variation in speed of bacterial growth during the exponential phase compared to the control. Time delay rate (Eq. (3)) was defined as the supplementary delay before the beginning of the exponential growth of the bacteria occurring in test samples compared to the control.

$$\text{Growth rate}\,(\%) = 100\,[(OD_c - OD_t)/OD_c] \quad (1)$$

$$\text{EPSV}(\%) = 100\,[(S_c - S_t)/S_c]$$

where

$$S = (OD_t - OD_{t-1})/OD_{t-1} \quad (2)$$

$$\text{Delay rate}\,(\%) = 100\,[(\lambda_c - \lambda_t)/\lambda_c] \quad (3)$$

where OD_c is the mean optical density of the bacterial controls; OD_t mean optical density of the test samples; S_c speed of development of the bacterial controls; S_t speed of development of the test samples; λ_c appearance time (min) of maximal growth speed of the control; and λ_t appearance time (min) of maximal growth speed of the test samples.

2.5 NMR spectroscopy

One-dimensional proton nuclear magnetic-resonance (1D ^{1}H NMR) spectra were recorded at 25 °C on a Bruker Avance 400 spectrometer equipped with an inverse probe 5 mm triple resonance TBI ^{1}H/(BB)/^{31}P. NMR analyses of water-soluble extracts were recorded on samples dissolved in 700 μl of 100% D_2O, according to standard Bruker program acquisitions. The spectra were obtained using a 2 s delay and scan 30 degree angle pulse. Chemical shifts are expressed in ppm relative to trimethylsilyl propionate (TSP) as an external reference.

2.6 Statistical analysis

Extractions and biochemical analyses were performed in triplicate and antibacterial assays in sixplicate. Mean and standard deviations were calculated for each experiment. All calculations were based on measured concentrations of extracts. To compare results obtained with *G. turuturu* and *P. palmata*, statistical analyses were performed using SigmaStat software (3.1) with two-way ANOVAs followed by Holm-Sidak procedures. A p-value less than 0.05 was considered significant.

3 Results

3.1 Biochemical composition

The annual average of water-soluble protein content was significantly higher for *P. palmata* (11.0 ± 0.9 mg g^{-1} dw) than for *G. turuturu* (6.6 ± 1.9 mg g^{-1} dw) (Fig. 1a). The maximal water-soluble protein content of *G. turuturu* was recorded in December and January (9.5 ± 0.4 and 9.3 ± 0.1 mg g^{-1} dw, respectively) and the minimal in March and April (4.2 ± 0.6 and 4.4 ± 0.3 mg g^{-1} dw, respectively). The water-soluble protein content of *P. palmata* was more stable over the seasons with a maximum determined in December (12.4 ± 0.1 mg g^{-1} dw) and a minimum in August (9.4 ± 0.9 mg g^{-1} dw). As depicted in Figure 1b, no monthly variation in the water-soluble carbohydrate content was detected for *G. turuturu* while values for *P. palmata* were significantly variable throughout the study period, with the highest content between May and December (from 99.9 ± 0.5 to 70.8 ± 0.1 mg g^{-1} dw).

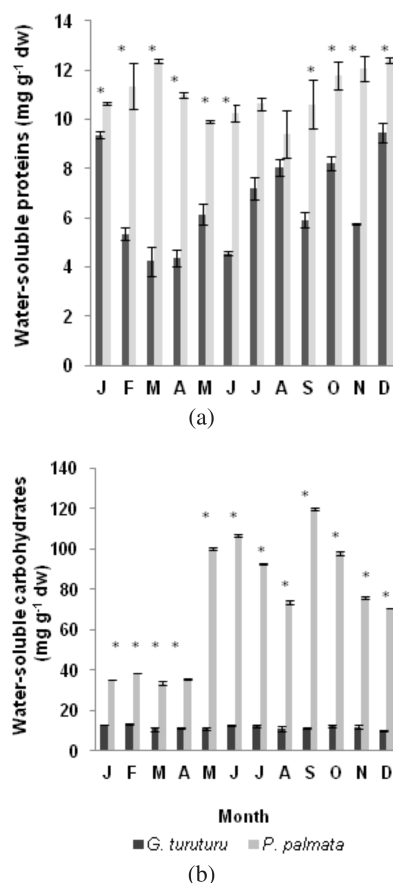

Fig. 1. Monthly mean water-soluble protein (a) and water-soluble carbohydrate (b) contents (mean ± SD, n = 3) in *Grateloupia turuturu* and *Palmaria palmata* extracts, *: indicates a significant difference (p-value < 0.05) between the two seaweeds obtained with the Holm-Sidak procedure after a two-way ANOVA.

3.2 Bacterial growth according to temperature

The ORM4 strain cultured at a higher temperature had faster growth rates and a greater bacterial concentration at the end of the experiment than the same ORM4 strain at 16 °C (Fig. 2). The ORM4 strain at 28 °C reached the stationary phase at around 8 h while the strain cultured at 20 °C reached it after 17–18 h. The strain cultured at 16 °C did not even reach the stationary phase by the end of the experiment. ORM4 at 28 °C needed only 4 h to start the exponential phase (period characterized by cell doubling) while the strain at 20 °C needed twice as long to reach this phase. Only the results of the antimicrobial screening assays at 20 °C were studied as this is the average temperature in the natural environment where *Vibrio harveyi* ORM4 lives.

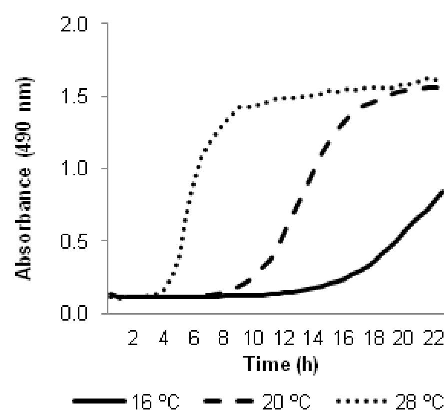

Fig. 2. Growth of *Vibrio harveyi* ORM4 strain at three different temperatures (16, 20 and 28 °C).

3.3 Activity of extracts

Crude extract dilutions did not lead to significant differences regarding the responses evaluated. For a better understanding of the figures, only the 10 μg μl^{-1} concentration is reported.

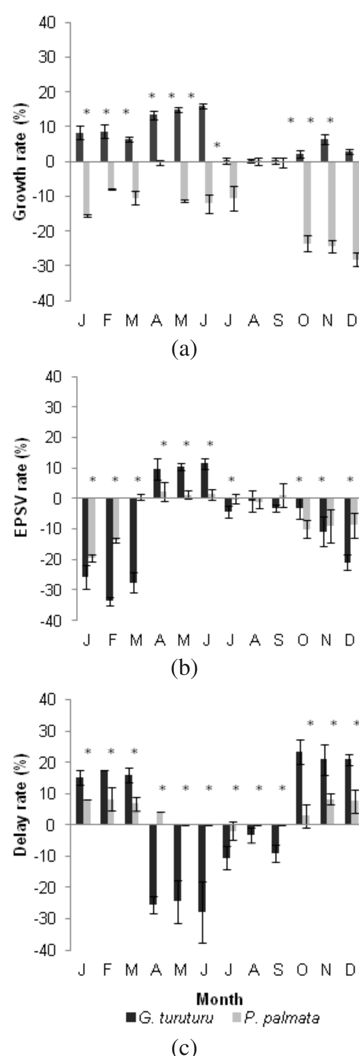

Fig. 3. Growth rate (a), exponential phase speed variation, EPSV (b) and delay rate (c) of *Vibrio harveyi* strain ORM4 at 20 °C induced by *Grateloupia turuturu* and *Palmaria palmata* water-soluble extracts at 10 μg μl^{-1}. Data are expressed as mean ± SD ($n = 6$), *: indicates a significant difference (p-value < 0.05) between the two seaweeds obtained with the Holm-Sidak procedure after a two-way ANOVA.

3.3.1 Evaluation of bacterial growth

A seasonal variation in inhibition of bacterial growth between 0 and 16% was observed for water-soluble extracts of *G. turuturu* (Fig. 3). The strongest antibacterial activity was obtained in spring extracts of *G. turuturu* with a percentage of inhibition around 16%, whereas the winter extracts showed a weaker antibacterial activity of around 5−7%. An almost nonexistent effect was recorded in July, August and September.

Conversely, extracts of *P. palmata* were not effective against the tested pathogenic organism *V. harveyi* Among the seasonal extracts tested, the autumn extracts exhibited the best growth of the tested bacterial species, of around 25%. The highest development of the pathogen ORM4 was observed in the December *P. palmata* extract, which induced a growth of around 30%.

3.3.2 Evaluation of speed variation during the exponential phase

Extracts from December to March of *G. turuturu* promoted cell doubling rate of around 25% compared with the control strain. Nevertheless, spring extracts generated a decrease in the cell doubling rate of approximately 12%. For the summer months, no change compared to the control was demonstrated (Fig. 2). For *P. palmata*, only the winter extracts tested promoted cell doubling rate 1D ^{1}H NMR at around 10% compared with the control.

3.3.3 Evaluation of delay during the exponential phase

Delay in the growth of *V. harveyi* decreased by approximately 25% in April, May and June with *G. turuturu* extracts. On the contrary, delay increased by around 20% with the winter and autumn extracts. The results observed for *P. palmata* extracts (Fig. 3) showed an increased delay of around 10% from November to March. No change was observed with other monthly extracts.

3.4 Nuclear magnetic resonance analysis of macroalgal extracts

^{1}H NMR spectra obtained from monthly water-soluble extracts of *G. turuturu* (Fig. 4a) showed the presence of osmolytes, like floridoside and isethionic acid, in water-soluble extracts of *G. turuturu* throughout the study period. Letters F, I, M and D_2O refer to the resonances of floridoside ((2-O-α-D-galactopyranosyl) 1→2' (glycerol)), isethionic acid (2-hydroxyethane sulfonic acid), N-methyl-L-methionine sulfoxide (4-methane sulfinyl-2-methylamino butyric acid) and deuterium oxide, respectively. The spectral composition was variable but did not explain the variations in inhibition of bacterial strain ORM4.

^{1}H NMR spectra obtained from water-soluble extracts of *P. palmata* (Fig. 4b) were very similar throughout the year. The major compound identified in this alga during the study period was floridoside.

4 Discussion

The two species, *P. palmata* and *G. turuturu*, demonstrated a similar biochemical composition corresponding to abalone feeding preferences (Mai et al. 1995, Garcia-Bueno et al. unpublished). Water-soluble extracts, used to avoid toxic solvents (methanol or chloroform), enabled the recovery of polysaccharides, oligosaccharides, proteins and peptides, which are the main biochemical compounds of seaweeds (Mabeau and Fleurence 1993). The water-soluble protein content of both seaweeds presented a monthly variation. Total protein contents of *P. palmata* were maximum in winter and minimum in summer, as previously shown by Galland-Irmouli et al. (1999). The water-soluble carbohydrates values were in agreement with those recorded in previous studies, demonstrating that the amount of water-soluble sugars in *G. turuturu*

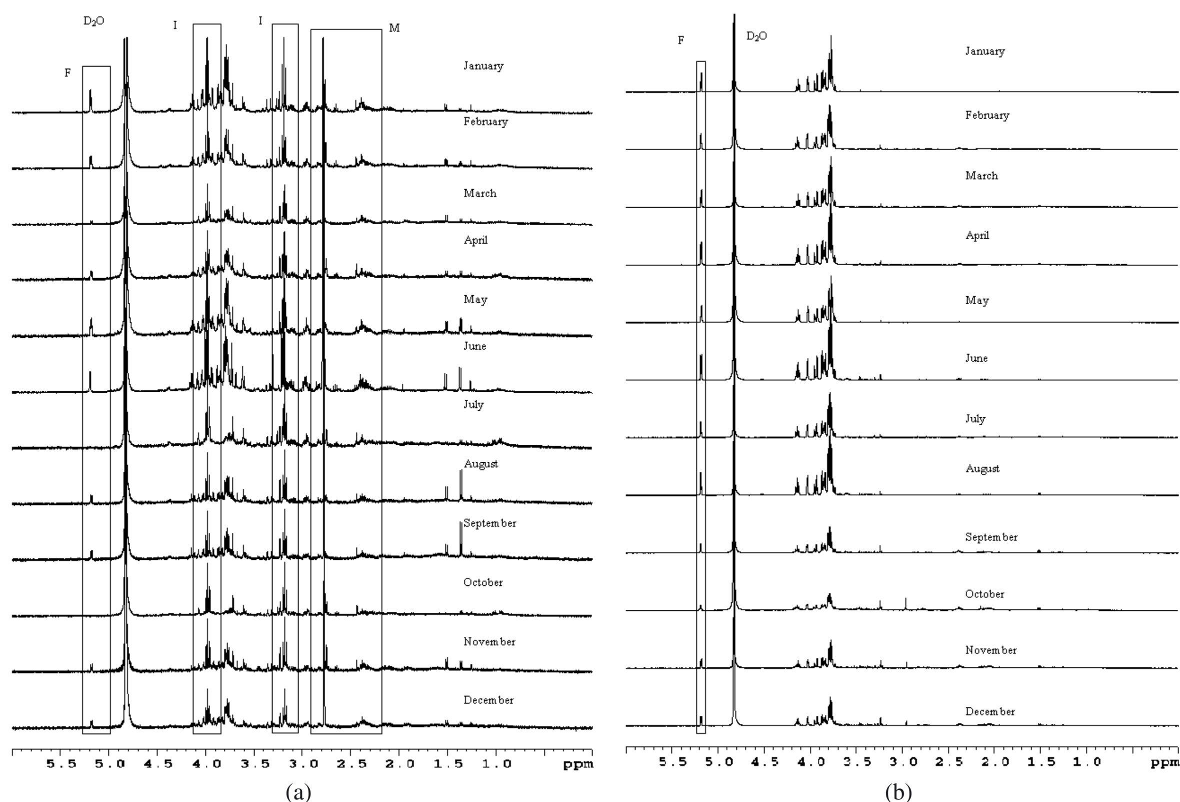

Fig. 4. Monthly *Grateloupia turuturu* (a) and *Palmaria palmata* (b) water-soluble extracts analyzed using ^{1}H NMR. Letters F, I, M and D2O refer to the resonances of floridoside (α-D-galacto-pyranosyl-(1-2)-glycerol), isethionic acid (2-hydroxyethane sulfonic acid), N-methyl-L-methionine sulfoxide (4-methane sulfinyl-2-methylamino butyric acid) and deuterium oxide, respectively.

(Denis et al. 2009; Munier et al. 2013) is lower than that recorded for *P. palmata* (Morgan et al. 1980; Morgan and Simpson 1981; Rosen et al. 2000).

Specific variability in the production of metabolites in seaweeds is generally related to seasonal variation, which can explain the difference revealed by the results of biological tests. This study confirmed the capacity of *G. turuturu* extracts to act as an antibacterial agent against *V. harveyi*. However, the inhibitory action of *G. turuturu* extracts was very variable according to the season. In the present work, maximal antibacterial activity against *V. harveyi* was recorded by *G. turuturu* extracts from April to June. This seasonal activity is consistent with the study carried out by Hornsey and Hide using Atlantic seaweed samples (Hornsey and Hide 1974; Hornsey and Hide 1976). Contrasting results have been reported in studies focusing on Indian (Rengasamy 2000) and Mediterranean seaweed samples (Martí et al. 2004) where autumn was the season with the highest percentage of inhibition. The worst level for antibacterial activity occurring in the summer can be explained by the increase in the seawater temperature, which modifies metabolite production (Bourgaud et al. 2001). Research carried out in various parts of the world on *G. proteus* (Caccamese et al. 1980), *G. filicina* (Vlachos et al. 1998) and *G. doryphora* (Val et al. 2001) identified high activity against Gram-positive and Gram-negative bacteria. However, results confirmed that *P. palmata* extracts did not exhibit activity against *V. harveyi* when compared to other studies where antimicrobial (Hellio et al. 2001), antifouling (Eguía and Trueba 2007) and antioxidant (Wang et al. 2010) activities were demonstrated. Here, in contrast, *P. palmata* extracts showed a better bacterial growth, which reached a value of 28%.

As mentioned in the study of Simon-Colin (2002), ^{1}H NMR spectra led to the characterization of three main organic solutes present in *G. turuturu*. The ^{1}H-NMR analyses revealed an annual stability in the presence of signals corresponding to the major compounds of both algae. It appears, therefore, that the major peaks of the present NMR spectra are not related to the very large variation in inhibition. The inhibitory effect of *G. turuturu* extract is probably due to other minor compounds not visible on the spectra and not characterized by our NMR analysis. Plouguerné et al. (2006) demonstrated the antibacterial activity of cholesteryl formate, which is a minor lipidic molecule found in *G. turuturu* from Brittany coasts. Secondary metabolites are factors modifying seaweed susceptibility to herbivores (Yates and Peckol 1993; Cronin and Hay 1996; Cronin et al. 1997). In future studies, it will be interesting to extend the research into the metabolites responsible for this antibacterial activity of *G. turuturu* extracts.

In conclusion, the maximum antibiotic activity recorded in the water-soluble extract of *G. turuturu* was ~16% of growth inhibition. This level is not high but it suggests the presence of a compound with an inhibitory effect on the growth of *V. harveyi*. Further studies, especially the purification of this molecule, will be necessary to characterize its chemical

nature. This purification should also improve the level of antibiotic activity reported initially. Thus, purified metabolites or extracts could be added to the abalone culture media. The best form in which to administer them remains to be defined and could be inspired by the study of Wu et al. (2011). As *G. turuturu* is an important biomass along the French Atlantic coast, the exploitation of this resource constitutes an ecological and economic challenge.

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