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Seasonal variation in the antivibrio activity of two organic extracts from two red seaweed: *Palmaria palmata* and the introduced *Grateloupia turuturu* against the abalone pathogen *Vibrio harveyi*

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Abstract – The wide polarity range and highly polar compounds of two selected red seaweed, *Grateloupia turuturu* and *Palmaria palmata* were extracted using two different types of solvent, dichloromethane/methanol and methanol/water. Monthly in vitro antibacterial activities were studied using the microplate method against the marine bacteria *Vibrio harveyi* strain ORM4, known to infect abalone. Inhibition, slowdown and delay of *Vibrio harveyi* growth were investigated. Polar compounds of seaweed showed an activity against the abalone pathogen. The best activity was recorded from *P. palmata* collected in spring, with an inhibition of 7.9% of the bacterial growth. Preliminary 1H NMR profiles identified the differences between the extracts.

Keywords: Antibacterial activity / abalone disease / *Haliotis tuberculata* / red seaweed / *Vibrio harveyi*

1 Introduction

Metabolites of seaweed are an important source of organic molecules with significant biological activities (El Gamal 2010). Crude extracts of some algae have demonstrated growth inhibition on bacteria and fungi (Hellio et al. 2000; Maréchal et al. 2004; Engel et al. 2006; Plouguerné et al. 2008). 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. In fact, diseases have a real impact on the profitability and the conservation of species in shellfish farming such as abalone (*Haliotis tuberculata*) in halioticulture (Huchette and Clavier 2004). Different infectious diseases have been identified in abalone caused by the herpes parasite *Haplosporidium* by bacteria of the *Rickettsia* genus (Azevedo et al. 2006; Balseiro et al. 2006) and by the bacteria *Vibrio harveyi* (Nicolas et al. 2002; Travers et al. 2009) which has caused cases of vibriosis in abalone in Australia Japan and France (Handlinger et al. 2005; Sawabe et al. 2007).

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Two seaweed species, *Palmaria palmata* and *Grateloupia turuturu* constitute an important resource in the north of France (Simon et al. 2001; Le Gall et al. 2004) and could be of interest in the abalone culture preservation. In fact, *P. palmata* is known to be among the best species of algae suitable for the growth of abalone (Viera et al. 2015) Sulfated polysaccharides extracted from *G. turuturu* have exhibited antiviral (Chattopadhyay et al. 2007; Wang et al. 2007) and anticoagulant (Shanmugam and Mody 2000) properties. Moreover, this alga has presented antibacterial activity against *Vibrio parahaemolyticus* (Pang et al. 2006). The study of the potential antibacterial activity of *P. palmata* and *G. turuturu* against a main abalone pathogen *V. harveyi* could then be of major interest.

According to the studies of Hornsey and Hide (1976) carried out in the south-west of England, the antibacterial activity of seaweed had shown four types of temporal variation: (1) uniform and constant activity throughout the year, (2) a peak of activity in the winter; (3) a peak of activity in the spring, and (4) a peak of activity in the summer. These variations were due to climate change affecting water temperature, salinity, currents, tides, waves, etc., which could probably be transposed to France.

The present study investigates the potential antibacterial activity of highly polar and wide polarity range extracts from *P. palmata* and *G. turuturu* against a main abalone pathogen *V. harveyi*, and the variations of this activity during the year. It complements the work on the water-soluble extract (Garcia-Bueno et al. 2014). The purpose is to determine and identify extracts presenting activity against the growth of pathogenic bacteria in the European abalone *H. tuberculata*.

2 Materials and methods

2.1 Seaweed sampling

Seaweed *Grateloupia turuturu* Yamada 1941 (Rhodophyta, *Halymeniaceae*) and *Palmaria palmata* Linnaeus 1805 (Rhodophyta, *Palmariaceae*) were collected monthly between May 2011 and April 2012 at Batz-sur-Mer (France). The harvested samples were successively rinsed with seawater, tap water and distilled water Samples were then freeze-dried and ground in liquid nitrogen before being frozen. The resulting fine powder was used for extractions.

2.2 Extractions

Two different extracts were obtained from algae: one from a polar solvent mix of methanol and water (highly polar extract (PE)) and one from a solvent mix of dichloromethane and methanol (wide polarity ranges extract (WPE)). Extractions were performed in triplicate

2.2.1 Extraction with methanol/water

An amount of 2.5 g of powder was transferred to a tube previously coated with aluminum to minimize the risk of oxidation related to light. Then 75 ml of the methanol/water solvent (1/1; v/v) was added to each tube. Subsequently, tubes were placed in an incubator for 3 h (Edmund Bühler® GmbH TH 30 and SM-30 CONTROL) at 40 °C under stirring (150 rpm). All samples were filtered to eliminate residues. The filtrate was collected in a previously weighed matrass and placed in a rotary evaporator (STUART RE300B Heidolph® LABOROTA 400). The solvents in the matrass were evaporated to dryness at 40 °C, then the matrass was immediately weighed. The matrass weight difference determines the amount of extracts. Lastly, compounds were recovered with methanol in a solution of 1 $g\,l^{-1}$.

2.2.2 Extraction with dichloromethane/methanol

An amount of 2.5 g of powder was transferred to a tube previously coated with aluminum to minimize the risk of oxidation related to light. Then 75 ml of the dichloromethane/methanol solvent (2/1; v/v) was added to each tube. Subsequently, tubes were placed for 3 h at room temperature (16–18 °C) on trays stirred with a magnet. All samples were filtered to eliminate residues. Filtration of the compounds extracted with dichloromethane/methanol was conducted with a vacuum pump due to the viscosity of *G. turuturu*, which is rich in polysaccharides. The filtrate was collected in a previously annotated matrass then weighed. The solvents in the matrass were evaporated at 40 °C, then the matrass was immediately weighed. The matrass weight difference determines the amount of extracts. Lastly, compounds were recovered with methanol in a solution of 1 $g\,l^{-1}$.

2.3 Bacterial strain

The pathogen *V. harveyi* strain ORM4 (Nicolas et al. 2002; Travers et al. 2008) from the Culture Collection of the IUEM of Brest (UBO, France) was used for the experiment. Luria-Bertani broth (LB, Sigma) supplemented with extra NaCl (20 $g\,l^{-1}$, final concentration) (LBS) was used for bacteria growing in a temperature-controlled shaker at 28 °C for 18 h at 180 rpm. Before preparing serial dilutions, the bacterial concentration was calculated with the following formula from Travers et al. (2008) CFU (colony-forming unit) = (ODt − ODc) × 6.109 + 2.108 by optical density (OD) at 490 nm with a Mithras LB 940 spectrophotometer, where ODt = main optical density of the strain ORM4 on LBS broth and ODc = main optical density of control test LBS broth. This formula was determined experimentally by OD measurements and control bacterial plating and is only used between OD 0.1 and 1 to remain in the linear part of the correlation. Each well in the Bioscreen plates had the same bacterial content of 10^5 CFU ml^{-1}.

2.4 Antimicrobial assays

Growth of the pathogenic strain ORM4 confronted with PE and WPE monthly extracts of *P. palmata* and *G. turuturu* was monitored using a Bioscreen (C BIOSCREEN® MBR) to measure optical density at 490 nm every 30 min for 24 h. The extracts were incubated with the bacteria in 96-well plates (Falcon® VWR) at 20 °C. The temperature was selected according to the mean temperature of seawater when declines in abalone stocks on the French coast were observed in 1999 (Nicolas et al. 2002). Two controls were performed for every assay, the LBS medium and algal extract (from *P. palmata* or *G. turuturu* according to the experiment) to ensure no influence of microbial growth. LBS medium + bacterial strain was used as a control. All the extracts and controls were tested on 6 replicates. From each extract, 10 μg of sample (PE and WPE) was transferred to plate wells in a hood and the solvent allowed evaporating for around 30 min. During evaporation, the plates were covered with aluminum to minimize the light effect. Then 200 μl culture samples at 10^5 CFU ml^{-1} were transferred to wells (6 wells per extraction/month/seaweed) for incubation for 24 h at 20 °C.

Bacterial growth curves produced by the Bioscreen are presented as OD plotted against time. After incubation, the intensity of growth in the presence of the tested compounds and controls was compared. Growth rate 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 (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.

$$\text{Growth rate}(\%) = 100[(\text{ODc} - \text{ODt})/\text{ODc}] \quad (1)$$

$$\text{EPSV}(\%) = 100[(\text{Sc} - \text{St})/\text{Sc}] \quad (2)$$

where $S = (\text{OD}_\text{t} - \text{OD}_{\text{t}-1})/\text{OD}_{\text{t}-1}$

$$\text{Delay rate}(\%) = 100[(\lambda\text{c} - \lambda\text{t})/\lambda\text{c}] \quad (3)$$

where ODc is mean optical density of the bacterial controls; ODt mean optical density of the test samples; Sc speed of development of the bacterial controls; St speed of development of the test samples; λc appearance time (min) of maximal growth speed of the control; and λt appearance time (min) of maximal growth speed of the test samples.

2.5 NMR

1D ^{1}H NMR (proton nuclear magnetic resonance) 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 to provide a global vision of the compounds forming the extracts. *G. turuturu* and *P. palmata* extracts were tested by NMR. Samples were selected based on their activity (inhibition, activation or no effect) on bacterial growth. PE extracts were dissolved in deuterated methanol (CD_3OD) and WPE extracts in deuterated chloroform ($CDCl_3$), and then transferred into an NMR tube for analysis according to standard Bruker program acquisitions.

2.6 Statistical analysis

Extractions and biochemical analyses were performed in triplicates and antibacterial assays in hexaplicates. 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 as significant.

3 Results

3.1 Evaluation of bacterial growth

G. turuturu PE induced the greatest inhibition comparing with the control with maximal values in March and December of 7.9% and 7.7%, respectively (Fig. 1a). However, PE samples between August and October showed little or no inhibition of bacterial ORM4 growth. The results for WPE showed a slight percentage of ORM4 growth inhibition for February and March of 0.8% and 2.0%, respectively. The PE of *P. palmata* inhibited bacterial growth more throughout the year than *G. turuturu* (Fig. 1b). Among the seasonal extracts tested, PE from January to May and also July, September and October exhibited an antibacterial activity toward the tested bacterial species, with an average of 4.5%.

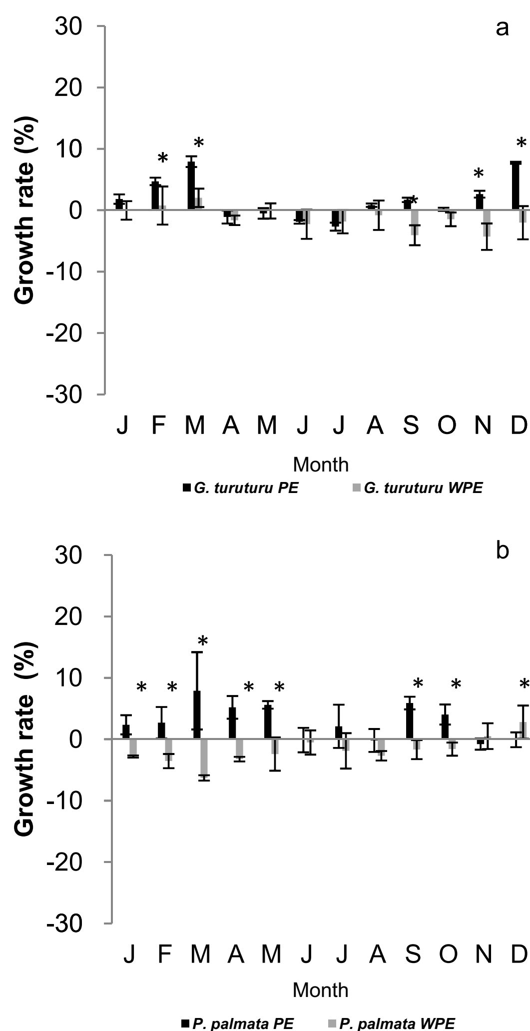

Fig. 1. Growth rate of *Vibrio harveyi* strain ORM4 at 20 °C induced by *Grateloupia turuturu* (a) and *Palmaria palmata* (b) PE (highly polar extract) and WPE (wide polarity ranges extract) at 10 $\mu g\,\mu l^{-1}$. Data are expressed as mean ±SD (n = 6), ⋆: indicates a significant difference (p-value < 0.05) between the two seaweeds obtained with the Holm-Sidak procedure after a two-way ANOVA.

Conversely, WPE of *P. palmata* improved bacterial growth throughout the year – except in November and December – with a maximal bacteria growth of 6.3% observed in March (Fig. 1b). As with *P. palmata*, *G. turuturu* WPE do not showed inhibition growth all year round, except in February and March (Fig. 1a).

3.2 Evaluation of slowdown during the exponential phase

The annual trend for PE and WPE of *G. turuturu* was a decrease in the cell-doubling rate with a peak reduction of 36.5% observed in July with polar compounds (Fig. 2a). All *P. palmata* PE generated a decrease in the cell-doubling rate with an average of 6.0% (Fig. 2b) while *P. palmata* WPE presented a large variation throughout the year, with a high decreased in

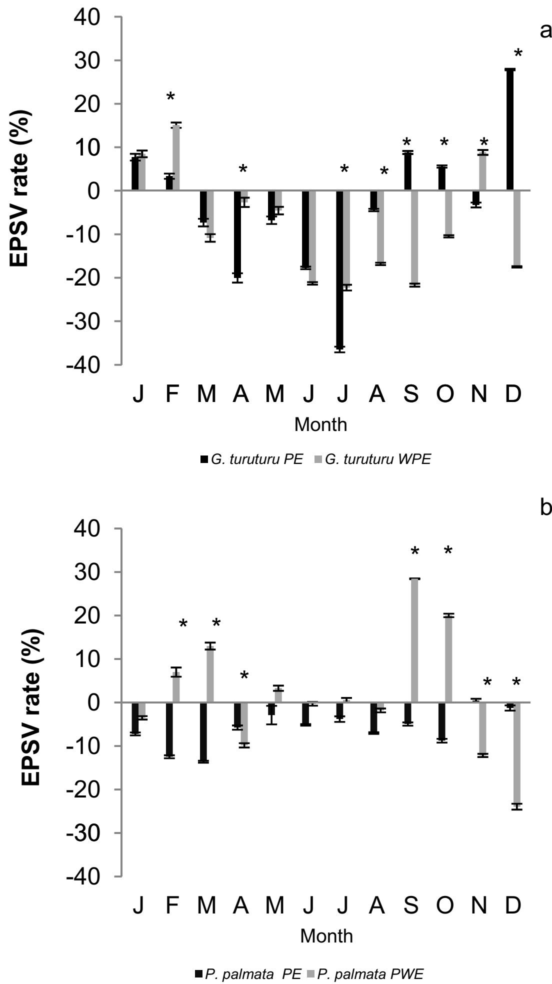

Fig. 2. Exponential phase speed variation (EPSV) of *Vibrio harveyi* strain ORM4 at 20 °C induced by *Grateloupia turuturu* (a) and *Palmaria palmata* (b) PE (highly polar extract) and WPE (wide polarity ranges extract) at 10 μg μl^{-1}. Data are expressed as mean ±SD (n = 6), ⋆: indicates a significant difference (p-value < 0.05) between the two seaweeds obtained with the Holm-Sidak procedure after a two-way ANOVA.

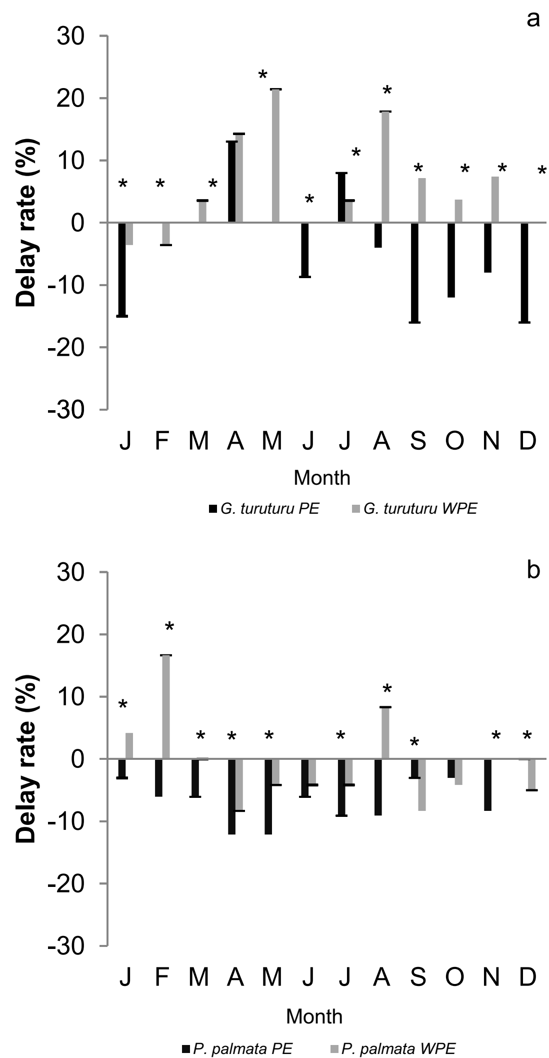

Fig. 3. Delay rate of *Vibrio harveyi* strain ORM4 at 20 °C induced by *Grateloupia turuturu* (a) and *Palmaria palmata* (b) PE (highly polar extract) and WPE (wide polarity ranges extract) at 10 μg μl^{-1}. Data are expressed as mean ±SD (n = 6), ⋆: indicates a significant difference (p-value < 0.05) between the two seaweeds obtained with the Holm-Sidak procedure after a two-way ANOVA.

cell doubling of 12.1% in November and 23.9% in December, respectively.

Conversely, January and February results of *G. turuturu* showed a promotion of cell doubling from both extracts of 7.7% and 3.4% for PE and 8.5% and 15.1% for WPE, respectively (Fig. 2a). Evenly, December PE showed a high peak of cell doubling of 27.9%, while September and October presented weaker percentages of 8.8% and 5.6%, respectively. *P. palmata* WPE showed a high peak of cell doubling of 28.5% and 20.0% in September and October, respectively. February and March results showed also a high peak of cell doubling for these extracts. For the summer months of June and July, there was no change compared to the control, as shown in Figure 2b.

3.3 Evaluation of delay during the exponential phase

A lag time decrease of between 8.0% and 16.0% relative to the control was observed in the growth of *V. harveyi* from September to January for *G. turuturu* PE (Fig. 3a). No difference in lag time appeared for February, March and May extracts compared to the control. January and February WPE of *G. turuturu* showed a weak decrease of around 3.5%. *P. palmata* results were more stable throughout the seasons (Fig. 3b) than those for *G. turuturu*. All PE showed a decrease in lag time with an average of 6.5%; only December extracts showed no effect in comparison with the control. WPE decreased the lag time with a weaker average of 0.6%. March and November extracts showed no change with regard to the control.

Conversely, only April and July *G. turuturu* PE showed an increase in lag time of 13.0% and 8.0%, respectively. The results observed for WPE of *G. turuturu* were an increase from March to November, except for June where no difference with the control was obtained (Fig. 3a). For *P. palmata* results only

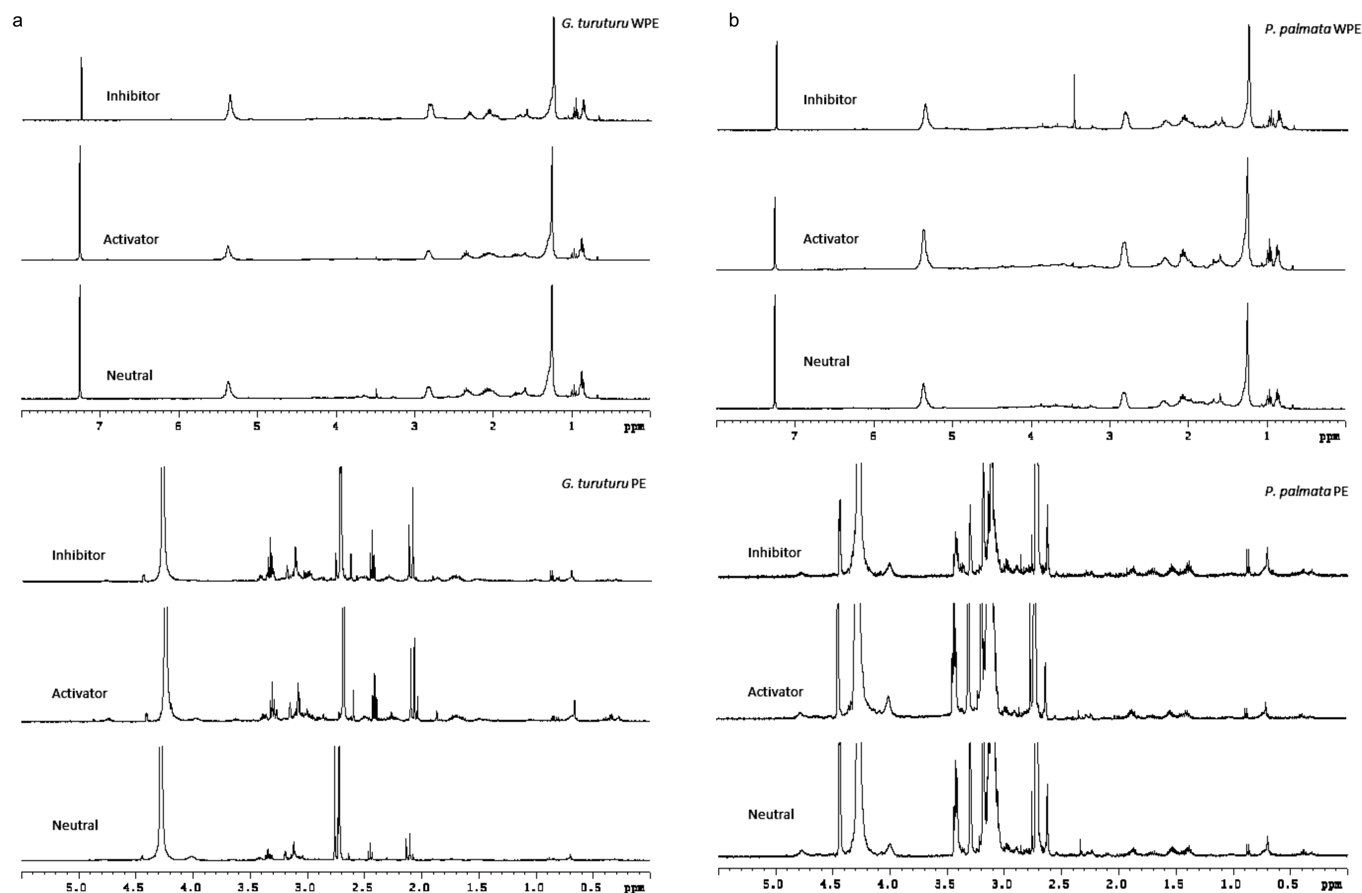

Fig. 4. PE (highly polar extract) and WPE (wide polarity ranges extract) inhibitor, activator and neutral extracts of *Grateloupia turuturu* (a) and *Palmaria palmata* (b) analyzed using ^{1}H NMR.

January, February and August WPE showed an increase in lag time with an average of 9.72% (Fig. 3b).

3.4 Nuclear magnetic resonance analysis on macroalgal extracts

^{1}H NMR spectra comparing inhibitor, activator and neutral samples obtained for PE and WPE of *G. turuturu* are shown in Figure 4a. All the profiles were similar, with only the neutral PE spectra of *G. turuturu* showing less intense peaks.

P. palmata ^{1}H NMR spectra obtained from inhibition, activation and no effect PE and WPE are shown in Figure 4b. Spectra were very similar for all profiles regarding PE. Polar inhibitor spectra showed little variation in some peak intensities compared with two others. Concerning WPE *P. palmata* spectra, a peak could only be observed in the extract presenting inhibition activity. Even so, this observation was not sufficient to explain the variations in growth of bacterial strain ORM4.

4 Discussion

The present study confirmed the presence of active substances in extracts from the seaweed studied that have the potential to be used as a substitute for commercial antibiotics in aquaculture. The study reports the activity of PE and WPE from *G. turuturu* and *P. palmata*. The response of PE (methanol/water) was found to be more effective than WPE (methanol/dichloromethane) on the growth of bacterial *V. harveyi*, in agreement with the research of Dubber et al. (2008). In previous studies investigating antibacterial and antifouling effects of marine seaweed, the former were detected in WPE (Hellio et al. 2001; Bansemir et al. 2006; Dubber and Harder 2008). In this study, the addition of WPE of *G. turuturu* and *P. palmata* in the culture medium resulted in an enhancement of the growth of *V. harveyi*. March samples of *P. palmata* showed an improved bacterial growth of 6.3% in comparison with the control strain ORM4. The rest of the WPE showed a small increase with an average of 1.3% for *G. turuturu* and 1.9% for *P. palmata* respectively.

In a recent investigation (Garcia-Bueno et al. 2014) demonstrated the antibacterial activity of water-soluble extracts from the red alga *G. turuturu* against the same strain, ORM4. In the present work, the inhibition is weaker than that presented by water-soluble extracts of *G. turuturu* with an average of 16.0%. *P. palmata* exhibited the highest inhibition in March and September with values of 7.9% and 5.9% against *V. harveyi* respectively. In this study, *G. turuturu* PE showed the best results between November and March, with a peak in March of 7.9%, thus these seasonal results accord with those

observed for *G. turuturu* water-soluble extracts where April, May and June presented the highest growth inhibition (Garcia-Bueno et al. 2014).

Identification by NMR of the effective compounds revealed that the major peaks present in the spectra are not the cause of the very large variation in inhibition. This is probably due to other minor compounds not visible in the spectra and thus not characterized by our NMR analysis. Same characterization had been already done regarding water soluble extracts of the same sample and even if some variations had been observed, they could not be related to the antibacterial activity (Garcia-Bueno et al. 2014). Hence, this characterization could only provide a fingerprint of the extracts. As the macroalgal extracts are not finely characterized, further research is needed to purify and identify the active molecules responsible for this activity. Antibacterial activity in the same algal species may vary in different geographic areas and could be an indicator of the capability of seaweed to synthesize bioactive metabolites. The intraspecific variability in the production of metabolites in seaweed is seasonal, which can explain the differences shown by the biological tests. This study has achieved an annual monitoring of the effects of polar and non-polar compounds on the growth of *Vibrio harveyi* and provided interesting results on the potential of natural molecules synthesized by *P. palmata* and *G. turuturu*, of interest to the aquaculture sector.

In conclusion, the above data provide a new way of valorizing these red algae, especially *G. turuturu*. A large amount of biological material is needed to use natural products in aquaculture disease, which may be an important constraint. In the case of *G. turuturu*, this is not a problem because this species is proliferative and very abundant on the French coast, as has already been described (Simon et al. 2001). In the future, it would be interesting to characterize the metabolites in the antibacterial activity of *G. turuturu* to confirm whether they play a multiple ecological role. Indeed, the antibacterial activities highlighted in this paper and in a recent study (Garcia-Bueno et al. 2014), make *Grateloupia turuturu* a promising model in the search for new solutions for disease in aquaculture.

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