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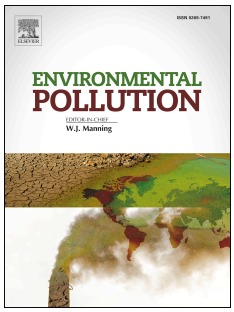

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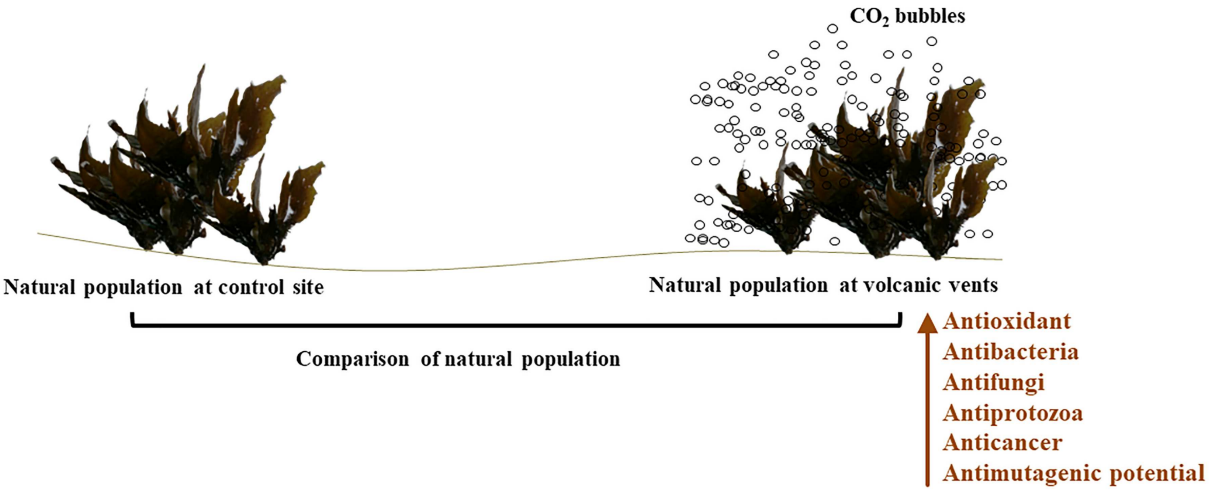

Ocean acidification affects biological activities of seaweeds: A case study of *Sargassum vulgare* from Ischia volcanic CO_2 vents

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Abstract

We utilized volcanic CO_2 vents at Castello Aragonese off Ischia Island as a natural laboratory to investigate the effect of lowered pH/elevated CO_2 on the bioactivities of extracts from fleshy brown algae *Sargassum vulgare* C. Agardh. We analysed the carbohydrate levels, antioxidant capacity, antibacterial, antifungal, antiprotozoal, anticancer properties and antimutagenic potential of the algae growing at the acidified site (pH ~ 6.7) and those of algae growing at the nearby control site Lacco Ameno (pH~8.1). The results of the present study show that the levels of polysaccharides fucoidan and alginate were higher in the algal population at acidified site. In general, extracts for the algal population from the acidified site showed a higher antioxidant capacity, antilipidperoxidation, antibacterial, antifungal, antiprotozoal, anticancer activities and antimutagenic potential compared to the control population. The increased bioactivity in acidified population could be due to elevated levels of bioactive compounds of algae and/or associated microbial communities. In this snapshot study, we performed bioactivity assays but did not characterize the chemistry and source of presumptive bioactive compounds. Nevertheless, the observed improvement in the medicinal properties of *S. vulgare* in the acidified oceans provides a promising basis for future marine drug discovery.

Capsule: Elevated DIC improves bioactivity in fleshy brown macroalgae at natural volcanic CO_2 off Ischia Island, Italy.

Keywords: Climate change; Bioactivity; Marine algae; Natural acidification; Marine drugs

1. Introduction

Volcanic CO_2 vents off Ischia Island in the Mediterranean Sea offer a unique setting to investigate the effects of ocean acidification (OA) on marine organisms (Foo et al., 2018). These vents are in particular useful to investigate benthic biota with less mobility and limited dispersal of propagules or larvae and that have occupied these areas for years (Foo et al., 2018). Venting activities have created a pH gradient along the rocky shores. Decreasing pH has caused a reduction in calcifying species (Hall-Spencer et al., 2018). At the most acidified site (pH ~6.7), calcareous algae has disappeared and species richness is decreased by 72% in comparison to control sites higher in the in the pH gradient (pH~8.1). The acidified site is dominated by fleshy seaweeds, in particular the brown algae *Sargassum vulgare* (Hall-Spencer et al., 2008; Porzio et al., 2011; Gao et al., 2019). This natural CO_2 gradient provides a unique set up to investigate how lowered pH/elevated dissolved inorganic carbon (DIC) may affect bioactivity of *S. vulgare* and associated microorganisms.

Theoretically fleshy seaweed should benefit from elevated carbon dioxide (CO_2) and bicarbonate (HCO_3^-) as it can be used for photosynthesis (Koch et al., 2013; Mackey, 2015). However, growing evidence from the literature suggests that the response of seaweeds to ocean acidification depend on which DIC source it uses as well as mechanisms of DIC acquisition (Koch et al., 2013). There are three major DIC in seawater: CO_2 (~1%), carbonate (CO_3^{2-} ~8%) and bicarbonate (HCO_3^- ~91%), of which only CO_2 and HCO_3^- can be used for photosynthesis (Roleda and Hurd, 2012). Though CO_2 can diffuse inside the cell, its low concentration and low diffusion rate in seawater limits its availability for photosynthesis. Hence, almost 95% of marine macrophytes have developed efficient carbon concentrating mechanisms (CCM) i.e. uptake of HCO_3^- from the surrounding water inside the cell for photosynthesis (Koch et al., 2013). CCMs includes the active uptake of HCO_3^- involving P-type H^+ ATPase pump, direct uptake of HCO_3^- through plasmalemma-located anion exchange (AE) proteins, and involvement of external and internal carbon anhydrase (CA)

enzyme activity (Fernández et al., 2014). External CA dehydrates $HCO3^-$ into CO_2, which diffuses inside the cell where internal CA interconvert CO_2 to HCO_3^- to maintain internal pH and avoid CO_2 leakage (Fernández et al., 2018). Furthermore, chloroplast CA ensures the supply of CO_2 to the active site of RuBisCO for carbon fixation (Fernández et al., 2018). Thus, seaweeds lacking CCMs are likely to benefit from additional aqueous CO_2 (Kübler et al., 1999), but even seaweeds with CCMs may benefit from excess aqueous CO_2 if they can simultaneously use CO_2 and HCO_3^- under ambient and ocean acidification conditions to reduce the energy costs (Fernández et al., 2015; Wu et al., 2008). In contrast to photosynthesis, other physiological processes including ion homeostasis, respiration, nutrient uptake, and enzymatic activities are inhibited by ocean acidification conditions (Fernández et al., 2015; Gutow et al., 2014; Hofmann et al., 2013; Roleda et al., 2012).

Around the world, seaweeds are utilized as food and medicines since prehistoric times (Mouritsen et al., 2018). They are rich sources of proteins, vitamins, lipids, sugars, dietary fibre, and nutrients essential for human nutrition (Perumal et al., 2019). They are also known for their importance in pharmacology, being a rich source of bioactive compounds against bacteria, fungi, virus, protozoa, and various cancers (Hao et al., 2019; Smit et al., 2004). Because of these benefits, seaweeds are gradually gaining importance as healthy food (Cherry et al., 2019). The algal genus *Sargassum* (Ochrophyta, Phaeophyceae), in particular has anticancer, antibacterial, antifungal, anti-viral, anti-inflammatory, anticoagulant, antioxidant, hypoglycaemic, hypolipidemic, antimelanogenic, antiboneloss, hepatoprotective and neuroprotective activities (Ghania et al., 2017; Liu et al., 2012; Pramesti et al., 2017; Torres et al., 2014). Bioactivities of the seaweeds are attributed to primary and secondary metabolites e.g. meroterpenoids, phlorotanins, polysaccharides, phytosterols, polyunsaturated fatty acids and glycolipids (Dore et al., 2013; Hao et al., 2019; Kolsi et al., 2017; Liu et al., 2012; Sousa et al., 2008; Torres et al., 2007).

The effects of global climate change mainly through ocean acidification (OA) and warming have the potential to alter the carbon physiology of seaweeds and levels of metabolites, including bioactive compounds and nutritional value (Yousefzadi & Miejalili, 2017). Increased CO_2 concentration increase level of sugars, essential amino acids, phenolic, and vitamins, which in-turn improves total antioxidant capacity, and bioactivity against several microbes and cancer cells (Al Jaouni et al., 2018; Ghasemzadeh, 2011; Idso et al., 2000; Jaafar et al., 2012; Saleh et al., 2018). So far only a handful of studies have reported nutritional changes in seaweed under acidified conditions. The level of proteins and lipids increased in the green algae *U. rigida* in response to ocean acidification (Gao et al., 2017). OA also increased the colour and flavour of red algae *Pyropia yezoensis* by increasing the levels of pigments, and amino acids (Gao et al., 2019). Several seaweeds exposed to OA conditions showed varying responses on the levels of phenols and flavonoid compounds (Arnold et al., 2012; Betancor et al., 2014; Celis-Plá et al., 2015; Celis-Plá et al., 2017; Kumar et al., 2018). Moreover, the severity of the impact of ocean acidification on seaweeds depends on other environmental parameters including nutrients (Gao et al., 2018). It is therefore important to better understand how OA will impact the metabolite and bioactive properties of seaweeds.

In the present study, we investigated bioactive properties of *S. vulgare* growing naturally at the most acidified site of the volcanic CO_2 vents and control site off the Ischia Island, Italy. Previous studies have shown *S. vulgare* can utilize both CO_2 and HCO_3^- as a carbon source for photosynthesis and may therefore not be carbon limited (Kumar et al., 2017a). Nevertheless, this alga showed significant increase in photosynthetic performance (relative electron transport rate and saturating irradiance) upon short term exposure to elevated DIC (Kumar et al., 2017a). They exhibit high plasticity to elevated DIC/lowered pH conditions by activating molecular and physiological strategies to grow in the acidified water (Kumar et al., 2017a, Kumar et al., 2017b). Primary and secondary metabolites of *S. vulgare* were strongly

influenced, in particular the level of soluble carbohydrates were increased (Kumar et al., 2018; Porzio et al., 2017). Because soluble carbohydrates can act as precursors for bioactive metabolites, the current study is aimed to investigate the possibility that OA increase bioactive properties of *S. vulgare.* Therefore, we analysed the levels of polysaccharides fucoidan and alginate and antioxidant capacity, antibacterial, antifungal, antiprotozoal, anticancer properties and antimutagenic potential of the algae growing at the acidified site (pH ~ 6.7) and compared to those of algae growing at the nearby control site Lacco Ameno (pH~8.1). The present work represents a snapshot study where we conducted bioactivity assays but did not characterize the chemistry and source of presumptive bioactive compounds.

2. Materials and Methods

2.1 Sampling locations and seaweed collection

Sargassum vulgare (Class Phaeophycea, Order Fucales, Family Sargassaceae) was collected from two different locations along Ischia Island, Italy: Castello Aragonese (40°43.87N, 013°57.78E) – the acidified site, and Lacco Ameno - the control site (40°45.35N, 013°53.13E, Fig. 1). Castello Aragonese site is characterized by acidified seawater due to continuous emission of CO_2 (90.1–95.3%) from underwater volcanic vents, for 2 millennia (Lombardi et al., 2011), with no detectable harmful sulfur gases. Though the venting activities are quite variable, on average pH values, where *S. vulgare* occurs are constantly around 6.72 (Porzio et al., 2011). Lacco Ameno almost 6 km northwest from Castello Aragonese, was used as a control site as algae are growing here at the same hydrodynamic and physical conditions but at current pH values (8.1). In both locations, *S. vulgare* grows at <1 m depth, in a sheltered bay with similar geographical exposure (Kumar et al., 2017b). Salinity fluctuates between 37 and 38.5 psu at both the sites, which is typically the case in Tyrrhenian coastal waters (d'Alcalà et al., 2004; Lorenti et al., 2005). Temperature varies between 14 - 28 °C during the year and shows no significant difference between the

sites (Kumar et al., 2017b). Photosynthetically active radiation (PAR) irradiances obtained by the PAR spherical sensor QSI-140 (Biospherical Insturments, San Diego, USA) as Kd coefficient from surface to 1 m depth was reported to be similar (0.18 and 0.20 at acidified and control site, respectively) (Lorenti & De Falco 2004). Therefore, we believe that depth, salinity, temperature, and light were not confounding factors. Carbon chemistry of acidified site has been assessed during the last 10 years (Calosi et al., 2013; Cigliano et al., 2010; Hall-Spencer et al., 2008; Kroeker et al., 2013; Kumar et al., 2017b; Ricevuto et al., 2014), and control site assessed during Apr-May 2013 and described by Ricevuto et al., (2015) is summarized in Table A.1 in Appendices. Total alkalinity was similar at both sites, while pCO_2, HCO_3^- , and DIC contents were higher at acidified site, and CO_3^{2-} was higher at the control site (Ricevuto et al., 2014). Nutrient levels at both sites varied, e.g. nitrate was similar in both sites, while phosphate was almost 40% lower and potassium was 27% higher in acidified waters than control site (Kumar et al., unpublished).

Seaweed thalli were collected at both sites on the same day (March, 2014 at midday) at the same time (11AM-12 PM) to avoid influence of unpredictable and difficult to measure environmental factors. A total of 9 individual thalli of similar frond length (8-10 cm) were handpicked along a 15 m coastal stretch to cover natural variability of the local population at each site. Upon collection, the algae were maintained in seawater from respective sites and transported to the laboratory within an hour. The algal tissues were washed with filtered sea water to remove visible epiphytes, flash frozen in liquid nitrogen and immediately stored at -80°C until further analysis. Determination of fucoidan, alginate and biological activity assessment, were performed on at least three independent thalli from both the locations, thus creating three biological replicates from each site.

2.2 Determination of Fucoidan and Alginate

Fucoidan in the algal tissue was determined according to Turan et al. (2017). Briefly, 50 mg of algal tissue was ground using a MagNALyser (Roche, Vilvoorde, Belgium) and 2

ml of 0.2 N HCl was added and the suspension was heated for 1 h at 70 °C with regular stirring followed by cooling so that particles were allowed to settle. The liquid was filtered, stored and then the retained particles were again extracted using 5 ml of 0.2N HCl. Finally, both liquids were combined together. 1ml of sample extract was mixed with 4.5 ml dilute H_2SO_4 in the ice-water bath for ~1 min followed by boiling for 10 min. Upon cooling to room temperature, 0.1 ml of Cysteine solution was added, mixed and incubated at room temperature for 30 minutes. The absorbance in the solution was observed at 396 and 427 nm for fucose content which was multiplied by a factor of 2 to obtain fucoidan content.

For alginate determination, the algal tissue (50 mg) was extracted in 2 ml 0.1 M sodium citrate and centrifuged for 30 min at 5000 rpm. To separate alginate, supernatant was treated with 1.5 M hydrochloric acid for 1 h. After precipitation, tubes were centrifuged and the precipitates were mixed with 500 µl citrate buffer, subsequently alkalinised with 500 µl of 0.8N NaOH, finally neutralized after 5 min by adding 60µl of 2.25N citrate. The colorimetric estimation was done by adding 20µl of 1, 9-dimethyl methylene blue (DMMB) in the homogenate, measured at 520 nm and 650 nm and calculated as described by Hallé et al. (1993).

2.3 Biological activity assessments

2.3.1 *In vitro* antioxidant activity

Total antioxidant capacity was determined utilizing ferric reducing antioxidant power (FRAP) and diphenylpicrylhydrazyl (DPPH) assays. 300 mg algal tissue was ground using a MagNALyser (Roche, Vilvoorde, Belgium) and mixed with 3 ml of 80% ice-cold ethanol. The homogenate was centrifuged for 30 min at 14000 rpm at 4 ^{0}C and the supernatant was collected. FRAP assay was performed according to AbdElgawad et al. (2015). Briefly, 20 µl of the ethanol extract was mixed with 180 µl of freshly prepared pre-warmed FRAP reagent (10 mM 2, 4, 6 Tris (2yridyl) s-triazine (TPTZ), 0.3 M acetate and 20 mM $FeCl_3$) and incubated for 30 min at 37 °C. Absorbance of the mixture was measured at 593 nm using a

208 microplate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA). Trolox was
209 used to prepare standard curve and using slope and intercept, antioxidant concentration was
210 determined which was expressed as μMTrolox equivalent g^{-1} frozen weight.

211 DPPH assay was performed according to Al Jaouni et al. (2015). Briefly, algal
212 extract was prepared by grinding 300 mg of tissue in 3 ml of 80% ice-cold ethanol and mixed
213 with 0.5 ml of DPPH solution (0.25 mM in 95% ethanol, followed by incubation at room
214 temperature for 30 min. Thereafter, absorbance of the mixture was measured at 517 nm to
215 calculate the inhibition percentage.

216 **2.3.2 Antilipid peroxidation assay**

217 The degree of lipid peroxidation was measured by the thiobarbituric acid reactive
218 substance (TBARS) method, using egg yolk homogenate as lipid rich substrate (Ohkawa et
219 al., 1979). Algal extract was prepared by grinding 100 mg algal tissue using a MagNALyser
220 (Roche, Vilvoorde, Belgium) and mixed with 2 ml of 90% ethanol. Algal extract and egg
221 homogenate (0.5ml of 10% v/v) were mixed with 15 mM ferrous sulphate (to induce lipid
222 peroxidation), after incubation for 30 min, 1.5 ml of 10% TCA was added. Following that,
223 the mixture was transferred to a tube containing 1.5 ml of 0.67% TBA and boiled for 30
224 min. The chromogen formed was measured at 535 nm.

225 **2.3.3 Antimicrobial activities**

226 Algal extract was prepared by mixing 100 mg of grinded tissue in 90% ethanol and
227 then evaporated using centrifugal rotor evaporator. The antibacterial activity was determined
228 using the disc diffusion method (7.5 mg of the algal ethanol extract per filter paper disc) on
229 Muller Hinton agar (MHA inoculated with 100 μl of a suspension containing 10^8 CFU/ml of
230 bacteria) (Selim et al., 2013). The plates were incubated at 35 °C for 24 h and the activity was
231 expressed as the diameter of the inhibition zone in mm. Negative controls were prepared
232 using the disc containing ethanol only.

233 **2.3.4 Antiprotozoal activities**

In vitro assessment of the antiprotozoal activity was performed according to Räz et al. (1997). *Trypanosoma cruzi* and *Leishmania donovani* were cultured in Minimum Essential Medium with additional supplements. Algal extract prepared in 90% ethanol and then dried in rotor evaporator (5 mg/ml) were used against *T. cruzi* and *L. donovani* and compared with control having no algal extract. The results were expressed as percentage of reduction in parasite load in treated and control conditions.

2.3.5 Antimutagenicity assay (Ames test)

An Ames assay (Maron and Ames, 1983) was performed to measure the antimutagenicity of β-D-glucogallin. Two strains of *Salmonella typhimurium* (TA98 and TA100) were used for the test. The assay was conducted in pre-incubation and co-incubation mode using 100 µl of overnight grown bacterial cultures with the addition of algal extract and mutagenic agents in triplicates. The algal extract was prepared by grinding 100 mg tissue in ethanol/ water (1:1 v/v) followed by filtration using 0.45 µm filter (Millipore, USA). Overnight bacterial culture, mutagen, algal extract and phosphate buffer were added to agar containing histidine/biotin (0.5 mM). As a positive control, Mutagen 4-nitro-o-phenylenediamine (NPD) and NaN_3 were used for TA98 and TA100 and ethanol/water (1:1, v/v) was used as negative control instead of algal extract. The mixture was poured onto minimal glucose plates. Histidine independent revertants colonies and viable cells were scored on plates after incubation at 37°C for 48 or 72 h. After incubation for 48 hrs at 37 °C, the number of revertants per plate was counted. The inhibition percent of mutageneic activity was calculated by observing the difference in the number of histidine revertants induced by mutagen alone, mutagen in the presence of the extract and extract alone**.**

2.3.6 Anticancer activities

The anticancer potential of the algal extract was assessed against 4 human cancer cell lines: hepatocellular carcinoma (HepG2), colon carcinoma (Colo205), embryonic kidney adenocarcinoma (293), and urinary bladder carcinoma (T24P). Algal extract prepared in 90%

ethanol and then dried in rotor evaporator. To assess the activity of the algal extract against these cell lines, cells were grown in Dulbecco's Modified Eagle Medium (DMEM, fetal calf serum, Na-pyruvate, 1mg/0.1 ml streptomycin, 1U/ 0.1ml penicillin) in the presence and absence of algal extract (2 mg/ml) at 37°C and 5% CO_2. Then, cells were harvested through trypsinization (Saleh et al., 2018). Cell viability was determined using a Cell Titer-Blue reagent as described by Solowey et al. (2014) and the results were expressed as percentage dead cells.

2.4 Statistical Analysis

Statistical analyses were performed using SPSS (v21 SPSS Inc, Chicago, IL, USA). The data were checked for normality by visual inspection and homogeneity of variance by Levene's test before applying independent sample t-test on the mean data values to determine significant differences between control and acidified site samples.

3. Results

To investigate the effect of ocean acidification on the metabolic composition of *S. vulgare*, we analysed the thalli growing naturally under acidified conditions and control conditions. The concentration of fucoidan was significantly higher in algae grown at the acidified site (46.71 ± 1.41 mg/FrozendryW) compared to those from the control site (34.96 ± 0.40 mg/gFrozendryW). Similarly, alginate content was also found to be higher in algae at the acidified site (93.3 ± 2.98 mg/gFrozendryW) than in those growing at the control site (74.1 ± 2.02, Fig. 2).

When compared to algae from control site, algae from the acidified site showed improved diphenylpicrylhydrazyl (DPPH) antioxidant scavenging activities (25.40 ± 0.23% vs 15.51 ± 0.11%, $P<0.0001$), however, no significant differences were observed in ferric reducing antioxidant power (FRAP) antioxidant and anti-lipid peroxidation activities (Fig. 3A, B, C).

Under elevated dissolved CO_2 conditions at the volcanic vents of Ischia, *S. vulgare* showed a general increase in the bioactivity against bacteria, fungi, protozoa, and cancer cells. The significant increase in antibacterial activity was recorded for *Pseudomonas aeruginosa* (p=0.00013), followed by *Klebsiella pneumonia* (p=0.008), *Escherichia coli* (p=0.003), *Proteus vulgaris* (p=0.002), *Serratia marcescens* (p=0.04), *Streptococcus sp.* (p=0.002). Only for *S. aureus*, no significant change (p=0.053) in the activity was observed (Fig. 4). The antifungal activities were significantly higher in the acidified samples for *Aspergillus parasiticus* (p=0.0003), and *Fusarium oxysporum* (p=0.008), however, no difference in activity was observed against *Candida albicans* (p=0.53)*, Penicillium expansum* (p=0.07)*, and Trichoderma harzianum* (0.559). The antiprotozoal activities showed that algal extracts from the acidified site exhibited significant lower values for IC_{50} than from control sites against *Trypanosoma cruzi* (p=0.001), and *Leishmania donovani* (p=0.0024) (Fig. 5). The anticancer activities against all four cancer cell lines, HepG2 (p=0.0001), Colo205 (p=0.0001), T24P (p=0.002), and 293 cells (0.0002) tested were more pronounced in the algal extracts from the acidified site (Fig. 6), In the antimutagenicity assays, performed with TA98 and TA100, the algal extract from the acidified site showed higher percent inhibition of revertants in TA 98 (p= 0.001) and TA100 (p=0.03) than algal extracts from the control site (Fig. 7). The output of t-test is given in Table A. 2 as appendices.

4. Discussion

In this study, we tested the hypothesis that increased carbohydrate availability in *S. vulgare* exposed to elevated DIC at Ischia CO_2 vents can increase the bioactivity against microbial pathogens and cancer cells. Our findings supporting this hypothesis includes the elevated levels of polysaccharides fucoidan and alginate and increased antioxidant capacity, antilipidperoxidation, antibacterial, antifungal, antiprotozoal, anticancer activities and antimutagenic potential in algal homogenate from the population at acidified site compared to the control population.

4.1 Elevated dissolved CO_2 increases the content of polysaccharides

Anionic polysaccharide alginates and fucoidan measured in the present study are major constituents of the brown algal cell wall. The elevated level of these sugars could be due to increased photosynthesis in elevated DIC conditions in the short term despite the presence of CCM in *S. vulgare* (Kumar et al., 2017a). Since, *S. vulgare* is a mixed CO_2 and HCO_3^- user, elevated CO_2 might have down regulated direct or indirect HCO_3^- acquisition and the energy saved by bypassing anhydrase activity could be used for enhanced growth (Fernández et al., 2015) in the volcanic CO_2 seeps. As such, there is no clear mechanistic understanding regarding CO_2 concentrations and cell wall sugars, however, it is reported that higher CO_2 concentrations can increase the activity of enzymes involved in the synthesis of cell wall uronic acid, thus increasing synthesis of cell wall polysaccharide (Cheng et al., 2015). In our previous molecular study, we found that genes encoding for the enzymes related to the formation and structures of cell walls and carbon storage were expressed at higher levels in *S. vulgare* at elevated DIC conditions than in control conditions (Kumar et al., 2017b). In other seaweeds, high carbohydrate concentration and improved photosynthetic rates were also reported under elevated CO_2 (Figueroa et al., 2014; Suárez-Álvarez et al., 2012).

4.2 Elevated dissolved CO_2 increases bioactivity

The bioactivity of seaweeds is attributed to its primary and secondary metabolites including fucoxanthin, alginates, laminarins, fucans, cellulose as well as unsaturated fatty acids and essential amino acids (Dore et al., 2013; Kolsi et al., 2017; Liu et al., 2012; Sousa et al., 2008; Torres et al., 2007). Acting as a substrate for photosynthesis, elevated CO_2 (up to certain physiological limit) has the potential to enhance carbon assimilation, thus improving the accumulation of non-structural carbohydrates and their breakdown through dark reaction (Leakey et al., 2009). This might provide increased levels of precursors and energy for the synthesis of different bioactive secondary metabolites (Kumar et al., 2018; Saleh et al., 2018).

In the current study, elevated CO_2 resulted in increased levels of polysaccharide fucoidans and alginates which are known to have antimicrobial and anticancer bioactivity (Costa et al., 2011; Jun et al., 2018; Sousa et al., 2008; Wu et al., 2016). In *S. vulgare* increased levels of fucoidans and alginates has been related to improved antimicrobial and anticancer activity (Wu et al., 2016) as well as antiprotozoal activity (Armeli Minicante et al., 2016). *S. vulgare* at acidified site has increased the level of photosynthetic pigments (Kumar et al., 2017a), which may be linked with improved antimutagenicity as pigments either suppress the metabolic activation of mutagens or inhibit activated mutagens (Osuna-Ruiz et al., 2016; Hayatsu et al., 1993).

We demonstrated that the antioxidant capacity of algal extracts varied depending on the assay used. With DPPH radical scavenging method, the antioxidant potential of algae of the acidified site was higher compared to control population, whereas no difference was observed with FRAP assay. This is not surprising considering that the two assays measured different parameters. Whereas the FRAP method is based on the ability of antioxidant to reduce ferric ion complex, the DPPH method measures the reduction of an organic radical by the capability of the antioxidant to donate a hydrogen (Csepregi et al., 2016). Antioxidant capacity in seaweeds is mostly attributed to polyphenols (Machu et al., 2015). Even though lowered levels of polyphenols and flavonoid were found in the algae at the acidified site in comparison to control site (Kumar et al., 2017a; Kumar et al., 2018), it is interesting that DPPH radical scavenging is higher in these algae. *Sargassum* contains more than 280 metabolic compounds including, pigments, primary and secondary metabolites with bioactive properties (Liu et al., 2012). Therefore, our result suggests that other bioactive molecules which are not yet identified may be contributing towards antioxidant activity of *S. vulgare* (Kumar et al., 2018). Another reason for dissimilar results between metabolites and bioactivity may be due to different sampling season. Moreover, temporal and spatial variation has been reported in biochemical composition and metabolite contents of seaweeds (Graiff et

al., 2015; Pavia et al., 2018; Roleda et al., 2019; Véliz et al., 2018). Seasonal variation in bioactivity of the genus Sargassum has been reported (Praioboon et al., 2015). It is also important to note that recent studies on seaweed associated microorganisms (holobiont) indicated that at least part of bioactivity observed in the present study could be from associated microbial communities (Singh and Reddy, 2014). Also, associated microbial communities are reported to be site and tissue specific in the seaweeds (Serebryakova et al., 2018; Campbell et al., 2011), and microbial community structure changes under acidified conditions (Aires et al., 2018). Hence, the involvement of microbes in these bioactivities cannot be ruled out in the present study. In future, manipulative laboratory experiments will be conducted by bringing samples from both sites and expose to ocean acidification to eliminate the role of associated microorganisms and to ensure the observed bioactivity is due to elevated DIC or lowered pH or any other factors.

In conclusion, the present work on *S. vulgare* expands our understanding of the bioactivity derived from seaweeds under a future climate scenario. As an outcome, this preliminary study can be seen as a positive sign for marine drug discovery considering the improvement in the medicinal properties of *S. vulgare* or associated microbial communities in the acidified oceans. It would be interesting to conduct a larger scale metabolite profiling analysis to understand which compounds contribute towards algal bioactivity, source of bioactive compounds (whether seaweed directly or associated microbes) and to investigate biochemical pathways leading to varying metabolite levels that are activated by ocean acidification.

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Figure Legends

Fig. 1. Study sites around the coast of the Ischia Island, Italy: Castello Aragonese (CO_2 vents; acidified site) and Lacco Ameno (control site; Image generated through QGIS version 2.12.2).

Fig. 2. Concentration of Fucoidan and Alginate in the extract of *S. vulgare* collected at the control and the acidified site. Values are mean ± SE, n= 3, ***p <0.001.

Fig. 3. *In vitro* antioxidant activity of the extract of *S. vulgare* collected at the control and the acidified site, A: antioxidant capacity using FRAP (ferric reducing/antioxidant power) assay; B. antioxidant activity using DPPH (diphenylpicrylhydrazyl) radical scavenging assay; C. anti-lipid peroxidation assay. Values are mean ± SE, n=3, *** p < 0.001.

Fig. 4. Antimicrobial activity of *S. vulgare* collected from the control and acidified site against human pathogens. Values are mean ± SE, n=3, *p< 0.05, ** p< 0.01, ***p< 0.001.

Fig. 5. Antiprotozoal activity of *S. vulgare* collected from the control and acidified site against *T. acruzi* and *L. donovani*. Values are mean ± SE, n=3, *p< 0.05, ** p< 0.01, ***p< 0.001.

Fig. 6. Anticancer activity of *S. vulgare* collected from the control and acidified site against four cancer cell lines hepatocellular carcinoma (HepG2), colon carcinoma (Colo205), embryonic kidney adenocarcinoma (293), and urinary bladder carcinoma (T24P). Values are mean ± SE, n=3, *p< 0.05, ** p< 0.01, ***p< 0.001.

Fig. 7. Antimutagenic activity of *S. vulgare* collected from control and acidified site against *S. typhimuriam* TA 98 and TA 100. Values are mean ± SE, n=3, *p< 0.05, ** p< 0.01, ***p< 0.001.

Appendices Table A. 1: Summary of water chemistry at the control site along Lacco Ameno and at the acidified site along Castelo Aragonese, Ischia Island, where *S. Vulgare* occurs.

[1] Hall-Spencer *et al.*, 2008; [2] Cigliano *et al.*, 2010; [3]Frieder *et al.*, 2013; [4] Kroeker*et al.*, 2011; [5]Calosi *et al.*, 2013; [6]Ricevuto *et al.*, 2014. *Point value on 29th sept 2014, obtained from Dr. Gil Rilov.

	Acidified site, Castello Aragonese							Control stie, Lacco Ameno
Parameters	**2007 (Apr-May)[1]**	**2008 (Apr-May)[2]**	**2009 (Sep)[3]**	**2010(Sep-Oct)[4]**	**2011 (May-June)[5]**	**2013 (Apr-May)[6]**	**2014 (Aug-Sep)**	**2013 (Apr-May)[6]**
Salinity	38	38	38.1±0.04	37±0.2	37.14±0.05	37.11±0.6	37±0.5	36.9±0.7
Temperature	18.5	19.44±1.98	25±0.63	17.5±2.8	23.31±0.12	19.02±1.21	15±0.4	19.10±1.23
pH	6.57±0.06	7.32±0.29	6.83±0.42	6.59±0.51	7.07±0.07	7.14±0.4	6.80±0.09	8.13±0.05
TA(µequi/kg)	2500	2566±12.98	2578±6.1	2563±13	2673.73±12.57	2597.40±75	2608*	2612.93±26.5
pCO_2(µatm)	20812±2253	3317.80±1744	15099±2997	23989±16638	5337.65±567.54	4266.2±2814	NA	165.8±24.6
HCO_3^- (µmol/kg)	2470±100	2418±113	NA	NA	2565.58±20.72	2518.9±101.4	NA	2067.3±53.9
CO_3^{2-}(µmol/kg)	100±10	62±44.94	NA	NA	38.30±2.93	31±40.1	NA	217.2±21.8
DIC(µmol/kg)	3160±80	2585.20±122.68	2564±108	3849±790	2781.21±20.15	2549.92±61.30	NA	2284.55±32.09

Appendices Table A2. T- test statistics summary for bioactivity of S. vulgare from acidified and control site.

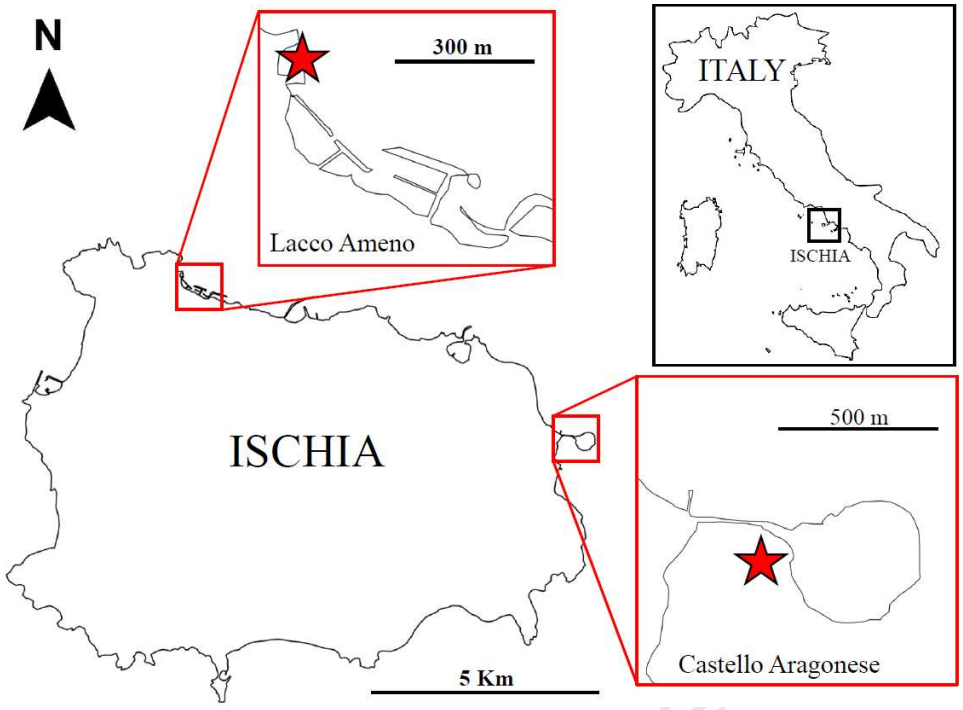
Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Antiprotozoal_TA	Equal variances assumed	0.698	0.435	5.737	6.000	0.001218	2.627	0.458	1.507	3.747
	Equal variances not assumed			5.737	5.166	0.002023	2.627	0.458	1.461	3.793
Antiprotozoal_LD	Equal variances assumed	1.234	0.309	3.007	6.000	0.002379	1.460	0.485	0.272	2.647
	Equal variances not assumed			3.007	5.163	0.028679	1.460	0.485	0.224	2.695
Antibac_Strepto	Equal variances assumed	1.797	0.229	-2.926	6.000	0.002643	-2.164	0.740	-3.974	-0.354
	Equal variances not assumed			-2.926	3.966	0.043440	-2.164	0.740	-4.224	-0.104

Antibac_E.coli	**Equal variances assumed**	**0.636**	**0.456**	**-4.670**	**6.000**	**0.003432**	**-5.774**	**1.236**	**-8.799**	**-2.748**
	Equal variances not assumed			**-4.670**	**5.034**	**0.005392**	**-5.774**	**1.236**	**-8.945**	**-2.602**
Antibac_Serretia	**Equal variances assumed**	**2.029**	**0.204**	**-2.514**	**6.000**	**0.045649**	**-2.991**	**1.190**	**-5.902**	**-0.080**
	Equal variances not assumed			**-2.514**	**3.542**	**0.073804**	**-2.991**	**1.190**	**-6.469**	**0.487**
Antibac_S.aureus	**Equal variances assumed**	**0.029**	**0.871**	**-2.402**	**6.000**	**0.053133**	**-4.043**	**1.683**	**-8.162**	**0.075**
	Equal variances not assumed			**-2.402**	**5.778**	**0.054716**	**-4.043**	**1.683**	**-8.200**	**0.114**
Antibac_K.pneumoniae	**Equal variances assumed**	**0.153**	**0.709**	**-3.862**	**6.000**	**0.008338**	**-4.955**	**1.283**	**-8.094**	**-1.816**
	Equal variances not assumed			**-3.862**	**5.421**	**0.010143**	**-4.955**	**1.283**	**-8.177**	**-1.733**
Antibac_P.vulgaris	**Equal variances assumed**	**0.500**	**0.506**	**-5.044**	**6.000**	**0.002347**	**-6.211**	**1.231**	**-9.223**	**-3.198**
	Equal variances not			**-5.044**	**4.708**	**0.004671**	**-6.211**	**1.231**	**-9.436**	**-2.986**

	assumed									
Antibac_P.aeruginosa	**Equal variances assumed**	**0.001**	**0.976**	**-8.662**	**6.000**	**0.000131**	**-4.268**	**0.493**	**-5.474**	**-3.062**
	Equal variances not assumed			**-8.662**	**5.998**	**0.000131**	**-4.268**	**0.493**	**-5.474**	**-3.062**
Antifungal_C.albicans	**Equal variances assumed**	**0.947**	**0.368**	**-0.665**	**6.000**	**0.530621**	**-1.123**	**1.688**	**-5.254**	**3.008**
	Equal variances not assumed			**-0.665**	**4.316**	**0.539770**	**-1.123**	**1.688**	**-5.678**	**3.432**
Antifungal_A.parasiticus	**Equal variances assumed**	**1.305**	**0.297**	**-7.341**	**6.000**	**0.000327**	**-4.556**	**0.621**	**-6.075**	**-3.037**
	Equal variances not assumed			**-7.341**	**3.610**	**0.002708**	**-4.556**	**0.621**	**-6.355**	**-2.757**
Antifungal_P.expansum	**Equal variances assumed**	**1.437**	**0.276**	**-2.164**	**6.000**	**0.073681**	**-4.606**	**2.128**	**-9.814**	**0.602**
	Equal variances not assumed			**-2.164**	**4.144**	**0.094086**	**-4.606**	**2.128**	**-10.435**	**1.224**
Antifungal_T.harzianum	**Equal variances assumed**	**0.088**	**0.777**	**-0.618**	**6.000**	**0.559508**	**-0.238**	**0.385**	**-1.181**	**0.705**

	Equal variances not assumed			**-0.618**	**5.311**	**0.562335**	**-0.238**	**0.385**	**-1.211**	**0.735**
Antifungal_F.oxysporum	**Equal variances assumed**	**1.661**	**0.245**	**-3.812**	**6.000**	**0.008845**	**-3.969**	**1.041**	**-6.516**	**-1.421**
	Equal variances not assumed			**-3.812**	**5.316**	**0.011116**	**-3.969**	**1.041**	**-6.598**	**-1.340**
Antimutagenic_S.typhimurium TA98	**Equal variances assumed**	**1.543**	**0.261**	**-5.354**	**6.000**	**0.001738**	**-11.767**	**2.198**	**-17.144**	**-6.389**
	Equal variances not assumed			**-5.354**	**3.664**	**0.007500**	**-11.767**	**2.198**	**-18.096**	**-5.437**
Antimutagenic_S.typhimuriumTA100	**Equal variances assumed**	**0.494**	**0.509**	**-2.754**	**6.000**	**0.033137**	**-8.626**	**3.133**	**-16.291**	**-0.961**
	Equal variances not assumed			**-2.754**	**5.212**	**0.038401**	**-8.626**	**3.133**	**-16.581**	**-0.671**
AntiCancer_HepG2	**Equal variances assumed**	**1.981**	**0.209**	**-8.483**	**6.000**	**0.000147**	**-25.583**	**3.016**	**-32.962**	**-18.203**
	Equal variances not assumed			**-8.483**	**3.432**	**0.002028**	**-25.583**	**3.016**	**-34.532**	**-16.633**
Anticancer_Colo205	**Equal variances**	**2.660**	**0.154**	**-8.438**	**6.000**	**0.000151**	**-13.828**	**1.639**	**-17.838**	**-9.818**

	assumed									
	Equal variances not assumed			**-8.438**	**3.445**	**0.002032**	**-13.828**	**1.639**	**-18.682**	**-8.974**
Anticance_293	**Equal variances assumed**	**0.534**	**0.492**	**-5.086**	**6.000**	**0.000225**	**-4.307**	**0.847**	**-6.379**	**-2.235**
	Equal variances not assumed			**-5.086**	**5.063**	**0.003683**	**-4.307**	**0.847**	**-6.475**	**-2.138**
Anticancer_T24P	**Equal variances assumed**	**1.907**	**0.216**	**-5.194**	**6.000**	**0.000202**	**-10.249**	**1.973**	**-15.078**	**-5.421**
	Equal variances not assumed			**-5.194**	**3.439**	**0.009798**	**-10.249**	**1.973**	**-16.099**	**-4.399**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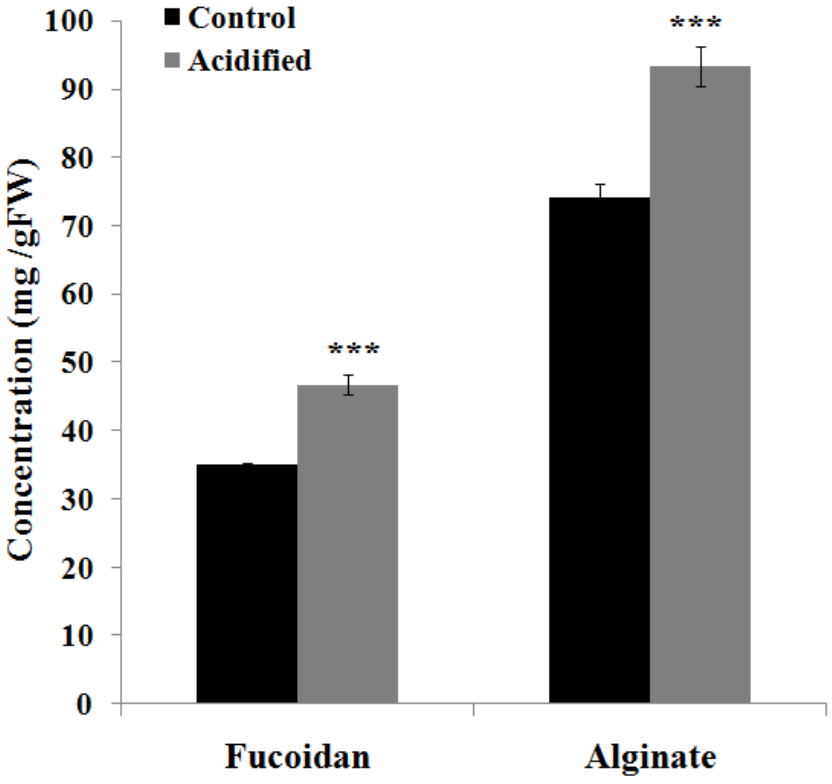

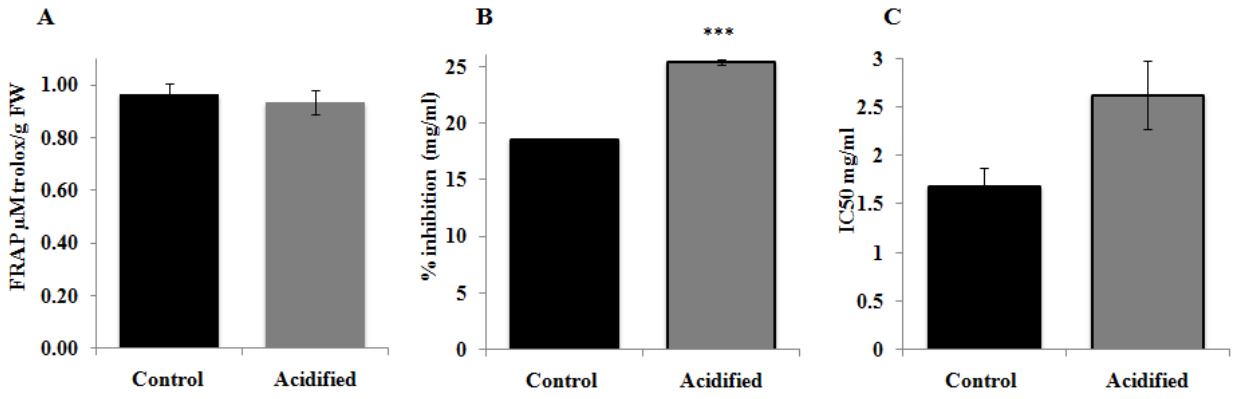

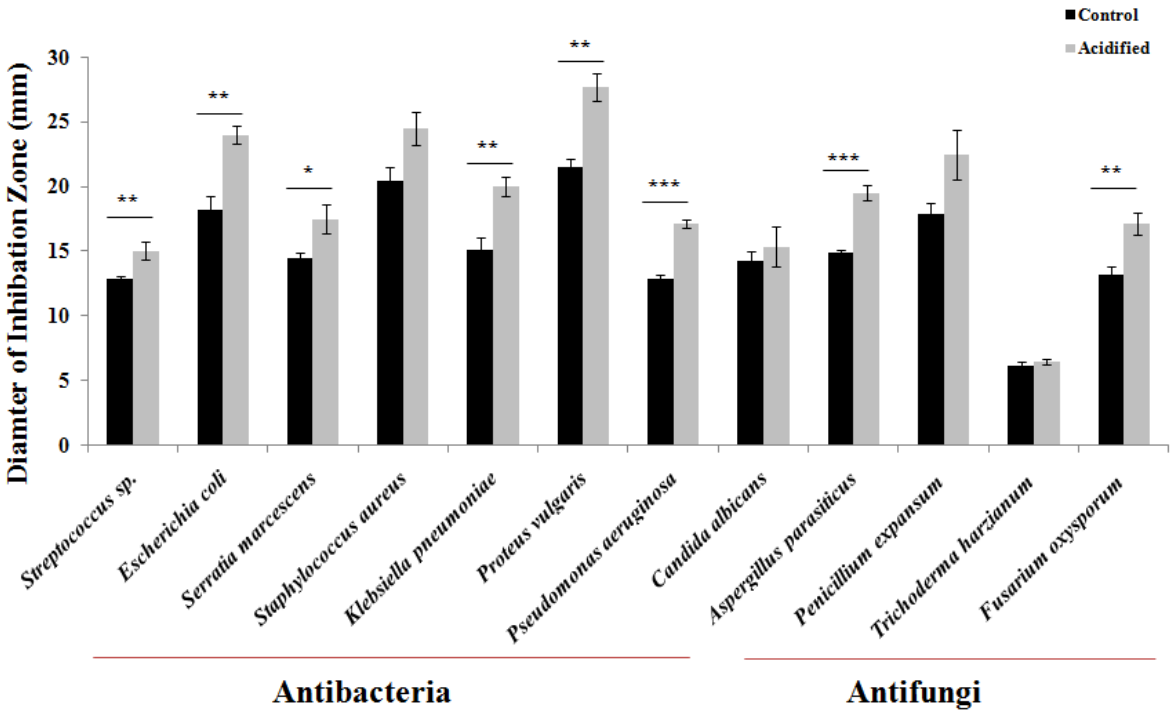

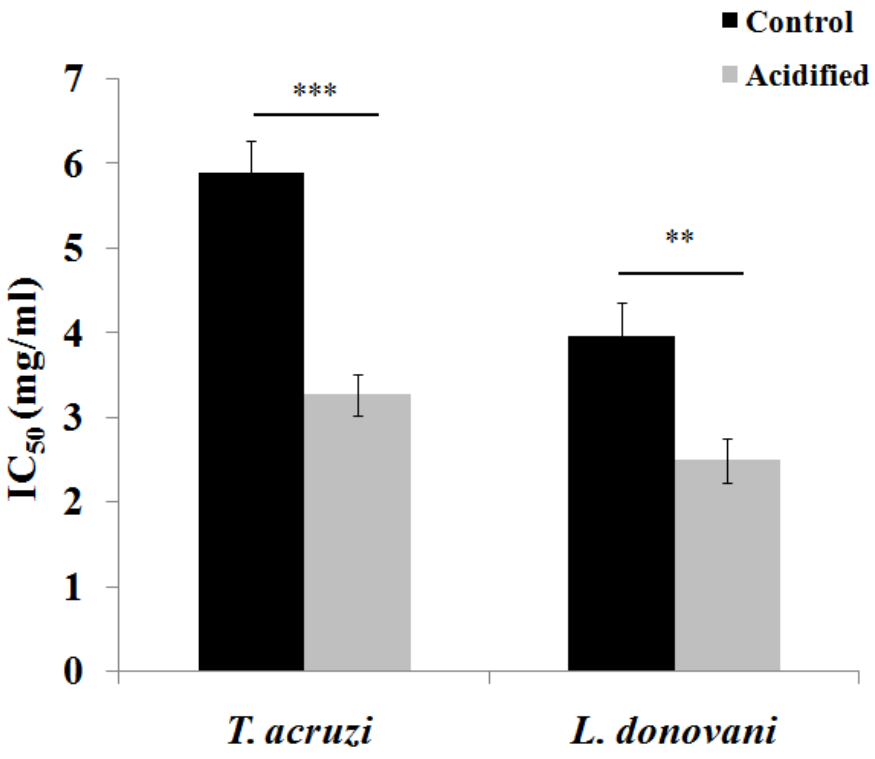

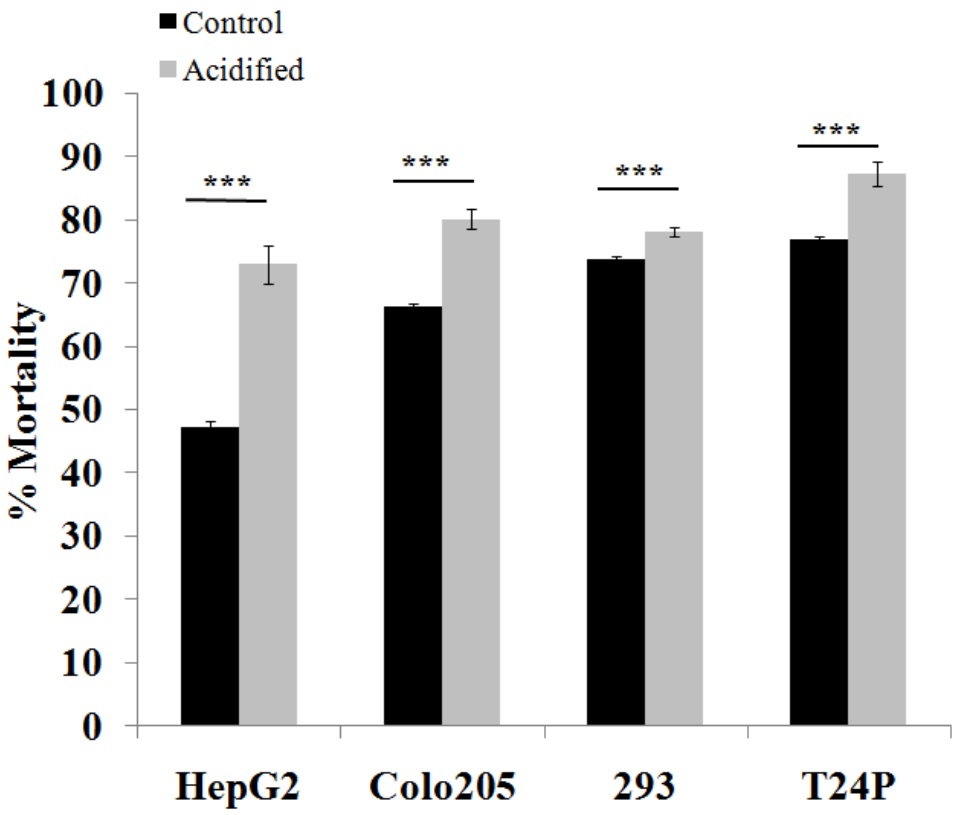
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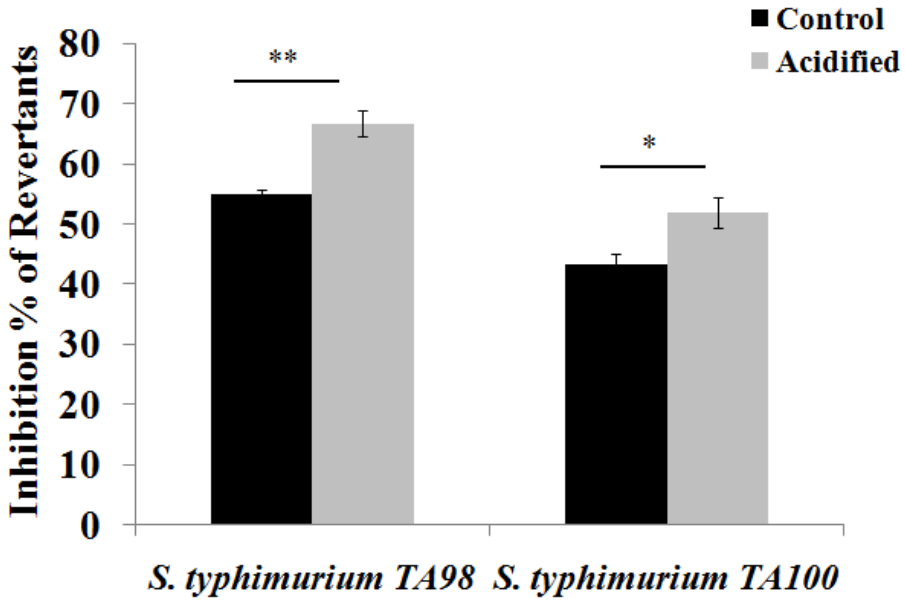
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S. typhimurium TA98
S. typhimurium TA100

Highlights

- Bioactivities of *S. vulgare* from Ischia CO_2 vents and nearby control site were analysed.
- Elevated DIC increases polysaccharide content in the algae at CO_2 vents.
- Algal extract from acidified population showed higher antimicrobial, and antiprotozoal activity.
- Acidified population showed pronounced antimutagenic potential and anticancer activities.

AUTHORSHIP STATEMENT

Manuscript title: **Ocean acidification affects biological activities of seaweeds: A case study of *Sargassum vulgare* from Ischia volcanic CO_2 vents**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Hong Kong Journal of Occupational Therapy*.

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Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

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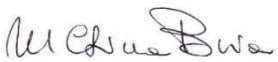
Maria Cristina Buia		**15/11/2019**
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Declaration of interests

[×] The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

[×] The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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