

ORIGINAL ARTICLE

Growth dynamics and bioactivity variation of the Mediterranean demosponges *Agelas oroides* (Agelasida, Agelasidae) and *Petrosia ficiformis* (Haplosclerida, Petrosiidae)

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Keywords

Agelas oroides; bioactivity variation; growth dynamics; natural population; *Petrosia ficiformis*; seasonality.

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This work is dedicated to the memory of Profs Michele Sarà, Fabio Cicogna and Gustavo Pulitzer-Finali.

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Accepted: 8 December 2008

doi:10.1111/j.1439-0485.2008.00278.x

Abstract

Growth dynamics and bioactivity variation of the Mediterranean demosponges *Agelas oroides* and *Petrosia ficiformis* were investigated over 15 months at Paraggi and Colombara within the Marine Reserve of Portofino Promontory (Mediterranean Sea, Ligurian Sea, Italy). For both species, growth rates varied between individuals and were unaffected by initial sponge size. The two species showed a different trend in growth pattern: *A. oroides* did not vary significantly between seasons, sites and depths; in contrast, some individuals of *P. ficiformis* showed a seasonal pattern, shrinking during winter as water temperature decreased and growing during summer when water temperature increased. Differences in growth between the two species may result from different reproductive cycles, food availability, species-specific thermophily and patterns of spatial competition. Moreover, spatial competition probably induced sponges to produce bioactive secondary molecules. Spatial and temporal variation of bioactivity of both species was examined for the first time by studying its effect on human neuroblastoma cells. The bioactivity of *A. oroides* extracts differed significantly between seasons, sites and depths, whereas the cytotoxicity of *P. ficiformis* differed significantly between seasons and depths (differences for sites were not determined). These results suggest the possible influence of environmental factors on bioactive metabolite biosynthesis.

Problem

Porifera are a significant element of many marine benthic communities, occurring worldwide from polar to tropical regions from intertidal to abyssal environments (Reiswig 1973; Dayton *et al.* 1974; Uriz *et al.* 1992; Hooper & van Soest 2002). Sponges are abundant in many environments, can have long life spans and may reach a large size, constituting much of the biomass of benthic

communities (Sarà 1970; Reiswig 1973; Dayton *et al.* 1974; Pansini & Pronzato 1990). The long-term study of the hard-bottom communities is an important source of information on sponge recruitment, competition for space, growth, predation and mortality (Pansini & Pronzato 1990; Turon *et al.* 1998; Duckworth & Battershill 2001; Lesser 2006). The growth rates and patterns are known for several tropical and temperate sponge species (Reiswig 1973; Johnson 1979; Fell & Lewandrowski 1981;

Ayling 1983; Barthel 1986; Hoppe 1988; Pansini & Pronzato 1990; Gaino *et al.* 1991; Duckworth & Battershill 2001). Sponge growth is generally considered indeterminate, as individual size varies depending on the ambient environmental conditions (Sebens 1987). However, the competition for space or trophic resources may limit the growth of adults and exponential growth rarely occurs in natural populations (Sebens 1987). Seasonal variations of sponge growth have usually been studied in intertidal or shallow waters and freshwater environments, and probably result from seasonal cycles of water temperature and food availability (Reiswig 1973; Fell & Lewandrowski 1981; Manconi & Pronzato 1991; Pronzato & Manconi 1995). However, seasonal growth patterns were not found in all species (Ayling 1983; Hoppe 1988; Pansini & Pronzato 1990; Gaino *et al.* 1991; Turon *et al.* 1998; Garrabou & Zabala 2001).

Sponges compete for space with other Porifera and organisms such as algae, bryozoans, tunicates and corals (*e.g.* Paul 1992; Pawlik 1993). Chemical ecology studies show that spatial competition has promoted the development in sponges of highly sophisticated defence systems by the production of bioactive secondary molecules (*e.g.* Green 1977; Bakus *et al.* 1986; Pawlik *et al.* 1995; Uriz *et al.* 1996). The biosynthesis of these sponge metabolites may vary between seasons (Green *et al.* 1990; Turon *et al.* 1996; Swearingen & Pawlik 1998; Duckworth & Battershill 2001; Page *et al.* 2005). Considering the pharmaceutical potential of these molecules, extensive investigations have been done to examine the effects of crude extracts and/or isolated compounds on several kinds of tumoral cell lines (*e.g.* Brown *et al.* 2001; Carballo *et al.* 2002; Aoki *et al.* 2003a,b; Choi *et al.* 2005; Ferretti *et al.* 2007).

In the present study we focused on the ecological and biological role of the Mediterranean demosponges *Agelas oroides* Schmidt, 1864 (Agelasida, Agelasidae) and *Petrosia ficiformis* (Poiret, 1789) (Haplosclerida, Petrosiidae). *Agelas oroides* is a massive, variably lobate-digitate sponge, generally 5–25 cm in height and common in 2–40 m water depth. This species is normally vivid orange in colour, but individuals living in dim light are generally pale. The tissue is firm but elastic and the surface is conulose. *Petrosia ficiformis* is a stiff and friable species because of its high mineral content. This species may have several morphotypes as a result of abiotic factors (Sarà & Vacelet 1973; Bavestrello & Sarà 1992; Bavestrello *et al.* 1993, 2003). The surface is rough, and the colour is violet to white depending on light exposure, which controls the presence of symbiotic cyanobacteria. This species is common from the surface to 50 m depth on rocks, walls and in caves.

Agelas oroides produces several bioactive metabolites with pharmaceutical potential such as the alkaloid

taurodispacamide A, which has antihistaminic activity (Fattorusso & Tagliatalata-Scafati 2000). Species in the genus *Petrosia* produce sterols and polyacetylenic molecules showing cytotoxic, antibacterial and antifungal activities (Cimino *et al.* 1990; Sepčić *et al.* 1997; Guo *et al.* 1998; Lim *et al.* 2001; Otha *et al.* 2003; Venkateshwar Goud *et al.* 2003). We have recently demonstrated that *P. ficiformis* markedly reduced cell viability of LAN5 neuroblastoma cells, and a time- and dose-dependent pro-apoptotic action of *A. oroides* extracts was observed (Ferretti *et al.* 2007). In the present work we focused on the growth dynamics and the temporal and spatial variation of bioactivity of the Mediterranean demosponges *A. oroides* and *P. ficiformis* to select a site and depth suitable for culturing the two sponge species for the production of biomolecules with pharmaceutical potential.

Material and Methods

Study area

This study was done in the Promontory of Portofino Marine Protected Area (NW Mediterranean Sea), which was established in 1999 (G.U.N. 131 07.06.1999). The Promontory protrudes 3 km into the sea and its coast line is 13 km long (Fig. 1). Study on the growth dynamics and bioactivity of *Agelas oroides* and *Petrosia ficiformis* was carried out in two sites, 9 km apart, along the Promontory cliffs: Paraggi (44°18'N, 9°09'E) and Colombara (44°18'N, 9°10'E) (Fig. 1). Two depths were chosen, 10 and 20 m, for both sites. The Paraggi rocky cliff is oriented northwest to southeast, and is exposed to southern winds and to the coastal current streaming out of Golfo Marconi. These conditions generate large amounts of sediment suspension, especially during winter. The cliff slope is between 60° and 90° and the maximum depth is 25 m. The Colombara rocky vertical cliff is oriented northeast to southwest and the maximum depth is 39 m. This area has strong hydrodynamics and a low amount of sediment suspension (Morri *et al.* 1986), with the main water current flowing in an east–west direction with an average speed of 25 cm·s⁻¹. This area is characterized by a biocoenosis (called precoralligenous) typical of the southern side of the Promontory (Morri *et al.* 1986). Table 1 shows the mean abundance per square meter (m²) of *A. oroides* and *P. ficiformis* at both sites at the two depths. To estimate the density of both species, three 1-m² fixed quadrates, 3 m apart, were positioned at both sites at two depths; thus 24 quadrates in total were used (12 quadrates for each species). The four corners of each quadrate were marked with tags hammered into the rock and the quadrates were delimited by a rope. Near each individual in each quadrate, a labelled tag was hammered into the rock.

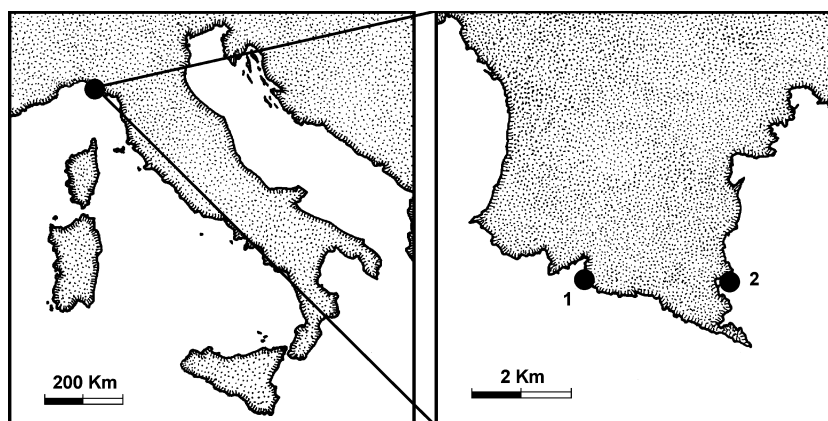


Fig. 1. Map of Italy showing the Portofino Promontory (Ligurian Sea) and the two study sites: Colombara (1) and Paraggi (2).

Table 1. Mean abundance (number of sponges per m²) of *Agelas oroides* and *Petrosia ficiformis* at Paraggi and Colombara at 10 and 20 m depth.

site	depth (m)	<i>Agelas oroides</i> (sponges per m ²)	<i>Petrosia ficiformis</i> (sponges per m ²)	No. of monitored sponges	
				<i>Agelas oroides</i>	<i>Petrosia ficiformis</i>
Paraggi	10	3	0.22	9	4
Paraggi	20	6	0.22	18	4
Colombara	10	2	0.03	6	0
Colombara	20	5.3	0.03	16	0

The Table also shows the number of tagged sponges to monitor the growth dynamics of both species.

The experiments ran for 15 months, from July 2004 (summer) to October 2005 (autumn), thus across all seasons.

Monitoring sponge growth

Sponge size was measured every 2–6 months. Bad sea conditions during autumn–winter prevented regular monitoring. Individuals of *A. oroides* and *P. ficiformis* generally had a massive and complex morphology. For this reason, sponge volume was measured according to the method previously used by Duckworth & Battershill (2001). At each monitoring time, each sponge was photographed with a Nikon V camera with 28-mm lens and macrophoto attachment. A frame outlining the picture area was held against the rock substrata of the sponges, ensuring the similar orientation and distance of each photo. The height of each sponge was measured *in situ* using a ruler. The images were then digitalized to trace the outline of each sponge and calculate the basal area in cm² using the computer programme IMAGEJ 1.33u (National Institutes of Health, USA). Basal area and height were multiplied to calculate the theoretical volume of each individual. The initial volume of *A. oroides* ranged from 0.59 to 92.07 cm³, and the initial volume of *P. ficiformis* from 21.27 to 160.90 cm³.

Monitoring bioactivity

To examine the influence of season, site and depth on the bioactivity, five individuals each of *A. oroides* were collected from Paraggi and Colombara at both 10 and 20 m depth for a total of 20 sponges. Because of low density at Colombara, *P. ficiformis* was collected only from Paraggi at 10 and 20 m of depth (n = 10). The sampling was done in September 2004 (summer) and March 2005 (winter). All samples were frozen as soon as possible at –20 °C. Sponge samples were then thawed and placed in 0.9% NaCl at room temperature overnight, homogenized and centrifuged at 15,000 g for 30 min. The supernatant was filtered, lyophilized and frozen again at –20 °C. Samples were extracted three times with methanol on a stirrer for 3 h or overnight. The supernatants were evaporated in Rotovapor at 40 °C to obtain the crude extracts of 12 samples.

To examine the effect of sponge crude extracts, LAN5 human neuroblastoma (NB) cell line was used as experimental model. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% non-essential aminoacid solution and 1% antimetabolic solution. Cells were grown on chamber slides (Iwaki Seiyaku Co., Tokyo, Japan) and then treated for 15 min

with 5 p.p.m. of the methanolic crude extracts of *A. oroides* and *P. ficiformis*. After the treatment, the cells were labelled with propidium iodide, which enters cells that have lost their membrane integrity (Vermees *et al.* 1995; Ferretti *et al.* 2007). Cells were observed and counted (300 cells for each condition and experiment) under a fluorescence Leica DIMRB microscope (Leica Microsystems AG, Wetzlar, Germany), using a filter for rhodamine. Cells that lost membrane integrity showed red staining throughout the nucleus. To evaluate the cytotoxic effect, propidium iodide (PI)-positive cells were considered.

Statistical analysis

The growth rate (GR, in %) of each individual of both species was calculated using the following formula adapted from Duckworth & Battershill (2001):

$$GR = \{[(V_m - V_{m-1})/V_{m-1}]/n\} \times 100,$$

where V_m is the volume of sponges measured at month m , V_{m-1} is the volume of sponges measured at the previous monitoring event and n is the number of months between the two monitoring events.

The effect of initial sponge size on growth was examined by comparing the mean growth rate of each sponge over its initial volume for each month-year group. The possible effect of initial sponge size on growth was determined using the Spearman rank correlation coefficient.

A hierarchical three-factor analysis of variance (ANOVA) was used to compare differences in GR between sites and depths at different times of the year. The factors were season (month-year), site and depth, and all factors fixed. ANOVA was also used to test whether the bioactivity of *A. oroides* crude extracts differed between seasons (month-year), sites and depths, and the bioactivity of *P. ficiformis* crude extracts differed between seasons (month-year) and depths. After each ANOVA, all factors were analysed with the Tukey-Kramer Multiple Comparison Test to determine which treatments were significantly different from each other ($\alpha = 0.05$). One-way ANOVA and Bonferroni's test for multiple comparisons were used to analyse the statistical significance of parametric differences among sets of experimental data from NB cell line tests. All the analyses were performed using Number Cruncher Statistical Systems 2004 (NCSS, Kaysville, UT, USA).

Results

Agelas oroides

The growth rate of *A. oroides* was similar between seasons, sites and depths, and there were no significant inter-

Table 2. Analysis of variance testing the effects of season, site and depth on growth rate of *Agelas oroides*.

source of variation	df	F-ratio	P-value
season	3	0.45	n.s.
site	1	0.41	n.s.
season × site	3	1.00	n.s.
depth	1	0.07	n.s.
season × depth	3	0.48	n.s.
site × depth	1	0.29	n.s.
season × site × depth	3	0.48	n.s.
residual	43		

Data were log-transformed prior to analysis. $\alpha = 0.05$. df, degrees of freedom; n.s., not significant.

actions (Table 2). *Agelas oroides* showed a high interindividual variability in growth, with a GR ranging from -8.9% to $28.9\% \cdot \text{month}^{-1}$ (Fig. 2). At Paraggi at both depths, *A. oroides* GR was similar over the experimental period (Fig. 2A,B) and, although not significant, a partial decrease in volume was observed during winter (Fig. 2A). Overall, the mean specific growth rate for this species was $0.05\% \cdot \text{day}^{-1}$ (SE, 0.06). Although the Spearman correlation coefficients showed that the initial size of a sponge did not influence its growth rate ($P > 0.05$), larger individuals generally had slower growth.

The cytotoxicity of *A. oroides* showed a significant sites*seasons*depth interaction (Table 3), being greatest (70% of propidium iodide-labelled NB cells) for individuals collected from the Paraggi site in winter at -20 m (Fig. 3A). During summer a similar cytotoxic effect (65% of propidium iodide-labelled NB cells) was registered when LAN5 cells were treated with the -10 m Paraggi extract (Fig. 3A). Comparing analyses showed that Colombara extract had a lower cytotoxic effect (8–20% of propidium iodide-labelled NB cells) than Paraggi one (Fig. 3A).

Petrosia ficiformis

Monitored specimens showed an almost constant trend in volume increase over time (data not shown). The growth rate of *P. ficiformis* was similar between seasons, but was significantly different between depths (Fig. 4; Table 4), being $0.73\% \cdot \text{month}^{-1}$ (SE, 1.60) at -10 m and $1.30\% \cdot \text{month}^{-1}$ (SE, 1.30) at -20 m. At -10 m, a similar trend was observed between water temperature and GR; in particular, when the water temperature fell to 12 °C the GR reached the minimum value ($-2.5\% \cdot \text{month}^{-1}$). In contrast, for the organisms living at -20 m a relation between the GR and water temperature was not found. Larger individuals generally had slower growth, although the Spearman correlation coefficients showed that the initial size of a sponge did not influence its growth rate ($P > 0.05$).

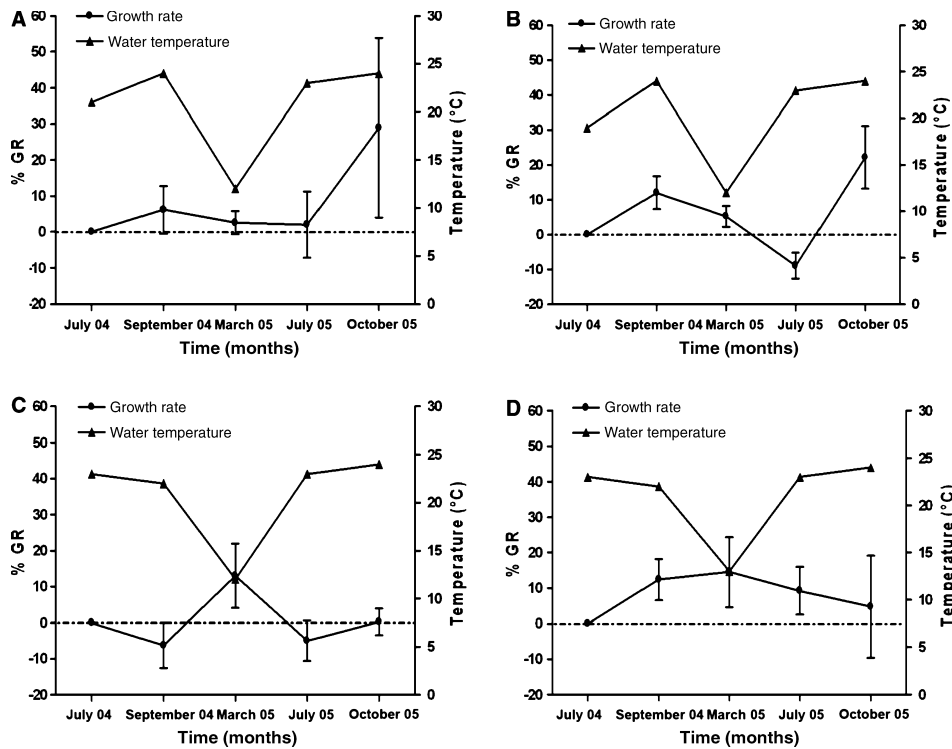


Fig. 2. Growth rates (GR) of *Agelas oroides* at Paraggi (PRG) at 10 m (A) and 20 m (B) depth and at Colombara (CLMB) at 10 m (C) and 20 m (D) depth from July 2004 to October 2005. Mean water temperature is also shown. Error bars represent variation between monitored individuals.

Table 3. Analysis of variance testing the effects of season, site and depth on cytotoxicity of *Agelas oroides* crude extracts on LAN5 cells.

source of variation	df	F-ratio	P-value
season	1	14.37	*
site	1	85.54	***
season × site	1	0.03	n.s.
depth	1	9.10	*
season × depth	1	2.51	n.s.
site × depth	1	3.06	n.s.
season × site × depth	1	10.89	*
residual	8		

Data were log-transformed prior to analysis. $\alpha=0.05$. df, degrees of freedom. n.s., not significant; * $P < 0.05$, *** $P < 0.001$.

The cytotoxic effect of *P. ficiformis* extracts significantly differed between seasons and depths (Table 5), being greatest for individuals collected in winter at -20 m (75% of propidium iodide-labelled NB cells) (Fig. 3B). In contrast, the -20 m summer extract did not affect the viability of LAN5 cells (Fig. 3B).

Discussion

At Paraggi at both depths, *Agelas oroides* GR was similar over the experimental period and, although not signifi-

cant, a partial decrease in volume was observed during winter. This is probably due to an increase of the sedimentation rate during the coldest season and to a consequent complete closure of oscula (Pansini & Pronzato 1990). These two events probably cause a reduction in the sponge pumping activity and volume. Similar to our results, many studies carried out for other sponges showed that the reduction of pumping activity is a consequence of a high sedimentation rate (Reiswig 1971; Wilkinson 1978; Gerrodette & Flechsig 1979; Pansini & Pronzato 1990; Tompkins & Leys 2006).

At -10 m, *Petrosia ficiformis* shrank during winter as water temperature fell and grew when the water temperature increased during summer-autumn. At -20 m the GR seemed to increase when the water temperature reached 12 °C. The different growth pattern between the two depths may have resulted from interindividual heterogeneity, or low replicate number may bias growth variation. *Agelas oroides* also showed high interindividual variability in growth. Seasonal growth patterns are found for some sponge species (Simpson 1968; Fell & Lewandrowski 1981; Leys & Lauzon 1998; Turon *et al.* 1998; Duckworth & Battershill 2001; Garrabou & Zabala 2001), but not for others (Ayling 1983; Hoppe 1988; Pansini & Pronzato 1990). The interindividual variation may be influenced by the sponge size at the onset of monitoring. In fact, for

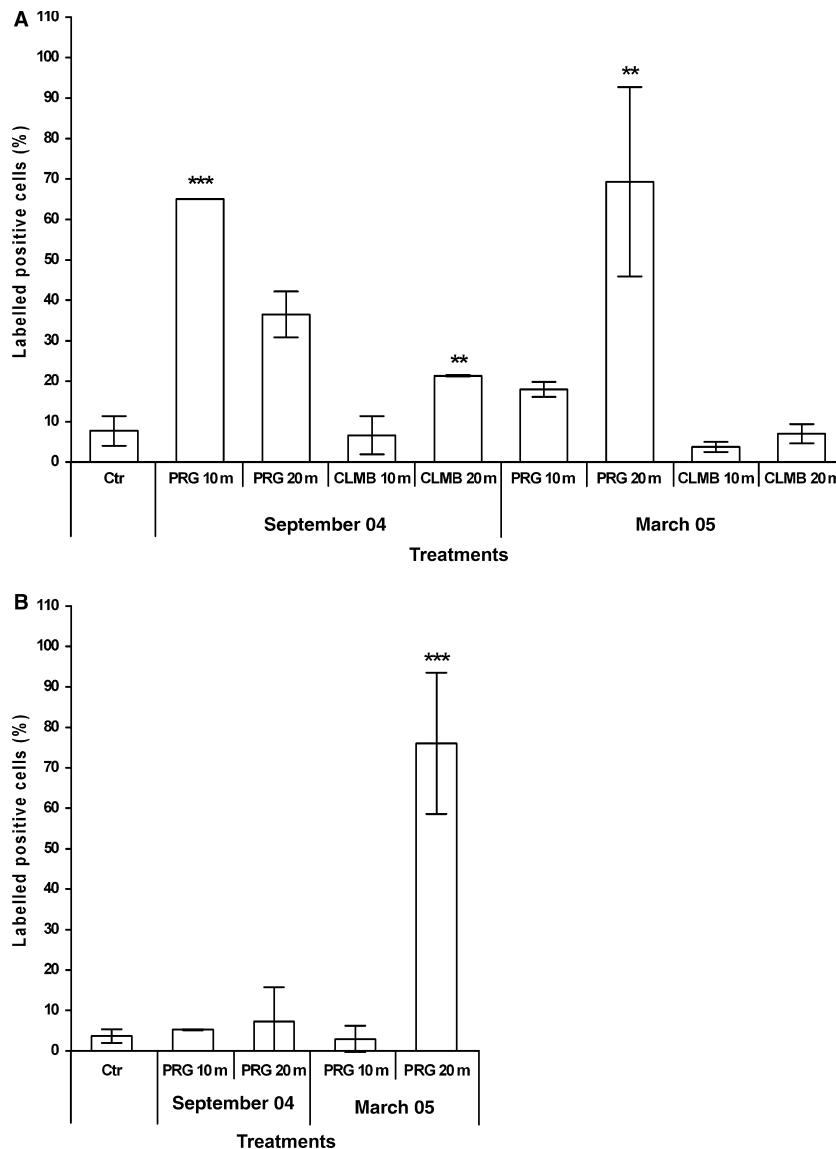


Fig. 3. The effect of methanolic crude extracts of *Agelas oroides* (A) collected from Paraggi (PRG) and Colombara (CLMB), and of *Petrosia ficiformis* (B) collected from Paraggi (PRG) at –10 and –20 m in September 2004 (summer) and March 2005 (winter) on the human neuroblastoma cell line LAN5. ** $P < 0.01$, *** $P < 0.001$ versus control. Error bars represent standard deviation.

several species, smaller sponges grow faster than larger ones (e.g. Hoppe 1988; Leys & Lauzon 1998), whereas for other sponge species, size does not influence growth (Duckworth & Battershill 2001). Similarly, we observed that the growth rates of *A. oroides* and *P. ficiformis* were not affected by the initial sponge size.

Temporal variation in growth of sponges may be related to their reproductive cycles, because reproductive cells originate from the feeding choanocytes, diverting energy from sponge growth (Reiswig 1973; Scalera-Liaci *et al.* 1973; Elvin 1979; Simpson 1984; Barthel 1986; Sebens 1987). The reproductive cycle of *A. oroides* is unknown, but more information is available for *P. ficiformis*. The oogenesis of *P. ficiformis* generally occurs in spring up to early autumn (Scalera-Liaci *et al.* 1973; Scalera-Liaci &

Sciscioli 1975). The short spermiogenesis (15–20 days) occurs during October–November (autumn) and involves only a small part of the entire population (Scalera-Liaci *et al.* 1973). The generally lower growth rate of *P. ficiformis* in autumn at –20 m may be a result of its reproductive investment. A similar trend in sponge growth was common in several species of Mediterranean sponges, in which the reproductive investment is highest between spring and summer, when spawning occurs (Corriero *et al.* 1996; Uriz *et al.* 1998).

Sites and seasons may also modulate the biosynthesis and the release of bioactive molecules (Green *et al.* 1990; Turon *et al.* 1996; Swearingen & Pawlik 1998; Duckworth & Battershill 2001; Page *et al.* 2005). In the present study, we have focused on the spatial and temporal variation of

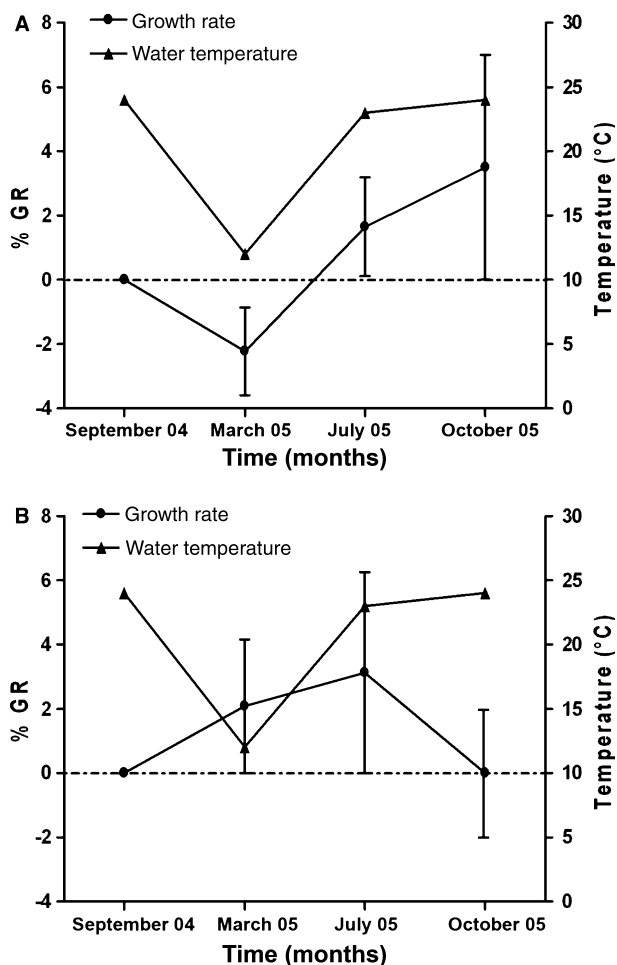


Fig. 4. Growth rates (GR) of *Petrosia ficiformis* at Paraggi (PRG) at 10 m (A) and 20 m (B) depth from September 2004 to October 2005. Mean water temperature is also shown. Error bars represent variation between monitored individuals.

Table 4. Analysis of variance testing the effects of season and depth on growth rate of *Petrosia ficiformis*.

source of variation	df	F-ratio	P-value
season	2	0.36	n.s.
depth	1	9.09	*
season × depth	2	0.39	n.s.
residual	18		

No data transformation prior to analysis. $\alpha=0.05$. df, degrees of freedom. n.s., not significant; *P < 0.05.

bioactivity of *A. oroides* and *P. ficiformis* extracts. Comparing the bioactivity between the two sites, biomolecules released by *A. oroides* from Paraggi (shaded habitat) at -20 m were more cytotoxic during winter, inducing the highest necrotic response in LAN5 cells; the Colombara

Table 5. Analysis of variance testing the effects of season and depth on cytotoxicity of *Petrosia ficiformis* crude extracts on LAN5 cells.

source of variation	df	F-ratio	P-value
season	1	1.01	n.s.
depth	1	4.22	n.s.
season × depth	1	6.53	*
residual	4		

Data were log-transformed prior to analysis. $\alpha=0.05$. df, degrees of freedom. n.s., not significant; *P < 0.05.

(more illuminated habitat) extracts had a lower cytotoxicity. These responses to environment may be related to different light habitat conditions. Our hypothesis is in agreement with that of Uriz *et al.* (1991) who reports that the most biologically active benthic organisms are found in shaded habitats as compared to highly illuminated ones, suggesting that allelopathy may play an important role in spatial competition interactions.

Because of the low density of *P. ficiformis* at Colombara, its bioactivity was examined only at Paraggi to avoid extensive harvesting from the wild population. Comparing the effects induced by bioactive molecules released by sponges collected at -20 m in different seasons, we observed that the summer extracts did not induce death of LAN5 cells, whereas the winter extract was more cytotoxic. In contrast, several other species are more bioactive in warmer seasons (Green *et al.* 1990; Turon *et al.* 1996; Duckworth & Battershill 2001). A higher bioactivity in summer/autumn may be the result of environmental factors such as the increase of fouling of the sponge surface (Duckworth & Battershill 2001) and/or an increase in spatial competitors (Turon *et al.* 1996).

Although further investigations on this field are needed to discriminate which single components are responsible for the different bioactivity shown by sponge extracts, our results suggest that the cytotoxicity of both species might be influenced by seasons, sites and depths, and that there might be an individual reaction through the molecule biosynthesis to particular conditions of the micro-environment or adaptations to spatial competition.

Acknowledgements

This work was supported by grants from the Italian Ministry of Agricultural and Forestry Politics, by Genova University, by Giannina Gaslini Institute and from the Italian Ministry of University: PRIN no. 2006065711_002. C. Ferretti was supported in part by a Fabio Cicogna grant. We would like to thank all our colleagues at DIPTERIS, DIMES, DCCI of Genova University and ISMAR-CNR (Genoa) for their precious technical

assistance. In particular, we thank Dr Mario Mori for his critical suggestions on the manuscript.

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