

Allelopathic interaction among rocky intertidal invertebrates: Sponge *Cinachyrella* cf. *cavernosa* and Zooxanthellatezoanths *Zoanthus sansibaricus*

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Abstract

Intertidal sessile invertebrates experience tough competition for space which they avoid by releasing secondary metabolites (allelochemicals). We investigated the allelopathic interaction between sponge *Cinachyrella* cf. *cavernosa* and its neighbour zoanths *Zoanthus sansibaricus* over a year on the rocky beach of Anjuna, (Goa) India. A major sterol (β -sitosterol) was isolated from the sponge and its natural variability was checked in tagged sponges with and without their competitor zoanths during the reproductive and non-reproductive months of the sponges. The sponge showed significant variability in the production of β -sitosterol owing to its spatial competition and asexual reproduction (budding) as predicted by the multiple regression analysis. Zoanths are known to have dynamic mutualistic association with their autotrophic endosymbionts' dinoflagellate (*Symbiodinium*, known as zooxanthellae), which are directly involved in their physiology. The phytigelTM based field assay of sponge's bioactive compound (β -sitosterol) was conducted to check its allelopathic impact on *Z. sansibaricus* and its symbiotic zooxanthellae (*Symbiodinium* sp.). Results showed significant bleaching (50% -80%) in the host *Z. sansibaricus*, resulting from a decrease in symbiotic zooxanthellae count, cell density, chl a and chl c content. This suggests the role of β -sitosterol as allelochemical of the sponge in preventing the overgrowth of aggressive *Z. sansibaricus*, possibly by affecting its symbionts. This investigation is not only important to understand the ecological roles of marine-derived allelochemicals, but also to allow sustainable bioprospecting of marine resources.

Keywords

Marine sponge, zoanths, zooxanthellae, allelopathic interaction, β -sitosterol, gel-based field assay, rocky intertidal region.

1. Introduction

In space-limited regions such as coral reefs and rocky intertidal areas, diverse sessile marine organisms adopt a variety of strategies to acquire a suitable space. One of the strategies is the production of secondary metabolites (also called allelochemicals) to hinder the growth and reproduction of their aggressive neighbours. The term “allelopathy” stands for both positive and negative interactions between two species in which one species uses bioactive compound to either influence or prevent the growth of another species. However, it is mostly used to describe the detrimental effect of one plant species on the other (Reigosa and Pedrol 2002). Similar to the terrestrial plants, marine sessile organisms also produce these notorious compounds to maintain their substratum clean and avoid competition for resources from their neighbours (Pawlik et al. 2007; Rasher and Hay 2010; Steinberg and De Nys 2002). The spatial competition among sponges and corals have been extensively studied by various researchers (López-Victoria et al. 2006; Pawlik et al. 2007; Porter and Targett 1988; Russell et al. 2003; Sullivan et al. 1983; Voogd et al. 2004).

Sponges are important members of benthic community worldwide with various functional roles, such as (a) bioerosion and reef regeneration (b) benthic-pelagic coupling and nutrient cycling and (c) role in primary and secondary production (Bell 2008). Sponges also exhibit various biological interactions with organisms, from microbes to vertebrates (Ávila et al. 2007; Webster and Taylor 2012), due to which any changes in their abundance and diversity can significantly influence overall functioning of the ecosystem (Peterson et al. 2006). They have been reported as the dominant fauna in regions with a decline in coral abundance (Fujii et al. 2011) due to accelerated ocean acidification, climate change and pollution (Aronson et al. 2002; Ward-Paige et al. 2005; Weil 2002). However, other cnidarians such as soft corals, corallimorpharians and zoanthids, which lack calcium carbonate skeleton have comparatively little effect of ocean acidification, climate change and pollution. These organisms can grow aggressively and provide tough competition for space to their neighbours such as sponges (Suchanek and Green 1981). Recent work on few epizoic zoanthids associated with Caribbean sponges showed that there was a significant reduction in pumping rate of the sponges due to mechanical blockage of ostia (Lewis and Finelli 2015). Many zoanthids such as *Palythoa caribaeorum* DUCHASSAING and MICHELOTTI, 1860 and *Zoanthus sociatus* ELLIS, 1768 have faster growth rates and can easily overgrow other sessile invertebrates (Pérez et al. 2005; Suchanek and Green 1981). Sponges and zoanthids are the two benthic invertebrates which are in tough competition for space. There are various examples to describe the detrimental impact of sponges on corals (who are like the cousins of zoanthids, belonging to same subphylum

Anthozoa)(Engel and Pawlik 2000; Pawlik et al. 2007; Singh and Thakur 2016). Thus, research work conducted on corals can provide useful insights into the competitive behaviour of their related cousins, zoanthids. Surprisingly, the allelopathic interaction among sponges and zoanthids has not been explored in details.

These allelochemicals have the potential to cause a direct influence on the physiology of interacting organisms as well as their symbionts (Pawlik et al. 2007). These zoanthids also harbour symbiotic dinoflagellates (zooxanthellae) belonging to the genus *Symbiodinium* (Reimer 1971b). Recently, this 'Symbiodinium –host relationship' has been receiving a special focus due to the increase in the rate of bleaching of corals and in other cnidarians such as soft corals, corallimorpharians and zoanthids. The phenomenon of bleaching (where *Symbiodinium* is degraded and/or expelled from hosts, resulting in drastic whitening and/or death of hosts) has been observed worldwide. Bleaching can occur due to various reasons: abnormally high (Glynn 1996) or low ocean temperatures (Lirman et al. 2011), high levels of solar radiation (Lesser 1997), increased terrestrial runoff (Done et al. 2007), changes in salinity (Goreau 1964; Reimer 1971a), pollution as well as other environmental stressors (Brown 1997). Interestingly, it was demonstrated through a field assay that the sponge's allelochemicals can affect health of zooxanthellae associated with few coral species by impairing photosynthesis, resulting in coral bleaching (Pawlik et al. 2007).

The production of allelochemicals by organisms is a costly biochemical process. It occurs at the expense of other primary processes (such as growth and reproduction) since the total energy reservoirs of an organism are limited (Leong and Pawlik 2010; Thoms and Schupp 2007). It is also influenced by various biological (growth, reproduction, natural enemies) and abiotic (e.g. temperature, nutrient availability) factors that determine the organism's ability to defend its space from encroaching neighbours and potential invaders (De Caralt et al. 2013). In this context, we studied the spatial competition between the globular solitary sponge *Cinachyrella* cf. *cavernosa* sensu BURTON, 1959 (in part) {not: *Cinachyrella cavernosa* LAMARCK, 1813 and it is likely to be a new species (JNA Hooper & KA Hall, pers. Comm.)} and the encrusting zoanthid *Zoanthus sansibaricus* CARLGREN, 1900 over a period of one year from October 2013 to September 2014. During our regular field trips to the rocky intertidal region of the study area (Anjuna, Goa), we observed that the globular sponge *C. cf. cavernosa* avoids being overgrown by zoanthids. As *C. cf. cavernosa* is a slow-growing, it is highly likely that this stand-off interactions between these two sessile marine organisms are driven by allelochemicals released by the sponges rather than their fast growth. The hypothesis behind this work was that in the presence of competitors such as fast-growing *Z. sansibaricus*, the sponge *Cinachyrella* cf. *cavernosa* should produce some

allelochemical (probably the cytotoxic compound) to avoid the overgrowth. This paper aims to answer the following questions: 1) what is the impact of the presence of zoanthids on the production of allelochemical compound (for instance, beta-sitosterol, in this study) by the sponge? 2) What are the effects of beta-sitosterol of sponge on zoanthids and its symbionts?

2. Materials and methods

2.1. Study site and organisms

The field experiment was conducted on the rocky shore of Anjuna beach, Goa, West Coast of India (15° 34' 34.986"N; 73° 44' 23.895" E), from October 2013 to September 2014. The sponge *Cinachyrella cf. cavernosa* and the zoanthids, *Zoanthus sansibaricus* are among the most dominant fauna in the lower inter-tidal region of this shore. The environmental variables such as temperature, salinity, dissolved oxygen, pH of the rock pool water containing the tagged organisms were measured every month, using the multiparametric probe, Oakton PCD 650 (OAKTON Instruments, Vernon Hills, IL, USA). Nutrient analysis (phosphate, silicate, nitrate, nitrite and ammonia) of the rock pool water was carried out using a continuous flow analyser (San++, SKALAR, Holland).

2.2. Experimental set up (Competitors' removal experiment)

To know the potential allelochemical involved in the spatial competition of the against its fast-growing neighbour zoanthids colonies, the similar method of competitors' removal was adopted as described in our previous work (Singh and Thakur 2015b). In brief, two sets of experiments were carried out (a) non-manipulative experiment (consisting of tagged sponges with the competitor, n=36) and (b) manipulative experiment (sponges without the competitor, n=36). Briefly, quadrats of 15 x 15 cm (n=36), each containing an adult sponge (mean diameter ~ 4 to 5 cm), surrounded by polyps of *Z. sansibaricus* were marked with four stainless steel nails then non-manipulative setup. Whereas, in the manipulative experiment, additional quadrats (n = 36) were marked in a similar way and polyps of *Z. sansibaricus* which surrounded the sponges were carefully removed using a scalpel. *C. cf. cavernosa* reproduced asexually by budding which can be observed as the small outgrowths (spherical balls) on its surface (Singh and Thakur 2015a) These buds detached from mother sponges and settled down, giving rise to a new sponge. To understand the effect of competitors, *Z. sansibaricus* on the budding of the sponge, the budding frequency of the tagged sponges from these two experimental sets up was estimated. Briefly, the surface images of tagged sponges were captured using an underwater camera (Canon Power shot A650) using the photo-quadrant method to capture images from the same angle and distance. The images of sponges buds were analysed in using Image-J as described previously to calculate monthly budding frequency (i.e.

the proportion of total individuals with buds)(Singh and Thakur 2015b). The whole methodology for this research work has been described schematically (Figure 1).

2.3. Isolation of the potent allelochemical (β -sitosterol) from sponge

For isolation of one of the potent allelochemicals, tissue sample was collected in the month of October 2013 from each of three tagged sponges, from two sets of experiments: 1) with and 2) without competitor. Each of the samples was immediately frozen in liquid nitrogen and lyophilized. 15 g of lyophilized samples (n=6) was separately extracted with chloroform: methanol (CHCl₃: MeOH, 1:1) three times at room temperature and concentrated under reduced pressure. The crude extract obtained from tagged sponge with competition was then subjected to column chromatography over silica gel (100-200 mesh) using a gradient of hexane and ethyl acetate (from 100% hexane to 100% Ethyl acetate) afforded three fractions (I–III) based on their analytical TLC data. Each fraction was subjected to bioactivity assay (cytotoxicity against Hela and PC-13 cell lines). The bioactive fraction was further subjected to column chromatography over silica gel and eluted with 5% ethyl acetate–hexanes. Mass spectra were obtained for the pure fraction on an Agilent 6540 (Q-TOF) high-resolution mass spectrometer, in the electrospray (ESIMS) mode. ¹H NMR spectra were recorded (Bruker Avance) DPX FT-NMR at 400 MHz and ¹³C NMR at 100 MHz in CDCl₃, chemical shifts values are reported in δ (ppm) units and coupling constants values in hertz. Tetramethylsilane (TMS) was used as the internal standard.

2.4. Impact of the zoanthids on the potent allelochemical (β -sitosterol) of the sponge

To study the monthly variation in the concentration of β -sitosterol in sponges with and without competitor, High-performance thin-layer chromatography (HPTLC) method was adopted (Murthy and Mishra 2009). The pure β -sitosterol was purchased from Sigma-Aldrich (Cat. No. S1270-10MG, $\geq 95\%$, Canada), other solvents and chemicals of analytical grade were used. For the preparation of crude extract, every month about 15 g of the lyophilized powder of sponge tissue (n=3 sponges \times 2 conditions) were extracted separately with (chloroform: methanol (CHCl₃: MeOH, 1:1) (3 \times 100 mL) using a sonicator (15 min each time). The combined extracts were filtered and concentrated on rotator vapour until complete dryness. A stock solution of β -sitosterol (100 μ g mL⁻¹) was prepared by dissolving 1 mg of accurately weighed β -sitosterol in methanol and making up the volume of the solution to 10 mL with methanol (MeOH). TLC was performed on a pre-coated TLC plate silica gel 60F254 (20 cm \times 20 cm), Merck-Millipore (Cat. No. 105553). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler (Linomat V, Camag, Muttenz, Switzerland) under a flow of N₂ gas. The linear ascending development was

carried out in a Camag twin trough chamber (20 cm × 20 cm) which was pre-saturated with 20 mL mobile phase toluene:chloroform:methanol (4:4:1 v/v). After the development; TLC plates were dried and post chromatographic derivatization was carried out in anisaldehyde–sulphuric acid. Quantitative evaluation of the plate was performed in absorption-reflection mode at 527 nm with a computerized TLC scanner-3 integrated with winCATS 4 software(Camag, Muttenz, Switzerland). Quantification of β -sitosterol in the crude extracts of the sponge was performed using pure β -sitosterol as standard. Stock solution (100 $\mu\text{g mL}^{-1}$) of the standard was prepared in methanol (MeOH) and different amounts (100–600 ng spot⁻¹) were applied on a TLC plate, using Linomat V for preparing six-point calibration graph of peak area versus concentration. Peak areas were recorded and the amount of β -sitosterol was calculated using the calibration plot. The specificity of the method was determined by analysing the sample of standard β -sitosterol and the unknown sample (crude extract). The spot for β -sitosterol in the sample was confirmed by comparing the Retardation factor (Rf) and spectra of the spot with that of the standard. The method was validated for precision, accuracy and repeatability (ICH Guidelines 2005).

2.5. Impact of potent allelochemical (β -sitosterol) of the sponge on the zoanthids and its symbionts

2.5.1 On zoanthids:

PhytigelTM-based field assay was used to find out the effect of sponge *C. cavernosa*'s metabolite (β -sitosterol) on the colonies of *Z. sansibaricus*(Chaves-Fonnegra et al. 2008; Pawlik et al. 2007). This method was the modified version of field antifouling assays (Henrikson and Pawlik 1995) in which potential allelopathic compounds were incorporated into stable gels (PhytigelTM) to allow the slow release metabolites i.e. 1–2% per day (Engel and Pawlik 2000; Henrikson and Pawlik 1995). It has been used to find out the contact-based allelopathic effect of sponge's metabolites on the corals by using zooxanthellae as proxy for coral health (Chaves-Fonnegra et al. 2008; Pawlik et al. 2007). For the gel-based allelopathic assays, the treatment gels (n= 5) were made by resuspending the potent allelochemical (in this study, β -sitosterol) in 1 mL methanol and finally adding the homogeneous solution to PhytigelTM (Sigma Aldrich, cat. no. P8169) at its mean natural concentration (i.e., 92 ppm). Control gels (n=5) were created in the same manner, including the addition of methanol without β -sitosterol. The gel was poured onto the rectangular mould of size (5.5 × 2 × 1 cm³) having four holes at the corner to tie nylon threads for subsequent attachment onto the zoanthids. The gel was allowed to solidify at room temperature and refrigerated in sealed plastic bags for use within 24 h. The gel in the mould containing the compound was placed upside down so gel gently rested against the top, flat area of the live polyps of *Z. sansibaricus*. The loose ends of nylon threads

(connected to four corners of the mould) were tied to nails, located outside the marked boundary of photo-quadrat. The mount was fixed properly without excess pressure onto the zoanthids so as not only it prevents any free movement but also creates the least pressure on the zoanthids. This process was done for both control (n=5) and treatment gels (n=5) similarly. The gels were left in place for 24 hours, after which they were lifted, and the presence/absence of dead zoanthids was recorded using the colour card reading. As zoanthids under control gels also showed little bleaching due to the masking effect of the gel, the measurements were taken after 3 days to determine the allelopathic level of the compound (β -sitosterol) based on the recovery of zoanthids from bleaching under both conditions. This was determined by employing a colour card reading. Marked quadrats (n=10, treatment =5 and control =5) were photographed using a digital camera (Canon Power shot A650) fixed on a photo-quadrat stand. A laminated 'coral watch –coral health chart' was included in each shot for unbiased comparative analysis and to avoid possible error due to variable light levels or camera equipment (Siebeck et al. 2006). Images were scored from 1 (low-intensity colour) to 6 (high-intensity colour) based on the gradation of the intensity of colour in the colour chart. To account for the subtle differences in shading for *Z. sansibaricus* colonies, an increment of 0.5 (for instance 1.0, 1.5, 2.0, 2.5 ... to 6.0) was added to the scale. Immediately after the photography, polyps of *Z. sansibaricus* from both treatment and control gels were carefully cut along with the attached substrate (i.e., rocks beneath the colonies) using a sharp scalpel and chisel. They were then placed individually in marked Nasco sampling bags (Himedia). The bags were then placed in a 10 L bucket filled with ambient seawater and immediately transferred to the laboratory for further analysis.

2.5.2. On symbiotic zooxanthellae

To find out the impact of sponges' allelochemical (β -sitosterol) on the symbiotic zooxanthellae (*Symbiodinium*) of *Zoanthus sansibaricus*, the collected polyps from treatment and control gels were processed to harvest zooxanthellae cells to study their morphological condition, cell densities and chlorophyll content.

i) Zooxanthella morphological condition and cell density: Zooxanthella cells were harvested from each of treated and control experimental sets. Briefly, the polyps of freshly collected zoanthids (n=5*2) were cut from the attached substratum using a sharp scalpel and were transferred to 1ml of 0.22 μ filtered sterile seawater in the vials. To release out the zooxanthellae cell, each collected polyp was macerated, homogenized using mortar and pestle and was filtered through a 100 μ m mesh (to remove debris). The filtrate was washed with 0.22 μ m filtered sterile sea water by centrifuging at 10,000rpm to get a brown pellet. The pellet was re-suspended in 0.22 μ filtered sterile

seawater (SSW) and the zooxanthellae cells (n=120 per samples) were observed under 400X magnification using a hemocytometer and photographed to classify them as healthy and unhealthy based on their morphological conditions (i.e., intact or deformed cell membrane using PI staining). %NZ stands for no. of healthy zooxanthellae cells and it was calculated for treated and control experimental sets (Kuroki and Van Woesik 1999; Mise and Hidaka 2003; Reimer et al. 2007). To calculate the zooxanthellae cell densities, additional tissue from the zoanthid polyps (n=5*2) (each of approximately 1 cm³) were processed and visualized similarly. Zooxanthellae cell density was calculated as the total number of cells observed divided by the total volume observed.

ii) Chlorophyll content of zoanthids: The pieces of zoanthid polyps (approx. 1 cm³) were macerated, homogenized for isolation of zooxanthellae cells. Samples were centrifuged (1,500 rpm, 3–5 min) to pellet zooxanthellae cells, which were then resuspended in 15 ml tubes containing seawater. It was kept at -20°C overnight to break open the cells. This mixture was extracted by adding (90%) acetone. The resulting supernatant was diluted to 10 ml using the same solvent for the spectrophotometric analysis. The absorbance was read at 750, 664, 647 and 630 nm. The chl a and chl c content was then calculated using the trichromatic equations of (Jeffrey and Humphrey 1975).

2.6. Data Analysis

The monthly variation in the concentration of β -sitosterol was expressed as the % of total volume. In the phytagelTM based field assay for allelopathy level determination of the sponge's compounds on the zoanthids, the colour score of *Z. sansibaricus* from both treated and control experimental sets was represented as mean \pm SD of the colour scores for each polyp in the colony. NZ%, cell density, chl a and chl c content from both treated and control experimental sets were also represented as mean \pm SD. Data were then log transformed to meet the assumptions of normality and equal variance. Student's *t* tests (for unpaired groups) were used to find out if there were significant differences among each pair of the studied variables from both treated and control experimental sets.

3. Results

3.1 Environmental variables of the study site and its impact on sponge's life history functions

(allelochemical production and budding): The average values and range (expressed as minimum and maximum value) of environmental variables (such as temperature, salinity, DO, pH, nitrate, nitrite, ammonia, silicate and phosphate) of the rock pool containing tagged sponges is shown in **Table 1**. The budding cycle occurred from December to September with the maximum budding frequency in March. No budding occurred from October to November. The stepwise multiple regression analysis of abiotic (temperature, salinity, DO, pH, nitrate, nitrite, ammonia, silicate and

phosphate) and biotic variables (budding, allelochemical production, zoanths count) yields two significantly different models, in which forward selection of variables resulted in Model I, predicting the variability in production of β -sitosterol as a function of three predictive variables. On the other hand, backward elimination step yield Model II which included only two predictive variables (budding frequency and abundance of competitor) (**Table 2**).

3.2 . Impact of the zoanthidson the potent allelochemical (β -sitosterol) of the sponge

The two-phase solvent extraction gave approximately 400 mg of crude extract from the sponge with zoanths. On silica column chromatography using solvent gradient, three fractions (I–III) were obtained based on their analytical TLC data. The bioactive fraction I (0.13 g) was a white amorphous solid and showed cytotoxicity against Hela and PC-13 cell lines. It was characterized as β -sitosterol (**Fig. 2 A**) based on the ^1H and ^{13}C NMR and mass spectral data, as these values well-matched with literature data (Goad and Akihisa 2012; Nes et al. 1992; Sadikun et al. 1996; Slomp and MacKellar 1962; Smith 1978). The bioactive compound β -sitosterol was identified by NMR which showed monthly variation as depicted by HP-TLC. The concentration of β -sitosterol in the crude extract of the sponge showed a monthly variation with C_{max} =169 to 172 ppm during October–November 2013. The lowest concentration was found during February–March, 2014 (C_{min} = 23–64 ppm). In the sponges without zoanths, the concentration of β -sitosterol was significantly reduced for all the months as depicted by Student's *t* tests ($t = 4.490$, $p=0.00018$). For the months (October–November 2013), our data showed 70–72% reduction in β -sitosterol production in the sponges without zoanths whereas for months (May –June 2014), there was only a 34–38% reduction in its production. The maximum decrease (88%) in the concentration of β -sitosterol in the sponges without zoanths was estimated for February 2014 (**Fig. 2B**).

3.2. Impact of allelochemical(β -sitosterol) of spongeon Zoanths

3.2.1. On zoanths

Colour scores ranged from 5.5 to 6.0 which is suggestive of healthy colonies of zoanths and corals (Hibino et al. 2013; Nishiguchi et al. 2018; Siebeck et al. 2008; Siebeck et al. 2006). Zoanths under-treated gels showed a significant deleterious effect (such as complete bleaching and a few cases of necrosis) after 24 hrs days of contact). Colour scores were 3.5 times lower in treated colonies as compared to controls (Student's *t* test: $t= 9.53$, $P=0.000012$, $N=5$). After 24 h and thereafter, however, there was a great deal of polyp recovery from bleaching in treated zoanths on the removal of gels (polyp lethality at 48h, 31.20 ± 9.28 and at 72 h, 15.4 ± 5.50 ; Student's *t* test: $t=11.60$, $p =0.001$). Colour chart readings from zoanthid tissue under control gels were also compared with those

taken from tissue immediately adjacent to control gels to monitor the masking impact of Phytigel™ against zoanths tissue, and in no case was there a significant colour difference in the polyps under control gels as compared to the adjacent polyps. Zoanths away from treated gels were always normal and healthy, which suggests that allelopathy could occur only through contact.

3.2.2. On symbiotic zooxanthellae

(i) **Zooxanthella morphological condition and cell density.** Student's *t* test showed significantly lower numbers of healthy zooxanthella and cell density under the treated conditions as compared to control conditions (NZ%, $t = 15.96$, $p = 0.00001$ and cell density, $t = 8.19$, $p = 0.000037$) (**Fig.3**).

(ii) **Chlorophyll content of zoanths.** Student's *t* test showed a significantly lower chl a and chl c content of *Z. sansibaricus* under-treated conditions as compared to control conditions (chl a, $t = 15.15$, $p = 0.00001$ and chl c, $t = 5.01$, $p = 0.001$) (**Fig.3**).

Discussion

Sponges release a variety of bioactive compounds to protect their occupied space in a crowded habitat. The biosynthesis of these bioactive compounds in sponges may vary between seasons (Duckworth and Battershill 2001; Green et al. 1990; Page et al. 2005; Swearingen III and Pawlik 1998; Turon et al. 1996a). Both abiotic (depth, salinity, pollutants, light, and temperature) and biotic (space competition, predation, fouling, and presence/absence of symbionts) variables can influence the production of secondary metabolites in marine sessile organisms including sponges (Becerro et al. 2003; Hay 1996; Martí et al. 2004; Rohde et al. 2012; Wulff 2006). In some cases, the temporal variation in the production of the secondary metabolites was correlated with temperature (Abdo et al. 2007; Duckworth and Battershill 2001; Sacristán-Soriano et al. 2012; Swearingen III and Pawlik 1998). There is a close link between the production of secondary metabolites and the organisms' life cycle (growth, reproduction) as well as its physiological state (Ferretti et al. 2009; Ivanisevic et al. 2011; Leong and Pawlik 2010; López-Legentil et al. 2006; López-Legentil et al. 2007; Pawlik et al. 2008).

In this study, we isolated a cytotoxic compound (β -sitosterol) from the sponge which showed variability in its natural concentration, owing to seasonal changes in reproductive ability (in terms of budding frequency) of the sponge and abundance of its zoanths (polyps count). However, it has always been very challenging to study the ecological role of the secondary metabolites in the field (Becerro 2008). Few studies have described the role of secondary metabolites of sponges in the spatial competition (Chaves-Fonnegra et al. 2008; De Caralt et al. 2013; Engel and Pawlik 2000; Turon et al. 1996b). The combinatorial approach of bioassay-guided fractionation and phytigel™-

based allelopathic assay enabled us to establish β -sitosterol of the sponge as the potent allelochemical compound against the zoanthids. The present study, the elevated production of the cytotoxic sterol β -sitosterol (up to 17.2 % of total sterols) by the sponge decreased the number and density of symbiotic zooxanthellae in the host zoanthids, thereby explaining the possible reason for the bleaching of zoanthids in the vicinity of sponge. The compound β -sitosterol is the common sterol of plants (Saeidnia et al. 2014) where it acts as allelochemical against insects by hampering their growth and development (Mukherjee 2003). It has also been reported from several marine organisms, such as the scallop *Placopecten magellanicus* GMELIN, 1791 (Napolitano et al. 1993), the sponge *Haliclona (Reniera)* sp. (Lawson et al. 1988) and the alga *Cladophora densa* (ROTH) KÜTZING, 1845 (Saeidnia et al. 2014). In *Haliclona (Reniera)* sp., β -sitosterol and its derivative sigmasitosterol, constituting 8.7% of total sterols, have been reported to be assimilated from a dietary source (such as macroalgae, *Cladophora densa*) or its photosynthetic symbionts, rather than *de novo* synthesis (Lawson et al. 1988). Sponges release specialized (spherulous) cells that store large amounts of bioactive compounds, through its osculum in a process known as "spherulization" (Ternon et al. 2016). This process allows the sponge to form a chemical shield around it, thereby avoiding the overgrowth by neighbouring organisms. It can be speculated that the spherulization could be the possible strategy that sponges might use for the storage and release of water insoluble allelochemical (β -sitosterol) in the present study. However, future research in the fields of metabolomics can provide a better understanding of the localization of allelopathic compounds and their release mechanisms.

The present research work extends the current knowledge regarding the response of sponges to the environmental stress with special emphasis on allelochemicals. Such studies also provide a new perspective on ecosystem functioning with management implications. For instance, in the protected reef areas (with prohibited fishing, collection and anthropogenic pollution), allelopathic interactions can work naturally to favour one species over another, thus maintaining an overall diversity and balancing the benthic ecosystem. In addition to the ecological management and restoration programs, understanding the ecological role and factors affecting the production of a particular compound in sponge communities can also benefit the blue biotechnology and blue economy. It helps in predicting the best harvesting time of bioactive metabolites and design of successful aquaculture of sponges, thus supporting a sustainable utilization of sponges for bio-prospecting.

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Compliance with Ethical Standards

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Conflict of interest: The authors declare no conflict of interest.

Ethics statement: The field collections carried out for the purpose of this paper did not involve endangered or protected species. No specific permission was required to collect the analysed sponge samples.

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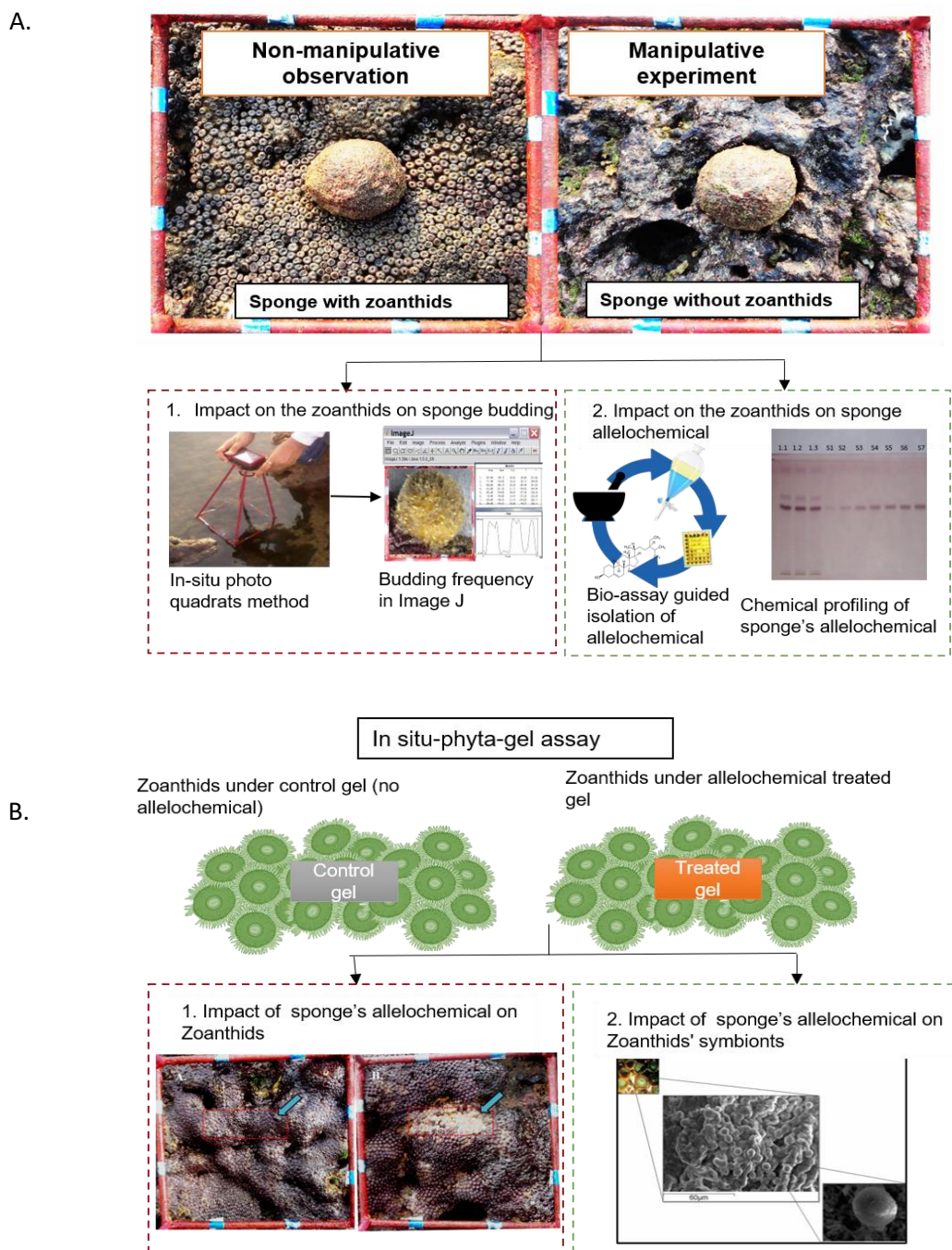


Fig.1. Schematic diagram of the methodology adopted for the present study. A. Non-manipulative and manipulative sets were made where in manipulative experiments, the zoanthids were removed from the vicinity of tagged sponges. The budding frequency was calculated using in-situ photo-quadrats method to find out the impact of zoanthids on the reproduction of sponge i.e., budding. The sponge tissue was collected from these two sets of experiments for determination of potent allelochemical component using bio-assay guided fractionation and its chemical profiling was done using HPTLC to understand the impact of zoanthids on sponge's allelochemical. B. In-situ phyta-gel assay was conducted to determine the allelopathic impact of sponge's compound on zoanthids and its symbiotic zooxanthella.

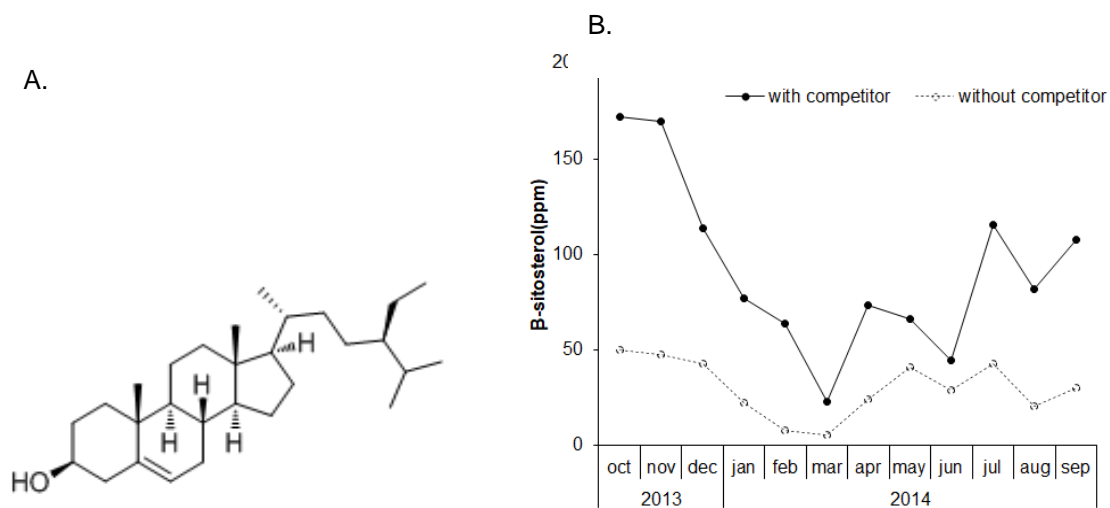


Fig.2.A. Structure of β -sitosterol (major sterol from sponge B. Monthly variations in concentration of β -sitosterol in sponge with and without zoanthids (sponge n=36 *2 experimental set up).

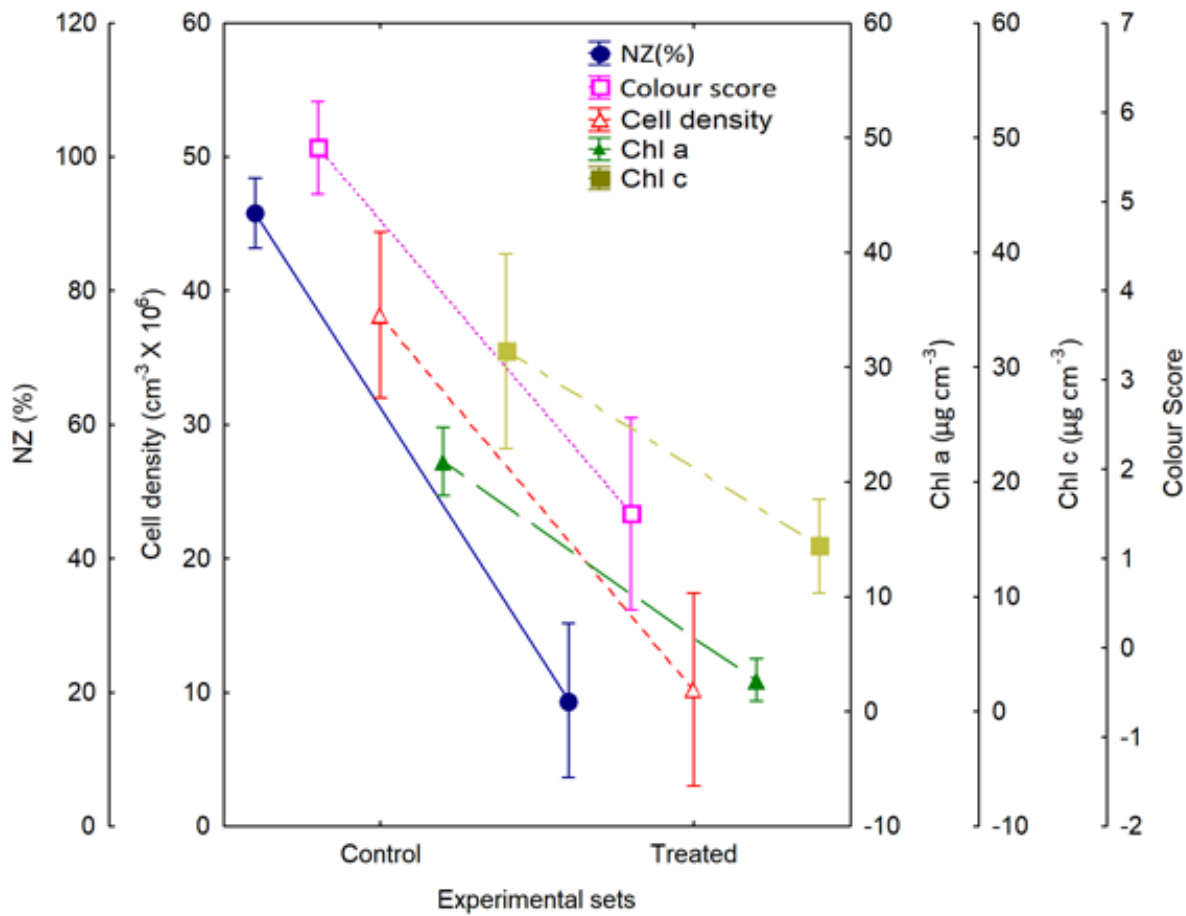


Fig.3. Whisker and box plot for zoanthids' colour core and its symbionts morphological condition NZ(%), cell density, chlorophyll a and c content under control and treated conditions. Colour scores NZ%, cell density, chlorophyll a and c content depicted the similar patterns, with significantly lower values for zoanthids under treated gels as compared to controls.

Table 1. Environmental measurements of the study area

	Mean \pm SD	Min.	Max.
pH	7.78 \pm 0.50	6.6	8.1
Temperature ($^{\circ}$ C)	29.35 \pm 1.15	27.6	31.2
Salinity (ppt)	32.85 \pm 2.36	26.82	34.91
Dissolved Oxygen (mg L ⁻¹)	4.31 \pm 0.95	2.19	5.23
Silicate (μ M)	8.17 \pm 4.86	2.76	19.12
Phosphate (μ M)	1.67 \pm 0.66	0.62	2.7
Nitrate (μ M)	6.25 \pm 2.36	3.48	11.16
Nitrite (μ M)	0.88 \pm 0.32	0.40	1.6
Ammonia (μ M)	3.19 \pm 1.35	0.97	5.12

Table 2. Estimated coefficients, standard errors, and p-values of multiple regression models for the “production of β -sitosterol” as predicted by abiotic and biotic environmental variables using a forward stepwise selection procedure (Model A, three explanatory variables, Multiple R= 0.96, $R^2 = 0.92$, adjusted $R^2 = 0.89$, $p < 0.00001$) and a backward stepwise elimination procedure (Model B, two explanatory variables, $R = 0.95$, $R^2 = 0.91$, Adjusted $R^2 = 0.88$, $p < 0.00001$).

	Estimate	Standard Error	p
Model I (Forward Selection)			
Intercept	-10.82	6.35	0.1054
competitor	0.01	0.00	0.0000
budding	-0.08	0.02	0.0001
salinity	0.73	0.18	0.0006
nitrate	-0.31	0.16	0.0599
pH	-0.76	0.69	0.2807
Model II (Backward Elimination)			
Intercept	-13.21	5.14	0.0183
budding	-0.08	0.02	0.0001
competitor	0.01	0.00	0.0000