

Light-Dependency of Growth and Secondary Metabolite Production in the Captive Zooxanthellate Soft Coral *Sinularia flexibilis*

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Abstract The branching zooxanthellate soft coral *Sinularia flexibilis* releases antimicrobial and toxic compounds with potential pharmaceutical importance. As photosynthesis by the symbiotic algae is vital to the host, the light-dependency of the coral, including its specific growth rate ($\mu \text{ day}^{-1}$) and the physiological response to a range of light intensities (10–1,000 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) was studied for 12 weeks. Although a range of irradiances from 100 to 400 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ was favorable for *S. flexibilis*, based on chlorophyll content, a light intensity around 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ was found to be optimal. The contents of both zooxanthellae and chlorophyll *a* were highest at 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. The specific budding rate showed almost the same pattern as the specific growth rate. The concentration of the terpene flexibilide, produced by this species, increased at high light intensities (200–600 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$).

Keywords *Sinularia flexibilis* · Light intensity · Specific growth and budding rates · *Zooxanthellae* · Flexibilide

Introduction

The symbiotic branching soft coral *Sinularia flexibilis* produces secondary metabolites, which have been found to possess a range of biological activities such as antimicrobial, anti-inflammatory, and cytotoxicity (e.g., Coll et al. 1982; Sammarco et al. 1987; Volkman 1999; Bhosale et al. 2002). For drug development from these compounds, large quantities of the coral are needed. One of the possibilities for that is to rear the corals in captivity. For this, the corals' physiological characteristics for cultivation need to be known.

S. flexibilis, like other symbiotic corals, harbors symbiotic algae, the zooxanthellae. They belong to the dinoflagellata and translocate photosynthates to the coral host (e.g. Muscatine et al. 1989). It is well documented, mainly in hard corals, that photoautotrophy becomes more efficient under optimal light conditions (e.g., Muscatine and Porter 1977; Muscatine et al. 1981) and that light enhances calcification (e.g., Al-Horani et al. 2003, 2007; Tentori and Allemand 2006). Hence, light is a major factor affecting both zooxanthellae and coral physiology and ecology, even at the scale of a single branch (Chalker et al. 1983; K uhl et al. 1995; de Beer et al. 2000). Additionally, symbiotic corals are able to acclimatize to environmental disturbance (e.g., light and temperature) through physiological and morphological responses, even though the range of acclimatization capacities within a species is not known (Gates and Edmunds 1999). In general, little is known about photoacclimation of corals (Titlyanova et al. 2001).

Moreover, it has been found that the photoreponse of corals to be species-specific (Titlyanov and Titlyanova 2002a,b). Because of the dependency of *S. flexibilis* on light (Fabricius

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et al. 1995a,b; De'ath and Fabricius 2000), it is important that light conditions are controlled in coral husbandry. However, the effect of irradiance on growth kinetics and biosynthesis of the major terpene of this species, flexibilide (e.g., Aceret et al. 2001), has not been clarified yet. Similarly, despite the fact that this soft coral can also propagate by budding (e.g., Fautin 2002), the link between both light-dependent specific growth rate and budding rate is unknown. Therefore, this long-term study was carried out under controlled laboratory conditions to verify (1) the influence of light intensity on the coral's specific growth and specific budding rates and also optimal irradiance for growth irradiance and physiology and (2) the influence of irradiance on the contents of zooxanthellae, chlorophyll *a*, protein, and flexibilide in the coral samples.

Materials and Methods

Organism

Parent colonies of the soft coral *S. flexibilis* were obtained from Burgers' Zoo, Arnhem, The Netherlands. They were transferred to rearing tanks (Eco-deco systems, Dymico-Model 1000, containing $\pm 1,300$ L saltwater) at 34 ± 0.5 salinity, $25.8\pm 0.2^\circ\text{C}$. The saltwater was made of the nutrient-free Instant Ocean Reef Crystals (Aquarium systems, Sarrebourg, France, 2007). Lighting (12 h light/12 h dark) was provided by VHO Halide, 10,000 K, HQI lamps (Aqua Medic aqualight 400, Aquaria Veldhuis, Enschede, The Netherlands).

Experimental Design

Experiments were performed in the laboratory under controlled conditions. Small coral colonies (5–7 cm) were taken, fixed on polyvinyl chloride platelets within ± 2 weeks, and placed in the tank. The experimental corals ($n=8$) were allocated to each light treatment. Different light intensities from 10 to $1,000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, measured by an underwater photo sensor (LI-COR, Li250 Light meter) were applied to the colonies at several locations in the tank. Because of the dependency of coral growth on water flow, the local mean water velocities for each experimental group in the tank was measured using a thermistor to ensure almost similar water flow for all colonies ($8\pm 2 \text{ cm s}^{-1}$), which is in the range of optimal flows for this species (Khalesi et al. 2007).

Specific Growth and Budding Rates

Growth of the experimental corals was calculated after a minimum of 2 weeks of adaptation to the lighting regimes, which would result in a complete photoacclimation (Anthony and Hoegh-Guldberg 2003). The weight gain of

each coral group ($n=8$) was measured weekly by buoyant weighing, using an analytical balance (Prolabo A&D HR300) with underweighing device. Average weekly μ were then calculated from: $\mu = (\ln W_2 - \ln W_1) / \Delta t$; where W_1 and W_2 are the weights at the beginning (t_1) and end (t_2) of each time interval. The mean of these specific growth rates was then calculated every week for the colonies at each light intensity. Changes in the mean μ were then plotted against light intensity. Statistical analyses included analysis of variance (ANOVA) and Student's *t* tests. To have a clear estimation of likely light effects on budding, the initial (time zero) and final (week 12) number of buds for coral samples in each group were counted to determine the specific budding rate using the same formula as for the specific growth rate.

Zooxanthellae, Chlorophyll *a*, Protein, and Flexibilide

At the end of the experiment, equal samples from each treatment were separated to measure the relative amounts of zooxanthellae, chlorophyll *a* (chl. *a*), protein, and flexibilide at various light intensities. Wet weight was determined by weighing dry blotted samples prior to freezing. Following freeze-drying, dry weight of the samples was also determined. In order to count the zooxanthellae, the samples were homogenized in tubes containing 2 ml filtered ($0.2 \mu\text{m}$) seawater. The homogenate (1 ml) was diluted and vortexed. The cells were counted using a hemocytometer under a microscope. Chlorophyll *a* was extracted based on Kinzie (1993), using chilled methanol/tetrahydrofuran (80:20 vol/vol) as the solvent at 4°C during night. The following day, absorbance of the supernatant was read at 665, 636, and 750 nm. The equation of Kinzie (1993) was also used to determine chlorophyll *a* concentration, which was normalized to coral's protein content to have a meaningful comparison of photosynthesis-dependent growth among the coral groups (Chalker et al. 1983). Protein normalization also provides a compatible measure of biomass (e.g., Zamer et al. 1989). Both the algae and chl. *a* were also normalized to the dry weight of the samples for comparison. The methanolic extraction was then used to quantify concentrations of flexibilide, based on Dmitrenok et al. (2003), using the standards kindly provided by Prof. B. Bowden, Queensland University, Australia. The flexibilide content was expressed as mg g^{-1} dry weight of the samples.

Results and Discussion

Light-dependent Growth

The light-dependency of the colonies of the soft coral *S. flexibilis* was investigated from low to high light intensities

(10 to 1,000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$). The raw data of buoyant weighing for the coral groups at each light intensity is shown in Fig. 1. The coral groups at moderate to relatively high light intensities (100–600 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) showed ascending growth, whereas at very low and especially at high light intensities (10 and 1,000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), the corals showed stunted growth. However, the growth at very low irradiance (10 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) was comparably higher than at 1,000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Weekly mean specific growth rates ($\mu \text{ day}^{-1}$) were calculated from buoyant weight data for 12 weeks (Fig. 2). Maximum value of μ was $16 \times 10^{-3} \text{ day}^{-1}$, which is similar to values in our previous study on this species (Khalesi et al. 2007). Paired t test showed significant differences in μ between the groups of 10 and 100 ($p=0.002$), 100 and 600 ($p=0.025$), 10 and 200 ($p<0.03$), and 200 and 600 ($p<0.02$). No significant differences in μ was found in the corals between 100 and 400 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (ANOVA, $p>0.05$). The optimal light intensity for rapid growth of *S. flexibilis*, therefore, appears to be in the range of 100–400 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The present light-dependent specific growth rate (μ) of *S. flexibilis* at light intensities of 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ is identical to the value that was established by Moya et al. (2006) for light-enhanced calcification (skeletal growth).

In addition, specific growth rates were also calculated from initial and final number of buds. A similar result was obtained as with buoyant weight data as a base for μ (Fig. 1). It is concluded that the number of buds per unit of buoyant weight is constant and independent of light intensity. Budding in *S. flexibilis* was discussed earlier as a mean of asexual reproduction both in the field (Bastidas et al. 2004) and in captivity (Khalesi et al. 2007).

Despite the coral survival (visual observation) at the extreme irradiances, their specific growth and budding rates were zero, indicating that they may survive but cannot thrive without suitable illumination. Hidaka et al (1982) found a relation between sunlight and budding frequency in

the scleractinian coral *Galaxea fascicularis*. To the author's knowledge, the current research is the first study of an optimal irradiance based on similar patterns of both specific growth and budding rates in corals.

The curvilinear light-growth pattern for *S. flexibilis* indicates no further growth increase at higher irradiances beyond the optimal range (100–400 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, Fig. 1). Hence, in addition to light availability (phototrophy) as the main source of energy for *S. flexibilis*, optimal light intensity also plays a fundamental role to maximize the utilization of available irradiance. This can also be of considerable attention on the ecological and physiological consequences of light intensity in the coral habitats, which are necessarily exposed to sunlight.

Photoacclimation

The relatively similar μ of the corals at light intensities of 100–400 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Fig. 2) suggests photoacclimation, which occurs within rather a long period (e.g., Barnes and Chalker 1990; Robison and Warner 2006). Photoacclimation improves the light tolerance, results in diminished light-induced injury, and maintains the maximum growth under these conditions. This result is in agreement with the earlier findings (Lambers et al. 1997; Titlyanov and Titlyanova 2002a) that (symbiotic) corals have a wide, species-specific range of light intensities in which, through physiological acclimation, they maintain a relatively stable level of production. In addition, *S. flexibilis*, being a shallow-water species, is considered to be more resistant to high radiation than deep-water corals (Siebeck 1988).

The flexible retractile structure of *S. flexibilis* probably was helpful to photoacclimate to supra-optimal levels. We observed that the corals at relatively high light ($>200 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) were always retracted once exposed and showed a delayed expansion when exposed again to low and moderate illuminations (10 and 100 $\mu\text{mol quanta m}^{-2}$

Fig. 1 Buoyant weight (g) of colonies of *Simularia flexibilis* at different light intensities within 12 weeks

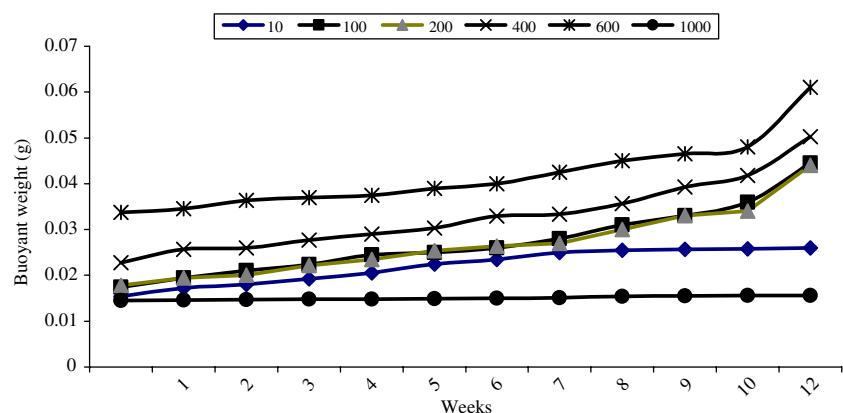
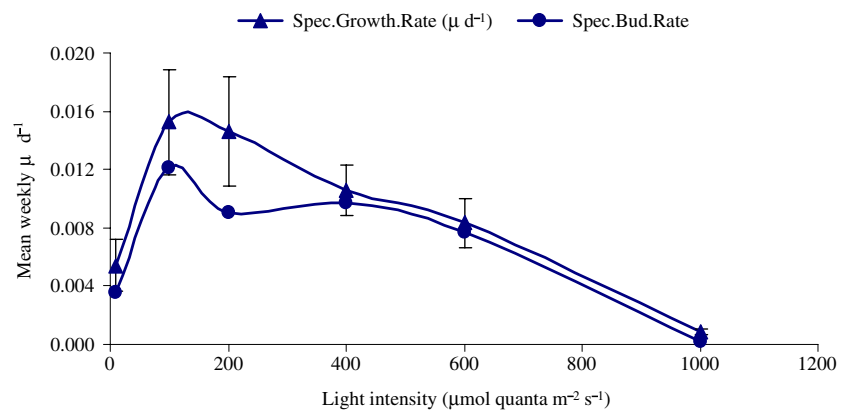


Fig. 2 Specific growth rates (μ day⁻¹) and specific budding rates day⁻¹ of *Simularia flexibilis* as a function of light intensity



s^{-1}). Besides, the yellowish appearance of the corals at high light intensities ($>100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) suggested the photoprotective xanthophyll cycle as a key factor in photoacclimation, which is a key photoprotective defense in shallow water corals (e.g., Long et al. 1994; Coles and Brown 2003). These features should have enabled this species to divert intense radiation and shield zooxanthellae, as was found for other symbiotic, retractile corals (Lasker 1979; Brown et al. 1994).

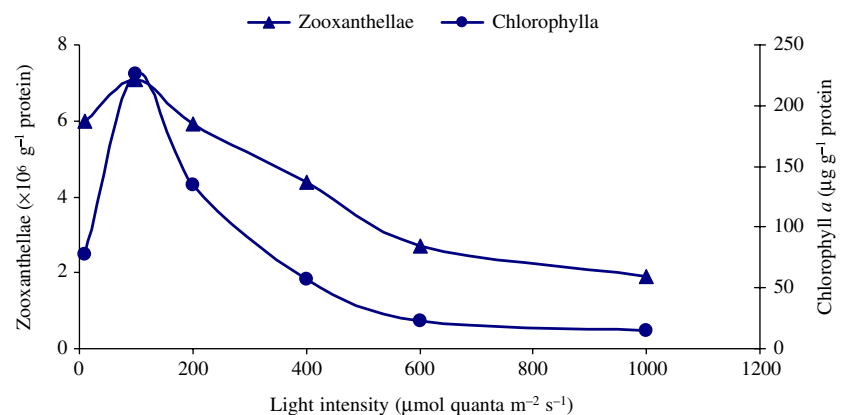
Zooxanthellae and Chlorophyll *a* Changes

Light-dependent growth of *S. flexibilis* shows the role of zooxanthellae and their distinctive light-absorbing pigment, chlorophyll *a* (Fig. 3). Zooxanthellae density increased two times at low light intensities (up to $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) compared to high irradiances. Chlorophyll *a* concentrations (g^{-1} protein) increased by a factor of 2 in the range of $10\text{--}100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and then decreased again by a factor of >2 times above $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Furthermore, normalization of the algae and chlorophyll *a* densities based on dry weight of the coral resulted in the same pattern. Despite the high zooxanthellae content at low

light intensity ($10 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) to maximize light absorption, compared to higher irradiances (200 and $400 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), they contain low chlorophyll because of low light availability. In contrast, the lower chlorophyll content at 200 and $400 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, as opposed to that of $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, led to the increased coral growth than at $10 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Fig. 2), suggesting an efficient photosynthesis. Therefore, photoacclimation through a decrease in the amount of photosynthetic units, while maintaining photosynthetic capacity, enabled *S. flexibilis* to thrive in a range of favorable light intensities ($100\text{--}400 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$). At this, the chlorophyll content and the photosynthetic light absorption capacity of the zooxanthella decreased; as a consequence, not all light was used in photosynthesis and a part was diverted. At higher intensities, the zooxanthella, as a result of photoinhibition, could not promote coral growth (Apprill et al. 2007; Fitt and Cook 2001; Iglesias-Prieto and Trench 1994, 1997).

Figure 3 is similar to the pattern of linearity of photosynthetic rates in corals at low light, deviation at certain irradiances, and reaching optimal values (maximal photosynthetic rate) at high irradiance (Chalker et al. 1983;

Fig. 3 *Simularia flexibilis*. Zooxanthellae and chlorophyll *a* contents based on protein content at varying light intensities after 12 weeks



Levy et al. 2004; Stambler and Dubinsky 2005). Hence, maximum algal photosynthesis corresponds to maximum growth of *S. flexibilis* at favorable light conditions.

Similarly, a negative correlation between zooxanthellae densities in *S. flexibilis* and solar downflux was found by Michalek-Wagner (2001). At a similar light intensity ($100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) as optimum growth irradiance, a higher content of chlorophyll than at other intensities was also found in a soft coral (Tsai and Liu 2005). Therefore, zooxanthellae act more efficiently under optimal light conditions, as was found earlier (Titlyanov et al. 2001; Titlyanov and Titlyanova 2002b).

Irradiances above $600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ apparently caused expulsion of zooxanthellae from the host (bleaching), i.e., the loss of a crucial energy source (Richter et al. 1990; Glynn 1993; Jones and Hoegh-Guldberg 2001) leading to the observed reduced growth. These phenomena are relevant at light intensities above $400 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, i.e., in shallow-water corals (Baker and Weber 1975; Lesser and Farrell 2004).

Flexibilide Content

The level of flexibilide increased up to irradiances of $600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ and then decreased again (Fig. 4). The flexibilide contents we found are in the same range as has been reported for this species before (Maida et al. 1993; Michalek-Wagner and Bowden 2000). Our data show that increased production of flexibilide in *S. flexibilis* presents a strategy to acclimatize the stressful situations through a chemical response to extensive long-term exposure to high light stress. Michalek-Wagner and Bowden (2000) have suggested such an energy investment into increased flexibilide under conditions of combined elevated temperature and solar irradiance for 12 days in the field. They, however, did not mention any range of light intensities nor a distinguishable effect of solar irradiance and temperature. Irrespective of the mechanism involved in flexibilide

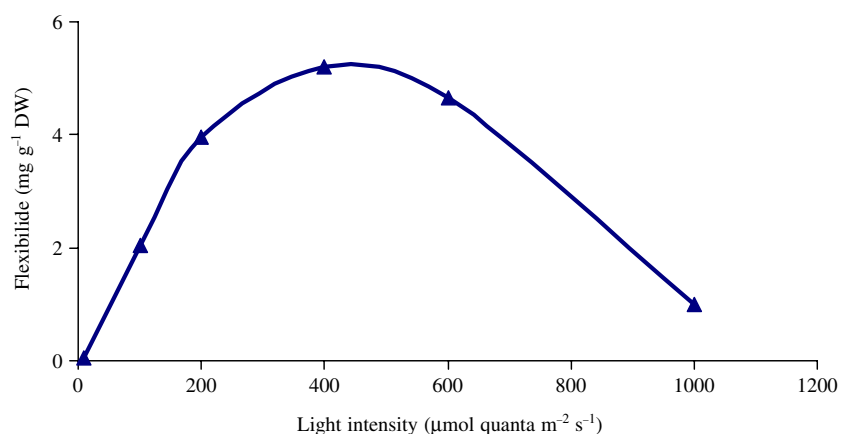
increase, both studies show that *S. flexibilis* has some capacity to overcome stressful conditions.

At very low light, reduced photosynthesis energy and the loss of zooxanthellae under high irradiances (10 and $1,000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) might have resulted in nutritional constraints leading to reduction of required energy for the biosynthesis of flexibilide. Although previously unknown, this mechanism has been proposed by Michalek-Wagner et al. (2001). Zooxanthellae in soft corals may also produce specific secondary metabolites (Papastephanou and Anderson 1982; Ciereszko 1989) or they may not (Michalek-Wagner et al. 2001; Frenz-Ross et al. 2008); zooxanthellae expulsion could therefore directly interrupt metabolite production in case the metabolites are really biosynthesized by the zooxanthellae or indirectly damage manufacturing the secondary metabolites through nutritional restrictions.

Conclusion

Our results show that phototrophy is decisive for the rapid growth of *S. flexibilis*. Both specific growth and budding rates of *S. flexibilis* showed a curvilinear dependency on light intensity. They were linear up to optimal light level, remained relatively constant at optimal range of irradiance, and then dropped at higher light intensities. These show a preference for optimum light and deterrence against high illuminations in this species. The photoacclimation of *S. flexibilis* and its symbionts helped the coral to sustain growth and acclimate a range of steady-state light intensities as optimal growth irradiances. Both the physiological and morphological acclimations led to a reduced photo-damage from high irradiances and to a maximized utilization of optimal range of irradiance. Because this soft coral apparently invests energy in the biosynthesis of metabolites against rather high illuminations, the optimal growth irradiance should be preserved at subsaturation levels. Thereby, the higher chlorophyll content will enable *S.*

Fig. 4 *Simularia flexibilis*. Flexibilide contents at varying light intensities after 12 weeks



flexibilis to optimize photosynthetically driven growth. This octocoral, therefore, has developed both physiological and morphological means to cope with various light conditions.

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