Ex Situ Cultivation of the Soft Coral *Sinularia flexibilis* for Biotechnological Exploitation

Mohammad Kazem Khalesi

Promotor

prof. dr. ir. R.H. Wijffels,
hoogleraar in de Bioprocestechnologie, Wageningen Universiteit

Co-promotor

dr. H.H. Beeftink,
universitair docent leerstoelgroep Bioprocestechnologie, Wageningen Universiteit

Samenstelling promotiecommissie

prof. dr. A.C. Smaal, Wageningen IMARES

prof. dr. ir. H.F.J. Savelkoul, Wageningen Universiteit

dr. R. Osinga, Wageningen Universiteit

dr. E.H.W.G. Meesters, Wageningen IMARES

Dit onderzoek is uitgevoerd binnen de VLAG OnderzoekSchool.

Mohammad K. Khalesi

Ex Situ Cultivation of the Soft Coral *Sinularia flexibilis* for Biotechnological Exploitation

PROEFSCHRIFT
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. M. J. Kropff,
in het openbaar te verdedigen
op maandag 28 april 2008
des namiddags te vier uur in de Aula

Khalesi M. K. (2008) *Ex Situ* Cultivation of the Soft Coral *Sinularia flexibilis* for Biotechnological Exploitation.

Ph.D. Thesis, Wageningen University, the Netherlands - With summary in English and Dutch

ISBN: 978 – 90 – 8504 – 912 – 8

To my beloved family

Acknowledgements

The completion of my Ph.D. thesis at Wageningen University during the last four years is undoubtedly indebted to mercy from Our Lord, and support from people. First of all, I praise Allah, the Almighty for providing me this opportunity and granting me the capability to proceed successfully.

I would like to thank the Iranian Ministry of Science, Research and Technology for funding this research. I also appreciate the support of Wageningen University for the last 6 months of my research.

René and Rik, I learned a lot from you. My cordial thanks for all fruitful discussions and useful supervision. You tried best to show me the appropriate means of research and to supervise me to express my findings in a proper scientific manner.

The supply of the corals for this research by the Burgers' Zoo, Arnhem, the Netherlands deserves great appreciation. My special thanks to Max Janse for this.

I express my deepest appreciation to my wife and my lovely daughter for their patience and spiritual support. Their presence and support here in Wageningen made it possible to perform this research with more morale.

To all my colleagues in Process Engineering, thank you for the pleasant environment. My roommates, Hassan and Sayam, thank you very much for your aid and for making the atmosphere of our room as friendly as possible.

To my colleagues in Marine Biotechnology group, Fred, Packo, Marcel, Rouke, Marzia, Marieke, Jan Willem and Maurice, many thanks for all the assistance you gave in my work.

Dear Natalia, you worked hard and have a big contribution in the 7th chapter of this thesis. I appreciate your efforts for achieving nice results.

I warmly thank Prof. Burice Bowden, Queensland Univ., Australia for kind provision of standards for secondary metabolites of *Sinularia flexibilis*.

Finally, thanks to all my Iranian friends here in Wageningen for the joyful gatherings and all their support. My special gratitude to Samad, Mohammad Ali, Hussein, Ali, Majid, Mostafa, Kaka, Mohammad and Mahmoud. We had lots of delightful, memorable get-togethers here, and I received kind help from you whenever needed.

Mohammad Kazem Khalesi
28 April 2008, Wageningen, the Netherlands

Contents

Chapter 1:	Introduction	1
Chapter 2:	The soft coral *Sinularia flexibilis*: potential for drug development	3
Chapter 3:	Light dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*	25
Chapter 4:	Phototrophy and heterotrophy in the captive symbiotic octocoral *Sinularia flexibilis*	39
Chapter 5:	Effects of ammonium, phosphate, and *Artemia* on the growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae	59
Chapter 6:	Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*	77
Chapter 7:	Cell cultures from the symbiotic soft coral *Sinularia flexibilis*	91
Chapter 8:	General Discussion and Conclusions	109
Summary		115
Samenvatting		119
Overview of educational activities		125
Curriculum Vitae		127
List of Publications		129

1 Introduction

The present thesis describes a study to culture a sessile marine invertebrate for the exploitation of potential marine pharmaceuticals, in particular, the *ex situ* cultivation of a soft coral known as *Sinularia flexibilis*. Over the past years, systematic screening of the toxins of this species has shown that its bioactive compounds have pharmacological important activities. Research has shown that several compounds isolated from *S. flexibilis* have anti-cancer, anti-microbial, and anti-fungal properties. Coral aquaculture could be a good method for sustainable continuous supply of these compounds.

We studied cultivation of *S. flexibilis* in captivity. In this thesis, the outcomes of our studies on the coral biology and metabolite production are presented. In the 2nd chapter, the biological and potential pharmacological applications of the coral metabolites, and also various means of biomass supply for drug development is reviewed. In the 3rd chapter, the light-dependency of both growth and secondary metabolite production in this symbiotic coral is studied. The 4th chapter deals with the extent of phototrophy and heterotrophy in this species. The topic of the 5th chapter is on the effects of nutrient enrichment on the coral growth and its algal symbionts. In the 6th chapter, influence of water velocity on the growth and physiology of *S. flexibilis* is discussed. And finally, the 7th chapter presents our study on the coral cell culture as an alternative to the coral supply.

In the end, the overall results of our research are discussed and accordingly, conclusions are made. Besides, approaches for future research in this field are recommended.

2 The soft coral *Sinularia flexibilis*: potential for drug development

Abstract

Evidence available to date suggests that the soft coral *Sinularia flexibilis* (Anthozoa, subclass Octocorallia, order Alcyonacea, family Alcyoniidae) offers a rich reserve of novel organic molecules, which can be useful as new drugs to combat diseases or as biochemical, physiologic and pharmacologic tools in biomedical research. In this article, over 210 studies till 2007 on the secondary metabolites isolated from known and unknown species of the genus *Sinularia* have been reviewed. A total of 42 researches about compounds from *S. flexibilis* are listed. Several compounds with special or selective activities have been described in more detail. It is important to investigate whether the compounds from *S. flexibilis* could be developed into future medical and industrial products. Cultivation of *S. flexibilis* under controlled conditions could be the solution to supply the biomass for pharmacological exploitation of some highly potent bioactive compounds.

This chapter is submitted as: Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. The soft coral *Sinularia flexibilis*: potential for drug development.

Introduction

Among Cnidaria (formerly Coelenterata), 21% of the species contain potential marine biomedical compounds (54). Almost 50% of soft corals (octocorals) as members of Cnidaria (phylum Cnidaria, class Alcyonaria, subclass Octocorallia) have been reported to produce toxins; about 60% of their extracts are bioactive molecules with medicinal potential (26, 30, 50, and 112). It has been stated that those compounds are promising to be used against diseases without the shortcomings of steroids and other anti-inflammatory drugs that are used presently as medicines (111).

Soft-bodied sessile invertebrates such as soft corals use a refined chemical weapon as they lack physical defenses; they have been the first target in screening programs for bioactive compounds because of their potential to provide molecules of use in pharmacology and as antifouling agents (e.g. 30, 124). Octocorals (class Anthozoa, subclass Octocorallia, order Alcyonacea, family Alcyoniidae) were one of the first marine groups that were systematically screened for secondary metabolites (127). These compounds, especially cembranoid diterpenes (51), have a function in chemical defense, in competition for space, against fouling and inhibit reproduction of other organisms (e.g. 17, 26–28, 30, 43, 58, 62, 70, 93, 105–112, 132, 133). In addition, corals produce mycosporine-like amino acids (MAAs) and other mycosporines referred to as true 'multipurpose' secondary metabolites. The most important function of MAAs and other mycosporines in nature is that they have a role as sunscreen against UV light (e.g. 94) in combination with other functionalities such as prevention of oxidation reactions (113).

From all marine-derived potential new drugs in preclinical stage in 1998 (78-80) and 2001-2 (81), 11-17% originate from soft corals. This shows that soft corals are an important source of active biological molecules and model compounds for drugs (23, 29, 108).

The soft coral genus *Sinularia* is one of the most widely distributed soft corals. About 60% of sinularin corals contain toxins (e.g. 30), including sesquiterpenes, diterpenes, norditerpenes, polyhydroxylated steroids, polyamine compounds with antimicrobial, anti-inflammatory and cytotoxic activities (5-7, 12, 13, 15, 16, 44, 51, 55-56, 68, 71, 72, 93, 99-102, 108, 112, 114, 117-120, 123, 138, 139). Our extensive literature

review over a period of more than 30 years has recorded 50 known species [out of a total of 90 (137)] and 23 unknown species of *Sinularia* (*S.* spp.) that have been chemically examined. To the extent of our review, over 210 papers have been published about the chemical constituents of *Sinularia* (both from known and unknown species), from which the majority report novel cytotoxic terpenoids.

The soft coral *Sinularia flexibilis* (Fig. 1) is cosmopolitan in its distribution and occurs in different seas (10, 30). Chemical examination of several collections of this species led to the earliest isolation of a range of cembranoid diterpenes (e.g. 18, 22, 118, 47) with potential anticancer activity (126). The current article reviews the secondary metabolites of *S. flexibilis*, their biological and pharmacological significance, and various means of the biomass supply for drug development.

Fig. 1. A colony of *Sinularia flexibilis*.

Secondary metabolites of *S. flexibilis* and their bioactive properties

Table 1 lists all the studies done on the bioactive compounds of *S. flexibilis* with their biological activities from 1975 up to date. In a review on the genus *Sinularia*, eight terpenoids of *S. flexibilis* in 15 studies from 1978 to 2002 have been surveyed (57).

The earliest isolated terpenoid was sinulariolide (126). This compound and two other compounds from this species that were isolated later, sinularin and dehydroxysinularin, had potential anticancer activity (136). The metabolite 7, 8-deoxyflexibilide that is present in low concentrations in *S. flexibilis*, was found to be toxic for the Japanese medaka fish (128). Alcyonin was purified from an Okinawan *S.*

flexibilis (69) with cytotoxic activity against Vero cells (kidney cell cultures from monkey). The same species was later reported to yield three diterpenes, lobatrientriol, acetoxylobaoxide, and lobatrienolide (47), but no biological activities were reported.

Many of the compounds are known to play important ecological roles in the defense against predation (feeding deterrence and ichthyotoxicity) and competition for space via allelopathy (reviewed by 30, 107). Diterpenes from *S. flexibilis*, for instance, were found to inhibit the development of eggs and larvae of two stony corals *in vitro* (1). The release of toxic secondary metabolites of this species into the surrounding water (26, 27) promoted inhibition of growth and mortality of neighboring scleractinian corals by altering their photosynthesis and respiration rates (99, 123). Low concentrations of some compounds from *S. flexibilis* have also been shown to cause expulsion of nematocysts and zooxanthellae, and subsequent death in scleractinian corals (1, 3).

These molecules, although lipophilic, are highly soluble in seawater (e.g. 21), and as allelopathic or anti-fouling agents are selectively absorbed onto biomembranes of fouling organisms. It has been found that the water around soft orals, specifically *S. flexibilis*, can contain between 1-5 mg l^{-1} of flexibilide and dihydroflexibilide (26, 28). This is precisely the concentration of toxin required to induce mortality in several scleractinian corals enabling the soft coral to exert influence on neighboring organisms in competition for space or fouling interactions (77). The identification of the potent algaecide 11-epi-sinulariolide from *S. flexibilis* (85) provides further evidence for the potential efficacy of released metabolites as anti-fouling agents. An antimicrobial compound described by Ref. (3) is expected to be used as future antibiotic.

Because *Sinularia flexibilis* is highly toxic (106) it is rarely overgrown by epibionts (bacteria or algae: 3, 130). Studies showed that antimicrobial properties of the diterpenes help protect the coral from competitors and predators. Two of the five tested diterpenes inhibited the growth of gram-positive bacteria, suggesting that this set of compounds may be an important source of new antibiotics (25). Nonetheless, of the many diterpenes isolated from *S. flexibilis*, only one (3) has been studied for antibiotic purposes.

Sinularia flexibilis yields a dichloromethane extract that typically contains approximately 8 mg of flexibilide (sinularin), 6 mg of dihydroflexibilide and 2 mg of sinulariolide per gram of coral dry weight (73). Flexibilide, the major terpene isolated from *Sinularia flexibilis* (e.g. 4) is a potent vector in allelopathy (28); it exhibited anti-inflammatory and anti-arthritic activity in rats (92), a property similar to the anti-inflammatory drug phenylbutazone (60). Flexibilide was also found to be an effective oral anti-inflammatory agent against rat paw oedema at 20-100 μmol/kg doses (21). In the same study, an advantage of flexibilide compared to betamethasone valerate, an anti-inflammatory drug, was that the rats treated with flexibilide did not loose weight, showed no side effects, being as healthy as untreated rats.

A broad range of biological activities have been reported for sinulariolide as being an algaecide with antifouling properties (127); it also showed marginal cytotoxic activities against a number of cell lines (121). Flexibilide, dihydroflexibilide, and sinulariolide were shown to be cardioactive, producing vasorelaxant responses in the isolated rat tissues, which may be useful for improved treatment of cardiovascular disease, especially heart failure (2). Flexibilide and sinulariolide were found to be effective potential anticancer agents (134); both compounds also exhibited antimicrobial activity and inhibited growth of Gram-positive bacteria (3); hence, they were reported as antibacterials being at preclinical research in 1998 (79).

More studies on this species revealed that organic extracts of *S. flexibilis* inhibited coral spat settlement (74, 75). A further study identified 11-epi-sinulariolide as the active algaecide exhibiting highly bioactive characteristics at many levels (85). In addition, cembranoid diterpenes isolated from *S. flexibilis*: sinuflexolide, dihydrosinuflexolide, sinuflexibilin, and sinuflexin showed significant cytotoxicity in human lung adenocarcinoma, human colon adenocarcinoma, human epidermoid carcinoma, and mouse lymphocytic leukemia cell cultures (P-388: 34, 35). An atrial stimulant compound was also reported in *S. flexibilis* (61).

Sinulariolone, a new highly oxygenated cembranoid, was obtained from a Philippine collection of *S. flexibilis* (46), and the trihydroxy cembranolide lactones, flexibiolide and dihydroflexibiolide were isolated from an Indian collection of *S. flexibilis* (9). Besides, five cembranolides with three new analogues from this species

were isolated (52), for which the cytotoxicity was also confirmed. Phospholipase A2, a toxic enzyme with a defensive role present in tissue homogenate of *S. flexibilis* was also identified (91). Conclusively, the antimicrobial activity of *S. flexibilis* diterpenes will not only add information to the growing pharmaceutical knowledge on marine compounds, but also indicate their potential as a source of antibiotics (3, 73).

In addition to the terpenes, *S. flexibilis* is also rich in steroids. Six new sterols were isolated and characterized in this species (55 and128). The marine sterols were reported to show a variety of biological and pharmacological activities (38, 87); those compounds were suggested to be potential candidates for anti-allergic drugs development (56).

Natural sunscreens

Mycosporine-like amino acids (MAAs) in corals are an important component of their photo-protective system against harmful UV radiation in shallow waters (e.g. 111). It has also been found that MAAs are biological antioxidants in coral tissue and zooxanthellae (135). The unique physical and chemical properties of MAAs as natural sunscreens prompted an investigation of their use in health-care applications and in the formulation of cosmetic products (129). MAAs in *S. flexibilis* are composed of six different components, with palythine as the major one (95%: 86). The major property of MAAs as photoabsorbents suggest potential commercial application in suncare products for skin protection and protection of non-biological materials as photostabilising additives in the plastic, paint and varnish industries (11).

We counted the number of publications related to certain toxic activities. In figure 2 it is shown that most activities reported are cytotoxic. This suggests that those compounds are expected to be promising anticancer drugs.

Table 1. Studies on the secondary metabolites of the soft coral *Sinularia flexibilis*

Metabolite	Activity	Ref.
7,8-epoxy-11-epi-sinulariolide acetate, 11-acetoxyl-15(17)-dihydrosinulariolide, 7,8-epoxy-11-sinulariolide acetate, and 3,4:8,11-bisepoxy-7-hydroxycembra-15(17)-dihydro-1,12-olide	Cytotoxic	52
Sinulariolide, flexibilide, dihydroflexibilide, and organic extracts	Cytotoxic, Algicidal, Cardiac vasorelaxant, Feeding deterrents	1, 2, 4, 8, 68, 73,130
Flexibiolide, dihydroflexibiolide sinularin, dihydrosinularin, sinuflexolide, sinuflexibilin, alcyonin, dihydrosinuflexolide, sinuflexin, and toxic extracts	Cytotoxic	34, 35, 9, 26, 69, 134
Flexibilide, sinulariolide, and 11-epi-sinulariolide	Antimicrobial Algaecide	3, 76, 85, 81, 118
Flexibilide	Anti-inflammatory	92, 21
Flexibilide,7,8-deoxyflexibilide and crude extracts	Ichthyotoxic	28, 70, 128
Toxic extracts	Allelopathic	26, 74, 75, 76, 105
Flexibolide, sinulariolone, 8,11-epoxy cembranolide, lobatrientriol, acetoxylobaoxide, lobatrienolide and flexibilene	Unknown	10, 46, 47, 49, 88
Aqueous extract	Antifouling	77
Flexibilide and dihydroflexibilide	Allomones*	110
2-Phenylethylamides	Atrial stimulants	61
Phospholipase A_2	Toxic compound (possibly cytolytic)	91
Mycosporine-like Amino Acids (MAAs)	Photoprotective (sunscreens)	86
5,8-epidioxysterols, and a cinnamide compound	Unknown	10, 137

* Any chemical released by one species for defense that affects the physiology of another species.

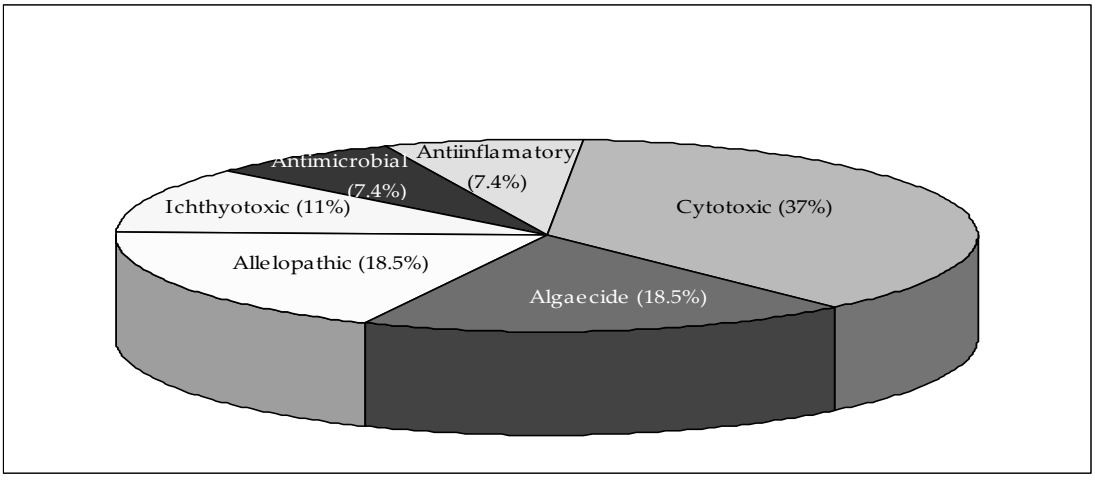


Fig. 2. Percentage of main activities of bioactive metabolites from *S. flexibilis* based on No. of reports.

The demand for coral biomass

Despite the fact that many natural products from marine invertebrates are promising drugs or drug leads, the inadequate supply of coral biomass as a raw material has delayed the development of these agents (40). This has been a major constraint in the development of the bioproducts from corals (e.g. sarcophytol: 37). A critical step is the inclusion of a sustainable, industrially feasible supply in order to overcome this main limitation and ensure a regular pathway of preclinical-clinical investigations, not to mention the market demand, for which a preliminary estimation is made below.

Possible means to overcome the supply problem are environmentally amenable ways of obtaining adequate supplies of the compounds of interest; these include aquaculture, cell culture, analogue development, chemical synthesis, and genetic manipulations (24). Synthesis of the bio-products as the commercial source of choice for pharmaceutical industry allows control of all aspects of production. But, unlike terrestrial bio-compounds, many bioactive marine natural products, particularly those used in the pharmaceutical field, are extremely complex in structure, and require intensive multi-step processes that are not amenable to economic, industrial-scale synthesis. Therefore, the complexity of the marine-derived chemical structures, difficulty to develop, low yields (89), and expensiveness can limit the development of synthesis processes. However, synthesis of two compounds from *S. flexibilis*, flexibilene (a short and simple path) and alcyonin (in 11 steps), were reported by 83 and 31.

The establishment of cell lines from marine invertebrates has encountered obstacles; no single valid marine invertebrate cell line had been developed by 1998 (103). For instance, in only one study (41), despite the establishment of long-term cell cultures from ten taxa of marine cnidarians (including octocorals), secondary cell cultures from corals were not fulfilling as in most cases, cells were maintained until 1 year without any signs of multiplication. However, five studies relating to the development or improvement of cell cultures from corals have been published from 1999-2004, which represented short-term experiments (<1 month) with significant implications for holding cnidarian cells (including corals) *in vitro* (Rewied by 103). Kopecky and

Ostrander (67), for example, reported a maximum of 300 hours of survival of cultivated hard coral cells, but did not succeed to develop continuous proliferating cell cultures. Although some encouraging reports on cniderian cell culture systems have been presented, the lack of vital information regarding cell requirements and their physiology and biochemical patterns *in vitro*, as well as improper comparisons between vertebrate-invertebrate cell requirements might account for the failure (104).

A long-term laboratory culture of the coral juvenile (planula or coral larvae) from the symbiotic hard coral *Acropora tenuis* has been developed recently (131). The coral juveniles maintained for at least three months, and exhibited size increment during that period. This could be encouraging for coral tissue culture.

Since most of these bioactive metabolites are found at low concentrations, large collections of the source coral are needed to isolate sufficient compounds for clinical trials (61, 98). For drug development or production, it is often not economical and would be environmentally destructive and expensive to supply drugs by large-scale harvesting from the environment (32). Aquaculture, as an alternative to natural harvest requires major advances in culture techniques. In some cases, it might be the only way to obtain sufficient amounts of compounds (90). A series of studies have been done on aquaculture (mari-and/or lab-culture) (e.g. 130 in aquarium systems; 14 *in situ*) to reduce the need for coral collection for ecotoxicological studies (32). Successful aquarium rearing of corals has been reported (48) and we think that aquaculture could be a satisfactory solution for the supply of certain species and phyla of marine invertebrates. It has been shown to be a feasible and manageable technology to meet, in part, the needs for commercial drug supplies (84, 98). Nevertheless, possible problems related to mass culture such as destruction of culture facilities and stocks by storms or by diseases are possible scenarios that will make reliable continuous supply difficult (98). A further advanced biotechnological approach, the genetic manipulation, by cloning and expression of the respective biosynthetic genes (if they are known which they are mostly not) provides the opportunity to spread biosynthesis of secondary metabolites.

Table 2. Summary of advantages and disadvantages of various means of mass production of the coral.

Approach	Advantage	Disadvantage
Aquaculture (closed system)	Environmental parameters are controllable; growth kinetics and the effects of culture conditions on the biosynthesis of the compounds is easily studied (64); year-long cultivation (115); feasibility is proven (e.g. 19, 115).	Possibly expensive, time consuming, and space intensive; requires knowledge of species-specific cultivation parameters.
Mariculture (open system)	Cost-effective (81); bulk supply of the compounds; feasibility is proven (14).	Complete control of environmental parameters and diseases not possible; likely destruction of culture facilities by storm; seasonality (115).
Tissue or cell culture	Establishing cell lines as models for in vitro production of bioactive metabolites; to study the factors controlling production to enhance process optimization.	Not successful to establish long-lasting, proliferating cell lines up until now;
Organic synthesis	Allows control of all aspects of production; successfully done for a number of compounds.	Multi-step process; not applicable for all compounds; low yield; expensive (98).
Genetic manipulation	Possibility to identify, isolate, clone, and express the genes for production of the compounds in a heterologous host.	The biosynthesis of the target compound is mostly not or poorly known; expensive; low feasibility; lack of effective techniques.

A summary of the advantages and disadvantages of various approaches of mass production of the coral is presented in table 2. Considering the pros and cons for each approach, it is concluded that those approaches with proven feasibility (captive aquaculture and mariculture) are more promising in short term. These approaches also provide valuable knowledge on coral biology to be used for biotechnological applications.

Estimating the annual demand for *S. flexibilis*

The average content of the major antibacterial terpene (flexibilide) in *S. flexibilis* was found to be 5×10^{-3} kg kg^{-1} coral dry weight (3, 26, and 28). The yearly demand for other commercial drugs vary from 1–5 kg for sponge halichondrins up to 45,000 tonnes for penicillin (53, 20). It can, therefore, be estimated that the annual coral need

for flexibilide production should lie between 10^3 to 10^{10} kg dry weight (10^4-10^{11} kg wet weight), depending on the type of the required compound. Assuming a regular annual supply of 10^{11} kg of the coral biomass yielding 50 tonnes flexibilide $ha.^{-1}$ y^{-1} from a cultivation system, we would need an arable area of 0.2×10^8 hectare. These very preliminary estimates of the annual biomass production for preclinical-clinical trials suggest that an annual production equates to huge amount of the coral that apparently can be partially supplied by aquaculture, as is also true for sponges (90). It has been estimated that sponge halichondrins necessitate an annual harvest of 3000–17000 tonnes of the source sponges (53). Even though, large-scale production of sponge metabolites by generation of biomass is not probable in many cases (115). Therefore, because of apparent uncertainty for large-scale coral cultivation, it can be directed to be an alternative for production of specialty drugs (e.g. anticancers) which have a lower market demand than antibiotics (halichondrins vs. penicillin).

A more exact estimate together with actual production variables and economics can be made via the required quantity of the compounds. However, culture of the source coral under artificial controlled conditions is considered to be a viable method for supplying metabolites for drug development (e.g. 32), and also a particularly challenging opportunity for marine bioprospecting to discover new bioproducts (97); such a system also allows growth parameters such as light levels and water flow, as well as compound biosynthesis to be optimized (115). Therefore, for in vitro culture to succeed, it is vital to develop appropriate biological regimes that promote growth and biosynthesis of the target metabolites (e.g. 33). In addition, in order to develop models for aquaculture, factors such as the standing stock of the organism, its growth and the factors that affect growth should be clarified (96).

Cultivation of *S. flexibilis*

With the goal of creating a sustainable resource, through a series of studies on the cultivation of *S. flexibilis* in captivity, we showed that colonies of this soft coral are able to fully adapt to laboratory conditions in a relatively short period. Our data on growth kinetics (64) and metabolite biosynthesis demonstrate that *S. flexibilis* responds

convincingly to the controlled conditions, being able to produce flexibilide, which, for instance, is influenced by light intensity (65).

Fig. 3. Eco-Deco cultivation system at Wageningen University.

The culture system (Eco–deco systems, Dymico–Model 1000, ± 1300 L, Fig. 3) was also a mechanised system that could be developed to a large-scale stocking- type culture system.

Our findings show that *S. flexibilis* could be grown successfully in small-scale trials and that the flexibilide continued to be biosynthesised, even after several years of culture. In addition, an advantage of *S. flexibilis* with a branching growth form is that more small fragments can be collected from the parent colony, without affecting its ability to recover from the collection. Another benefit of this species compared to some other symbiotic corals is its absolute light-dependency (66) independent of manual feeding in captivity.

Similarly, sustainable harvest has allowed researchers to obtain sufficient supplies of a gorgonian coral over a 15-year period without devastating local populations, ultimately leading to purification of a highly profitable product, pseudopterosins, by ensuring an adequate supply (19). It was also shown that a cultured soft coral *Eleutherobia caribaeorum* produces the secondary metabolite (eleutherobin) in captivity (122). Considering the fact that no pharmaceutical company has yet relied solely on

aquaculture for bulk supply of a compound (53), the above-mentioned evidences indicate that coral culture opens the way to aquaculture production of interesting pharmacological agents.

Economical considerations

The economics of soft coral farming is affected by many variables such as location, its size and market; the costs will vary greatly with the region (36). To obtain insight in the economics of *S. flexibilis* mass production, our estimation assumes the production costs comparing cost price of sponge *ex situ* cultivation (115) because of similar systems, and regional similarity. The feeding costs are excluded (because of the phototrophy of the coral) and instead, lighting expenses are included. The costs of our closed aquaculture (*ex situ* cultivation) have been compared to available respective resources on coral commercial mariculture (36, 125).

For the production of 1 kg of flexibilide, a minimum coral biomass of 2×10^3 kg (wet weight) is needed. If the cultivation tanks (5 m^3, table 3) yield 20 kg coral wet weight, the cost per kg flexibilide exceeds 5×10^6 € y^{-1}. This is lower than that of 1.9×10^7 € y^{-1} for production of sponge halichondrins through *ex situ* cultivation (115). Considering the lower annual cost for coral mariculture (table 3), the cost will be lower for the coral mariculture per kg of flexibilide y^{-1}.

Table 3. Summary of expenses for two approaches of coral mass production, extracted from Ref. 115 with some changes (left column). The right column displays the costs of coral mariculture (36, 125).

***Ex situ* aquaculture**		**Mariculture**	
Equipment	Price (€)	Equipment	Price (€)
Cultivation tank (5 m^3)	10,000	Green house (25×12)	9500
Laboratory	2000 m^{-2}	Heat pump	3390
Production space	800	Sump	300
Storage house	500	Pumps	600
Lighting bulb/tank	100	Electrical	850
Electricity/12 hrs d^{-1}/bulb	220 y^{-1}	SCUBA gear/2 people	150
Office	1200 m^{-2}	Boat and motor	1400
Employee	37000 y^{-1}	Land lease (if applicable)	2400
		Other costs (incl. employee)	12000
Total	**51,820 € y^{-1}**	**Total**	**30,600 € y^{-1}**

Based on expenses in table 3 and also estimations on flexibilide production, an economically feasible technique to culture coral on a relatively larger scale, can be a combination of off-sea (land-based) and controlled cultivation, depending on regional facilities. In that case, while taking advantage of lower costs, environmental parameters are also controlled.

Conclusions

In this review, the biomedical aspects and the application of secondary metabolites from the soft coral genus *Sinularia* have been broadly surveyed. Our overview covering a long period of over 30 years shows that the soft coral *Sinularia flexibilis* has attained the highest records of researches on its bioproducts. The evidence of novel and pharmacologically active compounds from *S. flexibilis* points out its biomedical significance for future therapeutic agents. As a consequence, increasing demands for such a pharmacologic and toxicological model for screening as well as for in depth studies might lead to overexploitation of the corals' natural reservoir. These facts, together with the proofs demonstrated in this review, would present a demand for sustainable approaches of long-term exploitation. This review tried to concentrate on pharmacological potential of some highly potent compounds, entirely called toxins, isolated from *S. flexibilis*, as well as the coral potential for *in vitro* cultivation. Growing the coral under controlled conditions with further expansion of the knowledge base for their aquaculture can be one alternative by which the demanding biomass is supplied.

References

1. Aceret, T.L., Sammarco, P.W., Coll, J.C., 1995. Toxic effects of alcyonacean diterpenes on scleractinian corals. J Exp Mar Biol Ecol 188, 1, 63-78.
2. Aceret T.L., Brown L., Miller J., Coll J.C., Sammarco P.W., 1996. Cardiac and vascular responses of isolated rat tissues treated with diterpenes from *Sinularia flexibilis*. Toxicon 34: 1167–1171.
3. Aceret T.L., Coll J.C., Uchio Y., Sammarco P.W., 1998. Antimicrobial activity of the diterpenes flexibilide and sinulariolide derived from *Sinularia flexibilis* Quoy and Gaimard. Comp Biochem Physiol (C) 120: 121–126.
4. Aceret, T.L., Sammarco, P.W., Coll, J.C., Uchio, Y., 2001. Discrimination between several diterpenoid compounds in feeding by *Gambusia affinis*. Comp Biochem Physiol Part C 128, 55–63.
5. Ahmed A.F., J.H. Su, R.T. Shiue, X.J. Pan, C.F. Dai, Y.H. Kuo, and J.H. Sheu, 2004a. New Caryophyllene-Derived terpenoids from the soft coral *Sinularia nanolobata*. J. Nat. Prod., 67, 4, 592-597.
6. Ahmed A.F., Y.H. Kuo, J.H. Sheu, 2004b. Scabrolides E-G, three new norditerpenoids from the soft coral *Sinularia scabra*. J. Nat. Prod., 67, 4, 592-597.
7. Ahmed A.F., S.H. Taia, Y.C. Wuc, J.H. Sheu, 2007. Sinugrandisterols A–D, trihydroxy steroids from the soft coral *Sinularia grandilobata*. Steroids 72, 4, 368-374.
8. Alino, P.M., Coll, J.C., Sammarco, P.W., 1992. Toxic prey discrimination in a highly specialized predator *Chaetodon melannotus* (Block et Schneider): Visual vs. chemical cues. J Exp Mar Biol Ecol 164, 2, 209-220.
9. Anjaneyulu A.S.R., Sagar K.S., 1996. Flexibiolide and dihydroflexibiolide, the first trihydroxycembranolide lactones from the soft coral *Sinularia flexibilis* of the Indian Ocean. Nat Prod Lett 9-12.
10. Anjaneyulu A.S.R., K.S. Sagar, and G.V. Rao, 1998. New cembranoid lactones from the Indian Ocean soft coral *Sinularia flexibilis*. J. Nat. Prod., 60, 9-12.
11. Bandaranayake W.M., 1998. Mycosporines: are they nature's sunscreens? Nat Prod Rep, 159–172.
12. Bhosale, S.H., Nagle, V.L., Jagtap, T.G., 2002. Antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomonas*. Mar. Biotech. 4,111–118.
13. Blunt J.W., B.R. Copp, M.H.G. Munro, P.T. Northcote, and M.R. Prinsep, 2006. Marine natural products Nat. Prod. Rep. 23 , 26–78
14. Bongiorni L., Shafir S., Angel D., Rinkevich B., 2003. Survival, growth and gonadal development of two hermatypic corals subjected to in situ fish farm nutrient enrichment. Mar Ecol Prog Ser 253, 137–144.
15. Bowden BF, JC Coll, W Hicks, R Kazlauskas and SJ Mitchell, 1978. Studies of Australian soft corals. X. The isolation of epoxyisoneocembrene-A from *Sinularia grayi* and isoneocembrene-A from *Sarcophyton ehrenbergi*. Aust. J. Chem 31, 12, 2707 – 2712.
16. Bowden BF, JC Coll, SJ Mitchell and R Kazlauskas, 1981. Studies of Australian soft corals. XXIV. Two cembranoid diterpenes from the soft coral *Sinularia facile*. Aust. J. Chem 34, 7, 1551 – 1556.
17. Bowden BF, JC Coll, Silva ED de and Costa MSL de, 1985. Studies of Australian soft corals. XXXI. Novel furanosesquiterpenes from several sinularian soft corals (Coelenterata, Octocorallia, Alcyonacea). Aust. J. Chem 36, 2, 371 – 376.

18. Bowden, B.F., Alino, P.M., Coll, J.C., 1992. Soft corals and their toxins. In: Watters, D., Lavin, M., Maguire, D., Pearn, J. (Eds.), Toxins and Targets. Effects of natural and synthetic poisons on living cells and fragile ecosystems. Harwood Academic Publ, Philadelphia, pp. 55-64.
19. Bruckner, A.W., 2002. Life-Saving Products from Coral Reefs. Issues in Science and Technology Online-issues.org.
20. Bruggink, A., 2001. Synthesis of β-lactam antibiotics: Chemistry, biocatalysis and process integration. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 19-20.
21. Buckle P.J., Baldo B.A., Taylor K.M., 1980. The anti-inflammatory activity of marine natural products-6-n-tridecylsalicylic acid, flexibilide and dendalone 3-hydroxybutyrate. Agents and Actions 10, 4, 361-367.
22. Campos, R.T., Alino, P.M., Montano, N.E., Cuaresma, N.T., 1995. Ichthyotoxicity of octocorals from Bolinao, Pangasinan. In: Sotto F.B., Young J.G., Baumgarner, J. (Eds.), Third National Symposium of Marine Sciences, Philippine Association of Marine Science (PAMS), Iloilo, Philippine Sciences, Suppl.
23. Carte B.K., 1996. Biomedical potential of marine natural products: Marine organisms are yielding novel molecules for use in basic research and medical applications. BioScience 46, 4, 271-286.
24. Clare A.S., D. Rittschof, D.J. Gerhart, I.R. Hooper, and J. Bonaventura, 1999. Antisettlement and narcotic action of analogues of diterpene marine natural product antifoulants from octocorals. Mar. Biotech. 1, 427–436.
25. Clark D., 2000. Counteracting resistance with research on natural antibiotic sources. Bio 25L Section 23.
26. Coll J.C., S. La Barrel, P.W. Sammarco, W.T. Williams, G.J. Bakus, 1982a. Chemical defences in soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef: A study of comparative toxicities. Mar. Ecol. Prog. Ser. 8, 271-278.
27. Coll, J.C., Bowden, B.F., Tapiolas, D.M. and Dunlap, W.C., 1982b. In situ isolation of allelochemicals released from soft corals (Coelenterata: Octocorallia): a totally submersible sampling apparatus. J. Exp. Mar. Biol. Ecol. 60, 293–299.
28. Coll, J.C., Sammarco, P.W., 1983. Terpenoid toxins of soft corals (Cnidaria, Octocorallia): their nature, toxicity, and ecological significance. Toxicon Suppl., 69–72.
29. Coll J.C., B.F. Bowden, D.M. Tapiolas, R.H. Willis, P. Djura, M. Streamer and L. Trott, 1985. Studies of Australian soft corals—XXXV: The terpenoid chemistry of soft corals and its implications. Tetrahedron 41, 6, 1085-1092.
30. Coll, J.C., 1992. The Chemistry and Chemical Ecology of Octocorals (Coelenterata, Anthozoa, Octocorallia). Chem. Rev. 1002, 92, 613-631.
31. Corminboeuf, O., Overman L.E., Pennington L.D., 2003. Total synthesis of the reputed structure of alcyonin and reassignment of its structure. Org. Lett., 5, 9, 1543-1546.
32. Davies P.S., 1995. Coral nubbins and explants for reef assessment and laboratory ecotoxicology. Coral Reefs 14, 267-269.
33. Duckworth, A., 2004. Ascidian, sponge culture supplies bioactive metabolites. Global Aquaculture Alliance, 66-67.

34. Duh C., Wang S., Tseng H., Sheu J., 1998a. A novel cytotoxic biscembranoid from the Formosan soft coral *Sinularia flexibilis*. Tetrahedron Lett 39, 7121–7122.
35. Duh C.Y., Wang S.K., Tseng H.K., Sheu J.H., Chiang M.Y., 1998b. Novel cytotoxic cembranoids from the soft coral *Sinularia flexibilis*. J Nat Prod. 26, 61, 844-847.
36. Ellis, S.C., Samson, G., 1998. Farming soft corals for the marine aquarium trade. Center for tropical and subtropical aquaculture, Waimanalo, Hawaii, USA. Publ. No. 140.
37. Fahmy et al., 2004. An improved synthesis of 7, 8-Epoxy-1,3,11-cembratriene-15R, 16-diol, a cembranoid of marine origin with a potent cancer chemopreventive activity. Mar. Drugs 2, 1-7.
38. Faulkner D.J., 1997. Marine natural products. Nat Prod Rep 14, 259–302.
39. Faulkner, D.J., 2000. Marine pharmacology, Anton. Leeuw. Int. J.G. 77, 135–145.
40. Fenical W., 2006. Marine pharmaceuticals: Past, Present, and Future. Oceanography, 19, 2, 110-119.
41. Frank, U., Rabinowitz, C., Rinkevich, B., 1994. In vitro establishment of continuous cell cultures and cells lines from ten colonial cnidarians. Mar. Biol. 120, 491–499.
42. Gateno D., Y. Barki, B. Rinkevich, 1999. Aquarium maintenance of reef octocorals raised from field collected larvae. Aq Sci Conserv 2, 227–236.
43. Gerhart D.J., Coll J.C., 1993. Pukalide, a widely distributed octocoral diterpenoid, induces vomiting in fish. J Chem Ecol 19, 11, 2697-2704.
44. Goto R., K. Muramoto, M. Yamazaki and H. Kamiya, 1992. Purification and characterization of an agglutinin of the soft coral *Sinularia* species. Develop Comp Immunol 16, 1, 9-17.
45. Grace K.J.S., D. Zavortink and R.S. Jacobs, 1994. Inactivation of bee venom phospholipase A_2 by a sesquiterpene furanoic acid marine natural product. Biochem Pharmacol 47, 8, 1427-1434.
46. Guerrero PP, Read RW, Batley M, Janairo GC, 1995. The structure of a novel cembranoid diterpene from a Philipine collection of the soft coral *Sinularia flexibilis*. J Nat Prod 58: 1185–1191.
47. Hamada T, Kusumi T, Ishitsuka MO, Kakisawa H, 1992. Structures and absolute configuration of new lobane diterpenoids from the Okinawan soft coral *Sinularia flexibilis*. Chem Lett 33–36.
48. Harriott V.J., 2003. Can corals be harvested sustainably? Ambio 32, 2, 130-133.
49. Herin M., Tursch B., 1976. Chemical studies of marine invertebrates. XXIV. Minor cembrane diterpenes from the soft coral *Sinularia flexibilis*. Bull Soc Chim Belg 85, 801-803.
50. Higa, T., Tanaka, J, Ohtani, I.I., Musman, M., M.C. Roy, Kuroda, I., 2001. Bioactive compounds from coral reef invertebrates. Pure Appl. Chem. 73, 3, 589–593.
51. Hirono, M., Ojika, M., Mimura, H., Nakanishi Y., Maeshima, M., 2003. Acylspermidine derivatives isolated from a soft coral, *Sinularia sp.*, inhibit plant vacuolar H+-Pyrophosphatase. J. Biochem. 133, 811–816.
52. Hsieh P.W.; F.R. Chang; A.T. McPhail; K.H. Lee; Y.C. Wu, 2003. New cembranolide analogues from the formosan soft coral *Sinularia flexibilis* and their cytotoxicity. Nat Prod Res, 17, 6, 409- 418.
53. Hunt, B., A.C.J. Vincent, 2006. Scale and sustainability of marine bioprospecting for pharmaceuticals. Ambio 35, 2, 57-64.
54. Jha, R.K., Zi-rong, X., 2004. Biomedical compounds from marine organisms. Mar. Drugs 2, 123-146.

55. Jia R., Y.W. Guo, E. Mollo, M. Gavagnin, and G. Cimino, 2006. Two new polyhydroxylated steroids from the Hainan soft coral *Sinularia* sp. Helvetica Chimica Acta, 89, 1330- 1336.
56. Jin P., Z. Deng, Y. Pei, H. Fu, J. Li, L. Van Ofwegen, P. Proksche, W. Lin, 2005. Polyhydroxylated steroids from the soft coral *Sinularia dissecta*. Steroids, 70, 8, 487-493.
57. Kamel H.N. and M. Slattery, 2005. Terpenoids of *Sinularia*: chemistry and biomedical applications. Pharmaceutical Biology 43, 3, 253–269.
58. Kamel H.N., F.R. Fronczek, S.I. Khalifa, and M. Slattery, 2007a. Microbial Transformation of 5-Episinuleptolide. Chem. Pharm. Bull. 55, 4, 537—540.
59. Kamel H.N., F.R. D. Ferreira, L.F. Garcia-Fernandez, and M. Slattery, 2007b. Cytotoxic diterpenoids from the hybrid soft coral *Sinularia maxima* × *Sinularia polydactyla*. J. Nat. Prod., in press.
60. Kazlauskas, R., P.T. Murphy, R.J. Wells, P. Schonholzer, 1978. Cembranoid constituents from an Australian collection of the soft coral *Sinularia flexibilis*. Aust. J. Chem. 31, 1817-1824.
61. Kazlauskas R., J.F. Marwood, R.J. Wells, 1980. 2-Phenylethylamides of a novel lipid acid; atrial stimulants from the soft coral *Sinularia flexibilis*. Aust. J. Chem. 33, 8, 1799-1803.
62. Kelman D., Y. Kashman, E. Rosenberg, A. Kushmaro, Y. Loya, 2006. Antimicrobial activity of Red Sea corals. Mar Biol 149, 357–363.
63. Kerr, R.G., Boehnlein, J., Newberger, N., Ranzer, L., 2004. Concentrations of anti-inflammatory compounds increased in culture studies of coral, microalgae. Global Aquaculture Alliance, Australia, 70.
64. Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. J Exp Mar Biol Ecol 351, 106 - 113.
65. Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Light–dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*. Submitted.
66. Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Effects of ammonium, phosphate, and *Artemia* on growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae. Submitted.
67. Kopecky, E., Ostrander, G. 1999. Isolation and primary culture of viable multicellular endothelial isolates from hard corals. In vitro Cell Dev Biol Anim 35, 616–624.
68. Kumar R., V. Lakshmi, 2006. Two new glycosides from the soft coral *Sinularia firma*. Chem. Pharm. Bull. 54, 12, 1650—1652.
69. Kusumi T., Uchida H., Ishitsuka M.O., Yamamoto H., Kakisawa H., 1988. Alcyonin, a new cladiellane diterpenes from the soft coral *Sinularia flexibilis*. Chem Lett 1077–1078.
70. La Barre, S.C., Coll, J.C., Sammarco, P.W., 1986. Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef. II. The relationship between toxicity and feeding deterrence. Biol. Bull. 171, 565-576.
71. Li G., Y. Zhang, Z. Deng, L. van Ofwegen, P. Proksch, and W. Lin, 2005. Cytotoxic cembranoid diterpenes from a soft coral *Sinularia gibberosa*. J. Nat. Prod. 68, 649-652.
72. Liyanage G.K., W.D. Patnasooriya and L.M.V. Tillekeratne, 1992. Post-coital contraceptive agents from extracts of Sri Lankan alcyonacean soft corals. Contraception 46, 3, 297-305.

73. Maida, M., Carroll, A.R., Coll, J.C., 1993. Variability of terpene content in the soft coral *Sinularia flexibilis* (Coelenterata: Octocorallia), and its ecological implications. J Chem Ecol 19, 10, 2285-2296.
74. Maida, M., Sammarco P.W., Coll J.C., 1995a. Preliminary evidence for directional allelopathic effects of the soft coral *Sinularia flexibilis* (Alcyonacea: Octocorallia) on scleractinian coral recruitment. Bul Mar Sci 56, 1, 303-311.
75. Maida, M., Sammarco P.W., Coll J.C., 1995b. Effects of soft corals on scleractinian coral recruitment. 1. Directional allelopathy and inhibition of settlement. Mar Ecol Prog Ser 121, 1-3, 191-202.
76. Maida, M., Sammarco, P.W., Coll, J.C., 2001. Effects of soft corals on scleractinian coral recruitment. II: allelopathy, spat survivorship and reef community structure. Mar Ecol 22, 4, 397–414.
77. Maida, M. , Sammarco P.W., Coll J.C., 2006. A diffusion chamber for assessing efficacy of natural anti-fouling defenses in marine organisms. J. Exp. Mar. Biol. Ecol. 337, 59–64.
78. Mayer A.M.S., 1999. Marine pharmacology in 1998: antitumour and cytotoxic compounds. The pharmacologist 41, 4, 159-164.
79. Mayer A.M.S., Lehmann K.B., 2000. Marine pharmacology in 1998: Marine compounds with antibacterial, anticoagulant, antifungal, antiinflammatory, anthelmintic, antiplatelet, antiprotozoal, and antiviral activities; with actions on the cardiovascular, endocrine, immune, and nervous systems; and other miscellaneous mechanisms of action. The pharmacologist 42, 2, 62-69.
80. Mayer A.M.S., K.R. Gustafson, 2003. Marine pharmacology in 2001–2: antitumour and cytotoxic compounds. Int J Cancer 105, 291-299.
81. Mayer A.M.S., K.R. Gustafson, 2004. Marine pharmacology in 2001–2: antitumour and cytotoxic compounds. European Journal of Cancer 40, 2676–2704.
82. Mayer A.M.S., K.R. Gustafson, 2006. Marine pharmacology in 2003–2004: Anti-tumour and cytotoxic compounds. European Journal of Cancer 42, 2241–2270.
83. McMurry J.E., Matz JR, Kees K.L., 1987. Synthesis of microcyclic terpenoids by intermolecular carbonyl coupling: flexibilene and humulene. Tetrahedron 43, 23, 5489-5498.
84. Mendola D., 2003. Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: process developments and economics. Biomol. Eng. 20, 441-458.
85. Michalek-Wagner K., Bowden, B.F., 1997. A natural algacide from soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia, Alcyonacea). J. Chem. Ecol. 23, 259-273.
86. Michalek-Wagner K., 2001. Seasonal and sex-specific variations in levels of photo-protecting mycosporine-like amino acids (MAAs) in soft corals. Mar Biol 139, 651–660.
87. Miyaoka H., M. Shinohara, M. Shimomura, H. Mitome, A. Yano, K. Iguchi and Y. Yamada, 1997. Aragusterols E-H, new 26, 27-cyclosterols from the Okinawan marine sponge of the genus *Xustospongia* and absolute configurations of xestoketols A and B. Tetrahedron 53, 5403–5413.
88. Mori K, Suzuki S, Iguchi K, Yamada Y, 1983. 8,11-epoxy bridged cembranolide diterpene from the soft coral *Sinularia flexibilis*. Chem Lett 10, 1515-1516.

89. Müller W.E.G., M. Böhm, R. Batel, S. De Rosa, G. Tommonaro, I.M. Müller, H. C. Schröder, 2000. Application of cell culture for the production of bioactive compounds from sponges: synthesis of avarol by primmorphs from *Dysidea avara*. J. Nat. Prod. 63, 1077-1081.
90. Munro, M.H.G., Blunt, J.W., E.J. Dumdei, S.J.H. Hickford, R.E. Lill, S. Li, C.N. Battershill, A.R. Duckworth, 1999. The discovery and development of marine compounds with pharmaceutical potential. J Biotechnol 70, 15–25.
91. Nevalainen T.J., H.J. Peuravuoria, R.J. Quinnb, L.E. Llewellync, J.A.H. Benzied, P.J. Fennere, K.D. Winkel , 2004. Phospholipase A2 in Cnidaria. Comp Biochem Physiol B 139, 731–735.
92. Norton R.S. and R. Kazlauskas, 1980. ^{13}C NMR-study of flexibilide, an anti-inflammatory agent from a soft coral. Experientia 36, 276-278.
93. Ojika M., Islam M.K., Shintani T., Zhang Y., Okamoto T., Sakagami Y., 2003. Three new cytotoxic acylspermidines from the soft coral, *Sinularia* sp. Biosci Biotechnol Biochem 67, 1410-1412.
94. Oren A., Gunde-Cimerman N., 2007. Mycosporines and mycosporine-like amino acids: UV protectants or multipurpose secondary metabolites? FEMS Microbiol Lett 269, 1–10.
95. Paul V.J., M.P. Puglisi, 2004. Chemical mediation of interactions among marine organisms. Nat. Prod. Rep. 21, 189-209.
96. Pomponi, S.A., 1999. The bioprocess–technological potential of the sea. J Biotechnol 70, 5–13.
97. Pomponi, S.A., 2004. Aquaculture supports expansion of marine-derived drugs. Global Aquaculture Alliance, 68-68.
98. Proksch P., R. Edrada-Ebel , R. Ebel, 2003. Drugs from the Sea - Opportunities and Obstacles. Mar. Drugs 1, 5-17.
99. Radhika P., Subba Rao P.V., Anjaneyulu V., Asolkar R.N., Laatsch H., 2002. Horiolide, a novel norditerpenoid from Indian Ocean soft coral of the genus *Sinularia*. J Nat Prod 65, 737-739.
100. Radhika P., M. Cabeza, E. Bratoeff, G. García, 2004. 5α-Reductase inhibition activity of steroids isolated from marine soft corals. Steroids, 69, 7, 439-444.
101. Radhika P., P.R. Rao, J. Archana, and N.K. Rao, 2005. Anti-inflammatory activity of a new sphingosine derivative and cembrenoid diterpene (lobohedleolide) isolated from marine soft corals of *Sinularia crassa* TIXIER-DURIVAULT and *Lobophytum* species of the Andaman and Nicobar islands. Biol. Pharm. Bull. 28, 7, 1311—1313.
102. Reddy NS, Goud TV, Venkateswarlu Y, 2002. Seco-sethukarailin, a novel diterpenoid from the soft coral *Sinularia dissecta*. J. Nat. Prod. 65, 1059-1060.
103. Rinkevich B., 1999. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. J Biotech 70, 133–153.
104. Rinkevich B., 2005. Marine Invertebrate Cell Cultures: New Millennium Trends. Mar Biotechnol 7, 429-439.
105. Sammarco P.W., J.C. Coll, S. La Barre, Willis B., 1983. Competitive strategies of soft corals (Coelenterata: Octocorallia): Allelopathic effects on selected scleractinian corals. Coral Reefs 1, 3, 173-178.

106. Sammarco P.W., S. La Barre, J.C. Coll, 1987. Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef III. The relationship between ichthyotoxicity and morphology. Oecologia 74, 93-101.
107. Sammarco, P.W., Coll J.C., 1988. The chemical ecology of alcyonarian corals (Coelenterata: Octocorallia). In: Scheuer, P.J. (Ed.), Bioorganic marine chemistry, Springer-Verlag, Berlin, pp. 87–116.
108. Sato A., W. Fenical, Z. Qi-tai and J. Clardy, 1985. Norcembrene diterpenoids from pacific soft corals of the genus *Sinularia* (Alcyonacea; octocorallia). Tetrahedron 41, 19, 4303-4308.
109. Sato, A., 1996. The search for new drugs from marine organisms. J Toxicol Toxin Rev 15, 171–198.
110. Schulte B.A., R. De Nys, G.J. Bakus, P. Crews, C. Eid, S. Naylor, L.V. Manes, 1991. A modified allomone collecting apparatus. J Chem Ecol 17, 7, 1327-1332.
111. Scripps, 2007. Institution of Oceanography, UC San Diego http://sio.ucsd.edu/About/Research_Overview/Marine_Biotechnology_and_Biomedicine.
112. Sheu J.H., A.F. Ahmed, R.T. Shiue, C.F. Dai, and Y.H. Kuo, 2002. Scabrolides A-D, four new norditerpenoids isolated from the soft coral *Sinularia scabra*. J. Nat. Prod. 65, 1904-1908.
113. Shick JM, Dunlap WC, 2002. Mycosporine-like amino acids and related gadusols: biosynthesis, accumulation, and UVprotective functions in aquatic organisms. Annu Rev Plant Physiol 64: 223–262.
114. Shindo T, Sato A, Horikoshi H, Kuwano H., 1992. A farnesyl arabinoside as an enhancer of glucose transport in rat adipocytes from a soft coral, *Sinularia* sp. Experientia 48, 7, 688-690.
115. Sipkema D., R. Osinga, W. Schatton, D. Mendola, J. Tramper, R.H. Wijffels, 2005. Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? Biotech Bioeng 90, 2, 201-222.
116. Skillings, D., 2005. Laboratory culture of the soft coral *Carijoa riisei*. Journal of Young Investigators 15.
117. Su J, Yang R, Kuang Y, Zeng L, 2000. A new cembranolide from the soft coral *Sinularia capillosa*. J Nat Prod 63, 1543-1545.
118. Su J.Y., Kuang Y.Y., Zeng L.M., Li H., 2005. New tetracyclic diterpenoid and new ceramides from the soft coral *Sinularia conferta*. J Asian Nat Prod Res. 7, 2, 107-113.
119. Su J.H., A.F. Ahmed, P.J. Sung, C.H. Chao, Y.H. Kuo, and J.H. Sheu, 2006a. Manaarenolides A-I, diterpenoids from the soft coral *Sinularia manaarensis*. J Nat Prod. 69, 8, 1134-1139.
120. Su J.H., Y.J. Tseng, H.H. Huang, A.F. Ahmed, C.K. Lu, Y.C. Wu, Y.H. Kuo, and J.H. Sheu, 2006b. 9,11-Secosterols from the soft corals *Sinularia lochmodes* and *Sinularia leptoclados*. J. Nat. Prod., 69, 5, 850 -852.
121. Sui-jian Q., Qi C., Wen-lie P., An-long X.J., 2002. Cytotoxic properties of cembranolides diterpenoids from soft coral *Sinularia tenella* Li. Trop Oceanogr 21, 87–91.
122. Taglialatela-Scafati O., U. Deo-Jangra, M. Campbell, M. Roberge, R.J. Andersen, 2002. Diterpenoids from cultured *Erythropodium caribaeorum*. Org. Lett. 4, 23, 4085-4088.
123. Takaki H, Koganemaru R, Iwakawa Y, Higuchi R, Miyamoto T, 2003. Inhibitory effect of norditerpenes on LPS-induced TNF-α production from the Okinawan soft coral, *Sinularia* sp. Biol Pharm Bull. 26, 3, 380-382.

124. Temraz T.A., Houssen W.E, Jaspars M., Woolley D.R., Wease K.N, Davies S.N., Scott R.H., 2006. A pyridinium derivative from Red Sea soft corals inhibited voltage-activated potassium conductances and increased excitability of rat cultured sensory neurons. BMC Pharmacol 6, 10, 1-19.
125. The Florida aquarium Caribbean coral farm project. AZA conservation endowment fund proposal: www.aza.org/ConScience/CEF_Examples/Documents/CEF_Expample_Coral.pdf. Accessed 2007.
126. Tursch B., J.C. Braekman1, D. Daloze, M. Herin, R. Karlsson, D. Losman, 1975. Chemical studies of marine invertebrates—XI: sinulariolide, a new cembranolide diterpene from the soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia, Alcyonacea). Tetrahedron 31, 2, 129-133.
127. Tursch, B., 1976. Some recent development in the chemistry of Alcyonaceans. Pure Appl Chem 48, 1–6.
128. Uchio, Y., Nakatani, M., Hase, T., Fukazawa, Y., Kodama, M., 1988. A new ichthyotoxic cembranolide from the soft coral *Sinularia flexibilis*. In: IUPAC, Kyoto Ed.., Proc. 16th Int. Symp. Chem. Nat. Prod. Pa 38, 640.
129. Volkman J.K., 1999. Australasian research on marine natural products: chemistry, bioactivity and ecology. Mar. Freshwater Res., 1999, 50, 761-779.
130. Wahl, M., 1989. Marine epibiosis: I. Fouling and anti-fouling: some basic aspects. Mar. Ecol. Prog. Ser. 58, 175–189.
131. Watanabe T., Utsunomiya, Y., Yuyama, I., 2007. Long-term laboratory culture of symbiotic coral juveniles and their use in eco-toxicological study. J Exp Mar Biol Ecol 352, 177-186.
132. Webb L., Coll J.C., 1983. Effects of alcyonarian coral terpenes on scleractinian coral photosynthesis and respiration. Toxicon 3, 485–488.
133. Webb, L., 1986. The biology and biochemistry of terpenes in alcyonacean corals from the central region of the Great Barrier Reef. Ph.D. dissertation, Chemistry and Biochemistry, James Cook University of North Queensland, Townsville, Qld., Australia.
134. Weinheimer A.J., J.A. Matson, M. B. Hossain, D. van der Helm, 1977. Marine anticancer agents: sinularin and dihydrosinularin, new cembranolides from the soft coral, *Sinularia flexibilis*. Tetrahedron Letters 18, 34, 2923-2926.
135. Yakovleva I., Bhagooli R., Takemura A., Hidaka M., 2004. Differential susceptibility to oxidative stress of two scleractinian corals: antioxidant functioning of mycosporineglycine. Comp Biochem Physiol B 139, 721–730.
136. Yates, K.R., Carlson, B.A., 1992. Corals in aquariums: How to use selective collecting and innovative husbandry to promote reef conservation. Proc. 7th Int Coral Reef Sym 2, 1091–1095.
137. Yu S., Z. Deng, L. van Ofwegen, P. Proksch, W. Lin, 2006. 5,8-Epidioxysterols and related derivatives from a Chinese soft coral *Sinularia flexibilis*. Steroids 71, 955–959.
138. Zhang C.X., S.J. Yan, G.W. Zhang, W.G. Lu, J.Y. Su, L.M. Zeng, L.Q. Gu, X.P. Yang, and Y.J. Lian, 2005. Cytotoxic diterpenoids from the soft coral *Sinularia microclavata*. J. Nat. Prod. 68, 1087-1089.
139. Zhang G.W., Ma X.Q., Su J.Y., Zhang K., Kurihara H., Yao X.S., Zeng L.M., 2007. Two new bioactive sesquiterpenes from the soft coral *Sinularia* sp. Nat Prod Res 20, 659-64.

3 Light–dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*

Abstract

The branching zooxanthellate soft coral *Sinularia flexibillis* 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 (μ d^{-1}) and the physiological response to a range of light intensities (10–1000 μmol quanta m^{-2} s^{-1}) was studied for 12 weeks. Although a range of irradiances from 100 to 400 μmol quanta m^{-2} s^{-1} was favorable for *S. flexibilis*, based on chlorophyll content, a light intensity around 100 μmol quanta m^{-2} s^{-1} was found to be optimal. The contents of both zooxanthellae and chlorophyll *a* were highest at 100 μmol quanta m^{-2} 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 μmol quanta m^{-2} s^{-1}).

This chapter is submitted as: Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Light dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*.

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, harbours symbiotic algae, the zooxanthellae. They belong to the dinoflagellata and translocate photosynthates to the coral host (e.g. Muscatine, 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; Kühl 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 photoresponse of corals to be species–specific (Titlyanov and Titlyanova, 2002a, b). Because of dependency of *S. flexibilis* on light (Fabricius et al., l995 a,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 effects

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 *Sinularia flexibilis* were obtained from Burgers' Zoo, Arnhem, The Netherlands. They were transferred to rearing tanks (Eco–deco systems, Dymico–Model 1000, containing ± 1300 L saltwater) at 34‰±0.5 salinity, 25.8±0.2 °C. The saltwater was made of the nutrient–free Instant Ocean Reef Crystals (Aquarium systems, Sarrebourg, France, 2007). Lighting (12h light :12h 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 PVC platelets within ± two weeks, and placed in the tank. The experimental corals (n= 8) were allocated to each light treatment. Different light intensities from 10 to 1000 μmol quanta m^{-2} 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± 2 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 two weeks of adaptation to the lighting regimes, which would result in a complete photoacclimation (Anthony and Hoegh–Gulderberg, 2003). The weight gain of each coral group 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 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 was 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 μm) seawater (FSW). The homogenate (1 ml) was diluted and vortexed. The cells were counted using a haemocytometer under a microscope. Chlorophyll *a* was extracted based on Kinzie (1993), using chilled methanol: tetrahydrafuran (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 metanolic 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 Univ., Australia. The flexibilide content was expressed as mg g^{-1} dry weight of the samples.

Results & Discussion

Light–dependent growth

The light dependency of the soft coral *Sinularia flexibilis* was investigated from low to high light intensities (10 to 1000 μmol quanta m^{-2} s^{-1}). Weekly mean specific growth rates (μ d^{-1}) were calculated from buoyant weight data for 12 weeks (Fig. 1). Maximum value of μ was 16×10^{-3} d^{-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– 400 μmol quanta m^{-2} 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 μmol quanta m^{-2} s^{-1}.

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).

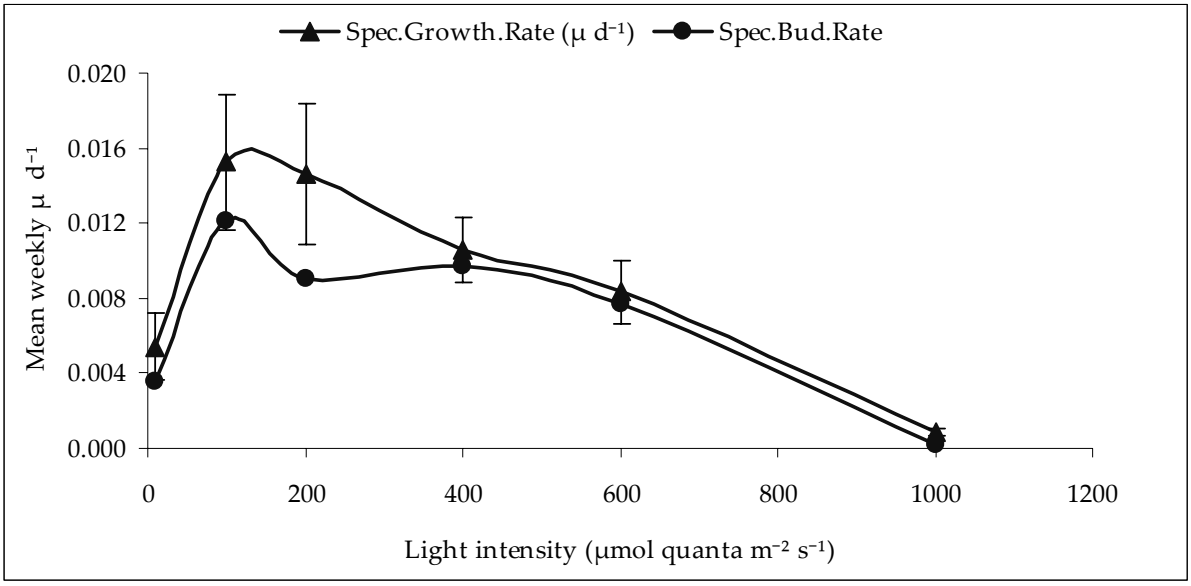


Fig. 1. Specific growth rates (μ d^{-1}) and specific budding rates $d^{-1}$of *Sinularia flexibilis* as a function of light intensity.

Despite the coral survival (visual observation) at the extreme irradiances, their specific growth and budding rates were zero, indicating that they may survive but can not 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 μmol quanta m^{-2} 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.

Light–enhanced calcification

The light-dependent specific growth rate (μ) of *S. flexibilis* was found to be maximal at light intensities of 100 μmol m^{-2} s^{-1} (Fig. 1). Since specific growth rates were calculated from buoyant weight data, they may be assumed to reflect both tissue production and calcification (Tentori and Allemand, 2006; Al-Horani et al., 2007; Titlyanov and Titlyanova, 2002b; Marubini et al., 2001; Gattuso et al. 1999). Interestingly, the present optimum illumination of 100 μmol m^{-2} s^{-1} is identical to the value that was established by Moya et al. (2006) for calcification rate alone. The fact that these two optima coincide may reflect the fact that both growth and calcification are light-dependent.

Photoacclimation

The relatively similar μ of the corals at light intensities of 100–400 μmol quanta m^{-2} s^{-1} (Fig. 1) 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. Also, *S. flexibilis*, being a shallow–water species, is considered to be more resistant to high radiation than deep–water corals (Siebeck, 1988).

Morphological and structural adaptations

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 μmol quanta m^{-2} s^{-1}) were always retracted once exposed, and showed a delayed expansion when exposed again to low and moderate illuminations (10 and 100 μmol quanta m^{-2} s^{-1}). Besides, the yellowish appearance of the corals at high light intensities (> 100 μmol quanta m^{-2} s^{-1}) suggested the photoprotective xanthophyll cycle as a key factor in photoacclimation, which is a key photoprotective defence 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

Growth of *S. flexibilis* depends on light and thus on zooxanthellae and their distinctive light–absorbing pigment, chlorophyll *a* (Fig. 2). Zooxanthellae density increased 2 times at low light intensities (up to 100 μmol quanta m^{-2} s^{-1}). Chlorophyll *a* concentrations (g^{-1} protein) increased by a factor of 2 in the range of 10-100 μmol m^{-2} s^{-1}), and then decreased again by a factor of > 2 times above 100 μmol m^{-2} s^{-1}. Also, 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 μmol quanta m^{-2} s^{-1}) to maximize light absorption, compared to higher irradiances (200 and 400 μmol quanta m^{-2} s^{-1}), they contain low chlorophyll because of low light availability. In contrast, the lower chlorophyll content at 200 and 400 μmol quanta m^{-2} s^{-1} as opposed to that of 100 μmol quanta m^{-2} s^{-1} led to the increased coral growth than at 10 μmol quanta m^{-2} s^{-1} (Fig. 1), 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- 400 μmol quanta m^{-2} s^{-1}). At these light intensities, the chlorophyll content and the photosynthetic light absorption capacity of the zooxanthella decreased;

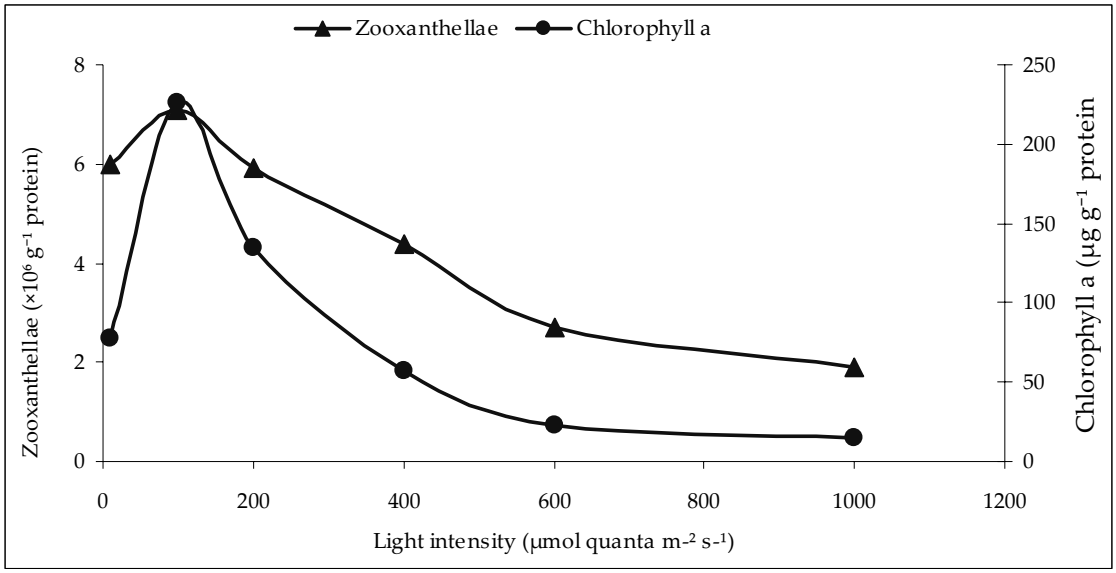


Fig. 2. *Sinularia flexibilis*. **A**) Zooxanthellae, and **B)** Chlorophyll *a* contents based on protein content at varying light intensities after 12 weeks.

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; Fit and Cook, 2001; Iglesias-Prieto and Trench, 1994, 1997).

Figure 2 would represent coral tissue growth and light–enhanced calcification as these processes are proportional with photosynthesis (e.g. Bak, 1983; Chalker et at., 1988; Al-Horani et al., 2003, 2007; Tentori and Allemand, 2006). This pattern is also similar to the 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; Levy et al., 2004; Stambler and Dubinsky, 2005). Hence, maximum algal photosynthesis corresponds to maximum growth of *S. flexibilis* at favourable 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 μmol quanta m^{-2} 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 μmol quanta m^{-2} 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 μmol quanta m^{-2} 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 μmol quanta m^{-2} s^{-1} and then decreased again (Fig. 3). 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).

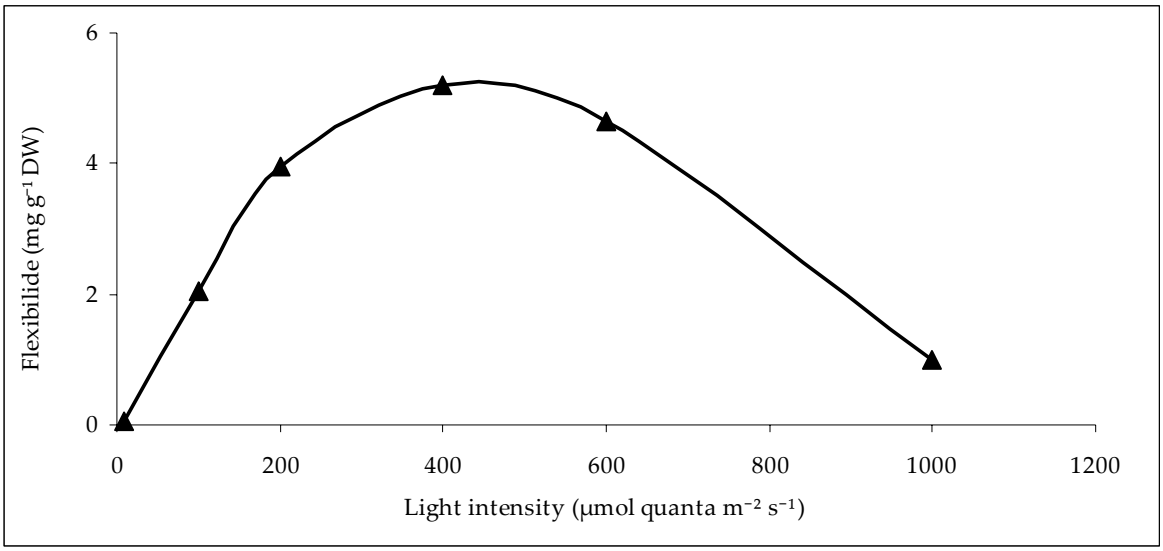


Fig. 3. *Sinularia flexibilis.* Flexibilide contents at varying light intensities after 12 weeks.

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 *Sinularia 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 1000 μmol quanta m^{-2} 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 and Bowden (2001).

Conclusion

Our results show that phototrophy is decisive for rapid growth of *Sinularia 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 maximize 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. flexibilis* to optimize photosynthetically–driven growth. This octocoral, therefore, has developed both physiological and morphological means to cope with various light conditions.

References

Aceret, T.L., Sammarcob, P.W., Coll, J.C., Uchio, Y., 2001. Discrimination between several diterpenoid compounds in feeding by *Gambusia affinis*. Comp Biochem Physiol Part C 128, 55–63.

Al-Horani, F.A., Al-Moghrabi, S.M., de Beer, D., 2003. The mechanism of calcification and its relation to photosynthesis and respiration in the scleractinian coral *Galaxea fascicularis*. Mar Biol 142, 419–426.

Al-Horani, F.A., Tambutté, É., Allemand, D., 2007. Dark calcification and the daily rhythm of calcification in the scleractinian coral, *Galaxea fascicularis*. Coral Reefs 26, 531-538.

Apprill A.M. Bidigare R.R., Gates R.D., 2007. Visibly healthy corals exhibit variable pigment concentrations and symbiont phenotypes. Coral Reefs 26, 387–397.

Bak, R.P.M., 1983. Neoplasia, regeneration and growth in the reef–building coral *Acropora palmata*. Mar Biol 77, 221–227

Baker, P.A., Weber, J.N., 1975. Coral growth rate – variation with depth. Earth Planet Sci Lett 27, 57–61.

Barnes, D.J., Chalker, B.E., 1990. Calcification and photosynthesis in reef–building corals and algae. In: Dubinsky Z (ed) Ecosystems of the world: coral reefs, 25, 109–131.

Bastidas, C., Fabricius, K.E., Willis, B.L., 2004. Demographic aspects of the soft coral *Sinularia flexibilis* leading to local dominance on coral reefs. Hydrobiologia 530/531, 433–441.

Bhosale, S.H., Nagle, V.L., Jagtap, T.G., 2002. Antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomonas*. Mar. Biotechnol. 4, 111–118.

Brown, B.E., Le Tissier, M.D.A., Dunne, R.P., 1994. Tissue retraction in the scleractinian coral *Coeloseris mayeri*, its effect upon coral pigmentation, and preliminary implications for heat balance. Mar Ecol Prog Ser 105, 209–218.

Chalker, B.E., 1981. Simulating light–saturation curves for photosynthesis and Calcification by reef–building corals. Mar Biol 63, 135–141.

Chalker, B.E., Dunlap, W.C., Oliver, J.K., 1983. Bathymetric adaptations of reef–building corals at davies reef, great barrier reef, australia. II. light saturation curves for photosynthesis and respiration. J. Exp. Mar. Biol. Ecol., 13, 31–56.

Chalker, E., Barnesd, J., Dunlop W.C. & Jokiel P.L., 1988. Light and reef–building corals. Interdisc. Sci. Rev., 13,222–237.

Coles, S.L., Brown, B.E., 2003. Coral bleaching - capacity for acclimatization and adaptation. Advances in marine biology, 46, 184–212.

Coll, J.C., La–Barre, S., Sammarco, P.W., Williams, W.T., and Bakus, G.J., 1982. Chemical defense in soft corals (Coelenterata: Octocorallia) of the Great Barrier reef: a study of comparative toxicities. Mar Ecol Prog Ser 8, 271–278.

De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: A powerful yet simple technique for ecological data analysis. Ecology, 81, 11, 3178–3192.

de Beer, D., Kühl, M., Stambler, N., Vaki, L., 2000. A microsensor study of light enhanced Ca^{2+} uptake and photosynthesis in the reef-building hermatypic coral *Favia sp*. Mar Ecol Prog Ser 194, 75–85.

Dmitrenok, A.S., Radhika, P., Anjaneyulu, V., Subrahmanyam, C., Subba Rao, P.V., Dmitrenok, P.S., Boguslavskya, V.M., 2003. New lipids from the soft corals of the Andaman Islands. Russ Chem Bull, Intern Ed 52, 8, 1868—1872.

Dubinsky, Z., Stambler, N., Ben–Zion, M., McClosky, L., Falkowski, P.G., Muscatine, L., 1990. The effects of external nutrient resources on the optical properties and photosynthetic efficiency of *Stylophora pistillata*. Proc R Soc Lond B 239, 231–246.

Fabricius, K.E., Benayahu, Y., Genin, A., 1995a. Herbivory in asymbiotic soft corals. Science 268, 90–92.

Fabricius, K.E., A. Genin, Y. Benayahu, 1995b. Flow dependent herbivory and growth in asymbiotic soft corals. Limnol Oceanogr 40, 1290–1301.

Fautin, D.G., 2002. Reproduction of Cnidaria.Can. J. Zool. 80, 1735–1754.

Fitt, W.K., Cook, C.B., 2001. Photoacclimation and the effect of the symbiotic environment on the photosynthetic response of symbiotic dinoflagellates in the tropical marine hydroid *Myrionema amboinense*. J Exp Mar Biol Ecol 256, 15–31.

Gates, D.M., 1980. Biophysical ecology. Spring Berlin Heidelberg, New York.

Gates, R.D., Edmunds, P.J., 1999. The physiological mechanisms of acclimatization in tropical reef corals. Amer. Zool., 39, 30–43.

Gattuso, J.P., Allemand, D., Frankignoulle, M., 1999. Photosynthesis and calcification at cellular organismal and community levels in coral reefs: a review on interaction and control by carbonate chemistry. Am Zool 39, 160–183

Glynn, P.W., 1993. Coral reef bleaching: ecological perspectives. Coral Reefs 12, 1–17.

Hidaka, M., Uechi, A., Yamazato, K., 1982. Effects of certain factors on budding of isolated polyps of a scleractinian coral, *Galaxea fascicularis*. Proceedings 4th Int Coral Reef Symp, Manila, 1982, 2. 229-231

Iglesias-Prieto, R., Trench, R.K., 1994. Acclimation and adaptation to irradiance in symbiotic dinoflagellates. I. Responses of the photosynthetic unit to changes in photon flux density. Mar Ecol Prog Ser 113, 163-175.

Iglesias-Prieto, R., Trench, R.K., 1997. Acclimation and adaptation to irradiance in symbiotic dinoflagellates. II. Response of chlorophyll-protein complexes to different photon-flux densities. Mar Biol 130, 23-33.

Jones, R.J., Hoegh–Guldberg, O., 2001. Diurnal changes in the photochemical efficiency of the symbiotic dinoflagellates (Dinophyceae) of corals: photoprotection, photoinactivation and the relationship to coral bleaching. Plant, Cell and Environment, 24, 1, 89–99.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow–dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. J Exp Mar Biol Ecol, 351, 106-113.

Kinzie,R.A., Hunter, T., 1987. Effect of light quality on photosynthesis of the reef coral *Montipora verrucosa*. Mar Biol 94, 95–109.

Kinzie, R.A., 1993. Effects of ambient levels of solar ultraviolet radiation on zooxanthellae and photosynthesis of the reef coral *Montipora verrucosa*. Mar Biol 116, 319–327.

Kühl, M., Cohen, Y., Dalsgaard, T., Barker, Jorgensen, B., Revsbech, N.P., 1995. Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studies with microsensors for O2, pH and light. Mar Ecol Prog Ser 117, 159–72.

Lambers, H., Chapin III F.S., Pons, T.L., 1997. Plant physiological ecology. Springer, Berlin Heidelberg, New York.

Lasker, H.R., 1979. Light dependent activity patterns among reef corals: *Monfastrea annularis*. Biol. Bull. 156, 196–211.

Lesser, M.P., Farrell, J.H., 2004. Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs 23, 367–377.

Levy, O., Dubinsky, Z., Schneider, K., Achituv, Y., Zakai, D., Gorbunov, M.Y., 2004. Diurnal hysteresis in coral photosynthesis. Mar Ecol Prog Ser, 268, 105–117.

Long, S.P., Humphries, S., Falkowski, P.G., 1994. Photoinhibition of photosynthesis in nature. Ann Rev Plant Physiol Plant Molec Biol 45, 633–662.

Maida, M., Carroll, A.R., Coll, J.C., 1993. Variability of terpene content in the soft coral *Sinularia flexibilis* (Coelenterata: Octocorallia), and its ecological implications. J Chem Ecol 19, 10, 2285-2296.

Marubini, F., Barnett, H., Langdon, C., Atkinson, M.J., 2001. Dependence of calcification on light and carbonate ion concentration for the hermatypic coral *Porites compressa*. Mar Ecol Prog Ser, 220, 153–162.

McCloskey, L.R., Muscatine, L., 1984. Production and respiration in the Red Sea coral *Stylophora pistillata* as a function of depth. Proc. R. Soc. Lond. B. 222, 215–230.

Michalek–Wagner, K., Bowden, B.F., 2000. Effects of bleaching on secondary metabolite chemistry of alcyonacean soft corals. J Chem Ecol, 26, 7.

Michalek–Wagner, K., Bowden, B.F., 2001. The effects of different strains of zooxanthellae on the secondary metabolite chemistry and development of soft coral host *Lobophytum compactum* . Mar Biol 138, 753–760.

Michalek–Wagner, K., 2001. Seasonal and sex-specific variations in levels of photo-protecting mycosporine-like amino acids (MAA5) in soft corals. Mar Biol 139, 651–660.

Moya A., Tambutté S., Tambutté E., Zoccola D., Caminiti N., Allemand D., 2006. Study of calcification during a daily cycle of the coral *Stylophora pistillata*: implications for 'light-enhanced calcification'. J. Exp Biol 209, 3413-3419

Muscatine, L., Porter, J.W., 1977. Reef corals: mutualistic symbiosis adapted to nutrient–poor environments. Bioscience 27, 454–460.

Muscatine, L., McCloskey, L.R., Marian, R.E., 1981. Estimating the daily contribution of carbon from zooxanthellae to coral-animal respiration. Limnol Oceanogr 26, 4, 601-611.

Muscatine, B.L., Falkowski, P.O., Dubinsky, Z., CooK, P.A., Mocloskey, L.R., 1989. The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc. R. Soc. Lond. B. 236, 311—324.

Porter, J.W., Muscatine, L., Dubinsky, Z., Falkowsky, P.G., 1984. Primary production and photoadaptation in light– and shade –adopted colonies of the symbiotic coral *Stylophora pistallata*. Proc. R. Soc. Lond. B. 222, 161– 180.

Richter, M., Rühle, W., Wild, A., 1990. Studies on the mechanism of photosystem II. photoinhibition I. The involvement of toxic oxygen species. Photosyn. Res 24, 237–243.

Robison, J.D., Warner, M.E., 2006. Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of *Symbiodinium* (pyrrhophyta). J. Phycol. 42, 568–579.

Sammarco P.W., S. La Barre, and J. C. Coll, 1987. Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef, 3: the relationship between ichthyotoxicity. Oecologia 74, 93–101.

Siebeck, O., 1988. Experimental investigation of UV tolerance in hermatypic corals (Scleractinia). Mar Ecol Prog Ser 43, 95–103.

Stambler, N., Dubinsky, Z., 2005. Corals as light collectors: an integrating sphere approach. Coral Reefs 24, 1–9.

Titlyanov, E.A., Shaposhnikova, M.G., Zvalinskii, V.I., 1980. Photosynthesis and adaptation of corals to irradiance. 1. Contents and native state of photosynthetic pigments in symbiotic microalgae. Photosynthetica 14, 413–421.

Titlyanov, E.A., 1991. The stable level of coral primary production in a wide light range. Hydrobiologia 216/ 217, 383–387.

Titlyanov, E.A., Titlyanova, T.V., Yamazato, K., Woesik, R. van, 2001. Photo-acclimation of the hermatypic coral *Stylophora pistillata* while subjected to either starvation or food provisioning. J Exp Mar Biol Ecol, 257, 163–181.

Titlyanov, E.A., Titlyanova, T.V., 2002a. Reef–building corals—symbiotic autotrophic organisms: 1. General structure, feeding pattern, and light-dependent distribution in the shelf. Russian J Mar Biol 28, 1, 1–15.

Titlyanov, E.A., Titlyanova, T.V., 2002b. Reef–building corals—symbiotic autotrophic organisms: 2. Pathways and mechanisms of adaptation to light. Russian J Mar Biol 28, 1, 16–31.

Tsai, C.H., Liu, L.L., 2005. Effects of light intensity on the morphology and physiology of the soft coral *Pachyclavularia violacea*. Master's thesis, a publication of Institute of Marine Biology, National Sun Yat–sen University, Taiwan.

Verde, E.A., McCloskey, L.R., 2002. A comparative analysis of the photobiology of zooxanthellae and zoochlorellae symbiotic with the temperate clonal anemone *Anthopleura elegantissima* (Brandt). Mar Biol 141, 225–239.

Volkman, J.K., 1999. Australasian research on marine natural products. Mar. Freshwater Res., 50, 761–79 .

Zamer, W.E., Shick, J.M., Tapley, D.W., 1989. Protein measurement and energetic considerations: comparisons of biochemical and stoichiometric methods using BSA and protein isolated from sea anemones. Limnol Oceanogr 34, 256–263.

4 Phototrophy and heterotrophy in the captive symbiotic octocoral *Sinularia flexibilis*

Abstract

The symbiotic octocoral *Sinularia flexibilis* is a producer of potential pharmaceutically-important metabolites and of mycosporine-like amino acids (MAAs). Its response to different light conditions and *Artemia* feeding was studied. *Artemia* feeding with and without illumination, confined corals in light (no continuous ambient foods from main stocking tank), and light without *Artemia* feeding for 7 weeks showed significant differences between specific growth rates (μ d^{-1}), and the contents of zooxanthellae, chlorophyll, and MAAs. All these parameters had minimum values in colonies that were exposed to light (without a continuous ambient nutrient supply from the stocking tank) and in *Artemia*-fed colonies in the dark. In a next experiment, coral respiration and photosynthesis ratio (P:R>1) reflected entire phototrophy of *S. flexibilis*. Similarly, the potential carbon contribution by zooxanthellae to animal (host) respiration (CZAR), being equal for both light and light plus food colonies, showed a high percentage of translocated photosynthetically fixed carbon to the host.

This chapter is submitted as: Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2008. Phototrophy and heterotrophy in the captive symbiotic octocoral *Sinularia flexibilis*.

Introduction

The branching symbiotic octocoral, *Sinularia flexibilis* (Quoy & Gaimard) produces and stores toxins (e.g. Coll et al., 1982; Sammarco et al., 1987, Aceret et al. 1995; Michalek-Wagner and Bowden, 1997; Maida et al., 2001) with pharmaceutical importance as well as mycosporine-like amino acids (MAAs) as intracellular natural sunscreens against increased UV radiation (e.g. Dunlap and Shick, 1998). The MAAs are expected to originate from zooxanthellae, which require nitrogen acquisition for their biosynthesis (e.g. Dunlap and Chalker, 1986; Michalek-Wagner, 2001). The production of pharmaceutically important compounds by *S. flexibilis* is an incentive to study growth kinetics of this species under captive cultivation as an alternative for continuous biotechnological exploitation.

Coral growth, in general, is affected by environmental variables such as light and plankton concentration (Sebens, 1984). In view of the coral-zooxanthellae symbiosis, light is assumed to be a primary limiting factor in the growth of symbiotic corals (e.g. Veron, 1995; Yapetal., 1998; Vermeij and Bak, 2002). Whereas phototrophy is well-documented in symbiotic soft corals (e.g. Muscatine et al., 1981) the literature on their heterotrophic feeding is contradictory and little is known about their nutrition (Fabricius and Klumpp, 1995; Fabricius et al., l995a, b; Anthony and Fabricius, 2000). Although the capture of particulate matter by various octocorals were reported (e.g. Lewis and Smith, 1971; Murdock, 1978; Lasker, 1981), it remains doubtful whether zooplankton is a major energy source for octocorals in general (Gili et al., 2001; Houlbre`que et al., 2003). Likewise, little information is available on light-dependency and on survival reactions in total darkness (Titlyanov et al., 2001a). In addition, an established phenomenon in zooxanthellate invertebrates is translocation of algal fixed carbon to the host. Since daily photosynthesis:respiration (P:R) ratio represents coral's dependency on phototrophic nutrition, it is highly important for studies on coral nutrition to realize contribution of both phototrophy and heterotrophy to carbon and energy budget in corals (Muscatine et al., 1981; Lesser et al., 2000).

A variety of factors such as irradiance and nutrition can change coral pigment and MAAs contents (Stambler et al., 1991). The link between light and feeding with growth and energetics of *S. flexibilis,* especially for long-term captive cultivation, as well as the

effects of light and nutrition on MAAs biosynthesis in captivity have not been clarified yet. Thus, in this study we investigated: (1) effects of light and *Artemia* feeding on the specific growth rate of *S. flexibilis*, its algal symbionts, and on MAAs, as well as on phototrophy and respiratory responses; and (2) the daily energetic requirements of the coral based on estimations of translocated carbon from zooxanthellae.

Materials and methods

Corals and experimental design

Colonies of the octocoral *Sinularia flexibilis* were obtained from Burger's Zoo, Arnhem, the Netherlands. These corals all originate from one stock and are, therefore, all genetically clones. They were placed in a main tank (Eco-deco systems, Dymico-Model 1000) containing ~1300 L saltwater, made of Instant Ocean, at 34‰±0.4 salinity, 25.8°±0.2 C) for rearing the coral stock. The tank was equipped with VHO Halide, 10,000 HQI lamps (Aqua Medic aqualight 400, Aquaria Veldhuis, Enschede, The Netherlands) adjusted for 12 hrs light: 12 hrs dark photoperiod. After fixing the coral pieces (nubbins) on small PVC plates following ±two weeks, 5 colonies (5-7 cm) were placed at each experimental treatment (light-food or LF, light only or L, starved (not fed) in light or LS, and dark-food or DF. Coral cuttings represent genetically identical replicates of a single coral colony (Stambler et al., 1994; Shafir et al., 2006). In addition, the treatments were applied to each of the tanks all with similar environmental conditions; hence, each colony is considered as pseudo-replicates (implicit pseudo-replication: Hurlbert, 1984; Heffner et al., 1996). Accordingly, coral responses observed during experiment were caused by the treatments. The LF, LS, and DF were all connected to main recirculation tank containing the coral stock. The L corals were in a aquarium connected to a filtration apparatus independent of main recirculation tank (without a continuous ambient nutrient supply from the main stocking tank).

Average light intensity was measured as photon flux density (PFD) using an underwater photo sensor (LI-COR, Li250 Light meter) being approx. 200 μmol quanta m^{-2} s^{-1}. The DF group was located in a corner covered by a PVC chamber to create darkness. The chamber was open at its two sides plus the side facing the flow pipe to allow water and food exchange. They were supplied with turbulent water flow from perforated PVC pipes mounted at two sides of the tank. The LF and the L groups were at two aquaria, under the same illumination and temperature as the tank. The average water velocity, charted by an Acoustic Doppler Velocity meter (Micro ADV 16 MHZ, Sontek), was 3 cm s^{-1} for the DF, L and LF groups.

Specific growth rates

Weight increments for each series of experiments were measured weekly using the underwater technique (buoyant weighing) by an analytical balance PROLABO A&D HR300 (bearing: 310 g; accuracy: ± 0. 01 mg) with underweighing device. Based on the weekly weight measurements, the specific growth rates (μ d^{-1}) were calculated for each coral group at each tank using the following formula: $\mu = (\ln W_2 - \ln W_1) / \Delta t$ (Kaufmann, 1982); where W_2= weight at end and W_1= weight at the beginning of each time interval. The mean of these specific growth rates was then calculated after 7 weeks for the colonies in each tank.

Feeding pattern

Live *Artemia* nauplii were used as food (e.g. Lewis, 1982; Sorokin, 1991), after being hatched according to Treece (2000). The number of the hatched nauplii after incubation for 24-26 hours were counted (Patterson, 1991) after the samples had been filtered on gridded Whatman GF/C filters. The DF group was fully fed in the dark chamber to maximize the food accessibility. The LF aquarium received 10 ml suspension containing ±1500 nauplii (≈ 10 ml^{-1} based on the water volume) for the whole period. Both the DF and the LF corals were allowed to feed for 2 hours three days $week^{-1}$.

Zooxanthellae, chlorophyll a, protein, and MAAs determinations

At the end of the 7-w period of the experiment, equal samples from each treatment were randomly selected to measure the relative amount of animal and algal biomass at various treatments. Wet weight was determined by weighing dry blotted samples. Following the 7-week treatment, the rest of the DF group was uncovered to be in light (50 and then gradually to 200 μmol quanta m^{-2} s^{-1}) to monitor their recovery. Their growth and survival was also compared to the other groups.

In order to count the zooxanthellae, an equal amount (30 mg) of tissue of each group was homogenized in tubes containing 3 ml filtered (0.2 μm) seawater (FSW). The homogenate (1ml) was diluted in FSW and vortexed. The cells were counted using a haemocytometer under a microscope. For chlorophyll *a* extraction, one ml of the homogenate was transferred to a sterile tube containing 9 ml acetone, vortexed for 10 min, and centrifuged for 5 min at 5000 rpm. The absorbance spectrum at 400–700 nm was measured using a spectrophotometer (Spectronic 20 Genesys, USA) and the concentration calculated using the equations of Jeffrey and Humphrey (1975), normalized to the coral wet weight.

After the extraction of chlorophyll and algae determination, the pellets were stored at -20 °C for later protein and total MAAs determination. Total protein of the thawed,

rehomogenized samples was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, 1979) and the absorbances were read at 595 nm. Bovine gamma globule was used as a standard (Chalker et al., 1983). MAAs were extracted according to Kinzie (1993) using chilled methanol: tetrahydrafuran (80:20 vol:vol) as the solvent at 4°C overnight. The following day, absorbance of the supernatant was read at 320 nm (Dunlap and Chalker, 1986), which is a qualification of the MAAs level (Kinzei, 1993). The presence of absorption peaks in the wavelengths of maximum absorbance was expressed as the ratio of optical density of 1 ml of extract per g protein of the corals (Yakovleva and Hidaka, 2004).

Measurement of photosynthetic and respiratory rates

In a separate experiment, rate of oxygen production in light was measured for corals colonies (n=4) in light and light-food (*Artemia*); respiration of the same corals was measured in dark with and without *Artemia*. This was done using cylindrical glass chamber (1000 ml volume) using oxygen electrodes, WTW Oxy 340i, coupled to a recorder and a magnetic stirrer. Coral samples (n=4) were set on a plastic grid, 3 cm from a magnetic stirring bar. The chamber was closed with a stopper that prevented any gas exchange with the atmosphere. The internal temperature was 26 ^{o}C, and the light intensity, measured with a Li-Cor underwater sensor (LI-COR, Li250 Light meter), was approx. 200 μM quanta m^{-2} s^{-1}. Oxygen flux was also measured in a control chamber without corals, and was corrected for photosynthesis and respiration. In order to assure accuracy, the oxygen probes were calibrated in air-saturated seawater before each test (n=3, each for 4 hrs). The results of the 3 tests were averaged and normalized to the buoyant weight of the samples. Oxygen production (photosynthesis) rate was calculated according to: P_{net}= (V × slope) / W; where P_{net} is the rate of net photosynthesis (mg O_2 g^{-1} h^{-1}), V is the volume of the chamber (l); slope is the dissolved oxygen variation (mg O_2 h^{-1}), and W is the buoyant weight of the colonies. Total 12 h respiration in the light (presumably equal to dark respiration) was calculated by multiplying hourly dark respiration by 12 h (McCloskey et al., 1994). Gross photosynthesis was calculated by summing the net O_2 and respired values (Titlyanov et al., 2001b). All hourly rates of oxygen produced by photosynthesis (P) and consumed by respiration (R) were converted to carbon equivalents (mg g^{-1} BW), using the carbon to oxygen ratio (12 : 32=0.375; Muscatine et al., 1981; McCloskey et al., 1994) to determine C assimilation and loss by the coral. To estimate CZAR (% contribution of zooxanthellae to animal respiration), the ratio of: [($C_{(fixed)}$ × $C_{(\%\ translocated)}$)/$C_{(respired\ by\ the\ animal\ in\ light)}$] was used, incorporated in the following equation (Muscatine et al., 1981):

CZAR = {[(P_g + R_c). PQ_z^{-1}] − [(1 − θ)R_c (day). RQ_c]}T / θ.R_c.RQ_c^{-1}; where P_g= Gross photosynthesis, R_c= Carbon respired by coral, PQ_z= Photosynthetic quotient for zooxanthellae

(0.8-1.1, arbitrarily set to 1), β= ratio of carbon respired by the animal to carbon respired by the coral, and RQ_c= Respiratory quotient for coral. All parameters are expressed as mg C g^{-1} buoyant weight (BW). Knowing that zooxanthellae respiration is up to 10% that of the coral (Leletkin, 2000), β and animal (host) respiration were estimated for 12 h as: $\beta = R_{(a)}/ R_c$ or $R_{(a)} = \beta.R_c$ (Muscatine et al., 1981). Alternatively, the equation of Steen and Muscatine (1984) was also used to compare the CZAR values: CZAR= (Net C fixed d^{-1}/Ra). T.

The respiration rates were converted into energy units of joules per hour using the oxy-joule equivalent of 19.63 to determine the energy equivalents (Elliot and Davison, 1975; Davis, 1991). Thereby, the daily 'light utilization efficiency' (Kishino et al., 1986) was estimated using the equation: ε=PSR/PAR,×K, where PSR is photosynthetically stored radiation (mg C d^{-1}), PAR is photosynthetically available radiation, and K (m^{-1}) is water depth. The light intensity (μM quanta m^{-2} s^{-1}) was divided by 4.6 to convert it into energy unit (Joules: Riddle, 2007).

Results and Discussion

Growth kinetics

Effects of light and *Artemia* feeding on growth of *Sinularia flexibilis* is shown in figure 1A&B. It shows that the dark (DF) colonies were not able to grow without irradiance; they eventually showed negative growth and death in the absence of light. Fig. 1B shows that colonies exposed to light (L, in confined tank without a continuous ambient nutrition from the main stocking tank) and fed colonies in dark (DF) initially grew, but growth declined after some weeks of treatment. The corals in both light-starved (LS) and light plus food (LF) had significantly higher (two-factor ANOVA, $P<0.05$) specific growth rates (μ d^{-1}) than the corals only treated with light (L) or food in absence of light (DF) (Fig.1A). Pairwise comparisons (paired t-test: LF vs. L and LS vs. L) also showed significant effect of light plus food (LF) and light without food (LS) vs. light (L), ($P<0.05$) and dark (D, $P=0.025$) treatments. Illuminated colonies exposed to *Artemia* feeding (LF) or not fed (LS) had similar μ. It is obvious that if either light or nutrients are limiting, initially a measurable growth occurs, but after some time there is a significant reduction in growth.

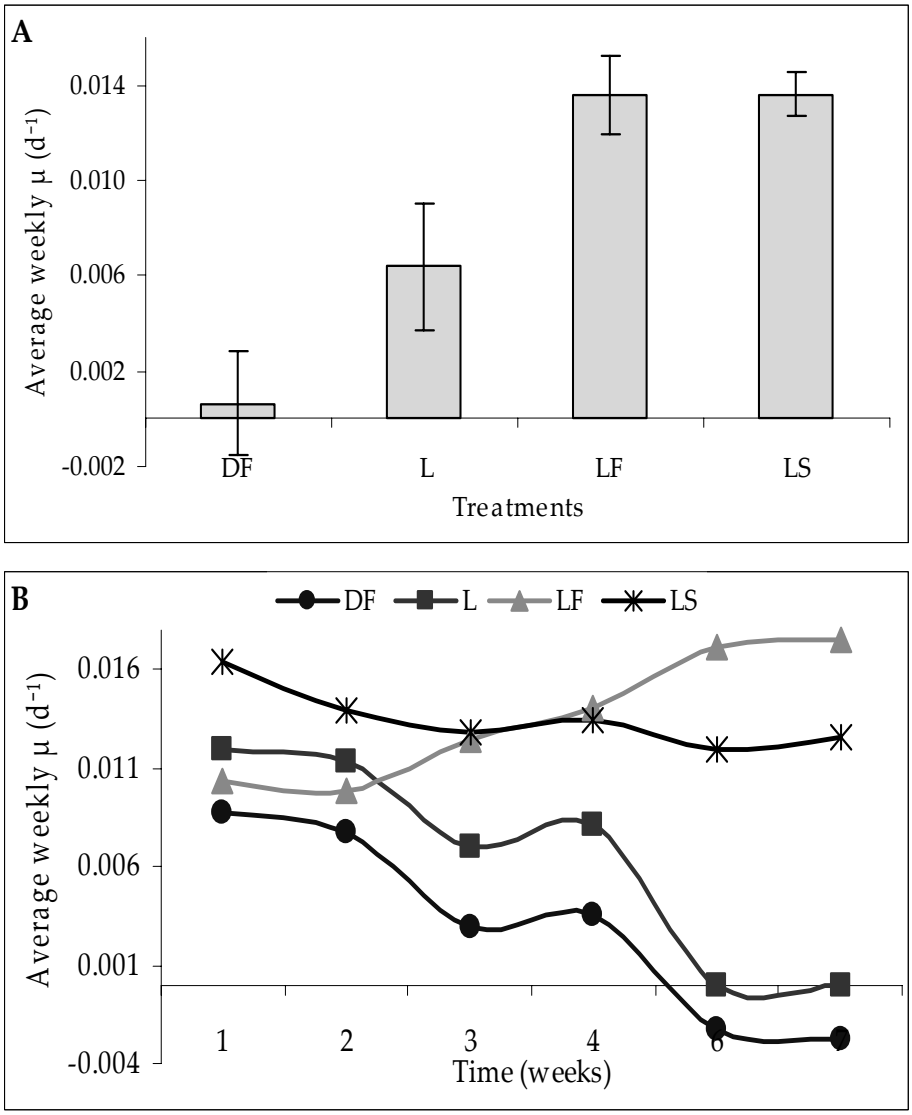


Fig.1. A) *Sinularia flexibilis*. Comparisons of average weekly μ during 7 weeks of dark, food, and light treatments. Error bars indicate standard errors.; **B)** Weekly μ of each group within 7 weeks.

The similar μ of the LF and LS colonies shows that *S. flexibilis* is able to thrive by phototrophy effectively, with the use of ambient nutritional resources independent of *Artemia* feeding.At the end of the 7-week period, LF and LS corals still kept growing, whereas the L and DF groups stopped growing (Fig. 1B).

Reduction of μ in the L corals (in confined tank) after some weeks probably shows the depletion of ambient nutrient sources in the water column, particularly nitrogenous compounds as limiting factors for coral growth in general (Davies, 1984). Besides ambient dissolved nutrients, the coral habitat normally contains small zooplankton (micro-, nano-, and picoplankton) and other heterotrophic inputs (protozoa, detritus and bacteria) as available food everywhere being readily available

for coral nutrition (Ferrier-Pagès et al., 1998; Orejaz et al., 2003; Palardy et al., 2006). Therefore, both irradiance and the availability of nutritional sources other than *Artemia* (e.g. food particles, bacteria, and dissolved organic matters) control the growth of *S. flexibilis*. This confirms the view of Bythell (1988), Dubinsky and Jokiel (1994), and Mills et al. (2004) that in addition to zooxanthellae photosynthates, heterotrophic nutrient uptake (e.g. particle feeding) is necessary for corals growth.

Our results indicate that *Artemia* feeding did not meet the colonies' metabolic needs. These results do not infer that *S. flexibilis* is incapable of heterotrophy entirely. In addition to species-specificity of phototrophy and heterotrophy in corals (Anthony and Fabricius, 2000), the photo- and/or heterotrophy of *S. flexibilis* may also be explained by its morphology. Polyp size was found to be a determining factor in nutritional habit of corals (Porter, 1976; Lasker et al., 1983). Branching, small-polyped species have increased light-capture surfaces over a larger body area, i.e. a higher surface to volume ratio (Sebens et al., 1996) and also a space limitation for ingestion with poor particle-feeding abilities (Anthony, 1999). Thus, the very small polyps of *S. flexibilis*, expanded all day as photosynthetic organs (Sebens et al., 1996) can be a further indicative of phototrophic dependency in this branching, shallow-water species. Nonetheless, small polyp size may restrict coral zooplanktivory to smaller prey sizes than *Artemia* and may not always correlate with prey capture abilities (Sebens et al., 1996).

The failure of *S. flexibilis* in the dark (DF) to utilize the ambient resources shows that heterotrophy is affected by irradiance. Hence, the low and then zero growth of the DF corals indicates that utilization of available nutrients (e.g. ammonium) requires light (Lasker et al., 1983). Similarly, feeding of the hard coral *Stylophora pistillata*, was influenced by irradiance (Ferrier-Pagès et al., 1998).

Zooxanthellae, chlorophyll and MAAs contents

The algal density varied widely from dark corals (DF) to illuminated colonies (L, LF and LS). Chlorophyll *a* content was higher in both the LF and LS than in the L, and was lowest in the DF corals (Fig. 2). It was highest in the "dark brown" or normal-looking corals (LF and LS) and lowest in the "pale" appearing colonies (slightly brown,

DF) as the most obvious change with very few zooxanthellae in microscopic examination.

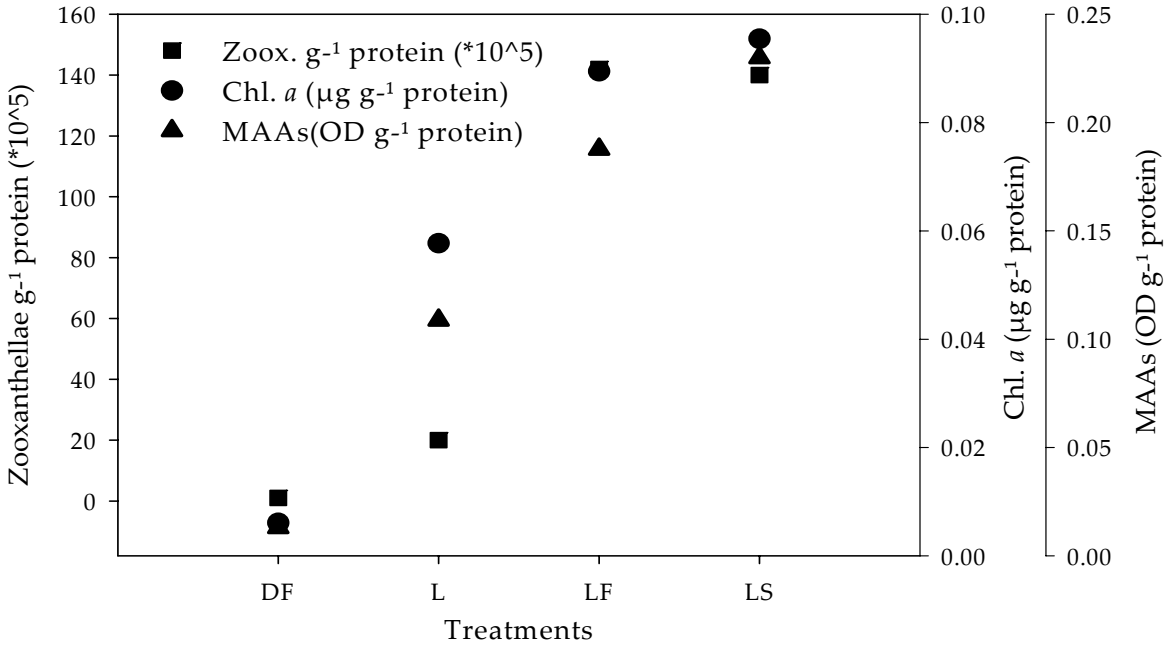


Fig. 2. *Sinularia flexibilis*. Zooxanthellae and chlorophyl *a* contents, and MAAs qualification (OD= optical density at 320 nm) in the coral colonies kept under different treatments of light-starved (LS), light-food (LF), light (L), and dark-food (DF) after 7 weeks.

These are indicators of pigmentation, color tone (pale, medium or dark) and coral health (Thieberger et al., 1995). Therefore, irradiance plus nutrient availability of the coral influenced photosynthetic pigment content under ambient conditions.

Because of the similar irradiance level for the illuminated colonies, differences in zooxanthellae and pigment contents between LS and LF on one hand and L on the other should be due to decreased nutrition, presumably of nitrogen (Cook et al., 1988). This also agrees with the dependency of zooxanthellae growth on light and mineral nutrition (Leletkin, 2000). As algal symbiont fuels coral growth, therefore, without nutritional sources (as dissolved organic or inorganic nutrients: Fabricius et al., 1995a) neither host nor symbiont is able to survive.

MAAs contents were higher in both the fed (LF) and starved (LS) than in the light only (L) and the dark-food (DF) corals (Fig. 2). As production of MAAs, in addition to other environmental conditions, involves a considerable nitrogen supply (Cook, 1983), hence, the nutrient availability of the coral resulted in the observed differences. For symbiotic tropical corals (as *S. flexibilis*) living at very low nutrient concentrations,

inorganic nutrients (particularly nitrogen) are an important source of nutrition (Mills and Sebens, 2004). This can explain a typical process in tropical corals, the 'tight nutrient recycling' (e.g. Wang and Douglas, 1998), which makes growth of *S. flexibilis* independent of extra feeding, depending on available nutritional resources.

Coral response in total darkness

The DF corals were uncovered after the 7-week period to monitor their recovery. Despite being exposed to gradually increasing light with a regular *Artemia* supply after being uncovered, they could no longer enhance their growth and seemed to be mostly dead within 3 to 4 weeks afterwards. They were, however, able to survive relatively longer (±10 weeks) than the corals in the Red Sea (Gohar, 1940), which died after 2 weeks in dark in spite of available zooplankton. These corals and also those of Franzisket (1970) survived in light without food. Franzisket (1970) and Titlyanov et al. (2001b) also observed death of hard corals in total darkness in spite of food provisioning. Similarly, Clayton and Lasker (1982) reported significant lower tissue biomass for the hard coral *Pocillopora damicornis* in darkness than in sunlight after two weeks.

The DF corals in this study gradually became pale and showed signs of bleaching after ±3 weeks, with greatly reduced polyps and invisible tentacles, i.e. an abnormal polyp structure. The lack of autotrophy in the dark corals being deprived of photosynthetic energy shows that utilization of available nutrients requires photosynthetic energy (Clayton and Lasker, 1982; Piniak, 2002). This means that *Sinularia flexibilis* cannot survive without irradiance.

Photosynthesis and respiration rates

Photosynthesis and respiration provide energy and form a highly active internal carbon cycle in the coral (Al-Horani et al., 2003). In a next experiment, rates of respiration and photosynthesis were determined to estimate daily carbon requirements and the contribution of phototrophy to meet the coral's carbon demands. In figure 3, oxygen production (gross photosynthesis) by the symbiont is quite high in both light and light-food colonies. O_2 is completely consumed in dark (respiration). The marked differences in the host light-dark metabolism demonstrate that the coral

host (animal) is totally dependent on symbiont photosynthesis (Lewis and Smith, 1971). Hence, as respiration reflects the animal's demand for ATP (Muscatine et al., 1981, Markager, 1993), the whole consumption of O_2 in dark (± *Artemia*) by the polyp and zooxanthellae (in both LF and L) and production by the symbiont in light show a close symbiosis in *S. flexibilis*.

The similar O_2 production of both fed (LF) and light only (L) corals (Fig. 3) shows that photosynthesis was a function of light, not feeding the coral as no effect of feeding on photosynthesis and respiration was found in the present experiments. The same result obtained for some stony corals (Szmant-Froelich and Pilson, 1984; Edmunds and Davies, 1986; Anthony and Fabricius, 2000; Reynaud et al., 2002). Therefore, the photosynthates of zooxanthellae are crucial for the energetics of *S. flexibilis*. The observed different rates of oxygen production and respiration (Fig. 3) probably reflect a behavior of expansion and contraction of the coral, causing such fluctuations in metabolic rates (e.g. Sebens and De Riemer, 1977).

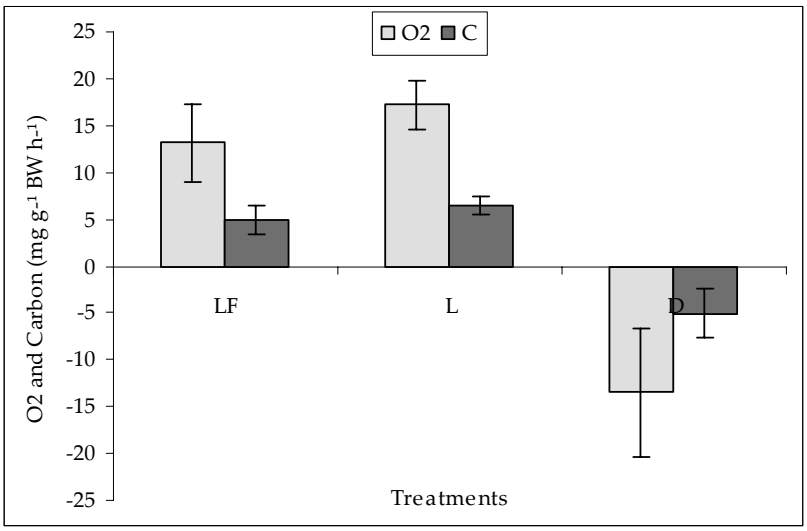


Fig. 3. Oxygen production (gross photosynthesis) and consumption (respiration) of *Sinularia flexibilis* colonies (n=4) at a light intensity of 200 μM q. m^{-2} s^{-1} in light-food (LF), light (L), and dark (D± food). Error bars indicate standard errors for No. of tests, each for four hrs. The side bars indicate the carbon equivalents per g buoyant weight of the coral.

- Photosynthesis-irradiance curve

Figure 4 shows the photosynthesis-irradiance (P/I) curve derived for *S. flexibilis*. There is an increase in oxygen production (photosynthesis) with light intensity up to about 150 μmol quanta m^{-2} s^{-1}. Photosynthesis is saturated at low irradiances; the lower and higher levels should lead to photo–damage and/or inability in optimal use of photosynthetic capacity (Anthony and Hoegh–Gulderberg, 2003).

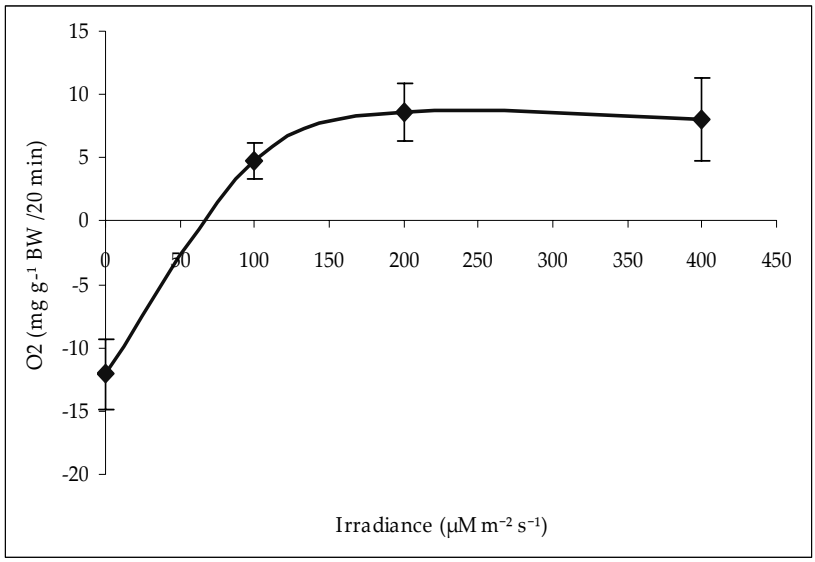


Fig. 4. *Sinularia flexibilis*. Averages of 3 measurements for dark respiration and P/I (Photosynthesis vs. Irradiance) at a range of light intensities (µmol q. m^{-2} s^{-1}. Error bars indicate standard errors for No. of tests, each for 20 min.

The relatively low light saturation *S. flexibilis* at around 150 mmol quanta m^{-2} s^{-1} (Fig. 4) lies in the range of those demonstrated in reef corals (50– 450 mmol quanta m^{-2} s^{-1}: Bosscher and Schlager, 1992). This P/I pattern is also similar to growth-irradiance pattern for *S. flexibilis* at the same light intensities (100-400 µmol quanta m^{-2} s^{-1}) that we observed before (Khalesi et al., 2007). Likewise, similar photosynthesis pattern was found by Ferrier-pagès et al. (1998).

- Estimating a daily energy budget for *S. flexibilis*

Table 1 shows average photosynthesis and respiration rates on a 12 h light (daily) basis. The average daily ratios of P:R >1.00, as is seen for both the L and LF corals, have been interpreted to mean that the coral is self-supporting with respect to carbon (Muscatine et al., 1981), i.e. *S. flexibilis* is entirely phototrophic, as was found for the hard coral *Porites porites* (Edmunds and Davies, 1986). This agrees with the finding (e.g. Wang and Douglas 1998) that translocated photosynthates of zooxanthellae is the main respiratory substrate. The majority of subtropical and tropical zooxanthellate species (as *S. flexibilis*) fuel their total metabolism phototrophically by assimilates of their symbionts and dissolved organic matters (Widdig and Schlichter, 2001).

By converting all the O_2 values to carbon equivalents, the average translocated carbon to the coral host approximates 75% (=T) of total fixed carbon for both the L and LF colonies.

Table 1. *Sinularia flexibilis*. Characteristics of average respiration and photosynthesis of the coral colonies under conditions of light-food (LF), light (L), and dark (D±food) on 12h light basis (±SE). BW= Buoyant weight; P:R= Photosynthesis : Respiration ratio; C= Carbon; CZAR= Contribution of Zooxanthellae to Animal Respiration.

	Gross photosyn. (mg O_2 g^{-1} BW 12 h LD)	Net photosyn. (mg O_2 g^{-1} BW 12 h L)	Respiration (mg O_2 g^{-1} BW 12 h)	P:R (12 h)	C(total) (mg C g^{-1} BW 12 h^{-1} LD)	C(respired)	C(host resp.) (mg C 12 h^{-1})	CZAR (%)
L	340±43	207±31	133 ±12	1.5±0.01	78±12	50±4.4	42± 3 (65)	127±16
LF	300±94	160 ±50	138 ±47	1.3±0.28	60±19	52±17	46±16 (85)	111±20
D	—	—	−161 ±82	—	− 61±31	− 130±63	—	—

Energy equiv.:	O_2 (J 12 h^{-1})	Irradiance (200 μM q. m^{-2} s^{-1}) (J 12 h^{-1})	Energy (O_2) : Irradiance (J 12 h^{-1}, %)	Light efficiency (ε) (mmol C(J)/Irr. (J) 12 h^{-1})
L	4056±612	1.88×10^6	0.2±0.0003	0.65±0.04
LF	3110±974	1.88×10^6	0.2±0.0005	0.85±0.18
D	− 3168±1610	—	—	—

Estimating daily contribution of zooxanthellae fixed carbon to coral host demands (CZAR) using Muscatine's equation (1981), suggests that an average of 111-127 % of the zooxanthellae photosynthates is translocated to the animal in *S. flexibilis* (Table 1). Our estimated CZAR (under steady-state irradiance) for *S. flexibilis* indicates that not only the entire daily respiratory carbon is supplied from zooxanthellae, but the photosynthetic energy exceeds the daily coral needs.

This is consistent with the general view that most shallow-water corals (as *S. flexibilis*) are able to produce more energy via photosynthesis than is required for whole-colony respiration (e.g. Davies, 1977; Muscatine, 1990); only a small fraction of fixed carbon (1-3%) are assimilated or respired by the zooxanthellae, with the majority being transferred to the host (Muscatine et al., 1984; Falkowski et al., 1984). In addition, *S. flexibilis* as a branching coral with a high S/V (surface to volume ratio) as well as higher growth rates, is expected to have higher energy requirements (per unit weight) than non-branching corals, as is generally accepted for such corals (Davies, 1980, 1991). For other symbiotic invertebrates including corals, CZAR values of 25-352% at various conditions have been reported (Muscatine et al., 1984, 1985; Davies, 1984; Edmunds and Davies, 1986; Hoegh-Guldberg and Hinde, 1986; McCloskey et al., 1994; Klumpp and Griffiths, 1994; Verde and McCloskey, 2002; Grottoli et al., 2006).

Major differences in the values of T and CZAR are associated with differences in the living conditions of the organisms studied (Leletkin, 2000). Similarly, using the approach of Steen and Muscatine (1986) resulted in the same CZAR percentages.

Contribution of CZAR to coral growth

An indication of P/R >1.0 as a surplus of photosynthetically fixed carbon, does not give any indication of how much of this is required for growth of the coral colony. To verify the extent of contribution of CZAR to coral growth, the carbon energy expenditure for growth can be used.

- **Energy equivalents**

The relations of photosynthesis to irradiance can also be studied on the basis of energetic values (joules: Morel and Smith, 1974). To have an insight into energy investment with respect to phototrophy, the oxygen values (12 hrs) were converted into energy equivalents (joules), using the oxy-joule coefficient for lipid of 19.63 (Davies, 1991). This is because carbon-rich lipids as the main product of carbon fixation through photosynthesis (Crossland et al., 1980; Davies, 1991).

Table 2 shows the daily estimates of energy expenditure in *S. flexibilis* based on energy equivalents for carbon. Our estimated range of energy allocation to the coral growth depends on the range of energy excretion (10-50 % of respired carbon by the host) that has been reported for some symbiotic corals (e.g. Cooksey and Cooksey, 1972; Crossland et al., 1980; Davies, 1984, Muscatine et al., 1985).

Table 2. *Sinularia flexibilis*. Characteristics of average daily energy expenditure (mg C g^{-1} buoyant w, ± SE) of the coral colonies under conditions of light-food (LF) and light (L).

$C_{(J, total)}$ = [$C_{(J, resp. zoox.)}$ + $C_{(J, resp. host)}$ + $C_{(J, excretion, 10-50\%)}$] + $C_{(J, coral growth)}$					$\%C_{(growth)}/C_{(tot.)}$
L	1521±230 =	152±23	826±63	83±6 - 413±32	130±101 - 460±115	9-30
LF	1166±365	117±37	896±311	90±31 - 448±156	186±140 - 256±12	16-22

The average consumption of daily energy for the coral growth (12-26%, table 2) averages 6-12 % of total photosynthesis energy (mg C g^{-1} BW d^{-1}) and about 0.02 % of the total daily radiant energy (table 1). This estimation (6-12 %) is in agreement to those of 0.9 to 8.9% daily photosynthetic input required by shallow-water corals to

support the biosynthesis of host tissue (Davies 1984; Muscatine et al., 1985; Edmunds and Davies, 1986).

Alternatively, the expression of Muscatine et al. (1985) displays available translocated C for the host growth: $C_{avail.} = (P_z net \,.\, T) - R_a$ ($P_z net$: net C produced by zooxanthellae, T: fraction of translocated $P_z net$, and R_a: respired C by the host). Based on this, the average carbon (7 mg C or 137 J g^{-1} BW d^{-1}) available for the host growth equals the minimum of mean energy values for growth in table 2. All daily carbon needed by the host is produced by the zooxanthellae and is supplied to the host by translocation.

The total C-based growth energy (table 2) suggests a low carbon energy allocation to general growth in *S. flexibilis*. This may be because nitrogenous compounds, which are mainly involved in overall coral growth (Davies, 1984), are deficient in photosynthates (Houlbre`que et al., 2003); hence, acquiring nitrogenous sources is necessary for growth. It can also be concluded that in soft corals, the 'carbon-specific growth rate' is low compared to that of hard corals as *Stylophora pistillata* due to involvement of C in carbonate formation for the coral skeleton, leading to higher CZAR values (Falkowski et al., 1984).

Light utilization efficiency

As is seen in table 1, at the light intensity of our experiments (200 μM quanta m^{-2} s^{-1} or 43.5 joules), considering the energy units for both O_2 and irradiance, the percentage of usable radiant energy is very low. It is higher than a minimum of 0.1% of available underwater irradiance necessary for net photosynthesis (Sakshaug et al., 1997). This shows that only a minor fraction of incident light is practically utilized for the coral energetics.

Similarly, it is also possible to estimate the photosynthetically usable radiation or 'Light utilization efficiency' (ε: Kishino et al., 1986) to estimate the fixed carbon through available irradiance. This is expressed as the ratio of photosynthetic rate (P_J in joules) to available radiant energy (E_J in joules) multiplied by a conversion factor of 112 on the assumption that glucose is the primary product of photosynthetic carbon fixation (Kishino et al., 1986; Davies 1984). Accordingly, having: $\varepsilon=(C_{(J)}. 112/E_{(J)}).K)/C$,

and comparing the result (107 mmol C) to the total photosynthetic C (144 mmol), an average ε value of 75% 12 h^{-1} is obtained. This is similar to the average translocated C (in % or T) by the host mentioned above. Light utilization efficiency (ε), therefore, suggests the requirement for photon flux to saturate photosynthesis, showing the coral's ability to utilize available light effectively.

Based on usable photosynthetic irradiance, and assuming a range of light intensities (or radiant energy), it is possible to determine a range of effective irradiances. Taking the values of our previous study (Khalesi et al., 2007) on the light-dependency of *S. flexibilis* (10-1000 μmol quanta m^{-2} s^{-1}) with optimum performance at 100-400 μmol quanta m^{-2} s^{-1} (as in Fig. 4), the phototrophic energy would lie in the range of 20- 80 joules s^{-1} or 0.1-0.3 % of radiant energy. Therefore, it can be concluded that lower and higher irradiances do not provide effective energy for the coral's phototrophy.

Conclusion

The results of this study showed that phototrophy plays a fundamental role in *Sinularia flexibilis* growth and its physiology. This species depends upon energy from the photosynthesis of zooxanthellae for survival and growth. The absence of light and available nutrients leads to starvation and also morphological changes. In short, it is evident that *S. flexibilis* can satisfy all of its carbon requirements via autotrophy. Therefore, in dark conditions, heterotrophy does not account for meeting energy requirements of this symbiotic octocoral. By supplying both optimum irradiance and ambient nutrient availability, *S. flexibilis* can thrive well in captivity.

References:

Aceret, T.L., Sammarco, P.W., Coll, J.C., 1995. Effects of diterpenes derived from the soft coral *Sinularia flexibilis* on the eggs, sperm and embryos of the scleractinian corals *Montipora digitata* and *Acropora tenuis*. Mar Biol 122, 317–23.

Al-Horani, F.A., Al-Moghrabi, S.M., de Beer, D., 2003. The mechanism of calcification and its relation to photosynthesis and respiration in the scleractinian coral *Galaxea fascicularis*. Mar Biol 142, 419–426.

Anthony, K.R.N., Fabricius, K.E., 2000. Shifting roles of heterotrophy and autotrophy in coral energetics under varying turbidity. J Exp Mar Biol Ecol 252, 221-253.

Anthony, K.R.N., Hoegh-Guldberg, O., 2003. Kinetics of photoacclimation in corals. Oecologia 134, 23–31.

Bythell, J.C., 1988. A total nitrogen and carbon budget for the elkhorn coral *Acropora palmata* (Lamarck). Proc. 6th Int Coral Reef Symp, Townsville, Australia, pp 535–540.

Clayton W.S., Lasker, H.R., 1982. Effect of light and darkness treatments on feeding by the reef coral *Pocillopora damicornis* (Linnaeus). J Exp Mar Biol Ecol 63,3, 269-279.

Cook, C.B., D'Elia, C.F., Muller-Parker, G., 1988. Host feeding and nutrient sufficiency for zooxanthellae in the sea anemone *Aiptasia pallida.* Mar Biol 98, 253-262 .

Cooksey, K.E., Cooksey, B., 1972. Turnover of photosynthetically fixed carbon in reef corals. Mar Biol 15, 289-292.

Davies, P.S., 1977. Carbon budgets and vertical zonation of Atlantic reef corals. Proc. 3rd Int Coral Reef Symp. Miami. 1, 391-396.

Davies, P.S., 1980. Respiration in some Atlantic reef corals in relation to vertical distribution and growth form. Biol Bull 158, 2, 187-194.

Dubinsky, Z., Jokiel, P.L., 1994. Ratio of energy and nutrient fluxes regulates symbiosis between zooxanthellae and corals. Pac Sci 48, 313–324.

Dunlap, W.C., Chalker, B.E., 1986. Identification and quantification of near-UV absorbing compounds (S-320) in a hermatypic scleractinian. Coral Reefs 5: 155–159

Dunlap, W.C., Shick, J.M., 1998. Ultraviolet radiation absorbing mycosporine-like amino acids in coral reef organisms: a biochemical and environmental perspective. J. Phycol 34, 418 - 430.

Edmunds, P.J., Davies, P.S., 1986. An energy budget for *Porites porites* (Scleraetinia). Mar. Biol. 92, 339-347.

Elliot, J.M., Davison, W. 1975. Energy equivalents of oxygen consumption in animal energetics. Oecologia (Berlin) 19, 195-201.

Falkowski, P.G., Dubinsky, Z., Muscatine, L., Porter, J.W., 1984. Light and the bioenergetics of a symbiotic coral. Bio Sci. 34, 11, 705-709.

Fabricius, K.E., Y., Benayahu, A. Genin, 1995a. Herbivory in asymbiotic soft corals. Science 268, 90-92.

Fabricius, K.E., A. Genin, Y. Benayahu, 1995b. Flow dependent herbivory and growth in asymbiotic soft corals. Limnol Oceanogr 40, 1290-1301.

Fabricius, K.E., Klumpp, D.W., 1995. Widespread mixotrophy in reef-inhabiting soft corals: the influence of depth, and colony expansion and contraction on photosynthesis. Mar Ecol Prog Ser 125, 195-204.

Fabricius, K.E., M., Dommisse, 2000. Depletion of suspended particulate matter over coastal reef communities dominated by zooxanthellate soft corals. Marine Ecology Progress Series 196, 157-167.

Ferrier-Pagès, C., Allemand, D., Gattuso, J.P., Jaubert, J., Rassoulzadegan, F., 1998. Microheterotrophy in the zooxanthellate coral *Stylophora pistillata*: effects of light and ciliate density. Limnol Oceanogr 43, 7, 1639-1648.

Franzisket, L., 1970. The atrophy of hermatypic reef corals maintained in darkness and their subsequent regeneration in light. Intr. Rev. Hydrobiologie 55, 1—12.

Gili, J.M., Coma, R., Orejas, C., López-González, P.J., Zabala, M., 2001. Are Antarctic suspension-feeding communities different from those elsewhere in the world? Polar Biol. 24, 473–485.

Grottoli, A.G., Rodrigues, L.J., Palardy, J.E., 2006. Heterotrophic plasticity and resilience in bleached corals. Nature 440, 1186-1189.

Heffner, R.A., Butler, M.J., Reilly, C.K., 1996. Pseudoreplication revisited. Ecology 77, 2558- 2562.

Hoegh-Guldberg, O., Hinde, R., 1986. Respiration and the translocation of newly fixed carbon by zooxanthellae in *Pteraeolidia ianthina*. Proceed Royal Soc Lond Ser B, Biological Sciences, 228, 1253, 493-509.

Houlbreque, F., Tambutte, E., Ferrier-Pagès, C., 2003. Effect of zooplankton availability on the rates of photosynthesis, and tissue and skeletal growth in the scleractinian coral *Stylophora pistillata*. J Exp Mar Biol Ecol 296, 145–166.

Hurlbert, S.H., 1984. Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54, 187- 211.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. J Exp Mar Biol Ecol 351, 106 - 113.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Light dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*. Submitted.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Effects of ammonium, phosphate, and *Artemia* on growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae. Submitted.

Kinzie, R.A., 1993. Effects of ambient levels of solar ultraviolet radiation on zooxanthellae and photosynthesis of the reef coral *Montipora verrucosa*. Mar Biol 116, 319-327.

Kishino, M., N., Okami, M., Takahashi, S., Ichimura, 1986. Light utilization efficiency and quantum yield of phytoplankton in a thermally stratified sea. Limnol Oceanogr 31, 3, 557-566.

Klumpp, D.W., Griffiths, C.L., 1994. Contribution to phototrophic and heterotrophic nutrition to the metabolic and growth requirement of four species of giant clam (Tridacnidae). Mar. Ecol. Progr. Ser., 115, 103–115.

Lasker, H.R., 1981. A comparison of the particulate feeding abilities of three species of gorgonian soft corals. Mar Ecol Prog Ser 5, 61-67.

Lasker, H.R., Gottfried, M.D., Coffroth, M.A., 1983. Effects of depth on the feeding capabilities of two octocorals. Mar Biol 73, 73-78.

Leletkin, V.A., 2000. The energy budget of coral polyps. Russ J Mar Biol 26, 6, 389–398.

Lewis, D.H., Smith, D.C., 1971. The autotrophic nutrition of symbiotic marine coelenterates with special reference to hermatypic corals: 1. Movement of photosynthetic products between the symbionts. Proc. R. Soc. Loud. Set B, 178, 111-129

Maida, M., Sammarco, P.W., Coll, J.C., 2001. Effects of soft corals on scleractinian coral recruitment. II: Allelopathy, spat survivorship and reef community structure. Mar Ecol, 22, 4, 397- 414.

Markager, S., 1993. Light absorption and quantum yield for growth in five species of marine macroalgae. J. Phycol. 29, 54-63.

McCloskey, L.R., Muscatine, L., Wilkerson, F.R, 1994. Daily photosynthesis, respiration, and carbon budgets in a tropical marine jellyfish (*Mastigias* sp.). Mar Biol 119, 13-22.

Michalek-Wagner, K., Bowden, B.F., 1997. A natural algacide from soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia, Alcyonacea). J. Chem Ecol 23, 259- 273.

Michalek-Wagner, K., 2001. Seasonal and sex-specific variations in levels of photo-protecting mycosporine-like amino acids (MAA5) in soft corals. Mar Biol 139, 651-660.

Mills, M.M., F. Lipschultz, K.P. Sebens, 2004. Particulate matter ingestion and associated nitrogen uptake by four species of scleractinian corals. Coral Reefs 23, 311–323.

Morel, A., Smith, R.C., 1974. Relation between total quanta and total energy for aquatic photosynthesis. Limnol Oceanogr 19, 4, 591-600.

Morel, A., 1978. Available, usable, and stored radiant energy in relation to marine photosynthesis. Deep-Sea Res. 25, 673-688.

Murdock, G.R., 1978. Circulation and digestion of food in the gastrovascular system of a gorgonian octocoral (Cnidaria, Anthozoa). Bull. mar. Sci. 28, 363-370.

Muscatine, L., 1980. Uptake, retention, and release of dissolved inorganic nutrients by marine alga- invertebrate associations. In C.B. Cook, P.W. Pappas, and E.D. Rudolph (Eds.). Cellular interactions in symbiosis and parasitism: 229-244. Columbus: Ohio State University Press.

Muscatine, L., McCloskey, L.R., Marian, R.E., 1981. Estimating the daily contribution of carbon from zooxanthellae to coral animal respiration. Limnol Oceanogr 26, 4, 601-611.

Muscatine, L., Falkowski, P.G., Porter, J.W., Dubinsky, Z., 1984. Fate of photosynthetic fixed carbon in light and shade-adopted colonies of the symbiotic coral *Stylophora pistillata*. Proc. R. Soc. Lond. B 222, 181- 202.

Muscatine, L., 1990. The role of symbiotic algae in carbon and energy flux in reef corals. In Z. Dubinsky (Ed.). Ecosystems of the World 25. Coral Reefs 4, 75-86. Elsevier, Amsterdam.

Palardy,J.E., A.G., Grottoli, K. A., Matthews, 2006. Effect of naturally changing zooplankton concentrations on feeding rates of two coral species in the Eastern Pacific. J Exp Mar Biol Ecol 331, 99-107.

Piniak, G.A., 2002. Effects of symbiotic status, flows peed, and prey type on prey capture by the facultatively symbiotic *Oculina arbuscula* temperate coral. Mar Biol 141, 449–455.

Porter, J.W., 1976. Autotrophy, heterotrophy, and resource partitioning in Carribean reef-building corals. Amer. Nat. 110, 731–742.

Reynaud, S., Ferrier-Pagès, C., Sambrotto, R., Julliet-Leclerc, A., Jaubert, J., Gattuso, J.P., 2002. Effect of feeding on the carbon and oxygen isotopic composition in the tissues and skeleton of the zooxanthellate coral *Stylophora pistillata*. Mar Ecol Prog Ser 238, 81–89.

Sakshaug E., A., Bricaud, Y., Dandonneau, P.G., Falkowski, D.A., Kiefer, L., Legendre, A., Morel, J., Parslow, M., Takahashi, 1997. Parameters of photosynthesis: definitions, theory and interpretation of results. J Plank Res 19, 1637-1670.

Sebens, K.P., DeRiemer, K., 1977. Diel cycles of expansion and contraction in coral reef Anthozoans. Mar Biol 43, 247-256.

Sebens, K.P., Vandersall, K.S., Savina, L.A., Graham, K.R., 1996. Zooplankton capture by two scleractinian corals, *Madracis mirabilis* and *Montastrea cavernosa*, in a field enclosure. Mar Biol 127, 303-317.

Shafir S., J. Van Rijn, B. Rinkevich, 2006 Coral nubbins as source material for coral biological research: A prospectus. Aquaculture 259, 444–448.

Stambler, N., Cox, E.F., Vago, R., 1994. Effect of ammonium enrichment on respiration, zooxanthellar densities, and pigment concentrations in two species in Hawaiian corals. PAC. SCI., 48, 3, 284-290.

Steen, R.G., L., Muscatine, 1984. Daily budgets of photosynthetically fixed carbon in symbiotic zoanthids. Biol. Bull. 167, 477-487.

Szmant-Froelich, A, 1981. Coral nutrition: comparison of the fate of ^{14}C from ingested labeled brine shrimp and from the uptake of $NaH^{14}CO_3$ by its zooxanthellae. J Exp Mar Biol Ecol 55, 133–144.

Tentori, E., 2005. http://www.aims.gov.au/pages/auscore/auscore-11.html.

Thieberger, Y., Kizner, Z., Achituv, Y., Dubinsky, Z., 1995. A novel, nondestructive bioassay for assessing areal chlorophyll *a* in hermatypic cnidarians. Limnol. Oceanogr., 40, 6, 1166-1173.

Titlyanov, E.A., Titlyanova, T.V., Yamazato, K., Van Woesik, R., 2001a. Photoacclimation dynamics of the coral *Stylophora pistillata* to low and extremely low light. J Exp Mar Biol Ecol, 263, 211–225.

Titlyanov, E.A., Titlyanova, T.V., Yamazato, K., Woesik, R. van, 2001b. Photoacclimation of the hermatypic coral *Stylophora pistillata* while subjected to either starvation or food provisioning. J Exp Mar Biol Ecol, 257, 163–181.

Treece, G.D., 2000. *Artemia* production for marine larval fish culture. SRAC Publication 702.

Verde E.A., McCloskey, L.R., 2002. A comparative analysis of the photobiology of zooxanthellae and zoochlorellae symbiotic with the temperate clonal anemone *Anthopleura elegantissima* (Brandt). II. Effect of light intensity. Mar Biol 141, 225-239.

Vermeij, M.J.A, Bak, R.P.M., 2002. How are coral populations structured by light? Marine light regimes and the distribution of Madracis. Mar.Ecol.Prog.Ser.233: 105-116

Wang, J.T., Douglas, A.E., 1998. Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? J Exp Biol 201:2445–2453

Widdig, A., Schlichter, D., 2001. Phytoplankton: a significant trophic source for soft corals? Helgol Mar Res 55, 198–211.

Yakovleva, E., Hidaka, M., 2004. Diel fluctuations of mycosporine-like amino acids in shallow-water scleractinian corals. Mar Biol, 145, 863–873.

5 Effects of ammonium, phosphate, and *Artemia* on growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae

Abstract

The effects of prolonged enrichment of ammonium, phosphorus and *Artemia* feeding on the specific growth rate (μ) and zooxanthellae content of the symbiotic soft coral *Sinularia flexibilis* was investigated during three series of experiments in captivity. The 1st series was done at six 3-week periods: no treatment, enrichments of various amounts of NH^{+}_{4} (0.6-50 μM) and PO_4^{3-}(2-30 μM), and again no treatment. In the control tank, μ remained nearly constant for the 1st and 2nd series of the experiments. With PO_4^{3-} enrichment, μ was nearly constant and equal to the control. With NH^{+}_{4} addition, however, μ decreased significantly at higher concentrations of 6-50 μM; it returned to the control value when the N stress was relieved at the 1st series. In the 2nd periodic experiment, supply of 0.5 μM of P plus 1 μM of N with or without *Artemia* feeding, feeding alone, or 1.5 μM of N, all resulted in a μ similar to the control corals. In the 3rd series of the experiments, enrichment of *S. flexibilis* with NH^{+}_{4} (1.5-50 μM) for three weeks resulted in an increase in both zooxanthellae and chlorophyll *a* contents after the 1st week of enrichment, followed by a decrease (equal to control) for the next two weeks, showing a temporary response. In conclusion, over a long period (18 weeks) growth of *Sinularia flexibilis* is adversely affected by high nutrient concentrations. Supply of both nutrients ± *Artemia* or *Artemia* only was found to show the same growth response as the control.

This chapter is submitted as: Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2008. Effects of ammonium, phosphate, and *Artemia* on growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae.

Introduction

The zooxanthellate octocoral *Sinularia flexibilis* Quoy & Gaimard 1833, harbors symbiotic dinoflagellates and produces pharmaceutically important diterpenes (e.g. Coll et al., 1986; Aceret et al., 1995; Maida et al., 2001; Kamel and Slattery, 2005). Substantial extraction and exploitation of these compounds require, as an alternative, rearing of the corals under artificial controlled conditions. For this purpose, it is necessary to study species-specific growth kinetics in captivity.

In reef waters, growth of symbiotic corals depends on inorganic nutrient availability (typically 0.1 to 0.5 μM of nitrate, 0.2 to 0.5 μM of ammonium and < 0.3 μM of phosphorus; Furnas, 1991); corals habitats are generally very low (< 2μM) in nutrients (Szmant, 1997). They also rely on a tight nutrient recycling between the host and its endosymbionts (Muscatine and Porter, 1977). Elevated nutrient levels were reported to have contrasting effects on growth of hard corals in both laboratory experiments (e.g. Stambler et al., 1991; Jokiel et al., 1994) and *in situ* observations (e.g. Grigg and Dollar, 1990; Genin et al., 1995; Bucher and Harrison, 2000). These include: lower linear growth rate at 15 μM of ammonium (Stambler et al., 1991), a 50% decrease in the rate of calcification with 5 μM of nitrate (Marubini and Davies, 1996), a higher growth rate due to ammonium excretion of fish (1 μM: Meyer and Schultz, 1985), and a very high growth rate upon addition of 7 μM of N and 0.6 μM of P (Atkinson et al., 1995). Finally, Steven and Broadbent (1997) observed a 30% growth increase in a hard coral upon enrichment with 20 μM of N and 4 μM of P. Some studies, however, did not observe significant reactions to raised nutrient accessibility (Dollar, 1994; Grigg, 1995).

High inorganic nutrient loads may have adverse effects on health such as reduced growth, increased susceptibility to bleaching, or mortality, since high nutrient loads may affect the coral-zooxanthellae symbiosis (Szmant, 2002), or, alternatively, may have toxic effects (Hoegh-Guldberg, 1994). It is well-known that zooxanthellae play a fundamental role in growth (skeletogenesis) of symbiotic corals (e.g. Muscatine, 1973; Langdon and Atkinson, 2005) and that photosynthesis and calcification are linked (e.g. Gattuso et al., 1999; Kangwe, 2006). Accordingly, it is assumed that stimulation of zooxanthellae growth by elevated nutrients leads to competition for carbon between

photosynthesis by algae and calcification by the corals (Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1989; Falkowski et al., 1993; Dubinsky and Stambler, 1996). As a result, the carbon source is reduced and photosynthesis decreases due to carbon limitation (e.g. Dubinsky et al., 1990; Titlyanov et al., 2000), resulting in reduced coral growth because less photosynthates are translocated from the zooxanthellae to the coral host.

Alternatively, increased inorganic nutrients have been reported (in hard corals) to have toxic effects on zooxanthellae alone, or on both the zooxanthellae and the coral host (e.g. Fitt et al., 1993; Hoegh-Guldberg, 1994; Belda et al., 1993a,b; Achituv et al., 1994); however, data on long-term effects on the growth of soft corals in captivity are absent. Moreover, all the above effects on the coral growth and physiology are less clear (Atkinson et al., 1995; Steven and Broadbent, 1997; Ferrier-Pagès, 2000; Bongiorni et al., 2003). Also, the outcome of N and P addition was found to be both dose- and species-dependent (Koop et al., 2001).

In the present research, therefore, the effects of nitrogen and phosphorus enrichment and of simultaneous *Artemia* feeding on the specific growth rate and on coral symbionts were studied in captivity. The main question was: what are the effects of nutrients (nitrogen, phosphorus) concentrations and *Artemia* feeding, alone or in combination, on the specific growth rate of *Sinularia flexibilis* and its symbionts?

Materials and methods

Parent colonies of the soft coral *Sinularia flexibilis* were obtained from Burgers' Zoo, Arnhem, The Netherlands. They were transferred to rearing tanks (Eco-deco systems, Dymico-Model 1000) at 34 ± 0.5‰ salinity, 25.8 ± 0.2 °C. Experiments were performed under controlled conditions. For all series of experiments, small colonies (5-7 cm) were taken, fixed on small PVC plates and left to adapt for ± two weeks. As coral cuttings represent genetically identical replicates of a single coral colony (Stambler et al., 1994; Shafir et al., 2006), four coral colonies were placed in three tanks (N, P and Control) all with similar environmental conditions. In addition, the treatments (e.g. nutrient level) were applied to each of N and/or P aquarium, hence, each colony is considered as pseudo-replicates (implicit pseudo-replication: Hurlbert, 1984; Heffner et al., 1996). Accordingly, coral responses observed during the nutrient or food additions were caused by the treatments.

Lighting (~200 μmol quanta m^{-2} s^{-1}, photoperiod 12h light :12h dark) was provided by VHO Halide, 10,000 K, HQI lamps (Aqua Medic aqualight 400, Aquaria Veldhuis, Enschede, The Netherlands). Because of dependency of corals to water flow, the local mean water velocities in the enrichment tanks, measured by Acoustic Doppler Velocity Meter (Micro ADV 16 MHZ, Sontek), were adjusted to that of the control tank. The saltwater was made of the nutrient-free Instant Ocean Reef Crystals (Aquarium systems, Sarrebourg, France, 2007). All tanks were aerated and maintained under identical temperature (26°C) and light.

Nutrient enrichment

The 18-week incubation time of the 1st series was divided into six 3-week periods: 1st period of pre-addition (control). From the 2nd to the 5th periods, calculated amounts of ammonium (NH_4CL: 0.6 – 50 μM) and phosphorus (KH_2PO_4: 2 – 30 μM) were added daily to the N or P tanks. Ammonium was used as an appropriate source of nitrogen for the corals (Wilkerson and Trench, 1985). Seawater was changed daily and the tanks were cleaned as necessary in order to minimize algal growth on the walls.

Artemia feeding with or without nutrients

These 2nd series of periodic experiments were performed during four 3-weekly periods (12 weeks) under the same experimental set up as the previous series. Stock solutions of NH_4C1 and KH_2PO_4 were prepared and continuously added to the tanks in pre-determined values (0.5- 1.5 μM). A suspension of *Artemia* nauplii (100 ml, n=± 1000) was fed to corals at only food and/or food plus nutrients tanks, 4 days per week. They were allowed to feed for two hours.

Response of zooxanthellae to ammonium enrichment

To verify the zooxanthellae reaction to high levels of NH^+_4, in a 3rd series of experiments new coral colonies (n=8 per tank) were incubated for 4 weeks in the same concentrations of NH^+_4 as the first series (1.5, 6, and 50 μM) plus a control group. Coral samples from pre-enrichment (1st week) and from each week (enriched) were weekly collected, and their tissue were homogenized to determine the density of zooxanthellae and chlorophyll *a* at each treatment and also in the control.

Specific growth rate

The total weight of each coral group (n=4) from each tank in experiments 1 and 2 was measured weekly by buoyant weighing, using an analytical balance (PROLABO A&D HR300, bearing: 310 g; accuracy: ± 0. 01 mg) with underweighing device. Buoyant weight represents body material of any coral and consequently changes in buoyant weight are primarily due to coral growth (Bak, 1973, 1976; Jokiel et al., 1978). This non-destructive method has been used

to measure small changes in growth of corals (Davies, 1989; Davies, 1990, 1995) and enabled us to measure short-term changes in growth of *S. flexibilis*, as we performed previously (Khalesi et al., 2007). Specific growth rates (μ d^{-1}) were then calculated weekly for each group in the experiments 1 and 2 according to: $\mu = (\ln W_2 - \ln W_1)/\Delta t$ (Kaufmann, 1982); where W_1 and W_2 are weight at the beginning (t_1) and end (t_2) of each time interval. The mean of these specific growth rates was then calculated every three weeks for the colonies in each tank. Changes in the mean μ were then plotted against time. Statistical analyses used were ANOVA and Student's t-Test.

Extraction of samples

The coral tissues homogenized in tubes containing 3 ml filtered (0.2 μm) seawater (FSW). The homogenate (1ml) was diluted in FSW and vortexed. One half was used to count the zooxanthellae using a hemocytometer at four replicate counting, and the average was recorded and expressed per coral wet weight. For the determination of chlorophyll *a*, the second half of the homogenate was transferred to a sterile tube containing 9 ml 90% acetone, vortexed for 10 min, and centrifuged for 5 min at 5000 rpm, and the absorbance spectra at 665, 647, and 630 nm were measured using a spectrophotometer (Spectronic 20 Genesys, USA). The amounts of chlorophyll were then calculated using the equations of Jeffrey and Humphrey (1975) and normalized to the wet weight of the samples.

Results and Discussion

The specific growth rates (μ d^{-1}) of *Sinularia flexibilis* were measured weekly in the control and enriched tanks, in which the influence of NH^{+}_{4} or PO_4^{3-} additions on the coral colonies was investigated. The μ values ranged from 0 to 15×10^{-3} d^{-1}, which, at its highest, is close to previous values for this species (Khalesi et al., 2007). The μ was constant during period 1 (no treatment, Fig. 1A, B, & C) and remained nearly constant in the control tank for 18 weeks (Fig. 1C). In the NH^{+}_{4}-enriched tank, the μ was almost constant when the NH^{+}_{4} concentration was increased from 0.6 to 1.5, and 6 μM (periods 2, 3, and 4, Fig. 1A). The subsequent increase in NH^{+}_{4} from 6 to 50 μM, however, reduced coral growth until it finally stopped altogether (period 5, Fig. 1A). The μ values during periods 1-4 were not different (ANOVA, P= 0.6). Comparison of each period (1, 2, 3, 4, and 6) and also the control with the 5th period (50 μM of NH^{+}_{4}) showed significant differences in μ (paired t–Test, $p< 0.05$).

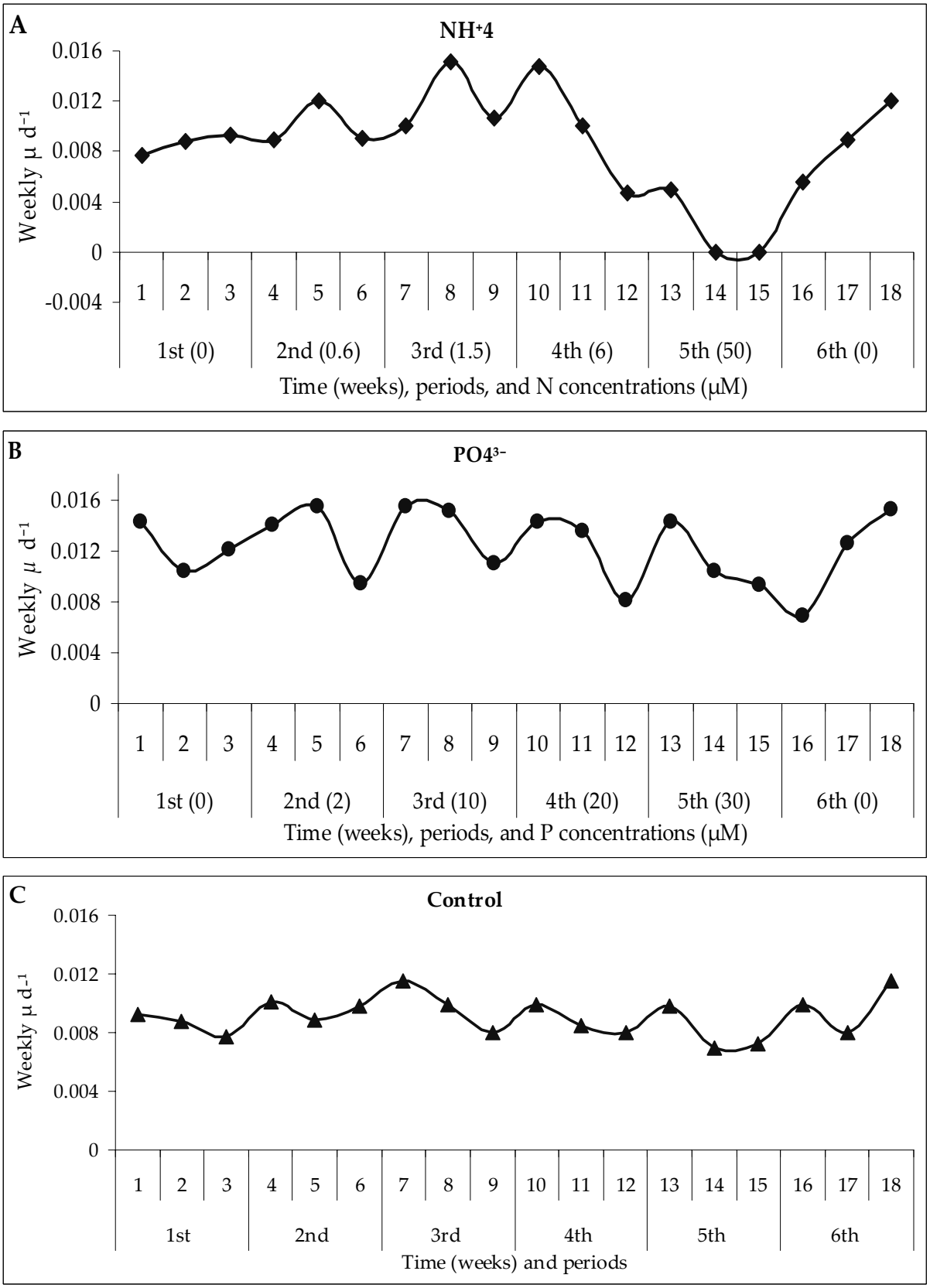


Fig. 1. *Sinularia flexibilis*. Specific growth rates (μ d^{-1}) calculated weekly for the colonies incubated in the experimental tanks of series 1 (**A**, N=NH^{+}_{4}; **B**, P=PO_4^{3-}; and C, Control). Periods (1st to 6th) indicate every 3 weeks. The numbers in parentheses show the concentrations (μM) at each period.

Apparently, a concentration in the range of 6 to 50 μM of NH^+_4 fully inhibits growth of this species, showing that this coral is able to endure very low ammonium concentrations only. This is in agreement with earlier reported results for hard corals (Koop et al., 2001) and McGuire (1997, at 5 and 10 μM of ammonium). Up until now, no comparable data were available on the growth response of captive soft corals to nutrient enrichment.

The reduced or zero μ at high NH^+_4 concentrations (6 and 50 μM of N, Fig. 1A) may be caused by either toxicity, competition for carbon, or both. Toxicity by elevated ammonium was also stated by Hoegh-Guldberg (1994). Besides, marine invertebrates in general are known to be susceptible to ammonium, i.e. nitrogen toxicity (Baird et al., 2006). Bruno et al. (2003) also showed that a moderate increase in nutrient concentrations (up to 6.4 μM nitrate, 4.6 μM phosphorus, and 11 μM ammonium) can cause coral diseases. Eventually, Szmant (2002) concluded that elevated ammonium negatively affects coral health and growth rate through indirect physiological effects, in addition to the effect on coral-zooxanthellae symbiosis.

During the recovery period without NH^+_4 addition (period 6, Fig. 1A) the specific growth rate increased again to its original value. Apparently, *S. flexibilis* was able to recover relatively fast from severe N stress. A recovery time of 5 weeks for the stony coral *Stylophora pistillata* was reported before (Ferrier-Pagès et al., 2000).

In the present study, *S. flexibilis* responded negatively to relatively elevated NH^+_4 concentrations and showed no positive reaction at lower levels. Results obtained by others on hard corals indicate variable effects on coral growth including positive, neutral (e.g. Taylor, 1978; Marubini and Atkinson, 1999) or negative effects (e.g. Marubini and Davies, 1996; Szmant, 2002). These contrasting results suggest that the effects of nitrogen enrichment on corals depend on concentration, coral species, and the enrichment duration (Ferrier-Pagès et al., 2000). However, biological relations between ammonium enrichment and both coral health and calcification, especially for soft corals, need to be further investigated.

In the PO_4^{3-}-enriched tank, the μ was almost constant and independent of the PO_4^{3-} concentration (2-30 μM), equal to the control (Fig. 1B, paired t-Test: $p > 0.05$). No significant differences in μ was found among the whole periods in the PO_4^{3-} tank

(ANOVA, p =0.9). Therefore, *S. flexibilis* tolerated relatively high loadings of PO_4^{3-}. Similarly, Stambler et al. (1991) did not find any changes in coral growth rate nor in zooxanthellae content at elevated levels of phosphate in the reef-building coral *Pocillopora damicornis*, whereas at relatively low values of 2 μM of PO_4^{3-}, Ferrier- Pagès et al. (2000) observed a 60% decrease in a stony coral growth rate following 9 weeks of exposure. In the latter case, the reduced coral growth at very low levels of P may be explained by the fact that phosphorus by acting as a 'crystal poison': By binding to calcium, it inhibits or slows down the calcification (skeletal growth) of hard corals (e.g. Snidvongs and Kinzie, 1994; Hoegh-Guldberg et al., 1997). Obviously, such an inhibitory action is particularly pronounced and relevant for hard corals and less so for soft-bodied corals (as *S. flexibilis*).

Effects of *Artemia* feeding and nutrients

In a second series of experiments, we investigated the growth response of *S. flexibilis* to various combinations of food (F, *Artemia* nauplii) and low concentrations of N and P (0.5- 1.5 μM) during four 3-weekly periods (12 weeks). The specific growth rate of control colonies was similar to the control group of the 1st series ($9\times10^{-3} \pm 0.002$ d^{-1}, data not shown). All the fed, enriched, and the control corals were not significantly different in μ (ANOVA, p> 0.05). Table 1 indicates that with nutrient enrichment and *Artemia* feeding, alone or in combination, the specific growth rate was altogether similar to the control as well as to the values of the slightly N-enriched, P-enriched, and control corals at the 1st series of enrichments (Fig. 1A, B, C).

Table 1. *Sinularia flexibilis.* Effects of feeding and lower additions of NH^+_4 or NH^+_4 plus PO^{3-}, alone or with *Artemia* feeding on the weekly μ (± standard deviations). The numbers in parentheses show the nutrients concentrations (μM).

Treatment	μ (d^{-1}) ± SD
P (0.5μM) + N (1μM)	0.011 ± 0.002
F + P (0.5μM) + N (1μM)	0.008 ± 0.001
Food (F)	0.010 ± 0.0004
N (1.5μM)	0.011 ± 0.0008

The corals in both the fed and enriched groups maintained their mean specific growth rates, as was also found by Muscatine et al. (1989). Our results indicate that

Artemia feeding did not account for the colonies' growth needs. This result does not infer that *S. flexibilis* is incapable of zooplanktivory entirely; it may take up smaller prey sizes than *Artemia* nauplii, as stated for some hard corals (Sebens et al., 1996). Moreover, the fact that μ does not change upon N or P enrichment or feeding probably reflects the sufficiency of ambient levels of nutrients as well as the tight nutrient recycling within this soft coral. In addition, in tropical corals (as *S. flexibilis*), because of nitrogen-poor products of zooxanthellae in the tropics, a process known as 'nitrogen conservation' predicts that they should conserve nitrogen compared to temperate corals (Szmant et al., 1990; Ferrier- Pagès et al., 1998). Hence, small amounts of nitrogen taken up from the surrounding environment can sustain coral growth (Grover et al., 2002). Accordingly, any further supply of N, P, or food does not increase μ, since no limitations are relieved.

Conclusively, more studied hard corals have been found to increase growth at low P concentrations (e.g. 0.5 and 0.6 μM: Stambler et al., 1991; Atkinson et al., 1995) and to decrease growth at higher levels (>2 μM of P: e.g. Kinsey and Davis, 1979; Ferrier-Pagès et al., 2000, Renegar and Riegl, 2005). The response of *S. flexibilis*, as a soft coral, to P addition was neutral, even at high levels (Fig.1B). Our results for N enrichment agree with Atkinson et al. (1995) that ammonium *per se* does not appear to inhibit growth of coral at concentrations up to at least 5μM of N, and that soft corals survive and apparently thrive under elevated nutrient conditions (Fleury et al., 2000). In general, increases of nutrient concentrations, particularly phosphate (for stony corals), by a factor of 2-3 above ambient levels has resulted in stress to coral nutrition, growth and, ultimately, survival (e.g. Smith et al., 1981; Walker and Osmand, 1982; Tomascik and Sander, 1985). Therefore, it has generally been recommended to keep inorganic nutrient concentrations at levels below 1 μM for maintaining (hard) corals in aquaria (Atkinson et al., 1995). Our main conclusion is that over a long period, growth of *Sinularia flexibilis* is adversely affected by high ammonium concentrations, and addition of both nutrients alone or with *Artemia* does not enhance coral growth.

Zooxanthellae and chlorophyll *a* concentrations

To verify the effect of high levels of NH^{+}_{4} on the density of zooxanthellae, possibly resulting in the observed reduced growth of *S. flexibilis*, new coral samples were enriched with the same concentrations (1.5, 6, and 50 μM) at three separate tanks. Figure 3 shows that following the 1st week of NH^{+}_{4} addition, both the algae and chl. *a* contents increased at all concentrations. After the 1st week, densities of the algae and chl. *a* dropped again to initial levels at 1.5 and 50 μM, but was almost constant at 6 μM.

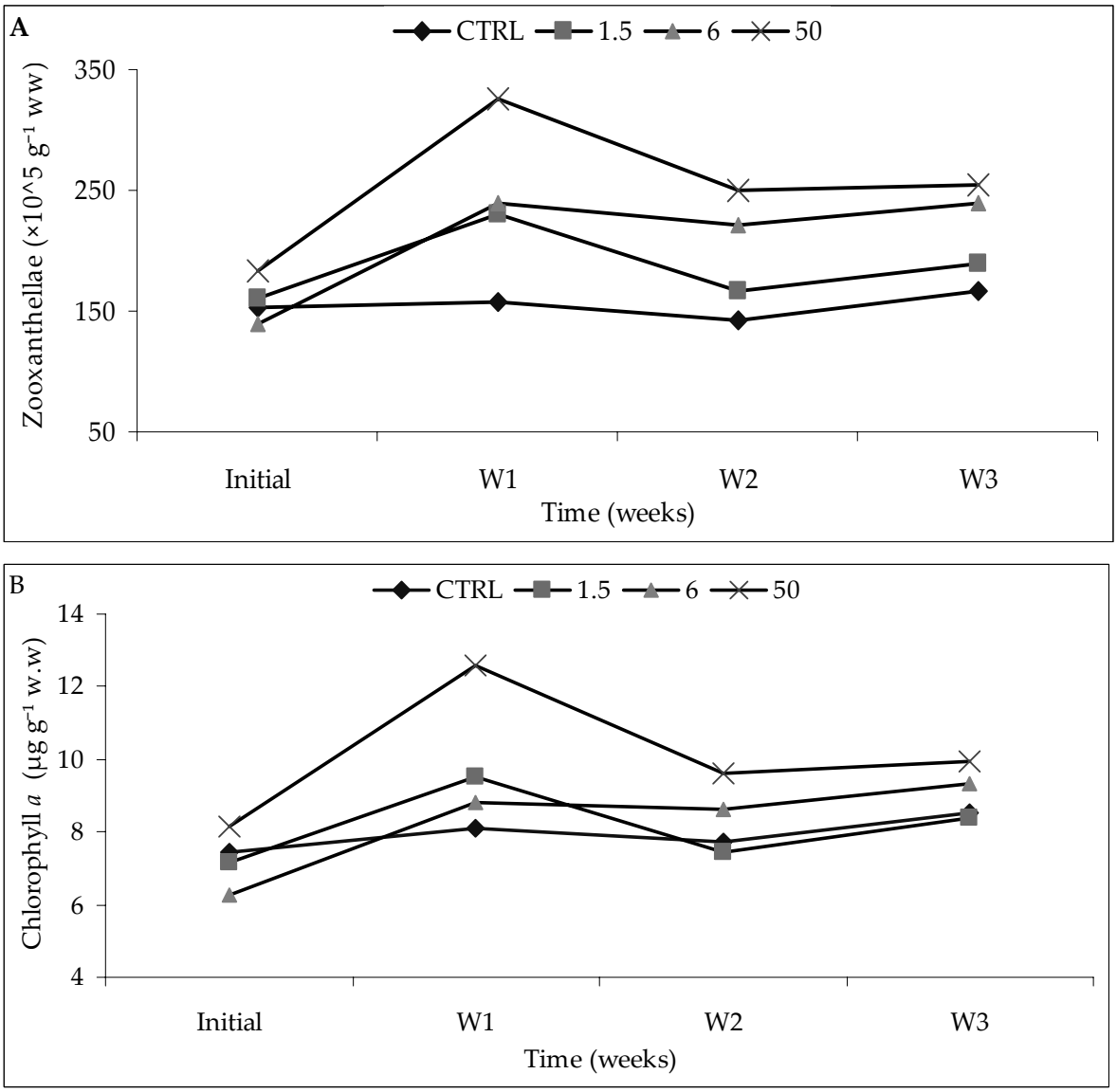


Fig. 2. *Sinularia flexibilis.* **A)** Zooxanthellae density, and **B)** Concentration of chlorophyll *a* in the enriched corals of the 3rd series of enrichment experiments vs. controls. The coral colonies enriched with NH^{+}_{4} (1.5, 6, and 50 μM) for 3 weeks.

In spite of decrease in both zooxanthellae and chlorophyll content after the 1st week of ammonium addition, especially at 50 μM (experiment 1), the harmful effect on coral growth continued as long as the enrichment persisted (Fig. 1A). Therefore, the observed stunted growth of the high N-loaded corals in this study after one week does not appear to be because of a coral-algae competition for substrates due to the increased zooxanthellae population, otherwise their proliferation should have increased constantly. Besides, based on our results, zooxanthellae in *S. flexibilis* do not seem to be limited by ammonium, and ambient levels seem to be sufficient probably because of a tight nutrient recycling as well as nitrogen conservation within this symbiotic association. This is in agreement with previous observations (Hoegh-Guldberg and Williamson, 1999; Grover et al., 2002).

The lack of a provable long-term effect of elevated NH^+_4 concentrations, despite an increase during the early enrichment, suggests a transitory and short-lived effect on the zooxanthellae density in *S. flexibilis*. The same effect was also observed by Cook et al. (1988), Hoegh-Guldberg and Smith (1989), Muscatine et al. (1989), and Schlöder and D'Croz (2004). For instance, Cook et al. (1988) reported increased zooxanthellae density with 20 μM NH^+_4 lasting for 10 days, which decreased afterwards. Tanaka et al. (2007) also observed both zooxanthellae and chlorophyll *a* duplication at 5 μM NH^+_4 in a hard coral only for 10 days of nutrient enrichment; the consequence for a longer period is missing. Our data for a longer period (3 weeks) show that external supply of NH^+_4 temporarily stimulates the growth of zooxanthellae in *S. flexibilis*, which is not long-lasting. This means that the zooxanthellae and the coral host do not outgrow each other, suggesting a regulated control to maintain a constant host to algal cell ratio, as has been shown by Jones and Yellowlees (1997). Similarly, Takabayashi (1996) and Muscatine et al. (after 2 weeks, 1989) did not detect significant differences among high-loading nutrient treatments for the population density of zooxanthellae in colonies of *Stylophora pistillata*; also, the zooxanthellae in this species were not nitrogen limited (Koop et al., 2001). In the same way, Nordemar et al. (2003) did not observe significant differences in zooxanthella density nor in chlorophyll *a* content of the scleractinian *Porites cylindrica* at 15 μM of nitrate after 2 weeks of exposure; they

reported enhanced algal density, while surplus algae were expelled. Correspondingly, the chlorophyll content of zooxanthellae in response to nitrogen supply seems to be different for particular coral species (Cook et al., 1997), in addition to possible influencing factors such as light intensity and feeding history (Nordemar et al., 2003).

The reduction of zooxanthellae population in this study following an increase may also reflect regulation of the algal density within the coral host through mechanisms such as expulsion and/or reduction of the algal proliferation rate by the host to maintain a steady-state population (Mucatine and Pool, 1979). Stimson and Kinzie (1991) also reported a 40% release of extra N-enriched zooxanthellae from the coral host as a mean of regulation. Moreover, Jones and Yellowlees (1997) suggest that space availability and the size of the algal symbionts determine the algal densities in the coral colonies.

Conclusion

The overall long-term results of this study show that nutrient availability might be advantageous to *S. flexibilis* over a very low concentration range only. Background levels of NH_4^+ and PO_4^{3-} are quite sufficient to meet the coral's demand for these essential nutrients. An additional supply of these nutrients beyond the micromolar levels is not expected to stimulate coral growth, nor a consistent effect on zooxanthellae. Moreover, with feeding on *Artemia* nauplii, plus or minus nutrient enrichment vs. nutrients alone, no difference in growth was obtained. Our results indicate that moderately nutrient addition adversely affects normal coral growth and, therefore, might reduce its potential for long-term exploitation. Therefore, sufficient ambient levels of ammonium appear to maintain an efficient growth and symbiosis in *S. flexibilis*.

References

Aceret, T.L., Sammarco, P.W., Coll, J.C., Uchio, Y., 2001. Discrimination between several diterpenoid compounds in feeding by *Gambusia affinis*. Comp Biochem Physiol Part C 128, 55-63.

Aquariumsystems: www.aquariumsystems.com/frames1.htm, accessed 2007.

Achituv, Y., Ben-Zion, M., Mizrahi, L., 1994. Carbohydrate, lipid, and protein composition of zooxanthellae and animal fractions of the coral, *Pocillopora damicornis* exposed to ammonium enrichment. Pacif Sci 48, 224-233.

Atkinson, M.J., Carlson B., Crow G.L., 1995. Coral growth in high- nutrient, low-pH seawater: a case study of corals cultured at the Waikiki Aquarium, Honolulu, Hawaii. Coral Reefs 14, 215 -223.

Baird, A.H., J.P. Gilmour, T.M. Kamiki, M. Nonaka, M.S. Pratchett, H.H. Yamamoto, H. Yawsaki, 2006. Temperature tolerance of symbiotic and non-symbiotic coral larvae. Proc 10th Int Coral Reef Sym, 38-42.

Bak, R.P.M., 1973. Coral weight increment in situ. A new method to determine coral growth. Marine Biology 20, 45–49.

Bak, R.P.M., 1976. The growth of coral colonies and the importance of crustose coralline algae and burrowing sponges in relation with carbonate accumulation. Netherlands Journal of Sea Research 10, 285–337.

Belda, C.A., Cuff, C., Yellowlees, D., 1993a. Modification of shell formation in the giant clam *Tridacna gigas* at elevated nutrient levels in sea water. Mar Biol 117, 251-257.

Belda, C.A., Lucas, J.S., Yellowlees, D., 1993b. Nutrient limitation in the giant clam-zooxanthellae symbiosis: effects of nutrient supplements on growth of the symbiotic partners. Mar Biol 117, 655-664.

Bongiorni L., S. Shafir, D. Angel, B. Rinkevich, 2003. Survival, growth and gonad development of two hermatypic corals subjected to in situ fish-farm nutrient enrichment. Mar Ecol Prog Ser 253, 137–144.

Bruno, J.F., Petes, L.E., Harvell, C.D., Hettinger, A., 2003. Nutrient enrichment can increase the severity of coral diseases. Ecol Lett, 6, 1056–1061.

Bucher, D.J., P.L., Harrison, 2000. Growth response of the reef coral *Acropora longicyathus* to elevated inorganic nutrients: do responses to nutrients vary among coral taxa? Proceed 9th Inter Coral Reef Symp, Bali, Indonesia 23-27.

Coll, J.C., Bowden, B.F., Konig, G.M., Braslau, R., 1986. Studies of Australian soft corals. The natural products chemistry of alcyonacean soft corals with special reference to the genus *Lobophytum*. Bull Soc chim Belg 95, 815-834.

Cook, C.B., D'Elia, C.F., Muller-Parker, G., 1988. Host feeding and nutrient sufficiency for zooxanthellae in the sea anemone *Aiptasia pallida*. Mar. Biol. 98, 253—262.

Cook, C.B., G., Muller-Parker, M.D., Ferrier, 1997. An assessment of indices of nutrient sufficiency in symbiotic dinoflagellates. Proc 8th Int Coral Reef Sym 903-908.

Davies, P.S., 1989. Short-term growth measurements of corals using an accurate buoyant weighing technique. Mar Biol 101, 389–395.

Davies, P.S., 1990. A rapid method for assessing growth rates of corals in relation to water pollution. Mar Poll Bull 21, 7, 346–348.

Davies, P.S., 1995. Coral nubbins and explants for reef assessment and laboratory ecotoxicology. Coral Reefs 14, 267–269.

Dollar SJ, 1994. Sewage discharge on coral reefs: not always pollution. Coral Reefs 13: 224.

Dubinsky, Z., Stambler, N., Ben-Zion, M., McCloskey, L.R., Muscatine, L., Falkowski, P.G., 1990. The effect of external nutrient resources on the optical properties and photosynthetic efficiency of *Stylophora pistillata*. Proc R Soc Lond B 239, 231 -246.

Dubinsky, Z., Stambler, N., 1996. Marine pollution and coral reefs. Global Change Biology 2, 511- 526.

Falkowski, P.G., Dubinsky, Z., Muscatine, L., McCloskey, L., 1993. Population control in symbiotic corals. BioScience 43, 606- 611.

Ferrier-Pagès, C., Gattuso, J.P,, Cauwet, G., Jaubert, J., Allemand, D., 1998. Release of dissolved organic carbon and nitrogen by the zooxanthellate coral *Galaxea fascicularis*. Mar Ecol Prog Ser 172, 265–274.

Ferrier-Pagès, C., Gattuso, J.P., Dallot, S., Jaubert, J., 2000. Effect of nutrient enrichment on growth and photosynthesis of the zooxanthellate coral *Stylophora pistillata*. Coral Reefs 19, 103-113.

Fitt, W.K., Heslinga, G.A., Watson, T.C., 1993. Utilization of dissolved inorganic nutrients in growth and mariculture of the tridacnid clam *Tridacna derasa*. Aquaculture, Amsterdam 109: 27-38

Fleury, B.G., J.C., Coll, E., Tentori, S., Duquesne, L. Figueiredo. 2000. Effect of nutrient enrichment on the complementary (secondary) metabolite composition of the soft coral *Sarcophyton ehrenbergi* (Cnidaria: Octocorallia: Alcyonaceae) of the Great Barrier Reef. Mar Biol 136, 63-68.

Furnas M.J., 1991. Nutrient status and trends in waters of the Great Barrier Reef Marine Park. In: Yellowlees D (ed) Land uses patterns and nutrient loadings of the Great Barrier Reef Region. James Cook Univ. Townsville, 162 - 179.

Genin, A., Lazar, B., Brenner, S., 1995. Vertical mixing and coral death in the Red Sea following the eruption of Mount Pinatubo. Nature 377, 507- 510.

Grigg R.W., Dollar S.J., 1990. Natural and anthropogenic disturbance on coral reefs. In: Coral reefs: ecosystems of the world 25, Dubinsky Z (ed), Elsevier, Amsterdam, 439- 452.

Grigg R.W., 1995. Coral reefs in an urban embayment in Hawaii: a complex case history controlled by natural and anthropogenic stress. Coral Reefs 14, 253- 266.

Grover R., Maguer J.F., Reynaud-Vaganay S., and Ferrier-Pagès C., 2002. Uptake of ammonium by the scleractinian coral *Stylophora pistillata*: Effect of feeding, light, and ammonium concentrations Limnol. Oceanogr. 47, 3, 782–790.

Heffner, R.A., Butler, M.J., Reilly, C.K., 1996. Pseudoreplication revisited. Ecology 77, 2558- 2562.

Hoegh-Guldberg, O., Smith, G.J., 1989. Influence of the population density of zooxanthellae and supply of ammonium on the biomass and metabolic characteristics of the reef corals *Seriatopora hystrix* and *Stylophora pistillata*. Mar Ecol Prog Ser 57, 173-186.

Hoegh-Guldberg, O., 1994. Population dynamics of symbiotic zooxanthellae in the coral *Pocillopora damicornis* exposed to elevated ammonium concentrations. PAC. SCI 48, 3, 263-272.

Hoegh-Guldberg, O., Takabayashi, M., Moreno, G., 1997. The impact of long-term nutrient enrichment on coral calcification and growth. Proc 8th Int Coral Reef Symp 1, 861-866.

Hoegh-Guldberg, O., Williamson J., 1999. Availability of two forms of dissolved nitrogen to the coral *Pocillopora damicornis* and its symbiotic zooxanthellae. Mar. Biol. 133: 561–570.

Hurlbert, S.H., 1984. Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54, 187- 211.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equation for determining chlorophyll a, b, c_1 and c_2, Biochem. Physiol. Pflanz., 167, 194-204.

Jokiel, R.L., Maragos, J.E., Franzisket, L., 1978. Coral growth: buoyant weight technique. In Coral Reefs: Research Methods. Monographs Oceanographic Methodology (UNESCO), eds. D. R. Stoddart and R. E. Johannes, vol. 5, pp. 529–542.

Jokiel P., Dubinsky Z., Stambler N., 1994. Nutrient limitation on the symbiotic association between zooxanthellae and reef building corals. Pac Sci 48, 215-218.

Jones, R.J., Yellowlees, D., 1997. Regulation and control of intracellular algae (= zooxanthellae) in hard corals. Phil. Trans. R. Soc. Lond. B 352, 457-468.

Kamel, H.N., Slattery, M., 2005. Terpenoids of Sinularia: Chemistry and biomedical applications. Pharmaceut Biol, 43, 253–269.

Kangwe, J.W., 2006. Calcareous algae of a tropical lagoon: primary productivity, calcification and carbonate production. Doctoral dissertation in plant physiology, department of botany, Stockholm university, Sweden.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. J Exp Mar Biol Ecol 351, 106 - 113.

Kinsey, D.W., P.J., Davies., 1979. Effects of elevated nitrogen and phosphorus on coral reel growth. Limnol Oceanogr 24, 935—939.

Koop, K., D., Booth, A., Broadbent, J., Brodie, D., Bucher, D., Capone, J. Coll, W. Dennison, M. Erdmann, P. Harrison, O. Hoegh-guldberg, P. Hutchings, G.B. Jones, A.W.D. Larkum, J. O'neil, A. Steven, E. Tentori, T. Ward, J. Williamson, D. Yellowlees, 2001. ENCORE: The effect of nutrient enrichment on coral reefs. Synthesis of results and conclusions. Mar Pol Bul 42, 2, 91-120.

Langdon, C., Atkinson, M.J., 2005. Effect of elevated pCO_2 on photosynthesis and calcification of corals and interactions with seasonal change in temperature/irradiance and nutrient enrichment. J. Geoph. Res. Oceans, 110, 1-54.

Maida, M., Sammarco, P.W., Coll, J.C., 2001. Effects of soft corals on scleractinian coral recruitment. II: allelopathy, spat survivorship and reef community structure. Mar Ecol 22, 4, 397–414.

Marubini F., Davies P.S., 1996. Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. Mar Biol 127, 319 -328.

Marubini, F., Atkinson, M.J., 1999. Effects of lowered pH amid elevated nitrate on coral calcification. Mar Ecol Prog Ser 188, 117—121.

McGuire, M. P. 1997. The biology of the coral *Porites astreoides*: reproduction, larval settlement behavior and responses to aminonium enrichment. Ph .D. Dissertation, University of Miami, Miami, Florida.

Meyer, J.L., Schultz, E.T., 1985. Tissue condition and growth rate of corals associated with schooling fish. Limnol Oceanogr 30, 157-166.

Muscatine, L., 1973. Nutrition of corals.— In: Jones, Q.A. & R. Endean (eds.): Biology and geology of coral reefs, vol. II: 77-115. London, New York.

Muscatine, L., Porter J.W., 1977. Reef corals: mutualistic symbioses adapted to nutrient-poor environments. BioScience 27, 454—460.

Muscatine, L., R.R. Pool, 1979. Regulation of numbers of intracellular algae. Proceedings of the Royal Society of London. Series B, Biological Sciences 204, 1155, The Class as a Habitat. 131-139.

Muscatine, L., Falkowski, P.G., Dubinsky, Z., Cook, P.A., McCloskey, L.R., 1989. The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc R Soc Lond B 236, 311 -324.

Nordemar, I., M., Nyström, R., Dizon, 2003. Effects of elevated seawater temperature and nitrate enrichment on the branching coral *Porites cylindrical* in the absence of particulate food. Mar Biol 142, 669–677.

Pearse, V.B., Muscatine, L., 1971. Role of symbiotic algae (zooxanthellae) in coral calcification. Biol. Bull. 141, 350-363.

Renegar, D.A., Riegl, B. M., 2005. Effect of nutrient enrichment and elevated CO_2 partial pressure on growth rate of Atlantic scleractinian coral *Acropora cervicornis*. Mar Ecol Prog Ser 293, 69-76.

Schlöder, C., D'Croz, L., 2004. Responses of massive and branching coral species to the combined effects of water temperature and nitrate enrichment. J. Exp. Mar. Biol. Ecol. 313, 255–268.

Sebens, K.P., Vandersall, K.S., Savina, L.A., Graham, K.R., 1996. Zooplankton capture by two scleractinian corals, Madracis mirabilis and Montastrea cavernosa, in a field enclosure. Mar Biol 127, 303-317.

Shafir S., Van Rijn, J., Rinkevich, B., 2006. Coral nubbins as source material for coral biological research: A prospectus. Aquaculture 259, 444–448.

Smith, S.V., Kirnnerer, W.J., Laws, E.A., Brock, R.E., Walsh, T.W., 1981. Kaneohe Bay sewage diversion experiment: Perspectives on ecosystem responses to nutritional perturbation. Pac. Sci. 35, 4, 279-385.

Snidvongs, A., Kinzie, R.A., 1994. Effects of nitrogen and phosphorus enrichment on in vivo symbiotic zooxanthellae of *Pocillopora damicornis*. Mar Biol 118, 705-711.

Stambler, N., Popper, N., Dubinsky, Z., Stimson, J., 1991. Effects of nutrient enrichment and water motion on the coral *Focillopora damicornis*. Pac Sci 45, 299- 307.

Stambler, N., Cox, E.F., Vago, R., 1994. Effect of ammonium enrichment on respiration, zooxanthellar densities, and pigment concentrations in two species in Hawaiian corals. PAC. SCI., 48, 3, 284-290.

Steven, A.D.L., Broadbent, A.D., 1997. Growth and metabolic responses of *Acropora palifera* to long-term nutrient enrichment. Proc 8th Int Coral Reef Symp 1, 867-872.

Stimson, J., Kinzie, R.A., 1991. The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (L.) under nitrogen-enrichment and control conditions. J Exp Mar Biol Ecol 153, 63-74.

Szmant, A.M., Ferrer, L.M., FitzGerald, L.M., 1990. Nitrogen excretion and atomic oxygen to nitrogen ratios in reef corals: evidence for conservation of nitrogen. Mar Biol 104, 119–128.

Szmant, A.M., 1997. Nutrient effects on coral reefs: A hypothesis on the importance of topographic and trophic complexity on nutrient dynamics. Proc. 8th Int. Coral Reef Symp. 2: 1527–1532.

Szmant, A.M., 2002. Nutrient enrichment on coral reefs: Is it a major cause of coral reef decline? Estuaries 25, 4b, 743 -766

Takabayashi, M., 1996. Impacts of in situ inorganic nutrient enrichment on the scleractinian corals, *Stylophora pistillata* and *Pocillopora damicornis*. M.Sc Thesis, University of Sydney, Sydney, Australia.

Tanaka, Y., T, Miyajima, I., Koike, T., Hayashibara, H., Ogawa, 2007. Imbalanced coral growth between organic tissue and carbonate skeleton caused by nutrient enrichment. Limnol. Oceanogr., 52, 3, 1139–1146.

Taylor, D.L., 1978. Nutrition of algal—invertebrate symbiosis. II. Effects of exogenous nitrogen sources on growth, photosynthesis and the rate of excretion by algal symbionts in vivo and in vitro. Proceed Royal Soc London 201, 401—412.

Titlyanov, E., Bil', K., Fomina, I., Titlyanov, T., Letekin, V., Eden, N., Malkin, A., Dubinsky, Z., 2000. Effects of dissolved ammonium addition and host feeding with *Artemia salina* on photoacclimation of the hermatypic coral *Stylophora pistillata*. Mar Biol 137, 463-472.

Tomascik, T., Sander, F., 1985. Effects of eutrophication on reef building corals: growth rate of the reef building coral *Montastrea annularis*. Mar Biol 87, 143-155.

Walker, D.I., Osmond, R.A.G., 1982. Coral death and phosphate pollution at Aqaba, Red,'Sea. Mar. Poll. Bull. 13, l, 21-25.

Wilkerson, F.P., Trench, R.K., 1985. Nitrate assimilation by zooxanthellae maintained in laboratory culture. Mar Chem 16, 385-393.

6 Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*

Abstract

Growth characteristics of colonies of the branching zooxanthellate octocoral *Sinularia flexibilis* with potential pharmaceutical importance were measured over a range of water velocities. The highest mean specific growth rate (μ d^{-1}) was found at a flow velocity of 11 cm s^{-1}. An optimal range of water turbulence was found at a Reynold's number of ≈10,000, with a minimum thickness of boundary layer for rapid mass transfer. There was a similar dependency on water velocity for the contents of zooxanthellae, chlorophyll *a*, and protein, indicating that photosynthesis also runs at an optimum rate at 11 cm s^{-1}, thus maximizing coral growth. Moreover, the corals showed morphological responses to the changes in water velocity: increase in the number of protruding branches (buds) in proportion to increased flow and then decrease at higher flows, as well as reduced sizes of the colonies at high velocities.

This chapter is published as: Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2008. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. *Journal of Experimental Marine Biology and Ecology 351, 106 - 113*.

Introduction

The upright branching (alcyonarian) soft coral, *Sinularia flexibilis* (Quoy & Gaimard, 1833) is highly abundant and a conspicuous species that commonly dominates inshore coral reefs of the Great Barrier Reef, thereby spanning a broad range of physical conditions. This zooxanthellate (harboring symbiotic dinoflagellates) species is one of the most prolific soft coral genera in the Indo-Pacific (110 species: Bastidas, 2001). *S. flexibilis* is known to contain biologically active diterpenes (Coll et al., 1982; Sammarco et al., 1987; Aceret et al., 1995; Duh et al., 1998; Kelman et al., 1999; Volkman, 1999; Maida et al., 2001; Bhosale et al., 2002; Kamel and Slattery, 2005) that possess a range of biological activities such as antimicrobial, anti-inflammatory, and cytotoxicity.

The flexible branching structure (Sanchez et al., 1997; Sanchez, 1999) of *Sinularia* allows it (as a shallow-water octocoral) to sustain, albeit not optimally, a range of hydrodynamic regimens and to colonize a variety of habitats. Branches are narrow to permit water to flow between them. Such forms achieve a degree of flexibility that permits them to adapt to multidirectional water movement (Riedi, 1971; Warner, 1977).

Growth rate responses to flow reflect the sum of flow effects on particle capture, nutrient uptake, photosynthesis, and respiration (Sebens et al., 2003). Water flow enhances photosynthesis by symbiotic algae and increases respiration rates of coral tissue as well as calcification (e.g. Dennison and Barnes 1988; Patterson et al., 1991), growth rate (e.g. Jokiel, 1978; Eckman and Duggins, 1993), uptake of dissolved nutrients (e.g. Thomas & Atkinson, 1997), and waste disposal from coral surfaces (Riedl, 1971). Ultimately, the rate of mass transfer and flux of nutrients to and from the coral is determined by the thickness of the boundary layer which varies with water velocity (Gardella and Edmunds, 2001). Water flow also induces expansion by corals (Sebens et al. 1997), and is one of the most important factors modifying coral morphology (e.g. Sebens, 1997; Gardella and Edmunds, 2001). Conversely, corals may modify water flow by posturing themselves. Low-flow conditions in coral reefs, like droughts in terrestrial habitats, cause nutrient deficiency and thus induce photorespiration and reduce photosynthesis (e.g. Finelli et al., 2006).

Although corals grow more rapidly when flow increases (e.g. Jokiel, 1978), the 'optimum' level of water motion has been found to differ for different species of hard corals. The 'calm water coral' *Montipora verrocosa*, the 'moderate water motion coral' *Pocillopora damicornis*, and the 'high water motion species' *P. meandrina* thrive at mean laboratory-measured water velocities of 1.7, 4.7, and 6.6 cm s^{-1}, respectively (Jokiel, 1978), or even higher velocities (10 cm s^{-1}, Sebens et al. 1998). On the coral zone of a reef flat in the Great Barrier Reef (Britta et al., 2003), average water flow rates range between 6 and 11 cm s^{-1}. Field aggregations of *S. flexibilis* occur on shallow channel sites with low wave action (De'ath and Fabricius, 1999).

Studies of water flow effects in the field might show combined effects with other inputs (e.g. light) that may result in difficulty or ambiguity in relating coral performance with water velocity. Controlled laboratory conditions may clarify these ambiguities, yielding knowledge of species-specific rearing conditions (Gateno et al., 1999). In aquaria, corals need adequate water motion for long-term maintenance (Adey, 1983). Similarly, Kaandorp (1996) indicates that the species-specific optimum water-flow rate remains unresolved, and is a subject for continuing research. Most of the studies on the effects of water velocity, however, have been done on hard corals, and relevant, extensive data on soft corals are inadequate because long-term effects in laboratory culture and such features as budding rate are frequently neglected. The characteristic morphology and flexibility of soft corals sets them apart from stony corals. Flexibility regulates the speed and pattern of the flow past the filtering structures, and thus filtering efficiency (Best, 1988; Patterson, 1991; Anthony, 1997). These characteristics are also expected to affect mass transfer and to modify such parameters as boundary layer thickness and water forces (e.g. Patterson, 1984; Harvell and Labarbera, 1985). Therefore, a better understanding of the relationship between octocoral behavior and water flow provides insights into their flow-dependent biology in captivity.

We therefore examined the long-term effects of water velocity upon growth kinetics of *S. flexibilis* and tried to establish optimal flow-dependent performance in captivity. We measured differences between the flow-exposed colonies in amounts of zooxanthellae, chlorophyll *a*, number of buds, and protein to determine their growth

variations as well as their morphological changes in relation to water velocities. Estimates of boundary layer thickness were used to identify the potential for mass transfer (flux of nutrients) as a key factor in the observed phenomena.

Materials and methods

The corals and their cultivation system

Colonies of the soft coral *Sinularia flexibilis* were obtained from Burgers' Zoo, Arnhem, The Netherlands, where they are reared in reef aquaria at 34‰±0.5 salinity and at 26°±0.2 C. The corals in this public aquarium all originate from one parent animal and are, therefore, genetically identical. The animal materials were transferred to tanks (Eco-deco systems, Dymico-Model 1000) containing 1300 L saltwater at 34‰±0.5 salinity, 25.8°±0.2 C. This cultivation system makes it possible to regenerate seawater and preserve plankton in closed systems in a completely natural way (Sipkema et al., 2005). The saltwater was made from Instant Ocean Reef Crystals (Aquarium systems, Sarrebourg, France) in deionized water. The system was equipped with two VHO Halide, 10,000 HQI lamps (Aqua Medic aqualight 400, Aquaria Veldhuis, Enschede, The Netherlands) adjusted for a 12 hours light: 12 hours dark photoperiod. Polypary pieces (polyp-bearing colonies, 5-6 cm long) were cut from the parent colonies and fixed onto PVC platelets, which attached within two weeks.

Experimental

Water flow was created by two parallel perforated PVC pipes, attached to one side of the tank wall. To provide higher flow speeds (> 15 cm s^{-1}) the tank was also supplied with an extra pump placed into a corner. This pump also created turbulent movements, with small eddies formed around the flow zones. Water velocity was measured ~5 cm above the coral head for 5 min using an Acoustic Doppler Velocity Meter (Micro ADV 16 MHZ, Sontek). The ADV current meter measures velocities with accurate mean values of water velocity, even at low-flow velocities (Garcia et al., 2005). Various water velocities were charted at several locations within the tank: 3 ± 1.6, 7 ± 3.1, 11 ± 3, 15 ± 3.8, and 19 ± 3.5 cm s^{-1}. Light intensity at the flow-determined locations was measured (approx. 200 μmol quanta m^{-2} s^{-1}) as photon flux density (PFD) using an underwater photo sensor (LI-COR, Li250 Light meter). At each experimental flow zone, five colonies were placed. After a few weeks of acclimation, the growth measurement was started. Because all the experimental samples were clones with nearly similar sizes, and all were in the same tank with similar controlled conditions except in water velocity, their response would be flow-dependent.

Turbulence

The Reynolds number for the flow around each colony was estimated. For coral colonies (Kaandorp et al., 2003) Re can be defined as: $\mathrm{Re} = uh/v$, where u is the average water velocity, h is the height of the colony, and v is the kinematic viscosity of seawater (0.685 × 10-² cm² s⁻¹). For flexible organisms, such as the branches of *S. flexibilis*, the convention is to use the thallus height (Koehl, 2001) perpendicular to flow (Hurd, 2000). For the estimations of Re numbers, our isolated branches (cuttings) were assumed to be cylinders (Koehl, 1977). As flows become turbulent at Re> 5,000 (Schlichting, 1979), our experimental microhabitats were turbulent at water velocities of 7- 19 cm s⁻¹ with a Re of approx. 10000 at the optimal velocity (11 cm s⁻¹). In addition, the thickness of the boundary layer (δ), which limits the rate of which mass transfer (Hurd, 2000), around a cylindrical object [as was done by Koehl (1981) for cylindrical setae of copepods] can be estimated by: $\delta = \frac{h}{\mathrm{Re}^{1/2}}$ where h is the height. To estimate δ, the average height of coral nubbins at each colony was measured.

Specific growth rates

Growth measurements were done at biweekly intervals for 12 weeks, whilst the PVC plates containing the coral nubbins were weighed using the underwater (buoyant weight) technique (e.g. Osinga et al., 1999). This non-destructive method gives information on the growth process of the animal (Bak, 1976). To improve weighing accuracies, each plate was first fully cleaned from fouling organisms and particles, and then weighed three times by an analytical balance PROLABO A&D HR300 with underweighing device (bearing: 310 g; accuracy: ± 0. 01 mg). Average weekly μ values were then calculated as: $\mu = (\ln W_2 - \ln W_1)/\Delta t$; where W_2 and W_1 are weight at the beginning (t) and end ($t+\Delta t$) of each time interval.

At the end of the experiment, the number of buds (not obvious in the beginning) for each group was counted to determine the final numbers of buds. To have a clear estimation of likely flow effect on budding, the initial small buds had already been removed. Wet weights of the groups were determined by weighing dry blotted samples prior to freezing. Following freeze-drying, dry weight of the samples was also determined. Measurements of wet and dry weights at the end of the experiment confirm the accuracy of the buoyant weighing. For the statistical analyses, one-way ANOVA was used.

Protein, zooxanthellae, and chlorophyll a

In order to determine the zooxanthellae density in the coral tissue, an equal amount of tissue of each group (0.3 g) was homogenized in tubes containing 3 ml filtered (0.2 μm) seawater (FSW). The homogenate (1ml) was diluted in 3 ml FSW and vortexed. The cells were

counted using a haemocytometer under a microscope. To determine algae dry weight per unit amount of total dry weight, the homogenate was filtered through 4 layers of cheese cloth, centrifuged (10 min, 1.5 *g*), and then washed with FSW. The dark brown algae pellets were lyophilized and then weighed.

For chlorophyll *a* extraction, 1 ml of the homogenate was transferred to a sterile tube containing 9 ml acetone, vortexed for 10 min, and centrifuged for 5 min at 5000 rpm. The absorbance spectrum at 400–700 nm was measured using a spectrophotometer (Spectronic 20 Genesys, USA) and the concentration calculated using the equations of Jeffrey and Humphrey (1975). Total protein of the samples was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, 1979) after the samples had been first extracted for chlorophyll. Bovine gamma globulin was used as a standard (e.g. Chalker et al., 1983). In order to quantify aqueous fractions of the zooxanthella and host (total protein) for each flow group all the data were then normalised to coral wet weight (g).

Results & Discussion

Specific growth rate

Specific growth rates (μ) of colonies of *S. flexibilis* in response to water velocities [low (3 cm s^{-1}), medium (7 & 11 cm s^{-1}) and high (15 &19 cm s^{-1})] are shown in figure 1. The observed differences in the mean μ during 12 weeks at the varying velocities were significant (ANOVA, $P< 0.05$; $R^2\approx 0.97$). The maximum mean μ was 0.019 d^{-1} at a velocity of 11 cm s^{-1}. The values of μ in this experiment are relatively high compared to field studies on hard corals (~1 × 10^{-3} to 2 ×10^{-3} d^{-1}: Vago et al., 1997).

Figure 1 shows that with increasing flow velocity, μ increases, and then decreases again at higher flow velocities probably because the polyps can not function efficiently. High velocities (15 & 19 cm s^{-1}) result in a 30% reduction of colony expanded height by retraction (e.g. Patterson, 1984). This reduces the coral's uptake area as well as exposure of the zooxanthellae to both light and nutrients, resulting in lower productivity and, therefore, reduced coral growth.

At low flows, the corals suffer from mass transfer limitations; as a result, they have difficulty in resource capture (e.g. Gardella and Edmunds, 2001) as well as waste disposal, resulting in lower specific growth rates. The observed pattern of flow effects is in agreement with the first theory of Eckman and Duggins (1993).

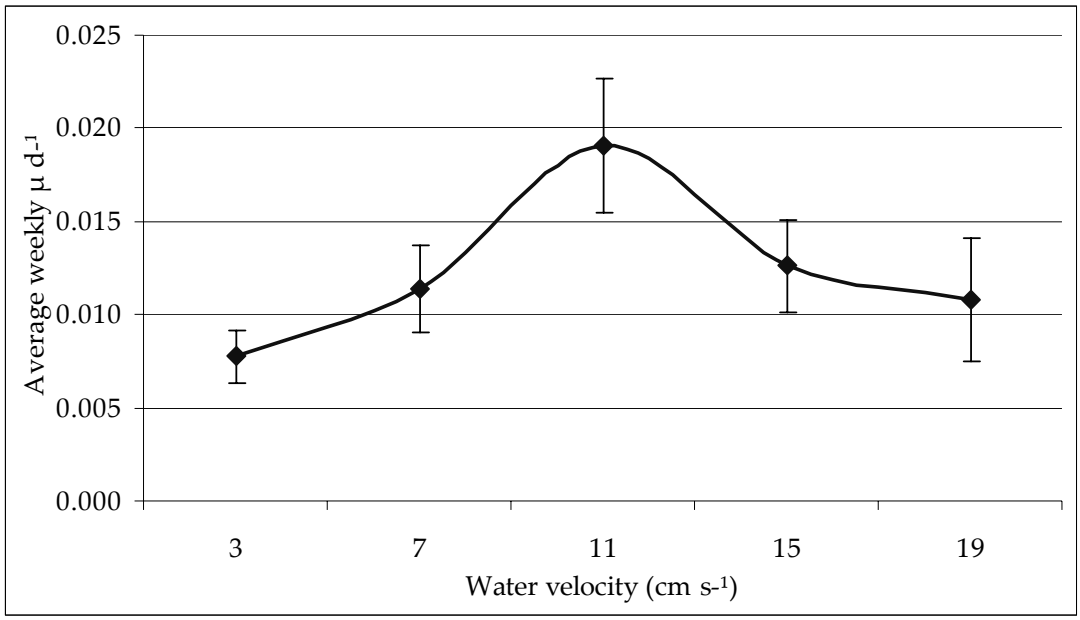


Fig. 1. *Sinularia flexibilis.* Average weekly specific growth rates (μ) as a function of water velocity. Error bars indicate standard errors.

In the field studies of Sebens et al. (1997, 1998) a distinct peak in prey capture at flows near 10 cm s^{-1} for single hard coral branches was reported. In *S. flexibilis*, the capture of light and nutrients seem to be the important factors for maximum growth, as is discussed further below.

Zooxanthellae and chlorophyll a content

Concentrations of both zooxanthellae and chlorophyll *a* within the coral tissue varied considerably with water velocity (Fig. 2). Density of zooxanthellae (and consequently chlorophyll *a*) showed a high correlation with the specific growth rate (R^2=0.99, P< 0.01). The highest amount found at 11 cm s^{-1} and the lowest were at the two extremes of the flow (3 & 19 cm s^{-1}). This may suggest maximum photosynthesis at an optimum water motion (e.g. Lesser et al., 1994). The estimated dry weight percent of zooxanthellae to the coral dry weight was >10%, which is in the range of 5-15% given by Muscatine & Porter (1977). This is higher that the ~7% observed in our previous study (Khalesi et al. unpublished) of light effects on this species, most probably because of optimal flow situation.

Similarly, protein concentration varied with water velocity and was maximal at ideal flow (11 cm s^{-1}, Fig. 2). From the correlation between N availability for zooxanthellae and protein accumulation found in the hard coral *Stylophora pistillata*

(Muscatine et al., 1989), it can be inferred that differences in density of zooxanthellae results in different protein concentrations, being highest at optimal flow.

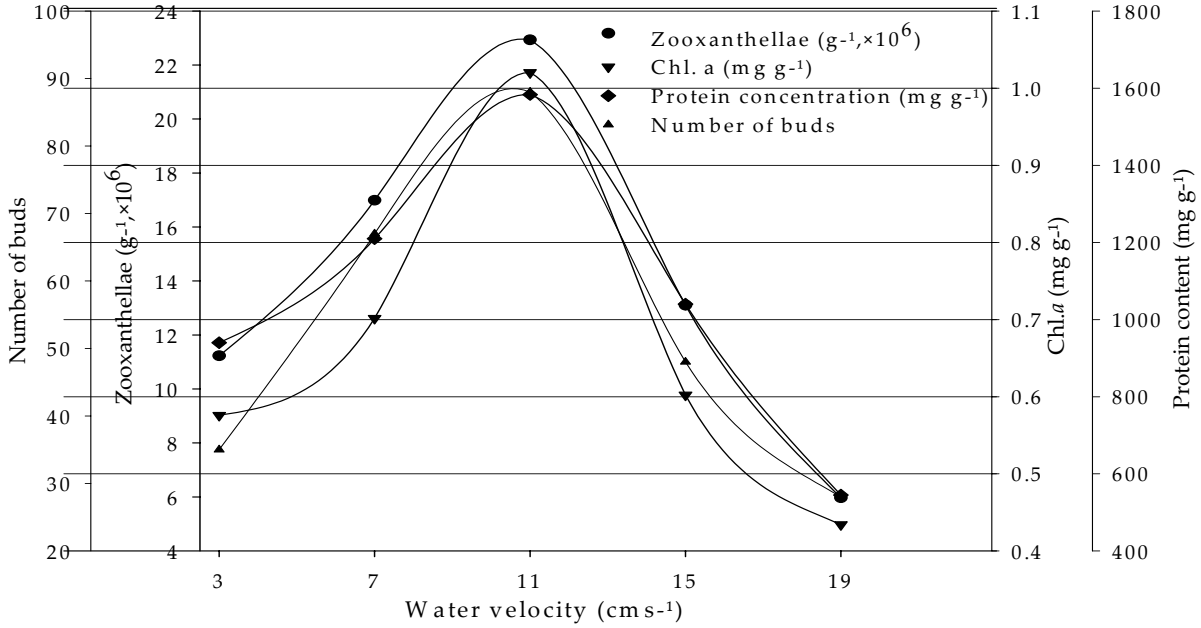


Fig. 2. *Sinularia flexibilis.* Changes in zooxanthellae, chlorophyll *a*, protein concentration, and final number of buds as a function of water velocity.

These findings may be an indicative of flow-dependent photosynthesis in *S. flexibilis*, which maximizes overall growth at most efficient flow (e.g. Helmuth et al., 1997b). This also agrees with observations on hydrodynamically modulated photosynthesis in symbiotic corals (e.g. Finelli et al., 2005; Patterson, 1985).

Differences in number of buds

The final number of small protrusions (buds) that emerged on the coral fragments (with nearly similar initial sizes) was maximal at a velocity of 11 cm s^{-1} (Fig. 2). Also, the budding rate was found to be proportional to the specific growth rate ($R^2 \approx 0.97$, $P > 0.01$). *S. flexibilis*, therefore, grows exponentially by asexual budding under optimal flow conditions to presumably capture more available resources (light, nutrients, food particles, etc: Fabricius et al., 1995). The polypary buds, eventually, result in a branching growth form, which is species specific (Merks et al., 2004).

The number of buds in the groups of *S. flexibilis* of 5 fragments after 3 months is high compared to Kramarsky-Winter and Loya (1996, 1998) in Fungiid hard corals in laboratory. They had a maximum of 15 buds out of 10 fragments after 2 months, with

no correlation between number of buds and size (growth). In *S. flexibilis,* correlation between number of buds and growth shows the accretive exponential development through clonal propagation (budding).

Reynolds number and boundary layer thickness

To characterize turbulence around the colonies, Reynolds numbers (Re) were estimated. An optimal turbulence with Re= 9600 was found at a water velocity of 11 cm s^{-1}, whereas lower values could not supply resources to the coral polyps, and higher values deformed the polyps. For our corals, this would appear to be the principal reason for the maximum μ at a water velocity of 11 cm s^{-1}. Up to this velocity, the increased turbulence thins the diffusive boundary layer (Fig. 3; e.g. Grant and Madsen, 1979). It also facilitates the efficient mass transfer of nutrients (Hurd, 2000).

The estimated thickness of the boundary layer (δ, Fig. 3) in relation to the calculated Re number was approx. 0.005 cm (50 μm) at the optimal velocity (11 cm s^{-1}) around the nubbins. Knowing the thickness of boundary layer makes it possible to relate the flow-influenced growth to this parameter (Fig. 3), which modulates delivery of dissolved substrates and products as a function of water flow. Nutrient uptake has been shown to be mass transfer limited (e.g. Thomas and Atkinson, 1997), and the boundary layer thickness and the mass flux are modified by water velocity.

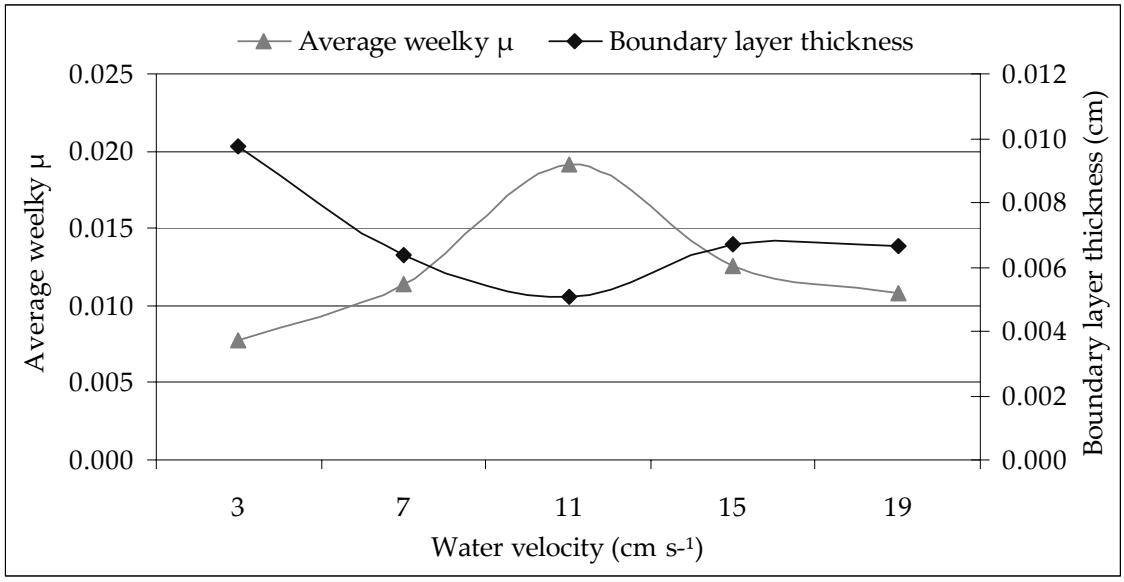


Fig. 3. *Sinularia flexibilis*. Relationship between water velocity, average weekly μ, and thickness of boundary layer (δ) as a function of water velocity.

It is, therefore, possible to explain the flow-dependent growth of *S. flexibilis* through optimized flux of nutrients because of reduction of boundary layer thickness at the optimal velocity (11 cm s^{-1}). This results in a rapid oxygen and nutrient diffusion (Sebens, 1987) leading to increased productivity of the algae from 3 to 11 cm s^{-1}.

As seen in figure 3, δ is minimal at 11 cm s^{-1} but thickens at higher Re numbers. This is because at high velocities, features such as coral shape, flexibility and swaying, which can modify the δ (e.g. Denny, 1988) decreases, resulting in bending. As a result, the characteristic size is reduced at high velocities (5 and 4 cm at 15 and 19 cm s^{-1} with mostly contracted nubbins) compared to larger sizes (6 cm at 7 & 11 cm s^{-1} with mostly open nubbins). Similarly, the thicker δ at 3 than at 15 and 19 cm s^{-1} caused a relatively lower mean μ (Fig. 3). Hence, the structural features (e.g. polyps, number of buds, overall shape, etc.: Patterson, 1992; Lesser et al., 1994; Helmuth et al., 1997a, b) as well as the extremely flexible and swaying skeleton of *S. flexibilis* can effectively optimize flows around the polyps, reducing deformation of polyps and giving them more time to function properly. At low flow (3 cm s^{-1}) no retraction was noticed in our samples. Retraction occurs naturally under conditions of low or high flow, which in the soft coral *Dendronephthya hemprichi,* for example, happened predominantly at flows of < 3 cm s^{-1} and > 25 cm s^{-1} (Fabricius et al., 1995).

Our results were obtained under controlled laboratory conditions, in large chambers providing much more realistic flows (e.g. Atkinson and Bilger, 1992), which would be more accurate than the response of a species in the field that might show combined effects of water flow and other inputs (light, etc.). This makes correlations of growth with water velocity difficult or unclear. Nevertheless, in field studies the same effects of low and high flow speeds were found in an azooxanthellate soft coral (Fabricius et al., 1995) and hard corals (Sebens et al., 2003; Jokiel, 1978).

Conclusion

Our work, overall, shows that water velocity has important consequences for *S. flexibilis.* A high flow-induced specific growth rate (μ) shows that this species is highly dependent on optimal flow to perform effectively. The results show that the response of this soft coral to a range of ambient water velocities is an outcome of its

physiological flexibility and morphological plasticity as well as physical phenomena (e.g. boundary layer thickness). These strategies for optimizing flow and resource acquisition in spatially varying flow regimes enabled the specimens of the coral to maximize their potential growth rate at each flow microhabitat. In summary, optimum turbulent flow as well as structural features of *S. flexibilis* facilitated thinning the boundary layer for efficient mass transfer of nutrients demanding for zooxanthellae photosynthesis to maximize coral growth. Therefore, this species can utilize energy efficiently by modifying the number of zooxanthellae as well as budding structures over a wide range of enclosed flow conditions.

References

Aceret, T.L., Sammarco, P.W., Coll, J.C., 1995. Effects of diterpenes derived from the soft coral *Sinularia flexibilis* on the eggs, sperm and embryos of the scleractinian corals *Montipora digitata* and *Acropora tenuis*. Mar Biol 122, 317–23.

Adey, W.H., 1983. The microcosm: a new tool for reef research. Coral Reefs 1, 193-201.

Anthony, K.R.N., 1997. Prey Capture by the Sea Anemone *Metridium senile* (L.): effects of body size, flow regime, and upstream neighbors. Biol. Bull. 192: 73-86.

Atkinson, M.J., Bilger, M.W., 1992. Effects of water velocity on phosphate uptake in coral reef-flat communities. Limnol. Oceanogr. 37, 426-435.

Bastidas, C., Benzie, A.H.J, Uthicke, S., Fabricius, K.E., 2001. Genetic differentiation among populations of a broadcast spawning soft coral *Sinularia flexibilis* on the Great Barrier Reef. Mar. Biol. 138, 517-525

Best, B., 1988. Passive suspension feeding in a sea pen: effects of ambient flow on volume flow rate and Filtering Efficiency. Biol. Bull. 175, 332-342.

Bhosale, S.H., Nagle, V.L., Jagtap, T.G., 2002. Antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomonas*. Mar. Biotechnol. 4, 111–118.

Bio-Rad Laboratories, 1979. Bio-Rad protein assay instruction manual. Bio-Rad Laboratories, Richmond, California, 17.

Britta, S., Uthicke, S., Klumpp, D., 2003. Water quality, sediment and biological parameters at four nearshore reef flats in the Herbert River Region, Gentral GBR. Great Barrier Reef Marine Park Authority Research Publication 82.

Dai, C.F., Lin., M.C., 1993. The effects of flow on feeding of three Gorgonians from southern Taiwan. J. Exp. Mar. Biol. Ecol. 173, 57-69.

De'ath, G., Fabricius, K.E., 1999. Classification and regression trees: A powerful yet simple technique for ecological data analysis. Ecology 81, 3178–3192.

Dennison, W.C., Barnes, D.J., 1988. Effect of water motion on coral photosynthesis and calcification. J. Exp. Mar. Biol. Ecol. 115, 67-77.

Denny, M.W., 1988. Biology and the Mechanics of the Wave-Swept Environment. Princeton University Press, Princeton, NJ.

Duh, C.Y., Wang, S.K., Tseng, H.K., Sheu, J.H., Chiang, M.Y., 1998. J. Nat. Prod., 61, 844.

Eckman, J.E., Duggins, D.O., 1993. Effects of flow speed on growth of benthic suspension feeders. Biol. Bull. 185, 28-41.

Fabricius, K.E., Genin, A., Benayahu, Y., 1995. Flow-dependent herbivory and growth in zooxanthellae-free soft corals. Limnol. Oceanogr. 40, 1290-1301.

Finelli, C.M., Helmuth, B.S.T., Pentcheff, N.D., Wethey, D.S., 2006. Water flow influences oxygen transport and photosynthetic efficiency in corals. Coral Reefs, 25, 47-57.

Gardella, D.J., Edmunds, P.J., 2001. The effect of flow and morphology on boundary layers in the Scleractinians *Dichocoenia stokesii* (Milne-Edwards and Haime) and *Stephanocoenia michilini* (Milne-Edwards and Haime). J. Exp. Mar. Biol. Ecol. 256, 279–289.

Gate˜no, D., Barki, Y., Rinkevich, B., 1999. Aquarium maintenance of reef octocorals raised from field collected larvae. Aqu. Sci. Cons. 2, 227–236.

García, C.M., Cantero, M.I., Niño, Y., García, M.H., 2005. Turbulence measurements with acoustic doppler velocimeters. J. Hydr. Engrg. 131, 1062-1073.

Grant, W.D., Madsen, O.S., 1979. Combined wave and current interaction with a rough bottom. J. Geophys. Res. 84, 1797-1808

Harvell, C.D., Labarbera, M., 1985. Flexibility: a mechanism for control of local velocities in hydroid colonies. Biol. Bull. 168, 312-320.

Helmuth, B.S.T., Stockwell, E.F., Brumbaugh, D.R., 1997a. Morphological and environmental determinants of mass flux to corals. Proc. 8th Int. Coral Reef Symp. 2, 1103–1108.

Helmuth, B.S.T., Sebens, K.P., Daniel, T.L., 1997b. Morphological variation in coral aggregations: branch spacing and mass flux to coral tissues. J. Exp. Mar. Biol. Ecol. 209, 233–259.

Hurd, C.L., 2000. Water motion, marine macroalgal physiology, and production. J. Phycol. 36, 453 – 472.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equation for determining chlorophyll a, b, c_1 and c_2, Biochem. Physiol. Pflanz., 167, 194-204.

Jokiel, P.L., 1978. Effects of water motion on coral reefs. J. Exp. Mar. Biol. Ecol. 35, 87–98.

Kaandorp, J. A., 1996. Modeling growth forms of the sponge *Haliclona oculata* (Porifera, Demospongiae) using fractal techniques. Marine Biology 110, 203-215.

Kaandorp, J. A., 1999. Morphological analysis of growth forms of branching marine sessile organisms along environmental gradients. Marine Biology 134, 295-306.

Kaandorp, J.A., Koopman, E.A., Sloot, P.M.A., Bak, R.P.M., Vermeij, M.J.A., Lampmann, L.E.H., 2003. Simulation and analysis of flow patterns around the Scleractinian coral *Madracis mirabilis* (Duchassaing and Michelotti). Phil. Trans. R. Soc. Lond. B. 358, 1551–1557.

Kamel, H.N., Slattery, M., 2005. Terpenoids of *Sinularia*: Chemistry and Biomedical Applications. Pharmaceutical Biology, 43, 253–269

Kelman, D., Benayahu, Y., Kashman, Y., 1999. Chemical defence of the soft coral *Parerythropodium fulvum fulvum* (Forskal) in the Red Sea against generalist reef fish. J. Exp. Mar. Biol. Ecol. 238, 127–137.

Koehl, M.A.R., 1977. Mechanical organization of cantileverlike sessile organisms: sea anemones. J. Exp. Biol. 69,127-142

Koehl, M.A.R., 1981. Feeding at low Reynolds number by Copepods. Lectures on Mathematics in Life sciences, 14.

Koehl, M.A.R., Helmuth, B., Carpenter, R., 2001. Growing and Flowing. In: The Algorithmic Beauty of Seaweeds, Sponges and Corals. J.A. Kaandorp and J.E. Kubler. Springer-Verlag, Heidelberg, pp. 17-29.

Kramarsky-Winter, E., Loya, Y., 1996. Regeneration versus budding in Fungiid corals: a trade-off. Mar. Ecol. Prog. Ser. 134, 179-185.

Kramarsky-Winter, E., Loya, Y., 1998. Reproductive strategies in two Fungiid corals from the northern Red Sea: Environmental constraints? Mar. Ecol. Prog. Ser. 174, 175–182.

Lesser, M.P., Weis, V.M., Patterson, M.P., Jokiel, P.L., 1994. Effects of morphology and water motion on carbon delivery and productivity in the reef coral *Pocillopora damicornis* (Linnaeus): diffusion barriers, inorganic carbon limitation, and biochemical plasticity. J. Exp. Mar. Biol. Ecol. 178, 153–179.

Maida, M., Sammarco, P.W., Coll, J.C., 2001. Effects of soft corals on Scleractinian coral recruitment. II: allelopathy, spat survivorship and reef community structure. Marine Ecology 22, 4, 397- 414.

Merks, R.M.H., Hoekstra, A.G., Kaandorp, J.A., Sloot, P.M.A., 2004. Polyp oriented modeling of coral growth. Journal of Theoretical Biology 228, 559–576.

Muscatine, L., Porter, J.W., 1977. Reef corals: mutualistic symbiosis adapted to nutrient-poor environments. Bioscience 27, 454-460.

Muscatine, B.L., Falkowski, P.O., Dubinsky, Z., CooK, P.A., Mocloskey, L.R., 1989. The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc. R. Soc. Lond. B. 236, 311—324.

Osinga, R., Tramper, J., Wijffels, R.H., 1999. Cultivation of marine sponges. Mar. Biotechnol. 1, 509–532.

Patterson, M.R., 1984. Patterns of whole colony prey capture in the octocoral *Alcyonium siderium*. Biol. Bull. 167, 613-629.

Patterson, M.R., 1985. The effects of flow on the biology of passive suspension feeders: prey capture, feeding rate, and gas exchange in selected Cnidarians. Ph.D. thesis, Harvard University, Cambridge, Massachusetts, 342.

Patterson, M.R., Sebens, K.P., Olson, R.R., 1991. In situ measurements of flow effects on primary production and dark respiration in reef corals. Limnol. Oceanogr. 36, 5, 936-948.

Patterson, M.R., 1992. A chemical engineering view of cnidarian symbiosis. Am. Zool. 32, 566–582.

Porter, J.W., 1976. Autotrophy, heterotrophy, and resource partitioning in Carribean reef-building corals. Amer Nat 110, 731–742

Riedi, R., 1971. Water movement-animals. Marine ecology, I, Part 2, edited by O. Kinne, John Wiley & Sons, New York, New York 1123-1156.

Sammarco, P.W., La Barre, S., Coll, J.C., 1987. Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef, 3: the relationship between ichthyotoxicity. Oecologia 74, 93–101

Sanchez, J.A., Diaz, J. M., Zea, S., 1997. Gorgonian communities of two contrasting environments from oceanic Caribbean atolls. Bull. Mar. Sci. 61, 61-72.

Sanchez, J.A., 1999. Black coral-octocoral distribution patterns on a deep-water reef, Imelda bank, Caribbean Sea, Colombia. Bull. Mar. Sci. 65, 1, 2 15-225.

Schlichting H., 1979. Boundary-layer theory. 7th ed. McGraw-Hill, New York.

Sebens, K.P., 1987. Coelenterate energetics. In Animal Energetics. F. J. Vernberg and T. J. Pandian, eds. Academic Press, New York. 4, 55-120.

Sebens, K.P., 1997. Adaptive responses to water flow: Morphology, energetics, and distribution of reef corals. Proc. 8th Int. Coral Reef Symp. 2, 1053 - 1058.

Sebens, K.P., Witting, J., Helmuth, B., 1997. Effects of water flow and branch spacing on particle capture by the reef coral *Madracis mirabilis*. J. Exp. Mar. Biol. Ecol. 211, 1–28.

Sebens, K.P., Grace, S.P., Helmuth, B., Maney, E.J., Miles, J.S., 1998. Water flow and prey capture by three Scleractinian corals: *Madracis mirabilis, Montastrea cavernosa* and *Porites porites* in a field enclosure. Mar. Biol. 131, 347-360.

Sebens, K.P., Helmuth, B., Carrington, E., Agius, B., 2003. Effects of water flow on growth and energetics of the Scleractinian coral *Agaricia tenuifolia* in Belize. Coral Reefs 22, 35–47.

Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., Wijffels, R.H., 2005. Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? Biotechnology and Bioengineering. 90, 2, 201- 222.

Sontek, www.sontek.com, accessed 2006.

Thomas, F., Atkinson, M., 1997. Ammonium uptake by coral reefs: effects of water velocity and surface roughness on mass transfer. Limnol. Oceanogr. 42, 81–8.

Vago, R., Dubinsky, Z., Genin, A., Ben-Zion, M., Kizner, Z., 1997. Growth rates of three symbiotic corals in the Red Sea. Limnol. Oceanogr. 42, 8, 1814 -1819.

Volkman, J.K., 1999. Australasian research on marine natural products. Mar. Freshwater Res. 50, 76-79.

7 Cell cultures from the symbiotic soft coral *Sinularia flexibilis*

Abstract

The symbiotic octocoral *Sinularia flexibilis* is a producer of potential pharmaceuticals. Sustainable mass production of these corals as a source of such compounds demands innovative approaches, including coral cell culture. We studied various cell dissociation methodologies and the feasibility of cultivation of *S. flexibilis* cells on different media and cell dissociation methodologies. Mechanical dissociation of coral tissue always yielded the highest number of cells and allowed subsequent cellular growth in all treatments. The best result from chemical dissociation reagents was found with trypsin-EDTA. Coral cells obtained from spontaneous dissociation did not grow. Light intensity was found to be important for coral cell culture showing an enduring symbiosis between the cultured cells and their intra-cellular algae. The GIM and GMIM media were found to be superior substrates. To confirm the similarity of the cultured cells and those in the coral tissue, a molecular test with Internal Transcribed Spacer (ITS) primers was performed. Thereby, the presence of similar cells of both the coral cells and zooxanthella in different culture media was confirmed.

This chapter is submitted as: Khalesi, M.K., Vera-Jimenez N.I., Aanen D.K., Beeftink, H.H., Wijffels, R.H., 2008. Cell cultures from the symbiotic soft coral *Sinularia flexibilis*.

Introduction

Soft corals are a promising source of cytotoxic agents as anticancer, anti-inflammatory, and analgesic compounds (Faith et al., 1997; Ata et al., 2003; Duque, 2004). The soft coral *Sinularia flexibilis* (family Alcyoniidae) harbours symbiotic dinoflagellate algae (zooxanthellae) and produces bioactive compounds with cytotoxic, antimicrobial, and anti-inflammatory activities (Dhu et al., 1998; Kamel and Slattery, 2005; Radikha et al., 2005; Yu et al., 2006; Chao et al., 2006). The increasing interest in these resources for drug development raises a conservation concern (Hunt and Vincent, 2006). To avoid overexploitation of coral ecosystems, the production of the source animal or its cells is important.

Amongst the approaches for sustainable exploitation is coral cell culture with applications in fields such as ecotoxicology and biotechnology (Rodgers, 2002). Techniques for cnidarian cell culture (including corals) have not been developed yet. Problems were encountered with cell dissociation, culture contamination, and medium (Odintsova et al., 1994; Frank et al., 1994; Rinkevich, 1999). Due to the lack of validated aquatic invertebrate cell lines, primary cell/tissue cultures for toxicology have been used (Reviewed by Villena, 2003).

Cell cultures from a gorgonian octocoral scleroblasts were maintained for more than 4 month (Kingsley et al., 1987). A review by Rinkevich (1999) indicates that between 1988 and 1998, only three studies on coral cell culture were published (Frank et al., 1994, Gates and Muscatine, 1992; Gates et al., 1992). In most cases the cells did not survive more than one month. Frank et al. (1994) observed that cryopreserved cell cultures after approximately one year were excluded by eukaryotic unicellular organisms. Again, between 1998-2004 there were no successful studies published on long term cnidarian cell cultures (Frank and Rinkevich, 1999, Kopecky and Ostrander, 1999, Schmid et al., 1999, Domart-Coulon et al., 2001, 2004,); even though, some encouraging reports were presented (reviewed by Rinkevich, 2005).

The failure to establish long-lasting and proliferating cell cultures has been attributed to the lack of vital information regarding cell requirements and their physiology and biochemical patterns *in vitro*, improper comparisons between

vertebrate-invertebrate cell requirements (Rinkevich, 1999) as well as insufficient information about the classification and identification of cell types (Puverel et al., 2005). To extend the functional survival of coral cells, cell culture protocols have to be improved (Domart-Coulon et al., 2004).

The principal aim of this study, therefore, was to investigate the establishment of long-lasting cell culture from the symbiotic soft coral *Sinularia flexibilis*. Because both substrate composition and cell dissociation methods are critical in the survival and prolongation of cells (Odintsova et al., 1994; Frank et al., 1994; Rinkevich, 1999), we examined various media and cell-dissociation methodologies. Eukaryotic endosymbionts might develop in invertebrate cell cultures instead of the target coral cells. Hence, specific tests such as genetic probes should be used to verify the nature of the cultured cells (Villena, 2003). Therefore, we have also used a genetic approach to identify coral cells in culture.

Materials and Methods

Coral cell culture

All cultivation procedures (in four stages) were executed with sterile materials; protocols were based on Frank et al. (1994), Kopecky and Ostrander (1999), Gates and Muscatine (1992), and Hurton et al. (2005).

Organism

Colonies of *Sinularia flexibilis* were obtained from Burgers' Zoo, Arnhem, The Netherlands, and were maintained in laboratory tanks (25 ± 0.5 °C, 12 hours light : 12 hours dark period, 34 ‰ salinity and a pH of 8 ± 0.5). Prior to dissociation, corals were rinsed at least 10 times with sterile sea water SSW (Instant Ocean Reef Crystals, Aquarium Systems, France) and sliced into 1 mm^3 pieces with a scalpel.

Tissue Dissociation

Spontaneous Dissociation. Coral samples (1-mm^2) were incubated in a 1% gentamycin-streptomycin (1:1) solution in SSW at room temperature during four hours, and transferred subsequently to the respective culture medium (see 'Culture media and conditions').

Mechanical Dissociation. Coral samples (1-mm^2) were rinsed with SSW and cut into small pieces using scalpel. Then, the samples were rinsed again and placed in a 100 μm nylon cell strainer (BD Falcon) and sieved by pressing them with a loose-fit plastic piston. Sieved cells

were resuspended in 1.5 ml of SSW in 2 ml sterile eppendorf tubes. Cell suspensions were centrifuged (300 *g*, 10 min); the supernatant was discarded and the pellet re-suspended in 1.5 ml 1% gentamycin-streptomycin solution (1:1) in SSW, this procedure was performed four times.

Chemical dissociation. Following rinsing, coral pieces (1-mm^3) were placed into eppendorf tubes (2ml) containing one of the following dissociation reagents: trypsin-EDTA 0.05% in SSW, pronase 0.1% in SSW or collagenase 0.05% in SSW and then shaken at 200 rpm for 40 minutes, 60 minutes and 2 hours respectively. Cell suspensions were centrifuged, re-suspended as explained for mechanical dissociation.

Culture media and conditions

Media. Four enriched media were used for cultivation: Dulbecco's modified Eagle medium (DMEM), medium 199 (M-199) (Frank et al., 1994), Grace's insect medium (GIM) and Grace's modified insect medium (GMIM) (Hurton et al, 2005). To avoid crystallization, supplements were not added (Frank et al., 1994). All media contained a 1% antibiotic cocktail (streptomycin/gentamycin 1:1). Cells in sterile sea water were used as a control.

Culture containers. After initial attempts with 25 cm^3 tissue culture flasks (T-flasks), cells were cultivated in 24 well plates with 1.5 ml of medium and 1% antibiotic cocktail (streptomycin/gentamycin 1:1).

Incubation conditions and culture maintenance

Cultures were preserved in a climate cabinet (Snijders Scientific) with a constant temperature of 24° C and 5% (v/v) of CO_2 in air. Fluorescent light (80 – 100 $\mu E\ m^{-2}\ s^{-1}$) was applied in a 12 h light:12 h dark photoperiod. Cultures were checked daily and culture media were refilled almost once per week or when the media were below 0.7 ml.

Culture analysis

Growth. Cultures were counted two times per week with an inverted microscope (Olympus CK40); pictures of all the treatments were taken using a digital camera (Olympus C-3030) with a lens (Olympus U-PMTVC) and the software AnaliSYS for photo extracting and scaling (Soft Imaging System GmbH). Three different counting methods were used: after the dissociation process and in the first stages of the experiment, counts were made by placing a graph paper transparency and counting the cells within a 1 mm^2. After appearance of cellular aggregates, the total area occupied by the cells was calculated with AnaliSYS software (Table 3). However, due to the low accuracy of this method in the end, counts were made based on image analysis using the software Image-Proplus for windows (version 4.5.0.29, Media cybernetics Inc.) with its manual tag tool.

Cell identification. To identify the cells present in the cultures, two approaches were used: histology slides and genetic test.

*Histology slides***.** A colony fragment of 3 mm² was rinsed in SSW at least five times and placed in a container with 1% agarose and 1% sugar in SSW. Subsequently, the sample was placed in a metal container and frozen with liquid nitrogen. Sections of 7 μm were made using a cryostat (type 2800 frigocut Reichert Jung), spread on slides, and fixated with 5 ml formaldehyde 40% neutral, 15 ml saturated picric acid and 1 ml acetic acid 100% during 10 min. Slides were then stained with haematoxylin and eosin (3 min each).

Genetic test using Internal Transcribed Spacer (ITS) primers

-DNA isolation. Coral DNA was extracted from both coral tissue (animal) and from coral cell cultures on GMIM, GIM and DMEM media. Either a piece of tissue or some cultured cells were placed in Eppendorf tubes together with 5 small glass beads. The tubes were frozen using liquid nitrogen and shaken in a bead-beater for 10 s. Subsequently, five extra small glass beads were added and the samples were frozen and shaken again for 10 s. Samples were then vortexed with 1 ml of LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris-HCl (pH 7.4), 0.2% SDS) and centrifuged for 5 min at 13.000 rpm. Of the supernatant, 700 μl was transferred into a fresh 2 ml Eppendorf tube and 5 μl of proteinase K (stock 20 μg/μl was kept at -20 ºC) were added; samples were incubated for 1 h at 37 ºC. Next, 300 μl phenol and 300 μl SEVAG were added and the samples were well vortexed and after that centrifuged for 15 min at 13.000 rpm; 600 μl of the supernatant were transferred to a clean 1.5 ml Eppendorf tube and 300 μl of SEVAG was added. Samples were mixed again and centrifuged for 15 minutes at 13000 rpm; subsequently, 500 μl of the supernatant was transferred to a clean 1.5 ml Eppendorf tube and 300 μl of isopropanol (0.6 x volume) added and samples were stored for 15 min at -20 ºC. Tubes were centrifuged once more for 15 min at 13.000 rpm and the supernatant was discarded, while the pellet was washed with 100 μl of ice-cold 70% ethanol and samples were centrifuged 1 min at 13.000 rpm. Then, the supernatant was discarded and the pellet dried under a vacuum. Finally the pellet was dissolved in 50 μl of Milli-Q.

- Polymerase chain reaction (PCR). The ITS of the coral animal was amplified using two different primer sets; the first set consisted of primers ITS 1s (5' - GGT ACC CTT TGT ACA CAC CGC CCG TCG CT - 3'), and ITS 2ss (5' - GCT TTG GGC TGC AGT CCC AAG CAA CCC GAC TC - 3') (McFadden et al., 2001); the primers belonging to the second set were ITS a (5'- GGG ATC CGT TTC CGT AGG TGA ACC TGC – 3') and ITS b (5'- GGG ATC CAT ATG CTT AAG TTC AGC GGG T – 3') (Coleman et al., 1994). The PCR protocol consisted of an initial denaturating step of 4 min at 94° C followed by 35 cycles of denaturation at 94° C (30 s), annealing at 52° C

(60 s), elongation at 72° C (90 s), and in the end a final elongation step at 72° C (10 min.) (McFadden et al., 2001). PCR reactions were performed in 25 ml volumes, using four DNA concentrations: undiluted and 10, 100, and 1000 times diluted. 5 ul of a pcr reaction was run together with an undiluted sample for each culture medium and also for the coral (animal) in a 0.6% agarose electrophoresis gel at 70 v/cm/s; the size of the fragments was established using the marker Orange Ruler 100bp DNA ladder.

Results and Discussion

Dissociation

Three different dissociation techniques to isolate free cells from coral tissue were tested (Table 1). Spontaneous dissociation yielded the lowest number of free cells. The low yield in comparison to the other techniques could be because of the absence of extensive mechanical procedures or enzymatic reactions to separate the tissue by pressure or chemicals. In addition, for spontaneous dissociation more washing steps are used; possibly some of the separated cells are lost in this process. With mechanical dissociation the highest number of free cells was obtained, which is consistent with Frank et al. (1994).

Three chemical dissociation agents were used, of which trypsin-EDTA was the most effective. Pronase was used only in two of the extractions because it caused a lot of damage to the cells and consequently low numbers of free cells were obtained. Pronase is a non-specific protease that hydrolyses proteins to individual amino acids and can lead to damage of the cellular membranes (Roche, 2006). The cell yield after treatment with trypsin-EDTA was much higher. Trypsin-EDTA cleaves the proteins into peptides, hence detaches the tissue into cell clusters, leaving more cells intact (Sigma-Aldrich, 2007).

Table 1. Cell counts (ml^{-1}) after dissociation

		Test	1 T-flasks	2 T-Flasks	24 well Plate	Agar assay
Spontaneous		8	2	4	0	2
Mechanical	without sieves	52	64	90	-	-
	with sieves	-	-	-	111	137
Chemical	trypsin-EDTA	7	45	42	52	74
	Pronase	-	36	25	-	-
	Collagenase	-	1	3	-	-

Collagenase treatment did not lead to positive results in our studies; this is in contrast with the studies of Frank et al. (1994) who found that collagenase was less harmful than trypsin-EDTA.

Growth of the coral cultured cells

After initial cultivation experiments with T-flasks, 24 well-plates were used for cultivation. In well plates we obtained higher cell densities and counting of cells was easier. An essential factor for cellular growth is that relatively high cell densities are used (Freshney, 1994; Verkerk, 2007). Results of 24 well-plate growth are displayed in figure 1.

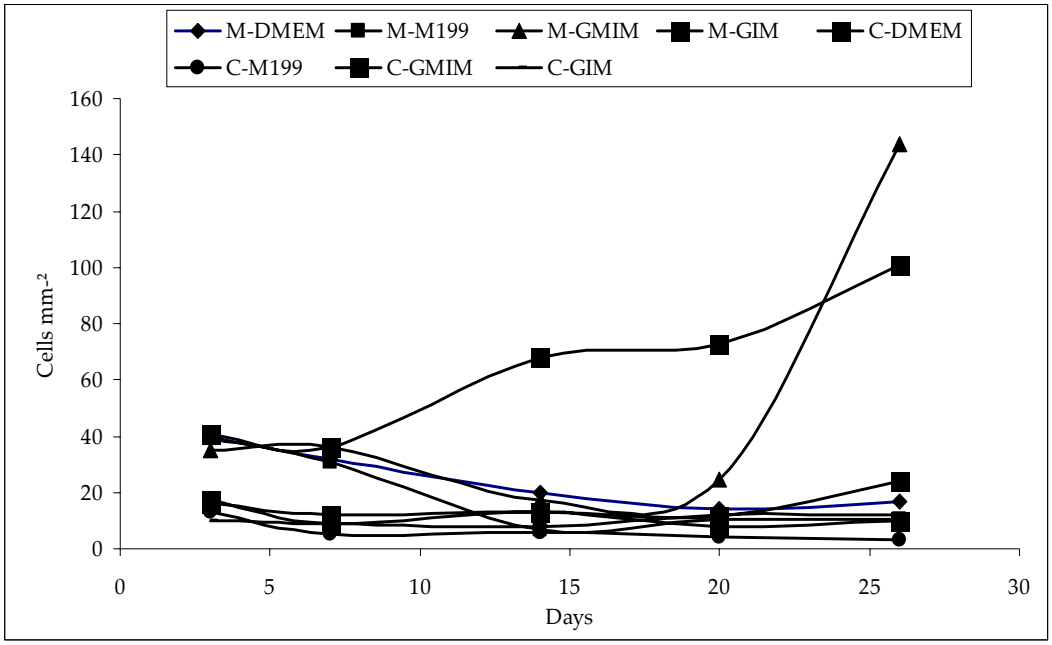


Figure 1. Growth of the cells of *Sinularia flexibilis* in 24-well-plates on different media with different dissociation methods. Units cell mm^{-2}; M-: mechanical dissociation, C-: Chemical dissociation.

During the first week of cell culture (24-well plate) viable cell numbers decreased in all media, probably due to the stress caused by the dissociation process (Freshney, 1994). On day 12 the light intensity was changed from 130 to 80 – 100 $\mu E\ m^{-2}\ s^{-1}$. After 14 days a significant increase in the number of mechanically dissociated cells in GIM medium (M-GIM) was observed (Figure 1). Cellular proliferation was also observed for mechanically dissociated cells grown in GMIM medium (M-GMIM) after 20 days of cultivation. Apparently, the coral cells and their intra-cellular zooxanthellae responded positively to decreased light intensity, indicating that they maintain a

strong symbiotic relationship even after tissue dissociation. A higher cell survival time (not growth) in light than in dark was reported previously for coral cells (Kopecky and Ostrander, 1999).

A slight growth of chemically dissociated cells in GMIM medium (C-GMIM) was observed on day 26. The rest of the wells even with a low cell density remained stable and in good conditions. DMEM and M199 were previously used in coral cell culture by Frank et al. (1994), who also tested Leibovits L-15 medium. However, the article's discussion is based on the last mentioned media and claims that coral cells could be maintained during one year using it. Data on DMEM and M199 performance were not shown, even though cellular growth was also reported. Based on the observations during that study, DMEM and M199 did not probably lead to better results than L-15 media. Kopecky and Ostrander (1999) also used DMEM, as the only culture medium; nevertheless, they just investigated the time that DMEM was able to keep the cells alive without changing the medium, establishing it up to 300 hours.

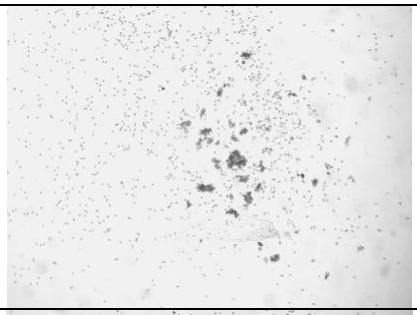
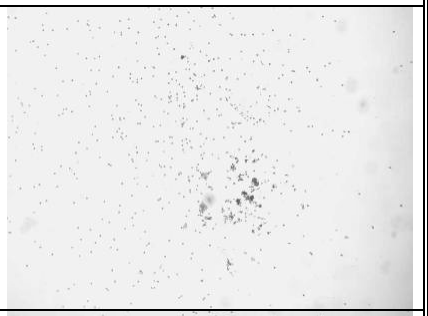
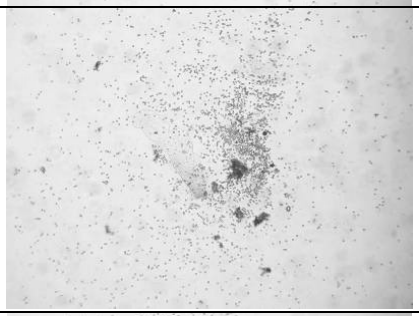
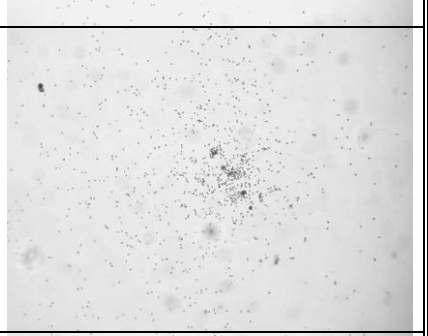
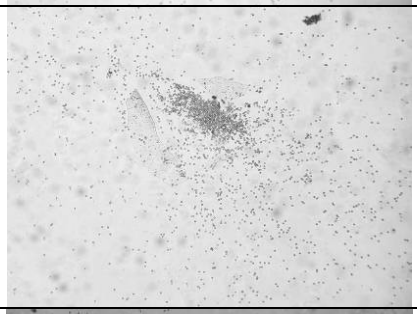
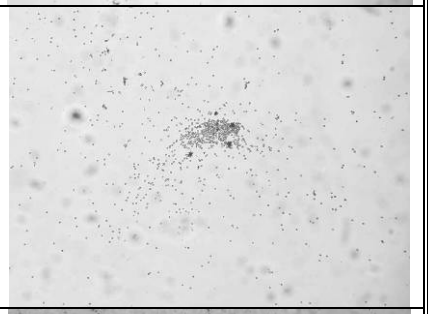
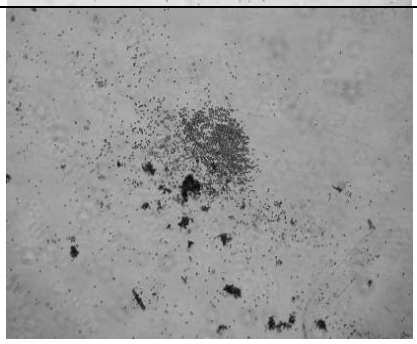
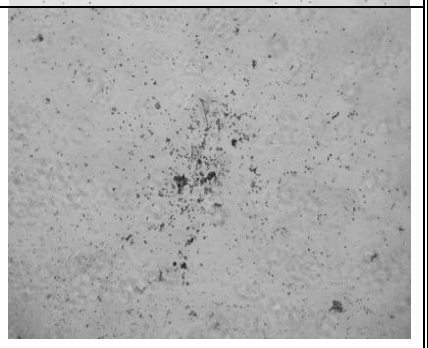
On the other hand, the GIM and GMIM media that performed best in the present study, have not been tested in coral cell culture before. They were used by Hurton et al. (2005) for primary cultures of amebocytes of the horseshoe crab *Limulus polyphemus*. In comparison, they had better results with GIM and GMIM than with L-15 media.

After the rather promising cell growth on GIM and GMIM media, a less time-consuming counting technique was used. It was not possible to use a counting chamber because the cells attached to the bottom of the wells. We determined the area occupied by the cells or cell aggregates instead (Table 2). With this method, growth could clearly be determined although the accuracy needs further improvement. In addition, the cells were partially spread through the well after the medium was changed, making the area monitoring impossible.

In order to find out if the lack of cell growth in the wells containing DMEM and M199 was due to the media, new wells were inoculated from the proliferating cultures

Table 2. Total cell aggregation area measurements (cells mm^{-2}) from GIM and GMIM wells.

Date / Treatment	C-GMIM 2	C-GMIM 3	C-GIM 1	C-GIM 2	C-GIM 3	M-GMIM 1	M-GMIM 2	M-GMIM 3
1-Jun	0	0.05	0	0	0	1.16	1.92	1.51
6-Jun	0	0.05	0	0	0	2.25	4.35	1.81

Days/Treatment	Mechanical GIM to GMIM	Mechanical GIM to GIM
2		
7		
8		
10		
30		

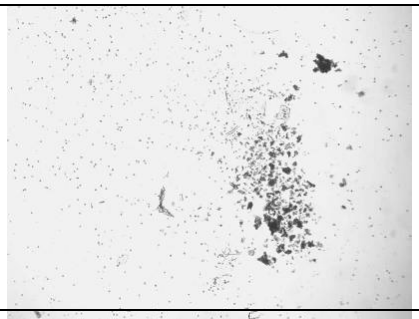
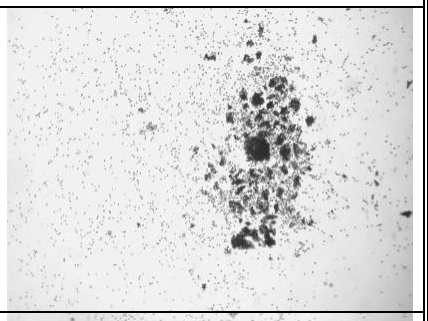
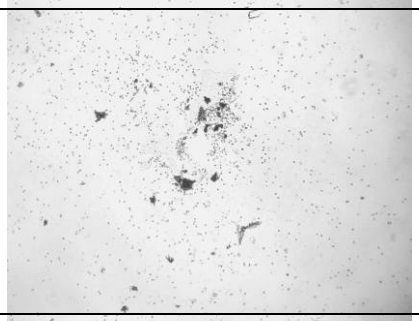
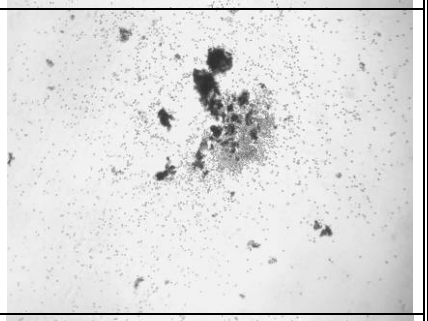
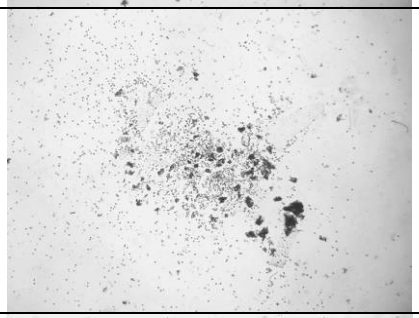
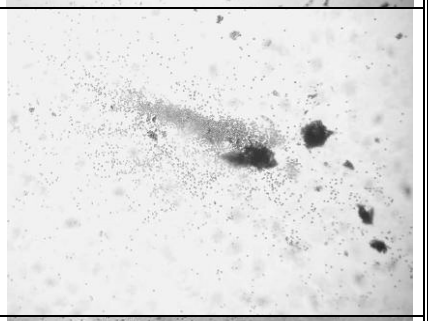
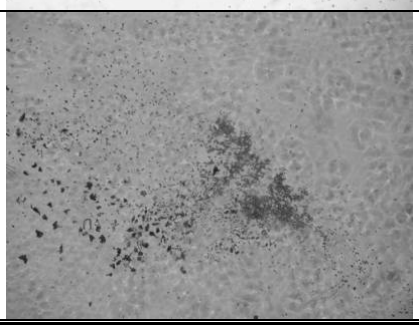
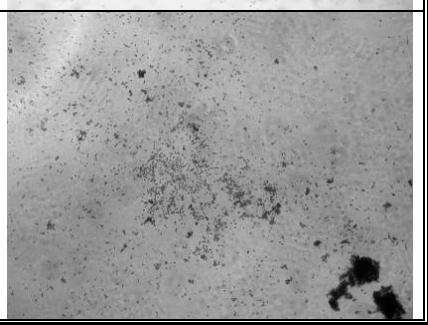
Days/ Treatment	Mechanical GMIM to GMIM	Mechanical GMIM to GIM
2		
7		
8		
10		
30		

Figure 2. Picture monitoring (4X magnification) on the cells of *Sinularia flexibilis* from 24-well inoculation plate using Image-proplus software.

(GIM and GMIM) and grown on the different media. Growth of these new cultures was evident from a series of general pictures at low magnification (4x, Figure 2). Again, cells did not grow on media M199 and DMEM and grew on GIM and GMIM. The number of cells in these general pictures was counted using the software Image-Proplus with its manual tag tool (Figure 2) as a reliable counting methodology, based on high-quality pictures and specific well-plates for microscopy. Because of the presence of other particles in the coral cell cultures, the manual tag tool was employed. An increase in the number of cells can be seen from day 2 till day 8. During the next two days, a decline of the number of cells is noticed in both GM-GIM and GM-GMIM wells, probably due to an overestimation in the preceding counts. Because the quality of the pictures employed during this stage was suboptimal, these low-quality pictures may be used as a visual estimation. This inoculation experiment showed the efficient performance of media GIM and GMIM. Therefore, other growth inhibitors in the wells or in the media of the remaining treatments were rejected.

From the first day of inoculation, a depletion in the number of viable cells in GIM wells for both the GM-GIM and TM-GIM was observed (Figure 2) that was caused by a microbial contamination. This is in agreement with Frank et al. (1994), who stated that microbial contamination may result in a rapid loss of over 80% of the coral cells. After day 16, a growth interruption and also a decrease in the number of viable cells on GIM media was observed due to a bacterial contamination and also a failure in the light manager device (the last picture on day 30, Figure 2); the cultures were unintentionally exposed to permanent light. Once more, due to the photosynthesizing symbiont (zooxanthellae), the importance of lighting period was observed through a decrease in the growth rates afterwards.

Cell identification

Because eukaryotic endosymbionts might develop in cell cultures (Pomponi et al., 1997) it is necessary to identify the target coral cells. Identification of cnidarian cell types based on morphology alone has proven to be very difficult (Puverel et al., 2005), mainly because cells adapt their shape to their endosymbionts. Thus, if a coral cell possesses single zooxanthellae, it will be round and approximately 10 μm in diameter,

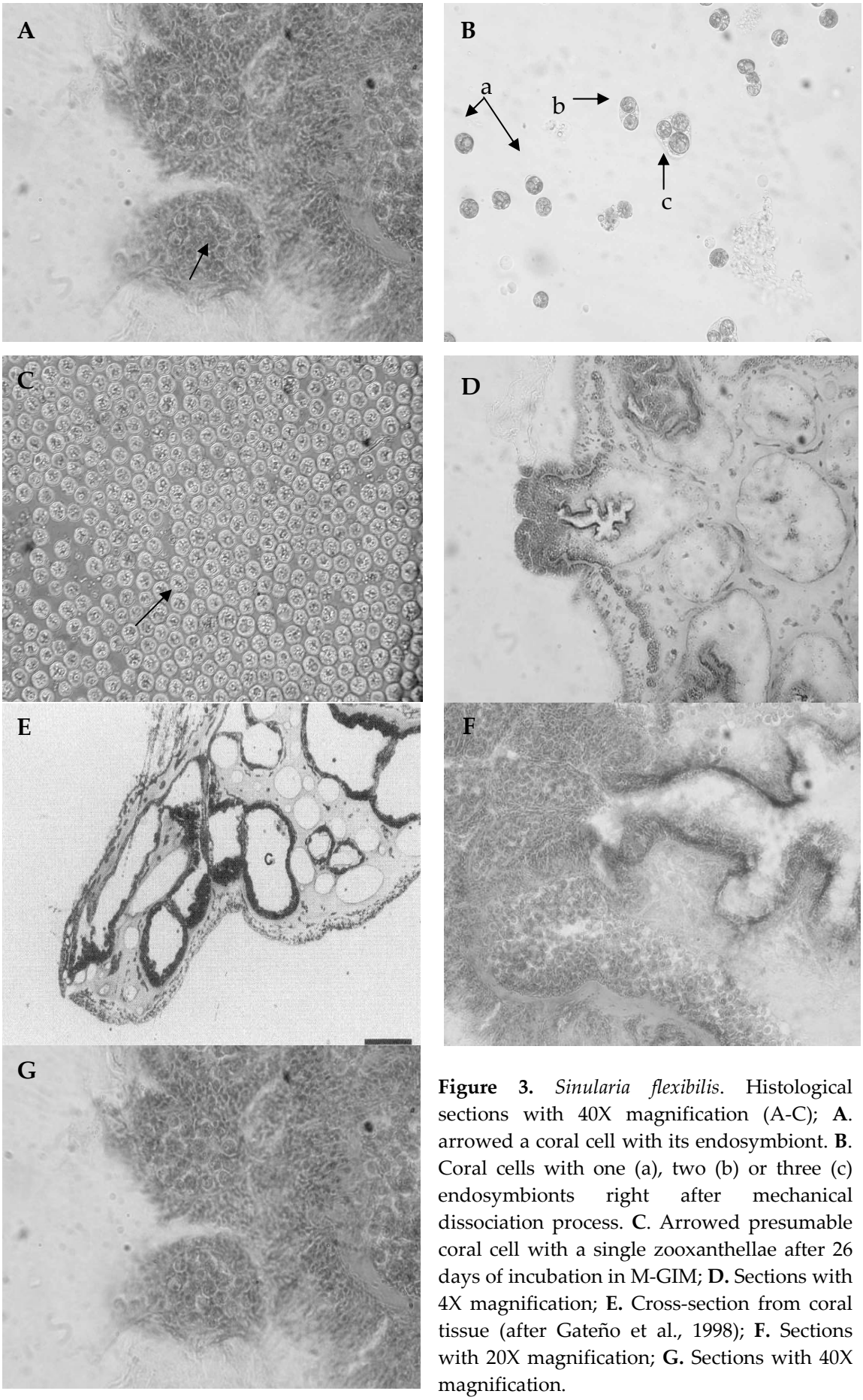


Figure 3. *Sinularia flexibilis*. Histological sections with 40X magnification (A-C); **A**. arrowed a coral cell with its endosymbiont. **B**. Coral cells with one (a), two (b) or three (c) endosymbionts right after mechanical dissociation process. **C**. Arrowed presumable coral cell with a single zooxanthellae after 26 days of incubation in M-GIM; **D.** Sections with 4X magnification; **E.** Cross-section from coral tissue (after Gateño et al., 1998); **F.** Sections with 20X magnification; **G.** Sections with 40X magnification.

but if it contains two or more alga, the host diameter will increase and its cell shape will conform to the shape in which the symbionts cluster (Gates and Muscatine, 1992). On the other hand, coral cells are colourless while their photosynthetic endosymbionts contain multiple pigments (Smith, 2001). This can help distinguish the cells by appearance.

Histological sections. To verify that the cultured cells were in fact zooxanthellae-containing coral cells, three histological sections were prepared from coral tissue, analyzed microscopically, and compared to cultured cells. Cells from coral tissue and those present in the cultures were found to be very similar (Figure 3 A- G).

Genetic test using Internal Transcribed Spacer (ITS) primers. To confirm the similarity of the cultured cells and those of the coral tissue, a genetic test using Internal Transcribed Spacer (ITS) was used. The conserved ribosomal genes (rDNA) in eukaryotes are separated by both highly variable external and internal TSs, one of which is the ITS used in this study. These typically include the 18S, 5.8 S and 25-28 S ribosomal RNAs. Primers designed for amplification of ITS regions will anneal to more conserved regions, but more specific primers can be defined, which amplify only specific groups of organisms (LaJeunesse, 2001). We used the primers 1s/2ss, designed for the genus *Alcyonium* (McFadden et al., 2001) closely related to *Sinularia*.

These primers flank a region of approximately 1400 bp of the nuclear ribosomal gene complex spanning the 3′ end of the 18 S subunit, internal transcribed spacer 1 (ITS-1), 5.8S subunit, ITS-2. If PCR products were obtained from the cell culture DNA extraction and from the coral tissue using these specific primers, and if these had the same size, the presence of coral and zooxanthellae cells will be confirmed.

Using this primer set 1s/2ss (McFadden et al., 2001) a fragment of approximately 1400 base pairs was obtained using PCR. The band was observed for all the undiluted samples, confirming the coral cells in the various cultures and in the intact animal to be identical. For the dilutions, in contrast, only the samples from the coral tissue presented a band for all of them and, as it is seen in figure 4, the higher the dilution, the lower its intensity; this may be explained by the lower amount of template DNA available for the PCR reaction in the dilutions. For GMIM, culture bands were visible

up to 100 times diluted, for GIM culture up to 10 times diluted and DMEM culture only showed a slight band for the undiluted sample (Figure 4).

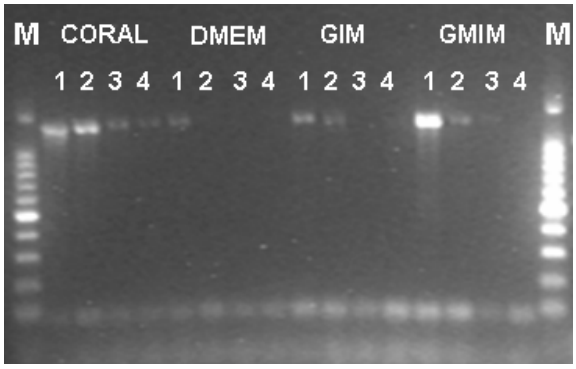


Figure 4. Electrophoresis gel showing 1s / 2ss primer combination. A band of approximately 1400-bp is visible for all the undiluted samples (1), confirmation of the presence of the same kind of coral cells in all the different cultures and the coral tissue sample. M= Marker, 1= undiluted sample, 2= 10 times diluted, 3= 100 times diluted, 4= 1000 times diluted.

Those results are in line with the cellular growth rates (Figure 1 and Table 3), where the higher cellular growth rates were obtained in GMIM medium and, to a lesser extent, in GIM medium. During the mentioned work, the medium DMEM did not show signs of growth but the cells were preserved in good conditions.

Using the primers ITSa/ITSb, specifically designed for volvocacean green algae (Coleman et al., 1994), also the region of the nuclear ribosomal gene complex spanning the 3′ end of the 18S subunit, internal transcribed spacer 1 (ITS-1), 5.8S subunit, ITS-2, and the 5′ end of the 28S subunit was amplified, a band of approximately 700 – bp was observed for all the undiluted samples, demonstrating once more the presence of the same kind of coral cells in all the cultures and coral tissue. Also, the algae symbiont (zooxanthellae) belongs most likely to the genus *Symbiodinium* (Loram et al., 2007).

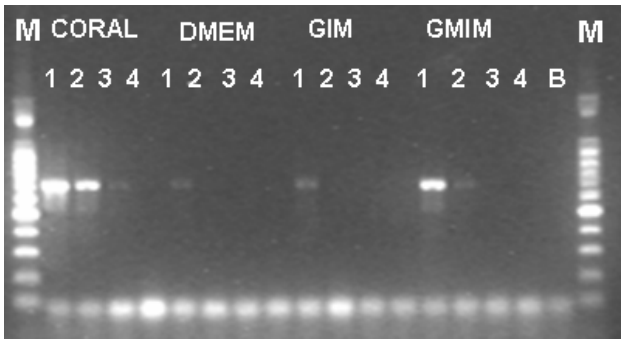


Figure 5. Electrophoresis gel showing ITSa / ITSb primer combination. A bands of approximately 700-bp are visible for all the undiluted samples (1), corroborating the presence of the same kind of symbionts in all the different cultures and the coral tissue sample. M= Marker, 1= undiluted sample, 2= 10 times diluted, 3= 100 times diluted, 4= 1000 times diluted B= Blank.

As it was explained previously, also the different dilutions had an effect on the PCR reaction which is reflected in the band intensity (Figure 5). Through this molecular test, the identity of the cultivated cells was established: they were found to be identical to native coral cells in all cases.

Conclusions

Mechanical dissociation of coral tissue for developing a primary coral cell culture was most successful. It yielded the highest number of extracted cells and allowed cell growth after subsequent cultivation. The best chemical dissociation agent was trypsin-EDTA. Coral cells obtained after spontaneous dissociation did not grow.

Due to their small area and easy handling, 24-well plates were found appropriate for the establishment of the first cultures. The antibiotics were found to be effective in disinfecting in 90% of the cultures. Light was a fundamental parameter for coral cell culture. The best growth of coral cells was obtained with the media GIM and GMIM. Although both DMEM and M199 preserved the cells in good conditions, no cell growth was observed.

The software Image-proplus was a helpful tool for the growth determination process, increasing the accuracy of the counts and partially reducing the time needed. The genetic test using ITS primers was a feasible technique to identify the similarity of the cultured cells and the original cells in the coral. Hence, it is applicable to future cell culture studies. Finally, the culture of coral cells seems to be possible; however, further investigations are needed in order to establish the optimum conditions for the culture development.

References:

Ata, A. Kerr, R., Moya, C. & Jacobs, R. 2003. Identification of anti-inflammatory diterpenes from the marine gorgonian *Pseudopterogorgia elisabethae*. Tetrahedron 59: 4215-4222.

Baird, G., Zacharias, D. & Tsien, R. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. PNAS 97 (22): 11984-11989.

Barnes, R. 1987. Invertebrate Zoology. Harcourt Brace Jovanovich College Publishers. 5th Edition. USA.

Bastidas, C., Benzie, J., Uthicke, S. & Fabricius, K. 2001. Genetic differentiation among populations of a broadcast spawning soft coral *Sinularia flexibilis*, on the great barrer reef. Mar. Biol. 13-525.

Begon, M., Townsend, C. & Harper, J. 2006. Ecology, from individuals to ecosystems. Blackwell publishing. 4th Edition. USA.

Chao, C., Hsieh, C., Chen, S., Lu, C., Daid, C. & Sheua, J. 2006. Sinularianins A and B, novel sesquiterpenoids from the Formosan soft coral Sinularia sp. Tetrahedron Letters 47: 5889-5891.

Coffroth, M., Lasker, H., Diamond, M., Bruenn, J. & Bermingham, E. 1992. DNA fingerprints of a gorgonial coral: a method for detecting clonal structure in a vegetative species. Mar. Biol. 114: 317-325.

Coleman, A., Suarez, A. & Goff, L. 1994. Molecular delineation of species and syngens in volvocacean green algae (Chlorophyta). J. Phycol. 30: 80-90.

D'Elia, C. Cook, C. 1988. Methylamine uptake by zooxanthellae-invertebrate symbiosis: Insigths into host ammonium environment and nutrition. Limnol Oceanogr 33(5): 1153-1165.

D'Elia, C., Domotor, S. & Webb, K. 1983. Nutrient uptake kinetics of freshly isolated zooxanthellae. Mar Biol 75:157-167.

Department of Plant and Microbial Biology University of California at Berkeley. 2002. Primer sequences. Accessed 2007. http://plantbio.berkeley.edu/~bruns/ primers.html.

Domart-Coulon, I., Elbert, D., Scully, E., Calimlim, P. & Ostrander, G. 2001. Aragonite crystallization in primary cell cultures of multicellular isolates from a hard coral Pocillopora damicornis. PNAS 98: 11885–11890.

Domart-Coulon, J., Sinclair, C., Hill, R., Tambutté, S., Puverel, S. & Ostrander, G. 2004. A basidiomycete isolated from the skeleton of Pocillopora damicornis (Scleractinia) selectively stimulates short-term survival of coral skeletogenic cells. Mar. Biol. 144: 583–592.

Duh,C., Wang, S., Tseng, H., Sheu, J. & Chiang, M. 1998. Novel cytotoxic cembranoids from the soft coral *Sinularia flexibilis*. J Nat Prod 61: 844-847.

Duque, C., Puyana, M., Narváez, G., Osorno, O., Hara, N. & Fujimoto, Y. 2004. Pseudopterosins P–V, new compounds from the gorgonian octocoral Pseudopterogorgia elisabethae from Providencia island, Colombian Caribbean. Tetrahedron 60: 10627-10635.

Faith, M., Tachibana, S., Chou, L. & Ding, J. 1997. Citotoxic and anticancer agents in mucus of *Galaxea fascicularis* purification and characterization. J Mar Biotech 5:50-57.

Faulkner, J. 1977. Interesting aspects of marine natural products chemistry. Tetrahedron 33: 1421-1443.

Frank, U. & Rinkevich, B. 1999. Scyphozoan jellyfish's mesoglea supports attachment, spreading and migration of anthozoans' cells in vitro. Cell Biol Int 23: 307– 311.

Frank, U., Rabinowitz, C. & Rinkevich, B. 1994. In vitro establishment if continuous cell cultures and cell lines from ten colonial cnidarians. Mar. Biol. 120: 491-499.

Freshney, R. 1994. culture of animal cells: a manual of basic technique. Wiley-liss. 3th edition. New York.

Gateño, D., Israel, A., Barki, Y. &Rinkevich, B. 1998. Gastrovascular circulation in an octocoral: Evidence of significant transport of coral and symbiont cells. Biol. Bull. 194: 178-186.

Gates, R., Baghdasarian, B. &, Muscatine, L. 1992. Temperature stress causes host cell detachment in symbiotic cnidarians: Implications for coral bleaching. Biol. Bull. 182: 324-332.

Goreau , T., Goreau, N. & Yonge, C. 1971. Reef corals: autotrophs or heterotrophs?. Biol. Bull., 141: 247-260.

Goulet, T. & Coffroth, M. Stability of an octocoral-algal symbiosis over time and space. Mar ecol prog ser 250: 117-124.

Hayes, R. & Bush, P. 1990. Microscopic observations of recovery in the reef-building scleractinian coral, *Monastrea annularis*, after bleaching on a Cayman reef. Coral reefs 8: 203-209.

Hunt, B. & Vincent, A. 2006. Scale and sustainability of marine bioprospecting for pharmaceuticals. Ambio 35 (2); 57-64.

Hunter, R., LaJeunesse, T. & Santos, S. 2007. Structure and evolution of the rDNA internal transcribed spacer (ITS) region 2 in the symbiotic dinoflagellates (*Symbiodinium*, Dinophyta). J Phycol 43: 120-128.

Hurton, L., Berkcson, J. & Smith, S. 2005. Selection of a standard culture media for primary culture of *Limulus polyphemus* amebocytes. In Vit Cell Dev Biol 41:325-329.

Johannes, R., Coles, S. & Kuenzel, N. The role of zooplankton in the nutrition of some scleractinian corals. Lymnol. Oceanogr. 15(4): 579-586.

Kamel, H. & Slattery, M. 2005. Terpenoids of Sinularia: Chemistry and Biomedical Applications. Pharmaceutical Biology 43 (3): 253–269.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. J Exp Mar Biol Ecol 351, 106 - 113

Kingsley R.J., A.M. Bernhardt, K.M. Wilbur, N. Watabe, 1987. Scleroblast cultures from the gorgonian *leptogorgia virgulata* (Lamarck} (Coelenterata: Gorgonacea}. In Vit Cell Dev Biol 23, 4, 297-302.

Kopecky, E. & Ostrander, G. 1999 Isolation and primary culture of viable multicellular endothelial isolates from hard corals. In vitro Cell Dev Biol Anim 35: 616–624.

LaJeunesse, T. 2001. Investigating the biodiversity, ecology, and phylogeny of endosymbiotic dinoflagellates in the genus *Symbiodinium* using the ITS region in search of a "species" level marker. J Phycol 37:866-880.

Loram, J., Boonham, N., O'toole, P., Trapido-Rosenthal, H. & Douglas, A. (2007) Molecular Quantification of Symbiotic Dinoflagellate Algae of the Genus Symbiodinium. Biol Bull 212: 259–268.

Loya, Y., Sakai, K., Yamazato, K., Nakano, Y., Sembali, H. & van Woesik, R. Coral bleaching: the winners and losers. Ecol lett 4: 122-131.

Mayer, A. & Hamann, M. 2004. Marine pharmacology in 2000: marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiplatelet, antituberculosis, and antiviral activities; affecting the cardiovascular, immune, and nervous systems and other miscellaneous mechanisms of action. Mar Biotech 6: 37-52.

McFadden, C., Donahue, R., Hadland, B. & Weston, R. (2001). A molecular phylogenetic analysis of reproductive trait evolution in the soft coral genus Alcyonium. Evolution 55 (1) : 54 – 67.

Michalek, K. & Bowden, B. 1997. Natural algaecide from soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia, Alcyonacea). J Chem Ecol 23 (2): 259-273.

Molina, N., Polverino, D., Minvielle, M., Apezteguía, M., Aguilar, M. & Basualdo, J. 2006. Comparison of lysis methods and DNA extraction of *Giardia lamblia* Trophozoites. Parasitologia Latinoamericana 61:133-137.

Muscatine, L. Cernichiari, E. 1969. Assimilation of photosynthetic products of zooxanthellae by a reef coral. Biol. Bull., 137: 506-523.

Nacional Oceans Services NOAA's web page. Corals. 2004. Accessed 2007. http://oceanservice.noaa.gov/education/kits/corals/coral01_intro.html

Odintsova, N.A., Ermak, A.V., Tsal, L.G., 1994. Substrate selection for long-term cultivation of marine invertebrate cells. Camp. Biochem. Physiol 107, 4, 613-619.

Pomponi, S.A., Willoughby, R., Kaighn, M.E. and Wright, A.E., 1997. Development of techniques for in vitro production of bioactive natural products from marine sponges. In: Maramorosch, K. and Mitsuhashi, J. (eds.), Invertebrate cell culture: novel directions and biotechnology applications. Science Publishers Inc., Enfield, pp. 231–238.

Puverel, S., E., Tambutte´, D., Zoccola, I., Domart-Coulon, A., Bouchot, S. Lotto, D., Allemand, S., Tambutte´, 2005. Antibodies against the organic matrix in scleractinians: a new tool to study coral biomineralization. Coral Reefs 24, 149–156.

Radhika, P., Rajeswara, R., Archana, J. & Koteswara, N. 2005. Anti-inflammatory activity of a new sphingosine derivative and cembrenoid diterpene (Lobohedleolide) isolated from marine soft corals of *Sinularia crassa* Tixier-Durivault and *Lobophytum* species of the Andaman and Nicobar Islands. Biolo. Pharm. Bull 28 (7): 1311-1313.

Rinkevich, B. 1999. Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. J Biotechnol 70: 133-153.

Roche applied Science. 2006. Pronase Nuclease-free, isolated from *Streptomyces griseus* Lyophilized powder. Accessed 2007. http://www.roche-applied-science.com/pack-insert/0165921a.pdf

Rodgers, C.J., 2002. Book review: Aquatic invertebrate cell culture, Mothersill, C. and B. Austin (Eds.). Springer-Verlag in association with Praxis Publishing, Ltd., Chichester, UK. 2000

Schmid, V., Ono, S. & Reber-Müller, S. 1999. Cell-substrate interaction in Cnidaria. Micros Res Tech 44: 254–268.

Sigma-Aldrich. 2007. Typsine-EDTA solution cell culture tested. Accessed 2007. http://www.roche-applied-science.com/pack-insert/0165921a.pdf.

Smith, D. 2001. Symbiosis research at the end of the millennium. Hydrobiologia 461: 49-54.

Strychara, K., Coatesa, M., Sammarcoc, P., Pivab, T. & Scott, P. 2005. Loss of *Symbiodinium* from bleached soft corals Sarcophyton ehrenbergi, Sinularia sp. and Xenia sp. J Exp Mar Biol Ecol 320: 159–177.

The George Washington University. 2007 The Evolution of Early Animal Complexity. Reference date: 09-06-2007. http://www.gwu.edu/~darwin/BiSc151/NoCoelom/early animal.html

Verkerk, M., van den End, F. & Martens, D. 2007. Animal cell technology (PRE 30806) practical guide. Food and bioprocess engineering group, Wageningen University. The Netherlands.

Villena, A.J., 2003. Applications and needs of fish and shellfish cell culture for disease control in aquaculture. Rev Fish Biol Fisheries 13, 111–140.

Webster, N. & Bourne, D. 2007. Bacterial community structure associated with the Antarctic soft coral, *Alcyonium antarcticum.* FEMS Microbiol Ecol 59: 81–94.

Wiedenmann, J., Ivanchenko, S., Oswald, F. & Nienhaus, U. 2004. Identification of GFP-like proteins in nonbioluminiscent, azooxanthellate anthozoa opens new perspectives for bioprospecting. Mar Biotech 6:270-277.

Yu, S., Deng, Z., van Ofwegen, L., Proksch, P. & Lin, W. 2006. 5,8-Epidioxysterols and related derivatives from a Chinese Soft Coral *Sinularia flexibilis*. Steroids 71: 955-959.

8 General Discussion and Conclusions

Introduction

The soft coral *Sinularia flexibilis* produces toxins with potential anticancer, antifungal, and antibacterial activities, as well as photoprotective mycosporine-like amino acids (MAAs). There is a growing interest in the development of these compounds into medical and industrial products and very large numbers of scientific publications (Chapter 2) deal with bioactive and pharmacologically active compounds from *S. flexibilis*. As a consequence, an overexploitation of these corals' natural reservoirs is imminent. The development of future therapeutic agents from this soft coral thus calls for sustainable means of long-term coral exploitation.

We established (Chapters 3, 4, 5, and 6) that coral cultivation under controlled conditions, with further expansion of the knowledge base for their aquaculture, may offer an alternative supply of coral biomass. Coral aquaculture, however, requires major advances in culture techniques as well as appropriate biological regimes that promote growth and biosynthesis of the target metabolites. Accordingly, growth kinetics and biosynthesis of secondary metabolites of *S. flexibilis* under controlled laboratory conditions were studied. The effects of environmental factors including light intensity, phototrophy, nutrients and feeding, and water flow have been examined (Chapters 3, 4, 5, and 6). The coral cell culture as an alternative to the cultivation of whole organisms was also studied (Chapter 7). Moreover, the biological and pharmacological significance of the coral metabolites, and various means of biomass supply for drug development were reviewed (Chapter 2).

Light intensity *(Chapter 3)*

S. flexibilis is a symbiotic coral, i.e. it co-exists with dinoflagellate algal partners that are known as zooxanthellae. This mutualism is beneficial to both organisms: the algae are provided with both shelter and the coral wastes (substrate), and the host (animal) is supplied with the photosynthates of zooxanthellae. Therefore, light availability is essential to maintain the symbiosis. Light intensity determines the photosynthetic

output, which decreases at both very low and high irradiances. Thereby, optimum irradiance becomes important for maximum performance of the coral.

Specific growth rate, measured from buoyant weight and from the number of buds over an extended period of time (12 weeks), was found to be dependent on light intensity (10 - 1000 μmol quanta m^{-2} s^{-1}). This is the first study of an optimal irradiance based on similar patterns of both specific growth and budding rates in corals. Based on both zooxanthellae and chlorophyll *a* contents, a light intensity around 100 μmol quanta m^{-2} s^{-1} was found to be optimal. In general, irradiance and calcification are linked in symbiotic corals; since little is known about this link in soft corals future work should be directed to understanding the relation between both calcification and tissue genesis with light intensity.

In this study, we found that the level of flexibilide, the major terpene of *S. flexibilis*, increased up to irradiances of 600 μmol quanta m^{-2} s^{-1} and then decreased again. Hence, increased flexibilide biosynthesis is the strategy by which the coral mitigates the stress from extensive long-term over-exposure to light. Because of a similar light-dependency of growth in the range of 100-400 μmol quanta m^{-2} s^{-1} without negative consequences, it should be practical to cultivate the coral at rather high irradiances (200-400 μmol quanta m^{-2} s^{-1}) to maximize flexibilide production. Future efforts can also focus on the irradiance-dependency of other terpenes and of MAAs of *S. flexibilis*.

The effect of the duration of the photoperiod on coral growth has received little attention. Our results (unpublished) showed that *S. flexibilis* grew optimally over a range of light-dark periods, from 12 h light up to 18 h light.

Phototrophy, nutrition, and CZAR *(Chapter 4)*

In addition to its zooxanthellate symbiosis, the shallow, illuminated natural habitats of S. *flexibilis* reflect its dependency on phototrophic nutrition. We studied the extent of phototrophy and heterotrophy in captive conditions, by incubating corals in the light and in the dark, with or without feeding. Results showed that *S. flexibilis* is not able to survive without light, despite the availability of ambient nutritional sources and/or manual feeding. For feeding, we used *Artemia* nauplii, which is rather large and possibly not suitable for the small polyps of this coral. To study the dependency

of coral planktivory on plankton size or particulate matter, labelling methods can be useful. However, as our main goal was to investigate the long-term biology of captive corals, we did not perform those short-term experiments.

Moreover, the ultimate aim of *S. flexibilis* cultivation is to utilize its secondary metabolites, of which the biosynthesis is affected by culture conditions. Thus, it will be conceivable to investigate whether nutritional sources influence metabolite production.

Nutrient enrichment and feeding *(Chapter 5)*

S. flexibilis is a tropical symbiotic soft coral that inhabits nutrient-poor waters. These habitat characteristics were an incentive to try to enrich the coral habitat with nitrogen and phosphorous in order to enhance growth. Colonies of *S. flexibilis* were supplied various levels of nitrogen and phosphorous, and also fed *Artemia* nauplii for a long period. Our results showed no positive effects of nutrient and/or *Artemia* additions on the coral growth. The density of zooxanthellae increased initially, but this increase was not sustained.

Incubation of the coral with labelled nitrogen and phosphorous might be a future tool to investigate the fate of the nutrients that are taken up, and their contribution to coral tissue formation. In addition, it may be a direct tool to measure the rate of zooxanthellae division, i.e. their 'mitotic index', thereby computing the specific growth rate of zooxanthellae under both standard and enriched conditions. These will provide more knowledge on the controversial topic of nutrient enrichment in literature and also on the mechanisms of zooxanthellae regulation following a blooming. Changes in zooxanthellae content have received less attention and thus deserve further research to increase the existing knowledge on the mechanism of algal population control by the coral to prevent overgrowth of its symbionts. Furthermore, the relation between nutrient enrichment and secondary-metabolite production should be researched in more detail.

Water velocity *(Chapter 6)*

Water velocity was found to be an important factor that affected growth and physiology of *S. flexibilis* under laboratory conditions. Optimal coral growth with high contents of zooxanthellae, chlorophyll, and protein was observed at an intermediate flow velocity of 11 cm s^{-1} with higher zooxanthellae, chlorophyll, and protein contents. We attributed this optimum in flow-dependent growth to availability of nitrogenous resources and to a minimum boundary layer thickness at optimal water velocity. These parameters facilitate substrate and product exchange for the coral metabolism.

In addition to dissolved organic and inorganic compounds, utilization of particulate food by symbiotic corals is species-specific; hence, it is feasible for future research to investigate the effect of different water flow rates on the rate of particle capture in *S. flexibilis*. An approach could be to supply the coral with labelled food particles such as *Artemia* nauplii and to measure flow-dependent prey capture rates. We also observed a flow-dependency of flexibilide production (Unpublished data). Moreover, measurement of the coral metabolic response (i.e. photosynthesis and respiration) to water flow rates, as well as rates of both tissue genesis and calcification at various velocities should also be interesting in further researches.

Finally, for our flow experiment, we used relatively small coral samples and measured round-about flow rates to examine the flow-dependency of the coral. Because coral morphology and water flow are mutually dependent, it is recommendable to perform future experiments on larger, more branched colonies of *S. flexibilis* and quantify the water flow among the branches and also near the polyps. This may provide more knowledge on the interactions between the coral branching morphology and inter-branch water velocities.

Measuring growth by buoyant weighing

For all our long-term experiments, we used the 'buoyant weight' method to measure coral growth. Besides being simple and non-destructive, this method enabled us to study the coral growth. Although it mainly reflects skeletal growth (calcification), the method, however, provided a credible measurement of specific growth rate (μ d^{-1}) for *S. flexibilis* within short intervals, depending on environmental

conditions. The specific growth rate shows actual effects of experimental treatments, independent of population size.

Coral cell culture *(Chapter 7)*

The ultimate objective of coral cell culture is to provide viable, long-lasting cell lines for various biotechnological applications. Most attempts for cellular culture of corals failed to establish durable cell cultures. We, therefore, tried to examine this approach for *S. flexibilis* using various methodologies. Depending on culture conditions, the coral's cultured cells were able to survive and proliferate at different durations. We used a culture medium (GMIM) that had not been tested in coral cell culture before, but was found to yield the best results for the maintenance of our coral cell cultures. Light intensity was revealed as a fundamental parameter to be taken into account for the coral cell culture. Future experiments should ascertain whether extended viability is also possible.

Interestingly, aspect of our study was that we developed a genetical cell identification test was, to our knowledge, applied for the first time in coral cell culture. Its result showed that the proliferating cells in culture media were indeed true cells of S. *flexibilis*. With the help of this approach, future attempts can reliably perform long-term studies on the coral cell culture.

Future study on the coral carotenoids

Symbiotic corals, especially their dinoflagellate partners, are a rich source of photosynthetic and photoprotective carotenoid pigments. Carotenoids in general have various industrial and pharmaceutical importances. For instance, the typical pigment of zooxanthellae, i.e. peridinin, and its by-products has been found to have anti-cancer properties. Because carotenoid content depends on environmental conditions such as irradiance and nutrition, future studies should concentrate on this topic as well. We were able to quantify some carotenoids in S. *flexibilis* (unpublished data). However, as very little information is available on the carotenoid composition of soft corals especially under enclosed situations, further research on the carotenoids of *S. flexibilis* will provide more knowledge in this field.

Conclusions

In conclusion, the main outcome of this thesis is that successful aquarium rearing of the soft coral S*inularia flexibilis*, after 4 years of captive husbandry, is possible. This species showed to be sturdy under laboratory conditions and to allow easy propagation due to its branching structure without loosing the ability of the parent colony to recover from collection. Our findings showed that the flexibilide continued to be biosynthesised, even after several years of culture. Therefore, coral culture opens the way to aquaculture production of interesting pharmacological agents.

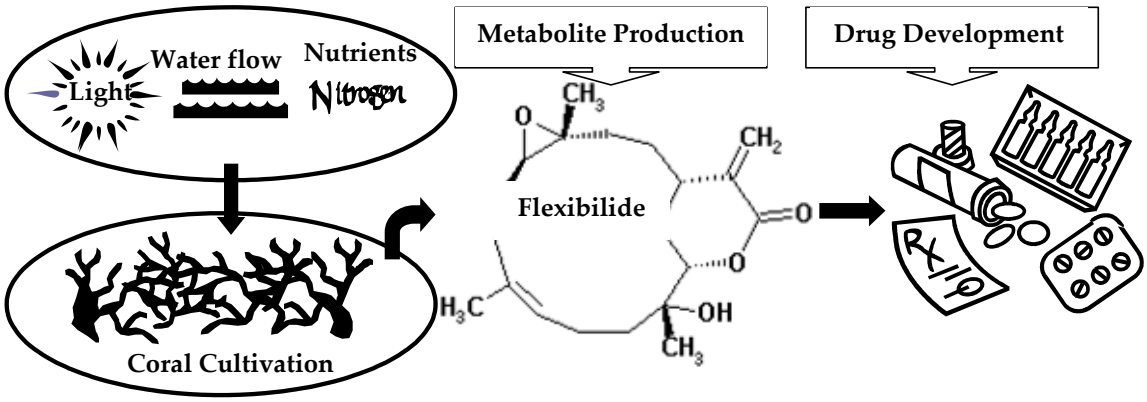


Fig. 1. Schematic overview of coral cultivation depending on environmental conditions, resulting in utilization of the coral's secondary metabolites for drug development.

Because growth of *S. flexibilis* is highly dependent on irradiance and also on ambient nutritional sources, aquaculture of this species could be an economic and manageable technology to meet, in part, the needs for coral-derived drug development (Fig. 1).

Summary

Introduction

Many of the marine sessile invertebrates such as soft corals produce toxins that help protect the coral from competitors and predators. These toxins are of medical importance (e.g. in cancer treatment). In recent years, there has been a focus to make use of marine organisms for this purpose. As a result, marine biotechnology is developing to meet the increasing demand. This thesis deals with one of the biomedically useful symbiotic soft corals, *Sinularia flexibilis* that co-exists with dinoflagellate algae or zooxanthellae, which together with the host (animal) form a holobiont. This species also produces compounds as intra-cellular photo-protective agents in shallow-water habitats; these compounds are called mycosporine-like amino acids (MAAs) with potential application in health care as sunscreens.

An advantage of this coral is that from a parent colony of coral, a lot of clones may be obtained via asexual reproduction (fragmentation). If this procedure is used natural marine resources can be preserved because excessive sea harvest of the source coral is not necessary. Because there is an increasing demand for drugs such as antibiotics and anticancers of marine origin, the main goal of this research was to culture *S. flexibilis* at optimized captive conditions, so that they can supply, in part, the future demand for drug development. To have an overview of scientific research on the metabolites of this species, we reviewed studies into these secondary compounds and listed various alternatives for the captive cultivation of corals (Chapter 2). In this research, we studied the impact of environmental parameters such as irradiance (Chapter 3), the relevance of phototrophy and heterotrophy (Chapter 4), the effect of nutrient enrichment (Chapter 5) and of water velocity (Chapter 6) on the coral growth and physiology in captivity, and on the biosynthesis of secondary metabolites. As an alternative to the cultivation of whole organisms, the cultivation of coral cells is described in Chapter 7.

- Secondary metabolites and cultivation opportunities (Chapter 2)

To have an overview of scientific research on the secondary metabolites of this species, we reviewed studies into these compounds. Our review showed that a high

percentage of these compounds are cytotoxic and may be promising as future anti-cancer drugs. To provide coral biomass, we discussed various approaches, including captive aquaculture.

- Light-dependency (Chapter 3)

As mentioned above, *S. flexibilis* is a symbiotic coral: it depends on the translocated photosynthates from its algal symbionts, the zooxanthellae. Accordingly, factors that promote photosynthesis of zooxanthellae should be given serious attention. Of these, light is being considered as the most important environmental factor affecting growth of symbiotic corals due to translocated photosynthates from zooxanthellae. Light intensity or photosynthetic photon flux density is important in promoting photosynthesis by zooxanthellae. Hence, finding optimum irradiance by applying various light intensities was the objective in the 3rd chapter of this thesis. Optimum specific growth rate (mu) of *S. flexibilis* was found at a range from 100 to 400 μmol quanta m^{-2} s^{-1}. More or less constant mu over a range of light intensities was attributed to photoacclimation of the coral. The content of flexibilide, a major terpene of *S. flexibilis*, increased with light intensity. The increase in flexibilide concentration was explained as the coral's response on stress

- Phototrophy and heterotrophy (Chapter 4)

The light-dependency of *S. flexibilis* and its zooxanthellae indicates that the algal photosynthates fuel metabolic requirements of the host coral. Our long-term findings verified this fact and showed that in the absence of light, even with addition of food, the coral was not able to grow nor to survive. Similarly, corals kept under optimal light without a continuous supply of ambient nutrient supply, were incapable to grow and survive. On the other hand, both colonies of *S. flexibilis* incubated at normal irradiances with or without extra feeding, grew well using available nutritional sources. In the same way, the coral physiology, i.e. the contents of zooxanthellae, chlorophyll, and MAAs reflected the effects of the above parameters. Therefore, both phototrophy and heterotrophy on ambient resources are important for *S. flexibilis* to perform optimally.

In short-term, measurements of metabolic rate (photosynthesis and respiration) and estimating daily energy budget for *S. flexibilis* showed complete phototrophy. Estimating the daily 'Contribution of Zooxanthellae to Animal Respiration (CZAR)' yielded high values of zooxanthellae contribution to the host metabolism. Using energy equivalents for photosynthetically fixed carbon, we established that a small fraction of translocated fixed carbon is allocated to coral growth. Based on dependency of *S. flexibilis* on ambient nutritional sources in addition to irradiance, we concluded that photosynthates of zooxanthellae are deficient in growth-enhancing nutrients, especially nitrogenous compounds, which indeed need to be fulfilled heterotrophically for overall coral growth.

- Nutrient enrichment and feeding (Chapter 5)

To study the effect of essential nutrients enrichment (nitrogen and phosphorous) on the specific growth rate of *S. flexibilis* and its zooxanthellae, we performed prolonged experiments (Chapter 5). Our results showed that ammonium addition did not have any positive effect on coral growth; it reduced the growth of *S. flexibilis* at high levels. The coral was able to recover after high ammonium loadings. The effect of phosphate enrichment on the coral growth was neutral. Enrichment of this species with ammonium for three weeks resulted in an increase in both zooxanthellae and chlorophyll content after the 1st week of enrichment, followed by a decrease (equal to control) for the next two weeks. We concluded that zooxanthellae multiplication because of ammonium addition was temporary.

- Flow-dependency (Chapter 6)

The importance of water flow for sessile organisms such as *S. flexibilis* is to provide them with nutrition (e.g. suspended food particles), remove the coral's wastes, and to facilitate exchange of substrates and products through modifying the boundary layer near their bodies. Long-term exposure of *S. flexibilis* to various water velocities resulted in the finding that the coral's growth, physiology, and morphology are affected by flow regimes. Specific growth rates were optimal at an optimum water velocity of 11 cm s^{-1}. Based on the contents of zooxanthellae, chlorophyll, and total

protein at the same water velocity for optimum growth, we concluded that optimal water velocity facilitated nutrient uptake through modification of the boundary layer thickness. This modification was aided by flexibility and swaying behaviour of *S. flexibilis* and also its polypary structure. High water velocities caused both coral and polyp retraction, which reduced exposure of its photosynthesizing surfaces together with decreased nutrient uptake resulting in low specific growth rates and also physiological parameters.

- Coral cell culture (Chapter 7)

We investigated cellular culture of *S. flexibilis* (Chapter 7) as an alternative to the coral supply, using different media and cell dissociation methodologies. A mechanical dissociation process provided the best method for the cell extraction procedure, maintaining always the highest number of cells extracted and subsequent cellular growth in all treatments. The best results from chemical reagents for dissociation was found using trypsin-EDTA. Coral cells obtained by spontaneous dissociation did not show signs of growth. Light intensity was revealed as a fundamental parameter to take into account for the coral cell culture. The media GIM and GMIM displayed the best results for the maintenance of coral cell cultures. By means of a molecular test using Internal Transcribed Spacer (ITS) primers, the similarity of cultured coral cells and zooxanthellae at different culture media with those in the coral tissue was confirmed. In addition to possibility of the coral cell culture depending on culture conditions and methodologies, our genetical cell identification test provided a reliable proof for the true cultured cells of *S. flexibilis*.

Samenvatting

Inleiding

Zachte koralen produceren – net als veel andere mariene sessiele invertebraten - toxinen die hen beschermen tegen concurrenten en predatoren. Deze toxinen zijn medisch vaak interessant, bijvoorbeeld voor kankerbestrijding. In de voorbije jaren heeft men dan ook, juist om deze reden, getracht deze organismen te kweken voor exploitatie; de mariene biotechnologie ontwikkelt zich meer en meer om aan deze groeiende vraag tegemoet te komen.

Dit proefschrift betreft het biomedisch interessante koraal *Sinularia flexibilis*. Dit zachte koraal leeft in symbiose met een dinoflagellaat, de zogenaamde zooxanthellae; samen met hun dierlijke gastheer vormen deze fotosynthetische algen een zogenaamde holobiont. Dit organisme produceert intracellulaire verbindingen die bescherming bieden tegen de hoge lichtintensiteiten in de ondiepe wateren waarin deze koralen groeien. Deze verbindingen worden mycosporine-achtige aminozuren genoemd (MAAs); ze zijn mogelijk toepasbaar als zonnebrandmiddelen.

Een voordeel van dit koraal is dat van één moederkolonie door ongeslachtelijke vermenigvuldiging een groot aantal klonen als nakomelingen verkregen kunnen worden (fragmentatie). Dank zij deze procedure kunnen natuurlijke mariene koraalpopulaties ontzien worden en is het oogsten uit de zee overbodig.

Gebaseerd op de toenemende vraag naar mariene geneesmiddelen zoals antibiotica en anti-kankermedicijnen, heeft het onderzoek in dit proefschrift zich gericht op het kweken van *S. flexibilis* onder geoptimaliseerde condities in gevangenschap, om zo tegemoet te komen aan de toekomstige vraag naar koraalbiomassa voor farmaceutisch onderzoek.

In Hoofdstuk 2 wordt een overzicht gegeven van wetenschappelijk onderzoek naar de secundaire metabolieten van dit koraal; tevens worden mogelijkheden tot kweek in gevangenschap verkend. In de navolgende hoofdstukken wordt onderzoek naar de invloed van omgevingsfactoren op de groei en fysiologie van koralen en op de productie van secundaire metabolieten gepresenteerd. In Hoofdstuk 3 wordt onderzoek naar de lichtsterkte gepresenteerd. Hoofdstuk 4 behandelt het relatieve

belang van fototrofie en heterotrofie. Hoofdstuk 5 is gewijd aan de invloed van de verrijking met nutriënten. In Hoofdstuk 6 wordt onderzoek naar de invloed van de watersnelheid gepresenteerd. In Hoofdstuk 7 wordt, als alternatief voor het kweken van holobionten, de kweek van koraalcellen onderzocht.

Secundaire metabolieten en kweekmogelijkheden (Hoofdstuk 2)

In Hoofdstuk 2 wordt een overzicht gegeven van het onderzoek naar de productie van secundaire metabolieten door *S. flexibilis*. Dit overzicht laat zien dat een groot deel van deze verbindingen cytotoxische eigenschappen hebben en derhalve veelbelovend lijken als toekomstige kankerbestrijdingsmiddelen. Uiteenlopende productiemethoden voor het produceren van koraalbiomassa, zoals aquacultuur, worden besproken.

Afhankelijkheid van licht (Hoofdstuk 3)

Als symbiotisch koraal is *S. flexibilis* afhankelijk van gereduceerde koolstofverbindingen zoals die door zijn fototrofe symbiont, de zooxanthellae, worden geproduceerd uit koolstofdioxide. Factoren die fotosynthese door zooxanthellae bevorderen verdienen dan ook serieuze aandacht; licht wordt beschouwd als belangrijkste factor die van invloed is op de groei. Lichtintensiteit, oftewel de fotonfluxdichtheid, is van rechtstreekse invloed op de fotosynthese van zooxanthellae. In Hoofdstuk 3 worden daarom uiteenlopende lichtintensiteiten getest teneinde een zo hoog mogelijke waarde van de specifieke groeisnelheid van het koraal te bewerkstelligen. In het gebied van 100 tot 400 μmol quanta m^{-2} s^{-1} bleek de specifieke groeisnelheid van *S. flexibilis* optimaal te zijn. De min of meer constante specifieke groeisnelheid binnen een scala aan lichtintensiteiten werd toegeschreven aan fotoacclimatie door het koraal.

Het gehalte aan flexibilide, de belangrijkste terpeen van S. flexibilis, bleek toe te nemen met de lichtintensiteit. De toename in flexibilidegehalte werd verklaard als een reactie op een 'stress'situatie.

Fototrofie en heterotrofie (Hoofdstuk 4)

De lichtafhankelijkheid van *S. flexibilis* laat zien dat de fotosyntheseproducten van de zooxanthellae een brandstof zijn voor de stofwisseling van het gastheer-koraal.

Onze langdurige experimenten bevestigen dit en lieten zien dat het koraal niet kon groeien of overleven in het donker, zelfs niet indien groeimedium werd toegediend. Koralen in optimaal licht, maar zonder een voortdurend aanbod aan groeimedium, waren evenmin in staat om te overleven en groeien. Kolonies van *S. flexibilis* in normaal licht, met of zonder extra groeimedium, groeiden echter goed en gebruikten daarbij de beschikbare voedingsstoffen. Op soortgelijke manier weerspiegelde de fysiologie van het koraal (de gehalten aan zooxanthellae, chlorofyl, en MAAs) het effect van bovengenoemde parameters. Daarom werd geconcludeerd dat zowel fototrofie en heterotrofie en hulpbronnen in de omgeving belangrijk waren voor het optimaal functioneren van *S. flexibilis*.

Uit kortetermijnmetingen aan metabole snelheden (fotosynthese en ademhaling) en uit schattingen van de dagelijkse energiebehoefte van *S. flexibilis* bleek complete fototrofie. Een schatting van de dagelijkse bijdrage van zooxanthellae aan de ademhaling van de gastheer leverde een hoge waarde. Als de fotosynthetisch gefixeerde koolstof werd uitgedrukt in energie-equivalenten bleek slechts een klein deel van de vanuit de zooxanthellae overgedragen koolstof gebruikt te worden voor groei van het koraal. Omdat *S. flexibilis*, naast licht, afhankelijk is van voedingsbronnen in zijn omgeving, concludeerden we dat fotosyntheseproducten van zooxanthellae tekort schieten in het aanleveren van groeiondersteunende nutriënten, in het bijzonder stikstof. Dit moet dan ook aangevuld worden door consumptie door het koraal van externe bronnen.

Verrijking met nutriënten en voeding (Hoofdstuk 5)

Het effect van verrijking met essentiële nutriënten (stikstof en fosfor) op de specifieke groeisnelheid van *S. flexibilis* en zijn zooxanthellae werd bestudeerd in lange-termijnexperimenten. De resultaten lieten zien dat toevoeging van ammonium geen positief effect had op de koraalgroei; bij hoge concentraties werd de specifieke groeisnelheid zelfs gereduceerd. Wel bleek het koraal in staat te recupereren na een dergelijke overbelasting met ammonium. Er werd geen effect gevonden van verrijking met fosfaat. Langdurige verrijking met ammonium resulteerde in een toename van zowel zooxanthellae als ook chlorofyl gedurende de eerste week van verrijking; in de

daarop volgende twee weken daalden beide gehalten echter weer. Wij concludeerden dan ook dat de toename in het zooxanthellaegehalte door ammoniumtoevoeging van voorbijgaande aard was.

Stromingsafhankelijkheid (Hoofdstuk 6)

Water is een transportmiddel dat, door stroming, sessiele organismen zoals *S. flexibilis* voorziet van een aanvoer van voeding (bijvoorbeeld gesuspendeerde deeltjes), een afvoer van afvalstoffen, en, meer in het algemeen, een uitwisselingsmogelijkheid van substraten en producten door een hydrodynamische grenslaag aan hun lichaamsoppervlak. Lange-termijnexperimenten met verschillende watersnelheden lieten zien dat de groei, de fysiologie en de morfologie van *S. flexibilis* afhankelijk waren van de stroomsnelheid. Specifieke groeisnelheden bleken optimaal bij een watersnelheid van 11 cm s^{-1}. Ook voor de gehalten aan zooxanthellae, chlorofyl en totaal eiwit bleek deze watersnelheid optimaal. Geconcludeerd werd dat deze optimale watersnelheid een efficiënte opname van nutriënten mogelijk maakte door een minimale dikte van de hydrodynamische grenslaag. Dit werd verder ondersteund door de flexibiliteit en het daarmee gepaard gaande wiegen op de stroming van *S. flexibilis* en bovendien door het gedrag en de structuur van de koraalpoliepen. Hoge watersnelheden lieten de koraalpoliepen en tevens het gehele koraal samentrekken. Door deze samentrekking werd de totale hoeveelheid fotosynthetiserend oppervlak verminderd en nam de nutriëntopname af; als gevolg hiervan daalden zowel de specifieke groeisnelheid als ook de gehalten aan zooxanthellae, chlorofyl en totaal eiwit.

Het kweken van koraalcellen (Hoofdstuk 7)

Celkweek van *S. flexibilis* werd onderzocht als een alternatieve productiewijze van koraalbiomassa. Hierbij werd gebruik gemaakt van verscheidene methoden tot celdissociatie en werden verschillende groeimedia getest. Mechanische dissociatie bleek de beste methode te zijn en leverde de hoogste celopbrengst en, aansluitend, de beste groei van deze cellen. Van de chemische reagentia bleek trypsine-EDTA de hoogste opbrengst te leveren. Cellen verkregen door spontane dissociatie vertoonden

geen groei. Licht bleek een essentiële parameter te zijn bij de kweek van koraalcellen. De GIM- and GMIM-media lieten de beste kweekresultaten zien. Met een moleculaire test met Internal Transcribed Spacer (ITS) primers, werd de overeenkomst aangetoond tussen gekweekte koraalcellen en hun zooxanthellae enerzijds en de cellen uit een koraal-holobiont anderzijds. Met deze genetische identificatie werd de identiteit van de gekweekte koraalcellen bevredigend vastgesteld.

Overview of completed training activities

Discipline specific activities

Courses		
Bioreactor Design and Operation	2006	1.0
Ethical Dilemas for life sciences	2007	3.0
Meetings		
Coral Husbandry Symposium (6 days)	2007	3.0
Netherlands Biotechnology Congress (4 days)	2006	2.4
Dutch Coral Research Symposium (1 day)	2007	0.3
EAZA Research Conference (4 days)	2007	2.4
General courses		
English intermediate course	2003	1.0
English Upper-intermediate course	2004	1.0
English Academic Writing	2005	1.0
English Fluency	2003	1.0
Personal Efficacy	2006	0.5
Information Literacy with Introduction to Endnote	2006	0.6
Project and Time Management	2006	1.5
Techniques for writing and presenting a scientific paper	2007	1.2
Creating your scientific network	2006	1.0
Optionals		
Preparation PhD research proposal		6.0
Brainstorm week	2005	1.0
Meetings		3.0
Total		**30.9**

Curriculum Vitae

Mohammad Kazem Khalesi was born on Auguest 17, 1967 in Babol, a northern city in I.R. of Iran. He completed his primary, intermediate, and high school in the same city, and achieved his diploma in Natural Sciences in 1984.

Afterwards, he continued his studies at Tehran University for over two years in Animal Sciences. After that, he performed a two-year military service in the Iranian armed forces. In 1990, he was employed at Mazandaran University in his native province as a technician. By resuming his studies in parallel to working at the university, he obtained his Bachelor in Animal Sciences in 1994. Later on, he was accepted in the entrance exam for MSc at Tarbiat Modarres University, and attained his degree in Biology of Marine Fishes in 2000.

In 2001, following aceptance in a nationwide exam, he was awarded a scholarship to do a Ph.D. at overseas universities. In 2002, he was admitted by Wageningen University, the Netherlands and finally started his Ph.D at the department of Agrotechnology and Food Sciences in April 2004. In his research, he investigated the "*ex situ* cultivation of the soft coral *Sinularia flexibilis* for biotechnological exploitation". After four years, he finished his Ph.D., funded by government of I.R. of Iran, in April 2008. He will return to Iran to resume work at Mazandaran University.

Email: mk_khalesi@yahoo.com

List of publications

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Flow-dependent growth in the zooxanthellate soft coral *Sinularia flexibilis*. Journal of Experimental Marine Biology and Ecology 351, 106–113.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. Light dependency of growth and secondary metabolite production in the captive zooxanthellate soft coral *Sinularia flexibilis*. Marine Biotechnology, submitted.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2007. The soft coral *Sinularia flexibilis*: Potential for drug development. Proseeding of the 1st International Symposium on Coral Husbandry, Arnhem, the Netherlands. In press.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2008. Phototrophy and heterotrophy in the captive symbiotic octocoral *Sinularia flexibilis*. Marine Biology, submitted.

Khalesi, M.K., Beeftink, H.H., Wijffels, R.H., 2008. Effects of ammonium, phosphorus and *Artemia* on the growth of the octocoral *Sinularia flexibilis* and its symbiotic zooxanthellae. Marine Biology Research, submitted.

Khalesi, M.K., Vera-Jimenez N.I., Aanen D.K., Beeftink, H.H., Wijffels, R.H., 2008. Cell cultures from the symbiotic soft coral *Sinularia flexibilis*. *In vitro* Cellular and Developmental Biology- Animal, submitted.

Abedian, A., **Khalesi, M.K.**, Shokri, M., Heidari, M.R., 2003. Investigation of laboratory reproduction and growth in the Caspian Sea gamarous (*Pontogammarus maeoticus*). Journal of Iranian Marine Science and Technology 2, 2-3, 51-54.

Sharifpour, E., Yousofian, M., **Khalesi, M.K.**, 2001. A study on the final ovarian maturation in cultured grey mullet *Mugil cephalus*. Journal of Iranian Marine Science and Technology 2, 1, 23-34.

This work was funded by the Ministery of Science, Research and Technology of I.R. of Iran.

The cover page was designed by Mohammad Kazem Khalesi.