Phylogenetic ecology of octocoral - gastropod associations

Reijnen, B.T.

Chapter 3

Bioactivity of Caribbean corals related to their associated fauna

Bastian T. Reijnen and Sancia E.T. van der Meij

Abstract

Natural products are commonly discovered in sessile coral reef organisms, after which their potential use in pharmacology is screened with the help of various bioactivity assays. In the present study a quick and easy assay is used to study the bioactivity of Caribbean corals, based on the luminescence of the marine bacterium *Aliivibrio fischeri*. EC_{50} values were determined for 77 anthozoan specimens collected from Curaçao (Octocorallia, n=52; Scleractinia, n=24; and one sponge species (Porifera)). In general sponge and octocoral specimens were more bioactive (= low EC_{50} values) than scleractinians, although some stony corals (e.g. *Madracis auretenra*) had EC_{50} values similar to some of the most bioactive soft corals. Scleractinians did not show specific bioactivity per family, genus or species, whereas some octocoral genera (*Antillogorgia*, *Gorgonia*, and *Pseudoplexaura*) had remarkable lower EC_{50} than other octocoral genera (*Pterogorgia*). There are discrepancies in bioactivity within a single coral species, which is in line with previous studies. Coral bioactivity was plotted on phylograms of Caribbean soft and stony corals, together with snail and crab associations (Ovulidae and Cryptochiridae, respectively). For the ovulid snails *Cyphoma gibbosum* and *Cymbovula acicularis*, both associated with Octocorallia, we found that host coral bioactivity is most probably unrelated to the snail's host specificity. The most bioactive host genera (*Antillogorgia* and *Gorgonia*) host two symbiotic species, and in contrast, less bioactive gorgonian genera are only predated by the generalist *C. gibbosum*. Cryptochirid crabs are more specific in their associations with certain scleractinian genera, but there is no clear association with the bioactivity of the corals. Nonetheless, the most bioactive coral (*Madracis auretenra*) does not have any cryptochirid symbionts.

Introduction

Many marine species are known to harbor secondary metabolites. These secondary organic compounds are not directly involved in the normal growth, development, or reproduction of an organism, contrary to primary metabolites. Sponges, ascidians, anemones, algae, soft corals and gorgonians, and stony corals all produce secondary metabolites (Chen *et al*., 2014; Liu *et al*., 2014; Daletos *et al*., 2015; Ebada *et al*., 2015). These compounds are subsequently screened by pharmacologists for useful properties in, for example, the biomedical sector and therefore referred to as 'marine drugs' (Marris, 2006; Benkendorff *et al*., 2015; De Jesus Raposo *et al*., 2015). The secondary metabolites or so-called marine natural products (MNPs) are divided into specific groups based on their chemical composition (e.g. terpenoids, lipids, and steroids), and many of these chemicals are cytotoxic, anti-inflammatory, antifouling or have anti-virulent effects (Rocha *et al*., 2011; Blunt *et al*., 2012). Discovery of new medicines or antibiotics from marine flora and fauna are, however, still uncommon. Two success stories are Prialt® (pain reliever) and Yondelis® (anti-tumor drug), which were produced from marine-derived precursors (Rocha *et al*., 2011).

Marine invertebrates, but also algae and bacteria, likely produce chemical compounds for defense against predators (fish, turtles, nudibranchs etc.) as well as for the competition for space with other sessile invertebrates (e.g. coral-coral interactions, coral-sponge interactions, overgrowing marine algae etc.) (McCook *et al*., 2001; Pawlik, 2012). These defensive metabolites are known to be toxic in pharmacological assays, but there is little evidence that specific unpalatable metabolites are toxic. Certain highly toxic metabolites tested in pharmaceutical assays are still quite palatable to fish and mollusks. No relationship was found between palatability and toxicity, but the number of available studies is still limited (Pawlik, 2012; and references therein).

The associated fauna of coral reefs makes up an important part of the diversity on reefs, and ranges from facultative to obligate associations (e.g. Zlatarski and Martínez-Estalella, 1982; Reijnen *et al*., 2010; Stella *et al*., 2011; Hoeksema *et al*., 2012; van der Meij, 2014). Species that live in close association with organisms such as sponges, corals and tunicates have to handle the chemical compounds of their host during their adult life (e.g. feeding), but also during larval settlement (Burke, 1986; Scheltema, 1986). Many obligate associations consist of species that are found on only one or a few species of closely related hosts, hence species-specific settlement cues are likely involved (Pawlik, 1992). The need to adapt to these compounds might be an important force in adaptive radiation and a driver in evolution for host-dependent marine organisms (Pawlik, 2012).

Here we used a quick assay based on light emitting bacteria to quantify the bioactivity of Caribbean anthozoans. Bioactivity is expressed by an EC_{50} value (half maximal effective concentration; expressed in mg/ml); a quantitative measure that indicates how much of a particular crude extract from biological origin is needed to inhibit half of the biological processes of the bacteria. With these EC_{50} values we identified differences in bioactivity between two different orders in the class Anthozoa (Scleractinia and Octo-

corallia), and between the genera and species in these orders. The results of the bioactivity assay were then used to study the link between the relative bioactivity of soft and stony corals and the host selection of associated fauna (false cowries – Ovulidae; gall crabs – Cryptochiridae). Cryptochirids are diminutive crabs that live in dwellings on their hosts, whereas Ovulidae snails roam over the branches of octocorals on which they prey, leaving large feeding scars behind. The snails and crabs are highly dependent on their host corals for survival, and therefore good candidates for a study on the effect of host bioactivity on associated fauna.

Material and methods

Collection and identification of specimens

Stony corals, soft corals and gorgonians, and a sponge sample were collected by SCUBA diving at the leeward site of Curaçao in November 2013. All samples were collected at 0-40 m depth and transported in large plastic bags with seawater to the Carmabi laboratory where the samples were drip-dried and stored in an -18°C freezer for further use. Stony coral identifications were based on Zlatarski and Martínez-Estalella (1982), Humann and DeLoach (2002), Coralpedia (http://coralpedia.bio.warwick.ac.uk) and the reference collections of Naturalis Biodiversity Center. Coral nomenclature was updated following Budd *et al*. (2012). Subsamples of the octocoral specimens were transported to Naturalis Biodiversity Center, where they were identified using Bayer (1961). Tissue of the octocorals was diluted in household bleach and the residual sclerites were washed with tap water and distilled water respectively. Sclerites were mounted on slides with Euparal for examination with an Olympus BX-53F microscope. The taxonomy of Caribbean octocorals is not yet completely resolved (Sánchez *et al*., 2003); hence not all specimens were identified to species level (nine specimens were identified to genus level). Additionally, octocoral samples were also subsampled for molecular analyses for the phylogeny reconstruction presented herein.

Background bioactivity assay

The luminometer method that was used in this study is developed to determine the toxicity of water-soluble samples in wastewater, fresh water and sediments and have been used as such since the 1980s (Chang *et al*., 1981; Dezwart and Slooff, 1983). The marine bacteria used in this assay (*Aliivibrio fischeri* (Beijerinck, 1889)) emit light by respiration. When the *A. fischeri* bacteria are exposed to toxic compounds, their respiration rate lowers or stops and the amount of emitted light decreases. In this study we use crude extracts from Anthozoa to measure the effect on the *A. fischeri* bacteria. By adding crude extracts obtained from stony corals and octocorals the bioactivity can be determined by measuring the decrease in light emission, which consequently is used to calculate the EC_{50} value. The lower the EC_{50} value, the more effect a sample has on the bacteria. Generally, only compounds displaying an $EC_{50} \leq 10.0$ µg/ml are considered, because these values are normally used in literature to ascertain relevant bioactivity (Rocha *et al*., 2011 and citations therein). Here we chose to show all bioactivity data related to the respective coral species.

Bioactivity assay materials and methods

Bioactivity assays on the anthozoan specimens were carried out using a Lumitester PD-20 (Kikkoman) in combination with an Aboatox BioTox™ Kit (1243-500) containing lyophilized *Aliivibrio fischeri* light emitting bacteria. The Lumitester PD-20 is a hand-held portable device, measuring light emission in Relative Light Units (RLU). Before bioactivity analyses can be started a 2% sodium chloride solution has to be prepared, which is used as buffer solution for the bacteria. The provided sodium chloride tablet was dissolved in distilled water (450 ml) to obtain the desired solution. The pH needs to be adjusted to 7 (± 0.2) with either HCl or NaOH. The *A. fischeri* bacteria need to be reconstituted before they can be used in the analysis. This involves adding the cold (4-7°C) reconstitution fluid (provided in the Aboatox BioTox™ Kit) to the bacteria and gently shake/swirl the vial until the solution is homogenous (no vigorous shaking). This reagent needs to stabilize at 15°C for at least 30 minutes. The reagent cannot be kept for long time intervals and should be used within 12 hours if kept at 4-7°C. To check if the bacteria are viable and emitting light at a stable rate, 500 μl of the bacteria solution was placed in a cuvette and measured with the luminometer. The bacteria should preferably emit >700 RLU and no less than 200 RLU. The same sample should be checked after 5 minutes to see if light emission has stabilized. If the RLU values are stable, they can be used for the bioactivity assay.

Samples (crude extracts of MNPs from anthozoans) were prepared in the field by scraping approximately 0.5 gram of soft tissue with a surgical blade from the samples. The amount of tissue was weighed using a digital analytical balance. A volumetric approach would be preferred above the gravimetric method (Pawlik, 2012), but this proved to be too problematic to collect tissue samples for the stony corals that could be used in the volumetric method. Soft tissue samples were ground up in 2 ml ethanol 96% for the stony corals and 3 ml for the soft corals and gorgonians using a mortar and pestle. Consequently this mixture was filtered (using a coffee filter) to obtain a clear solution without calcareous particles. The solution was then concentrated to 500 μl by evaporation and stored in micro vials before analyses (Tables 1-2). For every extracted coral specimen a dilution series was made. Several test runs showed that bioactivity differed greatly between Octocorallia and Scleractinia, hence two different dilution series were made with the 2% sodium chloride solution: 1:10, 1:20, 1:40, 1:80 for the Octocorallia and for the Scleractinia: 1:5, 1:10, 1:20, 1:40 and 1:80. Each cuvette was filled with 500 μl of the stabilized bacteria solution and their starting RLU values were measured and recorded. Consequently, the dilution series of the anthozoan extracts were added (also 500 μl) to their respective cuvettes and incubated for 30 minutes at approximately 15°C. For the negative control samples, 500 μl sodium chloride solution (2%) was added instead of anthozoan extract. All measurements were performed in duplicate. After the incubation period, samples were remeasured to record the light emission after incubation with the extracts. In some cases, the extracts proved to be hardly affecting the bacteria or were so toxic that the dilution series had to be adapted to measure their EC_{50} values.

Starting RLU values and values after incubation were entered into a spreadsheet (provided by Aboatox) to calculate the decrease of RLU and test whether the duplicate analyses were statistically sound (SD < 3% inhibition). Inhibition and concentration

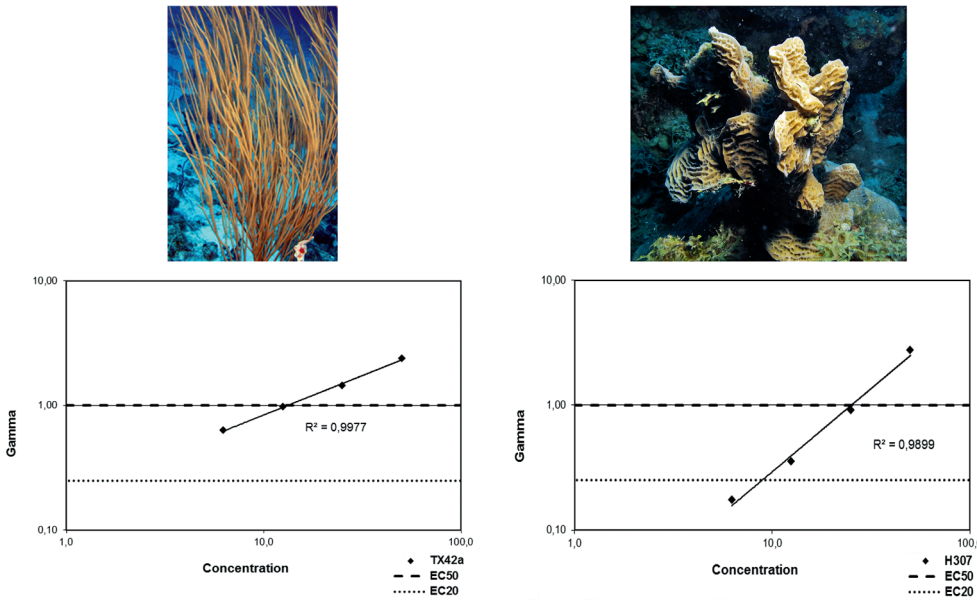

Fig. 1. Graphs showing anthozoan bioactivity data used for EC_{50} calculations: (left) the gorgonian *Ellisella barbadensis* (sample TX42a) and (right) the scleractinian coral *Agaricia agaricites* (sample H307). Gamma indicates log-transformed inhibition; concentration refers to the dilution series.

values were log transformed and consequently a fitted line of the linear regression was used to identify the EC_{50} values (Fig. 1).

Phylogeny reconstructions and host specificity

To study if there is a link between the bioactivity of the host corals and the phylogenetic relationships between the host species, phylogeny reconstructions were made of the host species. The octocoral phylogeny reconstruction was based on four markers (28S, COI, mtMutS and ND6) and the scleractinians on three markers (12S, CytB and COI). Octocorallia were sequenced at the Naturalis Barcoding lab, whereas the Scleractinia sequences were downloaded from GenBank (see supplementary material for the accession numbers).

A Maximum Likelihood (ML) analysis based on the GTR+I+G (Octocorallia) and HKY+G (Scleractinia) models were conducted in MrBayes (Ronquist and Huelsenbeck, 2003) (Octocorallia) and MEGA6 (Scleractinia) to infer phylogeny reconstructions for the host species. Both phylogeny reconstructions do not fully reflect the overall known relationships between corals (e.g. Fukami *et al*., 2004; Huang 2012; McFadden *et al*., 2006), for example due to paraphyletic relationships within the Caribbean (octo)corals and the relationships with Indo-Pacific corals. The phylogeny reconstructions are only used for the purpose of comparing the bioactivity of the Caribbean corals with symbiotic snails and crabs. Host data for the Ovulidae was based on Lorenz and Fehse (2009) and Reijnen *et al*., (2010), the host relations of the gall crabs were taken from a study by

van der Meij (2014). These associations were plotted on the phylogram to check if there is a relation with the bioactivity of the coral hosts.

Results

In total 77 specimens were analyzed, consisting of 52 Octocorallia, 24 Scleractinia and one Porifera sample. An overview of the measured ranges of toxicity values per genus shows that in general the Octocorallia have higher levels of bioactivity than the Scleractinia (Fig. 2). This is supported by the average EC_{50} calculated per anthozoan group. For the Scleractinia the average EC_{50} is 40.93 mg/ml (SD +/- 27.17), which is notably higher (= less bioactive) than the 10.17 mg/ml (SD +/- 8.13) average for the Octocorallia. Interestingly, some scleractinian corals have EC_{50} values close to those of some octocoral species. The sponge sample was among the most toxic samples in this study (EC_{50} = 1.62 mg/ml).

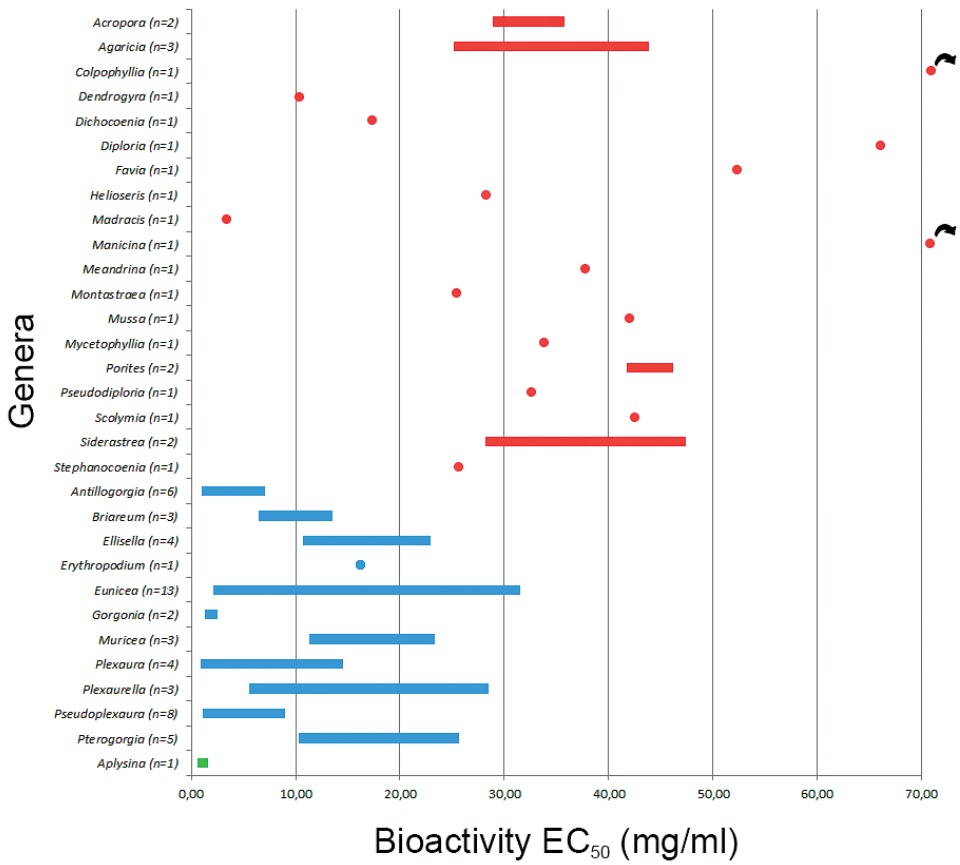

Fig. 2. Floating bar chart depicting the measured minimum and maximum EC_{50} values per genus (red = Scleractinia; blue = Octocorallia; green = Porifera). A circle shows a single data point. The EC_{50} value of *Colpophyllia* is 118.08 mg/ml and *Manicina areolata* is 117.18 mg/ml (off the chart).

Soft corals and gorgonians (Octocorallia)

Eleven genera of octocorals, comprising at least 22 species, were subjected to the bioactivity assay with the luminometer. Per species one to three specimens were analyzed (Table 1). It was observed that values between octocoral specimens of a single species had quite variable EC_{50} values, especially in the genera *Eunicea*, *Muricea* and *Plexaura* (Table 1, Fig. 2). It is therefore difficult to pinpoint the most bioactive octocoral species. The lowest EC_{50} value (most bioactive) for all octocoral specimens measured was 0.85 mg/ml for one of the *Plexaura homomalla* specimens. When the average bioactivity value was calculated over all *P. homomalla* specimens, the 'common sea fan' *Gorgonia ventalina* (n=2) turned out to have the lowest average EC_{50} value (1.91 mg/ml). The highest EC_{50} value (least bioactive) measured was 31.54 mg/ml for a specimen of *Eunicea mammosa*, but when EC_{50} values were averaged per species *Pterogorgia citrina* (n=3) had the highest EC_{50} value (23.81 mg/ml). However, the coefficient of determination (R^2) was low for *Plexaura homomalla* and might therefore be influencing the final EC_{50} value (Table 1). When samples with low R^2-values are not taken into account, *Pseudoplexaura* sp. and *G. ventalina* are the most bioactive species.

Differences in the average levels of bioactivity between coral genera were observed. The average EC_{50} values of unrelated *Antillogorgia* and *Pseudoplexaura* are low (ca. 4 mg/ml) when compared with *Pterogorgia* spp. (ca. 20 mg/ml). The average of all Octocorallia specimens in this study is 10.17 (SD +/- 8.13) mg/ml. Some species for which multiple specimens were analyzed showed a wide range of bioactivity. For example in *Eunicea flexuosa* EC_{50} values ranged from 2.5 to 17 mg/ml (Tables 1-2; Fig. 2).

Table 1. Overview of EC_{50} values and other metadata for Octocorallia samples analyzed in this study.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
Anthothelidae	*Erythropodium caribaeorum* (Duchassaing & Michelotti, 1860)	16.71	0.500	0.922
Briareidae	*Briareum asbestinum* (Pallas, 1766)	13.56	0.500	0.950
	Briareum asbestinum (Pallas, 1766)	9.31	0.500	0.827
	Briareum asbestinum (Pallas, 1766)	6.43	0.504	0.887
Ellisellidae	*Ellisella barbadensis* (Duchassaing & Michelotti, 1864)	11.97	0.513	0.962
	Ellisella barbadensis (Duchassaing & Michelotti, 1864)	13.19	0.492	0.998
	Ellisella barbadensis (Duchassaing & Michelotti, 1864)	10.72	0.505	0.837
	Ellisella elongata (Pallas, 1766)	22.89	0.500	0.892
Gorgoniidae	*Antillogorgia acerosa* (Pallas, 1766)	7.10	0.505	0.918
	Antillogorgia americana (Gmelin, 1791)	0.96	0.515	0.659
	Antillogorgia americana (Gmelin, 1791)	6.86	0.499	0.964
	Antillogorgia americana (Gmelin, 1791)	1.66	0.500	0.943
	Antillogorgia bipinnata (Verril, 1864)	5.36	0.509	0.844
	Antillogorgia bipinnata (Verril, 1864)	2.87	0.507	0.791

Table 1. Cont.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
	Pterogorgia citrina (Esper, 1792)	25.70	0.497	1.000
	Pterogorgia citrina (Esper, 1792)	23.46	0.502	0.883
	Pterogorgia citrina (Esper, 1792)	22.27	0.503	0.993
	Pterogorgia guadelupensis (Duchassaing & Michelin, 1846)	10.32	0.500	0.970
	Pterogorgia guadelupensis (Duchassaing & Michelin, 1846)	16.18	0.503	0.898
Plexauridae	*Eunicea calyculata* (Ellis & Solander, 1786)	8.14	0.502	0.936
	Eunicea calyculata (Ellis & Solander, 1786)	7.63	0.509	0.888
	Eunicea clavigera Bayer, 1961	12.39	0.508	0.911
	Eunicea clavigera Bayer, 1961	6.33	0.506	0.916
	Eunicea flexuosa (Lamouroux, 1821)	13.66	0.503	0.971
	Eunicea flexuosa (Lamouroux, 1821)	2.49	0.505	0.990
	Eunicea flexuosa (Lamouroux, 1821)	16.99	0.511	0.992
	Eunicea fusca Duchssaing & Michelotti, 1860	11.86	0.507	0.942
	Eunicea mammosa Lamouroux, 1816	31.54	0.504	0.906
	Eunicea pinta Bayer & Deichmann, 1958	0.00	0.497	0.588
	Eunicea succinea (Pallas, 1766)	2.15	0.504	0.932
	Eunicea succinea (Pallas, 1766)	2.15	0.518	0.958
	Eunicea succinea (Pallas, 1766)	5.06	0.508	0.963
	Gorgonia ventalina Linnaeus, 1758	2.48	0.513	0.964
	Gorgonia ventalina Linnaeus, 1758	1.34	0.496	0.986
	Muricea muricata (Pallas, 1766)	11.29	0.503	0.915
	Muricea muricata (Pallas, 1766)	23.35	0.504	0.828
	Muricea pinnata Bayer, 1961	12.05	0.511	0.986
	Plexaura homomalla (Esper, 1792)	7.15	0.505	0.944
	Plexaura homomalla (Esper, 1792)	0.85	0.505	0.600
	Plexaura homomalla (Esper, 1792)	4.64	0.513	0.980
	Plexaura nina Bayer & Deichmann, 1958	14.69	0.498	0.995
	Plexaurella dichotoma (Esper, 1791)	28.50	0.502	0.898
	Plexaurella dichotoma (Esper, 1791)	5.50	0.504	0.898
	Plexaurella dichotoma (Esper, 1791)	26.80	0.497	0.771
	Pseudoplexaura flagellosa (Houttuyn, 1772)	2.02	0.499	0.937
	Pseudoplexaura porosa (Houttuyn, 1772)	8.99	0.498	0.910
	Pseudoplexaura sp.	4.84	0.497	0.925
	Pseudoplexaura sp.	3.24	0.501	0.970
	Pseudoplexaura sp.	4.22	0.504	0.944
	Pseudoplexaura sp.	5.26	-	0.524
	Pseudoplexaura sp.	1.07	0.518	0.717
	Pseudoplexaura sp.	3.28	0.503	0.958

Table 2. Overview of EC_{50} values and other metadata for scleractinian corals analyzed in this study.

Family	Species (author)	EC_{50}	Wet tissue weight (gram)	R^2
Acroporiidae	*Acropora cervicornis* (Lamarck, 1816)	35.74	0.506	0.754
	Acropora palmata (Lamarck, 1816)	28.87	0.506	0.914
Agariciidae	*Agaricia agaricites* (Linnaeus, 1758)	25.15	0.506	0.990
	Agaricia lamarcki Milne Edwards & Haime, 1851	25.89	0.506	0.980
	Agaricia lamarcki Milne Edwards & Haime, 1851	43.90	0.515	0.932
	Helioseris cucullata (Ellis & Solander, 1786)	28.74	0.511	0.937
Astrocoeniidae	*Madracis auretenra* Locke, Weil & Coates, 2007	3.95	0.490	0.935
	*Stephanocoenia intersepta (*Lamarck, 1836*)*	26.09	0.495	0.987
Montastraeidae	*Montastraea cavernosa* (Linnaeus, 1767)	25.98	0.504	0.990
Meandrinidae	*Dendrogyra cylindrus* Ehrenberg, 1834	10.82	0.503	0.989
	Dichocoenia stokesi Milne Edwards & Haime, 1848	17.86	0.508	0.970
	Meandrina meandrites (Linnaeus, 1758)	38.19	0.499	0.997
Mussidae	*Colpophyllia natans* (Houttuyn, 1772)	118.08	0.495	0.953
	Diploria labyrinthiformis (Linnaeus, 1758)	66.64	0.497	0.989
	Favia fragum (Esper, 1795)	52.88	0.506	1.000
	Manicina areolata (Linnaeus, 1758)	117.18	0.506	0.400
	Mussa angulosa (Pallas, 1766)	42.40	0.498	0.989
	Mycetophyllia sp.	34.36	0.500	0.971
	Pseudodiploria clivosa (Ellis & Solander, 1786)	33.10	0.510	1.000
	Scolymia sp.	43.02	0.510	0.985
Poritidae	*Porites* cf. *divaricata* Le Sueur, 1820	41.71	0.511	0.980
	Porites porites (Pallas, 1766)	46.23	0.507	1.000
Siderastreidae	*Siderastrea siderea* (Ellis & Solander, 1786)	28.18	0.493	0.990
	Siderastrea siderea (Ellis & Solander, 1786)	47.45	0.501	1.000

Stony corals (Scleractinia)

Seventeen genera comprising 21 species (two specimens were not identified to species level) were analyzed. EC_{50} values varied greatly between species and ranged from 3.95 to 118.08 mg/ml (*Madracis auretenra* and *Colpophyllia natans*, respectively). Most stony coral species had EC_{50} values between ca. 25 and 45 mg/ml (average is 40.93 +/- 27.17 mg/ml). The least bioactive family appears to be the Mussidae, which is one of the largest Caribbean coral families. *Madracis auretenra* has the lowest EC_{50} value (3.95 mg/ml), but *Stephanocoenia intersepta* from the same family (Astrocoeniidae) does not appear to be very bioactive (26.09 mg/ml). In contrast to the Octocorallia, there are no clear similarities in EC_{50} values between families or genera (Table 2; Fig. 2).

As with the Octocorallia, we observed a range of bioactivity when multiple specimens of a single species were analyses. For *Agaricia lamarcki* and *Siderastrea siderea* the EC_{50} values ranged from 25.89 to 43.90 and 28.28 to 47.45, respectively (Table 2; Fig. 2).

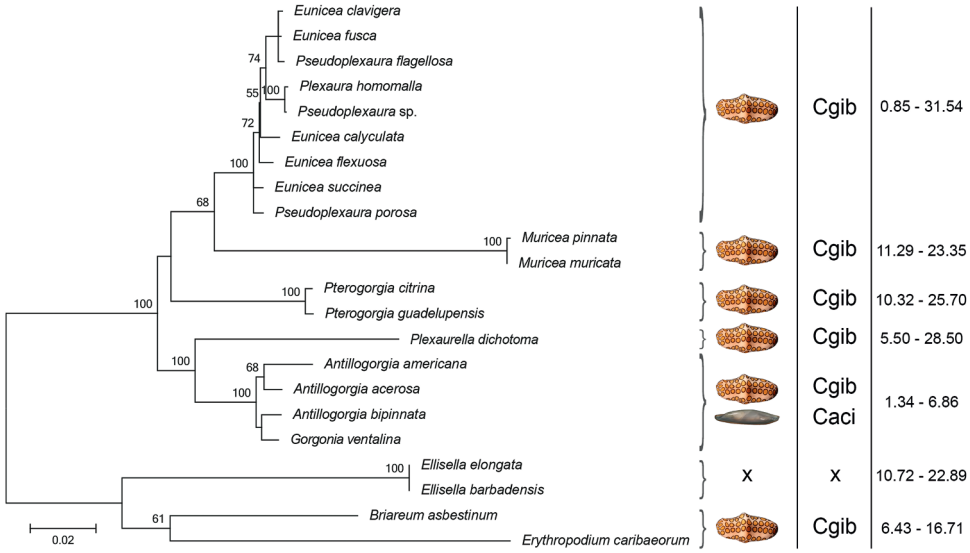

Fig. 3. Phylogeny reconstruction of Caribbean Octocorallia used in this study, including ovulid symbionts (Cgib = *Cyphoma gibbosum*; Caci = *Cymbovula acicularis*) and the octocorallian EC_{50} ranges as recorded in this study. Phylogeny reconstruction based on Bayesian inference analysis, bootstrap values > 50 are shown.

Sponge (Porifera)

For comparison with the cnidarian samples we also collected a specimen of the tube sponge *Aplysina archeri* (Higgin, 1875) as an outgroup. *Aplysina archeri* is highly bioactive with an EC_{50} value of 1.62 mg/ml ($R^2 = 0.992$).

Host specificity – Ovulidae

Cymbovula acicularis is a specialist predator associated with the bioactive octocoral genera *Antillogorgia* and *Gorgonia*, whereas the generalist species *C. gibbosum* is associated with a wide range of octocoral hosts, including the latter two octocoral genera (Fig. 3; Reijnen *et al*., 2010; Chapter 2).

By plotting the bioactivity values and host associations for the two symbiotic ovulid snails on a phylogram it becomes clear that there is no clear connection between these variables (Fig. 3). In fact, the two most toxic octocoral genera (*Antillogorgia*, *Gorgonia*) - which are closely related - have two instead of one ovulid symbiont (Fig. 3). The EC_{50} values as determined for *Ellisella* species are almost equal to the values of the genus *Muricea*, and in comparison with the other Octocorallia *Muricea* and *Ellisella* do not show high levels of bioactivity. *Ellisella* is the only genus included in this study that does not serve as a host for ovulid snails in the Caribbean, whereas *Muricea* is encountered with symbiotic *C. gibbosum* snails.

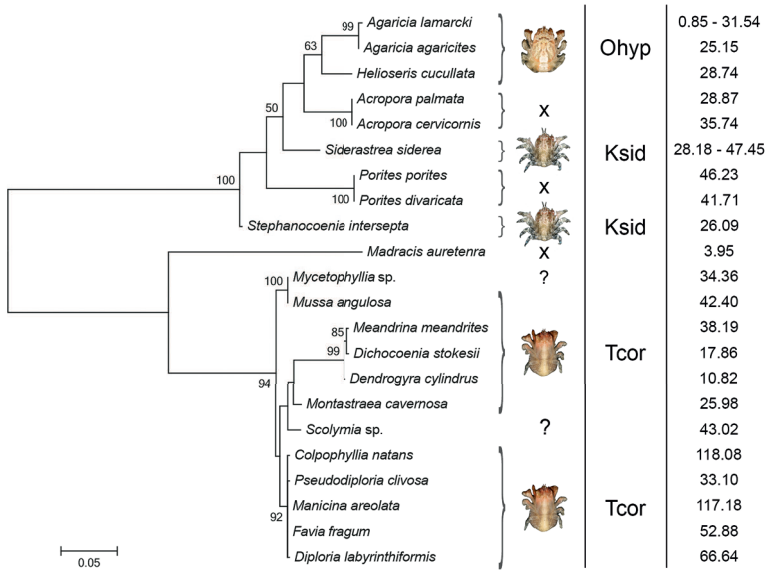

Fig. 4. Phylogeny reconstruction of the Caribbean stony corals used in this study, incl. their recorded cryptochirid symbionts (Ohyp = *Opecarcinus hypostegus*; Ksid = *Kroppcarcinus siderastreicola*; Tcor = *Troglocarcinus corallicola*; based on van der Meij, 2014) and the scleractinian EC_{50} values as recorded in this study. Question marks represent coral species for which gall crab specimens are recorded in literature (not found off Curaçao). Phylogeny reconstruction based on ML analysis, bootstrap values ≥ 50 are shown.

Host specificity – Cryptochiridae

Three shallow-water gall crabs species are associated with Caribbean stony coral hosts. *Opecarcinus hypostegus* inhabits corals of the family Agariciidae; *Kroppcarcinus siderastreicola* inhabits hosts of the genera *Siderastrea* and *Stephanocoenia*, and *Troglocarcinus corallicola* inhabits corals of a wide range of species - but does not occur in the former hosts. These associations were plotted on the phylogeny reconstruction of the Caribbean stony corals (Fig. 4). The most bioactive stony coral from this study (*Madracis auretenra*) is not a known host species for gall crabs.

Discussion

Bioactivity assay method

The luminescence bioassay has been used for some decades and provides scientist with empirical data on the bioactivity of compounds and products in a fast and easy way (Becerro *et al*., 1995; Marti *et al*., 2005). When using this bioactivity assay to determine EC_{50} values for eco-evolutionary research, three matters have to be considered.

Firstly, most marine invertebrates that act as hosts (e.g. corals, sponges, sea anemones) for associated fauna have zooxanthellae and bacteria in their tissue (Shnit-Orland and Kushmaro, 2013; Correa *et al*., 2013). The crude extract method therefore may

contain any possible secondary metabolites produced by these organisms. The exact degree of bioactivity of cnidarian species can therefore not be determined, as long as the source of the MNPs is unknown. It is likely that the coral tissue samples contained a mixture of compounds from different species groups, even beyond the animal kingdom. This cannot easily be overcome by any bioactivity assay.

Secondly, the density of calcareous skeletal parts from the stony corals and octocorals have effect on the amount of tissue containing MNPs subjected to the extraction. Pawlik (2012) mentions that calcite densities can differ per individual or even between locations in an individual. By using a volumetric approach instead of a gravimetric method the difference in skeletal tissue vs. actual tissue containing MNPs can be eliminated. This would be relatively easy for the octocorals, but proves to be almost impossible for the stony corals as most species have thin tissue layers. We therefore decided, despite the calcite density differences, to use the gravimetric method.

Thirdly, the bioactivity is measured on bacteria and not on the predators and associated fauna of the anthozoans, which are primarily vertebrates (fishes) and invertebrates (i.e. mollusks, crustaceans, worms). Since testing is performed on bacteria, some care has to be taken when extrapolating the results to the actual model organism. On the other hand, testing with bacteria also has the advantage of not becoming involved in any regulations for animal testing.

Bioactivity assay

As expected, soft corals are more bioactive that stony corals (Rocha *et al*., 2011), however, some stony coral species were more bioactive than certain soft coral species. When more specimens per species were analyzed for both the Scleractinia and the Octocorallia we observed that there is high intraspecific variety within species based on their respective EC_{50} values (Tables 1-2; Fig 2).

Octocorallia – It was not unexpected that *Gorgonia* and *Antillogorgia* are amongst the most bioactive octocoral genera. Pharmaceutical/chemical studies already focused on compounds obtained from these genera in their search for potential medicines because of high antimicrobial activity (Fenical, 1987). Both genera did not show a wide range of bioactivity whilst specimens from the genus *Eunicea* showed high variability in EC_{50} values and were amongst the most and least bioactive samples analyzed in this study. This fluctuating bioactivity can be the result of the differences in bioactive compounds from different tissue samples. Harvell and Fenical (1989) measured the bioactivity values in a single specimen between the polyps and the coenenchym of some Caribbean octocoral species (*Pseudopterogorgia = Antillogorgia*) and between the proximal and distal end of colony branches. They found that specific compounds observed in *Antillogorgia* could not be found in the coenenchym but were abundant in the polyp tissue or were found in significant higher doses at the distal end of a branch than at the colony base (which also harbors fewer polyps). Pawlik (2012), however, questions this result because a gravimetric method was used to weigh the animal tissue instead of a volumetric method, which would level out the variation in bioactivity values due to the effect of sclerite density vs. MNP containing tissue in the octocoral samples. As a result, it remains inconclusive what caused the

large variation observed between different specimens of the same species in some of the samples studied herein.

The variation in bioactivity among octocoral genera does not seem to reflect a phylogenetic relationship. Genera with similar EC_{50} values, for example *Antillogorgia* and *Gorgonia*, are phylogenetically closely related. In contrast, *Pseudoplexaura* clusters between *Plexaura* and *Eunicea*, which are not directly related to the genera *Antillogorgia* and *Gorgonia* (Fig. 3; Sánchez *et al*., 2003). Yet, the support values for the sister clades with *Muricea* and *Pterogorgia* species are not or moderately supported, which could indicate that species are actually more closely related than previously thought. The phylogenetic relationship between *Eunicea*, *Plexaura* and *Pseudoplexaura* is also unresolved. Recent molecular work by Lau *et al*., (unpubl. data) show that these genera cannot be separated easily based on four molecular markers and morphologically these genera are challenging to separate. We therefore analyzed the data generated for these genera as a single monophyletic group (Fig. 3). By grouping the data more genus-specific data might get lost, but future phylogenetic studies may reveal that only a single genus is involved.

Scleractinia – Scientific studies dealing with the bioactivity of scleractinians are a lot sparser than those available for Octocorallia and Porifera. The configuration of compounds obtained from *Tubastraea aurea*, *Cladocora cespitula* and *Tubastraea* sp. have been analyzed (Fusetani *et al*., 1986; Fontana *et al*., 1998; Marti *et al*., 2005; Meyer *et al*., 2009). Gunthorpe (1991) performed the first elaborate bioactivity assessment on scleractinian corals from different coral families at Heron Island, Australia. Additionally, Gunthorpe and Cameron (1990a, b) also investigated the intraspecific and intracolonial differences in bioactivity of scleractinian corals. Their findings of fluctuating EC_{50} values, within a nominal species, complement our findings.

Porifera – We only analyzed only one sample, which is among the most bioactive samples measured in this study.

Host specificity

Host species specialization is considered strong among insects, but not so much for marine invertebrates (Pawlik, 2012). This is primarily caused by the different life histories of insects and marine invertebrates. Insects such as butterflies have multiple generations in a season and have non-dispersive larvae, which are usually deposited by the adult on their host plant. In contrast, marine invertebrates have generation times measured in years and have larvae that are often pelagic and do disperse, sometimes over large distances. As a result, the evolutionary processes in insects are probably accelerated and more intense, when compared to most marine invertebrates (Pawlik, 2012). In the marine realm host specificity research between marine invertebrates is still scarce and only a few examples of tight associations are currently known (Lanterbecq *et al*., 2010; van der Meij, 2015). However, it is likely that many (marine) species associations still need to be discovered and that similar associations as described for insects will be revealed for (some) marine invertebrates.

Ovulidae – By plotting the host-symbiont data of the ovulids and the bioactivity data of the Octocorallia on the octocoral phylogeny it is shown that species symbiosis is not

based on the bioactivity of compounds in the host corals (Fig. 3). The octocoral genera with the lowest EC_{50} values are host to both ovulid species whereas one of the least bioactive genera, *Ellisella*, has no known ovulid symbionts in the Caribbean. *Ellisella* has similar-sized sclerites (approx. 0.10 mm) as *Gorgonia* which shows that structural defense is not the source for their lack of symbionts. Nonetheless, in the Indo-Pacific the family Ellisellidae is the most common host family for ovulid snails of the subfamily Aclyvolvinae (Lorenz and Fehse, 2009; Chapter 5).

One of the most iconic octocoral predators in the Caribbean is *Cyphoma gibbosum*. This brightly conspicuously coloured Caribbean snail has been found predating on a large number of different octocoral species (Lorenz and Fehse, 2009; Reijnen *et al*., 2010) and is considered a common generalist predator of Octocorallia. Another Caribbean ovulid is *Cymbovula acicularis*. This species is inconspicuous and is so far only found on the genera *Gorgonia* and *Antillogorgia* (Humann and Deloach, 2002; Lorenz and Fehse, 2009; Reijnen *et al*., 2010). The most bioactive octocoral genera in our assay (*Gorgonia* and *Antillogorgia*) are known to contain deterrent compounds against fishes and *C. gibbosum* (Van Alstyne and Paul, 1992). They used *C. gibbosum* in a feeding assay to test the chemical properties and structural defense of *Gorgonia ventalina*. Besides the repellent compounds in the crude extract of *G. ventalina* they also investigated the effect of *G. ventalina* sclerites (structural defense) in this assay. Their results showed that feeding was reduced by half when sclerites or crude extracts were introduced in the feeding pellets to *C. gibbosum* and the effect was even greater in fishes. Nevertheless, the deterrent effect by the crude extracts or the structural defense by sclerites does not seem hinder predation by *C. gibbosum* on *G. ventalina* in nature (Reijnen *et al*., 2010: Fig. 3). This is most probably because *C. gibbosum* can adapt to the chemical compounds due to biotransformation enzymes such as cytochrome P450 (Wahlen *et al*., 2010).

Cryptochiridae – As with the Octocorallia there is no clear connection between the coral phylogeny, bioactivity of the host and the symbiont fauna. Three shallow-water gall crabs species are associated with Caribbean stony coral hosts. Specialist species *Opecarcinus hypostegus* inhabits Agariciidae corals, whereas *Kroppcarcinus siderastreicola* inhabits the genera *Siderastrea* and *Stephanocoenia*. *Troglocarcinus corallicola* is a generalist species and inhabits a wide range of hosts (Fig. 4). The most bioactive stony coral from this study (*Madracis auretenra*) is not a known host of gall crabs (Kropp and Manning, 1987; van der Meij, 2014). It is unclear whether this is due to the presumed toxicity, the thin-branched, dendritic structure of the stony coral or the evolutionary history between the corals and crabs. Other genera that are not inhabited by gall crabs are *Acropora* and *Porites*. Caribbean *Acropora* is host to the xanthid crab *Domecia acanthophora* (Desbonne, in Desbonne and Schramm, 1867*)* that inhabit various types of structural deformations, somewhat similar to those of gall crabs (Patton, 1967). The genera *Madracis* and *Acropora* where, however, listed by Scott (1987) to be amongst the most hospitable corals, together with *Siderastrea* and *Agaricia lamarcki*. These species are ranked high on Lang's (1973) aggression hierarchy, used by Scott (1987), which is based on extracoelenteric digestion (Lang, 1973; Kropp, 1988). The absence of cryptochirid crabs in *Madracis* and *Acropora* is, therefore, likely linked to

other factors than the aggression or bioactivity of the host. In Scleractinia, presence of associates is significantly inversely correlated with polyp size according to Scott (1987). In his study, he included a wide range of infaunal associates of living coral, and many polychaetes, sipunculids and certain barnacles appear to be more common in (small-polyped) *Porites*.

Host specificity of associated fauna is perceived to be more specific in the Indo-Pacific (Stella *et al*., 2011), and hence the next step is to apply the described bioactivity assay on specimens from the Indo-Pacific to test whether the bioactivity of the host organism plays a role in the diversity of associated fauna.

Acknowledgements

We are grateful to Juha Lappalainen from Aboatox for his help in for setting up the bioactivity assay for anthozoans. The CARMABI Research Station and DiveVersity Curaçao supported this research. For financial support we would like to thank the TREUB-maatschappij (Society for the Advancement of Research in the Tropics), and Academy Ecology Fund (formerly KNAW Fonds Ecologie). For reading and commenting on the manuscript we thank Bert Hoeksema and Edi Gittenberger (Naturalis). Leen van Ofwegen (Naturalis) assisted with the Octocorallia identifications and commented on the manuscript. Rob van Soest (Naturalis) is acknowledged for confirming the identity of the sponge sample.

Suppl. mat. GenBank accession numbers for the sequences used in the phylogeny reconstruction of the Scleractinia (Fig. 4), n/a = not available.

	COI	CytB	12S
Acropora cervicornis	AY451340	AF099654	EF597094
Acropora palmata	AY451341	AB441331	EF597092
Agaricia agaricites	AY451366	n/a	EF597080
Agaricia lamarcki	AY451369	n/a	EF597076
Colpophyllia natans	AB117228	AB117306	EF596998
Dendrogyra cylindrus	AB117299	AB117384	EF597024
Dichocoenia stokesii	AY451360	AB117383	EF597020
Diploria labyrinthiformis	AB117224	AB117302	EF597002
Favia fragum	AB117223	AB117301	EF597005
Helioseris cucullata	AB441220	AB441305	n/a
Madracis mirabilis	AB441227	NC011160	NC011160
Manicina areolata	AB117227	AB117305	EF597012
Meandrina meandrites	AB117296	AB117381	EF597032
Montastraea cavernosa	AF108712	AB117374	EF597007
Mussa angulosa	AB117239	AB117316	EF597011
Mycetophyllia aliciae	AB117235	AB117312	EF597039
Porites divaricata	AY451381	n/a	EF597058
Porites porites	AY451384	DQ643837	EF597056
Pseudodiploria clivosa	AB117226	AB117304	EF597001
Scolymia sp.	AB117248	AB117325	n/a
Siderastrea siderea	AB441211	AB441296	EF597067
Stephanocoenia intersepta	AB441229	AB441313	EF597073