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Nephthea sp.: Correlation Between Natural Products Production and Pressure from Local Environmental Stressors

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Abstract

The presented study looked in a preliminary and speculative way for a correlation between production by a single soft coral species, *Nephthea* sp., of two secondary metabolites and local environmental stresses, pollution, around the Seribu Islands, Indonesia. The study region was selected because of the significant land-based run-off from the heavily populated Indonesian capital, Jakarta. A *Nephthea* sp., was chosen based on the well known bioactive cembranoid diterpene content of animals of the genus that is often involved in protecting them from predation. For the study, levels of the recently reported 3,4-epoxy-nephthenol acetate (**1**) and the previously known 15-hydroxy-cembrane (**2**) were monitored. Results showed levels of **1** and **2** to decrease as pollutants increased. A contour map of levels of **1** and **2** showed a likely different pattern of their occurrence, with **2** being found more in polluted, higher stress, areas and **1** likely only to occur in areas with low environmental stresses. It is postulated that low levels of anthropogenic stress, pollution, in the reef environment result in no inhibition of biodiversity and number of fishes that prey on *Nephthea* sp., likely causing enhanced secondary metabolite production in these species in order to protect them from predation. This may mean levels and/or presence or absence of **1** might be indicators of pollution entering a coral reef area, with the resultant threat to a reef's potential biopotency. Results also show where anthropogenic pollution increases in a reef system the overall level of biodiversity drops, as a consequence so does chemical diversity and with it the chance of discovering new and biological active secondary metabolites.

Keywords: *Nephthea* sp.; Cembranoids; Diterpenes; Secondary Metabolites; Environmental Stressors; Pollution; Seribu Islands; Indonesia

Introduction

In biotechnology and natural products research terms, coral reef communities are seen as one of the best sources of potentially new chemical entities that may have useful biological properties. Increasing anthropogenic pressures, pollution, and global warming are causing considerable damage to coral reef structures through direct and indirect means which leads to the conclusion that coral reef biodiversity is generally under threat [1]. The loss, reduction or unexpected appearance of any species to a natural system can change the overall dynamic of that system dramatically, in turn causing more and more change in that system and typically not in a positive way [2,3]. In other words, when populations are depleted genetic variation is reduced which in turn reduces an organisms' ability to adapt to new environmental changes and stresses [4]. And, due to the interdependencies among species, the demise of one can lead to the decrease or demise of others. Eventually this leads to either significant reduction or loss of biodiversity. Another way to look at this kind of change, particularly significant loss of species, is in terms of the likelihood that natural chemical diversity, or better said, natural products diversity, and everything this implies, that currently exists in reef communities is also threatened. The most obvious examples of this type of change would be through the loss of habitat, appearance of an invasive species [5] or rapid population growth of a predatory species [3] that may stop or enhance the establishment of certain sessile organisms like algae, soft corals and sponges, while allowing others to grow and thrive, while others die. These balanced predator prey relationships are key to the overall health and diversity of almost all living systems and dramatic external changes in these can be catastrophic [5]. Some species require challenges to stimulate the production of certain chemical defense substances [6,7], and if these challenges are not present the substances are simply not produced, this is known as an inducible or plastic response compared to a constant response where substances are produced continuously [8,9]. If the induced substances are not produced chemical diversity decreases and at some point the production of certain substance may stop altogether in some species. These negative responses may also occur as a result of the change of local conditions [10,11]. Thus, loss of predatory fish through overfishing or loss of other competitive organism in some other way, for example, by an environment becoming polluted with excessive amount of nutrients [12] will result in not just the reduction and or loss of biodiversity but also in the reduction and or loss of chemical diversity. Based on this premise, that local environmental stress, pollution, may affect chemical diversity, the research undertaken and reported in this current paper documents in a general and speculative way a possible relationship between natural product diversity in coral reef organisms and local environmental stresses placed on a given environment.

Materials and Methods

Study site and organism selection

The Seribu Islands is one of Indonesia's small island areas located in DKI (Special Capitol District) Jakarta province. Because of the islands' location close to the Indonesian capital, Jakarta, the marine

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habitat around these islands has local environmental stressors from land-based industrial and heavily populated domestic run-off that first enters Jakarta Bay and then flows out around them. In 2004, metal contaminants from Jakarta Bay were already considered to be above normal levels for marine organisms. At this time, the so called red tide phenomenon also occurred several times in Jakarta Bay as the concentration of phosphate and nitrate ions were high in that area [13]. In the Seribu Islands themselves there were also local domestic run-off and fish aquaculture conditions that could possibly trigger or be responsible for the high levels of phosphate and nitrate ions measured in the surrounding waters. In simple visual terms the amount of observable (July 2009) solid waste of all kinds flowing from Jakarta Bay to the Seribu Islands can only be described as significant. Based on these observations the Seribu Islands were chosen as the site for the current study. The organisms selected for investigation were *Nephthea* sp., based on their common occurrence on local coral reefs around these islands and their apparent successful deterrence of predators [14,15]. This successful defense strategy of soft corals is usually correlated with a defensive chemical anti-predatory system based on bioactive natural products known as terpenes, specifically cembranoid diterpenes [7,16]. The cembranoids focused on in this study were the recently reported 3,4-epoxy-nephthenol acetate (**1**) and the known 15-hydroxy-cembrane (**2**) [17].

Animal Material

All *Nephthea* sp., samples were collected on the basis of their morphological similarities to the sample used in the recently reported investigation of *Nephthea* sp. [17]. All samples were indentified on the basis of their overall morphological and spicule profiles. The samples' profiles were compared with the morphology and spicules profile database of soft corals [18]. From the profiles of sample spicules, all collected samples were identified as belonging to a single species within the genus *Nephthea*. Full taxonomic assignment of this likely new species will be the subject of a future discrete taxonomic investigation.

Samples were collected from twelve locations ranging from southern to northern Seribu Islands, Indonesia, during the period April 19-22, 2010. Precise locations were recorded using a GPS; Garmin GPSMAP 60CSXA. A plot of each site was made on maps from Holtrof [19], and are shown in Figure 1(a). At each site, at 5m depth, a 30m transect was laid based on established line methods [20] to estimate live coral cover. From each 10m section of the 30m line transect, a sample of the soft coral *Nephthea* sp., was taken giving a total of three from each transect. The fresh coral sample (ca. 5g) was immediately placed

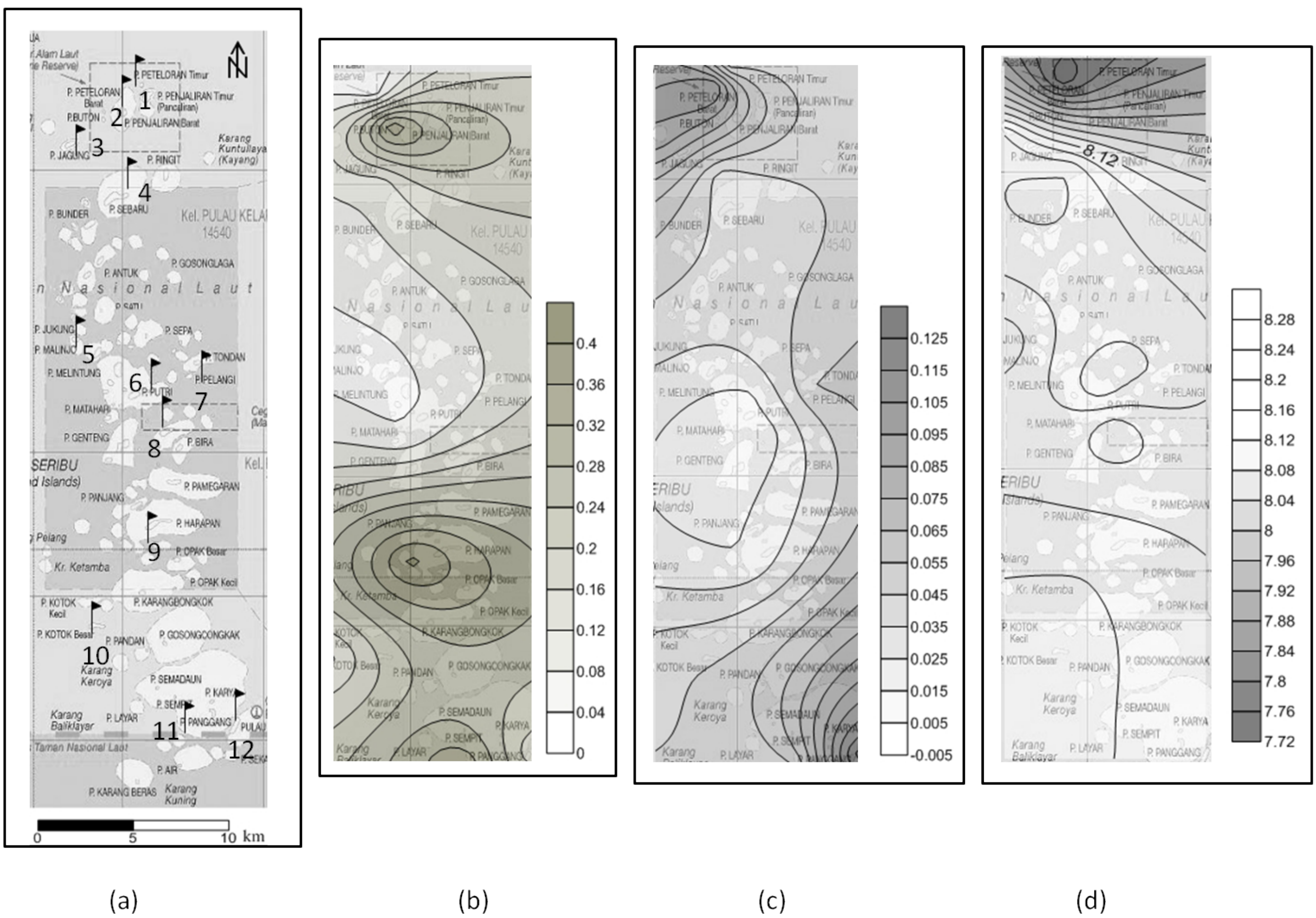

Figure 1: (a) Map of sampling sites in the Seribu Islands, and contour maps of; (b) active phosphate in ppm (c) inorganic nitrogen in ppm (d) pH

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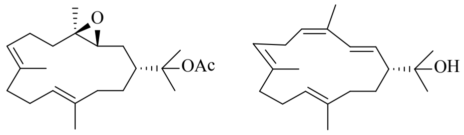


1 **2**

Figure 2: Cembranoid diterpenes quantified from *Nephthea* sp., collected from Seribu Islands; 3,4-epoxy-nephthenol-acetate (**1**) and 15-hydroxy-cembrane (**2**).

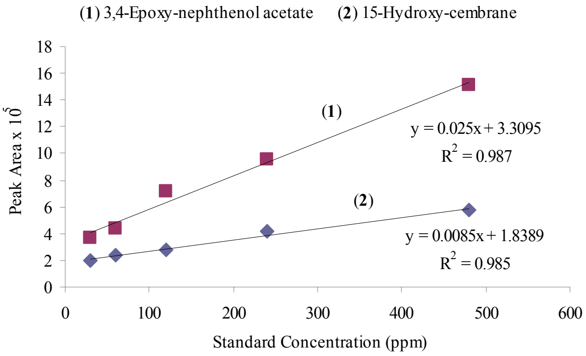


Figure 3: Regression curves of 3,4-epoxy-nephthenol acetate (**1**) and 15-hydroxy-cembrane (**2**) standards).

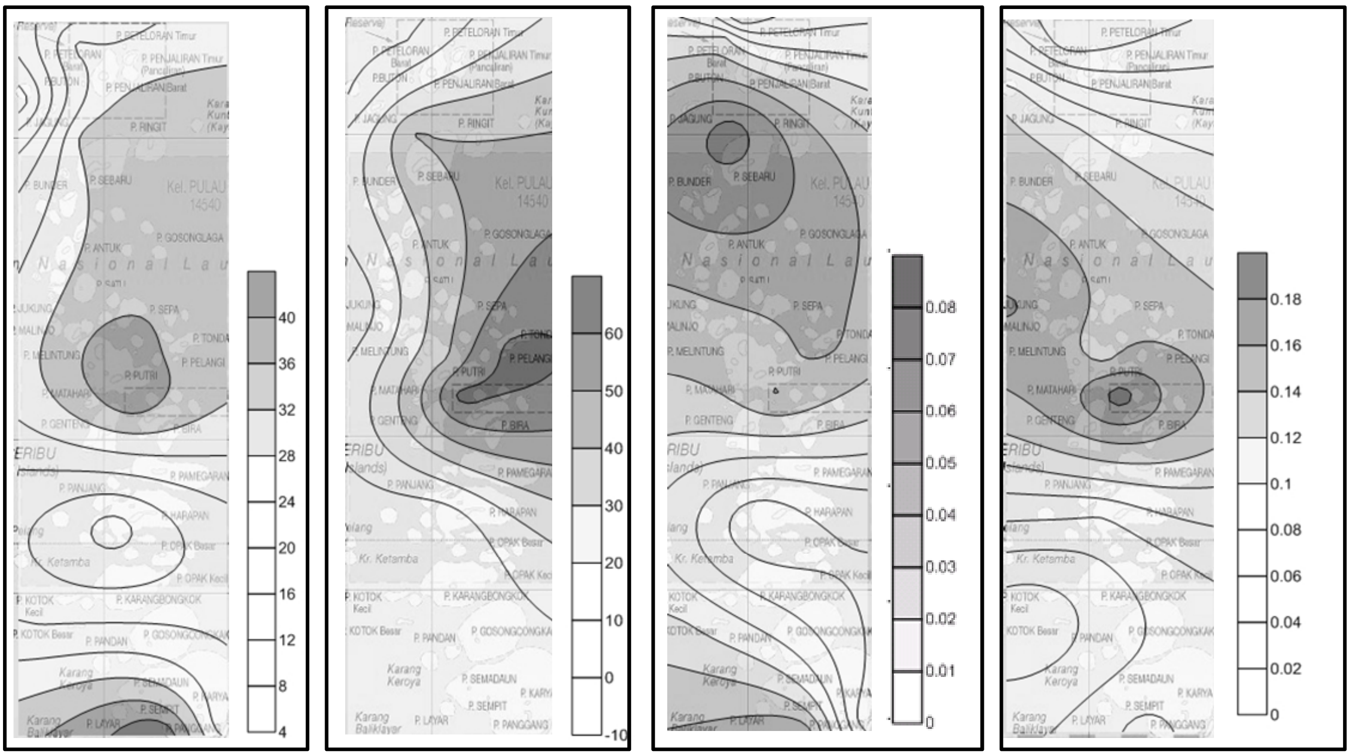
in 10mL of HPLC grade methanol and maintained at 0°C in a cool box containing ice water. On return to the laboratory, samples were exhaustively extracted with methanol to yield the extract that was used for further study.

Environmental Water Analysis

At each sampling site, water samples within one meter of *Nephthea* sp., (5m depth) were taken. Since three animal samples were collected from each site, three water samples were also collected at corresponding locations. Analysis of PO_4^-, NO_2^-, NO_3^-, NH_3, and pH were undertaken immediately on board above the sampling site using a portable colorimeter Hach DR-890 and a pH-meter. Dissolved inorganic nitrogen (DIN) concentration was determined as the sum of NO_2^-, NO_3^-, and NH_3 according to an established method [21].

Determination of Cembranoid Diterpenes

The two cembranoid diterpene standards were first isolated and characterized as 3,4-epoxy-nephthenol acetate (**1**) and 15-hydroxy-cembrane (**2**) (Figure 2) in a previous study [17]. For each compound, a dilution series ranging from 480–30ppm was prepared and a standard curve of concentration constructed (Figure 3). Quantification of cembranoid diterpenes in collected samples was achieved by injection of 20 µL of methanol extract *Nephthea* sp. (10mg/mL) into a Shimadzu 2010A HPLC system equipped with a Shimadzu ODS RP-C_{18} column (150×2.0 mm) and mobile phase gradient mixtures of water containing increasing amounts of acetonitrile (ACN). Determination of the concentration of each compound was achieved by comparison



(a) (b) (c) (d)

Figure 4: (a) Contour maps of % live coral cover in Seribu Islands waters (b) % Cytotoxicity of *Nephthea* sp., extracts (c) % Yield of 15-hydroxy-cembrane (**2**) (d) % Yield of 3,4-epoxy-nephthenol acetate (**1**)

of values obtained from regression plot standard concentration curves (Figure 3) and peak area of each correlated compound in resultant HPLC chromatograms.

Cytotoxic Testing

Screening of samples for their cytotoxic was conducted employing a single human cancer cell line, T47D (breast cancer), based on the methods of Zachary [22]. Each sample was analysed in triplicate at 30mg/L, based on limit inhibition potency from Fajarningsih *et al.*, [23].

Contour Maps

Each variable measured; phosphate concentration, inorganic nitrogen concentration, pH (Figure 1 b-d), live coral cover, extract cytotoxicity towards T47D cells, and concentrations of 3,4-epoxy-nephthenol acetate (**1**) and 15-hydroxy-cembrane (**2**) (Figure 4 a-d), was plotted to previously obtained GPS location coordinates. Contour maps of each variable were generated using Surfer Golden Software Version 9.9.78Results

Table 1 shows data for active phosphate concentration (PO_4^{3-}), dissolved inorganic nitrogen (DIN) concentration, and pH in sea water samples collected from twelve different locations during the time of this study; resultant contour maps are shown in Figure 1.

Figure 2 shows the chemical structures of the two cembranoid diterpenes (**1**-**2**) whose concentration in *Nephthea* sp., samples were analyzed in this study.

Table 2 shows percentage of live coral cover on reefs from where samples were collected, cytotoxicity of *Nephthea* sp., extracts towards T47D cells, and yields of **1** and **2**. Quantification of both cembranoid diterpenes was based on standard regression curves shown in Figure 3. Contour maps generated from these data are shown in Figure 4.

Discussion

It was evident at several study sites that levels of phosphate and dissolved inorganic nitrogen were above threshold values (0.1µM and 1µM, respectively) for a minimally impaired coral reef [24] (Figure 1). High levels of phosphate were probably caused by effluent from domestic run-off, as the majority occurred in southern areas where there are local communities living on the Seribu Islands. In northern regions of the islands, all parameters analyzed showed high concentrations of phosphate and inorganic nitrogen. The reason for high values in these areas is unknown, but it seems likely that the levels observed may come from non-point sources, such as movement of ions in local ocean currents. The northern part of Seribu Islands is an open sea area, which has oil drilling rig activities and is also a busy pathway for boat traffic to and from Jakarta province, which may have an influence on the variables being measured.

Secondary metabolite investigation of collected samples found them in most cases to contain both investigated compounds (**1**-**2**).

In a general sense, the results in Table 2 and Figure 4 (a) show the impact of anthropogenic stressors, pollutants, on the Seribu Islands coral reef environment. The contour map of live coral cover shows the maximum live cover in these areas was only around 40%. The biopotency, as measured by cytotoxicity, and not to be confused with the presence or absence of **1** and **2**, of extracts from *Nephthea* sp., loosely correlates with the observed coral coverage and vice versa, in all but one case, Site 10. In the southern region, where Site 10 was located, the cytotoxicity levels appeared not to show the same correlation with live coral cover as their central and northern counterparts.

Although speculative, the occurrence of cytotoxic compounds in marine invertebrates is usually correlated with an organisms attempt to win and or maintain living space in a given benthic area against their competitor(s) [7,25-27]. The high levels of nutrient ions detected in the southern part of the Seribu Islands, that are considered less than optimal to support a wide variety of life on the coral reefs, might be a cause of the lack of biopotency vs coral cover observed there and that the high coral cover was observed from only a few species that appear to be able tolerate such conditions, e.g., *Sarcophyton* sp., (Soft corals) and *Montipora* sp., (Hard corals) together with organisms from other groups, e.g., zoanthids, echinoderms, and algae, but the actual species diversity is low. The abiotic part of the environment in these regions was predominantly covered with rubble and sand.

Visual observation at sampling sites in the southern part of the Seribu Islands showed that these areas were dominated by soft corals

Site	Location	Phospate (ppm)		Dissolved Inorganic Nitrogen (ppm)		pH	
		Value	±SD	Value	±SD	Value	±SD
1	East Peteloran Island	0.043*	0.006	0.029	0.001	7.77	0.06
2	West Penjaliran Island	0.017*	0.006	0.097	0.016	7.73	0.06
3	Jagung Island	0.357	0.031	0.082	0.000	8.20	0.00
4	Sebaru Besar Island	0.103	0.015	0.020	0.003	8.23	0.06
5	Melinjo Island	0.033*	0.012	0.018	0.002	8.13	0.06
6	Putri Island	0.037*	0.012	0.004*	0.002	8.27	0.06
7	Pelangi Island	0.083	0.006	0.035	0.003	8.23	0.06
8	Kayu Angin Bira Island	0.047*	0.015	0.000	0.000	8.13	0.06
9	Kelapa Island	0.413	0.006	0.006	0.002	8.23	0.06
10	Kotok Island	0.110	0.000	0.033	0.010	8.27	0.06
11	Panggang Island	0.357	0.006	0.043	0.007	8.23	0.06
12	Pramuka Island	0.170	0.000	0.128	0.006	8.20	0.00

*Value below stated instrument detection limit (0.05 ppm for Phosphate and 0.005 ppm for nitrogen)

Table 1: Concentration of active phosphate (ppm) and inorganic nitrogen (ppm), and pH at sampling sites in the Seribu Islands.

Site	Location	Weight (%) 3,4-Epoxy-nephthenol acetate (1) in wet weight of *Nephthea* sp.		Weight (%) 15-Hydroxy-cembrane(2) in wet weight of *Nephthea* sp.		Inhibition effect of 30 ppm *Nephthea* sp. extract towards T47D cells (%)		Live Coral Cover (%)
		Value	±SD	Value	±SD	Value	±SD	Value
1	E. Peteloran Island	0.00	0.00	0.00	0.00	0.00	0.00	20
2	W. Penjaliran Island	0.01	0.00	0.04	0.01	0.00	0.00	25
3	Jagung Island	0.11	0.02	0.06	0.01	0.00	0.00	5
4	Sebaru Besar Island	0.16	0.02	0.09	0.01	40.52	0.78	30
5	Melinjo Island	0.13	0.02	0.06	0.00	0.00	0.00	25
6	Putri Island	0.14	0.01	0.05	0.01	37.71	1.56	35
7	Pelangi Island	0.19	0.01	0.06	0.00	61.92	0.62	30
8	Kayu A. Bira Island	0.17	0.04	0.06	0.01	63.10	1.98	35
9	Kelapa Island	0.00	0.00	0.00	0.00	0.00	0.00	10
10	Kotok Island	0.05	0.01	0.04	0.01	8.84	1.08	20
11	Panggang Island	0.07	0.00	0.07	0.00	10.13	1.51	40
12	Pramuka Island	0.05	0.00	0.00	0.00	0.00	0.00	20

Table 2: Yield of 3,4 epoxy-nephthenol acetate (**1**) and 15-hydroxy-cembrane (**2**) in wet weight of *Nephthea* sp., inhibition of 30 ppm *Nephthea* sp., extract to T47D cell lines, and live coral cover in each sampling location.

belonging to the genus *Sarcophyton*. *Sarcophyton* sp., are common on inshore reefs, and so it is not surprising that they could accommodate a wide range of nutrient conditions without showing adverse effects [28]. This proposed effect has also been discussed for some reefs on the Great Barrier Reef - Australia, where higher nutrient concentrations in inshore waters may contribute to the dominance and possible expansion by octocorals in that region [29]. The results of this latter study may also correlate in some way with those found for the soft coral *Sarcophyton ehrenbergi* that showed there was a general increase in the "physiological-change indicator", which is defined as the ratio of stress metabolites (terpenes) to energy storage metabolites (lipids), when local stress was increased by relocation next to a hard coral species but was variable when the nutrients nitrogen and phosphorus were considered [11]. Lower overall diversity may also mean lower competition for living space and so *Nephthea* sp., as a response might produce lower amounts of cytotoxic (antipredatory) compounds [8,9]. High cytotoxic activity of extracts observed in the central part of the Seribu Islands that has lower levels of nutrient ions compared to other sites combined with high coral cover supports the tentative correlation between high diversity of reef species and competition for living space [30]; in the particular case being studied the soft coral would produce higher levels of cytotoxic compounds in order to survive, win and, or maintain a space on the reef [31,32].

Generally, as seen in contour maps Figures 4(c) and 4(d), the cembranoid diterpenes **2** and **1** from *Nephthea* sp., occurred in a similar distribution pattern as that of live coral cover Figure 4(a). As there is a positive relationship between live coral cover and total number of fish species [33], it is probable that local coral fishes are in abundance in those areas with live coral cover. As a logical consequence, and based on the observation that many cembranoid diterpene compounds in soft corals are known to be toxic, i.e., serve as local coral fish feeding deterrents, as antifouling agents, and as agents assisting with successful competition for space with others corals [14,16], as well as being implicated in spawning [14], it is likely that *Nephthea* sp., growing in areas of high live coral cover will produce larger amounts of feeding deterrent cembranoid diterpenes than those from sites with less cover, so as to effectively deter fish feeding [9,31]. In contrast, in areas with higher levels of pollutants marine organisms would have difficulty surviving; that is, conditions might cause a reduction of hard coral cover and hence the number of reef fish species [34]. Under these circumstances, as the local number of predatory fishes decrease it is probable that *Nephthea* sp., would no longer require prior high levels of their antipredatory substances, like cembranoid diterpenes.

The finding of different concentration patterns of 3,4-epoxy-nephthenol acetate (**1**) and 15-hydroxy-cembrane (**2**) throughout the Islands [see Figures 4(c) and (d)] showed both of these compounds to likely serve as anti-predatory systems, and that each of them ma y address a different type(s) of predator(s) [8]. The known 15-hydroxy-cembrane (**2**) occurred more generally at every site that had live coral cover, while 3,4-epoxy-nephthenol acetate (**1**) occurred only in specific areas of the Seribu Islands, those with lower levels of environmental stressors. From these observations it might be likely that in the latter case the predator(s) or effector(s) that triggered this compounds production is (are) only found in what are considered "clean" environments. As production of **1** seems to be significantly compromised through pollution and other human activities, this study shows that one source of marine biodiversity may be being rapidly compromised or lost as a result of such occurrences [35].

In summary, the results of this study showed in a very preliminary and speculative way that *Nephthea* sp., sampled from the Seribu Islands produced lower amounts of cembranoid diterpenes as nutrification entered their habitats. This finding may be the result of anthropogenic stressors and its negative correlation on the total number of soft coral fish predators. In other words, when cembranoid diterpenes serve as an anti-predatory system, the production of those natural products might be conditional on predator challenge. As lower amounts of bioactive compounds were found in areas considered polluted, it might reasonably be concluded that anthropogenic pollution is a major threat to reef biopotency and hence, natural product discovery.

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