Original Article

LC-MS metabolomic analysis of environmental stressor impacts on the metabolite diversity in *Nephthea* spp.

Abstract

Context: The soft coral *Nephthea* spp. is a source of terpenoid class that potentially has pharmaceutical properties. However, metabolite diversity and cytotoxic activity of this species are varied among coral reefs from various sites. **Aim:** To analyze the water quality in *Nephthea* spp. environment as a possible factor causing a difference in its metabolite diversity. **Settings and Design:** *Nephthea* spp. from seven sites were taken in October 2010 at the Alor District of Marine Protected Area, Indonesia. **Materials and Methods:** Water quality assessment was analyzed *in situ* and indexed by Canadian Council of Ministry Environment-Water Quality Index (CCME-WQI) method. Meanwhile, metabolite diversity was analyzed by a LC-MS metabolomic method, using C18 reversed phase and gradient water-acetonitrile system. **Statistical Analysis Used:** Spearman's rho and regression analysis were applied to correlate the water quality index to ecological index (richness, diversity, and evenness) from LC-MS results. **Results:** The water quality index had a significant positive correlation and strong linear regression determinant to the total metabolite (R^2 = 0.704), particularly to semipolar metabolite richness (R^2 = 0.809), the area of terpenoid class in the organism. **Conclusion:** It can be concluded that water quality may serve as a major factor that affects the amount of richness in *Nephthea* spp. metabolites. When the water quality is lower, as environment stresses increases, it may affect the metabolite richness within direct disrupt of metabolite biosynthesis or indirect ecological means. Terpenoids are known as a soft coral antipredator (coral fishes), the amount of which depends on the water quality.

Key words:
Environmental stressor, metabolite diversity, metabolomics, Nephthea spp

Introduction

Nephthea spp. is one of the soft coral organisms that produce terpenoid compounds, a class of natural products, which have a wide range of pharmaceutical properties, such as cytotoxicity to cancer cell lines, antiviral, or anti-inflammatory.[1-6] However, the cytotoxic activity may vary from the same *Nephthea* spp. collected from different locations. This finding is suggested to due to the environmental characteristic variation among those species. As various anthropogenic environmental stressors, such as nutrient ions, are introduced to the coral reef community, the impacted *Nephthea* spp. may have a different amount of bioactive compounds or diversity pattern.[7] Therefore, this research had been done to analyze water quality of *Nephthea* spp. environment as a possible factor causing a difference in natural product diversity extracted from this organism.

Current technology of metabolomic liquid chromatography separation with a mass spectrometer detector was used as a rapid tool to do this research. Metabolomics is a powerful method to analyze metabolic shifted in an organism which adapts with its environment.[8] Commonly, metabolic fingerprint profiles are processed by statistical Principal Component Analysis.[9-11] Application of this method had been successfully applied to define metabolic shifted which is caused by toxic compounds, such as pesticides or heavy metals.[12,13]

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Different than the common processing method, this research used ecology index (richness, divesity, and evenness) as metabolic data processing. The application is based on T-RFs genetic studies, which generate similar graphical results as the mass chromatogram. Besides defining the variation between each chromatogram, the advantage of using ecology index is a further analysis such as correlation and regression could be applied, to analyze the connection between observed variables. The main subject of environmental stressors being analyzed was typical human anthropogenic pollutants, which were nutrient pollutants such as phosphate and inorganic nitrogen substances, at the Alor District Marine Protected Area, one of the famous coral reef area in Indonesia.

Materials and Methods

Animal materials

Nephthea spp. were taken from seven coral reef sites in the Alor District Marine Protected Area, Indonesia. The locations recorded in GPS Garmin GPSMAP 60CSXA and then were plotted in Bluechart Pacific v9.5 Map using MapSource v6.15.11, as shown in [Figure 1]. Sampling of the organisms was done with a careful inspection on their morphological similarities, to avoid misleading conclusion because of different species. At 5 m depth, 5 g of *Nephthea* spp. were taken and immediately preserved in 20 mL of JT Baker HPLC-grade methanol in dark-brown vials. All the samples were preserved in an iced cool box while transporting them from the field to the laboratory.

Sea water quality analysis

From the same sampling sites of *Nephthea* spp., three replicates of 200 mL of sea water was taken for water quality analysis. Dissolved nitrogen inorganic (nitrate, nitrite, and ammonia) and phosphate ions were analyzed by a colorimetric method using HACH DR-890 colorimeter. Besides that, pH and salinities were recorded using pH-meter and reflactometer. A Canadian Council of Ministry Environment—Water Quality Index (CCME-WQI) was calculated as CCME-WQI = $100-((\sqrt{(F_1^2+F^2_2+F_3^2)})/1.732)$,[14] where F_1 = (number of failed variables/total number of variables)×100, F_2 = (number of failed test/total number of tests)×100, and F_3 = nse/(0.01 nse+0.01).[14] Variable of nse was calculated from nse = ∑ excursions/number of tests, where the excursion = (failed test value/objective value) – 1 or = (objective value/failed test value) – 1.[14] The index was classified as poor (0–44), marginal (45–64), fair (65–79), good (80–94), and excellent (95–100).[14] The objective thresholds used in the calculation were the rule of sea water chemical properties limit for marine organisms by the Indonesian Ministry of Environmental (No. 51/2004), as described in [Table 1].

LC-MS metabolomic analysis

Metabolite diversity was analyzed by a LC-MS Shimadzu 2010A system, using 150×2.0 mm Shimadzu Shim-Pack

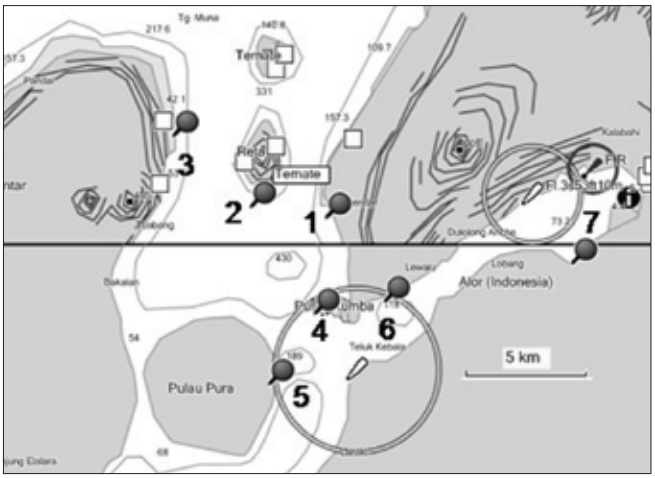

Figure 1: Sampling sites in the Alor District marine protected area: Sebanjar (1), Ternate (2), Jawatoda (3), Kepa (4), Pura (5), Ampera (6), and Moru (7)

ODS reversed phase and 50 minutes of gradient of water to an increasing acetonitrile mobile phase. The mobile phase was carried out in 10% acetonitrile and increased to 90% acetonitrile in 30 minutes. The last 20 minutes of the mobile phase system was done using an isocratic 90% acetonitrile in water to elute the nonpolar compounds. After a run was completed, the mobile phase was equilibrated in 10% acetonitrile-water before the next run. Electrospray ionization with low ionization (30 V in Q-array voltage and 150 in Q-array RF) was used in the system to minimize the fragmented ions, and only the molecule peak's ions were detected. The ions were detected in a wide range from 50 to 1000 m/z. Peaks from each chromatogram sample were identified by default qualitative processing from 5 to 50 minutes (to exclude solvent peaks). The peak's data from each LC-MS chromatogram were then subjected to calculate the ecology index: Richness (R), diversity (H'), and evenness (E) index. Furthermore, the richness was divided into three sections: Polar compound (5–10 minutes), semipolar compound (10–25 minutes), and nonpolar compound (25–50 minutes). The section of semipolar is an area of various bioactive terpenoid–cembranoid compounds.[15] Richness index was calculated as the total peak in the chromatogram.[16] Diversity index (H') was calculated from H' = $\sum p.i \cdot \ln(p.i)$, where p is the proportion of an individual peak area relative to the sum of all peak areas for each sample.[16] On the other hand, Evenness index (E) was calculated from E = H'/ln(R).[16] Diversity index was classified as low (H'<1.5), fair (1.5<H'<3.5), and rich (H'>3.5).[17] Meanwhile, if evenness index value was close to 1, it showed about an occurrence of dominant species or ion in the sample.[17] Meanwhile, richness index had no classification, as this value only counts the amount of peaks in MS chromatogram.

Statistical correlation analysis

Spearman's rho analysis using PAST ver. 2.00 Statistical

software had been done to correlate WQI index with the ecology index of *Nephthea* spp. metabolites. Furthermore, linear regression analysis using Microsoft Excel 2007 was applied to identify the fitness of WQI index as a predictor to the diversity of *Nephthea* spp. metabolites.

Results

The results of the sea water quality analysis at sampling sites are shown in [Table 1]. pH (7.7–8.2) was normal in all the sites, as their values were within the threshold values (7–8.5). OD (6.8–7.3 ppm) also showed that oxygen level was sufficient in all sites as they were higher than the threshold value (above 5). On the other hand, the salinity values (31–34) failed to fulfil the thresholds (33–34%). The coral reef areas that failed were Sebanjar and Jawatoda sites. This may have happened as both of these sites are near to domestic run-off. Meanwhile, phosphate and nitrogen ions analysis showed that high nutrient ions exceed the threshold levels at several sites. Due to these eutrophic conditions, the water quality index in all the sites was only within a range of poor to marginal (32–57).

Meanwhile, metabolite diversity differences are observed visually, as shown in [Figure 2]. Index of diversity, richness, and evenness *Nephthea* spp. metabolites from each chromatogram are shown in [Table 2]. Diversity (H') from all sites was considered to be fair (1.91–2.87) and its evenness (E) showed no significant ion dominance (0.69–0.94). An elevated index variable was shown only in the overall metabolite richness values (12–36). The Spearman's rho correlations of water quality index and ecology index are shown in [Table 3]. A significant correlation was seen between WQI index to the overall richness of *Nephthea* spp. metabolites (R=0.86 at P=0.01), particularly on the semipolar area (R=0.93 at P=0.005). Furthermore, as shown in [Figure 3], linear regression using WQI as an independent variable to total and semipolar richness as a dependent variable generated a mathematical formula below.

Total richness = 0.786 (WQI) – 9.046 (R^2=0.7.04)

Richness of semipolar compounds = 0.843 (WQI) – 22.89 (R^2=0.809)

Table 1: Sea water quality at sampling sites

Site	pH	Salinity (o/oo)	OD (ppm)	Phosphate (ppm)
Sebanjar	8.09±0.017	31±0	7.00±0.00	0.347±0.0115
Ternate	8.16±0.032	33±0	7.10±0.00	0.663±0.0058
Jawapoda	8.17±0.023	32±0	6.97±0.06	0.170±0.0000
Kepa	8.23±0.058	33±0	7.10±0.00	0.773±0.0058
Pura	8.14±0.012	34±0	7.10±0.00	0.093±0.0058
Ampera	7.73±0.058	34±0	6.80±0.00	0.027±0.0058
Moru	8.05±0.006	34±0	7.30±0.00	0.133±0.0058
Threshold value*	7–8.5	33–34	>5	<0.015
Site	**Nitrite (ppm)**	**Nitrate (ppm)**	**Ammonia (ppm)**	**WQI**
Sebanjar	0.0037±0.00058	0.00±0.000	0.087±0.0115	38
Ternate	0.0027±0.00058	0.00±0.000	0.000±0.0000	47
Jawapoda	0.0060±0.00100	0.00±0.000	0.000±0.0000	57
Kepa	0.0060±0.00000	0.01±0.000	0.017±0.0058	34
Pura	0.0060±0.00100	0.10±0.000	0.050±0.0000	32
Ampera	0.0000±0.00000	0.00±0.000	0.030±0.0100	50
Moru	0.0013±0.00058	0.01±0.000	0.027±0.0058	37
Threshold value*	<0.001	<0.002	<0.001	–

Mean value±SD from three replicates; *Values were derived from the rule of sea water chemical properties thresholds for marine organisms by the Indonesian Ministry of Environmental (No. 51/2004)

Table 2: Richness, diversity, and evenness index of *Nephthea* spp. metabolites from each sampling site

Site	R total	R of polar compounds (min 5–10)	R of semipolar compounds (min 10–25)	R of nonpolar compounds (min 25–35)	H'	E
Sebanjar	26	13	9	4	2.87	0.87
Ternate	33	5	22	5	2.74	0.78
Jawatoda	36	3	27	6	2.69	0.74
Kepa	21	1	8	13	2.65	0.87
Pura	12	6	4	2	2.33	0.94
Ampera	25	5	13	7	2.44	0.75
Moru	16	6	6	3	1.91	0.69

R – Richness, H' – Diversity, E – Evenness

Table 3: Spearman's rho correlations of water quality index and ecological index

Spearman's rho	R total	R of polar compound	R of semipolar compounds	R of nonpolar compounds	H'	E
WQI						
Correlation	0.86*	−0.27	0.93*	0.42	0.43	−0.57
Significance	0.01*	0.56	0.005*	0.33	0.34	0.18

*Significant and strong correlation; R – Richness, H' – Diversity, E – Evenness

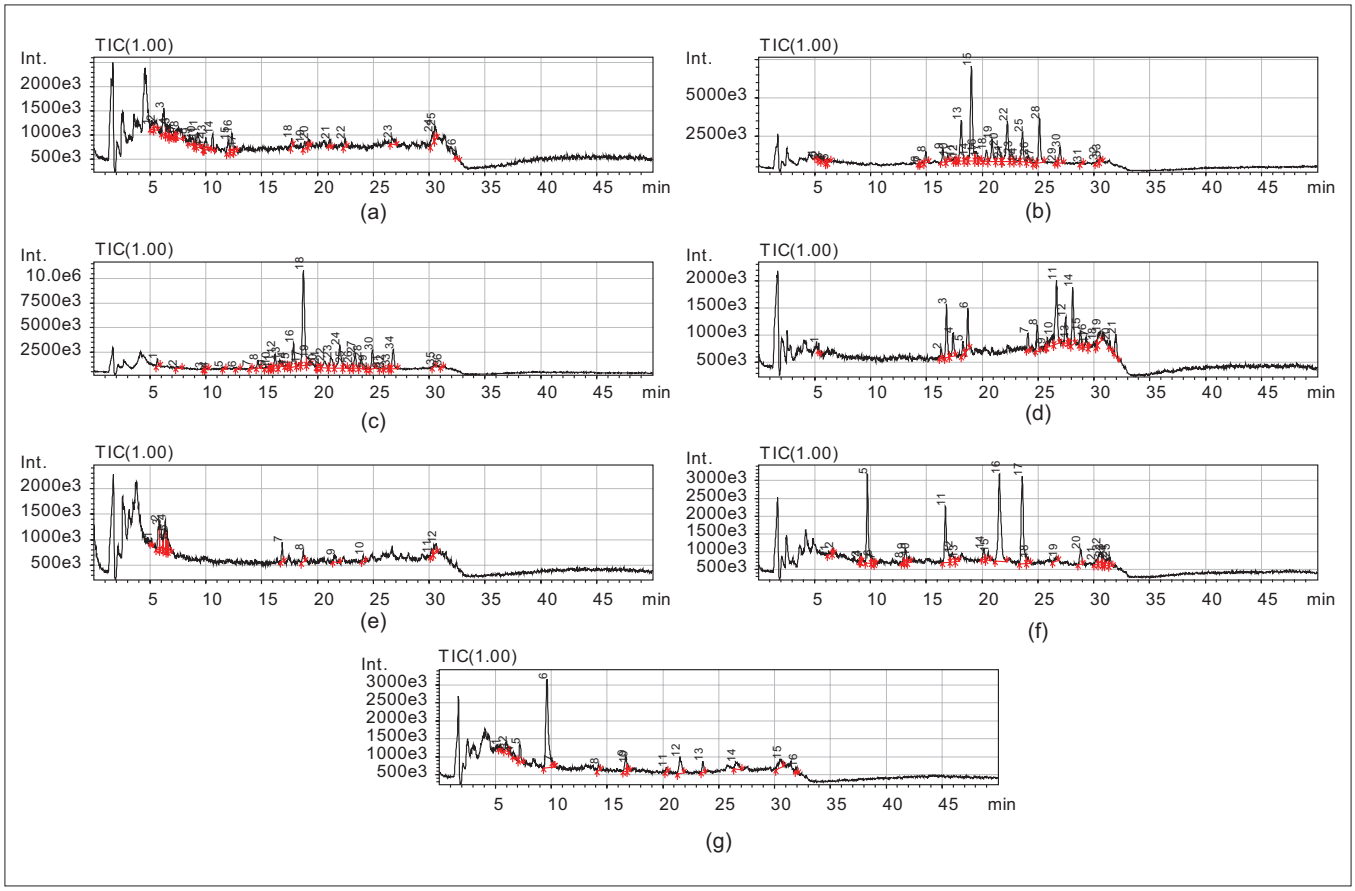

Figure 2: MS chromatogram of *Nephthea* spp. extracts from each sampling location: Sebanjar (a), Ternate (b), Jawatoda (c), Kepa (d), Pura (e), Ampera (f), and Moru (g)

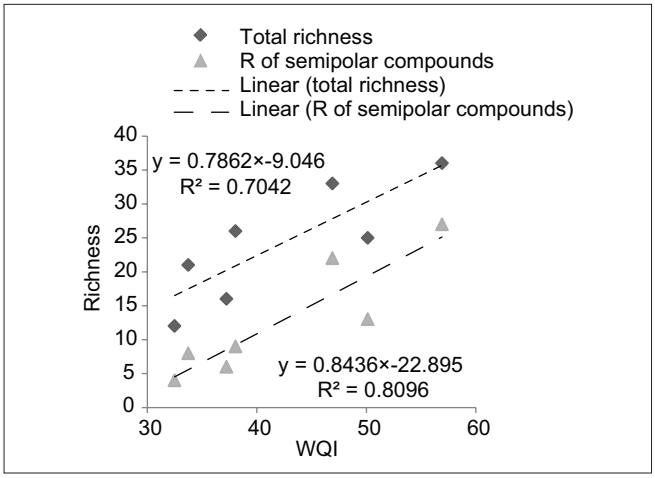

Figure 3: Linear regression of WQI index to total richness and semipolar compounds richness

Discussion

The WQI index, only within a range of poor to marginal, showed that even Alor District coral reef community is within a Marine Protected Area (MPA), which is stressed by an environmental pollutant factor. Probably, the major factor of nutrient addition into the reef area was due to domestic pollutant. These may imply that the MPA is not effective in protecting the coral reefs from environmental stressors. A domestic effluent processing is urgently needed to protect the reefs, as high anthropogenic nutrient level or eutrophication may cause a decline in the coral reefs.[18] A shifted hard coral to coralline algae or entirely algae overgrowth might happen as the result of these environmental stresses.[19]

However, *Nephthea* spp. survived in these eutrophic sampling sites. As a soft coral species, *Nephthea* spp. can

live within a wider range of nutrient level compared with hard coral.[20] Visually, there was not any difference between *Nephthea* spp. that lived in poor WQI compared with the one that lived at better index. The impacts of nutrient enrichment in coral reef area on *Nephthea* spp. were within its metabolite productions, as shown in [Figure 2], and defined in the ecology index given in [Table 2].

In general from all sites, diversities of metabolites were considered to be fair and no significant ion dominance as they were within the diversity (H') and evenness (E) index. As the peak's area in mass chromatogram directly showed a proportion of the ion's amount from each peak,[21] this may mean that in all chromatograms, there was no compound that dominates in the extract. The main difference, as visually shown in chromatograms, is the metabolite richness. The elevated overall and semipolar richness was significantly correlated to WQI. Furthermore, as shown in [Figure 3], linear regression analysis showed WQI may serve as a significant predictor to the overall and semipolar richness index. WQI determinant coefficients (R^2) as much as 0.704 (for total richness) and 0.809 (for richness of semipolar compounds) may mean that variations in both richness depend on 70.4% and 80.4% of the water quality. For about 30–20% more, the variation may depend on variables not included in this research, such as age or other biological stages. However, as the water quality was affected to such high percentages, this finding proves that ecologically, environmental conditions may be considered as the major factor in metabolites diversity in *Nephthea* spp.

There might be two considerations about how the environmental stresses affected the metabolite diversity in *Nephthea* spp. First, environmental stresses may directly disrupt several pathways of metabolic production in the organisms. The second is through indirect means, such as the ecological role of terpenoid's class in soft coral. The ecological role of bioactive metabolites in soft corals or sponges is within a chemical defensive weapon, such as to deterrent predator or win a living space competition.[22-26] Terpenoid class from soft corals known as anti-predator protect the organism from the predator coral reef fishes.[27,28] Fish such as *Chaetodon* spp. are known to feed on soft corals such as *Nephthea* spp.[29] Moreover, as the amount of coral reef fishes depends on the sea water quality,[30] the correlation of sea water quality to metabolite diversity in soft coral may be within an indirect link. It is probable that when the water quality is low, the amount of coral reef fish predators is also low and *Nephthea* spp. do not produce diverse chemical weapons to protect themselves. However, in a better water quality, the predator may present in higher amount and *Nephthea* spp. will produce active metabolites to defend themselves. From different soft coral species, *Sinularia* spp., an increase of antipredator compound caused by a different amount of fish predator was shown.[31] Therefore, the elevated diversity being found may also be due to a different amount of fish predator in each *Nephthea* spp. environment. However, neither one is true, and the study showed that in general, direct or indirect means, a diverse metabolite with potentially active compounds in pharmaceutical studies at the coral reefs organism may being compromised or lost, through the increase in marine environmental stresses.

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