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A new ecotoxicity tolerance index of macrobenthos associated with *Zoanthus sansibaricus* in the littoral zone of Hormuz Island, Persian Gulf, Iran

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Abstract

A general study with the objective to classify ecotoxicity status of macrobenthose associated with dominant toxic zoanthids in Hormuz Island is presented. Hence, a novel model based on hard bottom macrobenthos and the related substrate composition was suggested to be used for testing the accumulated palytoxin (PTX) in macrobenthose exposed to *Zoanthus sansibaricus*. Direct and rapid assessment index (Dara Index) of accumulated PTX in macrobenthos were re-evaluated for use in this classification. The new proposed ecotoxicity tolerance index (ETI) was tested and calculated based on the data of Dara Index. ETI was compared and evaluated against Dara Index for use in this classification. The macrobenthos groups of species included three categories, namely, sessile, sluggish and mobile species. The distribution of these three macrobenthose groups were grouped according to their presence or absence to associate with zoanthids and were weighted proportionately to obtain a formula rendering a six step numerical scale of ecotoxicity status classification. Its advantage against the former Dara Index lies in the fact that it reduces the clustering number of the sampling sites involved which makes it simpler and easier in its use. The usage of ETI as a classification tool of ecotoxicity status indicates that tolerance of marine animals to the PTX may enable it to enter food chains and to be followed by potential exposure to humans. Hence, the advantages of ETI include high discriminative power and simplicity in its use which make it a robust, simple and effective tool for application in the Persian Gulf.

Keywords: ETI, Dara Index, PTX, Zoanthid

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Introduction

All species of the order Zoantharia can produce a deadly chemical call palytoxin (PTX). It was first isolated and purified from corals belonging to the family Zoanthidae (Zoantharia: Coelenterata) in Hawaii (Moore and Scheuer, 1971). This toxic zoanthid was subsequently identified as *Palythoa toxica* (Walsh and Bowers, 1971).

PTX is widespread in the sea ecosystem, and it can importantly be found in the food chains including marine animals which prey on zoanthids or predators prey on these creatures (Hashimoto *et al*., 1969). Human can be exposed to PTX in many ways like eating seafood that contains PTX, and dermal interaction with zoanthids can be fatal to human and may be responsible for some of the human diseases (Tubaro *et al*., 2011).

Many studies on the effect of PTX have been done on different animals (monkeys, dogs, rabbits, guinea pig, rats and mice). However these studies are mainly based on PTX found in *Palythoa toxica*, *P. tuberculosa*, *P. caribeaorum*, *P. mammilosa*, *P. vestitus* (Munday, 2011) and *Zoanthus solanderi* and *Z. sociatus* (Gleibs *et al*., 1995).

To study the toxicity and the consequences of PTX found in zoanthids, it is needed to extract and purify the PTX, but in the case of rapid and urgent need to determine the toxicity effects of zoanthids on associated communities, mucus solutions can be useful for this purpose (Charlotte Chan, 2013), because PTX generally exists in mucus and gonads of zoanthids (Borneman, 2004).

Macrobenthos are found occupying the same ecological niche of zoanthids and therefore space competition is observed (Mirzabagheri *et al*., 2008). Macrobenthos are important indicators of environmental stress and they are sensitive to poison exposure (Holland *et al*., 1987). The information about the response of macrobenthos to biotoxins is still scant (Charlotte Chan, 2013), while attention is increasingly centralized on the response of macrobenthos to hydromorphological variation (Solimini *et al*., 2006).

In littoral reefs in the Persian Gulf, the highest number of toxic colonies per unit area is related to *Z. sansibaricus* which can have a significant impact on associated communities. Although zoanthid communities have been described in most Persian Gulf Islands (Noori Koupaei *et al*., 2014, 2015, 2016), there are no data on PTX of zoanthids in this area. The suggestions encourage the usage of biotoxic indices to determine the ecotoxicity status across the Persian Gulf (Mirzabagheri *et al*., 2008). For this aim, Dara Index, which is a new direct and rapid assessment index of accumulated palytoxin in macrobenthos associated with littoral zoanthids in the Persian Gulf, has been developed (Mirzabagheri *et al*., 2016).

The development and choice of the most appropriate tools for treating and evaluating benthic data for classification of macrobenthos groups is essential (Borja *et al*., 2003). Hence, this study presents the development of a new biotoxic index based on the initial idea of Mirzabagheri *et al*., 2016 to combine the estimated presence of three macrobenthose groups in a single formula resulting in a series of numeric values. The novelty of this new index called ecotoxicity tolerance index (ETI) endeavors a single value which decreases the big quantity of Dara Index parameters and represents data in a simple way. This preliminary rapid assessment was followed by a novel theoretical model of the ecotoxicity status based on ETI (Mirzabagheri *et al*., 2017), which is necessary to assess the quality of poisoning for ecosystem health.

ETI is an approach which minimizes the data volume to a great extent and simplifies the expression of ecotoxicity status. Calculation of ETI is based on the values of Dara Index within all sets of each transect. This study aimed to rapidly determine ecotoxicity status of accumulated PTX in macrobenthos associated with *Z. sansibaricus* in the littoral zone of Hormuz Island by applying ETI which makes it a robust, simple and effective tool for application in the Persian Gulf.

Materials and methods

Study area and specimen collection

This study was conducted at six transects, T1 (27°04'09.9"N, 56°25'30.1"E), T2 (27°04'00.3"N, 56°25'26.6"E), T3 (27°03'34.2"N, 56°25'15.3"E), T4 (27°03'21.1"N, 56°30'07.5"E), T5 (27°03'01.4"N, 56°29'53.9"E) and T6 (27°02'39.6"N, 56°29'44.8"E) situated along the west and the east from Hormuz Island in the Persian Gulf (Fig. 1). Geographical location of all transects was measured by Mobile GPS (Roy *et al*., 2012). Maximum abundance of *Z. sansibaricus* was found in the west and the east coasts of Hormuz Island. Hence, these coasts were selected for a horizontal Line Intercept Transect survey (English *et al*., 1997).

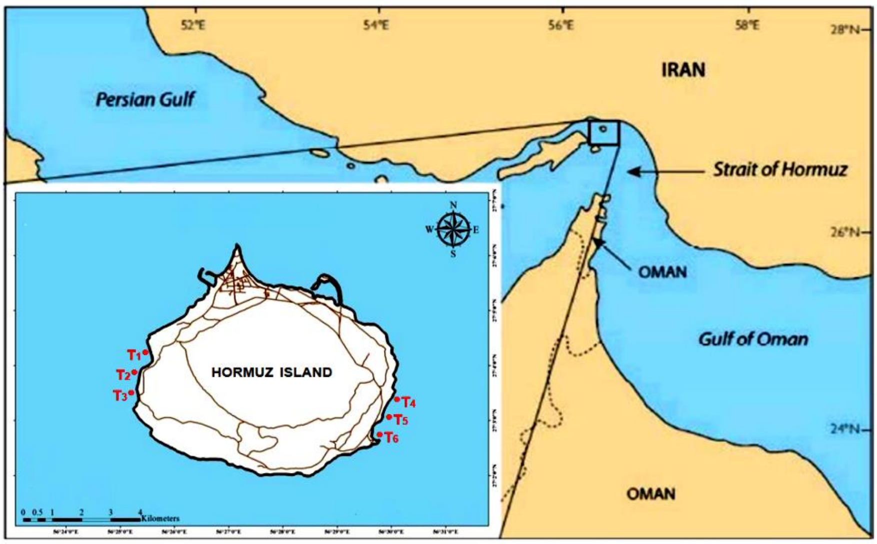

Figure 1: Map of Hormuz Island in the Persian Gulf, showing the sampling transects.

The predominant substrate composition of transects is classified: (1) Boulder in T1 and T4; (2) Cobble in T2 and T5; and (3) Pebble in T3 and T6. According to the novel model of Mirzabagheri *et al*. (2016) for each transect, 36 snails, *Planaxis sulcatus*, were collected from the rock for experimental purpose as they are very abundant on the reefs along the coast of the Hormuz Island. All snails were released back to their natural habitat as no snail died or was injured during this study.

Preparation process

The preparation procedure includes the following steps using Charlotte Chan's method (2013):

- Stock solution: Mucus was scribed using forceps or squeezed by hand with protective gloves on. A total of 0.5 ml of mucus was collected and put in a 10 ml tube. Mucus was then stored in the dark at fridge temperature (4 °C) for later use.
- Working solution: Dilute 0.5 ml of stock mucus solution with 4.5 ml of seawater to make working mucus solution (5 ml).
- Snail sample and setting: 36 snails (weight: 4-4.5 g) were used for each transect and they were placed separately in different plastic containers. 36 snails were divided into 4 set, each set will have 3 replicates. Set 1: Control group (Sea water), Set 2: Working mucus solution (Original solution), Set 3: Treatment 1 (Original solution + 2ml of sea water), Set 4: Treatment 2 (Original solution + 4ml of sea water).

It should be noted that in each transect, sea water samples were taken from the surrounding environment of the zoanthid colonies.

Injection process

The injection procedure includes the following steps using Charlotte Chan's method (2013):

- Two new 10 ml tubes and 1ml of working mucus solution was added into each of the tubes: For Treatment 1, add 2 ml of seawater and for Treatment 2, add 4 ml of seawater.
- Mix well, and place in the tube rack for later use.
- Two doses injected in each snail, the first dose was injected on the date 22/04/2016 and the next dose was injected 24 hours later.
- Hold the snail upside down to allow access to the foot.
- 1 ml^{-1} syringe fitted with a 30G needle were used to withdraw about 0.03 ml^{-1} of solution. New set of 1 ml^{-1} syringe and 30 G needle should be used for each solution.
- 0.03 ml^{-1} of solution in the syringe was then injected into the foot of the snail.
- Snails were then placed back into their own plastic container.
- 24 hours later, 2nd dose were injected in the same snail, following the same method as the 1st dose.
- Reaction of the snails was observed 24hrs after the 1st dose and 24 hours after the 2nd dose.

Metrics for ETI

Two metrics representing various features of macrobenthos associated with zoanthids were selected. Metric 1 was the Direct and rapid assessment index (Dara Index) and metric 2 was the abundance range of macrobenthos groups. The formula calculation of the metric 1 of Dara Index was as follows (Mirzabagheri et al., 2016):

$$\text{Dara Index} = (CT_{24} + CT_{48}) - (SB_{24} + SB_{48}) \quad (1)$$

CT_{24}: Number of the snails crawling up to the top 24hrs after the 1st dose

CT_{48}: Number of the snails crawling up to the top 24hrs after the 2nd dose

SB_{24}: Number of the snails staying on the bottom 24hrs after the 1st dose

SB_{48}: Number of the snails staying on the bottom 24hrs after the 2nd dose

It should be noted that after injection, if snails died it must be calculated as *SB* in formula 1.

According to the calculations on different values of Dara Index by Mirzabagheri *et al*. (2016), metric 1 generally varies between $-2n$ and $2n$ in each set (Table 1):

Table 1: Classification of boundaries among individual poisoning status based on Dara Index (Mirzabagheri *et al*., 2016)

Boundaries of Dara Index	Individual poisoning
Dara Index = $2n$	Minimum
Dara Index > n	Low
Dara Index < n	High
Dara Index = $-2n$	Maximum

n: Total number of snails into each set

In the calculation of the metric 2 of ETI, three major groups of macrobenthos associated with zoanthids were considered; Group 1 (G_1) includes sessile species, Group 2 (G_2) includes sluggish species and Group 3 (G_3) includes mobile species (Table 2).

The ecotoxicity tolerance index and its boundaries according to the ecotoxicity status

The formula of ecotoxicity tolerance index (ETI) is as follows (Mirzabagheri *et al*., 2017):

$$\text{ETI} = \sum \text{Dara Index} - M \quad (2)$$

M: Mortality of snails within all sets

N: Total number of snails within all sets

This index produces scores from less than $-2N$ to $2N$ and indicates the high status when it goes towards the score $2N$, and denotes the azoic status when it is less than $-2N$ (Fig. 2). The classification of boundaries among ecotoxicity status (from azoic to high status) were estimated using the changes of the existence of associated macrobenthos groups (G_1-G_3) across the boundaries of ETI, from the least

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impacted situation to the most impacted one (Table 2).

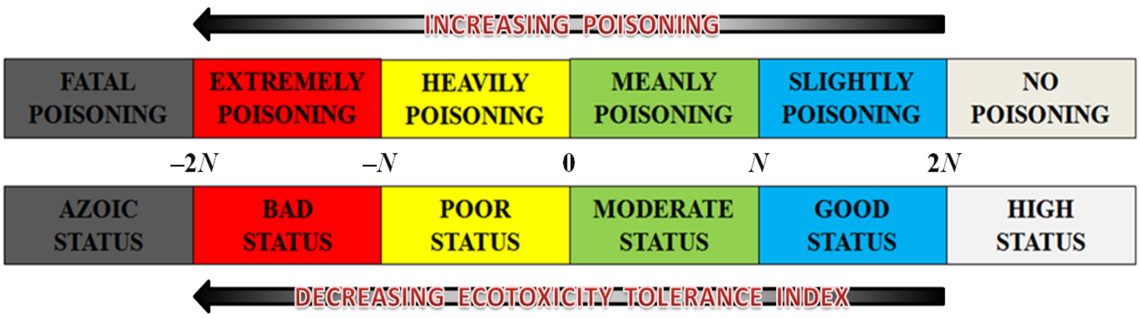

Figure 2: Theoretical model of the ecotoxicity status based on ETI (Mirzabagheri *et al*., 2017).

Table 2: Classification of boundaries among ecotoxicity status based on ETI (Mirzabagheri *et al*., 2017).

Boundaries of ETI	Associated macrobenthos poisoning	Associated macrobenthos health	Associated macrobenthos groups
ETI = 2N	No Poisoning	High	G_1,G_2,G_3
$N \leq$ ETI < 2N	Slightly Poisoning	Good	G_1,G_2,G_3
0 $\leq$ ETI < N	Meanly Poisoning	Moderate	G_2,G_3
–$N \leq$ ETI < 0	Heavily Poisoning	Poor	G_2,G_3
–2$N \leq$ ETI < –N	Extremely Poisoning	Bad	G_3
ETI < –2N	Fatal Poisoning	Azoic	Azoic

Validation of the ETI

ETI was evaluated by comparing it to the abundance pattern of macrobenthos associated with *Z. sansibaricus* at six transects in Hormuz Island (Table 3). The calculation of this index was made using a novel theoretical model given by Mirzabagheri *et al*. (2017).

Table 3: Abundance pattern of macrobenthos associated with zoanthids in the Hormuz Island (Mirzabagheri *et al*., 2017).

Site	Transect No.	Predominant substrate composition	Coverage range of *Zoanthus sansibaricus*			Abundance range of associated macrobenthos		
			Wide	Middle	Narrow	High	Mid	Low
West	T_1	Boulder	√				√	
	T_2	Cobble		√		√		
	T_3	Pebble			√			√
East	T_4	Boulder	√				√	
	T_5	Cobble		√		√		
	T_6	Pebble			√			√

Statistical analyses

Scores of Dara Index and ETI were calculated using the Microsoft Excel software. For analysis of Dara Index, a dendrogram was prepared to differences among 6 transects. graphically visualize the differences among the 4 set in each transect. Also for analysis of ETI, a dendrogram was prepared to graphically visualize the

Results

The purpose of this study was to investigate the mucus effects of *Z. sansibaricus* on associated macrobenthos in the littoral zone of Hormuz Island to determine a new ecotoxicity tolerance index. Results showed that no snails died during the study period. However, the changes of snail fitness were observed. Snails injected with the working mucus solution (highest concentration of mucus) and with treatment 1 became less active and the foot of the snails was less sticky compared to the control snails (injected with seawater).

Table 4 shows that more snails injected with seawater and treatment 2 crawled up to the top, whereas more snails injected with working mucus solution and snails injected with treatment 1 tend to stay on the bottom. The result from the fitness change with the calculation of Dara Index was enough to draw a significant outcome. As a result, there were sufficient evidences to conclude the mucus toxicity of *Z. sansibaricus*.

According to table 4, all ETI values are greater than or equal to 0 and less than *N*. Thus, according to Fig. 2 and Table 2, all ETI values show the mean poisoning of macrobenthos associated with *Z. sansibaricus*.

Table 4: The calculated scores of Dara Index after injection (Mirzabagheri *et al*., 2016) and the calculation of ETI based on the values of Dara Index.

Transect No.	Number of transect individual (*N*)	Set No.	Number of set individual (*n*)	Number of exposed individual				Dara Index	ETI
				24 hr		48 hr			
				CT*	***SB*****	***CT****	***SB*****		
T_1	36	S_1	9	9	0	8	1	16	2
		S_2	9	0	9	0	9	–18	
		S_3	9	4	5	2	7	–6	
		S_4	9	7	2	7	2	10	
T_2	36	S_1	9	9	0	9	0	18	8
		S_2	9	1	8	0	9	–16	
		S_3	9	5	4	3	6	–2	
		S_4	9	7	2	6	3	8	
T_3	36	S_1	9	9	0	9	0	18	14
		S_2	9	1	8	1	8	–14	
		S_3	9	5	4	3	6	–2	
		S_4	9	8	1	7	2	12	
T_4	36	S_1	9	8	1	8	1	14	0
		S_2	9	1	8	0	9	–16	
		S_3	9	4	5	2	7	–6	
		S_4	9	7	2	6	3	8	
T_5	36	S_1	9	9	0	8	1	16	6
		S_2	9	1	8	0	9	–16	
		S_3	9	4	5	2	7	–6	
		S_4	9	8	1	7	2	12	
T_6	36	S_1	9	9	0	9	0	18	14
		S_2	9	0	9	0	9	–18	
		S_3	9	5	4	4	5	0	
		S_4	9	8	1	8	1	14	

**CT*: Crawl Top ***SB*: Stay Bottom

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The dendrogram in Fig. 3 shows two different groups. In all transects, Set 1 and Set 4 were grouped together with a low poisoning; Set 2 and Set 3 were grouped together with a high poisoning.

The dendrogram in Fig. 4 shows three different groups. T_1 and T_4 were grouped together; these were clustered with T_2 and T_5. Also T_3 and T_6 form a third and separate group which is joined with the other two groups.

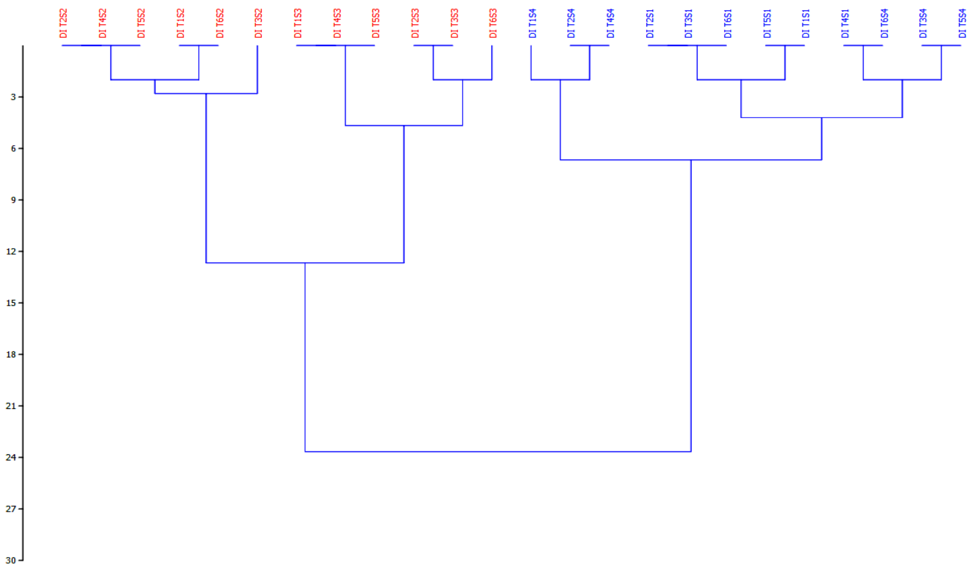

Figure 3: Dendrogram based on Dara Index (DI) with data from table 4; Transect (T) and Set (S).

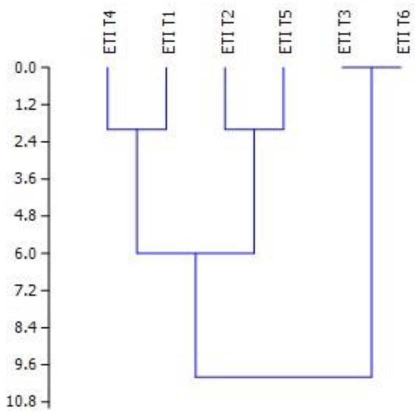

Figure 4: Dendrogram based on ETI with data from table 4; Transect (T).

Discussion

Palytoxin (PTX), one of the most potent marine natural products, which has been produced in zoanthids (Gleibs and Mebs, 1999), also occurred in other communities living in close association with their colonies from the same ecological region (Deeds and Schwartz, 2010), which accumulate high toxin concentrations in their organs, where PTX is stored as an active compound (Riobo and Franco, 2011).

This study provides some useful insights into the ecotoxicity status classification of macrobenthose associated with dominant zoanthids of the Persian Gulf. The analyses of this study represent a preliminary

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assessment of the overall predictive potential of macrobenthos–zoanthid relationships in the Hormuz Island, and a comparison of the performance of two new biotoxic indices. Macrobenthos–zoanthid relationships were assessed for 6 transects, based on macrobenthose groups, using a novel theoretical model given by Mirzabagheri *et al*. (2017). Preparation of the ecotoxicity status for this model was also a difficult task due to lack of previous studies on macrobenthose groups and some coverage range estimations in this ecosystem.

The concept of a biotoxic index based on macrobenthose associated with littoral *Z. sansibaricus* and using a formula which gives a series of continuous values has only been recently developed in the Hormuz Island. The first attempt to construct a single formula to obtain a continuous index was by Mirzabagheri *et al*. (2016). The main difference in the formula suggested in this study to derive ETI (Ecotoxicity tolerance index), from the formula of Mirzabagheri *et al*. (2016) is that the boundaries of Dara Index (Direct and rapid assessment index) are used for poisoning of individuals (Table 1), but the boundaries of ETI are used for poisoning of macrobenthose groups (Table 2) which include three categories, namely, sessile species (G1), sluggish species (G2) and mobile species (G3). Our results indicate that predictions based on macrobenthose groups, i.e. presence or absence groups, can be sufficiently accurate to be used in coastal protection programs and in other applications where estimates of the coverage range of species are relevant (Borja *et al*., 2003).

The results revealed by the dendrogram analyses in Fig. 3 showed two different groups, which imply individual poisoning status increases with decreasing Dara Index scores. The main difference of the dendrogram based on ETI in Fig. 4 from the dendrogram based on Dara Index in fig 3 is that the clustering number of the sampling sites is reduced which makes it simpler and easier for use. The studies imply that the geomorphology of the littoral zone plays an important role in the structure of the macrobenthos community associated with zoanthids (Trivedi *et a*l., 2014).

In this study, in the evaluation of ETI values with the coverage range of *Z. sansibaricus*, it clearly shows that there is a significant relationship between paired groups of dendrogram based on the ETI with predominant substrate composition of zoanthid habitat. In zoanthid habitats with boulder substrate, the coverage range of *Z. sansibaricus* is wide (Table 3) and thus PTX is high in the seawater surrounding the environments of zoanthid, because zoanthids are capable of squirting some PTX from their gastrodermis into the seawater as a defensive mechanism and also as a method for catching their prey (Hoffmann *et al*., 2008). Accordingly, in zoanthid habitats with boulder substrates, the poisoning power of associated macrobenthos is higher and ETI is lower than ETI calculated for macrobenthos associated with

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Zoanthids in cobble substrates (Table 3). Consolidated substrates are usually highly complex environments, which are important for sessile animals such as zoanthids (Denovaro and Fraschetti, 2002).

Littoral reef zones are the most dynamic environments and provide shelter to various species of invertebrates and vertebrates (Sukumaran and George, 2010). This area is mostly affected due to the changes in tidal influx. There are different compromises between the highest and lowest effects of water waves in a littoral ecosystem (Koehl, 1977). According to Table 3, the coverage range of *Z. sansibaricus* in pebbled substrate is narrow and ETI indicated the average poisoning of associated macrobenthos, but in the absence of proper shelter to hide from predators and being prevented from being carried away by waves, their abundance range is low (Perez *et al*., 2005). Thus, the decrease in species abundance at T3 and T6 could have resulted in dominance of pebbled substrates along these transects. It should be noted that in zoanthid habitats with substrate of cobble, coverage range of *Z. sansibaricus* is medium (Table 3), but because of the highly muddy sediments on and between its colonies, the effects of toxicity are reduced on the associated macrobenthos and thus their abundance range is high (Khushali *et al*., 2014).

The estimation of the average abundance range of macrobenthos associated with *Z. sansibaricus* shows moderate abundance in both sites along the west and the east coasts of the Hormuz Island which can have a significant relationship with moderate status in table 2. Also based on the results of ETI in all transects, the dominant macrobenthos associated with *Z. sansibaricus* are sluggish species (G2) and mobile species (G3), that is consistent with the results of other studies (Mirzabagheri *et al*., 2009 a,b, 2013, 2015).

The results of this study and those reported by Mirzabagheri *et al*. (2016) clearly revealed that the toxicity of *Z. sansibaricus* was not only non-acute for the associated macrobenthos, but there also was the possibility of *Z. sansibaricus* being eaten by them without causing any deaths. This eventually leads to the accumulation of PTX in their organs that was transferred to the food chain (Gleibs and Mebs, 1999). Therefore the transport and accumulation of marine toxins in food chains, especially in marine animals, is a natural phenomenon (Yasumoto and Murata, 1993). In order to get more reliable results, studies on the toxicity and the consequences of PTX found in the mucus of zoanthids will need to extract and purify PTX (Charlotte Chan, 2013). However, the results of the present study are only preliminary and further studies are required to verify the impacts of PTX in the whole ecosystem of the Persian Gulf.

We conclude that the ecotoxicity status in macrobenthos associated with *Z. sansibaricus* in the Hormuz Island can be successfully predicted using the theoretical model of the ecotoxicity status based on ETI. Also, the

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estimation of the average abundance range of macrobenthos associated with *Z. sansibaricus* should be considered due to the highest mean predictive performance found in each site from this study, especially for quantitative data. For the present model, accuracy increased with set size in transects. Hence, further studies are required to properly evaluate its strengths and weaknesses on a larger scale.

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