An aquarium hobbyist poisoning: Identification of new palytoxins in *Palythoa* cf. *toxica* and complete detoxification of the aquarium water by activated carbon

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ABSTRACT

Palytoxin (PLTX) is a lethal natural toxin often found in *Palythoa* zoantharians that, together with its congeners, may induce adverse effects in humans after inhalation of toxic aerosols both in open-air and domestic environments, namely in the vicinity of public and private aquaria. In this study, we describe a poisoning of an aquarium hobbyist who was hospitalized after handling a PLTXs-containing zoantharian hexacoral. Furthermore, we provide evidence for water detoxification. The zoantharian was morphologically and genetically identified as *Palythoa* cf. *toxica* (Cnidaria: Anthozoa). Palytoxin itself and two new PLTX congeners, a hydroxyPLTX and a deoxyPLTX, were detected and structurally identified by liquid chromatography high resolution multiple stage mass spectrometry (LC-HRMS^n, n = 1, 2). Total and individual toxins were quantified by LC-HRMS and sandwich ELISA both in the zoantharian (93.4 and 96.80 μg/g, respectively) and in the transport water (48.3 and 42.56 μg/mL, respectively), with an excellent mean bias of 1.3% between the techniques. Activated carbon adsorbed 99.7% of PLTXs contained in the seawater and this represents a good strategy for preventing aquarium hobbyist poisonings.

1. Introduction

Palytoxin (PLTX) (Fig. 1) is a lethal natural toxin originally discovered in the zoantharian *Palythoa toxica* (Cnidaria: Anthozoa: Hexacorallia: Zoantharia) from Hawaii (Moore and Scheuer, 1971). Subsequently, PLTX and/or its analogues have been detected in other *Palythoa* and *Zoanthus* species (Uemura et al., 1985) as well as in other marine organisms, such as dinoflagellates of the genus *Ostreopsis* (Ciminiello et al., 2014a), cyanobacteria of the genus *Trichodesmium* (Kerbrat et al., 2011), and in other various invertebrate and vertebrate species (Aligizaki et al., 2011; Birè et al., 2013).

Besides concerns regarding oral toxicity of PLTXs (Munday, 2011), there is growing evidence that PLTXs exert adverse effects through inhalation of toxic aerosols both in open-air and domestic environments as well as through cutaneous and/or ocular exposure to zoantharians or aquaria waters (Deeds and Schwartz, 2010; Tubaro et al., 2011; Ciminiello et al., 2014b; Pelin et al., 2016a; Tartaglione et al., 2016). In this frame, a number of case reports on human poisonings following manipulation of PLTX-contaminated zoantharians, widely used as aquaria decorative elements, have been reported, with a total of 53 people poisoned (Tubaro et al., 2011; Pelin et al., 2016a; Tartaglione et al., 2016). In most cases, exposure occurred through inhalation of steam generated during installation or cleaning of home aquaria containing

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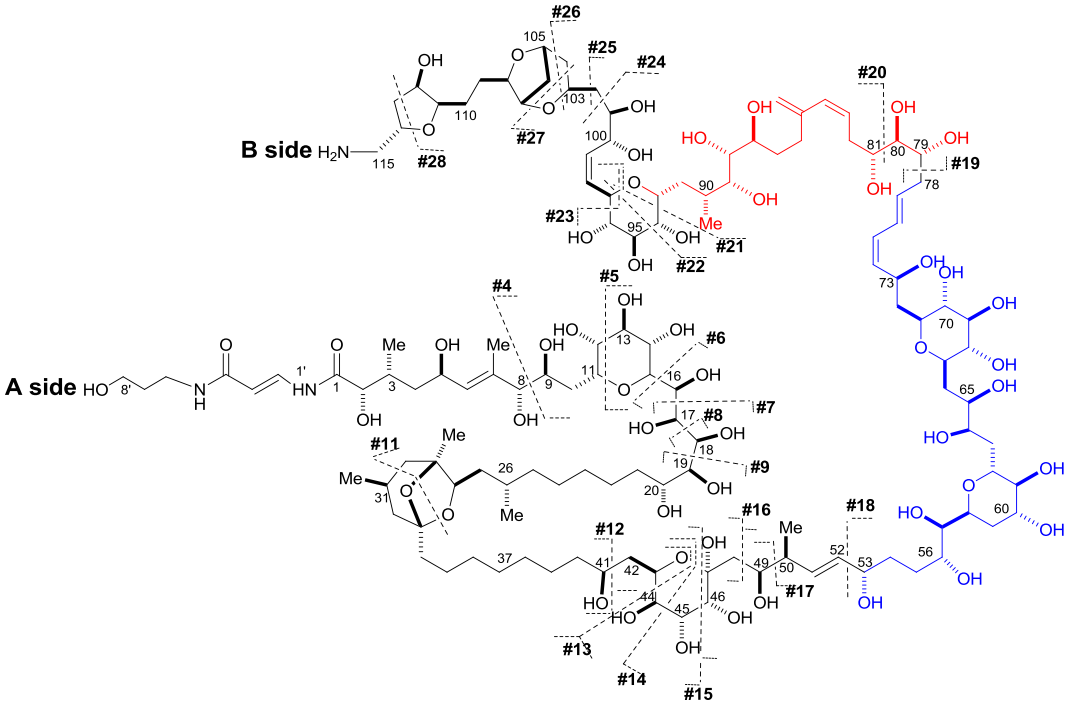

Fig. 1. Structure of palytoxin (PLTX) numbered following the convention of Uemura et al. (1985). HydroxyPLTX has an additional O atom in region C-79 to C-93 (highlighted in red) while deoxyPLTX lacks one O atom in region C-53 to C-78 (highlighted in blue). Cleavages resulting from HR CID MS^2 experiments are reported. Relevant ion fragments are reported in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Palythoa spp. (Moore et al., 1982; Majlesi et al., 2008; Deeds and Schwartz, 2010; Bernasconi et al., 2012; Dijkman and de Vries, 2012; Snoeks and Veenstra, 2012; Sud et al., 2013; Rumore and Houst, 2014; Wieringa et al., 2014; Hall et al., 2015; Hamade et al., 2015; Tartaglione et al., 2016). and only in a few cases through ocular exposure (Moshirfar et al., 2010; Ruiz et al., 2015) or skin contact (Moore et al., 1982; Hoffmann et al., 2008; Deeds and Schwartz, 2010; Nordt et al., 2011; Dijkman and de Vries, 2012) with zoantharians. The main signs and symptoms of inhalatory poisonings involved the respiratory tract (dyspnea, rhinorrhea, cough, sore throat), the skeletomuscular apparatus (myalgia, weakness, spasms, huge increase in creatine phosphokinase), the cardiovascular system (tachycardia), the gastrointestinal apparatus (dysgeusia, as bitter metallic taste, nausea and/or vomiting, diarrhea) and/or the nervous system (dizziness, paresthesia, ataxia, numbness, tremors). Fever $\geq$38 °C was observed in all cases (Tartaglione et al., 2016). It should be noted that for most reported poisonings, the involvement of PLTXs was only postulated (Majlesi et al., 2008; Nordt et al., 2011; Bernasconi et al., 2012; Dijkman and de Vries, 2012; Snoeks and Veenstra, 2012; Sud et al., 2013; Rumore and Houst, 2014; Hall et al., 2015; Ruiz et al., 2015) based on the symptomatology and on the assumption that some *Palythoa* spp. contain PLTXs, and not confirmed by chemical or biological means. In only five case-reports following cutaneous (Moore et al., 1982; Hoffmann et al., 2008) or inhalational exposure (Deeds and Schwartz, 2010; Wieringa et al., 2014; Hamade et al., 2015) to PLTXs-containing zoantharians or steamed water from aquaria were PLTXs confirmed to be the causative agents by hemolytic assay and/or liquid chromatography coupled with ultraviolet (LC-UV) or mass spectrometry (LC-MS) detection.

Aquarium hobbyist poisonings may be largely underestimated considering that *Palythoa heliodiscus* and related species' colonies containing high levels of PLTX and deoxyPLTX are commonly sold in the home aquarium trade (Deeds et al., 2011). In this study, we describe the first case in Italy of an aquarium hobbyist, who was hospitalized after handling a PLTXs-containing zoantharian that was morphologically and genetically identified as *Palythoa* cf. *toxica* (Cnidaria: Anthozoa: Hexacorallia). High Resolution LC-MS and a sandwich ELISA detected high amounts of PLTX and of new PLTX congeners, namely a hydroxyPLTX and a deoxyPLTX, both in the zoantharian and in the surrounding water. As well, the ability of activated carbon to detoxify the aquarium water was investigated through chemical means.

2. Materials and methods

2.1. Sample collection

A colony of a green-brown *Palythoa* species growing in the home aquarium of the poisoned patient was collected (See Supplementary material, Fig. S1-A). The polyps were readily contractile and their maximum oral disc diameter, without tentacles, was 15 mm. The colony morphologically resembled that of *Palythoa* sp. VAZOA responsible for a severe respiratory reaction in an aquarium hobbyist in Virginia (USA) in 2008 (Deeds et al., 2011). After photographic documentation (See Supplementary material, Fig. S1-B), 3 polyps were fixed in 96% ethanol for DNA analyses. A small colony with supporting substrate (approximately 10 individual polyps) was designated as specimen "NMS1" while a control specimen from a *Palythoa* colony of the Museum of Nature South Tyrol aquarium (See Supplementary material, Fig. S1-C) was sampled and designated as 'NMS2'. Both NMS1 and NMS2 samples were shipped alive in aquarium water for toxin analyses.

2.2. Species identification

High-resolution images of specimens NMS1 (See Supplementary material, Fig. S1) were examined, noting the following characters: colony and polyp form ('immersae', 'intermediae' or 'liberae'; see Pax, 1910), external coloration, oral disk coloration and

patterns, as well as tentacle coloration, numbers and length. Results were compared with descriptions of previously known *Palythoa* (including *Protopalythoa*) species (Reimer et al., 2004, 2014, 2015).

2.3. DNA extraction and phylogenetic analyses

DNA was extracted from tissue with a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Three different DNA markers were amplified. The host *Palythoa* nuclear internal transcribed spacer region (ITS-rDNA) was amplified using the primers Zoan-f 5′-CTT GAT CAT TTA GAG GGA GT-3′ and Zoan-r 5′-CGG AGA TTT CAA ATT TGA GCT-3' (Reimer et al., 2007), with a PCR thermal sequence of 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C, with a final elongation step of 10 min at 72 °C (Reimer et al., 2007). *Palythoa* mitochondrial 16S ribosomal DNA (16S-rDNA) was amplified using the primers 16Sant1a: 5′-GCC ATG AGT ATA GAC GCA CA-3′ and 16SbmoH: 5′-CGA ACA GCC AAC CCT TGG-3′ (Sinniger et al., 2005) with a PCR thermal sequence of 2 min at 94 °C, then 40 cycles: 30 s at 94 °C, 1 min at 52 °C, 2 min at 72 °C, with a final elongation step of 5 min at 72 °C (Sinniger et al., 2005). Finally, an approximately 300-base pair region of symbiotic *Symbiodinium* (=zooxanthellae) nuclear internal transcribed spacer 2 (ITS2) ribosomal DNA was amplified and directly sequenced using the following primers: ITSintfor2 (5′-GAATTGCAGAACTCCG TG-3′) and ITS-reverse (5′-GGGATCCATATGCTTAAGTTCAGCGGGT-3′) (Manning and Gates, 2008). PCR was performed under the following conditions: an initial denaturation for 5 min at 95 °C and 35 cycles of (94 °C for 45 s, 54 °C for 45 s, and 72 °C for 30 s), and a final extension at 72 °C for 10 min. All PCR mixes used HotStarTaq (Qiagen; Tokyo, Japan) following the manufacturer's instructions.

PCR products were visualized using 1.5% agarose gel. Gels were run for 20 min and then visualized using ethidium bromide. Successfully amplified products were purified by adding 3 μL of Exo-I + shrimp alkaline phosphatase (Takara) to 20 μL of PCR product, incubating at 37 °C for 15 min followed by 20 min at 80 °C. Sequencing reactions (both forward and reverse) were outsourced to Fasmac Japan (Kanagawa, Japan).

Sequences were initially checked by blastn (http://www.ncbi.nlm.nih.gov/BLAST) to confirm identity as Zoantharia or *Symbiodinium*, depending on the DNA marker. Newly acquired nucleotide sequences of *Palythoa* 16S-rDNA from samples were then manually aligned with previously published 16S-rDNA sequences from *Palythoa* species. A newly acquired nucleotide sequence of *Palythoa* ITS-rDNA from NMS1 was manually aligned with previously published ITS-rDNA sequences most similar to *Palythoa heliodiscus* or *P.* aff. *variabilis* from Reimer et al. (2007, 2012, 2013) and unpublished sequences from *P.* cf. *toxica* from Singapore and Japan. *Symbiodinium* ITS2 sequences were aligned with previously published ITS2 sequences of clade C *Symbiodinium*, primarily isolated from *Palythoa* spp. Outgroup sequences for the *Palythoa* 16S-rDNA and clade C *Symbiodinium* ITS2 trees were not designated, as both *Palythoa* spp. and *Symbiodinium* clade C have previously been shown to be monophyletic for these markers (Reimer et al., 2006, 2007). An outgroup (*Palythoa grandis*, GenBank accession number JX119129) was added to the *Palythoa* ITS-rDNA alignment as otherwise the target species group (*P. heliodiscus*/*P.* cf. *toxica*) was basal, and clear species groups were not recoverable. Alignments were inspected by eye and manually edited and all ambiguous sites were removed from the datasets for subsequent phylogenetic analyses. Three alignment datasets were generated: 1) 550 sites of 28 sequences (*Palythoa* 16S-rDNA), 2) 715 sites of 21 sequences (*Palythoa* ITS-rDNA), and 3) 319 sites of 24 sequences (*Symbiodinium* ITS2). The alignment data are available on request from J. D. Reimer. Novel sequences acquired in this study are registered under GenBank accession numbers KX712285-KX712295.

For phylogenetic analyses of the three alignments, the same methods were applied independently. Alignments were subjected to analyses with the maximum-likelihood method (MΛ) with PhyMΛ (Guindon et al., 2010) and the neighbor-joining (NJ) method using CLC Free Workbench 3 (http://www.clcbio.com). PhyML was performed using an input tree generated by BIONJ with the general time-reversible (GTR) model of nucleotide substitution, with invariable sites and a discrete gamma distribution (eight categories) (GTR + I + C). The proportion of invariable sites, discrete gamma distribution, and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1000 replicates) were performed using the same parameters as the individual ML trees. The distances were calculated using a Kimura's 2-parameter model (Kimura et al., 1972; Kimura, 1980) Support for NJ branches was tested by bootstrap analyses of 1000 replicates.

2.4. Toxin analyses

2.4.1. Chemicals

All organic solvents were of distilled-in-glass grade (Carlo Erba; Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd.; Bedford, MA, USA). Acetic acid (Laboratory grade) was purchased from Carlo Erba. PLTX was purchased from Wako Chemicals GmbH (Neuss, Germany). Multi-well strips were from Nunc (Langenselbold, Germany); horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit goat antibodies were from DakoCytomation (Milan, Italy). Other materials and chemicals were from Sigma-Aldrich (Milan, Italy).

2.4.2. Extraction

The rock (including polyps) and the aquarium water of sample NMS1 (rock wet weight 17 g, including 6 g of polyps; V_{water} = 50 mL) and NMS2 (rock wet weight 5 g, including 2.7 g of polyps; V_{water} = 45 mL) were separately extracted as follows. Each rock was suspended in an equal amount of methanol/water (8:2, v/v) and sonicated for 15 min in pulse mode, while cooling in an ice bath. The obtained extracts (V_{NMS1} = 30 mL, V_{NMS2} = 20 mL) were filtered through 0.45 μm centrifuge filters (Merck Millipore; Carrigtwohill, Ireland) and analyzed directly by LC/HRMS. For quantitative purposes, an aliquot of the NMS1 extract (100 μL) was diluted (1:1000) with the extraction solvent, whereas 800 μL of the NMS2 extract were concentrated down to 100 μL under nitrogen flow; both samples were re-analyzed by LC-HRMS. Each sample of aquarium water was extracted five times with an equal volume of *n*-butanol (extraction recovery 75%) (Tartaglione et al., 2016). The butanol layer was evaporated to dryness, dissolved in methanol/water (1:1, v/v) (V_{NMS1} = 3 mL and V_{NMS2} = 2 mL), filtered through 0.45 μm centrifuge filters and analyzed by LC-HRMS.

2.4.3. Liquid chromatography-high resolution mass spectrometry

LC-HRMS experiments were carried out on a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAX™ source (Thermo-Fisher; San Josè, CA, USA) coupled to an Agilent 1100 LC binary system (Palo Alto, CA, USA). A 2.7 μm Poroshell 120 EC-C18, 100 × 2.10 mm column (Agilent) was eluted at 0.2 mL/min with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. Gradient elution was 28–29% B in 10 min, 29–30% B in 10 min, 30–100% B in 1 min, and hold for 1 min. Re-equilibration time was 13 min. Injection volume was 5 μL. Under such conditions all the PLTXs chromatographically separated (Ciminiello et al., 2015). HR full MS experiments (positive ions) were acquired in the range *m*/*z* 800–1400 at a resolving power of 60,000. The following source settings were used in all LC-HRMS experiments: a spray voltage of 4.8 kV, a capillary temperature of 290 °C, a capillary voltage of 50 V,

a sheath gas and an auxiliary gas flow of 38 and 2 (arbitrary units). The tube lens voltage was set at 140 V. HRMS^2 data were acquired in collision induced dissociation (CID) mode at a resolving power of 30,000 by selecting the $[M + H + Ca]^{3+}$ ions of palytoxin (*m/z* 906.5), deoxypalytoxin (*m/z* 901.5), and hydroxypalytoxin (*m/z* 912.5) as precursors. A collision energy of 25%, an activation Q of 0.250, and an activation time of 30 ms were used. Quantitative determination of PLTXs in the extracts was carried out by using extracted ion chromatograms (XIC) of $[M + H + Ca]^{3+}$ ions of each congener (5 ppm mass tolerance) and calibration curve (triplicate injection) of PLTX standard at four levels of concentration (1000, 100, 25, 12.5 ng/mL), assuming for all the molecules the same molar response. Limit of detection (LOD) for PLTX on the day of analysis was 6.25 ng/mL.

2.4.4. Indirect sandwich ELISA

Quantitation of PLTXs by an indirect sandwich ELISA was performed as previously described (Boscolo et al., 2013). Briefly, anti-PLTX monoclonal antibody-coated multi-well strips were incubated for 2 h with 100 μL of PLTX or sample extracts (diluted 1:10 or 1:100 in PBS containing 0.1% Tween 20; PBS-Tw) after blocking with 200 μL of 2% skimmed milk (w/v). After washing, 100 μL of purified anti-PLTX rabbit polyclonal antibodies (0.17 μg/mL in blocking solution) were added in each well. After washing, 100 μL of horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:2000 in blocking solution) were added. After washing, 60 μL of 3,3,5,5-tetramethylbenzidine liquid substrate were added to the wells and the reaction was stopped after 30 min with 30 μL of 1 M H_2SO_4. The absorbance was read at 450 nm (Spectrophotometer Tecan; Milan, Italy). The amounts of PLTXs in zoantharian specimens and culture waters extracts were extrapolated from PLTX calibration curve. Results are the mean ± standard error (SE) of six independent experiments performed in duplicate.

2.4.5. Detoxification experiments

The NMS1 sample was used to test capability of activated carbon (Rowa Carbon in pellets) to detoxify the aquarium water. A sample of marine aquarium water (1 L) and 20 polyps were collected from the aquarium tank ($V_{aquarium} = 250$ L) where they had been kept alive after the poisoning incident. Two aliquots of the aquarium water (100 mL each) were then separated: aliquot #1 was mixed with 5 g of activated carbon while aliquot #2 was used as control. Both aliquots were kept under magnetic stirring for 24 h, centrifuged at 5000 rpm for 10 min and the supernatant was decanted ($V_{\#1} = 87$ mL; $V_{\#2} = 95$ mL). The supernatants were separately extracted 5 times with an equal volume of *n*-butanol. The butanol extracts were evaporated to dryness, dissolved in 5 mL of methanol/water (1:1, v/v) and analyzed by LC-HRMS versus matrix matched PLTX standards (Ciminiello et al., 2006) and by sandwich ELISA.

3. Results

3.1. Case report

A 47-year-old male marine aquarium hobbyist in Bolzano (Italy) was attempting to eradicate a colony of green-brown *Palythoa* from his aquarium by using a brush and pouring boiling water over the colony without any personal protective equipment. No detergent was used. The *Palythoa* colony, which had grown in the aquarium for two years, had arrived as a hitchhiker with live rocks and had overgrown more desirable organisms in the tank. The past medical history of the non-smoking patient revealed a family history of diabetes, hypertension treated with angiotensin-converting enzyme inhibitors, and no other significant pathology. During the eradication process, the patient inhaled steam from the boiling water. Within 2 h, he experienced coughing, difficulty in breathing and a temperature of 38 °C. The symptoms progressively worsened with pyrexia ≥39 °C, dyspnea and orthopnea. The physical thorax examination revealed no pathologic signs except for a diffusely reduced vesicular murmur, tachypnea with orthopnea at the rest and tachycardia. The thoracic RX revealed a sharpen costophrenic angle with a basal parenchymal hypodiaphania, not excluding a small bronchopneumonic outbreak. The blood gas analysis revealed a pH of 7.47, partial pressures of oxygen (PaO_2) and carbon dioxide ($PaCO_2$) of 55 mmHg and 31.5 mmHg, respectively, with 89% oxygen saturation. Hematochemical analyses evaluated the following parameters: K^+ (3.49 mmol/L), glycaemia (140 mg/dL), C-reactive protein (CRP, 15.27 mg/dL), pro-calcitonin (5.69 ng/mL), erythrocyte sedimentation rate (ESR, 33), D-Dimer (927.2 ng/mL), total bilirubin (3.7 mg/dL) and direct bilirubin (0.7 mg/dL). A neutrophilic leukocytosis (16,700 cells/mm^3) was found while liver and kidney parameters, electrolytes, lactate dehydrogenase, coagulation parameters and urinalysis were within physiological ranges.

The patient was hospitalized in semi-intensive therapy with a diagnosis of partial respiratory failure from a suspected bronchopneumonia. The pharmacological treatment consisted in broad-spectrum antibiotics (clarithromycin, piperacillin), oxygen mask therapy, aerosol therapy with beclomethasone and albuterol, ipratropium bromide, antithrombotic prophylaxis with low molecular weight heparin and antipyretic therapy with paracetamol.

The clinical symptomatology rapidly improved, with normalization of blood parameters (maximum peak of CRP: 30.5 mg/dL on day 2). The patient was discharged on the 6th day without fever and with only cough; the clinical and radiological pictures were completely fixed in the follow-up, 2 weeks after the onset of symptoms.

The atypical clinical, radiological and laboratory findings, the negative result of repeated blood cultures for aerobic and anaerobic germs as well as the negative serological tests for *Mycoplasma*, *Chlamydia*, *Klebsiella*, *Legionella* and *Streptococcus pneumoniae* excluded the most common bacterial pathogens for the etiological origin. The hypothesis of PLTX poisoning was suggested by the close temporal link between the onset of the symptoms and the possible inhalation of a toxic agent during the cleaning of the aquarium as well as by the detection of PLTXs in polyps of *Palythoa* and in the aquarium water.

3.2. Zoantharian collection

A portion of the aquarium rock colonized by *Palythoa* sp. (NMS1) was removed from the aquarium and suspended in artificial salt water. An aliquot of a *Palythoa* colony from the Museum of Nature South Tyrol (NMS2) was sampled and analyzed in parallel as control. Both samples NMS1 and NMS2 were subjected to morphological and phylogenetic analyses for species identification and to chemical and immunological analyses for determination of toxin content and profile.

3.3. Species identification

Based on gross external morphology, the *Palythoa* NMS1 coral was recognized as "liberae" (Pax, 1910) with polyps free and clear of the basal lamellar coenenchyme. The external colony coloration was generally brown, as seen in species of this genus, with an oral disk of mottled brown and olive green with occasional, randomly placed small whitish dots (See Supplementary material, Fig. S1A). The oral opening was also off-white in color. Tentacles were almost blue-green compared to the oral disk, but also somewhat mottled in patterning, with a dark brown tip on each tentacle, and 30–45%

in length of the oral disk diameter. Overall colony coloration became more brownish and less green once the colony was moved to the Museum of Nature South Tyrol's aquarium (See Supplementary material, Fig. S1B). The numbers of tentacles were between 74 and 88 (n = 4 polyps counted). Compared to previously described species, the identity of this specimen was difficult to ascertain. Oral disk coloration and toxic characteristics were similar to *P. toxica* from Hawaii (Walsh and Bowers, 1971), but the number of tentacles in *P. toxica* are given as up to 60, much lower than those observed in NMS1, and as toxicity has subsequently been reported in other *Palythoa* species, this character alone cannot identify *Palythoa* to species level. *Palythoa heliodiscus* (Ryland and Lancaster, 2003) does not morphologically match closely to this study's specimen, as it has no oral disk patterns and very short tentacles, although *P. heliodiscus* is described as having up to 80 tentacles or more. The NMS1 specimen had longer tentacles but looked similar to previously reported but not conclusively identified *P.* cf. *heliodiscus* specimens from the Central Indo-Pacific (Reimer et al., 2014), and to images of *P.* cf. *toxica* from the South China Sea (Reimer et al., 2015). As in Reimer et al. (2004, 2014, 2015), based on morphological data, NMS1 specimen was identified as *Palythoa* cf. *toxica*.

3.4. Phylogenetic analyses: 16S-rDNA, ITS-rDNA, and Symbiodinium ITS2 sequences

The 16S-rDNA sequence from specimen NMS1 formed a very well-supported clade (ML = 95%, NJ = 99%) with several identical sequences from previous studies, including four sequences from highly toxic aquarium trade *Palythoa* specimens reported by Deeds et al. (2011), two representative *P. heliodiscus* sequences from Japan (Reimer et al., 2006, 2007) and one *P.* aff. *variabilis* sequence from Florida (See Supplementary material, Fig. S2A).

The 16S-rDNA sequence from specimen NMS2 formed a moderately well-supported grouping (ML = 63%, NJ = 64%) with previously reported sequences from *P. mutuki* from Japan, *P. grandiflora* from Florida, and three weakly PLTX-positive aquarium trade specimens reported by Deeds et al. (2011) (See Supplementary material, Fig. S2A).

The ITS-rDNA sequence from the *P.* cf. *toxica* specimen NMS1 formed a small, weakly-supported subclade (ML = 62%, NJ = 63%) with specimen 1566 from the aquarium trade mentioned by Reimer et al. (2012). These two sequences clustered with other *P.* cf. *toxica* specimens, and were slightly different from *P. heliodiscus* and *P.* aff. *variabilis* sequences, which formed separate subclades (See Supplementary material, Fig. S2B).

The *Symbiodinium* ITS-rDNA sequence from specimen NMS1 was different from all previously reported *Symbiodinium* sequences (See Supplementary material, Fig. S3), and was derived from *Symbiodinium* subclade C20 sensu (LaJeunesse, 2002) forming a subclade with this sequence (ML = 81%, NJ = 63%). The *Symbiodinium* sequence from NMS2 was identical to numerous previously reported clade C3 sequences.

3.5. Chemical analyses by LC-HRMS^n (n = 1, 2)

Crude extracts of the zoantharians and of the surrounding water of NMS1 and NMS2 samples were analyzed by LC-HRMS in Full scan HRMS and HRCIDMS^2 modes versus a PLTX standard (from *P. tuberculosa*). A newly developed method for PLTXs which allows their chromatographic separation was used (Ciminiello et al., 2015), slightly modifying the gradient conditions in order to achieve a complete chromatographic resolution of PLTX congeners contained in the extracts.

Total ion chromatograms (TICs) of NMS1 extracts (zoantharian and water) were dominated by a chromatographic peak at 10.92 min (See Supplementary material, Fig. S4A) eluting at the same retention time as PLTX standard. The associated full HRMS spectrum contained all the characteristic triply and doubly charged ions of PLTX, including protonated and adduct ions (See Supplementary material, Table S1 and Fig. S4B), and it was superimposable to that of PLTX standard both in relative ion ratio, exact masses and isotopic ion patterns of individual ions. Confirmation of PLTX identity was provided by the diagnostic fragmentations (Fig. 1; Table 1) contained in the HRMS^2 spectrum of the $[M + H + Ca]^{3+}$ ion at *m/z* 906.8, which were the same as for PLTX standard (Ciminiello et al., 2012). The only difference was observed in the relative ion ratio of the fragment ions $[M + H + Ca\text{-}nH_2O]^{3+}$, n = 2–8 which dominate HRMS^2 spectra of all PLTX-like compounds: the $[M + H + Ca\text{-}2H_2O]^{3+}$ at *m/z* 894.8087 (mono-isotopic ion *m/z* 894.4757, $C_{129}H_{220}O_{52}N_3Ca$, $\Delta = -0.050$ ppm) was the most intense ion of the cluster in PLTX standard (from *P. tuberculosa*) while the $[M + H + Ca\text{-}3H_2O]^{3+}$ at *m/z* 888.8021 (mono-isotopic ion *m/z* 888.4681, $C_{129}H_{218}O_{51}N_3Ca$, $\Delta = -4.697$) was the most intense ion of the cluster in PLTX from specimen NMS1 (identified as *P.* cf. *toxica*). This suggested that some small structural differences (e.g. structural isomerism, stereoisomerism) might occur between the PLTX detected in NMS1 sample and PLTX standard. Further in-depth structural investigation by NMR would be needed to fully elucidate the stereo-structure of this compound.

Extracted ion chromatograms (XIC) of the $[M + H + Ca]^{3+}$ ions of all the PLTX-like compounds so far known (See Supplementary material, Table S2) highlighted the presence in the NMS1 extracts of two additional PLTX analogues, eluting at 10.01 min and 13.17 min. The former compound presented a full HRMS spectrum (See Supplementary material, Fig. S4C) dominated by triply-charged calcium and magnesium adduct ions and very weak doubly charged sodium and potassium adduct ions. A cross-check of elemental formulae of all these ions (See Supplementary material, Table S1) pointed to the molecular formula $C_{129}H_{223}N_3O_{55}$, suggesting that this compound was a hydroxyPLTX. A comparative analysis of its HRMS^2 spectrum (Table 1) versus that of PLTX standard indicated that the additional hydroxyl group was located in the region stretching from C-79 to C-93 (Fig. 1).

Thus, the hydroxyPLTX detected in the NMS1 sample was a new PLTX analogue isobaric with the 42-hydroxy-PLTXs previously isolated from *P. toxica* (Ciminiello et al., 2009) and *P. tuberculosa* (Ciminiello et al., 2014c).

Analysis of the full HRMS spectrum associated to the compound eluting at 13.17 min (See Supplementary material, Fig. S4D and Table S1) pointed to the elemental formula $C_{129}H_{223}N_3O_{53}$ consistent with a deoxyPLTX. Fragmentations emerging from its HRMS^2 spectrum (Table 1) suggested that deoxyPLTX, compared to PLTX standard, lacked one hydroxyl group in the region stretching from C-53 to C-78. This part structure does not undergo further fragmentation since likely a conjugated polyene structure is formed as suggested by Uchida et al. (2013).

Retention time and fragmentation pattern of the detected deoxyPLTX were different from those of other deoxyPLTX derivatives from *Ostreopsis* cf. *ovata* (ovatoxin-d and -e) for which a reference extract was available (Ciminiello et al., 2010; Dell'Aversano et al., 2014). Unfortunately, due to the lack of a reference sample of the other deoxyPLTX so far known (73-deoxyPLTX) (Uemura et al., 1985) it was not possible to check the identity of this compound. Other very minor PLTX analogues were detected in NMS1 extract and further studies are needed for their identification.

LC-HRMS analysis of the NMS2 extracts showed the presence of low levels of PLTX in the only zoantharian extract, with full scan HRMS and HRMS^2 spectra fully superimposable to those of PLTX standard. No PLTX congener was detected.

Table 1
Assignment of A-, B-side and internal fragments in HRMS^2 spectra of palytoxin (PLTX), HydroxyPLTX and DeoxyPLTX to relevant cleavages (#Clv) reported in Fig. 1.[a]

#Clv	PLTX		HydroxyPLTX		DeoxyPLTX	
	A-side	B-side	A-side	B-side	A-side	B-side
	m/z (-nH_2O) Formula (RDB)	*m/z* (-nH_2O) Formula (RDB)	*m/z* (-nH_2O) Formula (RDB)	*m/z* (-nH_2O) Formula (RDB)	*m/z* (-nH_2O) Formula (RDB)	*m/z* (-nH_2O) Formula (RDB)
#4	327.1900 ($^{1+}$) (-1H_2O) $C_{16}H_{27}O_5N_2$ (4.5)	1187.1172 ($^{2+}$) (-6H_2O) $C_{113}H_{195}O_{48}NCa$ (17.0) 791.7468 ($^{3+}$) (-3H_2O) $C_{113}H_{196}O_{48}NCa$ (16.5)	327.1898($^{1+}$) (-1H_2O) $C_{16}H_{27}O_5N_2$ (4.5)	1195.1142 ($^{2+}$) (-4H_2O) $C_{113}H_{195}O_{49}NCa$ (17.0) 791.0744* ($^{3+}$) (-1H_2O) $C_{113}H_{194}O_{48}NCa$ (17.5)	327.1897 ($^{1+}$) (-1H_2O) $C_{16}H_{27}O_5N_2$ (4.5)	1179.1192 ($^{2+}$) (-5H_2O) $C_{113}H_{195}O_{47}NCa$ (17.0) 786.4127 ($^{3+}$) (-3H_2O) $C_{113}H_{196}O_{47}NCa$ (16.5)
#11	446.2198($^{2+}$) $C_{40}H_{72}O_{17}N_2Ca$ (6.0)		446.2191($^{2+}$) $C_{40}H_{72}O_{17}N_2Ca$ (6.0)		446.2191($^{2+}$) $C_{40}H_{72}O_{17}N_2Ca$ (6.0)	
#12	544.2924 ($^{2+}$) (-2H_2O) $C_{52}H_{92}O_{19}N_2Ca$ (8.0)	807.8866 ($^{2+}$) (-1H_2O) $C_{77}H_{125}O_{32}NCa$ (16.0)	544.2923 ($^{2+}$) $C_{52}H_{92}O_{19}N_2Ca$ (8.0)	815.8830 ($^{2+}$) $C_{77}H_{125}O_{33}NCa$ (16.0)	544.2921 ($^{2+}$) (-2H_2O) $C_{52}H_{92}O_{19}N_2Ca$ (8.0)	790.8826 ($^{2+}$)* $C_{77}H_{123}O_{30}NCa$ (17.0)
#13	566.3055 ($^{2+}$) (-2H_2O) $C_{54}H_{96}O_{20}N_2Ca$ (8.0)	803.8837 ($^{2+}$) (-3H_2O) $C_{75}H_{125}O_{33}NCa$ (14.0)	566.3052 ($^{2+}$) $C_{54}H_{96}O_{20}N_2Ca$ (8.0)	n.d.	566.3051($^{2+}$) $C_{54}H_{96}O_{20}N_2Ca$ (8.0)	795.8834 ($^{2+}$) (-1H_2O) $C_{75}H_{125}O_{32}NCa$ (14.0)
#14	572.3054($^{2+}$) $C_{55}H_{96}O_{20}N_2Ca$ (9.0)	797.8834($^{2+}$) (-3H_2O) $C_{74}H_{125}O_{33}NCa$ (13.0)			572.3051($^{2+}$) $C_{55}H_{96}O_{20}N_2Ca$ (9.0)	780.8788 ($^{2+}$)*(-2H_2O) $C_{74}H_{123}O_{31}NCa$ (14.0)
#15	596.3159 ($^{2+}$) (-2H_2O) $C_{56}H_{100}O_{22}N_2Ca$ (8.0)	782.8784 ($^{2+}$) (-3H_2O) $C_{73}H_{123}O_{32}NCa$ (13.0) 1526.8034 ($^{1+}$) (-4H_2O) $C_{73}H_{124}O_{32}N$ (12.5)	596.3154 ($^{2+}$) (-2H_2O) $C_{56}H_{100}O_{22}N_2Ca$ (8.0)	790.8749($^{2+}$) (-3H_2O) $C_{73}H_{12}O_{33}NCa$ (13.0) 1542.7954 ($^{1+}$) (-3H_2O) $C_{73}H_{124}O_{33}N$(12.5)	596.3154 ($^{2+}$) (-2H_2O) $C_{56}H_{100}O_{22}N_2Ca$ (8.0)	774.8800 ($^{2+}$) (-2H_2O) $C_{73}H_{123}O_{31}NCa$ (13.0) 1510.8040 ($^{1+}$) (-4H_2O) $C_{73}H_{124}O_{31}N$ (12.5)
#16	633.3341($^{2+}$) (-3H_2O) $C_{59}H_{106}O_{24}N_2Ca$ (8.0)	745.8600 ($^{2+}$) (-5H_2O) $C_{70}H_{117}O_{30}NCa$ (13.0) 1452.7663 ($^{1+}$) (-6H_2O) $C_{70}H_{118}O_{30}N$ (12.5)	633.3334 ($^{2+}$) (-2H_2O) $C_{59}H_{106}O_{24}N_2Ca$ (8.0)	753.8571 ($^{2+}$) (-2H_2O) $C_{70}H_{117}O_{31}NCa$ (13.0) 1468.7604($^{1+}$) (-3H_2O) $C_{70}H_{118}O_{31}N$ (12.5)	633.3343($^{2+}$) (-3H_2O) $C_{59}H_{106}O_{24}N_2Ca$ (8.0)	737.8617 ($^{2+}$) (-4H_2O) $C_{70}H_{117}O_{29}NCa$ (13.0) 1436.7699 (-4H_2O) $C_{70}H_{118}O_{29}N$ (12.5)
#17	647.3319 ($^{2+}$) (-1H_2O) $C_{60}H_{106}O_{25}N_2Ca$ (9.0)	1406.7610 ($^{1+}$) (-3H_2O) $C_{69}H_{116}O_{28}N$ (12.5)	647.3209 ($^{2+}$) (-1H_2O) $C_{60}H_{106}O_{25}N_2Ca$ (9.0)	n.d.	647.3308 ($^{2+}$) (-1H_2O) $C_{60}H_{106}O_{25}N_2Ca$ (9.0)	1390.7629($^{1+}$) (-4H_2O) $C_{69}H_{116}O_{27}N$ (12.5)
#18		694.8264 ($^{2+}$) (-2H_2O) $C_{65}H_{107}O_{28}NCa$ (13.0)		n.d.		686.8291 ($^{2+}$) (-1H_2O) $C_{65}H_{107}O_{27}NCa$ (13.0)
#19	948.4955 ($^{2+}$) (-1H_2O) $C_{90}H_{156}O_{37}N_2Ca$ (14.0)	804.4337($^{1+}$) $C_{39}H_{66}O_{16}N$ (7.5)	948.4966 ($^{2+}$) (-1H_2O) $C_{90}H_{156}O_{37}N_2Ca$ (14.0)	820.4264 ($^{1+}$) $C_{39}H_{66}O_{17}N$ (7.5)	n.d.	804.4322($^{1+}$) $C_{39}H_{66}O_{16}N$ (7.5)
#20		744.4127 ($^{1+}$) $C_{37}H_{62}O_{14}N$ (7.5)		n.d.		744.4129 ($^{1+}$) $C_{37}H_{62}O_{14}N$ (7.5)
#21	1129.5916 ($^{2+}$) (-4H_2O) $C_{107}H_{186}O_{45}N_2Ca$ (16.0)	406.2205 ($^{1+}$) $C_{22}H_{32}O_6N$ (7.5)	1137.5878 ($^{2+}$) $C_{107}H_{186}O_{46}N_2Ca$ (16.0)	406.2197 ($^{1+}$) $C_{22}H_{32}O_6N$ (7.5)	1121.5923 ($^{2+}$) (-3H_2O) $C_{107}H_{186}O_{44}N_2Ca$ (16.0)	406.2205 ($^{1+}$) $C_{22}H_{32}O_6N$ (7.5)
#22	1144.5983 ($^{2+}$) (-2H_2O) $C_{108}H_{188}O_{46}N_2Ca$ (16.0)		1143.5878 * ($^{2+}$) (-2H_2O) $C_{108}H_{186}O_{46}N_2Ca$ (17.0)		1136.5984 ($^{2+}$) (-2H_2O) $C_{108}H_{188}O_{45}N_2Ca$ (16.0)	
#23	1174.6077 ($^{2+}$) (-3H_2O) $C_{110}H_{192}O_{48}N_2Ca$ (16.0)		n.d.		1166.6073 ($^{2+}$) (-2H_2O) $C_{110}H_{192}O_{47}N_2Ca$ (16.0)	
#24	1215.6312 ($^{2+}$) $C_{115}H_{198}O_{49}N_2Ca$ (18.0)		n.d.		1207.6290 ($^{2+}$) $C_{115}H_{198}O_{48}N_2Ca$ (18.0)	
#25	1222.6399 ($^{2+}$) (-4H_2O) $C_{116}H_{200}O_{49}N_2Ca$ (18.0)		1230.6329 ($^{2+}$) (-4H_2O) $C_{116}H_{200}O_{50}N_2Ca$ (18.0)		1214.6376 ($^{2+}$) (-3H_2O) $C_{116}H_{200}O_{48}N_2Ca$ (18.0)	
#26	1235.6446 ($^{2+}$) (-4H_2O) $C_{118}H_{202}O_{49}N_2Ca$ (19.0)		n.d.		1227.6468 ($^{2+}$) (-4H_2O) $C_{118}H_{202}O_{48}N_2Ca$ (19.0)	
#27	1236.6330 ($^{2+}$) (-2H_2O) $C_{117}H_{200}O_{50}N_2Ca$ (19.0)		n.d.		1228.6348 ($^{2+}$) (-1H_2O) $C_{117}H_{200}O_{49}N_2Ca$ (19.0)	
#28	n.d.		n.d.		n.d.	

#Clv	PLTX	HydroxyPLTX	DeoxyPLTX
	Internal fragments	Internal fragments	Internal fragments
	m/z (RDB) Formula (-nH_2O)	*m/z* (RDB) Formula (-nH_2O)	*m/z* (RDB) Formula (-nH_2O)
#4 + #12	372.1963 ($^{2+}$) $C_{36}H_{64}O_{13}Ca$ (5.0) 743.3853 ($^{1+}$) $C_{36}H_{63}O_{13}Ca$ (5.5)	372.1962 ($^{2+}$) $C_{36}H_{64}O_{13}Ca$ (5.0) 743.3851($^{1+}$) $C_{36}H_{63}O_{13}Ca$ (5.5)	372.1961 ($^{2+}$) $C_{36}H_{64}O_{13}Ca$ (5.0) 743.3848 ($^{1+}$) $C_{36}H_{63}O_{13}Ca$ (5.5)
#4 + #13	394.2095($^{2+}$) $C_{38}H68O_{14}Ca$ (5.0)	n.d.	394.2092($^{2+}$) $C_{38}H_{68}O_{14}Ca$ (5.0)
#4 + #15	424.2196 ($^{2+}$) (-1H_2O) $C_{40}H_{72}O_{16}Ca$ (5.0)	n.d.	424.2192 ($^{2+}$) (-1H_2O) $C_{40}H_{72}O_{16}Ca$ (5.0)
#4 + #16	461.2376 ($^{2+}$) (-1H_2O) $C_{43}H_{78}O_{18}Ca$ (5.0)	n.d.	461.23744 ($^{2+}$) (-1H_2O) $C_{43}H_{78}O_{18}Ca$ (5.0)
#5 + #12	657.3488 ($^{1+}$) $C_{32}H_{57}O_{11}Ca$ (4.5)	657.3471 ($^{1+}$) $C_{32}H_{57}O_{11}Ca$ (4.5)	657.3485 ($^{1+}$) $C_{32}H_{57}O_{11}Ca$ (4.5)

[a] Elemental formula of the monoisotopic ion peaks (*m/z*) are reported together with number of water losses observed in the spectra (-nH_2O) and relative double bonds (RDB). Mass errors were below 10 ppm in all cases. n.d. = not detected. * fragment appearing as water loss compared to the relevant fragment of the parent compound (PLTX).

3.6. Toxin quantitation by LC-HRMS and ELISA

LC-HRMS analyses provided information on total and individual toxin content and concentration. XIC of the $[M + H\text{-}Ca]^{3+}$ ions of the detected PLTX congeners were used for determining the total amount of the toxins in the zoantharian and in the water extracts. The ELISA assay recently developed for PLTXs (Boscolo et al., 2013) was used to estimate the total toxin content as μg of PLTX equivalents per g of zoantharian or mL of surrounding water. Table 2 reports both the LC-HRMS and ELISA results. It is noteworthy that in the NMS1 sample the total amount of toxins detected in the aquarium water by LC-HRMS was clearly higher (2417.2 μg) than the amount detected in the zoantharian (560.4 μg), with PLTX accounting for more than 80% of the total toxin content.

ELISA assay results fit well with those obtained by LC-HRMS, with a total PLTX equivalent concentration of 96.80 ± 3.13 μg/g in the zoantharian (versus a 93.40 μg/g measured by LC/HRMS) and 42.56 ± 2.28 μg/mL in the surrounding water (versus a 48.30 μg/mL measured by LC/HRMS). This indicates a bias between LC-HRMS and ELISA results of 3.6% and −11.9% for the zoantharian and the water, respectively. Considering that the aquarium water had been used as transport water during shipment of the zoantharians, the obtained results are in good agreement with data reported by Tartaglione et al. (2016) and confirm that under stress conditions, zoantharians actively release toxin into the surrounding water. In the NMS2 sample, very low levels of PLTX were measured in the zoantharian by both LC-HRMS (0.031 μg/g) and ELISA (0.035 ± 0.006 μg/g), with a bias of 12.2% between the techniques.

3.7. Detoxification

Most aquarium hobbyists use a filter unit filled with activated carbon in their aquaria as a device to absorb seawater contaminants. We examined whether activated carbon was a good strategy for water detoxification to limit risks of inhalation/contact exposure to PLTXs. Following the poisoning incident, NMS1 zoantharian was kept alive in the aquarium; a sample of the aquarium seawater with NMS1 polyps was then collected: one aliquot of water was separated and treated with activated carbon, while the other one was used as control. Extracts of the two aliquots were obtained and analyzed by LC-HRMS and ELISA. PLTXs concentration in the control aquarium water was 2.5 μg/mL, clearly lower than the concentration found in the transport water reported in Table 2. After treatment with activated carbon PLTXs content decreased to 8×10^{-3} μg/mL (LC-HRMS), indicating that the 99.7% of PLTXs in the seawater had been adsorbed. This result was confirmed by the ELISA assay that indicated a reduction of 99.4%.

4. Discussion

In the present study, we describe the first case from Italy of an aquarium hobbyist being hospitalized for a suspected idiopathic bronchopneumonia after handling a PLTXs-containing *Palythoa* zoantharian in his home aquarium and we provide experimental evidence for water detoxification through activated carbon.

Basing on morphologic and genetic analyses, the specimen NMS1 was designated as *P.* cf. *toxica* (Cnidaria: Anthozoa: Hexacorallia). Since aquarium trade animals (including zoantharians) are often sold without the geographical origin being known, and despite the NMS1 colony clearly being phylogenetically placed within the *P. heliodiscus*/*P. variabilis*/*P. toxica* group, we cannot conclusively state where the specimen was originally collected from, even to the level of Atlantic or Indo-Pacific Ocean, as sibling species from both oceans belong to the same phylogenetic species group (Reimer et al., 2012). However, based on the phylogenetic and toxicity results along with those reported by other authors (Hoffmann et al., 2008; Hamade et al., 2015), it is clear that highly toxic species of *Palythoa* are available in the aquarium trade both in Europe and in USA. Aquarium shop owners and dealers should regard any *Palythoa* spp. with morphological similarities to *P. heliodiscus*, *P. toxica* and *P. variabilis* with extreme caution. Nonetheless, other species, even very different in appearance, such as 'immersae' *P. tuberculosa*, have been demonstrated to contain very high levels of PLTXs as well (Kimura et al., 1972; Ciminiello et al., 2014c). Therefore, with *Palythoa* spp., there is no safe way to easily ascertain the toxicity of individual colonies for the average aquarium owner, and all the colonies should be treated with the utmost care. Moreover, although it is generally believed that zoantharians lose their toxicity after long times in aquaria (Friedrich, 2012), Stüber (2010) reported anecdotally of his own severe poisoning from *Palythoa* colonies that had grown in his aquarium for 20 years. Similarly, the colony of *P.* cf. *toxica* responsible for the severe respiratory reaction described in this study (specimen NMS1) had been living in an aquarium for several years and still was producing PLTXs in quite high amounts, as detected in the detoxification experiments.

LC-HRMS analyses revealed high amounts of PLTX and lower levels of two minor PLTX congeners, hydroxyPLTX and deoxyPLTX, both in the zoantharian and in the aquarium water. Analysis of the fragmentation pattern of the hydroxyPLTX pointed to an additional hydroxyl group in the PLTX part structure C-79 to C-93, a region that has never previously been reported to have structural modifications. The deoxyPLTX lacked an oxygen atom in the region C-53 to C-78. Deoxypalytoxin isomers have been previously detected in Mediterranean *O.* cf. *ovata* extracts (named ovatoxin-d and -e) (Ciminiello et al., 2010; Dell'Aversano et al., 2014) and isolated from *Palythoa* spp. (named 73-deoxypalytoxin) (Uemura et al., 1985).

Table 2
Individual and total toxin content and concentration of PLTXs (PLTX, HydroxyPLTX and DeoxyPLTX) in NMS1 and NMS2 zoantharians and surrounding waters obtained by LC-HRMS and sandwich ELISA.

		NMS1				NMS2			
		Zoantharian		Water		Zoantharian		Water	
		μg/g	μg total	μg/mL	μg total	μg/g	μg total	μg/mL	μg total
Individual PLTXs LC/HRMS	PLTX	82.8	497	40.7	2035	0.031	0.084	nd	nd
	Percentage	88.6%		84.2%					
	HydroxyPLTX	3.1	18.4	3.0	156	nd	nd	nd	nd
	Percentage	3.3%		6.5%					
	DeoxyPLTX	7.5	45.2	4.5	226	nd	nd	nd	nd
	Percentage	8.1%		9.3%					
Total PLTXs LC/HRMS		93.40	560.4	48.3	2417	0.031	0.084	nd	
Total PLTXs ELISA		96.80 ± 3.13		42.56 ± 2.28		0.035 ± 0.006		nd	

Since only a reference extract of *O.* cf. *ovata* was available, we could assess that the deoxyPLTX from NMS1 was a structural isomer of ovatoxin-d and -e but we could not exclude that it was 73-deoxypalytoxin.

Quantitative data obtained by LC-HRMS were in good agreement with those obtained by the recently developed sandwich ELISA (Boscolo et al., 2013), with an excellent mean bias of 1.3%, confirming that the latter technique is able to detect, besides PLTX itself, other PLTX congeners as well, and can be regarded as simple screening method to detect PLTXs. Likely, the antibodies used in the sandwich ELISA recognize a part of the structure common to some PLTX congeners, such as 42*S*-OH-50*S*-PLTX from *P. toxica* and ovatoxin-a (Ciminiello et al., 2014c; Pelin et al., 2016b). The high amounts of PLTXs measured in the zoantharian and in the aquarium transport water strongly suggest a correlation between the toxins and the symptomatology described, involving respiratory distress and cardiac alterations, as previously reported in similar poisonings (Pelin et al., 2016a; Tartaglione et al., 2016). Even considering that PLTXs released into the seawater by specimen NMS1 could have been increased by stressful conditions due to its manipulation/shipping, the total PLTXs amount detected in NMS1 water (1.8×10^{-5} M) was 6 orders of magnitude higher than the EC_{50} value inducing cytotoxicity in skin keratinocytes (10^{-11} M) (Pelin et al., 2011) and 4 orders of magnitude higher than that inducing their irreversible necrosis after a 4 h exposure (Pelin et al., 2014). To the best of our knowledge, toxicological evaluations at the respiratory level have not been carried out so far, except for an *in vitro* evaluation of PLTX tumor promotion on bronchial cells (Bonnard et al., 1998).

The identification of new PLTX congeners poses the need for characterizing the toxic potencies of individual PLTXs. Considering that just small structural and/or stereochemical changes in PLTX-like molecules significantly affect their toxic potential (Ciminiello et al., 2014c; Pelin et al., 2016b), the actual toxicity of the new compounds and their contribution to the toxicological risk related to PLTXs should be clarified.

The main risk due to exposure to PLTXs in home aquaria is associated to inhalation of steam from boiling water poured over zoantharian colonies in attempts to remove them. Although no fatal cases have been documented, inhalation of toxic steams thus far has sometimes involved entire families, with frequent hospitalization and supportive care for mild to severe respiratory problems (Pelin et al., 2016a; Tartaglione et al., 2016). The most common signs and symptoms reported so far included fever, cough, dyspnea, myalgia, headache, tachycardia, tachypnea and/or bronchoconstriction. Neutrophilic leukocytosis has also been recorded. These signs and symptoms are similar to those experienced by the Italian patient in this study. In fact, the patient developed cough, difficult breathing and a temperature of 38 °C within 2 h after exposure to the steam, which progressively worsened to pyrexia >39 °C, dyspnea, tachypnea with orthopnea at rest, tachycardia as well as neutrophilic leukocytosis and elevated blood levels of CRP. Most of these signs and symptoms are also concomitant with those of beachgoers following inhalation of marine aerosols in Mediterranean coastal areas during *O.* cf. *ovata* blooms (Tubaro et al., 2011; Ciminiello et al., 2014b; Pelin et al., 2016a; Tartaglione et al., 2016). The recent finding of some PLTX congeners in marine aerosols (Ciminiello et al., 2014b) further supports the hypothesis that PLTXs exert toxicity through inhalation.

Although documented cases of PLTX poisonings due to contact with zoantharians in home aquaria are limited, these probably represent only the tip of the iceberg. The risks of keeping *Palythoa* spp. in aquaria are largely unrecognized and underestimated by aquarium hobbyists, aquarium shop owners, and public aquaria staff. The trade of these animals, which may cause severe poisonings with notable sanitary and economic impacts, is not regulated at all. We believe that not only better consumer information is required (Bernasconi et al., 2012) but also that some regulation in trade is needed to avoid further poisonings and incorrect use of PLTXs-containing zoantharians as a threat to human health (e.g. chemical weapon). Although many marine aquarium hobbyists have handled zoantharians for years without any documented PLTX-related poisoning (Deeds et al., 2011), considering the extremely high toxicity of some *Palythoa* species, the dermotoxicity of PLTXs and its skin tumor promotion (Fujiki et al., 1986; Wattenberg, 2007; Pelin et al., 2011, 2013a, 2013b, 2014) we advise against handling zoantharians with bare hands and recommend the use of protective gloves, glasses and a breathing mask, equipped with active charcoal filters. Since disposable latex or nitrile gloves break easily in contact with sharp stone corals or rocks in aquaria, long robust rubber gloves with protection of the forearms are most suitable (Friedrich, 2012). Additionally, the use of boiling water or brushing to kill zoantharian colonies or polyps is dangerous and not recommended. We have shown in this study that activated carbon is able to almost completely detoxify aquarium water contaminated with PLTXs. Therefore, the safest current method of eradication is removing undesirable *Palythoa* colonies along with their substrates from aquaria and throwing them away, all while wearing personal protective equipment (Friedrich, 2012; Knop, 2012; Brockmann, 2013) and after water detoxification using activated carbon. Before handling zoantharians in aquaria, we recommend turning off protein skimmers, air bubblers and all pumps in the tank to reduce the potential formation of aerosols containing PLTXs.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

The paper represents a series of experiments carried out under the standard procedures of scientific ethics.

All authors have read the manuscript and agree to its publication in Toxicon.

The manuscript is an original paper and has not been submitted elsewhere.

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