Current Topics in Toxicology
Vol. 7, 2011

Original Communication

Investigation on fish gill cell toxicity and haemolytic activity of *Nemopilema nomurai* (Scyphozoa: Rhizostoma) from the northern Yellow Sea of China

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ABSTRACT

Incidences of increasing jellyfish mass occurrences necessitate fundamental investigation on their distribution, development and impacts. The potential toxic activity of the giant jellyfish *Nemopilema nomurai* has been determined by means of *in vitro* assays using fish gill cells and erythrocytes as responsive elements. Fishing tentacle and oral arm tissues were used for venom analysis. The whole tissue was lyophilized in order to ensure an efficient handling and storage. For testing the toxic activity, the dried material was resuspended and the intact nematocysts were discharged by ultrasound. Four different types of nematocysts have been described using light microscopy by means of their length, width and length to width ratio. The fish gill cells showed a remarkable high susceptibility to *N. nomurai* venom. A five times increased toxic activity has been measured compared to the boreal Scyphozoa *Cyanea capillata* corresponding to an EC_{50} value of 1.5 µg total venom protein per mL cell culture medium. The haemolytic activity of *N. nomurai* venom was also potentiated compared to *C. capillata* venom and was determined as HE_{50} 80.2 µg mL^{-1}.

KEYWORDS: *Nemopilema nomurai*, giant jellyfish, cnidom, venom, cytotoxicity, lysis

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1. INTRODUCTION

The giant jellyfish *Nemopilema nomurai* (Kishinouye, 1922) is considered to be one of the most problematic Scyphozoan species in East Asian Seas. Mass occurrences of the giant jellyfish *N. nomurai* were reported in the northern part of East China Sea and Yellow Sea in 2003 and Liaodong Bay in 2005 and 2007, in the sea of Japan in 2002, 2003 and 2004 [1, 2]. *N. nomurai* can cause economic threats by blocking fish nets but also ecological hazards by preying on fish fry and small fish.

N. nomurai was also responsible for most severe cases of jellyfish stings in Chinese seas. In total, 13 fatal cases of jellyfish *N. nomurai* stings were reported in medical journals [2]. Although Japanese authorities characterize *N. nomurai* as middle venomous and fishermen handle these animals without special care, there are reports on serious stings [3]. Due to its enormous amount of biomass it is reasonable to think about an exploitable use of the mesoglea. The identification of a glycoprotein Qniumucin obtained from the mesoglea of *N. nomurai* targeted to a therapeutic application against osteoarthritis [4, 5]. Further pharmacologically interesting substances could be an antihypertensive acting peptide and an unusual lectin [6, 7]. However, there are only few studies on the *in vitro* toxicity of *N. nomurai* venom. A study performed by Kang *et al.* (2009) showed an

increased susceptibility of heart myoblasts compared to skeletal myoblasts [8]. Additionally, *in vivo* experiments with rats supported the cardiotoxic activity [9].

Despite its toxic activity against mammalian cells, the toxic effects against marine organisms are of special interest. In the present study the fish gill cell toxicity and also the haemolytic activity of *N. nomurai* venom was investigated. *In vitro* fish gill cell toxicity was analyzed for the first time for *N. nomurai*, with a specific cell-based test which has been successfully established and applied for the comparison of the cytotoxicity of the dominant northern Scyphozoan species *Aurelia aurita* and *Cyanea capillata* [10]. Additionally, the cell organelles (nematocysts) harbouring the mixture of toxic substances were characterized microscopically, because type of nematocysts has been considered as an important systematic character. First insights into the complexity of the venom composition have been obtained by gel electrophoresis.

2. MATERIALS AND METHODS

2.1. Materials

Leibovitz L15 medium (L15) and Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Lonza group (Wuppertal, Germany). Penicillin/streptomycin solution and phosphate buffered saline (PBS) were purchased from Invitrogen (Karlsruhe, Germany). L-glutamin, Bradford reagent, bovine serum albumin (BSA) fraction V were obtained from Sigma (Munich, Germany) and foetal calf serum (FCS) from PAA Laboratories (Cölbe, Germany).

2.2. Organism

One medusa of the jellyfish *N. nomurai* with an umbrella diameter of approximately 1 m was collected in the Shidao Bay, Weihai, Shandong province, China, in August 2010. Major parts of the oral arms and fishing tentacles were removed immediately after sample collection, packed in polyethylene bags, and frozen at -80°C until further processing. At the laboratory, the frozen samples were freeze-dried using a lyophiliser (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

2.3. Venom preparation

The dried tissue was used for venom preparation. The dry material was thoroughly grounded in a mortar and a portion of 1 g was resuspended in ice cold 10 mM ammonium acetate buffer, pH 5.5. Prior to nematocyst lysis, the tissue material was carefully suspended and the containing nematocysts were discharged by ultrasound (4 x 1 min with 30 s breaks per each interval) in a cooled sonicator (Branson Sonifier 450, G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany). The discharge process was repeated and the breakage of nematocysts was controlled microscopically until more than 50% of the nematocysts have been discharged. The suspension was centrifuged twice at 11000 rpm for 5 min at 4°C and the supernatant was carefully removed, sterile filtered, and used for bioactivity assays. The protein content was measured by means of the Bradford protein microassay using BSA as standard protein [11]. Venom concentrations were based upon the total protein concentration in solution. In the following text, all mentioned venom concentrations refer to protein concentration expressed in units of µg mL^{-1}.

In order to detect a possible loss of bioactivity, a sample of *C. capillata* collected during a research cruise in 2005 at the Scottish Western Islands in the Bay of Stornoway/Lewis was used. The fishing tentacles of that sample (5 medusae; average umbrella diameter 33 cm) were immediately treated for the preparation of a purified nematocysts suspension as described in Helmholz *et al.* (2007) [12]. In parallel, a portion of the whole fishing tentacle tissue was freeze-dried. Both samples were stored at -80°C until further use and treated in the same way as described for the *N. nomurai* tissue.

2.4. Microscopy of nematocysts

The freeze-dried tissue suspension containing the intact nematocysts has been used for counting and measuring the different types of capsules by light microscopy (Olympus BX 51/M, Hamburg, Germany). Capsule lengths and widths were determined from photographs taken with a digital camera (Color View, Soft Imaging System, Olympus, Hamburg, Germany) mounted on an Olympus BX51/M microscope and the software analySIS (Olympus, Hamburg, Germany).

2.5. Gill cell toxicity assay

A cell viability assay (CellTiter-Blue®, Promega, Mannheim, Germany) was used to detect the acute cytotoxicity of the venoms. The rainbow trout cell line RTgill-W1 ATCC No: CRL-2523 (LCG Promochem, Wesel, Germany) was cultivated in Leibovitz L15 cell culture medium with 10% FCS, 2 mM L-Glutamine, 100 IU penicillin and 100 µg streptomycin per mL medium at 20°C modified after Bols *et al.* (1994) [13]. Cells of a continuous culture were sowed into a black microtiter plate at a density of 10^4 cells per well in 75 µL L15 medium without FCS and allowed to settle and reattach for 24 h. After this adaptation step, 25 µL of protein extracts were applied to each well in concentrations of 0.02 - 5.0 µg protein per well (corresponding to 0.2 - 50.0 µg protein mL^{-1}). Extracts were diluted with L15 medium. The following controls were used for the detection of the relative toxicity: a positive control with cells growing in 100 µL L15 medium, a negative control with the same volume of medium but without cells. After an incubation of 48 h at 20°C, 20 µL of CellTiter-Blue® reagent was added. The fluorescence intensity of the metabolized dye was recorded after 4 h incubation at 20°C at wavelengths 560 nm excitation/590 nm emission (Victor 3, 1420 Multilabel Counter, Perkin Elmer, Rodgau - Jügesheim, Germany). The assay was performed with eight replicates of each extract concentration and controls. The experiments were repeated independently at least three times. The percentage of viable cells was calculated by defining the fluorescence of positive controls minus the values of no-cell control as “100%”.

2.6. Haemolysis assay

The haemolytic activity was tested in a multiwell microtiter plate format. Rodent blood was obtained from Fiebig Nährstofftechnik (Idstein-Niederauroff, Germany). Erythrocytes were isolated from whole blood by centrifugation, washed in sterile PBS pH 7.2 and used in a final concentration of 4% (v/v). Samples were diluted with PBS to the effective protein concentration range (0.05 - 20.0 µg $well^{-1}$) and 50 µL of this dilution were filled into the wells of 96-well round-bottom microtiter plates. Erythrocyte solution measuring 50 µL was added and the plate was incubated 20 h at room temperature. After incubation the microtiter plate was centrifuged at 700 g for 15 min, and 70 µL of the supernatants were transferred into flat-bottom microtiter plates. The released haemoglobin was measured at a wavelength of 550 nm in a microtiter plate reader (Victor Multilabel Counter, Perkin Elmer, Rodgau-Jügesheim, Germany). Total lysis of erythrocytes was achieved by 1% Triton X-114 solution. PBS measuring 50 µL was used as negative control inducing no haemolysis. The absorbance of total haemolysis minus the value of the negative control was set as 100%. Samples were tested in 8 replicates in at least two independent experiments.

2.7. Gel electrophoresis

Tricine sodium dodecyl sulphate gel electrophoresis was performed with self-casted polyacrylamide gels according to Schaegger and von Jagow (1987) [14]. Separating gels of 10% were combined with a 4% stacking gel. An unstained SDS Page protein marker 6.5-200 kDa (Serva electrophoresis GmbH, Heidelberg, Germany) was used for the estimation of molecular weights.

The one-dimensional electrophoresis was performed with the Protein II electrophoresis system (BioRad, Munich, Germany). Imaging and gel analysis were conducted with an AlphaImager HP (Biozym, Germany) after silver staining and colloidal comassie staining (RotiBlue®, Carl Roth GmbH, Karlsruhe. Germany).

3. RESULTS

3.1. Morphometry of nematocysts

Utilizing bright field and differential interference contrast light microscopy, at least four different types of nematocysts could be distinguished. Microscopic images (Fig. 1a - c) show these major types. Length, width and shape of these cell organelles are summarized in Table 1. Small capsules, very probably isorhizas (Fig. 1a - i) were the most abundant type, but small or immature birhopaloids (Fig. 1a - brh) could not clearly be distinguished for the morphometric analysis. Large, mature birhopaloids (Fig. 1b - Brh) were the rarest type. They were identified due to their clearly visible thicker shaft with two dilatations on the shaft and larger spines at the second

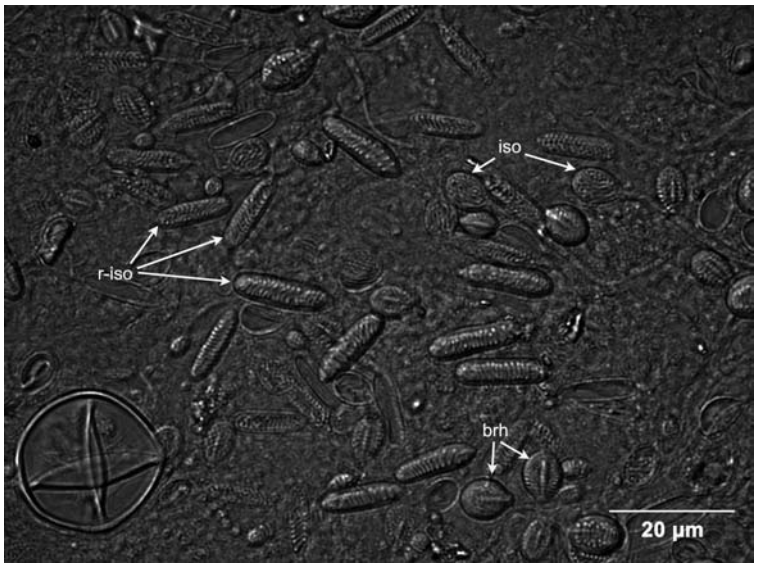

Fig. 1a. Micrograph of a nematocyst suspension obtained from *Nemopilema nomurai* tissue. Small isorhizas (iso), rod-like isorhizas (r-iso) and immature or small birhopaloide (brh) were labelled.

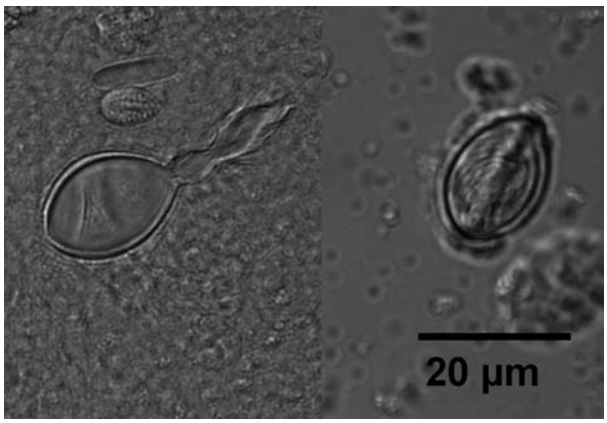

Fig. 1b. Micrograph of birhopaloide from *Nemopilema nomurai*, intact and discharged.

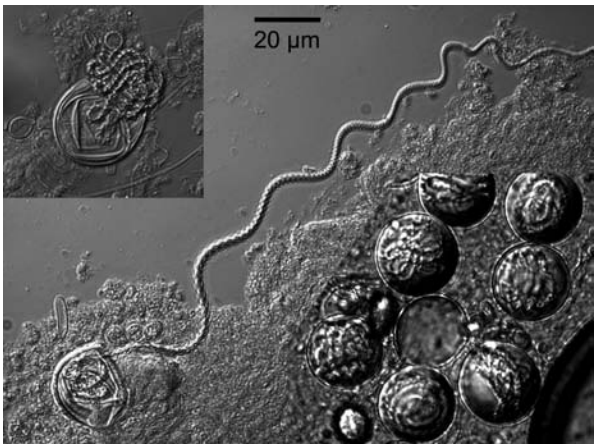

Fig. 1c. Micrograph of large round isorhizas from *Nemopilema nomurai*, intact and partly discharged.

extension of the filament. Large, round isotrichous isorhizas (Fig. 1c - I) could easily be discriminated from rod-like capsules (Fig. 1a - r-iso). This rod-like or spindle-shaped type was frequently abundant and has not been described for *Cyanea* species.

3.2. Impact of lyophilisation on toxic activity

The process of whole tissue lyophilisation was applied in order to reduce the sample volume, to ensure the sample stability during storage and to improve the sample handling. Since, most of the published investigation on toxic activities of jellyfish venoms had been performed with fresh or frozen material; the impact of the lyophilisation process on the biological activity has to be clarified. Therefore, the activity of venom obtained from freeze-dried *C. capillata* fishing tentacles (Ccap lyo) was compared to the activity of a venom sample prepared from a nematocyst suspension purified by means of fresh tissue maceration in distilled water (Ccap cyst). Both preparations

Table 1. Size and shape of the distinguishable capsule types of nematocysts obtained from whole tissue of *Nemopilema nomurai;* Mean (SD).

Proposed type	Measured n=	Length (µm)	Width (µm)	Length/width ratio
Small isorhizas/ immature birhopaloide	59	8.58 (0.89)	5.99 (0.61)	1.44 (0.13)
Birhopaloide	15	18.83 (1.53)	11.56 (1.47)	1.64 (0.14)
Large isorhizas	59	23.56 (1.62)	23.61 (1.73)	1.00 (0.04)
Rod-like isorhizas	53	12.67 (1.12)	3.77 (0.55)	3.41 (0.45)

Table 2. RT gill cell toxicity (EC_{50}) and haemolytic activity (HE_{50}) of *Cyanea capillata* venom obtained from freshly prepared nematocyst suspension (Ccap cyst), freeze-dried (Ccap lyo) fishing tentacle tissue and *Nemopilema nomurai* venom obtained from freeze-dried whole tissue (Nn).

	EC_{50} (µg mL^{-1})	HE_{50} (µg mL^{-1})
Ccap cyst	4.94	54.3
Ccap lyo	9.60	>> 200
Nn	1.53	80.2

(tissue maceration and lyophilisation) were performed with the same *C. capillata* catch in parallel and immediately after medusae collection. The impact of lyophilisation was analysed utilizing material from *C. capillata*, because only freeze dried material was available from *N. nomurai*.

In both *in vitro* test systems, the fish gill cell assay as well as in the haemolysis assay, a reduced activity of the lyophilised material has been observed. The resulting EC_{50} and HE_{50} values are compared in Table 2. The cytotoxic activity of the freeze-dried material against RT gill cells was measureable but reduced to about 50% of the activity of the venom from the fresh nematocyst suspension. There was only a marginal haemolytic activity observable by incubating rabbit erythrocytes with the extract of the freeze-dried material.

3.3. Toxic activities of *N. nomurai* venom

A definite dose-dependent cytotoxic activity against fish gill cells and the haemolytic activity against rabbit erythrocytes are presented in Figs. 2a and 2b. Corresponding EC_{50} values representing the venom protein concentrations that induce 50% loss of cell viability and integrity of erythrocytes (HE_{50}) are summarized in Table 2. A disintegration of gill cells can be observed already after 24 h incubation at concentrations of 0.5 µg protein equivalents per mL cell culture medium. At a dosage of 20.0 µg mL^{-1} a total loss of cell viability has been induced. The endpoint assay at 48 h incubation time resulted in an EC_{50} value of 1.5 µg mL^{-1}.

The effective concentration range for the haemolysis assay was determined as 10.0 µg mL^{-1} to 200.0 µg mL^{-1}. A lysis of 50% rabbit erythrocytes has been achieved by an average venom concentration of 80.2 µg mL^{-1}.

3.4. Venom analysis by gel electrophoresis

Although different sample preparations were run under various electrophoresis conditions, protein patterns with a high resolution could not be achieved (Fig. 3). Predominant protein bands were detected in molecular weight ranges of 140 kDa; 61 kDa; 43 kDa; 30 kDa and 22 kDa.

4. DISCUSSION

Cnidaria are in general a capable organism group regarding to their physiological and biochemical properties. They are widely distributed and increasing population developments have been predicted [15, 16]. The ecological and economic consequences of a high amount of jellyfish biomass for food webs, marine and fishery industries are not clear. Therefore fundamental ecological, toxicological and biochemical investigations are necessary in order to clarify potential effects on prey and other

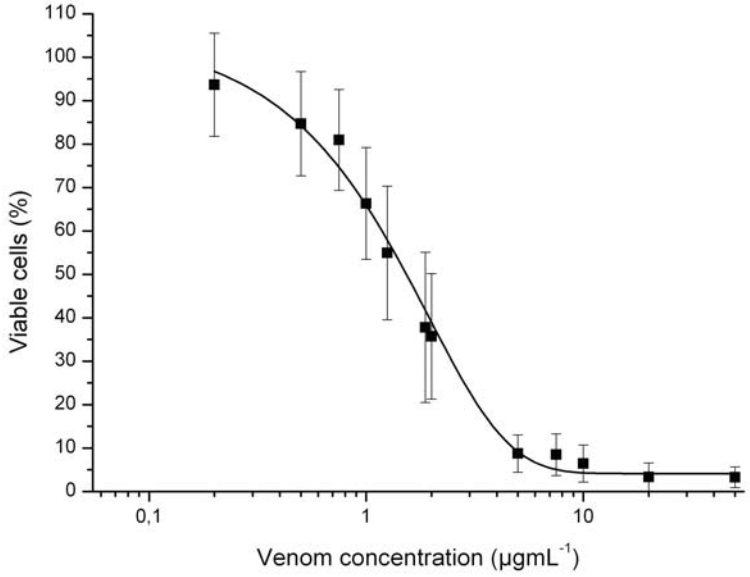

Fig. 2a. Dose - dependent cytotoxic effect of *Nemopilema nomurai* venom against the fish gill cells RTgill-W1, incubation time 48h at 20°C (Mean +/- SD, n = 16).

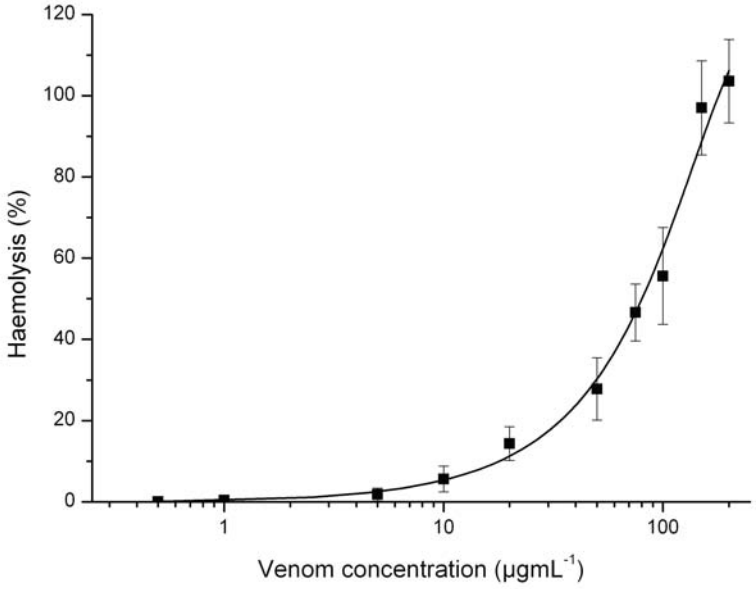

Fig. 2b. Dose - dependent haemolytic effect of *Nemopilema nomurai* venom against a 4% rodent erythrocyte suspension, incubation time 20h at room temperature (Mean +/- SD, n = 16).

pelagic organism and to predict the spatial and temporal occurrences.

Because of the huge amount of biomass and the ubiquitous distribution in Asian coastal and open waters the giant jellyfish *N. nomurai* is of special interest. Ecological studies target the ontogenetic development, the reproduction cycle and the migration in order to predict their mass occurrences [17-19].

The analysis of the distribution and morphology of the different types of the stinging nematocysts are considered as an important part of species descriptions. Due to their proposed different functions

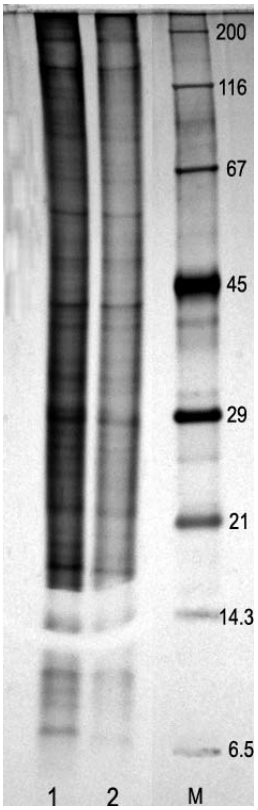

Fig. 3. SDS Page of a venom prepared from *Nemopilema nomurai* nematocyst suspension, silver staining; Lane M: Protein marker with labelled molecular weights (kDa) of standard proteins. Lane 1: Nn-venom 10µg protein; Lane 2: Nn-venom 5µg protein.

for prey capture and defence, nematocyst analysis can be helpful for an assumption of potential prey organisms and type of food [20, 21]. Mostly isorhizas with a supposed entangling function were found in the tissue material of *N. nomurai* whereas, only a small number of mature, penetrating birhopaloids, suitable for the paralysis of harder organisms like copepods was observed.

According to the guideline to nematocyst nomenclature and classification by Östman (2000) the peculiar rod-shaped nematocyst type was identified most likely as isorhizas because there was no visible shaft inside the undischarged capsule and the everted tubule appeared isodiametric [22]. In contrast to the large, round O-isorhizas, there were no spines visible along the whole everted tubule. Therefore this rod-shape type seemed to be atrichous. Although different types of isorhizas have been described for other Scyphozoa like *C. capillata,* this special small but very long type could be a special character for the giant jellyfish.

A loss of activity was expected during the sample preparation process utilizing lyophilisation. This hypothesis has been confirmed by using freshly prepared as well as freeze-dried material from *C. capillata*.

A significant impact was demonstrated for both applied test systems. The venom obtained from the freeze-dried *C. capillata* fishing tentacle tissue has nearly lost its haemolytic activity and its cytotoxic activity was diminished to about 50% compared to the venom extracted from purified nematocysts suspensions. These results indicated that the sample processing has evident impact on measured toxicity and should be considered when assessing the toxic potential of *N. nomurai* and discussing bioactivity results in relation to other Cnidarian species.

Up to now only a limited number of toxicological studies has been performed, primarily focused on cytolytic and myotoxic activities of *N. nomurai* venom. The fish gill cell toxicity of *N. nomurai* venom from lyophilised tissue is quite remarkable. Kang *et al.* (2009) detected EC_{50} values between 2 µg mL^{-1} and 12 µg mL^{-1} against different types of myoblasts and postulated a selective cardiotoxicity. However, the low EC_{50} of 1.5 µg mL^{-1} implied also a strong fish gill - toxicity. The detected gill cell toxicity of *N. nomurai* venom was stronger compared to other common jellyfish species like *A. aurita* and *C. capillata* [10], though, in the same order of magnitude.

Haemolysis is a common test system for natural toxins. Rodent erythrocytes have been turned out to respond very sensitive to jellyfish venoms obtained from *Cyanea* sp. and *A. aurita* [23]. Therefore, the lytic activity of *N. nomurai* venom has also been tested against rabbit erythrocytes. Compared to the results of Kang *et al.* (2009) a much stronger activity could be detected with HE_{50} values of about one magnitude lower. In that study also freeze-dried nematocysts have been utilized for venom preparation, but these cell organelles have been obtained from fresh tentacle tissue. In the present study the tissue from both toxigenic organs, fishing tentacles and oral arms

was freeze-dried. An increased lytic activity has been expected in oral arm venom, as it has been found for other Scyphozoan species. Therefore, the venom obtained from oral arms could contribute to the overall increased haemolytic activity compared to the results of Kang *et al.* (2009).

Considering the significant decrease of cytotoxic as well as haemolytic activity in the venom obtained from freeze-dried tissue an impressive toxic effect of the *N. nomurai* venom could be predicted for the nematocysts of living organisms.

Biochemical investigations analyzing venom components of *N. nomurai* are also very rare. Despite in the reference of Kang *et al.* (2009) mentioned major proteins in a molecular range of 10 - 15 kDa and 20 - 40 kDa, no further information was available. Apart from approving the major protein bands at 20 - 40 kDa additional protein bands in the higher molecular weight range of about 60 kDa and above 100 kDa have been detected. A toxin of such large molecular weight has been isolated from a related *Rhizostomae* species [24]. Cariello *et al.* (1988) isolated the Rhizolysin from *Rhizostoma pulmo* with a molecular weight of 143 kDa. A protein with a calculated molecular weight of 146 kDa has been detected in the venom of *N. nomurai* that could be similar to the Rhizolysin.

The present investigation documented the enormous potential of the giant jellyfish *N. nomurai* to produce a complex mixture of highly potent toxins that can express an impact on fish by affecting the integrity of their gills and blood cells. In order to prove these considerable *in vitro* effects, the ecologically relevant toxic potential against fish and other marine organisms has to be measured *in vivo*. This study showed also that further fundamental analysis has to be done to clarify the modes of action, elucidate causative substances and to estimate the potential economic impact by considering the huge amounts of biomass of *N. nomurai*.

ACKNOWLEDGEMENT

The cooperation was initialized by a funding of the Federal Ministry for Education and Research (CHW 09/024) in the framework of the “German-Chinese Year of Science and Education 2009/2010”. This work was supported by grants from Chinese Academy of Sciences (KZCX2-YW-Q07-04 and XDA05130703) and Yantai Science and Technology Bureau (O931041051).

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