A revisited hemolytic assay for palytoxin detection: limitations for its quantitation in mussels

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Abstract

Palytoxin (PLTX) and its analogues have been detected as seafood contaminants and associated with a series of human foodborne poisonings. Due to some fatalities ascribed to the ingestion of PLTX-contaminated marine organisms, the development of methods for its detection in seafood is recommended by the European Food Safety Authority (EFSA). Due to its feasibility, the spectrophotometric hemolytic assay is widely used to detect PLTX in different matrices, even though a standardized protocol is still lacking.

Thus, on the basis of available assay procedures, a new standardized protocol was set up using purified human erythrocytes exposed to PLTX (working range: $3.9x10^{-10}$-$2.5x10^{-8}$ M) in a K^+-free phosphate buffered saline solution, for 5 h at 41°C. An intra-laboratory characterization demonstrated its sensitivity (limit of detection, LOD=$1.4x10^{-10}$ M and quantitation, LOQ=$3.4x10^{-10}$ M), accuracy (*bias*=-0.8%), repeatability (RSDr=15% and 6% for intra- and inter-day repeatability, respectively) and specificity. However, the standardized method seems not to be suitable for PLTX quantitation in complex matrices, such as mussels (*Mytilus galloprovincialis*) extracts, below the limit suggested by EFSA (30 µg PLTXs/Kg shellfish meat). Thus, the hemolytic assay for PLTX quantitation in seafood should be used only after a careful evaluation of the specific matrix effects.

Keywords

Palytoxin, hemolytic assay, matrix effect, *Mytilus galloprovincialis*

Abbreviations: PLTX, palytoxin; LOD, limit of detection; LOQ, limit of quantitation; RSDr, relative standard deviation of repeatability; 42-OH-PLTX, 42-hydroxy-palytoxin; Ost-D, Ostreocin-D; OVTX, ovatoxin; EFSA, European Food Safety Authority; SAGM, Saline-Adenine-Glucose-Mannitol solution; ADSOL, Adenine-Dextrose Solution; D-PBS, Dulbecco's Phosphate Buffered Saline; EDTA, ethylenediaminetetracetic acid; O.D., optical density.

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1. Introduction

Palytoxin (PLTX) is a highly toxic non-polymeric complex molecule, originally isolated from zoanthids of the genus *Palythoa* (Moore and Scheuer, 1971), and later identified in benthic dinoflagellates of the genus *Ostreopsis* and cyanobacteria of the genus *Trichodesmium* (Ciminiello et al., 2008; Kerbrat et al., 2011). Over the years, several PLTX analogues have been detected in different marine organisms, including: (i) Ostreocin-D (Ost-D) in *Ostreopsis siamensis* (Usami et al., 1995); (ii) two stereoisomers of 42-hydroxy-PLTX (42-OH-PLTX), differing only for the configurational inversion at C50, identified in *Palythoa toxica* and *P. tuberculosa* (Ciminiello et al.,2014a); and (iii) ovatoxin-a (OVTX-a), the major toxin produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea (Ciminiello et al., 2012). In addition, a series of OVTX-a analogues have been identified, such as OVTX-b to -k and isobaric palytoxin (Brissard et al., 2015; García-Altares et al., 2015; Tartaglione et al., 2016). These toxins may enter the human food chain through their accumulation into different marine edible organisms, such as fishes, crustaceans, bivalves, gastropods, cephalopods, echinoderms, sponges and polychaete worms (Aligizachi et al., 2011; Biré et al., 2013; Gleibs and Mebs, 1999). In particular, a series of human poisonings characterized by general malaise, myalgia, cardiac problems, respiratory distress, and sometimes death, have been ascribed to the ingestion of PLTX-contaminated fishes and crabs in tropical areas (Deeds and Schwartz, 2010; Tubaro et al., 2011b; Wu et al., 2014).

In recent years, microalgae belonging to the genus *Ostreopsis* have frequently bloomed in the temperate Mediterranean Sea and the relevant toxins (mainly OVTX-a) have been detected in microalgae, aerosolized seawater and some marine edible organisms. However, no foodborne poisonings associated with PLTXs have yet been documented in this area, so far (Biré et al., 2013; Ciminiello et al., 2014a; Del Favero et al., 2012).

Although there is no official regulation for PLTXs in seafood, the European Food Safety Authority (EFSA) suggested a maximum limit of 30 μg PLTXs/Kg of shellfish meat, and recommended the development of specific, rapid, precise and accurate methods for PLTXs quantitation in seafood during monitoring programs (EFSA, 2009). Despite several methods for PLTXs detection have been already published (Riobò et al., 2011; Tubaro et al., 2014), they are not completely validated to be officially accepted, frequently requiring expensive equipments and highly qualified operators, and/or presenting limitations in terms of sensitivity, specificity and matrix effects.

Among them, the delayed hemolytic assay is one of the most used screening methods for PLTX quantitation, due to its simplicity, cheapness, rapidity and sensitivity. It is based on the toxin ability to convert the Na^+/K^+ ATPase of mammal erythrocytes to a nonspecific cationic channel, leading to a rapid loss of K^+ ions from cells and a delayed hemoglobin release that can be easily measured spectrophotometrically (Habermann et al., 1981). The hemolytic assay is usually carried out following the Bignami's protocol (1993) with or without modifications, but a standardized and universally accepted procedure has not been defined, so far. Most literature studies report the original assay with a series of modifications mainly concerning the origin of erythrocytes, the time and temperature of their incubation with PLTX, and/or the working buffer composition (Aligizaki et al., 2008; Biré et al., 2013; Brissard et al., 2014; Gleibs et al., 1995; Kim et al., 2002; Lenoir et al., 2004; Malagoli, 2007; Onuma et al., 1999; Pezzolesi et al., 2012; Riobò et al., 2006; Riobò et al., 2008; Taniyama et al., 2001; Taniyama et al., 2003; Volpe et al., 2014; Wachi and Hokama, 2001). Due to these experimental variables, a standardized protocol for the hemolytic assay to be considered as a reference procedure in PLTX quantitation is still lacking.

Moreover, edible marine species could contain hemolytic compounds different from PLTXs, tentatively interfering with the assay giving false positive results. For instance, a recently

developed biosensor to quantify PLTX in mussels, based on lactate dehydrogenase release from sheep erythrocytes, appeared to be influenced by a significant mussels matrix effect and low PLTX recovery (Volpe et al., 2014). Thus, the accuracy and precision of the hemolytic assay for spectrophotometric PLTX quantitation in shellfish needs to be properly evaluated. Hence, this study was carried out to set up a novel standardized protocol for the hemolytic assay useful as reference procedure and to characterize its suitability for PLTX quantitation in mussels (*Mytilus galloprovincialis*), often heavily contaminated by PLTXs (Brissard et al., 2014; Aligizaki et al., 2008; Aligizaki et al., 2011; Amzil et al., 2012).

2. Materials and methods

2.1 Toxins and other materials

PLTX was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan; purity > 90 %). 42*S*-hydroxy-50*S*-palytoxin and 42*S*-hydroxy-50*R*-palytoxin were isolated from *Palythoa tuburculosa* and *P. toxica,* as previously reported (Ciminiello et al., 2009; Ciminiello et al., 2014b). Ovatoxin-a and the mixture of ovatoxin-a,-d,-e were kindly provided by Prof. P. Ciminiello (Università di Napoli "Federico II", Naples, Italy). The mouse monoclonal anti-PLTX antibody 73D3 (mAb-PLTX) was produced by a hybridoma cell culture, as previously described (Bignami et al., 1992). All the other reagents were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy), if not otherwise specified.

2.2 Red blood cell purification

Blood samples from healthy human volunteers were obtained from the Transfusion Center, Azienda Ospedaliera Universitaria, Trieste, Italy. All donors signed an approved consent form giving permission for the collection and use of blood for research purposes (WMA Declaration of Helsinki). Blood was drawn by venipuncture between 08.00 a.m. and 10.00

a.m. to minimize variability due to circadian rhythms, and immediately processed. Blood, collected into standard triple bag systems, was fractionated following standard procedures to obtain buffy-coats, used to purify red blood cells. Buffy coats (50 ml) were diluted 1:1 (v/v) in the erythrocytes preservation solution (specified in section 2.3) and then centrifuged at 2400 rpm for 10 min at 4 °C. Red blood cells pellet (2 ml), suspended in the red blood cells preservation solution (10 ml), was washed three times by centrifugation at 1500 rpm for 5 min at 15 °C. Then, the final pellet was re-suspended 1:10 (v/v) in the preservation solution.

2.3 Red blood cells storage

Three storage solutions were used to evaluate red blood cells preservation at 4° C: Saline-Adenine-Glucose-Mannitol solution (SAGM, containing $5x10^{-2}$ M dextrose, 0.15 M NaCl, $2.4x10^{-3}$ M adenine and $2.9x10^{-2}$ M d-mannitol) (Zehnder et al., 2008), Adenine-Dextrose Solution (ADSOL, containing 0.12 M dextrose, 0.15 M NaCl, $3.9x10^{-3}$ M adenine and $4.1x10^{-2}$ M d-mannitol) (Moore, 1991) and Dulbecco's Phosphate Buffered Saline (D-PBS) containing 1mM ethylenediaminetetracetic acid (EDTA) and 5 mM glucose (Lowe et al., 1973). Red blood cells preservation was evaluated by means of erythrocyte resistance to spontaneous lysis and erythrocyte concentration. For the first parameter, 0.4 ml of red blood cells suspension was centrifuged at 1500 rpm for 5 min and the optical density of the supernatant was then measured at 405/540 nm (Microplate autoreader; Bio-Tek Instruments; Vinooski, VT). Red blood cell concentration was evaluated by cell counting following the Trypan Blue Exclusion Test. Acceptable thresholds were constant cells concentration and optical densities lower than 0.5, a value close to the average optical densities of the negative controls (125 µl of red blood cell suspension incubated with K^+-free D-PBS without toxin). Both parameters were assessed daily for 36 days.

146 *2.4 Experimental design*

147 The hemolytic assay was standardized considering the following parameters: the use of
148 purified human erythrocytes *vs* whole blood, the influence of selected ions (borates, calcium,
149 sodium, and potassium) on PLTX-induced hemolysis, and the incubation temperature of
150 erythrocytes exposed to PLTX. The standardized assay was subsequently characterized
151 according to the international principles, as described by the Eurachem Guide (Magnusson
152 and Örnemark, 2014).

153

154 *2.5 Standardized hemolytic assay*

155 After washing, red blood cells were pelleted by centrifugation (1500 rpm for 5 min) and re-
156 suspended in K^+-free D-PBS at the concentration of $1x10^8$ cells/ml. In 96-wells plates, 125 µl
157 of PLTX solution and 125 µl of the erythrocytes suspension were added to each well and
158 incubated for 5 h at 41 °C (PLTX final concentrations: $1.22x10^{-11}$-$4.00x10^{-7}$ M). As negative
159 controls, 125 µl of red blood cell suspension were incubated with 125 µl K^+-free D-PBS
160 without toxin. As a positive control, 100% hemolysis was achieved by incubating the
161 erythrocytes suspension with 125 µl of 0.1% Tween 20 (v/v) for 5 h at 41° C. After
162 incubation, the plate was centrifuged at 1500 rpm for 5 min at 15 °C and, using a
163 multichannel pipette, the supernatant was carefully transferred into a clear flat bottom 96-
164 wells plate avoiding pellet braking up. Optical density (O.D.) of supernatant was then
165 measured at 405/540 nm and the percentage of hemolysis calculated with respect to the
166 positive control by the following formula:
167 % of hemolysis = 100 x (O.D. PLTX exposed sample−O.D. negative control)/(O.D. positive
168 control – O.D. negative control).

169

170 *2.6 Evaluation of the matrix effect*

To assess the hemolytic assay suitability to quantify PLTX in mussels at levels below the suggested EFSA limit (30 μg PLTXs/kg edible parts, corresponding to about $11.2x10^{-9}$ mol/kg; EFSA, 2009), different extracts of *Mytilus galloprovincialis* edible parts were prepared. Each extract was analyzed by liquid chromatography high resolution mass spectrometry (LC-HRMS) to verify the absence of PLTX before the matrix effect evaluation. Mussels were collected in the Gulf of Trieste (Italy) and shucked meat (200 g) was homogenized (14000 rpm, 3 min) using an Ultra-Turrax (Ika-Werk; Staufen, Germany). The homogenate (1 g) was extracted three times with 3 ml of different solvents (80%, 50% or 20% aqueous ethanol or aqueous methanol). Each extractive solution was then centrifuged at 5500 rpm for 30 min, the corresponding supernatants were pooled and the volumes adjusted to 10 ml with the relevant extraction solvents to obtain six extracts at a final concentration of 0.1 g mussels meat equivalents/ml. The hemolytic activity of each extract was then evaluated at five dilutions (1:1, 1:10, 1:50, 1:100 and 1:1000, v/v) to assess background hemolysis.

At dilutions devoid of background hemolysis, extracts were spiked with different PLTX concentrations to prepare matrix matched-samples at PLTX levels ranging from $3.9x10^{-10}$ to $2.5x10^{-8}$ M. These matched samples were then analyzed using the hemolytic assay. The relevant hemolytic activity was compared to that induced by the same PLTX concentrations without matrix.

2.7 Statistical analyses

Results of the hemolytic assay are presented as mean±SE of at least three independent experiments performed in triplicate. Linearity (r^2) of the calibration curve was estimated by linear regression analysis, using the GraphPad Prism software version 5.0 (GraphPad Prism; GraphPad Software, Inc.; San Diego, CA). Concentration-effect curves were compared by two-way ANOVA statistical analysis and Bonferroni post test, and significant differences

were considered at $p < 0.05$. EC_{50} (effective concentration giving 50% hemolysis) was calculated by nonlinear regression using a four parameters curve-fitting algorithm of the GraphPad Prism software.

Hemolytic assay performance was characterized according to the international principles described by the Eurachem Guide (Magnusson and Örnemark, 2014). Briefly, limit of detection (LOD) and quantitation (LOQ) were expressed as PLTX concentration corresponding to the average of 10 blank values plus 3 or 10 times the standard deviations, respectively. Accuracy was measured as % Bias (n = 10), calculated as % difference between PLTX concentration measured by the assay and the theoretical concentration in the sample divided by PLTX theoretical concentration. Repeatability was expressed as relative standard deviation of repeatability (RSDr), measured as % ratio between the standard deviation of independent results and their mean value. Both independent results obtained by the same operator in one day (intra-assay RSDr; n = 10) and within a 6-month period by different operators (inter-assay RSDr; n = 10) were considered.

3. Results and discussion

3.1 Storage of purified human erythrocytes

The human erythrocytes model was chosen due to the easy availability of human blood, rapid isolation of significant cell numbers, and low cost. The use of purified human erythrocytes poses the need of a medium suitable to preserve the cells. To this aim, three cells storage solutions were evaluated: Saline-Adenine-Glucose-Mannitol Solution (SAGM), Adenine-Dextrose Solution (ADSOL) and D-PBS containing 1 mM EDTA and 5 mM glucose (PBS-EDTA-glucose), as described in section 2.3 (Lowe et al., 1973; Moore, 1991; Zehnder et al., 2008). Erythrocytes preservation in these solutions was daily evaluated, monitoring two

parameters up to 36 days: the spontaneous hemolysis (measured spectrophotometrically as hemoglobin release) and the erythrocytes concentration determined by visual cell counting. Acceptable thresholds were constant cell concentration in the storage solution and optical densities lower than 0.5. Only PBS-EDTA-glucose solution allowed mainteinance of red blood cells suitable for the assay for up to 3 weeks (Fig. S1), probably due to the presence of crucial constituents providing the proper energy source to erythrocytes (glucose) and preventing coagulation (EDTA). Hence, these conditions allow to prepare a batch of human erythrocytes suitable for the hemolytic assay up to 3 weeks, reducing the working time and avoiding to purify the erythrocytes before each single experiment.

3.2 Optimization of the hemolytic assay

The hemolytic assay was standardized considering different experimental parameters reported by previous published studies. In the first series of experiments, the hemolytic assay was carried out following the most recent published method, using human erythrocytes (Malagoli, 2007). Following Malagoli's protocol, the sensitivity of purified human erythrocytes to PLTX-induced hemolysis was compared to that of the whole human blood. The hemolytic activity of PLTX ($7.8x10^{-10}$-$5.0x10^{-8}$ M) towards purified human erythrocytes was significantly higher than that displayed by the whole human blood: 65% and 7% hemolysis were recorded at $5.0x10^{-8}$ M PLTX, respectively (Fig. 1A).

Previous studies demonstrated that borates, as H_3BO_3 or $Na_2B_4O_7$ (> $5.0x10^{-6}$ M), and calcium ions (> $2.0x10^{-5}$ M) in the buffer solution increases PLTX-induced hemolysis, probably by promoting the interaction between the toxin and its molecular target, the Na^+/K^+ ATPase (Ahnert-Hilger et al., 1982; Habermann, 1983). Thus, the influence of these factors on PLTX-induced lysis of purified erythrocytes was evaluated. As shown in Fig. 1B, no significant differences in PLTX-induced hemolysis were observed between buffer solution containing 1

246 mM H_3BO_3 and that containing 1 mM $Na_2B_4O_7$, in agreement with literature data, where
247 H_3BO_3 or $Na_2B_4O_7$ are interchangeably used. Regarding Ca^{2+} ions, no significant differences
248 were recorded between PLTX-induced hemolysis in D-PBS with or without $CaCl_2$ (Fig. 1C).
249 However, the Ca^{2+} concentration in D-PBS solution (137 mM) is higher than that (>20 μM)
250 reported to promote the interaction between the toxin and its target (Ahnert-Hilger et al.,
251 1982). Thus, additional Ca^{2+} ions at millimolar concentrations in the D-PBS buffer solution
252 containing 1mM H_3BO_3 are not necessary to increase PLTX hemolytic activity.
253 Based on the mechanism of action of the toxin (i.e. interaction with the Na^+/K^+ ATPase), the
254 influence of Na^+ and K^+ ions on PLTX-induced hemolysis was also evaluated. The presence
255 of Na^+ ions ($1.8x10^{-4}$M or $3.6x10^{-4}$M NaCl) in the buffer solution was associated to a
256 significant reduction of PLTX-induced hemolysis, probably due to the medium hypertonicity
257 causing erythrocytes shrinking and volume reduction (Kregenow, 1971) (Fig. 1D). In
258 contrast, while the K^+-free buffer containing H_3BO_3 did not significantly influence the
259 hemolytic activity of PLTX, the latter was significantly increased using K^+-free buffer
260 without H_3BO_3 (Fig. 1E).
261 Finally, the temperature influence on the hemolytic activity was evaluated. After erythrocytes
262 incubation with PLTX for 5 h at 41 °C, hemolysis was significantly higher than that recorded
263 at 37°C, with EC_{50} values of $6.2x10^{-9}$ M (95% confidence intervals, CI = 5.3 - $7.2x10^{-9}$ M)
264 and $4.9x10^{-8}$ M (95% CI = 4.1 – $5.9x10^{-8}$ M), respectively (Fig. 1F). This result is in
265 agreement to that reported by Habermann et al. (1981), suggesting that PLTX-induced
266 hemolysis is temperature-dependent.
267 On the whole, these results allowed to optimize a protocol for the hemolytic assay, using
268 purified human erythrocytes exposed to PLTX in a K^+-free D-PBS buffer at 41 °C for 5 h.
269

270 *3.3 Characterization of the hemolytic assay*

3.3.1 Calibration curve for PLTX

Using the standardized hemolytic assay, the calibration curve for PLTX represented in Fig. 2A was obtained. The working range for PLTX detection was $3.9x10^{-10}$ - $2.5x10^{-8}$ M, with a limit of detection (LOD) and quantitation (LOQ) of $1.4x10^{-10}$ M and $3.4x10^{-10}$ M, respectively. Analyzing the working range by linear regression, plotting the theoretical toxin concentrations against the PLTX concentrations measured by the hemolytic assay, a good correlation coefficient was found (r^2 = 0.9979; n = 10) (Fig. 2B). A mean *Bias* value (%) of -0.8% (range: -2.0% to 2.4%) highlights the accuracy of the measures (Table 1).

Intra-assay and inter-assay repeatability were then evaluated. A good correlation was observed, with r^2 values of 0.9736 for intra-assay (Fig. 3A) and 0.9977 for inter-assay repeatability (Fig. 3B). These data were confirmed by the intra-day and inter-day repeatability coefficients (relative standard deviation of repeatability, RSDr) of 15% (n=10) and 6% (six months period, n=10), respectively (Table 1).

The haemolytic effect of PLTX in these conditions (described in section 3.2; 94% hemolysis at $2.5x10^{-8}$ M) is much higher than that reported by Malagoli (2007) and Taniyama (2001): they recorded a maximum hemolysis lower than 50% at the highest tested PLTX concentrations (i.e. 10^3 and 10^2 ng PLTX/ml, corresponding to $3.7x10^{-6}$ M and $3.7x10^{-7}$ M PLTX, respectively). These concentrations were 1 to 2 orders of magnitude higher than the highest concentration of the assay working range presented in this study (i.e. $2.5x10^{-8}$ M, giving 94% of hemolysis). Thus, an improved PLTX-induced hemolysis was achieved by lowering the osmolarity of the working buffer (K^+ ions withdrawal) and increasing the assay temperature (41°C) in the optimized hemolytic assay protocol.

3.3.2 Cross-reactivity with PLTX analogues

The hemolytic assay was then evaluated for its ability to detect some PLTX analogues and N-biotinyl-PLTX within the working range set up for PLTX ($3.9x10^{-10}$ - $2.5x10^{-8}$ M). Although the hemolytic activity of nanomolar concentrations of 42*S*-OH-50*S*-PLTX (from *P. toxica*) was slightly lower than that of the same PLTX concentrations, its hemolytic potency (EC_{50} = $5.8x10^{-9}$ M, 95% CI = 4.1 - $8.2x10^{-9}$ M) was comparable to that of PLTX (EC_{50} = $6.2x10^{-9}$ M, 95% Cl = $5.3x10^{-9}$ - $7.2x10^{-9}$ M) (Fig. 4). The latter result is in agreement with a previous study demonstrating a similar hemolytic activity of the two compounds, using mouse erythrocytes (Tubaro et al., 2011a). On the other hand, hemolysis induced by 42*S*-OH-50*R*-PLTX (stereoisomer from *P. tuberculosa*) was significantly lower than that of PLTX (Fig. 4), in agreement with its lower cytotoxic effects, previously observed on HaCaT cells (Ciminiello et al., 2014b). Also the hemolytic activity of pure OVTX-a and of a mixture of OVTXs (OVTX-a, -d and –e) was significantly lower than that of PLTX, similarly to their cytotoxicity toward skin keratinocytes, as recently demonstrated by Pelin et al. (2016). In contrast, the latter result is not in agreement with other studies hypothesizing that PLTX and OVTXs possess similar hemolytic activity (Brissard et al., 2014; Pezzolesi et al., 2012). However, since these studies were carried out using sea urchins and *Ostreopsis ovata* extracts containing OVTX analogues other than OVTX-a, -d and –e as well as isobaric PLTX (Brissard et al., 2014; Pezzolesi et al., 2012), we can speculate that those mixtures of toxins could display a hemolytic activity different than that of OVTX-a or of the other single OVTXs. Moreover, since complete studies on this matrices were not carried out, a significant interference of the extract's matrix cannot be excluded.

In addition, the hemolytic potency of a semisynthetic PLTX derivative, biotinylated PLTX, was significantly lower than that of PLTX, inducing only 5% hemolysis at the highest concentration ($2.5x10^{-8}$ M). Intriguingly, a biotin linked to the terminal PLTX amino group reduces PLTX-induced hemolysis, indicating the importance of the primary amine for the

hemolytic effect via Na^+/K^+-ATPase interaction. This finding is in agreement with previous evidences for N-acetyl-PLTX which biological activity via Na^+/K^+-ATPase were over 100 times weaker than those of the parent compound PLTX, tentatively due to a change in the global toxin conformation that prevents its dimerization (Kudo and Shibata, 1980; Ohizumi and Shibata, 1980; Inuzuka et al., 2008). Moreover, this result suggests that the functional hemolytic assay detects only biologically active PLTX analogues. In contrast, other non-functional analytical methods can detect also biologically inactive PLTX-like compounds (Boscolo et al., 2013), which might not contribute the whole toxic potential of PLTXs-contaminated seafood samples.

In conclusion, these results demonstrated that the hemolytic assay has a good sensitivity for PLTX and 42*S*-OH-50*S*-PLTX from *P. toxica*. On the contrary, the stereoisomer 42*S*-OH-50*R*-PLTX from *P. tuberculosa*, as well as OVTX-a and OVTXs mixture have lower hemolytic activity. Anyway, all the tested natural PLTX analogues exert hemolytic effects, which suggest a common mechanism of action.

3.3.3 Hemolysis neutralization

It is known that the cardiac glycoside ouabain, which binds to the same molecular target of PLTX, inhibits the *in vitro* effects of PLTX (Habermann and Chhatwal, 1982; Pelin et al., 2013). Thus, to confirm the specific delayed hemolysis by PLTXs, excluding a possible hemolysis by other constituents of seafood samples, the standardized assay was carried out pre-incubating the red blood cells with 100 μM ouabain for 30 min at 37 °C (Aligizaki et al., 2008; Biré et al., 2013; Brissard et al., 2014; Gleibs et al., 1995; Malagoli, 2007; Onuma et al., 1999; Pezzolesi et al., 2012; Riobo et al., 2006; Riobo et al., 2008; Taniyama et al., 2001; Taniyama et al., 2003; Volpe et al., 2014). Similarly, the mouse monoclonal anti-PLTX antibody (mAb-PLTX, 50 μg/ml) was used to neutralize the hemolysis induced by PLTX. The

PLTX induced hemolysis was completely inhibited by ouabain and only partially by the mAb-PLTX (Fig. 5), in agreement with previous findings reported by Bignami (1993).

3.4 Matrix effect

To assess the interference of mussel matrix on PLTX-induced hemolysis, different extracts of *Mytilus galloprovincialis* were prepared. Firstly, 80%, 50% and 20% aqueous methanol and ethanol extracts of toxin-free mussels were tested after 1:1, 1:10, 1:50, 1:100 and 1:1000 (v/v) dilutions, to verify whether the matrix (mussel extracts) could produce false positive results. All the aqueous ethanol extracts required dilutions higher than 1:100 to avoid matrix effects in the hemolytic assay (Fig. S2). On the other hand, no significant interferences were recorded for the 80% aqueous methanol extract at 1:50 dilution or 50% and 20% aqueous methanol extracts at 1:10 dilution (Fig. S3). Thus, the subsequent studies were carried out using the aqueous methanol extracts at these dilutions. Specifically, 80% aqueous methanol extract (1:50), as well as 50% and 20% aqueous methanol extracts (1:10) were spiked with known amounts of PLTX (final concentrations ranging from 3.9×10^{-10} M to 2.5×10^{-8} M). Then, the spiked extracts were analyzed by the hemolytic assay in comparison to the same PLTX concentrations without matrices. The extract which did not interfere with the hemolytic assay was the 80% aqueous methanol extract diluted 1:50 (Fig. S4). Notably, this extraction method is the most suitable for PLTX extraction from mussels, giving 90-100% toxin recovery, by LC-HRMS and a sandwich ELISA (Ciminiello et al., 2011; Boscolo et al., 2013). Thus, 80% aqueous methanol at 1:50 dilution was subsequently used for the matrix effect study.

Fig. 6 shows the results of the linear regression analysis carried out comparing the theoretical PLTX concentrations in the spiked extract to those measured by the hemolytic assay. The LOQ for PLTX in the mussels extract was 1.3 ng/ml, corresponding to 13 µg/kg meat. Considering the 1:50 dilution, the LOQ for PLTX in mussels corresponds to 640 µg/kg meat,

a value about 20 times higher than the maximum limit suggested by EFSA (30 µg PLTXs/kg meat). Moreover, the linear regression analysis yielded a correlation coefficient (r^2 = 0.9259) and a *Bias* range from -27.0 to 33.6% (mean *Bias* = 3.7%, Table 2) index of high dispersion of the data, suggesting low accuracy and precision of the measurement. Thus, the standardized and characterized hemolytic assay suffers from a significant mussel matrix effect, which does not allow PLTX quantitation in *Mytilus galloprovincialis* at concentrations lower than that proposed by EFSA. Interestingly, a significant interference by mussels matrix was recently observed also for a biosensor exploiting lactate dehydrogenase (LDH) release from sheep erythrocytes as hemolysis parameter to quantify PLTX (Volpe et al., 2014): similarly to our study, due to the significant matrix effect, a 1:50 dilution of the mussels extract was required to quantify PLTX in mussels, not meeting the EFSA requirements (Volpe et al., 2014). Intriguingly, the observed interference of the mussels extract is not due to the solvent used for extraction (i.e. 80% aqueous methanol) since the relevant mussels-free solvent did not induce hemolysis (data not shown). On the contrary, low dilutions of mussels methanol extract (i.e. 1:1 or 1:10, Fig. S3) displayed an increased hemolytic activity as compared to that of higher extract dilutions, sometimes comparable to that of the positive control (0.1% Tween 20). Hence, the significant matrix effect could be due to hemolytic compounds different from PLTX, that could be at least partially extracted from mussels by aqueous methanol. Indeed, a series of potentially hemolytic compounds, such as glycoglycerolipids, lysophospholipids and unsaturated fatty acids, are known constituents of mussel edible parts. The concentration of these compounds in mussel meat could be also influenced by season, living site of mussels, dietary composition of phytoplankton as well as temperature and enzymes action during transportation and storage of seafood (Colles and Chisolm, 2000; Facchini et al., 2016; Ginsburg et al., 1989; Parrish et al., 1998; Pleissner et al., 2012).

All together, these results suggest that PLTX-induced erythrocytes lysis is not a suitable endpoint for the toxin quantitation in mussels. In addition, since the hemolytic assay has been worldwide used to detect PLTXs in different field marine samples (also concomitantly to potential human poisonings ascribable to PLTXs), those data should be carefully considered because of possible matrices effect that could have affected the analytical outcomes.

4. Conclusions

In conclusion, this hemolytic assay employing purified human erythrocytes is characterized by a good sensitivity, accuracy, specificity and repeatability. However, it does not allow any PLTX quantitation in mussels (*Mytilus galloprovincialis*) at concentrations below the maximum limit suggested by EFSA (30 μg/kg; EFSA, 2009), due to the high matrix interference. In fact, hemolytic substances different from PLTX could be extracted from mussels together with PLTX (Colles and Chisolm, 2000; Facchini et al., 2016; Ginsburg et al., 1989; Parrish et al., 1998; Pleissner et al., 2012), causing false positive results. Thus, the hemolytic assay for PLTX quantitation in seafood could be used only after a careful evaluation of the specific matrix effects in each natural sample.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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606

607

608 **Fig. 1.** Optimization of the hemolytic assay. **A**: PLTX-induced hemolysis using whole human
609 blood or purified human erythrocytes. **B**: PLTX-induced hemolysis on human erythrocytes in
610 presence of 1 mM boric acid or 1 mM sodium tetraborate. **C**: PLTX-induced hemolysis on
611 human erythrocytes with and without 1 mM $CaCl_2$. **D**: PLTX-induced hemolysis on human
612 erythrocytes with and without $1.8x10^{-4}$M or $3.6x10^{-4}$M NaCl, 2x or 4x. **E**: PLTX-induced
613 hemolysis on human erythrocytes with and without K^+ ions (2.7 mM KCl, 8.1 mM KH_2PO_4).
614 **F**: PLTX-induced hemolysis on human erythrocytes at 37° C and 41° C. Each point represents
615 the mean ± SE of 3 different experiments.*p <0.05; **p < 0.01; ***p < 0.001 (for D and E as
616 compared to PBS + H_3BO_3 + NaCl 1x; two-way ANOVA and Bonferroni post test).

617

618 **Fig. 2. A**: Calibration curve of the hemolytic assay for PLTX. Each point represents the mean
619 ± SE of ten different experiments. **B**: Linear regression analysis performed within the working
620 range of the hemolytic assay ($3.9x10^{-10}$ M - $2.5x10^{-8}$ M) by plotting the theoretical PLTX
621 concentrations against the toxin concentrations measured by the hemolytic assay (n = 10).

622

623 **Fig. 3.** Repeatability of the hemolytic assay. Linear regression analysis performed within the
624 working range of the hemolytic assay ($3.9x10^{-10}$ M - $2.5x10^{-8}$ M) by plotting the theoretical
625 PLTX concentrations against toxin concentrations measured by the hemolytic assay. **A**: Intra-
626 day repeatability (n = 10). **B**: Inter-day repeatability (six months period, n = 10).

627

628 **Fig. 4.** Hemolytic activity of N-biotinyl-PLTX, PLTX and its natural analogues within the
629 working range for PLTX ($3.9 x 10^{-10}$ M - $2.5x10^{-8}$ M). Each point represents the mean ± SE of
630 3 different experiments. *p <0.05; **p < 0.01; ***p < 0.001 as compared to PLTX (two-way
631 ANOVA and Bonferroni post test).

632

633 **Fig. 5.** Neutralization of PLTX-induced hemolysis within the working range of the hemolytic
634 assay ($3.9x10^{-10}$ M - $2.5x10^{-8}$ M) by ouabain and anti-PLTX monoclonal antibody, mAb.
635 Each point represents the mean ± SE of 3 different experiments.*p <0.05; **p < 0.01; ***p <
636 0.001 as compared to PLTX (two-way ANOVA and Bonferroni post test).

637

638 **Fig. 6.** Matrix effect. Linear regression analysis within the working range of the hemolytic
639 assay ($3.9x10^{-10}$ M - $2.5x10^{-8}$ M) performed on 80% aqueous methanol mussels extracts
640 diluted 1:50. Linear regression analysis was performed within the working range of the
641 hemolytic assay ($3.9x10^{-10}$ M - $2.5x10^{-8}$ M) by plotting the theoretical PLTX concentrations
642 against toxin concentrations measured by the hemolytic assay.

643 **Table 1.** *Bias* values (%) for PLTX analysis and intra-day (n=10, 1 day) and inter-day (n=10,
644 6 months) relative repeatability (RSDr %).

645

PLTX (M)	*Bias* (%)	Intra-day repeatability		Inter-day repeatability	
		Mean	RSDr %	Mean	RSDr %
$3.91x10^{-10}$	-1.2	$4.55x10^{-10}$	17	$3,86x10^{-10}$	10
$7.81x10^{-10}$	-1.7	$7.60x10^{-10}$	12	$7,68x10^{-10}$	3
$1.56x10^{-9}$	-2.0	$1.63x10^{-9}$	20	$1,53x10^{-9}$	8
$3.13x10^{-9}$	2.4	$2.47x10^{-9}$	24	$3,24x10^{-9}$	7
$6.25x10^{-9}$	0.3	$6.51x10^{-9}$	12	$6,27x10^{-9}$	3
$1.25x10^{-8}$	-1.4	$1.28x10^{-8}$	8	$1,24x10^{-8}$	7
$2.50x10^{-8}$	-1.7	$3.18x10^{-8}$	14	$2,50x10^{-8}$	7
Mean	-0.8		15		6

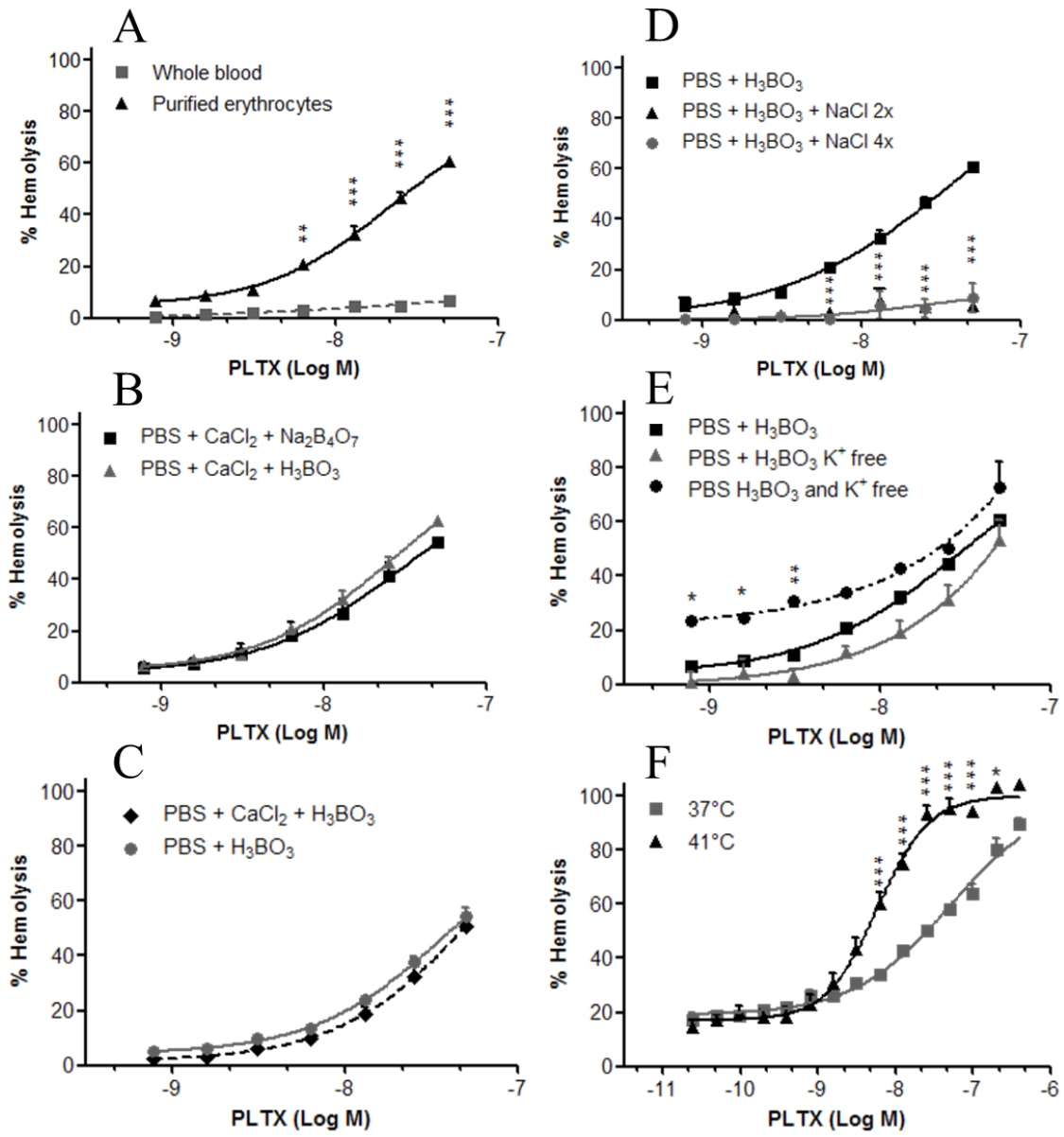
646

647

Table 2. *Bias* values (%) for PLTX detected in 80% aqueous methanol mussels extracts spiked with the toxin after 1:50 dilution in comparison to the theoretical PLTX concentrations (n=6).

PLTX (M)	*Bias* (%)
$3.91x10^{-10}$	-13.8
$7.81x10^{-10}$	-12.8
$1.56x10^{-9}$	12.9
$3.13x10^{-9}$	31.7
$6.25x10^{-9}$	-27.0
$1.25x10^{-8}$	33.6
$2.50x10^{-8}$	0.9
Mean	3.7

663 Figure 1



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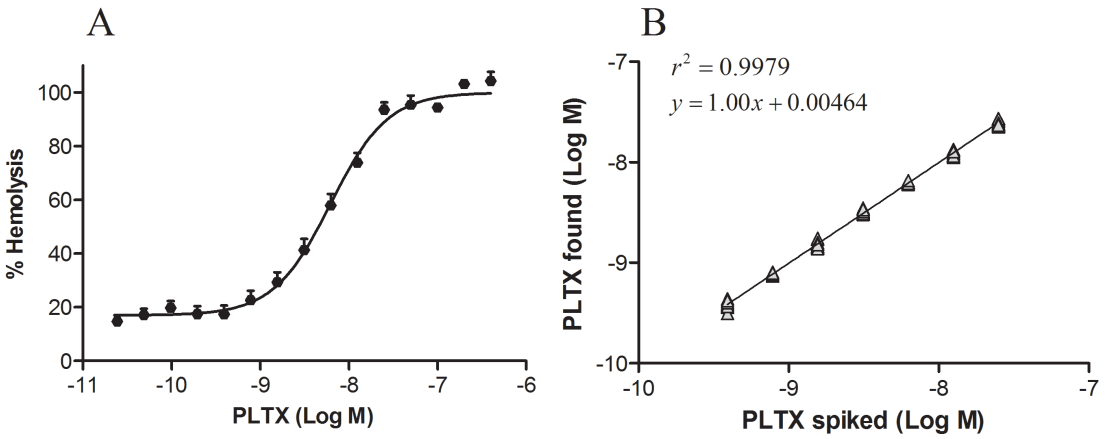
666 Figure 2



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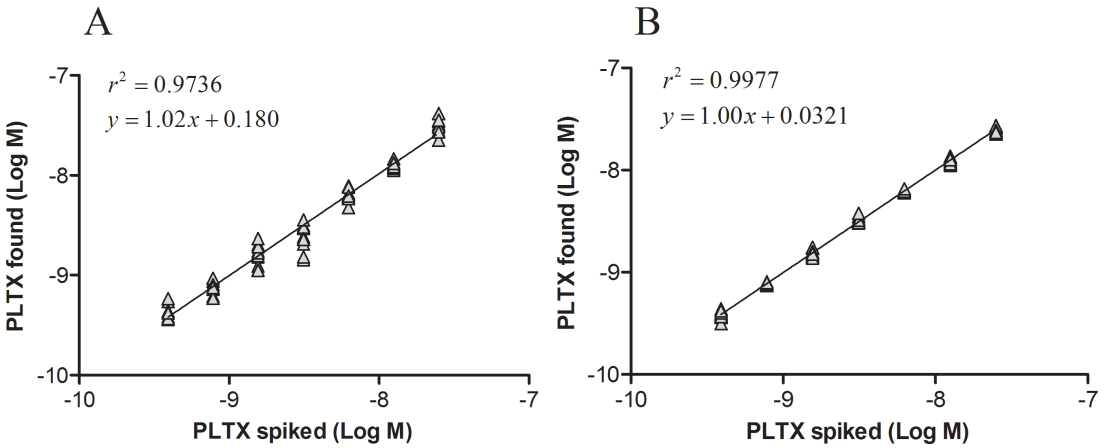
669 Figure 3



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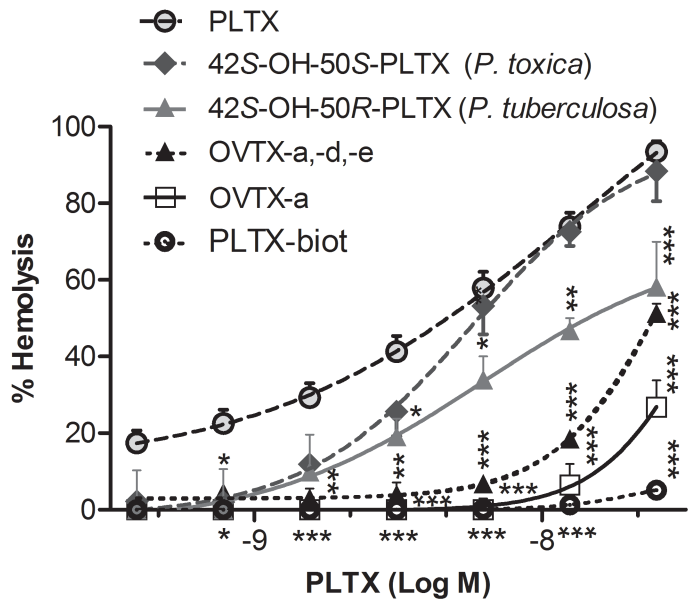
673 Figure 4



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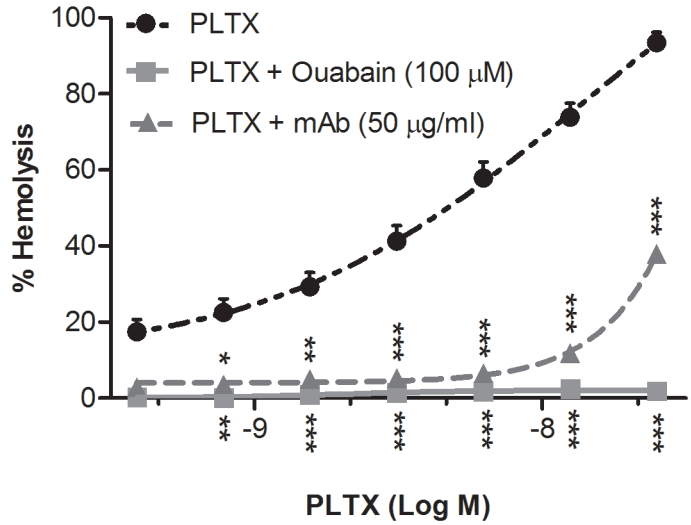
676 Figure 5



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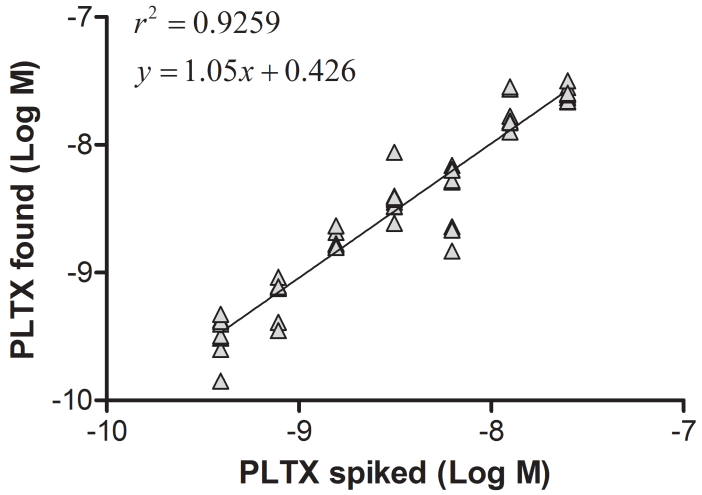
680 Figure 6



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