

# **Combined oral toxicity of azaspiracid-1 and yessotoxin in female NMRI mice**

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## **Abstract**

For many years, the presence of yessotoxins (YTXs) in shellfish has contributed to the outcome of the traditional mouse bioassay and has on many occasions caused closure of shellfisheries. Since YTXs do not appear to cause diarrhoea in man and exert low oral toxicity in animal experiments, it has been suggested that they should be removed from regulation. Before doing so, it is important to determine whether the oral toxicity of YTXs is enhanced when present together with shellfish toxins known to cause damage to the gastrointestinal tract. Consequently, mice were given high doses of YTX, at 1 or 5 mg/kg body weight, either alone or together with azaspiracid-1 (AZA1) at 200 µg/kg. The latter has been shown to induce damage to the small intestine at this level. The combined exposure caused no clinical effects, and no pathological changes were observed in internal organs. These results correspond well with the very low levels of YTX detected in internal organs by means of LC-MS/MS and ELISA after dosing. Indeed, the very low absorption of YTX when given alone remained largely unchanged when YTX was administered in combination with AZA1. Thus, the oral toxicity of YTX is not enhanced in the presence of sub-lethal levels of AZA1.

## Introduction

The presence of yessotoxin (YTX), a disulphated polycyclic ether toxin, was first reported in scallops (*Patinopecten yessoensis*) in 1987 (Murata et al., 1987). The toxin was then found together with okadaic acid group toxins (OA/DTXs) that are well known for causing diarrhetic shellfish poisoning (DSP) in man (Yasumoto et al., 1978). YTX and its analogues (YTXs) are produced by the dinoflagellate algae *Protoceratium reticulatum* (Satake et al., 1997; Satake et al., 1999; Ciminiello et al., 2003; Samdal et al., 2004), *Lingulodinium polyedrum* (Draisici et al., 1999) and *Gonyaulax spinifera* (Rhodes et al., 2006). In recent years, YTXs have been reported in shellfish from many countries, including Japan, New Zealand, Australia, Canada, Italy, France, Norway and the United Kingdom (FAO/IOC/WHO, 2004; Amzil et al., 2008). YTXs may contribute significantly to the outcome of the traditional mouse bioassay for DSP toxins when injected intraperitoneally (i.p.). However, the mechanisms of action of OA/DTXs and YTXs are different, since it has been found that YTXs, in contrast to OA/DTXs, do not inhibit protein phosphatases (Ogino et al., 1997). The only pathological changes observed upon i.p. injection of YTXs is in the heart muscle (Aune et al., 2002; Tubaro et al., 2003) and no diarrhetic effect is observed after either i.p. or oral administration (Aune et al., 2002; Tubaro et al., 2003; Tubaro et al., 2004).

The lack of documented cases of human poisoning with YTX is consistent with the very low acute oral toxicity of YTX generally demonstrated in experimental animals (Ogino et al., 1997; Aune et al., 2002; Tubaro et al., 2003; Paz et al., 2008; Munday et al., 2008; Tubaro et al., 2008), although varying results have been obtained with respect to the effect on the heart muscle, studied by electron microscopy, after repeated oral exposure to YTX. In one study, mice given 5 mg/kg YTX by gavage seven times in three weeks did not show adverse effects (Espenes et al., 2006) whereas, in a separate study, mice given 1 mg/kg YTX daily for one

week showed changes in cardiac muscle 24 h and 30 d after the last treatment (Tubaro et al., 2008).

The very low oral toxicity of YTX has prompted discussion as to whether the YTXs should be deregulated in the EU, and in a recent recommendation from the European Food Safety Authority (EFSA, 2008), an acute reference dose for YTX of 25 µg/kg was suggested, based on the no-observed-adverse effect level (NOAEL) of 5 mg/kg. Depending on the consumption of contaminated shellfish, the derived maximum acceptable level of YTX in shellfish will vary between 3.75 mg/kg (400 g portion) and 15 mg/kg (100 g portion). This recommendation is in strong contrast to today's EU regulatory level of 1 mg/kg YTX-equivalents in shellfish meat.

Before YTXs are considered for deregulation, it is important to know whether their low oral toxicity might be enhanced by simultaneous presence of shellfish toxins acting on the gastrointestinal tract, for example azaspiracids (AZAs) and OA/DTXs. Both the AZAs and the OA/DTXs cause damage to the small intestine (Terao et al., 1986; Ito and Terao, 1994; Ito et al., 2000; Ito et al., 2002a; Ito et al., 2002b). The main effect of AZAs in humans is severe diarrhoea, together with nausea, vomiting and abdominal cramps, similar to the symptoms of DSP (McMahon and Silke, 1996). However, unlike OA/DTXs, AZAs do not inhibit protein phosphatases (Twiner et al., 2005). The AZAs affect the cytoskeleton (Roman et al., 2002), and studies have shown that AZAs are able to disrupt the paracellular barrier in cells in the GI-tract, thus increasing fluid secretion (Ryan et al., 2006).

Both YTXs and AZAs has been observed in shellfish from the same locations in several countries (Twiner et al., 2008; Amzil et al., 2008; Ueoka et al., 2009; Hess, 2010) including Norway (Norwegian National Surveillance Program 2000-2010, unpublished data). Thus, an experiment was set up to study whether combined oral exposure (by gavage) of mice with

AZA1 and YTX would lead to increased uptake of either toxin, or to more severe toxic effects in the heart or other internal organs compared to the effects caused by each toxin alone.

## **Materials and Methods**

**Toxins:** AZA1 used in this study was an aliquot of the certified AZA1-stock produced in 2006, namely lot 2006/07/19 of NRC CRM-AZA1 (NRC-CNRC, 2007). The aliquot had been set aside after NMR-characterization of the whole lot and was stored in methanol at  $-80\text{ }^{\circ}\text{C}$ , a condition under which no degradation of AZA1 was observed during a 12-month stability study (Perez et al., 2010). The isolation procedure has been briefly described by Ronzitti et al. (2007). Gravimetry, LC-MS and NMR have been used in the certification of the purified AZA1, and the certification process and uncertainties are described elsewhere (Perez et al., 2010). The purified toxin, provided in ampouled aliquots of  $40.7\text{ }\mu\text{g/mL}$  in methanol, was used as a stock solution. Some impurities were identified at concentrations ranging from 0.4 to 2.8%, relative to AZA1. The major impurity (2.8%) was an isomer of AZA1, while AZA3 and dehydro-AZA3 were present at 0.4 and 0.7%, respectively. YTX was purified and stored in methanol solution to prevent decomposition as described by Loader et al. (2007). The purity of this material was estimated to be  $>95\%$  from NMR analysis (see supporting information from Loader et al. (2007)) and its concentration was determined by LC-MS/MS analysis relative to a certified standard of YTX from NRC (NRC-CNRC, 2006).

**Other chemicals:** Ethanol (96%, from Arcus, Norway), methanol and acetonitrile (HiperSolve, BDH Prolabo, VWR), formic acid (pro analysis, Merck, Germany) and ammonium formate (pro analysis, Carl Roth, Karlsruhe, Germany) were obtained from VWR, Norway. Saline solution (0.9 %) was prepared at the Norwegian School of Veterinary

Science. Deionised water (grade I) from a Milli-Q system (Millipore, MA, USA) was used in all preparations requiring aqueous solutions.

Animals: Female NMRI mice, weighing between 19 and 22.5 g were used. The NMRI mouse is a Swiss mouse strain, originating from Lynch, and obtained by the Naval Medical Research Institute, USA (NMRI). It was established at M&B A/S (now Taconic Europe, Bomholt, Denmark), in 1961 and again in 1985, where it is maintained as an outbred stock. The mice had access to feed and water ad libitum. Day-length (12/12), temperature (20-22 degrees C) and humidity was monitored. An ethical permit was obtained for the animal trials from the Norwegian Animal Research Committee.

Solutions for study of combined toxicity of AZA1 and YTX: Two 500 µg aliquots of YTX in MeOH were combined and the solvent was evaporated under nitrogen to give a white solid. This was re-dissolved immediately in MeOH to give 2000 µg/mL as a stock solution. The calculated volumes (Table 1) of the YTX stock solutions were dispensed into vials, which were capped and stored at -20 °C until required for preparation of doses. Immediately prior to dosing, the YTX solutions were evaporated to dryness (gentle stream of nitrogen at ca 35 °C). Then AZA1 stock (395 µL) was added as appropriate. The contents of all vials were evaporated to dryness under nitrogen and dissolved in 40 µL of 96% EtOH (vortex mixer) whereupon 960 µL saline was added with vortex-mixing. The clear colourless solutions were then taken directly to the animal facility and used in the dosing experiment. Volumetric transfers of toxin solutions and solvents were performed with 500 µL or 100 µL Hamilton syringes. Saline was dispensed with a 1 mL air-displacement pipette (Finnpipette).

***Animal experiments:***

Yessotoxin was given by gavage at 1 or 5 mg/kg, either alone or in combination with AZA1 at 200 µg/kg. A fifth group of mice received 200 µg/kg AZA1 alone while a group of control mice were given vehicle only. Each mouse was dosed a volume of 250 µL per 20 g b.w. The groups consisted of three mice except the control group which consisted of six mice. All mice were sacrificed after 24 h.

The mice were killed by cervical dislocation. Tissue samples were immediately taken from brain, heart muscle, lungs, thymus, liver, spleen, kidneys, stomach, small intestine (duodenum, middle and lower jejunum) and colon for fixation in formalin. One half of the brain was fixed *in toto* and cut in three transverse sections through the level of frontal cortex, midbrain and cerebellum. All the fixed tissues were dehydrated, embedded in paraffin wax, and sections were stained with haematoxylin and eosin. For analysis of AZA1 and YTX, blood (from large blood vessels in thoracic cavity) and samples from the tissues mentioned above were collected and frozen. For electron microscopy, two pieces of tissue from the heart muscle less than 1 mm in diameter were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, treated with OsO<sub>4</sub> and embedded in Epon. Ultrathin sections from each embedded tissue block were stained with uranyl acetate and lead citrate and examined in a Philips EM208S electron microscope.

All the tissue samples from stomach and intestines for ELISA and LC-MS/MS analysis had the digestive contents removed mechanically before they were homogenised and extracted.

### ***Chemical analyses:***

***Extraction:*** Samples of tissues were pooled all 3 animals in each group of mice receiving AZA1 and YTX, and 4 of 6 controls. Pooled samples of the organs in each group were transferred to capped pre-weighed 15-mL polypropylene centrifuge tubes (NUNC A/S, Roskilde,

Denmark), weighed, and stored at  $-80^{\circ}\text{C}$  until analysis. The tissues (0.5 g portion of pooled liver homogenate and 0.1–0.6 g of pooled tissue from the other organs) were homogenized in 2.0 mL MeOH with water bath cooling using an Ultra-turrax<sup>®</sup> (Ika, Germany) at 15,000 rpm for 30–60 s. The homogenised samples were centrifuged at  $\sim 1500$  g for 20 min, left in the refrigerator overnight and then centrifuged again at  $\sim 1500$  g for 20 min the following day. Aliquots of supernatants were transferred to HPLC vials for analysis, and the remainder stored in capped vials at  $-80^{\circ}\text{C}$  for later study (ELISA).

**LC-MS/MS:** Analysis was performed on an LC-MS/MS system consisting of an Agilent 1200 HPLC system connected to an Agilent G6410 MS/MS (Santa Clara, CA, USA). The column used was a Hypersil BDS C8 (3.5  $\mu\text{m}$ , 2.0  $\times$  50 mm) (Thermo Fisher Scientific, Waltham, MA, USA). A gradient elution was run with two mobile phases; water (A) and 95% acetonitrile (B), each containing 50 mM formic acid and 2 mM ammonium formate (Table 2), based on Quilliam et al. (2001). The AZA1 and YTX used for dosing were analysed by selected reaction monitoring (SRM) for the transitions characteristic for AZA1, AZA2 and AZA3 ( $m/z$  842.5, 856.5 and 828.5  $\rightarrow$  362.5) and YTX, 45-hydroxyYTX and carboxyYTX ( $m/z$  1141.5, 1157.5 and 1173.5  $\rightarrow$  855.5). The samples were quantified against standards for AZA1 and YTX from the CRM program of IMB, NRC, Halifax, NS, Canada.

**ELISA: Sample preparation:** Extracts for ELISA analysis were prepared by serial three-fold dilutions with phosphate-buffered saline with 0.05% Tween 20 (PBST) and sample buffer (10% methanol in PBST) to give a final methanol concentration of 10%. ELISA quantitation was performed at three dilutions out of 1:10, 1:30, 1:90, 1:270, 1:810 and 1:2430 (usually 1:10, 1:30, 1:90 or 1:270), as appropriate.

**Reagents:** ELISA analysis (Briggs et al., 2004) was performed with a quantitative YTX standard obtained by dilution of the dosing solution and calibrated by LCMS against the

certified NRC standard. Ovine antibodies to YTXs (serum #505) were obtained from AgResearch Ltd, Hamilton, New Zealand (Briggs and Miles, 1999; Briggs et al., 2004). Horseradish peroxidase (HRP)-coupled rabbit anti-sheep antibodies were purchased from Zymed Laboratories (61-8620; now distributed by Invitrogen, Camarillo, CA, USA). Substrate was K-Blue® Aqueous Substrate (TMB) from Neogen (Lexington, USA). OVA-BrYTX-coater was prepared as described by Briggs et al. (2004) and all other reagents for the YTX-ELISA were prepared according to Samdal et al. (2004).

**ELISA analysis:** The concentration of YTXs in each mouse tissue sample extract was determined by indirect competitive ELISA as described by Briggs et al. (2004), with only minor adjustments to plate coater and antibody concentrations to optimize the assay (Samdal et al., 2004). All incubations were performed at room temperature. YTX standard in methanol (1 µg/mL) was diluted in PBST and sample-buffer (10% methanol in PBST), to give a methanol concentration of 10%. Serial three-fold dilutions of the YTX standard were performed with sample buffer, giving 10 standards from 0.002–40 ng/ml. Serial dilutions of standards and samples were performed in duplicate. Absorbances were measured at 450 nm using a plate reader (Wallac 1420 Victor<sup>2</sup> multilabel counter, Wallac, Turku, Finland).

## **Results**

Very low levels of YTX were found in internal organs when mice were given YTX by gavage at 1 or 5 mg/kg (Fig. 1, Table 3). The level of quantification for YTX was exceeded only in kidneys, spleen, liver and lungs. Considerably higher levels of YTX were recovered from tissue samples from the GI tract. When mice were exposed to combinations of either 1 or 5 mg/kg YTX together with AZA1 at 200 µg/kg, the level of YTX in internal organs remained very low (Fig. 2, Table 3). In Fig. 2, only data from AZA1 together with the highest YTX

dose are given, due to the very low levels of YTX at 1 mg/kg. A very large increase in YTX-levels was observed in stomach tissue when YTX, at both levels, was combined with AZA1, whereas the levels found in the small and large intestines were similar to the levels when YTX was given alone.

An estimate of the total amount of YTX detected by LCMS in different internal organs 24 h after gavage is shown in Table 4. When given alone, the amount of YTX recovered from each internal organ was below 0.05% of the total amount given, regardless of the YTX dose given. The amount of YTX in the heart and brain were below the limit of quantification. When co-administered with AZA1 (200 µg/kg), the levels of YTX in organs remained essentially unchanged (Table 4).

The effect of the combination of YTX with AZA1 was also studied concerning effects on absorption of AZA1 from the GI-tract (Fig. 3). The absorption of AZA1 was considerably higher than that of YTX. Co-administration with YTX did not enhance the absorption of AZA1.

Analyses of YTX were performed both with LC-MS and ELISA, since only YTX and main analogues are quantified by the LC-MS, while a whole range of analogues/metabolites are detected by ELISA (Aasen et al. 2005, Briggs et al., 2004; Samdal et al., 2005). Results of both methods corresponded well to each other in samples from the GI tract, whereas the YTX levels in internal organs were consistently higher by ELISA than by LC-MS (Table 3 and Supporting Information). However, YTX levels in internal organs were very low, increasing the uncertainty in both methods. Nevertheless, the correlation between the two methods for the different tissues was examined using linear regression. The correlation ratios were 1.1 ( $R^2$  0.999) for the stomach, 1.4 ( $R^2$  0.99) for the duodenum, 1.2 ( $R^2$  0.94) for the jejunum, 1.5 ( $R^2$  0.97) for the ileum, and 1.9 ( $R^2$  0.94), suggesting a low level of metabolism during passage

through the GI tract. The correlation ratios for internal organs were 1.3 ( $R^2$  0.89) for lung, 2.2 ( $R^2$  0.85) for spleen, 1.6 ( $R^2$  0.74), and 3.8 ( $R^2$  0.90) for kidney. These results suggest a degree of metabolism of YTX within the internal organs of the mouse.

The mice were observed for 24 h, and no clinical signs of disease were observed for the period, either for those exposed to AZA1 or YTX alone or for those exposed to the combination of the two compounds.

Macroscopical pathology: Mice that had received AZA1 alone showed considerable retention of material in the stomach and the upper  $\frac{1}{3}$  of the small intestine was dilated with increased fluidity of the content compared with control animals. Mice that received a combination of YTX and AZA1 showed similar changes to those that had received AZA1 only, at both 1 and 5 mg/kg YTX. In mice that had received only YTX at 1 and 5 mg/kg, no changes were observed compared to the control mice.

Microscopical pathology: In mice that had received YTX at 1 or 5 mg/kg in combination with AZA1 at 200  $\mu$ g/kg, the villi in the duodenum appeared contracted, blunt and occasionally club-shaped, the cryptal compartments were extended and an extensive infiltration of polymorphonuclear granulocytes (neutrophils) were noted in the *lamina propria*. These changes were similar to the changes observed in the duodenum of mice that had received AZA1 alone (Fig. 4). In mice that had been given YTX alone, the duodenum was normal and similar to the duodenum in control mice. By light microscopy, no changes were observed in any of the other organs examined including the other intestinal segments.

By electron microscopy, no changes were found in the heart muscle, either in the group given a combination of AZA1 and YTX or in the group given YTX alone. The myocardial cells surrounding the capillaries showed no signs of cytoplasmic swelling or abnormal

mitochondria (Fig. 5) and the general structure of the myofibrils and distribution and structure of the mitochondria were found normal (Fig. 6).

## **Discussion**

The present study examined the effect of oral exposure of mice to the marine algal toxins AZA1 and YTX and combinations thereof. Such co-exposure could potentially have led to new or enhanced toxic effects that are not observed when the toxins are administered separately. In particular, we wondered if the pathological changes caused by AZA1 in the intestine (Ito et al., 2000; Ito et al., 2002b; Aasen et al., 2010) could damage the mucosal barrier and increase the absorption of YTX, and thereby cause systemic effects similar to those observed after intraperitoneal (i.p.) injection of YTX (Terao et al., 1990; Aune et al., 2002; Tubaro et al., 2003). Very low concentrations of YTX were measured in kidneys, spleen, liver and lungs, when YTX at 1 or 5 mg/kg was given alone (Fig. 1, Table 3). In blood, the YTX level was below the LOQ by LC-MS in all groups except in the group given YTX at 5 mg/kg together with AZA1, where the YTX level was very low. When using ELISA, the YTX concentration in blood was slightly higher, and corresponded well with the results reported by Tubaro et al. (2008).

The level of YTX in the heart, the putative target organ when YTX is given i.p., was below the level of quantification by LC-MS. This corresponds well with previous data showing no significant difference between mice exposed to YTX and controls after repeated oral exposures, i.e. a dose of up to 5 mg/kg YTX given seven times during three weeks (Espenes et al., 2006). However, other studies have indicated that YTX can induce ultrastructural changes in the heart muscle of mice both after short term exposure (Aune et al., 2002) and after repeated daily exposures of 1 or 2 mg/kg for one week (Tubaro et al., 2004; Callegari et

al., 2006; Tubaro et al., 2008). In the present study, the YTX levels in internal organs were very low, suggesting that only a small proportion of YTX is transported across the intestinal barrier. However, only one time point and a limited number of organs were analysed, so detailed information on the absorption and distribution of YTX could not be obtained. The total fraction of YTX in the internal organs measured by LC/MS accounted for less than 0.1% of the administered dose (Table 4). Most of the toxin presumably passed through the animals since most of the YTX was recovered from the lower intestine and the faeces, while only trace amounts were found in blood, urine and tissues. (Munday et al., unpublished results).

The level of YTX in the stomach was around two orders of magnitude higher when YTX was given in combination with AZA1, compared to when YTX was given alone (Table 3). Since the stomach content was only mechanically removed before the toxin level was measured, a sharp distinction between toxin level in the content and the stomach wall could not be made.

The observed increase in retention of the stomach contents, suggest an inhibitory effect of AZA1 on the motility of the stomach and duodenum, which would extend the period with high YTX levels in the stomach. Another possible explanation is that the combination induced absorption of YTX into the stomach wall, but this is less likely since such an absorption ought to have resulted in higher toxin levels in the liver and/or other internal organs. However, since the YTX concentration in the distal parts of the intestines was similar to when YTX was given alone (Table 3 and Fig. 2), it seems that the motility of the stomach and intestines is not severely inhibited due to AZA1 but rather slowed. It may be assumed that the part of the intestine with pathological changes has been exposed to a substantial amount of YTX.

The concentrations of AZA1 found in the different tissues when given in combination with YTX were similar to the levels when AZA1 was given alone at 200 µg/kg (Fig. 3). These results were also similar to those obtained in Aasen et al. (2010) for AZA1 at 200 µg/kg. Consequently, YTX does not seem to influence the absorption of AZA1.

In mice exposed to a combination of AZA1 and YTX, the pathological changes in the intestine were similar to the changes caused by AZA1 alone. The damage to the intestine did not appear to increase the absorption of YTX as shown by the quantification of YTX in blood and internal organs by LC-MS. The levels found in the blood by LC-MS and ELISA (Table 3) corresponded with the levels reported by Tubaro et al., (2008). The low YTX levels in the internal organs were consistent with the histological investigations by light microscopy showing normal morphology in the same organs.

It is possible that the YTX concentration in internal organs could have been higher at a time point earlier than 24 h, or that YTX could have been modified in the tissues to analogues not detectable by the LC-MS method. However, lack of pathological changes strongly indicates that a high YTX concentration in the tissues had not been present during the first 24 h as this has been shown to be within the time limit to induce changes in tissues (Aune et al., 2002; Tubaro et al., 2003). The ELISA measurement of YTXs did not show the presence of a high proportion of analogues in most tissues, supporting the LC-MS data obtained in the present study but indicating the potential for a degree of metabolism in internal organs of mice.

To more precisely address possible changes in the presumed target organ for YTX, the heart muscle, ultrastructural studies were performed. As early as 1990, Terao et al. reported the toxic effects of YTX upon i.p. exposure of mice. The animals died within a few hours at doses of 300 µg/kg. Subsequent studies have confirmed the high lethality of YTX when given via the i.p. route (Terao et al., 1990; Ogino et al., 1997; Aune et al., 2002; Tubaro et al., 2003; Aune et al., 2008). YTX given i.p. caused changes in the cardiac muscle with oedema and vacuolation (Aune et al., 2002). The degree of damage to the heart has varied in different studies. Terao et al. (1990) reported swelling and degeneration of the endothelial lining cells of the left ventricle and swelling of most cardiac muscle cells at 500 µg/kg of YTX when given i.p., and Aune et al. (2002) reported less severe changes in the heart after i.p. doses of

1,000 µg/kg one hr after injection, with swelling and separation of myofibrils and mitochondria of myocardial cells in the left ventricle, most pronounced near the capillaries.

The effects of oral exposure have been more variable, as Terao et al. (1990) found no effects on the heart 21 h after exposure to YTX at 0.5 mg/kg whereas Aune et al. (2002) described such changes in mice 24 h after oral dosing with 7.5 and 10 mg/kg and 1 h after dosing of 2.5, 5 and 10 mg/kg, although in the latter group the changes were moderate for the lowest dose. Even doses of 1 and 2 mg/kg YTX given orally have been reported to induce ultrastructural changes in heart muscle cells 24 h after administration although no signs of toxicity and no indication of cardiac tissue damage based on plasma enzyme measurements, were noted (Tubaro et al., 2003). In the present study, no ultrastructural changes were seen in the heart muscle 24 h after 1 or 5 mg/kg of YTX, either alone or in combination with AZA1, findings that were consistent with the very low level of YTX that was detected in the heart muscle (Figs. 5 and 6, Tables 3 and 4). In this study, the concentration of toxins found in the internal organs of the mice was measured by LC-MS based on the method of Quilliam et al. (2001). This method has some limitations in that it has not been optimised for mouse tissues (Aasen et al., 2010). However, the method has been used for the analysis of several matrices in our lab, and was the best alternative available for quantification of low concentrations of AZA1 and YTX.

The LC-MS analysis could potentially be influenced by matrix effects from the different organs, but we were not able to study this phenomenon in the present work. However, since one of the major aims in this study was to compare the effects of different treatments on the concentration of YTX in the same organ, the possible matrix effects are likely to be minor. The ELISA method for YTX is expected to give higher figures than the LC-MS method if other analogues or metabolites of YTXs are present. With the LC-MS method, only YTX and two main analogues were measured, while with ELISA, all those YTX analogues that have an

intact part of the ladder were measured (Briggs et al., 2004; Samdal et al., 2005). The results obtained with ELISA indicate that metabolism of YTX was generally modest, as the results were close to those obtained by LC-MS where YTX was the only analogue detected.

The limit of quantification (LOQ) for AZA1 with the LC-MS method was approximately 0.35 ng/mL and for YTX 0.5 ng/mL, while the limit of detection (LOD) was below 0.1 ng/mL for both toxins. The tissue samples were pooled from all animals treated, but the total weight was still relatively low for some of the organs. This low sample mass increases the uncertainty of the analysis, since the concentration in the extracts (ng/mL) was multiplied by extraction volume and divided by weights of the sampled organs to estimate the concentrations of toxins ( $\mu\text{g}/\text{kg}$ ).

In conclusion, we report that oral exposure of mice to 1 or 5 mg/kg YTX, either alone or together with AZA1 at 200  $\mu\text{g}/\text{kg}$  (a level that causes damage to the small intestine (Aasen et al., 2010)), does not lead to enhanced absorption of YTX nor to toxic effects in the heart or other internal organs. These results support the suggestion to deregulate YTXs in shellfish, especially since the most recent risk assessment by the EU Panel on Contaminants in the Food Chain (2008) recommend a considerably higher acute reference dose for the YTX-group.

### **Supporting Information**

Plot of ELISA (YTXs) versus LC/MS (YTX) results for tissues from mice dosed with YTX (1 and 5 mg/kg) alone and in combination with AZA1 (200  $\mu\text{g}/\text{kg}$ ), with linear least squares correlations fitted in Excel.

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### **Conflict of Interest**

On behalf of all authors I declare that there is no conflict of interest related to the publication of the manuscript.

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## Captions for Figures and Tables

Figure 1. Concentrations of YTX (by LC/MS) in  $\mu\text{g}/\text{kg}$  in tissues of female NMRI mice, after dosing 1 mg/kg and 5 mg/kg YTX by gavage.

Figure 2. Concentrations of YTX (by LC/MS) in  $\mu\text{g}/\text{kg}$  in tissues of female NMRI mice, when given alone at 5 mg/kg and in combination with 200  $\mu\text{g}/\text{kg}$  AZA1.

Figure 3. Concentrations of AZA1 in  $\mu\text{g}/\text{kg}$  in tissues of female NMRI mice after dosing of 200  $\mu\text{g}/\text{kg}$  AZA1 alone and in combination with 1 mg/kg and 5 mg/kg YTX.

Figure 4. Representative micrographs of duodenum from mice killed 24 h after oral exposure to either (A) vehicle, (B) AZA1 alone, 200  $\mu\text{g}/\text{kg}$ , (C) YTX 5 mg/kg alone or (D) a combination of AZA1, 200  $\mu\text{g}/\text{kg}$  and YTX, 5 mg/kg. Mice exposed to AZA1 alone or the combination of AZA1 and YTX showed shortened villi, elongated crypts and accumulation of inflammatory cells in lamina propria (arrows). Mice exposed to YTX alone did not show changes. HE-stain. Bar: 100  $\mu\text{m}$ .

Figure 5. Representative electron micrographs of heart muscle of mice. Normal structure of capillaries (cap) and adjacent myocardial cells of a control is shown in A. There were no changes in myocardial cells close to the capillaries, either in mice orally exposed to YTX alone (B) or to a combination of AZA1, 200  $\mu\text{g}/\text{kg}$  and YTX, 5 mg/kg (C). In all groups, the myocardial cells contained many mitochondria (arrows). Bar: 1  $\mu\text{m}$ .

Figure 6. Electron micrographs of heart muscle. There were no changes in myocardial cells related to the structure of the myofibrils or the morphology or distribution of mitochondria (arrows) either in mice orally exposed to a combination of azaspiracid-1 at 200 µg/kg and yessotoxin at 5 mg/kg (B) nor as compared to mice exposed to vehicle only (A). Bar: 2 µm.

Table 1. Calculated volumes of AZA1 and YTX stock solutions for dilutions

Table 2. HPLC gradient program with two-phase gradient of water (A) and 95% acetonitrile (B), each containing 50 mM formic acid and 2 mM ammonium formate

Table 3. Levels of YTX (µg/kg) found by LC-MS/MS and by ELISA in mouse tissues. LOQ values for each sample given

Table 4: Average weight of internal organs of 20 g female NMRI mice and estimates of total amount of YTX (ng) determined by LCMS in internal organs of mice 24 h after oral intubation at two doses, with and without AZA1 (200 µg/kg), and estimated percentage of dosed YTX recovered in each organ. LOQ in ng for each organ in separate column.