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ACCUMULATION AND DEPURATION OF YESSOTOXIN IN TWO BIVALVES

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ABSTRACT Contamination of bivalves with yessotoxins (YTXs) has been reported since 1987 in several coastal environments. In our study we investigated the accumulation and the metabolism of YTXs in 2 bivalve species important to German fisheries and aquaculture. Mussels and oysters, *Mytilus edulis* and *Crassostrea gigas*, were exposed to the YTX-producing dinoflagellate *Protoceratium reticulatum* isolated from the North Sea. Toxin profiles from algae and bivalves were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). It was evident that 45-hydroxy YTX (45-OH YTX), carboxy YTX (COOH YTX), and the putative 45-hydroxy-carboxy YTX (45-OH-COOH YTX) were the dominant YTX analogues in *M. edulis*. The oyster *C. gigas* displayed a different toxin profile. YTX, 45-OH YTX, and 45-OH-COOH YTX were also present, but COOH YTX was not detectable. Furthermore, 2 unidentified analogues with the same MS/MS transition ($[M\text{-}H]^- > [M\text{-}H\text{-}SO_3]^-$) occurred in both bivalve species. Homo YTX and 45-hydroxy-homo YTX, which are regulated together with YTX and 45-OH YTX in 2002/225 EC were not detected. In general, we confirmed former field investigations on accumulation and metabolism of YTXs in *M. edulis*; however, this is the first study on accumulation and metabolism of YTXs in the oyster *C. gigas*.

KEY WORDS: marine toxins, shellfish toxicity, yessotoxin, YTX, 45-hydroxy yessotoxin, carboxy yessotoxin, keto yessotoxin, *Protoceratium reticulatum*, *Gonyaulax grindleyi*, blue mussels, *Mytilus edulis*, Pacific oysters, *Crassostrea gigas*, LC-MS/MS, metabolism

INTRODUCTION

Yessotoxin (YTX), a disulfated polyether toxin was first isolated by Murata et al. (1987) from the digestive glands of Japanese scallops (*Patinopecten yessoensis*). To the present, YTX has been detected in phytoplankton and shellfish from New Zealand (MacKenzie et al. 1998), Japan (Suzuki et al. 2007), Italy (Ciminiello et al. 1997, Ciminiello et al. 2003), Chile (Yasumoto & Takizawa 1997), Norway (Aasen et al. 2005), Canada (Paz et al. 2008), the United States (Paz et al. 2007), Spain (Paz et al. 2004, Paz et al. 2007), and Africa (Krock et al. 2006).

Three species of dinoflagellates have been identified as causative organisms: *Protoceratium reticulatum* (Claparede & Lachmann) Buetschli (=*Gonyaulax grindleyi*) (Satake et al. 1997), *Lingulodinium polyedrum* (Stein) Dodge (=*Gonyaulax polyedra*) (Tubaro et al. 1998, Draisci et al. 1999), and *Gonyaulax spinifera* (Claparede & Lachmann) Diesing (Rhodes et al. 2006, Riccardi et al. 2009). Meanwhile, the production of YTX by the dinoflagellate *P. reticulatum* has been reported in New Zealand (Satake et al. 1997), Japan (Satake et al. 1999), Norway (Ramstad et al. 2001, Samdal et al. 2004), and Africa (Krock et al. 2006).

Many different YTX analogues were detected until now, but of about 100 YTXs, only 40% are characterized concerning their exact chemical structure (Paz et al. 2008). The best known modifications at the YTX backbone are insertion of a methyl group between C-1 and C-2 (e.g., 1a-homo YTX) as well as between C-41 and C-42 (e.g., 41a-homo YTX), methylation of C-9 (e.g., 9-Me-41a homo YTX), glycosylation of C-32 (e.g., glyco YTX A), several modifications at the unsaturated side chain (e.g., carboxylation (COOH YTX), hydroxylation (45-OH YTX, 44,55-di OH YTX), addition of amides (e.g., trihydroxylated amide of 41a-homo YTX), elimination of different parts (e.g., trinor YTX, keto YTX, ketohomo YTX), absence of ring A (e.g., nor-ring A YTX), desulfonations (e.g., 1-ds YTX, 4-ds YTX), and many variations of the aforementioned (Miles et al. 2004, Finch et al. 2005, Miles et al. 2005a, Miles et al. 2005b, Souto et al. 2005, Miles et al. 2006a, Miles et al. 2006b, Ciminiello et al. 2007). Beside these structural variations, another YTX-like toxin (adriatoxin; ATX) was isolated from mussels of the Adriatic Sea. The structure of ATX is similar to YTX for ring A–ring J but, in contrast, the 11th ether ring (ring K) is absent. In addition, ATX contains a hydroxyl group at C-36 and a 3rd sulfate group at C-37 at ring J (Ciminiello et al. 1998).

YTX is mainly produced by *P. reticulatum*, albeit some strains have been found with homo YTX as the prominent analogue (Paz et al. 2008). The different YTXs are produced by dinoflagellates to a considerable extent (Suzuki et al. 2007); however, some of them are metabolized in the shellfish tissue (Miles et al. 2004).

The chemical structure of YTX is closely related to other ladder-shaped polyethers like ciguatoxin (CTXs), maitotoxin, brevetoxins (PbTXs), and some diarrhetic shellfish toxins (DSTs). CTXs and (PbTXs) are potent activators of voltage-gated sodium channels in mammals (Dechraoui et al. 1999, Denac et al. 2000, Naar et al. 2007). In contrast, YTX neither causes an activation of this type of ion channels (Inoue et al. 2003) nor inhibits protein phosphatases 1 and 2A, which is typical for okadaic acid and dinophysistoxin-1 (Takai & Mieskes 1991, Takai et al. 1992, Honkanen et al. 1994, Windust et al. 1996). In addition, YTX does not cause fluid accumulation and it is not hemolytic or ichthyotoxic (Ogino et al. 1997). Nevertheless, YTXs are potent cytotoxins (Paz et al. 2008), able to cause damage of the myocardium (Aune et al. 2002, Tubaro et al. 2004). Furthermore, YTX affects Ca^{2+} influx in human lymphocytes (De la Rosa et al. 2001), produces a Ca^{2+}-dependent decrease of cyclic adenosine monophosphate (Alfonso et al. 2003, Pazos et al. 2005), slows down the disposal of an E-cadherine degradation product ($ECRA_{100}$) (Callegari & Rossini 2008), and causes neurotoxicity in rat cerebellar neurons (Pérez-Gómez et al. 2006).

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Referring to the classic mouse bioassay, YTX was classified into the group of DSTs. However, Satake et al. (1999) discovered that YTX given *per os* does not cause symptoms comparable with other DSTs. As a result of these findings, YTX and its analogues were separated from other DSTs into their own toxin category by the scientific community.

In 2002 the European Commission decided that the limit of YTX and its analogues has to be set up to 1 mg/kg of shellfish meat (European Commission 2002). It was considered, that this threshold is valid for YTX and 3 of its analogues: 45-hydroxyYTX (OH YTX), 1a-homo YTX (homo YTX), and 45-hydroxy-1a-homoYTX (OH-homo YTX). Furthermore, some countries established toxic equivalence factors for these YTXs (YTX = 1, homo YTX = 1, OH YTX = 1, and OH-homo YTX = 0.5 (European Food Safety Authority 2008). Recently, the European Food Safety Authority published that a 400-g portion of shellfish meat should not contain more than 3.75 mg YTX eq/kg, a concentration higher than that of the current regulation limit (European Food Safety Authority 2008).

Independent of the problem of regulation of upper limits for YTXs in seafood, there is still a lack of information about the metabolism of YTXs in shellfish. Aasen et al. (2005), Ciminiello et al. (2000), and MacKenzie et al. (2002) reported an accumulation of YTXs in mussels after blooms of *P. reticulatum*. The results indicated that the accumulation, metabolism, and depuration of YTXs depend directly on the toxin composition of the bloom-forming dinoflagellates. In this context, temperature and food availability are additional factors that influence the depuration of shellfish contaminated with YTXs (Blanco et al. 1999).

The aim of this study was to elucidate the accumulation and depuration of YTXs in *Mytilus edulis* and *Crassostrea gigas* of the North Sea, which are important for German fishery and shellfish industries. Furthermore, we wished to clarify the possible metabolic pathway of YTX in different shellfish species.

MATERIALS AND METHODS

Experimental Protocol and Sample Extractions

P. reticulatum Cultures

The dinoflagellate *P. reticulatum* used for the experiments was isolated by M. Hoppenrath from the North Sea. Several cultures were grown sequentially in f/2 medium without silica (Guillard 1975) at 60–90 µmol/sec/m^2 and 14°C, using a 14-h/10-h light/dark regime in 10-L borosilicate flasks. After 21 days, the cultures reached the mid exponential growth phase, and 2 L of the culture was withdrawn for inoculating each bivalve tank. In addition, 90 mL was filtered on GF/C filters (Whatman, Great Britain) for toxin analysis and 10 mL was used to determine cell densities. For determination of the cell density, cells were fixed in Lugol's iodine solution (Carl Roth GmbH & Co, Germany) and counted using Sedgwick rafter's counting chamber under light microscopy (10× magnification); a minimum 400 cells were counted.

Bivalves and Experimental Conditions

Mussels, *M. edulis*, were collected from Helgoland Roads in the North Sea by scuba diving. Oysters, *C. gigas*, were collected from the tidal zone of the island Sylt (Germany). The toxification of the bivalves with *P. reticulatum* was carried out in tanks filled with 25 L natural seawater, and 2 × 24 samples per species were placed in separate tanks. Because of the smaller size, we pooled 6 individuals of *M. edulis* and handled them in our analyses as 1 sample.

Bivalves with similar sizes were selected and the size was recorded (*M. edulis*: length, 5.9 ± 0.7 cm; width, 2.8 ± 0.4 cm; *C. gigas*: length, 8.6 ± 1.2 cm; width, 6.4 ± 1.2 cm). To ensure constant water conditions, oxygen content and temperature were measured over the entire project (OxyScan Graphic; Umwelt-, Membran- und Sensortechnik Melningen; Germany). The oxygen concentration was 8 ± 0.3 mg/L (saturation, 95 ± 1.4 %) and the water temperature was 18 ± 0.5°C.

The experiment was subdivided into 3 parts, the toxification period (16 days), a break (4 days), and the detoxification period (16 days). The break was inserted because earlier experiments showed that mussels need time for depuration. The sampling was carried out during toxification ($n = 2$) and detoxification ($n = 1$) every 2nd day. The tanks were cleaned, supplied with new seawater, and filled with bivalves to the prior amount. We fed 2 L of the midexponential algal culture, containing 7,000 cells/mL on average, per tank (25 L) during the toxification period. Hence, the total concentration in the tank was approximately 520 cells/mL. During the break and detoxification periods, bivalves were fed with natural plankton from the North Sea. Bivalve samples were lyophilized and stored in the freezer at –20°C until extraction.

Extraction of Yessotoxins

Lyophilized filters of *P. reticulatum* were extracted with methanol (MeOH) using an ultrasonic bath for 15 min and centrifuged at 14,000*g* for 10 min. Extracts were placed in 2-mL single-use syringes and filtered through a 0.45-µm nylon filter (Carl Roth GmbH & Co., Germany). They were stored at –20°C in a brown autosampler vial until analyses by HPLC-MS/MS.

For the extraction of the bivalve samples, lyophilized material was ground using a mortar. After determination of dry weight, 30 mL MeOH was added to *M. edulis* and 20 mL MeOH was added to *C. gigas*. The samples were vortexed for 1 min and subsequently extracted using a cooled ultrasonic bath for 15 min. To guarantee a good recovery, we allowed soaking overnight and extracted them again using an ultrasonic bath (15 min). The extracts were centrifuged at 12,000*g* for 45 min (Sorvall RC-5B Superspeed Centrifuge, Du Pont Instruments). The supernatant was evaporated to dryness under nitrogen and resolved in 3 mL MeOH by vortexing again for 1 min, and afterward centrifuged for 10 min at 4,500*g* (CR-412 refrigerated centrifuge; Jouan). Finally, extracts were filtered through 0.45-µm nylon filters (Carl Roth GmbH & Co.) and stored at –20°C until analysis by LC-MS/MS in brown autosampler vials.

LC–MS/MS Analysis

Certified YTX standard solution dissolved in MeOH was obtained from the National Research Council Canada. HPLC was carried out with 2 LC-10 AT pumps, a SIL-10 AD VP autosampler, an SCL-10 AT VP controller, and a CTO-10 AS VP column oven (Shimadzu GmbH, Germany). Liquid chromatography was performed using a Hyperclone C8 column (3 µm, 130

Å, 50×2.0 mm) with security guard (Phenomenex, Germany) by gradient elution at a flow of 0.3 mL/min. The injection volume was 10 µL. The mobile phase consisted of eluent A (5 mM ammonium formate in acetonitrile/water (10:90)) and eluent B (5 mM ammonium formate in acetonitrile/water (90:10)). The gradient elution was done with 100% A for 1.5 min, followed by linear gradient to 40% A over 3.5 min, held over 5.0 min, and within 1.0 min to 100% B, held for 9.0 min and at least within 1.0 min back to 100% A, held for 24 min.

MS/MS analyses were performed using a Triple Quadrupole API 365 LC-MS/MS (Applied Biosystems GmbH, Germany) with electrospray ionization by ESI Turbo Ion spray Interface (SCIEX, Canada). The following parameters were used: nebulizer gas, 13 L/min; curtain gas, 12 L/min; ion spray voltage, –4,500 V; desolvation temperature, 450°C; collisionally activated dissociation gas, 8 L/min; declustering potential, –12 V; focusing potential, –180 V; entrance potential, –8 V; collision energy, –45 V; and collision cell exit potential, –51 V.

After several scans in the Q1 (stepwise from 500–1,800 amu) and measurements in the single ion monitoring mode, neutral loss mode (loss of 80 amu [SO_3]), and product ion mode (parent ion: m/z 1,141.5, scan for fragments m/z 500–1,200), qualitative analyses were carried out in multireaction monitoring mode with negative ionization. Selected transitions were (precursor ion $[M\text{-}H]^-$ > fragment ion $[M\text{-}H\text{-}SO_3]^-$): YTX m/z 1,141.5 > 1,061.5, homo YTX m/z 1,155.5 > 1,075.5, 45-OH YXT m/z 1,157.5 > 1,077.5, COOH YTX m/z 1,173.5 > 1,093.5, the putative 45-OH COOH YTX m/z 1,189.8 > 1,109.5, m/z 1,047.5 > 967.5 (probably keto YTX), and m/z 1,191.5 > 1,111.5.

The YTX content was estimated using a 3-point calibration curve. It was determined with certified YTX standard solution (National Research Council Canada). Because of the lack of standard solutions for the YTX analogues it was not possible to calculate the absolute amount of YTXs in the shellfish samples. Hence, the LC-MS/MS results for YTXs were calculated with reference to the YTX standard solution. The limit of detection using a signal-to-noise ratio of 3:1 was 0.02 ng YTX on column. As a result of eventual variations in sensitivity for the different YTXs by LC-MS/MS, it was not possible to calculate the amounts of YTXs with the molecular weight (MW) of the metabolites. Therefore, the concentrations were expressed as YTX equivalents.

RESULTS

Toxins in P. reticulatum *Cultures*

YTX (MW, 1,142 Da) was detected in *P. reticulatum* by application of LC-MS/MS, whereby the cells contained 5.58 ± 1.66 pg YTX/cell (n = 6). Traces (<0.3 pg YTX eq/cell) of COOH YTX and the putative keto YTX were also found. The total amount of YTX, 605.97 µg YTX/tank, fed over the entire

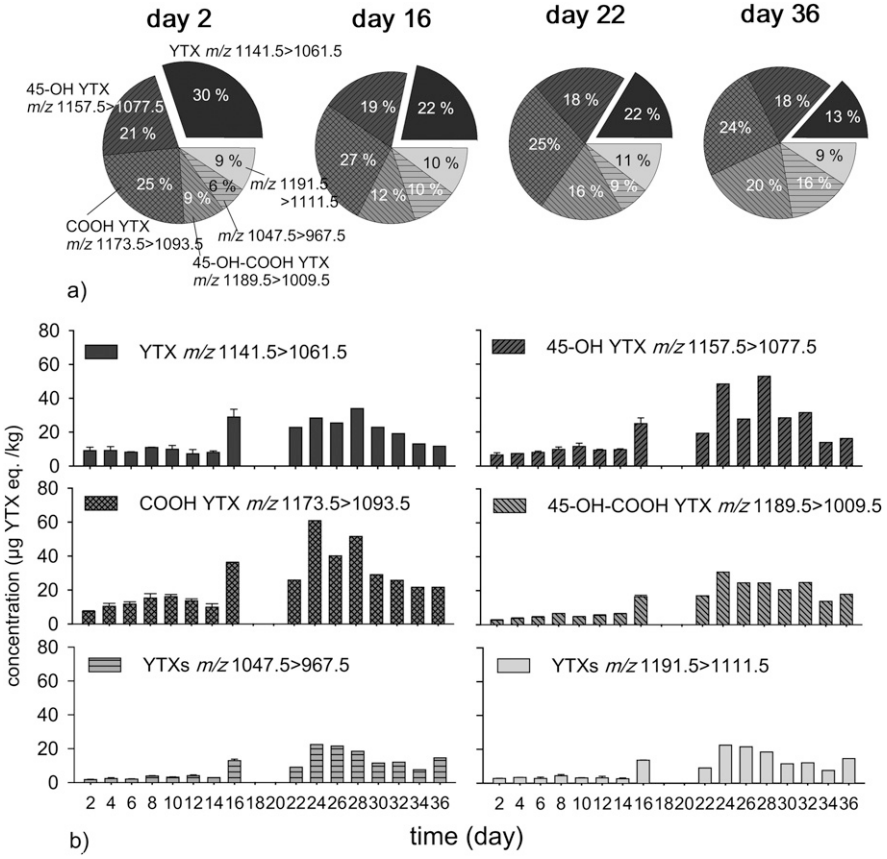

Figure 1. (A, B) Concentration of YTXs in *M. edulis* as percentage (A) and as a total concentration in micrograms YTX equivalents per kilogram wet weight (B). Analogues in YTX equivalents (YTX Rt, 21.3 min; 45-OH-YTX Rt, 19.4 min; COOH YTX Rt, 18.5 min; 45-OH-COOH YTX Rt, 18.7; m/z 1,147.5 > 967.5 with an Rt of 19.5 min and m/z 1,147.5 > 967.5 with an Rt of 18.2 min).

TABLE 1.

Concentration of YTXs in *M. edulis* in microgram YTX equivalents per kilogram wet weight.

Transition	*m/z* 1,141.5 > 1,061.5	*m/z* 1,157.5 > 1,077.5	*m/z* 1,173.5 > 1,093.5	*m/z* 1,189.5 > 1,089.5	*m/z* 1,047.5 > 967.5	*m/z* 1,191.5 > 1,111.5
Retention time (min)	21.3	19.4	18.5	18.7	19.5	18.2
Time (day)			concentration (μg/kg BW)			
2	9.12	6.50	7.42	2.66	1.77	2.81
4	9.18	7.30	10.44	3.69	2.35	3.35
6	8.14	8.01	11.69	4.44	2.03	2.83
8	10.95	9.73	15.24	6.54	3.89	4.42
10	9.89	11.50	16.18	4.74	3.20	3.09
12	7.20	9.38	13.61	5.55	4.07	3.15
14	8.03	9.64	9.91	6.43	2.88	2.63
16	28.92	24.98	36.29	16.50	12.93	13.63
22	22.84	19.24	25.88	17.01	9.06	11.45
24	28.35	48.27	60.84	30.98	22.51	14.17
26	25.40	27.63	40.17	24.64	21.50	14.63
28	33.93	57.86	51.55	24.57	18.42	11.20
30	22.86	28.38	29.07	20.60	11.51	7.95
32	19.14	31.42	25.74	24.86	12.04	13.09
34	13.06	13.85	21.68	13.75	7.57	7.78
36	11.67	16.18	21.64	17.89	14.58	7.98

toxification period, was calculated on the basis of cell densities and YTX load per cell.

Toxins in Bivalves: Accumulation, Metabolism, and Depuration

M. edulis contained YTX, 45-OH YTX, COOH YTX, and the putative 45-OH COOH YTX. The retention times (Rt) of the detected YTXs were YTX *m/z* 1,141.5 > 1,061.5 with an Rt of 21.3 min, 45-OH YTX *m/z* 1,157.5 > 1,077.5 with an Rt of 19.4 min, COOH YTX *m/z* 1,173.5 > 1,093.5 with an Rt of 18.2 min, and 45-OH-COOH YTX *m/z* 1,189.8 > 1,109.5 with an Rt of 18.7 min, *m/z* 1,047.5 > 967.5 with an Rt of 19.5 min, and *m/z* 1,191.5 > 1,111.5 with an Rt of 18.2 min. No strong increase of YTXs in the tissue was observed directly after start of the inoculation. The amount of all detected YTXs increased significantly after 16 days (Fig. 1, Table 1). We stopped feeding at day 16. After a break of 4 days, the concentration of the YTXs did not decrease. Subsequently, the levels of YTX, 45-OH

TABLE 2.

Concentration of YTXs in *C. gigas* in microgram YTX equivalents per kilogram wet weight.

Transition	*m/z* 1,141.5 > 1,061.5	*m/z* 1,157.5 > 1,077.5	*m/z* 1,173.5 > 1,093.5	*m/z* 1,189.5 > 1,089.5	*m/z* 1,047.5 > 967.5	*m/z* 1,191.5 > 1,111.5
Retention time (min)	21.3	19.4	18.5	18.7	19.5	18.2
Time (day)			concentration (μg/kg BW)			
2	23.00	32.96	20.77	n.d.	5.55	4.06
4	26.39	60.32	25.87	n.d.	10.04	4.54
6	23.66	20.23	16.93	n.d.	9.72	5.53
8	48.13	28.48	31.11	1.86	15.78	5.21
10	140.11	130.16	67.32	8.28	47.75	13.94
12	58.69	58.31	45.17	3.38	28.85	8.48
14	16.07	13.66	14.95	2.35	46.51	4.88
16	120.25	224.55	49.50	11.92	72.84	25.49
22	18.11	10.34	15.66	2.19	19.88	4.99
24	18.40	6.67	27.37	2.29	46.95	7.89
26	19.48	11.81	23.19	2.58	65.67	9.16
28	40.79	30.22	19.23	4.21	79.18	10.20
30	15.63	9.61	n.d	1.70	33.77	3.64
32	25.57	8.37	4.47	n.d	102.18	4.35
34	21.92	11.12	11.16	3.10	142.84	7.22
36	2.89	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

YTX, and COOH YTX declined during the detoxification period, whereas the concentration of 45-OH-COOH YTX remained in the same range.

C. gigas showed a different profile of YTXs compared with *M. edulis*. YTX *m/z* 1,141.5 > 1,61.5, 45-OH YTX *m/z* 1,157.5 > 1,077.5, and 45-OH-COOH YTX *m/z* 1,189.8 > 1,109.5 were also present, and 45-OH-COOH YTX was detectable in low amounts (Table 2, Fig. 2); however, COOH YTX *m/z* 1,173.5 > 1,093.5 was not found at an Rt of 18.2 min, but 2 signals with the same multireaction monitoring characteristics (same transition) were observed at an Rt of 18.5 min and 18.9 min, respectively. After the break of 4 days, the dominant YTX analogues (YTX and 45-OH YTX) were almost degraded/eliminated. Approximately 15% YTX and less than 5% 45-OH YTX remained. Hence, the detoxification of these YTXs occurs very fast in *C. gigas*.

In addition, we found 2 YTX analogues with the typical loss of the sulfo group (80 amu) in both bivalves (YTX analogues *m/z* 1,047.5 > 967.5 with an Rt of 19.5 min and *m/z* 1,191.5 > 1,111.5 with an Rt of 18.2 min). However, these YTXs were present in *M. edulis* as minor components, and less amounts of the latter (YTXs *m/z* 1,191.5 > 1,111.5) were accumulated in *C. gigas*. Interestingly, the concentration of the putative YTXs with *m/z* 1,047.5 > 967.5 increased over both time periods.

DISCUSSION

In the current study, we confirmed the results of former field investigations about the metabolism of YTX in blue mussel (*M. edulis*; Fig. 3). New data are presented concerning accumulation and detoxification in the Pacific oyster (*C. gigas*). Oysters showed a different response to toxification by YTX, and a possible metabolic pathway—especially in *C. gigas*—is under discussion.

Aasen et al. (2005) reported 45-OHYTX (MW, 1,058 Da), COOH YTX (MW, 1,174 Da), and the putative 45-OH-COOH YTX (MW, 1,190 Da) as the dominant YTX analogue in *M. edulis*. The metabolism of YTX to 45-OH YTX takes place relatively rapid in the shellfish tissue; a half-life time of YTXs of 20–24 days was observed. Furthermore, COOH YTX was more abundant than YTX and 45-OH YTX. Consequently, it was concluded that detoxification of COOH YTX occurs more slowly than YTX and 45-OH YTX (Aasen et al. 2005, Samdal et al. 2005). All YTXs mentioned previously were detectable in *M. edulis* in low concentrations until day 14. These results are in accordance with former studies (MacKenzie et al. 1998, MacKenzie et al. 2001, Koike et al. 2006). In our study, YTX was the dominant analogue only over a short time period; however, after 4 days, the concentration of COOH YTX was higher than the concentration of YTX in *M. edulis*. In addition, 45-OH-COOH YTX was long-lasting in the tissue of *M. edulis*, and the concentration of 45-OH-COOH YTX measured at the last day of sampling was not lower than the concentration present at the beginning of the detoxification period. This result confirms the assumption that 45-OH-COOH YTX originates from 45-OH YTX (Aasen et al. 2005) and/or COOH YTX (Table 1, Figs. 1A and 3A). The concentration of YTXs in *M. edulis* and *C. gigas* was low in comparison with the concentration

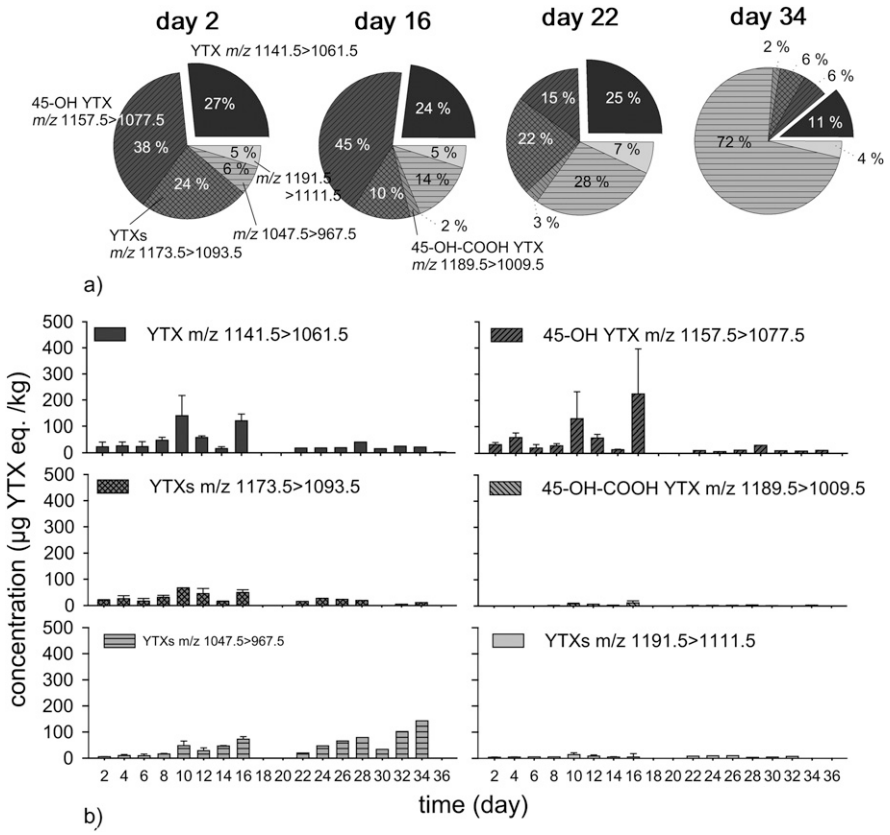

Figure 2. (A, B) Concentration of YTXs in *C. gigas* as a percentage (A) and as a total concentration in micrograms YTX equivalents per kilogram wet weight (B). Analogues in YTX equivalents (*m/z* 1,173.5 > 1,093.5). Only 1 of the 2 YTX analogues with an Rt of 18.7 min is shown. For all other retention times, see Figure 1.

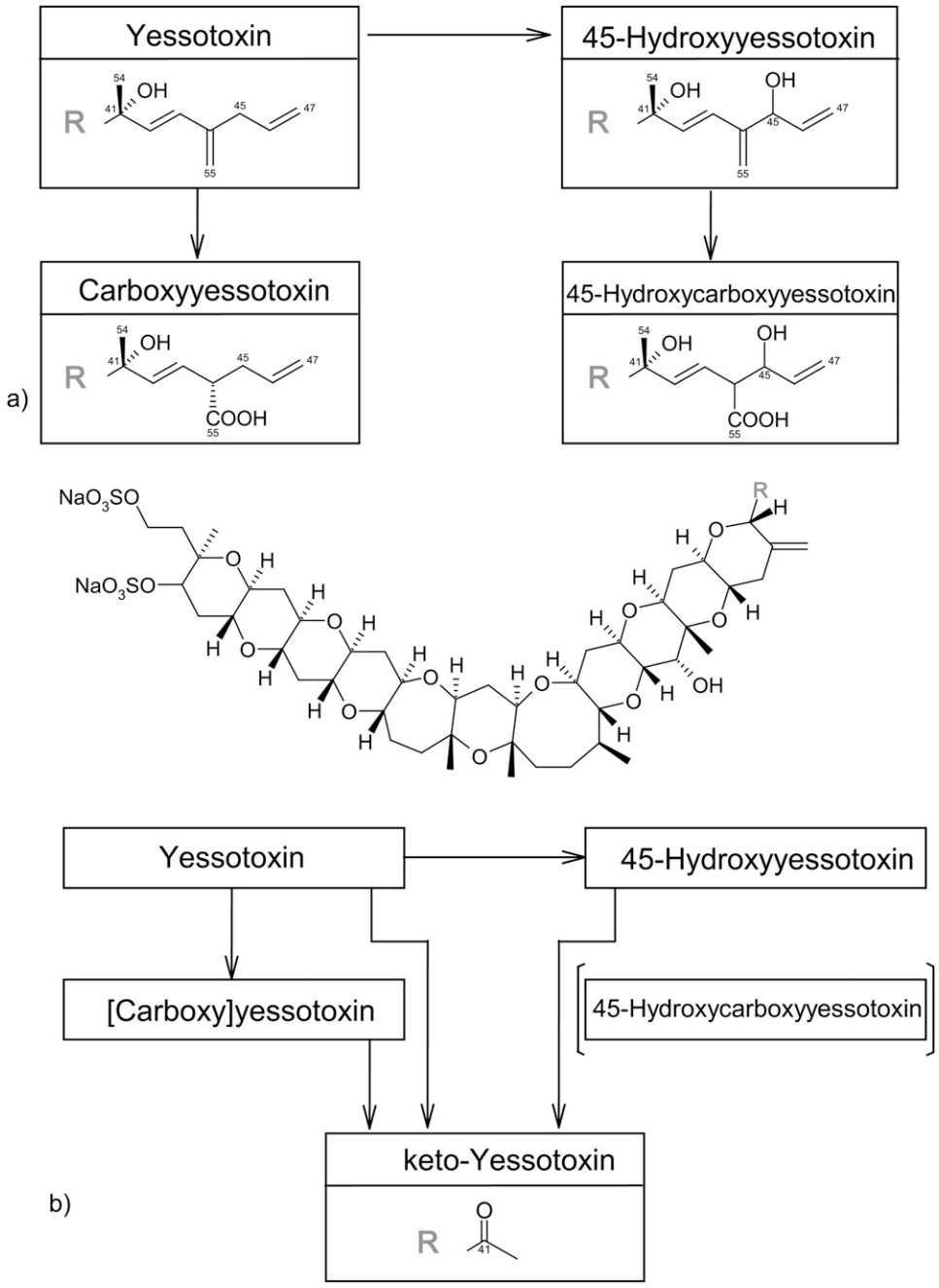

Figure 3. (A) Metabolism of YTX in *M. edulis* (Aasen et al. 2002), modified. (B) Putative metabolism of YTX in *C. gigas*.

of YTXs reported in the literature. Potentially, the lower concentrations measured during our experiments were caused by the relative low concentrations of YTXs in the culture of *P. reticulatum* (5.58 ± 1.66 pg/cell) and were associated with a smaller amount of cells per liter in comparison with natural blooms. Several authors stated that the concentration of YTX in *P. reticulatum* ranged from 0.9–79 pg/cell, whereas the toxin profile of the dinoflagellate can differ (MacKenzie et al. 2002, Paz et al. 2004, Samdal et al. 2006, Paz et al. 2007).

As reported by Aasen et al. (2005), a reason for the variation of the data concerning concentrations of YTXs obtained by LC-MS/MS and ELISA analyses could be that additional analogues are present in the mussel samples. In this context, 2 YTX analogues (*m/z* 1,047 and *m/z* 1,191) could be detected, but the exact chemical structure is not elucidated so far. We assume that *m/z* 1,047 could be a keto YTX (heptanor-41-oxo YTX; 40-epi-keto YTX or keto YTX-enone) with an MW of 1,048 Da.

Both bivalve species fed on the same dinoflagellate culture, yet differences in the toxin profiles were observed. Apparently, accumulation and detoxification of YTXs in *C. gigas* is faster than in *M. edulis*. Furthermore, *C. gigas* did not produce/accumulate COOH YTX, and 45-OH-COOH YTX and a substance with *m/z* 1191 were present only in small amounts. The identification of the peaks with COOH YTX-like transitions is

foreseen for later analyses; however, there is evidence that the structure is probably related to stereo-isomers of COOH YTX. Surprisingly, the concentration of the YTX analogue with *m*/*z* 1,047 increased during the detoxification period. Potentially, this substance is formed at the end of the detoxification process (Table 2, Fig. 3B).

The metabolism in the greenshell mussel (*Perna canaliculus*) seems to be similar to the metabolism in *M. edulis*, albeit the process of metabolism is somewhat slower (half-life of 49 days). LC-MS/MS analyses by MacKenzie et al. (2002) demonstrated the presence of YTX and 45-OH-YTX in the greenshell mussel. Interestingly, the authors found YTX also analogues with 1,173 and 1,189. These more polar metabolites of YTX gave the characteristic loss of daughter ions (80 amu) by fragmentation in the mass spectrometer. MacKenzie et al. (2002) supposed the YTX substances are COOH YTX and OH-COOH YTX. Given at the results by Aasen et al. (2005) and our data, this assumption is supported.

Independent of similarities in profiles of YTXs in the mussels *M. edulis* and *P. canaliculus*, more YTXs have been detected in the Mediterranean mussel (*Mytilus galloprovincialis*), which is more closely related to *M. edulis* (Ciminiello et al. 2000). These authors reported on the accumulation of ATX and homo YTX besides YTX, OH YTX, and COOH YTX in *M. galloprovincialis*, whereas Morton et al. (2007) detected YTX, OH YTX, and homo YTX in *M. galloprovincialis*. Finch et al. (2005) reported the presence of YTX, OH YTX, COOH, and di-OH YTX in all mussel species under investigation with YTX as the main component in *M. edulis* and *P. canaliculus* (in every case more than 60%), and with COOH YTX (40%) and YTX (32%) as main components in *M. galloprovincialis*.

To date, knowledge concerning the relationship between YTX-producing dinoflagellates and shellfish with respect to the resulting toxin profile is incomplete. In this study, the YTX producer *P. reticulatum* forms mainly YTX, together with very low amounts of YTX analogues. Therefore, we assume that YTX is preferentially metabolized in the shellfish tissue after uptake of *P. reticulatum*. On the other hand, it cannot be discounted that the putative keto YTX, which was present in the culture in low amounts, was accumulated by *C. gigas*, and consequently the concentration of this YTX analogue increased in the tissue of *C. gigas* also in absence of *P. reticulatum*.

Currently, the mouse bioassay (MBA) is the standard method for testing shellfish with regard to lipophilic marine biotoxins in Europe (European Commission 2005); however, other methods (e.g., chromatographic methods and bioassays) could be applied alternatively or as a supplement. Yet, differences between results obtained by application of ELISA, MBA, or HPLC-MS methods were observed (Miles et al. 2005a, Samdal et al. 2005). One reason is that, in contrast to chromatographic methods, the total toxin concentration is analyzed by application of ELISA and MBA. Currently, regulatory limits exist for YTX, OH YTX, homo YTX, and OH-homo YTX (European Food Safety Authority 2008). However, the potential presence of about 100 YTX analogues in different shellfish species is problematic with regard to seafood safety, because their toxicity seems to be different. Because YTXs and more toxic lipophilic marine biotoxins, DSP toxins (e.g., okadaic acid and dinophysistoxins) cause similar reactions when injected intraperitoneally in mice, other extraction methods were established for their differentiation, but it is also evident that some desulfo YTXs were coextracted with okadaic acid and dinophysistoxins (Ciminiello et al. 2007).

In summary, the presence of YTX, OH YTX, COOH YTX, and OH-COOH YTX in *M. edulis* fed with *P. reticulatum* from the North Sea confirmed the results of studies by Aasen et al. (2005). The exact structure of the detected YTX analogues with *m*/*z* 1,047.5 > 967.5 and *m*/*z* 1,191.5 > 1,111.1 should be elucidated in the future. As a result of this first study regarding accumulation and metabolism of YTXs in *C. gigas*, it was obvious that differences exist between mussels (*M. edulis*) and oysters (*C. gigas*) concerning the toxin profiles and the rates of detoxification. In comparison with *M. edulis*, YTX accumulates faster and is more quickly eliminated in *C. gigas*. Consequently, our investigations indicate that accumulation and metabolism of YTXs in different species depend on the toxin profile of the YTX-producing dinoflagellates as well as on the filter feeders.

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