

LC-MS-MS aboard ship: tandem mass spectrometry in the search for phycotoxins and novel toxigenic plankton from the North Sea

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Abstract Phycotoxins produced by various species of toxigenic microalgae occurring in the plankton are a global threat to the security of seafood resources and the health of humans and coastal marine ecosystems. This has necessitated the development and application of advanced methods in liquid chromatography coupled to mass spectrometry (LC-MS) for monitoring of these compounds, particularly in plankton and shellfish. Most such chemical analyses are conducted in land-based laboratories on stored samples, and thus much information on the near real-time biogeographical distribution and dynamics of phycotoxins in the plankton is unavailable. To resolve this problem, we conducted ship-board analysis of a broad spectrum of phycotoxins collected directly from the water column on an oceanographic cruise along the North Sea coast of Scotland, Norway, and Denmark. We equipped the ship with a triple-quadrupole linear ion-trap hybrid LC-MS-MS system for detection and quantitative analysis of toxins, such as domoic acid, gymnodimine, spirolides, dinophysistoxins, okadaic acid, pectenotoxins, yessotoxins, and azaspiracids (AZAs). We focused particular attention on the detection of AZAs, a group of potent nitrogenous polyether toxins, because the culprit species associated with the occurrence of these toxins in shellfish has been controversial. Marine toxins were analyzed directly from size-fractionated plankton net tows (20 µm mesh size) and Niskin bottle samples from discrete depths, after rapid methanolic extraction but without any further clean-up. Almost all expected phycotoxins were

detected in North Sea plankton samples, with domoic acid and 20-methylspirolide G being most abundant. Although AZA was the least abundant of these toxins, the high sensitivity of the LC-MS-MS enabled detailed quantification, indicating that the highest amounts of AZA-1 were present in the southern Skagerrak in the 3–20 µm size-fraction. The direct on-board toxin measurements enabled isolation of plankton from stations with high AZA-1 levels and from the most suspicious size-fraction, i.e. most likely to contain the AZA-producer. A large number (>100) of crude cultures were established by serial dilution and later screened for the presence of AZAs after several weeks growth. From one crude culture containing AZA, a small dinoflagellate was subsequently isolated and brought into pure culture. We have thus proved that even sophisticated mass spectrometers can be operated in ship laboratories without any limitation caused by vibrations of the ship's engine or by wave movement during heavy seas at wind forces up to nine Beaufort. On-board LC-MS-MS is a valuable method for near real-time analysis of phycotoxins in plankton for studies on bloom dynamics and the fate of toxins in the food web, and for characterization and isolation of putatively toxigenic organisms.

Keywords North Sea · Plankton · Azaspiracids · AZA · Phycotoxins · Shellfish toxin · LC-MS-MS

Introduction

Tandem mass spectrometers, in particular hybrid instruments such as triple-quadrupole linear ion-trap systems, are regarded as highly sensitive and delicate equipment, requiring controlled laboratory conditions for reliable operation. Modern systems incorporating liquid chromatographs coupled with

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tandem mass spectrometers (LC-MS-MS) have relatively large orifices that require high pumping capacities. This pumping capacity is accomplished by differential pumping systems, including rotary vane and turbo-molecular pumps, the latter operating at several thousand rpm and thus having enormous torsional moment. Such systems are clearly not engineered for ship-board operation, where instruments may be subject to damage in transport and installation, vibration and erratic rocking motion when underway, unstable power supply, and corrosive effects of salt water and high humidity. Given these limitations, the traditional approach to LC-MS analysis of seawater and plankton matrices is to collect the samples at sea and to archive them by freezing, lyophilization, or solvent extraction for later laboratory analysis with advanced instrumentation. This procedure has a major drawback in that valuable in situ information on chemical composition is not available for optimization of the sampling programme or to conduct more detailed guided investigations—the strategy is essentially “blind”. This temporal separation between sampling and chemical analysis has been a critical limitation of the wider application of LC-MS methodologies for field screening of phycotoxins in seawater and plankton matrices, because toxic plankton bloom are typically of relatively short duration (days to weeks) and are also spatially patchy.

Nevertheless, if technically feasible, operation of an on-board, highly sensitive LC-MS-MS system would be a very useful tool in field marine science and oceanography, particularly in studies of toxic bloom dynamics and toxin distribution in food webs where transient effects are critical to our understanding of the underlying processes. We are aware of one previous instance where an LC-MS system was deployed on a scientific cruise for shipboard analysis of phycotoxins [1], but this approach was a compromise between obtaining spectral information in near real-time while sacrificing sensitivity. The instrument was a small single-quadrupole mass spectrometer, operated in the selected-ion-monitoring (SIM) mode. This scan type is less sensitive than MS-MS modes, due to increased background noise and relatively low selectivity. Yet high sensitivity, especially for field measurements, is a key issue because secondary metabolites such as phycotoxins in seawater and plankton matrices are present only at very low concentrations (sub-nanomolar) and there are only limited means of sample concentration.

Here we report on the deployment of a triple quadrupole-linear ion trap hybrid LC-MS-MS system on a research vessel for a four week cruise on the North Sea. One major aim of this expedition was to investigate the potential use of advanced on-board LC-MS-MS systems for near real-time analysis of a wide variety of marine phycotoxins in plankton and seawater. We focused particularly on known phycotoxins, including domoic acid and lipophilic toxins,

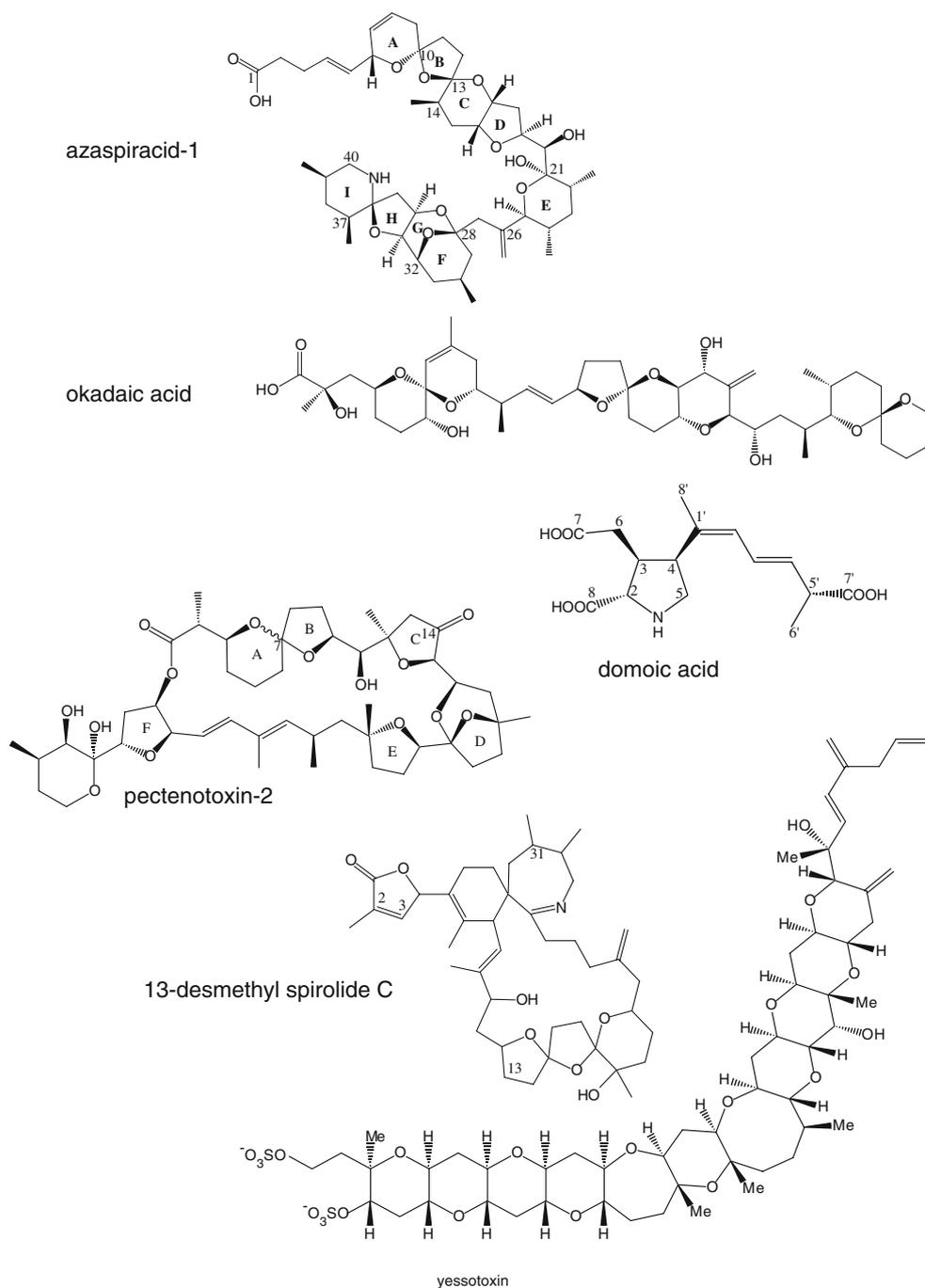
such as spirolides, dinophysistoxins, pectenotoxins, and yessotoxins associated with various shellfish toxin syndromes. We also intended to opportunistically exploit this on-board analytical capability to determine the hitherto unknown or unconfirmed identity of toxin producing plankton, such as the source of azaspiracids (AZAs) (Fig. 1), a group of potent nitrogen-containing macrocyclic polyether toxins first found in shellfish.

The first azaspiracid poisoning (AZP) was that of eight people in the Netherlands who became ill in November 1995 after consumption of mussels from the Irish west coast [2]. Symptoms of the affected persons—nausea, vomiting, severe diarrhoea, and stomach cramps—were typical of diarrhetic shellfish poisoning (DSP), and, indeed, at first the toxicity was erroneously associated with this syndrome. However, the only DSP toxins present in the mussels were okadaic acid and dinophysistoxin-2 and at very low concentrations, which could not explain the severity of the human poisonings.

Several years after the initial poisoning incident, the lipophilic macrocyclic polyether azaspiracid-1 (AZA-1) was finally isolated from shellfish and structurally elucidated as the causative compound [3]. Other structural variants of AZA-1 have been isolated from shellfish and characterized, and 24 AZAs are now known [4]. The toxins associated with AZP are not restricted to Ireland; AZA-contaminated mussels have also been found on the English east coast and on the Norwegian west coast [5]. Other cases of human poisoning from the consumption of mussels in Ireland, France, and Italy have now been unambiguously attributed to AZP [6]. Nevertheless, the ultimate source of AZP remained elusive. The heterotrophic dinoflagellate *Prorocentrum crassipes* has been proposed as the causative organism [7], but this was somewhat unexpected because thus far only autotrophic plankton have proven to be producers of polyether phycotoxins [8]. Recently the Irish Marine Institute reported that there was no correlation between occurrence of AZAs in shellfish and blooms of *P. crassipes* during a four year monitoring period along the Irish coast (2002 to 2006) [9].

Plankton material from the water column along the North Sea coast was harvested by size-fractionation of plankton net tows and from Niskin bottle samples. Plankton fractions were extracted for direct on-board analysis of a broad spectrum of phycotoxins, including AZA, to determine the biogeographical distribution and to identify the AZA-producer(s). Our application demonstrates for the first time the feasibility, robustness, and efficacy of on-board direct measurements of phycotoxins by use of highly sensitive triple-quadrupole linear ion-trap LC-MS-MS. As illustrative of the more general application of on-board LC-MS-MS to such toxin studies, we also discovered a link between AZAs and an associated toxic planktonic organism.

Fig. 1 Chemical structures of the different classes of phycotoxins



Materials and methods

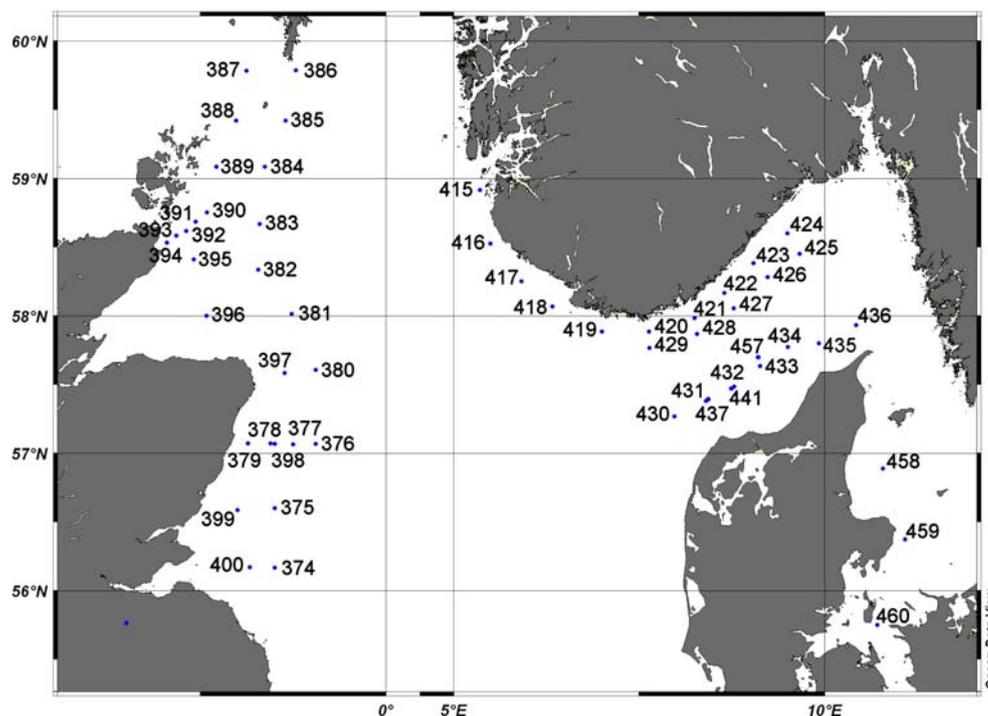
Plankton collection and size-fractionation

Plankton samples were collected during the FS Poseidon 352 (ship dimensions: length 60.8 m, width 11.4 m, 1049 gross registered tons) cruise in the North Sea from 10th June to 4th July 2007 at stations along the Scottish east coast and on the south coast of Norway and the north coast of Denmark in the Skagerrak (Fig. 2). Vertical net tows were conducted at each station through the upper 20 m of

the water column with a 20 μm -mesh plankton net. Net tow concentrates were adjusted with filtered seawater to a volume of 850 mL. A 50 mL sub-sample was removed for microscopic analysis of plankton and the remainder was sequentially filtered through Nitex mesh of 200, 50, and 20 μm .

Discrete seawater samples were collected in Niskin entrainment bottles mounted on an automatically triggered rosette sampler at 3 m and 10 m depth. Seawater was passed through Nitex gauze of 20- μm mesh-size and 1 L of each filtrate was then filtered through polycarbonate filters

Fig. 2 Geographical locations of the sampling stations during the Poseidon 352 expedition in the North Sea



(25 mm diameter, Millipore) with 3 μm pore-size. Filters were stored in 50-mL centrifuge tubes (Sarstedt, Nümbrecht, Germany) until analysis.

Toxin extraction

The cell pellets from the plankton net tows were harvested by centrifugation ($3,220\times g$, 15 min at 4 °C), suspended in 500 μL methanol, and subsequently transferred to a FastPrep tube containing 0.9 g lysing matrix D (Thermo Savant, Illkirch, France). The samples were homogenized by reciprocal shaking at maximum speed (6.5 m s^{-1}) for 45 s in a Bio101 FastPrep instrument (Thermo Savant). After homogenization, samples were centrifuged (Eppendorf 5415 R, Hamburg, Germany) at $16,100\times g$ at 4 °C for 15 min. The supernatant (400 μL) was transferred to a spin-filter (pore-size 0.45 μm ; Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 s at $800\times g$. The filtrate was transferred to an autosampler vial and analyzed by LC-MS-MS. Polycarbonate filters were repeatedly rinsed with 1 mL methanol until complete decolouration of the filters. The methanolic extract was filtered through spin-filters as described above.

Analytical reagents and standards

Water was deionized and purified (Milli-Q; Millipore) to $18\text{ M}\Omega\text{ cm}^{-1}$ quality or better. Formic acid (90%, p.a.), acetic acid (p.a.), and ammonium formate (p.a.) were purchased from Merck (Darmstadt, Germany). The solvents

methanol and acetonitrile were high-performance liquid chromatography (HPLC) grade (Merck).

A reference solution of AZA-1 and a mussel extract containing AZA-1, AZA-2, and AZA-3 were kindly donated by Philipp Hess, Marine Institute, Galway, Ireland.

On-board installation of the LC-MS-MS system

The complete LC-MS-MS system was placed on a bench in the rear dry laboratory of the Poseidon and lashed with retaining belts to fix the instrument. Ship electricity was accessed directly without further modification for voltage spikes or surge protection. Compressed air was supplied by the ship engine and purified with a charcoal filter (Option Carbon Absorber P/N 159.003678; Parker Filtration and Separation, Etten-Leur, The Netherlands) for a nitrogen generator. A N2-22 nitrogen generator (Parker Balston Analytical Gas Systems, Maidstone, UK) delivered nitrogen at 7 bar (99.5% purity at a flow rate of 19 L min^{-1}).

Lipophilic toxin measurement of field samples by LC-MS-MS

Mass spectral experiments were performed on an ABI-Sciex 4000 Q Trap triple-quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo V ion source coupled to an Agilent (Waldbronn, Germany) 1100 LC liquid chromatograph which included a solvent reservoir, in-line degasser (G1379A), binary pump (G1311A), refrigerated autosampler (G1329A/G1330B), and temperature-controlled column oven (G1316A).

Table 1 Mass spectrometric parameters used for the multi phycotoxin method

	Period 1 (0–8.75 min)	Period 2 (8.75–11.2 min)	Period 3 (11.2–18.0 min)
Curtain gas	20 psi	10 psi	10 psi
CAD	Medium	Medium	Medium
Ion-spray voltage	5500 V	5500 V	5500 V
Temperature	275 °C	Ambient	Ambient
Nebulizer gas	50 psi	10 psi	10 psi
Auxiliary gas	50 psi	Off	Off
Interface heater	On	On	On
Declustering potential	50 V	50 V	50 V
Entrance potential	10 V	10 V	10 V
Exit potential	15 V	15 V	15 V
MRM transitions (collision energy, V)	312 → 161 (30) 312 → 266 (20)	508 → 490 (40) 692 → 164 (55) 692 → 150 (55) 694 → 164 (55) 694 → 150 (55) 706 → 164 (55) 708 → 164 (55)	822 → 223 (55) 836 → 237 (55) 946 → 223 (55) 874 → 213 (55) 892 → 213 (55) 894 → 213 (55) 842 → 824 (55) 1160 → 965 (55)

After injection of 5 µL of sample, separation of lipophilic toxins was performed by reversed-phase chromatography on a C₈ phase. The analytical column (50×2 mm) was packed with 3-µm Hypersil BDS 120 Å (Phenomenex, Aschaffenburg, Germany) and maintained at 20 °C. The flow rate was 0.2 mL min⁻¹ and gradient elution was performed with two eluents, where eluent A was water and eluent B was acetonitrile-water (95:5 v/v), both containing 2.0 mmol L⁻¹ ammonium formate and 50 mmol L⁻¹ formic acid. Initial conditions were 12 min column equilibration with 5% B, followed by a linear gradient to 100% B in 10 min and isocratic elution until 15 min with 100% B. The system was then returned to initial conditions until 18 min (total run time: 30 min).

The chromatographic run was divided into three periods:

- 1) 0–8.75 min for domoic acid,
- 2) 8.75–11.20 min for gymnodimine and spirolides, and
- 3) 11.20–18 min for okadaic acid, dinophysistoxins, pectenotoxins, yessotoxin, and azaspiracid.

Measurements were carried out in positive-ion mode by multiple reaction monitoring (MRM) experiments. Mass spectrometric parameters are summarized in Table 1. Dwell times of 100–200 ms were used for each transition.

Results and discussion

Despite the calculated risk to the equipment and without precedent for ship-board operation, we report the successful and trouble-free functioning of the triple quadrupole linear ion-trap hybrid mass spectrometer under sea-going con-

Table 2 Mass transitions and retention times of toxins monitored on board

Toxin ^a	Mass transition (m/z)	Retention time (min)
Domoic acid	312 → 266; 312 → 161	7.58
Gymnodimine	508 → 490	9.83
Spirolide A	692 → 150	
Spirolide B	694 → 150	
13-Desmethylspirolide C	692 → 164	10.42
Spirolide G	692 → 164	
13-Desmethylspirolide D	694 → 164	
Spirolide C	706 → 164	
20-Methylspirolide G	706 → 164	10.72
Okadaic acid	822 → 223	11.85
DTX-1	836 → 237	12.83
DTX-2	822 → 223	12.15
PTX-1	892 → 213	11.33
PTX-2	876 → 213	12.40
PTX-2sa	894 → 213	11.93
PTX-4	892 → 213	
PTX-8	892 → 213	
PTX-11	892 → 213	12.12
PTX-12	874 → 213	12.59
PTX-13	892 → 213	11.96
PTX-14	874 → 213	
AZA-1	842 → 824	13.88
YTX	1160 → 965	12.45

^a DTX= dinophysistoxin, OA-d8 = okadaic acid C₈ diol ester, PTX = pectenotoxin, sa = seco acid, AZA = azaspiracid, YTX = yessotoxin

Table 3 Limits of detection (LOD, $S/N=3$), and minimum and maximum amounts of phycotoxins found in North Sea plankton samples

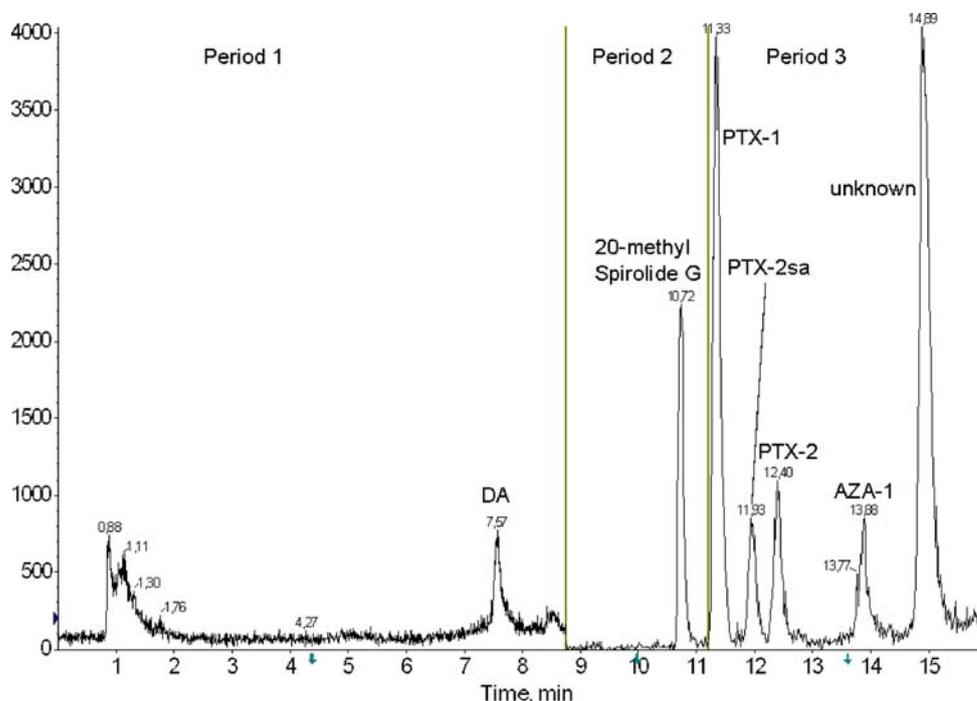
Toxin ^a	LOD (ng/NT)	Min (ng/NT)	Max (ng/NT)
Domoic acid	0.3	0.5	12,800
13-Desmethylspirolide C	0.015	n.d.	3,670
20-Methylspirolide G	0.07	n.d.	83
Okadaic acid	0.085	0.085	41.7
DTX-1	0.15	n.d.	24.4
DTX-2	0.15	n.d.	60.9
PTX-2	0.25	n.d.	207
PTX-2 seco acid	0.07	n.d.	69.8
PTX-11	0.13	n.d.	11
YTX	0.05	n.d.	56
AZA-1	0.015	n.d.	6.7

^aDTX = dinophysistoxin; PTX = pectenotoxin; YTX = yessotoxin; AZA = azaspiracid

ditions. The system proved to be both robust and absolutely stable throughout the cruise. Several power-supply failures, vibration caused by the ship engines, and heavy seas (3–6 m waves) with wind force of up to Beaufort 9 had no effect on the performance of the instrument, which was continuously operated throughout the entire expedition. Even after four weeks of ship-board operation, followed by venting and transport to the institute, no tuning or mass calibration were necessary.

During the Poseidon 352 expedition, 423 samples at 48 geographical stations were analyzed for phycotoxins. An overview of the toxins surveyed by the LC-MS-MS method, including their mass transitions and retention times, is presented in Table 2. The range of phycotoxins detected in plankton net tows is given in Table 3. Domoic acid, a rare neurotoxic amino acid produced by the pennate diatom *Pseudo-nitzschia* spp. [10] and associated with amnesic shellfish poisoning (ASP) in humans, was found at all stations over a wide concentration range from 0.5 to 12,800 ng per plankton net tow (approximately 2500 L of seawater filtered). The macrocyclic imine gymnodimine and related analogues, which occur primarily in temperate waters around New Zealand [11] but also the Mediterranean [12], in association with the dinoflagellate *Karenia brevesulcata*, were not detected. The related group of macrocyclic imine toxins known as spirolides and originating from the dinoflagellate *Alexandrium ostenfeldii* was detected at some stations. Cultured isolates of *A. ostenfeldii* may produce a diversity of spirolides [13, 14], but only 20-methylspirolide G and to a lesser extent 13-desmethylspirolide C were found in the North Sea field plankton samples. Somewhat surprisingly, spirolides A, B, C, D, and G were completely absent. Several species of the genus are believed to produce the dinophysistoxins (DTXs) [15], and okadaic acid and pectenotoxins (PTXs) [16, 17], believed to originate from the planktonic dinoflagellate genus *Dinophysis*. These toxins are often collectively (and erroneously in the case of pectenotoxins) associated with the diarrheic

Fig. 3 LC-MS-MS chromatogram obtained from the 20–50 μm plankton fraction of a net tow collected at St 376 east of Aberdeen, Scotland



shellfish poisoning (DSP) syndrome. In our North Sea samples, PTX-2 was the most abundant variant among members of the PTX-group, whereas ratios of okadaic acid to DTX-1 and DTX-2 varied significantly among sampling stations. Yessotoxin and azaspiracid-1 were the least abundant of the detected phycotoxins. A typical chromatogram from a net tow size-fraction (50–200 μm) is shown in Fig. 3.

The azaspiracid AZA-1 was detected in plankton net tows at all sampling stations in the North Sea (Fig. 2). On the Scottish coast, however, AZA-1 concentrations north of 58 ° latitude were very low and abundance was generally higher at sampling stations closer to the shore. The AZA-1 levels were particularly high at two stations St 374 and 398 on the Scottish coast. Interestingly, the highest abundances were found in the 50 μm fraction, whereas at all other stations AZA-1 was more evenly distributed among the three size-fractions. Although several *Protoperdinium* species were present in the 50–200 μm size-fractions, albeit at relatively low cell numbers, *P. crassipes* (Kofoid) Balech, the previously reported source of AZA, was very rare.

Crude on-board cultures initiated by serial dilution from natural plankton samples (Krock et al. submitted for publication) revealed the presence of AZAs in one of the crude cultures. A small dinoflagellate was subsequently isolated from that well and brought into unialgal culture. A detailed taxonomic description of this dinoflagellate is in progress (Tillmann et al. in preparation). Azaspiracid production by this isolate has subsequently been confirmed by toxin measurements by LC-MS-MS of various subcultures in the laboratory.

In conclusion, without access to the near real-time data provided by such an advanced instrument at sea, we would not have been able to accomplish many objectives of the cruise—including mapping the biogeographical distribution of phycotoxins in the water column and tracing the movement of phycotoxins through the lower levels of the food web from natural samples and on-board grazing experiments. Except when occasionally present as highly concentrated blooms, toxigenic microalgae and hence their toxins are usually present at low ambient levels. The high sensitivity of the LC-MS-MS system enabled the detection and quantification of a wide variety of phycotoxins present in seawater samples containing only a few toxic cells per litre. Finally, the use of LC-MS-MS on board enabled the guided isolation, culture, and identification of the causative organism of AZP from field samples and the measurement of AZAs in various plankton fractions containing putative vector organisms for these toxins.

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