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Determination of new algal neurotoxins (spirolides) near the Scottish east coast

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Summary

Spirolides were discovered by Hu et al. 1995 in scallops from Canada. They represent eight macrocyclic polyethers with molecular weights ranging from 691 to 711. In 2000, Cembella et al. identified the dinoflagellate *Alexandrium ostenfeldii* as producer of these toxins. In European waters the presence of spirolide producing organisms was proven by investigation of a cultured isolate of *A. ostenfeldii* from Limfjord, Denmark. To obtain further information about the occurrence of spirolides in Europe some 200 phytoplankton samples were taken from surface water during a reasearch cruise in May 2000. Samples were analysed on board of the research vessel for several algal toxins by LC-MS. Spirolides were detected in 75% of the samples collected in a large area (~45.000 km^2) in the North Atlantic and along the Scottish east coast. The contamination differed concerning both the amounts and the relative abundance of the spirolide toxins. Conventional extraction of the phytoplankton samples resulted in low spirolide concentrations. Furthermore,

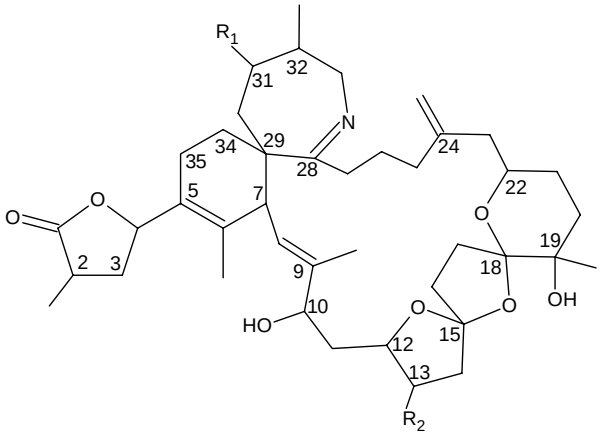
similar structures (differing often only in one double bond) and the lack of commercially available external standards rendered especially quantification of spirolide toxins. Therefore, additional clean-up and concentration steps using size-exclusion chromatography were introduced enabling identification of individual spirolides by application of LC-MS-MS.

Introduction

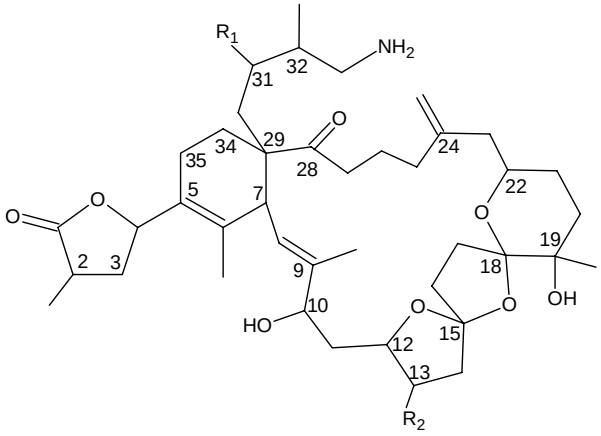
After the discovery of an unusal “fast acting toxin” in lipophilic extracts of scallop (*Placopecten magellanicus*) and mussels (*Mytilus edulis*) from the southeast coast of Nova Scotia, Canada, the structures of two of the causing substances, spirolides B and D, have been determined [1]. Due to their macrocyclic structure containing a spiro-linked tricyclic ether ring system and a spiro-linked cyclic imminium moiety this new group was named “spirolides”. Due to the latter structure element, spirolides are closely related to pinnatoxins [2] and gymnodimine [3]. Macrocyclic spirolides can be classified in two major groups: first the cyclic imines and the second, consisting of spirolide E and F [4] and showing an open keto- amine structure, which can be seen as hydrolysis products of the first group’s members (Figure 1). Further structural differences within these groups concern the double bond between carbon 2 and 3 as well as the rests R_1 and R_2, which can be a methyl group or a hydrogen. Due to theses variations spirolides are named spirolide A to spirolide F. It has to be mentioned that so called desmethyl variants, which lack a methyl group in another position than carbon 31 were found, of which 13-desmethyl C has recently been characterized [5].

As pharmacological active substances (in mouse bioassays) spirolides with the exception of spirolides E and F cause death with characteristic neurotoxic symptoms after intraperitoneal injection. These findings lead to the idea, that the cyclic imminium moiety represents the active pharmacophore, but today the mechanism of toxicity is still not fully understood [4].

The origin of spirolides remained unclear until 2000, when the dinoflagellate *Alexandrium ostenfeldii* was identified as (yet the only known) producer of these toxins [6], although it cannot be excluded that also other taxa might be able to form these toxins. During the annually recurring blooms in Graves Shoal and Ship Harbour, two important shellfish aqua-culture locations in Eastern Canada, differences in the spirolide profile were observed at both places [7]. The objectives of the presented study were to investigate whether or not spirolides are also present in European coastal waters.



No.	name	R_1	R_2		MW
1	spirolide A	H	CH_3	$\Delta^{2,3}$	691
2	13-desMe spirolide C	CH_3	H	$\Delta^{2,3}$	691
3	spirolide B	H	CH_3		693
4	desMe spirolide D*	CH_3	?		693
5	spirolide C	CH_3	CH_3	$\Delta^{2,3}$	705
6	spirolide D	CH_3	CH_3		707



No.	name	R_1	R_2		MW
7	spirolide E	H	CH_3	$\Delta^{2,3}$	709
8	spirolide F	H	CH_3		711

Figure 1: Chemical structure of spirolides (structures with an asterisk are not described yet)

Experimental

Standards

Spirolide standards are not commercially available. A purified solution of spirolide D and an extract obtained from a spirolide producing *Alexandrium ostenfeldii* strain was kindly provided by Michael A. Quilliam and Allan D. Cembella from Institute of Marine Biosciences, National Research Council of Canada, in Halifax, Nova Scotia, Canada.

Materials and chemicals

Acetonitrile and methanol from Baker (Baker BV, Netherlands) were HPLC-grade. Water was purified to HPLC-grade quality with a Millipore-Q RG Ultra Pure Water system (Millipore, USA). Nitrogen for the Turbo ion-spray interface of the LC-MS system was generated using a Nitrox UHPLCMS nitrogen generator (Domnick Hunter, Germany). All other chemicals used were at least analytical grade (p.a.).

Sample material

Bulk plankton was obtained during a cruise with the reasearch vessel "Heincke". The precise locations of sampling sites (S) were determined using the on-board global positioning system (data not shown). Bulk plankton was sampled with a plankton net (20 µm mesh size) from sub-surface water (approximately 1 to 3 m water depth), rinsed on 0.45 µm filters (50 mm diameter; Schleicher & Schuell, Germany) using seawater and stored in 2 mL Eppendorf vials. From each S several filters were obtained. While most of them were analyzed immediately on board, additional filters from each site were stored in a freezer (-28°C) to be re-analyzed under laboratory conditions ashore. Since neither the bio-mass nor the species composition of the bulk plankton samples was exactly determined, only semi-quantitative results regarding the spirolide concentrations were obtained.

Extraction of spirolides

Filters were extracted using methanol/water (1.0 mL, 50:50, *v*:*v*) by sonication (1 min) with an ultrasonic probe (Sonopuls HM 70, Bandelin, Germany). The raw extracts were centrifuged for 10 min. (2980 g) and the supernatant was passed through 0.45µm nylon filters. The extracts were directly subjected to LC-MS analysis.

Clean-up of raw extracts and enrichment of spirolides

To increase the detection sensitivity in MS-MS experiments raw extracts were subjected to a clean-up and an enrichment by size-exclusion chromatography (SEC). The method was slightly modified from recently described protocols for clean-up and enrichment of diarrhetic shellfish poisoning (DSP) toxins and microcystins containing extracts [8,9].

SEC was performed isocratically with a mixture of 0.1 N acetic acid and acetonitrile (80:20, *v*:*v*, flow 1.0 mL min^{-1}) on a Superdex® Peptide column (HR10/30, ID = 1 cm, V = 24 mL, Pharmacia Biotech, Germany) using a LC-10 AT/VP pump, and a SIL-10AD/VP autosampler, (both from Shimadzu, Germany) and a fraction collector (Model 202 Gilson-Abimed, France). 100 µL of the raw extracts were injected. All spirolides eluted from the SEC column in a small time window (18.2

to 21.2 min). The spirolide-containing fraction was evaporated to dryness with a speed-vac (Christ RVC 2-18, Germany) equipped with a vacuum pump (Vacu-Brand, MZ 2C, Germany) and the residue was re-dissolved with methanol/water (100 µL; 50:50; *v:v*). The enrichment of spirolides was achieved by repeated SEC of raw extracts. Spirolide containing fractions were combined, evaporated to dryness, and the residues were re-dissolved with methanol/water.

LC-MS determination of spirolides

Liquid chromatography with MS detection of spirolides was performed with a PE Series 200 Quaternary Pump and a PE Series 200 autosampler (both from Perkin-Elmer, Germany). The separation of spirolides was carried out on a reversed phase column (Luna 3 µm C18, 150 x 4.60 mm ID, Phenomenex, Germany) with an ammonia formate/formic acid buffer system. The mobile phase was slightly modified from Cembella et al. [6] and was composed of 50 mM formic acid and 2 mM ammonia formate in water (eluent A), 50 mM formic acid and 2 mM ammonia formate in water/acetonitrile (5:95, v:v)(eluent B), and methanol (eluent C). Flow rate was 1.0 mL during the entire run. The quota of eluent C was set to 10% during the entire analysis time. The quota of eluent B was linearly increased from 20% to 40% within 15 min. After 10 min of equilibration with the starting mixture the system was ready for the next injection. Detection of spirolides was carried out using an API 165 mass spectrometer equipped with a pneumatically assisted atmospheric pressure ion (API) source operating in turbo ion spray mode (Applied Biosystems, Canada). The LC flow from the column was transferred to the MS using a split ratio of 5:1 (volume wasted / volume transferred). Nitrogen heated to 400°C (620 kPa, 7.5 L min^{-1}) was used to dry the ion-spray and also applied as nebulizer gas with 0,6 L min^{-1} (operating pressure approximately 520 kPa). The ionization voltage of the turbo ion-spray interface was set to 5.2 kV.

Selected ion monitoring (SIM) was used for the spirolide determination. Ions *m/z* 692.5, 694.5, 706.5, 708.5, 710.5 and 712.5 $[M+H]^+$ were monitored, according to the molecular weight of spirolides published to date [1,4,4,6,6].

A rough estimation of the spirolide content in the bulk plankton samples was possible. The summarized signal abundance (all ions measured) of S 189, i.e. the sample with the highest spirolide content, was set as 100%. Spirolide contents higher 70% of S 189 are called “high”, those higher 30% were ranked as “medium”, and those below 30% were regarded as “low” Sampling sites with negative results were marked with a cross.

MS-MS identification of spirolides

To identify suspicious compounds as spirolides, selected extracts were investigated by LC-MS-MS. Therefore, the chromatographic system described above was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Germany). Fragmentation spectra of spirolides were obtained using a pneumatically assisted atmospheric pressure ionization (API) source operated in electrospray mode. Daughter ion spectra from dominating peaks $[M+H]^+$ *m/z* 692.5 (RT 7.7) and *m/z* 706.5 (RT 9.6) (data not shown) were measured in the mass range *m/z* 100 to 750 (mass differences *m/z* 0.2) in positive mode using collision activated dissociation (CAD) with nitrogen target gas in the second quadrupole (60 eV collision energy).

Results

The total ion current (TIC) chromatogram obtained from a solution of spirolide D (Figure 2a) is dominated by one large peak eluting at RT 9.0 min. The extraction of selected ions revealed more detailed information. The main peak at RT 9.0 min originated from ions of *m/z* 708.5 and *m/z* 710.5 as expected for spirolide D, whereby *m/z* 710.5 (with the same RT) is most likely the isotope signal of *m/z* 708.5 and not caused by spirolide E. The second small peak with *m/z* 708.5 (RT 9.6 min) may represent an isomer of spirolide D. Two peaks with *m/z* 694.5 were obtained at RT of 8.3 and 8.9 min, respectively. Both peaks showed poor intensity and their ion mass *m/z* 694.5 referred to spirolide B and desmethyl spirolide D compounds. No signals with *m/z* 692.5 appeared. Therefore, the presence of spirolide A and /or desmethyl spirolide C in the solution of spirolide D can be excluded definitely.

In the LC-MS chromatogram of a bulk plankton extract from S 161 (Figure 2b) no peak appeared at RT 9.0 min in the chromatogram, thus spirolide D was not present in this sample. Signals with higher intensity were observed only at RT of 7.7 and 9.6 min, whereby the SIM chromatogram revealed characteristic spirolide composition. The peak with *m/z* 692.5 at RT 7.7 min was likely originated from spirolide A or desmethyl spirolide C. Consequently, *m/z* 694.5 was the isotope peak. The highest signal, however, was observed at RT 9.6 min. At this RT high intensity for *m/z* 706.5 characteristic for spirolide C was obtained, whereas the signal *m/z* 708.5 at RT 9.6 min was most likely caused by the isotope of the compound related to *m/z* 706.5. The signal *m/z* 708.5 eluting with RT 9.9 min might be a spirolide D isomer different from the spirolide D in figure 2a (RT 9.0 min). A lately eluting compound with *m/z* 710 at RT 14.6 min could be a spirolide E isomer.

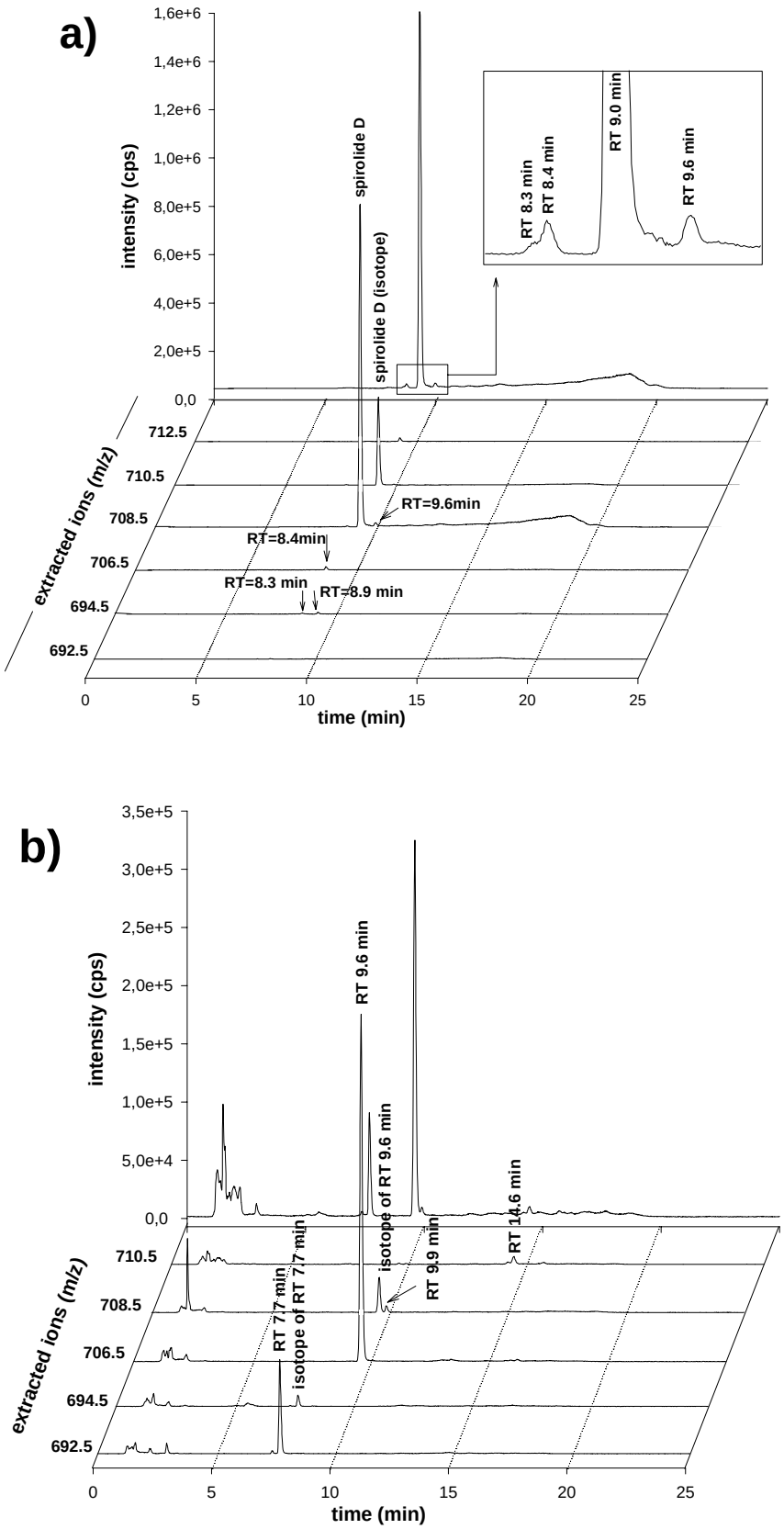


Figure 2 a) LC-MS TIC chromatogram of a spirolide D standard solution

b) LC-MS TIC chromatogram of a plankton extract obtained at S 161

The comparison of all LC-MS TIC chromatograms obtained from different S revealed remarkable differences regarding the spirolide composition. Varying spirolide profiles were observed, of which three are exemplary shown in figure 3 with TIC chromatograms from S 15, S 100, and S 161. Compounds with RT of 7.4, 7.7, and 9.6 min were detectable at all S mentioned above, whereby the peak at RT 9.6 min dominated in all TIC chromatograms. In addition, two minor compounds eluted at RT of 7.4 and 7.7 min. Here, differences in the peak area ratios of these earlier eluting substances were observable. S 15 and S 100 were characterized by dominanance of peak with RT 7.4 min in comparison to peak with RT 7.7 min and at S 161 the peak with RT 7.7 min showed the higher abundance.

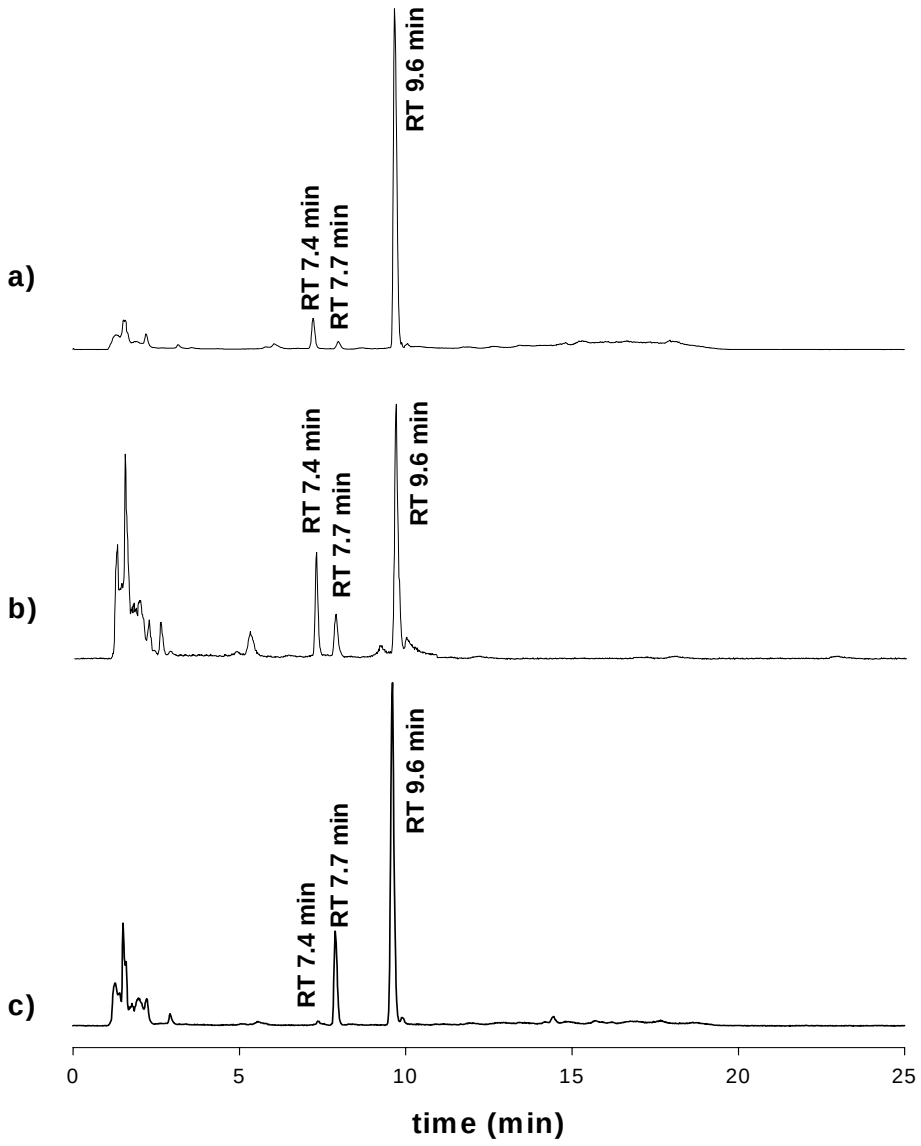


Figure 3 LC-MS TIC chromatograms obtained from a) S 15, b) S 100 c) S 161

To visualize the TIC chromatograms' various peak patterns originating from different sites at the Scottish east coast, figure 4 shows typical relative distributions of peaks with *m/z* ratios and their RTs. In the southern sampling area relatively constant spirolide profiles were found. At S 137, S 144, and S 172 spirolide profiles were identical, while the profile at S 161 shows some slight variations.

In all samples the profiles were dominated by ion *m/z* 706 with RT 9.6 min. A peak with the same RT, but with *m/z* 708, was most probably caused by the isotope of ion *m/z* 706, which is related to spirolide C. In addition, two compounds with *m/z* 692 eluting after 7.4 min and 7.7 min representing either spirolide A or desmethyl spirolide C were found. The higher concentrated compound with *m/z* 692 eluted after 7.7 min. Therefore, its isotope peak (*m/z* 694) was detectable.

The profiles found at S 15 and S 189 were clearly different from the one described above. The concentrations of compound with *m/z* 706 (RT 9.6 min) were even slightly higher, but the change in the ratio of the two compounds with *m/z* 692 at RT 7.4 and RT 7.7 min, respectively, was more obvious. The earlier eluting peak was now the dominant compound. In addition, a substance with *m/z* 708 at RT 9.9 min was present, which could not be an isotope of *m/z* 706, since no such compound appeared at this RT. The same substance was present at S 161 and S 172, but not at S 137 and S 144. This contaminant is probably an isomer of spirolide D.

The third distinct profile was found at S 100. The percentage of peak *m/z* 706 at RT 9.6 amounted only 52%, and thereby it was the lowest in all samples analyzed. As a consequence, the percentages of the compounds with *m/z* 692 were higher than in all other samples (23% at RT 7.4 and 11% at RT 7.7, respectively). Spirolide were found in approximately 75% of the samples, and their contents were expressed by different sized dots and crosses in figure 4.

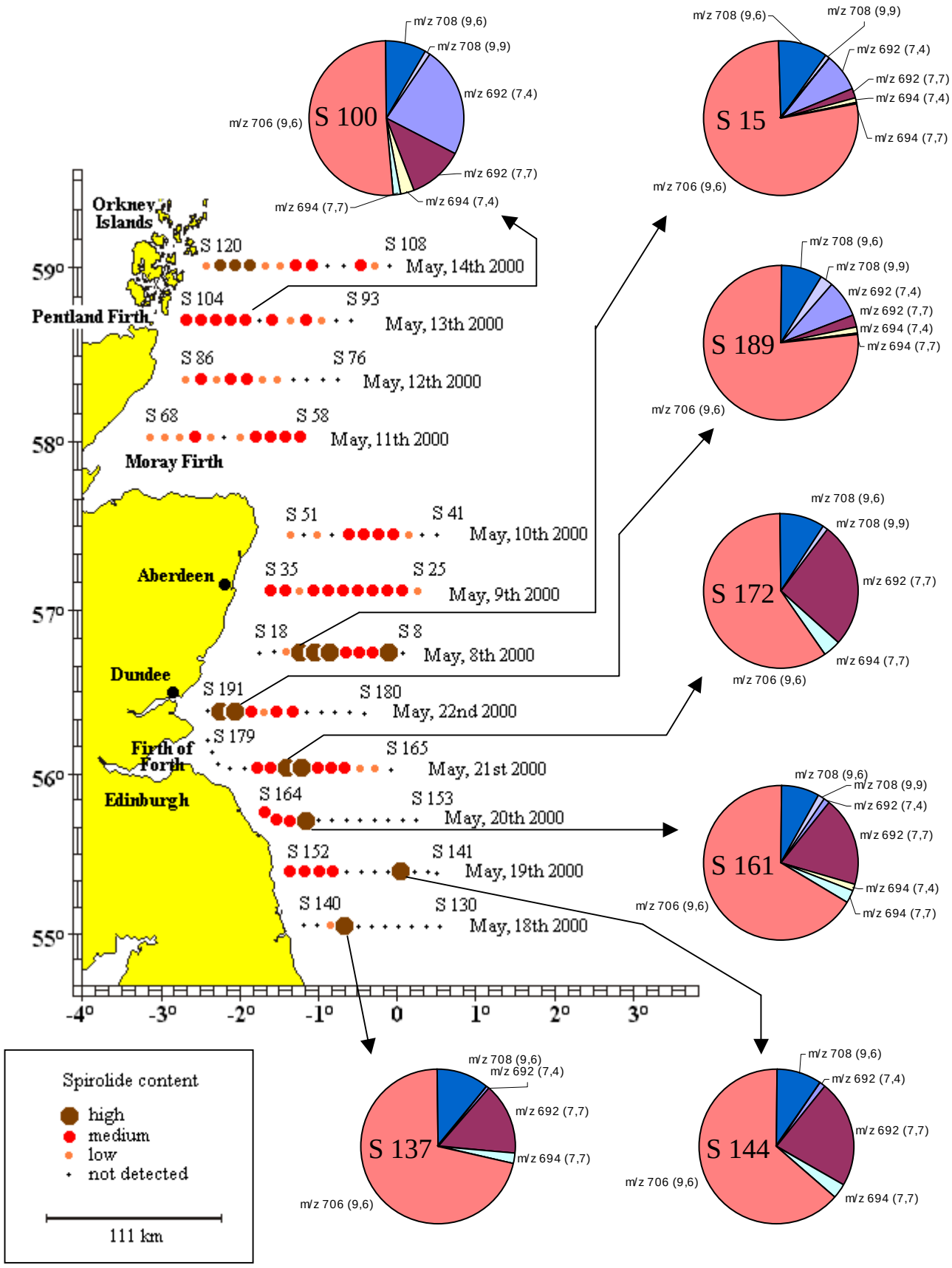


Figure 4 Sampling area, sampling dates, spirolide contents and -profiles (found at selected S). For the different compounds, mass: charge ratios and RTs (in brackets) are given.

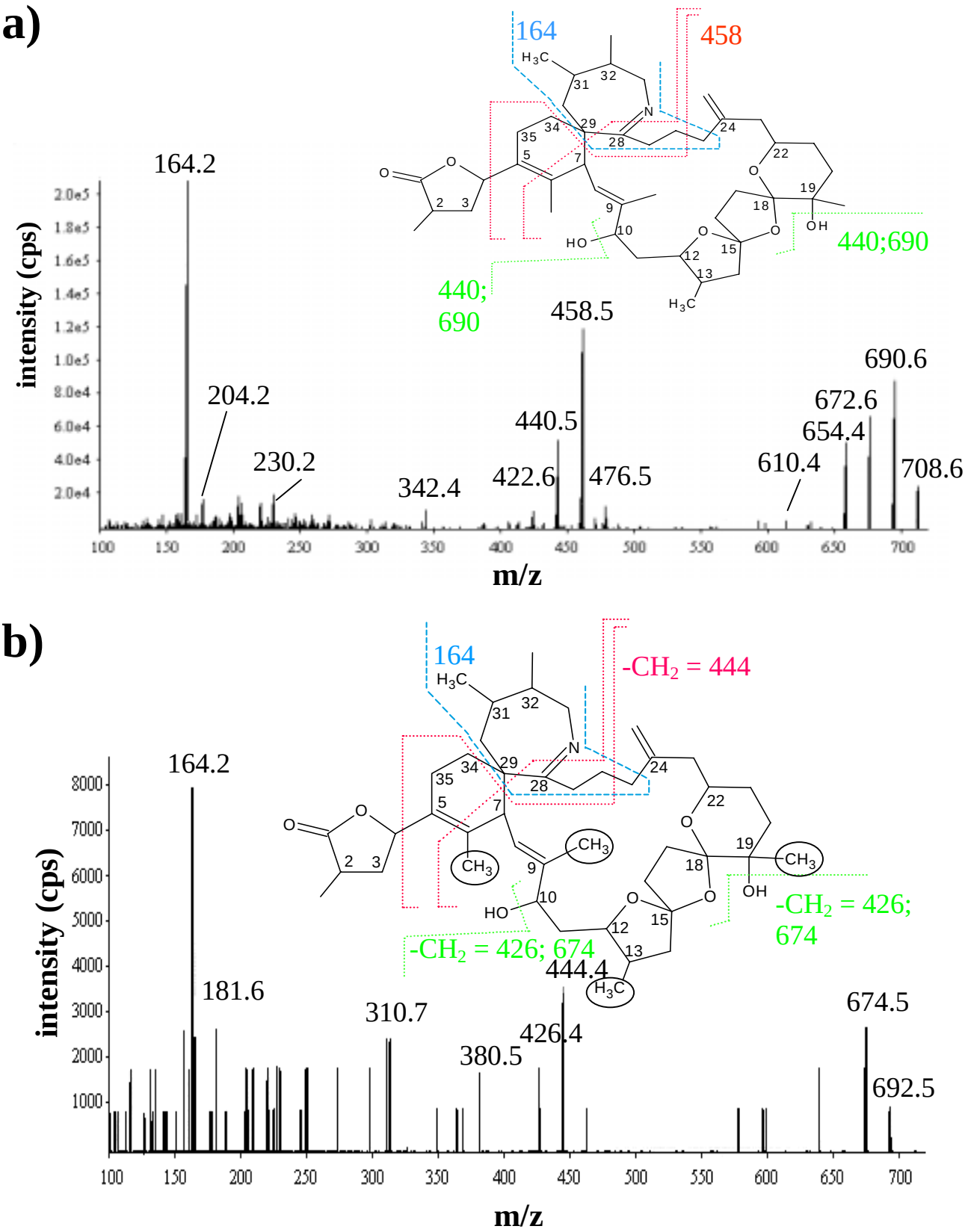


Figure 5 a) LC-MS-MS spectrum obtained from a spirolide D solution

b) LC-MS-MS spectrum obtained from peak *m/z* 692 (RT 7.7 min) of pooled extracts (S 169 – S 174)

The MS-MS spectra of spirolide D (Figure 5a), and of a suspicious peak originated from pooled sample material of S 169 – S 174 (Figure 5b) are shown. The chemical structures of spirolides are added to the MS-MS spectra, whereby possible fragments are indicated by lines.

The dominating fragment in the spectrum of spirolide D is *m/z* 164. It is typical for C/D type spirolides, which carry a methyl group at carbon 31 [1,6]. The loss of one or two water molecules at carbon 10 and 19 results in the fragments 690 and 672, respectively. Two mechanisms explain fragment *m/z* 458: The cleavages of double bonds between carbon 28 and nitrogen of the cyclic imine moiety and between carbon 5 and 6, 28 and 29, and 29 and 7 form fragment *m/z* 458. However, the second possibility to form *m/z* 458 is cleavage of bonds between the following carbon atoms: 25 and 26, 7 and 29, 29 and 34, 4 and 5 (loss of lactone ring). Fragments *m/z* 440 and 422 are, therefore, corresponding to loss of one or two water molecules from fragment *m/z* 458.

Figure 5b shows the MS-MS spectrum of peak *m/z* 692 $[M+H]^+$ at RT 7.7 min. The fragment *m/z* 164 is dominating, too. The molecular weight 691 can be related to both spirolide A and desmethyl spirolide C. However, the fragment *m/z* 164 clearly indicates that this spirolide must contain a methyl group at carbon 31. In MS-MS spectra of spirolide A type fragment *m/z* 150 dominates instead of *m/z* 164 [5]. Therefore, the presence of spirolide A in this sample material can be excluded. On the other hand, it can be assumed that the compound with *m/z* 692 represents a desmethyl spirolide C. This theory is supported by appearance of *m/z* 444 instead of *m/z* 458 (the latter was detectable from spirolide D, see figure 5a). In this case the loss of one or two water molecules leads to fragments with *m/z* 426 and *m/z* 674, respectively.

The first desmethyl spirolide C, which has yet been characterized, is 13-desmethyl spirolide C [5]. Nevertheless, in figure 5b the chemical structure of spirolide C is shown, whereby in (possibly occurring) different desmethyl spirolide C molecules one of four methyl groups labelled with a circle has to be absent. Unfortunately the fragmentation spectrum does not provide any information, which of these methyl groups is absent, thus here the presence of 13-desmethyl spirolide C cannot definitively be proven without further investigations.

Discussion

During May 2000 spirolides were detected in a Scottish coastal zone of approximately 45.000 km^2. The results show clearly that the occurrence of spirolide like toxins is not restricted to Canadian coastal waters. Therefore, this toxin class is not only a North American problem.

In addition, the total content of spirolides differed in a broad range and the profiles of toxins showed clear variations. The spirolide composition at the sampling sites south of the Firth of Forth (S 130 – S 179) appeared to be relatively constant, whereby all sample from this area were obtained within 4 days from the 18th of May till the 21st of May. One day later a different profile was

observed on the latitude of Dundee at S 189, and the toxin composition here was similar to that obtained two weeks earlier at S 15. The estuary system in front of the Firth of Forth seems to be a borderline between two different spirolide producing plankton communities.

The profile variations were obviously not time related, because only 24 hours passed between sampling at S 172 and S 189. Nevertheless, different profiles were found. On the other hand, similar spirolide profiles were found at S 189 and at S 15, although two weeks passed between sampling at these sites. It can be stated that the spirolide content in sample material obtained north of Aberdeen was much lower than in the south. However, characteristic spirolide profile could also be determined east of the Orkney Islands.

Spirolide related problems are not restricted to small coastal areas or bights such as Graves Shoal and Ship Harbour in East Canada. Here, the spirolide production is closely connected to one dominant strain of *A. ostenfeldii*, which produces a distinct toxin profile [6,10]. The results presented in this study indicate that large coastal zones and off-shore habitats are also contaminated with spirolides. However, in the open sea plankton organisms are widely distributed and more diverse plankton communities could form distinct spirolide profiles. Consequently, in open sea areas the formation of identical recurring spirolide profiles as reported from Canada is rather unlikely.

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