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ORIGINAL ARTICLE

First records of the benthic, bloom-forming, non-toxic dinoflagellate *Thecadinium yashimaense* (Dinophyceae) in Europe: with special emphasis on the invasion in the North Sea

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Abstract *Thecadinium yashimaense* was recorded for the first time in France, Great Britain, The Netherlands, and Germany. The invasion and establishment of the species in the German Bight was documented reliably and is presented here. The geographic expansion of the species from the North Pacific to the North Atlantic Ocean is discussed. This bloom-forming, marine, sand-dwelling dinoflagellate was shown to be non-toxic. Also *Thecadinium kofoidii*, the type species of the genus, was analyzed for potential toxin production and turned out to be non-toxic as well.

Keywords Benthos · Dinoflagellate · Invasion · Plankton · *Thecadinium* · Toxins

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Introduction

The number of introduced and established marine species of the North Sea coasts is about 80 and comprises invertebrates, macroalgae, and protists (Reise et al. 1999). It is a very difficult task to distinguish between real exotics and misinterpreted native species (pseudo-exotics), which were discovered late but may have been around long before without being noticed (Reise et al. 1999). This is especially true for small organisms like phytoplankton and protist taxa. Methodological problems and extrinsic factors (Lee and Patterson 1998) make it difficult to prove the exotics-status of protists (Elbrächter 1999). There are only a few examples of well documented invasions of planktonic diatoms into the North Sea, e.g., *Odontella sinensis* (Greville) Grunow (as *Biddulphia sinensis* Greville, in Ostenfeld 1908), *Thalassiosira punctigera* (Castracane) Hasle (Dürselen and Rick 1999), and *Coscinodiscus wailesii* Gran et Angst (Boalch and Harbour 1977; Dürselen and Rick 1999; Rick and Dürselen 1995; Robinson et al. 1980). Flagellates have rarely been documented as invasive species (Elbrächter 1999), and the only known examples are *Karenia mikimotoi* (Miyake et Kominami ex Oda) Hansen et Moestrup (as *Gyrodinium aureolum* Hulburt, in Braarud and Heimdal 1970; Hickel et al. 1971) and *Alexandrium leei* Balech (Koeman 1997).

The possible vectors for the dispersal of non-indigenous species are aquaculture, migratory birds, and ocean going vessels. The transport of protists via ballast water is documented (Galil and Hülsmann 1997; Hallegraeff and Bolch 1991, 1992; Hallegraeff et al. 1990). Surviving the harsh conditions inside ballast water tanks is a prerequisite for a successful invasion, especially for phototrophic species. Most of them will die because of darkness, but a few cells and cysts can stay alive (Dickmann and Zhang 1999; Hallegraeff and Bolch 1992; Yoshida et al. 1996). The ability to form resting cysts is only known for a minority of dinoflagellate species (Hallegraeff and Bolch 1992). The awareness of a possible survival of resting cells of phototrophic species is relatively new (e.g., Dickmann and Zhang 1999; Zhang and Dickmann 1999). Resting cells can survive for lengthy periods in ballast water tanks (C.J.S. Bolch, personal communication). There is also the possibility that species may naturally expand their range via ocean currents.

Galil and Hülsmann (1997) showed that the protists found in ballast water tanks belong mainly to the benthic communities. Deciding whether a newly recorded benthic protist species is introduced to the North Sea habitat or has been overlooked in the past, is nearly impossible in most cases because knowledge about the species assemblage is missing for nearly all taxa (for example, see Houpt and Hoppenrath 2006). There are no monitoring programs for benthic microalgae/protists, as there are for the phytoplankton community. Moreover, intensive taxonomic investigations and published accounts are missing in many cases. Sand-dwelling dinoflagellates, euglenoids, and ciliates of the North Frisian Wadden Sea are an exception (Hoppenrath 2000a; Hartwig 1973), and dinoflagellates and euglenoids are also documented for the Danish Wadden Sea (Larsen 1985, 1987).

Thecadinium yashimaense Yoshimatsu, Toriumi et Dodge was described from Japan (Yoshimatsu et al. 2004). There was some taxonomic confusion about this species (Hoppenrath et al. 2005). The taxon has been known for nearly 40 years, first recognized at the North American Pacific coast, but misidentified at that time (Baillie 1971; Hoppenrath et al. 2004). Six marine, sand-dwelling *Thecadinium* species were observed in the German Wadden Sea (Hoppenrath 2000b; Hoppenrath et al. 2004). Here we present the first records of *T. yashimaense* in the North Sea and the reliably documented invasion. Shortly before submitting the manuscript we learnt about an isolate of *T. yashimaense* from France, and we have integrated this information too.

Methods

Samples and cultures

Benthic sand samples were collected with a spoon during low tide, and dinoflagellates were separated from the sand by extraction with melting seawater-ice (Uhlig 1964) through a fine filter (Hoppenrath et al. 2004). They were accumulated in a Petri dish beneath the filter and were then identified with a Leitz Fluovert FS invert-microscope at 40 to 250× magnification. Sampling sites were in bare, eulittoral regions, south of List Harbor (55°00.85′N; 08°06.30′E; tidal flat of 100 m) and at the "Oddewatt" northeast of List (55°01.80′N; 08°26.00′E; tidal flat of 500 m). For more details on the sampling sites, see Hoppenrath (2000c).

Net samples from surface water near List (55°01.30′N; 08°27.10′E) and of the Helgoland Reede Station (54°11.30′N, 7°54.00′E) were collected weekly (Drebes and Elbrächter 1976; Hoppenrath 2004). Nets of 20 and 80 μm mesh size were used. The samples were brought to the laboratory and living organisms were identified in small Petri dishes with an inverted light microscope equipped with seawater-immersion objectives.

Cells were isolated by micropipetting and cultivated in Petri dishes supplied with f/2 medium (Guillard and Ryther 1962) at 16°C under a 14:10 LD cycle at about 10 μmol/s m^2 light intensity.

Thecadinium yashimaense isolated from the Pacific coast of British Columbia, Canada, was available as culture in the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP 1890, see Hoppenrath et al. 2004) and was also used for the toxin analysis. Two new isolates from Sylt and Helgoland, Germany, were deposited in the CCMP (culture numbers CCMP 2726 and CCMP 2667).

The observations of *T. yashimaense* in The Netherlands were made in two water-bodies in the southwestern part of the country: Lake Grevelingen, a semi-stagnant saltwater lake, and the Oosterschelde estuary, which is a marine tidal basin. Locations were Lake Grevelingen (DREISR: 51°42.88′N, 3°59.96′E) and Oosterschelde (HAMMOT: 51°39.61′N, 3°51.16′E; LODSGT: 51°29.66′N, 4°07.92′E; WISSKKE: 51°36.10′N, 3°43.24′E; ZIJPE: 51°38.68′N, 4°05.82′E). On these locations samples were collected at least monthly. Recently the species was also observed in the north-eastern part at Rottumerplaat (ROTTMPT3: 53°33.58′N, 6°33.51′E), west of the island of Borkum. For storage reason the samples of 1l were fixed with acidic Lugol's solution. Algae were enumerated by analyzing 1–40 ml of the fixed samples with the aid of an inverted microscope after sedimentation of the cells in a counting chamber (Utermöhl 1958). Thecate dinoflagellates were counted after rinsing with distilled water and staining with Calcofluar-white with an epifluorescence microscope. When necessary a small needle was used to manipulate individual cells.

The *Thecadinium/Amphidiniopsis* culture (strain M1408) from the Culture Collection of Algae at the University of Cologne (CCAC; Surek and Melkonian 2004) turned out to be *T. yashimaense* as well. The species was isolated from a sand sample in the tidal flat at the south-east coast of the Ile de Batz, Brittany, France. Sampling date was September 3, 1996 and the culture was established in the weeks after sampling.

Thecadinium kofoidii was isolated at Helgoland (Düne), Germany in August 2002. Culture conditions were as described above.

Toxin analyses

The toxin groups

The marine biotoxin analyses determined the presence of paralytic shellfish poisoning (PSP) toxins, diarrhetic shellfish poisoning (DSP) toxins and the amnesic shellfish poisoning (ASP) toxin domoic acid. The applied methods were based on chromatographic separation of the toxins followed by their selective and sensitive detection using HPLC/FD or LC/MS devices. These methods allow unambiguous determination of the various marine biotoxins which exhibit different chemical structure and polarity.

The PSP toxins are hydrophilic compounds grouped in carbamate toxins [saxitoxin (STX), neosaxitoxin (NEO) and gonyautoxins (GTX1-GTX4)], N-sulfocarbamoyl toxins (C1-C4, B1, B2), and decarbamoyl toxins (dcSTX, dcNEO, dcGTX1-dcGTX4), whereas DSP toxins are lipophilic toxins including okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), yessotoxins (YTXs), and azaspiracides (AZAs). The ASP toxin domoic acid (DA) is a water soluble amino acid.

Sample preparation

Independent of the determination method, the toxins have to be quantitatively extracted from the biomass. Filters with algal material were homogenized with 1 ml methanol/water (50:50) using an ultrasonic probe. The suspension was centrifuged and filtrated (Hummert et al. 2002). In addition, for determination of PSP toxins the extraction was carried out with 0.03 N acetic acid (Luckas 2000).

Determination of PSP toxins

The common methods applied for PSP determination are based on ion-pair chromatography of underivatized PSP toxins followed by post-column oxidation and fluorescence detection (Luckas et al. 2003). Recently, such an HPLC/FD method was modified to lower the limits of detection especially for GTX 2/3, dcSTX and STX (Diener et al. 2006). This method was applied for investigation of the *Thecadinium* cultures concerning PSP toxins.

Determination of ASP and DSP toxins

New LC/MS methods have been fully validated for quantitative determination of both ASP and DSP toxins. The methods provide advantages in speed, specificity and sensitivity. These needs are particularly urgent for the broad groups of DSP toxins where, in addition to okadaic acid and dinophysistoxins, there have been rapid advantages in knowledge on yessotoxins, pectenotoxins, and azaspiracids (Holland et al. 2003).

The ASP and DSP toxins were separated on a C18 HPLC column (150 mm × 2.0 mm) from Phenomenex with gradient elution. Both eluents contained 53 mM formic acid and 5 mM ammonia formate as buffer, whereby the eluents were a water/acetonitrile solution. The quota of acetonitrile was 10% in eluent 1 and 90% in eluent 2.

The HPLC system was equipped with a PE series 200 quaternary pump and a PE series 200 autosampler from Perkin Elmer, Germany. All measurements were performed on an API 165 SCIEX mass spectrometer with pneumatic assisted atmospheric pressure ion (API) source operating in turbo ion-spray mode (PE Sciex, Canada). The MS responses were obtained from the protonated molecules $[M + H]^+$, $[M + NH_4]^+$, $[M - H_2O + H]^+$, and $[M + Na]^+$.

Results

A so far unidentified sand-dwelling dinoflagellate, tentatively identified as *Amphidiniopsis* sp., isolated in September 1996 from the Ile de Batz, Brittany, France, and cultivated since that time (CCAC strain M1408), was identified as *T. yashimaense* (Fig. 1a). This culture represents the first proof of the species for the North Atlantic Ocean (Fig. 2).

Thecadinium yashimaense was documented from samples of the Lake Grevelingen in The Netherlands in 2000, but not identified at that time (Fig. 1b–f). This was the first record of the species from the North Sea (Figs. 2, 3a). Once identified the species were counted during the routine monitoring program (Table 1). It was encountered in two different waters at five different Dutch collecting sites in the south-western part of the country (Fig. 3a, b). Cell numbers were up to 2,000 per liter (Table 1).

The first observation of *T. yashimaense* in the German Bight took place on September 5, 2002 in a net-phytoplankton monitoring sample from List, Sylt (Figs. 1g–i, 2, 3a, c). It was the first record of this marine sand-dwelling species in Germany. Since that day, during the weekly monitoring of living species, *T. yashimaense* was found regularly in phytoplankton samples from the North Frisian Wadden Sea at the Wadden Sea Station Sylt. The data of *T. yashimaense* recordings from 2002 to 2005 are listed in Table 2. Shortly after the first record in the plankton, a bloom (sand discoloration) of the species was observed in a tidal sand flat close to an oyster mariculture ("Blidselbucht", Sylt, Germany) in October 2002. In mid November 2002 an extensive discoloration was registered in the sand flat south of List Harbor (G. Klein, personal communication). Further blooms in the tidal sand flats of the island of Sylt were observed in the summer of 2003 in the "Königshafen" northeast of List (J. V. Beusekom, personal communication), south of List Harbor, and in the "Blidselbucht", and in summer 2004 in the "Oddewatt" northeast of List. Sand-dwelling dinoflagellates co-occurring with *T. yashimaense* south of List Harbor in 2003 were *Amphidinium operculatum*, *A. semilunatum*, *A. poecilochroum*, *Gymnodinium* cf *danicans*, *Togula britannica*, and *Sinophysis ebriolum*.

The first record of *T. yashimaense* for Helgoland (open North Sea; Figs. 2, 3a, d) was on May 27, 2004 in a net-phytoplankton sample. It should be mentioned that the days before were stormy. The species was observed again on June 10, 2004. Cultures were established from the samples of the first records for Sylt and Helgoland.

Most recently (April 19, 2005) one *T. yashimaense* cell was seen in a sample from the West Frisian Wad-

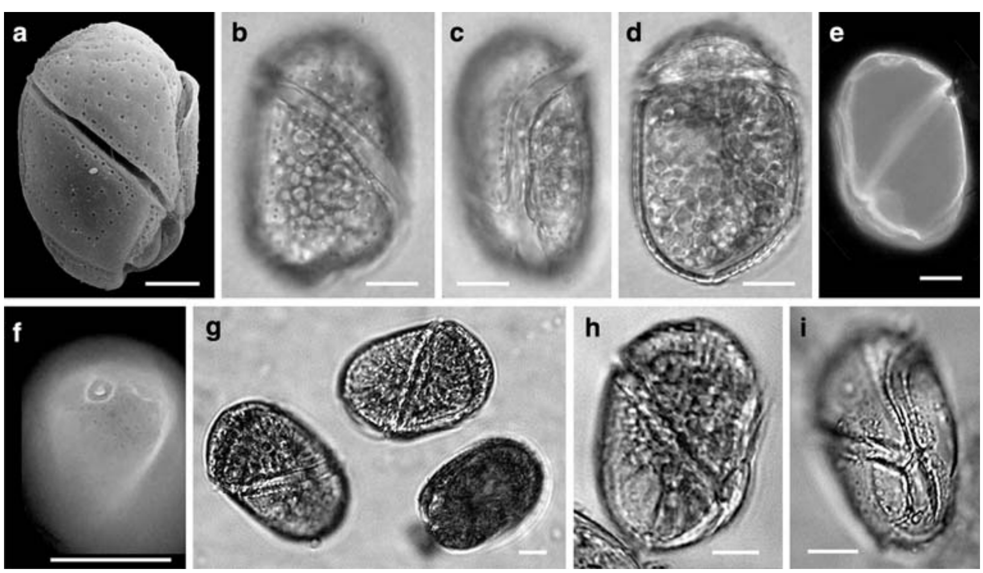

Fig. 1 Images of *Thecadinium yashimaense* from the different sites. **a** Scanning electron micrograph from the culture isolated at the Ile de Batz, France; **b**–**f** light micrographs from a sample from The Netherlands; **b** right lateral view; **c** ventral view; **d** median focus, showing the cingulum on the left lateral side too; **e** epifluorescence microscopy of a calcofluor-white stained cell showing the right lateral side with strongly descending cingulum and the apical pore plate (side reversed image); **f** epifluorescence microscopy of a calcofluor-white stained cell showing the epitheca with the apical pore plate and the border of the first intercalary plate; **g**–**i** light micrographs from a sample from Sylt, Germany; **g** three cells in different views; **h** right lateral view; **i** ventral view; *scale bars* 10 μm

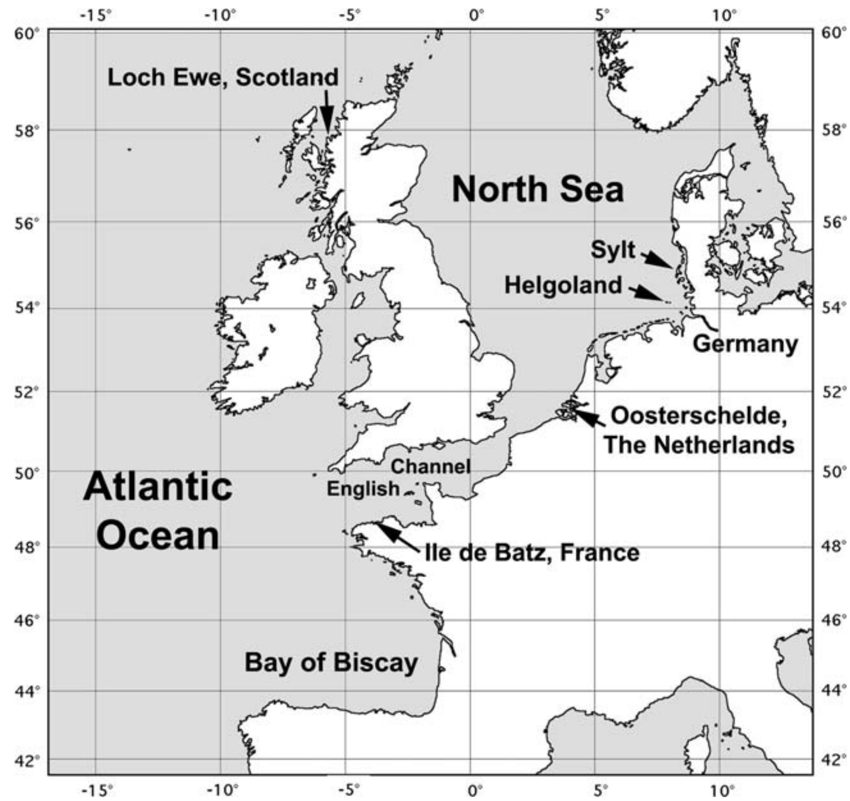

Fig. 2 Map showing the European sites of *Thecadinium yashimaense* recordings

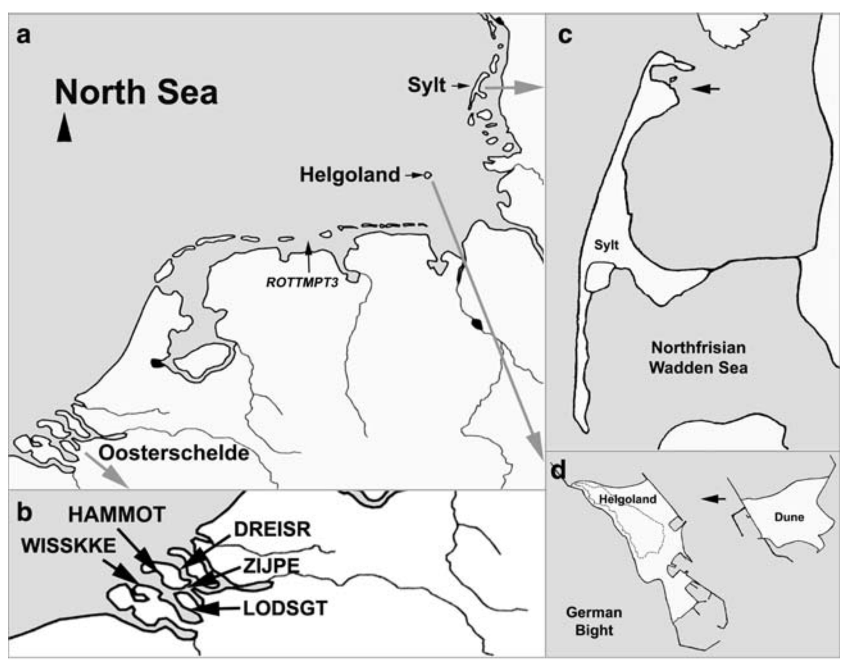

Fig. 3 Maps showing the sampling sites in the North Sea. **a** Coastline of the North Sea from The Netherlands over Germany (German Bight) to Denmark; **b** sampling sites in the south-western part of The Netherlands; **c** sampling site at Sylt in the North Frisian Wadden Sea, Germany; **d** sampling site at Helgoland in the German Bight, Germany

den Sea, The Netherlands, at Rottumerplaat, west of Borkum (Table 1, Fig. 3a).

The cultures of Sylt and Helgoland together with the culture of *T. yashimaense* from British Columbia, Canada, were analyzed for potential toxin production in order to check possible health risks of consumers, as this species showed mass occurrence in the direct neighborhood of mariculture at Sylt. The cultures were analyzed for amnesic shellfish poisoning toxin (domoin acid), diarrhetic shellfish poisoning toxins [okada acid, dinophysistoxin-1, -2, pectenotoxins (PTX-1, -2), yessotoxin (YTX-1), azaspirazides (AZA-1, -2, -3)], and paralytic shellfish poisoning toxins [N-sulfocarbamoyl toxins (C1-4, B1, B2), gonyautoxins (GTX1-4), neosaxitoxin,

Table 1 *Thecadinium yashimaense* counts in Dutch waters 2003–2005

Location	Sample date	Relative depth	Cells/l
Lake Grevelingen			
DREISR	March 17, 2003	Surface	140
	April 14, 2003	Surface	120
	April 28, 2003	Thermocline	64
	March 15, 2004	Surface	78
	April 26, 2004	Thermocline	76
	January 17, 2005	Surface	435
	March 17, 2005	Surface	1,538
	March 29, 2005	Surface	1,538
	April 13, 2005	Surface	769
	August 29, 2005	Bottom	204
	August 29, 2005	Thermocline	65
Oosterschelde			
HAM-MOT	March 30, 2004	Surface	147
LODSGT	March 05, 2003	Surface	153
	April 28, 2004	Surface	77
	April 26, 2004	Surface	51
	May 10, 2004	Surface	137
	January 18, 2005	Surface	177
	February 16, 2005	Surface	425
	April 12, 2005	Surface	769
	December 21, 2005	Surface	1,000
WISSKKE	March 02, 2004	Surface	304
	February 16, 2005	Surface	57
	April 26, 2005	Surface	69
ZIJPE	March 30, 2004	Surface	223
	May 10, 2004	Surface	2,000
	April 26, 2005	Surface	65
Rottumerplaat			
ROTT-MPT3	April 19, 2005	Surface	76

For location codes, see Methods

decarbamoyl toxins (dcSTX, dcNEO, dcGTX1-4), saxitoxin]. None of these toxins could be detected. In addition *T. kofoidii*, the type species, was analyzed in the same way and was also found to be non-toxic. These are the first toxin analyses of species of the benthic genus *Thecadinium*, whose photosynthetic species can show mass occurrence in tidal flats.

Discussion

In Europe, in the North Atlantic Ocean, *T. yashimaense* was first recorded in a sample from the Ile de Batz, Brittany, France, in 1996 (CCAC culture, Figs. 1a, 2). Balech (1956) investigated the sand-dwelling dinoflagellates from Roscoff, Brittany, France—an area not too far away from the Ile de Batz. He did not publish a complete list of identified species, however, he described new thecate species from that area. Since he did not mention *T. yashimaense*, we believe that he did not observe it. Because of its cell size, the clearly thecate appearance, specific morphological features, and the phototrophic mode of nutrition, we are quite confident that Balech would have had included this species, if it had been present in his samples.

The recordings of *T. yashimaense* from three different areas of the North Sea—from the south-western part of The Netherlands and from the islands of Sylt (North Frisian Wadden Sea) and Helgoland (open North Sea, German Bight), Germany—documented the appearance of a new species in these dinoflagellate communities. The species was first recorded in phytoplankton net-samples because there are regular collections for monitoring reasons. In fact, it is a marine, sand-dwelling species (Hoppenrath et al. 2004; Yoshimatsu et al. 2004), but seems to be tychopelagic as well. In our interpretation *T. yashimaense* could be observed in plankton samples taken at shallow coastal waters (e.g., Sylt and The Netherlands), while blooming occurs in the sediment. Appearance of the species in open sea samples (e.g., Helgoland) will happen only rarely, after strong mixing of the water column with benthic material. We are confident that *T. yashimaense* is an invader of the German Wadden Sea at least, probably also of the marine waters in the south-western part of The Netherlands, because of the exceptional monitoring situation of these habitats. The site at Sylt has been studied weekly for about 20 years and the same person has always identified the dinoflagellates. Moreover, an intensive study of the sand-dwelling dinoflagellate species composition for the North Frisian Wadden Sea, especially around Sylt, has been conducted from 1996 to 2000 (Hoppenrath 2000a), without any record of this conspicuous taxon. Therefore, we may rule out that the species was overlooked in the past. *Thecadinium yashimaense* also was not detected before May

Table 2 *Thecadinium yashimaense* records in the North German Wadden Sea plankton, 2002–2005

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
2002									5/12/19/26	2	7/28	5/19
2003	2/9/23	–	5	–	1	12	3/10/17/24	7	4/18	–	2	–
2004	8/15/22/29	11/19	4	22	27	10/17	22/29	5/19	15/22/30		25	16/23
2005	26	–	–	–	12/19	30	–	–	–	–	–	22

2004 during samplings at Helgoland (Hoppenrath 2000a, 2004). No study has been conducted on benthic, sand-dwelling dinoflagellates of the Dutch coast (Houpt and Hoppenrath 2006), and therefore we cannot be sure that *T. yashimaense* occurred in 2000 for the first time. It may have been abundant enough to be encountered in a plankton sample for the first time in 2000.

If we agree that *T. yashimaense* is an exotic invader, how was the species introduced? The observation of *T. yashimaense* blooming in the neighborhood of oyster aquacultures seems striking. Oysters are cultured in the Bay of Morlaix, France, close to the Ile de Batz, in The Netherlands, and also at Sylt close to the sampling sites. This vector is well known for exotics (e.g., Reise et al. 1999). The fact that *T. yashimaense* has been known for nearly 40 years, first recognized at the North American Pacific coast, underlines the observation that phytoplankton and macroalgal exotics are almost all of Pacific origin (Reise et al. 1999). The original description of *T. yashimaense* is based on samples collected in southern Japan from 1997 to 1999, also a locality within the Pacific Ocean, but on the western side (Yoshimatsu et al. 2004). These data support the hypothesis of the introduction of the species from the Pacific into the North Sea/North Atlantic Ocean. Bolch and Campbell (2004) found *T. yashimaense* in the north eastern shore of Loch Ewe, Scotland (Fig. 2) in 2001 and described it as *Thecadinium foveolatum* (Hoppenrath et al. 2005). During our discoveries of the species in The Netherlands and then in the German Bight, it was already established in Scotland, UK.

Our data suggest that *T. yashimaense* was originally introduced to France after the year 1956, but before 1996. From there a natural migration to the north is a plausible explanation. For the introduction into the North Sea there are two different scenarios: (1) *T. yashimaense* was transported with water currents around the northern tip of Scotland and the Orkney Islands into the North Sea, or (2) it was transported through the English Channel and migrated via the West Frisian coast north-east to Helgoland and Sylt. *Thecadinium yashimaense* is able to divide in the plankton and is growing slowly in culture. Therefore, the duration of a transport along water currents must not be deadly but the survival is possible, in our view. However, we cannot rule out that multiple invasions via aquaculture organisms like oysters took place, and that the occurrences in France, Scotland, The Netherlands, and Germany were independent events. It would be interesting to investigate samples from the East Frisian Wadden Sea, Germany and from the Danish Wadden Sea. We expect *T. yashimaense* inhabiting the East Frisian Wadden Sea sediments, because that would be the logical way of spreading from the estuaries in the south-western part of the Netherlands to the North Frisian Wadden Sea. This scenario is supported by the latest record of *T. yashimaense* west of the island of Borkum in the West Frisian Wadden Sea (Table 1). Dinoflagellates from the Danish Wadden Sea sand samples were studied over a period of 1 year from 1980 to 1981 (Larsen 1985), without a single record of *T. yashimaense*. After the establishment in the Sylt area, only a few kilometers from that Danish area, it is likely that this invader has already arrived, or will appear during the next years in the Danish Wadden Sea. The species appears to have migrated in a northern direction. The southern coast of England should be checked for *T. yashimaense*. The species could have migrated also in southern direction. It should be searched for also south of the first recorded site in France, e.g., in Arcachon, also an oyster aquaculture site.

Another possibility is that climate-induced changes in the species composition of marine benthic habitats may have occurred, possibly due to global warming. The temperature tolerance of *T. yashimaense* and also that of all other sand-dwelling dinoflagellates is not known. It appears that organisms from eulitoral habitats may have a high tolerance for higher temperatures. We do not know the tolerance of these organisms for lower temperatures.

The known distribution of *T. yashimaense* is restricted to the northern hemisphere in the Pacific and Atlantic Ocean. Comparable habitats are very well studied in Australia (Al-Qassab et al. 2002, western Australia; Murray 2003, southern Australia; S. Murray and M. Hoppenrath, unpublished observations for the Broome area, northwestern Australia), but *T. yashimaense* was not recorded.

Toxin producing dinoflagellate species are mostly known to be planktonic, but also epibenthic and some sand-dwelling species could be toxic (e.g., Besada et al. 1982; Faust 1995a; Taylor et al. 2003). Knowledge about sand-dwelling, toxic dinoflagellate species is scarce and very few papers mentioned them (Faust 1995b; Holmes et al. 1998; Murray and Patterson 2002; Ten-Hage et al. 2000). Until today, species of five genera have been found to produce toxic compounds—*Amphidinium*, *Coolia*, *Gambierdiscus*, *Ostreopsis* and *Prorocentrum*. The species are all phototrophic. The genus *Thecadinium* includes three phototrophic species, which are able to discolor the sand, but so far their ability for toxin-production was never checked. *Thecadinium kofoidii* and *T. yashimaense* do not produce PSP-, DSP-, and ASP-toxins. Other toxins such as ciguatoxins and fast acting toxins (FAT) have not been checked so far.

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