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SHORT COMMUNICATION

Pigment profile of the ichthyotoxic dinoflagellate *Gymnodinium* sp. from a massive bloom in southern Chile

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The pigment profile of the ichthyotoxic dinoflagellate Gymnodinium sp. from a bloom occurred in southern Chile contained fucoxanthin and 19′-acyloxyfucoxanthin derivatives, including novel forms of these latter compounds. This profile resembled those found in other ichthyotoxic Gymnodinium species.

A massive outbreak of phytoplankton was observed in Southern Chile during March and April 1999. This event caused a high mortality in shellfish (clam, sea urchins, abalone), farmed salmon and wild fish species. The large mortality of salmon constituted a severe loss for the farmers involved (Clément *et al.*, 1999). Their studies revealed that the predominant phytoplankton species, more than 99%, was *Gymnodinium* sp., with a density of up to 8 to 9 million cells per litre of seawater in the coloured patches. Some small cells looking like chlorophytes (1–2 μm) were also observed in fresh samples. This unidentified species showed strong haemolytic and allelopathic activity. This explains one of the modes of action of the toxin(s) and the almost monospecific nature of the blooms (Clément *et al.*, 1999). These properties resemble those of other known ichthyotoxic *Gymnodinium* species (*G. mikimotoi*, *G. breve*, *G. galatheanum*) that contain fucoxanthin and its 19′-acyloxyderivatives (19′-hexanoyloxyfucoxanthin and 19′-butanoyloxyfucoxanthin) as main carotenoids (Jeffrey *et al.*, 1975; Bjørnland and Tangen, 1979; Tangen and Bjørnland, 1981; Bjørnland, 1989; Bjørnland and Liaaen-Jensen, 1989; Bidigare *et al.*, 1990; Suzuki and Ishimara, 1992; Johnsen and Sakshaug, 1993; Millie *et al.*, 1995; Hansen *et al.*, 2000). In addition to fucoxanthin and its 19′-acyloxyderivatives, a novel carotenoid first described in the haptophyte *Emiliania huxleyi* (Garrido and Zapata, 1998) and recently characterized by Egeland *et al.*, (Egeland *et al.*, 2000) as 4-keto-19′-hexanoyloxyfucoxanthin (Zapata *et al.*, 2000) were also detected in the ichthyotoxic dinoflagellate *G. breve* (Zapata *et al.*, 1998). In the same work they reported the presence of a non-polar chlorophyll *c*-like pigment *in G. breve* (Zapata *et al.*, 1998), which was initially described in the haptophyte *Chrysochromulina polylepis*.

In this communication we describe the pigment profile of the ichthyotoxic populations of *Gymnodinium* sp. found in southern Chile in the autumn of 1999. The high-performance liquid chromatography (HPLC) method used (Garrido and Zapata, 1997), allowed the separation of new pigment markers, which could be useful for the detection of this toxic species in natural samples.

Samples of *Gymnodinium* sp. from the coloured patches found during the bloom were cultured at 13.5 ± 0.5°C, under 30 ± 10 μmol quanta m^{-2} s^{-1} photosynthetically active radiator (PAR) during 14 days prior to the pigment analyses. The cells were approximately 35 μm wide, with a large nucleus (10 μm) located opposite the apical extremity. Numerous small chloroplasts were visible (Figure 1). Cells were gently filtered onto 45 mm Whatman GF/F filters. Pigments were extracted from the filters with 100% methanol at –20°C for 24 hours during transportation to Mar del Plata, Argentina. The extracts

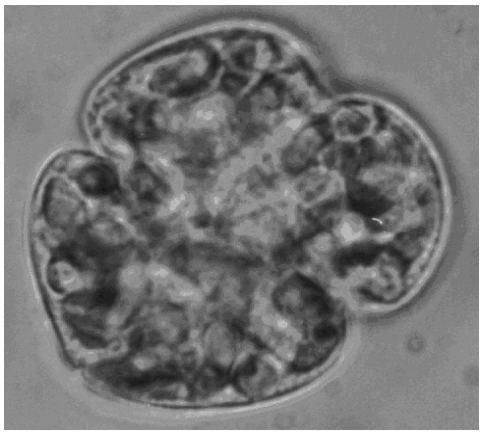

Fig. 1. Cell morphology of *Gymnodinium* sp.

were filtered through GF/F filters to remove cell debris. Immediately before injection, water was added to the extract to get a dilution of 80% methanol (Jeffrey *et al.*, 1997). Aliquots of the sample solution were automatically injected into an HPLC system Shimadzu LC 10 A. For pigment elution we use the method of Garrido and Zapata (Garrido and Zapata, 1997) slightly modified. The column was a Vydac 201 TP 54, 250 × 4.6 mm internal diameter, protected with an Opti-Guard™ C18 guard column. Mixing chamber and column were thermostatted at 27°C by means of a CTO-10 AC (Shimadzu) column oven. Eluent A was a 45 : 35 : 20 mixture of methanol, acetonitrile and aqueous solution (0.25 M pyridine, pH adjusted to 5.3 with acetic acid), and eluent B was always acetone. Peak detection was carried out using a model SPD-M10Avp diode array detector and a FR-10Axl spectrofluorometer. Pigments were identified by on-line diode array spectra and by co-chromatography with pure standards purchased from VKI (The International Agency for ^{14}C Determination, Denmark) or isolated from cultures of *Emiliana huxleyi* (clone CCMP 370).

Gymnodinium sp. showed a complex pigment profile (Figure 2). Pigments included the light-harvesting chlorophyll *a* (23), chlorophyll c_2 (17) and chlorophyll c_3 (16), fucoxanthin (11), 19′-hexanoyloxyfucoxanthin (13) and 19′-butanoyloxyfucoxanthin (9). In addition to fucoxanthin and its 19′-acyloxyderivatives, two carotenoids (7 and 10) with the spectral properties of 19′-acyloxyderivatives were also detected. One of them (10) was identified as the novel carotenoid 4-keto-19′-hexanoyloxyfucoxanthin (Egeland *et al.*, 2000), first described in the haptophyte *E. huxleyi* (Garrido and Zapata, 1997) and also detected in the haptophytes *Chrysochromulina polylepis* and *Phaeocystis* sp. and in the toxic dinoflagellate *G. breve* (Zapata *et al.*, 1998). This pigment has not been previously detected in the European isolates of *Gyrodinium aureolum* [= *Gymnodinium mikimotoi* (Miyake et Kominami) ex Oda according to (Hansen *et al.*, 2000)], in the Japanese *Gymnodinium mikimotoi* (Suzuki and Ishimara, 1992), in *G. breve* (Millie *et al.*, 1995), and in *G. galatheanum* (Johnsen and Sakshaug, 1993), probably because they co-elute with fucoxanthin using HPLC protocols based on monomeric octadecyl silica (ODS) columns (Zapata *et al.*, 1998). Recently however, a peak eluting immediately prior to 19′-hexanoyloxyfucoxanthin and with a similar absorption spectrum, was also found in two (Japanese and Norwegian) strains of *G. mikimotoi* (Hansen *et al.*, 2000). The other carotenoid (7) with spectral properties (Figure 3) of 19′-acyloxyfucoxanthins and higher polarity than 19′-butanoyloxyfucoxanthin, has not been previously characterized. However, their polarity is similar to that of an unidentified carotenoid, detected by Zapata *et al.* (Zapata *et al.*, 1998) in the toxic dinoflagellate *G. breve* and in the haptophyte *Phaeocystis* sp.

In *Gymnodinium* sp. fucoxanthin is the main carotenoid. The next most abundant fucoxanthin derivatives were 4-keto-19′-hexanoyloxyfucoxanthin (10), 19′-butanoyloxyfucoxanthin-like (7), 19′-butanoyloxyfucoxanthin (9) and 19′-hexanoyloxyfucoxanthin (13). Previous studies of the carotenoid composition of ichthyotoxic *Gymnodinium* species (Tangen and Bjorland, 1981; Suzuki and Ishimara, 1992; Johnsen and Sakshaug, 1993; Zapata *et al.*, 1998; Hansen *et al.*, 2000) used different methods of detection, making it difficult to draw a direct comparison. Furthermore, the relative contribution of these acyloxyfucoxanthins not only varied between strains (Jeffrey and Wright, 1994), but also appear to be dependent on the light intensity (Johnsen and Sakshaug, 1993; Schlüter, *et al.*, 2000) and nutrient conditions (Buma *et al.*, 1991; Van Leeuwe and Stefels, 1998) to which the cells are exposed.

Magnesium-2,4-divinyl pheoporphyrin a_5 monomethyl ester (MgDVP), a chlorophyll *a* biosynthetic precursor widely distributed in several algal groups (Zapata *et al.*, 1998), was also detected in our chromatograms (12). In addition to chlorophylls c_2 and c_3, trace amounts of the non-polar chlorophyll *c*-like (27 and 28), of the type described in the haptophyte *C. polylepis* (Zapata *et al.*, 1998) and in the dinoflagellate *G. breve* (Zapata *et al.*, 1998), also occurred in the *Gymnodinium* sp. studied here. Several minor peaks (1, 2, 3, 4, 5, 6, 8) with spectral properties (absorption and fluorescence spectrum) similar to chlorophyll *c* were also detected in trace amounts. This type of compound has been observed in several algal species (Zapata *et al.*, 1998),but their structure, function and metabolic roles are largely unknown.

Photoprotective carotenoids in *Gymnodinium* sp. include diadinoxanthin (18) and diatoxanthin (19). In our shade-adapted cells, diatoxanthin was present in trace amounts, in accordance with the findings of Demers *et al.* and

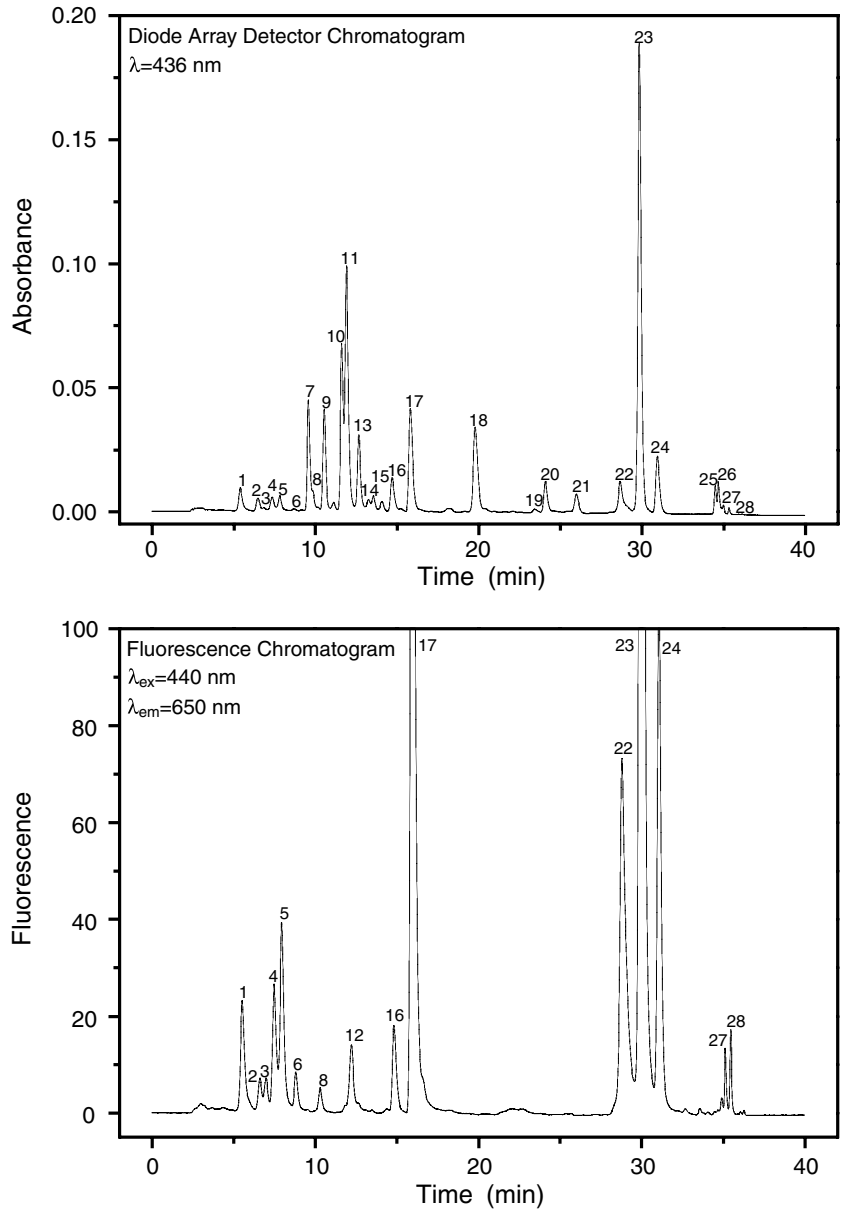

Fig. 2. HPLC chromatograms of the *Gymnodinium* sp. extract. Detected pigments**.** 1, 2, 3, 4, 5, 6 and 8: unknown (chlorophyll *c*-like), 7, 19′-butanoyloxyfucoxanthin-like; 9, 19′-butanoyloxyfucoxanthin; 10, 4-keto-19′-hexanoyloxyfucoxanthin; 11, fucoxanthin; 12, MgDVP; 13, 19′-hexanoyloxyfucoxanthin; 14, 15 and 19, unknown carotenoids; 16, Chl c_3; 17, Chl c_2; 18, diadinoxanthin; 20, gyroxanthin-diester; 21, gyroxanthin-diester-like; 22, Chl *a* allomer; 23, Chl *a*; 24, Chl *a* epimer; 25, α-carotene; 26, β-carotene; 27 and 28, non-polar chlorophyll *c* (C. polylepis type).

Willemoës and Monas (Demers *et al.*, 1991; Willemoës and Monas, 1991). Moreover, diatoxanthin was not detected in shade adapted cells of *G. aureolum* (= *G. mikimotoi*) and *G. galatheanum* (Johnsen and Sakshaug, 1993). As *G. breve* (Zapata *et al.*, 1998) and *G. mikimotoi* (Johnsen and Sakshaug, 1993; Hansen *et al.*, 2000), *Gymnodinium* sp. had both α-carotene (25) and β-carotene (26) as minor components, whereas *G. galatheanum* only has β-carotene (Bjørnland and Tangen, 1979; Johnsen and Sakshaug, 1993).

Gyroxanthin-diester (20) whose light-harvesting and/or photoprotective function are unknown, was also present in *Gymnodinium* sp. In addition, an unidentified carotenoid (21) that eluted after gyroxanthin-diester and displayed a similar absorption spectrum, was also found. The easy formation of a 9-*cis* isomer during manipulation

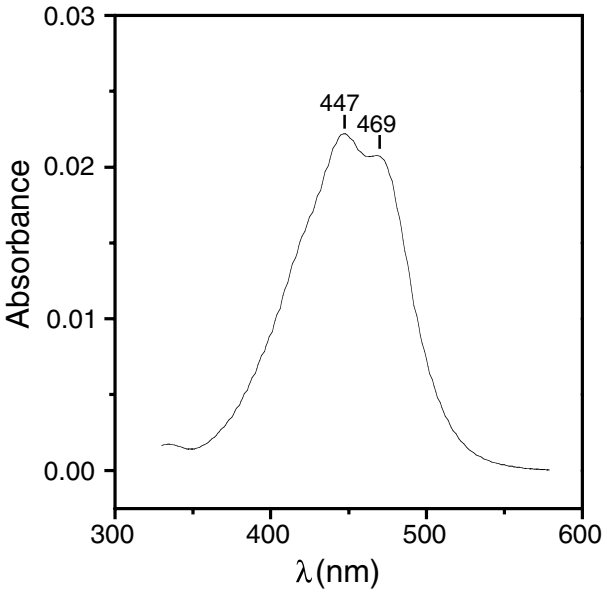

Fig. 3. DAD absorption spectra of 19′-butanoyloxyfucoxanthin-like (peak 7).

and storage of gyroxanthin-diester (Bjørnland, 1989) may have accounted for the presence of this peak. Other unknown carotenoids (14, 15) were present only in trace amounts.

Gyroxanthin-diester has been observed in several toxic fucoxanthin-containing dinoflagellates (Bjørnland and Tangen, 1979; Bjørnland, 1989; Bjørnland and Liaaen-Jensen, 1989; Johnsen and Sakshaug, 1993; Hansen *et al*., 2000). This xanthophyll has not been detected in other species so far, including those members of the *Crysophyceae* and *Prymnesiophyceae*, also reported to contain 19′-acyloxy-fucoxanthins (Bjørnland, 1989; Jeffrey and Wright, 1994; Jeffrey *et al*., 1997). Recently, Bjørnland and Tangen (Bjørnland and Tangen, 2000) reported that gyroxanthin-diester is also present in the chloroplast pigmentation of the pelagophyte *Pelagomonas calceolata*. These authors, guided by the taxonomic distribution of gyroxanthin-diester and fucoxanthins, extended the original hypothesis concerning the phylogenetic evolution of these toxic dinoflagellates, through endosymbiosis of colourless flagellates with prymnesiophytes to include a possible pelagophycean origin (Bjørnland and Tangen, 2000). Notably, an isolate from the Pettaquamscutt River, USA, which probably represents what Hulburt (Hulburt, 1957) described as *Gyrodinium aureolum*, contained peridinin as the major carotenoid (Hansen *et al*., 2000). Analysis of small subunit rRNA from the plastid and the nuclear genome of *G. aureolum* (= *G. mikimotoi*), *G. breve* and *G. galatheanum* also indicates that they have acquired their plastid via endosymbiosis of a prymnesiophyte (Tengs *et al*., 2000). However, there is a considerable molecular and morphological divergence between the three species, with *G. galatheanum* as the most divergent taxa. (Tengs *et al*., 2000) This divergence can explain the minor qualitative differences in their carotenoid pigmentation that include the presence of several esters related to halocynthiaxanthin (Bjørnland, 1989, Bjørnland and Tangen, 2000).

In conclusion, our results showed that the pigment composition of *Gymnodinium* sp. resembled those reported for other ichthyotoxic *Gymnodinium* species. However, in comparison with previous studies on pigment composition of *G. breve*, *G. mikimotoi* and *G. galatheanun* using HPLC methods based on monomeric ODS columns, the pigment profile of *Gymnodinium* sp. was more complex. We were able to detect this complex pigment composition thanks to the enhanced selectivity of the HPLC method used (Zapata *et al*., 1998). Indeed the pigment profile of *Gymnodinium sp.* found here, was very similar to that reported, using the same method by Zapata *et al*., (1998), for the dinoflagellate G. *breve*. These dinoflagellates show some common pigments with some prymnesiophytes (*E. huxleyi*, *C. polylepis* and *Phaeocystis* sp.), (Zapata *et al*., 1998), and therefore, these new pigments cannot be considered as pigment markers for ichthyotoxic dinoflagellates. The only exception is gyroxanthin diester, which could be potentially a useful indicator for the detection of this, or eventually other, toxic *Gymnodinium* sp. in the coastal waters of southern Chile. However, as has been discussed for *G. breve* bloom detection (Millie *et al*., 1997), due to the small contribution of this pigment to total cellular absorption and the fact that their absorption maxima are similar to those of other carotenoids, distinction of this pigment through remote sensing technology is not feasible.

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