Nodularia spumigena blooms and the occurrence of hepatotoxin in the Gulf of Gdańsk*

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Abstract

Nodularia spumigena forms extensive summer blooms in the Baltic Sea. The occurrence of the blooms is determined by water temperature, light intensity and nutrient concentration; levels of nitrogen and phosphorus in particular are critical. The time of the seasonal maximum and intensity of the *Nodularia* bloom in the Gulf of Gdańsk vary significantly from year to year. In 2001 a rapid and massive proliferation of *N. spumigena* was observed in late June – early July. The concentration of nodularin in water ranged from 90 to 18 135 μg dm^{-3} and in lyophilised phytoplankton samples from 3000 to 3520 μg g^{-1} d.w. (dry weight). Such a high concentration of toxin in the recreational waters of the Gulf of Gdańsk constitutes a health risk for users of bathing areas. In 2002, the *N. spumigena* bloom was less dense, but lasted longer, with a maximum in late July – early August. In 2002 the concentration of nodularin did not exceed 12.6 μg dm^{-3} in water and 919 μg g^{-1} d.w. in lyophilised phytoplankton samples. Other cyanobacterial toxins – microcystins and anatoxin-a – were also detected in the coastal waters of the Gulf of Gdańsk.

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1. Introduction

The substantial input of nitrogen and phosphorus compounds from municipal and industrial wastes as well as from agriculture promotes the growth of phytoplanktonic organisms, which are the most frequently used indicators of eutrophication. In the Gulf of Gdańsk the summer bloom of the cyanobacteria *Nodularia spumigena* and *Aphanizomenon flos-aquae* is an annual occurrence favoured by a low N:P ratio, calm weather and high solar radiation; the latter two factors are conducive to the formation of the thermocline. A halotolerant organism, *Nodularia* has a strong potential for growth in estuaries, where it benefits from land-derived nutrients. Extensive cyanobacteria blooms seriously affect water quality in that they cause deoxygenation and produce hydrogen sulphide, a process that is usually accompanied by unpleasant odours. The presence of *Nodularia* in coastal, recreational waters is of particular concern as it produces nodularin, a cyclic pentapeptide with hepatotoxic activity (Fig. 1). The LD_{50} of nodularin is approximately 50 μg kg^{-1} b.w. (body weight) in mice when administrated intraperitoneally (Carmichael et al. 1988). With such a low LD_{50} nodularin is one of the most potent natural toxins. The hydrophobic Adda side-chain and the cyclic structure of nodularin are essential for the molecule's biological activity. At the cellular level the toxin inhibits the activity of protein phosphatases (PP), which are key regulatory enzymes (Yoshizawa et al. 1990). In liver, the inhibition of PP 1 and 2 A leads to cellular disruption and promotes tumour formation (Ohta et al. 1994). Most of the data on nodularin toxicity have been obtained from experiments on rodents.

In Baltic coastal areas several cases of terrestrial animal poisoning associated with a bloom of *N. spumigena* have been reported (Edler et al. 1985, Nehring 1993). Symptoms of poisoning were observed within a few hours after exposure to toxic cyanobacteria and included weakness, vomiting and diarrhoea. Some of the affected animals died as a result of circulatory shock induced by pooling of blood into the liver. The toxin has also been proved to have a negative impact on aquatic biota (Christoffersen 1996). The most pronounced effects have been found in Baltic flounders, which suffer from liver tumours in areas with large-scale blooms of *Nodularia* (Wiklund & Bylund 1994). Nodularin accumulation has been observed in the Baltic mussel, flounder, cod, stickleback, herring and salmon (Sipiä 2001, Sipiä et al. 2001), but exactly how the toxin is transferred from cyanobacteria to fish is unclear. The detected level of nodularin in mussels and fish was quite low and did not exceed the Tolerable Daily Intake (TDI) (0.04 μg kg^{-1}). So far there have been no confirmed reports of human

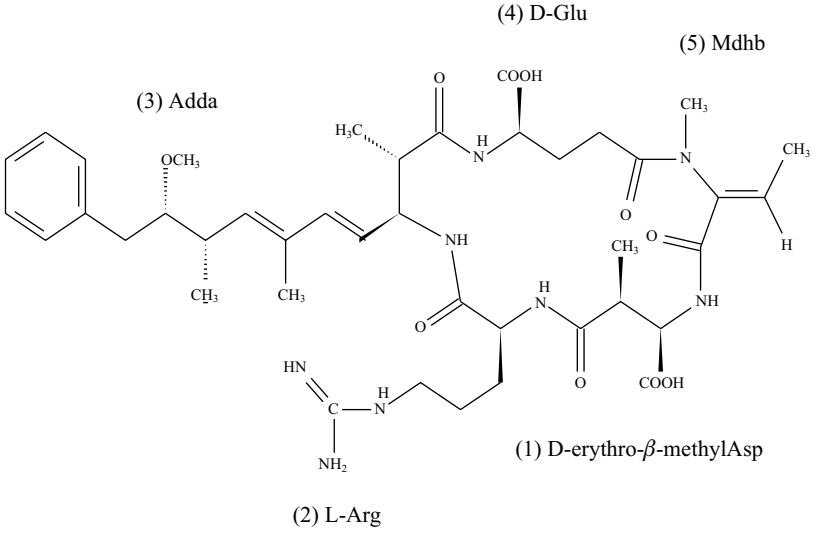

Fig. 1. The chemical structure of nodularin (NOD)

fatalities attributable to the *N. spumigena* toxin. However, people who have bathed in an area with a cyanobacteria bloom have suffered from allergic reactions, skin irritation and ingestion-related illnesses (Codd et al. 1999).

On average, the concentration of nodularin in lyophilised phytoplankton samples from the Baltic Sea ranged from less than 100 to 2400 μg g^{-1} d.w. (dry weight) (Sivonen et al. 1989, Kankaanpää et al. 2001). Hepatotoxin concentrations as high as 18 100 μg g^{-1} d.w. were recorded in samples from the Gulf of Bothnia (Kononen et al. 1993). The average cell-bound concentration of nodularin in water varied between 150–220 μg dm^{-3}, while in filtered water samples it was from 0.01 to 18.7 μg dm^{-3} (Kankaanpää et al. 2001). Elevated concentrations of the toxin, a thousand- to a million-fold higher than in water, can be found in surface scums. These are wind-driven on to the shore where they accumulate, presenting a threat to humans and animals. Interestingly, experiments on mice have demonstrated that these animals prefer water containing toxic cyanobacterial strains to distilled water (Rodas & Costas 1999).

Numerous workers have studied the influence of environmental conditions, such as salinity, light intensity, temperature and nutrient concentration, on toxin production by *N. spumigena*. The results are contradictory and strain-specific (Lehtimäki et al. 1994, Blackburn et al. 1996, Hobson et al. 1999, Repka et al. 2001). Attempts have also been made to describe and understand the complex biological, chemical and physical interactions

that determine the dynamics of *N. spumigena* blooms. Some of the preconditions for blooms to occur are known, but the available knowledge is still too patchy to permit the forecasting of future bloom scenarios.

2. Materials and methods

2.1. Sampling

In the summer months of 2001 and 2002 (June–September), cyanobacteria samples were collected from one onshore station situated at the end of the promenade in Gdynia and during cruises 1 nautical mile off the coast at Gdynia, Sopot, Gdańsk and Świbno near the Vistula river mouth. Fig. 2 shows a map of the study area in the Gulf of Gdańsk (southern Baltic). Surface water samples with suspended organisms were collected and on the same day passed through a Whatman GF/F glass microfibre filter. The volume of the samples ranged from 5 to 200 cm^3, depending on the intensity of the bloom. Additionally, concentrated cyanobacteria samples for toxin analyses were collected with a phytoplankton net (mesh size 100 μm) towed horizontally through the surface layer. The plankton was freeze-dried and, like the other samples, stored at –20°C prior to toxin extraction and analysis. Sub-samples, live or preserved with Lugol solution, were analysed under the microscope in order to determine the proportion of cyanobacteria in the phytoplankton population.

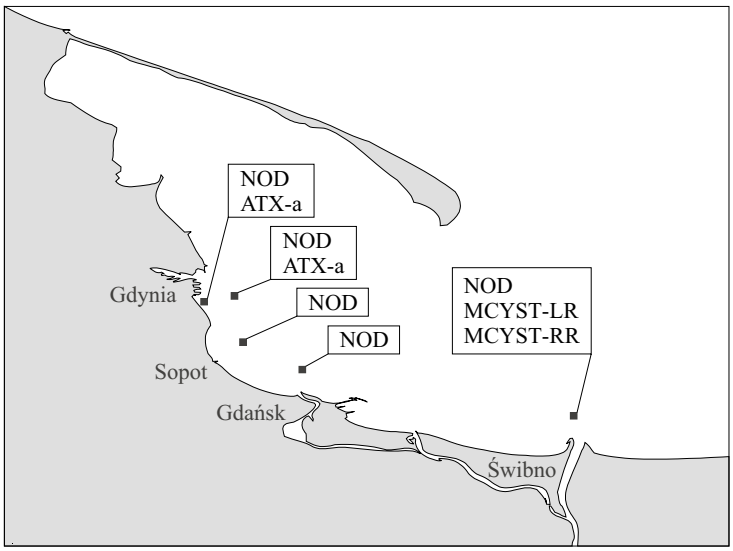

Fig. 2. Sampling locations of *Nodularia spumigena* in the coastal waters of the Gulf of Gdańsk. Sites where different cyanobacterial toxins were found are indicated (NOD – nodularin; MCYST – microcystin; ATX-a – anatoxin-a)

2.2. Extraction and analyses

To determine the total concentrations of cell-bound and extracellular nodularin, a known volume of a water sample was sonicated for 5 min with an HD 2070 Sonopuls ultrasonic homogeniser (BANDELIN, Berlin, Germany) and passed through a glass microfibre filter (Whatman GF/C). The filtrate was concentrated by solid phase extraction (SPE) on 500-mg Sep-Pak tC_{18} cartridges (Waters, Milford, MA, USA), which had been activated with 10 cm^3 of 100% methanol, followed by 20 cm^3 of MilliQ water from an ultra-pure water system (Millipore, Bedford, MA, USA). The filtered water sample was introduced into the cartridge and allowed to flow through it at a rate of about 5 cm^3 min^{-1}. The cartridge was then rinsed with 10 cm^3 of MilliQ water and the toxin eluted with 20 cm^3 of 100% methanol. The solvent was evaporated to dryness *in vacuo* at 35°C and the residue dissolved in 1 cm^3 of 100% methanol for HPLC analysis.

For analyses of cell-bound nodularin, filters with cyanobacteria cells or lyophilised cyanobacteria cells (50 mg) were placed in microcentrifuge tubes. 1 cm^3 of 90% methanol was added to the samples, which were sonicated for 30 s with an HD 2070 Sonopuls ultrasonic homogeniser. After 1 hour, the samples were centrifuged at 13 000 × g for 15 min, following which the supernatants were collected and analysed by HPLC. The methanol extracts from the phytoplankton samples retained on the GF/C filters were additionally analysed for chlorophyll *a* content. Absorption was measured with a Shimadzu UV-1202 UV–Vis spectrophotometer (Australia) at 665 and 750 nm. To optimise the toxin extraction procedure, 50 mg of lyophilised material was extracted sequentially once, twice and three times with 1 cm^3 of 90% methanol. After centrifugation, the supernatants were pooled and evaporated to dryness *in vacuo* at 35°C. Then, 1.0 cm^3 of 90% methanol was added to the residue and the extract analysed with the Waters 3HPLC system. This system consisted of a model 626 pump with model 600S controller, model 917plus auto-sampler and a model 996 photodiode-array detector (PDA) operating in the 200–300 nm range. Separations were performed on a LiChrospher 100 RP-18 column (25 cm 0.4 cm I.D., 5 μm particle size) and a 100 RP-18e LiChroCart cartridge (Merck, Darmstadt, Germany). The mobile phase was a mixture of 10% aqueous acetonitrile:100% acetonitrile (60:40), both containing 0.05% trifluoroacetic acid (TFA). The flow-rate was maintained at 1 cm^3 min^{-1} and the auto-injection volume was 20 μl. The nodularin standard was purchased from Calbiochem Novabiochem (La Jolla, CA, USA). Concentrated nodularin solution (10 μg cm^{-3}) was prepared in methanol. All reagents used for analyses were of HPLC grade. Methanol and acetonitrile were purchased from J. T. Baker (Deventer,

The Netherlands). Deionised-distilled water was obtained using a MilliQ ultra-pure water system (Millipore, Bedford, MA, USA). The toxin was identified by its retention time and characteristic absorption spectrum with a maximum at 238 nm, which is due to the conjugated diene in the structure of the unusual amino acid Adda (Fig. 3). All data were collected and processed using Waters Millennium software. Quantitative analysis of the toxin was carried out using a calibration curve based on peak area measurements for the standard solution. It was confirmed that in a single-step extraction of lyophilised cells with 1 cm^3 of 90% methanol almost the same amount of toxin (1.41 μg mg^{-1} d.w.) was recovered as in two (1.41 μg mg^{-1} d.w.) or three-step (1.44 μg mg^{-1} d.w.) extractions.

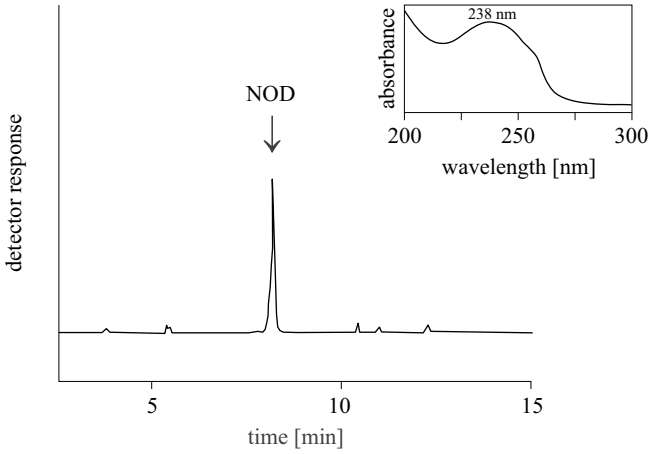

Fig. 3. HPLC chromatogram of a *Nodularia spumigena* sample from the Gulf of Gdańsk. The absorption spectrum of the peak matches the spectrum of authentic nodularin

3. Results and discussion

Blooms of *N. spumigena* and their production of toxin in the coastal waters of the Gulf of Gdańsk were studied in 2001 and 2002; there was a marked difference in the bloom dynamics between the two years. In June 2001, a highly intensive *N. spumigena* bloom developed in a very short space of time. In samples collected at the end of the promenade in Gdynia, a high concentration of *Nodularia* filaments and a toxin concentration 0.6 μg dm^{-3} were recorded at the end of June. On 5 July the wind pushed the floating cyanobacteria towards the shore. This resulted in a sudden increase in surface chlorophyll *a* concentration, from 100 μg dm^{-3} in the morning to 5800 μg dm^{-3} in the afternoon (Fig. 4). On the next day

a further increase in chlorophyll *a* concentration up to 17 625 μg dm^{-3} was measured. Since the hepatotoxin-producing *N. spumigena* made up about 80–90% of the phytoplankton biomass, a dramatic rise in nodularin concentration from 90 μg dm^{-3} on 5 July (morning) to 18 135 μg dm^{-3} on the following day was also recorded (Fig. 4). During this intensive bloom of *Nodularia*, cell-bound nodularin made up about 80–90% of the total toxin concentration. In the lyophilised phytoplankton samples collected in 2001, the nodularin concentration varied between 3000–3520 μg g^{-1} d.w. and was higher than the average values for the Baltic Sea (100–2400 μg g^{-1} d.w.). These were the highest concentrations of nodularin recorded in our studies. According to Falconer et al. (1999), cyanobacterial hepatotoxin at such concentrations poses a high risk of adverse health effects for water users. The situation in 2001 forced the local authorities to close temporarily all the beaches along the Gulf of Gdańsk. From 6 to 8 July the nodularin concentration decreased significantly and, after torrential rains on 9 July, the *N. spumigena* bloom suddenly ceased. Despite the later improvement in the weather and the high water temperature, blooms of *N. spumigena* did not recur in 2001. In all samples collected during the dense bloom, the ratio of nodularin to chlorophyll *a* concentration was close to 1 (0.9–1.25). In culture experiments on *N. spumigena* isolated from the Gulf of Gdańsk, this ratio never reached such a high value and, depending on the culture conditions, varied between 0.2–0.65 (Mazur, unpublished).

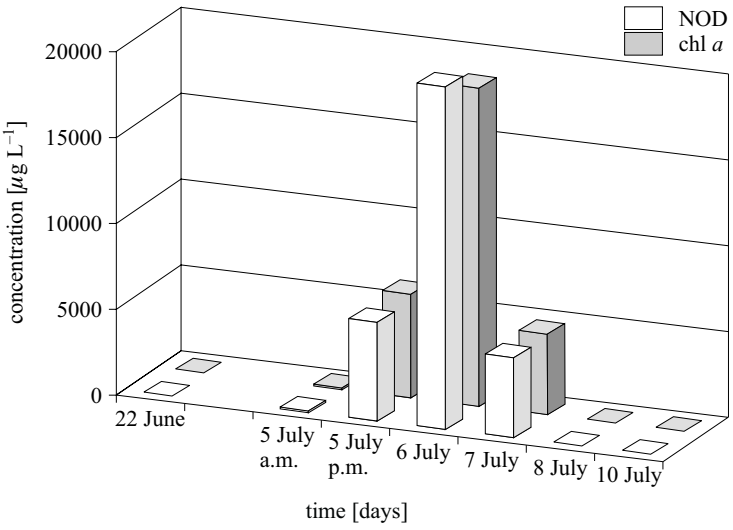

Fig. 4. Nodularin and chlorophyll *a* concentrations in samples collected in 2001 from the promenade site in Gdynia

In 2002 elevated concentrations of *N. spumigena* filaments and a nodularin concentration of 0.3 μg dm^{-3} were recorded in the Gulf of Gdańsk as early as 10 June. In that year the total concentrations of nodularin in the water ranged from just traces to 12.6 μg dm^{-3} (Figs. 5 and 6). Nodularin concentrations in lyophilised phytoplankton samples were also low and ranged from 5 to 919 μg g^{-1} d.w. This level of hepatotoxin concentration represented only a moderate health risk to water users (Falconer et al. 1999). Even the highest concentrations of nodularin measured in the coastal waters of the Gulf of Gdańsk in late July and early August (Fig. 6) were three orders of magnitude lower than those in the 2001 samples. In 2002 the bloom did not reach the intensity recorded in the previous year, but it lasted much longer – for over two months. The prolonged *Nodularia* bloom in 2002 occurred during favourable weather conditions – it was an exceptionally warm and sunny summer with water temperatures in excess of 20°C. Depending on the sampling time and site *N. spumigena* made up from 10% to 80% of the phytoplankton population and mostly co-occurred with *A. flos-aquae* and *Anabaena*.

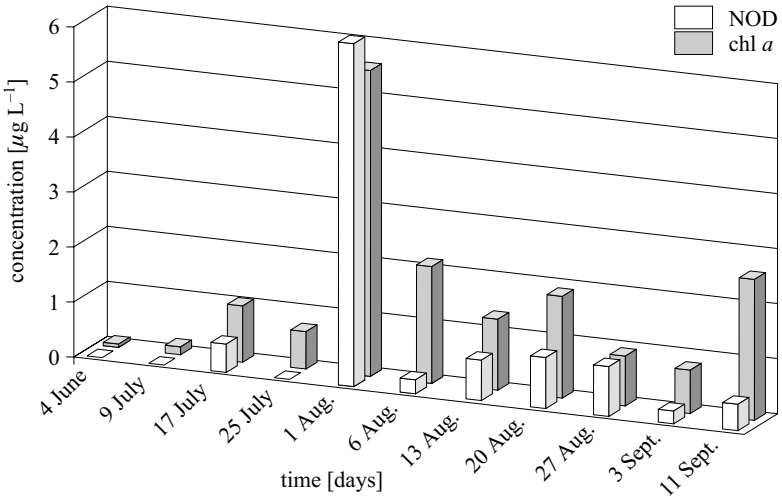

Fig. 5. Nodularin and chlorophyll *a* concentrations in samples collected in 2002 from the promenade site in Gdynia

According to Lahtimäki (2000) *N. spumigena* is the only toxin-producing cyanobacterium in the Baltic Sea. In the coastal waters of the Gulf of Gdańsk some other toxic or potentially toxic cyanobacteria from fresh water have been recorded (Pliński & Jóźwiak 1996, Pliński et al. 1998, Witek & Pliński 1998). The occurrence of fresh water species in brackish and marine waters is a result of well-developed adaptive strategies in

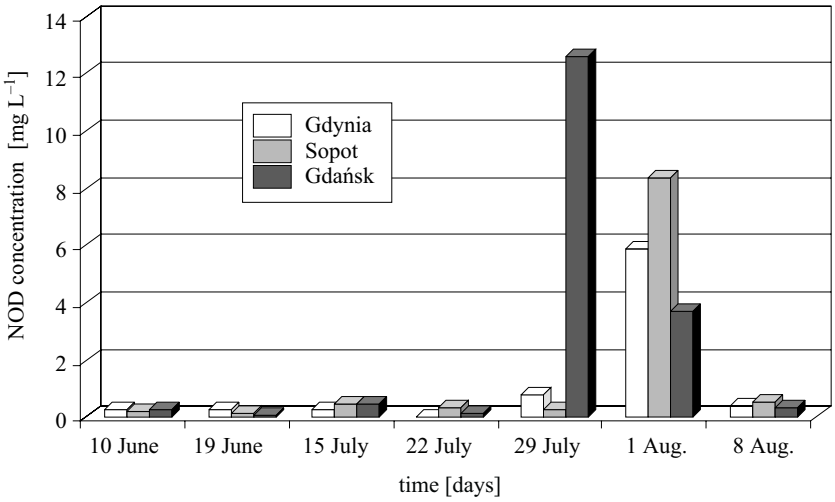

Fig. 6. Nodularin concentrations in samples collected in 2002 during cruises 1 nautical mile off the coast at Gdynia, Sopot and Gdańsk

cyanobacteria, which allow them to survive, grow and even flourish in different environmental conditions. Phytoplankton samples collected near the Vistula river mouth off Świbno at the end of July exhibited a more complex hepatotoxin profile than did the samples from other stations. In this region *N. spumigena* made up about 35% of the phytoplankton and the concentration of nodularin was 0.6 μg dm^{-3} in water and 712 μg g^{-1} d.w. in a lyophilised phytoplankton sample. Apart from nodularin, there were some microcystins present as well; two of them were identified as microcystin-LR (MCYST-LR) and microcystin-RR (MCYST-RR) (Fig. 2). Microscopic analysis of the samples revealed a 10% presence of the fresh water cyanobacterium *Microcystis aeruginosa*, which is a major producer of cyclic heptapeptide hepatotoxins known as microcystins. Another cyanobacterial toxin, identified as anatoxin-a (ATX-a), was found in samples collected off the coast at Gdynia at the beginning of September (Fig. 2). This cyanobacterial neurotoxin is produced mainly by *Anabaena*, which made up 20% of the phytoplankton population in those samples.

No correlation between the nodularin concentration and the proportion of *Nodularia* in the phytoplankton biomass was found for the 2002 samples. This discrepancy may be due either to the presence of various *Nodularia* strains characterised by different rates of nodularin synthesis, or to the impact of different environmental conditions. In our laboratory experiments on *N. spumigena* isolated from the Gulf of Gdańsk, the cell content of nodularin rose with increasing salinity of the culture medium (from 3 to 35 PSU), while a greater light intensity (from 10 to

80 μmol photon m^{-2} s^{-1}) caused the rate of nodularin synthesis to fall significantly (Mazur, unpublished). Under natural conditions, the interrelations between the different biotic and abiotic factors affecting biological processes in cyanobacteria are much more complex and their role in toxin production in nature is still not fully understood.

In a way, the *N. spumigena* blooms in the last two years were exceptional: in 2001 because of their high intensity and in 2002 because of their duration. The two-year studies revealed an earlier start to cyanobacteria blooms than previously reported (Sivonen et al. 1989, Kononen et al. 1993, Kankaanpää et al. 2001). According to Pliński & Jóźwiak (1996), *N. spumigena* blooms occur in the Gulf of Gdańsk mainly in July and August. In 2001 and 2002 we observed high concentrations of *N. spumigena* filaments as early as the beginning of June. As *Nodularia* can form toxic blooms at any time in summer, cyanobacteria and cyanotoxins in coastal recreational waters need to be monitored on a regular basis.

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