RESEARCH ARTICLE   **ARAŞTIRMA MAKALESİ**

An assesment of domoic acid levels as a function of salinity-temperature variations in the Sea of Marmara

Marmara Denizi'ndeki domoik asit seviyelerinin tuzluluk-sıcaklık değişimlerinin bir fonksiyonu olarak değerlendirilmesi

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Abstract: This study presents the domoic acid (DA), responsible from Amnesic Shellfish Poisoning (ASP), levels of plankton net samples in the Sea of Marmara during April, July and October 2019. Plankton net samples were collected from 13 stations, which were located close to the Istanbul metropolitan area, and along the east and southwest parts of the Sea of Marmara. DA measurements were done by high-performance liquid chromatography (HPLC), using the diode-array detection (detection limit (LOD): 0.108 µg mL⁻¹). Twenty-two out of the 26 plankton net samples (84.6%) were found to contain DA during the study period and the concentrations varied between 0.22 and 3.45 µg mL⁻¹. Possible relationships between physical parameters (salinity-temperature) and DA concentrations were investigated. DA production was mainly controlled by salinity (r:0.47, p<0.05; n:26) along the study period. These data may be used to consider the probability of finding similar conditions in different parts of the Sea of Marmara in order to determine the potential risks of DA to local mussel aquaculture and fisheries.

Keywords: Domoic acid, *Pseudo-nitzschia*, Sea of Marmara, HPLC

Öz: Bu çalışmada Nisan, Temmuz ve Ekim 2019 dönemlerinde Marmara Denizi'nden alınan plankton kepçe örneklerinde Amnezik Kabuklu Zehirlenmesi'ne (AKZ) neden olan denizel biyotoksin, domoik asit (DA), seviyeleri sunulmaktadır. Plankton kepçe örneklemeleri, Marmara Denizi'nin doğusunda, güneybatısında ve İstanbul metropol bölgesinin yakınında bulunan toplam 13 adet istasyondan yapılmıştır. Domoik asit ölçümleri, diode-array dedeksiyonu kullanılarak Yüksek Performanslı Sıvı Kromatografisi yöntemiyle gerçekleştirilmiştir (Belirleme limiti: 0,108 µg mL⁻¹). Çalışma dönemi boyunca alınan toplam 26 adet plankton kepçe örneğinden 22'sinin (%84,6) DA içerdiği tespit edilmiş ve bu örneklerdeki konsantrasyon aralığı 0,22 – 3,45 µg mL⁻¹ olarak belirlenmiştir. DA konsantrasyonları ile fiziksel parametreler (tuzluluk ve sıcaklık) arasındaki olası ilişkiler incelenmiş ve çalışma dönemi boyunca DA üretiminin genel olarak tuzluluk (r:0,47, p<0,05; n:26) tarafından kontrol edildiği tespit edilmiştir. Çalışma sonucunda elde edilen bu veriler, Marmara Denizi'nin farklı bölgelerinde de aynı koşulların bulunma olasılığının değerlendirilmesinde kullanılabileceği gibi, yerel midye yetiştiricilik ve balıkçılık alanlarındaki potansiyel riskleri belirlemek açısından da kullanılabilecektir.

Anahtar kelimeler: Domoik asit, *Pseudo-nitzschia*, Marmara Denizi, HPLC

INTRODUCTION

Domoic acid (DA), a naturally occuring neurotoxin, was first isolated from the red macroalgae *Chondria armata* (Takemoto and Daigo, 1958). This tricarboxylic neurotoxin is a crystalline water-soluble amino acid with a secondary amino group and belongs to the kainoid class of compounds (Saeed et al., 2017). DA can be transferred via the food web and cause amnesic shellfish poisoning (ASP) in humans. ASP, is a kind of intoxication with gastrointestinal symptoms (vomiting and diarrhea) and following neurological disorders (short time memory loss and coma) on humans (Lefebvre and Robertson, 2010). Perl et al., (1990) reported the first ASP case in Prince Edward Island, Canada in 1987, over 100 people were sickened and three died, after the consumption of DA contaminated blue mussels (*Mytilus edulis*). Also, DA accumulated in filter feeders can be harmful to higher trophic levels, such as sea lions, whales and birds (Fire et al., 2010; Scholin et al., 2000). Since the incident in Canada, growing number of cases has been reported and DA widely investigated in many regions of South America (Buck, 1992; Hallegraeff, 2004), Europe (Klein et al., 2010; Vrieling et al., 1996), Australia (Lapworth et al., 2001), North Africa (Sahraoui et al., 2012) and Asia (Likumahua et al., 2019).

Pseudo-nitzschia, a chain forming diatom genus, is among the most widely distribute members of marine phytoplankton communites around the world (Almandoz et al., 2008; Hasle, 2002; Kudela et al., 2004; Ljubešić et al., 2011; Teng et al., 2014; Tenorio et al., 2016). The last reported total number of identified *Pseudo-nitzschia* species is 52 of which 26 are thought to be capable of producing DA (Bates et al., 2018). Moreover, many studies have reported that the toxic species of the genus *Pseudo-nitzschia* are frequent members of the phytoplankton community in the Golden Horn Estuary and Sea of Marmara in recent years (Dursun and Tas, 2019; Tas et al., 2016; Tas and Lundholm,

2017; Tas and Yilmaz, 2015). Furthermore, many studies have shown the importance of a variety of factors such as temperature (Lewis et al., 1993), salinity (Thessen et al., 2005), pH (Lundholm et al., 2004) and nutrients (Bates et al., 1991). Thus, it is essential to understand the factors that stimulate *Pseudo-nitzschia* growth and DA production.

The Sea of Marmara, an inland sea, is a transition zone between the Black Sea and the Mediterranean Sea (Figure 1). It is located between Europe and Asia, and together with the Dardanelles and Bosphorus forms the 'Turkish Strait System'(Beşiktepe et al., 1994). The characteristic of the system is the permanent stratification seperating the Black Sea originated less saline upper layer (~18 psu) from the saline Mediterranean waters (~38 psu) of the lower layer (Ünlülata et al., 1990). It is under dense anthropogenic pollution pressure and it has numerous fragile bays where frequent algal blooms have frequently been observed. Furthermore, the industrialized regions of the Gulf of Izmit, and Gemlik, Bandirma, Erdek and Tekirdag Bays are some of the most densely populated coastal regions in Turkey. Thus, these regions constitute a unique and complex experimental site for examining the relation between natural and anthropogenic factors influencing the development of harmful algal blooms in coastal waters. Phytoplankton monitoring is conducted in the region since 1990s revealing potentially toxic genera, such as *Alexandrium*, *Gymnodinium*, *Dinophysis*, and *Pseudo-nitzschia* has been by microscopy (Aktan et al., 2005; Deniz and Tas, 2009). However, the monitoring programs was only focused on the abundance and distribution of phytoplankton with particular attention to harmful and toxic species, nor did it include associated toxin analyses.

Until now, DA production has been reported in Golden Horn Estuary, Turkey (Dursun et al., 2017, 2018; Tas et al., 2016). Only one study has revealed the relationship between DA and environmental factors and it focused on the smaller parts of the Sea of Marmara (Dursun et al., 2016). On the other hand, environmental factors that promote the production of DA are very complex and can be unique to regions (bays, gulfs, coastal zones or open seas) and seasons (Trainer et al., 2012). This complexity and varying environmental factors needs broader areas and further investigations. To our knowledge, no detailed and particular study addressed DA levels in the Sea of Marmara, including the Istanbul metropolitan area, east and southwest part of the region. On the other hand, the coast along the Sea of Marmara has a large human population that consumes fish and other seafood products, making them potentially vulnerable to ASP.

Given the complete lack of information on potential DA levels in the Sea of Marmara, the present study firstly addressed the possible presence and variability of domoic acid in plankton net samples. Secondly, physical parameters that might regulate the production and variability of DA were investigated. For this purposes, seasonal cruises were conducted at 13 stations, which represents spring (April 2019), summer (July 2019) and autumn (October 2019) conditions, water column properties as well as DA concentrations were analysed.

MATERIALS AND METHODS

Field sampling

Seasonal cruises were conducted in the Sea of Marmara during spring (April 2019), summer (July 2019) and autumn (October 2019). Plankton net samples were collected from 13 stations (Table 1) which were located close to the Istanbul metropolitan area (ST1-ST4), and along the east and (ST5-ST9) southwest parts of the Sea of Marmara (ST10-ST13) (Figure 1). The selection of the sampling sites was based on different criteria related to the hydrological regime and the localization of urban and industrial discharges. Plankton net samples were obtained from the 20 m depth to the surface (upper layer) vertically with a Nansen net (57 µm mesh size, 55 cm Ø). A sample volume between 25 and 180 mL was filtered through a Whatman GF/C (0.45 µm, 47 mm Ø) glass fiber filter then kept at - 20°C until extraction and HPLC analysis (Bates et al., 1991). As physical indicators, temperature and salinity measurements were recorded using a SBE 911 plus CTD system and a multi-parameter probe (YSI Professional Pro Plus) to trace changes in physical conditions, which could act as a trigger for domoic acid production (Dursun et al., 2016). All environmental data were used as the average of 0-20 m depths.

Toxin extraction and analysis in plankton samples

Duplicate portions of plankton net samples were filtered through a 47mm Whatman GF/C filter to determine domoic acid concentrations in plankton samples. The filter was rolled, placed in a test tube and kept frozen until analysis. Samples were extracted according to the procedure described by Milley et al. (1990), that is, extracted with 5 mL 50% (v/v) aqueous methanol, vortexed for 2 min and centrifuged at 4000 rpm for 10 min at 20°C. The supernatant was filtered through a Millex-GS 0.22 µm disposable filter (if necessary) into a vial and 20 µL were injected into the column.

Table 1. List of the sampling stations

Sampling date	Station	Region	Depth (m)
April, October 2019	ST1	Kucukcekmece	38
April, October 2019	ST2	Buyukcekmece	50
April, October 2019	ST3	Princes' Islands	96
April, October 2019	ST4	Tuzla	93
April, October 2019	ST5	Yalova coast	57
April, October 2019	ST6	Cinarcik	1267
April, October 2019	ST7	Gemlik Bay	100
April, October 2019	ST8	Imrali Island (Northwest)	200
April, October 2019	ST9	Imrali Island (West)	46
April, July 2019	ST10	Marmara Island (North)	90
April, July 2019	ST11	Marmara Island (West)	70
April, July 2019	ST12	Pasalimani Island (South)	40
April, July 2019	ST13	Avsa Island (Southwest)	60

Chromatographic analysis were performed using a Hewlett-Packard (HP) Model 1100 equipped with an inline degasser, quaternary pump, autosampler and diode-array detector; data collection and results processing were performed using the HP Chemstation software. Toxins were separated on a Macherey-Nagel Nucleodour 100 RP-C18 (250 mm × 4.6 mm, 5 µm) column. Detection wavelength was set at 242 nm with a 10 nm band width; the reference wavelength was 450 nm with a 100 nm bandwidth. The mobile phases were Milli-Q water plus 0.1% (v/v) trifluoroacetic acid (TFA) and acetonitrile (MeCN) plus 0.1% TFA. The flow rate was set at 0.4 mL min^{-1}. Gradient elution was programmed at 25% MeCN, maintained for 15 min and increased to 50% over 15 min, which was maintained for 5 min. Elution was then followed by an increase to 100% MeCN over 5 min before programming back to initial conditions over 5 min. Initial conditions were maintained for a further 5 min, resulting in a total cycle time of 30 min according to Tas et al. (2016). DA has a retention time of 8.8 min. The seven-point calibration curve was linear for the range 0.125 –10 µg mL^{-1} (r^2: 0.99, n: 3). The LOD, based on a signal to noise ratio of 3.3, was 0.108 µg mL^{-1} and limit of quantification (LOQ) was 0.326 µg mL^{-1}. DA concentrations were examined by Pearson Product-Moment Correlation analysis for their relationships with physical parameters. Data analyses were conducted using the Software package STATISTICA 6.0.
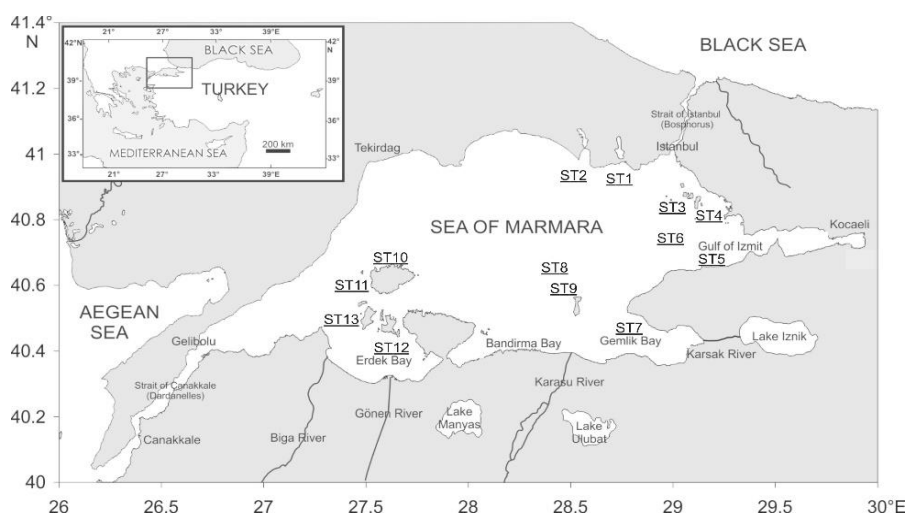

Figure 1. Map of the study area showing the location of the sampling stations in the Sea of Marmara

RESULTS AND DISCUSSION

Average (0-20 m) salinity showed a slightly spatial and remarkably seasonal pattern during the study period, ranging between 16.60 (ST11, July 2019) and 28.10 (ST4, October 2019) (Figure 2a). Average salinity was relatively stable between 24.40 and 26.00 during April 2019, decreased slightly to 22.10 in July 2019 and started to increase 26.90 at the end of the sampling period (October 2019).

Average (0-20 m) water temperatures showed a seasonal pattern during the study period, ranging between 10.7°C (ST13, April 2019) and 23.4°C (ST12, July 2019) (Figure 2b). During April 2019, the average temperature was 11.9°C, increased remarkably to 21.2°C in July 2019 and started to decrease 19.4°C at the end of the sampling period (October 2019). The highest difference between the stations were recorded at stations ST10, ST11, ST12 and ST13, which were located at southwest part of the Sea of Marmara.

HPLC chromatograms clearly demonstrated the presence of DA in plankton net samples (Figure 3). Twenty-two out of the 26 plankton net samples (84.6%) were found to contain DA during April and October 2019, but no DA was detected during July 2019 at the southwest part of the Sea of Marmara.
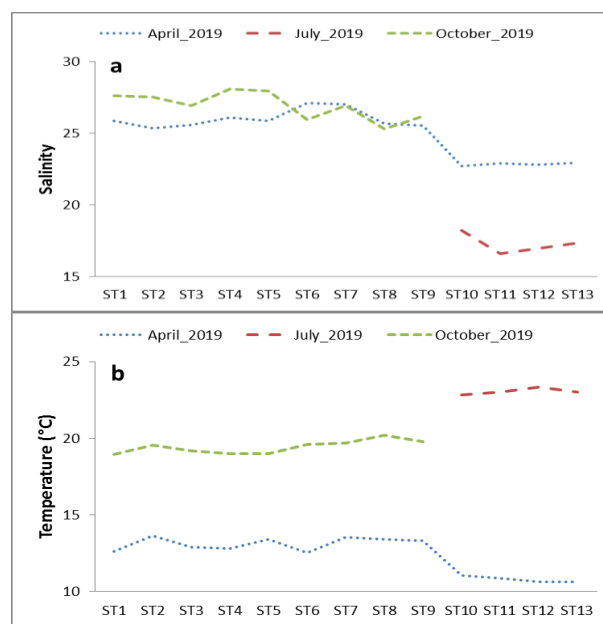

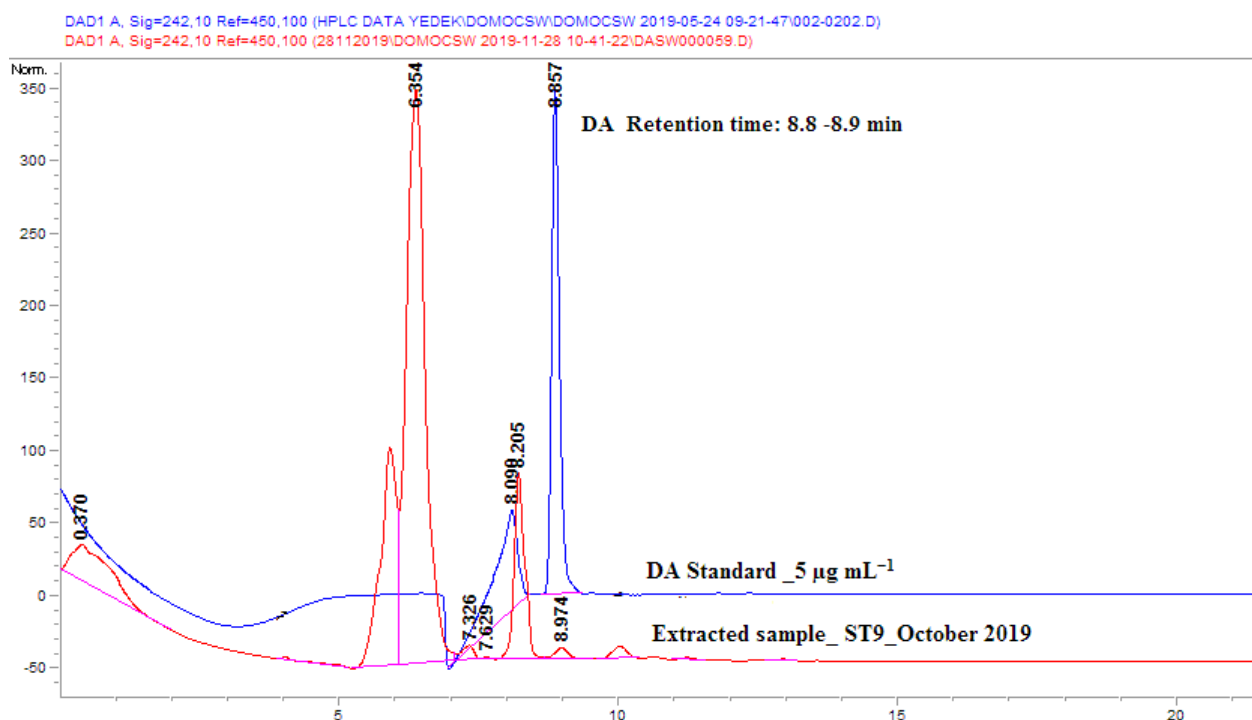**Figure 2.** Changes in salinity (a) and temperature (b) during the study period

Figure 3. The HPLC-DAD chromatograms of DA standard (blue line) and plankton net sample extract (red line)

DA levels showed significant seasonal variation, and the concentrations varied between 0.22 and 1.27 µg mL^{-1} in April 2019 and 0.89-3.45 µg mL^{-1} in October 2019 (Figure 4). DA levels were generally higher during October 2019 and the highest concentrations (2.66 and 3.45 µg mL^{-1}) were observed at stations ST8 and ST9, which were located along the east part of the Sea of Marmara (Figure 4). Lower range of DA concentrations were measured in April 2019, with a mean of 0.89 µg mL^{-1} at the stations ST10, ST11, ST12 and ST13, which were located southwest part of the Sea of Marmara. When compared with other stations, ST8 (Imrali Island, Northwest) and ST9 (Imrali Island, West) are not close to the Istanbul metropolitan area and under the effect of inputs via industry and/or rivers. Also these results agree with the Bates et al. (1991) which reported the higher DA concentrations at offshore stations. DA distribution is not associated with highly elevated nutrient concentrations and chlorophyll-*a* peaks characteristics of the coastal waters immediately affected by the river inputs (Bates et al., 1991). Thus, these differences may stimulate the production of DA at these stations in the Sea of Marmara.

In recent years, some studies have reported DA concentrations, collected samples by plankton net, from different regions of the world. Toxic species of *Pseudo-nitzschia* have been reported from Lisbon Bay, Portugal (Vale and Sampayo, 2001), Queensland, Australia (Takahashi et al., 2007), Nha Phu Bay, Vietnam (Dao et al., 2009) and Sea of Marmara (Dursun et al., 2016) with highest values of 700, 120, 12 and 5.25 µg mL^{-1}, respectively. Sampling depth and region, sample volume and collection methods can affect DA

levels. In this study, plankton sampling was conducted with 55 µm mesh size of plankton net instead of commonly used 20 µm mesh size and maximum value was measured as 3.45 µg mL^{-1}. Our results are expected to be more similar to the results of Takahashi et al. (2007) and Dursun et al. (2016), which may be due to the fact that phytoplankton samples were collected in both studies with similar mesh-sized plankton nets. However, in this study, DA concentrations were detected as high as in different regions (Schnetzer et al., 2007; Trainer et al., 2000) faced with DA toxicity events, with maximum values of <1.5 µg mL^{-1}.

It has previously been reported that *Pseudo-nitzschia* genus is the regular and frequent member of phytoplankton assemblages in the Sea of Marmara and, abundances varied between 1×10^3 and 186×10^3 cells L^{-1} (Deniz and Tas, 2009; Taş et al., 2011). Some species of the genus have the ability to produce DA (Dursun et al., 2016). On the other hand, no ASP case has ever documented in the Sea of Marmara, in spite of the presence of toxic *Pseudo-nitzschia* species. Despite the current conditions appearing to be unfavourable for the formation of dense and highly toxic blooms of *Pseudo-nitzschia* in the Sea of Marmara, it is known that physical factors can stimulate DA production in different ways, as Granéli and Flynn (2006) and Macintyre et al. (2011) stated. It can be said that differences in DA levels between the regions due to the changes in environmental conditions including temperature and salinity. Pearson correlation analysis showed that DA production was mainly controlled by salinity (r:0.47, p<0.05; n:26) along the study period. But no correlation was found between temperature and DA. This

relationship is similar to study which is reported by Busse et al. (2006) for San Diego, California and Dursun et al. (2016), for the Sea of Marmara during February 2011; but also it differs from the data given for the Golden Horn Estuary between August 2011 and July 2012 by Dursun et al. (2018).
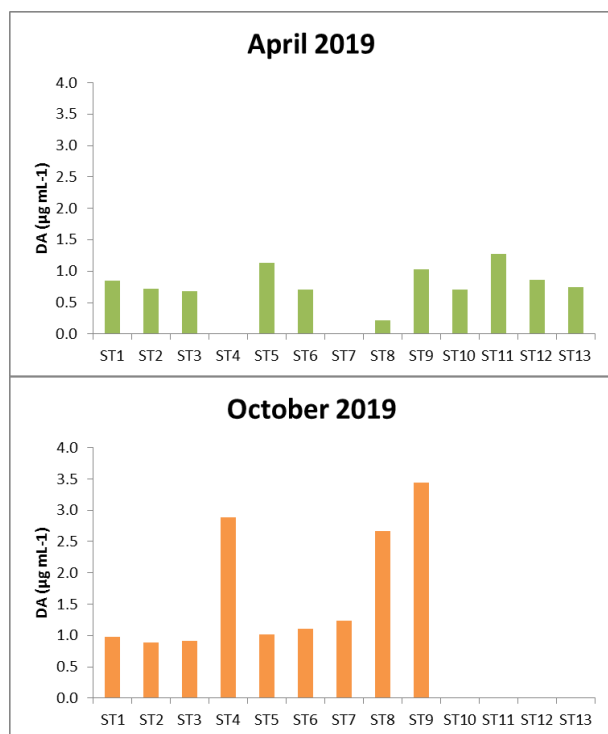

Figure 4. The spatio-temporal distributions of domoic acid levels in plankton net samples

Here, the results of preliminary research related to the DA production in the Sea of Marmara were presented. Even when the sampling was not exhaustive, domoic acid was found to be present in plankton samples collected during April and October 2019. This means that the human population and the shellfish resources could be affected by amnesic shellfish poisoning. This study shows the importance of understanding the DA production process in relation to the physical conditions in plankton net samples. However, more investigation containing all environmental variables is needed to get a clear understanding of DA production.

This work did not include the detection of DA accumulation in shellfish and fish species, but that would be a logical step for further studies on DA production in the Sea of Marmara. There have not yet been any reports of illness in humans or marine life due to DA poisoning in the region. Even if the DA concentrations are generally low, there is a potential for toxin production to occur in this region. Thus, more research is needed to confirm the capacity of the genus *Pseudo-nitzschia* in coastal waters of Turkey to produce DA, since this species exists in phytoplankton blooms. It is very important to understand better and manage the impact of potential harmful algal blooms, and in order to prevent human intoxication and losses to aquaculture.

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