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The use of diatoms in forensic science: advantages and limitations of the diatom test in cases of drowning

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Abstract: Diatoms are unicellular, photosynthetic, eukaryotic organisms often classified as among the algae. There are around 15 000 known species, but many more have yet to be described. Their uniqueness lies in the siliceous covering of the cell, each being encased in a pair of silica valves. Silica is virtually inert and indestructible, so after the organism's death the silica parts remain. The silica parts provide information for the classification of these diverse organisms. Diatoms have been used in forensic science in a variety of ways, the most frequent being the diagnosis of death by drowning. When a person drowns, water will enter the lungs and then enter the bloodstream through ruptures in the peripheral alveoli before being carried to the other organs such as the liver and heart. Naturally, the microscopic contents of the water, which will include diatoms, will pass into the blood as well. The detection of diatoms in the organs can contribute to a diagnosis of death by drowning, a process referred to as the 'diatom test'. We will discuss this test in more detail, illustrating our discussion with real examples.

Diatoms are unicellular, photosynthetic, eukaryotic organisms that inhabit almost all bodies of water. They are found in springs, rivers, ponds, lakes, ditches and in freshwater, brackish and marine waters (Smol & Stoermer 2010) and occur in terrestrial habitats such as wet rocks, mosses and soils, even caves (Falasco *et al.* 2014; Tofilovska *et al.* 2014). They are either planktonic (living in the open water) or benthic (growing associated with or attached to a particular substrate); they can, on occasion, be found in tap water (Schneider 1980), bathwater (Ago *et al.* 2011), well water Nikolova *et al.* 2002) and even airborne (Geissler & Gerloff 1966; Dayan *et al.* 1978; Romero *et al.* 1999).

These specific qualities have meant that diatoms have been used in forensic science in a variety of ways, the most frequent being the diagnosis of death by drowning (Peabody & Cameron 2010). When a person drowns, water will enter the lungs and then the bloodstream through ruptures in the peripheral alveoli and be carried to the other organs, such as the liver and heart (Pollanen 1998). Naturally, the microscopic contents of the water, which will include diatoms, will pass into the blood. Detection of diatoms in various organs can contribute to the diagnosis of death by drowning, a process referred to as the 'diatom test'. The use of diatoms (the 'diatom test') to diagnose a cause of death by drowning is often one of a number of independent techniques utilized by the forensic pathologist. We discuss the test and its limitations below, along with some experimental studies, but first we provide an overview of these complex but fascinating organisms.

Diatoms: a brief introduction to their classification, ecology and diversity

Diatoms are photosynthetic so traditionally have been classified as a group of algae, but a more accurate (phylogenetic) placement is among a group of organisms named stramenopiles (Heterokonta). These include an array of diverse organisms, photosynthetic and as well as non-photosynthetic, such as kelps (the large classical examples of seaweeds) and water-moulds (e.g. Oomycetes such as *Phytophthora*) (Adl *et al.* 2005). While being morphological and physiologically diverse, all stramenopiles share a distinctive 'hairy' flagellum (in diatoms this is restricted to their gametes) and have distinctive molecular signatures. For a recent review of relationships amongst the stramenopiles see Riisberg *et al.* (2009); for a lengthy discussion of problems in naming organisms in this heterogeneous group see Blackwell (2009).

From: Williams, M., Hill, T., Boomer, I. & Wilkinson, I. P. (eds) 2017. *The Archaeological and Forensic Applications of Microfossils: A Deeper Understanding of Human History*. The Micropalaeontological Society, Special Publications. Geological Society, London, 261–277.

Each diatom cell is enclosed in a silica case (called the frustule) which have distinctive shapes and ornamentations. The frustule is composed of several parts, namely two valves (the two might be almost identical or completely different, varying in shape and ornamentation), which are separated by a series of silica hoops referred to as the girdle bands. A detailed discussion of the parts of the silica frustule can be found in Anonymous (1975), although this is now a little dated and has since been supplemented by numerous studies (e.g. Round *et al.* 1990); some examples of diatom valves can be seen in Figures 1–4. The various ornamentations found on the silica frustule, in particular the valves, is often species-specific and can therefore provide important information for their identification and classification (e.g. Round *et al.* 1990).

Diatoms have traditionally been classified according to the shape of their valves, divided into two major structural groups: the 'centric' diatoms

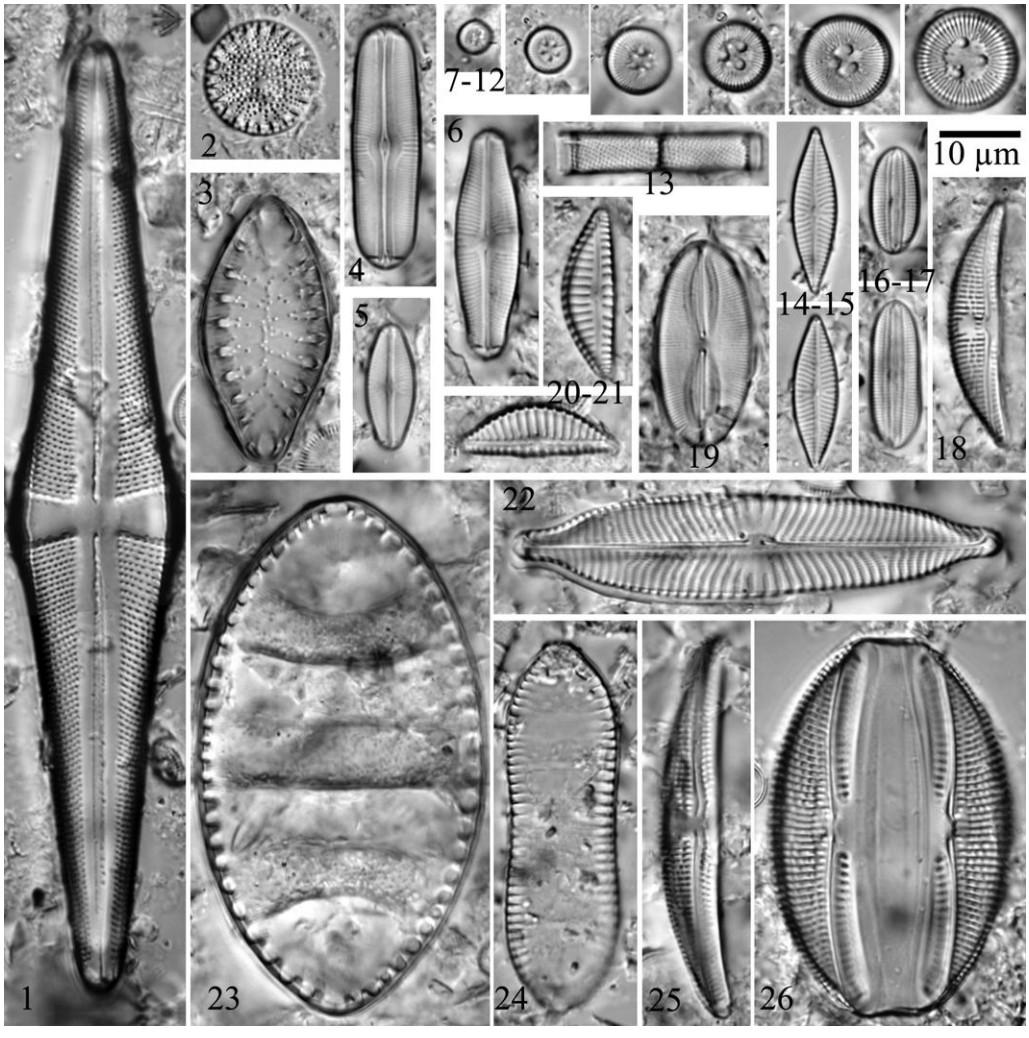

Fig. 1. Images of species present in the sample used for treatment of rats during the experimental studies. 1, *Stauroneis acuta* W. Smith. 2, *Cyclotella radiosa* Grunow. 3, *Surirella helvetica* Brun. 4, *Sellaphora bacillum* (Ehrenberg) D.G. Mann. 5, *Sellaphora* sp. 6, *Sellaphora pupula* (Kützing) Mereschkowsky. 7–12, *Cyclotella ocellata* Pantoscek. 13, *Aulacoseira italica* (Ehrenberg) Simonsen. 14, 15, *Navicula cryptotenella* Lange-Bertalot. 16, 17, *Diploneis oculata* (Brébisson) Cleve. 18, *Amphora copulata* (Kützing) Schoeman & Archibald. 19, *Fallacia pygmaea* (Kützing) Stickle & D.G. Mann. 20, 21, *Encyonema minutum* (Hilse) D.G. Mann. 22, *Navicula viridula* (Kützing) Ehrenberg. 23, *Cymatopleura elliptica* (Brébisson) W. Smith. 24, *Cymatopleura solea* (Brébisson) W. Smith. 25, *Amphora alpestris* Levkov. 26, *Amphora ovalis* (Kützing) Kützing.

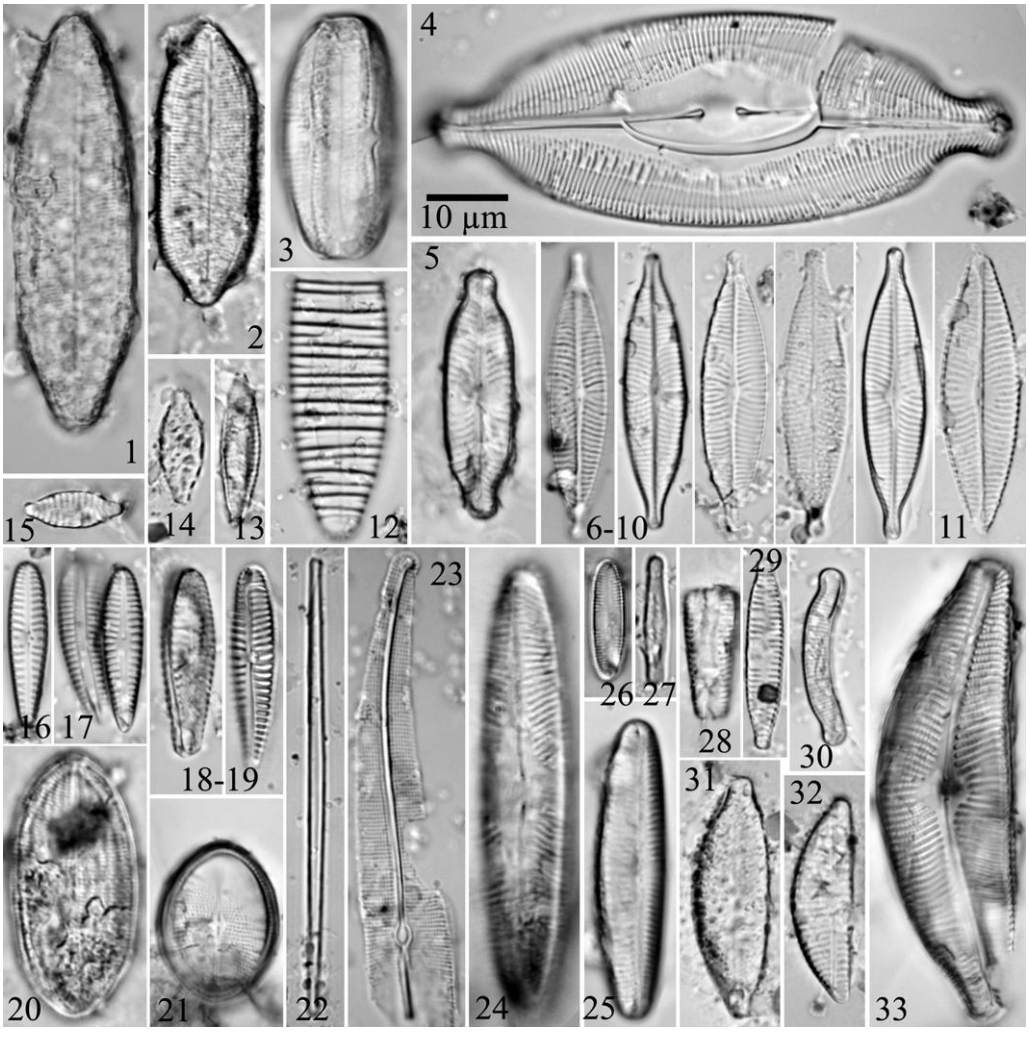

Fig. 2. Images of widespread diatom taxa recovered from the internal organs in case no. 4 (Table 1). 1, *Neidium ampliatum* (Ehrenberg) Krammer. 2, *Neidium dubium* (Ehrenberg) Cleve. 3, *Amphora copulata* (Kützing) Schoeman & R.E.M. Archibald. 4, *Caloneis amphisbaena* (Bory) Cleve. 5, *Placoneis elginensis* (W. Gregory) Cox. 6–10, *Navicula capitatoradiata* H. Germain. 11, *Navicula lanceolata* (C. Agardh) Kützing. 12, *Diatoma vulgare* Bory. 13, *Navicula cryptotenella* Lange-Bertalot. 14, *Navicula* sp. 15, *Fragilaria vaucheriae* (Kützing) J.B. Petersen. 16, 18, 19, *Gomphonema minutum* (C. Agardh) C. Agardh. 17, *Rhoicosphenia abbreviata* (C. Agardh) Lange-Bertalot. 20, *Cocconeis placentula* var. *lineata* (Ehrenberg) Cleve. 21, *Cocconeis pediculus* Ehrenberg. 22, *Fragilaria* sp. 23, *Gyrosigma* sp. 24, *Pinnularia brebissonii* (Kützing) Rabenhorst. 25, *Cymbopleura subaequalis* (Grunow) Krammer. 26, *Caloneis fontinalis* (Grunow) Lange-Bertalot & Reichardt. 27, *Achnanthidium minutissimum* (Kützing) Czarnecki. 28, *Gomphonema* sp. 29, *Fragilaria capucina* Desmazières. 30, *Eunotia nymanniana* Grunow. 31, *Cymbopleura naviculiformis* (Auerswald) Krammer. 32, *Encyonema silesiacum* (Bleisch) D.G. Mann. 33, *Cymbella tumida* (Brébisson) Van Heurck. Scale bar = 10 µm.

(with valves exhibiting a radial symmetry) and the pennate diatoms (with valves exhibiting bilateral symmetry; in Figs 1–4, only the specimens in Figs 1: 2, 7–13 and 4: 11–12 are centric diatoms). During the last two decades, however, advances in diatom classification have largely rejected the first group – the 'centric' diatoms – as being monophyletic and therefore not a natural systematic group. For an account of past diatom classifications see Williams (2007); for a summary of progress with respect to DNA evidence see Theriot *et al.* (2010).

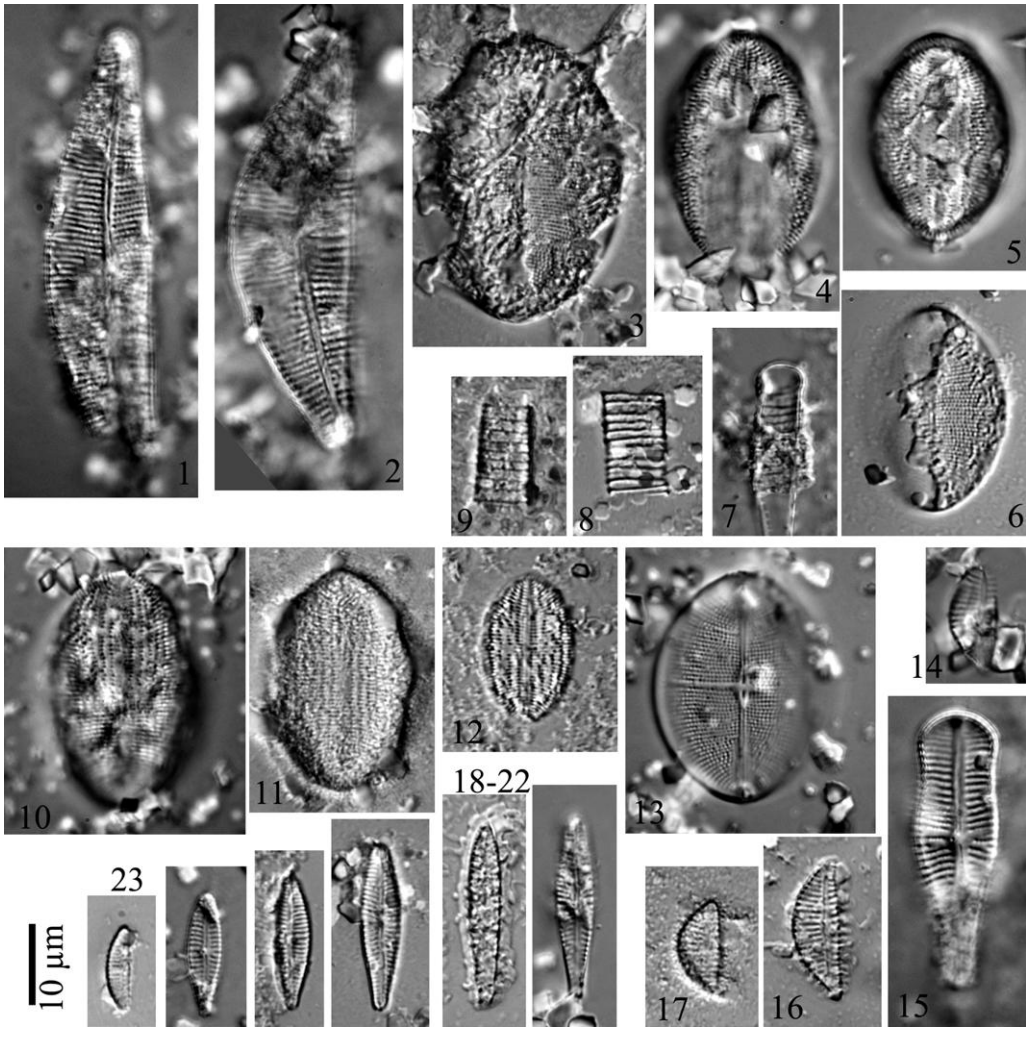

Fig. 3. Highly corroded specimens recovered from internal organs in case no. 2 (Table 1). 1, 2, *Cymbella* sp. 3–6, *Cocconeis* sp. 7–9, Fragments of *Diatoma* sp. 10–13, *Cocconeis* sp. 14, 16, 17, *Encyonema* sp. 15, *Gomphonema truncatum* Ehrenberg. 18–22, *Gomphonema* spp. 23, *Amphora indistincta* Levkov. Scale bar = 10 µm.

Different estimates of diatom diversity have been offered. For example, Mann & Droop (1996) suggested there may be in excess of 200 000 species of diatoms (extant and fossil), but more recently Guiry (2012) gave the much reduced estimate of 20 000 species, with 12 000 already described (and accepted) and a further *c.* 8000 still to be discovered. Using modern diatom species concepts, and with the increased use of fine ultrastructural features and molecular analyses for species discrimination, a more realistic total number of species could be *c.* 30 000–100 000 (Mann & Vanormelingen 2013). It is probably agreed that around 15 000 are currently known and accepted.

Diatoms occur everywhere on the planet; some are widely dispersed but many have narrow ecological preferences and are therefore useful for biomonitoring (Collins *et al.* 2012; Bennion *et al.* 2014), palaeoecological reconstructions (Smol & Stoermer 2010; Snyder *et al.* 2013; Cvetkoska *et al.* 2015), biogeographical (Williams & Reid 2006) and evolutionary studies (Khursevich *et al.* 2001). Some species have restricted distributions as they are found only in a particular area and are considered to be endemic (Vanormelingen *et al.* 2008; Williams & Kociolek 2017); many endemic diatom species have been found in large or ancient lakes (Cocquyt 1998; Williams & Reid 2006;

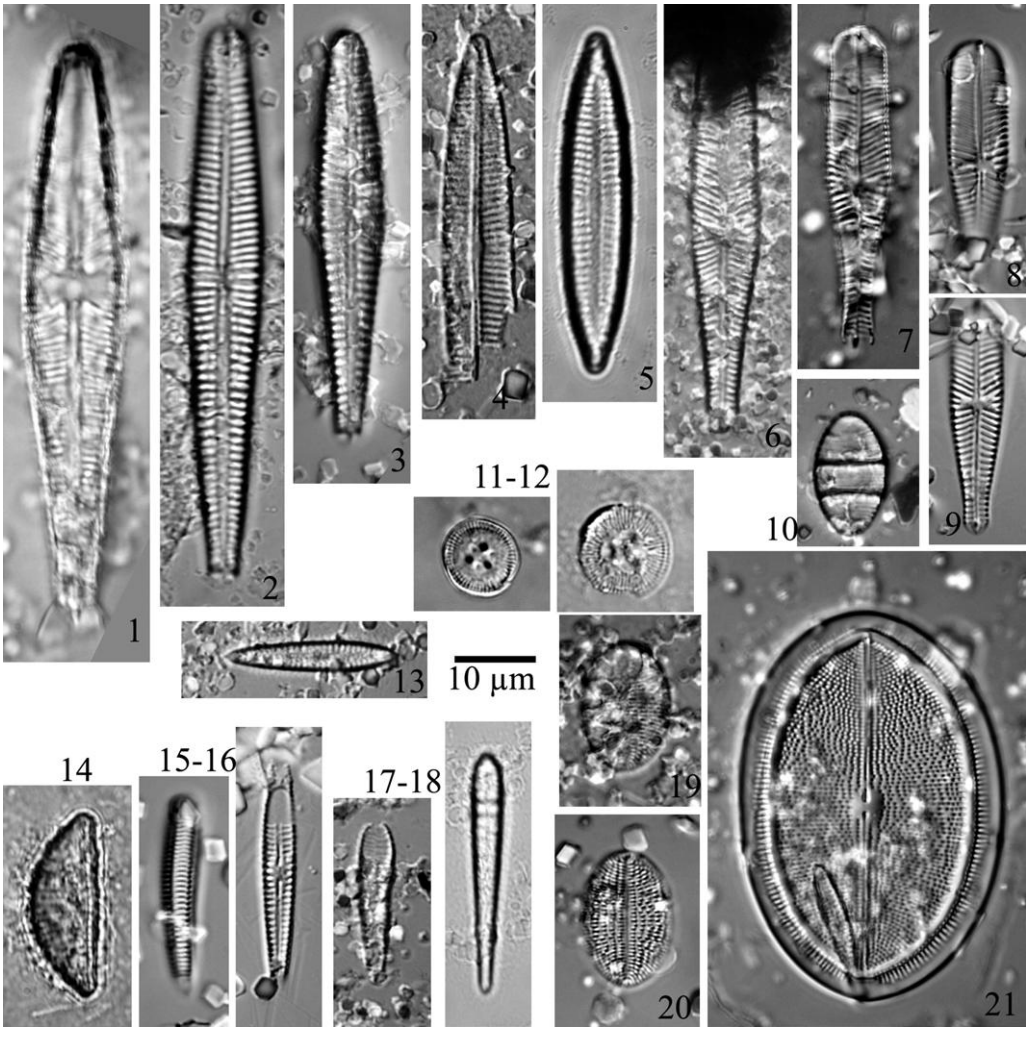

Fig. 4. Endemic and typical species from Lake Ohrid recovered from internal organs in case no. 6 (Table 1). 1, *Gomphoneis ohridana* Levkov. 2, 3, *Gomphonema pratense* Lange-Bertalot. 4, *Navicula tripunctata* (O.F. Müller) Bory. 5, *Navicula* sp. 6–9, *Gomphonema fonticolum* (Hustedt) Levkov & Krstic. 10, *Odontidium minutum* Levkov & D.M. Williams. 11, 12, *Cyclotella ocellata* Pantoscek. 13, *Gomphonema lychnidum* Levkov, Mitic-Kopanja & E. Reichardt. 14, *Encyonema ochridanum* Krammer. 15, 16, *Rhoicosphenia tenuis* Levkov & Nakov. 17, 18, *Meridion* sp. 19–21, *Cocconeis placentula* var. *lineata* (Ehrenberg) Cleve. Scale bar = 10 µm.

Kulikovskiy *et al.* 2012; Levkov & Williams 2012). Diatoms are very sensitive to ecological fluctuation, and their response to changes can be noted in the species composition and abundance. Many species are specialized to inhabit particular kinds of habitat (e.g. plankton, epiphyton, epilithon) and have specific environmental preferences that allow indicator values to be set for several important environmental variables such as light, moisture conditions, temperature, current velocity, salinity, pH, oxygen, inorganic or organic nutrients (Van Dam *et al.* 1994).

While it has been noted that diatoms found in tap water and bathwater, as well as those that are airborne, can be sources of entry into human tissues, in general diatoms enter humans (or other animals) via three pathways: the gastrointestinal tract (Yen & Jayaprakash 2007), the respiratory organs or external injuries. Additionally, diatoms may also enter post-mortem into the internal organs as a result of degradation during prolonged submersion of the body in water (Krstic *et al.* 2002). Several studies have shown that diatoms can be present

in human bodies when the cause of death is attributed to factors other than drowning. In one of five cases analysed by Levkov (pers. obs.), several centric diatoms (mostly from the genus *Cyclotella*) were observed. Much earlier, Foged (1982) recorded diatoms from seven mummies. In five cases he detected many freshwater diatoms, but also detected many freshwater diatoms in both drowned and non-drowned humans. Later, Foged (1983) noted that diatom valves are normally present since he found many valves in non-drowned persons, but their numbers vary from person to person. Interestingly, the majority of species he found belonged to the pennate diatoms, although some centric diatoms were present in low abundance.

Several studies have investigated different parts of the human internal system to detect whether diatoms were present. Some examples are: blood ('from the left atrium', Kärkolä & Neittaanmäki 1981, p. 150; 'samples of left ventricular blood', Aghayev *et al.* 2005, p. 66), internal organs such as the liver and kidney (Matsumoto & Fukui 1993; Pachar & Cameron 1993; Krstic *et al.* 1996; Taylor 1994; Ludes *et al.* 1999; Hürlimann *et al.* 2000), stomach contents (Peabody 1977; Hürlimann *et al.* 2000), brain (Pachar & Cameron 1993; Krstic *et al.* 2002) and bone marrow (Peabody 1977; Schellmann & Sperl 1979; Pollanen 1997; Hürlimann *et al.* 2000). The most frequently examined parts are the lungs (Kobayashi *et al.* 1993; Matsumoto & Fukui 1993; Ludes *et al.* 1999; Krstic *et al.* 2002; Aghayev *et al.* 2005; Horton *et al.* 2006; Takeichi & Kitamura 2009; Ago *et al.* 2011; Lin *et al.* 2014).

All these factors make diatoms suitable organisms for application to forensic investigations: they are often locality/environment specific; silica is virtually indestructible and so can be detected with some relative ease; they are present in every waterbody; and they can pass through lung tissue into the blood supply and be carried to other organs if the heart is still pumping.

The diatom test

Numerous studies have shown that diatoms are valuable supportive evidence in cases of drowning (Pollanen 1998; Lunetta & Modell 2005; Horton *et al.* 2006; Bortolotti *et al.* 2011; Delabarde *et al.* 2013). The results of several studies with diatoms confirmed as present in human tissue were used as the basis for the so-called 'diatom axiom' or 'diatom test' (Pollanen 1998). According to Pollanen, the 'axiom' states that if diatoms are detected in bone marrow then 'drowning caused death or was contributing factor and the individual was breathing upon the entry into the water' (Pollanen 1998). The concept is based on the simple postulate that diatoms will enter the lungs with the inhalation of any liquid and, if there is effective cardio-vascular circulation, they can be carried to other internal organs (Pollanen 1998). In contrast, if a body is already dead when it is transferred to water, or the cause of death is other than drowning, then diatoms might still be present in the lungs (via passive penetration) but not present in any other internal organ. However, the axiom might be better if reversed: if diatoms are not detected in any internal organs then the cause of death is not drowning or, if other signs of drowning are present, the drowning may not have occurred in natural water but in some alternative non-natural source, such as bathwater or a swimming pool.

Limitations of using diatoms as indicators of drowning

Analyses of drowning cases performed by Pollanen (1998) demonstrated that diatoms were found in human tissues in only 28% (205 from 738) of the cases studied. Similarly, Auer & Möttönen (1988) found no diatoms in 11.2% (12 cases) of the drowning cases they studied. There are several explanations for these results: there was an absence or low abundance of diatoms in the drowning medium; rapid death occurred due to other circumstances; or there were methodological drawbacks such as inefficient methods of extraction, dissolution of the diatom valves during processing, and a low sample weight or an inappropriate location for tissue sampling.

Diatom abundance. In cases of drowning, the presence of diatoms in high abundance in the water is essential for positive findings, but there have been very few attempts to estimate the lowest diatom concentration required for a positive diatom test. Most of the experimental studies, not surprisingly, have been performed on animals. Mueller (1959) calculated the number found to be 20 000 valves per 100 mL suspension for rats, and 13 500 for rabbits. In most of the cases where diatoms were analysed from human tissues data for their concentrations were not provided, yet most studies do contain information about the composition of diatoms in the drowning media (Ludes & Coste 1996; Ludes *et al.* 1999) but rarely their abundance (Kakizaki *et al.* 2011). According to Hürlimann *et al.* (2000), diatom density significantly decreases (10–100 fold) from that found in the drowning medium when compared to that found in the lungs. The decrease of diatom density is even more remarkable when comparing the lungs to other internal organs (in the order of 100–1000 decrease).

The other important factor when considering the diatom concentration in the media is the type of

water habitat. Several authors have analysed cases from rivers and canals (Krstic *et al.* 2002; Bortolotti *et al.* 2011; Delabarde *et al.* 2013). In these habitats the diatoms are usually attached to the bottom sediment or to macrophytes. It is also worth noting that diatoms in rivers can be passively transported by the water course and can therefore directly attach to the surface of the body or passively enter the body. Most of the cases studied by Pollanen *et al.* (1997) and Pollanen (1998) came from Lake Ontario; Ago *et al.* (2011) analysed cases from bathwater; several other studies provided data from corpses found in different habitats from marine to freshwater (Yorulmaz *et al.* 2003; Kakizaki *et al.* 2011; Lin *et al.* 2014) as well as wells (Esiyok *et al.* 2006). Examining these data, it can be concluded that most of the positive findings originate from either the rivers or marine sites. The results from shallow freshwater lakes and rivers are to be expected since in these environments diatoms often have a low abundance in the plankton, especially in oligotrophic lakes. It is important to point out that in oligotrophic ancient lakes (such as Lake Ohrid), maximum diatom productivity occurs in the deeper waters (20–40 m), while the surface-water diatoms are represented with only a few cells per millilitre throughout the year. In such cases the drowning medium will contain a small number of diatom cells and the probability of observing some in human tissues is low. This might explain the relatively low percentage of positive cases observed by Pollanen *et al.* (1997) and Pollanen (1998) in Lake Ontario.

Rapid death. According to Pollanen (1998), the diatom test was positive in only 13% of cases where drowning occurred in the winter months (when the diatom bloom is present). Such results might be explained by death being sudden or rapid and caused by shock or cardiac dysfunction (reflex cardiac arrest) due to the low water temperature (Saukko & Knight 2004), sometimes referred to as dry drowning (Peabody & Cameron 2010). Several reports have also shown that rapid death can occur due to the presence of diseases and might result in a low number or even the total absence of diatoms in human tissues, even in the lungs (Krstic *et al.* 2002). Additionally, the use of alcohol or drugs might influence the test (Lawler 1992; Yorulmaz *et al.* 2003; Ago *et al.* 2011) by possibly decreasing the length of time taken to drown and the quantity of inhaled water.

Methods of extraction. One of the most important issues of the diatom test is the efficiency of the extraction method and the identification of diatoms in human tissues. There are several different methods used for extraction and detection of diatoms, all of which have advantages and disadvantages. Extraction of diatoms from the internal organs requires the complete destruction of the organ tissues. Some earlier methods of diatom extraction involved direct microscopic analysis of tissue sections (Weinig & Pfanz 1951), the detection of diatoms in blood with direct observation of diatoms on a membrane filter (Möttönen & Ravanko 1971) after blood haemolysis by sodium dodecyl sulphate (SDS, formula $CH_3(CH_2)_{11}SO_4Na$, Yoshioka & Takahashi 1986), or by a combination of haemolysis, 5 mm pore-membrane filtering, digestion with HNO_3 and re-filtration (Funayama *et al.* 1987).

The most frequently used method of extraction, however, is acidic digestions. There are several different versions based on the use of nitric (HNO_3), sulphuric (H_2SO_4) and hydrochloric acids (HCl) as well as hydrogen peroxide (H_2O_2). For example, Auer & Möttönen (1988) used a combination of HNO_3 and H_2O_2 to digest the tissues of lungs, kidney, liver and brain; Bortolotti *et al.* (2011) used HNO_3 heated to 60°C for 48 hours to digest lungs and sternum; and Krstic *et al.* (2002) and Fucci (2012) used H_2SO_4 for extraction of diatoms from several internal organs (kidney, liver, lung and brain). DiGiancamillo *et al.* (2011) performed several experiments using different concentrations of HCl and H_2O_2 with different duration times of digestion. According to their results the 'best' results (highest number of diatoms recovered) occurred when using 20–37% HCl and H_2O_2, while a combination of H_2SO_4 and HNO_3 increased the presence of damaged and fragmented diatom valves and also left a high level of precipitates on the glass slide after specimen preparation. There has also been some effort to construct a special instrument ('can') for acid digestion (Yange *et al.* 1999). It consists of three parts: a can body, an inner cover and an outer cover. The internal wall of the can mouth is connected to the inner cover through the thread and the outer wall of the can mouth is also connected to the outer cover through the thread.

Ming *et al.* (2007) examined four different extraction methods based on four criteria:

(i) time-consumption for complete digestion;
(ii) digestive capability;
(iii) reclaiming ratio of diatoms; and
(iv) destruction of diatom valves.

According to these authors, the best results were obtained using proteinase K (enzymatic digestive method) since it has a higher reclaiming ratio for diatoms (Kobayashi *et al.* 1993; Takeichi & Kitamura 2009). The disadvantages of this approach are that it is a time-consuming process, it is more expensive than other methods and there is presence of a higher concentration of residuals. Another method that can be applied to diatom extraction is the use of Soluene® 350 (a strong organic base,

formulated with toluene). This approach is simple, fast and less hazardous than other methods (Fukui *et al.* 1980) but, according to Sidari *et al.* (1999), it is not effective when extracting marine diatoms because frustules of seawater diatoms are solubilized by Soluene-350.

With the development of molecular biology, techniques for the extraction and detection of genes are becoming more readily available and easier to use, and therefore more frequently applied in the discovery of planktonic species in the human tissues. The most frequently used gene has been 16S rRNA subunits of ribosomal RNA (Kane *et al.* 1996, 2000; He *et al.* 2008). According to He *et al.* (2008) plankton DNA was identified from several organs including lung, liver, kidney, blood and brain of the drowned victims, and might become a more accurate method for the detection of diatoms in the human tissues.

Appropriateness of organs

It is important to consider the organ used in the diatom test and the quantity and location of the samples taken for analyses. As mentioned above, various authors have used different organs for the detection of diatoms in cases of suspected drowning. Kärkolä & Neittaanmäki (1981) and Funayama *et al.* (1987) analysed 'left heart blood' from which several diatoms were extracted. This method can only be applied if the bodies are relatively fresh and the material is taken over a period not longer than 12–24 hours, however. In almost all studies the lungs are considered as the first and most important organ for the detection of diatoms. Interestingly, data originating from studies undertaken between 1960 and 1980 suggest that diatoms have been frequently found in the lungs of persons that have not drowned (Tomonaga 1960; Neidhart & Greendyke 1967; Reh 1970; Foged 1982, 1983). Most of the more recent studies show that diagnosis of death by drowning can however be supported by analyses of the diatoms found in the lungs (Kobayashi *et al.* 1993; Ludes *et al.* 1999; Krstic *et al.* 2002; Aghayev *et al.* 2005; Takeichi & Kitamura 2009; Ago *et al.* 2011; Lin *et al.* 2014). Horton *et al.* (2006) showed that if a correlation between the diatom composition in the drowning media and the lungs is found, then a diagnosis of drowning can be supported. According to Krstic *et al.* (2002), determination of qualitative diatom composition in the organs and on the surface of the body provides valuable information regarding the possible place of drowning. The same conclusion has been made in several other studies (Pollanen *et al.* 1997; Ludes *et al.* 1999; Horton 2007; Farrugia & Ludes 2011).

Besides the lungs, other organs have been analysed with varying degrees of success. The heart has shown positive results with the diatom test (Krstic *et al.* 2002), but the number of studies is limited. Other organs that have been taken into consideration for diatom analyses are the kidney and liver. For instance, Giri *et al.* (1993) detected 20 diatom valves in the liver, while Krstic *et al.* (2002) observed 37 valves. According to Hürlimann *et al.* (2000), these organs can be considered as substitute samples.

The bone marrow matrix is of great potential interest in forensic investigation from a toxicological viewpoint (Cartiser *et al.* 2011), but is also frequently considered as important for forensic science (Gruspier & Pollanen 2000; Bortolotti *et al.* 2011; Delabarde *et al.* 2013). In some countries there have been attempts to standardize this method (Díaz-Palma *et al.* 2009), but in all studies the number of observed valves is very low. For instance, Hürlimann *et al.* (2000) found only a single valve in bone marrow in contrast to 104 in lungs; Delabarde *et al.* (2013) found only two valves.

Positive v. negative test results: numbers and sizes

One of the most important questions relates to the validity of the test itself, and how many valves need to be recovered/counted to yield a true positive result. According to Farrugia & Ludes (2011), the diatom test can be considered positive when at least 20 diatom valves are identified per 100 μL of sediment extracted from a 2 g lung sample, and identification of more than 5 complete diatom valves per 100 μL of sediment extracted from a 2 g tissue samples such as brain, kidney, liver and bone marrow. The same values were suggested by both Ludes *et al.* (1994) and Bortolotti *et al.* (2011). In some case studies, however, the presence of low numbers of valves from cosmopolitan diatoms in bone marrow (1–2) have been considered positive. In contrast to the numbers suggested above Hürlimann *et al.* (2000) proposed much higher values: 20–40 diatoms/5 g for bone marrow. While numbers of valves recovered vary according to the organ sampled, some judgment is therefore required to confirm a positive test. It is imperative to establish a standard (or standards) that will be applied and accepted among those in the forensic diatom community.

It is also worth considering the size of the diatom valves recovered. Available data for valve sizes of identified diatoms in human tissues are very rare. According to Lunetta *et al.* (1998) the maximum length of diatom valves that can pass through the alveolo-capillary barrier is around 110 μm. Tomonaga (1960) recorded species in blood with a maximum size of 100–160 μm. Most of the species

recorded by Hürlimann *et al.* (2000) had a valve length smaller than 15 μm, while almost 90% of all recorded species have a valve length shorter than 40 μm. Similar results were presented by Pachar & Cameron (1993) where the size of diatom valves in the internal organs was smaller than 30 μm. Most of the diatoms observed by Krstic *et al.* (2002) had valve sizes smaller than 50 μm, with the dominant size class being 15–40 μm. According to Kakizaki *et al.* (2011), the average size of diatoms in natural habitats is 16.1–59.2 μm.

Experimental studies

Material and methods

Two types of samples were analysed in this study: human tissues and organs from experimental animals (rats). (Permission for the treatment of rats was received from the Animal Welfare Committee at Ss Cyril and Methodius University in Skopje; 02-557/1.)

Human cases. Sample tissue from 10 cases was received from the Institute of Forensic Medicine, Criminalistics and Medical Odontology in Skopje. For all cases analysed in this study the cause of death was drowning, or drowning was a contributing factor; only cadavers found in freshwater were available. The analysed group comprised 10 individuals who died during the period 2008–12 from various localities and habitats (mostly from River Vardar and Lake Ohrid). A portion of the organs (lungs, brain, liver, heart and kidney) weighing around 10–15 g were taken from each cadaver. For diatom analyses, 2 g of tissue were used. The samples were washed thoroughly with distilled water and then cut into smaller fragments using knives washed with distilled water. The samples were transferred into a 100 mL glass beaker and 10 mL of 30% hydrogen peroxide (H_2O_2) was added. The glass beakers were heated for 30 minutes in a water bath at 60°C. After 10 mL of potassium permanganate ($KMnO_4$) was added, the sample was stored at room temperature (20–25°C) for 24 hours. The following day 20 mL of hydrochloric acid (HCl) was added and the sample heated for 30–45 minutes at a temperature of 90–95°C. The samples were then transferred to a centrifuge tube and washed repeatedly with a large amount of water. At the end of the fifth washing cycle, the remaining sediment was resuspended in 1.0 mL distilled water and a few drops of the suspension deposited on a glass microscope slide and air dried. The slides were mounted using Naphrax. Light microscopy (LM) was performed using Nikon E80i (Nikon Corp., Tokyo, Japan) equipped with Nikon Coolpix 6000 on magnification ×1000 or ×1500.

Experimental cases. Six groups of rats were analysed, each group containing 3–5 individuals. The diatom suspension used for this experiment was collected as sediment from the Prilep reservoir, Macedonia and prepared as above. The sample yields more or less equal numbers of centric and pennate diatoms (Fig. 1: 1–26) with the dominant species being *Cyclotella ocellata* (valve diameter 4–26 μm, average 11 μm). In the first group of rats a suspension saturated with diatoms was given *ad libitum* during a period of 10 days. The second group consisted of rats that had been drowned in the saturated diatom suspension and immediately analysed. The third group consisted of rats that had been drowned in the saturated diatom suspension and left for one week in the suspension. Individuals in the fourth group were killed (with narcotics) and then submerged into the diatom suspension for a week. For the fifth group, dead rats with opened stomachs were placed in the River Treska for 48 hours. Finally, the sixth group comprised dead rats with opened stomachs that were first placed in the River Treska for 24 hours and then transferred to the River Vardar and submerged for an additional 24 hours. After extraction, the following organs were analysed: lungs, brain, liver, heart and kidneys. The diatom extraction process and preparation of slides was the same as for human cases.

Results

Human cases. Of the ten cases analysed, three samples from the brain and liver were not available for the diatom test and four samples from the kidney were not available (see Table 1). In case 1 only four diatom valves were detected in the lungs, while the number of diatom valves was significantly higher in brain and heart. Similar results were obtained in case 2 where the highest number of diatom valves was recorded in the heart (Fig. 3: 1–23). In cases 3 and 4 (Fig. 2: 1–33) the situation is the opposite, with the number of diatom valves being much higher in the lungs when compared to the brain or heart. Still, the number of diatom valves in the heart is high. In case 5, as well as in the lungs a high number of diatom valves were recorded in the kidney, while in the brain no diatoms were found. In the sixth and seventh cases relatively low numbers of diatoms were observed in the lungs, while a large number of diatoms were recorded in the liver and kidney (case 6, Fig. 4: 1–21) and the heart (case 7). In case 8 no diatom valves were found in either the lungs or the heart, while a significant number of diatom valves were found in the liver and kidney. In the ninth case the brain and liver contained a large number of diatom valves, even larger than those recorded in lungs. In the last case (10) only two organs were available

Table 1. *Observed diatom valves in ten cases of drowning*

Case	Lungs				Brain				Liver				Heart				Kidney			
	No. valves	No. species	Size (μm) min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P
1	4	4	22–55	1–3	26	14	20–117	4–22	17	8	21–107	3–14	28	13	15–68	5–23	6	6	18–52	1–5
2	15	8	17–118	4–11	NA	NA	NA	NA	39	6	17–167	22–17	41	13	13–82	5–36	NA	NA	NA	NA
3	49	13	9–38	17–32	2	1	15–28	0–2	NA	NA	NA	NA	24	12	13–50	11–13	5	3	11–28	1–4
4	46	26	16–71	5–41	NA	NA	NA	NA	28	20	13–62	2–26	15	13	14–64	2–13	NA	NA	NA	NA
5	48	15	17–67	6–42	/	/	/	/	NA	NA	NA	NA	7	7	8–65	1–6	35	14	18–72	1–34
6	19	11	27–118	17–2	2	2	18–95	0–2	38	16	17–187	6–32	4	4	10–85	0–4	17	14	16–67	0–17
7	15	10	9–122	0–15	3	3	16–53	0–3	24	14	15–113	2–23	74	25	13–77	4–70	NA	NA	NA	NA
8	/	/	/	/	2	2	23–34	0–2	23	13	19–132	6–17	/	/	/	/	13	7	16–122	1–12
9	15	10	17–65	0–15	23	19	7–55	6–17	29	14	20–51	5–24	5	5	12–55	1–4	1	1	42	0–1
10	15	14	18–56	1–14	NA	NA	NA	NA	NA	NA	NA	NA	1	1	24	0–1	NA	NA	NA	NA

The table includes 10 cases examined for number of valves recovered ('No. valves'), number of species recovered ('No. species'), their size ('Size min–max') and their shape, contrasting centric with pennate diatoms ('Shape C–P'). These are given for lungs, brain, liver, heart and kidney in each case unless otherwise stated. Where tissue/organ was not available, this is indicated with NA ('not applicable'). No diatoms observed is indicated with '/'.

as the corpse was highly decomposed. However, a relatively large number of valves were observed in the lungs (the details of these 10 cases are summarized in Table 1).

Experimental cases. In the first group (I) the largest number of diatom valves was observed in the heart, but other organs also contained a large number of diatoms (see Table 2). In almost all of the organs examined, centric diatoms (mostly valves of the species *Cyclotella ocellata*) were abundant. In the second group (II) (rats drowned in the diatom suspension) the number of observed diatom valves found in all organs was significant, with the largest number found in the liver. However, the number of centric diatoms was lower when compared to pennate diatoms, but also lower when compared to the previous group (I) which was treated with the same suspension. Between the third group (III) and second (II) group there is no appreciable difference in number and composition of diatom valves found in the organs. A relatively small number of diatom valves was found in the fourth (IV) group, with the largest number of valves found in the lungs. The fifth (V) and sixth (VI) groups acted as proxies for dead bodies with external injuries that are transferred to water and intact bodies with injuries caused by some mechanical impact or animal grazing while in the water. A relatively large number of diatoms were found in the lungs from the fifth group (V) indicating the passive transport of diatoms to the lungs, especially in terms of the number of diatoms found in the liver (23) and kidney (15). However, these tissues were not previously cleaned (washed) with diatom-free water and a small portion of sediment was present on the surface of the organs.

Implications

One of the main challenges of the diatom test is how to define positive v. negative results; crucial to this is the determination of the number of valves needed to confirm a positive test. As noted above, various authors have proposed different numerical criteria to obtain a positive test. According to the experimental study above all cases can be considered positive, with the exception of case 8 where no diatoms were observed in the lungs. There are two explanations for this negative result: either the position from which the tissue was sampled was unsuitable, or the effects of the pre-treatment and extraction process led to the loss of diatom valves. Since human organs are too large for them to be used whole in the analyses, it becomes important to determine the position where the tissue is sampled for treatment. However, in most of the literature information about the location of sampling from human organs is not provided. There are three possible approaches: tissue should be taken near the large blood vessels; tissue should be taken from the peripheral part of the organs; or replicates should be taken from across the organ.

In the first approach, 'contamination' from blood is possible. However, a positive aspect of that outcome is that if diatoms are present in the blood, then they will be recorded. The results from the second approach might be more representative of sampling in the diatom test. Limitations here, however, can be that lower numbers of diatom valves will be observed, since lower quantities of blood are transported to those parts.

Tissue pre-treatment usually comprises 'washing thoroughly with distilled water'. Diatom valves are not usually firmly attached to human organs, although some experimental studies show that diatoms might be 'stuck' in the tissue (Lunetta *et al.* 1998). Washing with distilled water might result in the removal of diatoms from the tissue. However, such a procedure might be necessary if the recovered body was injured and ambient water entered the abdomen or thorax. In such cases, diatom valves may attach to the organ surface.

Problems might also occur during the diatom extraction process. In general, two types of difficulties occur during this process. Some lung and brain tissue will not be fully digested when using acid treatments and they usually simply foam at the top of the suspension. When HCl is added to the suspension, the oxygenation reaction is intensive and a portion of sample might be lost. Alternatively, in some cases the extraction process might result in heavy corrosion of the valves or the amount of residuals becomes very high, preventing proper identification of the species (see Fig. 3: 1–23).

One possible solution or approach in further studies is to use the replicates from the tissue taken from across the organ. This might be important for organs such as brain and kidney, and particularly the heart where different parts of the organ have special functions that might be intensified or suppressed during the stress caused by the drowning. Because of that it is recommended to use replicates from different parts of the organs for diatom analyses. This approach might increase the sensitivity of the diatom test and its reliability as supportive evidence in cases of drowning.

It should be noted that this study was designed to obtain positive results. During the autopsy all cadavers had clear signs of drowning and in such cases the percentage of success is higher. Additionally, eight of the cases comprise bodies found in rivers (Vardar) and only two were drowned in relatively shallow waters (at 5 m depth in Lake Ohrid from a sunken boat). From previous experience (Krstic *et al.* 2002) it was known that in cases where the

Table 2. *Observed diatom valves in six experimental groups*

Exp. Gr.	Lungs				Brain				Liver				Heart				Kidney			
	No. valves	No. species	Size (μm) min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P	No. valves	No. species	Size min–max	Shape C–P
I	28	13	4–58	16–12	18	10	6–44	12–6	29	14	4–48	10–19	61	18	17–39	21–40	3	1	4–10	3–0
II	26	7	9–45	9–17	15	11	10–118	5–10	31	12	10–55	8–23	18	12	9–88	7–11	16	11	10–72	5–11
III	25	15	10–59	3–22	14	7	4–56	6–8	14	5	16–48	1–13	22	8	17–29	3–19	0	0	/	/
IV	10	8	18–45	2–8	0	0	/	/	5	4	15–45	1–4	2	1	5–25	0–2	2	2	15–62	0–2
V	29	11	8–128	13–16	2	2	9–22	1–1	23	10	8–63	2–21	9	7	20–90	2–7	15	9	22–40	3–12
VI	8	7	9–48	2–6	0	0	/	/	16	5	9–31	1–15	12	7	18–28	2–10	3	3	22–40	1–2

The table includes 6 experimental cases examined; column headers as for Table 1.

body was found in rivers, there was a greater probability that a positive diatom test would be obtained.

The greatest disadvantage of this study is the lack of cases where drowning occurred in open lake waters (Macedonia is a closed country without any coastal waters), especially from Lake Ohrid. The ecological studies of plankton communities in Lake Ohrid show that diatoms live mainly at the border between metalimnion (thermocline) and hypolimnion (25–40 m depth of water column), while abundance in the surface waters is very low. In many oligotrophic lakes, diatoms in plankton communities are dominant during the spring and autumn seasons (during mixing) and the prevalence of positive cases might be higher (Pollanen 1998). The ecology and distribution of planktonic diatoms will influence the sensitivity of the diatom test. As seen in Pollanen (1998), positive diatom tests in Lake Ontario is only *c.* 25%. In this study two cases from Lake Ohrid were analysed (nos 6 and 7, see Table 1). Both originated from boats that sunk with tourists on board in *c.* 5 m water depth.

Several endemic diatom taxa from Lake Ohrid were identified in the internal organs (Fig. 4: 1–21). When endemic diatoms are found in the internal organs, not only does it represent useful evidence for drowning as the cause of death but it can also help to determine the place of drowning. In most of the cases analysed however, not just this study but worldwide, it is mostly cosmopolitan (widespread) diatoms that are found which cannot be used to determine the place of drowning.

A number of previous studies have investigated not only the species composition but also the shape and size of the diatom valves recovered from the bodies. It is assumed that because of their linear (or lanceolate) shape pennate diatoms are more able to penetrate the lung tissue and enter the circulation. In most studies where species composition is given, the pennate diatoms are always found in greater number than the centric diatoms. There are two possible explanations: that centric diatoms are present but with a lower abundance; or centric diatom valves have greater difficulty penetrating the lung or intestine tissue.

Nikolova *et al.* (2002) observed more than 200 valves in 2 g of organs (lungs, heart, brain, kidney, pancreas and liver) from an old non-drowned woman who used water from an open well. Most of the diatom valves found belonged to the centric species *Aulacoseira granulata*; pennate taxa were present but with significantly lower abundance. These findings raise important questions about the morphology of deposited diatom valves in human tissues, regarding the centric/pennate ratio. In previous reports of non-drowned persons, the most frequently encountered valves in the internal organs belong to centric diatoms. This is the opposite of the most frequently observed valves in cases of drowning, which belong to pennate diatoms. In general, there are two explanations: most human cases that were examined in this study (8 out of 10) originated from bodies found in rivers where the dominant diatom communities are benthic: epilithon, epipelon and epiphyton. These communities are generally dominated by motile or sessile pennate diatoms, while the centric diatoms are in low abundance. In two cases of drowned persons in Lake Ohrid pennate diatoms were also dominant, although in plankton community centric diatoms prevail. The second factor that might influence the dominance of centric diatoms in non-drowned persons is the morphology of the valves. It might be supposed that circular-shaped diatoms cannot be removed from the internal organs. Such a hypothesis has been tested with the rats and the results provide support for this hypothesis. In cases where the rats were treated with the oral suspension of diatoms, the most dominant species observed in the internal organs was *Cyclotella ocellata*, a centric diatom. In contrast, in the experimental study with drowned rats it was shown that the percentage of pennate diatoms was much larger than that of centric diatoms. Interestingly, in a case presented by Ago *et al.* (2011), *Cyclotella* sp. diatoms were detected in the internal organs of the drowned person, but not in the bathwater drowning medium. Similarly, Pollanen (1998) frequently found species of the benthic *Cocconeis* species in bone marrow but not in drowning medium, which may be due to these species having a broadly elliptical to almost round valve outline, similar to many centric diatoms. In several other studies the number of pennate diatoms in the drowning cases is much higher than the number of centric diatoms (e.g. Lin *et al.* 2014). As mentioned above it might be assumed that, due to their shape, centric diatoms hardly penetrate the internal organs, but they can hardly be removed from the tissue.

Several studies where the surface of the body (skin, hair) was analysed for diatoms (e.g. Krstic *et al.* 2002) show that large number of diatoms can be attached to it. Similarly, diatoms can be attached to the internal organs if they are exposed to the ambient water. The study with injured rats (group V and VI) demonstrated that diatom valves might be attached to internal organs if they are in contact with the ambient water. After the rats were exposed to a 48-hour period in the river, several diatoms were recorded in the analysed organs (Table 2). In such cases the origin of the diatoms is a result of post-mortem contamination instead of via the drowning process. As was mentioned above, if the organs are exposed directly to the ambient water due to injuries of the body, then the procedure of vigorous cleaning of the surface with distilled water is necessary. Otherwise, it is

recommended to use samples from the inner parts of the organ and avoid sampling from the external surface of the organ.

Several studies have indicated that post-mortem contamination might occur from laboratory equipment used during analyses (e.g. Krstic *et al.* 2002), and it was pointed out that post-mortem diatoms might penetrate the body and be recorded in the lungs. This study showed that this is quite possible. The experimental studies with rats (group IV, V and VI) showed diatoms might penetrate the body. The results from group IV (rats sacrificed with narcotics and then submerged into the diatom suspension for a week) indicate that diatoms might be observed in the lungs from intact bodies, but not found, or found in very small numbers, in other organs. In a previous study of a body found in water, Krstic *et al.* (2002) also found that diatoms were recorded in the lungs but not in the internal organs, the cause of death being a massive heart attack.

Conclusions

We have described the major use of diatoms in forensic science: their ability to provide supporting evidence for the cause of death when a body is found in water. The 'diatom test' is simply based on the fact that diatoms will enter the lungs with the inhalation of any liquid when a person is drowning. They may penetrate the wall of the lungs and, if the cardio-vascular circulation is efficient, it will carry them to other internal organs (heart, liver, kidney, etc.) where they will remain. If a person is already dead before entering the water, although some diatoms may still be present in the lungs they will not be present in any other internal organ and the cause of death may then be other than drowning. Examination of lungs and other internal organs for the presence of diatoms will therefore yield supporting evidence for drowning if diatom valves are found.

Drawbacks have been discussed and new experimental data provided to explore some of the possible limitations of the test. It is clear that contamination, concentration of diatom valves in the medium, the abundance of diatoms in the medium and recovered in the organs and even the shape of the diatom valve will have an effect on the efficiency of the test. All these factors require further study, but in general the diatom test is an efficient way of confirming the cause of death of victims found in water.

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