Int J Legal Med (2017) 131:1573–1580
DOI 10.1007/s00414-017-1565-y

ORIGINAL ARTICLE

Diatoms in drowning cases in forensic veterinary context: a preliminary study

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Received: 4 November 2016 / Accepted: 16 February 2017 / Published online: 17 March 2017

Abstract In this preliminary study, a digestive method used in forensic context to extract diatoms has been applied in organs taken from ten wildlife animals belonging three species of mammals (a deer, a roe and five otters) and one species of birds (two magpies). Only four carcasses were recovered from aquatic environments (bath trough for animals, bathtub irrigation, river) and only in three cases out of ten that the cause of death was ruled out as drowning. In seven cases, the death was due to other causes: gunshot injuries for one otter, blunt trauma for a magpie, and traumatic injuries followed by motor vehicle collision in other four otters and a roe. Post-mortem examination was performed in all carcasses. The diatom test protocol was performed according to the Italian guidelines for analysis of benthic diatoms for ecological status assessment of inland waters. Five grams of lung, liver, and kidney was taken from all the animal carcasses. In some cases, additional tissue samples were also available among which brain, heart, spleen, and bone marrow. In all four cases found in water, the drowning medium was also available. Hydrogen peroxide (H_2O_2) digestion was performed, and identification of 21 genera of diatoms was obtained. The method proved to be suitable for the identification of diatoms in the organs of the animals drowned supporting the final diagnosis of drowning. Only in otters, all died for causes other than drowning, diatoms did not prove to be suitable for the diagnosis of drowning since their presence in the internal organs was mainly related to their main diet based on fishmeal. The authors believe that this first trial is very promising, and the results suggest that diatom test can be easily applied in forensic veterinary context.

Keywords Diatoms · Forensic veterinary · Drowning · Forensic pathology

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Introduction

Forensic veterinary is part of the broader field of comparative forensic medicine [1] which covers area of both human and veterinary interest. The principles of investigation on an animal's carcass closely follow those used in human forensic pathology [2] in order to determine the circumstances of the death. Determining the cause of death in animals recovered in aquatic environments (river, lake, swimming pools, or other water-containing vessels) is a real challenge even for veterinarians [3]. Although the drowning process has been reviewed extensively in the medical literature [4, 5], drowning is still a difficult autopsy diagnosis as it is usually a diagnosis of exclusion [6] requiring reliable information from the recovery scene, the medical history, or witness reports.

At autopsy, forensic pathologists are aware that there are no pathognomonic findings both in humans and in animals to indicate drowning as cause of death [4–6]. All the macroscopic and microscopic pathological signs are non-specific and, in combination, are merely suggestive of a violent asphyxial process caused by drowning [4–6]. A key point is to determine if the individual/animal was alive before entering the water [7] as victims may fall in water after death due to other causes. Many biological and thanato-chemical markers of drowning have been proposed [8–14], but they are still controversial and not yet widely accepted by the scientific community neither routinely applied [15, 16]. Additional different approaches have been studied as potential diagnostic tools such as post-mortem computed tomography [17] and microbiology [18] including, in particular, the detection of bacterioplankton [19, 20], as well as using real-time PCR assays with TaqMan probes [21] or metagenomic 454-pyrosequencing [22].

However, among the several ancillary tests available in the diagnosis of drowning, no other has received as much attention as the diatom analysis [23–27]. The diatom test has been considered as the "golden standard" for the diagnosis of drowning [6], although it is still debated and not routinely applied because of the lack of a standard protocol and the technique itself is rather labor-intensive and time consuming. It is based on the assumption that the victim must be alive in order to aspirate the diatoms along with the drowning medium, entering the circulation and the internal organs. In fact, diatoms are one of the most common types of phytoplankton, photosynthesizing microscopic algae that live in fresh and saltwater, ranging in size usually between 20 and 200 μm (although sometimes their length can be greater than 2 mm), as they can be mostly unicellular but form chains or colonies [28–31]. Although diatoms are greater than marine bacteria, they can easily enter the bloodstream along with the drowning medium [18]. The capacity of diatoms to penetrate the alveolar-capillary barrier along with the drowning medium has been experimentally demonstrated by electron and fluorescence microscopy [32, 33] and should occur following the inhalation of large volumes of water. Based also on such findings, the presence of any aquatic microorganism derived from the drowning medium in the tissues of a drowning victim (such as liver, kidney and bone marrow) is considered sufficient to prove the hematogenous dissemination of particles from the lung requiring a beating heart during the violent asphyxial process, especially if these diatoms are observed in the drowning medium [19, 20, 34]. In this matter, the value of water samples for diatom analysis has been strongly emphasized for a comparative evaluation with those particles found in tissues [24–27]. In fact, seasonal variation of diatom community (in term of abundance and composition) can provide information on location, drowning medium, and the period of drowning due to their ecology characteristics [29–31, 34].

Although very promising, the diatom test is still controversial showing relevant limitations related to the interpretation of data analysis. In fact, diatoms have been found in the tissues of humans and animals who died of causes other than drowning [35–37] and may be absent in case of drowning [4–6]. Ante-mortem contamination has been ascribed mainly due to the widespread distribution of diatoms throughout the environment, in soil, water supplies, food [5, 15, 26, 27, 34], and in air [23–25, 38]. In particular, the ingestion of seafood such as shrimps and clams has been proven to increase the concentration of diatoms in tissues [39] commonly affecting the sensitivity of diatom testing with positive results also on not-drowned subjects [23–25]. Some authors have also observed that more conservative methods may preserve diatom patterns not representative of the diatom population in the drowning medium because of ante-mortem contamination [24, 36, 40]. On the other hand, false-negative results can also occur due to a prolonged digestion procedure that may destroy the diatoms, especially in the case of slightly silicized diatom species [24, 41]. The risk of contamination has raised a wide discussion on the accuracy and reliability of diatom analysis also based on the lack of data concerning the concentration of diatoms in tissues before drowning [36]. The possibility of post-mortem contamination has been also demonstrated by a pilot study on three piglet carcasses that died by natural causes and immersed in aquatic environment after death. The diatom testing showed the presence of diatoms in all samples isolated from two decomposed carcasses, immersed in water, respectively, for 1 and 2 months after death.

In this study, we applied a digestive method used in forensic context to extract diatoms in organs taken from animals for different cause of death. The method was just validated in a previous study [42] in bodies that required post-mortem examination. The protocol was performed according to the Italian guidelines for analysis of benthic diatoms for ecological status assessment of inland waters as required by Water Frame Directive 2000/60/CE [43, 44]. The goal of the present study is to investigate the possibility of ante-mortem contamination (due to diet during life) and post-mortem contamination (due to passive diffusion of water once in aquatic environment) in the veterinary context on animal carcasses belonging to different species of wildlife mammals, found in aquatic and terrestrial environments. Moreover, the authors share the idea about an absolute necessity of standardization of methodologies both in human and in veterinary context.

Materials and methods

The death of ten carcasses of four species of wildlife mammals and non-mammals (two magpies, one deer, two roes, and five otters) was investigated. Only four carcasses (one magpie, one otter, one deer, and one roe) were recovered from aquatic environments (bath trough for animals, bathtub irrigation, river) while the others along the side of roads due to traffic collisions. Post-mortem examination including a medicolegal necropsy was performed in all carcasses. Only in three cases out of four found in water the cause of death was ruled out as drowning. In seven cases, the death was due to other causes as follows: gunshot injuries for one otter found in the riverside, blunt trauma for a magpie, and traumatic injuries followed by motor vehicle collision in other four otters found on the road and a roe. For each carcass, information regarding sex, weight, site of recovery, stage of decay, post-mortem interval, and cause of death are available and summarized in Table 1 and Fig. 1.

A diatom test was performed in all carcasses. A digestive method previously used in human bodies during forensic death investigation was applied [42] according to the Italian guidelines for analysis of benthic diatoms for ecological status assessment [43, 44]. For all the carcasses, 5 g of lung, liver, and kidney was taken. In some cases, additional tissue samples were also available among which brain, heart, spleen, and bone marrow (this latter available only in one case). All autopsy samples were taken at random from organs using cleaned and separate instruments for each sample. Samples from each organ were collected separately into glass containers, previously checked for the absence of diatoms. Drowning medium was available in all four cases found in aquatic environment. Samples were stored at −20 °C until the diatom analyses by using the following hydrogen peroxide (H_2O_2) digestion. Five-gram aliquots of each sample were placed in a Schott Duran bottle, previously checked to confirm the absence of diatoms, cleaned with free phosphorous detergent, soaked in 20% HCl for 24 h, and rinsed with diatom-free distilled water (DFDW). Tissue samples and drowning medium were treated by H_2O_2 (40%). After overnight sedimentation and decanting of excess liquid, the sediment was centrifuged three times at 1000 rpm and then washed with DFDW to eliminate all trace of HCl. The supernatant was aspirated to obtain a 300-μl sample. The pellet was transferred onto microscope slides ±76 × 26 mm (Kaltek S.r.l., Padova, Italy), allowed to dry on five serially marked microscopic slides, and then mounted permanently with Naphrax resin (Brunel Microscopes Ltd., Chippenham, UK). After placement, the Naphrax was covered with cover slides (Laltek) and slight pressure was applied. Slides were positioned above a heating plate at 150 °C for at least 30 min to evaporate the solvent inside the Naphrax. Once the resin was solidified, the slides were examined using a light microscope at ×1000 magnification (Motic BA 410) with image software (ICCapture). A quantitative diatom test was also performed. Diatom counts were then performed on slides with the light microscope by calculating the number of diatoms in each milliliter of material.

Results

Twenty-one genera of diatoms were identified. The distribution pattern of diatoms and diatom genera identified along the ten animal carcasses is summarized in Table 2. The dominant genera were *Cyclotella*, *Nitzschia*, *Navicula*, *Gomphonema*, and *Amphora*. In Fig. 2, some photomicrographs of the most common occurring diatoms identified in case no. 8 (a deer found in aquatic environment) are depicted. They are *Amphora* sp., *Cocconeis pediculus*, *Navicula tripunctata*, *Nitzschia* sp., *Rhoicosphenia abbreviata*, and *Cymbella sp*. A semi-quantitative diatom test was applied in all cases according to Ludes et al. [48] and considered positive when a minimum number of at least 5 diatoms per 100 μl of sediment

Table 1 Series of ten animal carcasses investigated, found in terrestrial and aquatic environments

Species no.	Sex	Weight (kg)	Cause of death	Site of recovery	Stage of decay	PMI
Magpie (*Pica pica*)						
1	F	0.250	Drowning	Aquatic environment (bathtub irrigation)	Advanced	5 days
2	M	0.275	Blunt trauma[a]	Terrestrial environment (road)	Bloat	24 h
Otter (*Lutra lutra*)						
3	F	4.950	Gunshot injuries	Aquatic environment (river)	Fresh	08 h
4	M	5.200	Blunt trauma[a]	Terrestrial environment (road)	Advanced	48 h
5	F	5.000	Blunt trauma[a]	Terrestrial environment (road)	Advanced	72 h
6	F	4.800	Blunt trauma[a]	Terrestrial environment (road)	Advanced	4 days
7	M	6.300	Blunt trauma[a]	Terrestrial environment (road)	Bloat	72 h
Deer (*Cervus elaphus*)						
8	M	150	Drowning	Aquatic environment (river)	Advanced	10 days
Roe (*Capreolus capreolus*)						
9	M	24	Drowning	Aquatic environment (bath trough)	Advanced	72 h
10	M	26	Blunt trauma[a]	Terrestrial environment (road)	Fresh	08 h

[a] Blunt trauma due to vehicle collision

Fig. 1 Some of the carcasses investigated depicted on site of recovery or at necropsy

was observed, except for lungs where a number of 20 diatoms was requested. The diatom presence and minimum number of diatoms found for each carcass is summarized in Table 3. No diatoms were found in tissues collected from only two not-

Table 2 The distribution pattern of diatoms and diatom genera along the ten animal carcasses

Case number	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10
Diatom genera and species										
Cyclotella (Kützing) Brébisson sp.	+	–	+	+	+	+	+	–	+	–
Cyclotella ocellata Pantocsek	+	–	+	–	+	–	–	–	–	–
Denticula tenuis Kutzing	–	–	–	–	–	–	–	–	+	–
Nitzschia dissipata Kutzing	–	–	–	–	–	–	–	+	–	–
Nitzschia sigmoidea Kutzing	–	–	–	–	–	–	–	–	+	–
Nitzschia Hassall sp.	+	–	–	+	+	–	–	+	+	–
Cocconeis pediculus Ehrenberg	–	–	–	+	–	–	–	+	–	–
Amphora inariensis Krammer	–	–	–	–	–	–	–	+	–	–
Rhoicosphenia abbreviata (Agardh) Lange-Bertalot	–	–	–	–	–	–	–	+	–	–
Nitzschia hungarica Grunow	–	–	–	–	–	–	–	+	–	–
Nitzschia frustulum (Kutzing) Grunow	–	–	–	–	–	–	–	+	–	–
Navicula pupula (Kutzing) Grunow	–	–	–	–	–	–	–	+	–	–
Cymbella tumida (Brebisson) Van Heurck	–	–	–	–	–	–	–	+	–	–
Amphora inariensis Krammer	–	–	–	–		–	–	+	–	–
Navicula tripunctata (Muller) Bory	–	–	–	–	–	–	–	+	–	–
Melosira varians Agardh	–	–	–	–	–	–	–	+	–	–
Navicula capitatoradiata Germain	–	–	–	–	–	–	–	+	–	–
Gomphonema Ehrenberg sp	+	–	+	+	+	+	+	+	+	–
Gomphonema subclavatum (Grunow) Grunow	–	–	–	–	–	–	–	–	+	–
Navicula Bory de Saint-Vincent	+	–	+	+	+	+	+	+	+	–
Amphora (Ehrenberg) ex Kützing sp)	+	–	+	–	–	+	+	–	–	–
Amphora ovalis (Kutzing) Kutzing	–	–	–	–	–	–	–	+	–	–
Staurosira brevistriata Grunow	–	–	–	–	–	–	–	–	+	–
Total number of diatoms recovered	8	0	5	5	5	5	5	17	9	0

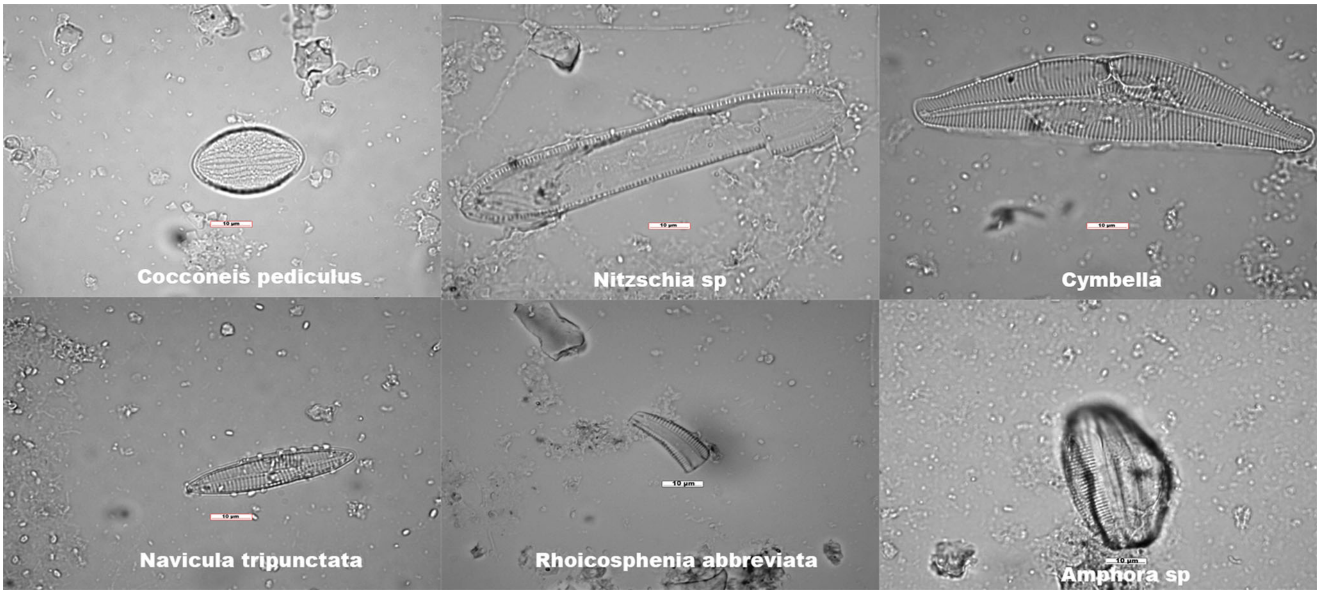

Fig. 2 The most common occurring diatoms identified in case nos. 8 and 9 (a deer and roe found in aquatic environment)

drowned cases (a magpie and a roe). In all five otters not-drowned, at least five diatoms per 100 μl were observed. In three carcasses (one magpie, a deer and, a roe) found in aquatic environment, at least eight diatoms per 100 μl in all additional necropsy samples other than lungs were counted. The maximum number of diatoms (over 15) was recovered in case no. 8 related to a deer found in aquatic environment 10 days approximately after death. In these latter three cases, the diatom population was consistent with that one recovered from the drowning medium supporting the final diagnosis of drowning.

Discussion

Diatom frustules can be easily extracted from internal organs of a drowning victim, usually whole and intact, sometimes fragmented but still recognizable [30], just because all diatoms have a siliceous skeleton frustule (made up of two valves fitted together by a connective tissue) resistant to putrefaction and degradation via enzymatic and acid digestion [42, 45, 46]. Most of the diatom sample treatment is based on the digestion of tissue samples by strong acid because it is cheap and known to be a reliable technique [24–27, 34, 42, 45–47].

Table 3 The distribution of diatom presence and minimum number of diatoms found in each carcass

Species	Cause of death	Additional necropsy samples[a]	Drowning medium	Diatom presence
Magpie (*Pica pica*)				
1	Drowning	Brain/skeletal muscle/heart/bone marrow	Yes	++
2	Blunt trauma			–
Otter (*Lutra lutra*)				
3	Gunshot injuries	Spleen/heart	Yes	+
4	Blunt trauma	Spleen/heart		+
5	Blunt trauma	Spleen/heart		+
6	Blunt trauma			+
7	Blunt trauma			+
Deer (*Cervus elaphus*)				
8	Drowning		Yes	++
Roe (*Capreolus capreolus*)				
9	Drowning		Yes	++
10	Blunt trauma			–

+ at least five diatoms per 100 μl, ++ at least eight diatoms per 100 μl, *Yes* water samples were also collected

[a] Samples collected in all species: lung/liver/kidney

The results demonstrate that diatom testing using H_2O_2 digestion can be applied on tissue samples taken also from animal carcasses, and it is suitable for the identification of diatoms. It seems a valuable diagnostic tool for routine forensic veterinary procedures in the extraction and detection of diatoms. The final diagnosis of drowning can be further supported by this diatom test as in a magpie, a deer, and a roe all found in aquatic environments. In those cases, the necropsy was negative for causes of death other than drowning, although all the three carcasses were in advanced stage of decay with a post-mortem interval ranging between 3 and 10 days. The possible effect of post-mortem contamination cannot be excluded totally, in particular, for the deer carcass in which the maximum number of diatoms (over 15) was also recovered. However, the positive quantitative diatom testing in all tissues samples (among which also bone marrow for the magpie) seems against this mere hypothesis. In fact, a large amount of diatom frustules was observed in all necropsy samples consistent with the diatom population found on drowning medium.

Qualitative and quantitative distributions of diatoms [25, 38] in the tissues are mainly dependent by the diatom density (number, species, and dimensions of such organisms) in the drowning medium and the filtration with respect to size that occurs as the diatoms pass from the lungs into the blood [48]. It seems that only quite small diatom valves can penetrate as far as the bone marrow during drowning, and most of the diatoms or their fragments have a maximum length of less than 20 μm.

The quantitative diatom test is sometimes performed to aid in excluding post-mortem contamination due to passive diffusion of water into the lungs and other organs [34, 38], and it can be considered positive when a minimum number of 20 diatoms per 100 μl of sediment from enzymatic digestion of 10 g of lung tissue or at least 5 diatoms from other tissues extracted similarly have been found [34]. In the present study, only in three carcasses (a magpie, a deer, and a roe), at least 10 diatoms per 100 μl of sediment derived from necropsy tissues other than lungs were observed supporting the hypothesis of an hematogenous dissemination of particles during the asphyxial process.

The possibility of ante-mortem contamination is also confirmed by the present pilot study. In all five cases related to not-drowned otters, the presence of diatoms was also demonstrated but in less number (only five per 100 μl of sediment). This result can be easily explained with the feeding behavior of this semi-aquatic carnivorous mammal whose diet is mainly based on fish, crabs, lams, shellfish, and other shelled creatures.

These results are consistent with a previous pilot study [36] showing a strong evidence of the ante-mortem contamination during life as represented by a sea-diatom (belonging to a different pattern from the expected one) found in a muscle sample of a piglet immersed in water after death. The authors explained such findings on the presence of fish flours in fodders for animal-breeding as the sea-diatom could be derived from the feeding of piglet usually by fishmeal [36]. According to such interpretation of diatom presence in tissues, the report of diatoms also in routine cytology [49] has been attributed to contamination of the tap water. The diatom presence in a cytologic specimen of a tracheal wash in a non-human mammal (a female German Wirehaired Pointe), with aspiration pneumonia, was attributed to inhalation of surface water [50]. Previous animal experimentations have demonstrated that the survival time is inversely proportional to the volume of fresh or salt water aspirated in dogs [51, 52] since the aspiration of as little as 5 mL of salt or fresh water in dogs can cause rapid and persistent hypoxia without significant hypercarbia.

The absence of diatoms in two not-drowned cases is also worth mentioning. In a magpie and a roe deer, which all died due to blunt injuries, no diatoms were observed according to their diet. Roe deer is a well-known herbivorous mammal, widespread in Europe that feed on grass, leaves, and young shoots. Magpies are birds of the Corvidae family, opportunistic omnivore, eating not only many types of insects, carrion, and rodents but also garbage depending on their habitat (urban, suburban, farmlands, forest, etc.). No trace of diatoms was found in the tissue samples taken from these two carcasses according to their daily diet including no seafood at all. Based on the above findings, the application of forensic techniques in the veterinary context can give further information on the limits of diatom testing, useful for a reliable interpretation of the results. Most of information on drowning process is derived from experiments in dogs or other animals, drowned just to determine physiological and biochemical responses to various volumes, temperatures, and salinity of water or other liquids [8, 53–56]. Experimental animal models of drowning have been also used to develop PCR testing for detection of diatoms [57], for determining submersion intervals [58], and to document diatom penetration of the alveolo-capillary barrier by electron microscopy [5]. According to Knight [59], unfortunately, the vast amount of published material, using animal experimentation, has obtained little practice relevance. The lack of knowledge is still relevant in the mechanism of drowning, and there is a strong need of further research. In particular, Knight [59] criticized the "classic research" on drowning using un-anesthetized and conscious dogs as not only an example of questionable correspondence between scientific data on animals and humans but also an example of the great lack of consideration for the welfare of animals, actually not acceptable and disturbing [3, 60].

It is the authors' opinion that there is no need for experimental animal models of drowning as very useful information can be derived from the application of a medicolegal approach in death investigation even in animal carcasses from routine forensic veterinary casework. Drowning is not anymore accepted as method of euthanasia by the American Veterinary Medical Association and others [61, 62].

In the veterinary context, the results of the present study confirm some limitations of the diatom test mainly related to the ante-mortem contamination in aquatic mammals (such as otters). On the other hand, negative diatom testing has been also found as expected on herbivorous mammals (roe deer) and omnivore birds (magpie). A quantitative diatom test can be useful to support a final diagnosis of drowning when no other cause of death is demonstrated by necropsy findings. Post-mortem and ante-mortem contamination has to be considered according to diet during life stage of decay and post-mortem submersion interval [63, 64].

There are no guidelines or standard procedure for the monitoring of the biological quality elements (diatoms) in the veterinary field, as well as for cases of human death. Standard protocols are applied only in the environmental field in order to standardize the methods of collection of the sampling data of bodies and methods of analysis [43, 44]. It would be desirable that the same analytical protocol could be applied in a forensic context in cases of suspected drowning and/or body was found near waterways. It would be appropriate to indicate how the sampling was carried out by operators intervened during the inspections, type and weight of the sample, site of event, and drowning medium. The digestive method we have adopted in this study includes the use of hydrogen peroxide according to the Italian national protocols [43, 44], already used by a diatomologist for analysis of diatoms of rivers and lakes to assess the ecological status of water bodies. The method proved to be suitable also for the search of the diatoms in the organs of the bodies of animals autopsied in the veterinary field to identify the cause of death. The authors believe that the application of the diatom test to confirm drowning in animals requires additional rigorous validation studies especially in the harmonization of procedures [65].

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