DIATOMS IN FORENSIC ANALYSIS. A PRACTICAL APPROACH IN RATS.

Isaac K. Badu[1], Eloy Girela[1], Cristina M. Beltrán[1], Ignacio Ruz-Caracuel[2], Ignacio Jimena[2].

1. Section of Legal and Forensic Medicine. Faculty of Medicine. University of Córdoba, Spain
2. Department Morphological Sciences (Section of Histology). Faculty of Medicine. University of Córdoba, Spain

Corresponding author: Prof. Dr. Eloy Girela

Department of Legal Medicine, Faculty of Medicine.

University of Córdoba, 14004 Córdoba, Spain.

Telephone: +34-957-218156 Fax: +34-957-218279

e-mail: ft1gilpe@uco.es

Abstract

A diagnosis of drowning is a challenge in legal medicine as there is generally a lack of pathognomonic findings indicative of drowning. Diatom analysis has been considered very supportive for a diagnosis of drowning, though the test is still controversial for some investigators. We assessed diatoms association with drowning in the peripheral tissues of drowned rats and effects of the drowning medium on the diatom yield. A modified acid digestion method was optimised for diatom recovery in water and rat tissues. 18 Wistar rats were employed for the study; sub-divided into six groups of three rats. Groups 1, 3, and 5 were drowned in sea, lake, and river water respectively, while groups 2, 4, and 6 were controls (immersed after death in sea, lake, and river water respectively). Water samples were taken from the sea, lake, and river in Málaga and Córdoba (Spain) for the purposes of diatomological mapping and drowning of the rats. Diatoms were successfully recovered from all water samples and matched with tissues of the drowned rats. There were significant differences in diatom numbers between control and test samples for all the tissues studied as well as within test samples. Histological investigations conducted on lung samples obtained from drowned rats provided complementary and valuable information. This study demonstrates the feasibility of the diatom test as a reliable method for the diagnosis of drowning, especially if adequate precautions are taken to avoid contamination, and if interpretation of the analysis is performed in light of other complementary investigations.

Key words:

Drowning, Diatom test, Post-mortem submersion, Diatomological mapping, Rats

Introduction

When bodies are retrieved from water, the usual questions asked are if the person died from drowning, from other causes while in the water, or was submerged after death. Drowning is an asphyxial death caused by respiratory impairment from submersion/immersion in a fluid, whether or not the fluid is aspirated into the lungs. Its diagnosis, however, is still a very difficult task in legal medicine [1]. It lacks pathognomonic autopsy findings that are diagnostic of drowning [2]. Several authors have postulated various diagnostic tests, most of which are merely suggestive or supportive of the diagnosis. There are four main objective data sources that can be examined for the diagnosis of drowning: autopsy findings, chemical markers, microscopic/histologic findings, and diatom analysis.

At autopsy, drowning decedents may show frothing in the mouth, nostrils and airways, oedematous and heavy lungs, emphysema (inflation), sub pleural petechial, water in the nasal sinuses, and pleural effusion. Nevertheless, signs of drowning are scanty and not absolutely specific. Furthermore, these signs may be lost

when a considerable period has elapsed between death and retrieval of the body [3]. It also is not uncommon to observe microscopic findings in lung that include intracellular swelling, emphysema aquosum, rupture of terminal air spaces, alveolar dilatation, thinning of inter-alveolar septa, a marked increase in alveolar macrophages, and increased phagocytic activity [4]. Chemical ion markers such as chloride, strontium, magnesium, and iron concentrations have been investigated for evidence of haemodilution and haemoconcentration. For instance, Gettler [5] suggested that in freshwater drowning, chloride ion concentration was diagnostic of haemodilution if the difference between the right and left ventricles was above 25mg/100ml blood, although this finding has been refuted by others [6]. This test is flawed by a lack of sensitivity and limited by putrefactive changes [7] suggested that blood strontium was diagnostic of haemoconcentration in seawater drowning if the concentration difference was more than 75 µg/l. Recently, Pérez-Cárceles et al. [8] found that strontium was the best serum trace elements for the diagnosis of drowning in seawater (significantly higher in left ventricle than in right). The drawbacks, however, include the non-specificity of the tests and variation in strontium levels seawater of varying salinity and at different locations.

The diatom test has been proposed as a marker for the diagnosis of drowning. It may be especially useful for the diagnosis of drowning in putrefied bodies which are impossible to diagnose anatomically and useful for the ecological mapping in relation to the water types and locations [3, 9, 10]. The test is based on the theory that it was possible to recover diatoms in the peripheral organs of drowned decedents if they aspirated water containing diatoms into the lungs which eventually enter the blood circulation.

Since the first discovery of diatoms in the lungs by Hofmann in 1896 and its diagnostic use by Revenstorf in 1904, the method has been used and improved over the years. The test, however, is not without controversy; some authors [11-13] question its reliability as it is prone to contamination due to the ubiquitous nature of diatoms. Moreover the possibility of diatoms entering the body through the intestinal tract into the bloodstream also has been proposed.

Our study aimed to establish evidence of a diatom association with drowning by conducting drowning and non-drowning experiments in rats to investigate the effects of water type on diatom recovery.

Materials and methods

Water samples

Samples of water were obtained from three sites; the Mediterranean Sea at San Luis de Sabinillas, Manilva-Malaga; Lake Navallana at Alcolea-Cordoba and River Guadalquivir in Cordoba, Spain between 8th February, 2013 and 27th April, 2013. Samples were obtained with plastic bottles from the seashore and close to the banks of the lake and river respectively. The water samples were for diatomological mapping of the three sites and the drowning experiments.

Experimental Series of Animals

Eighteen (18) adult Wistar rats (average weight 453.9 ± 52.6 grams) were randomly assigned to one of six groups, each consisting of 3 animals. The groups included Group 1: drowned in sea water, Group 2: control post-mortem submersion in sea water, Group 3: drowned in lake water, Group 4: control post-mortem submersion in lake water, Group 5: drowned in river water, and Group 6: control post-mortem submersion in river water. The rats were maintained under the same laboratory conditions at the University of Cordoba, School of Medicine, under the supervision of an animal technician prior to the experiment.

Batch drowning was conducted according to the allocated groups. Groups 1, 3 and 5 were drowned in sea, lake and river water respectively. Each rat belonging to the groups was caged and completely submerged in plastic container filled with water.

The control groups (2, 4 and 6) were anaesthetized by injection with ketamine 75mg/Kg (Imalgene 100mg/ml, Merial Laboratorios) and sacrificed by elongation of the cervical spine before submersion in the water. All drowning/submersion procedures were performed at room temperature for a total period of 30 minutes with intermittent movement of the water to ensure adequate interaction between the diatom-rich water and the submerged rats.

The rats were washed thoroughly with deionised water prior to necropsy. Cross-contamination was prevented by using separate sets of surgical instruments for the dissection of external and internal organs for each rat and all excised tissues were washed with diatom-free deionized water before storage in sterile capped tubes for freezing.

Approximately 10 g of muscle from the superior portion of the posterior limbs and whole samples of liver, kidneys, spleen, lungs, brain, and one femur were placed in 15 ml plastic capped tubes and frozen at -21 degrees Celsius. A part of the lung was removed and fixed in diatom-free 10% saline-buffered formalin solution for histological examinations.

Acid Digestion of tissues

The criteria for selecting the best digestion method was based on the cleanest sample with least organic matter, integrity of the diatom frustules, high diatom density and availability of diagnostic features for species identification. The optimum procedure was determined by the modification of generally accepted and widely reported methods in literature [3, 14, 15].

The modified nitric acid digestion method was developed and optimised (Table 1). All glassware used for the experiment were thoroughly washed with detergent and rinsed several times with deionised water. Approximately 2-4 g of tissue was transferred into a 100 ml beaker in a fume chamber. Concentrated (65%) nitric acid (1:4) was added and incubated on a hotplate for an hour. Drops of 1M HCl were added to decalcify the sample which were further oxidised with equal volumes (1:4) of 30% H_2O_2. $K_2Cr_2O_7$ grains were added to

accelerate the process with additional heating for 2-3 hours for concentration. All these reagents were from Panreac Quimica SLU (Barcelona, Spain). Samples were allowed to cool and then transferred into 15 ml graduated centrifuge tubes. The tubes were centrifuged at 1500 rpm for 10 minutes. All but 0.5 ml of supernatant was drawn off without disturbing the pellet at the bottom. Approximately 10 ml of deionised water (Option 4 water purifier, ELGA-Gemini BV, Netherlands) was added to the tube and vortexed gently. The sample was re-centrifuged for 10 minutes at 1500 rpm and the process repeated at least 4 times. Deionised water was also examined simultaneously to rule out possible contamination resulting from the reagents or the environment.

50µl of well mixed final cleaned sample was pipetted onto the coverslip (24 x 60mm) and dried on a slide warmer (50-60°C) and mounted. A smear of nail polish was applied to the edges of a clean pre-labelled slide (26 x 76mm) and coverslip were mounted on the slide with the sample side down and pressed gently. Diatoms were examined using an Optical Microscope fitted with high definition camera (Nikon Eclipse E1000) at magnifications 400 for diatom count and 1000 for diatom identification, both in the water samples and tissue samples.

Diatom quantitation and statistical tools

Diatoms were identified and classified by comparison to available literature [16, 17, 18]. Diatoms were counted and measured by using the Image-Pro plus (Version 4.5.0.29) imaging software. This program was used to assist the observer in the counting and measuring process, who was blind to the samples. The criterion for inclusion of destroyed diatoms in the count was if more than half of the frustule was still intact.

Data analysis was performed using Microsoft Excel and STATA applications. Statistical tools employed included ROC Curve in order to choose the cut off points, and non-parametric test such as Kruskal-Wallis One Way ANOVA, Mann-Whitney Rank Sum Test and the Tukey Test for the experimental samples. We considered statistically significant differences for p-values less than 0.05.

Histological analysis

Lung samples were fixed in 10% saline-buffered formalin and parafin-embedded. Sections of 6 µm thickness were obtained and stained with Haematoxilin and Eosin (H & E) for light microscope studies under bright-field illumination on a Nikon Eclipse 1000 microscope coupled with a high-definition camera at magnifications of x100 and x400. The histologists responsible for the analysis were blinded to the groups.

Ethical issues

The experiment was performed according to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, and was ethically approved by the University of Cordoba bioethics committee before commencement.

Results

Numbers, sizes and types of diatoms in water samples

The ranges of diatom counts per 50 µL pellet of Lake Navallana, River Guadalquivir and the Mediterranean Sea samples collected between the 8th and 14th of February, 2013 were 97-200 (150 ± 67), 597-859 (700 ± 313) and 166-1007 (404 ± 181) frustules, respectively. Diatom counts from samples collected on 27th April, 2013 were 291-459 (354 ± 56) for lake; 18-123 (50 ± 23) for river, and 128-239 (183 ± 82) diatom frustules/50µl for sea. These counts demonstrated a considerable decrease in sea and river counts between the two different sampling periods; the lake, however, showed an increase in the diatom count.

Diatom sizes for the lake, river, and sea from the first sampling period (8th to 14th February) were 57.53 ± 47.27 µm, 9.73 ± 5.17 µm and 8.50 ± 4.91 µm, respectively. The second batch (collected on 27th April) contained diatoms with sizes of 52.51 ± 46.05 µm, 39.27 ± 23.92 µm and 7.62 ± 6.23 µm respectively for lake, river, and sea. This indicates fairly larger diatom sizes in the lake samples relative to the sea and river (see Fig.2). A decrease in diatom size was observed between the two sampling periods in the lake and river. The sea, however, showed a considerable increase in diatom sizes within the same period.

Sea samples contained characteristic centric and pennate diatoms predominantly *Cyclotella* sp, *Nitzschia* sp, *Navicula* sp, *Cymbella* sp, and *Lyrella* sp. Also, lake diatoms consisted predominantly of *Nitzschia* sp, *Navicula* sp, *Melosira roseana, Gomphoneis herculeana* and *Gomphonema* sp. Diatoms recovered from the river samples were mainly *Gomphonema* sp, *Suriella* sp, *Amphora* sp, *Bacillria* sp, *Synedra* sp and *Fragilaria* sp.

Diatom quantitation in animal tissues

Diatom numbers per 50µl recovered from rat tissue samples are shown in Table 2. All diatoms were further classified based on morphology [19] and one obvious finding was the abundance of centric and symmetrical biraphid diatoms in all samples. Commonly identified diatoms included Navicula, Nitzschia and Fragilaria spp. (see Fig 1).

Our study adopted a cut-off value for classifying diatom count as positive based on a ROC curve. A minimum of 4 diatoms / 50µl in lung (sensitivity- 89%, specificity-78%) and 1 diatom / 50µl in other organs (sensitivity and specificity-100%) were accepted.

This study tested for significant differences between diatom counts in the tissues of control and test rats (see Table 3). The test revealed a significant difference ($p<0.05$) between the diatom numbers in all the tissues.

The Kruskal Wallis test and the Tukey test also demonstrated general and specific differences in diatom numbers between the drowned tissue samples ($p<0.05$), indicating a significant difference in diatom numbers between the lung and the other organs observed except for muscle and spleen.

Estimated diatom sizes recovered from rat tissues were 19.0 ± 13.5 µm for sea, 33.7 ± 26.4 µm for lake and 38.6 ± 32.8µm for river.

Histologic findings

Histological analysis was performed on lungs samples of test and control rats in seawater and freshwater.

Sea Control samples: The most striking findings were inflammatory infiltrates perivascularly and in the alveolar interstitium. In some zones the alveoli were collapsed with congestive vessels (see Fig. 2).

Fresh Control samples: The findings were similar to those in the control sea group (inflammatory infiltrates around vessels and in the interstitium). Nevertheless, in some cases, dilated alveoli, ruptured walls, thickening of the alveolar septa and reduction of the intra-alveolar space were observed (see Fig. 2).

Sea test samples: The most remarkable findings were intra-alveolar haemorrhages and emphysema aquosum, with rupture of several alveolar walls and flattened inter-alveolar septa. Perivascular and intra-alveolar spaces infiltration of inflammatory cells and macrophages were noticeable as well as foreign material in alveolar spaces, probably algae and diatoms (see Fig.3)

Fresh test samples: The changes observed were similar to those in the drowning sea cases; intra-alveolar haemorrhage and emphysema aquosum. There was also inflammatory cell infiltrates mainly inter-alveolar, with increasing numbers of macrophages within alveolar spaces, some of them pigmented with hemosiderin.

Discussion

Diatom counts in water samples varied between the two sampling periods with a decrease in diatom numbers in the sea and river samples but an increase in the lake samples. For diatom sizes, the river samples were the smallest for both sampling periods.

Analysis of the water samples indicated larger numbers of intact diatoms recovered in the river and lake compared to the sea. The considerable destruction of sea diatom frustules has been observed by other authors who employed alternative methods [14, 20, 21]. This phenomenon had been attributed to thinner and less resistant siliceous cell walls of sea diatoms rendering them fragile and vulnerable to harsher treatments [21]. The number of diatoms recovered and identified is influenced by the sampling location, digestion method, and microscopic magnification [22, 23]. Moreover, the variations in diatom counts from the same location are

affected by the tidal flow of the sea, conditions of the river and lake, and prevailing changes in weather conditions such as the onset of rainfall [24]. This is in accordance with the findings of our study, suggesting that the changes in diatom numbers and sizes were influenced by changes in weather conditions, especially the onset of rainfall during the collection periods.

In relation to the diatoms recovered from rat tissues, the experiment detected the highest diatom counts and largest sized diatoms in tissues from lake water; conversely the tissues from sea water yielded low diatom counts and the smallest sizes. The ability of diatoms to percolate the alveolar walls of the lungs into pulmonary circulation has been attributed to size and morphology [21]. The considerable reduction in diatom counts in test sea samples despite their relatively small sizes supports the assertion that perhaps most of the diatom frustules were destroyed by the acid digestion method.

The results showed generally low diatom counts in the tissues of control rats. The numbers of the diatoms counted in test samples ranged from 10-221 frustules / 50µl, while those in control (post-mortem submersion) samples ranged from 0-5, respectively. Furthermore, all the diatoms observed in the tissues of control rats were in the lung. It was notable that all diatoms in tissues matched the respective drowning media. We want to highlight the fact that the observer was blind to the samples in order to avoid a bias.

A common trend observed was that for samples with positive diatom count for organs such as kidney, liver, muscle, femur, brain, and spleen also showed positive diatom counts for lungs; conversely, samples with no diatoms in the lungs also had no diatoms in the other organs as well. This trend was observed by Auer and Möttönen [14] when they performed a diatom analysis using 107 human cadavers.

In this study we adopted a cut-off value for classifying diatom counts as positive based on a ROC curve. A minimum of 4 diatoms / 50µl in lung and 1 diatom / 50µl in other organs were accepted, according to our experimental conditions. These values are slightly different from other studies [3, 25]. However this study adopted a different criterion for diatom counts in tissue samples. This was due to the different parameters such as the smaller mass (3 g samples) and volume (50µl pellet) of sample used.

Two of 9 (22%) control samples were positive for diatoms in the lungs based on the cut-off values used in this study. The numbers, though not high, are important as it may affect the interpretation of drowning cases. One of the main challenges in the inclusion of lungs for diagnosis of drowning is the possibility of passive movement of water into the lungs [25-27]. It is therefore not the best tissue for diatom analysis, and requires other peripheral organs for more reliable interpretation. This study tested for significant differences between diatom numbers in the tissues of control and test rats using the Mann-Whitney Rank Sum test. The test revealed a significant difference ($p<0.05$) between the diatom numbers in lung and other tissues of control and test rats. In doubtful cases, when organs contained only a few diatoms, or when the density of diatoms was low in water at discovery sites, we could use an additional confirmatory method, such as bacterioplankton detected by PCR, which provides important information about the type of aspirated water [28].

Macroscopic and histological findings in the test samples indicated some classical signs of asphyxia, mainly sub-pleural haemorrhages, pulmonary congestion and oedema, though these are mostly non-specific

findings of death. The histological findings were mostly similar to those published in the literature for the diagnosis of the "drowned lung" [4], such as emphysema aquosum, thinning and tearing of the alveolar septa, intra-alveolar haemorrhages, and a leading amount of macrophages within alveolar spaces, some of them pigmented with hemosiderin (the latter more evident in fresh water samples). Some results however were difficult to interpret, such as some emphysema in control samples (although some authors have claimed that this change can result simply from the hydrostatic pressure caused by submersion in a water depth of 4 m or more in humans [29], and even the presence of pigmented macrophages (probably haemosiderin is a finding not related to drowning because the short period between submersion and death does not permit to activate this change). Also, perivascular and interstitial inflammatory infiltrates were noticed both in control and test samples. We observed some foreign material including probable algae, and diatoms in the intra-alveolar spaces both in control and test samples.

The above described findings indicate vital submersion and support a diagnosis of drowning in test samples, even though there were diatoms in histological lung slides of both test and control samples.

Conclusions:

This study was successful in using diatoms analysis to differentiate drowning from post-mortem submersion in rats. Diatom counts in the control and test groups statistically differentiated the two. Moreover the diatom yield depended on the technique used including the tissue size, digestion method, and counting method. It also is important to use condition-dependent cut-off values. Finally, the interpretation of the analysis in conjunction with other tests in a contamination-free environment produces reliable results.

A few recommendations include the following: Contamination should be avoided as much as possible, tissues used should be adequate, and alternative less-harsh techniques should be used to analyse seawater diatoms. Finally, lungs should be assessed for diatoms together with other organs.

Limitations of the study:

There were specific setbacks and challenges which included the limited sample size used for the experiment. A total of three rats per experimental group seem inadequate to show outcome patterns exhaustively. Moreover, most of the organs used, including the brain, kidney and spleen were of low quantity (weight).

Acknowledgment

The authors wish to acknowledge Prof. José L. Ubera (Department of Botanic, Ecology, and Plant Physiology. University of Córdoba) for his technical assistance in classification and taxonomy of the diatoms.

References

1. Piette, M.H. and De Letter, E. Drowning: still a difficult autopsy diagnosis. Forensic Sci Int. 2006;163(1-2):1-9.
2. DiMaio, V.J. and DiMaio D. Death by drowning. In: DiMaio VJ and DiMaio D (eds). Forensic Pathology. 2nd Edition. CRC Press LLC, Boca Raton, Florida. 2001. pp. 399-407.
3. Saukko, P and Knight, B. Immersion deaths. In: Saukko P and Knight B (eds). Knight's forensic pathology. 3rd ed. London: Arnold; 2004. pp. 395–411.
4. Dettmeyer, R. B. Histopathology of selected trauma. In: Dettmeyer RB (ed.). Forensic Histopathology: Fundamentals and Perspectives. Edición. Heidelberg: Springer Berlin; 2011. pp. 37-65.
5. Gettler, A.O. A method for the determination of death by drowning. JAMA. 1921;77:1650-2.
6. Durlacher S, Freimuth H, Swan H. Blood changes in man following drowning. Arch Pathol. 1953;56:454-460.
7. Azparren JE, De la Rosa I, Sancho M. Biventricular measurement of blood strontium in real cases of drowning. Forensic Sci Int. 1954;69:139-148.
8. Pérez-Cárceles MD, Del Pozo S, Sibón A, Noguera JA, Vizcaya MA, Luna A. Serum biochemical markers in drowning: Diagnostic efficacy of Strontium and other trace elements. Forensic Sci Int. 2012;214:159-166.
9. Thakar, M.K. and Singh, R. Diatomological mapping of water bodies for the diagnosis of drowning cases. J Forensic Leg Med. 2010;17(1):18-25.
10. Xu, G., Hu, B., Shen, R., Pan, X., and Zhou, X. Applications for Drowning Identification by Planktonic Diatom Test on Rats in Forensic Medicine. Procedia Engineering. 2011;18:417-421.
11. Schellmann, B. and Sperl, W. Diatomeen Nachweis im Knochenmark (Femur) Nichtertrunkener. Z. Rechtsmed. 1979;83:319-324.
12. Foged, N. Diatoms and Drowning-Once More. Forensic Sci Int. 1983;21:153-59
13. Yen, L.Y. and Jayaprakash, P.T. Prevalence of diatom frustules in non-vegetarian foodstuffs and its implications in interpreting identification of diatom frustules in drowning cases. Forensic Sci Int. 2007;170(1):1-7.
14. Auer, A. and Möttönen, M. Diatoms and drowning. Z Rechtsmed. 1988;101(2):87-98.
15. Pollanen, M.S. The Diagnostic Value of the Diatom Test for Drowning, II Validity: Analysis of Diatoms in Bone Marrow and Drowning Medium. J Forensic Sci. 1997;42(2):286-90.
16. Vinyard, W.C. Diatoms of North America. 2nd Edition. California: Mad River Press Eureka; 1979.
17. Bourrelly, P. Les algues d'eau douce. Algues jaunes et brunes. París: Société Nouvelle des ëditions Boubée.; 1981.
18. Stidolph, S.R., Sterrenburg, F.A.S., Smith, K.E.L., and Kraberg, A. *Diatom Atlas*: U.S. Geological Survey Open-File Report 2012–1163, 2012. Available online at http://pubs.usgs.gov/of/2012/1163/.
19. Spaulding, S.A., Lubinski, D.J. and Potapova, M. Diatoms of the United States. 2010. http://westerndiatoms.colorado.edu (2010; accessed 1 July 2013).

20. Terazawa, K. and Takatori, T. Isolation of intact plankton from drowning lung tissue by centrifugation in a colloidal silica gradient. Forensic Sci Int. 1980;16(1):63-66.
21. Sidari, L., Di Nunno, N., Costantinides, F., and Melato, M. Diatom test with Soluene-350 to diagnose drowning in sea water. Forensic Sci Int. 1999;103(1):61-5.
22. Hürlimann, J., Feer, P., Elber, F., Niederberger, K., Dirnhofer, R. and Wyler, D. Diatom detection in the diagnosis of death by drowning. Int J Legal Med. 2000;(114):6-14
23. Ming, M., Meng, X., and Wang, E. Evaluation of four digestive methods for extracting diatoms. Forensic Sci Int. 2007;170(1):29-34.
24. Kakizaki, E., Kozawa, S., Sakai, M., and Yukawa, N. Numbers, Sizes, and Types of Diatoms around Estuaries for a Diatom Test. Am J Forensic Med Pathol. 2011;32(3):269-274.
25. Ludes, B., Coste, M., Tracqui, A., and Mangin, P. Continuous River monitoring of the diatoms in the diagnosis of drowning. Journal of Forensic Sciences. 2016;41(3):425-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8656181.
26. Singh, R., Singh, R., Kumar, S. and Thakar, M. K. (2006) Forensic Analysis of Diatoms- A Review. Internet Journal of Forensic Medicine and Toxicology. 2006;7(2).
27. Lucci, A., Campobasso, C. P., Cirnelli, A., and Lorenzini, G. A promising microbiological test for the diagnosis of drowning. Forensic Sci Int. 2008;182(1-3):20–6.
28. Uchiyama T, Kakizaki E, Kozawa S, Nishida S, Imamura N and Yukawa N. A new molecular approach to help conclude drowning as a cause of death: Simultaneous detection of eight bacterioplankton species using real-time PCR assays with TaqMan probes. Forensic Sci Int. 2012; 222: 11–26.
29. Janssen, W. Gewebe-und Fremdkörperembolien, in: Autores. *Forensische Histologie*, Edición. Lubeck: Ed. Schmidt-Römhild; 1977.

TABLES

Table 1. Method development and optimization

Digestion technique	Concentration	Digestion time (h)	Observation
HNO_3	2M	12, 24 and 48	Slide surface completely coated by organic matter, digestion incomplete, impossible to identify diatoms
	9M	12, 24 and 48	Less organic matter coating slide surface, difficult to identify and or count diatoms, incomplete digestion of organic matter
	14.5M (65%)	12, 24 and 48	Less organic matter though still difficult to reliably identify and count diatoms in 12 hour samples. 24 and 48 hours samples showed improved background as there was less organic matter coating the surface. It was possible to identify and count diatoms. There was less organic matter in the 48 hours samples than the 24 hours samples, but there were more broken diatom frustules in the 48 hour samples.
H_2O_2 + heat	Concentrated (30%)	4	Organic matter less digested, difficult to identify and count diatoms
Combined (HNO_3 + H_2O_2 + $K_2Cr_2O_{7)}$	Conc. HNO_3, Conc. H_2O_2	24	Slides show clearer background with less organic matter, diatoms are identifiable and countable. No significant differences between direct mounted slides and glycerol mounted slides
		*4 hours + heat	Organic matter mostly digested, diatoms easily identified and counted. Fewer broken frustules

* Optimum conditions achieved.

Table 2. Diatom numbers per 50µl recovered from rat tissue samples.

Rat samples	Nº diatoms / 50 µl sample							Total
	Femur	Brain	Lung	Spleen	Kidney	Liver	Muscle	
[a]**G1.1**	**2**	**3**	**4**	**1**	**3**	**1**	**1**	**15**
G1.2	0	1	11	0	0	0	2	14
G1.3	0	6	2	0	1	0	1	10
[b]**G2.1**	0	0	5	0	0	0	0	5
G2.2	0	0	3	0	0	0	0	3
G2.3	0	0	2	0	0	0	0	2
G3.1	0	0	82	0	0	0	2	84
G3.2	0	0	16	5	4	5	2	32
G3.3	3	4	194	5	2	2	11	221
G4.1	0	0	5	0	0	0	0	5
G4.2	0	0	2	0	0	0	0	2
G4.3	0	0	3	0	0	0	0	3
G5.1	2	3	25	7	0	1	6	44
G5.2	4	0	62	2	0	9	3	80
G5.3	4	5	26	5	6	7	3	56
G6.1	0	0	2	0	0	0	0	2
G6.2	0	0	0	0	0	0	0	0
G6.3	0	0	0	0	0	0	0	0

[a] Groups 1 (G1), 3 (G3) and 5 (G5) refer to test rats drowned in the sea, lake and river water respectively.
[b] Groups 2 (G2), 4 (G4) and 6 (G6) refer to control rats submerged in the sea, lake and river water respectively.

Table 3. Mann-Whitney Rank Sum Test of control and test samples

	Groups						
	Lung	Liver	Brain	Kidney	Spleen	Muscle	Femur
Median (Control groups 2, 4 and 6)	2.00	0.00	0.00	0.00	0.00	0.00	0.00
Median (Test groups 1, 3 and 5)	25.00	5.50	3.00	1.00	2.00	2.00	2.00
[a]**P-value**	0.004	0.005	0.005	0.014	0.005	<0.001	0.014

[a] For p-values less than 0.05, the difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference

FIGURES

Fig.1 Light microscope photographs of some diatoms recovered from liver (A), spleen (B, F), Lung (C, E) and kidney (D) in test groups. Magnification: 100x

Fig. 2 Control sample (seawater) with (A) perivascular and (B) interstitial infiltration of inflammatory cells and macrophages. Control sample (freshwater) with some ruptured walls, dilated alveoli, and perivascular and interstitial infiltration (C) and thickening of the alveolar septa showing intra-alveolar diatom (D). Stain: H/E, magnification: (A)-10x, (B)-40x, (C)-10x, (D)-40x.

Fig. 3 Test sample (seawater); rupture of alveolar walls and flattened inter-alveolar septa (A); inflammatory infiltrate around vessels (B), and many macrophages infiltration into alveolar spaces (C) and foreign material in alveolar space (D and E), probably algae and diatoms. Stain: H/E, magnification; (A)-10x and (B-E)-40x.