Diatom extraction: A new technique with heated H2O2. A technical note

Legal Medicine

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Abstract:	The best method of diatom identification in animal and human tissues is still an important discussion topic, in terms of effectiveness and reliability. In this technical note, authors propose a new method of extraction of diatoms using heated hydrogen peroxide from animal and human tissue samples. This method has been compared with the traditional method of digestion with acids. The results of the comparison show that heated hydrogen peroxide extraction is more efficient in terms of reduction of sediment, extraction of the material and preservation of diatoms proving to be a viable alternative to conventional approaches with acids in terms of costs and operator safety.
Suggested Reviewers:	Reinhard B. Dettmeyer reinhard.dettmeyer@forens.med.uni-geissen.de
Response to Reviewers:	Following technical observations, the method has not been modified but has been explained in greater detail in order to offer a gratifying result.

Cover Letter

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Parma, 12th June 2020

Dear Editor,

We would like to submit a short communication entitled "*Diatom extraction: a new technique with heated H2O2. A technical note*" authored by Dr Cristina Marchetti, Mr. Giovanni Lanzi, Dr. Andrea Lusetti, Dr. Alessia Bertani, Prof. Rossana Cecchi and myself, which we hope can be suitable for the publication on *Legal Medicine*.

In this work a new method of digestion of diatoms is proposed. The novelty is the use of hydrogen peroxide heated to 70°C. This procedure is characterised, besides a best quality of the tissue digestion, by a lower cost and greater safety for operators compared to more traditional methods such as acid digestion.

We aim to contribute to the relevant thanatological field of forensic science by adding valuable technical information on the management of diatoms.

We hope that the paper could be of interest for its publication on *Legal Medicine*.

Best Regards,

Dr. Francesca Marezza

Once again, we would like to thank you for your interest in our research. In response to the comments of the reviewers, the text changes are written in red.

Reviewer comments and authors' answers:

Reviewer #1: I hope that your new H_2O_2 method will help to diagnose a death by drowning in the near future.

We kindly appreciate the genuine interest and the trust expressed in our work.

Reviewer #2: General comments. I have extracted the diatom actually several times according to information on the addition from you. However, I did not obtain the great result. In addition, I changed the amount of the added alcohol, and did the extraction. The fat layer was not lost. The microscopic specimen did not transparently become clear either. I did not obtain the great result, though I will do according to your instruction and additional improvement. Therefore, I judge this thesis to be reject to our regret.

We are extremely apologetic that reviewer 2 did not achieve a satisfactory result by performing our improvements. However, we still believe that our diatom extraction method can offer a suitable and safe alternative to the acid one.

Reviewer #3: A diatom test of tissue samples from the victim found in water is crucial in a forensic practice. Although Italian investigators have reported cold hydrogen peroxide (H2O2) method with or without a drop of hydrochloric acid [1, 2], authors describe a novel method using heated H2O2 without acid reagent. This method seems to be more desirable than the traditional strong acid digestion in the point of effectiveness.

We kindly appreciate the genuine interest and the trust expressed in our work.

Reviewer #3: However, I have some concern about this manuscript. I also re-examined heated H2O2 method. Because 30w/v% of H2O2 is mainly used in my country, I put 26 mL of 30% H2O2 to only 2 grams of human spleen which were chopped into small pieces. A large amount of foam was generated (without a stirring rod); I had to aspirate gas under foam using disposable pipette. It may be better to add H2O2 in several batches, not to put H2O2 and tissue sample directly together (page 3, line 20).

Group B: to obtain a more satisfactory result, especially in the presence of particularly voluminous specimens, it may be useful to coarsely fragment them into smaller pieces, using a disposable scalpel, before putting them into the beaker. Then 20 ml of reagent (H2O2, hydrogen peroxide 40%) and tissue sample was slowly put together in a 200 ml beaker, to achieve a better control of the reaction.

Reviewer #3: In my re-examination, I could not obtain complete digestion, which is shown in Figure 1 in the manuscript, with 4 hours using heated H2O2; Twenty-four hours duration was not sufficient. If either of small amount of acid [2] or additional fresh H2O2 is needed to complete digestion, the details should be clarified in the Materials and methods section. Is it necessary to use 40% H2O2 instead of 30% one?

We strongly suggest the use of 40% H2O2 as we do not know the outcome with other concentrations.

Reviewer #3: The authors show the effectiveness of heated H2O2 method in figures and tables; however "heated and cold H2O2" and "heated H2O2 and acids" are compared separately. The authors may present the advantages of heated H2O2 method by adding tissue status with acid digestion to Figure 1 (and diatoms treated with cold H2O2 to Figure 2), if these are desirable. The authors assert the safety of heated H2O2 method, however scarcely mentioned in the manuscript. Is this method really safe? Concentrated H2O2 is corrosive and it may cause explosion at elevated temperatures and pressures [3].

The aim of this work is to suggest an alternative method to the acid digestion, with the same effectiveness, but safer and cheaper.

Reviewer #3: The degree symbol and the word "degree" are duplicated as "at 70 C° degrees" (page 2, line 15).

Therefore, a new protocol, involving heating hydrogen peroxide at 70 C°, was experimented.

Reviewer #3: I'm afraid the authors mean "56" instead of "fiftx-six" (page 2, line 18). However, Tables 1 and 2 indicate 61 samples from human and animal tissue.

We analyzed sixty-one tissue samples from five human cases of suspected drowning in rivers, whose samples had been collected and stored through the years by the Unit of Legal Medicine of the Department of Medicine and Surgery at the University of Parma, and five animal cases, including a coypu, a fallow deer, a mink, a dog and a fox, provided by the Department of Veterinary Science at the University of Parma, all suspected cases of drowning deaths in fresh water. All samples were stored at – 20°C.

Reviewer #3: Because of the revision, it is unclear what does "these two techniques" mean (page 4, line 26).

At first, the significant agreement of results between the new technique and the one with acids has shown that they both represent an effective method of diatom extraction. Furthermore in one case, coypu's liver, the new method with peroxide detected diatoms while the one with acids did not.

Reviewer #3: The citation of Table 3 may be inserted to the Discussion section (page 4, line 30) as "… a smaller amount of end-stage sediment (Table 3)."

Comparing these techniques, the hydrogen peroxide heated method almost always showed a more satisfying tissue digestion, because of a smaller amount of end-stage sediment. Regularly, with both methods, the liver, the fattest organ, showed worse digestion and a higher end-sediment rate (table 3).

Reviewer 3#: The legend beneath Figure 2 is incorrect, please correct as "Figure 1."

We have fixed this mistake.

Highlights

1. The new digestion method with heated hydrogen peroxide: a safe and low-cost approach.
2. Diatoms provide a reliable diagnosis of drowning.
3. The value of pathologist and forensic veterinarian teamwork in the diagnosis of drowning.

DIATOM EXTRACTION: A NEW TECHNIQUE WITH HEATED H_2O_2. A TECHNICAL NOTE

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Declarations of interests: none

Diatom extraction: a new technique with heated H2O2. A technical note

Abstract

The best method of diatom identification in animal and human tissues is still an important discussion topic, in terms of effectiveness and reliability. In this technical note, authors propose a new method of extraction of diatoms using heated hydrogen peroxide from animal and human tissue samples. This method has been compared with the traditional method of digestion with acids. The results of the comparison show that heated hydrogen peroxide extraction is more efficient in terms of reduction of sediment, extraction of the material and preservation of diatoms proving to be a viable alternative to conventional approaches with acids in terms of costs and operator safety.

Keywords

Diatoms extraction, heated hydrogen peroxide method, forensic pathology, forensic veterinary, drowning.

1. Introduction

A reliable diagnosis of drowning is the final result of a logical comparison between manifold elements and observations. Only a few circumstantial evidences and clinical signs on the drowned body proved to be pivotal by themselves in this diagnosis, one of the toughest challenges for the forensic pathologist [1].

Diatoms have a leading role in this scenario [2, 3]. They are a group of a few microns in size unicellular microalgae, classified as eukaryotes, usually lacking of flagella. Diatoms are often autotrophic and they live in both fresh and saltwater [4]. A peculiar feature of diatom structure is to be surrounded by a cell wall made of silica, called frustule [5].

When water or any other fluid reaches the lungs, the diatoms penetrate the alveolar capillary barrier and through the pulmonary venous circulation arrive in the heart [6].

If the heartbeat is effective, they can reach the other organs through the bloodstream.

The finding of these organisms in the peripheral tissues demonstrates that the circulatory system was still working when the drowning medium filled the lungs and, therefore, the victim was still alive. This data often distinguishes between drowning and submersion of corpse [7, 8, 9].

In literature are described many researches about methods of diatoms identification in drowning deaths. Nevertheless, there is a lack of unanimous consensus. Each protocol relies on the specific resistance of diatom's external layer composed by silica. Indeed, the cell wall can resist different types of reagents, although if too aggressive they can also destroy the diatom itself.

The best procedure should ensure a fair compromise between tissues digestion and structural integrity of diatoms.

Over the years there have been many different protocols, with slight changes or additions in the choice of reagents. Currently, the most widely used methods are: digestion with proteinase k, [10, 11], with papain [12], with strong acids [13] (alone or combined with hydrogen peroxide), with Soluene 350 [14] and microwave digestion [15, 16], etc.

The strong acid digestion is definitely one of the best methods in the diatoms identification [17]. However, it implicates substantial safety problems for the operator and adequate laboratory equipment (like vacuum system). Moreover, excessive digestive power may damage the structure of diatoms [18].

The aim of this work is to suggest an alternative method to the acid digestion, with the same effectiveness, but safer and cheaper. At first, the consolidated cold hydrogen peroxide protocol [19, 20] was used. However, the results obtained in the first samples were not satisfying in terms of end-stage sediment.

Therefore, a new protocol, involving heating hydrogen peroxide at 70 C°, was experimented.

2. Materials and methods

We analyzed sixty-one tissue samples from five human cases of suspected drowning in rivers, whose samples had been collected and stored through the years by the Unit of Legal Medicine of the Department of Medicine and Surgery at the University of Parma, and five animal cases, including a coypu, a fallow deer, a mink, a dog and a fox, provided by the Department of Veterinary Science at the University of Parma, all suspected cases of drowning deaths in fresh water. All samples were stored at – 20°C.

According to availability of the various cases samples of lungs, liver, spleen, heart and kidney were taken, and, wherever possible, divided in two equal portions between a maximum of 5 and a minimum of 1.5 grams each.

The original idea was to apply to the first group (Group A) the traditional acid digestion and to the second group (Group B) the cold hydrogen peroxide digestion.

However, due to the poor results obtained with cold hydrogen peroxide, i.e. abundant end-stage sediment, a digestion with heated hydrogen peroxide was tested. Thanks to the better results obtained, Group B was treated with the new digestion method using heated hydrogen peroxide (Table 1). As negative control, a sample of double distilled water "*Carlo Erba*" was chosen to rule out possible contaminations during procedures.

All samples were collected from organs using cleaned and separate instruments for each one.

At every stage, we used disposable tools, as tweezers, scalpels and gloves to prevent sample contamination. Besides, the glass instrumentation was washed with double distilled water "*Carlo Erba*", previously tested diatoms free, and well dried.

Samples were stored in separate glass containers and frozen at -20° C until we performed the procedures. Then, we applied the following protocols:

Group A: We put each fragment in a 200 ml beaker, while in a different glass container we made a blend with 10 ml 65% nitric acid and 5 ml 96% sulphuric acid using graduated glass pipettes and a rubber bulb.

The addition of the acid mixture to the tissue sample triggered a strong boiling reaction. Therefore, it was necessary to use a stirring rod to prevent an overflow. The reaction finished at the end of the boiling. All these procedures were performed under a vacuum system. The sediment was transferred in a Corning centrifuge tube and then was centrifuged five times at 1200 rpm for three minutes. At every stop of the centrifuge the supernatant was removed. At each centrifuges, for the correct balance of the instrument the volume of at least 40 ml of liquid in the Corning centrifuge tubes was reached by adding double-distilled water tested diatom free. After completing the last centrifuge and eliminating the supernatant, the sediment was suspended in a few drops of double-distilled water in order to clean it.

Group B: to obtain a more satisfactory result, especially in the presence of particularly voluminous specimens, it may be useful to coarsely fragment them into smaller pieces, using a disposable scalpel, before putting them into the beaker. Then 20 ml of reagent (H_2O_2, hydrogen peroxide 40%) and tissue sample was slowly put together in a 200 ml beaker, to achieve a better control of the reaction. Then, instead of leaving the mixture to rest overnight, as in the traditional method with cold hydrogen peroxide, the mixture was heated up to 70°C, using a heating plate. This temperature was chosen because it improves the digestive capacity of the reagent without destroying the silica wall of the diatoms. After an initial phase of foaming, while heating the beaker, the digestion continued steadily. In our experience, unlike the acid method, the formation of the foam was slight. However, in case of overfilling a stirring rod can be used to control the reaction. It took mostly one or two hours for a complete digestion of some tissues, and in a few cases almost three or four hours. A continuous supervising was requested in order to identify the complete digestion, stop the reaction, remove the beaker from the heating plate and allow the solution to rest overnight. After the overnight samples were centrifuged and treated as in group A. Then in both groups, a graduated pipette was used to collect 300 µl of digested samples. At the end, this small amount of material was transferred onto microscope slides.

Finally, the slides were left to dry and then coated with cover slides.

3. Results

As shown in (Figure 1), the heating of hydrogen peroxide has led to an important improvement in tissue digestion, justifying the rapid adoption of this method.

In both heated hydrogen peroxide and acids technique in five cases diatoms were found (human case 1, human case 3, coypu, mink and fox). In all cases only the lungs were positive, except for the Coypu where all the organs tested where positive with the hydrogen peroxide technique (table 2). In only one case (coypu liver) a discrepancy between both techniques was found: no diatoms with acids and presence of diatoms with hydrogen peroxide technique. The sample of double distilled was found negative by both digestion method.

4. Discussion

The purpose of this study was not to make a drowning diagnosis, which can be made through a diatom quantitative test [21]. This test a sample can be considered positive when a minimum of 20 diatoms per 100 µl of sediment extracted from digestion of 2 g of lung or 5 complete diatoms per 100 µl of sediment extracted from 2 g of other tissues has been found. The aim of the study was to compare two diatom extraction techniques, a more traditional strong acid digestion and a new method with hydrogen peroxide heated to 70° C. Indeed, this work is a pilot study with the purpose of describing a new diatom extraction technique. The positivity of the diatom test is influenced by several factors of confusion, however, not related to the extraction method. First of all, not all aquatic habitats are suitable for these microorganisms. Other elements, such as temperature, salinity, presence of nutrients, affect the abundance or absence of these microorganisms in water and, consequently, the spread in tissues.

The samples used belong to old cases from the institute's casuistry, preserved over time. It was not always possible to establish the environmental conditions at the time of discovery of the bodies.

In addition, all the specimens analyzed came from cases of suspicious but not certain drowning deaths. It has to be remembered that for a positive diatom test, the fluid medium must reach the lung tissues, through the airways, or peripheral tissues, through the bloodstream. These eventualities occur in drowning but not, for example, in the submersion of a corpse or when death occurs without or with reduced inhalation of liquids. At first, the significant agreement of results between the new technique and the one with acids has shown that they both represent an effective method of diatom extraction. Furthermore in one case, coypu's liver, the new method with peroxide detected diatoms while the one with acids did not.

Comparing these techniques, the hydrogen peroxide heated method almost always showed a more satisfying tissue digestion, because of a smaller amount of end-stage sediment. Regularly, with both methods, the liver, the fattest organ, showed worse digestion and a higher end-sediment rate (table 3). This can make the analysis of the slides of this organ more difficult. . To solve the problem of high fat content, an additional centrifuge with ethyl alcohol at 96 degrees (instead of double-distilled water) has been added to the centrifuges already described.

After completing this last centrifuge and eliminating the supernatant, the sediment was suspended in a few drops of 96-degrees ethyl alcohol. This has greatly improved the cleanliness of the microscopic field.

In addition, as far as the observation of the final preparations is concerned, the new method has resulted in fewer interfering elements on the slide and less damage to the siliceous skeleton, allowing a more satisfying view (fig 2).

Nevertheless, the new technique is not immune to some problems. For example, the reaction time is much longer than the few hours needed to complete digestion with acids. Furthermore, the reaction with hydrogen peroxide is not self-limiting, thus requiring continuous and careful supervision by the operator, while in acid digestion, the end of the reaction is clearly identified by the end of the boiling process.

In conclusion, despite these technical issues, the new digestion protocol with hydrogen peroxide heated up to 70°C has an excellent digestive and diatom-preserving capacity and can therefore be considered a low-cost and safe alternative to strong acid digestion. Aware that this is a pilot study that needs further research to be validated, we believe that this work offers a technical improvement in the forensic field.

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	DIGESTION METHOD WITH ACID					DIGESTION METHOD WITH HEATED HYDROGEN PEROXIDE				
Tissue	**Lung (g)**	**Liver (g)**	**Spleen (g)**	**Kidney (g)**	**Heart (g)**	**Lung (g)**	**Liver (g)**	**Spleen (g)**	**Kidney (g)**	**Heart (g)**
Human 1	5	5	/	/	/	5	5	/	/	/
Human 2	/	5	/	5	/	/	5	/	5	/
Human 3	5	5	/	5	/	5	5	/	5	/
Human 4	1.5	5	/	5	/	1.5	5	/	5	/
Human 5	/	/	/	/	/	/	1.5	/	1.5	/
Coypu	/	5	/	4	/	1.5	5	1.5	4	1.5
Dog	5	5	/	5	5	5	5	/	5	5
Fallow deer	5	5	5	5	5	5	5	5	5	5
Mink	/	/	/	2.5	3	1.5	/	1.5	2.5	3
Fox	5	/	3.5	5	5	5	/	3.5	5	5

Table 1: Types of tissue and amounts used for the digestion method with hydrogen peroxide and acids

	DIGESTION METHOD WITH ACID					DIGESTION METHOD WITH HYDORGEN PEROXIDE				
Tissue	**Lung**	**Liver**	**Spleen**	**Kidney**	**Heart**	**Lung**	**Liver**	**Spleen**	**Kidney**	**Heart**
Human 1	Pos.	Neg.	/	/	/	Pos.	Neg.	/	/	/
Human 2	/	Neg.	/	Neg.	/	/	Neg.	/	Neg.	/
Human 3	Pos.	Neg.	/	Neg.	/	Pos.	Neg.	/	Neg.	/
Human 4	Neg.	Neg.	/	Neg.	/	Neg.	Neg.	/	Neg.	/
Human 5	/	/	/	/	/	/	Neg.	/	Neg.	/
Coypu	/	Neg.	/	Pos.	/	Pos.	Pos.	Pos.	Pos.	Pos.
Dog	Neg.	Neg.	/	Neg.	Neg.	Neg.	Neg.	/	Neg.	Neg.
Fallow deer	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Mink	/	/	/	Neg.	Neg.	Pos.	/	Neg.	Neg.	Neg.
Fox	Pos.	/	Neg.	Neg.	Neg.	Pos.	/	Neg.	Neg.	Neg.

Table 2: Detection of diatoms in the analysed samples. Abbreviations: pos. instead of positive, neg. instead of negative

	Initial weight of the tissue for digestion with acid	Sediment produced with acid method	Sediment produced with hydrogen peroxide method
Human 1: lung	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 1: liver	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 2: liver	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 2: kidney	5 g and 5 g	0.5 ml	Less than 0.5 ml
Coypu: lung	/ and 1.5 g	/	Less than 0.5 ml
Coypu: liver	5 g and 5 g	Less than 0.5 ml	Less than 0.5 ml
Coypu: spleen	/ and 1.5 g	/	Less than 0.5 ml
Coypu: kidney	4 g and 4 g	Less than 0.5 ml	Less than 0.5 ml
Coypu: heart	/ and 1.5 g	/	Less than 0.5 ml
Dog: lung	5 g and 5 g	1 ml	Less than 0.5 ml
Dog: liver	5 g and 5 g	2 ml	Less than 0.5 ml
Dog: kidney	5 g and 5 g	1 ml	Less than 0.5 ml
Dog: heart	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: lung	5 g and 5 g	2 ml	Less than 0.5 ml
Fallow deer: liver	5 g ad 5 g	3 ml	2 ml
Fallow deer: spleen	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: kidney	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: heart	5 g and 5 g	1 ml	2 ml
Mink: lung	/ and 1.5 g	/	0.5 ml
Mink: spleen	/ and 1.5 g	/	Less than 0.5 ml
Mink: kidney	2.5 g and 2.5 g	1 ml	Less than 0.5 ml

Table 3: Amount of end stage sediment of tissue samples with both strong acid digestion and hydrogen peroxide heated digestion.

Figure 1: (A) Tissue status at the end of digestion with cold hydrogen peroxide. The sample, a fallow-deer heart, shows the presence of a significant amount of undigested material. (B) Tissue status at the end of digestion with heated hydrogen peroxide. The sample, a fallow deer spleen, shows a more complete digestion.

Figure 2: (A), (B), (C) diatoms from tissues treated with acids, the bigger amount of undigested organic material not always allows for a satisfactory microscopic observation; (D), (E), (F) diatoms in tissues treated with heated H_2O_2, the small amount of undigested organic material allows an easy observation.

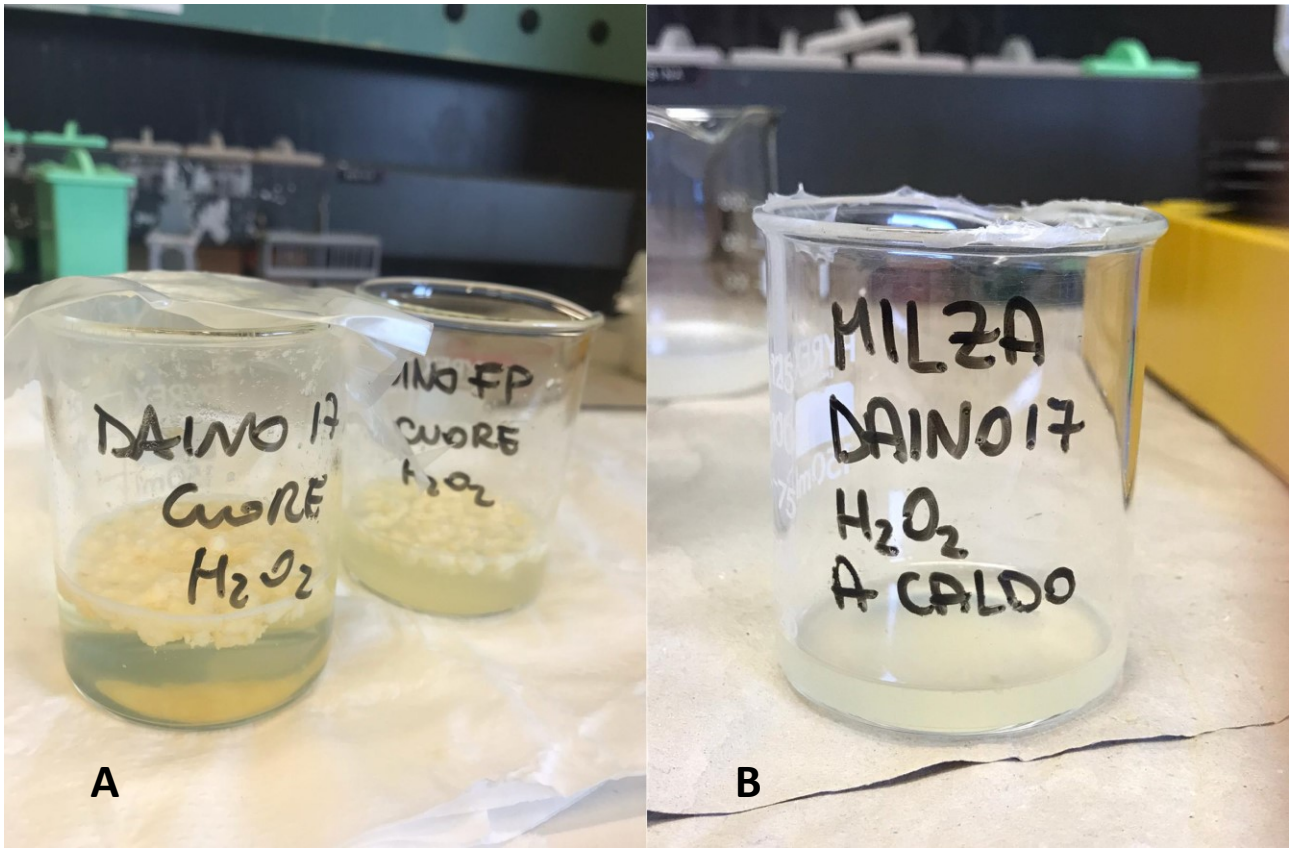

Figure 1: (A) Tissue status at the end of digestion with cold hydrogen peroxide. The sample, a fallow-deer heart, shows the presence of a significant amount of undigested material. (B) Tissue status at the end of digestion with heated hydrogen peroxide. The sample, a fallow deer spleen, shows a more complete digestion. (**Colours should be used for this image**)

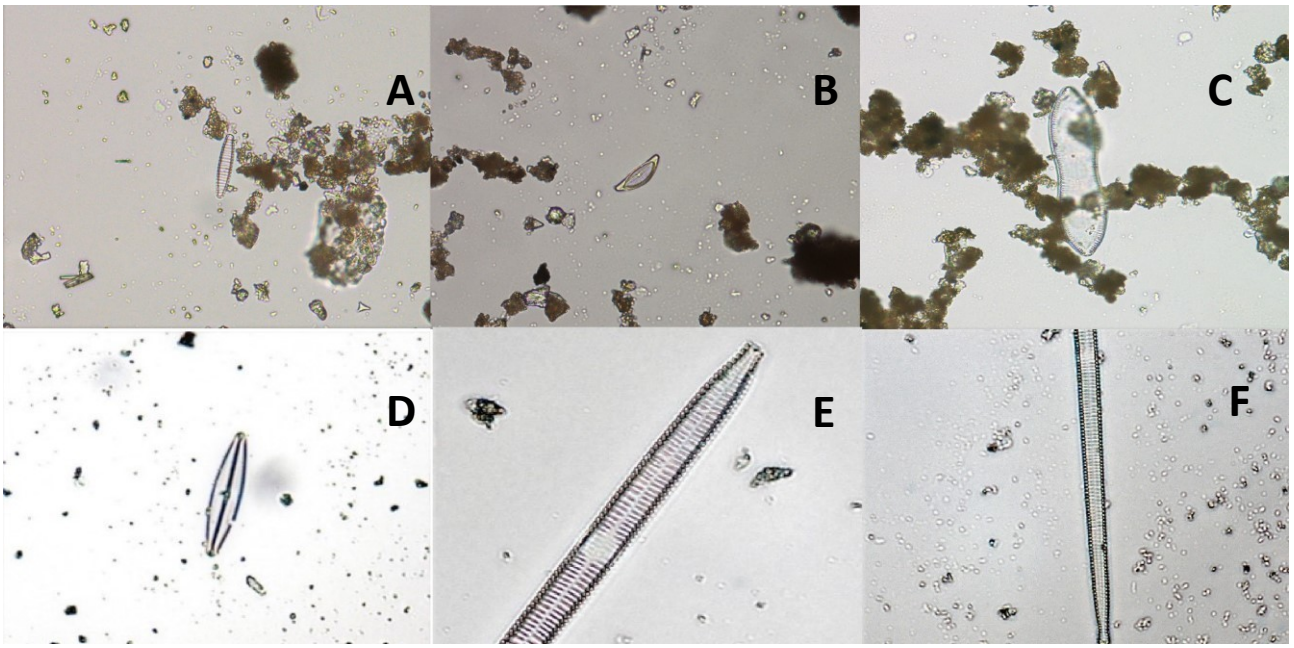

Figure 2: (A), (B), (C) diatoms from tissues treated with acids, the bigger amount of undigested organic material not always allows for a satisfactory microscopic observation; (D), (E), (F) diatoms in tissues treated with heated H_2O_2, the small amount of undigested organic material allows an easy observation.(**Colours should be used for this figure**)

	DIGESTION METHOD WITH ACID					DIGESTION METHOD WITH HEATED HYDROGEN PEROXIDE				
Tissue	**Lung (g)**	**Liver (g)**	**Spleen (g)**	**Kidney (g)**	**Heart (g)**	**Lung (g)**	**Liver (g)**	**Spleen (g)**	**Kidney (g)**	**Heart (g)**
Human 1	5	5	/	/	/	5	5	/	/	/
Human 2	/	5	/	5	/	/	5	/	5	/
Human 3	5	5	/	5	/	5	5	/	5	/
Human 4	1.5	5	/	5	/	1.5	5	/	5	/
Human 5	/	/	/	/	/	/	1.5	/	1.5	/
Coypu	/	5	/	4	/	1.5	5	1.5	4	1.5
Dog	5	5	/	5	5	5	5	/	5	5
Fallow deer	5	5	5	5	5	5	5	5	5	5
Mink	/	/	/	2.5	3	1.5	/	1.5	2.5	3
Fox	5	/	3.5	5	5	5	/	3.5	5	5

Table 1: Types of tissue and amounts used for the digestion method with hydrogen peroxide and acids

	DIGESTION METHOD WITH ACID					DIGESTION METHOD WITH HYDORGEN PEROXIDE				
Tissue	**Lung**	**Liver**	**Spleen**	**Kidney**	**Heart**	**Lung**	**Liver**	**Spleen**	**Kidney**	**Heart**
Human 1	Pos.	Neg.	/	/	/	Pos.	Neg.	/	/	/
Human 2	/	Neg.	/	Neg.	/	/	Neg.	/	Neg.	/
Human 3	Pos.	Neg.	/	Neg.	/	Pos.	Neg.	/	Neg.	/
Human 4	Neg.	Neg.	/	Neg.	/	Neg.	Neg.	/	Neg.	/
Human 5	/	/	/	/	/	/	Neg.	/	Neg.	/
Coypu	/	Neg.	/	Pos.	/	Pos.	Pos.	Pos.	Pos.	Pos.
Dog	Neg.	Neg.	/	Neg.	Neg.	Neg.	Neg.	/	Neg.	Neg.
Fallow deer	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
Mink	/	/	/	Neg.	Neg.	Pos.	/	Neg.	Neg.	Neg.
Fox	Pos.	/	Neg.	Neg.	Neg.	Pos.	/	Neg.	Neg.	Neg.

Table 2: Detection of diatoms in the analyzed samples. Abbreviations: pos. instead of positive, neg. instead of negative.

	Initial weight of the tissue for digestion with acid and with hydrogen peroxide	**Sediment produced with acid method**	**Sediment produced with hydrogen peroxide method**
Human 1: lung	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 1: liver	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 2: liver	5 g and 5 g	0.5 ml	Less than 0.5 ml
Human 2: kidney	5 g and 5 g	0.5 ml	Less than 0.5 ml
Coypu: lung	/ and 1.5 g	/	Less than 0.5 ml
Coypu: liver	5 g and 5 g	Less than 0.5 ml	Less than 0.5 ml
Coypu: spleen	/ and 1.5 g	/	Less than 0.5 ml
Coypu: kidney	4 g and 4 g	Less than 0.5 ml	Less than 0.5 ml
Coypu: heart	/ and 1.5 g	/	Less than 0.5 ml
Dog: lung	5 g and 5 g	1 ml	Less than 0.5 ml
Dog: liver	5 g and 5 g	2 ml	Less than 0.5 ml
Dog: kidney	5 g and 5 g	1 ml	Less than 0.5 ml
Dog: heart	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: lung	5 g and 5 g	2 ml	Less than 0.5 ml
Fallow deer: liver	5 g ad 5 g	3 ml	2 ml
Fallow deer: spleen	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: kidney	5 g and 5 g	0.5 ml	Less than 0.5 ml
Fallow deer: heart	5 g and 5 g	1 ml	2 ml
Mink: lung	/ and 1.5 g	/	0.5 ml
Mink: spleen	/ and 1.5 g	/	Less than 0.5 ml
Mink: kidney	2.5 g and 2.5 g	1 ml	Less than 0.5 ml

Table 3: Amount of end stage sediment of tissue samples with both strong acid digestion and hydrogen peroxide heated digestion.

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