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Fatal poisoning of four workers in a farm: distribution of hydrogen sulfide and thiosulfate in 10 different biological matrices

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Abstract:	We evaluate the distribution of sulfide and thiosulfate (TS) in biological samples of four dairy farmers died inside a pit connected to a manure lagoon. Autopsies were performed 4 days later. Toxicological analyses of sulfide and TS were made using an extractive alkylation technique combined with gas chromatography/mass spectrometry (GC/MS). Autopsies revealed: multiorgan congestion; pulmonary edema; manure inside distal airways of three of the four victims. Sulfide concentrations were cardiac blood: 0.5-3.0 µg/mL, femoral blood: 0.5-1.2 µg/mL, bile: <0.1-2.2 µg/mL; liver 2.8- 8.3 µg/g, lung: 5.0-9.4 µg/g, brain: 2.7-13.9 µg/g, spleen: 3.3- 6.3 µg/g, fat: <0.1-1.5 µg/g, muscle: 2.6-3.5 µg/g. TS concentrations were cardiac blood: 2.1-4.9 µg/mL, femoral blood: 2.1- 2.3 µg/mL, bile: 2.5-4.4 µg/mL, urine: <0.5-1.8 µg/mL; liver <0.5-2.6, lung: 2.8-5.4 µg/g, brain: <0.5-1.9 µg/g, spleen: 1.2-2.9 µg/g, muscle: <0.5-5.6 µg/g. The cause of death was assessed to be acute poisoning by hydrogen sulfide (H 2 S) for all the victims. Manure inhalation contributed to the death of three subjects. The measurement of sulfide and TS concentrations in biological samples contributed to better understand the sequence of the events. Subjects 3 provided the highest concentration of sulfide in brain, thus, supporting the hypothesis of a rapid loss of consciousness and respiratory depression. One by one, the other farmers entered the pit in attempts to rescue the coworkers but collapsed. Despite the rapid death, subject 3 was the only one with TS detectable in urine. This could be due to differences in metabolism of H 2 S.
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Dear Editor,

we would like to submit for publication on Forensic Science International the study entitled "**Fatal poisoning of four workers in a farm: distribution of hydrogen sulfide and thiosulfate in 10 different biological matrices**".

In the present article, we report the toxicological investigations applied in an accidental poisoning by hydrogen sulfide (H_2S) inhalation involving four dairy farmers. We evaluate the distribution of sulfide and his metabolite thiosulfate (TS) in several biological matrices of the four victims.

Hydrogen sulfide represents a not rare cause of fatal events in workplaces. However, detailed toxicological analysis in these accidents are generally not rigorously defined. Furthermore, there is a shortage of postmortem data regarding the different distribution of H_2S and TS (in many reported cases not measured) in the tissues.

In our case, sulfide and TS were found in several biological fluids and tissues, even if not homogeneous toxicological values were detected in samples. The concurrent measurement of sulfide and thiosulfate concentrations helped us to better understand the circumstances of the deaths by supporting the timing and the supposed sequence of the events.

Thank you for your attention

Best regards

Matteo Moretti

Fatal poisoning of four workers in a farm: distribution of hydrogen sulfide and thiosulfate in 10 different biological matrices

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All authors contributed to the study conception and design. Material preparation, sample collection and data collection were performed by Matteo Moretti, Marco Ballardini and Chiara Siodambro. Analysis were performed by Francesca Freni, Claudia Vignali, Luca Morini. The first draft of the manuscript was written by Matteo Moretti, Chiara Siodambro and Francesca Freni. Luca Morini, Claudia Vignali, Livio Tronconi, Antonio Marco Maria Osculati supervised, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare that they have no conflict of interest.

Highlights

- We report an accidental poisoning by hydrogen sulfide involving 4 farmers.
- Sulfide and thiosulfate were evaluated in 10 different biological matrices.
- We discuss the not homogeneous toxicological values detected in samples.
- Toxicological analyses contributed to understand the sequence of the events.

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Abstract

We evaluate the distribution of sulfide and thiosulfate (TS) in biological samples of four dairy farmers died inside a pit connected to a manure lagoon.

Autopsies were performed 4 days later. Toxicological analyses of sulfide and TS were made using an extractive alkylation technique combined with gas chromatography/mass spectrometry (GC/MS).

Autopsies revealed: multiorgan congestion; pulmonary edema; manure inside distal airways of three of the four victims.

Sulfide concentrations were cardiac blood: 0.5-3.0 µg/mL, femoral blood: 0.5-1.2 µg/mL, bile: <0.1-2.2 µg/mL; liver 2.8-8.3 µg/g, lung: 5.0-9.4 µg/g, brain: 2.7-13.9 µg/g, spleen: 3.3- 6.3 µg/g, fat: <0.1-1.5 µg/g, muscle: 2.6-3.5 µg/g.

TS concentrations were cardiac blood: 2.1-4.9 µg/mL, femoral blood: 2.1- 2.3 µg/mL, bile: 2.5-4.4 µg/mL, urine: <0.5-1.8 µg/mL; liver <0.5-2.6, lung: 2.8-5.4 µg/g, brain: <0.5-1.9 µg/g, spleen: 1.2-2.9 µg/g, muscle: <0.5-5.6 µg/g.

The cause of death was assessed to be acute poisoning by hydrogen sulfide (H_2S) for all the victims. Manure inhalation contributed to the death of three subjects. The measurement of sulfide and TS concentrations in biological samples contributed to better understand the sequence of the events. Subjects 3 provided the highest concentration of sulfide in brain, thus, supporting the hypothesis of a rapid loss of consciousness and respiratory depression. One by one, the other farmers entered the pit in attempts to rescue the coworkers but collapsed. Despite the rapid death, subject 3 was the only one with TS detectable in urine. This could be due to differences in metabolism of H_2S.

Keywords: hydrogen sulfide; thiosulfate; intoxication, distribution,

INTRODUCTION

Hydrogen sulfide (H_2S) is a gas produced wherever sulfur-containing compounds decompose under reducing conditions, either chemical or by sulphate-reducing bacteria action [1].

These conditions are common during decomposition of organic matter in places like drains, latrines, septic tanks, sewers and deposits of dung. H_2S is also a byproduct of many industrial processes such as rayon dye production, heavy water production, petroleum refining, natural gas, asphalt, waste management, and the fishing industry [2].

In addition, natural sour gas also contains sulfur compounds [3, 4].

In the organism, endogenous H_2S is a neuromodulator that is actively synthesized in the tissues and is involved in the regulation of vascular tone, neuromodulation, cytoprotection, inflammation and apoptosis [5-9].

Catabolism of H_2S occurs in mitochondria by sulfide:quinone oxidoreductase, persulfide dioxygenase, and sulfite oxidase to thiosulfate (TS), sulfite, and sulfate [10-12]. The formation of TS is believed to be catalyzed by both hemoglobin and hepatic enzymes [13]. The catabolism is influenced by factors such as oxygen pressure, mitochondria density, or efficacy of mitochondrial electron transport [10, 12].

H_2S is a toxic, flammable, moderately water soluble, colorless gas. It has a characteristic rotten egg odor. Following inhalation, H_2S is distributed to the blood, brain, lungs, heart, liver, spleen and kidneys and is quickly metabolized to thiosulfate, sulfite, and sulfate, especially in the liver [10-16]. Renal excretion is the major route of elimination of H_2S which, after oxidation, is excreted as free sulfate or as a conjugated sulfate in urine [7, 15].

The toxic effect of H_2S is probably related to inhibition of different enzymes [17-19]. For long time it was assumed that the main toxic effects were due to inhibition of cytochrome c oxidase, as with cyanide [19]. However, there are many variances in the clinical presentation of poisoning by H_2S and cyanide, suggesting different modes of toxicity [17, 18, 20-23]. These secondary mechanisms, still not totally understood, are probably the cause of manifestations of sulfide poisoning such as hyperventilation and apnea [22, 24, 25], or coma [20].

Concentration is much more important than duration of exposure: for hydrogen sulfide, higher concentrations are much more toxic, even with proportionally shorter exposure levels [26].

The H_2S irritating concentration is reported as 10 ppm [15, 27], and exposure to 10 ppm for 1 hour is indicated as the ceiling concentration that will not cause irreversible harm [28]. At 50–100 ppm airway irritation, dizziness, nausea, vomiting, keratoconjunctivitis, corneal ulceration could be observed [26, 29].

The National Institute for Occupational Safety and Health states that H_2S concentration of 100 ppm could be immediately dangerous to life and health [30].

Olfactory nerve endings become rapidly fatigued or paralyzed at concentrations between 100 and 150 ppm [15, 27, 29].

Pulmonary edema is a common consequence of poisoning, already at 250-500 ppm [31, 32].

Exposition to hydrogen sulfide at 500 ppm or higher can induce acute neurotoxicity and neurodegeneration that can affect brain as primary target, and the victim could become unconscious rapidly, in only few breaths [33-35]. This circumstance is termed “slaughterhouse sledgehammer” effect or “knockdown” effect [25] [25, 36, 37]. This effect appears far too quickly to be attributed to hypoxia and is likely a consequence of a direct toxic effect of H_2S on the brain and on respiratory center in the brainstem [23, 37]. Knockdown can lead to death but, if exposure is promptly terminated, cases of collapse could be followed by rapid recovery [29, 38].

At 1000-2000 ppm or greater, knockdown, apnea and death occur rapidly [23, 25, 33-35] and, in this case, "*Death may come on like a stroke of lightening*" [39].

Given that H_2S is heavier than air, it accumulates in low-lying areas or in confined spaces . For this reason, it represents a not rare cause of fatal events in workplaces [29, 32, 40-46].

However, exposure concentration and duration as well as detailed toxicological analysis in these accidents are generally not rigorously defined [47]. Furthermore, there is a shortage of postmortem data regarding the different distribution of H_2S and TS (in many reported cases not measured) in the tissues.

In the present article, we report the toxicological investigations applied in an accidental poisoning by H_2S inhalation involving four dairy farmers. We evaluate the distribution of hydrogen sulfide and thiosulfate in biological fluids and tissues of the four victims.

CASE HISTORY

In 2019, four dairy farmers were found dead inside a small reception pit, connected to a manure lagoon. Another colleague saw them, for the last time, at 7.00 a.m., while they were feeding the cattle, and discovered the bodies floating in the sewage at 12.30 a.m., when he returned to the dairy farm. He found that the cover of the pit had been removed, and that an iron ladder had been placed into it. A manure spreader tank truck was parked near the pit.

The pit measured 1,5-meter x 1,5-meter x 2,6-meter-deep, half full of manure.

A schematic representation of the scene is shown in figure 1 and 2.

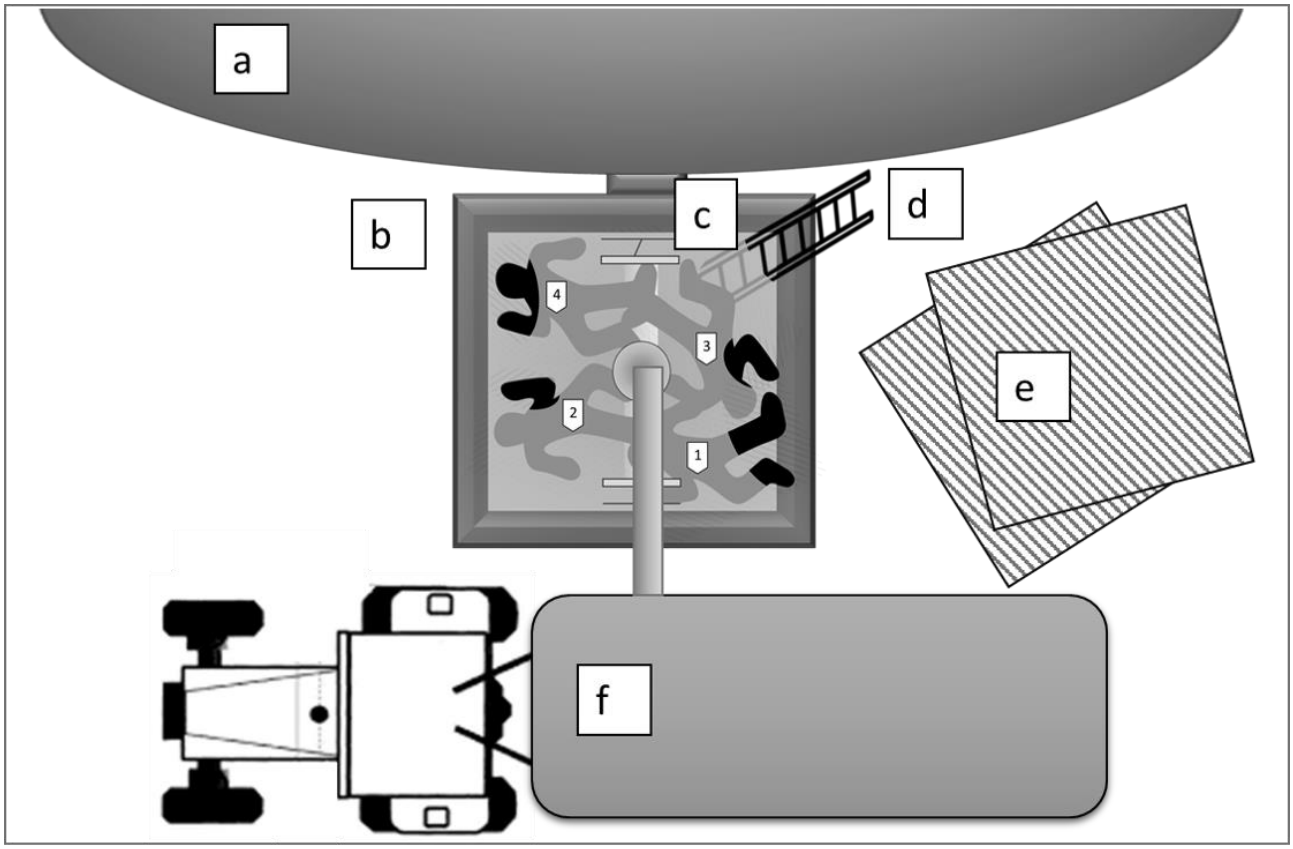

Figure 1: schematic representation of the scene.

a: Manure lagoon. b: Pit (1,5-m x 1,5-m x 2,6 m deep). c: Valve that allows the sewage to exit from the manure pool to the reception pit. d: Ladder. e: Removed cover of the pit. f: Manure spreader tank trunk

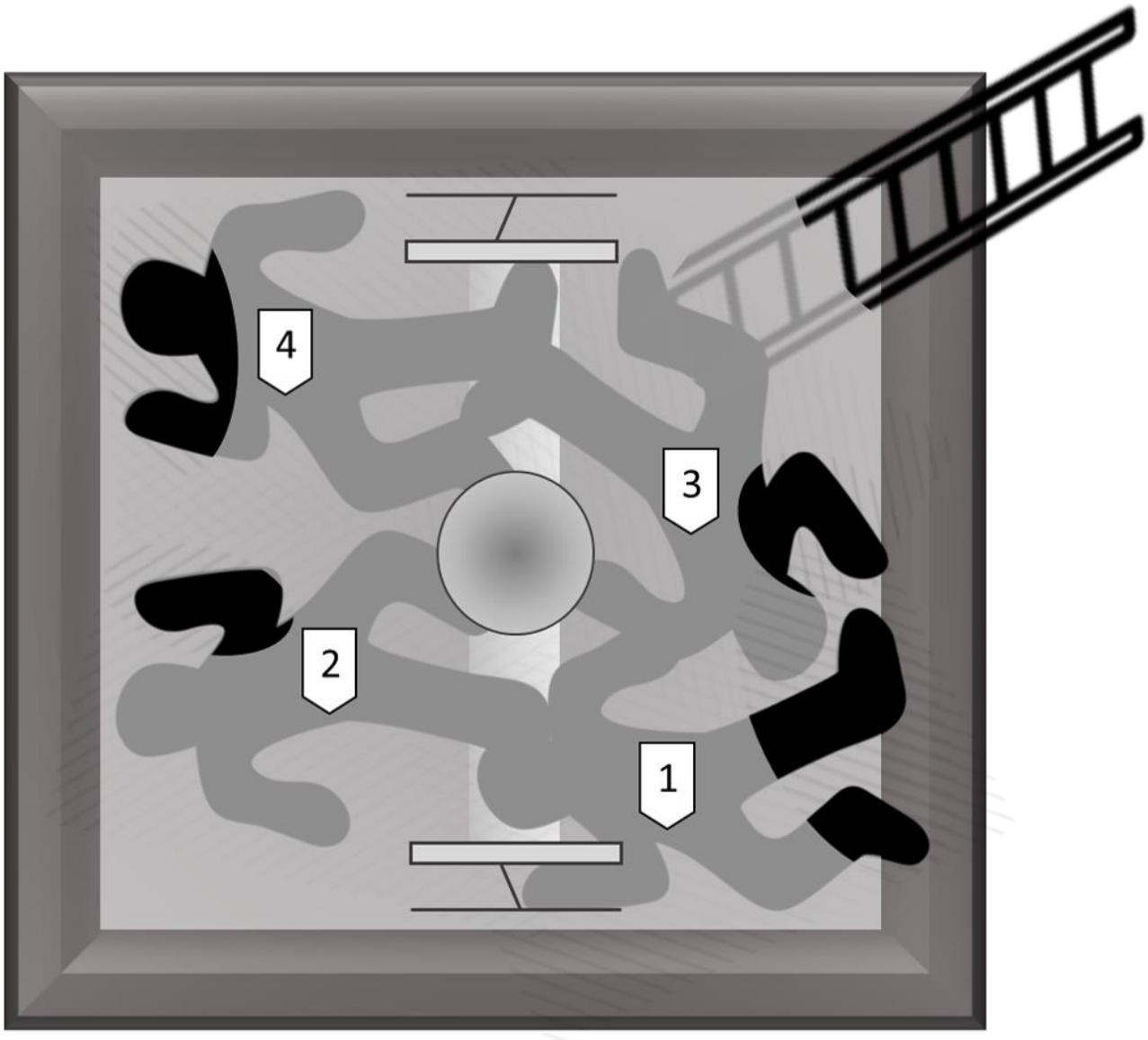

Figure 2: schematic representation of the position of the four dairy farmers inside of the pit half-full of manure.

After having excluded the presence of toxic gases in the atmosphere within the pit, Fire Department personnel removed the victims from the manure, using specialized equipment. A total of 4 death bodies were found.

MATERIAL AND METHOD

EXAMINATION OF THE BODIES

The bodies were preliminary examined at the scene and then refrigerated until autopsies, which were performed 4 days later.

Samples of cardiac and femoral blood, urine, bile, brain, lung, kidney, liver, spleen, muscle and fat were collected in sealed vials and submitted to the toxicology laboratory for testing. All samples were stored at −20 °C until analyses.

In accordance with Italian Law, this research was performed on small portions of biological samples routinely taken during autopsies that were already examined for diagnostic and/or forensic purposes.

TOXICOLOGICAL ANALYSES

Toxicological analyses were performed on all the samples using an extractive alkylation technique combined with gas chromatography/mass spectrometry (GC/MS), set up in FULL SCAN mode. All the analytical procedure was previously published [41, 48, 49].

Chemicals

All solvents were purchased from Carlo Erba Reagents SRL (Milan, Italy). Water was purified by filtering deionized water on a Milli-Q filtration system from Merck Millipore (Milan, Italy).

Sodium sulfide, sodium thiosulfate pentahydrate, iodine, pentafluorobenzyl bromide (PFBBr), 1,3,5-tribromobenzene (TBB), l-ascorbic acid, sodium chloride, ethyl acetate, tetradecyldimethylbenzylammonium chloride, sodium tetraborate and potassium dihydrogenphosphate were purchased from Sigma–Aldrich (Milan, Italy).

Instrumentations

GC/MS analyses were carried out on an HP6890 gas chromatograph (Agilent Technology Inc., CA, USA) equipped with a Model 5975 Mass-Selective Detector (Agilent Technology Inc., CA, USA) and a Model 7673 automatic injector (Agilent Technology Inc., CA, USA). MS conditions: source temperature 230 °C, operating in Full Scan Mode. Quantitative determination was performed using m/z 314, 394 and 426 for internal standard, derivative of sulfide and derivative of thiosulfate respectively.

Injections were performed on an Agilent Ultra 2 (5% phenyl, methylsiloxane) fused-silica capillary, 12 m column (0.2 mm i.d. and 0.33 µm film). Helium was used as the carrier gas at a flow-rate of 1 mL/min (constant flow mode). The programmed operative temperatures were the following: injector, 220°C; column, maintained at 100°C for 2.00 min, then at 10°C/min to 220°C; transfer line, 280°C.

GC/MS determination of sulfide ion and TS in biological samples

Sulfide was detected as bis(pentafluorobenzyl)sulfide. Briefly, an amount of 0.2 mL for the biological fluids (or 0.2 g for tissues, homogenized with a Precellys system, Bertin, Genova, Italy) was added to a mixture consisting of 0.5 mL of 20 mM PFBBr solution in ethyl acetate, 2.0 mL of internal standard (I.S.) ethyl acetate solution (10 µM TBB) and 0.8 mL

of 5 mM tetradecyldimethylbenzylammonium chloride solution in bidistilled water. Water was previously maintained under vacuum condition for 20 minutes, in order to remove oxygen and, eventually, was saturated with sodium tetraborate. Treated solutions were vortexed for 1 min and centrifuged at 5000 rpm for 5 min; after centrifugation 100 mg of potassium dihydrogenphosphate were added to the mixture. The preparation was again vortexed for 10 s and centrifuged at 3000 rpm for 10 min. Organic phase was separated, evaporated and recovered in 100 µL ethyl acetate. 1 µL of the solution was then injected onto a GC/MS apparatus.

Thiosulfate was detected as bis(pentafluorobenzyl)disulfide, as follows: a mixture consisting of 0.5 mL of 20 mM PFBBr solution in acetone, 0.05 mL of 200 mM ascorbic acid solution and 0.05 mL of 5% sodium chloride was initially prepared; then, 0.2 mL (or 0.2 g for homogenized tissues) of the sample were added to the solution. The sample was vortexed for 1 min, and 2 mL of 25 mM iodine solution in ethyl acetate, 0.5 mL of I.S. solution (40 µM TBB in ethyl acetate) were added to the preparation. After vortexing the solution for 30 s the mixture was centrifuged at 3000 rpm for 15 min and kept at room temperature in the dark for about 1 hour. Finally, 1µL of the organic phase was injected onto a GC/MS apparatus.

The limits of detection (LOD) and quantitation (LOQ) were established to be 0.1 and 0.5 µg/g or µg/mL for sulfide, and 0.5 and 1.0 µg/g or µg/mL for thiosulfate, respectively. Linearity was measured using a six-point calibration curve within the range 0.5-20.0 µg/g or µg/mL for sulfide and 1.0-20.0 for thiosulfate. The curves fitted using a linear least-squared regression model for the two molecules. Coefficients of determination were above 0.99. Accuracy and precision, measured at two quality control levels (1.0 and 10.0 µg/mL) were always lower than 15% for both the molecules.

RESULTS

AUTOPSIES FINDINGS

The four workers were aged between 27 and 48 years and were all in good health. Autopsies revealed multiorgan congestion, petechiae, hyperinflated and overexpanded lungs, pulmonary and cerebral edema. The ocular conjunctiva showed marked hyperemia. Massive manure aspiration in the upper and lower airways was found in three individuals (subject 1, 2 and 4). Abundant foam at the mouth and in the trachea was observed in subject 3. No greenish discoloration of the skin and of the organs was remarked. A summary of the four cases is reported in **Table 1.**

Table 1. Summary of the main pathological findings together with the characteristic of the subjects. Postmortem interval: 4 days.

Subject n°	Age (years)	Sex	BMI (kg/m^2)	Macroscopic findings	Histological findings

1	48	M	28.3	- Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage. - Pulmonary and cerebral edema.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization. - Hepatic steatosis.
2	45	M	29.9	- Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage. - Pulmonary and cerebral edema.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization.
3	30	M	23.2	- Foam at the mouth and in the trachea. - Latex glove on one hand. - Glottis and trachea with submucosal hemorrhage. - Diffuse congestion and petechial hemorrhage of the internal organs. - Pulmonary and cerebral edema.	- Intra-alveolar edema and dilation of the alveolar spaces. - Hemorrhagic pulmonary edema. - Multiorgan congestion. - Subarachnoid hemorrhage. - Myocardial vacuolization. - Multiorgan congestion.
4	27	M	26.2	- Few abrasions on the body. - Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization.

EXAMINATIONS OF THE SITE

A technician monitored the emissions of toxic gases at the death scene several days after the accident, inside the emptied pit. After opening the valve connected to the manure lagoon (simulating what happened the day of the accident), the sudden increase of H_2S levels to lethal values was confirmed.

TOXICOLOGICAL RESULTS

The concentrations of sulfide and TS in the biological samples obtained at the time of autopsy from the four cadavers are reported in Table 2.

Table 2. Sulfide and thiosulfate (TS) concentration in biological samples.

	Subject 1	**Subject 2**	**Subject 3**	**Subject 4**

	Sulfide µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g
Liver	3.7	<LOD	3.0	<LOD	2.8	1.9	8.3	2.6
Lung	9.4	5.4	5.0	3.5	5.2	2.8	8.0	4.0
Brain	2.7	<LOD	3.7	<LOD	13.9	1.9	6.6	1.8
Spleen	5.0	1.2	3.3	2.0	4.1	1.5	6.3	2.9
Fat	<LOD	<LOD	1.5	<LOD	1.5	<LOD	<LOD	<LOD
Muscle	2.7	<LOD	2.6	<LOD	3.2	1.5	3.5	5.6
	Sulfide µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL
Urine	<LOD	<LOD	<LOD	<LOD	<LOD	1.8	<LOD	<LOD
Bile	2.2	3.3	1.6	4.4	<LOD	2.6	<LOD	2.5
Cardiac blood	2.2	4.0	2.7	3.7	0.5	2.1	3.0	4.9
Femoral blood	0.5	2.1	N/a	N/a	0.8	2.2	1.2	2.3

N/a sample not available
Sulfide LOD: 0.1 µg/g(mL)
Thiosulfate LOD: 0.5 µg/g(mL).

Routine toxicological analyses were performed on blood and urine samples of each subject. All specimens resulted negative for alcohol, psychotropic, common medicines and illegal drugs. Blood and brain samples were tested also for volatile hydrocarbons, such as methane, but provided negative results.

DISCUSSION

After carbon monoxide, H_2S is the most common cause of occupational gas exposure deaths, and several fatal intoxications in confined spaces are reported, particularly in oil and gas, manure processing, sanitation, fishing, and farming industries [21, 29, 32, 40-46, 50-52].

As in the case here described, casualties usually occur in confined spaces and in multiples, as would-be rescuers rush to save their co-workers, neglecting to protect themselves [42].

The macro- and microscopic findings of the four victims of the present case report, as typically happens in similar situations, were unspecific and represented by pulmonary edema and multiorgan congestion [37, 43, 48, 53]. Manure was found inside distal airways of subject 1, 2 and 4.

No greenish discoloration of the skin and of the organs was found, even if this condition (attributed to postmortem formation of sulfhemoglobin) has been reported in several previous cases of H_2S poisoning [54, 55].

The lack of pathognomonic findings necessitates toxicological investigation to assess similar intoxication case.

Earlier publications reported that physiological levels of sulfide in blood plasma range between 0.96 - 3.2 µg/mL [56, 57] and that its concentration in the animal brain is around 1.6 - 5.13 µg/g [7]. However, these values have been criticized as they could be influenced by the analytical methods and by the changes that occur in the samples. For this reason, it is highly probable that the physiological levels of sulfide are orders of magnitude lower than previously reported values [58, 59].

In a cadaver, sulfide concentration in blood could be affected by the postmortem interval and environmental temperature, as hydrogen sulfide is produced during the putrefaction of sulfur-containing organic substances [51, 60]. Other situations may result in the artifactual absence or presence of sulfide in biological specimens, obfuscating interpretation of laboratory values. For instance, hydrogen sulfide rapidly disappears from blood during life [13], and it has been shown to either increase or decrease in vitro depending on the nature of the specimen, storage temperature, and initial sulfide concentration [60].

For this reason, in post-mortem evaluations, sulfide detection is not enough to establish if the cause of death was the exposure to this gas. As recommended by some members of the American Office of Occupational Safety and Health Administration [61], in accordance with several authors [49, 52, 54, 62], it is important to test also thiosulfate in postmortem blood to document occupational fatalities from H_2S exposures. This aspect is frequently overlooked in case reports concerning fatal poisoning [32, 51, 63, 64]. Thiosulfate is more stable than H_2S in vitro, as it has been reported, for example, that it remained stable in urine for at least 8 h at room temperature and for up to 18 months when the urine was stored at –20°C [65, 66].

In the fatal cases described in the present study, concentrations of sulfide were consistent with values found in fatal cases of hydrogen sulfide poisoning [4, 52, 67, 68]. Refrigeration of the bodies during the interval from death to autopsy and the frozen storage of specimens in airtight containers until analysis have reduced possible postmortem production or decay of H_2S. However, a loss of H_2S during the period between death and autopsy (4 days), cannot be excluded.

Sulfide levels in cardiac blood (2.2-3.0 µg/mL) were higher than those in femoral blood (0.4-1.2 µg/mL), except for subject 3 (0.4 µg/mL in cardiac blood *vs* 0.8 µg/mL in femoral one).

Sulfide were detected at levels of 5.0-9.4 µg/g in lungs, 2.6-3.5 µg/g in skeletal muscles, 2.8-8.3 µg/g in liver, 2.7-13.9 µg/g in brain, 3.3-6.3 µg/g in spleen, <0.1-2.2 µg/mL in bile. Sulfide was not detected in urine (<0.1 µg/mL). In general, more highly perfused organs (brain, lungs, spleen, muscles) showed higher concentrations of sulfide, while relative low levels were found in less perfused ones, such as fat (<0.1-1.5 µg/g). In fact, due to its lipophilic property, in such tissues sulfide requires higher distribution time. It was observed that the higher concentration in lung, the higher was the ratio of cardiac to peripheral blood concentrations (C/P ratio), thus suggesting a postmortem redistribution of sulfide.

In the present cases, TS was detected at levels of 2.1-4.9 µg/mL in cardiac blood, 2.1-2.3 µg/mL in femoral blood, 2.5-4.4 µg/mL in bile, <0.5-1.8 µg/mL in urine, <0.5-2.6 µg/g in liver, 2.8-5.4 µg/g in lung, <0.5-1.9 µg/g in brain, 1.2-2.9 µg/g in spleen, <0.5-5.6 µg/g in muscle. TS was not detected in any fat tissues (<0.5 µg/g).

In blood of healthy persons not exposed to H_2S, the normal level of TS is about 0.3 µg/mL [61]. TS levels in our samples were low, suggesting that the death of the four men occurred quickly. Some differences could be observed between TS concentrations detected in tissues and those measured in blood (in liver and, for 3 of the 4 subjects, in muscle, lower concentrations were found).

Only in a few cases thiosulfate has been measured in brain and muscles [69-71], in liver and fat tissues [70, 71] while more data, consistent with our results, are available for lung [49, 55, 69, 72].

The reported incident was unwitnessed, but toxicological evidence (in accordance with postmortem and on-scene investigations) suggests the following sequence of events:

- One of the victims wanted to pump the sewage from the manure pool into a manure spreader tank. Probably, there was a problem with the valve that allows the sewage to exit from the manure pool to the reception pit. So, one of the workers descended into the pit to manually open/unblock the valve (he was the only one that was wearing gloves). When sewage began to flow out of the pipe, a concentrated emission of H_2S gas occurred. The high H_2S concentration immediately led to brain respiratory center paralysis, asphyxia and cardiac failure.
- Subjects 3 provided the highest sulfide concentration in brain (13.9 µg/g) and the lowest concentration in blood, suggesting the hypothesis of a rapid loss of consciousness and respiratory depression ("slaughterhouse sledgehammer" effect). It can be assumed that subject 3 was the first to enter in the pit and that he was exposed directly to H_2S higher concentrations compared with the other workers.
- One by one, the other farmers entered the pit in attempts to rescue the coworkers but were overcome and became unconscious from the high concentration of sulfide. By this stage, the sludge had started to flow out through the unblocked valve. So, the unconscious workers died by a combination of intoxication and drowning in the sludge.
- Subject 1 and subject 2 had a similar time of exposure and death, considering that they had similar sulfide and TS levels.

- Subject 4 was the last to enter in the pit, when H_2S levels, although still lethal, had decreased compared to the first few moments after the opening of the valve. This hypothesis is supported by the fact that he presented the highest sulfide concentration in tissues.

Despite the rapid death, subject 3 was the only one with TS detectable in urine (1,8 µg/mL). This is in contrast with previously reported data, considering that it is assumed that urine thiosulfate increase does not occur with rapid fatalities [48].

A possible explanation is that the presence of TS in urine of subject 3 is due to differences in metabolism of H_2S, potentially age and BMI related (he was young and slimmer than the others). In fact, it is known that the response of thiosulfate to H_2S exposure may vary significantly between individuals [55, 73].

An alternative hypothesis is that thiosulfate in urine does not represent, in this case, a signal of acute poisoning but a sign of a sub-acute or chronic exposure. In fact, subject n° 3 might have been exposed to low levels of H_2S in the period prior to exposure to high and lethal concentrations (maybe during attempts to unlock the valve inside the pit or during its routine work activities). It should be said that levels up to 3.4 µg/mL of thiosulfate in urine have been reported in apparently healthy people not exposed to H_2S [66].

CONCLUSIONS

We evaluated the distribution of sulfide and TS in biological fluids and tissues of four dairy farmers accidentally died inside a pit connected to a manure lagoon. The cause of death was assessed to be acute poisoning by H_2S for all the victims. Manure inhalation contributed to the death of subjects 1, 2 and 4.

Even today, despite the several cases reported in the literature, similar tragedies continue to occur, suggesting the need for more stringent training and prevention procedures.

Interestingly, sulfide and TS could be found in several biological fluids and tissues, even if not homogeneous toxicological values were detected in samples.

In similar cases, the concurrent measurement of sulfide and thiosulfate concentrations is fundamental to better understand the circumstances of the deaths, the timing and the real sequence of the events.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure

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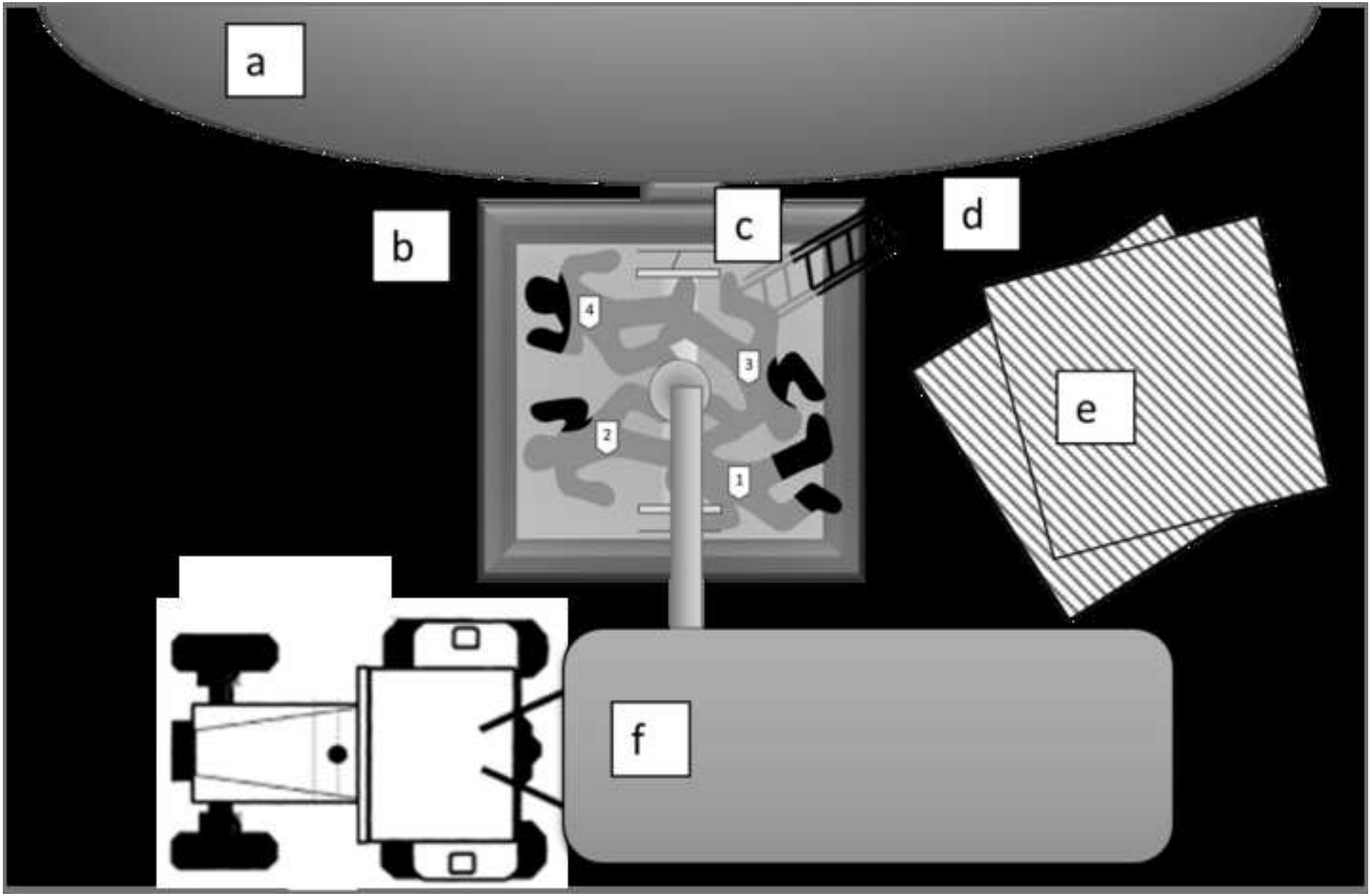

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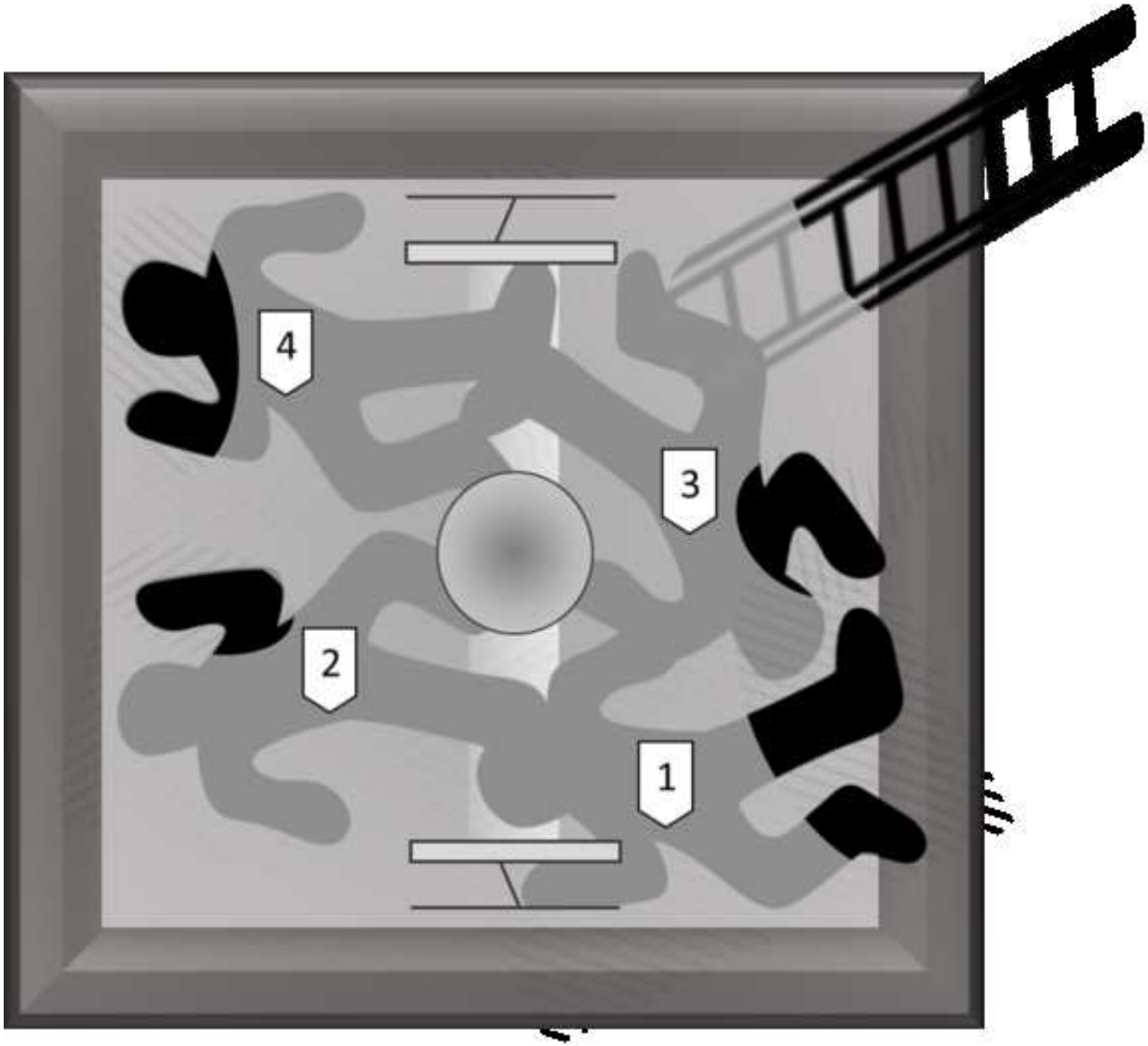

Table

Table 1. Summary of the main pathological findings together with the characteristic of the subjects. Postmortem interval: 4 days.

Subject n°	Age (years)	Sex	BMI (kg/m^2)	Macroscopic findings	Histological findings
1	48	M	28.3	- Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage. - Pulmonary and cerebral edema.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization. - Hepatic steatosis.
2	45	M	29.9	- Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage. - Pulmonary and cerebral edema.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization.
3	30	M	23.2	- Foam at the mouth and in the trachea. - Latex glove on one hand. - Glottis and trachea with submucosal hemorrhage. - Diffuse congestion and petechial hemorrhage of the internal organs. - Pulmonary and cerebral edema.	- Intra-alveolar edema and dilation of the alveolar spaces. - Hemorrhagic pulmonary edema. - Multiorgan congestion. - Subarachnoid hemorrhage. - Myocardial vacuolization. - Multiorgan congestion.
4	27	M	26.2	- Few abrasions on the body. - Manure in the upper and lower airways. - Multiorgan congestion with petechial hemorrhage.	- Alveolar overinsufflation. - Pulmonary edema. - Presence of vegetable and amorphous matter (manure) and bacteria in the bronchioles as well as in the alveoli. - Multiorgan congestion. - Myocardial vacuolization.

Table 2. Sulfide and thiosulfate (TS) concentration in biological samples.

	Subject 1		**Subject 2**		**Subject 3**		**Subject 4**	
	Sulfide µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g	**Sulfide** µg/g	**TS** µg/g
Liver	3.7	<LOD	3.0	<LOD	2.8	1.9	8.3	2.6
Lung	9.4	5.4	5.0	3.5	5.2	2.8	8.0	4.0
Brain	2.7	<LOD	3.7	<LOD	13.9	1.9	6.6	1.8
Spleen	5.0	1.2	3.3	2.0	4.1	1.5	6.3	2.9
Fat	<LOD	<LOD	1.5	<LOD	1.5	<LOD	<LOD	<LOD
Muscle	2.7	<LOD	2.6	<LOD	3.2	1.5	3.5	5.6
	Sulfide µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL	**Sulfide** µg/mL	**TS** µg/mL
Urine	<LOD	<LOD	<LOD	<LOD	<LOD	1.8	<LOD	<LOD
Bile	2.2	3.3	1.6	4.4	<LOD	2.6	<LOD	2.5
Cardiac blood	2.2	4.0	2.7	3.7	0.5	2.1	3.0	4.9
Femoral blood	0.5	2.1	N/a	N/a	0.8	2.2	1.2	2.3

N/a sample not available
Sulfide LOD: 0.1 µg/g(mL)
Thiosulfate LOD: 0.5 µg/g(mL).

Credit Author Statement

All authors contributed to the study conception and design. Material preparation, sample collection and data collection were performed by Matteo Moretti, Marco Ballardini and Chiara Siodambro. Analysis were performed by Francesca Freni, Claudia Vignali, Luca Morini. The first draft of the manuscript was written by Matteo Moretti, Chiara Siodambro and Francesca Freni. Luca Morini, Claudia Vignali, Livio Tronconi, Antonio Marco Maria Osculati supervised, reviewed and edited the manuscript. All authors read and approved the final manuscript.