

STUDY OF STRONTIUM AS A MARKER OF VITALITY IN DEATHS FROM SUBMERSION BY ICP-MS


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Estando cerca del final de mis estudios de medicina en la *UdG*..... me siento muy feliz por haber pertenecido a esta promoción de estudiantes en esta comunidad que nos ha acogido tan gratamente y que nos ha hecho sentirnos integrados y que tanto nos ha enseñado en lo personal y en lo profesional.

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1. ABBREVIATIONS

WHO: World Health Organization

INE: Instituto Nacional de Estadística

IMLCF: Instituto de Medicina Legal y Ciencias Forenses

LECR: Ley de Enjuiciamiento Criminal

s: Seconds

min: Minutes

CO₂: Carbon Dioxide

O₂: Oxygen

INTCF: Instituto Nacional de Toxicología y Ciencias Forenses

h: Hours

mL: Milliliters

CO: Carbon monoxide

BOE: Boletín Oficial del Estado

μL: Microliters

g: grams

Sr: Strontium

μg/L: Micrograms per liter

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy

mL: milliliters

VR: Right Ventricle

VL: Left Ventricle

μg Sr/L: Micrograms of strontium per liter

PMI: Post-mortem immersion interval

Km: Kilometers

cc: Centiliters

EDTA: Ethylenediaminetetraacetic acid

°C: Degrees celsius

Na₂EDTA: Disodium salt dihydrate

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

UdG: Universitat de Girona

SRT: Serveis Tècnics de Recerca

HNO₃: Nitric acid

HCl: Hydrochloric acid

H₂O₂: Hydrogen peroxide

LoD: Detection limits

LoQ: Quantitation limits

mg: Milligrams

H₂O miliQ: ultrapure water

SPSS: Statistical Package for the Social Sciences

IC: Confidence Interval

SD: Standard Deviation

AMM: Asociación médica mundial

CDI: Comité de Docencia e Investigación.

IMLC: Instituto de Medicina Legal de Cataluña

H₂O₂ SP: Hydrogen peroxide solution

HNO₃ SP: Nitric acid solution

2. ABSTRACT

BACKGROUND

Submersion is one of the most frequent death causes encountered and constitutes a public health problem. Its prevalence is increasing worldwide due to the proliferation of aquatic activities and natural disasters.

Sometimes, the lack of specificity of macroscopic findings rendered by the death by submersion autopsy, forces the introduction of complementary examinations to achieve an accurate diagnosis.

Besides the histopathological and biological studies (diatom testing), the use of biochemical parameters such as Sr has been proposed as a good vitality marker in death by submersion cases.

However, since the quantity of Sr may vary depending on the type of water, we do not know data linked to Sr concentration in both internal water and seawater within our own geographical medium.

GOALS

We want to determine Sr levels in freshwater and saltwater submersions by ICP-MS.

Compare Sr data with other types of death caused by mechanical asphyxia (suffocation, hanging, strangulation).

Compare Sr data with other types of death caused by different kinds of asphyxia (cardiovascular, gunfire, car crash).

METHOD

We will carry out analytical research on case-controls. Samples from deaths by submersion in Girona from 2017 to 2018 will be gathered. The autopsies will be performed at the *Intituto de Medicina Legal y Ciencias Forenses de Catalunya división de Girona*

KEY WORDS

Strontium
Blood
Submersion
Drowning

3. INTRODUCTION

3.1. DEFINITION

Submersion can be defined in several different ways. Amongst the most classic definitions is the one provided by *Roll* on 1918 ^{(1),(2)} who defines submersion as "the result derived from breathing impairment by both mouth and nose obstruction caused by a fluid medium, generally water."

Furthermore, *Simonin* ⁽³⁾ defines submersion as "the mechanism that brings about death by the respiration impediment experienced through an immersion into liquid."

The First World Congress on Drowning, focused on Asphyxia by Submersion and held in Amsterdam in 2002 ⁽²⁾, generated guidelines and definitions of submersion aimed at improving clarity in the realm of scientific communication. The consensus definition of submersion stated is "a process resulting in primary respiratory impairment from submersion or immersion into a liquid interface present at the entrance to the victim's airway which prevents the individual from breathing oxygen. The victim might live or die after this process."

Idris et al on 2003 ⁽²⁾, define submersion as "the process that results in a secondary respiratory failure due to immersion or submersion into a fluid medium, usually water". This definition implies the fact that the liquid-air interface is present at the entrance of the airway, hindering adequate respiration. Regardless of whether the victim passes away or survives after going through this process, the victim has suffered a submersion event.

En 2004, *Corcheiro y Suárez*, ⁽⁴⁾ defined submersion within the clinical-legal context as "death or pathological trauma caused by the introduction of a liquid medium, usually water, into the respiratory passageway".

The following terms have now been abandoned due to the confusion they generated: "near-drowning", "wet drowning", "dry drowning", "active or passive drowning", "secondary drowning" and "silent drowning". ⁽⁴⁾ Terms like "complete submersion" or "partial submersion" are still used to describe the event that presents the body utterly immersed into water in the former case, or only the head in the latter, as well as terms descriptive of the water type such as salt water or freshwater.

Knight ⁽⁵⁾, specifies at least six different types of bodies within the group of those retrieved from water:

1. Died from natural disease before falling into the water (agonal or periagonal state).
2. Died from natural disease while already in the water.
3. Died from injury before being thrown into the water. This type comprehends cases of traumatic death caused by facts such as the body striking rocks or a cliff wall before hitting the water.
4. Died from injury while in the water (damage from boats, ship's propellers, etc).
5. Died from effects of immersion other than drowning (so-called "dry drowning").
6. Died from drowning (positive signs of typical death by submersion).

In 2013, *Delgado Bueno* ⁽⁶⁾, classifies submersion into several kinds on the basis of different parameters (Table 1).

Table 1 Classification of submersion (*Delgado Bueno 2013*)

| | |
|---|--|
| Kinds of submersion on the basis of the degree of immersion of the body into the liquid medium | - Complete: the whole body is submerged -Partial Submersion: at least the mouth and respiratory orifices are submerged. |
| Kinds of submersion on the basis of the type of the liquid medium | -Freshwater: rivers, lakes, swimming-pools, bathwater, groundwater, irrigation ditches etc. -Salt water: oceans - Other liquid medium: industrial containers, wine, etc. |
| Kinds of submersion on the basis of its differents consequences | -Primary: death occurs immediately after submersion. -Secondary: pathological trauma that may eventually cause death. |

Certain cases present difficulties in determining whether the cause of death was the submersion itself or any other previous or simultaneous cause (acute myocardiac arrest, cranio-encephalic traumatism, etc.) . It is important in these cases to describe the submersion predisposing factors to facilitate the specification of the circumstances of death in order to obtain an accurate diagnosis.

The predisposing factors are:

- a) Syncope or convulsions, epilepsy.
- b) Altered or diminished consciousness and/or functioning of the motor areas of the brain caused by alcohol, drugs, hypothermia and/or cranio-encephalic traumatism.
- c) Circulatory arrest (loss of electrical activity or ventricular fibrillation).
- d) Unconsciousness resulting from any other cause (ischemic cardiopathy, arrhythmogenic channelopathies like the long QT syndrome, etc).

3.2. EPIDEMIOLOGICAL DATA

According to the latest *WHO* estimates, drowning is considered to be the third cause of accidental death in the world and responsible for up to 7% of all deaths associated with traumatisms.

Estimates in 2012 state that 372,000 people died by drowning that year, making out of drowning a huge public health problem.

Risk of drowning is higher for children, men and people with easy access to water ⁽⁷⁾. Global estimates are likely to be significantly underestimating the actual magnitude of drownings. Nowadays, the increase of migration via maritime routes in extremely precarious conditions has considerably increased the number of drowning casualties. In many cases recovery of the corpses is impossible. Over the last few decades more than 20,000 migrants have died by drowning while attempting to reach the Spanish shoreline, which accounts for the underestimation of deaths by drowning⁽⁸⁾.

Spain has experienced an exponential increase of drowning deaths since 2010 to 2014, a total of 2370 deaths registered in the latter year,⁽⁹⁾ (Table 2, Figure 1).

Table 2 Number of deceased by drowning, submersion and accidental suffocation in Spain from 2010 to 2014 (INE)

| | Todas las edades | | | | |
|---|--------------------|--------------------|--------------------|--------------------|--------------------|
| | 2014 | 2013 | 2012 | 2011 | 2010 |
| 093 Ahogamiento, sumersión y sofocación accidentales | | | | | |
| Total | 2.370 ³ | 2.208 ³ | 2.257 ³ | 2.200 ³ | 2.067 ³ |

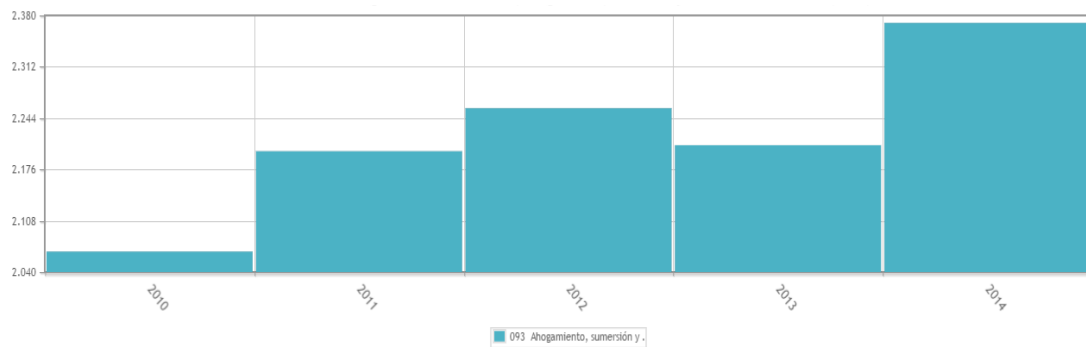


Figure 1 Number of deceased by drowning, submersion and accidental suffocation in Spain from 2010 to 2014 (INE)

Data provided by the *División de Girona del IMLCF de Catalunya* states that the Girona región registered 21 cases of death by submersion in 2014, and 24 death by submersion cases in 2015.

3.3. CLINICAL LAW ETIOLOGY

From a clinical-legal point of view, and according to its typology, death by submersion is considered to be a violent death: accidental, suicidal or homicidal.

The most frequent clinical-legal etiology is the **accidental**, with a high impact on children of 5 and young people between 14-25, predominantly male, during the summertime, a season that facilitates access to water and increased aquatic activity. It is also frequently associated to alcohol consumption and other toxic substances. Prevalence varies geographically, 2, 208 accidental submersion, drowning and suffocation deaths were registered in Spain in 2013, making out of it the third cause of death⁽¹⁰⁾.

The **suicidal** clinical-legal etiology has a significantly weaker impact than the accidental one, since asphyxia by submersion is not a process subject to choice in our medium. It has greater impact on women and ages above the stretch mentioned in the accidental etiology paragraph; it is also associated to toxic drugs and alcohol consumption. Researchers agree on that it is 10-20% of the total of submersions deaths and represents 1% of suicide cases⁽⁴⁾.

The **homicidal** clinical-legal etiology is exceptional, unless the victim has been previously rendered unconscious by traumatisms or alcohol and/or toxics consumption, or a great disparity of physical strength is present between the aggressor and the victim. Throwing a corpse to the water is a relatively frequent strategy of concealment, hence, it is always a possibility that must be considered when a body is retrieved from the water ^{(1),(4),(6)}.

Se desconoce la cifra exacta ya que en ocasiones pueden pasar por accidentes o suicidios dada la dificultad en su resolución policial y judicial.

The exact figure is unknown due to the fact that they are occasionally mistaken for suicides or accidents because of the degree of difficulty police and judicial resolutions entail. In any case, when a death is caused by drowning, etiology aside, it will be prescriptive to open police and judicial investigations. Since we are dealing with violent deaths, the *LECR* in its articles 340 y 343 establishes that a judicial autopsy must be performed in order to determine the cause and circumstances of death.⁽¹¹⁾

3.4. PHYSIOPATHOLOGY

The physiopathologic mechanism that determine death by submersion can be divided into 5 phases of specific characteristics whose features can be seen in the following table ⁽⁶⁾ (Table 3):

Table 3 Phases, duration and characteristics of the pathophysiological mechanisms of submersion (Delgado Bueno 2013)

| Fases | Duración | Características |
|--|-----------------|---|
| Surprise | 5-10s | Period of breath-holding and laringospasms due to the presence of water in the airway. |
| Respiratory Arrest | 1 min | (Hypoxia, hypercapnia, acidosis stimulate-through chemoreceptors-the respiratory bulb centre increasing the frequency of breathing movements). |
| Deep Respiration/Inspiration (froth formation) | 1 min | The victim swallows a great deal of water with active respiratory movements (which enable even greater aspiration of water) |
| Respiratory Arrest (convulsions) | 1 min | No gas Exchange (laryngeal obstruction). Oxygen arterial tension drops, and with it the laryngospasm ceases, giving way to the victim's active aspiration of liquid in amounts that vary from victim to victim, and can eventually determine death. |
| Terminal Phase: Vital signs fade away | between 3-4 min | Progressive fading away of vital signs that results in death. |

When a person falls into the water the first automatic reaction is to inspire deeply before sinking.

Afterwards, a willing deep apnea until the O_2 being depleted and the CO_2 not being eliminated force inspiration. Water inspiration continues and convulsions might be experienced. Respiratory arrest follow and irreversible cerebral anoxia sets in precipitating death (Figure 2).

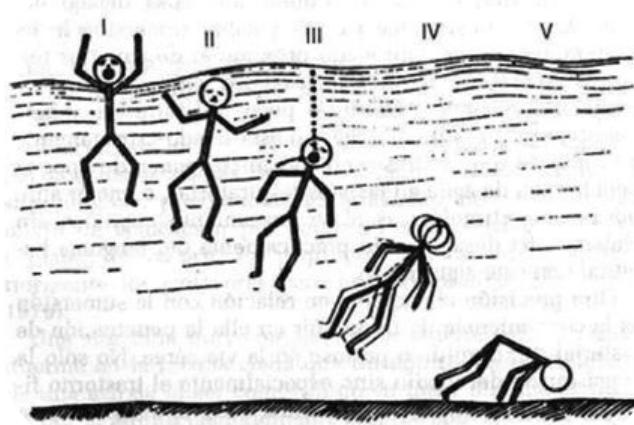


Figure 2 Phases of submersion (Gisbert Calabuig 2004)

Irreversibility depends on age, water temperature and the limit established between 3-10 minutes.

Anoxic anoxia is the classic mechanism explaining deaths by submersion. Nowadays a lot of importance is given to electrolyte changes prompted by the entrance of water into the circulatory torrent.

Submersion in freshwater cases, which is hypotonic as regards plasma, find the submersion liquid that reaches the alveoli is absorbed fast, passing into the pulmonary circulation. This prompts a marked hemodilution and hypervolemia that, in extreme cases, may cause a hyponatremic situation, with circulatory overload that facilitates cardiac failure.

Submersion in saltwater cases, which is hypertonic as regards plasma, find the submersion liquid occupying intra-alveolar light, attracting water to the airway from the pulmonary circulation causing hypovolemia, hemoconcentration and severe pulmonary edema. The level of blood viscosity increases and acute hypernatremia ensue; on a different note, the pulmonary edema increases even more the hypoxia and the hypercapnia situation, putting the heart in a condition of bradycardia and asystolia.

There are also submersion death cases in which no entrance of liquid into the lungs is observed. It is believed that the mechanism of death consists of laryngospasm blocking ventilation, but this theory has not yet been proven correct, since during several minutes the larynx should be kept closed in order for the victim to be killed.

Another type of death that would be included within this group of "deaths in the water with dry lungs" is the death caused by vagal inhibition. Death can occur suddenly in the water due to a reflex cardiac inhibition prompted by the vagal nerve stimulation that induces hyperactivity in the parasympathetic nervous system resulting in bradycardia and ending in cardiac arrest ^{(1),(4),(5)}.

3.5. MEDICO-LEGAL AUTOPSY

The *Recomendación n°(99)3 del Consejo de Ministros de los Estados miembros* ⁽¹²⁾, establishes a uniform methodology to perform medico-legal autopsies. This recommendation states that in those cases in which the cause of death may be due to other than natural causes, the relevant authority, accompanied by one or more medico-legal expert, must investigate thoroughly the place where the facts happened, must examine the body, and must decide whether or not an autopsy should be performed.

There is a set of specific recommendations for each type of death, and in autopsies of death by submersion it is recommended to annotate the following findings: plume of froth at the mouth, cutis anserina, cutaneous maceration, alga and mud, secondary lesions caused by aquatic animals or elements (rocks, boats, etc.), nail loss, skin tissue, lividity location specification.

As regards complementary tests, the need to examine the following samples is emphasized: samples of gastric contents, exact description of the lungs (weight, measurements, emphysema expansion); samples of pulmonary fluid, liver and other organ tissues, aimed at obtaining evidence of likely traces of diatoms and other contaminants.

It is recommended to take samples of the submersion medium (e.g. river water, bathwater, etc.).

In each case, the samples must be gathered, prepared and forwarded in a precise and specific manner. In order to regulate this process, the law *Orden JUS/1291/2010, de 13 de mayo* ⁽¹³⁾, was passed. It states the importance of regulating the retrieval, preparation and forwarding of samples to be analyzed by the *INTCF*. An important aspect focuses on the sample forwarding forms. They must be clear and contain all necessary data to identify, unequivocally, the packets and samples, correctly instruct the analyses, ensure the safeguarding of the chain of custody, and, furthermore, facilitate sample control and return or destruction once the relevant process is completed.

3.6. AUTOPSY MACROSCOPIC FINDINGS

Autopsy performance must follow a specific protocol in order to gather all the macroscopic findings presented by submersion deaths. There are three different autopsy phases:

1) **External exam** ^{(1),(5),(14)}. Is described as:

A. Corpse permanence-in-the-water signs:

- a. **Cadaveric Spasm:** Specifically affecting, at the hand level, the fingers clenched in a rigid flexion, usually retaining traces of sand or algae from the location where the submersion took place. It is meaningful as a sign of clear vital reaction.
- b. **Cadaveric Lividity:** They present a paler coloring than usual, probably due to the dilution of the blood and the low temperature of the submersion medium. The corpse usually adopts a position similar to the prone position and, therefore, the lividities should be spotted in the upper torso.
- c. **Cutis anserina:** It is a consequence of the rigidity process suffered by the hair erectile muscles, and it is favoured by the low temperature of the medium.
- d. **Body Temperature Drop** (the lower the temperature, the faster the process).
- e. **Cutaneous Maceration:** It is suffered at the stratum corneum level and it is most heavily evident in the palms of the hands and the soles of the feet. A whitening process appears initially progressing towards a swelling and wrinkling of the epidermis ending with its loosening off along with the hair and the nails.
- f. **Cadaveric Putrefaction Evolution:** It is slower due to the influence of the temperature of the medium. Once the body is retrieved from the water, the putrefaction process is accelerated.
- g. **Saponification:** It is a putrefaction modification process that uses hydrolysis and hydrogenation of a fat tissue by an alkali that leads to the formation of a substance of oily-waxy initial appearance (adipocere).

B. Signs of Vital Submersion:

Vital Submersion is the submersion suffered by a person who was alive when it took place, dying by drowning due to the water entering the airway, which as further consequence leaves a series of specific traces. Agony is understood as the stretch of time between the moment the victim falls into the water and the moment death occurs.

- a. **Plume of Froth:** Identification of the plume of froth at the mouth and nostrils is pathognomonic of this kind of death, although it is not an utterly consistent finding. It usually appears 2-3 hours after the body retrieval from the water took place. The froth is normally pinkish-white with homogeneous small-calibre bubbles, and is the product resulting from a mixture of mucus, proteins, surfactant of the airway, and the water of the drowning medium.

C. Corpse external lesions:

External lesions may lead to interpretation problems, mostly in those cases of lesions caused by corpse dragging through the bottom, water currents, a propeller's parts, etc. There may even be soft parts lesions caused by aquatic animals.

2) **Internal Exam** ^{(1),(6),(14)}

A. Cranial Autopsy:

- a. **Middle Ear:** The middle ear and the mastoid region can experience hemorrhagic infiltration transparent through the petrous bone and of bluish coloring, but it is considered to be unreliable.
- b. **Sinuses:** Watery fluid present in the sinuses, although it can be considered as a consequence of the water entering the body post-mortem.

B. Autopsy of the thorax:

- a. **Intramuscular haemorrhages:** In the neck and back due to the agony convulsions overwhelming anoxic muscle effort, although this feature is subject to controversy.
- b. **The lungs:** weight and volume augmented so much they may even hide the pericardial sac and frequently present marks left by the ribs on the pulmonary surface. Ecchymosis and Paltauf spots can also be found scattered over the pulmonary surface, subpleurally located and of light coloring.
Applied pressure brings up crepitation caused by the emphysema and the edema, with diminished elasticity revealing fovea.
When cut, they crepitate and release air, revealing a massive pulmonary congestion, abundant blood of foamy appearance mixed with the submersion fluid flowing through the section surfaces.
Intrapulmonary haemorrhages are also apparent.
The trachea and bronchia are full of pinkish-white froth originated by the plume of froth. Sometimes traces of vomit or vegetation can be seen.
- c. **Pleural Cavity:** It often appears filled with serous or serohaematic liquid. This feature is of no value in cases of advanced putrefaction because it is believed to be a consequence of the postmortem diffusion of pulmonary liquids.
- d. **Heart:** Some cases present the dilation of the right ventricle caused by hypervolemia and pulmonary resistance, although this is a subjective and non-specific sign.

- e. **Blood, hemodilution:** Blood fluidity increases as a consequence of the dilution process. The blood contained by the left cardiac cavities is more diluted than the blood present in the right cardiac cavities.

D. Abdominal autopsy:

- a) **Digestive System:** Submersion medium and foreign bodies present in the stomach and duodenum. >500 mL is considered to be the quantity that is a determinant sign of intravital submersion in an adult body. Occasionally, gastric mucosa tear can be observed at the cardia point caused by vomit.
- b) **Spleen:** It may be found shrunk and anemic, but this fact is believed to be a postmortem phenomenon in prolonged submersion cases.

In order to establish a macroscopic diagnosis of death by submersion, the presence of macroscopic findings as described above might proved to be sufficient in correlation with the circumstances of death (traumatic pathology aside). However, quite often it is not possible to establish the diagnosis because the macroscopic findings are not always conspicuous and they can be ambiguous and/or unspecific, without correlation with the circumstances of death. In these cases the diagnosis of death by submersion must be either dismissed or confirmed, and in order to do that we perform several complementary examinations that allow us to achieve a diagnosis with certainty.

3.7. COMPLEMENTARY TESTS

The main complementary tests are the following ^{(1),(4),(6),(14)}:

- A. **Radiological Examination:** They enable appreciation of the opacity of paranasal sinuses prompted by the water content that may be postmortem.
- B. **Toxicological Examination:** It aims at determining the presence or absence of drugs, ethanol, pharmacological and/or other volatile compounds, CO, and other gases used to assess its determinant role or contribution to the cause or mechanism of death.
- C. **Histopathological Examination:** in order to find out:
 - a. “Aqueous enphysema” with alveolar dilation, thinning of the alveolar wall and compression of the alveolar capillaries.
 - b. Pulmonary congestion, edema, alveolar haemorrhage, alveolar walls rupture.

- c. Alveolar macrophages diminished in recent victims of drowning, as they are dragged or washed by the submersion liquid.
- d. Alveolar haemorrhage, significant in cases of submersion without putrefaction and asphyxial deaths compared with subjects whose death took place due to other causes.

D. Biological Examination:

Diatoms: They are eucaryotic unicellular algae that can be found in both water, soil and air. It is important to emphasize they are resistant to decomposition and to the action of strong acids. Hence they are considered to be a good vitality marker as they penetrate circulation together with the submersion liquid, unable to gain entry passively. They can be found in organs such as the bone marrow, the liver, the brain and the kidneys. It is conclusive in quantities of 20 diatoms per 100 µl per sediment obtained through 10 g of another tissue. Although a good vitality marker, it presents technical detection difficulties and the possibility of contamination throughout people's lifetimes.

a) **Estroncio:**

Strontium: Strontium (Sr) is a metal commonly present in sea salt, found in concentrations of approximately 8000 µg/L in oceans and seas, variations depending on the degree of salinity of the water with the possibility of reaching a maximum of 13000 µg/L. However, its concentration in freshwater may vary wildly, as it can be similar to the one found in the blood or ten times higher, a fact that requires the retrieval of samples of the water surrounding the corpse to enable comparison with the water found in the corpse itself. The use of Sr as indicator in submersion cases was first proposed by *Icar* in 1932⁽¹⁵⁾, but it was *Abdallah* who first analyzed it as vitality marker. *Piette y Timperman* in 1989⁽⁴⁾ recommended serum Sr as indicator of intravital submersion in saltwater.

Sr concentration in human beings varies between 16-43 µp/L (although it can be modified by nutritional habits and certain pharmacological compounds), and the quantity found in seawater is so big (8000 µp/L) that the difference of Sr concentration between blood and seawater is considered to be a vitality marker.

⁽¹⁶⁾

When water enters the respiratory system in the deep inspiration phase, suspended particles do so too, along with the Sr, provoking not only a

hemoconcentration variation or hemodilution of the blood according to the type of water, but also impacting this element capable of piercing through the alveolar capillary membrane to enter systemic circulation, which indicates intravital penetration.

To be regarded as a good marker, *Piette y De Letter* refer to a series of requirements that must be met, which Sr meets:

- It must be capable of piercing through the alveolar capillary membrane and penetrate circulation.
- It must be present in considerable quantities in the submersion medium, and in very small quantities in the blood of healthy individuals.
- It must not penetrate circulation via other routes (digestive system) or via diffusion postmortem phenomena.

It is important to emphasize that the levels of Sr are not affected by resuscitation maneuvers, hemolysis or putrefaction induced changes, hence its importance as vitality marker in submersion as well. The studies reviewed until the date rejected them samples > 72 h.

3.8. STRONTIUM AND SUBMERSION: CURRENT SITUATION

M. Piette en 1994 ⁽¹⁷⁾, was amongst the first to study strontium, having previously studied Sr in the ICP-AES technique, and determining that only 10 ml of blood were needed as sample for the detection of Sr levels.

One of the most active research groups working with this marker is led by *J.E. Azparren*, who during that same year ⁽¹⁶⁾, studied Sr in VR/VL with an spectrophotometer of atomic absorpsion with Zeeman effect.

He concluded that the combined Sr measure in the two heart ventricles (VR/VL) helped submersion diagnosis in seawater with a value of >75 µg Sr/L in "typical drowning" cases.

River specifications were helpful only in certain cases. It evinced that Sr is the element with the greatest concentration ratio seawater/blood concentration, serum, plasma, as shown in Table 4 ⁽¹⁵⁾ depicting various elements as compared to Sr on the basis of its ratios. This is why it is regarded as a better indicator than any other elements studied so far ⁽¹⁵⁾.

Table 4 Ratios between concentration of those most abundant elements in seawater and in human blood (J.E. Azparren 2000)

| Element | Seawater (µg/l) | Blood (µg/l), Serum (µg/l)*, Plasma (µg/l)** | Ratio: Seawater/blood or serum* seawater/plasma** |
|-----------|-----------------|--|---|
| Chlorine | 19,353,000 | 3,651,350** | 5.3** |
| Sodium | 10,770,000 | (3.5–2.9)×10 ⁶ * | 3.7–3.0* |
| Magnesium | 1,290,000 | 18,500±25,800* | 29.3* |
| | | ~135,760 | 9.5 |
| Calcium | 412,000 | 100,000* | 4.1* |
| Potassium | 399,000 | (215,000–140,000)* | (2.8–1.8)* |
| Bromine | 67,300 | 3900* | 17.2* |
| Strontium | 8000 | ~30 | ~267 |
| Boron | 4450 | 365–39 | 114.1–12.2 |
| Silicon | 4000 | 3040–880 | 4.5–1.3 |
| Fluorine | 1300 | 190** | 6.8** |
| Lithium | 185 | 5 | 37 |
| Rubidium | 120 | 2.700 | 0.04 |
| Iodine | 64 | 63** | 1** |

In 1998, the same author ⁽¹⁸⁾, compared Sr with other markers (chlorine, hemoglobin and diatoms) in blood of VR/VL. The results indicated that both hemoglobin and chlorine are not good indicators of drowning in blood samples presenting high degree of decomposition, and that Sr values such as > 500 µg/L in the VL or VR can be used successfully as indicators of death by submersion.

A new study was published in 2000 ⁽¹⁵⁾, in which he argued that the quantity of Sr in the blood is linked to the agony period in drowning cases.

The Sr intervals in the blood would indicate the approximate length of the agony period and would provide information used in the differential diagnosis. However, it can only be applied to seawater drowning cases, given that freshwater cases present a higher level of Sr entry in the circulatory system.

The high ratio [Sr seawater]/[Sr in the blood] translates into an intense Sr penetration into the circulatory system during the agony period, allowing in an approximate fashion the differentiation between long agony periods and shorter agony periods in the liquid water medium.

Three types of agony period have been defined:

- Sr lower levels in the blood are linked to shorter agony periods, that can take place in death by hypothermia cases, traumatism, ischemic cardiopathy or any other cause that presents a markedly shortened agony period.
- Higher levels are normal in asphyxia-submersion cases.

- Levels close to the baseline levels would define a type of death with a null period of agony in the water, either due to instantaneous death, or death occurring before the victim was submerged (1)(6).

Table 5 ⁽¹⁵⁾, shows the difference between "instantaneous death", "fast vital-submersion drownings" and "common vital-submersion drownings".

Table 5 Proposed limit values for LVSr an LVSr-RVSr in seawater drowning cases grouped by the length of the agonal period (post-mortem immersion interval (PMI) was less than 5 days in every drowning cases) (J.E. Azparren 2000)

| Groups of drownings | LVSr | LVSr-RVSr | Peripheral blood |
|---------------------|----------------|--------------|------------------|
| ID | <125 µg/l | <30 µg/l | Site dependent |
| FVSD | (125–172) µg/l | (30–70) µg/l | Site dependent |
| CVSD | >172 µg/l | >70 µg/l | >172 µg/l |

J.E. Azparren studied in 2003 ⁽¹⁹⁾, the use of Sr in the blood of the VR/VL as diagnosis enabler of freshwater drowning. The effectiveness of Sr as marker depends on its level of concentration in the aqueous medium. It was observed that 32% of bodies retrieved from freshwater can be diagnosed, which means Sr can be considered a good marker also in freshwater if the restrictive criteria is taken into account throughout the analysis process.

In 2006, M. Piette ⁽²⁰⁾ one of the pioneers in the study of Sr, analyzed the difficulties entailed by the performance of autopsy in drowning cases. There are still cases whose cause remains a mystery because of unspecific macroscopic and microscopic findings which due to putrefaction fade away quickly. Putrefaction consequences in these cases must be studied in detail. Furthermore, it has been established that the combination of autopsy results and complementary examinations in putrefactive bodies can provide helpful indications for provisional drowning diagnosis.

J.E. Azparren's latest research in 2007 ⁽²¹⁾, studied the factors affecting the absorption of Sr in drowning cases. A series of variables -such as sex, age, salinity, and water temperature- that had been ignored before were taken into account, yielding results that did not showed any significant difference.

J.L. Romero Polanco in 2007 ⁽¹⁾ facing the great deal of studies carried out, decided to revise and update the information dealing with death by submersion. He gathered together all the information available from different authors.

M.D. Pérez-Cárceles is another author interested in this subject ⁽²²⁾ that published in 2008 an essay focused on the study of the Sr levels in diverse causes of death to achieve an accurate

drowning diagnosis. He also took into account drownings in saltwater and seawater, other types of asphyxia, and other causes of death such as "gunshot" and "jumping from a height. Thus it was confirmed that the analysis of Sr in blood VR/VL is a good complementary tool assisting drowning diagnosis.

This same author compared, in another essay dating from 2012 ⁽²³⁾, Sr with other element traces to assist drowning diagnosis. The same conclusion was reached emphasizing Sr as the best marker in seawater drowning cases, although other element traces such as magnesium's and chlorine's can be useful. Freshwater drowning cases Sr use, aided by other element tracing such as Iron, can increase the possibility of obtaining an accurate diagnosis.

A recent study (*Fortes, 2014*) ⁽²⁴⁾ investigated the possibility of using Sr as vitality marker in a matrix different from blood, such as teeth. The goal was to ascertain the differences in Sr intensity distribution in the teeth of individuals whose death was caused by submersion in order to compare the result with teeth coming from other types of death; thus, lack of information about the postmortem Sr diffusion encountered in other studies was compensated.

The perfect vitality marker in submersion is yet to be identified, so, even if it is possible that Sr is the best one discovered so far, further research needs to be carried out.

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5. STUDY JUSTIFICATION

Submersion is one of the most frequent death causes encountered and constitutes a public health problem.

Its prevalence is increasing worldwide due to the proliferation of aquatic activities and natural disasters.

Girona province, with its 300 kms shoreline, its tourism boom, and its aquatic activities increase, registers an average of 22 deaths by drowning annually.

Sometimes, the lack of specificity of macroscopic findings rendered by the death by submersion autopsy, forces the introduction of complementary examinations to achieve an accurate diagnosis. Besides the histopathological and biological studies (diatom testing), the use of biochemical parameters such as Sr has been proposed as a good vitality marker in death by submersion cases. However, since the quantity of Sr may vary depending on the type of water, we do not know data linked to Sr concentration in both internal water and seawater within our own geographical medium.

The ideal vitality marker in submersion has not been identified yet. Sr may well be a good marker to this end, but we need more research.

6. HYPOTHESIS

Strontium assessment through ICP-MS is a good marker to diagnose deaths by submersion.

7. GOALS

A. **Primary:**

- a) Sr levels determination in freshwater and saltwater submersions by ICP-MS.

B. **Secondary:**

- a) Compare Sr data with other types of death caused by mechanical asphyxia (suffocation, hanging, strangulation).
- b) Compare Sr data with other types of death caused by different kinds of asphyxia (cardiovascular, gunfire, car crash).

8. METHODOLOGY

8.1. STUDY DESIGN

We will carry out analytical research on case-controls. Samples from deaths by submersion in Girona from 2017 to 2018 will be gathered. The autopsies will be performed at the *Intituto de Medicina Legal y Ciencias Forenses de Catalunya divisi3n de Girona*.

8.2. STUDY POPULATION

All cases will be selected via simple random sampling method, except the submersion cases from 2017 to 2018, that will all be included in the study.

8.3. INCLUSION AND EXCLUSION CRITERIA

This study will comprehend:

1. All deaths by submersion from 2017 to 2018 in the province of Girona, regardless of the postmortem evolution time.

In order to avoid false positives related to analysis of Sr, we have excluded the following cases:

1. Cases in which no submersion water sample could be obtained.
2. Cases in which resuscitation maneuvers aided by cardiac substances injections were performed.
3. The case-controls in drowning cases will be chosen according to age and gender criteria.

8.4. SAMPLING

The size of the sampling has been calculated with the *GRANMO* calculator. We intend to compare the SR concentration in the group of deaths by submersion with other types of death.

Assuming an alpha risk of 0.05, and a beta risk of 0.2 in a bilateral contrast, 16 subjects are needed in the group of deaths by submersion and 32 subjects are needed in the other group to

enable the detection of a Sr concentration difference of $\geq 15 \mu\text{g/L}$. The standard deviation is understood to be 17.4. Data loss and dispersion estimate is 0%.

The cases will be classified into three groups on the basis of the cause of death:

1. Submersion (16 subjects)
 - a. Freshwater submersion
 - b. Seawater submersion
2. Other types of asphyxia (16 subjects)
 - a. Hanging
 - b. Suffocation
 - c. Strangulation
3. Other causes of death (16 subjects)
 - a. Car Crash
 - b. Gunfire
 - c. Cardiovascular disease

8.5. VARIABLES

The variables that will be taken into account in this study are:

A. Independent Variable:

Death by submersion, which is a qualitative dichotomous variable ("Yes or No").

B. Dependent Variable:

Sr concentration in the cardinal cavities, measured in $\mu\text{g/L}$, which is a quantitative continuous variable.

C. Covariables:

1. **Gender:** It can be introduced as a qualitative dichotomous variable (Man/Male or Woman/Female).
2. **Age:** Measured in years is a quantitative discrete variable.
3. **Postmortem Interval:** Period of time stretching from the time of death to the finding of the corpse, and also, the period of time the corpse has been exposed to the environment, measured in hours, even if it surpasses 72 hrs. It is a quantitative continuous variable.

8.6. TECHNIQUE

In order to prevent contamination of samples, 10cc of VR and VL blood will be extracted, and preserved in vials at the EDTA. The process will take place at the Autopsy Theatres of the *IMLCFC división de Girona*.

Blood from the cardiac cavities will be obtained through intracardiac puncturing with an sterilized needle of the vena cava or the right auricle, plus the left ventricle or the aorta.

All samples will be appropriately labelled and carefully numbered, protecting the anonimity of the corpse. They will be refrigerated at -20°C if they are expected to be used more than 48hrs later

To avoid blood coagulation is added 100 μg to 3% of Na_2EDTA .

Once sufficient samples have been gathered, they will be transported at the same required temperature to the laboratory *Servei Tècnics de Recerca (SRT) de la UdG* and the analysis process based on the ICP-MS (Inductively Coupled Plasma-Mass) technique will be applied following these steps:

Analysis will start as soon as the samples arrive from the *SRT de UdG*.

The machines will be tuned up to be ready to start work as the samples and reactants to be used are removed from the fridge in order to reach ambient temperature.

The analysis scale (*METTLER TOLEDO AX205*) preparation consisting internal callibration is implemented before starting the work session. Annotation of temperature and test with weight-controls (one weighing 2,000 mgs.; the other weighing 10 mgs.) are also performed. See Figure 4.

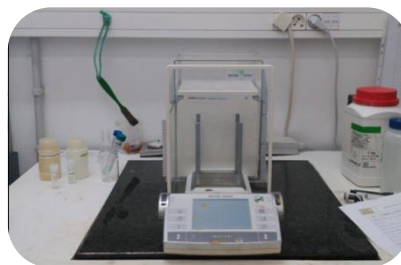


Figure 4 Analytical scale *METTLER TOLEDO AX205 (SRT UdG)*

Falcons preparation is carried out, as they must be identified both in the stopper and the body. The weight is determinated by the scale without reactants or samples.

Before measuring the Sr in the samples it must be a process of digestion to eliminate due to organic matter and ensure the stability of plasma in the ICP. The microwave (*ETHOS PLUS*) is used for this.

Samples are weighed, in duplicate, in the microwave reactors and, under in showcase hood (*OR-ST 1500*), see Figure 5, adds a certain amount of acid (HNO_3 , HCL) and other oxidants (H_2O_2).



Figure 5 Showcase Hood OR-ST 1500 (STR UdG)

Once prepared the samples, they wave to make them homogeneous. Reactors are covered with security

valves, and closes with a dynamometer calibration key. The next steps is to put them in a program in the microwave where they are subject to a time-temperature ramp, increasing the temperature over a period of time. See Figure 6 y 7.



Figure 6 Microwave *ETHOS PLUS*
(STR UdG)

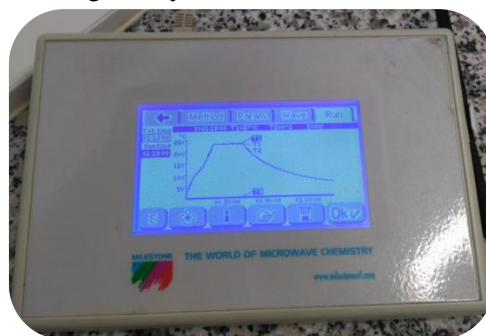


Figure 7 Time-temperature microwave program
(STR UdG)

Usually, with samples of unknown nature, its evaluates the ease with it can be attacked testing with small quantities at ambient temperature, to see how they react.

Once the program of microwave is finished, the samples are diluted with a miliQ quality water, washing them reactors three times and transfer the contents to the falcons and leaving cool to ambient temperature. See Figure 8.

Once cooling has taken place, analytical scale weighting is performed again, the previously annotated weight subtracted to obtain the exact quantity of reactant+sample.

This stage completed, they will remain inside the fridge until the following day.



Figure 8 MiliQ water tank (STR UdG)

For *ICP-MS* technique, we must verify working conditions by measuring several machine parameters besides sensitivity to ascertain it meets specified minimum requirements. This requires a controlling solutions with lithium, yttrium, thallium with measured their concentration, also measure the oxide percentage and double charges to evaluate the response of them.

Then pretends to helium inside the machine to avoid interference with other elements and turns to look at the concentrations of the solution that must be within a certain range o values.

A recuperation of Sr in the blood and water samples is also carried out to check we have the concentration we are looking for (both the minimum and the maximum). The process to follow now is the same we follow for the processing of the samples. The point is to check Sr behaves in the same fashion in each phase, and it not faked the results matrix interference.

Again, the first step consists of removing the samples from the fridge so they achieve ambient temperature while the precision analytics scale is tuned for use, as explained above. An internal standard is added to the samples to correct the matrix effect.

The blank analitical are prepared, namely vials, with H_2O_2 , HNO_3 and HCL to calcúlate the limits of detection (LoD) and quantification (LoQ), the calibration standards (a series of vials containing known concentrations of Sr, covering the range of analysis to do the calibration curve), and samples that we want to analyze, and to identify correctly.

The blank go first to avoid contamination. Automatic pipettes will add acids and H_2O miliQ. After weight annotation the data is used by Excel to calculate the concentration values.

Once they are ready the samples are introduced into autosampler to the *ICP-MS*, we introduce the method and calibrate the machine.

For the analysis in the *ICP-MS* (AGILENT 7500 C), we prepare ranges of concentrations of approximately 0-500 $\mu\text{g/L}$, the samples of seawater ($\geq 800 \mu\text{g/L}$) must dilute them to avoid concentrations of salts than can stop the system of introduction of samples. See Figure 9.

The *ICP-MS* process is simple, the samples will be collected by a tract and introduced in the *ICP*. Subsequently, the will go through a nebulizer and through an Argon source that will form plasma (that is, the matriz will be deleted and form ions which reach to *MS*).

Once the plasma is formed, it will go through a series of cones that will guide it towards the ionic lenses to pass the *MS*.



Figure 9 ICP-MS AGILENT 7500 C (SRT UdG)

It will analyze the amount of ions that come through with the mass-charge target. The data will be provided in ratios (mass-charge relationship of the element of interest and the internal standard one).

Finally, a report of results will be delivered.

9. STATISTICAL ANALYSIS

Statistical analysis is carried out by using the *SPSS versión 24.0 software*.

A safe interval of 95% (IC 95%) will be estimated and a $p < 0,05$ will be considered statistically significant.

The following analyses will be performed:

- Descriptive Analysis:
 - For qualitative variables results will be expressed in percentages and proportions.
 - For quantitative variables with normal distribution medium \pm SD, and for those with normal distribution the median and the interquartile range will be calculated.
- Bivariable Analysis:

The dependent variable (quantitative continuous) is compared to the independent variable (qualitative dichotomous). We will use the *Student T* statistical test if it presents a normal distribution or the *Mann-Whitney test* if it does not.
- Multivariable Analysis:

To avoid confusion a multivariable analysis will be performed taking into account the covariables gender, age and postmortem interval. This analysis will be adjusted by covariables with a lineal general model. The criteria of statistical significance was established in $p < 0,05$.

10. ETHICAL CONSIDERATIONS

This project will be presented to the *Comisión de Docencia e Investigación del IMLCFC* to request and obtain authorization, as we intend to analyze biological samples from judicial corpses. Finally, the project will be presented to the *Comisión de Ética del Hospital Universitario de Bellvitge (Barcelona)* to request its endorsement.

This project follows the guidelines stated in the *Declaration of Helsinki*, the *Convención de Derechos Humanos y Biomedicina (Oviedo 2007)* and the *Ley 14/2007, de 3 de julio, de investigación biomédica*.

In every single case, the informed consent form document (please see annexe 1) will be requested from the relevant families before the sampling collection process begins. If a case arises that offers no possibility of contacting a relative or a victim's approved contact, we will proceed with the approval of the *Ethic Commission*.

The anonymity of the samples sources is guaranteed and identification will not be possible without previous consent from the relevant individual/s. All information shared with collaborators will be encrypted or codified.

Personal and clinical data protection of all participants and collaborators involved with the study is guaranteed, in accordance with the *Ley Orgánica 15/1999 de Protección de Datos de Carácter Personal*.

Samples will be collected and stored in maximum quality conditions, in harmony with the quality standards held up throughout the study. Data maintenance and custody will be implemented by the *IMLCFC (División de Girona)*.

11. STUDY LIMITATIONS

The limitations to be encountered by the implementation of this study will be systematic mistakes in the case selection process, as, unlike other studies, we do not have a number of previously established cases available. Therefore, we will have to apply inclusion and exclusion criteria.

Data collection will also be limited due to the occasional lack of water samples available for comparison of Sr levels in the water, the body, and other subjects that will have to be excluded from the study.

The time of duration of the study will also be a limitation since we don't know when there will be a case of submersion, in the *IMLCFC división de Girona* are recorded an average of 12 cases per year.

The study timeline will also be limited and constrained by the unforeseeability involving submersion cases occurrences, even if submersion is the third cause of death, and even more frequent during the summer months than the rest of the year.

The average cost of the study may also pose an obstacle in the form of presenting the need to employ more resources due to the project's considerable duration.

12. WORK PLAN

The research team will carry out all coordination, interpretation, and results diffusion tasks. The sequence of activities to be developed either individually or by the whole research team is detailed below:

1. **Stage 0:** Study Design (3 months): this stage has already been completed.
 - 1.1. Bibliographical Research.
 - 1.2. Protocol Elaboration.
2. **Stage 1:** Ethical Assessment of the Protocol (1 month).
3. **Stage 2:** Study Implementation (1 month).
 - 3.1. Application Design.
 - 3.2. Application Submission.
 - 3.3. Official Authorization waiting time.
 - 3.4. Authorization granted for the development of our study that allows us to access all information available at the *Instituto de Medicina Legal y Forense de Cataluña División de Girona* y su información.
4. **Stage 3:** Coordination (1 month). Before the study begins:
 - 4.1. Kickoff meeting.
5. **Stage 4:** Sample collection (12 months).
 - 5.1. Se recogerán las muestras de los casos que vayan llegando al *Instituto de Medicina Legal y Forense División de Girona*, del lugar la sumersión así como de los casos controles que se precisen. Samples from the submersion cases of the *Instituto de Medicina Legal y Forense División de Girona* will be collected as they occur, as well as from the submersion places, and the case-controls that may require it.
 - 5.2. Samples will be analyzed at the laboratory *Serveis Tècnics de Reserca de la UdG*.
6. **Stage 5:** Data Interpretation and Statistical Analysis (1 month).
 - 6.1. Statistical Analysis will be carried out by the Statistics Department staff at the *Instituto de Medicina Legal y Forense División de Girona*.
7. A meeting aimed at analyzing and interpreting the study results with the Statistics Department staff and the researchers will take place at this stage.

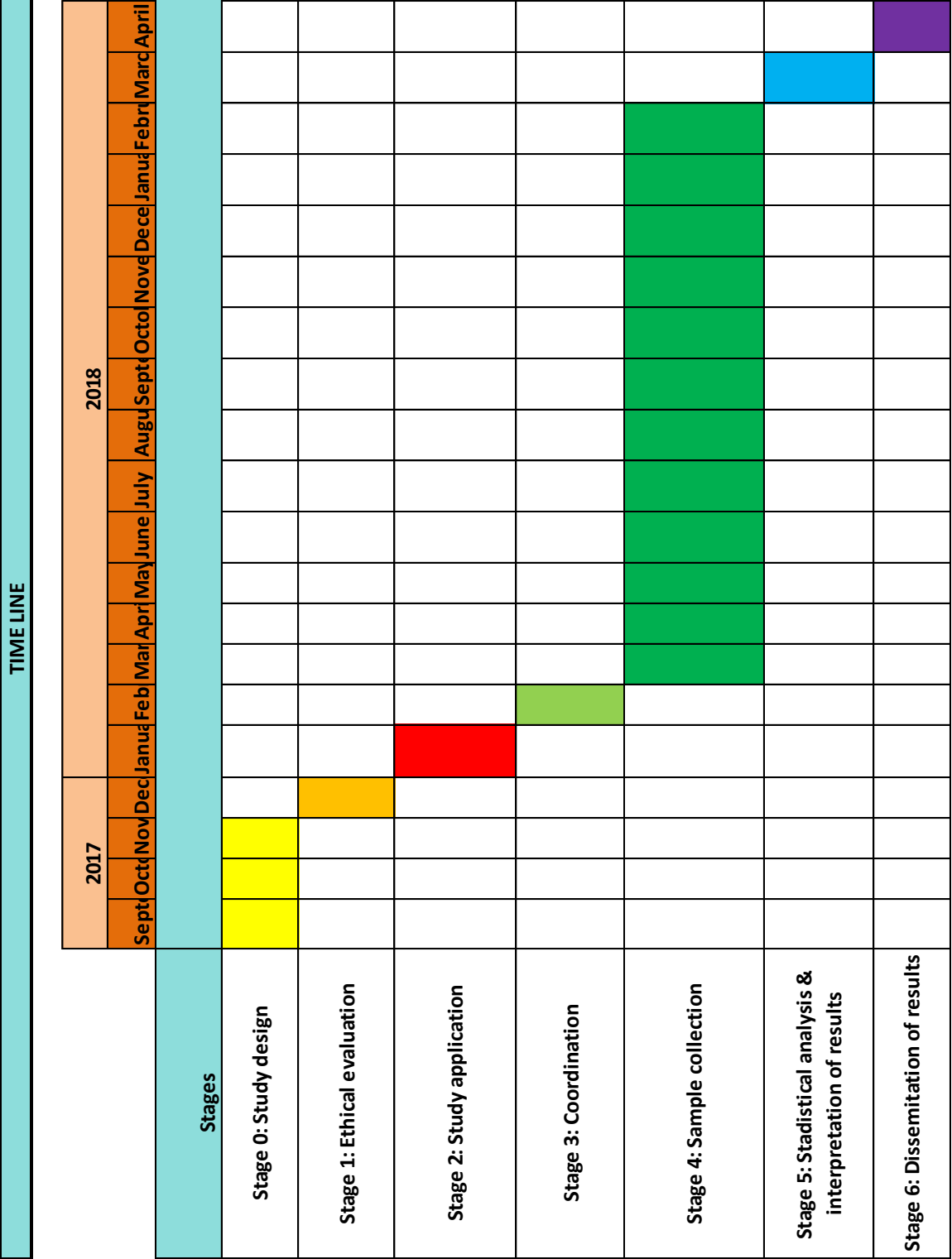
8. **Stage 6:** Results Publication (1 month).

8.1. Study Essay Writing.

8.2. Study Publication.

8.3. Diffusion Plan: the study results will be included and presented in conferences, presentations, meetings, essays and other media.

13. CHRONOGRAM



14. BUDGET

| STUDY BUDGE | COST |
|---|---------------------|
| <u>Staff cost</u> | |
| Laboratory Staff (28h/47,41€/h) | 1.327 € |
| Qualifed statistician (30h x 35€/h) | 1.050 € |
| | Subtotal 4.050 € |
| <u>Materials cost</u> | |
| Blood containers (100 ud) | 14 € |
| Syringe (100 ud) | 10 € |
| Document printing (0,30cent/100 copies) | 30 € |
| | Subtotal 54 € |
| <u>Services Procurement</u> | |
| Microwaving (96 samples) | 1.572,86 € |
| ICP-MASA (96 samples) | 2.122,51€ |
| H ₂ O ₂ SP | 115 € |
| HNO ₃ SP | 188 € |
| | Subtotal 3.998,37 € |
| <u>Publication cost</u> | |
| - | 800 € |
| | Subtotal 800 € |
| | |
| <u>TOTAL COST</u> | 8.902,37 € |

15. ANNEXES

15.1 INFORMED CONSENT

Como representante legal del participante, he sido informado por un responsable del estudio sobre la secuencia operacional, propósitos, objetivos y riesgos del estudio, así como de las expectativas por participar. He leído y comprendido la hoja de participación en el estudio. Para más información o dudas podré contactar con el responsable quien está obligado a responderme.

Consiento a participar en el proyecto “**Estudio del estroncio como marcador de vitalidad en muertes por sumersión con ICP-MS**”, por lo que estoy de acuerdo con lo siguiente:

1. Que los datos médicos estén guardados anónimamente y siguiendo las leyes nacionales de protección de datos, y estoy de acuerdo que sólo se utilicen para propósitos de investigación.
2. Que los datos médicos se analicen en *El Instituto de Medicina Legal y Ciencias Forenses de Catalunya (División de Girona)* y que los datos se puedan ser transmitidos, de forma anónima, para estudios similares.
3. Que los datos médicos se almacenen en los archivos del *El Instituto de Medicina Legal y Ciencias Forenses de Catalunya (División de Girona)* y que pueda ser consultado en caso de duda o cuestiones
4. Entiendo completamente la información referente al estudio y deseo colaborar en el estudio con esta participación. Permito oficialmente a los responsables del estudio recopilar los datos requeridos
5. Estoy informado que mi participación no me proveerá de ningún beneficio directamente
6. Declaro que estoy de acuerdo con los puntos mencionados anteriormente.

☐

SI

☐

NO

- 7 Mi consentimiento a participar puede ser revocado totalmente o parcialmente, mediante una comunicación escrita o primero verbal y después escrita, sin necesidad de más explicaciones.

Su deseo de participar en el estudio es una contribución muy positiva para el avance médico. Agradecemos y apreciamos su participación.

Nombre_____. Fecha de nacimiento_____

(Representante legal)

Fecha_____.

15.2 BUDGET SERVEI TÈCNICS DE RECERCA DE LA UdG

| | | | |
|---|--------|--------|------------|
|  | Oferta | Codi | STR082 |
| | | Versió | 1 |
| | | Data | 26/07/2013 |
| | | Pàgina | 1 del 1 |

Oferta: (4161) UAQIE Determinació de Sr en un lot de 20 mostres de sang
Client: Grup de Recerca d'Anatomia Clínica, Embriologia i Neurociència (NEOMA)
Projecte: (BG0204867) UAQIE-16-ICP-NEO1-01

Planificació econòmica

| Unitats | Descripció | Import u. | Import |
|--------------------|------------------------------------|------------------------|------------------|
| 8,00 procés/essoss | Digestió per Microones | 40,96€ | 327,68€ |
| 28,00 hora/es | Personal tècnic | 47,41€ | 1.327,48€ |
| 7,00 hora/es | Plasma d'Acoblament Inductiu (ICP) | 63,17€ | 442,19€ |
| | H2O2 SP (preu/mL) | | 115,00€ |
| | HNO3 SP (preu/mL) | | 188,00€ |
| | | Base imposable: | 2.400,35€ |
| | | IVA (0%) | 0,00€ |
| | | TOTAL: | 2.400,35€ |

Observacions: Aquesta oferta suposa:
 -Digestió d'un lot de 20 mostres (per duplicat) de sang
 -Digestió de mostres d'aigües de dues procedències (amb duplicats)
 -Determinació per ICP del contingut en Sr a totes les mostres
 -Avaluació de la recuperació de Sr
 -Determinació del LoD i LoQ
 -Informe de resultats

Contracte de serveis STR

