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UMinho | 2023

July 2023

Universidade do Minho
Escola de Engenharia

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Master's Dissertation
Integrated Master's in Physics Engineering
Devices, Microsystems and nanotechnologies

Thesis performed under supervision of
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July 2023

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ACKNOWLEDGEMENTS

Em primeiro lugar, desejo agradecer ao meu orientador, Professor Luís Gonçalves, pelos conselhos, apoio e ideias que me ofereceu ao longo do desenvolvimento deste projeto.

Uma palavra de agradecimento ao João e à Camila, por toda a ajuda no laboratório e pela paciência que tiveram comigo e à Sofia, que realizou o teste final ao dispositivo, comprovando a sua utilidade.

Agradeço de coração à minha mãe, por ser a pessoa mais incansável e forte que já conheci, e nunca deixou que me faltasse nada. Ao meu irmão, por ser um companheiro que, de vez em quando, arruma a louça.

Quero expressar minha gratidão aos que foram um comigo, aos fizeram isso acontecer, e aos que se tornaram um depois. Esta jornada conjunta é o que considero mais valioso destes anos. Um agradecimento especial ao meu parceiro de estudo e aventuras, Farisca. E aos meus 3 preferidos.

Ao Arsca – Pedro, Sr. Lopes e à D. Dolores, pelos momentos de diversão que ajudaram a passar as semanas de estudo e trabalho.

Aos meus amigos de longa data, que estiveram ao meu lado já antes do início desta jornada, quero expressar minha profunda gratidão. Vocês têm sido pilares de apoio, fontes de alegria e cúmplices em todas as aventuras ao longo dos anos

E, com profunda emoção, agradeço ao meu pai, sinto saudades a cada dia.

Muito obrigado a todos que, de alguma forma, tornaram esta conquista possível. O vosso apoio e a vossa presença foram fundamentais para o sucesso deste projeto e para a pessoa que sou hoje.

DECLARATION OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

Deep Sea Biological Sampling

With the advancement of Environmental DNA (eDNA) monitoring methods, there is a growing interest in developing new, simple, and cost-effective devices to collect eDNA from seawater, particularly from the deep sea.

This thesis provides a theoretical introduction to the Environmental DNA concept and existing methods for sample collection, followed by a state-of-the-art review highlighting the key features of each analysed device.

In the scope of this work, a novel device was designed and constructed, capable of filtering water at different depths and preserving the filters for subsequent analysis, by the addition of ethanol. The device can collect up to four independent filters. To clean the equipment, the user simply needs to fill a container a decontaminating liquid (10% sodium hypochlorite ($NaClO$) (bleach), per example) and place the device in cleaning mode.

The constructed equipment consists of two aquarium pumps, one of them serving for the intake of external liquid, while the other one directs the alcohol from a reservoir to the system; and a valve system electronically controlled by a microcontroller that determines the state of each component based on the depth measured by a pressure sensor during sample collection. In cleaning mode, the microcontroller opens all valves, except the alcohol valve, sequentially and activates the intake pump for ten minutes. Once the ten minutes have elapsed, the pump is turned off, and the valves are closed sequentially.

Finally, conclusions are presented, weaknesses of the equipment are revealed. Additionally, indications for possible future projects, aiming to optimize the device, are presented.

RESUMO

Amostragem Biológica em Mar Profundo

Com o avanço dos métodos de monitorização baseados na análise de DNA Ambiental (eDNA), há um crescente interesse no desenvolvimento de dispositivos simples e acessíveis para recolha de DNA ambiental da água do mar, em particular do mar profundo.

Esta tese realiza uma introdução teórica do conceito de DNA Ambiental e dos métodos existentes para a recolha de amostras, seguida de uma revisão do estado da arte, destacando os pontos mais relevantes de cada dispositivo analisado.

No âmbito deste trabalho, foi projetado e construído um novo dispositivo com a capacidade de filtrar água em diferentes profundidades, preservando os filtros para análise posterior, através da adição de etanol. Esse dispositivo é capaz de recolher até quatro filtros independentes. Para a limpeza do equipamento, o utilizador deve apenas encher um recipiente com uma solução descontaminante (hipoclorito de sódio (NaClO) (lixívia) 10%, por exemplo) e nele inserir o dispositivo em modo de limpeza.

O equipamento desenvolvido é composto por duas bombas, em que uma delas serve para a introdução de líquido do exterior, enquanto a outra dirige o álcool de um reservatório para o sistema; e um sistema de válvulas eletronicamente controladas por um microcontrolador, que ajusta o estado de cada componente com base na profundidade medida por um sensor de pressão durante a recolha de amostras. No modo de limpeza, o microcontrolador abre todas as válvulas, exceto a válvula do álcool, sequencialmente, e aciona a bomba de alimentação por um período de dez minutos. Após o término desses dez minutos, a bomba é desligada e as válvulas são fechadas sequencialmente.

Finalmente, as conclusões obtidas a partir deste estudo são apresentadas, juntamente com uma análise das limitações do equipamento. Adicionalmente, são apresentadas orientações para potenciais projetos futuros, com o propósito de aperfeiçoar o dispositivo.

Palavras-chave: DNA Ambiental; Recolhedor de amostras; Profundidade.

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LIST OF ABBREVIATIONS/ ACRONYMS

3G-ESP	Third generation environmental sample processor
ADC	Analog-to-digital converter
AFP	Acid flush path
ANEMONE-11	Advanced Natural Environment Monitoring Equipment
AUV	Autonomous underwater vehicle
CN	Cellulose Nitrate
DC	Direct current
DNA	Deoxyribonucleic acid
DOT	Dartmouth ocean technologies
eDNA	Environmental DNA
F	Female
GF	Glass fibre
GND	Ground
HCL	Hydrochloric acid
LED	Light emitting diode
M	Male
NA	Not applicable
NC	Normally closed
NO	Normally open
PCTE	Polycarbonate Track Etched
PES	Polyethersulphone
pH	Potential of hydrogen
PVDV	Polyvinylidene Difluoride
RAS	Remote access sampler
RS232	Recommended Standard 232
TTL	Transistor-transistor logic
USB	Universal serial converter
WFP	Water flush path

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LIST OF SYMBOLS

µm Micrometre

ml millilitre

l litre

mM Millimolar

g Gram

º C Degrees Celsius

% Percent

cm centimetres

s second

V Volt

mA Milliampere

min Minutes

Kg Kilograms

W Watt

″ Inches

KΩ Kiloohm

h Hour

nF nano Faraday

Ω Ohm

LIST OF TABLES

1. INTRODUCTION

1.1 The importance and challenges of marine ecosystems study

The oceans have long served as rich ecosystems, housing a vast array of species. However, it is believed that a significant number of these species remain undocumented by scientists, especially when compared to terrestrial species (Mora et al., 2011).

Monitoring marine ecosystems has become crucial, particularly due to human activities contributing to increased extinction rates. By closely monitoring these environments, we can proactively respond to anomalies before they lead to irreversible consequences.

Numerous challenges hinder the study and monitoring of marine ecosystems, including factors such as size, depth, pressure, water temperatures, pollution, and logistical difficulties in accessing remote areas. Conventional methods often involve direct sampling, fishing, and animal tagging, but these methods require trained personnel and can be invasive and destructive. Certain species, especially gelatinous ones, can escape fishing nets, while others actively avoid capture.(Govindarajan et al., 2021) .

In recent years, a more modern approach gaining prominence involves the collection and analysis of genetic material from water samples. DNA-based monitoring tools have proven highly valuable, enabling large-scale taxonomic identification of species, which has been a significant challenge in biological evaluation (Nagler et al., 2022).

Utilizing DNA-based monitoring techniques addresses some of the limitations of traditional methods and provides a more comprehensive understanding of marine ecosystems. This approach offers promising opportunities for advancing knowledge of marine biodiversity and implementing effective conservation measures to safeguard these vital environments.

1.2 Objectives

The main objective of this project is to design, construct, and evaluate a compact (lower volume than 10 cubic decimetres) and reusable device, for automatic or semi-automatic seawater eDNA sampling at various depths, with a primary focus on depths up to 50 meters in a single dive. The purpose of this device is to capture genetic material present in seawater for subsequent analysis.

An essential feature of the device is its ability to ensure sample preservation, maintaining the integrity of the eDNA in each sample without any cross-contamination between different depths. This preservation guarantees the immediate conservation of eDNA samples obtained from discrete depths, safeguarding them from degradation or breakdown, as well as isolated from water from other depths, thereby preserving the genetic material's authenticity.

The goal of implementing such a device is to create an accessible and cost-effective tool that can be deployed on a large scale for bio monitoring purposes. Additionally, the potential for discovering new species and conducting vertical and/or horizontal profiling based on the analysis of the collected samples opens exciting possibilities for further research.

In conclusion, this thesis focuses on the development of an innovative and practical solution for eDNA sampling from seawater. The successful realization of this device holds the potential to revolutionize biomonitoring efforts, providing valuable insights into marine ecosystems, and potentially leading to significant discoveries in the field of marine biology.

1.3 Organization of the dissertation

The present document is structured into five distinct phases, each serving a specific purpose in this project.

In the introductory stage, the general problem that motivated this project's undertaking is explained, providing context for the work. Additionally, the purpose and objectives of this project are clearly outlined.

The second phase comprises a comprehensive theoretical review of the environmental DNA (eDNA) concept, covering aspects related to extraction techniques and methods for preserving it for subsequent analysis. This review is crucial for a deeper understanding not

only of the device's chosen specifications but also for emphasizing the importance of involving a biology specialist as a co-adviser in the project.

The third chapter presents a review of the state of the art, offering an overview of existing devices relevant to the topic of this project. This survey of related technologies provides valuable insights into the current advancements and informs the development process.

The fourth chapter details the actual labour of the thesis, encompassing explanations of the device's working principle and the materials used in its construction. This section is a pivotal part of the document, as it describes the key aspects of the device's design and implementation.

Finally, the fifth and concluding chapter delves into the tests conducted on the device, presenting the results obtained and engaging in discussions around them. Additionally, this section presents the conclusions drawn from the overall work, summarizing the project's achievements, potential implications, and future possibilities.

By following this structured approach, the document offers a comprehensive and coherent account of the entire project, from its inception to the implementation and testing phases, culminating in meaningful insights and findings.

2. ENVIRONMENTAL DNA – EDNA

2.1 General considerations

DNA exists in various states, and its treatment and interpretation depend on the specific context in which it is collected. In this project, environmental DNA is defined as genetic material that can be extracted from environmental samples, specifically from water. A crucial aspect of eDNA is that it comprises both organismal DNA, originating from microscopic organisms like bacteria, as well as extra-organismal DNA, which comes from macroscopic organisms. (Mauvisseau et al., 2022).

The temporal and spatial interpretation of extra-organismal DNA poses significant challenges due to the potential distance, layers, and time it may have travelled from its initial release point (Cristescu & Hebert, 2018; Francisco Chavez et al., n.d.). The movement of DNA in the environment can be influenced by various factors such as water currents, wind, and other environmental conditions (Office for the Environment FOEN, 2020). As a result, the DNA collected at a particular location and time may have originated from organisms that are distant and from a different period.

Furthermore, the low concentration of extra-organismal DNA in environmental samples demands careful handling and safety measures during the collection and preservation processes. This is crucial to minimize the risk of contamination, which could lead to erroneous results or misinterpretations. Even minute traces of external DNA, if introduced during sampling or analysis, could skew the findings, and compromise the accuracy of the study.

To address these challenges, rigorous protocols must be followed during the entire process of eDNA collection, handling, and analysis. Proper precautions need to be taken to prevent cross-contamination from external sources, both during fieldwork and in the laboratory.

When collecting eDNA samples, some mandatory precautions are:

- The sampling equipment should not be in direct contact with independent samples towards preventing contamination. This does not mean that the sampling device must be disposable, although it should be subjected to a decontamination process before it is used again.

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- When sampling in a non-flowing water body, going in the water during or immediately before sampling should be avoided. In the other hand, when sampling in a flowing water body, going in the water might be necessary, and in that case, it should be done after (downstream to) the sampling point. Again, this is a way of mitigating contamination risk deriving from DNA transfer from shoes or clothing.

- When the sampling process is happening from a boat, the bow is the best place to collect samples. Once more, this should be done to evade contamination. In lotic bodies of water, sampling should be performed against the flow, as the previous point explained.

- It is advised to wear disposable gloves, anticipating DNA transfer to the sample and, moreover, decreasing the cross-contamination chance.

- Negative controls must be as frequent as possible, particularly when there are decontamination processes for reutilization purposes.

- In the lab where the eDNA is being processed, there are some requirements such as having clean rooms specifically for this kind of work, meaning that, for example, the different stages of the process must be physically separated, the workflow is unidirectional, decontamination processes should be constantly happening as well as negative controls, etc.

2.2 Sampling Strategy

Within the realm of environmental DNA (eDNA) sampling, a diverse range of strategies exists, each offering varying levels of detection probability. These strategies aim to optimize the collection of genetic material from waterbodies to gain valuable insights into the presence and distribution of organisms.

One such strategy involves continuous sampling across the entire area of a waterbody for a predetermined time interval. This method provides a comprehensive and continuous snapshot of the genetic diversity within the water body during the sampling period. However, it may require substantial resources and time, especially for larger water bodies, and can pose challenges in its implementation.

Another approach entails the merging of subsamples obtained from different points within a specific area. This method allows for spatial distribution and occupation analysis, offering valuable information about the presence of organisms across the sampled area. It has

the advantage of being relatively more cost-effective compared to continuous sampling, yet still requires meticulous planning and coordination to ensure representative subsamples.

On the other hand, researchers may opt for analysing multiple samples taken from different locations along the waterbody. This method offers a detailed spatial assessment, enabling a comprehensive understanding of organism distribution in various areas. However, the cost increases linearly with the number of samples analysed.

The spatial distribution, which is a key-feature when working with eDNA, incorporates the distance travelled since it was released from an organism and how it is integrated in the water column.

Obviously, the intended result when sampling is de detection of eDNA, and with that in mind, the sampling design must be optimized for heightened detection probability. Figure 1 illustrates the ideal sampling designs for the major types of waterbodies, considering the spatial distribution (Bruce et al., 2021).
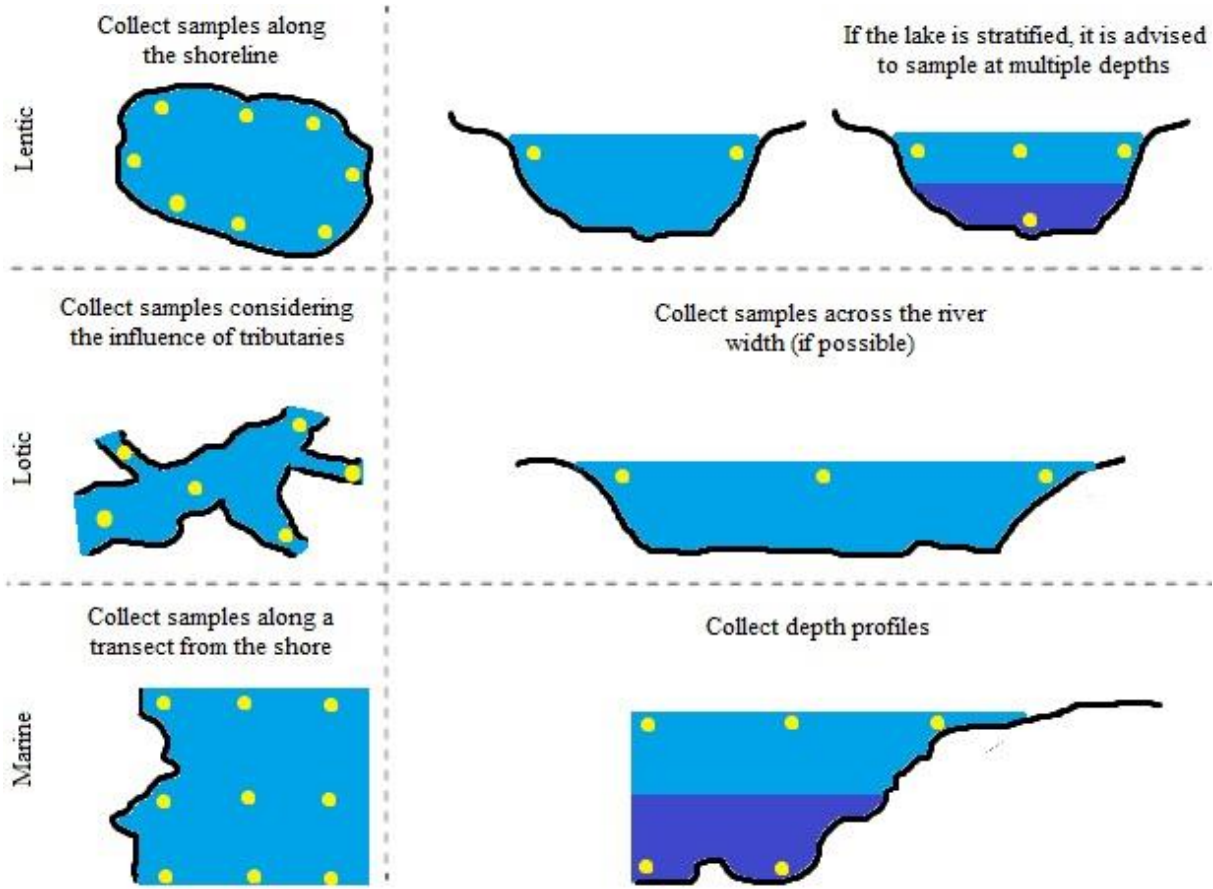

Figure 1 – Ideal sampling design for the main water body types

In lentic water areas such as lakes, environmental DNA is irregularly distributed, however, detection probability can be increased by increasing the sample/ subsample number and having a good sampling design. Additionally, the seasonal circulation influences the vertical distribution of the genetic material in the water column. Water temperature is the main agent in this phenomenon and determines if the water column is stratified or mixed (Hervé et al., 2022). The eDNA is regularly distributed in the water columns in the thermal circulation periods, and because of that, all the column is represented by surface samples. On the other hand, when the water is stratified, water is almost divided into layers and each layer contains distinct species genetic material, consequently, for a complete sampling process, samples should be collected from the different layers.

In lotic waterbodies like rivers, eDNA is more homogeneously mixed in the water column, but in contrast, water flow, tributaries influence on the concentrations must be kept in mind in the sampling design. Of course, the objective of the monitoring is the key to decide if the samples should be collected upstream or downstream to a tributary. The spatial distribution of eDNA modelling is particularly difficult (Jo et al., 2019) and there is no unanimity on how to conventionally do it and that is the reason behind the general acceptance of uncertainty when working with environmental genetic material in rivers. For instance, the flow rate directly impacts the upstream area represented by a single sample, in a slow flowing stream, the sample can represent an extent of hundreds of meters, whereas, in fast flowing river it can be representative of tens of kilometres.

In marine environment, there are less certainties about the influence of hydrological systems on eDNA distribution and transportation. Nonetheless, various studies have already shown that communities found through the processing and interpretation of eDNA are better representative of the environment where it was collected that it was expected. In a similar way to lentic waterbodies, the water column being stratified means that samples should be collected in different depts to achieve a better description of the marine community at a given location (Jensen et al., 2023).

eDNA does not last long because it suffers constant degradation, nevertheless, the degradation rate is influenced by physical/chemical properties of the environment such as the season, mass and size of the water body, temperature, depth, substrate, chemical composition of the water, flow, and stratification. The effects on the DNA degradation of these

elements are nearly impossible to distinguish, especially because eDNA is sampled in a non-controlled environment, yet these are the ones that disturb detection probability the most (Joseph et al., 2022).

Table 1 summarizes general considerations to have in mind when sampling in the main water bodies (Bruce et al., 2021).

Physical-chemical phenomena affect how long the genetic material lasts in the environment by being catalysers in its degradation and consequently, detection probability. Although a faster degradation meaning a shorter time interval for collection and that sounding completely disadvantageous, it inevitably brings the benefit of reducing the chance of collecting eDNA nowhere near where it was released, incurring in a better temporal and spatial interpretations. To be indubitable that this advantage can overcome the smaller time window, the sampling should be more frequent and/ or to be better scattered in the sampling design for a full characterization of a community.

From the numerous factors that can be related to the degradation rate, the next ones are highlighted:

Acidity – Low pH is usually associated to a higher degradation rate (Bohara et al., 2022). Despite the lack of a focused study on measuring DNA degradation in different pH conditions, it is speculated that the more acid conditions correlate to the faster degradation, since positively charged enzymes are catalysing agents on DNA degradation, besides being low pH indicators. Plus, eDNA will degrade in water because of acidic hydrolysis, especially bellow pH 7.5. This is also why DNA preservation buffers are alkaline.

Temperature – A temperature increase is accompanied by microbial activity intensification that facilitates eDNA degradation (Tsuji et al., 2017). Despite the temperature itself being an indicator of an increased degradation rate, it is also an indicator of greater quantity of eDNA because an increased activity, especially on animals.

Oxygenation – This could as well be in the forementioned point since water oxygen saturation level drops as temperature increases. Nevertheless, this is a point in its own because DNA structure is extremely stable when the aquatic system does not contain dissolved oxygen and swiftly degrades in oxygenated environment through hydrolysis (Bruce et al., 2021).

Table 1 - Important considerations while sampling

	All types	Lentic	Lotic	Marine
When to sample	When the target species is in the waterbody based on the literature. During summer and spring there is greater algal and bacterial load that might impact the study	Consider thermal stratification classification patterns for in stratified lakes, if the water is well mixed, sampling from depth is not necessary.	Avoiding drought and flood conditions. Consider migration patterns.	Consider migration patterns, matting and breeding sites. Consider season.
Where to sample	Avoid entering the water before and during the sampling process. If necessary, lotic water should be entered downstream to the sampling point. Consider human activity as to avoid contamination	Near the shoreline. If there is a large variation in depth or if it is stratified, sampling in the middle is advised.	Across the river width. Fixed intervals along the network. Consider tributaries and altitude variations.	Consider vertical profiles, tidal influences, and habitat heterogeneity. Several depths
Sample number	Should represent spatial complexity, system size and access to the area. Consider subsampling.	More samples required for complex habitats	Flowing water should be sampled at even intervals.	Depends on the spatial scale. Deeper water forces bigger sampling effort for better water column information. Consider dilution factor.
Sample Volume	Most studies involving filtration present 500ml to 5l of water per sample filter. Pooled subsamples require less replication	Consider turbidity – increasing sample volume is advised	Methodical sampling/ subsampling is recommended	Sample volume should be maximized for it to be representative. There were obtained good results with 2l to 5l per sample although it depends on the target
Turbidity	Turbidity could constrain sampling	Substrate disturbing can cause problems. Prefiltering with a larger pore is advised. Avoid sampling right after rainfall and during algal blooms		Disturbing seafloor might cause turbidity in inshore areas.

Nutritional load – High nutritional loading is, once again, typically linked with high microbial activity and therefore, it is projected that the degradation rate increases, however this was never proven.

Biotic factors that may also implicate detection probability (Rourke et al., 2022), not so much by increasing degradation rate, but rather by increasing or decreasing the eDNA quantity in the environment. In this category can be included:

Season migration – The patterns and species movements impact the probability of eDNA to be in determined area.

Species life-history – The fact that a determined species does not release a lot of genetic material when an organism is in the adult stage of life, means that the best time to try and detect them is in the breeding season, since adults are more active and eggs or sperm being eDNA sources. Besides, new-borns may be moulting (Takeuchi et al., 2019).

Physiological attributes – Animals with exoskeletons or with dry or scaly skin typically release a minor quantity of genetic material (Andruszkiewicz Allan et al., 2021) than the animals that produce mucous or shed skin. Furthermore, faeces are a main source of eDNA, making the target species toilet habits be an important object of study in order to improve detection probability. This additionally implies that the more frequent feeding increases the detection probability and that cold blooded organisms typically shed a smaller amount of genetic material than warm blooded ones.

Behaviour factors – There is evidence that animals usually shed more DNA when they are active, warm, or stressed (Harrison et al., 2019).

In short, there is a multitude of environmental and species attributes that will impact detectability and increasing the sample collection may be vital for a good representation of a community. It is worth noticing that some of the previously mentioned factors are part speculation because there were not yet conducted sufficient of studies on them, subsequently, it is challenging to understand how much they will disturb detection probability, but certainly it will depend on the environment and target species. Table 2 condenses the attributes more likely to imply detectability and advises solutions to some of them. Incontestably, increasing the sampling effort is always an effective solution, so it was omitted from the table (Bruce et al., 2021).

Table 2 - Common factors that influence detection probability

	Factor	Implication reason	Mechanism	Offsetting factors	Possible Solution
Habitat properties	Cold water	Low eDNA production and mixing	Low animal activity and stratification	Slow eDNA degradation and better detection probability for some groups	Sampling at different depts
	Warm water	Higher eDNA degradation rate	High microbial activity	Higher activity levels (leading to higher eDNA production)	
	Great volume of water	Low eDNA concentration	Dilution		
	Acid water	Higher eDNA degradation rate	Positively charged enzymes		
	High nutritional load	Higher eDNA degradation rate	High microbial activity		
Target species properties	Exoskeleton	Low eDNA production	Physical barrier to *eDNA* shed	Reproductive cells shed and moulting	Sample during and right after breeding season
	Ectothermic	Low eDNA production	Slow metabolism and low shedding	Mucous production or skin/ scales shedding	
	Low activity levels	Low eDNA production	Slow metabolism and low shedding	eDNA accumulation	
	Terrestrial latrine	Low eDNA input to water	Lack of eDNA source	After rainfall it may have better detection probability.	Sample after rainfall
	Not fully aquatic	Low eDNA input to water	Inconsistent eDNA shedding	May be seasonal	Sample when the species is in water

2.3 Filtration method

Filtration is the preferred technique for capturing eDNA in water samples, however, this was not always the case. Formerly, it was used a very clever precipitation technique where the water containing the genetic material was added to a sodium acetate or other ethanol and salt solution at low temperature, which made the DNA change its charge and become

hydrophobic and precipitate. Extraction was applied to the pellet where the DNA was then forced into. Although this smart technique was the one that the first eDNA related studies used, it is believed now that filtration is the most effective process in species detection, for the best part of the systems and the exceptions are for small volume samples (Muha et al., 2019). Filtration not only ensures better sensitivity, logistic and safer working conditions but also guarantees an easier disposition and lower contamination possibility.

A vast range of options is presented when considering eDNA capture in aquatic environment. This happens due to the different membrane materials, pore sizes, filtration mechanisms, transportation and preservation methods and DNA extraction protocols. With all this options, it is hard to address the significance of the decisions at any stage. Besides, a given membrane material may be the best to work with determined pore size or DNA extraction technique, while others are ideal with a different combination of options (Cooper et al., 2022).

This means that that, in order to make the best decisions, a list of possible challenges, problems, logistic options (budget, available equipment, distance and deadlines to name a few) and target system properties should be made firstly. From there, optimization of the remaining workflow should be prioritized not only for a better assessment of the advantages and disadvantages of different combinations but also because studies have shown that this optimization is more important than the actual choices, because even with different options, eDNA analysis was successful.

The filter systems used in eDNA capture could be set into three different categories (Bruce et al., 2021):

- Open filters are filters where the membrane is exposed to air while filtering (even if filtration is performed in the lab). This kind of system is typically used with the aid of vacuum pumps when filtering in the lab and with peristaltic pumps in the field. Contamination risk is the highest for open filters not only for its air exposure, but also because they commonly force its handling during preservation and extraction processes. In the light of this facts, it is easy to understand that handling should be performed with extreme care by qualified personnel and negative controls should be done as frequently as possible. On Figure 2, the use of open filters is shown.

Figure 2 – Open filter examples

- Housed Filters are filters in which the membrane is protected within a solid housing while filtering. The greatest advantage in this system is that the filter housing could be opened, and the membrane removed for preservation and posterior processing, however, when this is done in the field, it must be done carefully to avoid contamination. Preservative solutions may be added directly inside the housing, so that the outside can be cleaned in the lab and opened in clean room conditions. The filter transfer brings contamination risk and therefore it is imperative that it is done attentively, and it is also recommended that negative controls procedures are conducted in order to be certain that there was no contamination. This encapsulation system is the most flexible one to different membrane materials and extraction methods. Figure 3 portrays a housed filter system (Thomas et al., 2019).
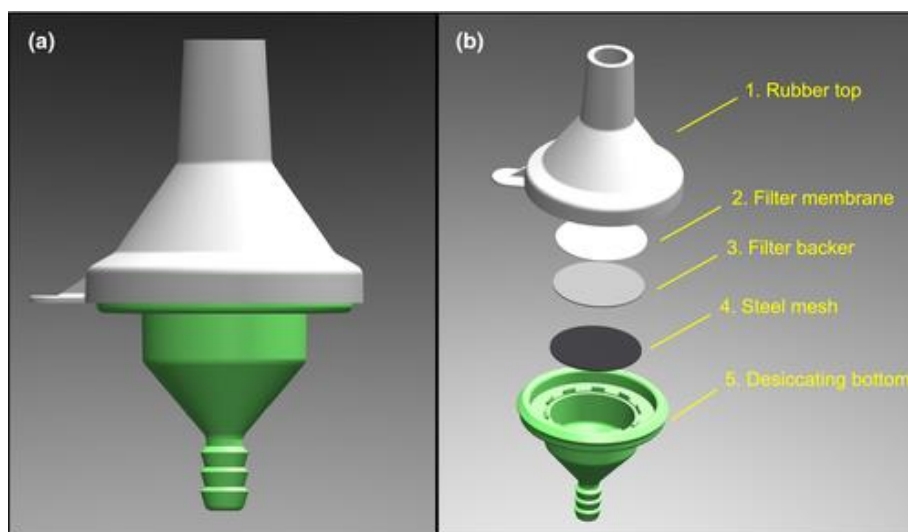
Figure 3 – Housed filter example

- Enclosed filters are those where the membrane is always protected within an outer housing. This means that there is no handling of the membrane and consequently, no contamination risk from it. Again, preservative solutions may be added directly in the membrane. This system is the one that mitigates contamination risk the most and probably the best option for large scale studies due to the possibility of samples being collected by non-qualified people. The major downsides to this system are related to the unit cost and the shortage of options for membrane material and pore size. Furthermore, mechanical lyses, required for DNA extraction from diatoms, cannot be performed with these filters. Two distinct enclosed filter systems are shown in Figure 4.

Figure 4 – Enclosed filter examples

Table 3 provides a summary of the aspects observed for different filter systems.

The combination of membrane material and pore size is critical to determine the volume of water that each unit can filter and for a larger filtered volume, the membrane should be made of a hydrophilic material.

The most used membrane materials (Rourke et al., 2022) are:

- Cellulose Nitrate (CN) (Schabacker et al., 2020)
- Glass fibre (GF) (Bott, 2015)
- Polycarbonate Track Etched (PCTE) (Takahara et al., 2012)
- Polyethersulphone (PES) (Hunter et al., 2019)
- Polyvinylidene Difluoride (PVDF) (Truelove et al., 2022)

Table 3 - Summary of the filter systems

Filter System	Advantages	Disadvantages	Overall Characteristics
Open Filters	Simple and easy to use. Allows for easy filter replacement. Minimal setup and maintenance required.	More susceptible to contamination from external sources. Exposed to environmental factors. May not be ideal for deep-water or harsh environments. Potential loss of genetic material during sampling.	Cost-effective. Suitable for small-scale sampling. Limited protection for genetic material.
Housed filters	Provides partial protection from contaminants. Offers some protection from external elements. Can be used in a variety of environments.	More complex setup and maintenance. Requires occasional filter replacements. Limited protection compared to enclosed systems.	Intermediate cost and protection. Suitable for moderate-scale sampling. Ensures some level of genetic material preservation.
Enclosed filters	Maximum protection from contamination. Ideal for deep-water and harsh environments. Prevents exposure to external elements.	More expensive and complex to build. Filter replacement may be challenging. Requires specialized maintenance.	High-cost, high-level protection. Suitable for large-scale sampling. Ensures best preservation of genetic material.

Despite any of the previously mentioned materials being a valid choice, each one has its advantages and disadvantages. (Bruce et al., 2021; Patin & Goodwin, 2023; Rheyda & Hinlo, 2018).

CN filters are cost-effective and widely available, making them a popular choice for eDNA studies. However, they are prone to clogging, which can hinder the filtration process and result in reduced DNA recovery.

PVDF filters have high porosity, making them suitable for capturing small DNA fragments. They offer good DNA retention, but one potential drawback is that they may lead to higher DNA loss during extraction, impacting the overall yield of DNA samples.

PCTE filters exhibit a high DNA binding capacity, ensuring efficient DNA retention during eDNA sampling. However, compared to other materials, they can be relatively more expensive, which may impact the cost-effectiveness of large-scale eDNA studies.

GF filters are known for their excellent retention capacity, making them ideal for capturing DNA from water samples. However, one concern with glass Fiber filters is their susceptibility to sample cross-contamination, which can compromise the accuracy of results.

PES filters have low protein binding capacity, reducing the likelihood of DNA loss due to adsorption. This makes them well-suited for eDNA studies. However, it's important to note that they may not retain small DNA fragments as effectively as other materials.

Table 4 presents a comparative analysis of various filter membrane materials commonly used in filtration processes. Each material comes with its unique set of advantages and disadvantages, influencing their suitability for specific applications.

Table 4 - Filter membrane advantages and disadvantages

Filter Membrane Material	Advantages	Disadvantages
CN	Cost-effective and widely available	Prone to clogging
GF	Excellent retention capacity	Prone to sample cross-contamination
PES	Low protein binding	May not retain small DNA fragments
PCTE	High DNA binding capacity	More expensive
PVDF	high porosity suitable for small DNA fragments	May have higher DNA loss during extraction

Although typically bellow the micrometre mark, pore size is one of the parameters with most options. An increase in the pore size often leads to a larger volume of water filtered, however, by doing this, it is also increased the information loss possibility, due to the inability of capturing the smaller eDNA containing particles. Most particles that contain eDNA are bigger than $1\mu m$ and rarely over $10\mu m$, meaning that for pore sizes up to $1\mu m$ the information loss is marginal. Even though not being problematic for pore sizes in the μm mark, the loss of information could become a serious problem when using a larger pore size, however, reports have shown that it is more efficient to filter a large volume of water with a large pore size than filtering a small volume with smaller pore size (Schabacker et al., 2020). It is clear though, that smaller pore size should be prioritized when targeting species like macroinvertebrates due to the reduced eDNA shedding.

The most used pore sizes are $0.22\mu m$ and $0.45\mu m$ and there are plenty of good reasons to use these smaller pore sizes, the main one is the lack of commercial options with greater pore size.

When a smaller pore size is required, it is recommended that a prefiltration is performed, using a larger pore size. The main reason for this is to delay filter clogging, and by doing this, increasing the volume of water that is filtered. Moreover, as some particles containing eDNA might get stuck in the first filter, DNA extraction from the prefilter is also advised.

2.4 Filtration in the lab or in the field

Several articles on the matter report the sampling step as simply collecting water in sealed container for posterior filtration (Geerts et al., 2018; Kumar et al., 2022) . Despite the simplicity and speed being a very interesting feature of this technique, the fast DNA degradation forces not only sample refrigeration during transportation, but also its process or freezing on the same day, which can prove to be too expensive and unrealistic, especially for large scale monitoring.

The other option is to filter water in the field, and it can be performed manually with manual pumps and syringes or with a vacuum or peristaltic pump. Manual filtration has the advantage of being a cheap solution and it can be implemented in basically every system. Nonetheless, it could be difficult physical labour and take a long time, depending on the water volume, sample number, sampling design, etc., therefore, it is only logical that powered pumps are employed as they facilitate the whole process. The problem with the use of powered pumps may be related to logistical issues. For instance, if there are multiple teams collecting samples or if a power supply cannot be connected, this technique quickly becomes impracticable. This is the reason for the on growing investment in the development and innovation of sampling equipment aimed at the acquisition of portable and fully integrated eDNA sampling systems and making it available for future studies.

Figure 5 – Methods for the collect and filtering of water samples

It should be noticed that eDNA retention on the filter membrane decreases as the filtering pressure intensifies since more particles are forced through it, however, this effect is not as noticeable as the advantages coming from an enlarged volume of water per sample. Figure 5 illustrates sampling collection processes and in site filtration (Bruce et al., 2021). Note that, ideally, samples should be collected without entering water as it is shown in a. and b., however, when necessary, water should be entered downstream to the sampling point, as in c. or d.

Peristaltic pump filtering is presented in e. and the manual method is exemplified in f., g., and h. along with the use of disposable syringes.

2.5 Volume of water

Although the most used sample volumes are in the $500ml$ to $5l$ range, there are successful published reports that collected only $15ml$ (Forsström & Vasemägi, 2016) samples and others with over $100l$ (Strand D, n.d.) samples, and this happens because there is still no certainty as to the minimum significant volume for each sample, since it also depends on external factors such as sampling design, turbidity, water body size, monitoring purpose and efficiency of extraction and analysis processes.

Increasing the volume of water in each sample usually increases the quantity of genetic material collected and therefore, greater detection probability comes along, nevertheless, the fact that this is not exactly proportional should be enhanced and it should additionally be noted that good results can be obtained even using small volumes samples.

Consequently, it is relatively safe to say that sampling design and strategy has a greater influence on the overall result than the actual volume of water, especially when the study is in lentic water bodies where eDNA is not homogeneously scattered.

In spite of the previously mentioned, when eDNA concentration is scarce, considerable volumes of water should be preferred.

Environmental, physical, or biological conditions often have a negative impact on detectability, so, sampling effort should be extended accordingly, meaning that either volume of water filtered per sample or sample number should be increased, however, increasing the volume of water could prove to be innocuous due to the possibility of filter clogging. Besides, a higher number of samples allows for frequency and occupancy assessment.

2.6 eDNA preservation in filters

Genetic material can be preserved for storage or transportation through three main processes:

- Freezing is very efficient and it can have positive impact in eDNA recovery when compared to samples immediately extracted after filtering. The downside to this method is that it means investing freezing equipment that can be taken into the field so that the filter can be frozen right after it is used. Also, it must remain frozen during transportation because freeze-thaw cycles have negative effects (Yamanaka et al., 2017).

- Drying is an attractive method due to its simplicity. It enables storage for weeks at room temperature (Majaneva et al., 2018), however, this process requires silica gel, desiccators, or paper to absorb water and it is impracticable to this when working with enclosed filters.

Table 5 - Preservative solutions properties

Preservative	Recipe	Cell lysing	Microbe exterminating	RNA preserving	Important considerations
Ethanol	NA		X		Highly flammable May prevent downstream reactions.
RNAlater	Sodium Citrate $25 mM$ EDTA $10\ mM$ Ammonium Sulphate $70\ g/100ml$ solution pH *5.2*		X	X	Requires specific optimization. Complicates DNA extraction.
Longmire's buffer	Tris-HCL *0.1M* pH 8 *0.1 M* EDTA *0.1 M* NaCl *0.5% w/v* SDS	X			Precipitates bellow $10°C$ but returns to solution when heated
Sarkosyl buffer	Tris *100 mM* EDTA *100 mM* NaCl *10 mM* *Sodium N-lauroylsarcosinate 1%*	X	X		Attractive alternative to Longmire's buffer as it does not precipitate at low temperatures

Preservative liquids – the possibility of adding positive control DNA after filtering is a major advantage because it can be used to check if eDNA has been effectively preserved. Ethanol or RNAlater the most used examples of pure preservatives, one of the two categories that exist when considering preservative liquids. The other category includes Longmire's buffer (Williams et al., 2016) and Sarkosyl buffer (Mauvisseau et al., 2021), that besides preventing degradation, release DNA into solution. A vital aspect when using preservative liquids is being aware of the impact on cells or organelle membrane, as DNA extraction processes will be affected. In short, there are several preservative liquids available, each one

has its advantages, disadvantages and each one may suit best a given target. Table 5 reviews the most used preservative liquids properties (Bruce et al., 2021).

Of course, the choice on the preservative solution will depend on the budget and available options for the project. Figure 6 presents the best options for each context (Minamoto et al., 2016).
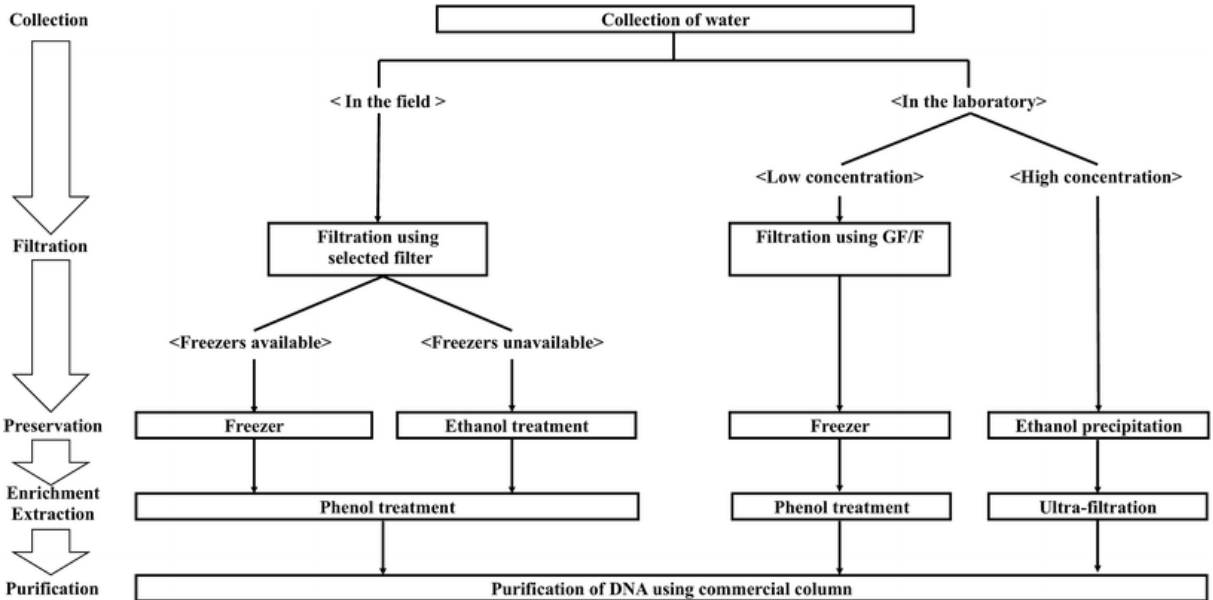
Figure 6 – Best options for sampling contexts

2.7 eDNA extraction from filters

There are several eDNA extraction kits available for purchase in which protocols could be based on, and although this is, typically, the best option, custom column phased methods and liquid phase methods are also possibilities. The reason behind the preference for the extraction kits is the expected standardization. Furthermore, and despite the effectiveness of some of the other options, the health and safety concerns they bring along are other reasons for not choosing them. This does not mean that the commercial kits are free of health and safety concerns and still the must be handled carefully and need adequate disposal of waste, for instance, bleach is highly reactive with some components that are used in the best part of extraction kits to produce poisonous gases. This is a health risk because bleach is generally the preferred decontamination product used in laboratories, so the staff must clean with a mild detergent before using bleach.

As aforementioned, filter type, conservation method and the target may implicate some constrains in DNA extraction, so, adequate optimization, particularly in the initial phases, is in order.

The first step is to select a lysis method, considering the target species – For most animals, chemical lyses is appropriate for extracting genetic material, nonetheless, mechanical lysis is vital to disrupt the cell walls of certain unicellular groups. As mechanical lysis cannot be applied to enclosed filters inhibits their utilization for this kind of targets.

Posteriorly, lysis is affected also by the preservative liquid, for instance, if the liquid is a pure preservative, it can be discarded leaving the filter membrane. Furthermore, if ethanol was the chosen one, it should be completely evaporated in order to prevent inhibition on the later stages of the extraction. It is also possible to add a precipitation step despite it increasing costs. On the other hand, when a lysis agent was preferred, a significant amount of DNA is in solution when this phase begins and therefore, this solution cannot be discarded. The right procedure is to incubate the filter in the liquid which is used in the latter stages of the extraction. It should be considered the concentration of salt on the lysis agent, to grant compatibility between kit and lysis agent. (Bruce et al., 2021)

3. STATE OF THE ART

As the field of eDNA research experiences exponential growth, new studies are conducted regularly, leading to the continuous design and development of novel devices. Existing devices are also subject to constant upgrades and enhancements to meet evolving research needs.

The foundation for the design and assembly of the new device in this study was based on three previously established devices. These devices served as crucial starting points, providing valuable insights and lessons for the development of the new instrument.

Conversely, the final device under consideration is a more recent innovation that was not available when this project commenced. Being a state-of-the-art device, it incorporates the latest advancements in eDNA sampling technology and presents exciting possibilities for more efficient and accurate data collection.

3.1 Third Generation Environmental Sample Processor
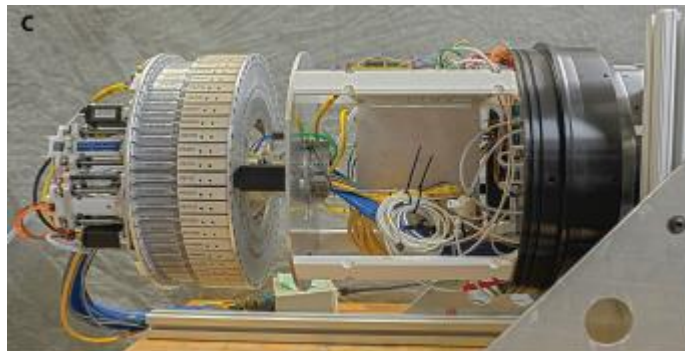
Figure 7 – 3G-ESP

The functional details of the 3rd Generation Environmental Sample Processor (3G-ESP) were documented in (Pargett et al., 2016), and a brief overview will be provided here to comprehend the key aspects to be integrated into the new device.

The 3G-ESP, seen in Figure 7 employs a system of reusable 60 cartridges, as the one portrayed in Figure 8, each containing the required filter and reagents for a sampling event. These cartridges are mounted on a toroid valve, which facilitates the selection of the designated cartridge for filtration during the sampling process. This design ensures efficient

and precise filtration while offering the advantage of reusability, making the system cost-effective and environmentally friendly, (Yamahara et al., 2019).
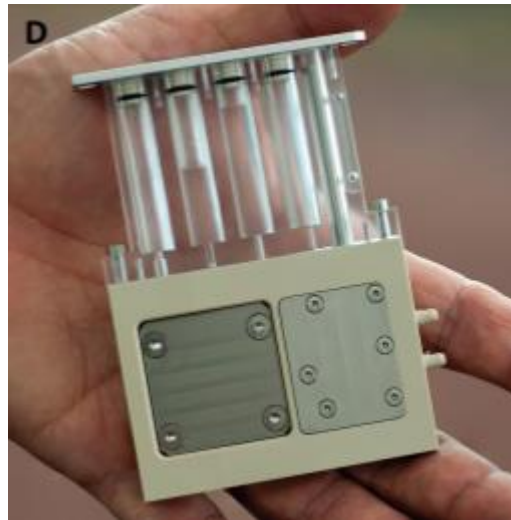
Figure 8 – One of the cartridges of the 3G-ESP

The entire system, comprising the sampling pump, cartridges, actuators, and other necessary electronics, can be compactly housed within a cylindrical space measuring $30cm$ in diameter and $60cm$ in length. This streamlined design allows for integration and assembly at the front of an autonomous underwater vehicle (AUV), as demonstrated in Figure 9, enabling efficient and autonomous underwater sampling operations.

Figure 9 – 3G-ESP mounted in an AUV

For eDNA sampling, assembled cartridges contain a filter for particulate sample collection and RNAlater for sample preservation. Before it is deployed, the AUV-ESP housing is filled with ultra-pure nitrogen gas to create an innocuous atmosphere. When a sampling

event begins, water is pumped through, to flush residual water out of the system. After flushing, a cartridge is selected, and water is directed through a cartridge for filtration. Target sample volumes are predetermined by the user. Sample collection is terminated when the target volume is reached, the filter shows signs of clogging (flow rates under $0.2\ ml/s$), or the maximum filtration time is reached. After this, cartridge syringe actuators deliver RNAlater into the filter chamber, transferring the residual liquids to a waste chamber inside the cartridge. The preservative left in contact with the sample filter remains to saturate/incubate the particulates captured on the filter. After soaking, the RNAlater is expelled from the filter chamber to waste chambers with nitrogen gas or ambient air; the processed filter remains moist but is not flooded. For a deployed AUV-ESP, cartridges remain in a nitrogen environment until the instrument is returned to the lab. Figure 10 visually represents the hydraulic system and sampling protocol.

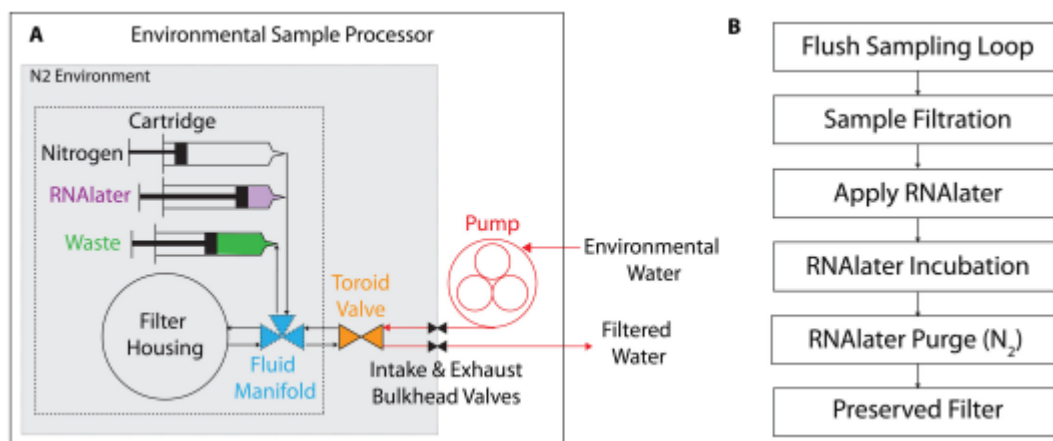*Figure 10 – A - Hydraulic system; B - Filtration flowchart*

The (3G-ESP) possesses a fascinating feature in that it collects filters instead of water samples. This design choice renders it more compact and lightweight, as it eliminates the need to store large volumes of water. However, despite its advantages, the complexity and cost associated with this device make it impractical for large-scale monitoring applications.

3.2 Remote Access Sampler

The Remote Access Sampler (RAS) is not categorized as an eDNA sampling device per se. Instead, it is primarily a water sampling device that garnered attention due to the impressive simplicity and effectiveness of its hydraulic system. A visual depiction of the device, along with its hydraulic system, is presented in Figure 11 (Honda & Watanabe, n.d.). The RAS has drawn interest for its capability to efficiently collect water samples, making it a valuable tool for various aquatic research and monitoring applications.
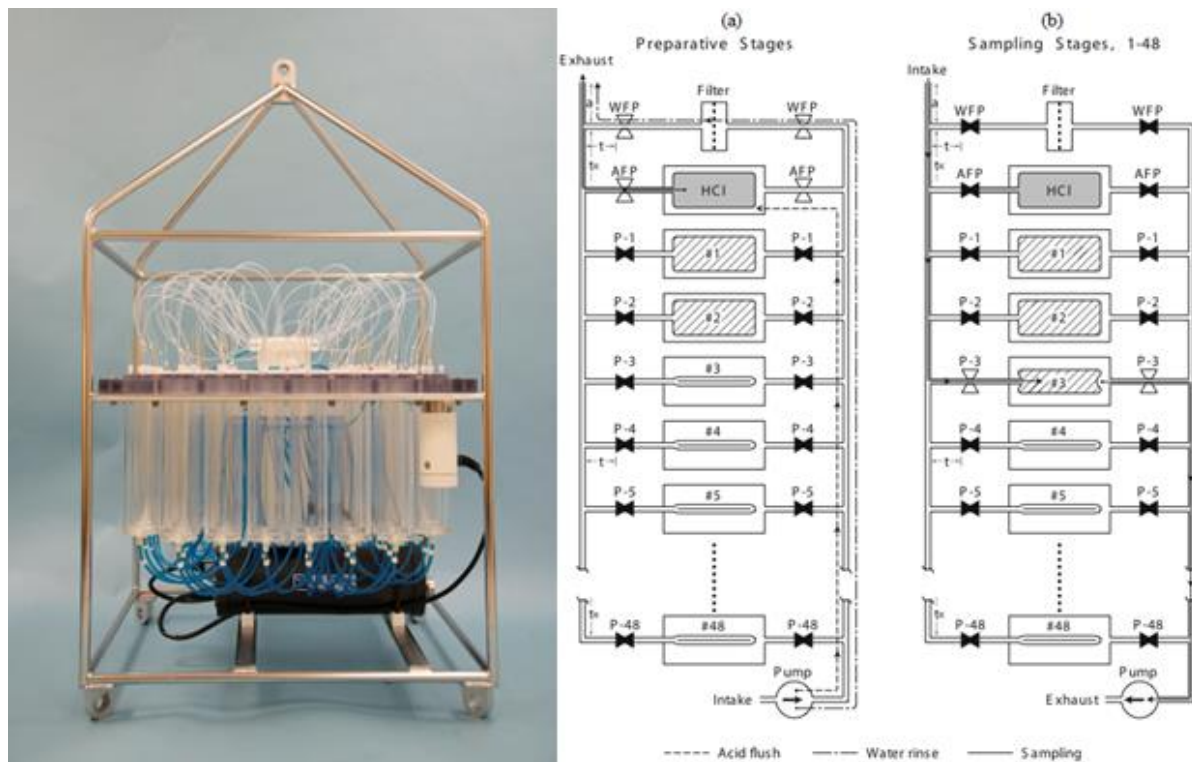
Figure 11 – The RAS and its Hydraulic system

During the preparative stage, the Remote Access Sampler (RAS) carries out two essential functions to prevent contamination effectively. Firstly, through the acid flush path (AFP), a pair of AFP valves opens, and a pump applies pressure to a sample bag containing hydrochloric acid (HCl). This HCl flushes the intake manifold "a," after which the AFP valves close to isolate the HCl bag. Subsequently, the Water Flush Path (WFP) valves open, allowing filtered in situ seawater to be pumped through, rinsing out any remaining HCl from "a." Once rinsing is complete, the WFP valves close, and the RAS is now ready for water sampling through the sampling path.

During the water sampling process, a microprocessor commands the opening of twin valves on the next empty bag, which is pre-loaded with a preservative. The pump runs in reverse, drawing in a programmed volume of in situ seawater into the bag. Once the sampling is complete, the bag is isolated for preservation by closing corresponding pair "P" valves until the next programmed cycle, which initiates the HCl flushing process again. In an actual RAS setup, all valves are arranged along two circumferences, facilitating the efficient and precise execution of these stages (Honda & Watanabe, n.d.).

This careful and systematic process in the preparative stage of the RAS ensures accurate and uncontaminated water sampling, enabling reliable data collection for various research and monitoring purposes.

The RAS holds potential for eDNA research if a method of preserving genetic material from the water it captures can be incorporated. While freezing water samples is a theoretically viable preservation technique, implementing such an alteration to the device would significantly increase its weight and cost. This modification may render the RAS impractical for its intended purposes as a lightweight and cost-effective water sampling tool.

3.3 Advanced Natural Environment Monitoring Equipment-11

Once again, it's worth noting that the Advanced Natural Environment MONitoring Equipment-11 (ANEMONE-11) is not an eDNA sampler; rather, it serves as a water sampler.

This device is equipped with a pump unit consisting of 4 piezoelectric pumps operating in parallel, collectively providing a flow rate of $40ml/min$. Additionally, there are 4 valve units, each connected to 4 sets of 32 sampling cylinders. Within each valve unit, 32 electromagnetic valves direct approximately $40ml$ of in situ water to one of the $50cm$ long cylinders with a $1cm$ diameter.

The battery unit features either 9 or 18 cells of lithium batteries, supplying the required $24V$ and $100mA$ power for the ANEMONE-11's operation. With 9 cells, the device can operate continuously for 12 hours.

Figure 12 displays the ANEMONE-11, including the pump unit, valve units, control unit, and battery unit. This water sampling equipment is well-equipped to efficiently collect water samples, making it a valuable tool for various aquatic research and monitoring applications.
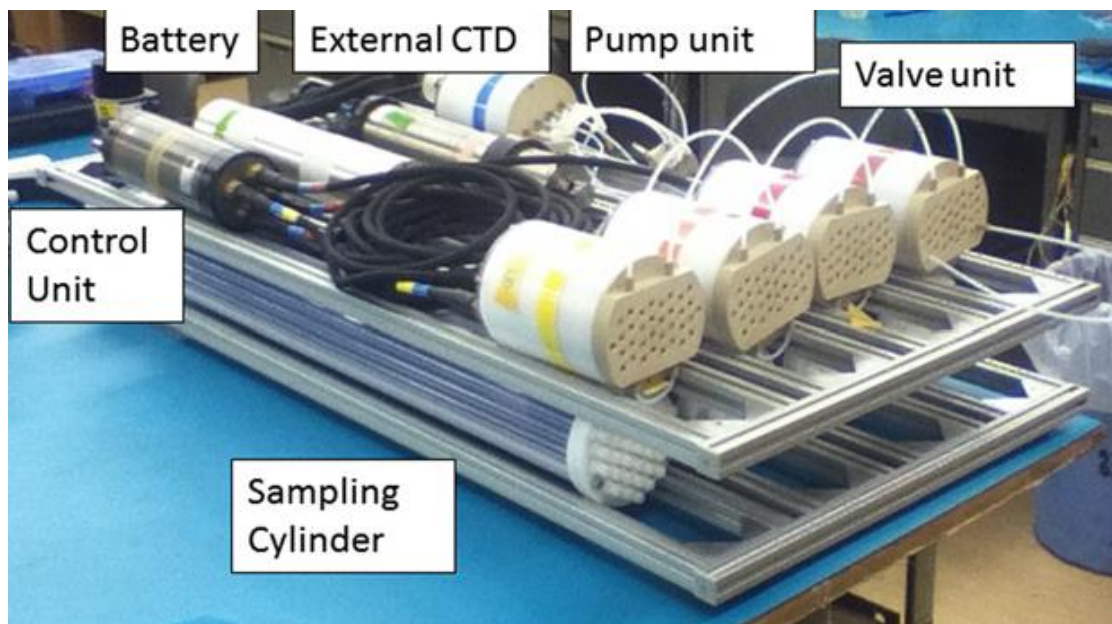
Figure 12 – ANEMONE-11

The control unit of the Advanced Natural Environment MONitoring Equipment-11 (ANEMONE-11) plays a crucial role in the sampling process. It is responsible for selecting which valve should be opened and effectively controls the piezoelectric pump, considering the parameters dictated by the Recommended Standard (RS-232) transmission.

To ensure precise sampling, the ANEMONE-11 is equipped with a pressure sensor that continuously monitors the depth of the water body. Once the predefined depth is reached, the control unit initiates the sampling operation, enabling the device to carry out its water sampling functions accurately.

Figure 13 presents the schematic diagram of the ANEMONE-11 equipment, offering a visual representation of its essential components and their interconnected workings (Okamura et al., 2013).
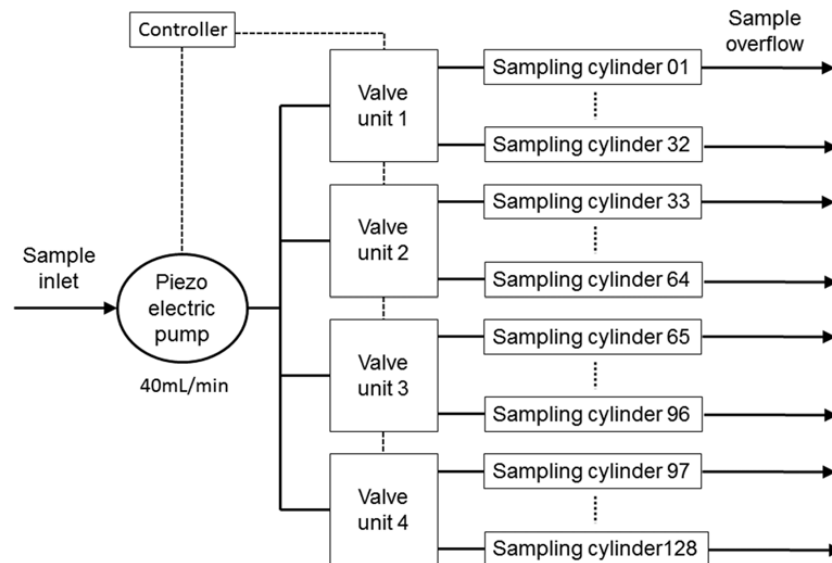
Figure 13 – Schematic diagram of the ANEMONE-11

This device is interesting because, to ensure utmost precision and prevent contamination, the outlet of each cylinder is equipped with a 20cm Teflon tubing. This tubing serves as a protective measure, safeguarding the collected samples from any potential contamination during the sampling process.

By incorporating this specialized valve and Teflon tubing setup, the device maintains the integrity and purity of the collected samples, making it a reliable tool for accurate water sampling in various environmental monitoring and research applications.

3.4 Dartmouth Ocean Technologies eDNA sampler

The Dartmouth Ocean Technologies (DOT) eDNA sampler was introduced at the end of March 2023, and it holds the potential to revolutionize eDNA sampling devices. While currently, its cost may limit its widespread adoption as a global monitoring tool, it is anticipated that with time, advancements in technology will lead to the development of new, simpler, and more cost-effective devices with similar capabilities.

The introduction of the DOT eDNA sampler, presented in Figure 14 and 15 (Hendricks et al., 2023), signifies a significant step forward in the field of eDNA research, showcasing the continuous progress and innovation in environmental monitoring technologies. As technology evolves and becomes more accessible, it is likely that eDNA sampling will become more widely employed, contributing to a greater understanding of aquatic ecosystems and biodiversity.
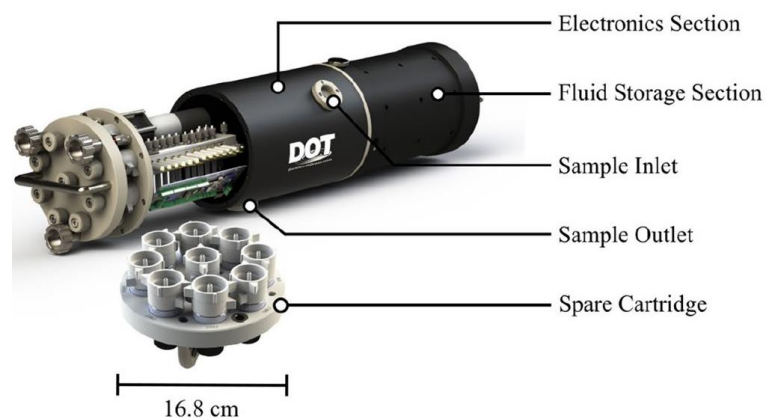
Figure 14 – DOT eDNA sampler

Weighing $11.3Kg$ (on air and $3.3Kg$ on salt water), sizing only $72.1cm$ in length and $16.8cm$ of diameter, the sampler is easily portable. Moreover, the fact that it can clean itself between sampling events and preserve the sample can only increase the value of this device. The standard version has 9 filter holders with $25mm$ in diameter. The filter membrane material and pore size can be chosen according to the target species.

9 Discrete Filters

Circuit Board
(Control and Logging)

Solenoid Valve Tree

Syringe Pump

In-Line Filter

Fluid Bags

Fluorometer (Optional)
PCB
LEDs
Lens
Filters
Acrylic Faceplate

72.1 cm
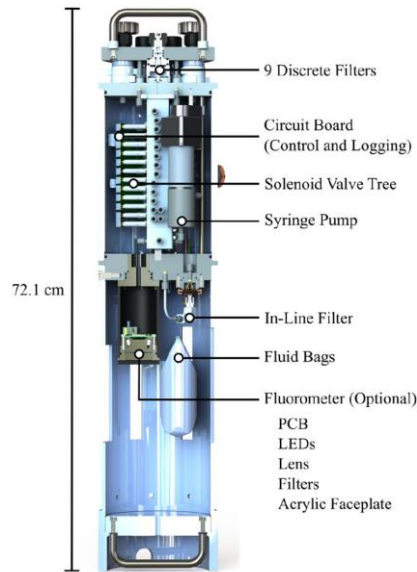
Figure 15 – Cut view of the device.

The filter cartridge, constructed from Polyetheretherketone, serves as the container for all filter holders. Once the filters are loaded into the cartridge, it can be easily attached to the electronics section of the device.

The electronics section comprises several key components, including a pump, a custom valve tree, and a printed circuit board responsible for automation and data logging. The valve tree functions as a fluid routing manifold, incorporating a pressure sensor, tubing inter-connections, and solenoid valves. Additionally, the valve tree is equipped with ports to connect to both the filter cartridge and fluid section. These detachable connections allow for convenient replenishment of filters and fluids during the sampling process.

The fluid storage section is dedicated to housing all the essential fluids required for various purposes, such as cleaning, sample preservation, rinsing, and waste management. This well-designed fluid storage arrangement ensures smooth and efficient operations, facilitating precise water sampling and maintaining the integrity of collected samples throughout the entire sampling process. In Figure 16 (Hendricks et al., 2023), the hydraulic system of the equipment is depicted, showcasing the integration of control scripts crucial for the coordinated operation between solenoid valves and the syringe pump.
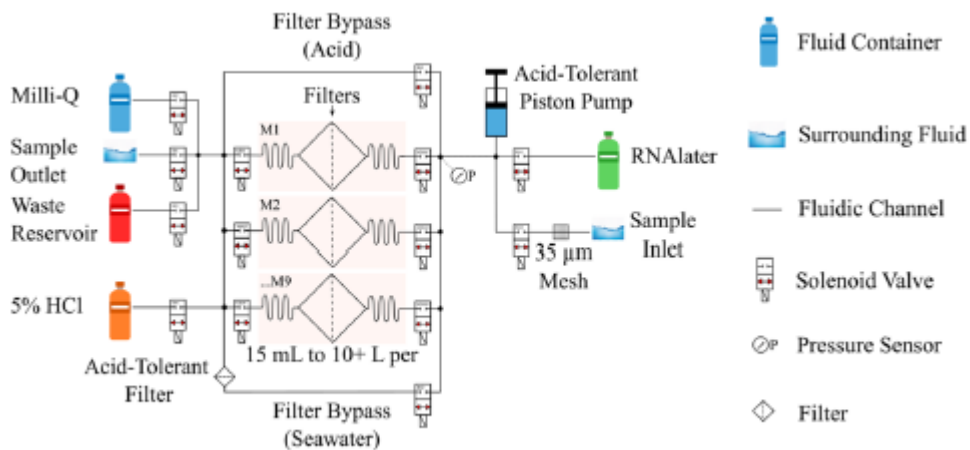
Figure 16 – Hydraulic system of the DOT eDNA sampler

The custom script plays a pivotal role in executing the sampling protocol, which defines essential parameters, including the number of active valves, collection volume, time limit, and minimum flow rate for each step of the sampling process. The sampling protocol, illustrated in Figure 17 (Hendricks et al., 2023) (a), offers a detailed outline of the sequential steps involved in the sampling procedure, ensuring precision and efficiency in water sample collection.
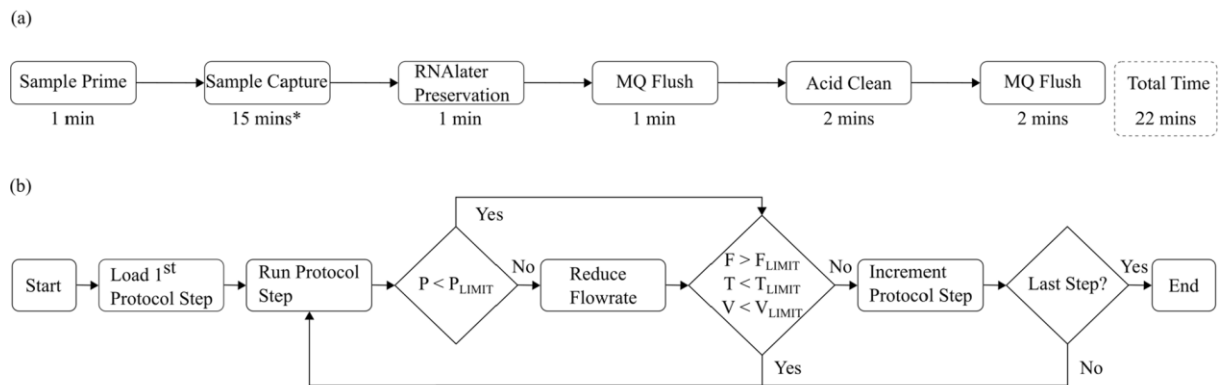
Figure 17 – (a) – Sampling protocol; (b) – Thresholds flowchart

Below each step, the estimated completion time is shown. The sampling protocol starts with the Sample Prime step, that prepares the sampler by flushing its internal channels with the intended sample fluid. Thereafter, the Sample Capture step pushes the sample fluid through the selected filter membrane for sample capture. To preserve the material collected on the filter, the RNAlater Preservation step pushes RNAlater through the selected filter

membrane. The MQ Flush step then uses Milli-Q filtered water to flush the RNAlater from the system to avoid it being in contact with 5% HCl to be used in the Acid Clean step, that cleans the internal fluidic channels of contaminant, by using 5% HCl. Next, the MQ Flush step flushes the 5% HCl from the channels using Milli-Q. This process cleans the sampler and prepares it for the next sample capture. The protocol also pushes $9ml$ of Milli-Q through the sample inlet to flush the lines and inlet of acid. This will force the dilute 5% HCl further away from the sample inlet and permit convective flow to remove localized acid before the next sampling event. The algorithm shown in Figure 17 (b) is executed whenever sample capture is triggered. This algorithm runs the steps shown in the sampling protocol and monitors volume, pressure, and time to ensure that the sampler stays in a tolerable running condition and this procedure repeats until there are no steps left in the sampling protocol. The sampler then enters a low-power state and waits for an interrupt to trigger the sampling protocol once more.

The eDNA sampler's system architecture is shown in Figure 18 (Hendricks et al., 2023).
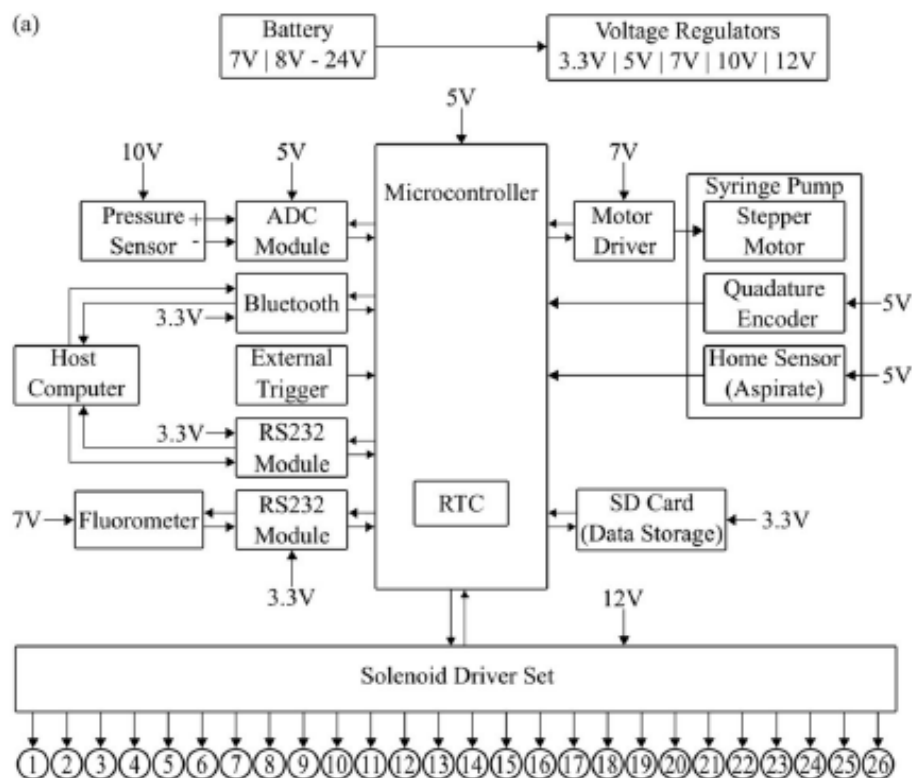
Figure 18 – Schematic of electronic system of the DOT eDNA sampler

Due to the varied electrical requirements of the components, the system features several regulators for generating multiple voltages ranging from $3.3V$ to $12V$ Direct Current (DC), all sourced by a battery or power supply input of $7V$ to $24V$ DC. The wide range of voltage input

allows for flexibility in the platforms used for deployment. The single microcontroller manages the syringe pump, data logging, communication, and protocol execution. The syringe pump is powered with a stepper-motor driver circuit along with an optical quadrature encoder to aid the precise tracking of the volume used. The 26 solenoid valves used by the system are driven by several motor drivers that allow the powering of the 32 valves without current overload. The eDNA sampler makes use of a 16–bit analog-to-digital (ADC) module that can read the pressure sensor in a Wheatstone bridge configuration with its built-in programmable gain amplifier. Users interface with the eDNA sampler through either Bluetooth (via a smartphone application) and/or through RS-232 and a personal computer terminal. These both permit operational commands to be sent to the sampler and are also conduits for transferring data to/from the system like setting scheduled sampling times via the real-time clock and/or for retrieving pressure and fluorometer data per membrane/sample. At idle the system draws $1W$ and $10W$ peak while sampling. All these components are powered by a battery. The battery can support a maximum of $33.7l$ of pumping, assuming no blockage of the membranes. Beyond battery or power consumption, fluids are currently the limiting factor for continual use as the standard reagent reservoirs are good for 1 filter cartridge (9 filter captures), with plans to have an enhanced fluid capacity version that would have to support 3 filter cartridges (27 filter captures). The eDNA sampler can also be powered from a typical $7V$ to $24V$ DC supply. Given the low power consumption of the device, it is highly flexible to the hotel load available on most autonomous vehicles and solar powered buoys or installations (Hendricks et al., 2023).

4. PROJECT DEVELOPMENT

As previously mentioned, the objective of this work is to design and assemble a semi-automatic device capable of conducting eDNA sampling in deep seawater. After extensive study and discussions on the state of the art, it was determined that the most practical and efficient approach would involve collecting eDNA samples directly onto a filter, rather than traditional water sampling methods. This necessitates a device design that allows for easy filter substitution and ensures the preservation of each filter's integrity, so that they remain undamaged and uncontaminated until analysis.

To achieve this, an electronically controlled valve system was deemed the most suitable solution, where the valves open and close based on data from a pressure sensor. In combination with an aquarium pump, this configuration guarantees a fast and stable filtration rate when the path to a filter is open. A battery provides the necessary power to operate all these components effectively.

Considering the various elements involved, the device can hold multiple filters, denoted as "N" in the hydraulic system aspect. Although the sampler is designed to accommodate four filters in this work, the concept of N filters is essential in the hydraulic system's discussion. However, specific material limitations and enhancements in the electronic hardware segment made it impractical to discuss N filters, which will be elaborated on in the respective section.

In summary, the critical components of this device are the hydraulic system, electronic hardware, and software. The goal is to create an instrument that allows users to utilize as many filters as needed, ensuring efficient and accurate eDNA sampling for comprehensive environmental monitoring and research applications.

4.1 Hydraulic

The most important aspects in the planning of the hydraulic system were to design it in a way that the filters are isolated from water until, and after they are used to avoid contamination, and, because the goal is for the device to be submerged, air inside it is something to be avoided as much as possible in order mitigate implosions or any other damage caused by the pressure.

The design of the previously described RAS (Figure 11) was the base of the hydraulic flowchart for the new equipment, presented on Figure 19.
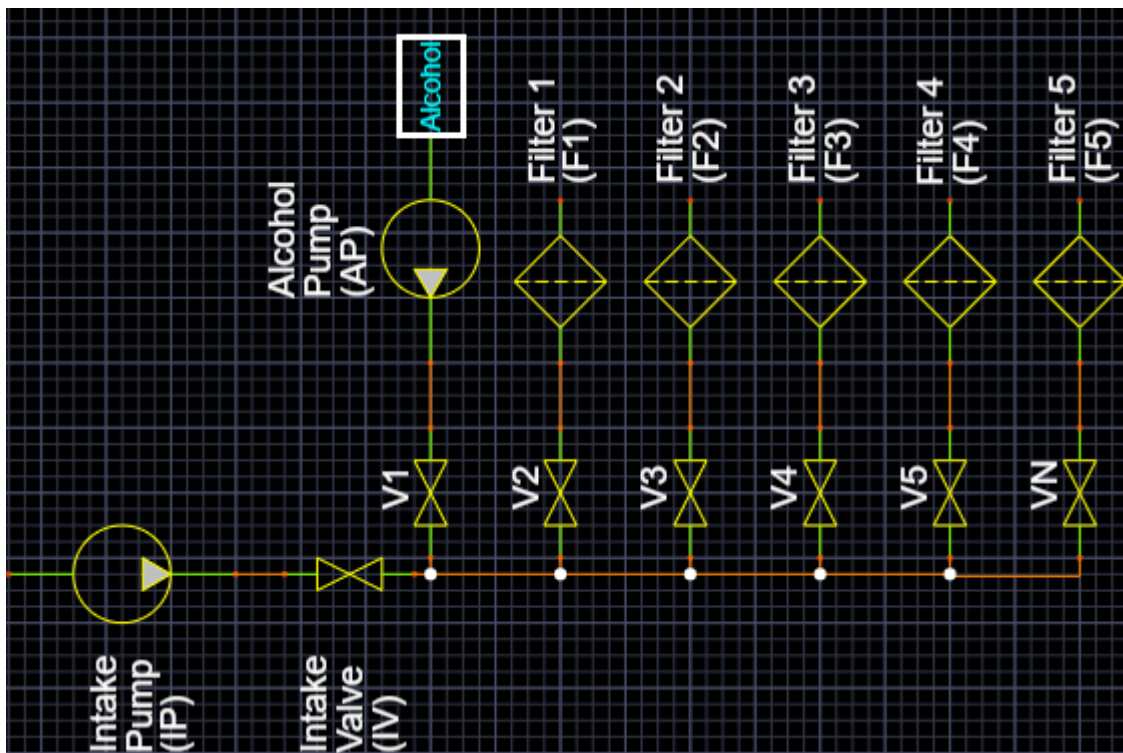
Figure 19 – Designed hydraulic system.

The instrument incorporates N+1 Female(F)/F electro valves with half an inch diameter inlet and outlet (1/2'') to direct water flow. Two pumps are utilized to enhance fluid flow rate. The setup comprises N tee (F/Male(M)/F) 1/2'' connections, one 1/2'' F/M elbow connection, N+1 1/2'' M/M connections, and two (1/2'')/(0.3'') M/M connections, which interconnect all valves and pumps. Additionally, N sets, each consisting of 1 (1/2'')/(3/4'') M/M connection, 1 (3/4'')/(1'') F/M connection, 1 1'' F/F connection, 1 (1'')/(1/2'') connection, 1 (1/2'')/(3/8'') M/F connection, and 1 (1/2'')/(0.3'') M/M connection, create a housing for the filters and establish connections between the housing and the valves. The device employs approximately 1 meter

of 0.3" rubber hose to link the pumps to the remaining hydraulic components and extend the distance between the filters and the water.

The alcohol compartment is designed as a balloon, motivated by two key reasons. Firstly, the detachable balloon allows for easy replacement, facilitating alcohol refilling as needed. Secondly, as the alcohol volume decreases, the balloon's size adjusts accordingly, preventing pressure differences and potential damages resulting from such variations.

Notably, this design features only one valve for each filter, in contrast to the two valves in the RAS. This might appear as a limitation, but two essential factors ensure compliance with the mentioned conditions. The critical aspect lies in the requirement that the device be filled with distilled water before the sampling process. By doing so, the existing air in the device is minimized, leaving only the air inside the electronic components. Furthermore, the filters will not be contaminated by contact with water from other depths. This is due to the absence of pressure difference between the interior of the device and the external environment, effectively making the flow of water negligible and preventing any unintended mixing of water from different depths.

This design approach showcases a well-thought-out and practical solution to optimize the hydraulic setup of the instrument. By carefully considering the use of various connectors, pumps, hoses, and the unique alcohol compartment, the device can efficiently and accurately perform its intended functions. The emphasis on using distilled water before sampling ensures data accuracy and eliminates the risk of cross-contamination between samples from different depths. This well-engineered hydraulic design, shown in Figure 20, contributes to the instrument's reliability and suitability for its designated underwater sampling applications.
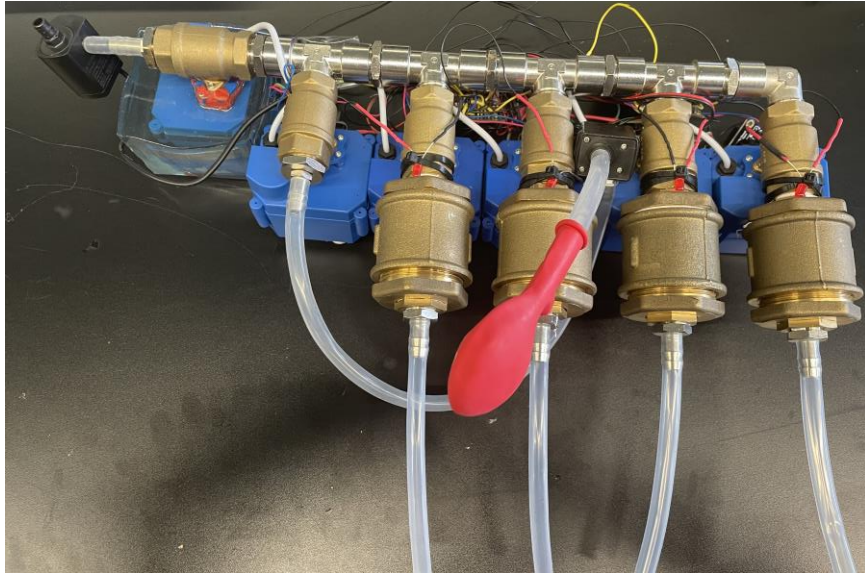
Figure 20 – Assembled hydraulic system

All junctions in the device were connected and isolated to ensure optimal performance and prevent any potential leakage or contamination. To achieve this, Teflon tape was employed extensively during the assembly process. This approach not only aids to ensure a secure and watertight system but also contributes to the overall efficiency and accuracy of the device during eDNA sampling in deep seawater.

4.2 Electronic circuit / hardware

Considering the hydraulic system, it was essential to ensure electronic control of the valves and pump, activating them only when the device reaches predetermined depth ranges and deactivating them thereafter. This requirement called for the incorporation of a microcontroller and a pressure sensor into the system. Additionally, to enable autonomous operation in water, the inclusion of a battery was deemed necessary for power supply.

Considering the available resources for the project and product options, 12V electro-valves and pumps were selected for integration into the device. Consequently, the battery needed to supply 12V to ensure their proper functioning. However, the microcontroller available for the project was incapable of supplying the required current or the 12V voltage, necessitating the incorporation of a driver. This introduced the requirement for a power manager to handle the two different voltages required for the multiple electronic components. Additionally, the power manager would also be responsible for managing the battery charging process.

To facilitate easier control of each electro valve, a relay was added to the circuit for every valve in the device. These relays required a supply of 5V from the microcontroller to toggle effectively.

Considering that the device would operate at various depths depending on the user's intentions, there was a possibility of the device not reaching specific depth ranges, rendering some filters unused. To address this, a Light Emitting Diode (LED) was included for each filter holder, serving as an indicator when the corresponding filter was used. This LED illumination provided valuable feedback to the user, aiding in data interpretation and device monitoring.

Furthermore, to facilitate user input of the depth ranges for sampling, a serial communication accessory was essential. In this case, a universal serial bus (USB) to transistor-transistor logic (TTL) cable was incorporated to enable seamless communication with the device, simplifying the configuration process.

In summary, the system operates in accordance with the configuration depicted in Figure 21.
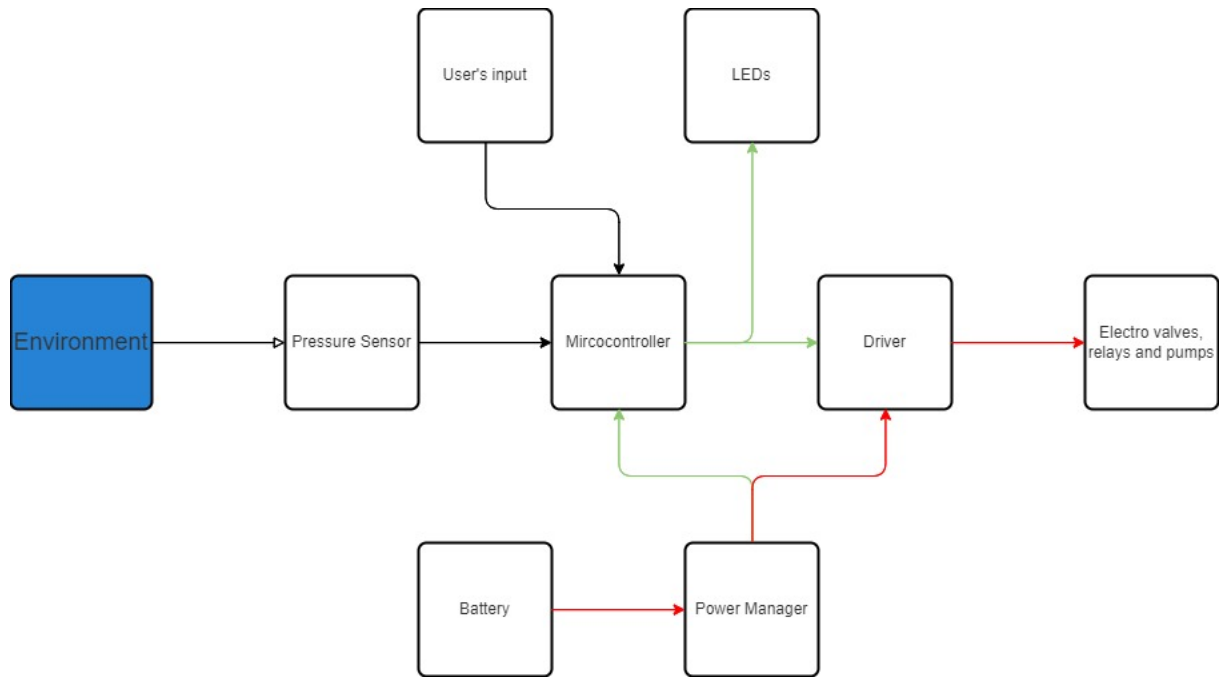
Figure 21 – Block diagram of the electronic system

The operation of the pumps is straightforward, as they merely necessitate a 12V potential difference between their terminals to function efficiently. Figure 22 illustrates the schematic diagram of a switch-controlled pump. The pump initiates its operation solely when the switch is closed, allowing the electric current to flow through and activate the pump mechanism.
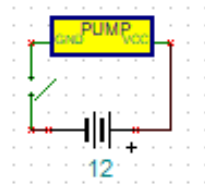
Figure 22 – Schematic diagram of a switch-controlled pump

The functioning of the valves is controlled by a 12V potential difference between specific wire combinations. When there is a 12V difference between the red and yellow wires, the valves open, allowing the flow of fluid. Conversely, the valves close when a 12V difference exists between the blue and yellow wires.

Figure 23 presents the schematic diagram of a switch-controlled valve, demonstrating the electrical connections responsible for regulating the valve's opening and closing mechanisms.
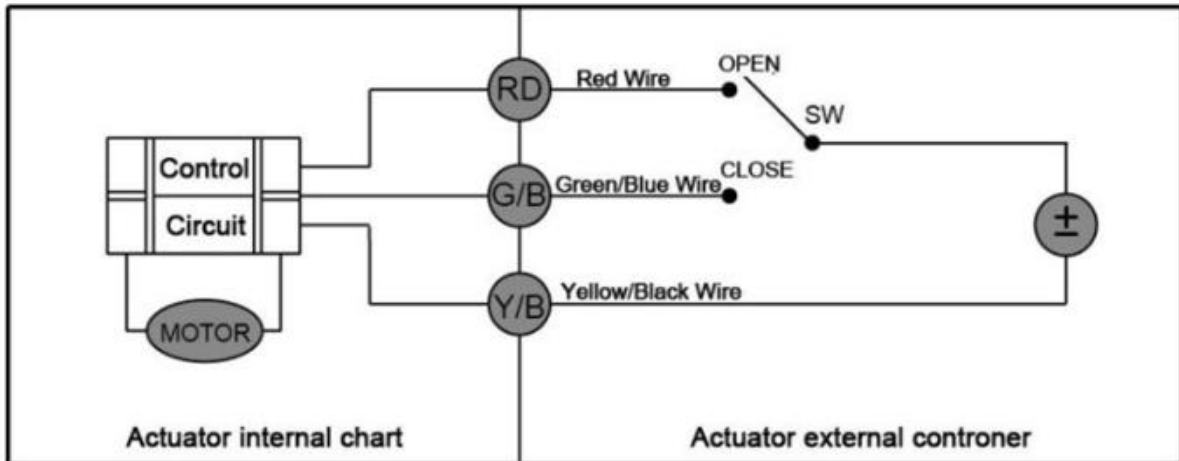
Figure 23 – Schematic diagram of a switch-controlled valve

The relay serves as a switch, akin to the one depicted in the previous image, responsible for toggling the 12V potential between the normally closed (NC) and normally open (NO) contacts, contingent on the voltage difference between the green and black wires. When 12V is applied in RD wire, valve opens, and when voltage is applied in the G/B wire, valve closes. For the sake of simplicity, the relays were interconnected in a manner that would open the valves when they were turned on and close the valves when they were turned off.

Figure 24 presents the schematic diagram of a switch-controlled set comprising a valve and relay. When the switch in the figure is in the open position, the internal switch of the relay connects the 12V from the power source to the NC pin, since there is no 5V difference between the black and green wires. However, upon closing the switch, the relay promptly switches to the NO pin, thereby opening the valve. The valve will remain open until the 5V difference between the green and black wires dissipates.
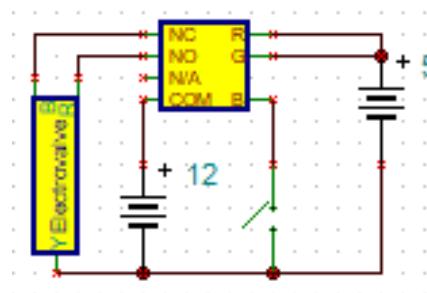
Figure 24 – Schematic diagram of a switch-controlled set of valve and relay

For the integration of the pressure sensor into the present project, the typical application circuit, as outlined in the datasheet available on the internet, was followed as a

reference. Figure 25 illustrates the schematic of the typical application circuit for the pressure sensor.

In our implementation, to match the available resistors and voltage source, the 10KΩ resistors specified in the typical circuit were substituted with 9.1KΩ resistors. Additionally, the 3V source was replaced with a 3.3V source, as the microcontroller utilized in the project operates on a 3.3V source instead of a 3V source.

It is important to note that this minor deviation in resistor values and voltage source does not impact the accuracy or reliability of the pressure sensor's readings. The chosen resistors and voltage source closely approximate the specified values, ensuring the sensor's effective performance in monitoring and measuring pressure.

By implementing this modified application circuit for the pressure sensor, the project ensures the precise and dependable acquisition of pressure data, a crucial component in the hydraulic system's functionality. The adjusted circuit configuration seamlessly integrates the pressure sensor with the microcontroller, contributing to the device's overall efficiency and accurate data collection capabilities.
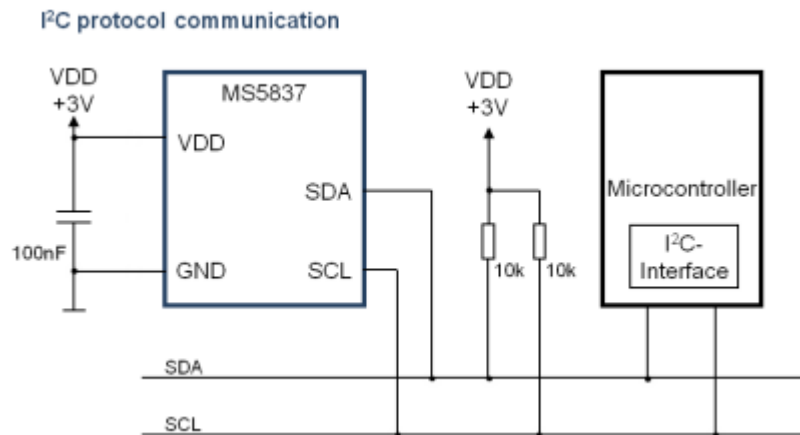
Figure 25 – Schematic of the typical application circuit of the pressure sensor

The driver plays a vital role in bridging the gap between the components that require 12V to operate, the microcontroller, and the 12V power supply. Figure 26 illustrates the schematic diagram depicting the effect of the driver in a switch-controlled pump. For this purpose, a Darlington transistor array was chosen as the driver, reference ULN2803A.

This component functions as a switch between the respective component and the ground (GND) connection. In the scenario depicted in the figure, when the switch is closed, the pump initiates its operation since the connection to GND is reestablished. The

microcontroller acts as the controller of this switch, executing the necessary actions to activate or deactivate the pump.

By utilizing the Darlington transistor array as the driver, the device can efficiently manage the flow of current to the components requiring 12V, under the control of the microcontroller. This driver serves as an essential interface, ensuring seamless communication and control between the microcontroller and the various components, thus facilitating the smooth functioning of the hydraulic system.
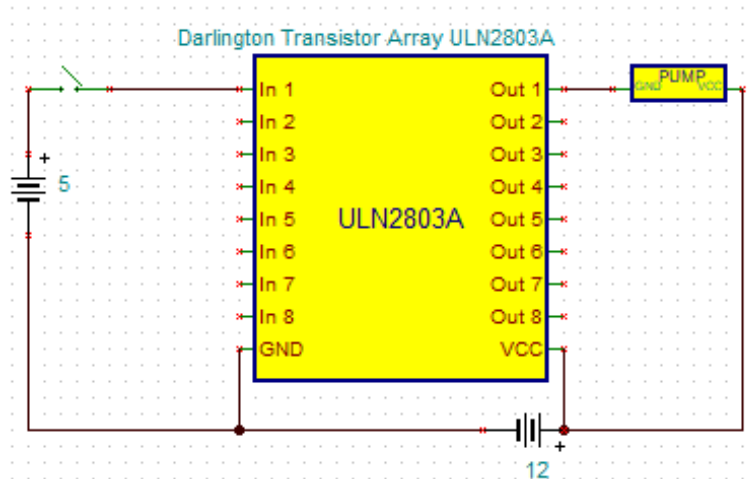
Figure 26 – Schematic diagram of the effect of the driver in a switch-controlled pump

Adding all the components together, the schematic in Figure 27 is obtained.
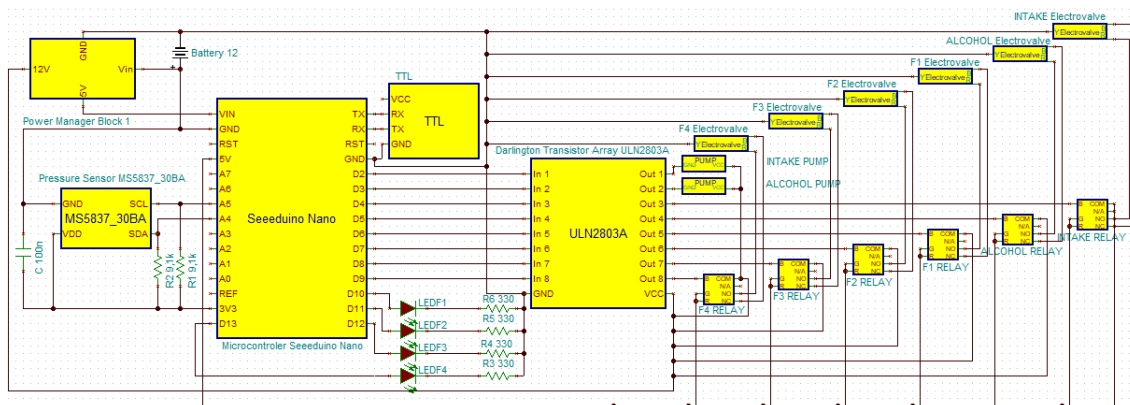
Figure 27 – Schematic of the device

The device features 1 microcontroller (seeeduino nano), 1 pressure sensor (MS5837_30BA), accompanied by 2 $9.1K\Omega$ resistors and a $100nF$ capacitor, 1 8 Darlington channels driver (ULN2803A), 2 pumps, 6 sets of relay and electro-valve, 4 red LEDs, each of them accompanied by a 330Ω resistor, 1 TTL to USB converter cable, 1 $12V, 2.4AH$ battery and 1 load manager.

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The fact that the seeeduino nano only has 22 pins where it can be connected an electro-valve, limits the number of possible filters to 14 because 2 of them are occupied by the pressure sensor, another 2 by the pumps, 2 more for the serial communication and 2 of the electro-valves are not directly connected to a filter.

In this configuration, the LEDs addition also reduces the maximum number of filters to a half, because there is a LED for each filter holder and, because of the blinking function, they cannot be connected in parallel with the valves.

In the light of this information, the LED addition, or at least, the blinking function, can be seen as superfluous, for a large-scale optimized device, but, in this stage, it was immensely useful because it allowed for checking the functionality of the instrument during tests.

The TTL block in figure interfaces the microcontroller with the user, using a serial port connection.

The power consumption is heavily influenced by the pumps, that can reach a theoretical peak $350mA$ each or $4.2W$. The electro valves can reach a peak $2W$, however, the only occasion in which they consume any power is in the change of state, meaning that, this value only has an effect for the about eight seconds that the valves take to fully open or close, making the power consumption practically irrelevant. The relays reach a peak $0.35W$ and are only on when the respective valve is supposed to be open. Finally, the LED and resistor set consumes around $10mA$ or $0.05W$ each, and the remaining components consume even less than the that.

Considering all of this, the most efficient way to tell the autonomy of this device is in litres pumped. To be able to calculate the autonomy of the battery, its energy must be calculated first:

$$P = VI; \; E = P\Delta t \rightarrow E = VI\Delta t$$
$$E_{battery} = 12V \times 2.4Ah = 28.8Wh$$

This means that the battery would have an autonomy of 1 hour if the load power was rated at $28.8W$. Bearing in mind that the pumps can pump a maximum $240l/h$ and that at least 2 valves must be opened (and therefore, the corresponding relays must be on) for the pump to be able to make water flow, we have:

$$Autonomy_{pumping} = \frac{E_{battery}}{P_{pump} + 2P_{relay}} = \frac{28.8Wh}{4.2W + 2 \times 0.35W} = 5.82h = 5h49min$$

$$Autonomy_{in\ litres} = 5.82h \times 240l/h = 1396.36l$$

Of course, these calculations are made with theoretical values, in perfect conditions, and with no filter obstructing the flow of water, so, even though the pump automatically regulates its power consumption depending on the flowrate it can provide, it is expected that this autonomy drops considerably in the real world.

4.3 Software

The software for the device is a collection of carefully crafted instructions and commands that enable the device to carry out its intended functions effectively.

The flowchart presented in Figure 28 provides an insightful overview of the software's logic and operation, allowing for a clear explanation of its functioning without delving into the actual code, present in the appendix.
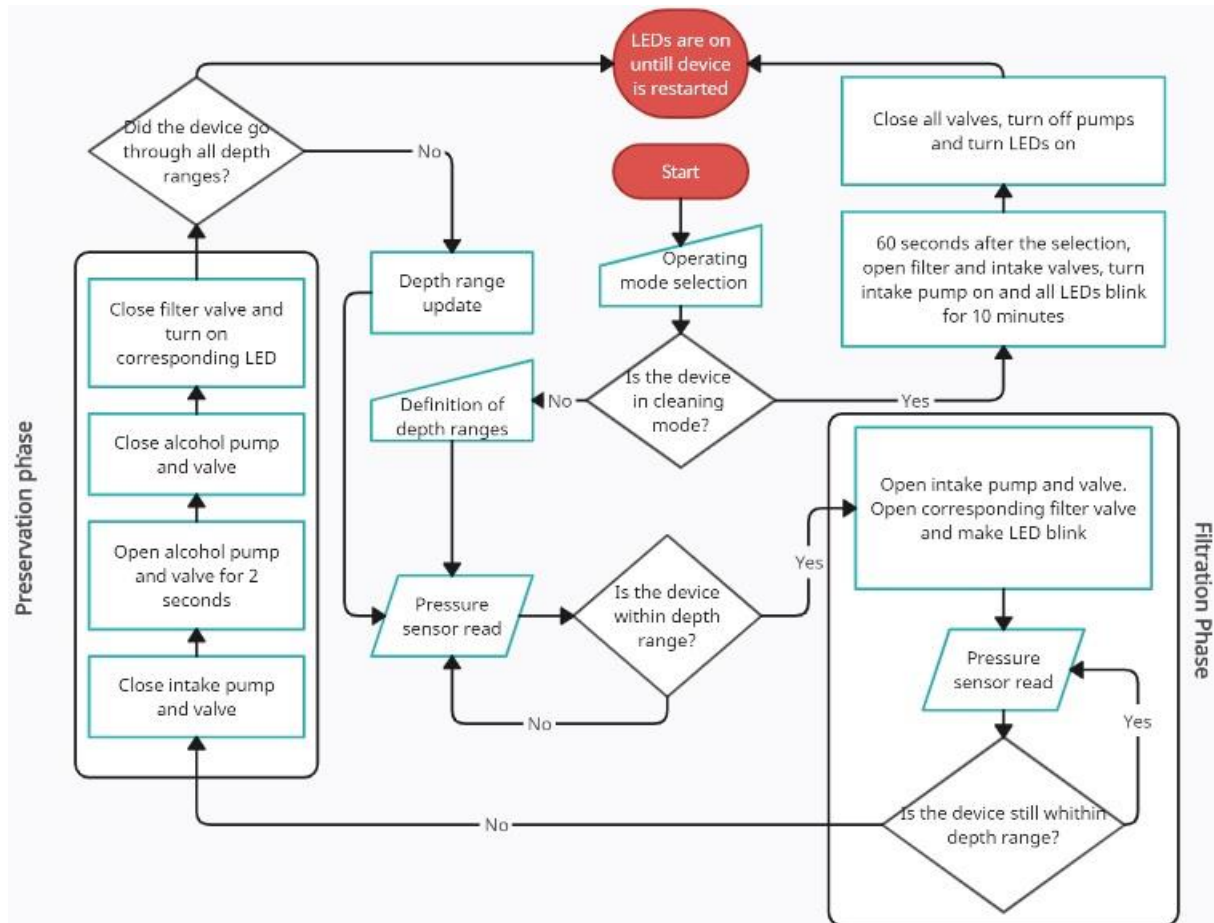
Figure 28 – Flowchart of the device's software

As illustrated in the preceding image, the device initiates its operation by prompting the user, transmitting a message through serial port, to select the desired mode: cleaning or sampling.

In the cleaning mode, upon user selection, the device enters a 60-second waiting period to allow the user to disconnect the serial port, close the tap, and submerge the device in a container filled with decontaminating liquid, such as bleach. After this preparatory interval, the valves leading to the filter holders and the intake valve and pump are activated.

Each filter holder is equipped with an LED that blinks while its corresponding valve remains open. The pump and valves operate for a duration of 10 minutes, after which the pump is deactivated, and all valves are sequentially closed. As each valve is closed, its corresponding LED stops blinking and remains steadily lit. Notably, the alcohol valve and pump remain inactive in this mode since no liquids other than alcohol flow through these components, eliminating the need for decontamination. The continued illumination of the LEDs post-cleaning signifies that the filter holders are now decontaminated.

Alternatively, in the sampling mode, upon user selection, the device requests specific information regarding the depth ranges for sampling. Once the depth ranges are specified, the sampler commences periodic checks to determine if it has reached the next most superficial range as per the user's specifications. This is accomplished using a 2-command cycle that begins with reading the current depth and subsequently verifying if it falls within the desired range. Once the target depth range is reached, the filtration process initiates.

The filtration process involves a new 3-command sequence. It begins by activating the corresponding filter valve, intake valve, and pump, which in turn triggers the blinking of the relevant LED. Simultaneously, the device continues to monitor its depth using the 2-command cycle mentioned earlier. If it is found that the depth no longer falls within the desired range, the filtration process is terminated, and the preservation process commences.

The preservation process consists of a 4-step set of instructions. It starts by closing the intake pump and valve. Subsequently, the alcohol valve and pump are briefly activated for a period of 2 seconds, following which both the alcohol valve and pump are turned off, and the filter valve is closed. The LED corresponding to the preserved filter remains steadily lit, indicating that the filter has been utilized and preserved for future extraction.

Upon completing the preservation stage, the device evaluates if all pre-defined depth ranges have been filtered. If any range remains unfiltered, the device automatically updates the range to the next most superficial one and repeats the process of checking for the new range. However, if the device has successfully filtered all the pre-defined ranges, it enters a standby mode, awaiting user input to restart the operation. Importantly, the device can be interrupted at any time with an input, provided it is not actively engaged in the filtration or preservation processes.

4.4 Other materials and methods

As demonstrated in the previous sections, the device incorporates a wide range of essential elements and components, each playing a significant role in its overall performance. However, there are certain critical elements that have not yet been discussed but are equally vital to the functionality of the device. This segment aims to shed light on these unmentioned elements and elucidate their integration into the device, along with their crucial importance. Among these pivotal components is the Veroboard, illustrated in Figure 29.
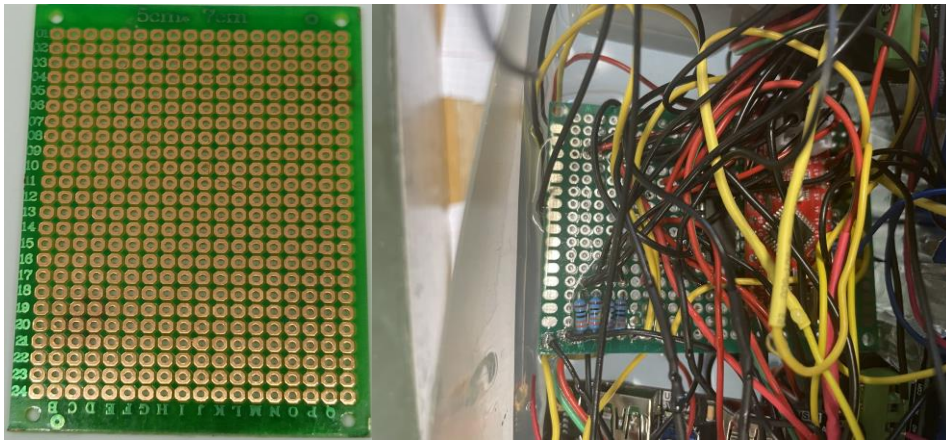
Figure 29 – Veroboard in the device

The integration of the Veroboard in the device's architecture proved to be instrumental in facilitating the assembly and organization of electronic components. Its versatility, reliability, and space-efficient design are key factors contributing to the overall performance and functionality of the device. As a result, the Veroboard stands as an indispensable element that empowers the successful operation of the device's electronic circuitry, elevating its capabilities and reinforcing its efficacy in the intended applications.

Another critical element in the construction of the device is the epoxy resin and hardener, as depicted in Figure 30.

Figure 30 – Epoxy resin and Hardener

Epoxy resin (A) is a liquid plastic compound that, when combined with the hardener (B) in a weight proportion of 10:3, undergoes a chemical reaction and transforms into a solid coating. This epoxy resin plays a pivotal role in enabling the device to function underwater with minimal concern about potential damage to its electrical components.

Before encapsulating the device in resin, a preliminary test was conducted on one valve and relay, both yielding successful results. For one of the electro-valves, approximately 4 layers of resin were applied, with each layer comprising $65g$ (50A + 15B) of the mixture. This test was undertaken to ensure that the valve could withstand the heat and pressure generated during the hardening process of the epoxy resin. Similarly, the relay was tested with only 2 layers of resin (10A + 3B), adequately covering the most delicate contacts, which were of particular concern.

Given that epoxy is an excellent electronic insulator, meticulous attention was given to achieving the best possible contacts between the electronic elements. It was crucial to ensure optimal connectivity before applying the resin, as any subsequent adjustments would become impractical once the resin had hardened.

The remaining portion of the device was then immersed in multiple layers of epoxy resin, each layer consisting of $150g$ of epoxy resin (A) and $45g$ of hardener (B). To facilitate a robust and reliable encapsulation, each layer was allowed to cure for a minimum of 7 hours before applying the subsequent one. The initial 5 layers were strategically applied to the bottom section, encompassing the bottom of the valves and the battery, providing a secure foundation for the device's components. Subsequently, the electronic components were

oriented downwards, and the epoxy resin was carefully applied, encapsulating, and safeguarding the delicate electronics.

The integration of epoxy resin and hardener reinforces the durability and resilience of the device, rendering it capable of withstanding challenging environmental conditions, particularly when submerged underwater. The precise and thorough encapsulation process ensures the longevity of the electronic components and safeguards against potential water-related damage, a critical consideration for the device's intended underwater operation.

In summary, the combination of epoxy resin and hardener serves as a protective shield, preserving the integrity of the device's electronics and enabling its successful operation in submerged conditions. Through meticulous testing and precise application, the device's internals are securely encased, providing a reliable and efficient solution for the designated applications.

Two identical switches with led indicator were employed in the device, outside of the epoxy resin, and their schematic diagram is presented in Figure 31.
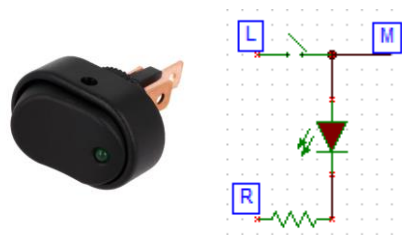
Figure 31 – Switches used and its schematic diagram

The first switch serves as the power on/off switch, enabling the user to control the device's activation and deactivation. To integrate this switch into the system, a GND voltage wire was soldered to the contact represented by "R" in the schematic diagram (Figure 30). The "Vin" of the power manager board was connected to the "M" contact, while the positive terminal of the battery was linked to the "L" contact. This configuration allowed for the interruption of the power flow to the load manager, effectively turning the device on or off as desired.

The second switch facilitates the reprogramming of the device, providing a means to reset the microcontroller when necessary. This switch required only two wires for its implementation. One wire connected the GND voltage to the "M" contact, and the other wire linked the microcontroller's RESET pin to the "L" contact. By establishing this connection, the

microcontroller could be easily reset, enabling reprogramming and updates to its firmware or configuration.

The integration of these switches adds an essential user interface aspect to the device. The power on/off switch ensures convenient and efficient control over the device's operational status, allowing the user to conserve energy and prolong battery life when the device is not in use. On the other hand, the reprogramming switch offers a straightforward mechanism to initiate the microcontroller's reset, facilitating updates and modifications to the device's functionality as needed.

By strategically incorporating these witches and their respective connections, the device achieves enhanced user-friendliness and accessibility. Users can effortlessly power the device on or off and easily reprogram it for future adaptations and improvements, contributing to the overall versatility and efficiency of the device.

In addition to the previously discussed elements, the integration of jack adaptors, as depicted in Figure 32, was essential for the device's functionality.

Figure 32 – Jack adaptors

The jack adaptors served a crucial purpose in enabling the device's battery charging process. A female connector, jack adaptor, was directly connected to the second input terminals of the power manager board. This connection involved soldering two wires to the female connector, with the positive wire linked to the central pin and the negative wire connected to the outer ring.

The male adaptor, designed to fit a 15V computer charger, when plugged into the female connector, provides a convenient and straightforward way to charge the device's battery.

The incorporation of a USB F/F connector, as depicted in Figure 33, was an essential requirement for enabling seamless serial communication between the user's terminal and the device.

Figure 33 – F/F USB adaptor

The USB F/F adaptor served a crucial purpose in simplifying the device's connectivity and enhancing user convenience. Instead of necessitating an extremely large cable hidden within the device or requiring users to purchase an uncommon M/F USB cable, the F/F USB adaptor offered a practical solution. This adaptor enabled the device to be used with a much more common M/M USB cable, readily available and widely used for various electronic devices.

By employing the USB F/F connector, the device could easily establish a direct and reliable connection to the user's terminal. This streamlined communication facilitated data exchange, configuration adjustments, and other interactions between the user and the device's microcontroller.

Moreover, the USB F/F connector's versatility contributed to the device's overall usability and compatibility with standard USB cables commonly found in the market. This approach ensured that users did not encounter unnecessary challenges in establishing a connection between their terminal and the device, promoting a seamless and user-friendly experience.

The integration of the USB F/F connector exemplifies the attention given to practicality and user-centric design in the development of the device. By choosing this accessible and widely compatible connector, the device's communication capabilities were enhanced without compromising on the convenience of users.

The device features two sets of connection and stopper, illustrated in Figure 34, which play crucial roles in enabling user access to the interrupter switches and facilitating the connection of various cables to the device.

Figure 34 – Sets of connection and stopper

By strategically positioning the jack plugs and switches within the connector set, and subsequently embedding it in resin, the device achieved a secure and organized configuration.

The connector set serves as a central hub, housing the jack plugs and switches in a compact and accessible manner. This thoughtful arrangement allows users to easily access the interrupter switches, granting them control over device activation and reprogramming functionalities.

Furthermore, the inclusion of the connector set eliminates the need for complex and cumbersome cable management. Users can effortlessly connect different types of cables to the device, thanks to the strategically placed jack plugs. This design choice enhances the device's versatility, making it compatible with a wide range of communication cables and facilitating data exchange and configuration procedures.

4.5 Total expenses

An important factor to consider during the examination of the expenses incurred in the design of this prototype device is its prototypical nature. Furthermore, it should be noted

that the materials incorporated into the device, in several instances, were selected based on availability rather than specific preferences.

Table 6 provides a comprehensive breakdown of the expenditure associated with different types of materials utilized in the development of the device.

Table 6 - Estimated expenditures on the prototype

Type of material	Value (€)
Hydraulic Material	100
Electronic Material	75
Epoxy Resin	280
Electro-valves	210
Water pumps	21
Others – Auxiliary Miscellaneous Material	15
Total	**701**

It is essential to recognize that the figures presented in the financial analysis are approximations. This approximation is due to the complex nature of procurement processes, which involved transactions with various vendors, the utilization of materials from existing stock, and the absence of the necessity to procure certain materials.

4.6 Preliminary tests in non-aquatic environment

Prior to embedding the device in resin, comprehensive functionality tests were conducted in non-aquatic environment. The device was activated in the cleaning mode, allowing for the verification of key components. The pressure sensor's functionality was confirmed as it displayed atmospheric pressure, altitude, and temperature at the time of testing. Similarly, the electro-valves and pumps operated as expected, demonstrating their proper functionality.

To assess the responsiveness of the valves to pressure sensor readings, a sampling mode was initiated with the depth set from 0 meters to various depths as the initial range. This test yielded successful results, as the pressure sensor effectively triggered the opening of

the valves when subjected to external airflow. The intake valve promptly closed shortly after, initiating the preservation phase.

Furthermore, a second non-aquatic test was performed with one set of valve and relay already embedded in resin. This test was essential to ensure that the resin did not interfere with the set's functionality. Once again, all components performed as expected, reaffirming the device's operational integrity.

The meticulous air testing provided valuable insights into the proper functioning of the device's critical components, inspiring confidence in its ability to conduct eDNA sampling in underwater conditions. These successful outcomes paved the way for the subsequent embedding process, where the device's potential would be fully realized, ensuring its reliability and effectiveness in marine environments.

4.7 Final tests in air

Following the complete epoxy resin coating of the device, the initial phase involved evaluating its performance in a non-aquatic setting. Originally, a series of planned tests included initiating the device in cleaning mode to assess the functionality of the filter valves, intake valve, and pump. However, during these trials, it was discovered that the last uploaded sketch to microcontroller, omitted the pump's programming. Consequently, the first test primarily involved addressing the hard reset switch, as the pumps were not activated.

Regrettably, the hard reset switch did not function, posing potential issues related to either a faulty wire connection or the infiltration of resin, which might have hindered its operation. Luckily, the two wires that operated the switch were still outside the resin, so, those wires were cut, and the device was reprogramed by contacting them while uploading a new sketch to the microcontroller.

With the device duly reconfigured, the evaluation proceeded with the device set to cleaning mode. During this phase, every component was tested, excluding the pressure sensor, alcohol valve, and pump. Notably, one of the valves displayed irregular behaviour, with suspected resin infiltration obstructing its smooth operation. While a suboptimal connection was considered, the distinct sound produced during activation ruled out this

possibility. Despite this setback, the test was deemed a success, as the filter associated with the non-functional valve served a crucial role as a negative control.

The final non-aquatic test involved setting the device in sampling mode with only one filter, starting at the surface (0 meters deep). This test proved highly successful, as it exhibited rapid valve response to external airflow, indicating the pressure sensor's efficiency. Subsequently, the intake valve closed promptly, and the alcohol valve and pump initiated the preservation phase, concluding as anticipated.

After this testing phase, the exposed wires were placed inside two new junctions and stoppers. These two new sets, along with a handle, were placed on top of the device, and new layer of resin was added, so that these features were truly incorporated in the device. However, this last layer of resin did not optimally adhere well to the remaining resin, and it broke, leaving the device, for the time being, because it can be redone, without a handle and without a way of reprogramming if necessary.

In summary, while the epoxy resin embedding process did present challenges with two components, specifically the switch and valve, the switch issue can be addressed with a new layer of resin, and the valve's problem does not constitute a critical device failure. It is important to note that the non-functional valve can still serve a valuable role as a negative control, which holds a pivotal position in the interpretation of results.

4.8 Tests in water

The final stage of testing the device involved simulating its performance in a deep-sea-like environment. The device was positioned within a small makeshift aquarium, initially filled with a cleaning solution containing bleach while set in cleaning mode. Following the predefined ten-minute cycle, the aquarium was emptied and thoroughly cleaned. Subsequently, it was refilled with ultra-pure water, and the device was once again placed in cleaning mode. As anticipated, these two phases transpired without unexpected developments, as it was already known that one of the valves was non-functional.

Continuing the evaluation, the aquarium underwent a thorough cleaning before being filled with fifteen Liters of deionized water, to which $100 \mu l$ of lamprey DNA at a concentration of $90 ng/\mu l$ was introduced, resulting in an environment with approximately $600 ng/l$ of

lamprey DNA. In this test scenario, the device was submerged in sampling mode, with the objective of filtering water from the surface down to a depth of one meter. It is worth noting that the device did not reach the intended depth and continued filtering until manually removed from the aquarium.

While the device was engaged in the filtration process, the flow rate was estimated by measuring the time taken to fill a $50ml$ tube, which was completed in less than 9 seconds, indicating a flow rate of $5.5ml/s$. Two separate ten-minute tests were conducted, each utilizing one test filter and one negative control filter. The results yielded promising outcomes, as the lamprey DNA was successfully detected in both test filters, while none was detected in any of the control filters. This confirms the device's effectiveness in identifying the presence of genetic material while ensuring the integrity of the remaining filters, preventing contamination.

Furthermore, the device was operated by the individual responsible for the extraction and analysis, demonstrating the device's user-friendly interface. Despite these positive aspects, some limitations were identified. Firstly, the alcohol system proved to be impractical, primarily due to the cumbersome nature of filling and assembling the balloon. Secondly, the device took longer than expected to conclude the filtration process and initiate the preservation phase, mainly because it had been set to filter water from the surface before being submerged in the aquarium. However, it is essential to acknowledge that this delay may not be detrimental, considering the device's intended application in deep-sea environments. Lastly, the device's substantial weight, coupled with the absence of a handle, rendered it challenging to retrieve from the water.

5. CONCLUSION

As DNA-based monitoring tools continue to evolve, there is an increasing demand for new, simple, and cost-effective sampling devices. In this project, a new equipment was designed and constructed to meet this need. The device at hand can be likened to a prism weighing approximately thirteen kilograms and measuring $57cm \times 12cm \times 15cm$ (excluding the sections extending beyond the resin coating). This makes it denser than water at just under $1.27g/cm^3$. After a comprehensive testing phase, several key conclusions were drawn:

Firstly, while the device was anticipated to function correctly based on its performance prior to the epoxy resin embedding, there was a potential risk associated with poorly soldered contacts that might fail when exposed to resin, causing isolation. Despite the cessation of one valve's operation, it is more likely that resin infiltration into the valve's pathway is the cause of this issue. This assumption is supported by the audible sound of the valve's motor during activation, suggesting potential physical hindrance within the valve.

Moreover, it's important to acknowledge that this device, in its current form, represents more of a prototype than a finalized product and therefore, there is significant room for improvement and enhancement.

The foremost area for improvement pertains to the device's physical attributes. It is relatively large and heavy, considering its capacity to collect only three samples. Weight and size reduction can be achieved by exploring alternative materials for the hydraulic system, adopting smaller and lighter electro valves, and reconfiguring the arrangement of electronic components to minimize the overall size and weight.

Additionally, the current utilization of a balloon as the alcohol reservoir is less than ideal in terms of professionalism and practicality. Investigating alternative solutions for this component would be a valuable avenue to explore.

Third, the LEDs blinking prevents the device from making more than one depth read every two seconds, and although this point can be changed at any time, this feature compelled the LEDs to be connected to another microcontroller pin, instead of being connected in parallel with each electro valve.

Despite these evident areas for improvement, the device did not fall short of its primary objective. Its capability to assist in the detection of genetic material while effectively isolating the remaining filters underlines its suitability for deployment in the monitoring of marine ecosystems.

Notwithstanding not fully achieving the originally proposed size, the construction of this device has successfully demonstrated the feasibility of designing a cost-effective equipment capable of collecting eDNA samples on filters and preserving them. This project marks a valuable step towards advancing eDNA sampling technology, opening new possibilities for effective environmental monitoring and research.

5.1 Personal critical opinion

Undertaking the design and construction of the device was undoubtedly the most challenging aspect of this thesis, but it also served as a tremendous source of motivation. The fear of making irreversible errors loomed large throughout the project's development, and unfortunately, a mistake did occur in the electronics part, necessitating a repetition of soldering and connections. However, this setback turned out to be a valuable learning opportunity, enabling me to refine and miniaturize the connections on the Veroboard.

The journey of building this equipment was filled with surprising engineering tricks and methods that I had not encountered before. It pushed me to apply and enhance my skills in electronics, hydraulics, programming, and biology. I came to realize that the evolution the world of science and engineering is a lot faster than I expected, with new methods and approaches being created and studied.

Despite any imperfections, flaws, or potential enhancements that the device may have, this project exemplified the essence of being a physics engineer. The amalgamation of knowledge from various distinct areas is what defines the role, and though it may not have required an exhaustive expertise in any field, I believe this project was a perfect fit for someone with my background.

In conclusion, despite the challenges and uncertainties, I am proud of the progress made and the skills honed throughout this project. It has been an extraordinary opportunity to explore multiple disciplines and engage in hands-on engineering work. This experience has further solidified my passion for science and engineering, motivating me to continue pursuing innovative projects and contributing to advancements in the field.

5.2 Future work

Future work in this field presents a promising avenue for enhancing the device's performance by addressing its current limitations and integrating the suggested improvements. By conducting a comprehensive study of recent devices and identifying their strengths, it becomes possible to create a refined version of this equipment.

One potential focus for future work involves mitigating the device's weak points. Incorporating the suggested enhancements plays a crucial role in advancing the device's

capabilities. By carefully evaluating the proposed improvements and their potential impact, the research can build upon existing knowledge and optimize the device's design. Whether it involves incorporating state-of-the-art components, refining algorithms, or introducing novel features, these enhancements can significantly elevate the device's effectiveness and user experience.

Thorough research and experimentation can pinpoint the specific areas where the current device falls short, be it in terms of efficiency, accuracy, or functionality. With a detailed understanding of these shortcomings, researchers and engineers can explore innovative solutions and alternative approaches to bolster the device's performance and overall reliability.

Furthermore, by studying recent devices in the same domain, researchers can gain valuable insights into cutting-edge technologies and design methodologies. Analysing successful devices can inspire new ideas and shed light on potential breakthroughs. By merging the strengths of these contemporary devices with the present one's design, researchers can create a hybrid approach that capitalizes on the best features of each system.

Ultimately, the goal of future work is to develop an improved version of the equipment that surpasses the current devices in various aspects. This endeavour requires a comprehensive approach, involving both theoretical analysis and practical experimentation. Rigorous testing and validation of the proposed enhancements will be essential to ensure that the improved device meets the desired performance standards and effectively addresses the identified weak points.

In conclusion, future work holds immense potential for refining and advancing the device in question. By strategically focusing on mitigating weaknesses and integrating suggested enhancements, along with drawing inspiration from recent devices, researchers can pave the way for a superior version of this equipment that will undoubtedly contribute to the field's progress and make a positive impact on its applications.

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ANEX: ARDUINO PROGRAM

```
#include <Wire.h>
#include "MS5837.h"

MS5837 sensor;

#define P1 9      //Intake Pump
#define P2 2      //Alcohol Pump
#define V1 8      //Intake Valve
#define V2 7      //Alcohol Valve
#define V3 6      //Filter 1 Valve
#define V4 5      //Filter 2 Valve
#define V5 3      //Filter 3 Valve
#define V6 4      //Filter 4 Valve
#define LED1 10  //Filter 1 LED
#define LED2 11  //Filter 2 LED
#define LED3 12  //Filter 3 LED
#define LED4 13  //Filter 4 LED


float depth, atmosphericpressure, depth_lim1, depth_lim2;  //y is an auxiliar int, x is the most useful
variable
int y, x = 4, fluidDensity = 1029;                         //for it is the difference between functional
modes and
int depth_limits1[4], depth_limits2[4];                    //it is very useful in the filtering mode as
well.
int auxarray[8];                                           //The arrays are used to store the depth
ranges information

void setup() {

  pinMode(V1, OUTPUT);
  pinMode(V2, OUTPUT);
  pinMode(V3, OUTPUT);
  pinMode(V4, OUTPUT);
  pinMode(V5, OUTPUT);
  pinMode(V6, OUTPUT);
  pinMode(P1, OUTPUT);
  pinMode(P2, OUTPUT);
  pinMode(LED1, OUTPUT);
  pinMode(LED2, OUTPUT);
  pinMode(LED3, OUTPUT);
  pinMode(LED4, OUTPUT);

  //Start serial communication

  Serial.begin(9600);
  Wire.begin();
```

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```
  //Start pressure sensor
  while (!sensor.init()) delay(1000);
}


//sensor.depth calculates depth with the same formula, but it uses the mean atmospheric pressure value

float actualdepth() {
  return (sensor.pressure() - atmosphericpressure) / (fluidDensity * 9.80665);
}


//This funcion is used to help to sort the auxiliar array before defining pressure ranges

int cmpfunc(const void* a, const void* b) {
  return (*(int*)a - *(int*)b);
}


void loop() {
  while (x == 5) {                          //Happens when cleaning is finished.
    while (!Serial.available()) delay(100); //The device must be restarted for further use.
    y = Serial.parseInt();                  //To restart the device, the user must send any
    Serial.println("The device is clean."); //message through the serial port. This will
    Serial.println("Restarting.");          //clean its buffer and restart the device.
    while (!Serial.available()) delay(100); //The user can also restart the device manually
    y = Serial.parseInt();                  //through one of the switches.
    x = 4;
    digitalWrite(LED1, LOW);
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    digitalWrite(LED4, LOW);
  }

  while (x == 6) {                                           //Happens when sampling is
finnished.
    while (!Serial.available()) delay(100);                           //The device must be restarted
for further use.
    delay(100);                                                       //To restart the device, the user
must send any
    y = Serial.parseInt();                                            //message through the serial port.
This will
    Serial.println("The device has finished filtering.");            //clean its buffer and restart
the device.
    Serial.println("Please clean the device before sampling again.");  //The user can also restart the
device manually
    Serial.println("Restarting.");                                   //through one of the switches.
    x = 4;
    digitalWrite(LED1, LOW);
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    digitalWrite(LED4, LOW);
```

```
  }

  Serial.println("\n\n\nStarting");
  sensor.read();
  delay(40);
  atmosphericpressure = sensor.pressure();
  Serial.print("Current atmospheric pressure is: ");
  Serial.print(atmosphericpressure);
  Serial.print(" mbar.\nCurrent temperature is: ");  //Not necessary but could be interesting
  Serial.print(sensor.temperature());
  Serial.print(" degrees C.\nCurrent Altitude: ");
  Serial.print(sensor.altitude());
  Serial.println(" m above mean sea level.");
  delay(300);


  //Select operating mode

  Serial.println("\nPlease, insert 1 for cleaning mode or 2 for sampling mode.");
  Serial.println("If anything else is inserted, the device will restart.");
  while (!Serial.available()) delay(100);
  y = Serial.parseInt();
  delay(200);
  Serial.print("You entered ");
  Serial.println(y);


  if (y == 1) {
    Serial.println("\nThe device will start cleaning itself in 60 seconds.");
    Serial.println("Please place the device in a decontaminating liquid (bleach).");
    Serial.println("Enter anything to restart.\n");
    x = 7;                    //1 for cleaning mode. Before it starts cleaning,
    for (y = 59; y; y--) {        //the device waits 1 minute to be placed in a
      if (Serial.available()) {  //recipient full of decontaminating liquid.
        y = Serial.parseInt();   //During this minute, the user has the option of
        x = 4;                   //restarting the device, cancelling cleaning mode
        delay(100);
        y = 1;
        Serial.println("Restarting\n\n");
      }
      delay(1000);
    }
  } else if (y == 2) {  //2 for sampling mode.
    x = 0;

    //If sampling mode is selected, user selects freshwater or seawater

    Serial.println("\nPlease, insert 0 if you are going to use the device in fresh water. Anything else
for seawater");
    while (!Serial.available()) delay(20);
    y = Serial.parseInt();
```

```
    delay(200);
    if (!y) fluidDensity = 997;  //0 for fresh water
    Serial.print("Fluid density chosen: ");
    Serial.println(fluidDensity);

    //When fresh or seawater is selected, determine depth ranges for filtering
    for (y = 0; y < 8; y++) auxarray[y] = 12000;
    Serial.println("\nHow many filters are you going to use? (1 to 4)");
    while (!Serial.available()) delay(20);
    y = Serial.parseInt();
    delay(200);
    if (y > 4) y = 4;  //This device has 4 available filters, so that's the maximum.
    if (y < 0) y = 0;
    Serial.print(y);
    Serial.println(" Filters will be used");
    Serial.println("\nPlease, insert depth values, in meters, one at a time.");
    for (y = y * 2; y; y--) {                      //For each filter, there is a range of depth between 2
vallues.
        while (!Serial.available()) delay(20);  //The user chooses the ranges and the depth values are
stored in
        auxarray[y - 1] = Serial.parseInt();    //the auxiliar array, in this phase, for them to be sorted
and
        delay(300);                              //then they are stored in the depth_lim arrays.
        Serial.println(auxarray[y - 1]);
    }

    qsort(auxarray, 8, sizeof(int), cmpfunc);  //Sorting phase

    for (y = 0; y < 8; x++) {              //This cycle is the transfer of values from the auxiliar array
to
        depth_limits1[x] = auxarray[y];      //the ones used in filtering mode. If the user chooses not to
        depth_limits2[x] = auxarray[y + 1];  //use all the filters, the value 12000 will be stored in the
remaining
        y = y + 2;                           //positions in the depth_lim arrays. this will be used as an
indicator
    }                                        //that the objective filters were already used.
    x = 0;                                   //x was used as auxiliar and now it is 0 again for filter
selection

    for (y = 0; depth_limits2[y] < 12000 && y < 4; y++) {   //This cycle provides the user with the
range's prints
        Serial.print("Range number ");                      //for in order for them to be checked.
        Serial.print(y + 1);                                 //Note that even if the user chooses not to
use all the
        Serial.print(" : ");                                 //filters, only the selected ranges will be
shown
        Serial.print(depth_limits1[y]);
        Serial.print(" - ");
        Serial.println(depth_limits2[y]);
```

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```
    }
    Serial.print("Fluid Density = ");
    Serial.println(fluidDensity);
    Serial.println("Please confirm if ranges and fluid density are correct.");
    Serial.println("If something is wrong, enter 0 to restart.\nIf everything is correct enter 1 to
proceed.");
    while (!Serial.available()) delay(10);
    y = Serial.parseInt();
    delay(10);
    if (y == 0) x = 4;  //Restart
  }


  //Serial communication done.

  while (x < 4) {              //Sampling mode
    sensor.read();            //Update pressure values
    delay(40);               //This is done to avoid errors. The pressure read is then
    depth = actualdepth();   //used to determine the at what depth the device is

    //Filtration phase

    if (depth >= depth_limits1[x] && depth < depth_limits2[x]) {//If the device is in the depth range,
filtration starts,
      if (x == 0) digitalWrite(V3, HIGH);   //otherwise, the while cycle restarts. Note that the x
      if (x == 1) digitalWrite(V4, HIGH);   //variable is also very useful to avoid 4 different while
      if (x == 2) digitalWrite(V5, HIGH);   //cycles because we are able to select the filter to be used
      if (x == 3) digitalWrite(V6, HIGH);   //with it.
      digitalWrite(V1, HIGH);
      digitalWrite(P1, HIGH);
      while (depth >= depth_limits1[x] && depth < depth_limits2[x]) {  //Only while in range, filtration
        if (x == 0) digitalWrite(LED1, HIGH);       // is enabled. These ifs are used to make each
        if (x == 1) digitalWrite(LED2, HIGH);       //filter's LED blink while the filter is being used.
        if (x == 2) digitalWrite(LED3, HIGH);       //LEDs turn on for 1 second and then turn off for
        if (x == 3) digitalWrite(LED4, HIGH);       //another second.
        delay(1000);
        if (x == 0) digitalWrite(LED1, LOW);
        if (x == 1) digitalWrite(LED2, LOW);
        if (x == 2) digitalWrite(LED3, LOW);
        if (x == 3) digitalWrite(LED4, LOW);
        delay(1000);
        sensor.read();          //Pressure values are read every 2 seconds
        depth = actualdepth();  //And pressure is updated.
      }
      digitalWrite(V1, LOW);                        //When the device is out of the destined depth range,
intake
      digitalWrite(P1, LOW);                        //valve and pump are closed, to stop filtration.
      for (y = 4; y; y--) {                         //This for cycle is useful, once again, to make
        if (x == 0) digitalWrite(LED1, HIGH); //the LED blink while the intake valve closes.
        if (x == 1) digitalWrite(LED2, HIGH); //This cycle lasts about 8 seconds, that is about
```

```
  if (x == 2) digitalWrite(LED3, HIGH);   //the time the valve takes to fully close
  if (x == 3) digitalWrite(LED4, HIGH);
  delay(1000);
  if (x == 0) digitalWrite(LED1, LOW);
  if (x == 1) digitalWrite(LED2, LOW);
  if (x == 2) digitalWrite(LED3, LOW);
  if (x == 3) digitalWrite(LED4, LOW);
  delay(1000);
}


//Preservation phase

digitalWrite(V2, HIGH);                   //To avoid contamination, valve 2 and pump 2 are opened
digitalWrite(P2, HIGH);                   //to pump alcohol through the used filter, preserving it
for (y = 4; y; y--) {                     //This for cycle has the same purpose of the previous one
  if (x == 0) digitalWrite(LED1, HIGH);   //purpose of the previous one, but this time, while the
  if (x == 1) digitalWrite(LED2, HIGH);   //filter is being preserved, the LED blinks at a higher
  if (x == 2) digitalWrite(LED3, HIGH);   //rate. This is useless in the field but very useful
  if (x == 3) digitalWrite(LED4, HIGH);   //during test phase, because the user is able to tell
  delay(500);                             //when filtration end and preserving starts.
  if (x == 0) digitalWrite(LED1, LOW);    //Preservation phase is very short. the valve does not
  if (x == 1) digitalWrite(LED2, LOW);    //even reach its fully open state and the order to
  if (x == 2) digitalWrite(LED3, LOW);    //close it is given because enough alcohol to preserve
  if (x == 3) digitalWrite(LED4, LOW);    //the filter passes in that period and it would be a
  delay(500);                             //waste to continue pumping it.
}
if (x == 0) digitalWrite(V3, LOW);
if (x == 1) digitalWrite(V4, LOW);
if (x == 2) digitalWrite(V5, LOW);
if (x == 3) digitalWrite(V6, LOW);
digitalWrite(P2, LOW);
digitalWrite(V2, LOW);
for (y = 4; y; y--) {                     //This cycle is to keep the LED blinking while valves
  if (x == 0) digitalWrite(LED1, HIGH);   //are being closed.
  if (x == 1) digitalWrite(LED2, HIGH);
  if (x == 2) digitalWrite(LED3, HIGH);
  if (x == 3) digitalWrite(LED4, HIGH);
  delay(500);
  if (x == 0) digitalWrite(LED1, LOW);
  if (x == 1) digitalWrite(LED2, LOW);
  if (x == 2) digitalWrite(LED3, LOW);
  if (x == 3) digitalWrite(LED4, LOW);
  delay(500);
}
if (x == 0) digitalWrite(LED1, HIGH);     //After preservation stage
if (x == 1) digitalWrite(LED2, HIGH);     //the corresponding LED
if (x == 2) digitalWrite(LED3, HIGH);     //stays on until device
if (x == 3) digitalWrite(LED4, HIGH);     //is restarted.
x++;                                      //Now that one of the ranges is done, x value is updated
```

```
      if (x == 4 || depth_limits1[x] > 11999) {  //if the device has finished filtering in all ranges:
        x = 6;                                    //Filtration mode over
      } else {
        depth_lim1 = depth_limits1[x];  //If the device has not yet filtered in all predefined
        depth_lim2 = depth_limits2[x];  //ranges, ranges are updated with help of the x variable
      }
    }
    if (Serial.available()) {
      y = Serial.parseInt();
      delay(100);
      Serial.print("The device couldn't enter range ");
      Serial.println(x + 1);
      digitalWrite(LED1, LOW);
      digitalWrite(LED2, LOW);
      digitalWrite(LED3, LOW);
      digitalWrite(LED4, LOW);
      Serial.println("Restarting");
      x = 4;
    }
  }


  //Cleaning mode

  if (x > 6) {                    //x > 6 only happens to get the device in cleaning mode.
    digitalWrite(V1, HIGH);       //in cleaning mode, all valves but alcohol valve are opened,
    delay(8000);                  //sequentially, so that bleach or other decontaminating liquid
    digitalWrite(V3, HIGH);       //can be pumped throughout the device and preventing future
    for (y = 4; y; y--) {         //sample contamination. The purpose of these for cycles is to
      digitalWrite(LED1, HIGH);   //get each valve's LED to blink while the valve is open or
      delay(1000);                //being opened. Again, the LEDs turn on for 1 second and turn
      digitalWrite(LED1, LOW);    //off for another second.
      delay(1000);
    }
    digitalWrite(V4, HIGH);
    for (y = 4; y; y--) {
      digitalWrite(LED1, HIGH);
      digitalWrite(LED2, HIGH);
      delay(1000);
      digitalWrite(LED1, LOW);
      digitalWrite(LED2, LOW);
      delay(1000);
    }
    digitalWrite(V5, HIGH);
    for (y = 4; y; y--) {
      digitalWrite(LED1, HIGH);
      digitalWrite(LED2, HIGH);
      digitalWrite(LED3, HIGH);
      delay(1000);
      digitalWrite(LED1, LOW);
```

```arduino
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    delay(1000);
  }
  digitalWrite(V6, HIGH);
  for (y = 4; y; y--) {
    digitalWrite(LED1, HIGH);
    digitalWrite(LED2, HIGH);
    digitalWrite(LED3, HIGH);
    digitalWrite(LED4, HIGH);
    delay(1000);
    digitalWrite(LED1, LOW);
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    digitalWrite(LED4, LOW);
    delay(1000);
  }
  digitalWrite(P1, HIGH);     //The Pump is the last things to be turned on and first to be
  for (y = 300; y; y--) {     //turned off because of their energy consumption. Again, this
    digitalWrite(LED1, HIGH); //cycle is useful to get the LEDs blinking through all the
    digitalWrite(LED2, HIGH); //cleaning phase that lasts about 10 minutes. Each of these
    digitalWrite(LED3, HIGH); //loops take about 2 seconds, so, they are repeated 300 times
    digitalWrite(LED4, HIGH); //to reach the 600 seconds that correspond to 10 minutes.
    delay(1000);
    digitalWrite(LED1, LOW);
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    digitalWrite(LED4, LOW);
    delay(1000);
  }
  digitalWrite(V3, LOW);      //Again, all valves are closed sequentially.
  for (y = 4; y; y--) {       //So, these cycles keep each valve's LED blinking while the
    digitalWrite(LED1, HIGH); //valves are closing
    digitalWrite(LED2, HIGH);
    digitalWrite(LED3, HIGH);
    digitalWrite(LED4, HIGH);
    delay(1000);
    digitalWrite(LED1, LOW);
    digitalWrite(LED2, LOW);
    digitalWrite(LED3, LOW);
    digitalWrite(LED4, LOW);
    delay(1000);
  }
  digitalWrite(LED1, HIGH);  //When one valve's path is clean and ready to be used, the
  digitalWrite(V4, LOW);     //corresponding LED turns on until device is restarted.
  for (y = 4; y; y--) {
    digitalWrite(LED2, HIGH);
    digitalWrite(LED3, HIGH);
    digitalWrite(LED4, HIGH);
```

```
      delay(1000);
      digitalWrite(LED2, LOW);
      digitalWrite(LED3, LOW);
      digitalWrite(LED4, LOW);
      delay(1000);
    }
    digitalWrite(LED2, HIGH);
    digitalWrite(V5, LOW);
    for (y = 4; y; y--) {
      digitalWrite(LED3, HIGH);
      digitalWrite(LED4, HIGH);
      delay(1000);
      digitalWrite(LED3, LOW);
      digitalWrite(LED4, LOW);
      delay(1000);
    }
    digitalWrite(LED3, HIGH);
    digitalWrite(V6, LOW);
    digitalWrite(P1, LOW);
    for (y = 4; y; y--) {
      digitalWrite(LED4, HIGH);
      delay(1000);
      digitalWrite(LED4, LOW);
      delay(1000);
    }
    digitalWrite(LED4, HIGH);  //Intake valve is the last thing
    digitalWrite(V1, LOW);     //to be closed and when this is finished, cleaning
    x = 5;                     //mode ends and nothing else needs to be done, so
  }                            //the device awaits input to restart.
}
```