Università degli Studi di Ferrara

DOCTORAL COURSE IN

ENVIRONMENTAL SUSTAINABILITY AND WELLBEING

CYCLE XXXV

COORDINATOR Prof. Spinozzi Paola

Circular Tutelage: a Sustainable Approach Toward Remediation and Enhancement of Endangered Areas

Scientific/Disciplinary Sector (SDS) CHIM/01

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Year 2019/2023

Preface

This is dedicated to:

Father and Grandfather, I wish you were here to witness this achievement of mine.

my lovely Mother, Grandmother and my little sisters. Thank you for supporting me.

Giulia, despite some incongruences, you have been the polar star through these hard years, always listening and, sometimes, supporting.

My "yet to meet" daughter, you have been the energy surge which allowed me to keep going over the last months of this path.

This road has been very harsh to me, and at a first glance it may seem that I lost far more than I gained, if we consider the daily cocktail of anxiety, stress and uncertainty, the pandemic, and also the war, I am quite surprised to be at the end of this journey in one piece. However, during this Ph.D. I failed, I struggled, I learned, I achieved, and I failed again in the endless, and painful, loop of growth. But after all of that suffering and failure, I feel stronger and more confident of who I am and what could be my place in the world.

For that, while my journey continues, I feel the necessity to look back and open my heart to express a "thank you" to my Tutor Luisa Pasti, the scientific board of the Ph.D. course and its coordinator Paola Spinozzi, my colleagues, my family and my friends.

Thank you.

Sincerely, Dr. Roccia, PhD

Be Smart, Be Better

Sommario

Introduction and Thesis Purpose

"Sustainability is the process of living within the limits of available physical, natural and social resources in ways that allow the living systems in which humans are embedded to thrive in perpetuity".

The phrase above is the "Academic Advisory Committee for the Office of Sustainability" working definition of sustainability at the University of Alberta, which is a readapted and more recent version of the sustainable development definition from the Brundtland Commission report "Our Common Future" i.e., the "*development that meets the needs of the present without compromising the ability of future generations to meet their own needs*" (Commission on Environment, 1987). In the most recent one the emphasis is plenty shifted on "sustain" rather than "development"; however, despite the differences in the semantic and syntax structure among the two, both definitions share the concept of the three pillars of sustainability, namely: environmental, social, and economic. These pillars represent the holistic way of action that is recommended to adopt while facing nowadays problems and planning development toward the future (Purvis, Mao and Robinson, 2019).

In detail, Environmental Sustainability represents the ecological integrity preservation: all of earth's environmental systems should be kept in balance while natural resources are consumed by humans with respect to natural production rates.

Economic Sustainability concerns the capacity of human communities across the globe of being able to maintain their independence whilst have access to the resources, finances and other, in order to meet their needs.

Social Sustainability regards the goal to grant to all people universal human rights and basics, while granting access to enough resources to keep their families and communities healthy and secure.

These are majestic guidelines, but unfortunately as a society we are far from being sustainable, since we fail to be balanced in almost every single aspect concerning the sustainability pillars. The abuse of natural resources, anthropogenic impacts, and pollution are reshaping environments and posing threats far beyond general wellbeing; in other words: human development has reached a scale where it can affect the planetary processes that sustain life. Whilst social and economic iniquities among countries are issues of primary concern, they also lead to ecological inequalities given the disproportion in CO_2 emissions participation (which is a climate change contributor indicator), and consumption of resources. As it can be observed in the newly "world inequality report" (Piketty *et al.*, 2022): "*the top 10% of emitters are responsible for close to 50% of all emissions, while the bottom 50% produce 12% of the total*".

Just to make an example, the "Overshooting Day by Country" (figure 1) is a good picture of how much our society is failing to face the ideal of sustainability; in fact, as the image shows, in each case of the whole planet behaving like a single country, there would be used yearly more resources than nature can provide, also it is possible to note the unbalances between the richest and the poorest countries, therefore a correlation between lifestyle quality and overconsumption of resources, therefore CO_2 emissions, is also present.

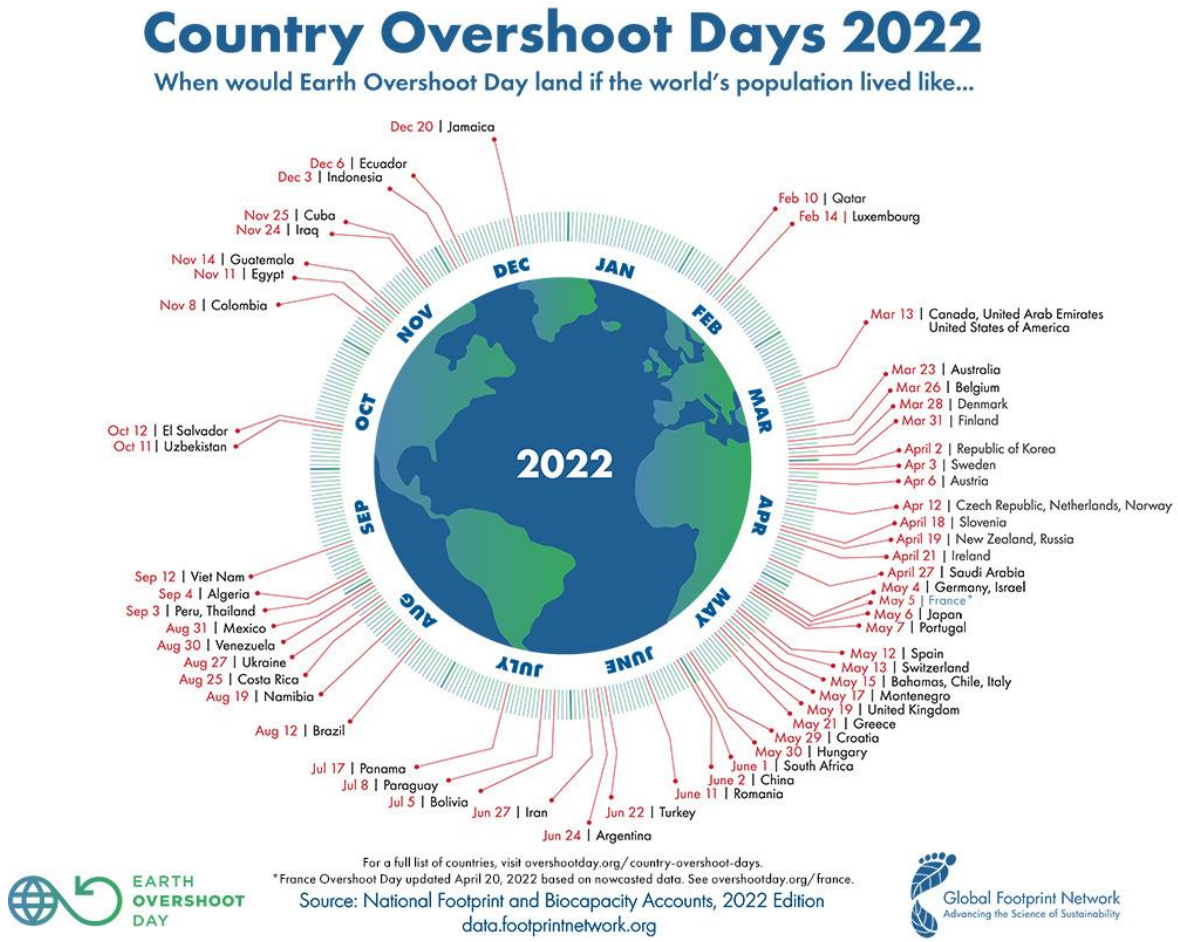
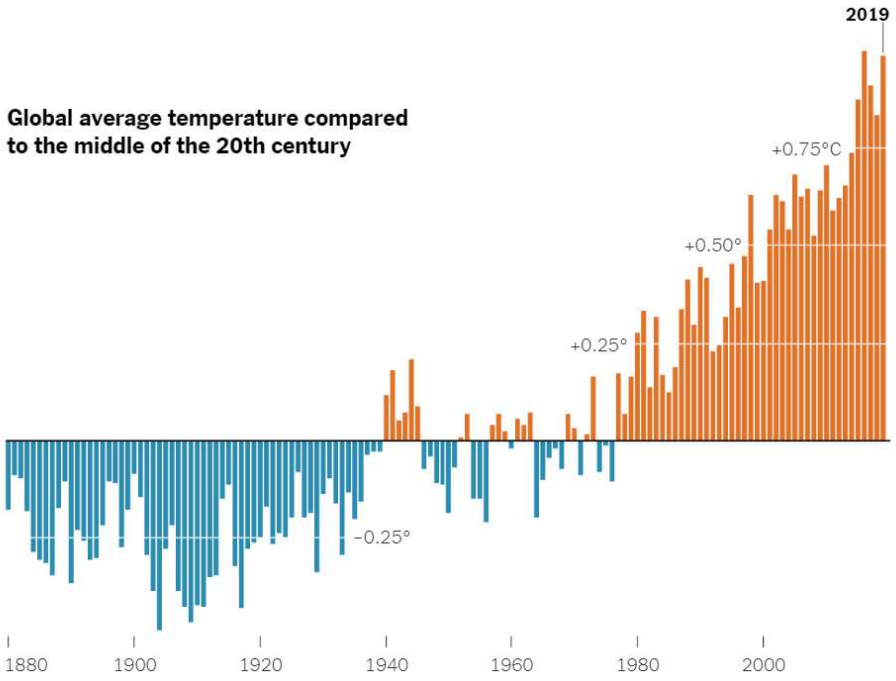


Figure 1) Overshoot Day Calendar. When all the yearly natural resources are depleted if all the world behaves like a particular country.

Acknowledging the situation, many are debating on what humankind should do in order to guarantee its survival (and to thrive, in the very meaning of the term), whether to change the paradigm to a degrowth approach aimed to an overall reduction in consume and production, or a green growth, which aims to keep the growth-centered paradigm, but with sustainable, therefore "green", accent (Sandberg, Klockars and Wilén, 2019). Whichever the case, the modification of our unsustainable reality is necessary, demanding time and efforts.

Whilst humanity has always easily faced the issue of "effort" during its development, it is now confronted with the issue of "time", as the path toward sustainability i.e., against global crises, can be seen as the end of the "mean temperature rise in Celsius degrees by 2050". For instance, the temperature threshold by 2050 is set to 2° higher than the pre-industrial mean temperature; in fact, beyond a 2° warmer world it is reasonable to expect profound alterations of its equilibria possibly leading to unpleasant scenarios, such as: disrupted climate with higher sea levels, animals and plants major extinctions, ecosystems disruption, fierce storms, floodings, desertification, and eventually more people dying from heat, bad weather, air quality, famine and infectious diseases (Bruce and Tin, 2006; King and Karoly, 2017; Nikulin *et al.*, 2018). The graph below, graph 1, reports the globe's average thermal anomalies, as it can be seen we are around the plus 1° Celsius increase in comparison to pre-industrial level.



Graph 1) Global Thermal Anomalies graph. By "The Learning Network".

In order to tackle the crises that we have paved the way for, several agreements among government took place with increasing incidence. Among many multilateral agreements, the most iconic are the "Kyoto Protocol" (1997) as the first legally binding document among countries, which drives the attention on a relatively small reduction of CO_2 emissions, the "Paris Agreement" (2016) which asks for more commitment in CO_2 reduction and introduces the goal of lesser than 2° mean temperature increase by 2050, and the "United Nations Agenda for 2030".

The latest introduces a series of targets for sustainability and development, the so called "sustainable development goals (SDGs)" (expansion of the millennium development goals); an ambitious action plan with plenty of "common targets" in order to face not only climate change, but also poverty, water shortage, and affordable energy, to cite some. The image below, figure 2, reports the seventeen main areas of interventions where the 160+ goals are grouped.

Thanks to its ambitions, the "Agenda 2030" is also a call for approaching issues in a multidisciplinar way, highlighting the importance of a "holistic" approach, which incarnates the essence of the sustainability pillars.

Figure 2) Sustainable Development Goals (SDGs).

Following the "Agenda 2030" inspiration, in this work of thesis, each issue faced is approached with strong multidisciplinar directions, considering aspects usually under the competences of socio-economic disciplines, other than chemistry, which is the main specialization field of mine. The crucial point is the multidisciplinarity itself and the message that it conveys while responding to challenges i.e., to be open and accommodating toward the "outside"; which is not to be seen as someone else's problem, but simply common matter to be resolved for the common good.

Thus, the main projects to favor sustainability that will be discussed in further sections, revolve around the criteria of Environment and Ecosystem Tutelage, Improved Circularity of Resources and Wellbeing with considerations on social aspects either; where the action can be divided into multiple categories, for example: environmental quality assessment and safeguard, remediation, optimization of processes, communication and simplification of complex decisional processes among decision makers and stakeholders.

Circular economy

One of the main common factors among the projects discussed later in the thesis concerns the concept of circularity and its enhancement. According to the definition from the European Parliament: "*The circular economy is a model of production and consumption, which involves sharing, leasing, reusing, repairing, refurbishing and recycling existing materials and products as long as possible. In this way, the life cycle of products is extended*".

In practice, it implies the reduction of waste to a minimum by applying the "Rs" i.e., reduce, reuse, and recycle (plus other "R"- words). However, despite the valuable strategy of optimizing the life cycle of goods and resources (Christopher Holt, no date; Jiang, 2006; Shaaban and Nasr, 2020), when a product reaches the end of its life, its materials should be kept within the economy wherever is possible. These should be productively used again and again, thereby creating further value, in a circular way.

This is a departure from the traditional, linear economic model, which is based on a take-make-consume-throw away pattern (Frosch and Gallopoulos, 1989). This linear model (or linear culture?) is also cause and consequence of factories' and industries' typical business model, where the interest for primary resources is limited to the "entrance gate" and the responsibility upon the product is mainly at the "exit gate"; whereas the circular model asks for a cradle-to-cradle approach, gaining knowledge and attribute more value to the goods and resources throughout all of their life cycle. However, there are some limits to the circularity by the terms that nowadays even a very good circular system will inevitably have some wastes produced, or resource wasted. Then, this model heavily relies onto innovation, smart processes, large quantities of cheap, easily accessible materials and energy other than a market capable of trading and accepting secondary raw materials and restored goods (green market). Ultimately, this model is not free from critiques (Valenzuela and Böhm, no date; Korhonen, Honkasalo and Seppälä, 2018; Corvellec, Stowell and Johansson, 2022), yet again the importance lies within the message that is transmitted, which aims to push for wider and for more sustainable projects' conception.

A couple examples from the Italian excellences in sustainability are represented by the "Dal Maso Group s.r.l." and "Feralpi Group", where the first operates in environmental services by stabilizing special and hazardous industrial solid wastes, and the second is a steel-forge industry.

The first, given its purpose and location in a tannery district in the northern Italy, handles animal leftovers from the leather processing in tanneries and salt wastes. Salt is necessary to maintain leather quality until the refinement process happens in the tannery; once removed, salt, is extremely impure, because it is accompanied by other wastes belonging to the animal, such as hair, fat, dung, nylon, flesh, woods, blood, nails, and more. Eventually, the precious salt couldn't be recovered given its dirtiness, and was sent to landfill; until the company patented a high temperature process, in 1995, to recover salt with a purity near to 100%. This process led to a three times sustainable achievement, given the usage of recovered salt to be used upon frozen roads in winter times instead of fresh salt from mines, also avoiding the energetic expenditure and pollution that mining operation causes, and less landfill usage. The organic byproducts of this process could be composted to obtain soil conditioners, but there is a normative hole surrounding this last issue that the company is trying to overcome. The company was awarded with the first prize as "best performer in circular economy for 2019/2020" by "Confindustria".

The steel production sector is usually pointed at "as an extremely polluting and energy consuming industry", which is partially true, given its purpose. However, "Feralpi group" is adopting smart measures to improve its impact, where the first is to use scrap metal from wastes to create new steel; secondly, they

blend plastics with normal fuel, thereby reducing the fuel usage itself while burning plastics avoiding landfill usage. Also, the slag by-produced along with steel, is stabilized trough thermal and chemical processes in order to produce the “Greenstone” which is a patented material, suitable for construction purposes, achieving a triple circular goal.

However, to find more ways of improving circularity is a complex and multidisciplinar task; thus, to facilitate the put in practice of circular models, some tools are available for the recognition of weak spots and for making solid sustainable choices; among many: the Life Cycle Assessment (LCA), Cost Benefit Analysis (CBA), and Foresight analysis allow for comparative studies and assessments “a priori”, therefore optimize choices and growths.

Life Cycle Assessment (LCA)

The LCA is a tool for evaluating environmental effects of a product, process, or activity throughout its life cycle or lifetime, which is also known as “cradle to grave” analysis. Theoretically, this tool should help in shifting the –cradle to grave- toward a –cradle to cradle- concept; but this needs a deep knowledge of the matter under study; in fact, LCA is based on Life Cycle Impact Assessment (LCIA) analysis, which on turn is based on Life Cycle Inventory (LCI) analysis (Miettinen and L~, 1997; Roy *et al.*, 2009).

LCI is the most labor intensive analysis to be carried over because of precise data collection needed; thus, for what concerns products, data should include all inputs and outputs of the processes where inputs are energy (renewable and non-renewable), raw materials, water, etc. and outputs are the products, co-products, by-products, and gaseous emissions (CO_2, CH_4, NOx and CO and SO_2) in air, water and soil (measured as: chlorinated organic compounds, total suspended solids, chemical oxygen demand and biological oxygen demand) and eventually solid waste generation (municipal solid waste: and landfills usage) (Suh and Huppes, 2005).

LCIA comes right after the LCI and aims to understand and evaluate environmental impacts based on the inventory output. In this phase, the LCI results are assigned to impact categories, relative to the expected repercussions on the environment. Thus, data are manipulated in few steps of classification, characterization, and normalization, in order to make the contribution to impacts comparable and aggregable, eventually producing the LCIA. To clarify and make examples, the most common impact categories include global contribution (global warming, ozone depletion, etc.); regional effects (acidification, eutrophication, photo-oxidant formation, etc.); and local effects (nuisance, working conditions, effects of hazardous waste, effects of solid waste, etc.) (Cavalett *et al.*, 2013).

Eventually, the purpose of an LCA is to draw conclusions that can support a decision or can provide a critical view on a particular product or process; whereas the tool of the Cost Benefit Analysis is suited and developed to make decisions, the right ones (if the models are well specified).

Cost Benefit Analysis (CBA)

“*The broad purpose of CBA is to help social decision-making. More specifically, the objective is to facilitate the more efficient allocation of society's resources*” (Boardman *et al.*, 1998).

A CBA is an ex-ante systematic process conducted to pinpoint hotspots of profitability. In other words, it is mainly adopted to help decision making process to allocate resources (A. Boardman, 1998), by the analysis of which decisions to make and which to avoid or postpone. Therefore, to evaluate the best choice among different investments or projects in uncertain situations on their payback and overall effects. CBA has a long history in management and economic studies, both for private and public investments, and since the 30's it has been used also for policy and environmental projects (Pearce, Atkinson and Mourato, 2006; Hanley and Barbier, 2009). Generally, this method helps to avoid being swept by the fashions of the moment (Prest and Turvey, 1966) by relying on economic data and evaluating the economic return, or loss, on a fixed timespan, with proper discount rates. The selection of a proper discount rate is fundamental to actualize future cash flows at present value (Tiwari, 1994; Zhuang and et al, 2007).

Therefore, in presence of multiple choices for a project development, an ex-ante CBA helps and guides the selection of the most profitable one, which maximizes the value of the investment by the comparison of net cash flows (discounted benefits minus costs) generated along a selected time frame. These values are expressed in monetary terms and reported to present values by applying a discount rate (equation1). In other words, Discounting reflects a social opportunity cost such as the return on the private or corporate investment displaced by government funding, the rate at which society is willing to trade-off consumption today for consumption tomorrow, the rate at which society expects wealth to increase in the future (and marginal utility of future benefits to decrease) thanks to economic growth; in this work of thesis it will also intercept a measure of risk. In fact, a higher discount rate generally means that there is more risk associated with the investment opportunity. Therefore, future cashflows should be discounted by a greater percentage because they are less likely to be realized. Conversely, if the investment is less risky, then theoretically, the discount rate should be lower on the discount rate spectrum.

A rational decision maker should opt for the investment with the highest economic return (Pearce, Atkinson and Mourato, 2006). Every cost should be considered, such as direct, indirect, fixed, variable, and also the intangible costs should be taken into account, where it's somehow complicated to express an economic value. Because even if it does not have a monetary value, it does not mean that it is free or meaningless, therefore a fictional value, based on solid arguments, should be attributed. Among the benefits, out of the economic ones, there could be intangibles benefits as well, thus they should be treated like the intangible costs (Zhuang *et al.*, no date; Atkinson and Mourato, 2008), and attribute a value.

$$NPV = \sum_{t=0}^{n} \frac{Rt}{(1+i)^t} - \sum_{t=0}^{n} \frac{Ct}{(1+i)^t}$$

Equation 1) Net Present Value Formula (Perman, 2003), where Rt are revenue, Ct are costs, i is the discount rate and t is the year of the timeframe.

Intangible benefits and costs are especially the case when environment and society are considered, which are usually hardly quantifiable and generally complexes; therefore, it is not rare to give monetary value to intangible benefit and costs on the basis of parametrized experts' opinions and/or surveys. Then if the model is well specified and solid in its considerations it is possible to evaluate the various outcomes. Eventually the decision makers should opt for the best solution in monetary terms.

Foresight Analysis

A foresight is a systemic, interactive and creative process of strategical evaluations to go beyond the visible, to perceive the utility of new choices, to gain awareness of hidden threats and problems, to develop visions for the future in the medium long period (even over 10 years), and thus detect possibilities innovations and further development, to optimize decision-making and policy interventions towards targets (Cariola and Rolfo, 2004). Then, the role of a foresight is to help and drive the allocation of limited resources toward solid targeted investments. Given that the opportunity for a business' success requires a perfect timing and the fruitful management of investments, since today is winning not only who comes first to an innovation, but mainly who is able to commercialize it first with products and services readily accepted by the market (Martin, 1995; Martin and Johnston, 1999). It is therefore important to develop a business strategy in agreeance with environmental and social context, future, and markets.

There are many viable methods in literature to perform a foresight analysis; the method selection will depend upon several factors, most notably available time, financial resources, and the objectives of the analysis (Georghiou, 1996; Popper, 2008; Rialland and Wold, 2009). The main approaches used in Foresight analysis are: Environmental Scanning, SWOT Analysis, Trend Extrapolation, Simulation Modelling, Genius Forecasting, Delphi, Scenario's analysis, Brainstorming, Expert Panel, Cross-Impact, Critical (and Key) Technologies, and Technology Road mapping (Georghiou, 1996; Popper, 2008; Rialland and Wold, 2009).

SWOT Analysis

SWOT analysis is a particularly helpful strategic management tool in acquiring a solid awareness of "Strengths", "Weaknesses", "Opportunities" and "Threats" (SWOT) of a project (company, issue, product), thus helping to overcome challenges and determine what measures to adopt to effectively pursue the target. Hence, the primary objective of a SWOT analysis is to develop a full awareness of all the factors involved in making decisions (Jain and Ewurum, 2015). Data and information to perform a SWOT analysis can come from general knowledge, conceptualization, reflections, and evaluation of impacts, of situations ranging from theoretical models through case studies to practical experience, and more. However, there are some limitations to this technique, where the main two are the need to link SWOT analysis to other strategic tools and methodologies for further theory building, and the lack of precisely quantifiable findings (Helms and Nixon, 2010; Leiber, Stensaker and Harvey, 2018).

Detach from linear paradigm.

Leaving the linear paradigm is a wide challenge that asks for contemporaneity of action in the deployment of circular innovations and models, while reworking the already existing linear processes into new circular ones through gradual processes. Since our entire economic system is based on fossil resources and their derivates, a complete change "out of the blue" is not even thinkable; rather the route to sustainability involves a gradual transition, in order to allow and concede time to businesses and people to adapt to change (turnover time), possibly turning problems into solutions. For example, while waiting for the complete transition to the "impact zero" in the automotive sector, it is possible to use biofuels to blend fossil fuels, both reducing fossil greenhouse gasses emissions and introducing in the market circular fuels, such as bioethanol, biomethane, and biodiesel. Biofuels are great examples of circularity, because they rely

on the natural carbon cycle, without emitting new fossil CO_2 in the atmosphere, in fact they are produced from biomass (also some biomasses can be directly used as biofuels) and organic wastes, after fermentation processes via microorganisms. Thus, with a change in perspective, there is the transformation of a waste into a vital resource, also with the advantage of producing small sized digestate that occupies smaller volumes in landfill or can be used as soil conditioner in cultivated camps. Eventually, with a relatively simple boost to biofuels technologies and a first step of blending, the benefits are multiple, aiming to optimization, exploitation and reduction.

However, when considering innovation and changes, especially in this historical era, it is no more likely to be possible to not consider people's wellbeing. The biggest challenge of the transition is then to put in practice the changes, without stressing too much the population, who are usually not willing to see deprivations to their lifestyle. An iconic example of this mindset can be found in the answer by the former U.S. president George H.W. Bush, during the "Rio Earth Summit" in June 1992, when asked which actions Americans were about to adopt in order to tackle environmental problems, he replied: "*The American way of life is not up for negotiations. Period*". Which highlights that the wellbeing is not a tradable good and should only increase in time. Therefore, an exemplificative composter for biomass, despite its various circular perks, is not well accepted by people because it "smells"; which also eradicates the possibility of deploying innovative "not-smelling" composters, since people will be diffident toward the technology that has the potential to harm their routine. Other parallel cases can be found in a wide range of situations, where the common baseline can be found in a "cognitive bias" induced by the lack on a proper knowledge on the topic, fear of the unknown, chemophobia, and "bad habits". Thus, effective communication becomes a crucial factor for the enhancement of social acceptance toward circular models and innovations.

Confirmation bias and NIMBY Syndrome

To detach from linear culture could be the hardest challenge, with no exaggerations, that human beings have ever faced, as a consequence of the effort needed to go against the exploitative, selfish, and greedy human nature. To clarify, despite the number of philosophical variants and attempts to produce a single definition of "human nature", yet not succeeded (Stenmark, 2012), human beings are, without space for discussions, part of the animal kingdom, therefore subjected to instincts and behavior rooted in the evolutionary path. Therefore, Instincts are from the most ancient "reptilian" or "primitive" brain, setting a plethora of automatic responses based on perceptions. With the passing of time the brain evolved, and the instincts were developed as well as emotions (Campbell, 2020); which include fear that is one of the most powerful tools for our survival (Angell, 2006), but it's also the root for many behaviors, some of which can be classified as "relatively bad". Among which there are the cognitive biases, especially the confirmation bias.

Confirmation Bias

"*Confirmation bias is the tendency to search for, interpret, favor, and recall information in a way that confirms or supports one's prior beliefs or values*" (Nickerson, 1998), and revolves around the need of cornerstones and strong points to anchor our place in the world and our beliefs; questioning, arguing, and changing them requires a marked strength in character (Campbell, 2020), which is not the case for the majority of people, on the basis of ego and arrogance blinding capacity. Thereby, an input like stories and

facts are more likely to be accepted by one, only if that matches its already existing strong points and beliefs alignment. Meaning that, if the acceptation process has been successful, then the input was not rejected, the one's belief are reinforced and so is the narrative. But what if the inputs or one's belief are corrupted, i.e., based on false data? In fact, false data can be acquired by misunderstanding topics and arguments, and / or through false narratives diffusion, and / or through fake news (twisted stories for morally despicable purposes or personal interests); thus, the consequence is that believes turns into misbeliefs. Whichever it is, it is not likely to be questioned, but only reinforced through acquiring other confirming inputs. Since there is a psychological mechanism of satisfaction that triggers when accomplishing a progress in cementing the cornerstones, a reward mechanism for having increased the stability in self-esteem and/or control, as well as a psychological defense mechanism, based on fear, which manifests in the unwanted will to account the possibilities that beliefs could be wrong or incorrect (Morry, 2005; Campbell, 2020); this mechanisms will be a crucial part for the first chapter of the thesis.

A very common example of confirmation bias is the habit of doing things in "the old-fashioned way" because "it has always been made like this", which eradicates the possibility for critical thinking; thus changes, progresses, and development, also showing distrust, skepticism, and suspicion toward what is "new".

NIMBY Syndrome

When facilities, installations, and new practices are to be introduced, a major stress comes along with changes in habits and contexts usually taken for granted by communities. Thus, imposed physical changes in landscapes, boundaries and surroundings, and non, may be seen and lived as oppressive and invasive, generally detrimental for the economic value of their properties and lifestyle quality. Thus, social rejection in forms of opposition committees and boycotting are deployed by communities. This phenomenon can find answers in the "Not In My BackYard" (NIMBY) syndrome theory (Peter Margulies, 1992; Rasmussen, 1992; Wexler, 1996; Xu and Lin, 2020), which refers to a particular condition where people and communities are willing to accept change and innovation, only if that change do not directly impact their daily life by being "not in their backyard". There are other variants of this social phenomenon, such as the LULU effect (local-unwanted-land-uses), the BANANA effect (building-anything-at-all-near-anyone), and the most radical NIABY effect (not-in-ANY-back-yard) (Pol *et al.*, 2006); however, NIMBY seems to be the most common one.

NIMBY syndrome theory can be analyzed from social-psychological perspectives through the study of classical theories of this field, like the "social exchange theory" proposed by Homans (1961) (Cook *et al.*, 2013), the "equity theory" by Adams (1965) (Adams and Freedman, 1976), "the attribution theories" in the tradition of Heider (1958), Jones and Davies (1965) and Kelley (1967) (Pol *et al.*, 2006; Bertram, 2011); however the crucial aspect of interest consists in its repercussions upon the shifting of the paradigm, from a linear culture, to a circular one; and as will be better described in a further chapter of this manuscript, the nimby syndrome, along with false narratives and wrong beliefs, played a crucial role in dismantling a win-win waste algal biomass correct handling project.

Pollution

Another main common factor within the thesis regards pollution issues; but what is pollution? The definition found on "Encyclopedia Britannica" is quite general and yet, the most complete: "*pollution, also called environmental pollution, is the addition of any substance (solid, liquid, or gas) or any form of energy (such as heat, sound, or radioactivity) to the environment at a rate faster than it can be dispersed, diluted, decomposed, recycled, or stored in some harmless form. The major kinds of pollution, usually classified by environment, are air pollution, water pollution, and land pollution. Modern society is also concerned about specific types of pollutants, such as noise pollution, light pollution, and plastic pollution. Pollution of all kinds can have negative effects on the environment and wildlife and often impacts human health and well-being*".

Interestingly enough, this definition states that modern society is mostly concerned by noise, light, and plastic pollution, despite the fact that there is plenty of pollutants within our daily life, for example in tap water and breathing air. However, this definition could be expanded by adding a layer regarding the source of pollution, if natural or anthropogenic and if "point source" or "non-point source", where the difference between the latest two lies in the possibility to exactly identify the source of pollution, e.g., a point-source could happen when a facility is discharging toxicants in a river, in opposition to the case of atmospheric particulate and pesticides, where the source is almost ubiquitous and not discriminable.

The "Environmental Protection Agency" (EPA) definition introduces a more pollutant-focused definition driving the attention on its chemical composition or quantity: "*The presence of a substance in the environment that because of its chemical composition or quantity prevents the functioning of natural processes and produces undesirable environmental and health effects*". Which completes the previous definition by also specifying the concept of "quantity", because even chemical compounds known to be nutrients, if overabundant can lead to unbalances in the ecosystems and thus causing problems and damages of various nature. This latest case of pollution is called: "nutrient pollution" and is typical, for example, of transitional waters, seas and coastlines when there is an abundancy of Phosphorous and Nitrogen that lead to noxious events of fish deaths, blooms of jellyfish, and harmful algal blooms, to cite some (Anonymous, 2000).

From industrial revolution, people have been exposed to a broad spectrum of substances of various nature, also due to the rapidly evolving technology, as well as technological clear conveniences; therefore, pollution may be seen as the progress' by product. In general, when discussing pollution phenomena, it is more likely to refer to classes of compounds rather than single substances, and this is due to the fact that chemical agents with similar composition and structure tends to behave in a similar way, diffusing through natural paths in water, air and ground with the possibility of being absorbed by living creatures and in the food chain.

Before introducing a context for the most concerning classes of pollutants, it is necessary to state that some pollutants are labelled as "priority" and "known", while other are "of growing concern" or "emerging"; this means that it exist a regulation procedure to introduce new entries on the pollutants' lists, for example the newly "Hazardous Air Pollutants" list from EPA (which is an updated version of the "1990 Clean Air Act Amendment") or the "1977 Priority Pollutant" list from EPA (updated, but yet quite old, version of the "toxic pollutant list"), or the "Clean Water Act" list always from EPA. Therefore, along with the pollutants lists, there are developed standardized analysis methods for quantitative determination and defined limit thresholds of concentrations, above which it is necessary to intervene and remediate. For what concerns the emerging pollutants, the US EPA defines emerging pollutants as "*new chemicals without*

regulatory status and which impact on environment and human health are poorly understood", because still to be studied in depth.

Bioaccumulation and Biomagnification

When a living organism absorbs in his tissues certain substances over time, so that their concentration is higher than that of the environment in which the organism lives in, it is called bioaccumulation. Problems arise if the compounds absorbed and accumulated show detrimental properties toward the organism, interfering with its biology, therefore reducing his health quality and wellbeing. Thus, this accumulation of pollutants, and the consequent quality of life reduction, is more marked for the top of the food chain's apex predators, in fact they can be directly subjected to pollutants from the environment, but mostly because of the constant and daily feeding on polluted prey, which are carrying a lifetime of bioaccumulated pollutants from feeding on the lower trophic level, which on their turn fed on polluted even lower trophic level, and so on until primary producers; this amplification phenomenon is called biomagnification within a trophic web. Needless to say that Humans are at the top of multiple food chains (Skarphedinsdottir *et al.*, 2010; Ali and Khan, 2019; Miller, Hamann and Kroon, 2020).

The interactions between organisms and chemicals depends mainly on two factors, namely physical retention and/or chemical affinity. As previously mentioned, the compounds' diffusion in the environment (air, soil, and water) depends on its chemical/physical properties, therefore it is possible to classify groups of pollutants on the basis of their nature, behavior, composition, and/or structure, thus on stability and reactivity in the environment, toxicant activity, bioaccumulation capacity, presence/absence of natural destroyers, and organic or inorganic nature, which are some of the main criteria.

Pollutants Classes

In order to better understand the fate and role of pollutants, it could be of use to clarify and discuss some of the major pollutant classes; where the most compelling examples may be found in the following list: Persistent Bioaccumulative and Toxic substances (PBTs), Persistent Organic Pollutants (POPs), PolyChlorinated Biphenyls (PCB), Pesticides, Polycyclic Aromatic Hydrocarbons (PAH), Volatile Organic Compounds (VOC), heavy metals (such as cadmium, mercury, and arsenic), organotin, and Organic Halogenated Pollutants (OHP), ozone, particulate matter (PM), Environmental Persistent Pharmaceutical Pollutants (EPPP), plastics and microplastics.

PBTs

Among many, one of the most infamous is the class of pollutants named PBTs, that stands for species that show Persistent, Bioaccumulative and Toxic behavior. Where "persistent" generally identifies that there is no natural disruptor or natural sink for that species, so that it stays in the environment for very long time, plastic is the easiest example of persistence. Whereas "toxic" means that the presence of that substance within an organism, given its nature, is detrimental for its biology, especially when endocrine disruption activity is present. Therefore, PBTs are species that have high resistance to degradation from abiotic and biotic factors, high toxicity, and high mobility in the environment. Consequently, PBTs have been observed

to have a high order of bioaccumulation and biomagnification, very long retention times in various media, and widespread distribution across the globe (Snyder *et al.*, 2000; Lipnick and Muir, 2001; Dórea, 2008a; Check and Marteel-Parrish, 2013).

POPs

POPs are another wide class of pollutants, also called “silent killers” or “forever chemicals”, specifically they can be identified mainly with organic substances, usually halogenated, with marked persistent behavior. Within this class is possible to find Pesticides, industrial chemicals like PCBs and diphenyl ethers, and industrial by-products like dioxins and furans (Alharbi *et al.*, 2018). POPs are typically hydrophobic and lipophilic, usually partitioning on solids both in water and soils, avoiding the aqueous phase. Therefore, when inside an organism, they partition into lipids, thus becoming stored in fatty tissue (Jones and de Voogt, 1999), favoring bioaccumulation and biomagnification. Known health repercussions, on humans and animals, due to POPs are diabetes, obesity, endocrine disturbance, cancer, cardiovascular and reproductive problems.

PCBs

Polychlorinated biphenyls is a very specific class because it is basically a single aromatic chemical, the biphenyl, with chlorine as a substituent in multiple sites and different combinations, which yield a congeners series of chemicals. PCBs were used blended with other chemicals as plasticizers and fire retardants and used in a range of products including caulks, adhesives, plastics, and carbonless copy paper; but historically, they had been used as electrical insulating fluids for transformers and capacitors, and also as hydraulic, heat transfer, and lubricating fluids (Erickson and Kaley, 2011). These chemicals degrade slowly in environment, and repercussions on health seem mild, however not correlated with serious adverse effects like cancer, or endocrine disturbance (Longnecker, Rogan and Lucier, 1997; Ross, 2004; Kimbrough, 2008). However, PCBs may be contaminated with the highly toxic family of the polychlorinated dibenzofurans (Kimbrough, 2008).

Pesticides

Pesticides are synthetic substances used for crop and livestock protection, thus, to minimize the yield loss due to pests (Özkara, Akyıl and Konuk, 2016). However, this term is quite wide and does not give any particular information upon the chemicals, but only the purpose i.e., to eliminate, destroy or regulate pests; among which: insecticides, herbicides and fungicides. Thus, pesticides vary in their chemical and physical properties depending on the target (Hassaan and el Nemr, 2020). Despite the numerous sub-categories, the most concerning pesticides are the ones that show toxicity, bioaccumulation and/or persistent behavior, e.g., organochlorine pesticides (such as: DDT and its analogs including dichlorodiphenyldichloroethylene (DDE) and Dichlorodiphenyldichloroethane (DDD), hexachlorocyclohexane (HCH), lindane, cyclodienes including aldrin, dieldrin, endrin, and heptachlor, chlordane, and endosulfan (Hassaan and el Nemr, 2020); in which, yet again, the presence of halogens bonded to carbon chains produces lipophilicity and chemical stability. This category induces health’s risks correlated to problems at the level of the central nervous system, by altering enzymatic nerve membranes

and electrophysiological properties (Tordoir and van Sittert, 1994). However, in the case of the Aral sea (United Nations Environment Programme, 2000; Hassaan and el Nemr, 2020), the contamination from pesticides is so hazardous that a plethora of other health problems correlated to cancer or other major disfunctions of the pulmonary, epithelial, and immune systems, rise.

Heavy Metals (HM)

More than half of the periodic table is made by metals and depending on their location in it, they can be, namely: alkaline, alkaline earth, transition, lanthanides (rare earth) and actinides. Though "Heavy" has little to do with the position in the periodic table, instead it labels those metals, and metalloids, with atomic number greater than 20 which have a density > than 5g /cm^3 (Ali and Khan, 2018). They occur as natural constituents of the earth's crust and are naturally persistent species since they cannot be degraded or destroyed. Hence, heavy metals can be essentials or non-essentials; the essentials are also known as micronutrients because the biological requirement lies in the magnitude of traces concentration (ppm, part per million mg/L and ppb, part per billion: µg/L); whilst the non-essentials are to be avoided (Duruibe and Egwurugwu, 2007). Therefore, "more than traces" concentration of essential and the presence of non-essential heavy metals constitute a pollution phenomenon; the main actors for heavy metals, given the hazardous consequences of their presence, are Cd, Cu, Pb, Zn, Al, Cr, Fe, Hg, Mn, Ni, As, Se (the latest two are not technically HM (Ali and Khan, 2018), but they are included as well in this list because of their chemistry and behavior) (Cheng, 2003). However, each metal has its own chemistry and speciation within different medium and conditions, thus also the detrimental effect on health and environment is proper of each metal. In general, HM's toxic effects can manifest through gastrointestinal disorders, vomiting, diarrhoea, stomatitis, tremors, paralysis, ataxia and convulsions, depression, and also neurotoxic, carcinogenic, mutagenic or teratogenic effects (Duruibe and Egwurugwu, 2007). Sources of this pollution is mainly through mining operations (Duruibe and Egwurugwu, 2007), industrial emissions, wastewaters, burning fuels and solid wastes vectors (Cheng, 2003).

Particulate Matter

A pollutant typical of both indoor and outdoor air composition is the Particulate Matter (PM) (Zagatti, Russo and Pietrogrande, 2020; Pietrogrande *et al.*, 2021), which is a class of pollutants that share the classification based on diameter dimensions, rather than chemical behavior or nature. Therefore, with Particulate Matter, solid material dispersed in the gaseous medium is intended; it is commonly indicated with the acronym PM, with a number as a subscript, e.g., PM_{10} and $PM_{2.5}$, which indicates the diameter of the particles expressed in µm (micrometers) (for comparison, a hair has a diameter of about 80 µm). The value of PM refers to the quantity of particulate material, per cubic meter (m^3) of air, with a hydrodynamic diameter (the diameter of a perfect solid sphere that would have the same hydrodynamic friction of the molecule of interest) lower than that reported in the PM subscript. The permanence, and diffusion, of particulate in the atmosphere depends on its chemical nature (density) and size, fine particulate tends to remain in the atmosphere for longer. Also, even health's risks are directly connected to dimensions, in fact, as the diameter decreases, a greater penetration into the respiratory system occurs, eventually reaching the blood circulatory system (Donaldson *et al.*, 2005; Romano *et al.*, 2020). PM of various sizes can be emitted naturally e.g., soil erosion, marine spray, volcanoes, forest fires, pollen dispersion, or anthropogenically through industries, heating, traffic and combustion processes in general. Particulate can

be of primary type, therefore emitted as is, or it can be of secondary type i.e., consequence of chemical physical processes of coagulation and condensation of smaller particles (ultrafine particles, down to molecules and atoms).

PAH

The pollutants in this class share structural patterns of fused aromatic rings and do not contain heteroatoms or other substitutes, thus weights can widely vary depending on the number of rings merged together. These differences in masses lead to different vapour pressure among PAHs (Callén *et al.*, 2008); therefore, the lighter ones can be very mobile through a multitude of deposition and re-volatilization processes, while the heavier are more likely to be more persistent and connected to the emission area and its surroundings (Wilcock *et al.*, 1996; Traven, 2013). Given their hydrophobicity (water solubility 10^{-10} and 10^{-13} mol/L), these pollutants are more likely to be adsorbed onto surfaces like suspended particulate, plastics and sediments; In this form they are more persistent to biodegradation in comparison to dissolved PAHs (de Luca *et al.*, 2005). In general, it is possible to state that PAHs are formed whenever substances are burned, more specifically they can be of biogenic, pyrogenic, petrogenic and diagenic origin; however, only the petrogenic and pyrogenic ones are present at biologically relevant concentrations in marine environments (ultimate sink for most pollutants) (Neff, Stout and Gunster, 2005; Booth *et al.*, 2007; Traven, 2013), therefore the main sources consist of industrial, traffic and domestic settings. For what concerns health's dangers, PAHs are characterized as priority pollutants since some of them are known, or suspected, carcinogens, mutagens and teratogens, with genotoxic and reproductive repercussions (Chen and Liao, 2006; Traven, 2013).

Plastic

The last class of pollutants is plastic, which is a generic term to identify material with synthetic origin made by polymers chains of carbon molecules; differences in the carbon molecules monomers lead to different physical properties in plastic products; plastics are mainly made with fossil carbon and petrochemical derivates. Then, given its flexibility of use and cheap costs, from its discovery plastic has been widely, and abundantly, used in every aspect of life; in fact, the invention and proliferation of plastics usage was not properly driven by a real need to develop and deploy new technologies, rather, to simply replace already existing objects, but with a price and quantity that helped to instantiate a regime defined by consumption (Davis, 2019). Modern society uses plastics to eat, to clothe, as soothers for babies, and also to support technology and internet, with thousands of underwater and underground cables sealed with plastic coating. Thus, what made plastics so attractive is also its major issue: that is its resistance to degradation, which lead to a strong persistence behavior in the environment. Moreover, given the variety of colors, shapes and forms, some plastics are constantly ingested by wildlife causing harm or agony, eventually killing animals by blocking their gastrointestinal or respiratory trait, or by physically choking and/or limiting movements, which leads to death by starvation or to be easily preyed upon. Plastics diffuses in ground and sediments and distribute along the water column and air, given the different densities and dimensions of the various polymers; however, in the long time it deteriorates due to physical stimulus, friction, and/or solar radiation, thus it fragments into smaller pieces, invisible to naked eye, known as microplastics. Although the topic of microplastics was first presented in 1972 by Carpenter and Smith(Carpenter and Smith, 1972), the scientific research on the issue did not show significant growth until 2013. Microplastics

show the same persistence of plastics, but higher mobility and more bioaccumulation, given its deeper penetration in living tissues. Hazardous effects of plastic on human health depend on its chemical nature and size of the debris, upon which depends the penetration in the bloodstream and tissues (Hwang *et al.*, 2019; Çobanoğlu *et al.*, 2021). In the latter sections of this thesis, a published paper of which I am co-author is reported; it is focused on the issues of plastic and microplastic ingestion by marine creatures and the consequences on the organism's health and endocrine disturbances (Chenet *et al.*, 2021). Another work, which I also participated, regards the study of the presence of plastics and microplastics in the gastrointestinal trait within a sample of one hundred "small-spotted catsharks" (Scyliorhinus canicula), one of the most abundant elasmobranchs in the Mediterranean Sea. The results obtained from this study indicates that the ingestion of plastics is a widespread phenomenon, with microplastics abundantly present in all samples and macro-plastics (dimension > 25 mm) in approximately 18% of the specimens collected (Mancia *et al.*, 2020).

"The present is conditioned by the accumulated traces of the past, and the future of the earth will bear the marks of our present. While the manufacture of plastics destroys the archives of life on the earth, its waste will constitute the archives of the twentieth century and beyond."

—Bernadette Bensaude-Vincent

Mobility

One last point to stress about pollutants regards their mobility that is characteristic for each species and varies in relation to which sphere (atmo-, geo-, hydro-) they are diffusing in; therefore, the main actors for pollution mobility is wind, rainfall, solids leaching, and temperature (which helps volatilize some, or precipitate others); but mobility may be also enhanced by the presence of carrier materials and/or organisms. In fact, migratory species that bioaccumulate pollutants act as vectors to improve mobility around the globe, as well as solid inorganic supports that can adsorb (i.e., superficial retention) pollutants.

For instance, plastics and micro-plastics in the sea are known to have the ability to carry hydrophobic pollutants (e.g., PBTs, POPs, PAH), this interaction produces roaming "super-pollutants" with enhanced ability to endanger life. In fact, plastic and micro-plastics are able to penetrate in living organisms with relatively ease, thus acting like a Trojan Horse for other pollutants that would have not entered so easily in the food chain (Wei *et al.*, 2019; Agboola and Benson, 2021). This should set a spotlight on the urge of removing plastic from our diet.

A tragicomic story regarding plastic pollution, which helps to relate with its mobility in oceans, is "MOBY DUCK", a book from Donovan Hohn; which is a story of a ship accident happened in the middle of the pacific ocean: where a container full of plastic ducks, turtles, frogs and beavers, broke and fell in the ocean. Precisely, 28800 plastic toys wandered and roamed upon tides and waves and throughout the years have been making their reappearance on shores and beaches of the whole planet: from Alaska and Russia to Washington and even in an otter lair.

More serious literature about diffusion regards the presence of microplastic in human blood (Leslie *et al.*, 2022) and in Antarctic's fresh fallen snow (Allen *et al.*, 2019; Aves *et al.*, 2022), this should be indicative of how deep and global the problem is, given the harmful effects proper of plastics, also in combination with the trojan horse effect introduced above. Settling even more urge to switch to other bio-acceptable materials, enhancing circularity and recycle, and removing existing plastics from the seas, because, at the moment, it can be considered like a poisonous phleboclysis.

Fragility and threats to transitional waters ecosystems services

Ecosystems services.

Ecosystems are “naturally balanced” systems, where the interactions between the different organisms (flora and fauna) that constitute the ecosystem contribute to a certain stability. Thus, an ecosystem must contain producers, consumers, decomposers, dead and inorganic matter, and also an energy requirement from an external source, usually the Sun. Vegetal life need sunlight and CO_2 to make photosynthesis and produce glucose, providing an energy and building source for themselves, those are called primary producers. Other organisms that depends on primary producers are the consumers, also organisms that relies on consumers are consumers as well; and eventually there are the decomposers which relies on dead organisms. Each ecosystem has its own peculiarity and interdependencies; however, the common baseline is the complex network of interactions going on: anything that impacts on one aspect of the ecosystem will, in turn, reflects on others. Chemical-physical factors are fundamental characters in these equilibria as well. For a water ecosystem, temperature, salinity, pH, and dissolved oxygen might be determining factors that, if altered, induces imbalances, stress, crises, and so on; for example one of the factors contributing to the coral reef ecosystems going extinct is indeed the rise in the ocean’s temperature (Jokiel and Coles, 1990).

Human race, during its classification as a dominant species, emerged from the concept of small medium ecosystems, to place itself at the top of the global system, picking and exploiting natural resources at will, sometimes overexploiting. Natural resources coming from an ecosystem can be defined as its productivity, which, according to the Dictionary of Ecology, “*refers to the rate of generation of biomass in an ecosystem, usually expressed in units of mass per volume (unit surface) per unit of time, such as grams per square meter per day (g m^{-2} d^{-1}). The unit of mass can relate to dry matter or to the mass of generated carbon. The productivity of autotrophs, such as plants, is called primary productivity, while the productivity of heterotrophs, such as animals, is called secondary productivity*” (Allaby, 2015).

Therefore, given the almost free availability of natural resources, ecosystems were, and are, assumed like services to enhance our wellbeing, since there is no “price to pay for picking”, only the “fatigue in doing the act”, thus the name Ecosystem Services (ES). Meanwhile some debate, with worthy reasons, the name itself of “ecosystem services”, since it poses the environment in a lower step than humans, favoring the anthropocentric view of the world and strengthening the already deeply rooted, exploitative relationship among human and environment (Schröter *et al.*, 2014). The reality of facts is that humans depend entirely on ES, but very little has been made to keep the equilibrium balanced. Moreover, after decades of growing stress and pressure, worldwide ecosystems are becoming dysfunctional and lesser suitable to sustain economic activities and the constant human growth, providing less resources than in the past (D.J. Rapport, R. Costanza and A.J. McMichael, 1998).

Pollution, climate change, ocean acidification, and desertification, are stressors and equilibrium changer for ecosystems, adding weight to the already existing pressure deriving from anthropic activities (including overexploitation of resources). Then, the fate of a stressed ecosystem, if not recovered or restored, is to get deteriorated, which leads to its compromissions and utter changes, eventually, in the worst case, the formation of a dead spot. However, each system is different from one another, given the number of variables into play, therefore its decline route and timing depends on its organization, resilience to stress and general vigor of equilibria (D.J. Rapport, R. Costanza and A.J. McMichael, 1998; Costanza and Mageau,

1999). Thus, a healthy ecosystem is the one that is sustainable (Costanza and Mageau, 1999), even though some ecosystems can be healthy but also more fragile than others.

Hence, given both our direct and indirect negative impacts on ecosystems, their overall value should be re-evaluated in the light of other factors that today are taken for granted (such as a shelter action) and also considering remediation costs. In fact, the value that we attribute to a certain ES is in function of the economic volumes that revolves around it, which is a partial economic consideration, since, yet again, the "price to pay for picking" is not included, but only the "fatigue of the act" is monetized.

In recent year economists and ecologist are trying to attribute realistic monetary value to ES in order to address the importance of natural features, translating them into relatable forms of capital (Tallis and Kareiva, 2007). However, it is not an easy task given the problem of attributing monetary value to something natural, free and granted, as it can be the protection from storms typical of forested ecosystems. Hence, in some cases, an additional layer of complexity may derive from assumptions upon the people's willingness to pay for a common service. The first issue is connected to the willingness to pay, which is individual dependent, thereby subjective; whereas the second issue regards the consequences of an equivalent of the "tragedy of the common" economic theory, when applied to a change in the socio-economic paradigm. More specifically, given that "*the tragedy of the commons refers to a situation in which individuals with access to a public resource (also called a common) act in their own interest and, in doing so, ultimately deplete the resource*" (1833, William Forster Lloyd; Harvard Business School insights) where the "public resources" are instead "services provided naturally" and possibly not exploited equally ("in respect to their own interest"). Therefore, attributing valor and capital to a priorly free ES (changing the paradigm) can lead to social problems and unpleasant speculations by individuals who may feel "robbed" and/or "tricked", because they haven't exploited that service as much as others, when it was valueless.

However, there is not a consensus on a proper ES' taxonomy, though within the "Millennium Ecosystem Assessment" it is possible to find a groupings approach that align ecosystem functions with marketable goods and services. For example, the functions are identified as provisioning, for food, water, fuel, fiber; regulating for prevention of soil erosion, flood control; cultural for recreation, spiritual value, sense of place; or supporting for soil formation, nutrient cycling, oxygen from photosynthesis (Tallis and Kareiva, 2007).

In addition it is stated in the report: "*the objective of the Millennium Ecosystem Assessment was to assess the consequences of ecosystem change for human wellbeing and to establish the scientific basis for actions needed to enhance the conservation and sustainable use of ecosystems and their contributions to human wellbeing*", thus the focus is to quantify on a realistic economic scale the change of the health status of an ecosystem, acknowledging gains and losses.

Fragile Ecosystems.

In the previous section, the concept of "fragile ecosystem" was introduced, and it may seem a simple concept, but in reality, its background is quite complex, since there is not a general agreement on its definition; instead, there are various considerations. The main approach to the concept of fragility can be found in these two main perspectives: "*areas that are naturally fragile because of large, natural, and internal successional changes (i.e., they change by themselves during natural evolution of landscapes)*", and "*areas that change as a result of external pressures, induced by humans*" (Nilsson and Grelsson, 1995).

Thus, the common ground, among these two, regards a mutation process; while, the difference lies in the nature of this process, therefore the perspective of fragility which considers human impacts might be the most useful one, since it accounts the causes and may open for remediation possibilities upon repercussions.

Hence, as previously introduced, fragility represents the degree of response to a change process; then, a fragile ecosystem has a scarce ability to address changes. However, the definition is still not exhaustive, in fact a change may be triggered by the variation of one or multiple factors or even their consequences. Thus, a change could be measured in four scales: temporal, spatial, taxonomic resolution, and numerical resolution (Nilsson and Grelsson, 1995). Where the first two scales regards the time and space coordinates, whilst the latest two regards modifications to the participants of the ecosystem: including invasion of alien species and extinction phenomena. In fact, the effects of external stressor, on a proper portion of the ecosystem for a proper time, may lead some participants of the ecosystem to contextual extinction, compromising the balances among trophic groups; or to leave, which can represent, in turn, a stressor effect for other ecosystems.

Out of pollution issues, climate change is the multifaced factor that adds a noticeable weight on ecosystems on a global scale: on land by favoring desertification processes, while in seas and oceans with the increase in water temperature.

Transitional waters.

Through history, cities have developed along coastlines due to various socio-economic benefits: transports, defense, trades, and access to food are some of the determining factors. Thereby, the interdependence between our society and coastal cities is multifaced and fundamental, especially for what concerns high productivity regions, which can be found in transitional waters ecosystems (TW, TWE). In fact, within the transition between the land and the sea, and hence, from freshwater to marine environment, a number of ecosystems with different physiographic forms can be found, and if the following characteristics are matched (McLusky and Elliott, 2007), then it is possible to identify a TW environment:

- Classical estuary, tidally dominated at the seaward part; salinity notably reduced by freshwater inputs; riverine dominance inward.
- Fjord, land freshwater seepage or markedly seasonal riverine inputs; limited tidal influence; stratified; long narrow, glacially eroded sea inlet, step side, sill at mouth.
- Lentic non-tidal lagoon, limited exchange with the coastal area through a restricted mouth; separated from sea by sand or shingle banks, bars, coral, etc. shallow area, tidal range ≤ 50 cm.
- Lentic microtidal lagoon, as above but with tidal range ≥ 50 cm.
- Ria, drowned river valley, some freshwater inputs; limited exchange.
- Fjard, glacially carved embayment, sea inlet, smaller than fjord; limited freshwater inputs. River mouth, river outlet as well-defined physiographic coastal feature.
- Delta low energy, characteristically shaped, sediment dominated, river mouth area, estuary outflow.
- Coastal freshwater/ brackish, water plume outflow of estuary or lagoon, notably diluted salinity.

It comes along that TWs are often characterized by shallow waters that are rapidly influenced by external environmental changes like salinity and chemical changes like nutrients concentrations, thus they are considered to be naturally stressed (Facca, 2020). In these systems biota have adapted to cope with this "natural" stress; therefore, the species' taxonomic richness tends to be limited compared to the adjacent sea or freshwater environments (Facca, 2020). Also, the morphological structure and the relative isolation provide a sheltered habitat for numerous species of flora and fauna, and a remarkable productivity, making TWs valuable ecosystems, also from the perspective of services provided.

A special focus is set upon estuaries, deltas, and coastal "transitional" lagoons, which are widely known to be elective habitats for abundant and diversified potential for fishery and/or aquaculture operations (Cavraro *et al.*, 2019). "Transitional" was specified for lagoons because, given the European Environment Agency's definition for TWs: "*Bodies of surface water in the vicinity of river mouths which are partly saline in character as a result of their proximity to coastal waters, but which are substantially influenced by freshwater flows.*", not all lagoons match the "*substantially influenced by freshwater flows*" prerequisite of the above definition, thereby showing other peculiarities.

TWEs show remarkably high productivity, given the constant shuffle of nutrients and organisms from inland freshwater and seawater, physicochemical factors (e.g., variation in salinity), peculiar morphological habitat features, and the fluctuating water levels caused by tides; these environments are allowed to play several ecological functions, such as feeding grounds or spawning and nursery areas for many fish species (Cavraro *et al.*, 2019), mussels and bivalve, and hosting non-fish animals, some of which are of conservation or economic value; delivering an extensive range of ecosystem services that provide many ecological, cultural, and socioeconomic benefits, in terms of provisioning, regulating services, supporting services, such as oxygen production from photosynthesis; and cultural services, such as recreation and ecotourism (Newton *et al.*, 2014).

TW's fauna is a key component of aquatic ecosystems, mainly for its important ecological role in maintaining aquatic food webs and more. Hence, the importance of these locations is a consequence of the ESs that they provide, which are highly valued by society in terms of economic and social terms (el Mahrad *et al.*, 2020). Beyond the natural beauty of TWs, that is subjective, they may be home of iconic animal species, and peculiar landscapes, fundamental for local's belonging feeling and pride, and also for tourism activities. See images below, figure 3 and 4, as examples of the iconic species of pink flamingos, and landscape proper of "Goro's Lagoon", belonging to the UNESCO's protected "park of the Delta".

Figure 3) Pink Flamingos, iconic animal of the park of the Delta.

Figure 4) Suggestive landscape proper of Goro's lagoon.

For what concerns the economic sphere, the main sectors involving lagoon ecosystems may include, broadly speaking, aquaculture activities (fishing, shellfish harvesting, and algae farming), salt and sand extraction, and maritime transports/tourism. Whereas areas surrounding the lagoons may present subsidiary activities to support fishing and aquaculture, agriculture, tourism, recreation, and industrial development. Also, from a geoscientific perspective, i.e., geological, and geomorphological, it is important to emphasize that lagoon water bodies are to be considered as: "naturally subjected to changes", given their features at the sea interface with land, as reported in the following quotation: "*There are three broad classes of coastal inlet – lagoons, estuaries, and deltas – which form a continuum and are difficult to define*

as entities. At one end of the continuum are lagoons produced solely by marine action and lying between some sort of barrier feature and the original coast. Then come estuarine lagoons where a river emerges into a lagoon which still owes most of its form to the sea. In the middle of the continuum are estuaries which are essentially the lower courses of rivers more or less invaded by the sea and which may or may not be partly blocked by marine barriers. Further along still are estuarine deltas in which there has been appreciable infilling of the estuary and the river bifurcates around the fill. Finally at the other end of the continuum come deltas in which river action is so strong that it causes progradation in one of many forms" (Davies, 1972). Therefore, in order to maintain lagoon services at the top of their efficiency, given their ephemerality, changes in hydrology and sedimentology, inlet modifications and coastal engineering are measures usually deployed (Duck and da Silva, 2012). Despite the ecosystem's fragility, these measures are to be considerate as a harmless anthropogenic intervention (if wisely projected), since aimed to maintain the lagoon's health and status constant.

Aquaculture activities importance.

The aquaculture sector represents a very diverse group of different aquatic plant and animal species cultivation spanning from unicellular Chlorella algae within inland bioreactors to the production of Atlantic salmon (Salmo salar) in outdoor floating net cages (Tacon, 2020), transitional waters are particularly prone to host some forms of aquacultures. However, in order to better figure the role and importance of aquaculture activities, it is possible to commence by looking at its definition and, also for this case, multiple variants, slightly different among themselves, can be found. The following is from Wikipedia, but also "FAO" (in 1988) and the "National Ocean Service" provided their definition, "*aquaculture is the controlled cultivation of aquatic organisms such as fish, crustaceans, mollusks, algae, and other organisms of value such as aquatic plants (e.g., macroalgae, lotus etc. etc.). Aquaculture involves cultivating freshwater, brackish water, and saltwater populations under controlled or semi-natural conditions*". Thus, aquaculture encompasses a wide range of species and cultivation methods, resulting in various social, economic, nutritional, and environmental outcomes. Broadly speaking, it involves the cultivation of various foodstuffs and biomasses with the advantage to make them in salt-, brackish, waste-, and fresh-water; with little, or none, cultivation and farming efforts (e.g., adding nutrients and freshwater). These two key factors may be the most outstanding advantages of these practices.

As a matter of fact, to grow nutrient-rich food goods without using dryland and soil is per se a winning situation, moreover there is no need (in most cases) to use freshwater (because of the cultivation's nature itself) and to add nutrients, because marine and brackish ecosystems can provide all the necessary energy; yielding a particularly sustainable formula (e.g., mariculture). Thereby, over than 95% of global aquaculture production is currently realized by developing countries, in clear contrast to most terrestrial agricultural food production systems, and with an increasing production trend. Also, given the rising profitability (global production valued at over US$250 billion) (Tacon, 2020), aquaculture activities are fast developing businesses, started back in 1950, that are now growing sources of income and wellbeing; as it can be seen in the dataset: "Aquaculture Production (Quantities and values) 1950-2020 (Release date: March 2022)" within the "fishstatj, software for fishery and aquaculture statistical time series", belonging to FAO (Food and Agriculture Organization) (FishStatJ, 2022), more and more countries are implementing, enhancing, and intensifying those activities; with Asia leading the way. This growing trend is not going to change soon, since the demand for seafood will increase significantly over the medium term (2030–2050) if historical trends of income and urbanization are maintained (Willett *et al.*, 2019; Gephart *et al.*, 2020). Also, given the

needs related to a fast-growing population, estimated ten billion by 2050, and facilitate the shift in dietary trends: from a more polluting and poorly resource-intense food and with lesser nutritional value toward a healthier and more sustainable one (Subasinghe, Soto and Jia, 2009; Tilman and Clark, 2014).

Despite its growth, aquaculture development is hindered and threatened by a number of pressure and stress sources. These include, overexploitation, technology limitations, and multi-use conflicts (Goldburg and Triplett, 1997; Frankic and Hershner, 2003). Starting from the latter, the "multi-use conflict" is a social issue occurring when tensions happen between two or more sectors that overlap economic interests between characters and their respective industries, or even aspects of local tradition (Walters, 2007). On the other hand, technology in this field is still at its embryonic level and thus may play the role of limiting factor for growth. Overexploitation is one of the major anthropogenic stressors, since it depletes resources and weakens the ecosystem, in fact aquaculture can be seen as the exploitation of a particular ring of an ecosystemic chain; therefore, insisting and stressing too much may lead to its break, while ought to be balanced, as everything should be.

Detrimental factors for transitional waters.

The ecosystem's productivity is linked to the aquaculture exploitation potential, thus transitional waters are most suitable to host activities of aquaculture, especially that of lagoons, estuarine, and deltas; given their marked productivity. However, along with productivity, also fragility is an important character for these environments; therefore, human impacts can influence the transitional water by exerting multiple pressures on ecosystems and inducing physical changes in the environment, affecting salinity, dissolved oxygen, and erosion as well; leading to changes in the ecology, with loss of biodiversity; and changes in the delivery of valuable ecosystem services (Geijzendorffer *et al.*, 2019). Overexploitation is among the first source of pressure that leads to resource depletion and environmental stress; not to mention that TW could be among those environments most affected by the ongoing climate change (Harley *et al.*, 2006), alien diffusion, pollution issues, and a combinations of these stressors. Coastal waterbodies then, will be subjected to the combined modifications taking place in the atmosphere, in the oceans, and over the land surface (Raimonet and Cloern, 2016; Pesce *et al.*, 2018).

Alien diffusion

The term "alien" refers to something not belonging to a certain context; speaking of flora and fauna an alien may be represented by a foreign species, outside of its normal distribution, that has the potential to alter normal equilibria of the host ecosystem. Alien species can diffuse spontaneously and/or intentionally or unintentionally by anthropic interventions. Usually, among various definitions, this term has an overall negative connotation, indicating an invasive effect with detrimental repercussions, it may also be referred as "biological pollution" (Elliott, 2003); however, this is not always true since alien species can show beneficial effects as well. Special case is the Mediterranean Sea, which is one of the most affected seas around the globe, both in terms of duration that invasives have been present and in numbers of alien species detected (Occhipinti-Ambrogi, 2007); in fact, for example, since ancient times species were unintentionally diffused from ships, both in cargo and upon the ship's hull, or the unintentional and spontaneous introduction of Erythrean species, whom entered the Mediterranean through the Suez Canal, and gained commercial interest for local markets. However, despite some isolated cases of beneficial

repercussions or successful introductions for economic returns, detrimental effects usually manifest in the alteration of equilibria, such as the outcompeting of local species in predation or feeding activities, or by the elimination of a certain trophic level, eventually leading to reduction or even extinction of local species, with compromission of the ecosystem and loss of biodiversity.

Climate change

The ecological consequences of climate change on transitional waters will largely depend on the rate and magnitude of change in two critical environmental drivers, which are namely, temperature and water availability from precipitation and runoff; these factors regulate many ecological processes in aquatic ecosystems, both directly and indirectly (Poff and Day, 2002). Temperature directly affects many processes essentials for life, and a change in the thermal regime (e.g., extreme temperatures, their duration, and seasonal rates of temperature change) directly affects the growth rate and reproduction behavior for species. Since individual aquatic and wetland species are adapted to a specific range of temperatures, global warming may induce these species to undergo stress and lose population or to move elsewhere, possibly becoming invasive alien species in other stressed ecosystems (Poff and Day, 2002). On the other hand, the water volume for an aquatic ecosystem directly influences its characteristics and processes, by determining the amount of available habitat and many aspects of water quality. Therefore, precipitation and runoff pattern dictates how the volume of water in transitional ecosystems changes over time. Hence, ecosystem services are in equilibrium with the hydrogeological regime (seasonal pattern of magnitude, frequency, duration and timing of runoff) of that specific area. Hence, seasonal variation in water volumes strongly influences what kinds of species can thrive and flourish in the aquatic system; thus, a change in local climate that alters the existing hydrogeological regime has the potential to greatly modify habitat suitability for many species and cause significant ecological changes (even if the thermal regimes remain unchanged) (Poff and Day, 2002).

Climate change and the consequent rising temperature and hydrogeological changes are actively modifying the natural cycles of biotic marine life, becoming one of the major and ubiquitous stressors toward ecosystems around the globe. Many variations induced by climate change are especially effective for what concerns TWEs, which, as previously introduced, are particularly fragile. In fact, they rely on trophic webs with relatively low taxonomic richness, therefore the lack of even one trophic level may compromise the whole ecosystem; also, TWEs equilibria are heavily influenced by freshwater apports; nonetheless, the shallow water with poor hydrodynamic circulation tends to badly handle heat energy dissipation i.e., water temperature rise, which is an important environmental cue for migration and regulates many ecological processes (Poff and Day, 2002). These effects play fundamental roles in eutrophication phenomena, especially when combined with nutrient pollution.

Pollution

Given their location at the interface between land and sea, transitional waters' dynamics are controlled by hydrological factors which operate locally at several temporal scales, ranging from daily to seasonally and even annually. Thus, mixing and transporting processes in TW systems are driven by tides, river flows and winds; where river's flow could be regarded as the main factor that controls physicochemical parameters in the TWEs (Zaldívar *et al.*, 2008). Thus, pollution has multiple vectors of interaction toward transitional ecosystems from the seawater, freshwater, and the coastline.

Coastline waters can be seen as a buffer region dependent on tides and river's outflow, there could also be the presence of both point and nonpoint pollution sources (point source like industries and activities, and non-point-source intended as garbage and litter in general); further in the thesis, a situation of detriment caused by waste materials' bad handling will be discussed. Vectors from the sea and freshwater are more complex. In fact, sea pollutants diffusion is connected mainly to salinity gradients, temperature gradients, winds, currents, and tides (Renzi *et al.*, 2020). As introduced in the "pollutants mobility chapter" for what concerns plastics and microplastics diffusion, the Moby Duck story is iconic and goes in parallel with the actual situation of continents made of plastics in our oceans (Figure 3), also called trash vortexes and garbage patches, as a result of the above factors. Therefore, seawater contribution of pollutants toward transitional waters can be highly variable and of multiple magnitude.

Figure 3) Garbage continents in our oceans. (Blue-Growth, 2018)

Instead, freshwater contribution is particular because it regards the whole hydrogeological basin of the rivers afferent to the water course which carries the freshwater to the TW. The freshwater pollutants load, in terms of species and quantities, is directly correlated to the anthropic activities carried on land; for example, areas greatly interested by anthropic presence and activities such as factories, populous cities, and intensely cultivated fields, contribute to a multifaced pollution situation. This happens because, given anthropic presence and activities, it is normal to find various classes of pollution sources, which contribute, alone, or in combination, to harm flora and fauna locally, and while reaching water courses and rivers.

As a matter of fact, the fate of the inland generated pollutants is to follow the river's flowing waters up to its outfalls. Throughout the river's course it is possible to spot, among many, macroscopic piece of trash and gross solids, consisting of materials such as styrofoam, metal containers and plastic packaging, which are unsightly and persist in the environment. Moreover, sand, grit and other fine solids litters and debris can be washed by stormwater, or run-off from farms and roads, into the rivers. Microscopically, instead, there could be microbiological risks, chemicals and micropollutants (e.g., PBTs, heavy metals, pesticides, PAH, PCB, drugs and their metabolites), and nutrient pollution (Chaudhary, Mishra and Kumar, 2017; Grizzetti *et al.*, 2017).

As mentioned in previous sections, the introduction of chemical substances, micro and macroscopic, adds pressure and stress to the river's outfall, i.e., the transitional ecosystem, its services, and human wellbeing as well (Booth *et al.*, 2007; Dórea, 2008b; Suja, Pramanik and Zain, 2009; Rastogi, Mahmoud and Kümmerer, 2017); with an immediate and catastrophic time scale, or else, it can build slowly by accumulating in plants and animals tissues.

Some evaluation measures and techniques can be deployed to assess the stress and pressure upon marine and transitional ecosystems, among which there is the measurement of physicochemical parameters, such as, dissolved oxygen, temperature, chlorophyll, salinity, conductivity, and more (Giordani, Zaldívar and Viaroli, 2009). Also, it is possible to identify species with certain prerogatives, to exploit them as monitoring agents for their respective ecosystem's health status. For example, studying the genic expressions of dolphins (Mancia *et al.*, 2007) can highlight differences in the response of the organism toward external stress, in comparison to the genic expression of a healthy specimen, as a consequence, the heavier the stress the deeper the modifications in its genic expression.

Combined effects: eutrophication and Harmful Algal Bloom.

The process leading to an enrichment of nutrients, like nitrogen (N), phosphorous (P), minerals, organic matter, and other chemicals in a water body, is called eutrophication (Poff and Day, 2002; Zaldívar *et al.*, 2008); this phenomenon affects primary producers, thereby the first consequence is a bloom in algal organisms, with various repercussions on the whole ecosystem.

When occurring naturally, eutrophication is a very slow process in which the above-mentioned nutrients, accumulate in water bodies. These nutrients derive from the slow degradation and dissolution of minerals from rocks, naturally and/or by the effect of lichens, mosses and fungi, that actively scavenge rocks for nutrients (Sawyer, 1966). Anthropogenic eutrophication is often a much more rapid process in which nutrients are added to a water body from a wide variety of polluting sources, i.e., nutrient pollution, is a primary cause of eutrophication of surface waters, in which excess nutrients stimulate algal and aquatic plant growth.

The shallow depths of TWs implies that benthic communities and benthic biogeochemical processes are the major character driving the nutrient cycles. Thus, Submerged aquatic vegetation (phanerogams and macroalgae) and microphytobenthos are those that will respond to nutrient overabundance and control the overall primary production (Zaldívar *et al.*, 2008). As such, entity and patterns of primary production should depend on the dominant components within the primary producers community; and a combination of hydrological and geomorphologic factors could greatly influence the dominance of the primary production extent. For example, with phytoplankton and macroalgae in semi closed and restricted lagoons and benthic phanerogams in leaky ecosystems (Zaldívar *et al.*, 2008), reacting to the over enrichment of P and N.

The excess of P can find its non-point sources among detergents rich in phosphates, industrial and domestic runoffs, and soil conditioners. However, after the phasing out of phosphate-containing detergents, back in the seventies, and despite the improved wastewater treatments, industrial and domestic wastewaters, sewage and agriculture's run-offs have emerged as the dominant contributors to eutrophication (Werner, 2009). While, The main N non-point sources, beside the natural fixation processes, are from agricultural run-offs (fertilizers and animal wastes), from sewage and from atmospheric deposition of nitrogen originating from combustion (NOx).

The anthropogenic algal bloom is then a rapid growth of algal biomass; though, “algae” is a general term which include photosynthetic oxygen-emitting aquatic organisms with a simple structure without stems, roots, or leaves. Also, they cannot be easily defined since they don’t fit under a single monophyletic group, in fact they exist in various forms and sizes, macro- and micro-algae, where few possess motility perks, like bacteria; otherwise, they simply follow the currents, or floats on the water surface; some grow in soils, trees, and animals by establishing a symbiotic bond with other organisms.

In the trophic network they are primary producers, therefore supporting several communities of herbivorous animals (invertebrates, such as some sea urchins and/or gastropods, and vertebrates, such as herbivorous fish), also macroalgae may act as a shelter against their predators, the carnivores. Moreover, In order to escape herbivory, which is sometimes intense (in natural reefs or rocky walls of the continental shelf), many macroalgae have been improving defense strategies, an example with calcification (common with red algae) being one of the most common; also, they can produce secondary metabolites, such as terpenes, aromatic substances, and polyphenols that act as demotivators for their ingestion by the predators (Pereira, 2021). Despite some similarities in trophic functions, there are important differences among micro- and macro-algae.

In detail, microalgae lack complex multicellular structures with great variation in their internal cell structure (Vaishnavi *et al.*, 2019), basically being planktonic forms of life; critical food for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) and for larvae of commercially important crustaceans and finfish. In most cases, the proliferation of plankton algae (up to millions of cells per liter), i.e., the algal bloom, is beneficial for aquaculture and wild fisheries operations.

However, the term “algae” is commonly used to refer to “marine macro-algae” or “seaweeds”. There are three major types of macroalgae in the marine environment, divided on turn in smaller categories: approximately 1800 different brown, 6200 red, and 1800 green macroalgae. Although there are substantial differences, all of them are organisms that perform photosynthesis, and some can reach up to 50 meters in size (Pereira, 2021). Green macroalgae are included in the Chlorophyta phylum, and their pigmentation is identical to that of vascular plants (for the presence of chlorophylls and carotenoids). While Red macroalgae belong to the Rhodophyta phylum, and have mostly phycobilins and carotenoids as photosynthetic pigments, chlorophyll- “a” is also present. Ochrophyta is the belonging phylum of brown macroalgae, where the pigmentation is due to chlorophylls “a” and “c” and carotenoids (where fucoxanthin predominates, responsible for their brownish color) (Pereira, 2021).

Other than the peculiar cellular structure, size, behavior, and morphology of the different phyla, algae show characteristic amounts of carbohydrates, lipids, minerals, and proteins, where this chemical composition varies across species and also along seasons (Renaud and Luong-Van, 2006). In addition, chemical composition is influenced by environmental factors (temperature and irradiation) and by the composition of the medium in which algae grow (Sutkowy, Lenarczyk and Kłosowski, 2018). Also, they can selectively accumulate substances, especially heavy metals, from the environment; for this reason, the possibility of using them as bio-remediator is nowadays studied; some cases from literature are reported (Bishnoi and Pant, 2004; Chekroun, ben Chekroun and Baghour, 2013; Hanbali, Holail and Hammud, 2014; Mahmood, Mirza and Shaheen, 2015; McGaughy *et al.*, 2019; Nguyen *et al.*, 2020).

However, in some situations algal blooms can show negative repercussions, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts (Hallegraeff *et al.*, 1995). This happens when some of the aforementioned effects of TW quality depletion, or their synergic combination, e.g., nutrient pollution and global warming, gains enough

relevance to trigger strong eutrophication phenomena, also known as: “Harmful algal bloom” (HABs). Probably, the first written reference to a harmful algal bloom appears in the Bible (1000 B.C.), Exodus 7:20 / 21: *‘... water of the Nile, and all the water was changed into blood. The fish in the Nile died, and the river smelled so bad that the Egyptians could not drink its water*’. During the 1960s and 1970s, harmful algal blooms were thought to be linked to nutrient over-enrichment resulting from anthropogenic activities such as industry, agriculture, and sewage disposal (Michael F. Chislock, 2013). For example, blooms of blue-green algae (i.e., cyanobacteria diatoms and dinoflagellates) may lead to the known consequences of eutrophication, including tainted drinking water supplies, degradation of recreational opportunities, hypoxia (Michael F. Chislock, 2013) and production of highly dangerous toxins (Hudnell, 2010). $2.2 billion annually is the estimated cost of damage mediation for eutrophication damages in the U.S. alone (Michael F. Chislock, 2013).

Generally, the effects of strong eutrophication include the formation of dense blooms of noxious, foul-smelling phytoplankton that reduce water clarity and harm water quality. Moreover, this dense film limits light penetration, reducing ecosystem productivity and growth, thus causing plants die-offs whilst also lowering the success of predators that need light to pursue and catch their prey. In addition, high rates of photosynthesis associated with eutrophication depletes dissolved inorganic carbon and raises pH during the day; altered pH can, in turn, limit the survivability of organisms that rely on perception of dissolved chemical cues by impairing their chemosensory abilities. Eventually, when these dense algal blooms enter the decline phase, microbial decomposition severely depletes dissolved oxygen, creating a hypoxic or even anoxic “dead zone” or “dead spot” by the lack of the necessary oxygen to support most organisms (Michael F. Chislock, 2013). Dead spots are hardly remediable and reversible, given that the ecosystem is broken, and only the passing of time and/or anthropic interventions can restore it, with the introduction of a whole new ecosystem.

HABs are also known to occur with macroalgal character, especially in temperate and tropical waters, and are particularly prevalent in productive estuaries and shallow coastal waters where they are triggered by strong eutrophication effects, poor water flushing, low wave action and high temperature and irradiance; lastly, macroalgal blooms can be exacerbated by the removal of herbivorous grazers (Joniver *et al.*, 2021a). Thus, the rising of mean global temperatures have resulted in an increased macroalgal biomass blooms nuisance both in intertidal and subtidal eutrophic estuaries. Alike the other type of microalgal blooms, once established, macroalgal blooms can lead to declines in seagrass populations (e.g., the angiosperm Neptune grass) and oxygen depletion altering zoobenthic communities. Other than the formation of anoxic dead spots, the natural end of an HABs is also connected with further problems, occurring when the biomass starts to decay. Therefore, noxious gases can be released, such as hydrogen sulphide, carbon disulphide and methyl sulphide, which can, in turn, lead to animal fatalities and social disturbance. Decaying beached algae can therefore lead to expensive clean-up operations, loss of amenity value, reduction in tourists recreational visits and inaccessibility for users such as sailors and fishers in particularly affected areas (Joniver *et al.*, 2021a). Phenomena of aggressive and severe eutrophication are happening worldwide with growing intensity, some examples, with storyline, can be found in these publications (Moore, Mantua and Salathé, 2011; Lewitus *et al.*, 2012; Glibert *et al.*, 2014; Townhill *et al.*, 2018; Xu, Zhang and Zhou, 2019; Al-Yamani, Polikarpov and Saburova, 2020; Filote *et al.*, 2021; Joniver *et al.*, 2021a). Nonetheless it is predicted that the frequency and severity is likely to increase, also in consideration of the global warming contribution (Townhill *et al.*, 2018).

Circular Tutelage

The strategic role that transitional waters ecosystem's services will play in the near future, the complexities of the interrelations among social, economic, and environmental spheres, and the pressure sources that are inducing decadence and disfunctions to said ecosystems, are the main topics of previous chapters. In light of which, a concrete urgence to develop a multidisciplinar methodology of action is needed to pave the way for a sustainable reality; thus by restoring and boosting natural equilibria, I would like to call this: Circular Tutelage.

A circular tutelage approach should aim to support the wellbeing and welfare of an endangered area through actions and means of prevention and remediation tailored on the local environmental-social-economic fabric, while enhancing circularity and sustainability. For example, exploiting wastes as a secondary raw material for the production of valuable market goods, thus creating value, reducing the usage of primary resources, and reducing GHGs emissions; while involving the social sphere by communicating science, facts, data, circularity, and innovations so to maximize the social acceptance, i.e., the "engine of change".

Forward chapters involve articles, works, and studies concerning marine life stress caused by plastics and the circular tutelage of the Goro's lagoon in the northern Adriatic sea, known as "*Sacca di Goro*", which is one of the most productive TWE. It is a 25 km^2 wide lagoon upon the delta of the "Po" river; it accounts for more than 55% of the whole Italian bivalve production, with an economic volume of more than fifty million euros yearly. However, major concerns regarding this area are the severe eutrophication phenomena that destroys the products and heavily endangers local economy. Given the bivalves' production there are also issues correlated with seashells handling, a waste that creates even further problems to environment. Therefore, circular tutelage actions should be deployed to tackle this complex situation, proper of a fragile transitional ecosystem, upon which rely the social and economic context of people depending on aquaculture, both directly and through indirectly supportive companies.

These topics are the fulcrums of the next chapters of the thesis, which are also published or being published articles.

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Chapter 1: Environmental Monitoring and a Cost Benefit Analysis for Waste Material Valorization

The topics discussed in the introduction section of the thesis represents the tools and the rational drivers that led to this multidisciplinar work which is basically composed of two sub-sections: the following section 1 that is an article and an empirical follow-up, section 2, with a composition determination and a pollution monitoring of algal biomass from the Goro's lagoon case study. This should yield a refined analysis on the best way to exploit algal biomass in a circular tutelage perspective toward the lagoon's environmental, economic, and social contexts' welfare.

The article is titled:" *Exploitation of Waste Algal biomass in Northern Italy. A Cost Benefit Analysis*", and it represent a joint effort of two main disciplines, namely economy and chemistry.

Section 1: Exploitation of Waste Algal biomass in Northern Italy. A Cost Benefit Analysis.

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Abstract

The need for improved circularity of resources is nowadays a crucial issue that must be tackled in order to achieve a more sustainable lifestyle; aquaculture and marine waste valorization could represent strong first steps. Transitional waters are valuable ecosystems due to the effective services in terms of aquaculture potential and biodiversity, but sensitive toward detrimental anthropogenic activities and pollution. This study aims to optimize and simplify the decision-making processes for the valorization of marine waste (natural and from aquaculture) as secondary raw materials for the production of market goods. In fact, waste like seashells and algal biomasses are prone to be transformed into valuable products; however, significant concentrations of pollutants may be present within wastes (e.g., heavy metals, PCBs, PAHs, and pesticides), and social issues can mine their usage. Goro's lagoon was chosen as a case study, where the relations between the ecosystem services, the thriving bivalve economy, and social aspects are deeply rooted and intertwined. Hence, the threats coming from Harmful Algal Blooms to the ecosystem productivity is causing severe stress to locals. Thus, algal biomass is suitable for marketable compound extraction and biofuel productions. Hence, the goal is to perform a cost benefit analysis and foresight analyses to determine the best usage for algal biomass, also in consideration of the environmental pollution status and social acceptance. Eventually leading to faster, and easier, decision-making processes.

Introduction

It is known that anthropic impacts are deeply influencing the planet's equilibria in negative ways, some effects and consequences are already observable in an overall detrimental perspective. Among the most concerning there are climate change, natural resource depletion, pollution, biodiversity and ecosystem services losses, that are increasingly posing threats to environment and biosphere especially toward delicate and fragile ecosystems (Toth and Szigeti 2016; Kassas 1995; Gerald et al 2009). A crucial issue for the future of humanity is related to the achievement of a global food and resources security in a scenario of growing world population, while reducing environmental impact and conserving ecological services (Tallis and Kareiva 2005.).

Hence, this work aims to improve resource efficiency use, to stimulate the transition towards a more circular economy (Korhonen, Honkasalo and Seppälä, 2018), and to relieve stress from endangered transitional ecosystems (Facca, 2020); hence, matching the Sustainable Development Goals "affordable and clean energy", "industry, innovation and infrastructure", and "life below water" (United Nations, 2022). Thus, the focus of this paper is set on the possible tutelage for transitional waters (TW), which are fragile coastal ecosystems home of aquaculture activities, alongside with their ecological and socio-economic context, while exploiting sea wastes to produced marketable goods (Khoo *et al.*, 2019).

Throughout human history, cities have developed along coastlines and TWs for the natural ecosystem services (ES) provided freely, such as trade, fishing, and aquaculture activities (Bawa and Kaufman, 1997; Costanza *et al.*, 1997); which have been of extreme importance and will play a major role in present and future development (Butler and Oluoch-Kosura, 2006; Newton et al. 2014).

TWs like rias, fjords, estuaries, deltas, and lagoons are considered fragile ecosystems despite their productivity; since they are often characterized by shallow waters, a limited and specialized taxonomic richness, and a geomorphological isolating and sheltering structure; thus, a protected shuffle point for nutrients and species, sited between freshwater and marine environments (Facca, 2020). However, all these factors are rapidly affected by external changes, thereby TWs are considered valuable and productive ecosystems and also naturally stressed, i.e., fragile. (Facca, 2020).

Unfortunately, TWs are becoming more and more vulnerable, deteriorated, and stressed environments as a consequence of anthropogenic pressure, losing ES' potential, due to over-exploitation of coastal resources, aquaculture, overfishing, the introduction of alien species (Poff and Day, 2002), and mass tourism (Facca, 2020). Other factors contributing to the depletion of TW ecosystem quality and productivity can be identified in climatic changes (Harley *et al.*, 2006), and pollution problems, which can lead to further pressure and imbalances on ecosystem equilibria (Raimonet and Cloern, 2016; Pesce *et al.*, 2018). For example, climate change and the rise in temperature are modifying the natural cycles of biotic marine life, since water temperature is a regulator for many ecological processes and the distribution of many coastal wetland species (Poff and Day, 2002).

Plus, the introduction of chemical substances, micro and macroscopic, such as, plastic debris, inorganic, and organic species which show Persistent, Bioaccumulative, and Toxic (PBT) behaviour, is causing direct and long-term damages to the ecosystem's health (Booth *et al.*, 2007; Dórea, 2008b; Suja, Pramanik and Zain, 2009; Check and Marteel-Parrish, 2013; Murray, 2014).

Nonetheless, another anthropogenic issue is linked to nutrient pollution, which is due by enrichment of nutrients, coming from households, agriculture, and industries, like nitrogen (N), phosphorous (P),

minerals, organic matter, and other chemicals in a water body (Werner, 2009). Nutrient pollution can affect primary producers with eutrophication phenomena (i.e., bloom of algal organisms), with various repercussions on the whole ecosystem (Poff and Day, 2002; Zaldívar *et al.*, 2008).

Generally, microscopic planktonic algae are critical food-sources for filter-feeding bivalve shellfish (oysters, mussels, scallops, clams) and for commercially important crustaceans and finfish larvae (Vaishnavi *et al.*, 2019). In most cases, the proliferation of plankton algae (up to millions of cells per litre) i.e., the algal bloom, is beneficial for aquaculture and wild fisheries operations. However, in some situations, algal blooms can exponentially grow, inducing negative effects, among which: severe economic losses to aquaculture, fisheries operations, and tourism, reflecting on major environmental and human health impacts (Hallegraeff *et al.*, 1995). This happens when some of the above-mentioned phenomena of TW quality depletion, or their synergic combination, gains enough relevance leading to a "Harmful algal bloom" (HABs), especially with overabundance of nitrogen and phosphorous nutrients. HABs are also known to occur with macroalgal composition, especially in temperate and tropical waters, and are mostly triggered by strong eutrophication, poor water flushing, low wave action and high temperature and irradiance; lastly, macroalgal blooms can be exacerbated by the removal of herbivorous grazers (Joniver et al. 2021), such as turtles, limpets, and common fish. In addition, the rising of mean global temperatures has resulted in an increased macroalgal biomass blooms nuisance.

Thus, the most threatening effect is the formation of dense blooms of noxious, foul-smelling photosynthetic organisms that harm water quality by limiting light penetration, reducing growth, causing declines in plants population like seagrass (e.g., the angiosperm Neptune grass) and oxygen depletion altering zoobenthic communities (Pesce *et al.*, 2018), depleting dissolved inorganic carbon, and raising pH. Hence, tainted drinking water supplies, degradation of recreational opportunities, hypoxia (Michael F. Chislock, 2013), and the production of highly dangerous toxins (Hudnell, 2010). When the HAB enters the decline phase, microbial decomposition severely depletes dissolved oxygen, creating an hypoxic or even anoxic 'dead zone' (or dead spot), as a consequence of the necessary oxygen lacking to support most organisms, also releasing noxious gases, such as hydrogen sulphide, carbon disulphide and methyl sulphide, which can, in turn, lead to animal fatalities and social disturbance (Michael F. Chislock, 2013). Eventually, decaying beached algae can lead to expensive clean-up operations, loss of amenity value, reduction in tourists visiting affected areas and inaccessibility for recreational users such as sailors and fishers (Joniver *et al.*, 2021a). $2.2 billion annually is the estimated cost of damage mediation for eutrophication damages in the U.S. alone (Michael F. Chislock, 2013).

Phenomenon of aggressive and severe algal blooms are happening worldwide with growing intensity, some examples with storyline can be found in the works of: (Moore, Mantua and Salathé, 2011; Lewitus *et al.*, 2012; Glibert *et al.*, 2014; Townhill *et al.*, 2018; Xu, Zhang and Zhou, 2019; Al-Yamani, Polikarpov and Saburova, 2020; Filote *et al.*, 2021; Joniver *et al.*, 2021a); nonetheless, it is predicted that the blooms frequency and severity is likely to increase (Townhill *et al.*, 2018). In Italy, one of the most susceptible areas from early 2000 is located in the northern Adriatic sea, in the transitional lagoons upon the Po river's delta, the Goro's lagoon.

This work aims to match the need for a multidisciplinar and holistic approach toward the resolution of problems according to all aspects of sustainability: environmental, economic, and social. Hence, in this study a Cost Benefit Analysis (CBA) and Foresight analyses are performed to systemically determine the best possible ways to exploit the excessive and problematic waste algal biomasses from HABs; thus, to restore and safeguard the TW's ecosystem services effectiveness, in consideration of socio-cultural,

historic, and environmental contexts. Therefore, the CBA is used to compare various industrial processes to transform biomass into marketable goods, while the SWOT Foresight analyses enrich the CBA output by also considering social factors and pollution status. This is applied to the valuable Goro's lagoon case study and could represent a strategic tool for stakeholders, leading to simplified and shortened decision-making processes. This work can represent an approach to develop a sustainable and circular strategy for the tutelage of a fruitful, but endangered, location.

In section 2 and 3 the background of the research, and the case study will be introduced. The options will be described in section 4, as well as the comparison method of the cost benefit analysis and the foresight technique. Chapters 5 and 6 will be a discussion of the results for both CBA and foresight. And lastly, in chapter 7 and 8 there are discussions and conclusions with final remarks, and suggestions for further analysis.

Background

Ecosystems Services (ES) are of fundamental importance toward our wellbeing (Bawa and Kaufman, 1997); hence, to preserve ES quality and productivity should be a primary interest also for improving economic returns, savings, and lifestyle quality. However, ecosystems are becoming so degraded that many regions in the world risk ecological collapse (Tallis and Kareiva, 2007); thus, the first move should be acting toward the stress reduction / elimination, possibly turning problems into solutions. Among various stressors the increased frequency of nuisance macroalgal blooms is unlikely to change in the immediate future, the challenge is then to exploit the problematic algal biomasses, coming from HABs (not exclusively), as secondary raw material to produce marketable goods, promoting the sustainable use of biomass, innovation, and development, also restoring the health and quality of the ecosystem, especially when many natural resources are becoming increasingly scarce.

Algae

Algal biomass is a resource suitable for producing a wide panorama of products of multiple purpose; fuels, drugs, food, integrators, and platform chemicals are some examples of the markets in which the agal products can fit (Park *et al.*, 2009a; Bruhn *et al.*, 2011a; Yildiz *et al.*, 2011a; van der Wal *et al.*, 2013a; Saeid and Chojnacka, 2015; Gupta, Malik and Bux, 2017; Koçer and Özçimen, 2018a). Readers may find deeper information for further utilizations that algal biomass might have in the studies of (Henriques *et al.*, 2019a; Luo *et al.*, 2020), (Raja *et al.*, 2016), and (Kartik *et al.*, 2021).

However, there are some limiting agents which stands against the exploitability of this re-discovered resource, among others, the immaturity of the infrastructures and a functioning bioeconomy. In fact, the lack of market readiness for bio-products acceptance represents an important obstacle to algal products diffusion. Moreover, the chemical composition of the biomass itself, and the presence of pollutants that can interfere with the exploitation routes are also important limiting factors. However, CO_2 sequestration, renewable energy production, improved circularity of resources, environmental protection, bioremediation, and the opening of new markets are some of the benefits deriving from a strong algal bioeconomy.

In general, algae show great variability and the term itself “algae” is commonly used to refer to “marine macroalgae or seaweeds”, where three major types of macroalgae in the marine environment are present, divided in smaller categories: approximately 1800 different brown macroalgae, 6200 red macroalgae, and 1800 green macroalgae (Pereira, 2021). Although there are substantial differences, all of them are not vascularized organisms that perform photosynthesis.

Other than the peculiar behaviour and morphology of the different phyla, algae show characteristic amounts of carbohydrates, lipids, minerals, and proteins; this chemical composition varies across species, seasons, and with environmental factors such as solar irradiation, water temperature and composition (Renaud and Luong-Van, 2006; Sutkowy, Lenarczyk and Kłosowski, 2018). Algae can, also, selectively metabolize (Pilatti *et al.*, 2016) or accumulate substances, especially heavy metals, from the environment, in Henriques et al (2019b) 6 grams per litre of water are used to deplete concentration of As, Cd, Pb, Cu, Cr, Hg, Mn and Ni, with an efficiency ranging from 48% for arsenic to 98% for mercury, in a timeframe of 12 hours for 50% removal. For these reasons, the possibility of using them as bio remediator is nowadays studied; some examples from literature are reported (Bishnoi and Pant, 2004; Chekroun, ben Chekroun and Baghour, 2013; Hanbali, Holail and Hammud, 2014; Mahmood, Mirza and Shaheen, 2015; McGaughy *et al.*, 2019; Nguyen *et al.*, 2020).

Biomass and Biorefinery

Biomass is the crude oil most promising substitute, since it is an abundant and renewable carbon-neutral source to produce energy, platform chemicals and biomaterials. Biomass may be made of organic wastes, by-products, and residues. Therefore, biomass feedstocks are classified by their origin in “generations” (Linares-Pastén, Andersson and Karlsson, 2014; Nanda *et al.*, 2018).

First-generation biomass mostly includes food crops viable for human consumption, hence the direct use as “biomass” is unjustifiable because of the food-versus-fuel controversies. In fact, this category includes the common edible plant materials and crops such as corn, wheat, sugarcane, and food grains (Nanda *et al.*, 2018).

Unlike first-generation biomass, that can be directly processed in biorefineries for fuel production with cleaner processes, the utilization of second-generation biomass requires additional processing steps and more operational costs for biofuel production. Examples of second-generation biomass can be found in the tremendous amounts of agricultural crop residues variety that are obtained, globally, as a result of agricultural and farming practices and from forest residues and wood processing[1] (Nanda *et al.*, 2018).

The third-generation biomass includes micro and macroalgae, animal manure (e.g., poultry litter, dairy manure, and swine manure), municipal solid waste, industrial effluent (textile effluents, paper and pulp industry wastes, tannery effluents, pharmaceutical wastes, etc.), and sewage sludge (Nanda *et al.*, 2018). This third kind is “dirtier” than the previous two and carrying conspicuous amount of phosphorous, nitrogen, and/or pollutants and/or pathogens. Therefore, aquatic biomass is considered a third-generation biomass, but also advanced biofuel feedstock due to its perennial and inherent growth.

Biomass can be transformed in a Biorefinery (BR), which is a general term that indicates an industry where the feedstock input is of biological nature i.e., biomass (Linares-Pastén, Andersson and Karlsson, 2014),

[1] Wheat straw, barley straw, corn stover, cotton stalk, sugarcane bagasse, softwood and hardwood, stems, barks, twigs, needles, sawdust, wood chips, lumps, bales, pellets, briquettes, and many others.

therefore it should reintegrate and maintain the carbon cycle. In fact, BR are being identified as the biological and green version of an oil refinery; however, while the oil refinery is defined by a core process of fragmented distillation, biorefineries are more complex to define, since there is not a common ground among facilities. Moreover, BRs are in an embryonic technological stage, thus the number of operating biorefineries is quite exiguous, therefore, to find and confront similarities among facilities is not an easy task. Furthermore, there is not a generally accepted classification criteria for BRs, and in literature a number of different naming strategies can be found; for example, according to the feedstock used, the transformation processes, or the type and quantity of products. However, there is a strategy to summarize some factors by introducing the "phase" description, a classification that considers the input and output of the BR to identify the plant's complexity and flexibility (Linares-Pastén, Andersson and Karlsson, 2014):

- Phase 1: one feed source / nonflexible main processes / only one product
- Phase 2: one feed source / rather flexible main processes / multitude of products
- Phase 3: various feed sources/ flexible main processes / multitude of products

Nowadays BR are mainly phase 1, whereas very few are phase 2, and lastly, phase 3 facilities exist only on a conceptual level.

In this work the focus is set on the usage of algae, both micro and macro, as biomass feedstock for processes typical for BR, such as the biofuel production. Unfortunately, the literature on BR technologies for biofuels is still immature, as it can be seen in figure 1, and table SM1 (appendix 1) and its presence is rising solely in the last two decades. For industrial purposes, it should be noted that transforming microalgae is easier and their composition is more controllable, the drawback is that the growing structure for them is quite always necessarily on land, while macroalgae can be grown in seawater, but then the biomass may be more polluted and with lesser control on its composition.

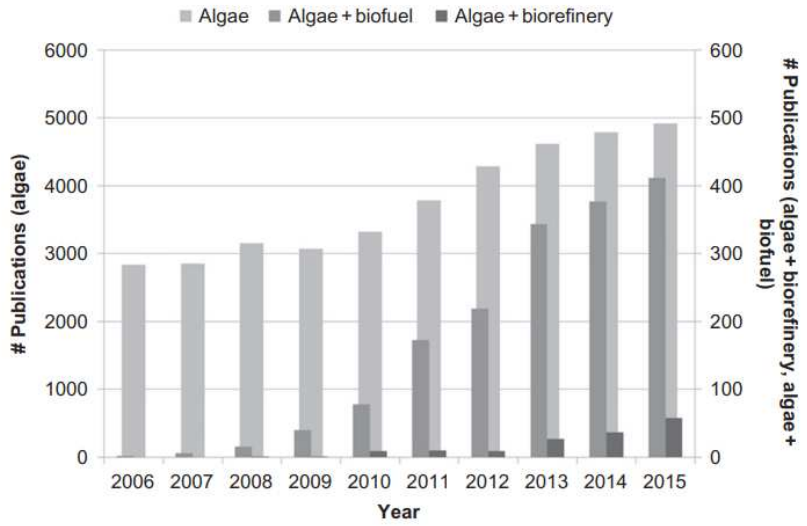


Figure 1) Publications related to algae biorefineries based on Web of Science in 2016 (Bastiaens *et al.*, 2017).

Thus, given the exiguous number of papers in the literature on biorefinery and algal biomass management, to study solutions compatible with the cascade utilisation of natural seaweeds to produce high-value intermediate and end products is a complex matter. Also, if compared to fossils, biorefinery products are hardly economically competitive. However, technical publications on the topic are more and more accompanied by economic feasibility analysis and Lifecycle Cost Analysis (Stevens *et al.*, 2015; Suganya *et al.*, 2016; Ingle *et al.*, 2018; Ahmad Ansari *et al.*, 2020; Dickson *et al.*, 2020; Greene *et al.*, 2020; Joniver *et al.*, 2021b; Zhang and Thomsen, 2021a). Nevertheless, bio products' impact evaluations and market acceptability are being made in literature with increasing frequency (Konda *et al.*, 2015a); which is a good sign for the installations increase of bio-plants. Biorefineries also hold a tremendous potential for innovation and are compatible with renewable sources of energy.

The Case Study

The area chosen for the case study is the Goro's Lagoon; the so called "Sacca di Goro", which is an iconic and valuable transitional water region upon the *Po* river delta in the northern Adriatic sea in Ferrara's province in the North-East of Italy (figure 2 and 3). The productivity of the area is remarkable since it accounts for 14/15 thousand tons of mussels yearly, 54% percent of Italian production, and 40% of European production (Paoletti, 2008; National Order of Biologists, 2018).

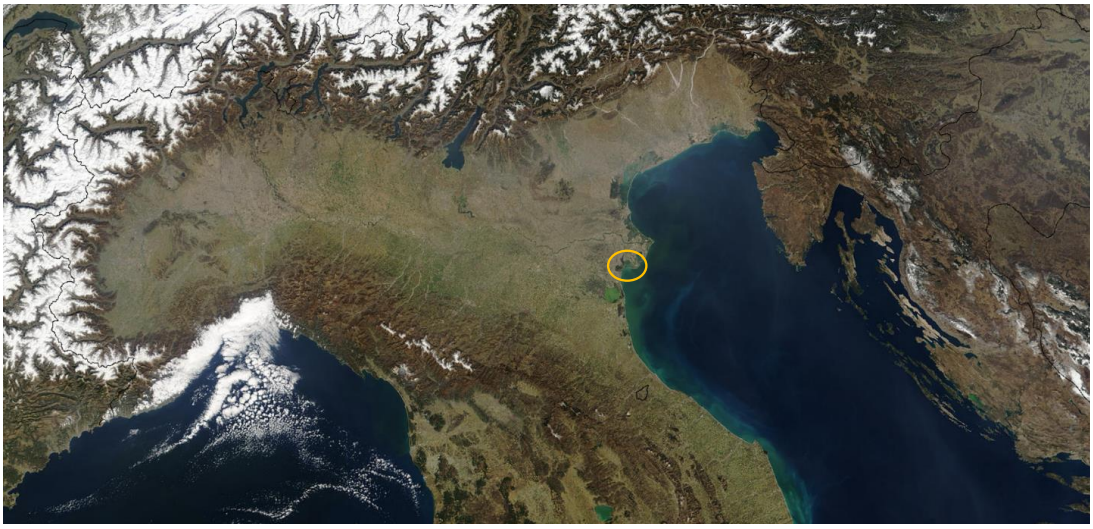


Figure 2) Satellite view of northern Italy and northern Adriatic sea. ***(Nasa, 2022).***

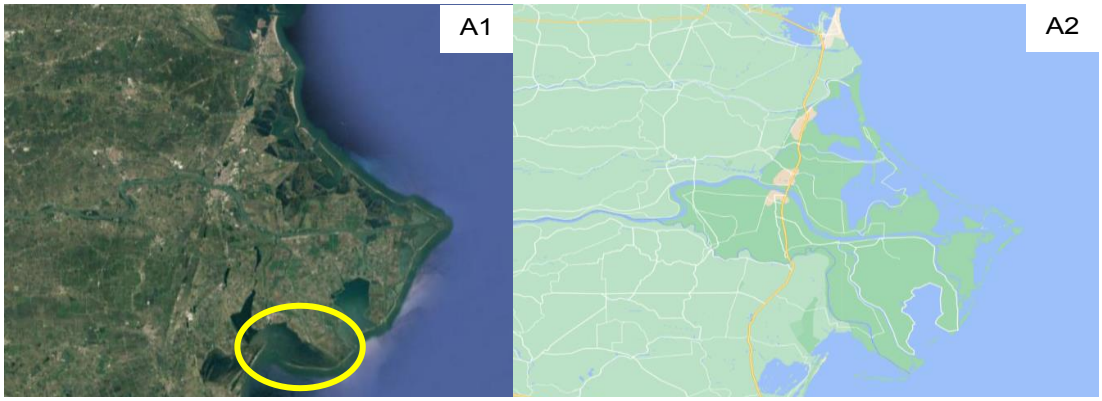


Figure 3) Images of the Po delta from google maps, A1, A2.

The Goro's lagoon (in Italian "Sacca di Goro") along with other nearby lagoons, is part of the "*Delta del Po*" protected park (UNESCO) and is a water surface extending for 20 square kilometers, with sandy bottoms on average 60-70 cm deep, with some 2 m deep zones ("Parco Del Delta Del Po".). The lagoon environment is an evolving one, which means that it's slowly changing and mutating; and although these natural changes

are due to the natural water circulation between freshwater and tides, also human intervention introduces morphological modifications in order to slow down these naturally occurring processes. One of the most recent anthropic interventions was made to improve the hydrodynamic circulation by the opening of additional freshwater outputs in the lagoon, for example the ones in the south-western side of the lagoon.

As previously mentioned, the productivity is remarkable and it stands as the solid base for a strong bivalve aquaculture economy, boasting several million euros per year. Thus, it exists a profound and rooted bond between social and economic context with this transitional location. Since the early 2000s, this area has been suffering from extraordinary algal blooms phenomena (e.g., Figure 4), of growing proportion; with a peak in the 2014-15 years; with consequent damages to the ecosystem and mussel's die-offs, severely affecting the socio-economic local tissue.

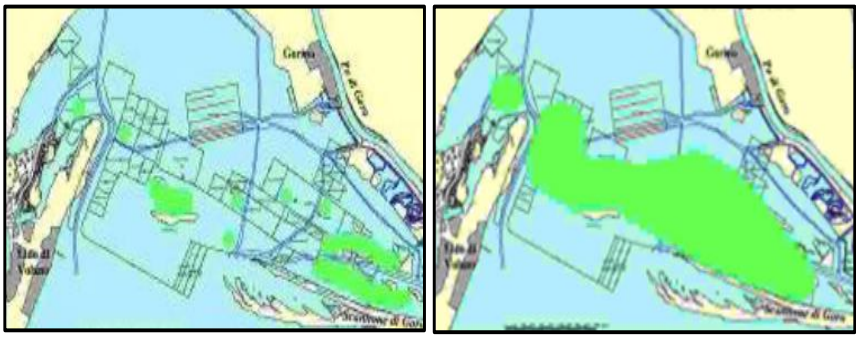


Figure 4) satellite reconstruction of a severe bloom happening in the 2005; left: May the 26th, right: May the 31ST. It took only five days to cover, almost completely, the lagoon (Anna Gloria Angonese, 2007).

In this transitional lagoon, algal presence is mostly made by the two macroalgal species of Ulva Lactuga and Gracilaria (Lenzi and Birardi, 2006), which are the two species majorly present as well during HABs in this location, of which an example from local newspapers (Perini Andrea, 2015).

The seaweed Ulva Lactuga belongs to the Chlorophyta phylum and shows a deep green pigmentation due to its chlorophyll content. The biomass of this origin performs well as soil conditioner, fertilizer and as a feedstock for aquaculture organisms, although it is especially suitable for the extraction of high added value chemicals and the production of biofuels. In particular this alga has a peculiar mix of lipids, which can be isolated or turned into biodiesel, phenols, which can be extracted and used as antioxidant, polysaccharides and saccharides which have a plethora of uses, among which the production of biopolymers or the transformation into bioethanol (Bruhn *et al.*, 2011b; Suganya, Nagendra Gandhi and Renganathan, 2013; van der Wal *et al.*, 2013b; Dominguez and Loret, 2019; Mhatre *et al.*, 2019; Sari-Chmayssem *et al.*, 2019). Other uses for Ulva can be found in additional references reported in appendix 1.

Gracilaria belongs to the Rhodophyta phylum and is characterized by a brownish-red pigmentation due to the major presence of phycoerythrin (red algae version of chlorophyll) and carotenoids. This biomass instead, shows a more fibrous structure and is already used in production plants for edible goods (in China and Japan mostly), like the phycocolloid known as "agar" used worldwide for culinary purpose, and the extraction of alginate (polysaccharide), other than fodder for organisms (Pramanick *et al.*, 2016). However, the exiguous amount of lipids and the high amount of carbohydrates makes Gracilaria suitable to produce

bioethanol (Habig, Debusk and Ryther, 1984; Hani Norziah and Yen Ching, 1998; Marinho-Soriano and Bourret, 2003; Guimarães *et al.*, 2007; Yildiz *et al.*, 2011b; Francavilla *et al.*, 2013; Kumar *et al.*, 2013; Meinita *et al.*, 2018).

Unfortunately, the exploitation of the biomass may not be a straightforward and clean process for multiple reasons. First of all, the marine biomass comes as a mixture of seaweeds and not a single phylum, also sand, salt, and solid debris e.g., shells, exoskeletons, organisms, and also plastic debris and microplastics must be washed away, adding extra costs. Moreover, the biomass may have accumulated not negligible amounts of pollutants from the water column and from sediments. This latter issue is discussed in the foresight analysis in chapter 6.

Methods:

Cost Benefit Analysis

To develop an eco-industrial system, ex-ante studies are often conducted to pinpoint hotspots of profitability with significant economic costs and benefits data. The Cost Benefit Analysis (CBA) is a standard method to evaluate the best choice among different investments or projects in uncertain situations on their payback and overall effects. CBA is a systematic process for calculating and comparing benefits and costs of alternative choices (e.g., scenarios, projects, investments); thus, it is mainly adopted to help decision making process to allocate resources in the most profitable way (Boardman 1998). Therefore, CBA has a long history in management and economic studies, both for private and public investments, and since the 30's it has been used also for policy and environmental projects (Pearce, Atkinson and Mourato, 2006; Hanley and Barbier, 2009). Generally, this method helps to avoid being swept by the fashions of the moment (Prest and Turvey, 1966) by relying on economic data and evaluating the economic return, or loss, on a fixed timespan, with various discount rates.

Therefore, in presence of multiple choices for a project development, an ex-ante CBA helps and guides the selection of the most profitable one, which maximizes the value of the investment by the comparison of net cash flows (discounted benefits minus costs) generated along a selected time frame. These values are expressed in monetary terms and reported to present values by applying a fixed discount rate (equation1), in this analysis five discount rates have been chosen for further comparisons, namely: 0,5%,1%, 3%, 5%, and 10% to simulate different investment opportunities and risks, and to match a realistic timeframe of thirty years for the operative lifespan of medium-big industries. More specifically, discounting reflects a social opportunity cost, such as the return on the private or corporate investment displaced by government funding, the rate at which society is willing to trade-off consumption today for consumption tomorrow, the rate at which society expects wealth to increase in the future (and marginal utility of future benefits to decrease) thanks to economic growth. However, in this specific case the discount rates are the expression of the risks connected to biomass exploitation. Eventually, a rational decision maker should opt for the investment with the highest economic return (Pearce, Atkinson and Mourato, 2006). The formula of the CBA is shown in equation 1.

$$NPV = \sum_{t=0}^{n} \frac{Rt}{(1+i)^t} - \sum_{t=0}^{n} \frac{Ct}{(1+i)^t}$$

Equation 1) Net Present Value Formula (Perman, 2003),
where Rt are revenue, Ct are costs, i is the discount rate and t is the year of the timeframe.

Hence, in this study different approaches for biomass exploitation are confronted on the structural basis of bio-plants costs and products, in order to determine the best manner to approach the exploitation of a biological waste, in such a delicate socio-economic context. However, because of the young age of these technologies and the little literature surrounding this topic, approximations and assumptions are necessary. As previously mentioned, the CBA collects and confronts different scenarios, among which: a "status quo"

option, as the real baseline, which is, relatively the cheapest, but is not solving any problem nor creating circular value, and various biorefinery approaches for biofuel production and more.

However, this is also a case where it's somehow complicated to express an economical value to community goods or fears, especially for the ecosystem's service quality (that allow for mussels to grow and thrive) and the intertwined social dynamics of people relying on it; nonetheless also pollution can represent a complicated parameter to consider economically. Therefore, scenarios including pollution and social aspects are built with Foresight techniques, thus they are analyzed and discussed, starting from the results of the CBA and by considering a real failed case.

Foresight analysis

A foresight analysis is a systemic, interactive and creative process of strategical evaluations to go beyond the visible, to perceive the utility of new choices, to gain awareness of hidden threats and problems, to develop visions for the future in the medium long period (over 10 years), and thus detect possibilities innovations and further development, to optimize decision-making and policy interventions towards targets (Cariola and Rolfo, 2004). Then, the role of a foresight is to help and drive the allocation of limited resources toward a solid target. The call for a business success requires a perfect timing and the management of investments, since today is winning not only who comes first to an innovation, but mainly who is able to commercialize it first with products and services readily accepted by the market (Martin, 1995; Martin and Johnston, 1999). It is therefore important to develop a business strategy in agreeance with transitional water ecosystem services improvement and safeguard, in present and future, and also with its surrounding economy, people and markets.

There are many viable methods in literature to perform a foresight analysis, the one adopted in this work is the SWOT analysis, with the purpose of "identifying the issue" (Georghiou, 1996; Popper, 2008; Rialland and Wold, 2009). SWOT is chosen as most proper tool for refining the CBA output by taking into consideration social and pollution issues upon algal biomass exploitation, evaluating "Strengths", "Weaknesses", "Opportunities" and "Threats" (SWOT). Basically, a refinement process that helps overcome challenges and determine what measures to adopt to effectively pursue the target. Hence, the primary objective of a SWOT analysis is to develop a full awareness of all the factors involved in making decisions (Jain and Ewurum, 2015).

In the following subsections the different alternative projects for algae management are shown.

Case Zero, the "Status Quo"

The "status quo", or case zero, is not entering in the CBA, but it is only reported as a baseline of how the situation is being managed, i.e., without circularity actions toward algae's aggressive blooms (HABs), then undergoing related damages and losses, whereas only a physical removal by the hands of the locals, aqua-cultivators, and fishermen is made. The biomass collected is then transported to a compost plant in the nearby (Ostellato around 50 km), to produce bio-stabilized to be sent in landfill, or to be used as a covering layer always in landfill. Thus, little energy is recovered, and the Carbon Cycle is interrupted, not to mention the marginal utilization of two facilities, a composter, and a landfill, with related costs and soil usage.

However, despite being the less impactful case in a social perspective, it might not be the best solution given the loss from HABs damages to mussels' population and collection, maintenance, disposal, and transportation costs; in Figure 5 the routes from Goro's city to Ostellato's composter, with a 50 km and 50 minutes mean mileage; plus, an equal distance and time from the composter to the landfill.

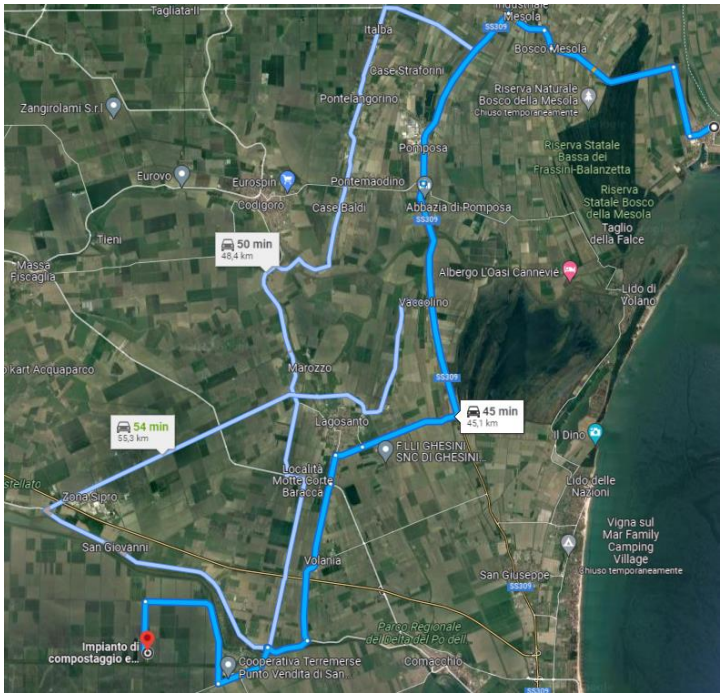


Fig.5) Routes from Goro to Ostellato; Google Maps.

Hence, the "do nothing" option has been working well in standard situations, but in case of HABs is not of use at all, given the multitude of anoxic crises detected from 2000 (e.g., Anna Gloria Angonese 2007) and the consequent bivalve die-offs with a drop in productivity even down to an 80% loss. However, the monetary losses coming from cultivation destruction is not quantifiable precisely, but to get an idea, it is possible to confront it with an annual income of more than 50 million euros for a single subject (e.g., consortia and cooperatives), when at 100% productivity (Gian Omar Bison, 2012).

Therefore, the income reduction from the lack of product to sell and the expenditure for disposal and restoration accounts heavily on the relatively small reality of this transitional waterbody both on the economic and social tissue. In table 2 the clean-up, disposal, and maintenance costs are reported from 2016 up to 2021. In those years, some modifications have been made in the lagoon's hydrodynamic circulation and the HABs problem has been, temporarily, appeased.

Case 1: Do-Nothing (status quo)

Products in HERA's plant in Ostellato: Bio-stabilized for LANDFILL

Biomass (tonn)	912,00	95,44	19,74	71,94	0	120,00
year	2016	2017	2018	2019	2020	2021
Maintenance cost	32.150,00 €	14.280,00 €	19.600,00 €	15.600,00 €	0	36.750,00 €
sea-transport cost	40.162,50 €	17.325,00 €	0	12.800,00 €	1.800,00 €	10.800,00 €
land-transport cost	14.350,00 €	1.684,00 €	2.800,00 €	1.400,00 €	0	2.100,00 €
Disposal cost (HERA)	5.000,00 €	5.000,00 €	5.000,00 €	1.798,50 €	0	3.003,50 €
total expenditure costs	91.662,50 €	38.289,00 €	27.400,00 €	31.598,50 €	1.800,00 €	52.653,50 €

Table 2) Algal biomass in Goro's lagoon clean up, disposal, and maintenance costs are reported from 2016 up to 2021. These data from CO.SA.GO. cooperative were kindly provided by the administration of Goro's town – ref: Matteo Zappaterra.

From table 2 it is possible to note that in 2016 the amount of algal biomass is higher than in the subsequent years, possibly confirming the success of the 2016/17's interventions to improve hydrodynamic circulation.

However, in 2020 there isn't excess biomass to be removed; thus, it could be interesting to highlight, in further studies, the possible correlations among the absence of excess biomass and the restrictive measures adopted to respond to the covid-19 pandemic.

During 2022 another dangerous bloom (caused by drought and high temperatures) caused severe troubles in the lagoon's ecosystem, with anoxic crises that led to fauna die offs, along with the correlated economic losses, the locals' general worries and preoccupations, which are forced to "fish algae" in order to save the residual low levels of dissolved oxygen (ANSA 2022; Bovenzi Mario 2022).

Given that this real scenario relies on the presence of a landfill and a composter, in the appendix1 are reported, for indicative reasons, capital costs per hectare for a landfill structure installation and its end-life costs (Daniel P. Duffy, 2016) (table 3, 4, and 5). Whilst the composter's installation capital costs are available indicatively in this work (Craig Coker, 2020); however, for the end-life costs both for composter and for the scenarios' bio-plants discussed in the next sections, a table with demolition expenditure is reported at the end of appendix 1 (table 6).

Scenarios Common Grounds

The scenarios analyzed in this study consist of various biorefineries approaches, which differ in terms of processes, complexity, capacity, flexibility, feedstock/s, and product/s; in other words, there is not a common structure or process. So, to avoid redundancies, in this first part, some common grounds are discussed. Where the idea is to stress the determination of the best approach toward algal biomass usage.

The first point is that scenarios capacities for algal biomass largely outmatch the Goros' lagoon's potential production, in fact during an HAB situation the biomass removed is averagely 160 tonn/day (ANSA, 2022a). Therefore, the focus of the comparison should be set on what is better to do with the biomass, rather than calculating the proper plant scaling. However, it must be pointed out that, given a standard scale and capacity, the industrial complexity will set a difference among cases by having a proportional, and not negligible, impact only for the end life demolition costs (table 6). Speaking of costs, the only adjustment to data is the actualization to 2021 euros[2].

Secondly, to make confrontations among cases, the starting biomass composition: "macro- or micro-algae" and "chloro-, rhodo- or ochro- phytha", is relatively not considered; since, in general, the processes are based on bromatological composition rather than the phylum. However, a crucial hidden factor is that algal biomass in each case is acquired as a resource; thus, the selling price of the final product/s will depend mainly on this expenditure, and since algae production is an uncommon activity, it is rather expensive. In our case, instead, the biomass' costs are mainly the ones for removing excess biomass, of which we have some cues in table 2, thus implying cheaper final product/s, more competitive on markets.

Another point concerns the adaptability of all bio plants with symbiotic satellite structures which push the biomass exploitation even further. For example, incinerators for heat and energy production and fermenters to produce biogas that can (fully or partially) respond to the factory's energy demand. Also, it is possible to have a small structure to pack and prepare the incinerator's ashes, with possibility to recover metals prior to be sent in landfill, or to prepare the completely exhausted biorefinery's organic waste, like a digestate cake (from satellite fermenters), to be used as soil conditioner, or to be sent in landfill. Also, they are easily connectable with innovations and renewable in general, for example with a hypothetical wind / wave-and-macroalgae marine farm combination plant.

Scenarios Description

In this subsection the approaches are described, at the end a resume table (table 7), with technicalities is available. Benefits and Costs for the CBA, come from the following techno-economic papers (Davis *et al.*, 2013; Konda *et al.*, 2015a; Nazemi *et al.*, 2021; Zhang and Thomsen, 2021b). These scenarios are chosen on the basis of a "biomass usage" criteria, starting with the solely production of biofuels, then biofuels plus a single compound of interest, then a full biorefinery that produces biofuels and multiple products, and lastly a biorefinery without biofuels.

1) First Scenario (Davis et al.*, 2013)*

It is quite straightforward: the bio-plant produces biodiesel starting from algal biomass, with a satellite that produce bioethanol through fermentation. Basically, it exploits the biomass' fatty component and the final

[2] Also considering inflation on dollars for each case (Bureau of Labor Statistics, 2022) and the change rate with 2021 euros.

exhausted biomass to produce biofuels. Despite the relatively simple structure and efficiency, this kind of approach is not very resilient on the market given the “one in /one out” formula typical of the phase I structure, which is also rather inflexible toward changes, but well performing.

2 a/b) Second Scenario (Konda et al.*, 2015b)*

This case consists of two scenarios, where there is a common industrial base which produces only ethanol, and another which has also a line for the side stream production of a functional product accordingly to the biomass composition under study, in this case alginate. Therefore, this case brings by itself a phase I and a phase “I/II” confrontation (meaning that there is an extra layer of industrial complexity, placing the overall complexity somewhat in between a phase I and II). However, in this paper some data are lacking, therefore some approximations are made to carry out the CBA. Specifically, we approximated the values of “fixed and variable operating costs” by proportioning on the other scenarios’ trend of these costs, then adjusting for the operating total, which was available. Unfortunately, also the total capital investment was not available, thus it was approximated by rescaling the values of cases 2c and 2d (which are the most similar) based on the feedstock annual capacity. Eventually, the bio-plant dimensions and capacities introduce the problem of market saturation, since the alginate produced by that single plant will outmatch the whole global demand (even though it is increasing annually).

2 c/d) Third Scenario (Nazemi et al.*, 2021)*

This case is quite similar to the previous one, but in this case, there are more side stream functional product lines, which lead to a full phase II biorefinery. It is to be noted, however, that this kind approach is extremely dependent on the biomass composition and phylum/phyla’s biological peculiarities in terms of active biomolecules, which can find use as food supplements, anti-inflammatory drugs and more. In this case: bioethanol, mannitol, alginate, proteins, and soil conditioner are produced.

3) Fourth Scenario (Zhang and Thomsen, 2021b)

This last case is not fuel oriented, instead it represents a refinery approach to produce food, food supplements, fodder, and fertilizers starting from various algal biomasses. This alternative is completely a phase II biorefinery, which exploits different feedstocks and produces multiple products such as, laminarin and fucoidan. Alike the previous multiproduct case, also this structure is resilient toward market volatility and changes, but since the idea is to use natural algae, these can collect pollutants from environment, which might represent a problem in terms of products for human consumption.

	Alternative 1: Biofuels	Alternatives 2a,b,c,d: Biorefineries				Alternative 3: Food / fodder
Phase (complexity for DEMOLITION): I, II, III	I	I	I/II	I	II	II
Product/s	Biodiesel, bioethanol, biogas	Bioethanol	bioethanol + alginate	bioethanol	bioethanol + mannitol, alginate, proteins, and soil conditioner	food supplement: laminarin + fucoidan, and fertilizers
building time (months); no gain	6 (planning) + 24	6 (planning) + 24	6 (planning) + 24	36	36	12
months for startup;	6: 50 % gain with 75% variable expenses	6: 50 % gain with 75% variable expenses	6: 50 % gain with 75% variable expenses	3	3	none (paper assumption)
Variable operating costs (M€/Y)	237,39	186,23	695,93	63,37	107,96	49,61
Fixed operating cost (M€/Y)	15,01	11,58	43,27	3,87	10,80	0,54
Total operating costs (M€/Y)	252,40	197,81	739,19	67,24	117,58	50,15
total direct cost (M€)	298,24	257,51	514,14	181,06	361,54	3,12
total indirect cost (M€)	178,88	154,48	308,43	108,66	216,86	1,87
total capital investments (M€)	503,05	435,94	870,37	307,09	613,13	5,40
Total annual sale (M€/Y)	695,33	218,63	832,89	29,22	370,46	166,15

Table 7) resume table for all scenarios, actualized at 2021 million euros.

Cost Benefit Analysis, Results

The Net Present Values, in million euros, are reported in the right side of the table below (table 8), in relation to the discount rates, from 0.5 to 10%, for each alternative, from one to three.

	discount rate	NPV (million euros)
Alternative 1) Biofuels	0.50%	10,572.75
	1.00%	9,707.21
	3.00%	7,012.87
	5%	5,192.06
	10%	2,676.16
Alternative 2.a) Bioethanol	0.50%	63.89
	1.00%	25.57
	3.00%	-91.87
	5%	-168.65
	10%	-266.46
Alternative 2.b) Biorefinery, single by-product	0.50%	1,118.20
	1.00%	977.99
	3.00%	532.37
	5%	223.15
	10%	-211.01
Alternative 2.c) Bioethanol	0.50%	-1,240.05
	1.00%	-1,164.32
	3.00%	-927.37
	5%	-765.58
	10%	-536.55
Alternative 2.d) Biorefinery multiproducts	0.50%	5,605.41
	1.00%	5,114.74
	3.00%	3,589.83
	5%	2,562.79
	10%	1,154.43
Alternative 3) No Biofuels and Biorefinery products	0.50%	3,103.44
	1.00%	2,873.61
	3.00%	2,155.87
	5%	1,667.65
	10%	983.20

Table 8) Cost Benefit Analysis; Net Present Value (equation 1) for the alternatives with various discount rates.

It can be observed that the most remunerative scenario is the biofuels only oriented, followed by multiproduct biorefinery; the third one for economic convenience is the plant that produces multiple products except biofuels. Instead, producing only bioethanol appears to be a failure path, since in both cases the NPV is mostly negative; while bioethanol plus a single component appears a little more competitive, but still not very profitable in the long run, given the major, and more realistic, discount rate. Below a resume table (Table 9) with the alternatives ranked based only on the NPV from the CBA.

Alternative 1: Biofuels
Alternative 2d: Biorefinery multiproducts
Alternative 3: No Biofuels and Biorefinery products
Alternative 2b: Biorefinery, single by-product
Alternative 2a/2c: bioethanol only

Table 9) Resume table of CBA alternatives' outputs ranked for profitability

Fixed discount rate

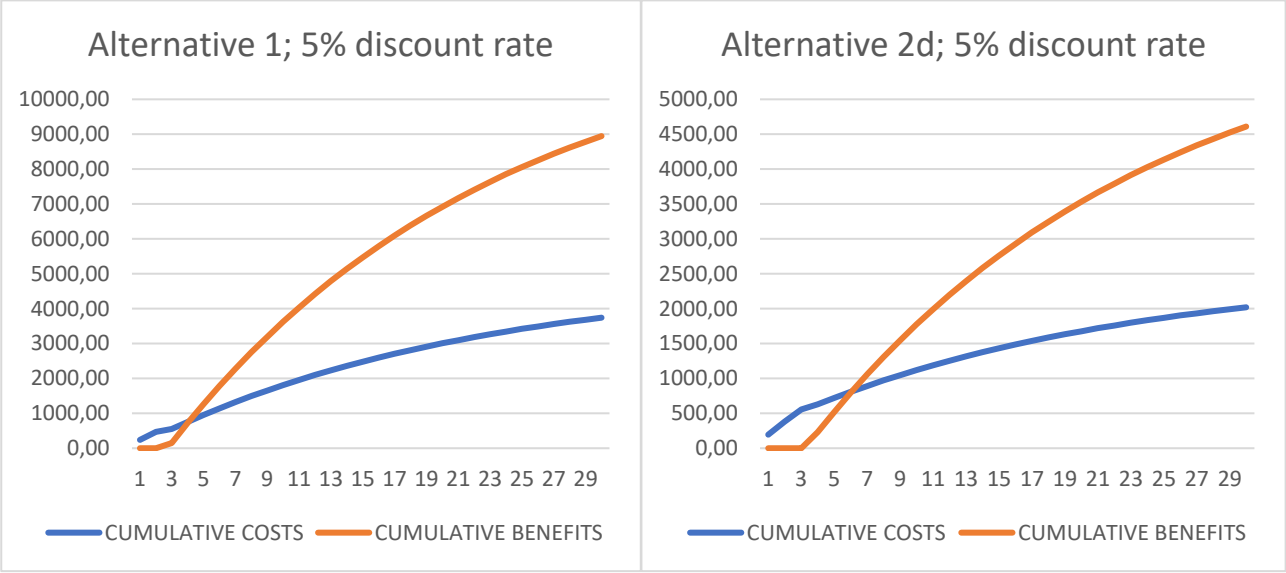
Knowing that this case study can contain numerous and varied obstacles, it was necessary to analyse the various alternatives through a spectrum of discount rates, from 0.5% to 10%, similarly to a sensitivity analysis for testing the sustainability of the circular tutelage project. Hence, with a 0.5% discount rate, a virtual income for the first alternative of more than ten billion is projected, in comparison to slightly more than two and a half billion with a discount rate of 10%: basically, a 75% incomes reduction. The comparison with other alternatives shows losses up to 80%, and some that are not enough resilient to produce positive incomes throughout the various discount rates; thus, little or not sustainable.

In other words, the discount rate is substantially the representation of the level of confidence that future income streams will equal what it is projected today; hence, it is a measure of risk. In fact, a higher discount rate generally means that there is more risk associated with the investment opportunity. Therefore, future cashflows should be attuned to a greater discount rate percentage because they are less likely to be realized. Conversely, if the investment is less risky, then theoretically, the discount rate should be lower on the discount rate spectrum. Thus, given the uncertainties related to the markets' acceptability of bioproducts, social fears and distrust, pollution, and regulations, a high risk for the investment is present. Therefore, a discount rate of 10% should be chosen to compensate this multifaced risk. However, the overall sustainability and the economic and environmental positive effects coming from the restored ecosystem quality, and its services, can stabilize these uncertainties; then lowering the overall risk and fear for the investments. Eventually, a discount rate of 5% is chosen for further analyses and comparisons among alternatives, as the most representative for the trade-off between risks and benefits proper of this case study.

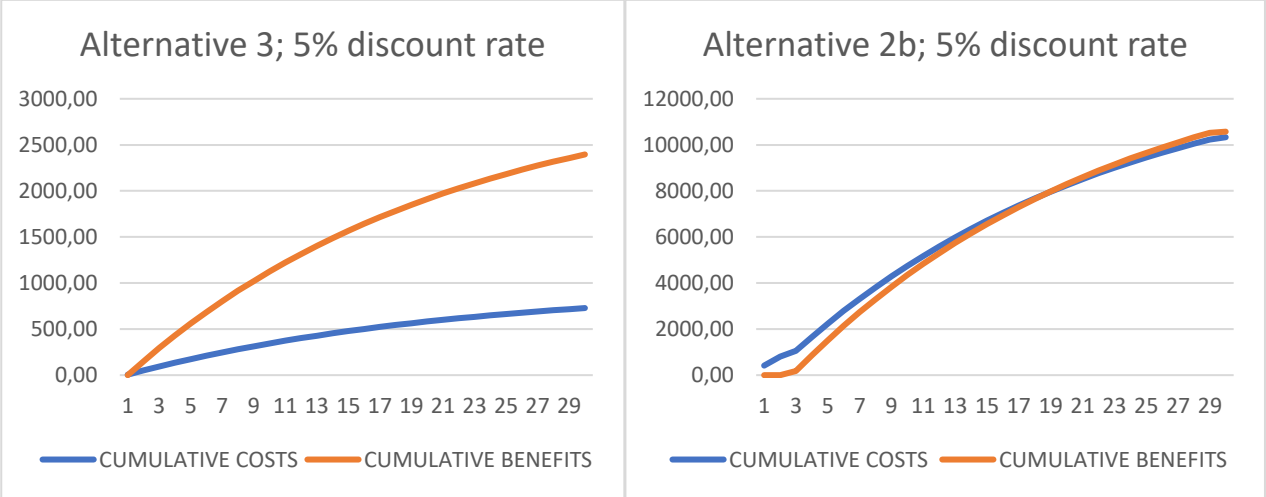
Therefore, according to the CBA' results, over a fixed timeframe of thirty years, the best scenario (alternative 1) is supposed to return roughly 5.19 billion euros, while the second-best choice (alternative 2d) returns slightly less than 2.6 billion euros, around half of the first alternative, whereas the third most remunerative (alternative 3) returns 1.6 billion, less than a third of the first alternative. These three best scenarios have also a breakeven point that falls within the short period of the fifth (graph 1), the seventh (graph 2), and the second year (graph 3) respectively, which are also independent by the discount rate applied (same year for all the discount rates). Other cases match the breakeven point in the nineteenth year for the alternative 2b (graph 4), and none for the alternative 2a (graph 5) and alternative 2c (graph 6); hence, highlighting the no profitability of these latter alternatives, which are not reaching a positive

balance. Hence, as previously introduced, the rational decision maker should opt for the first alternative, given the highest economic returns and faster breakeven, and lesser demolition costs; however, some limitations such as biomass composition, pollution, social aspects, and industrial flexibility can influence this choice.

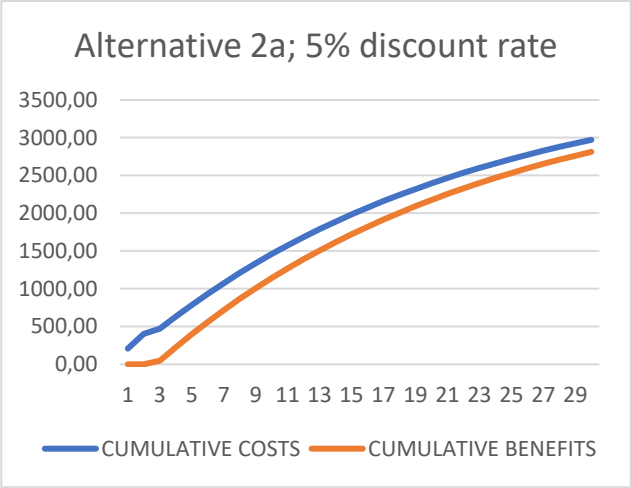
Below, the cumulative costs vs cumulative benefits graphs, with a 5% discount rate applied over thirty years, are reported according to table 9; the match point of the two lines is the breakeven year.

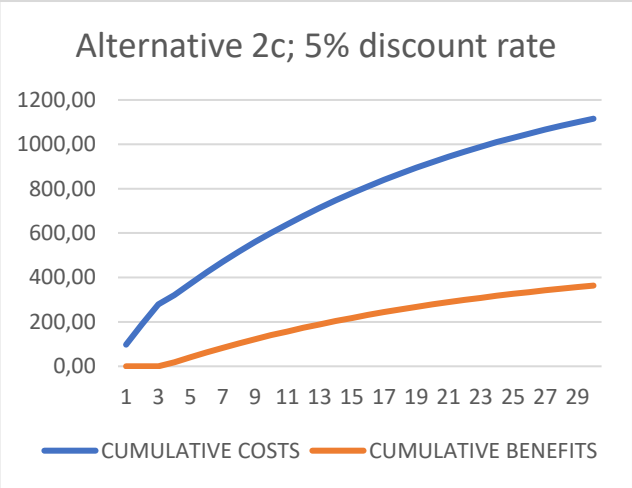


Graph 1,2) On the left: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 1. On the right: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 2d. Both with a 5% discount rate over a period of 30 years.



Graph 3,4) On the left: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 3. On the right: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 2b. Both with a 5% discount rate over a period of 30 years.

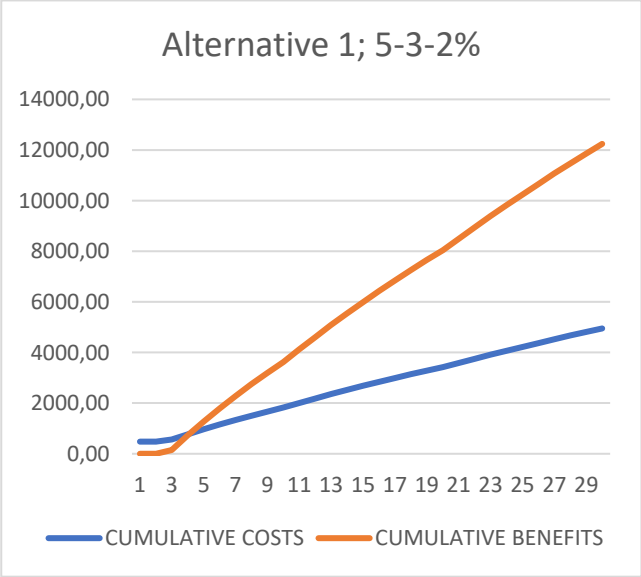


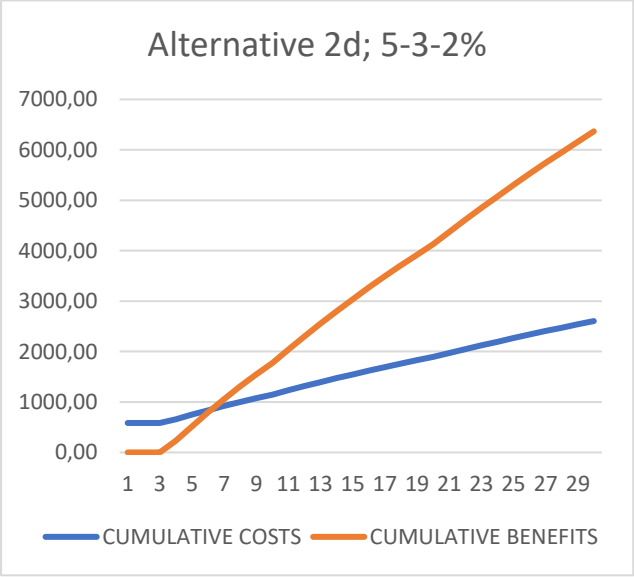


Graph 5,6) On the left: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 2a. On the right: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 2c. Both with a 5% discount rate over a period of 30 years.

Variable discount rate

Given the context's complexity and the long term of this investment, It is also possible to study the NPV with a decreasing discount rate over time to match the need of increased sensitivity. This is made by dividing the future into three different sub-periods of ten years each, characterized by a decreasing discount rate; in detail, 5%, 3%, 2% were chosen inspired by Weitzman (2001). Eventually, the output is 7.3 billion 2022 dollars for alternative 1 and slightly less than 3.8 for alternative 2d, with a breakeven falling at the fifth and seventh year respectively (graph 7,8), as with previous setup.





Graph 7,8) On the left: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 1. On the right: the cumulative costs vs cumulative benefits with the breakeven point for the Alternative 2d. Both with a decreasing discount rate over a period of 30 years.

Given that the ratio among these two alternatives is somewhat constant, as the first doubles the second, with both fixed (5%) and decreasing (5-3-2%) discount rate settings; a decreasing discount rate could be more suitable to intercept the increasing social comprehension, the increasing circular and sustainability trends of biofuels and bioproducts on market, as well as the benefit consequent to the environmental relief due to this remediation act. Thus, allowing for stronger economic considerations, also with the support of the foresight analysis based on social and environmental parameters.

Foresight Results

The second part of this study considers the key factors of pollution and social aspects for strengthening the CBA's best scenario using SWOT foresight techniques, which is at the end of the next two sections.

Chemical Pollution

In this section the focus is set on the pollution presence that threaten algal biomass exploitation. As a matter of fact, as described in previous sections, the usage of algal biomass is not a single and straightforward process and various processes could co-exist with the purpose to extract, isolate, transform, and produce a single compound, or family of compounds. Therefore, the presence of pollutants in the feedstock biomass can lead to interferences with the transformation and isolation processes' performances, compromising the overall plant conversion efficiency. Pollutants, then, might be also present in the final product (e.g., co-extraction), causing extra costs for cleanup and purification, also in the by-products and wastes. A couple of examples: the overabundance of metals can force the biomass residues to the landfill, rather than being used in cultivation; or the extraction of organic bioactive molecules can be altered by the presence of pesticides.

For what concerns our case study, the biomass from Goro's lagoon may be affected by pollutants belonging to near shores, sea, and also freshwater, where the latter involves the whole river's hydrogeological basin. In detail, the Po river's basin is very wide and covers almost the totality of the northern Italy, the so called "Pianura Padana", or Padanian flatland. Moreover, this area (figure 6), is greatly interested by anthropic presence and activities i.e., stress sources.

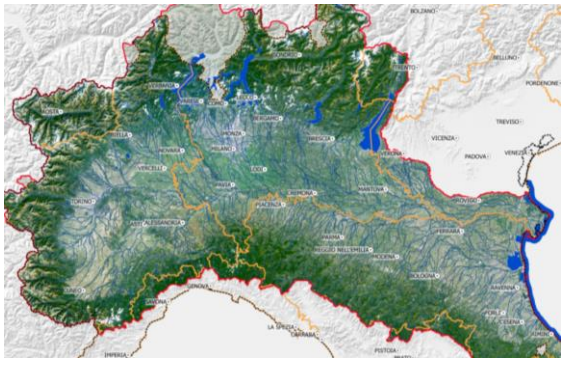


Fig. 6) Po river's hydrogeological basin (blue). In yellow the regional limits and in red the competence limits.

Thus, given the variety of the anthropic presence and activities, it is not unusual to find a variety of macro- and micro-scopic pollutants classes, in the forms of large solid trash fragments, sand, grit, and other fine solids, that can be washed by stormwater and wind into the rivers. Other than microbiological hazards, nutrient and chemical pollution can imbalance ecosystems, which are from industrial facilities, households, wastewater treatment plants, runoffs from farms and roads (Chaudhary, Mishra and Kumar, 2017; Grizzetti *et al.*, 2017).

As mentioned in previous sections, these chemicals (mainly PBT class) can damage the environment immediately and catastrophically, or else, can build threats slowly, accumulating in plants and animals' tissues. Eventually, the fate of the inland generated pollutants is to follow the freshwater's flowing up to the outfall in the sea or ocean. For what concerns the Po River, among the major players of this insidious category, it is possible to find, heavy metals, pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, drugs, and their metabolites (Murray, 2014; Rastogi, Mahmoud and Kümmerer, 2017; Midwest Consortium for Hazardous Waste Worker Training, 2018).

Given the variety of pollutant within the biomass, the biorefinery should be carefully evaluated in order to maximise the processes cost/effectiveness, and also the fate of the residues.

Social Failure

This last section is based on a failed attempt of exploiting algal biomass; where the failure came as a consequence of a single, poorly managed, weakness, despite the number of "strong" beneficial points with plenty of opportunities, as reported in the SWOT resume table in the next section, leading to a complete failure: environmental, social, and economic.

In order to face the growing issue of algal blooms, in 2012-13, the Goro's city administration opted for a different approach than the previously reported "Case zero". Therefore, a biogas plant was studied in collaboration with the company named "CCGL Group" and a group of biologists, as reported in the following newspaper articles of that period (Messina, 2013; Valentini, 2013).

The hypothetical combinate plant should have used washed algae, along with other biomasses to dilute the high content of Phosphorous and Nitrogen within algal masses, as feedstock for anaerobic digestion for biogas production. Therefore, biogas is transformed in heat i.e., hot water and electricity, to be introduced directly into the city's grid, and the remaining digested could be transformed into soil conditioner through composting process. This project was mainly financed by the company itself (CCGL group) with European funds, as it should have become a reference case for further development of innovative technologies and methodologies, for example the sea-cultivation of biomass to meet the winter months' needs for feedstock.

The plant should have been built directly on the Goro's lagoon and starting to operate in 2014/2015, also lifting the management expenses from local administration, seen previously in case zero, but unfortunately something went wrong.

In fact, the biogas plant once seen by the locals as a "saviour" for local economy suddenly turned into a deadly threat for the ecosystem, in locals' opinion. The main reason for this change in perspective is reported by word of mouth and appears to be for a political climb to power. However, in grey literature it is

possible to find few information on how the story developed, from discontent to strong opposition committee.

In the article of Forti (2014), the motivations and reasons to oppose the biogas plant are reported: whilst the many positive impacts are nullified, the possible and potential negative impacts and risks are over-magnified, without much accuracy and or scientific bases, but mainly with a fear instiller approach, such as:

- Suspicious sources of the other biomasses, with dilution purpose, and consequent release of unknown and deadly substances in the atmosphere,
- Suspected structural fragilities and hypothetic plant's lack of resilience toward natural disasters and rare phenomena,
- Suspected enormous microbiological and biological damages in case of un-stabilized compost misuse,
- Suspects regarding the "unknown" air emissions (usually air emitting plants are transparent and produce real time data, with law limits, like in this incinerator example nearby to the case study's area: (HERAmbiente, 2022)),
- Personal, biased, uncheckable experiences, inconsistent and exacerbated facts, like: "thirty noisy trucks each 12 minutes to and from the plant".

However, the potential benefits of this particular bio-plant would have been several, for instance:

- ✓ Solution to HABs issue,
- ✓ Rise of new activities for bio products commercialization,
- ✓ Openings for more workplaces,
- ✓ Increased circularity of resources, and carbon cycle with lesser "new fertilizer" bought.
- ✓ Renewable production of energy and heat for local consumption.

Eventually, in this last article of Dall'Oca (2014) it is reported that "citizens have won" against the "bad biogas plant", confirming the complete change in perspective, and the overall failure of the project as well as the people involved.

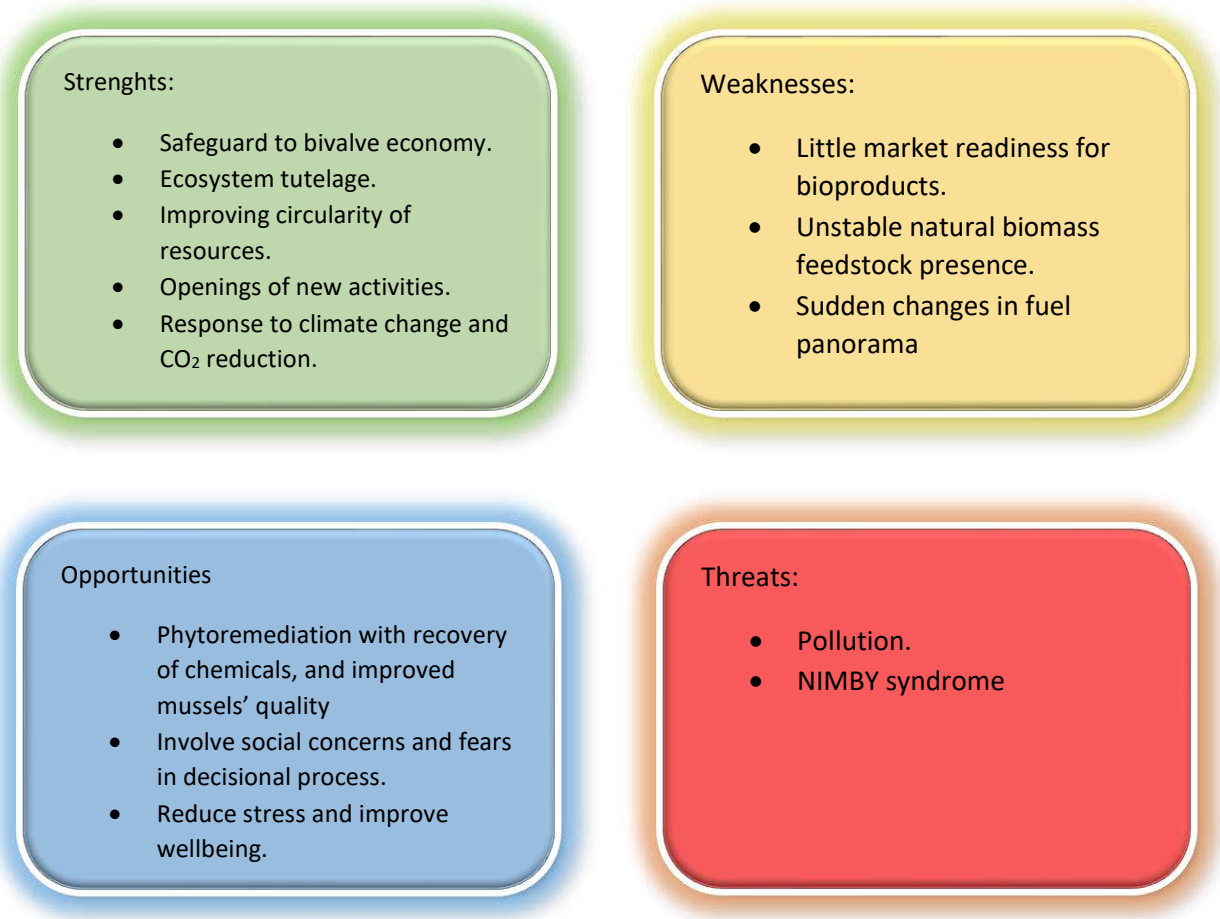
Whilst economic data for an effective comparison in the CBA are not available, from these reports it is possible to draw that the hidden social factors were a crucial weakness that should have been taken into consideration when trying to estimate the overall profitability, both environmental and economic, of the project.

In fact, major stressors for communities (i.e., threats for projects) are possibly connected to the fear of the unknown, and therefore from changes in habits and contexts usually taken for granted. Thus, imposed changes in landscapes, boundaries and surroundings may be seen and lived as war declarations. This phenomenon can find roots in the "Not In My BackYard" (NIMBY) syndrome (Peter Margulies, 1992; Rasmussen, 1992; Wexler, 1996; Xu and Lin, 2020), which refers to a particular condition where peoples and communities are willing to accept change and innovation, only if that change do not impact directly their daily life by being "not in their backyard".

The “Best Position” was rightfully economically evaluated to minimize or nullify the transportation expenditure, leaving only the biomass collection costs; however, it would have affected the landscape by placing the bio-industry directly upon the shores of the lagoon, which is the locals’ most precious good given the economic returns and wellbeing that it provides; possibly, this was the mistake that triggered fear in local stakeholders and the change in perspectives toward the beneficial role of the facility.

In general, whichever the alternative chosen, the social problems should be tackled by choosing a best position in agreeance with the local communities, since the problems rise with the “where” not the “what”. Thus, promoting a piercing campaign of effective communication regarding the mutual benefits for people’s wellbeing and their pockets, along with the positive outcomes of a functioning bioeconomy that generates incomes and creates workspaces, while relieving expenditure from administration, lowering environmental impacts, and improving ecosystems services quality.

SWOT Analysis



General SWOT analysis for Goro’s lagoon case study

Strenghts:

- Bivalve economy tutelage.
- Ecosystem tutelage.
- Improving circularity of resources.

Weaknesses:

- Unstable natural biomass feedstock presence.

Opportunities

- Involve social concerns and fears in decisional process.
- Reduce stress and improve wellbeing.

Threats:

- Pollution.
- NIMBY syndrome
- Sudden changes in fuel panorama

Targeted SWOT analysis for Alternative 1

Strenghts:

- Bivalve economy and ecosystem tutelage.
- Improving circularity of resources.
- Openings of new activities and workplaces.
- Response to climate change and CO_2 reduction.
- Resilience over time

Weaknesses:

- Unstable natural biomass feedstock presence.
- Pollution.

Opportunities

- Phytoremediation with recovery of chemicals, and improved mussels' quality
- Involve social concerns and fears in decisional process.
- Reduce stress and improve wellbeing.

Threats:

- NIMBY syndrome
- Little market readiness for bioproducts.

Targeted SWOT analysis for Alternative 2d

Discussions

The output of the CBA indicates the best alternative as the full biofuel approach (alternative 1), which involves a systemic conversion of fats, carbohydrates, and residues into biodiesel, bioethanol, biogas, and digestate; hence the feedstock biomass should have a minimum fatty composition that allows for such a processing. However, the solely production of biofuels, mainly biodiesel, can be little resilient to changes, as for example the European policy proposal for the stop to internal combustion vehicles production fixed to 2035 (ANSA, 2022b), or unforeseen disturbances in environmental and climatic parameters that reflects on algae quality and quantity. All factors that can represent tombstones for this phase 1 facility, since there is still little understanding of the capacity of biofuels industries to respond to rapid, nonlinear, unpredictable changes, and exogenous shocks, such as technological innovation, bio-markets availability, societal perspective, and environmental factors (Mu *et al.*, 2011).

For these reasons, even if less remunerative, a biorefinery approach (alternative 2.d) should be considered, because it allows for various long-term benefits, given the more strengths and opportunities as the SWOT analysis suggests, such as the improved adaptation to the context and to the waste algal biomass composition and availability, and also to unforeseen and unknown factors. This alternative can also face more effectively the pollution issues, discussed in the foresight analysis.

In fact, as described in previous sections, some classes of pollutants tend to be stored in algae because of the pollutants' bioaccumulative behavior. Thus, this can represent a disabling and/or compromising effect toward the biofuels' quality and/or forcing the exhausted biomass to landfill rather than to agriculture, because of the excessive concentration of pollutants in the digested residues. However, as previously anticipated, algae are already being used as remediators for polluted areas given peculiar affinities toward some classes of pollutants (Bishnoi and Pant, 2004; Chekroun, ben Chekroun and Baghour, 2013; Hanbali, Holail and Hammud, 2014; Mahmood, Mirza and Shaheen, 2015). This particular way of remediating to environmental pollution is called phytoremediation, and it is a process where pollutants are removed from the environment and stored within algae or degraded to lesser or unharmful species (Kandasamy *et al.*, 2021).

Therefore, to perform ex-ante qualitative and quantitative analyses on micropollutants within algal biomass to acknowledge and monitor the overall health and pollution status of the area, could lead to the strategic development of a bio-plant that turns weaknesses and threats of polluted biomass into opportunities for further ecosystem's quality improvements, and optimized biomasses exploitations. Generally, avoiding to waste resources in processes that would yield polluted products due to the interference of pollutants, that must be eliminated in further purification processes prior to the commercialization of the final (refined) product, adding costs and equivalent CO2 emissions. Heavy metals (such as lead) are a perfect example for this process given the natural affinity that seaweeds show toward them; hence, ideally projecting a bio-industry that produces primarily biofuels and metals as by-products (Cheng *et al.*, 2019).

The hypothetical decision-making process, then, should result simplified by emphasizing the production of biofuels (alternative 1) and the multiproduct biorefinery (Alternative 2d) as the most promising ways to face the stress sources that are threatening the lagoon, both with a fixed discount rate of 5% and a decreasing one (5-3-2%). At the same time the possibilities to be avoided are highlighted (mainly the bioethanol-only productions). However, the second-best option has the lower economic returns but shows other advantages in terms of flexibility over time and synergy with other technologies and activities as the phytoremediation, which is crucial for improving aquaculture products quality. However, the best

alternative to be chosen should be fitting to the pollution situation proper of the case study, then the third best option (Alternative 3), even if it is the least remunerative, should not be discarded a priori since, in case of certain contaminations, interfering with biofuels production, could still be a valid opportunity to exploit biomass.

Eventually, the totality of the benefits discussed until this point should be presented to the decision makers and stakeholders e.g., local and regional administrations, local people, fishermen, and aquaculture workers, in order to stimulate participation and increase acceptance toward the bio-plant, while avoiding fears and resistance. Thus, it should be considered a more costly, but socially acceptable, "Best Position" i.e., the furthest from social awareness and nearest to the operative area; to achieve a position with relatively low compromission of natural landscapes and lesser NIMBY Syndrome, without wasting too much in transportation costs and CO_2 emitted. Furthermore, local administration can also participate by fostering science-based information and develop incentives to increase the social acceptance of the alternative chosen. Hopefully, paving the way for a sustainable and circular action; with the cooperation of locals rather than opposition phenomena, as it has been for the failed case seen in the foresight section.

Unfortunately, the exiguous number of working biorefineries and articles in literature were a major limitation to this work, hence limiting the confrontations for finding the best way to handle algal biomass to macroscopic and superficial differences. Hence a future study should involve a focus on biorefineries that are most compatible with remediation and phytoremediation techniques.

Conclusions

In this work a Cost Benefit Analysis for the valorization of waste biomass to improve circularity of resources and environmental tutelage has been proposed; with the addition of a Foresight analysis to better fit in the social and environmental contexts of Goro's lagoon, a highly valuable but endangered transitional water ecosystem that provide fruitful services, economic returns, and sustains local activities.

The goal is to promote a multidisciplinar approach to obtain innovative and long-term sustainable ways for algal wastes valorisation and the development of more competitive bio-products on the markets, with the collaboration of locals and the respect of their wellbeing. Therefore, monitoring activities could play a major role as a KPI toward environmental health and as a base for ecosystem services quality improvement, given the opportunity to bioremediate pollution, while recovering valuable chemicals and exploiting biomasses for biofuels and bioproducts. In addition, given the long lifespan of a bio-plant (thirty years) in these times of climate challenges and changes, the flexibility requirements could be a key-factor for the project's success; hence, the best alternative may not be the most remunerative, as the CBA indicates, but the one which better adapts to the context, as the foresight and SWOT analyses suggest. In these regards, the multiproduct biorefinery has the potential to be the most promising way to face the stressors acting upon the lagoon, over its whole lifetime; whilst, the production of biofuels is a direct and simpler alternative, belonging to the high-risk high-reward paradigm, producing much more incomes on the chart, but may suffer from the passing of time, by the means of market needs and policies.

However, given the historical reality of this location toward bio-plants, the decision-making process should take place involving locals, to foster a sustainable way of circular tutelage.

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Section 2: Investigations Upon Waste Valorization Suitability

The previous section was focused on the crucial role of the ex-ante discrimination among ways of valorization for algal biomass, toward the tutelage of the stressed and endangered Goro's Lagoon, in the northern Adriatic sea. The following is the second part of the first chapter of the thesis; where are reported the peculiar insights of the environmental monitoring activities that were performed, which complete the output of the previous section.

Introduction

Monitoring activities allows for the production of key drivers in order to make rational decisions based on the local peculiarities of biomass, pollutant distribution, and their presence. Some preliminary analyses have been conducted to get an overall view of the Goro's lagoon ecosystem's health, through the determination of certain micropollutants in water, sediments, mussels and algae, belonging mainly to the volatile, organic, and heavy metals classes. These pollutants have been chosen because of their ability to bioaccumulate in living tissues, hazardous behaviors, and for the possibility to represent obstacles for the waste algal biomass' valorization process. Also, the bromatological composition of algae have been determined in different periods of the year, looking for macroscopic compositional differences among yearly and seasonal periods.

For each pollutant class the most common and dangerous chemicals have been investigated; the target analytes' are usually listed and noted as "most concerning", "emerging", and "priority" pollutants by ISPRA (Istituto Superiore per la Ricerca e Protezione Ambientale), ICRAM (Istituto Centrale per la Ricerca Scientifica e Tecnologica applicata al Mare), IRSA (Istituto di Ricerca Sulle Acque), OGS (Istituto Nazionale di Oceanografia e Geofisica Sperimentale), and EPA (Environmental Protection Agency); along with sample preparation from various matrixes and instrumental analysis procedures; which were suited to our instruments and laboratory equipment. In appendix 2 the technical procedures are reported and described in detail.

For what concerns the volatilome, the pollutant classes of halophenols, halomethanes, and haloacetonitriles, with relative chemicals, have been investigated. The nonvolatile pollutants class includes various sub-classes of organic chemicals known as haloacetic acids, PAH, PCBs, pesticides, organotins, and the inorganic class of heavy metals.

Experimental

Samples investigated were collected in two sites of the Goro's lagoon, one near a small dock and one at the center of the lagoon, over the 2020-2021 years, with the purpose to highlight the differences, if present, between the two spots, the years, and the bioaccumulation in algae and mussels. Fresh samples were homogenized, kept in dark, and refrigerated; a fraction was freeze dried and stored in dark.

To ensure robust quality control and assurance, multiple replicates were analyzed, when possible, along with procedural and instrumental blanks to minimize interferences from glassware (washed carefully with ultrapure water), instruments, and other chemicals; in addition, the analysis of certified reference materials (CRM) were performed to evaluate the analysis procedure's robustness, through the overall recovery factor. Also, samples were added with internal and surrogate standards, inert to the analytes and tactical for analyses, thus achieving a better quantification, accounting for the preparation and analysis inevitable losses of analytes and foreigner presence.

The analyses were carried with various instruments connected with proper revelators, specific for the target analytes class. Given the complexity of the matrixes and the number of analytes, the analytical techniques were mainly of separative nature; hence gas chromatography, and liquid chromatography paired with mass filters, electron capture, or fluorescence analyzers, depending on the analyte's nature. Therefore, the analytes must be isolated from the sample's matrix, in order to obtain more accurate signals and consequently smaller errors in the analytes' quantification.

Sample preparations involve various procedures to transfer the analytes from the sample to a form compatible with the instrument. Thus, extraction, derivatization, concentration, and purification techniques are common procedures to achieve this goal. The extraction is the process of adding a solvent mixture to which the analytes have particularly affinity with, so that they transfer spontaneously from the solid sample to the solution; this process can be performed multiple times and can be enhanced with ultrasounds and high temperatures. In some cases, it could be necessary to modify the analytes' chemical structure through chemical reaction; this process is needed when some characteristics of the analytes must be enhanced or discouraged, such as the volatility or the solubility. The concentration process literally means to increase the concentration of the analytes, usually achieved by reducing the volume of the extracts, which can be done by rising the temperature, however, given some analytes' relatively high volatility, the risk is to lose them to the air through evaporation processes. That is the reason why solvents are usually depleted with a stream of pure nitrogen in a steady temperature bath; however, the lightest analytes still can be partially lost. Eventually there is the purification step that is meant to further remove residues, co-extracted from the matrix, such as other chemicals not belonging to the investigated analytes class and water. Hence, the addition of specific removal agents is needed, e.g., copper to remove Sulphur or sodium sulphate to remove water, or by passing the extracts through a resin that retains the analytes, to be further eluted with fresh solvent after washing away the interferents. The purified is then ready for the instrumental analysis. Whilst the previous set of procedures is generally exemplificative for the investigation of organic pollutants, heavy metals followed a different preparation path, consisting of a digestion with strong acids under microwave heating assistance in order to mineralize all metals into solution, allowing for instrumental analysis. The instrumentations used are described below.

ICP-MS

This technique is the combination of the sample's atomization with the inductively couple plasma paired with mass spectroscopy; in other words, the sample is atomized by the plasma flame and then analyzed with a mass filter, in our case a quadrupole. The inductively coupled plasma (ICP) is a highly ionized gas generated by the interaction of a strong magnetic field with Argon gas flowing through a quartz glass torch. The sample is transferred, as an aerosol, into the central region of the plasma (6500K), where it is rapidly dried, dissociated, atomized and ionized; then it is directed, through vacuum to the mass filter. The mechanisms of the mass analyzer is to filter out non-analyte, matrix, and interfering ions and selectively transmit analyte ions of a single mass-to-charge ratio (m/z) to the detector one at a time, and thus to acquire signals. Given the destruction of compounds into basic elements, this technique is used majorly to make speciation studies and determination of isotopic composition, metals, and rare earth elements in trace concentrations (part per billion) with very low detection limits (Ammann, 2007). If the samples show high concentrations of analytes, or interferents, it is possible to dilute them or to use another technique, the ICP-OES.

ICP-OES

The inductively coupled plasma-optical emission spectroscopy is an analytical instrument capable of measuring the light (optical emission) produced by a liquid sample when introduced into an inductively coupled argon gas plasma. Through this mechanism it is possible to quantify the metals contained in the sample by measuring, for each, the intensity of the light emitted with a specific optical system. Then, this technique makes use of the unique photophysical signals of each element to successfully detect the type and relative amount of each element within a solution (Khan *et al.*, 2022). When using the ICP technique the sample is irreversibly destroyed and reduced to its elementary components; metals are elements therefore this technique is most suitable for this analyte class. However, complex mixture of chemical compounds cannot be characterized with these instruments. Hence, separative apparatus of gas and liquid chromatography are used to investigate the above listed classes of pollutants.

GC-MS

In chemical analysis, chromatography is a laboratory technique for the separation of a mixture into its components. The mixture is dissolved in a fluid solvent (gas or liquid) called "mobile phase", which carries it through a system (a column, a capillary tube, a plate, or a sheet) on which a material called the stationary phase is fixed. The different constituents of the mixture tend to have different affinities toward the stationary phase and thus are retained for different times depending on their interactions (modality and strength) with its surface sites. The separation is then based on the analytes' differential partitioning between the mobile and the stationary phases. Gas chromatographic separations happens when the mobile phase is a gas and is always carried out in a column, which is "capillary". Capillary columns generally have great resolution and are especially used for resolving complex mixtures. The stationary phase can be adsorbed or chemically bonded to the column walls or both (support particles adhered to column walls, but those particles have liquid phase chemically bonded onto them); a GC capillary column is measured in meters-wise length with an inner diameter of commonly 0.53 – 0.18mm. In other words, GC is based on the

analytes' partitioning equilibria between a solid or viscous liquid stationary phase and a mobile gas. This instrument is then paired with a mass filter that scans for mass to charge ratio that collects structural information for each compound separated through GC (Al-Rubaye, Hameed and Kadhim, 2017). This technique is used for volatile analytes.

GC-ECD

GC paired with electron capture detector (ECD) is extremely sensitive toward chemicals compounds that contains electronegative elements in the structure, such as the halogens; therefore PCB, pesticides, and haloacetic acids are suitable to be accurately quantitate with this technique. The ECD consists of a source of electrons, a beta emitter radionuclide with a made-up gas of nitrogen that releases electrons. The electrons are then accelerated towards a positively charged anode, generating a current; as the sample is carried into the detector by the carrier gas, electron-absorbing analyte molecules capture electrons and thereby reduce the current between the collector anode and a cathode, producing the output signal (Selvi *et al.*, 2012). PCB and pesticides were analyzed together and given the number of analytes a parallel double column instrumental set-up was adopted; the two columns were the DB-5 and the DB-1701, which are chemically slightly different. Thus, when the sample is injected, it splits into the two columns where the analytes will be eluted with different retention times, allowing for the quantification of co-eluted substances in a column which are not in the other.

HPLC-FLD

The separation in this instrument happens between the stationary phase and a liquid mobile phase under pressure to enhance performances; hence HPLC stands for High Pressure /Performances Liquid Chromatography. The column is centimeters long with a millimetric inner diameter. The revelator exploits the excitation of the electrons in molecules of certain compounds using a beam of light, usually ultraviolet, causing them to emit light back, that is measured to produce output signals. Therefore, given the aromatic structure of delocalized conjugated π systems that easily interact with light, this technique is most suitable to investigate PAH analytes (Pang, Yuan and Huang, 2018).

Bromatological composition and lipidic profile

Bromatology is the science that studies the composition, characteristics and chemical, chemical-physical and physical properties of food. It examines the individual nutritional factors (proteins, carbohydrates, lipids, vitamins, minerals) within the food sample (Varelis, 2016). Hence, in this study the bromatological analysis was conducted on the two species of algae, in order to determine the characteristic percentages of proteins, ash, lipids and soluble and insoluble fiber. Proteins were determined with the Kjeldahl standard procedure (Varelis, 2016), after digestion in concentrated sulfuric acid, the total organic nitrogen is converted to ammonium sulfate. Ammonia is formed and distilled into boric acid solution under alkaline conditions. The borate anions formed are titrated with standardized hydrochloric acid, by which is calculated the content of nitrogen representing the amount of crude protein in the sample. Most proteins contain 16% of nitrogen, thus the conversion factor is 6.25 (Varelis, 2016). Lipids can be quali/quantified

through GC-MS once extracted from the matrix, purified, and trans-esterified. The extractions are possible with various techniques depending on the fragility of the lipids in the sample; thus, for Gracilaria and Ulva algae two different techniques were deployed, respectively the Randall procedure with Soxhlet extraction (Kochneva *et al.*, 2019) and an extraction with methanol/chloroform. The ashes represents the inorganic residue after the sample's combustion, under certain conditions (CILIANA *et al.*, 2022). The total fiber, or carbohydrates, represents the totality of polysaccharides and lignin within the sample, it could be classified as soluble fiber (pectins, pentosans, β-glucans and other hydrocolloids) or insoluble fiber (cellulose, hemicellulose and lignin). The quantification of the total fiber is made with the "Megazyme Total Dietary Fiber Kit" method (Megazyme, 2017), which involves the addition of chemicals and enzymes followed by filtrations, extractions, and weight to quantify and sum the soluble and insoluble fibers; these values must be corrected by performing protein and ashes analysis on the fiber residues.

Results

In this section, the most relevant and significant results are reported and where necessary are commented; additional results are reported in appendix 2. The value expressed are mean values with instrumental confidence upon the estimations, unless otherwise specified. Calibration curves were built to widely include the quantity of the analytes.

Algae

Bromatological determination

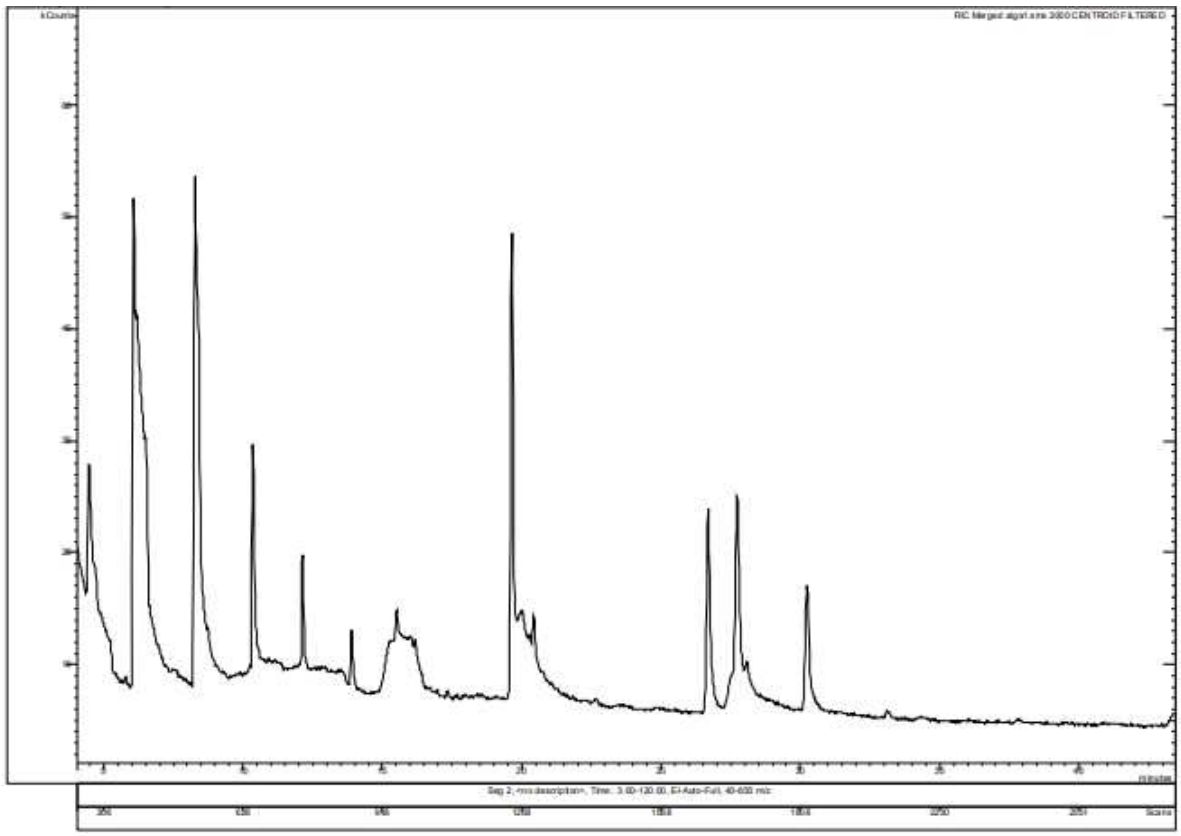
The next table (Table 1) reports the bromatological composition of both Ulva and Gracilaria, the latest over two different seasons. Also, the lipidic profile graphs (Graph 1, 2), with percentage composition tables (Table 2 and 3) are reported below. It is possible to note that Gracilaria's composition varies substantially among seasons, hence among the various stages of growth, however it is not showing lipids if compared to the Ulva algae whose instead has appreciable amounts. Data found are more or less in line with existing literature (Lourenço-Lopes *et al.*, 2020) except for some outliers that are possibly due to differences in the geo-physical-chemical environmental parameter of their habitat, such as temperature, irradiation, pH, salinity, and nutrients.

	Winter							
	Proteins		**Ashes**		**Fibers**		**Lipids**	
Gracilaria	28.00	±0.72	17.00	±0.30	55.00	±3.50	<1	\
	Summer							
	Proteins		**Ashes**		**Fibers**		**Lipids**	
Ulva Lactuga	18.12	±3.53	20.30	±0.069	51.52	±3.54	7.56	±0.026
Gracilaria	20.34	±0.68	13.02	±0.20	60.19	±3.75	<1	\

Table 1) Bromatological content for Ulva and Gracilaria with errors esteem; Gracilaria was also investigated in winter season.

The ash content is in line with literature that defines the range between 8 and 40% in marine algae. Generally, algae show high amounts of ash due to the polysaccharides that make up the cell walls and functional groups (carboxyls, sulphates and phosphates) which appear to be excellent bonding sites for metals, resulting in a large supply of mineral components (Mwalugha *et al.*, 2015).

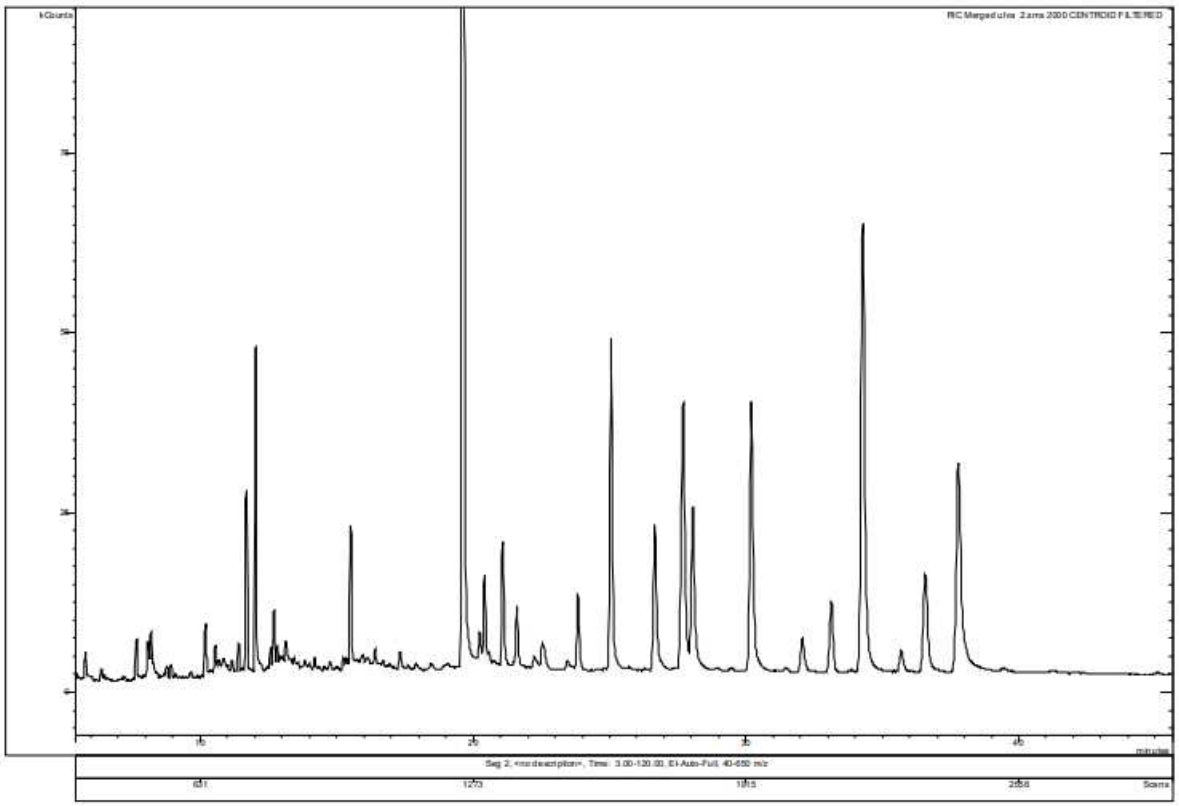
For the protein content various reference values are available, ranging between 7% and 13% for Gracilaria and between 4.2% of Ulva sampled in the Philippines and 27.2% of that sampled in Chile, indicating that these variations are strongly influenced by the seasonality, the growth stage, environmental conditions, and the presence of nutrients (such as nitrogen and phosphorous) in the water (Tabarsa *et al.*, 2012).



Graph 1) Chromatogram for the Gracilaria's lipidic profile

Compound name	Retention Time (min.)	Peak area	%
C16: 0 - palmitic acid	19.644	311735	35.18
C18: 0 - stearic acid	26.690	185098	20.89
C18: 1 - oleic acid	27.732	259917	29.33
C18: 2 - linoleic acid - ω6	30,235	129383	14.60

Table 2) Lipidic fractions for Gracilaria algae, referred to the peaks in Graph 1



Graph 2) Chromatogram for the Ulva's lipidic profile

Compound name	Retention Time (min.)	Peak area	%
C12: 0 - lauric acid	12.701	45728	1.13
C14: 0 - myristic acid	15,513	115022	2.83
C16: 0 - palmitic acid	19.631	1.68E $^{+06}$	**41.25**
C16: 1 - palmitoleic acid	20.246	22589	4.79
	20,42	69 249	
	21.090	102955	
C16: 2	21,608	56036	1.38
C16: 3	23,849	70020	1.72
C18: 0 - stearic acid	26,659	183113	4.51
C18: 1 - oleic acid	27.694	358741	**14.39**
	28.051	225972	
C18: 2 - linoleic acid - ω6	30.194	359418	8.85
C18: 3 - linolenic acid - ω3	34.282	650696	**16.02**
C20: 5 - EPA - ω3	51,536	58516	1.44
C22: 6 - DHA	60,382	40037	0.99
C22: 3	61,299	24543	0.60
C22: 0	63.119	4159	0.10

Table 3) Lipidic fractions for Gracilaria algae, referred to the peaks in Graph 2

In terms of lipidic profile, Gracilaria and Ulva Lactuga were tested with two different methods: the first with a hot extraction using automatic Soxhlet, while the second by hot extraction with chloroform and methanol 2:1. The number of fatty acids present in Gracilaria was found to be extremely low, less than 1% on 100g of dry sample. In the table, the percentage column (Table 2) represents the percentage of each fatty acid on the total of fatty acids found. Therefore, concluded with a semi-quantitative analysis, in which the quantification was made through the ratio between the peaks (Graph 1).

Ulva Lactuga, on the other hand, shows a presence in fatty acids equal to 7.45g per 100g of sample's dry weight; The greater amounts of fatty acids whose identification and quantification could be determined thanks to the chromatogram (Graph 2) is proof of the higher lipid content present in Ulva compared to Gracilaria. Similarly to the previous case, the percentage column (Table 3) represents the quantity of each fatty acid with respect to the totality of fatty acids found.

Both in Gracilaria and Ulva, palmitic acid (C16: 0) is the one present in higher percentages, as confirmed by the literature, which reports in these two species a percentage of C16: 0 around the 35% (Tabarsa *et al.*, 2012). In agreeance with the literature there are also high quantities of C16 and C18 fatty acids (with 16 and 18 carbon atoms) (Abomohra, El-Naggar and Baeshen, 2018); this reference data derive from in-depth studies on the usage of Ulva for the production of Biodiesel, which confirms higher percentages of fatty acids in Ulva Lactuga also compared to other species of Ulva (Abomohra, El-Naggar and Baeshen, 2018). In both Gracilaria and Ulva it was possible to identify and quantify some of the so-called ω3 and ω6, fatty acids essential for humans, that must be necessarily introduced with the diet. The literature confirms, in fact, that the consumption of algae in the diet can strongly contribute to the intake of ω3 and ω6, with a consequent influence in the regulation of blood pressure, in inflammatory responses and in the prevention of heart disease (Tabarsa *et al.*, 2012).

As already mentioned, the fiber determination turns out to be the most complicated and time-consuming analysis, since it requires a data correction through further analysis of protein and ash determination. The results derived from the filtration steps of soluble and insoluble fiber alone, provides a net value of fiber's grams obtained. However, these data must be corrected by subtracting protein and ash values ("Prosky method", AOAC Method 985.29 / 9 91.43), performed as follows:

- calculate the percentage of protein present in each replica, and, by multiplying the percentage value obtained with the net weight of the fiber, divided by one hundred, we obtain the grams of protein.
- the same calculation will be made for the ashes, calculate the percentage of ashes present and, consequently, the grams.
- once the grams of protein and ash have been obtained, we can subtract them from the experimental net fiber value (g), obtaining a new value in grams of fiber, corrected.
- it will now be possible to determine the percentage of soluble and insoluble fiber of each sample following the corrections, using the following formula:

$$\frac{corrected\ fiber\ (g) * 100}{mass\ sample\ used\ (g)}$$

- the sum of IF% and SF% will yield the total fiber%.

Hence, total values are quite higher than the mean in the literature, and yet the reasons can be attributable to habits and seasons differences.

In fact, according to Rosenberg and Ramus (Rosenberg and Ramus, 1984), the synthesis of carbohydrates in the periods of maximum growth, increase in photosynthetic activity and decrease in protein and nitrogen content. The synthesis of carbohydrates seems to be favored, for example, by strong solar radiation and high temperatures, at which instead corresponds a decrease in proteins, which seem to show the highest concentrations in the period between the end of winter and the beginning of spring (Tabarsa *et al.*, 2012; Mwalugha *et al.*, 2015).

Volatilome

Outside of bromatological investigations, the volatilome in Ulva has been analyzed; it was found that all pollutants included in the study were absent or present in concentrations below the lower limit of quantification (appendix 2, Tables 4, 5).

Haloacetic acids

Among the compounds searched, only two were distinctively found in Ulva algae: Dichloroacetic Acid (DCAA) and Dibromoacetic Acid (DBAA) (Appendix 2, Table 6).

PCB and Pesticides

Appreciable amounts of PCBs and Pesticides were found in Gracilaria seaweed (Table 7, 8).

PCB	ng/g
PCB31	**4.26**
PCB28	**0.46**
PCB52	**0.91**
PCB35	**0.18**
PCB101	<LOQ
PCB110	<LOQ
PCB81	<LOQ
PCB77	<LOQ
PCB118	**0.52**
PCB153	**0.46**
PCB105	<LOQ
PCB138	**0.48**
PCB126	**0.62**
PCB128	<LOQ
PCB156	<LOQ
PCB180	<LOQ
PCB169	<LOQ
PCB170	<LOQ

Table 7) PCB content in nanograms per gram of sample (Gracilaria) ±0.02 ng/g

Pesticides	ng/g
α-HCH	**11.53**
HCB	**1.17**
γ-HCH	**4.87**
β-HCH	<LOQ
Aldrin	<LOQ
2,4'-DDE	<LOQ
4,4'-DDE	<LOQ
2,4'-DDD	<LOQ
Dieldrin	<LOQ
2,4'-DDT	<LOQ
4,4'-DDD	<LOQ
4,4'-DDT	<LOQ

Table 8) Pesticides content in nanograms per gram of sample (Gracilaria) ±0,03 ng/g

Heavy Metals

Below, (Table 9) metals in seaweeds from different locations.

µg/g	Ulva Docks	Gracilaria Docks	Gracilaria Open Waters
51 V	7.44	2.85	4.60
52 Cr	9.95	3.84	5.27
55 Mn	264.48	150.64	384.66
63 Cu	9.12	5.85	4.01
66 Zn	40.06	35.02	26.52
208 Pb	3.93	0.67	2.26
60 Ni	8.94	3.42	5.04
137 Ba	18.67	4.22	8.60
56 Fe	3223.98	949.76	1487.31
27 Al	3170.21	1139.74	1630.29

Table 9) Metal content in algae from different locations, docks and open waters in milligrams per kilogram of sample (Ulva and Gracilaria) ±0.02 µg/g

Water

In this section the main results for water analysis are reported.

PAH

Acenaphtene was the only PAH found in both water locations (Appendix 2, Table 10)

Metals

Below the results of metal determination with two different preparation methods in ICP-MS are shown. The first method involves the concentration of target metals on a proper resin, the second instead is the direct injection of the diluted water samples, with the addition of acids and internal standards. Drawbacks for each method are present: the first one concerns the pH at which each metal interacts with the resin, hence with a buffered pH of around 5, some metals are effectively bound, some are mildly bound, and some are poorly or not bound, therefore lost in the purification process. The second method is straightforward with natural and fresh waters, but seawater requires, other than the elimination of sand, inorganic, and organic residues, the dilution of salinity. In fact, if above a certain threshold, it damages the instrumental apparatus; therefore the dilution step unavoidably affects the analytes, that are further diluted, with increased difficulties in their detection by the revelator.

Below, the quantification of metals in seawater are with both techniques, the concentration on cartridge (Table 11) and the dilution (Table 12).

µg/L	Water docks 2020
27 Al	<LOQ
51 V	<LOQ
52 Cr	<LOQ
55 Mn	<LOQ
56 Fe	**2955.21**
60 Ni	**12.76**
63 Cu	**15.75**
66 Zn	**34.15**
75 As	<LOQ
111 Cd	**0.08**
118 Sn	<LOQ
137 Ba	<LOQ
201 Hg	<LOQ
208 Pb	**6.10**

Table 11) metal content in micrograms per liter of a 2020 sample (seawater from docks), obtained with preparation with resin. ±0.02 µg/L

µg/L	Water docks 2021	Open Water 2021
27 Al	**202.09**	**88.90**
51 V	<LOQ	<LOQ
52 Cr	<LOQ	<LOQ
55 Mn	**39.48**	**17.12**
56 Fe	**306.98**	**145.96**
60 Ni	**2.40**	**2.07**
63 Cu	<LOQ	<LOQ
66 Zn	<LOQ	<LOQ
75 As	**0.29**	<LOQ
111 Cd	<LOQ	<LOQ
118 Sn	<LOQ	<LOQ
137 Ba	**25.69**	**30.13**
201 Hg	<LOQ	<LOQ
208 Pb	<LOQ	<LOQ

Table 12) metal content in micrograms per liter of 2021 samples (seawater from docks and middle lagoon), obtained with preparation with filtration and dilution. ±0.02 µg/L

Sediments

Organotins

All organotins, mono-di-tri-tetrabutil-Tin, were absent or in concentration below the lower limit of detection.

PAH

The following table reports the investigated PAH belonging to the sixteen priority PAH pollutants.

ng/g	Dock Sediments	Mid Lagoon Sediments
Naphthalene	**3.17**	<LOQ
Acenaphthene	**12.57**	<LOQ
Fluorene	**12.38**	**0.07**
Phenanthrene	**120.60**	<LOQ
Anthracene	**36.63**	<LOQ
Fluoranthene	**178.85**	**3.69**
Pyrene	**140.50**	**2.84**
Benzo (a) anthracene	**61.72**	**0.63**
Crisene	**76.49**	**0.97**
Benzo (b) fluoran	**74.64**	**4.67**
Benzo (k) fluoran	**37.76**	**0.38**
Benzo (a) pyrene	**73.00**	**3.55**
Dibenzo (a, h) ant	**13.68**	**21.42**
Benzo (g, h, i) per	**39.68**	**2.21**
Indeno	**37.72**	<LOQ

Table 13) PAH content in nanograms per gram of sample (sediments from docks and middle lagoon). ±0.02 ng/g

PCBs and Pesticides

These pollutants were investigated over two years in both locations, to get an overview of the variations between years.

ng/g	Docks Sediment	Mid Lagoon Sediments
α-HCH	<LOQ	<LOQ
HCB	<LOQ	<LOQ
γ-HCH	**0.23**	**0.11**
β-HCH	**0.29**	<LOQ
Aldrin	**0.16**	<LOQ
2,4'-DDE	**0.11**	<LOQ
4,4'-DDE	**0.73**	**0.18**
2,4'-DDD	<LOQ	<LOQ
Dieldrin	<LOQ	<LOQ
2,4'-DDT	**0.35**	**0.49**
4,4'-DDD	<LOQ	<LOQ
4,4'-DDT	<LOQ	<LOQ

Table 14) 2020 Pesticides content in nanograms per gram of sample (sediments from docks and middle lagoon). ±0.03 ng/g

ng/g	Docks Sediment	Mid Lagoon Sediments
PCB31	<LOQ	**0.31**
PCB28	**0.88**	**0.36**
PCB52	**0.35**	**0.17**
PCB35	**0.04**	<LOQ
PCB101	**0.23**	**0.27**
PCB110	**0.05**	**0.05**
PCB77	**0.24**	**0.20**
PCB81	<LOQ	**0.03**
PCB118	**0.24**	**0.15**
PCB153	**0.52**	**0.42**
PCB105	**0.17**	**0.05**
PCB138	<LOQ	<LOQ
PCB126	**0.07**	<LOQ
PCB128	<LOQ	**0.09**
PCB156	**0.12**	<LOQ
PCB180	**0.59**	**0.30**
PCB169	<LOQ	<LOQ
PCB170	**0.13**	**0.05**

Table 15) 2020 PCB content in nanograms per gram of sample (sediments from docks and middle lagoon. ±0.02 ng/g

ng/g	Docks Sediment	Mid Lagoon Sediments
α-HCH	**0.22**	<LOQ
HCB	**0.07**	<LOQ
γ-HCH	**0.02**	**0.35**
β-HCH	<LOQ	<LOQ
Aldrin	<LOQ	<LOQ
2,4'-DDE	<LOQ	<LOQ
4,4'-DDE	**0.43**	**0.25**
2,4'-DDD	<LOQ	**0.07**
Dieldrin	<LOQ	<LOQ
2,4'-DDT	<LOQ	<LOQ
4,4'-DDD	<LOQ	<LOQ
4,4'-DDT	**1.20**	**5.14**

Table 16) 2021 Pesticides content in nanograms per gram of sample (sediments from docks and middle lagoon). ±0.03 ng/g

ng/g	Docks Sediment	Mid Lagoon Sediments
PCB31	<LOQ	<LOQ
PCB28	**0.32**	**0.49**
PCB52	<LOQ	**0.13**
PCB35	**0.17**	**0.25**
PCB101	**0.09**	**0.25**
PCB110	**0.07**	**0.26**
PCB81	<LOQ	**0.08**
PCB77	**0.02**	**0.13**
PCB118	**0.14**	**0.24**
PCB153	**0.19**	**0.37**
PCB105	**0.66**	**1.03**
PCB138	<LOQ	<LOQ
PCB126	**0.04**	**0.03**
PCB128	**0.03**	**0.06**
PCB156	**0.10**	**0.02**
PCB180	**0.05**	**0.13**
PCB169	**0.58**	**0.10**
PCB170	<LOQ	**0.03**

Table 17) 2021 PCB content in nanograms per gram of sample (sediments from docks and middle lagoon. ±0.02 ng/g

Heavy metals

Heavy metals were investigated over two years. ICP-OES was used for the determination, however the second year, As and Cd were analyzed with GF-AAS (Graphite Furnace Atomic Absorption Spectroscopy) (Lewen and Schenkenberger, 1999), while mercury was analyzed with SMS-100 (Habiba *et al.*, 2017); instruments that are more suitable for those analytes.

	Docks Sediments	Mid Lagoon Sediments
V (mg/kg)	27.00	21.30
Cr (mg/kg)	74.40	82.70
Mn (mg/kg)	622.00	472.80
Cu (mg/kg)	32.90	5.70
Zn (mg/kg)	72.90	48.40
Pb (mg/kg)	6.98	6.30
Ni (mg/kg)	48.70	69.00
Ba (mg/kg)	40.30	15.90
Fe (mg/kg)	14664	16154.30
Al (mg/kg)	11675	8131.80
As (mg/kg)	3.90	3.30
Cd (mg/kg)	0.10	0.10
Hg (mg/kg)	<LOQ	<LOQ

Table 18) metal content in milligrams per kilogram of 2020 samples (sediments from docks and middle lagoon). ±0.02 mg/Kg

	Docks Sediments	Mid Lagoon Sediments
V (mg/kg)	24.00	24.55
Cr (mg/kg)	82.40	99.05
Mn (mg/kg)	610.00	515.50
Cu (mg/kg)	20.10	4.295
Zn (mg/kg)	59.75	48.15
Pb (mg/kg)	4.765	3.27
Ni (mg/kg)	61.85	103.75
Ba (mg/kg)	32.95	20.60
Fe (mg/kg)	15200	16200
Al (mg/kg)	10700	10950
As (mg/kg)	5.404	3.770
Cd (mg/kg)	0.0715	0.0925
Hg (ng/Kg)	0.0153	0.0378

Table 19) metal content in milligrams per kilogram of 2021 samples (sediments from docks and middle lagoon). ±0.02 mg/Kg for the ICP-OES analytes; ±0.002 mg/Kg for As and Cd (GF AAS); and ±0.002 ng/Kg for Hg (SMS-100).

Mussels

Unfortunately, the samples from the docks location were available only during 2020.

PAH

There is no evidence of a clear distribution pattern over years and locations, except for Pyrene, which is always found in each sample (Table 20,21).

ng/g	Dock Clam	Mid Lagoon Clams
Naphthalene	<LOQ	<LOQ
Acenaphthene	<LOQ	<LOQ
Fluorene	<LOQ	<LOQ
Phenanthrene	<LOQ	<LOQ
Anthracene	**4.05**	**3.98**
Fluoroanthene	**13.65**	<LOQ
Pyrene	**9.41**	**1.28**
Benzo (a) anthrax	**0.77**	**0.25**
Chrysene	<LOQ	**0.49**
Benzo (b) fluoran	<LOQ	<LOQ
Benzo (k) fluoran	**10.89**	**2.70**
Benzo (a) pyrene	**6.19**	**3.89**
Dibenzo (a, h) ant	<LOQ	<LOQ
Benzo (g, h, i) per	<LOQ	<LOQ
Indeno	<LOQ	<LOQ

Table 20) 2020 PAH content in nanograms per gram of sample (clams from docks and middle lagoon). ±0.02 ng/g.

ng/g	Mid Lagoon Clam
Naphthalene	**5,23**
Acenaphthene	<LOQ
Fluorene	<LOQ
Phenanthrene	<LOQ
Anthracene	<LOQ
Fluoroanthene	<LOQ
Pyrene	**5,89**
Benzo (a) anthrax	<LOQ
Chrysene	<LOQ
Benzo (b) fluoran	<LOQ
Benzo (k) fluoran	<LOQ
Benzo (a) pyrene	<LOQ
Dibenzo (a, h) ant	<LOQ
Benzo (g, h, i) per	<LOQ
Indeno	<LOQ

Table 21) 2021 PAH content in nanograms per gram of sample (clams from middle lagoon). ±0.02 ng/g

Heavy Metals

Also for these analyses, during 2021, it was used for Hg the SMS-100 and for As and Cd the GF-AAS.

µg/g	Dock Mussles	Mid Lagoon Mussels
V	0.70	2.04
Cr	6.69	4.92
Mn	41.87	55.52
Cu	9.24	6.04
Zn	226.28	87.41
Pb	0.43	1.08
Ni	11.62	13.02
Ba	3.41	5.29
Fe	374.62	976.80
Al	382.75	866.77

Table 22) 2020 metal content in micrograms per gram of sample (clams from docks and middle lagoon). ±0.02 µg/g

µg/g	Mid Lagoon Mussels
V	1.80
Cr	4.02
Mn	32.82
Cu	7.43
Zn	110.76
Pb	0.89
Ni	24.49
Ba	4.78
Fe	865.49
Al	775.47
As	9.27
Cd	0.423
Hg (ng/g)	0.055

Table 23) 2021 metal content in micrograms per gram of sample (clams from middle lagoon). ±0.02 ng/g for the ICP-OES analytes; ±0.002 mg/Kg for As and Cd (GF AAS); and ±0.002 ng/Kg for Hg (SMS-100).

Haloacetic acids

Only Monobromoacetic Acid was found in samples from open waters location in 2021 (appendix 2, Table 24)

Volatilome

As for the analysis on Ulva, it was found that all pollutants included in the study upon the mussels were absent or present in concentrations below the lower limit of quantification (appendix 2, Table 25, 26).

PCBs and Pesticides

ng/g	Mid Lagoon Clams
α-HCH	**0,67**
HCB	**5,03**
γ-HCH	**12,29**
β-HCH	**0,88**
Aldrin	<LOQ
2,4'-DDE	**6,69**
4,4'-DDE	**7,00**
2,4'-DDD	<LOQ
Dieldrin	**3,72**
2,4'-DDT	**1,96**
4,4'-DDD	<LOQ
4,4'-DDT	**21,54**

Table 27) Pesticides content in nanograms per gram of sample (Clams from middle lagoon). ±0.03 ng/g

ng/g	**Mid Lagoon Clams**
PCB31	**3,82**
PCB28	**3,78**
PCB52	**2,48**
PCB35	<LOQ
PCB101	**2,15**
PCB110	**3,91**
PCB81	**1,18**
PCB77	**0,28**
PCB118	**1,17**
PCB153	**2,39**
PCB105	**1,89**
PCB138	**3,32**
PCB126	**0,50**
PCB128	**0,68**
PCB156	<LOQ
PCB180	**1,09**
PCB169	**2,38**
PCB170	**0,07**

Table 28) PCB content in nanograms per gram of sample (Clams from middle lagoon). ±0.02 ng/g

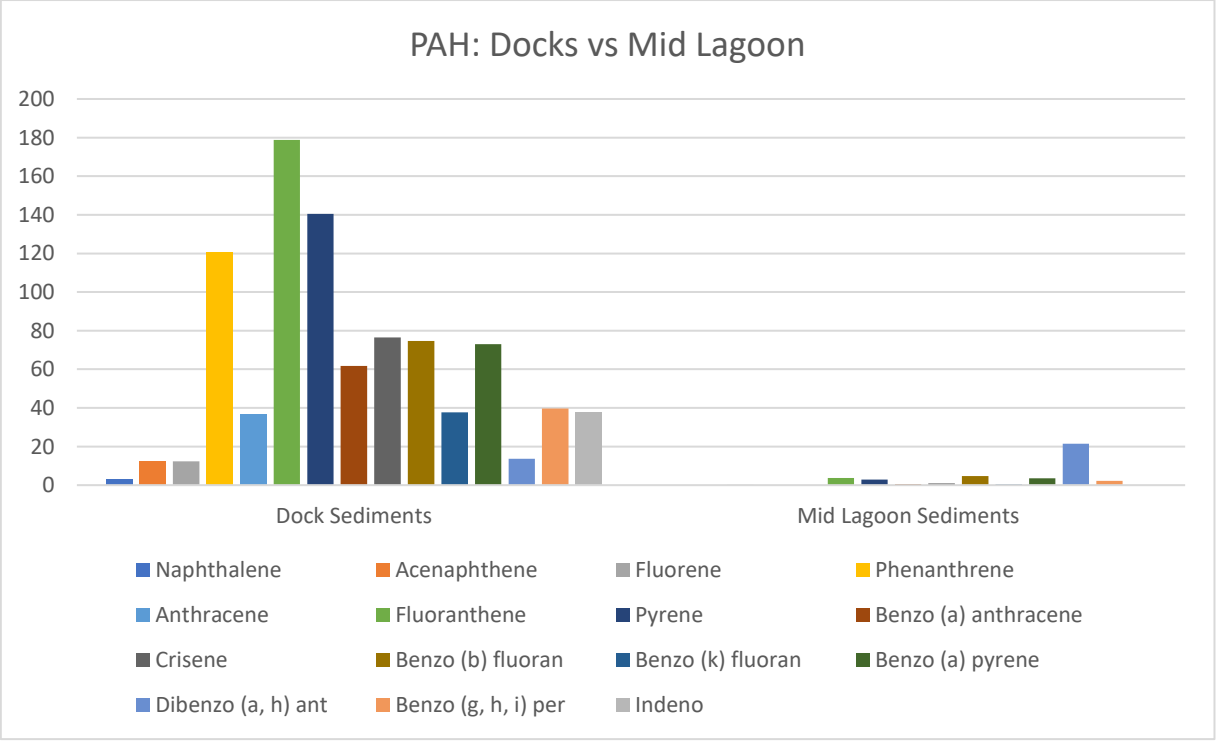
Graphic Elaborations

In the following paragraph, relevant data are briefly discussed and confronted internally and with literature, in order to highlight phenomena of bioaccumulation/absorption of pollutants toward biota, exploiting sediments values as reference for the "*environmental presence of the pollutant*" in which both seaweeds and mussels grow.

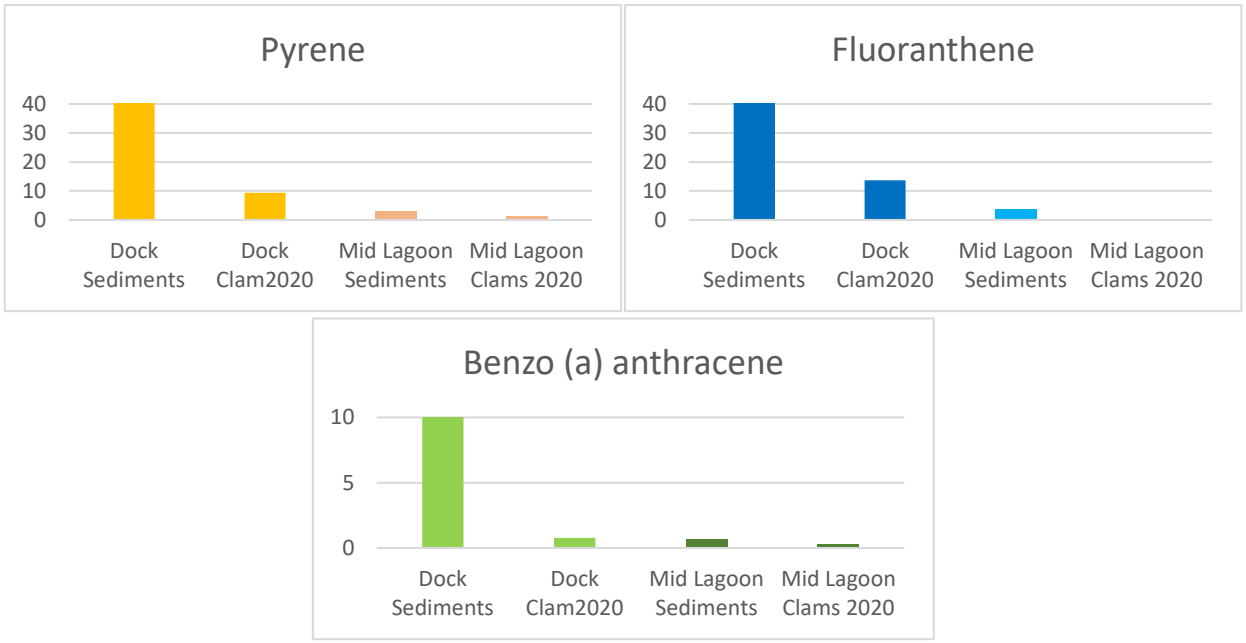
In all samples investigated, the volatile pollutants were not quantifiable at the "parts per billion" (PPB) level, which is in agreeance with recent literature upon the Adriatic sea (Romanelli *et al.*, 2019), where the only ones found were above the quantification threshold by little.

For what concerns haloacetic acids, only monobromoacetic, monochloracetic, and dibromoacetic acids were found in Ulva and clams samples respectively, with comparable concentrations (<20 ng/g) to the ones found in the monitoring study of Romanelli et al. (2019); in which it is reported that in mussels only two chlorine-based haloacetic acids were found in mussels. This can be an indication for point source pollution of those substances, or possibly identifying different processes for water disinfection.

PAHs, despite their concentration in sediments (Graph 3), do not seem to be bioaccumulated in mussels samples, however some PAHs are absorbed quite proportionately, some examples are reported below (Graph 4, 5, 6). As it can be seen in the graph below (Graph 3) there is a clear difference between the trafficked harbor and the lagoon's center; possibly given the pyrogenic and hydrophobic nature of this pollutants class. The total concentrations in sediments are widely comparable and generally lower to those found in other references regarding northern Adriatic sea (Bihari et al., 2006; Bihari, Fafandel and Piškur, 2007).

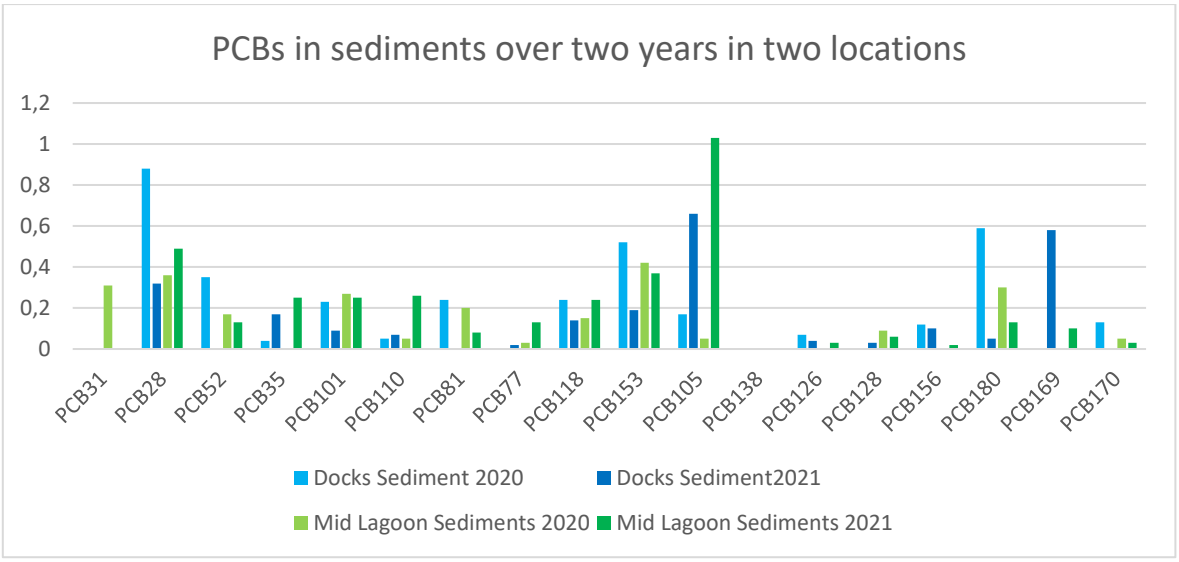


Graph 3) PAHs within docks' sediments compared to mid lagoon in ng/g



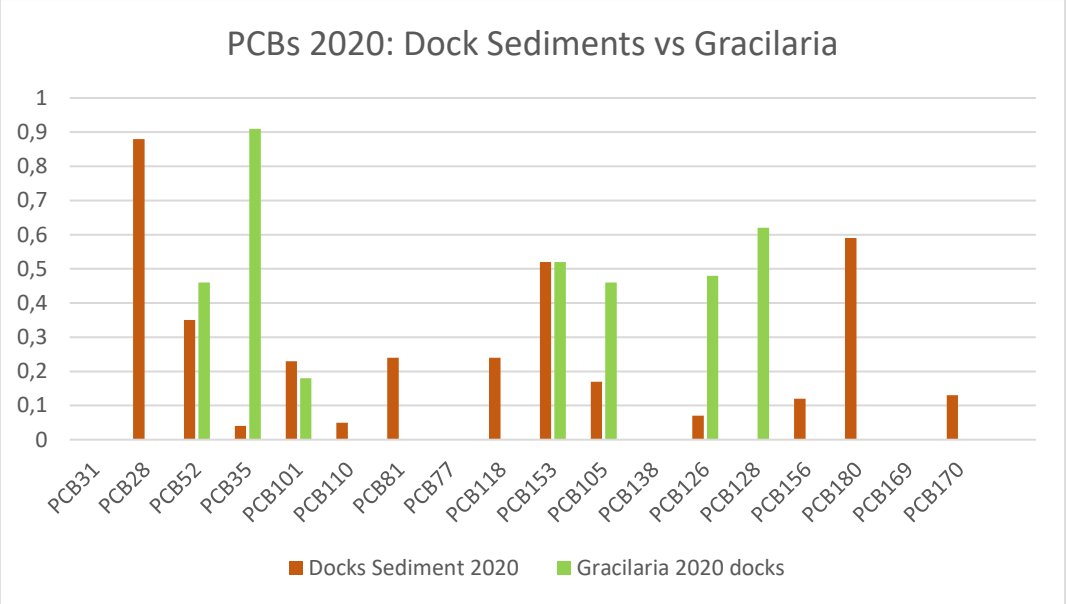
Graph 4, 5, 6) Examples of PAHs absorbed within clams in ng/g.
**Docks quantifications are cut from scale to better appreciate other participants.*

The data produced shows that PCBs are unevenly distributed in sediments over the year and location criteria, highlighting no particular patterns of PCBs' distribution (Graph 7); however, the concentration ranges in PPB scale are in line with other findings in Adriatic sea sediments (Combi *et al.*, 2020), mostly between LOQ and 3 ng/g, an exception is represented by PCB 138 that wasn't detected. In fact PCB 138 and PCB 180 account for the most predominant PCBs in Combi et al. (2020), differently than this study where it was found that the PCBs 28, 105, and 153 are generally the ones which are most present and still in line with the ranges of concentration between the LOQ and 3 ng/g.

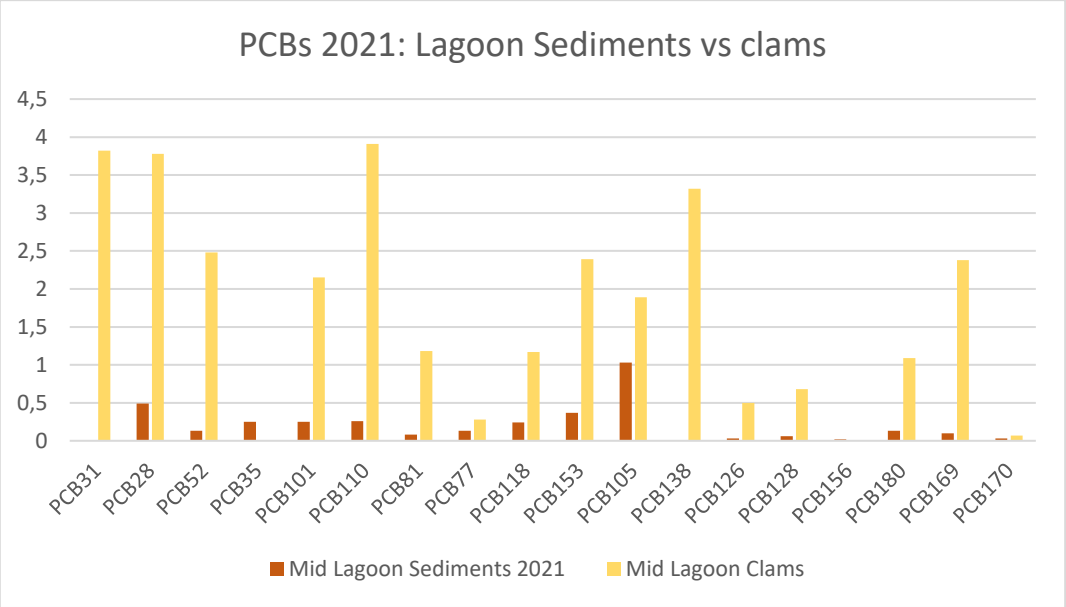


Graph 7) PCBs distributed in sediments over years and locations in Goro's lagoon. in ng/g.

Gracilaria algae seems to interact and bioaccumulate some PCBs; among which, the PCBs 35, 105, 126, and 128 are the preferred ones (Graph 8), of which some are congeners with six and four Chlorine atoms. Instead, Clams widely bioaccumulates these pollutants (Graph 9), seemingly with far less selectivity; hence, the concentrations found in mussels are all below 4 ppb, quite higher but on the same magnitude of what was found on Croatian shores by Milun, Lušić and Despalatović (2016), except for PCB 138 and 153 which are the main character in that study.

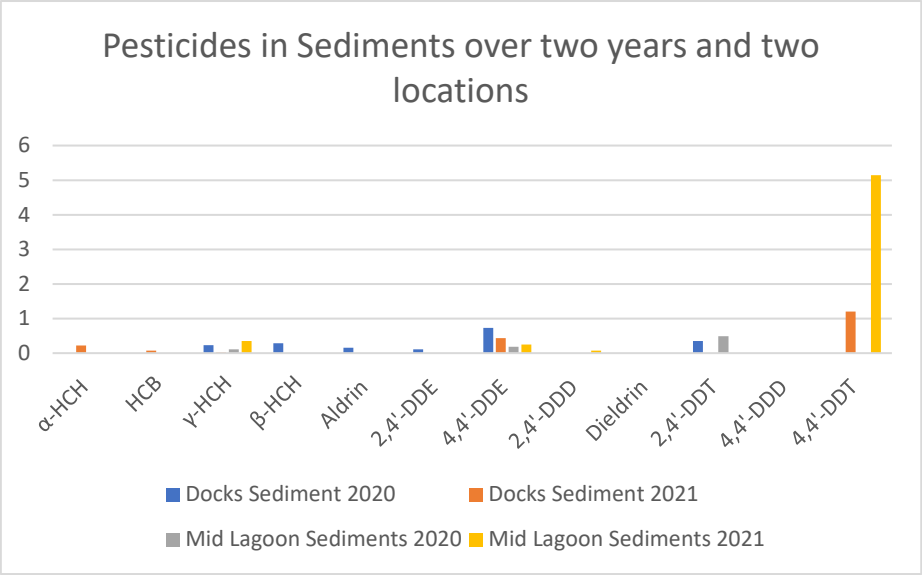


Graph 8) PCBs bioaccumulated in Gracilaria. in ng/g.

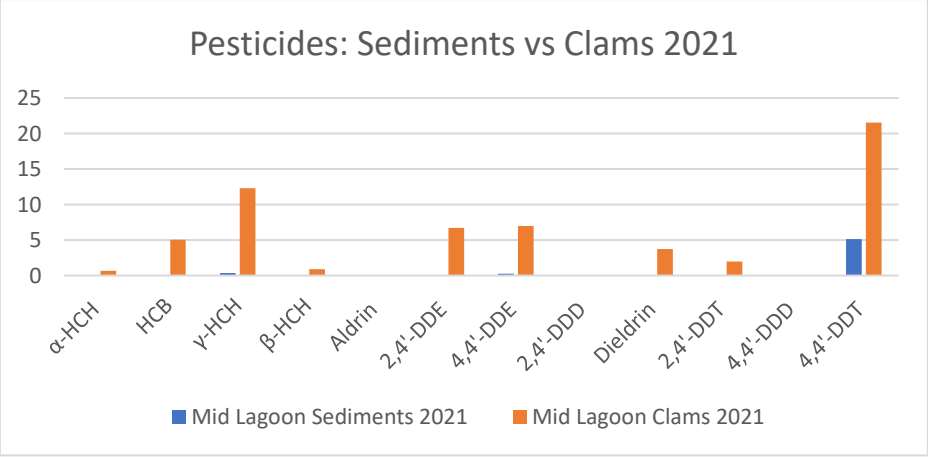


Graph 9) PCBs bioaccumulation in Clams. in ng/g.

Also pesticides are distributed with no specific patterns in low concentrations in sediments over years and locations (Graph 10), however they are abundantly present and strongly bioaccumulated in Clams, especially the infamous DDT (Graph 11) which is the most detectable pesticide in sediments and bioaccumulates in clams with 21.54±0.02 ng/g, along with DDE, which is the aerobic degradation product of DDT. These findings upon DDT are in line with recent literature focused on organochlorine pollutants on the Po river delta and Croatian shores (Milun, Lušić and Despalatović, 2016; Combi *et al.*, 2020) even though the concentrations of DDT found within Goro's lagoon mussels is four times higher. In these regards the sampling location can play a crucial role given that the engulfed nature of the lagoon may be more suitable to foster the interactions between biota and chemicals if compared to an open sea location; or it could be index of local intensive usage in past years. Aside from DDT, In Gracilaria seaweed there were found significant amounts only for "α" and "γ"– hexachlorocyclohexane.



Graph 10) Pesticides in sediments over years and locations. in ng/g.

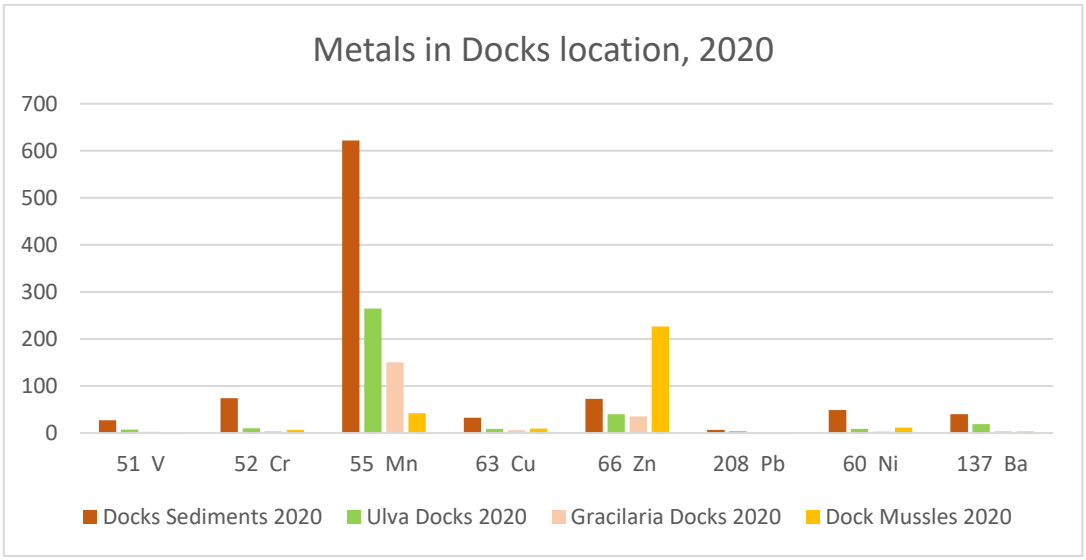


Graph 11) Pesticides bioaccumulation in Clams. in ng/g.

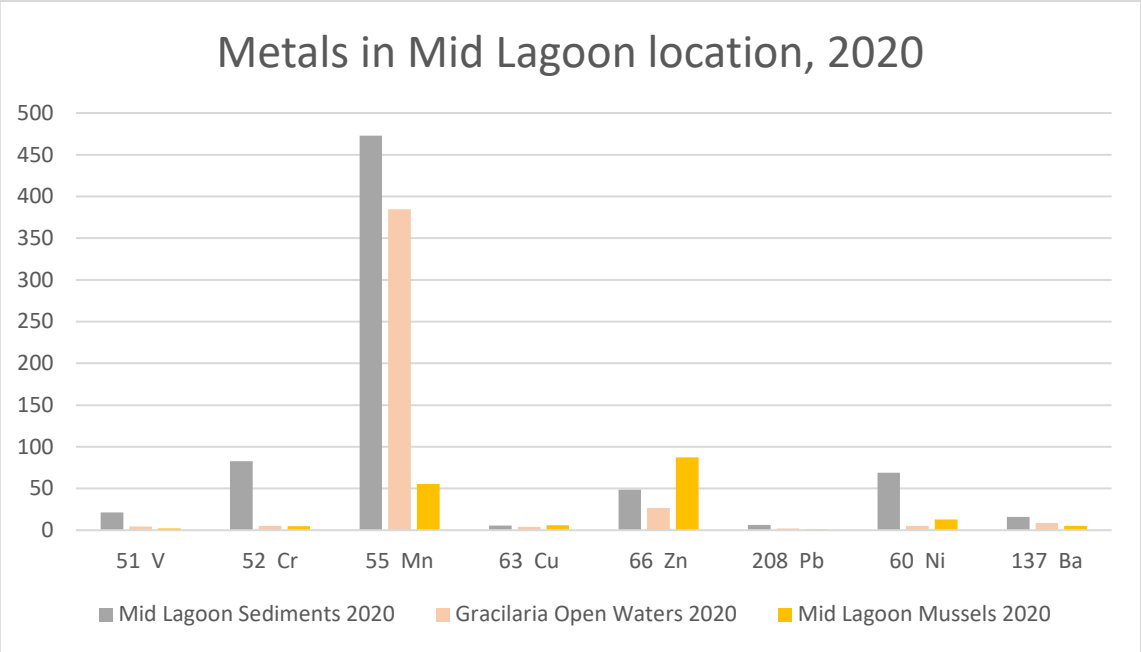
For what concerns the metal's class, Iron and Aluminum were not computed in the graphs, unless if otherwise specified, given their structural role in the sediment composition and thus higher concentrations in samples in comparison to the other elements, which instead are in trace concentrations of micrograms per gram of sample. Generally, metals concentration for Goro's mussels is on the same magnitude and thus comparable, with the ranges of mussels samples from Milun, Lušić and Despalatović (2016); except for slightly lower concentrations of Cd and Hg, and higher concentrations of Cr and Ni.

Heavy metals found in sediments are fundamental to evaluate the local situation and to make inferences about contaminations, pollution, and bioaccumulation phenomena; however, it is necessary to draw thresholds of concentration for each analyte; in Neff (2002) some thresholds to identify contamination are drawn. The data regarding Goro's lagoon falls below those thresholds, also in comparison to other works of Donazzolo et al. (1981, 1984) and Acquavita et al. (2010) especially for what concerns Cadmium. In fact for this Metal species a typical value for noncontaminated sediments ranges from 0.1 to 0.6 µg/g, and the Goro's lagoon shows a noncontaminant value of 0.10±0.02 µg/g. However, the northern Adriatic sea was found to be generally interested by low Cd concentrations (mean value 0.24 ± 0.07 µg/g), although the distribution showed three distinct peaks (from 0.33 to 0.45µg/g) (Donazzolo *et al.*, 1984). Probably, these hot spots were related to "Trieste" and "Monfalcone" industrial areas; also "Venice" lagoon was found with 0.50 µg/g (Donazzolo *et al.*, 1981). Also, it was found that the Cd level increases at rivers mouths in parallel with the percentage of carbonates; so that more carbonic and particularly calcitic sediments contain more Cd (Acquavita *et al.*, 2010). The interactions between Cadmium and carbonates are the main topics for the next section of the thesis.

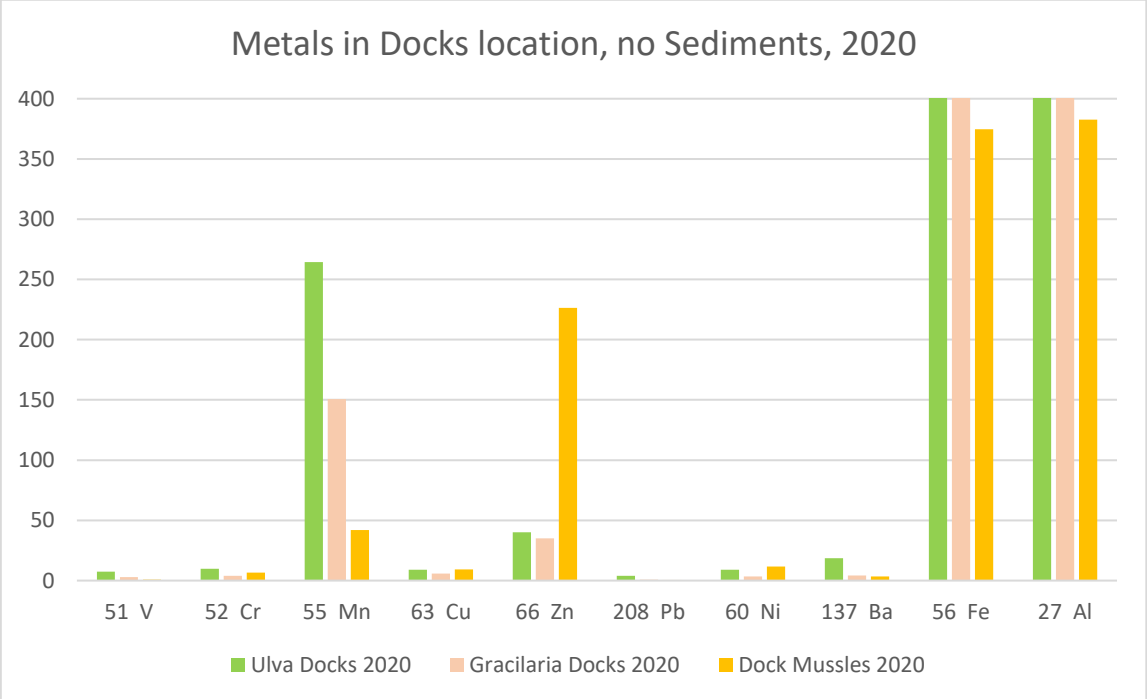
Considering the data then, in the dock location, with the exception of the zinc, no bioaccumulation is present in both algae and the mussels; Nickel and Copper also interact more with mussels than Gracilaria algae in both locations. Generally, Ulva is absorbing each metal with increased incidence over Gracilaria and clams, with the exception of Ni and Zn (Graph 12, 13). Graph 14 is reported to highlight the concentrations of metals within biotic samples, including Iron and Aluminum analytes. Whereas Graph 15 is there to highlight the metals concentrations belonging to sediments and mussels, with a focus (Graph 16) upon the dangerous, but well below the contamination thresholds, Arsenic, Cadmium, Mercury.



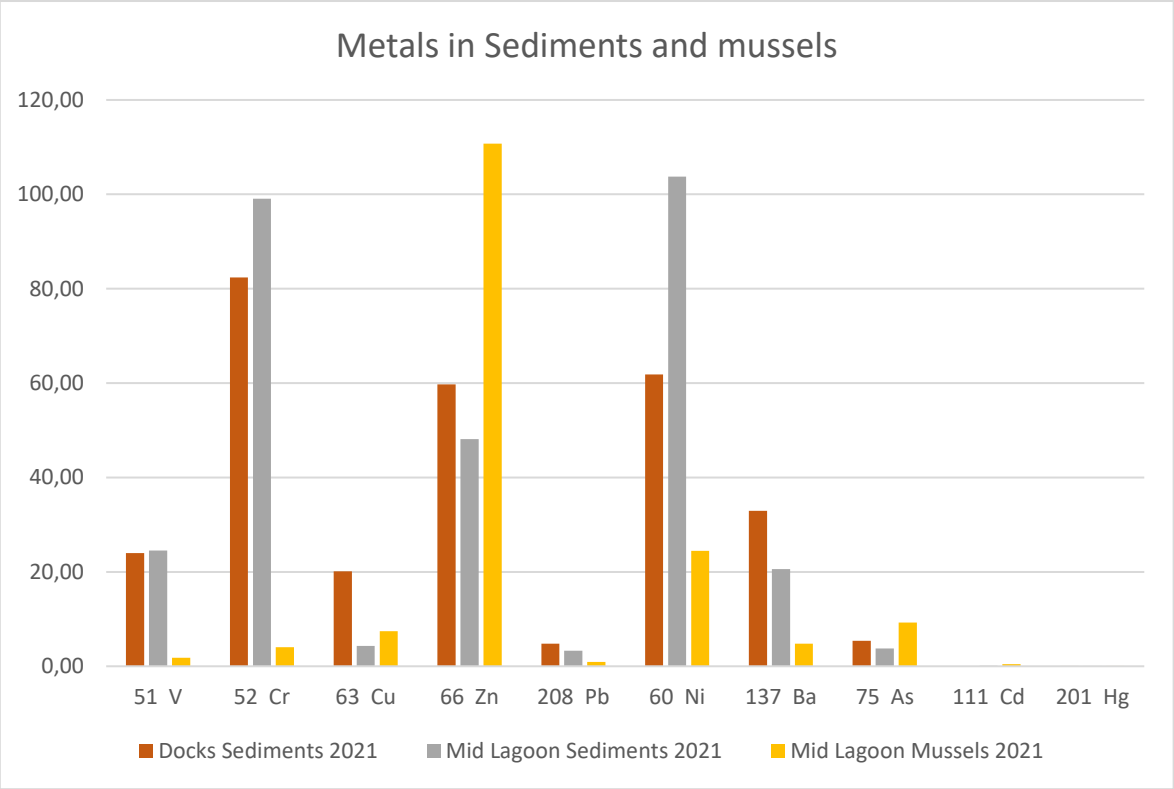
Graph 12) Metals in all samples in the docks location. in ng/g.



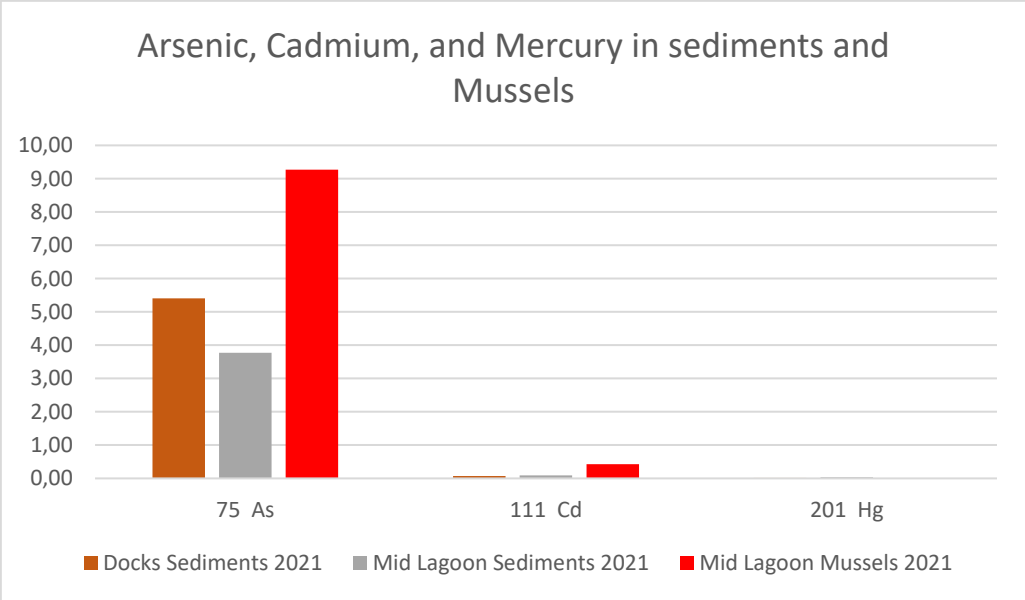
Graph 13) Metals in samples from the mid lagoon location. in ng/g.



Graph 14) Metals in biotic samples from the docks location. in ng/g.
Iron and Aluminon are not in scale for algae



Graph 15) Metals in sediments and mussels. in ng/g.



Graph 16) Extremely hazardous metals in sediments and mussels. in ng/g.

Except the volatile chemicals, every other pollutant class was found; in detail, it was possible to determine some bioaccumulation phenomena, especially for clams toward PCBs, pesticides and metals. In particular, it can be noticed that Cd is bioaccumulated by mussels (Graph 16); this issue is further investigated in the

next section of this manuscript, by studying the interactions between Cadmium, the mussel and its shell. Also, it is possible to confirm that areas closer to densely trafficked docks and/or freshwater outputs seems to be more affected by pollution, which is the perfect case for the PAH pollutants class; therefore, the biomass grown in those location seems to be more affected as well. Generally, Ulva algae is keener to uptake metals if compared to Gracilaria; which, on turn, seems to bioaccumulate targeted PCBs and Pesticides.

Discussion

The analyses conducted are not to be meant as a systemic investigation over the totality of Goro's lagoon; instead, the purpose is to obtain an overall view of the pollutants distribution among various trophic levels in comparison to sediments in order to determine the presence of bioaccumulation phenomena, and whether to invest for further systemic analysis. Hence, these monitoring activities were a first attempt to approach the relatively unknown pollution situation of Goro's Lagoon, not easily identifiable given the many routes of diffusion for chemicals, such as the rivers from south, the anthropic operations on the area, the coastline, and the main freshwater flows from the Po river.

Unfortunately, pandemic restrictions posed numerous challenges and problems both to the access to the laboratory and instruments, and to the quality of the sampling process; in fact 2020 and 2021 were terrible years for algae presence in the lagoon possibly due to the pandemic restrictions and lesser nutrients in the form of "nutrient pollution", as previously discussed. However, some interesting correlations can be found with the data produced. In general, the overall situation is not a dramatic picture given the mostly trace concentration levels of the investigated chemical classes, however bioaccumulation from algae and biota is a real factor that could primarily affect the quality of the mussels, which is the main revenue stream for the local economy and for the majority of the activities in that area; secondly, algal biomass quality is affected by the presence of proportionally acquired metals and organic pollutants like PCBs and Pesticides with some that are effectively bioaccumulated.

Thus, the bromatological investigation suggests that it is possible to exploit Gracilaria for bioethanol production and/or the extraction of hydrocolloids (such as agar), given the high percentage of fibers. Instead, Ulva shows a limited percentage of lipids, possibly due to the growth stage at which it was sampled and/or the period of the year and/or particular chemical-physical conditions in the area. Hence, despite its eligibility for biodiesel productions it could be more remunerative to extract lipids, such as the Palmitic, Oleic, and Linoleic acids for further use, rather than their conversion into fuel, for example given the beneficial effects for human diet.

In fact, the tight homeostatic control of Palmitic acid tissues concentration in human body is related to its fundamental physiological role to guarantee cellular membrane physical properties and, in the lungs, as an efficient surfactant, particularly in infants it seems to play a crucial role (Carta *et al.*, 2017). In order to maintain these physiological balances, it may be crucial an optimal intake of Palmitic acid, especially in the cases of unbalanced diets and sedentary life, which could alter its natural biogenic production (Carta *et al.*, 2017). Oleic acid is widely used as an excipient in pharmaceuticals and as an emulsifying / solubilizing agent in aerosol products; also, it may hinder the progression of adrenoleukodystrophy, a fatal disease that affects the brain and adrenal glands, and it may help boost memory (Choulis, 2011). Oleic acid may also be responsible for the hypotensive (or blood pressure reducing) effects of olive oil (Choulis, 2011). Linoleic acid is an essential (indispensable) nutrient, which is involved in the maintenance of the transdermal water

barrier of the epidermis. Deprivation of linoleic acid can result in scaly skin lesions, growth retardation, and altered plasma fatty acid patterns and thrombocytopenia (Whelan and Fritsche, 2013). It is typically provided in enteral, parenteral, and infant formulas where the fat content can vary depending on the specific use; similarly, topical applications can also provide linoleic acid, helping to treat skin-related disorders linked to its deficiency (Whelan and Fritsche, 2013).

However, the fat percentage could be higher for both algae in other periods of the year, and investigations upon their composition over seasons, in this lagoon context, should be performed to take accountability for the best usage of this algal biomass. The protein content superior to 15-20% suggests the presence of vitamins, therefore their extraction can lead to the production of integrators and food supplements, as well as antioxidants (e.g., tocopherol) and anti-inflammatory substances depending on the nature of the vitamins and the presence of phenols and polyphenols (Kinger, Kumar and Kumar, 2018), which are known in literature, to be present in extracts from Ulva algae (i.e., Ulvans) (Kidgell *et al.*, 2019).

However, interferences from organic pollutants towards bioactive compounds extraction processes should be avoided by carefully evaluating the technology and chemicals used for the extractive processes. The residues could be further used to produced bioethanol / biogas through fermentation processes and the final digestate could be used as soil conditioner. In these regards, Ulva can present high concentrations of metals, of which some can be detrimental for the soil conditioning purpose; hence, it can be sent to landfill, given the small volume and the exhausted potential, or some innovative techniques could be applied to recover metals within the final digestate (Cheng *et al.*, 2019).

The hazardous metals of As, Hg, and Cd were found in non-contaminant concentrations or below the detection limits in sediments, however As and Cd are likely to be bioaccumulated in mussels soft tissues given the two- and four-times higher concentrations respectively against sediments (Graph 16). Despite the distance of this insight from the topic of algal biomass exploitation, it could be interesting to investigate more the relations among Cadmium, the mussel, and their shells that are made of biogenic carbonate, which is known to interact well with Cd.

Conclusions

Pollution monitoring activities were performed with a double-edged purpose, the first was to gain an overview of the health and quality status of the Lagoon and its ecosystem, the second was to collect data to support the output of the previous chapter, the CBA-Foresight. In fact, the presence of pollution was identified both as one the weaknesses and as one of the threats in the Foresight SWOT analyses for the algal biomass exploitation. With these preliminary data it is already possible to make some discriminations among the various possible processes, however a robust investigation on seasonal composition distribution could be of assistance to better define the exploitation of these natural resource/waste, especially during the more and more frequent HABs.

Hence, Ulva and Gracilaria algal biomasses, despite their nutritional content, may not be good resource for direct consumption, given the presence of PCBs, Pesticides, and heavy metals; however, in order to infuse valor upon this bulky waste biomass prior to the deployment in landfill, single components or families of component can be extracted, while the rest can be turned into biofuels, metals can be collected from the

exhausted residues which are to be stored in landfill, or used as soil conditioner if not bearing excessive residual pollutants.

From these activities, it has also emerged that Cadmium is bioaccumulated in mussels, thus its presence is further investigated in the next chapter, since it paves the way for the resolution of a further problem negatively affecting the canals connected to the lagoon and the lagoon itself, impacting on ecosystems quality, a clams' processing by-product, the empty seashells. Which is another bulky waste that can be re-used in circular perspective. In particular, the determination of the relation among Cadmium and the mussels' shell could be a crucial factor for the determination of further uses for this material, such as fertilizer, biosensor, and bioremediator for heavy metals, among which Cd.

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Chapter 2: Scallop Shells Characterization for Improved Circularity of Resources and Remediation Purposes

The following chapter consists of an article that is currently being published on journal and involves the systemic study of the seashells' composition when exposed to various concentration of Cadmium, a highly dangerous heavy metal pollutant. Hence, the main goal is to determine the interactions between the biogenic shells and this heavy metal in order to better identify and design the most effective usages for those waste shells, such as remediators, biofilters, and soil conditioners functional goods, which will hopefully gain an increasing presence on markets.

This work fits within a circular framework of biogenic wastes' exploitation as valuable materials, aligned toward the environmental restoration and quality improvement by the means of remediation and pollution removal, through the study of functional peculiarities of these materials. Hence, the main findings indicates that the presence of organic components in the scallop shell matrix, among which pigments, enhance the contaminant uptake. Therefore, the preferential uptake of pollutants from coloured shells can play a crucial role in the design of biofilters and soil conditioning mixtures made by these bulky waste materials, for example preferring the brighter as soil conditioner and the darker as biofilters and remediators, given the different interactions with heavy metals and cadmium. Eventually, increasing circularity of resources, remediating to polluted areas, and contributing to the overall sustainability of aquaculture activities, which are going to play a crucial role for the near-future human development.

The article is attached at the end of the thesis in its submitted format.

Scallop Shells as Biosorbents for Water Remediation from Heavy Metals: Contributions and Mechanism of Shell Components in the Adsorption of Cadmium from Aqueous Matrix

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Abstract

To ascertain their potential for heavy metal pollution remedy, we studied the adsorption mechanism of cadmium onto scallop shells and the interactions between the heavy metal and the shell matrix. Intact shells were used to investigate the uptake and diffusion of the metal contaminant into the shell carbonatic layers, as well as to evaluate the distribution of major and trace elements in the matrix. LA-ICP-MS measurements demonstrate that Cd is adsorbed on a very thin layer on the inner and outer surfaces of the shell. Structural and thermal analyses showed the presence of 9 wt.-% of CdCO3 phase indicating that the adsorption is mainly a superficial process which involves different processes, including ion exchange between Ca and Cd. In addition, organic components of the shell could contribute to adsorption as highlighted by different metal uptake showed by shells with various colouration. In particular, darker shells adsorbed more contaminant than the white ones. The contribution of the organic shell components on the adsorption of heavy metals was also highlighted by the element bulk content which showed higher concentrations of different metals in the darker specimen. Raman spectroscopy allowed to identify the pigments as carotenoids, confirmed by XRD measurements which highlighted the presence of astaxanthin phases. This work indicates that the presence of organic components in the scallop shell matrix enhances the contaminant uptake. The results presented here provide new insights into the Cd adsorption mechanism highlighting the important contribution given by the organic components present in the biogenic carbonate matrix. Furthermore, the high efficiency of Cd removal of scallop shells, supported by adsorption kinetic and isotherm studies, has been demonstrated.

Introduction

Environmental pollution caused by heavy metals is one of the major global problems leading to adverse effects on ecosystems, biodiversity and human health (Jaishankar *et al.*, 2014). Among the heavy metals, cadmium (Cd) is one of the most toxic pollutants even at low concentrations. Furthermore, Cd has been classified as human carcinogen by the International Agency for Research on Cancer ('Cadmium and cadmium compounds.', 1993). The main source of Cd pollution in surface waters is from anthropogenic activities. On a global scale, smelting of non-ferrous metal ores has been regarded as the largest anthropogenic source of Cd input into the aquatic environment (Pan *et al.*, 2010). Cd tends to accumulate in the sediments by adsorption or precipitation as insoluble salts (Morford and Emerson, 1999). However, under certain conditions Cd can be mobilised and its concentration in the aqueous medium can increase. This is particularly the case when the salinity of the water body increases and dissolved Cd is stabilised in solution through the formation of chloro-complexes (Dabrin *et al.*, 2009).

Among the most common physical and chemical approaches for the removal of heavy metals from water, adsorption is an effective and economic technique, offering flexibility in the design and operation, and a vast variety of adsorbent materials (Carolin *et al.*, 2017). In the last years, the use of natural or waste materials as adsorbents has been largely studied to favour eco-friendly approaches in environmental remediation applications (Singh *et al.*, 2018). Among the waste products generated by food industry, mollusc shells have composition and structure characteristics suitable for the removal of heavy metals dissolved in water bodies.

Indeed, many studies had reported the capability of molluscan and crustacean shell powder to adsorb heavy metals from water aqueous matrices considering the effect of the adsorbent grain size (Köhler *et al.*, 2007; Du, Zhu and Shan, 2012; Ismail and Aris, 2013), different $CaCO_3$ shell structure (Wu *et al.*, 2014), or after the adsorbent material had been acid-pretreated (Liu *et al.*, 2009) or calcined (Peña-Rodríguez *et al.*, 2010; Alidoust *et al.*, 2015; Yen and Li, 2015). It has been demonstrated by Tudor et al. (2006) (Tudor, Gryte and Harris, 2006) that some seashells are more efficient in the uptake of Pb and Cd compared to non-biogenic calcareous materials. Mollusc shells can also be employed as marine sediment amendments for immobilisation of potentially toxic elements, in this way the water and sediment quality would be guaranteed without introducing extraneous materials in the lagoon system, since they are native of the area of interest. Furthermore, adding biogenic carbonate in marine sediments could possibly mitigate the effect of sea acidification, as reported in Drylie et al. (2019) (Drylie *et al.*, 2019). The use of mollusc shells for environmental remediation can contribute to improve the sustainability of shellfish farms since they are classified as waste material, and as such they require adherence to applicable policies and procedures to be correctly disposed (Yao *et al.*, 2014). In addition, mollusc shells are important environmental indicators. Indeed, they can accumulate heavy metals. Their concentrations in shells provide a time-integrated degree of metal availability, over long periods of time, thus providing an archive of past seawater. Therefore, the chemistry of shells can provide useful information on environmental conditions; shells can provide a more precise symptom of pollution and environmental change than soft tissue, due to the possibility of investigating the incorporation of elements over the entire period of shell formation, higher preservation potential even after the organism's demise, and relatively cheap and easy storage. Besides, the lower metal concentrations in the shells with respect to soft tissue can be overcome by the development of sensitive analytical techniques (Yap *et al.*, 2003; Binkowski *et al.*, 2019).

The evaluation of the interactions of dissolved metal contaminants with the mollusc shell matrix and its components would provide additional information on the mechanism of the metal uptake process and can

contribute to the development of the use of shells as bioadsorbents and bioindicators in environmental remediation and monitoring fields, respectively.

Herein, we present a study on the investigation of the mechanism of Cd adsorption and diffusion in the scallop shell carbonatic layers employing different analytical techniques, considering the interaction of heavy metals not only with the carbonate phase, but also with organic components present in the matrix. To detect trace elements concentrations, high spatial resolution techniques such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been employed. LA-ICP-MS allows to determine the element distribution across heterogeneous and multiphase solid samples with low µm spatial resolution. In this work, LA was applied in combination with ICP-TOFMS, which allows fast and quasi-simultaneous detection of most elements across the elemental mass spectrum (Gundlach-Graham *et al.*, 2015; Neff *et al.*, 2020), obtaining distributions of selected areas on the cross section of mollusc shells. Furthermore, it provides short analysis times with minimal sample preparation required (Günther and Hattendorf, 2005) and by using LA-ICP-TOFMS in the line scan mode, continuous elemental profiles of the sample can be achieved. Many studies employed this technique to obtain natural trace elements patterns along the shell direction of growth in order to use them as proxies to retrace variations of temperature and salinity of the water body (Hathorne *et al.*, 2003; Elliot *et al.*, 2009; Marali *et al.*, 2017; Warter and Müller, 2017; Warter, Erez and Müller, 2018). Despite the proven applicability of LA-ICP-MS in line scan mode for retrospective natural trace elements monitoring, only few works applied this technique to investigate metal contaminants in mollusc shells (Risk *et al.*, 2010; Holland *et al.*, 2014; Cariou *et al.*, 2017) and, to the best of our knowledge, there is no study relating to the diffusion of the metal through the shell layers in mollusc shell treated with the contaminant.

Since it has been demonstrated that the adsorption of metal can induce structural modification in the crystalline components of shells, thermogravimetric and X-ray powder diffraction analyses were carried out. The results indicate that scallop shells having different colourations show differences in Cd adsorption characteristics. This finding motivated us to investigate the role of pigments through micro-Raman and LA-ICP-TOFMS imaging (Neff *et al.*, 2020).

The insights gathered from this study provide information to better understand the distribution and interactions of metal contaminants with the shell matrix when mollusc shells are used as adsorbents or environmental indicators.

Materials and methods

Adsorbent material preparation

One hundred scallop shells (Aequipecten opercularis, Linnaeus 1758) were collected from a shell deposal site in Sacca di Goro (Northern Adriatic Sea, Italy). The shells were first brushed to remove any residual mollusc tissue, cleaned thoroughly with deionised MilliQ water (Millipore, MA, USA) and then dried in an oven at 50°C over night.

A total of 50 shells were selected and milled using a grinder (Retsch GmbH, Germany) to obtain a fine powder which was used for the determination of the mean calcium (Ca) content, particle size distribution, Cd adsorption experiments for kinetics and isotherm determination, and for thermal and X-ray powder diffraction analyses.

The material characterisation regarding minor and trace elements bulk composition was performed on scallop shells with different colouration, milled separately into a fine powder.

28 shells were kept intact and sorted by colour (white, pink and brown) for the metal diffusion investigation by LA-ICP-MS and for pigment characterisation with micro-Raman spectroscopy.

For the overall experimental workflow see Supplementary Figure S1 in the Supplementary Information.

Characterisation

The particle size distribution of the scallop shell powder used for batch adsorption experiments was determined with a Malvern Mastersizer 2000 Particle Analyser (Malvern instruments, UK).

For the determination of the bulk concentration of Ca, 0.1 g of scallop shell powder was digested in 10 mL of HNO_3 10%, filtered with PVDF membrane 0.45 µm (ACRODISC, New York, USA), diluted and analysed by solution based ICP-OES (Perkin-Elmer, Waltham, MA, USA) (for the measuring parameters see below in the *Batch adsorption* section).

To determine the trace elements bulk composition, three scallop shells were sorted by colour, milled into a fine powder, digested in HNO_3 10%, then filtered using syringe filters with PVDF membrane 0.45 µm (ACRODISC, New York, USA). After dilution, the analyses were carried out using an Agilent 7500 ICP-QMS (Agilent Technologies, Santa Clara, CA, USA); the instrument operating parameters were: 1450 W RF power, 4.2 mm sampling depth, 15 L min^{-1} coolant gas, 1.00 L min^{-1} carrier gas, the isotopes measured were ^{25}Mg, ^{39}K; ^{49}Ti, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{113}Cd, ^{137}Ba, and ^{208}Pb.

Batch adsorption

Cd solutions were prepared by dissolving $Cd(NO_3)_2{\cdot}4H_2O$ (Sigma-Aldrich, Steinheim, Germany) in ultrapure water (Millipore, MA, USA). The adsorption kinetics and isotherm were determined using the batch method, as explained below in the respective sections.

The Cd uptake (expressed as q_e for isotherm determination, and q_t for kinetic experiments) was calculated as follow:

$$q = \frac{(C_0 - C_e) \cdot V}{m} \quad (1)$$

where q (mg g^{-1}) is the quantity of Cd adsorbed, C_0 (mg L^{-1}) is the concentration of Cd in the initial solution, C_e (mg L^{-1}) the residual Cd concentration at a time t in kinetic experiments or at equilibrium for isotherm determination, V (L) the volume of the solution in the batch, and m (g) the quantity of scallop shell powder.

Adsorption kinetics

The kinetic experiments were conducted at three different initial Cd concentrations (5, 10 and 15 mg L^{-1}), 1 L of solution was prepared in a polypropylene bottle where 5 g of shell powder was added. The batches were kept under stirring and at a temperature of 21.0 ± 0.5°C using a refrigerating bath circulator (Jeio Tech, Daejeon, Republic of Korea). Samples (0.5 mL) were collected at different time intervals: every minute for the first 10 minutes, every 5 min up to 1 h, every 30 min up to 3 h, and every 60 min up until 5h. The samples were filtered with 25 mm syringe filters with PVDF membrane 0.45 µm (Agilent Technologies, Santa Clara, CA, USA) and the Ca and Cd residual concentration in the solution was evaluated by ICP-OES Optima 3100XL (Perkin-Elmer, Waltham, MA, USA) (axial view) equipped with a solid-state charge-coupled device detector (CCD), a peristaltic pump and a low-flow GemCone nebuliser coupled to a cyclonic spray chamber. Analytical lines 317.933 nm and 226.502 nm were selected for quantitative determination of Ca and Cd, respectively. The ICP-OES measuring parameters were: 15 L min-1 plasma flow, 0.5 L min-1 auxiliary flow, 0.65 L min-1 nebuliser flow, 1350 W RF power.

Adsorption isotherm

20 mL of Cd^{2+} solution was added to crimp top reaction glass flasks sealed with PTFE septa (Supelco, Bellefonte, PA, USA) with 40 mg of shell powder. The initial concentrations of Cd^{2+} in the solutions were in the range of 1-350 mg L^{-1}.

Adsorption experiments were performed with a contact time of 20 h, which is considered sufficiently longer than the equilibration time (30 min) as determined in the adsorption kinetics experiments.

After 20 h equilibration under stirring (600 rpm) and at a controlled temperature of 21.0 ± 0.5°C, the adsorbent was separated from the solution by filtration using 25 mm syringe filters with PVDF membrane 0.45 µm (Agilent Technologies, Santa Clara, CA, USA), the pH of the solution was recorded for each solution after equilibration.

The concentration of Ca^{2+} and Cd^{2+} in the solution, before and after the contact with the adsorbent material, was determined by ICP-OES, as described before.

The same procedure was followed to obtain the adsorption isotherm of Cd onto commercial pure calcium carbonate powder (Sigma-Aldrich, Steinheim, Germany).

Cd uptake across shell layers

For the study of the metal diffusion through the shell layers, the intact mollusc valves were put in contact with solutions containing Cd^{2+}, the concentrations of the metal ions were 1.0 and 5.0 mg L^{-1}. After a contact time of 20 h to ensure that the equilibrium had been reached, the shells were separated from the solution, rinsed twice with ultrapure water, air-dried and conserved for the LA-ICP-MS experiments. The Cd concentration in the solution before and after the contact with the shells was measured by ICP-OES (for the measuring parameters see *Adsorption kinetics* section) and the Cd uptake was calculated (equation 1).

To investigate the diffusion of Cd through the sample cross section, the shells were cut along the axis of maximum growth with a low-speed saw (Isomet 11-1180 Low Speed Saw, Buehler LTD, CH), embedded into an epoxy resin (Epofix, Struers, Birmensdorf, CH), ground to the desired thickness with a polishing machine (Forcipol 1V Grinder Polisher, Metkon, TR) equipped with a 120 grit diamond disc, then polished with 600 and 800 grit SiC powder. In order to obtain the trace elements distribution through the shell layers, laser ablation was conducted on the cross-sections of the samples, with the ablation direction oriented perpendicular to the direction of shell growth, from the outer to the inner surface layer (Supplementary Figure S2).

The high-resolution line scans and element imaging analyses were conducted with a 193 nm ArF Eximer laser system (GeoLas C, Lambda Physik, DE) equipped with the parallel flow ablation cell (Neff, Becker and Günther, 2022) and was coupled to an ICP-TOFMS (icpTOF2R, Tofwerk AG, CH). Ablation spots with 5 µm diameter were used in an edge to edge arrangement. The imaging mode “hole drilling with cleaning pulse” was applied using the imaging control system according to Neff et al (Neff *et al.*, 2020). The acquisition of one sampling position was conducted using one cleaning pulse which was not acquired, then 25 laser pulses at 100 Hz repetition rate which were acquired and a fluence of 15-20 J cm^{-2}. The ICP-TOFMS measuring parameters were: 16 L min^{-1} plasma flow (Ar), 0.8 L min^{-1} auxiliary flow (Ar), 0.6-0.7 L min^{-1} make-up gas flow (Ar), 1.4-1.6 L min^{-1} carrier gas flow (He), 1400 W RF power. The carbonate reference material MACS-3 (U.S. Geological Survey, USA) was used as external standard and a 100% mass normalisation approach(Liu *et al.*, 2008) was applied for the quantification assuming all metals are present as carbonates. The isotopes ^{23}Na, ^{24}Mg, ^{44}Ca, ^{55}Mn, ^{56}Fe, ^{66}Zn ^{88}Sr, ^{114}Cd and ^{208}Pb were evaluated. The mass resolving power of 4500-5000 achieved by the icpTOF2R in the lower mass range (24-56 m/z) (Hendriks, 2019) allows to separate $^{24}Mg^+$ from $^{12}C_2^+$, $^{44}Ca^+$ from $^{12}C^{16}O_2^+$, $^{55}Mn^+$ from $^{40}Ar^{14}NH^+$, and $^{56}Fe^+$ from $^{40}Ar^{16}O^+$ and $^{40}Ca^{16}O^+$. No significant interference of $^{114}Cd^+$ with $^{114}Sn^+$ was observed. This allowed to evaluate higher abundance isotopes to increase the sensitivity with low background intensities and therefore to lower the limits of detection.

Micro-Raman analyses

Raman spectra were recorded with a LabRam HR800 micro-Raman instrument (Horiba Scientific, FR) equipped with an air-cooled CCD detector at -70°C, an Olympus BXFM microscope, a 600 groove/mm grating and a 50 X objective to collect the Raman scattering signals. The excitation source was a He-Ne laser

(632.8 nm line) with a maximum laser power of 20 mW. A minimum spectrum accumulation of 10 times per second was used; if a high background was recorded, the accumulations were increased to a maximum of 100 times per second to improve the signal-to-noise ratio.

Thermo- analyses

Thermogravimetric analyses (TG) and differential thermal analysis (DTA) of both raw scallop shell powder and Cd-loaded scallop shell powder were performed on a STA 409 PC LUXX (Netzsch, DE). The measurements were carried out in air flow with a heating rate of 10°C min^{-1} from room temperature to 1000°C.

X-ray powder diffraction

The X-ray powder diffraction patterns of untreated and treated with Cd solution scallop shell powder were recorded on a D8 Advance diffractometer (Bruker, USA) equipped with a Si (Li) solid-state detector, (Cu $Ka_{1,2}$ radiation, 3–110 2θ range, counting time of 12 s per 0.02 2θ step). The unit cell parameters were refined together with the coefficients of the pseudo-Voigt function modelling the profile function of the Bragg peaks and of the function modelling the background (a six-term cosine Fourier series). Refinements were carried out by the Rietveld method using the GSAS (Larson and von Dreele, 2000) and EXPGUI (Toby,2001) packages. Structural models for all the phases were taken from the ICSD database (ICSD Database). Optimised parameters in final refinement were: background coefficients, cell parameters, zero shift error, peak shape parameters, preferred orientation, and phase fractions.

Results and discussion

Adsorbent material characterisation

The bulk composition of the shells was determined to evaluate the total cation contents of the untreated shells. The mass fractions determined by ICP-MS after acid digestion of ground shells are reported in Table 1 (Horwitz, 1988). Many metals, in particular V, Mn, Fe, Co, Cu, Zn, Ba, and Pb were detected in the samples. Among these, Fe was the most abundant element. The mass fractions of Cd were between the limit of detection and quantification of the employed method, thus indicating that the environmental habitat of these seashell was not severely polluted by Cd (Riminucci *et al.*, 2022), or that the replacement of Ca^{2+} in the calcium carbonate structure during the shell formation occurs to a lesser extent. Indeed, bioaccumulation rate of ions from seawater seems to be a function of many environmental and biological factors (Rainbow, Phillips and Depledge, 1990; Wright, 1995; Wang and Fisher, 1997), and different habitats, species, or even individual specimens at different stages of development, may present different patterns of metal uptake. Furthermore, only few published investigations (Freitas *et al.*, 2006; Barats *et al.*, 2007; Metian *et al.*, 2008; Poitevin *et al.*, 2020) deal with chemical composition of scallop shells and none of these in a habitat similar to that of the samples herein investigated (i.e., a lagoon on the Po River Delta). In this aspect, the present study on one hand can provide new data that can contribute to the knowledge on metal distribution in biota, On the other hand, however, the data cannot be compared with literature data.

Recently, the concentration of metals in sediments of Laguna di Goro have been investigated (Natali and Bianchini, 2018), and it has been found that trace-metals, such as Ni, Co, V and Cu, show concentration levels associated to the geological nature of the Po River alluvial sediments, whereas Pb and Zn show an enrichment in Sacca di Goro, when compared to the background alluvial sediments, probably due to anthropogenic sources. Indeed, these trace-metals present in the lagoon sediments were found in all the scallop shell samples analysed.

A particular feature that can be observed from the elements bulk composition is the fact that whereas for heavy metals such as Pb, Fe and Zn there is a higher concentration in the more coloured shell, the content of elements found naturally in the shells, such as Sr and Mg which are incorporated during the mollusc growth, does not vary significantly between shells of different colouration. These findings suggest that the components responsible for the shell colouration contribute significantly to the uptake of heavy metals from the aqueous environment surrounding the mollusc.

	White shell	Pink shell	Brown shell	LOD
^{25}Mg	92.8 ± 1.3	76.85 ± 0.75	106.82 ± 0.85	0.17
^{39}K	100.7 ± 3.6	118.6 ± 6.2	185.7 ± 4.5	9.80
^{49}Ti	<LOD	<LOD	0.91 ± 0.11	0.46
^{51}V	0.050 ± 0.040	0.0542 ± 0.0062	0.971 ± 0.047	0.054
^{55}Mn	24.45 ± 0.53	24.05 ± 0.65	178.4 ± 4.1	0.080
^{56}Fe	8.18 ± 0.39	10.60 ± 0.36	476.5 ± 8.7	0.22
^{59}Co	0.164 ± 0.021	0.0994 ± 0.0092	1.225 ± 0.018	0.0030
^{62}Ni	0.546 ± 0.089	2.20 ± 0.32	2.56 ± 0.25	0.37
^{65}Cu	1.270 ± 0.042	1.84 ± 0.10	17.40 ± 0.34	0.11
^{66}Zn	16.56 ± 0.30	16.30 ± 0.63	90.1 ± 2.8	0.60
^{88}Sr	1013.6 ± 5.8	947.2 ± 8.6	1067.7 ± 8.7	0.012
^{107}Ag	<LOD	<LOD	<LOD	0.036
^{111}Cd	<LOD	0.38 ± 0.13	0.54 ± 0.22	0.30
^{113}Cd	0.24 ± 0.11	0.304 ± 0.089	0.54 ± 0.17	0.17
^{137}Ba	1.74 ± 0.11	1.897 ± 0.069	8.40 ± 0.36	0.080
^{208}Pb	0.776 ± 0.041	0.685 ± 0.055	3.564 ± 0.080	0.030

Table 1) Bulk composition (µg g-1) with standard deviation (n = 5) and limit of detection (LOD) obtained from solution based ICP-MS analysis of powdered shells.

To further investigate on the scallop shell composition, specimens of shells were analysed before the contact with solution of Cd^{2+}. Compositional trace elemental variability in biogenic carbonates can be assessed by static layer by layer removal of material to obtain a depth-compositional profile. Alternatively, it can be carried out by LA along a defined section oriented perpendicular to the accretionary growth direction (Cariou *et al.*, 2017). The latter method was applied in the present study.

The investigation of the causes of the variability in the distribution of shell elemental components lays beyond the aim of this study, any correlation to seasonal environmental variations or pollution events cannot be made since the history of the shells, collected from a disposal site, is unknown. Nevertheless, information on the distribution of major and trace components of the shells used in this study can be obtained, highlighting the capability of the technique employed.

In Figure 1 the profile of the ratios of trace elements (i.e., Fe, Zn, Pb and Cd) with respect to Ca (Krause-Nehring, Brey and Thorrold, 2012; Holland *et al.*, 2014; Cariou *et al.*, 2017) of three scallop shell specimens are shown.

Lead was detected in every shell analysed, showing different distribution patterns across the shells, with peaks of higher abundance suggesting that during the formation of those layers, the organism was surrounded by a polluted environment.

Natali and Bianchini (2018) (Natali and Bianchini, 2018) reported that the sediments of Sacca di Goro are particularly enriched in Pb, with enrichment factors up to three with respect to the natural composition of alluvial sediments of the Padanian plain (di Giuseppe *et al.*, 2014), taken as representative of the geogenic

local background. The high Pb enrichment factor found in the lagoon sediments suggests anthropogenic contributions, possibly related to atmospheric emissions (Natali and Bianchini, 2018). Similar behaviours were found for Fe and Zn in the shells analysed, possibly related to the composition of the alluvial sediments (Wong *et al.*, 2017)

Regarding the metal contaminant considered in the present work, Cd/Ca concentration profiles for the three shells before treatment show that there is no appreciable Cd distribution across the shell layers; therefore, the results on the contaminant distribution obtained after the treatment of the shells with Cd-containing solution (see below) are entirely ascribable to the treatment performed.

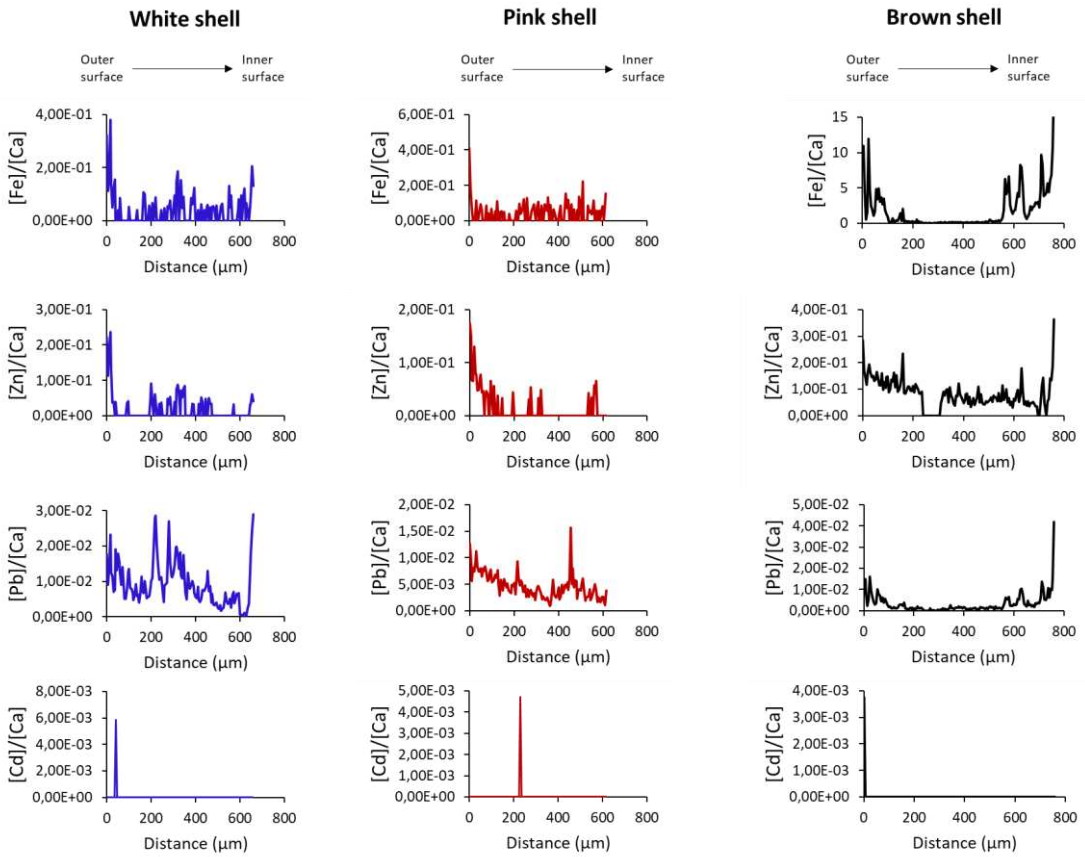

Figure 1. Trace element concentration profiles expressed as element/Ca ratio (mmol mol-1) obtained from LA-ICPTOFMS line scan carried out on not treated scallop shells; (the x-axis ranges from the outer to the inner surface of the shells).

In Supplementary Figure S3 the profile of the ratios of Na, Mg and Sr with respect to Ca of three different specimens is reported. Variations in compositions could originate from shells growth(Steinhardt *et al.*, 2016). These ratios, generally reported as mmol mol^{-1} (Mouchi *et al.*, 2013; Marali *et al.*, 2017), are biogeochemical parameters reflecting the Earth–ocean–atmosphere dynamic exchange of elements (Lebrato *et al.*, 2020) and are commonly employed to investigate trace elemental and isotopic inventor in successively secreted carbonate layers related to past environmental conditions of seashells (Lebrato *et al.*, 2020).

Generally, there is variability for all three elements in the element/Ca between the scallop shells, even though they belong to the same species. In addition, slight variations were observed within the same shell

across the different regions (see the profiles of Na/Ca of all the three shells and Mg/Ca of the brown one). Regarding strontium, the Sr/Ca profiles were characterised by distinct variations, especially in the white and brown specimens, suggesting an alteration in the incorporation rate of this element during the shell layers deposition, possibly related to seasonal temperature variations which most likely influence the shell calcification and hence the Sr/Ca ratio (Freitas *et al.*, 2006; Sosdian *et al.*, 2006).

Moreover, the brown shell shows a significant variation of the content of all three elements in a region between 230 and 315 µm; in particular, Mg decreases while Na and Sr increase. The investigation of the causes that led to this particular variation is beyond the aim of the present study, and correlations with seasonal variations cannot be made since the history of the shell is unknown. Nevertheless, such alteration in the distribution of these major components suggests that the mollusc was subjected to a stress condition that possibly led to an alteration in the metabolic processes or of the growth rate, resulting in a different incorporation of these elements during the formation of the shell (Freitas *et al.*, 2006; Sosdian *et al.*, 2006).

Adsorption of Cd from aqueous solution onto powdered scallop shells

The effectiveness of seashells in the adsorption of several heavy metals cations has already been demonstrated (Köhler *et al.*, 2007; Liu *et al.*, 2009; Peña-Rodríguez *et al.*, 2010; Du, Zhu and Shan, 2012; Ismail and Aris, 2013; Wu *et al.*, 2014; Alidoust *et al.*, 2015; Yen and Li, 2015).

In this work, we focus on the interaction of Cd on surface scallop shells, however, to quantify the adsorption of this material for the comparison with literature data, adsorption experiments on powdered scallop shells were carried out as well. The aim of these experiments was to investigate the capability of the powdered shell to adsorb Cd from solution. The kinetics and thermodynamics of adsorption can depend on particle dimensions, however in this study we were mainly focusing on the mechanisms of the adsorption instead of in maximising the adsorption capability.

In order to evaluate the adsorption properties of powdered scallop shells employed in the present study, the kinetics and thermodynamics of the adsorption process was investigated, details of the obtained results are reported in the Supplementary Information.

The experiments were carried out with powdered scallop shells characterised by particle dimensions spanning over a broad range, with an average value of 15 µm (see Supplementary Figure S4).

The adsorption properties of shells towards Cd^{2+} were evaluated at pH 7.0-8.5, measured at the end of the equilibration time of the batch experiments to account for shells dissolution (see Supplementary Discussion S1). The experiments were conducted at two constant temperatures, 21.0 ± 0.5°C and 9.0 ± 0.5°C, the latter to mimic seawater temperature in the region more closely.

The adsorption kinetics was investigated in a series of experiments for which the uptake was measured at different contact times. The adsorption kinetics obtained at three different initial concentrations are reported in Figure 2a.

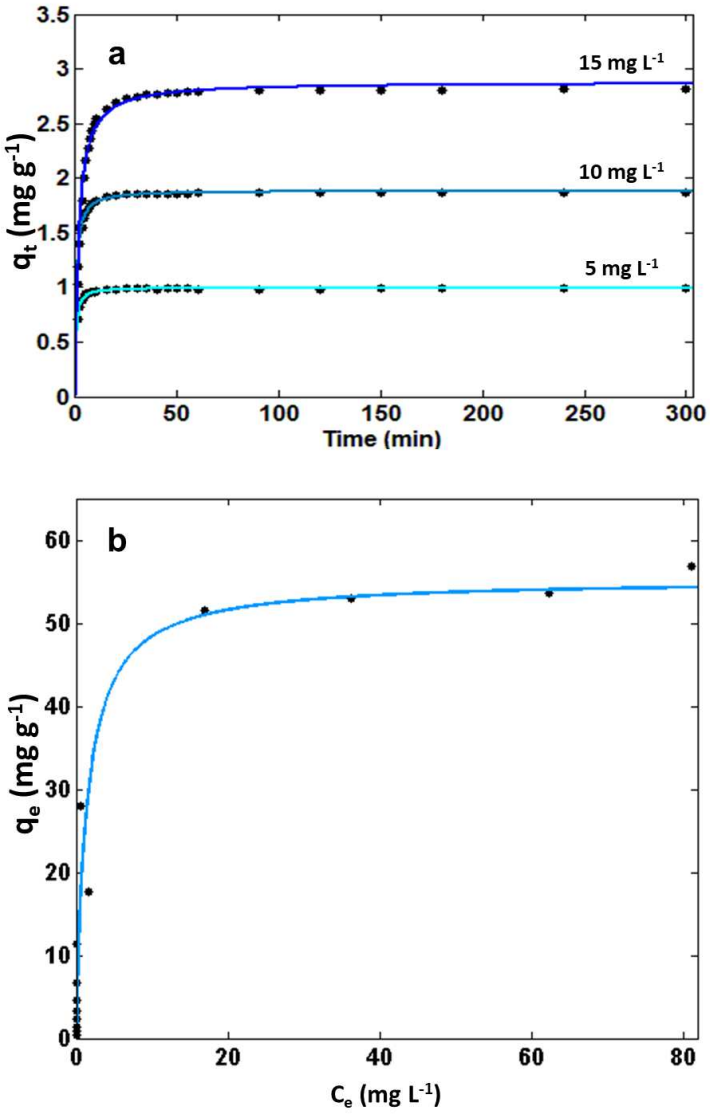

Figure 2.a) Effect of contact time on the adsorption of Cd onto scallop shell powder at three different initial metal concentrations; b) Adsorption isotherm of Cd onto scallop shell powder (21°C).

The adsorption was very fast, with the equilibrium reached within 30 min, and the majority of Cd removed in the first 15 min, for all the initial concentrations considered. These results are comparable with those found by Jeon(Jeon, 2018) for the adsorption of Cd onto grinded pen shells.

The very fast Cd uptake highlights the applicability of scallop shells as adsorbents in water remediation technologies; moreover, the short time needed for reaching equilibrium makes this material suitable also for continuous flow systems (Beni and Esmaeili, 2020).

The experimental data were fitted to the pseudo second order (PSO) kinetic model (Du, Zhu and Shan, 2012; Alidoust *et al.*, 2015; Monteiro *et al.*, 2016):

$$q_t = \frac{k_2 \cdot q_e^2 \cdot t}{1 + k_2 \cdot q_e \cdot t} \quad (2)$$

Where q_t is the amount of Cd adsorbed at time t, q_e is the quantity adsorbed when the equilibrium is reached, and k_2 the pseudo second order rate constant. The parameters obtained from the fitting are reported in Supplementary Table S1.

The good applicability of this model is usually associated with the situation when the rate of direct adsorption/desorption process (seen as a kind of chemical reaction referred to as ‘‘surface reaction’’) controls the overall sorption kinetics(Plazinski, Dziuba and Rudzinski, 2013); for further details see Supplementary Discussion S2.

Adsorption isotherm

The data obtained from batch experiments at the constant temperatures of 21°C and 9°C were fitted using the Langmuir isotherm model(Foo and Hameed, 2010):

$$q_e = \frac{q_{max} \cdot b \cdot C_e}{1 + b \cdot C_e} \quad (3)$$

where q_e is the amount of Cd adsorbed at the equilibrium (mg g^{-1}), C_e is the equilibrium concentration (mg L^{-1}), q_{max} is the saturation capacity of shell powder (mg g^{-1}) and b the adsorption constant (L mg^{-1}).

Fig. 2b shows the isotherm of Cd on scallop shell powder at 21°C (see Supplementary Figure S7 for the isotherm at 9°C): the curve has a concave shape, and it is characterised by a steep initial zone and a saturation plateau. The shape of the isotherm indicates a favourable adsorption of the metal onto the adsorbent material. This information, together with the relatively fast adsorption kinetics (see supra), confirms that scallop shell is a promising adsorbent for Cd. The Langmuir isotherm model refers to homogeneous adsorption without taking in consideration adsorbate-adsorbate and adsorbate-solvent interactions, moreover, the adsorption sites are energetically equivalent.

The parameters obtained from the fitting are summarised in Supplementary Table S2.

Moreover, the saturation capacity of the scallop shell powder (55.3 ± 7.4 mg g^{-1}) is much higher compared to that obtained with pure calcium carbonate powder (12.8 ± 1.6 mg g^{-1}, see Supplementary Figure S8) demonstrating that the composition of the biogenic shell matrix significantly enhances the capability of removing Cd from water matrices.

Structural and thermal analyses

The mineral phases occurring in scallop shells were identified by examination of XRD patterns. Qualitative mineralogical analysis revealed that calcite was detected in all the analysed samples. The coexistence with

otavite, $CdCO_3$, in the Cd-treated scallop shells can be inferred from inspection of the 20–60° 2**θ** region (i.e. 2θ=23.56, 30.35, 36.52, 43.92, 49.60 50.02°) showing the increased complexity of the diffraction pattern of samples containing two carbonates with respect to sample with only calcite (Fig. 3). Consequently, the process involved in Cd uptake by calcite was adsorption and Cd diffusion into the calcite crystal, leading to the formation of $(Cd_xCa_{1-x})CO_3$ solid-solution. At the same time, the occurrence of otavite in the Cd-loaded Scallop shells sample indicated that shell calcite substrate immersed in aqueous solutions containing Cd^{2+} acted also as passive surface, leading to dissolution of calcite and nucleation of otavite(Hay, Workman and Manne, 2003). Finally, the presence of additional and weak reflections in the 20–35° 2θ range (i.e. 2θ = 18.2, 20.5, 22.7, 25.5 and 32.2°) in Cd-scallop shells is reasonably related to polyenes (i.e. astaxanthin) (Allmann and Hinek, 2007; Pan, Wang and Gu, 2018), in the carbonate matrix. This result is in good agreement with the Raman spectra indicating the presence of a low fraction of pigments increasing the Cd adsorption efficiency of the biogenic $CaCO_3$ compared to geologic one (Warren and Haack, 2001; García-Sánchez and Álvarez-Ayuso, 2002).

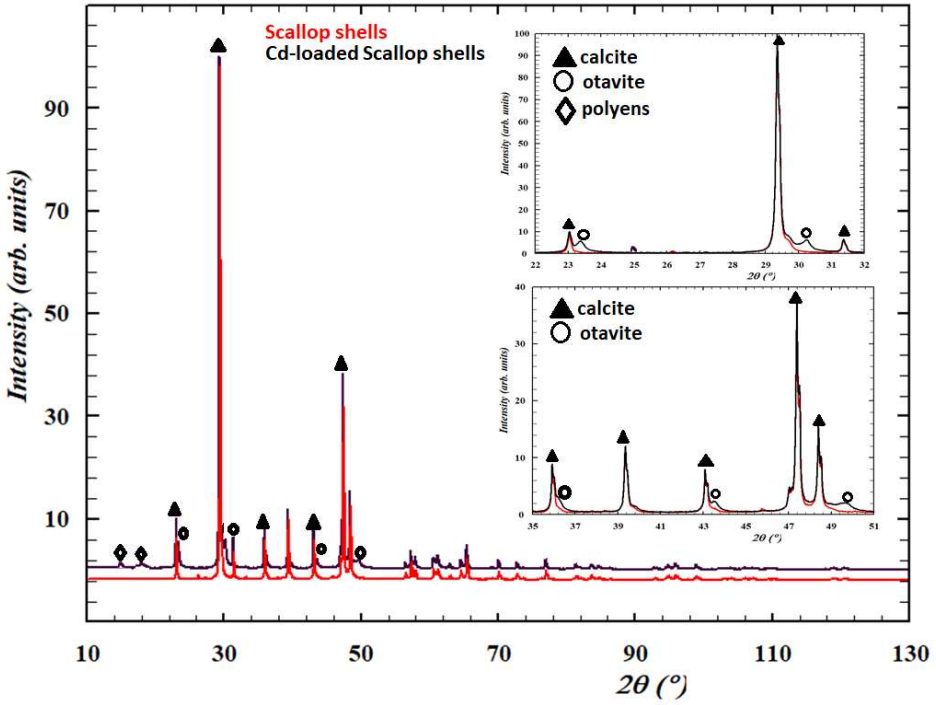

Figure 3. Comparison between XRD patterns of scallop shells (red line) and Cd- scallop shells (black line) respectively.

In order to quantify the phases content in Cd-loaded Scallop shells, quantitative phase analysis (QPA) by use of the Rietveld method was performed. It is well known that this approach gives rise to more accurate values compared to any other single technique, such as Fourier transform infrared spectroscopy (FTIR), chemical analysis and electron microscopy (Bish and Post, 2018).

In this procedure, the weight fraction w*i* of each i^{th} crystalline component in the multiphase system is calculated from the corresponding refined scale parameter *Si*, according to the equation:

$$wi = \frac{SiMiVi}{\sum_j SjMjVj} \quad with\ the\ normalisation\ condition \sum wi = 1.0 \tag{4}$$

with *Mi* and *Vi*, the unit cell mass and volume, respectively (Gualtieri, 2003). Firstly, quantitative refinement was performed by assuming calcite as the only carbonate present in scallop shells. In Cd-treated samples, the quantitative refinement was including both otavite and astaxanthin; the mass fractions obtained by Rietveld refinements are 90 wt% calcite, 9 wt% otavite and 1 wt% astaxanthin.

In addition, a correlation between the refined unit cell parameters of calcite and the Cd incorporation was noted (Table 2). In order to explain these variations, we suggest that in our samples the compression of the refined unit cell volume can be mainly ascribed to an increase of the Cd content due to the smaller ionic radius of Cadmium (0.95 Å) with respect to Calcium (1.06Å) (Shannon and Prewitt, 1970).

Calcite	Space Group	a (Å)	c(Å)	α=β(º)	γ(º)	Volume(Å^3)
	$R\bar{3}c$	4.9987(1)	17.1099(3)	90	120	370.24(1)
Cd-loaded Scallop shells						
	Space Group	a(Å)	c(Å)	α=β(º)	γ(º)	Volume(Å^3)
Calcite	$R\bar{3}c$	4.9981(2)	17.1109(5)	90	120	370.18(2)
Otavite	$R\bar{3}c$	4.9642(2)	16.2980(8)	90	120	347.83(3)

Table 2 Refined unit cell parameters for scallop shells not treated and scallop shells treated with solution containing Cd.

The trend of DTA and TG curves for both scallop shells samples are represented in Fig 4. Below 100 °C, DTA curves showed an endothermic peak weakly indicating low amounts of absorbed water. The occurrence of otavite was confirmed by a broad endothermic peak between about 370 and 460°C due to the decomposition of $CdCO_3$ into CdO under atmospheric pressure (Janeković and Matijević, 1985; Bultosa and Mulokozi, 1995). In the same temperature range the polyenes decomposition also occurred (Yuan *et al.*, 2008; Pan, Wang and Gu, 2018). Endothermic effects in the 720–900°C temperature range are ascribable to the CO_2 releasing by $CaCO_3$ decomposition. Lastly, the exothermic peak above 900 °C can be interpreted as the crystallisation of newly formed minerals.

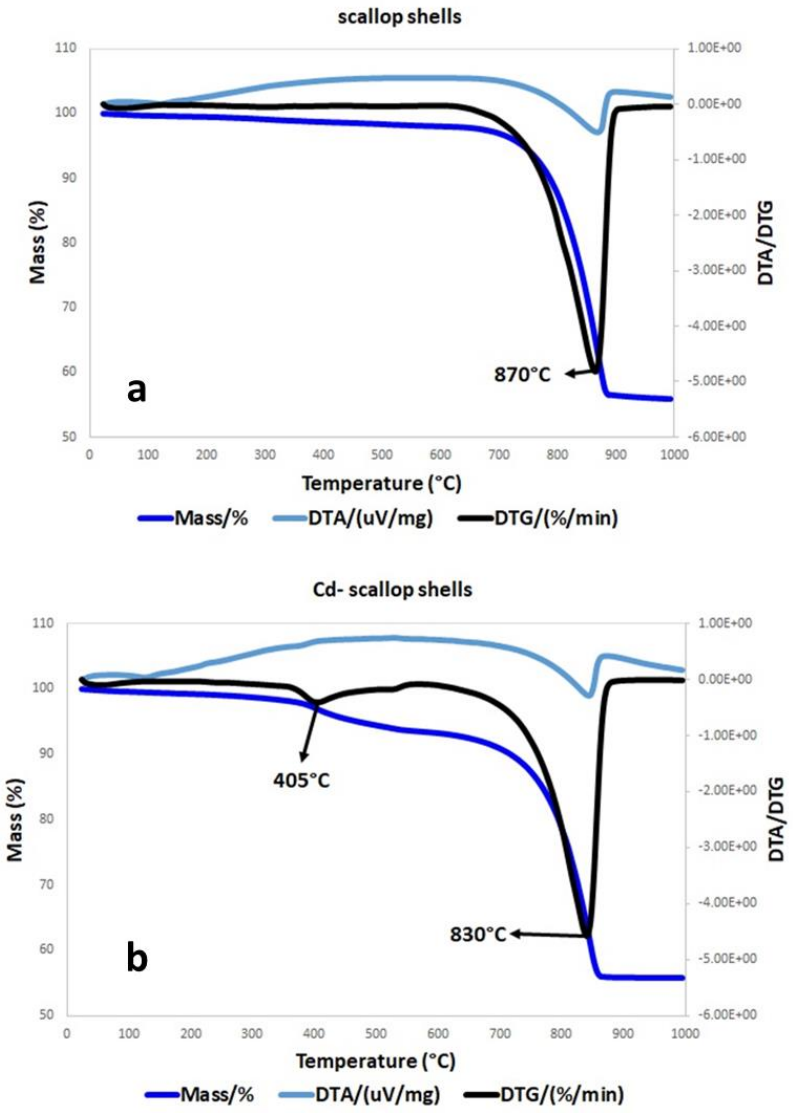

Figure 4. Comparison between TG, DTG and DTA curves of scallop shells (a) and Cd- scallop shells (b) one in the 25-1000°C temperature range, respectively.

The adsorption experiments performed using powdered scallop shells illustrated above were carried out to determine the mean values of Cd uptake obtained for a given particle distribution. To investigate the contaminant distribution and interactions with the shell matrix, intact scallop shell valves were used.

Cd profiles after adsorption

To study the distribution of the metal contaminant through the shell layers, Cd profiles were obtained from scallop shells treated with aqueous solutions containing Cd. Since scallop shells having different colours showed difference in composition, we selected scallop shells with three different colourations (white, pink and brown) to investigate potential differences in Cd adsorption characteristics. The colouration of mollusc shells is mainly due to the presence of organic pigments, mostly tetrapyrroles, carotenoids and melanins (Williams, 2017). Not only the colour and pattern of shells vary between species, but different colouration can also occur among a single species, or even a single shell can present differently coloured areas (Barnard and de Waal, 2006).

LA-ICP-TOFMS Imaging

In Figure 5 the Ca, Sr and Cd distribution images of three scallop shell samples, white, pink and brown, treated with Cd solution, are reported. Calcium carbonate ($CaCO_3$) consists of 40.0% calcium. The images indicate that Ca, most probably present as calcium carbonate, is the main component of the shells. Slight variations of the Ca content were observed and have been already reported for Ca in bivalves (ROSENBERG, 1973). Generally, they can be related to seashell metabolic variations or different environmental conditions. Sr is present in concentrations around 1000 mg kg^{-1} on the entire section considered and it shows more marked variations in the concentration along the shell cross section. The high-resolution images show distinct layers with different Sr concentration parallel to the inner and outer surfaces, suggesting a different incorporation rate for Sr. It could be correlated to the temperature influence on the shell growth rate (Sosdian *et al.*, 2006).

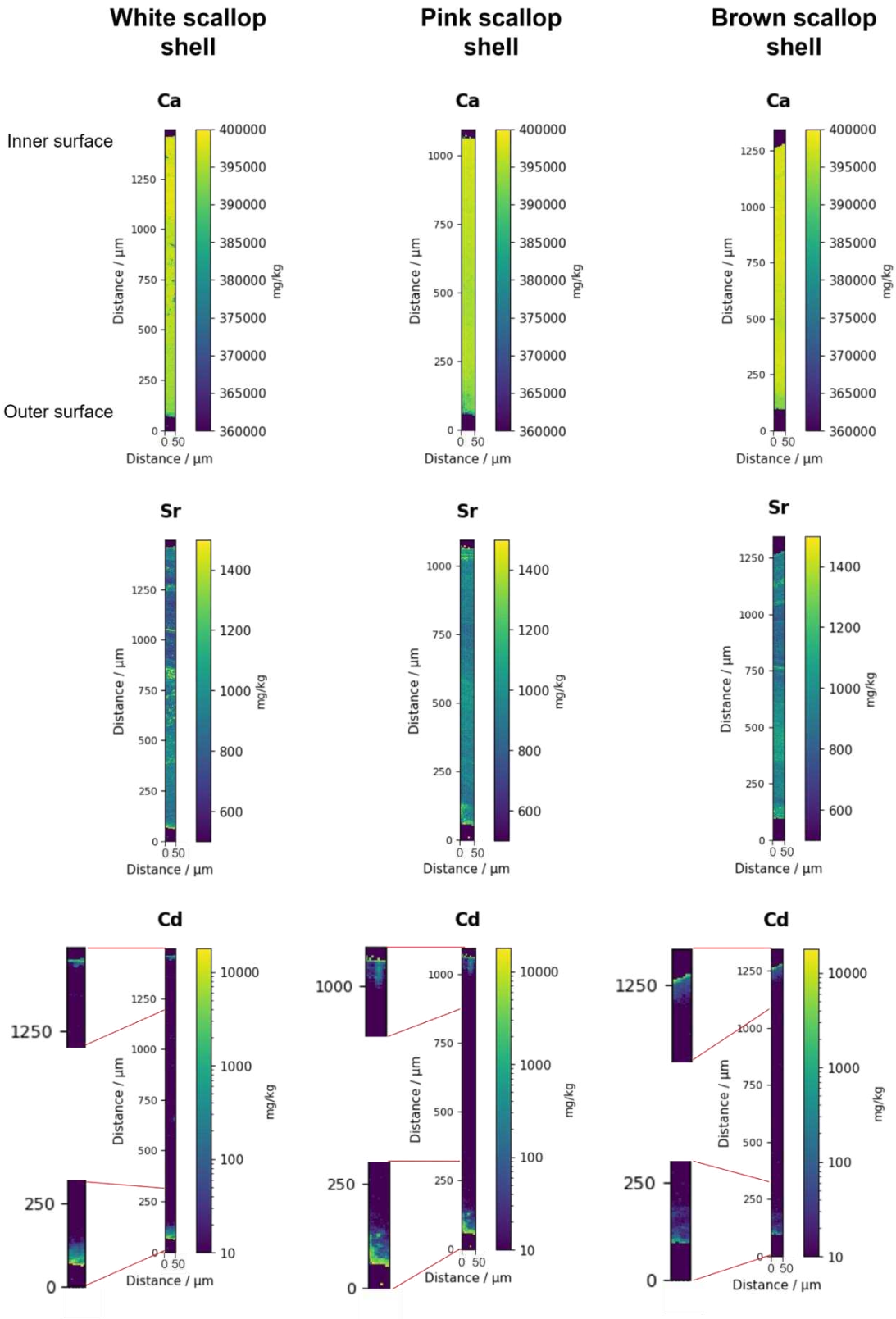

Figure 5. Ca, Sr and Cd images of the cross section of three scallop shells, treated with 1 mg L-1 Cd solution. After adsorption, Cd is located on a thin layer on the surfaces of the shells.

The Cd images of the three specimens show the metal distribution, with high concentrations on a thin layer located on the surfaces of the shell. Most of the adsorbed Cd was within the first 10 µm on both the inner

and outer surface. More specifically, Cd is present in high amounts on a thin layer on both the inner and the outer surface of the shell; however, a minor concentration of Cd is found in the first 50 µm towards the internal layers on both the boundary surfaces.

Cd bulk concentration and micro-Raman

To further investigate the role of shell pigment on adsorption, batch experiments were carried out by using scallop shells with three different colourations (white, pink and brown). As reported in Fig. 6, the Cd uptake (q_e) is lower for the white shells and it increases for the pink and brown shells, with the latter showing the highest uptake values. The difference in metal adsorption, especially between white and brown shells, is more evident when the Cd concentration in the aqueous solution increases (Figure 6b). These findings suggest that the components of the shell matrix responsible for its colouration enhances the metal adsorption. Evidence of the higher uptake of metals by the coloured shells rather than the white ones is also accentuated by the bulk concentration analyses (Table 1) which showed higher content of many metals, including Fe, Cu, Zn, and Pb, in the pink and especially in the brown shells.

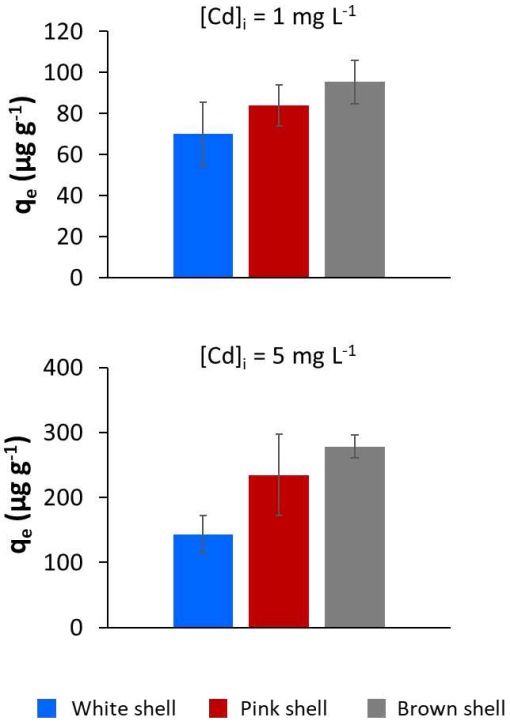

Figure 6. Cd uptake (qe) with standard deviation (n = 3) of scallop shells with different colouration treated with 1 mg L-1 (a) and 5 mg L-1 (b) Cd aqueous solution.

To examine in depth this particular feature, we investigated the nature of the pigments contained in our samples employing the micro-Raman technique (Barnard and de Waal, 2006; Soldati *et al.*, 2008; Thompson *et al.*, 2014; Gaspard *et al.*, 2019).

In Fig. 7 the Raman spectra acquired on the outer surface of scallop shells presenting different colours are reported. The white shell spectrum (Fig. 7a) presents only the bands at 1083, 707 and 274 cm^{-1}, belonging to the calcite matrix, while the spectra of the pink and brown samples (fig. 7b and 7c respectively) show two more bands, with higher intensities for the darker shells. The bands observed at ca. 1120 and 1500 cm^{-1} can be attributed to the stretching modes of the C=C double bond (v_1) and the C-C single bond (v_2) due to the presence of molecules characterised by a polyacetylenic chain (Barnard and de Waal, 2006; HEDEGAARD, BARDEAU and CHATEIGNER, 2006; Soldati *et al.*, 2008). In the Raman spectrum of the brown shell, we observed to more bands with much lower intensity at 1010 cm^{-1} and 1292 cm^{-1}, the former can be attributed to the CH=CH out-of-plane wagging mode (v_4) and the latter to the CH=CH in-plane rocking mode (v_3)(Barnard and de Waal, 2006).

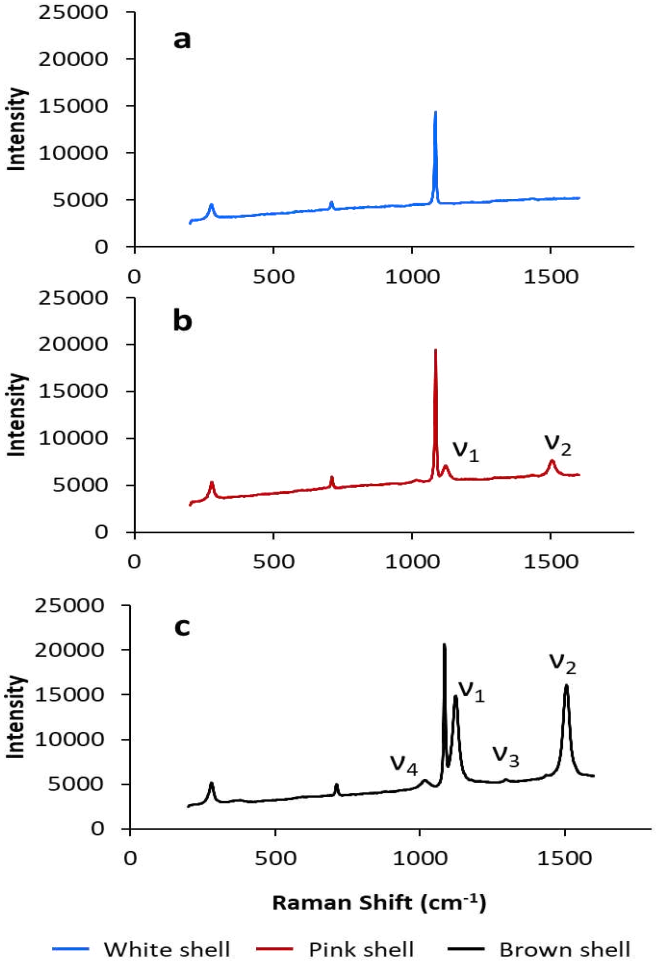

Figure 7. Micro-Raman spectra of white, pink and brown shells

The use of micro-Raman technique allowed us to identify the nature of the scallop shell pigments as polyenes, possibly belonging to the class of carotenoids by comparison with literature spectra (Barnard and de Waal, 2006; Soldati *et al.*, 2008; Thompson *et al.*, 2014; Gaspard *et al.*, 2019). The presence of these components in the carbonate matrix results in the enhancement of the adsorption efficiency of the biogenic $CaCO_3$ towards Cd compared to commercial $CaCO_3$ (Tudor, Gryte and Harris, 2006). This result is in

good agreement with the XRD data indicating the presence of a low fraction of pigments increasing the Cd adsorption efficiency of the biogenic $CaCO_3$ compared to geologic one (Warren and Haack, 2001; García-Sánchez and Álvarez-Ayuso, 2002).

Summary

The current work focused on the study of the mechanism of adsorption of the heavy metal cadmium onto scallop shells; in particular, the interactions between the metal and the components of the shell matrix were evaluated.

The bulk composition of shells taken from a deposit site was determined with regards to their colouration. In general, the brown shell showed higher amounts of Mn, Fe, Co, Cu, Zn, Ba, and Pb than the white and pink ones; regarding the contaminant investigated, Cd content was close to the limit of quantification of the method, indicating that the environment in which the shells were collected was not severely polluted by Cd.

LA-ICP-TOFMS was employed to investigate the distribution of major components and trace metals along the shell cross-section; Na, Mg and Sr showed minor variability between the different shells and within the layers of the same shell; in particular, Sr showed distinct layers with different Sr concentration parallel to the surface, possibly correlated to environmental factors such as temperature. Trace metals, such as Fe, Zn and Pb showed variations in their distribution across the shells cross section, especially for the brown specimen.

Powdered scallop shells, with an average particle dimension of 15 µm, were employed to investigate the capability to adsorb Cd. The kinetic experiments highlighted that the adsorption is a very fast process, reaching the equilibrium within the first 30 minutes.

The determination of the adsorption isotherm, obtained at 9 and 21°C, allowed to determine the saturation capacity of the material which resulted to be temperature dependent. In particular, at 21°C the saturation capacity resulted to be 55.3 ± 7.4 mg g^{-1}, much higher than that obtained using commercial $CaCO_3$ (12.8 ± 1.6 mg g^{-1}).

The analysis of XRD patterns of the scallop shell powder before and after Cd adsorption showed that the material is mainly composed of calcite, while, after the contact with the Cd containing solution, the presence of otavite ($CdCO_3$) phase was observed, as confirmed by DTA and TG curves. These findings, in addition with the kinetic experiments where an increase of Ca concentration in solution was observed during the adsorption, demonstrate that the adsorption process involves mainly an ion exchange between Ca and Cd.

The distribution and interactions of the Cd contaminant with the shell matrix were investigated using intact shell valves sorted by colour. High-resolution LA-ICP-TOFMS imaging allowed to assess Cd distribution after adsorption; an enrichment of Cd was found on a thin layer (10 µm) on both the inner and outer surfaces of the shell, with a minor Cd concentration found in the first 50 µm towards the internal layers.

For each shell, the total Cd uptake was evaluated: the white shells showed the lower Cd adsorption, while for the pink and brown shells the metal uptake increased, with the brown shells showing the highest adsorption values.

The nature of the pigments contained in the differently coloured shells was determined by micro-Raman spectroscopy; the Raman spectra show the bands typical of polyenes, possibly carotenoids, as also highlighted by the astaxanthin phase present in the XRD patterns.

Conclusions

The results reported provide new information on the mechanism of adsorption of Cd onto biogenic $CaCO_3$. Structural and thermal analyses highlighted the presence of cadmium carbonate phases in the scallop shells treated with aqueous solutions containing the metal contaminant, indicating that the adsorption is predominantly a superficial process involving the partial dissolution of superficial calcite and the nucleation of cadmium carbonate.

Cd is adsorbed on the inner and outer surfaces when intact scallop shells are used as adsorbent. The bulk concentration values showed that the metal adsorption depends on the mollusc species and on the quantity of organic substances (i.e. pigments) present in the carbonatic matrix. Indeed, we found higher cadmium uptake for scallop shells containing larger amounts of organic substances, even if the exchange with carbonate is the dominant adsorption mechanism. The pigments were identified as carotenoids, in particular, the XRD pattern showed the presence of astaxanthin phases which can act as complexing agent towards Cd, increasing its removal from aqueous media.

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Chapter 3: Pollution as a Stress Source Toward Marine Life; the Case of Plastics and Microplastics Within Mediterranean Sea.

The third section of the thesis is a published article on Environmental Pollution journal (https://doi.org/10.1016/j.envpol.2021.117449), regarding the plastic ingestion by marine life and the consequences on the organisms in terms of stress and disfunctions. That is a fundamental gusset in order to understand the complex consequences of anthropogenic stress on the ecosystems.

Plastic litter, in marine environments, can break up into pieces with various sizes, colors, and shapes, due to sunlight, the constant friction of waves, and collisions with other plastic litter. Thus, these objects are selected for ingestion by marine organisms, as the small and micro- plastics are eligible to resemble prey or food particles. Moreover, plastic polymers may contain chemical additives and contaminants (e.g., PBTs, POPs), including known endocrine disruptors that may be posing threats for the marine organisms; in turn posing potential risks to marine ecosystems, biodiversity, food availability, and the food chain itself.

Hence, this work is fundamental to understand how the plastic pollution bioaccumulation is a widespread and multifaced problem affecting basic functions of living beings, introducing “stress” and disorders that can be observed through the genic expressions in organs like the liver. Moreover, the fauna samples are from the Mediterranean Sea, which, as previously introduced, is a sea particularly interested by fishing and aquaculture activities and economies. Additionally, the climb of the trophic network is allowed given the persistent nature of this pollutant class; eventually impacting also on human health, as the apex of multiple food chains.

The article is attached at the end of the thesis in its published version.

Plastic ingestion by Atlantic horse mackerel (Trachurus trachurus) from central Mediterranean Sea: A potential cause for endocrine disruption

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Abstract

Plastics in the oceans can break up into smaller size and shape resembling prey or particles selected for ingestion by marine organisms. Plastic polymers may contain chemical additives and contaminants, including known endocrine disruptors that may be harmful for the marine organisms, in turn posing potential risks to marine ecosystems, biodiversity and food availability. This study assesses the presence of plastics in the contents of the gastrointestinal tract (GIT) of a commercial fish species, the Atlantic horse mackerel, Trachurus trachurus, sampled from two different fishing areas of central Mediterranean Sea. Adverse effect of plastics occurrence on T. Trachurus health were also assessed quantifying the liver expression of vitellogenin (VTG), a biomarker for endocrine disruption. A total of 92 specimens were collected and morphometric indices were analysed. A subgroup was examined for microplastics (MP < 1 mm) and macroplastics (MaP >1 cm) accumulation in the GIT and for VTG expression. Results indicated that specimens from the two locations are different in size and maturity, but the ingestion of plastic is widespread, with microplastics (fragments and filaments) abundantly present in nearly all samples while macroplastics were found in the larger specimens, collected in one of the two locations. Spectroscopic analysis revealed that the most abundant polymers in MP fragments were polystyrene, polyethylene and polypropylene, whereas MP filaments were identified mainly as nylon 6, acrylic and polyester. MaP were composed mainly of weathered polyethylene or polypropylene. The expression of VTG was observed in the liver of 60% of all male specimens from both locations. The results of this study represent first evidence that the ingestion of plastic pollution may alter endocrine system function in adult fish T. Trachurus and warrants further research.

Introduction

The occurrence and accumulation of plastic debris in the environment, especially in water, is recognized as a problem of major concern all around the world. The threat that plastics represent to the water ecosystem and, eventually, to human health, has been gaining more attention over the last few years. Plastics in large quantities have been detected in accumulation zones (e.g. the North Atlantic and the South Pacific subtropical gyre) (Law et al., 2010; Eriksen et al., 2013), and, in less amounts, in the remote polar regions (Cincinelli et al., 2017; Morgana et al., 2018).

After the first testimony in 1980 of visible plastic in the Mediterranean Sea (Morris, 1980), many studies have described the presence of plastic debris in this semi-enclosed basin (Collignon et al., 2012; Suaria and Aliani, 2014; Cozar et al., 2015; Simon-Sanchez ´ et al., 2019; Garofalo et al., 2020) thus identifying the Mediterranean Sea as a great accumulation zone for plastic particles. This is likely related to its densely populated coasts (Cozar et al., 2015; Vlachogianni et al., 2020) and its long water residence time, up to a century (Lacombe et al., 1981). Plastic pollution, linked to the high concentration of economic activities characterising the Mediterranean Sea, is expected to negatively affect the variety and abundance of the marine species populating this basin, as well as human health (Avio et al., 2017).

Plastic debris is generally sorted based on their size and their origin. According to the classification suggested by Hartmann et al. (2019), plastic particles can be divided into nanoplastics (1–1000 nm), microplastics (1–1000 µm), mesoplastics (1–10 mm) and macroplastics (1 cm and larger).

According to their origin plastic particles can be divided in primary microplastics, manufactured to be already microscopic for various industrial or domestic applications such as in toothpaste, cosmetics and in clothing in fishing nets, or in air blasting technology to remove rust and paint from machinery, and secondary microplastics, originating from the fragmentation of bigger plastic debris caused by photo-degradation, physical fragmentation, and biological and chemical processes (Auta et al., 2017).

Microplastics, both primary and secondary, occur in the marine environment in a variety of shapes and colours (Botterell et al., 2019), and the most common synthetic polymers likely to be found are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC) and polyethylene terephthalate (PET) (Andrady, 2011).

Large plastic debris can be a threat to marine species by entanglement whereas smaller plastic particles can be ingested by fish accidently or because they resemble natural food sources due to their shapes and colours (Foekema et al., 2013). Indeed, microplastics have been identified in several marine organisms, from fish (Lusher et al., 2013; Bellas et al., 2016; Mancia et al., 2020), to seabirds (Cole et al., 2011) and mammals (Nelms et al., 2019). The macro and microplastics accumulated in the gastrointestinal tract (GIT) after ingestion can cause not only physical damage, such as blockage, reduced feeding stimuli and inhibition of gastric enzyme secretion, but also can lead to chemical effects as a consequence of the release of toxic plastic additives or waterborne persistent organic pollutants (POPs) adsorbed onto the plastic debris (Li et al., 2016). In fact, the lipophilic properties of plastic materials make them capable to adsorb and transport organic pollutants that could leach once the plastic particles have entered the organism, with possible adverse effects. The most common polymer particles, such as PE, PP, PS and PVC, have demonstrated to be efficient adsorbents for organic contaminants like polycyclic aromatic hydrocarbons (PAH), dichlorodiphenyltrichloroethane (DDT), chlorinated benzenes and pharmaceuticals (O'Donovan et al., 2018).

Adverse effects such as tissue damages, neurotoxicity and endocrine disruption produced by ingestion of plastics by marine organisms have been reported (Rochman et al., 2014; Mak et al., 2019; Wang et al., 2019; Barboza et al., 2020) but the contribution that adsorbed contaminants may have to the toxicity of ingested plastics is not yet clearly understood. A study in adult medaka fish reported the higher effects in endocrine disruption after the ingestion of marine plastics in comparison with virgin plastics, suggesting that chemicals associated with the complex matrix of marine plastic debris compete with the endogenous oestrogen for the binding to the oestrogen receptor (Rochman et al., 2014).

The Atlantic horse mackerel (Trachurus trachurus, Linnaeus, 1758) is a gregarious bentho-pelagic species belonging to the Carangidae family. This species is commonly distributed in the Nort-heast Atlantic, from Cape Verde Islands to the Norwegian and North Seas, as well as in the Mediterranean and Black Seas, on shelf seas reaching depths down to 200 m (Comesana et al., 2008; Ferreri et al., 2019). The Atlantic horse mackerel is a zooplanktophagous fish feeding mainly on euphausiid crustaceans. Fish larger in size can be found at lower depths where they also feed on teleosts (Rumolo et al., 2017; Santic et al., 2005).

Atlantic horse mackerel is abundant in many pelagic and demersal food webs (Juan-Jorda et al., 2013; Carrozzi et al., 2019). Based on this, this species is vulnerable to plastic accumulation when feeding on lower trophic levels, as well as it can be a plastic vector towards the higher trophic levels such as elasmobranches and larger Scombridae (Karakulak et al., 2009).

Most of the studies on the uptake and the outcomes of microplastics ingestion on marine species are conducted under precise laboratory conditions, using model organisms such as medaka and zebrafish, feeding the specimen with virgin or treated plastics (Rochman et al., 2014, 2017; Limonta et al., 2019; Mak et al., 2019; Wang et al., 2019).

To the best of our knowledge, only few works study the potential adverse effects of marine plastics ingested by marine biota in wild populations (Alomar et al., 2017; Barboza et al., 2020; Mancia et al., 2020).

Herein, we present a study on the presence and toxicological effects of micro- and macroplastics in Trachurus trachurus (TT), an important fish species both under the economic and ecological point of view, caught by the large Italian fleet engaged in trawl fishing in two important fishing areas of south-central Mediterranean.

The gastrointestinal tract (GIT) of TT was treated to isolate, characterise, and quantify the plastic debris. Additionally, a potential link between the occurrence of plastics and adverse effects on TT health status was evaluated by investigating the expression of vitellogenin (VTG), a lipoprotein produced in female fish and used as a biomarker for endocrine disruption (García-Reyero et al., 2004; Biales et al., 2007).

Materials and methods

Samples collection

A total of 92 specimens of TT were collected in the Strait of Sicily and caught by 2 commercial fishing trawlers and more precisely 50 (29 females and 21 males) south of Mazara del Vallo (hereinafter MDV) and 42 (13 females and 29 males) south of Lampedusa island (hereinafter LMP) between April and May 2018.

According to the FAO General Fisheries Commission for the Mediterranean (GFCM), the two fishing grounds, MDV and LMP, are located in the Geographical Sub-Area GSA 16 and GSA 13, respectively. (Mancia et al., 2020; see SI-1 Fig. S1, Table S1). After collection, the samples were frozen and stored at − 20 ◦C. Once in the laboratory, the samples were carefully washed with MilliQ water and sectioned to remove the entire GIT (from oesophagus to vent), the liver and the spleen. To avoid plastic contamination from the container, the GIT was wrapped in aluminium foil, whereas the liver and spleen were fixed in RNAlater and stored in separated containers. All the samples were kept at − 20 ◦C until the analysis.

Biometric data

Fish evisceration was followed by biometric data recording: total length (TL), body weight (BW), liver, spleen and GIT weight (LIV W, SPL W, GIT W, respectively), gender and maturity stage. Visceral weight (VW) was calculated by subtracting the carcass weight (CW) to the BW. All the weights were measured using Sartorius balance (model: MSEE6202P-000-D0) to an accuracy of 0.01 g.

The Medits (International Bottom Trawl Survey in the Mediterranean) scale (with some modification) was used to define the sexual maturity by 7 stages of gonadic development: Stage 1 - immature; Stage 2 - virgin-developing; Stage 3 - recovering; Stage 4 - maturing; Stage 5 - mature; Stage 6 – spawner/spent; Stage 7 – resting (Anonymous, 2017).

Morphometric indices

The condition factor (CF), the spleno-somatic index (SSI), the hepato-somatic index (HSI), the GIT somatic index (GSI), the visceral somatic index (VSI) and the carcass somatic index (CSI) were calculated as previously described (Mancia et al., 2020) (see SI-1 Table S1).

Isolation and identification of plastic

Microplastics from the GIT were isolated following one of the protocols proposed by Dehaut et al. (2016). The procedure, described in detail in a previous work (Mancia et al., 2020), involved the digestion of the GIT in KOH 10% at 60 ◦C for 24 h, followed by a density-based separation using a NaCl hypersaline solution. The supernatant collected after the separation was filtered with 8 µm nitrocellulose filter (Whatman); filters containing undigested organic residues or minerals compromising the visualisation of MP, were further digested with a basic solution following the procedure reported in Roch and Brinker (2017) and rinsed with a HCl 0.1 M solution. The suspension was then collected and filtered.

Contamination was avoided washing and rinsing thoroughly with MilliQ water all glassware equipment before use, covering with aluminium foils all the materials, keeping filters in glass petri dishes. Personal protective equipment (gloves, lab coats and glasses) were used during the entire procedure. To account for airborne MP contamination, blank samples were prepared and analysed as controls following the same procedure as the samples (the control procedure was repeated ten times, n = 10).

The filters obtained (samples and blanks) were observed with a stereo microscope (Nikon SMZ745T stereomicroscope equipped with a Nikon Digital Sight DS-F12 camera) to visualise, count and sort the MP. The blank filters contained only white fibres with an average value of 4.7 (±0.5) fibres per filter.

The identification of MP fragments and filaments to determine the type of plastic material was carried out via Raman spectroscopy, with a LabRam HR800 micro-Raman instrument (Horiba Scientific) as described in Mancia et al. (2020). MaP debris composition was determined via Fourier-transform infrared spectroscopy (FT-IR) with a Bruker Vertex 70 FT-IR instrument, the FT-IR spectra were recorded between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and scan number of 128.

Sample ID	Gender	MAT	TL	BW	CW	VW	CF	SPL W	SSI	LIV W	HSI	GIT W	GSI	Date 2018	LS
TT1	F	5	19.50	63.10	55.30	7.80	0.85	0.05	0.0008	1.65	0.026	2.70	4.28	17-Apr	MDV
TT2	F	5	19.50	59.60	51.60	8.00	0.80	0.09	0.0015	1.12	0.019	2.10	3.52	17-Apr	MDV
TT3	M	5	19.00	55.60	50.70	4.90	0.81	0.06	0.0011	1.02	0.018	2.11	3.79	17-Apr	MDV
TT4*	F	5	20.00	64.10	56.30	7.80	0.80	0.08	0.0012	1.51	0.024	2.74	4.27	17-Apr	MDV
TT5	M	4	20.50	69.80	64.10	5.70	0.81	0.08	0.0011	0.95	0.014	2.85	4.08	17-Apr	MDV
TT6	F	6	17.50	45.40	41.80	3.60	0.85	0.03	0.0007	0.61	0.013	2.73	6.01	17-Apr	MDV
TT7*	F	5	20.00	57.30	51.60	5.70	0.72	0.03	0.0005	0.77	0.013	2.10	3.66	17-Apr	MDV
TT8*	M	4	19.00	50.80	46.60	4.20	0.74	0.08	0.0016	0.88	0.017	1.70	3.35	17-Apr	MDV
TT9*	M	5	18.50	45.50	41.53	3.97	0.72	0.03	0.0007	0.51	0.011	1.75	3.85	17-Apr	MDV
TT10*	M	4	20.00	60.10	54.50	5.60	0.75	0.08	0.0013	0.93	0.015	2.38	3.96	17-Apr	MDV
TT11*	M	5	19.00	50.79	46.90	3.89	0.74	0.04	0.0008	0.64	0.013	1.20	2.36	17-Apr	MDV
TT12*	F	6	21.00	70.10	65.80	4.30	0.76	0.05	0.0007	0.75	0.011	2.85	4.07	17-Apr	MDV
TT13*	M	5	19.00	52.70	47.38	5.32	0.77	0.02	0.0004	1.02	0.019	2.29	4.35	17-Apr	MDV
TT14*	F	4	21.00	73.80	66.30	7.50	0.80	0.07	0.0009	1.50	0.020	2.87	3.89	17-Apr	MDV
TT15*	F	5	23.00	90.90	81.64	9.26	0.75	0.03	0.0003	1.00	0.011	2.96	3.26	17-Apr	MDV
TT16*	M	5	21.50	76.70	70.40	6.30	0.77	0.03	0.0004	1.04	0.014	2.23	2.91	17-Apr	MDV
TT17*	M	5	20.50	74.10	65.80	8.30	0.86	0.08	0.0011	1.18	0.016	2.60	3.51	17-Apr	MDV
TT18*	M	4	20.00	65.70	61.10	4.60	0.82	0.11	0.0017	1.25	0.019	2.63	4.00	17-Apr	MDV
TT19*	M	4	20.00	66.19	59.25	6.94	0.83	0.03	0.0005	1.23	0.019	2.31	3.49	17-Apr	MDV
TT20*	M	4	20.00	68.20	62.40	5.80	0.85	0.10	0.0015	0.95	0.014	3.02	4.43	17-Apr	MDV
TT21*	F	4	19.50	65.40	57.70	7.70	0.88	0.07	0.0011	1.28	0.020	2.84	4.34	17-Apr	MDV
TT22*	F	4	20.50	68.26	61.80	6.46	0.79	0.04	0.0006	1.62	0.024	2.52	3.69	17-Apr	MDV
TT23*	M	5	17.50	46.30	41.21	5.09	0.86	0.05	0.0011	0.66	0.014	1.49	3.22	17-Apr	MDV
TT24*	M	4	20.00	54.80	51.71	3.09	0.69	0.05	0.0009	0.72	0.013	1.79	3.27	17-Apr	MDV
TT25*	F	4	19.00	56.40	49.37	7.03	0.82	0.06	0.0011	1.12	0.020	2.59	4.59	17-Apr	MDV
TT51*	M	4	38.00	466.70	423.16	43.54	0.85	1.30	0.00279	4.00	0.0086	23.19	4.97	21-Apr	LMP
TT52*	F	4	36.00	414.60	374.38	40.22	0.89	0.48	0.00116	4.56	0.0110	15.42	3.72	21-Apr	LMP
TT53*	M	4	37.00	395.90	366.14	29.76	0.78	1.09	0.00275	2.59	0.0065	15.81	3.99	21-Apr	LMP
TT54*	F	4	37.50	450.90	410.20	40.70	0.86	0.56	0.00124	4.09	0.0091	23.68	5.25	21-Apr	LMP
TT55*	F	4	36.00	386.70	349.73	36.97	0.83	0.40	0.00103	4.21	0.0109	13.42	3.47	21-Apr	LMP
TT56*	M	4	36.00	429.60	386.07	43.53	0.92	0.50	0.00116	4.66	0.0108	26.94	6.27	21-Apr	LMP
TT57*	F	5	38.00	479.97	428.22	51.75	0.87	0.31	0.00065	5.61	0.0117	18.35	3.82	21-Apr	LMP
TT58*	M	4	35.50	367.80	338.68	29.12	0.82	0.31	0.00084	2.81	0.0076	19.12	5.20	21-Apr	LMP
TT59*	M	4	37.00	427.24	390.48	36.76	0.84	0.44	0.00103	3.69	0.0086	21.88	5.12	21-Apr	LMP
TT60*	F	4	33.50	346.34	311.73	34.61	0.92	0.54	0.00156	3.49	0.0101	17.22	4.97	21-Apr	LMP
TT61*	F	4	39.00	506.77	457.35	49.42	0.85	0.57	0.00112	6.15	0.0121	16.16	3.19	21-Apr	LMP
TT62*	M	7	32.00	267.10	246.94	20.16	0.82	0.24	0.00090	2.66	0.0100	10.76	4.03	21-Apr	LMP
TT63*	M	6	21.50	87.80	83.45	4.35	0.88	0.05	0.00057	1.27	0.0145	2.42	2.76	07-May	LMP
TT64*	M	6	22.00	87.30	81.80	5.50	0.82	0.07	0.00080	0.68	0.0078	2.41	2.76	07-May	LMP
TT65*	M	6	21.50	79.90	75.90	4.00	0.80	0.06	0.00075	0.95	0.0119	2.56	3.20	07-May	LMP
TT66*	M	6	22.50	89.30	83.50	5.80	0.78	0.04	0.00045	0.86	0.0096	3.16	3.54	07-May	LMP
TT67*	M	6	20.50	73.90	68.80	5.10	0.86	0.06	0.00081	0.82	0.0111	1.99	2.69	07-May	LMP
TT68*	M	6	21.50	81.80	77.60	4.20	0.82	0.04	0.00049	0.84	0.0103	2.64	3.23	07-May	LMP
TT69	M	6	21.50	88.10	82.54	5.56	0.89	0.05	0.00057	0.75	0.0085	2.64	3.00	07-May	LMP
TT70*	M	6	21.00	80.95	75.96	4.99	0.87	0.02	0.00025	0.85	0.0105	2.86	3.53	07-May	LMP
TT71*	M	6	22.50	90.75	86.11	4.64	0.80	0.02	0.00022	0.85	0.0094	2.44	2.69	07-May	LMP
TT72	M	4	20.50	78.50	73.55	4.95	0.91	0.04	0.00051	1.17	0.0149	2.55	3.25	07-May	LMP
TT73	M	6	22.00	98.12	90.38	7.74	0.92	0.05	0.00051	1.50	0.0153	3.83	3.90	07-May	LMP
TT74*	M	6	21.50	79.30	75.38	3.92	0.80	0.06	0.00076	0.70	0.0088	1.61	2.03	07-May	LMP
TT75*	M	6	23.50	112.44	105.21	7.23	0.87	0.06	0.00053	1.57	0.0140	4.61	4.10	07-May	LMP
TT76*	F	6	22.00	87.96	83.11	4.85	0.83	0.03	0.00034	0.61	0.0069	2.57	2.92	07-May	LMP
TT77*	F	6	23.50	103.80	98.20	5.60	0.80	0.10	0.00096	1.15	0.0111	2.77	2.67	07-May	LMP
TT79*	F	6	23.50	102.90	95.94	6.96	0.79	0.09	0.00087	1.36	0.0132	2.77	2.69	07-May	LMP

*Table 1) Sample subset of T. trachurus used in the morphometric, plastic quantification and identification, and gene expression analyses (samples denoted by *).*

Gene expression analysis

RNA Extraction: Total RNA from 30 mg of liver samples of 50 randomly selected samples was extracted using the Qiagen RNeasy Plus Mini Kit (Hilden, Germany) following the manufacturer's instructions. Tissue lysis, homogenization and quantification was carried as previously described (Mancia et al., 2020). RNA samples that did not meet the absorbance ratio cutoff were extracted more than once and/or excluded from further analysis. The total number of good quality RNA samples (1.8–2.0, 280/260; 2.0–2.2, 260/230) used in the following steps was N = 44 (Table 1).

Quantitative real time PCR (q-PCR): TT Vitellogenin (VTG) gene was amplified and quantified using primers designed with Primer 3 on conserved region of the sequence. Primers were designed using the sequences for the Vitellogenin gene publicly available at https://www. ncbi.nlm.nih.gov from 8 species of bony fish (KJ804266, Syacium gunteri; EF582607, Hippoglossus hippoglossus; JQ283442, Dicentrarchus labrax; MF370511, Scophthalmus maximus; AJ416328, Pleuronectes platessa; AJ416327, Platichthys flesus; MG934701, Sciaenops ocellatus; HQ846510, Morone saxatilis). The primer sequences 5'->3' were: Fw: GGATCCCTGCAGTACGAGTT; Rev: CTTCAGAGGGGCATCTTCGT).

1 µg total RNA was retrotrascribed and 10 ng cDNA were used in qPCR as described before (Mancia et al., 2020). reaction was performed in triplicates in 96 wells plates, using the EvaGreen Dye Master mix (Bio-Rad) on CFX Connect Real-Time Detection system (Bio-Rad). qPCR efficiencies of each primer couple were calculated using a five points standard curve with serially diluted 1:5 cDNA from 4 samples (MDV: TT02, F; TT10, M - LMP: TT67, M; TT79, F) (Dhar et al., 2009). qPCR reactions were run as previously described and were run in triplicate with triplicate no-template controls. The average Ct values were normalised to the values of the housekeeping 18 S rRNA (primer sequence 5'->3': Fw: ACCACCCACAGAATCGAGAAA; Rev: GCCTGCGGCTT AATTTGACT) (Filby and Tyler, 2007). PCR amplicons were sequenced, and the identity of the products was confirmed; sequences were submitted at GenBank with Acc. Nos: MT862539 and MT862540 for VTG and 18 S, respectively.

Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control (males not expressing VTG, with VTG Ct values ≥ 33; specifically, 10 M from LMP: TT70, TT64, TT51, TT59, TT62, TT56, TT65, TT53, TT58, TT67 and 5 M from MDV: TT24, TT11, TT17, TT09, TT23) and all other samples on CFX connect manager software 3.1 (Bio-Rad). The Pfaffl method based on primer efficiencies was used to calculate fold (Pfaffl, 2001).

Statistical analysis

Data were analysed within and between locations (MDV and LMP) and gender (male and females) and in relation to presence of plastic (fibres and fragments) in the GIT using Student t-test and ANOVA as previously described (Mancia et al., 2020). Differences between gonadal stage at two sites MDV and LMP in males were analysed using Student t-test with Welch's correction. Spearman correlation was used to identify significant associations ($p > 0.05$) between biometric data.

Results and discussion

Morphometric analysis

Samples were analysed within location (1: MDV; 2: LMP), by gender (3: M; and 4: F), as a whole (5: MDV + LMP) and in relation to high or low plastic load (6: plastic).

In the analysis 1, MDV samples (29 F and 21 M) were used. Females VSI was higher (12%), probably in relation to the liver. HSI is in fact significantly higher as well (12%). A slightly smaller CSI observed in females vs males was probably due to the cyclical effect of reproduction on females muscle mass. In fact, it is known that when the females reach the maturity stage of maturing/mature/spawner, and our MDV samples were mainly at the stage "maturing/mature", their overall body condition appears visibly suffering due to the reproductive investment that, during eggs production, uses the body muscle mass as energy reserves (Mion et al., 2018).

For analysis 2 were used LMP samples (13 F vs 29 M). Similar to the MDV samples, in the LMP females vs males comparisons, VSI was higher (23%) and CSI was slightly smaller (− 2%) in females. Both males and females were in advanced gonadal maturity (stage 5 "mature", and 6 "spawner/spent") with a slight forward shift in favour of males (Table 2, A).

In analysis 3 all males of both fishing areas were analysed (50 M). Specimens sampled in the two locations were very different in weight and size. Specifically, MDV males specimens had shorter total length and lower weight, with a smaller CF, smaller organs, and a reduced gonadal maturity than males (i.e. in the stage of maturing and mature) of LMP found in the stage of spawner/spent. The correlation analysis of maturity (gonadal stage) and biometric data in all M sample set (n = 50) has shown a statistically significant association with VSI and HSI (VSI, rs (50) = - 0.685, p = 0.001; HSI, rs (50) = - 0.461, p = 0.001). To avoid a bias in the data related to the higher prevalence of lower maturity in MDV vs LMP site, we analysed a subgroup of individuals M from both MDV and LMP, with a weight between 60 g and 90 g and the same CF (12 M in MDV vs 11 M in LMP). We obtained two groups with larger specimens in LMP, and in the MDV vs LMP analysis, we observed that the carcass is heavier in LMP (− 4%, CSI). However, the viscera weighted more in males from MDV, a difference that was not linked to gonadal maturity (− 26%), but rather to the weight of liver (53%), spleen (51%) and GIT (23%).

For the analysis 4 all females were used (42 F) and, similarly to male samples, difference between MDV and LMP were observed: MDV specimens had shorter length and a minor weight, with a smaller CF, minor weight of the viscera, at an earlier gonadic stage (Table 2, A). However, there was no significant association of maturity and VSI/HSI, and maturity as observed in the 3 - all males - study ($p < 0.05$).

The analysis 5, using all the available samples (M and F from both locations MDV + LMP), highlighted the difference among the specimens in the two locations, including their maturity (Table 2, B). Females in MDV were smaller (in length and weight) than males, and the total weights and organs weight indexes reflect this difference. These results suggest that, overall, the presence of larger specimens (TL > 300 mm) in the LMP sample could be linked to shallower water that characterise this fishing ground, as well as other correlated oceanographic factors such as higher temperature and salinity of water masses, other than food availability (mainly anchovies and sardines) (Rumolo et al., 2017).

However, morphometric data from the males in MDV may show complications: here, females and males are almost at the same gonadal maturity, and females are expected to have a bigger liver and a smaller GIT than males (which is what we observed in LMP). Instead, we see MDV males with a small GIT and a big liver. A summary of the morphometric results is in Table 2, A and B (see details in SI-2). Furthermore, exposure to contaminants may impact the physiology of fish on many levels and could impact on the reproductive states of the fish populations. Plastic products may be one of the driven causes.

In analysis n. 6 (plastic load), 30 samples (15 from MDV and 15 from LMP, see SI-2) of the 53 analysed for GIT plastic (fibres and fragments) contents were divided in 2 groups: the high exposure group (H) with a total number of fibres + fragments > 90 and the low exposure group (L) with a total number of fibres + fragments < 90. Three specimens from LMP (TT61, TT62 and TT75 with a BW of 506 g, 267 g and 112 g respectively) were removed from the analysis to keep the sample set more homogeneous (BW < 100 g). The H group presents 40% more plastics in the GIT compared to the L group. In the H group, there were n = 8 samples (3 MDV, 2 F and 1 M; 5 LMP all M); in the L group there were n = 18 samples (11 MDV, 4 F and 7 M; 7 LMP all M). Animals from the H group have a bigger spleen (14%). Spleen is a lymphoid organ, and its production of lymphocytes is crucial to the activation of the humoral and cellular immune response against pathogens or foreign bodies, such as plastic objects (Mancia et al., 2020).

A

MDV vs LMP	CF	CSI	VSI	SSI	HSI	GISI	MAT
Females	-5%	-2%	29%	14%	54%	15%	-14%
Males	-5%	-3%	42%	17%	61%	4%	-23%
ALL	-4%	-3%	39%	14%	61%	10%	-20%
F vs M	CF	CSI	VSI	SSI	HSI	GISI	MAT
LMP	1%	-2%	23%	5%	18%	-4%	-11%
MDV	1%	-1%	12%	2%	12%	6%	-2%

B

F vs M	HSI	GISI	MAT
LMP	18%	-4%	-11%
MDV	12%	6%	-2%

Table 2) Percentage of statistically significant changes in the morphometric measurement of MDV samples compared to LMP samples (A) and of F vs M (A and B).

In order to remove bias linked to the unbalance gender specimens in the H vs L group analysis, we removed the 6 females (H group, n = 6: 1 MDV, 5 LMP; L group, n = 14 samples (7 MDV; 7 LMP). In this comparison, in addition to a significant increase of the spleen in the H group samples, we also see the increase of the VSI (26%) (see SI-2).

Plastics isolation and composition

The GIT of 53 specimens (25 from MDV and 28 from LMP) were selected to evaluate the occurrence of plastic particles, their quantity and type. The plastic debris detected by visual inspection was classified as MP fibres, MP fragments or MaP according to the classification suggested by Hartmann et al. (2019). Moreover, the MP fibres characterised by a regular diameter along the entire length and with sharp ends were referred to as MP filaments (Fig. 1A) to distinguish them from textile microfibres that could be composed of natural or semi-synthetic materials. As MP fragments were considered that debris with an irregular shape (Fig. 1B), whereas the debris with dimensions larger than 1 cm that was not degraded by the digestion process was considered MaP (Fig. 1C).

The number of ingested particles and the frequency of ingestion were employed to consider the presence of plastics (Avio et al., 2020). Microfibers characterised by irregular diameter and frayed ends were detected in all the specimen analysed from both locations, they were considered as anthropogenic particles that could cause adverse effects in the organisms, even though their basic composition may be natural (e.g. cellulose), because they underwent further processes such as dyeing (Collard et al., 2018).

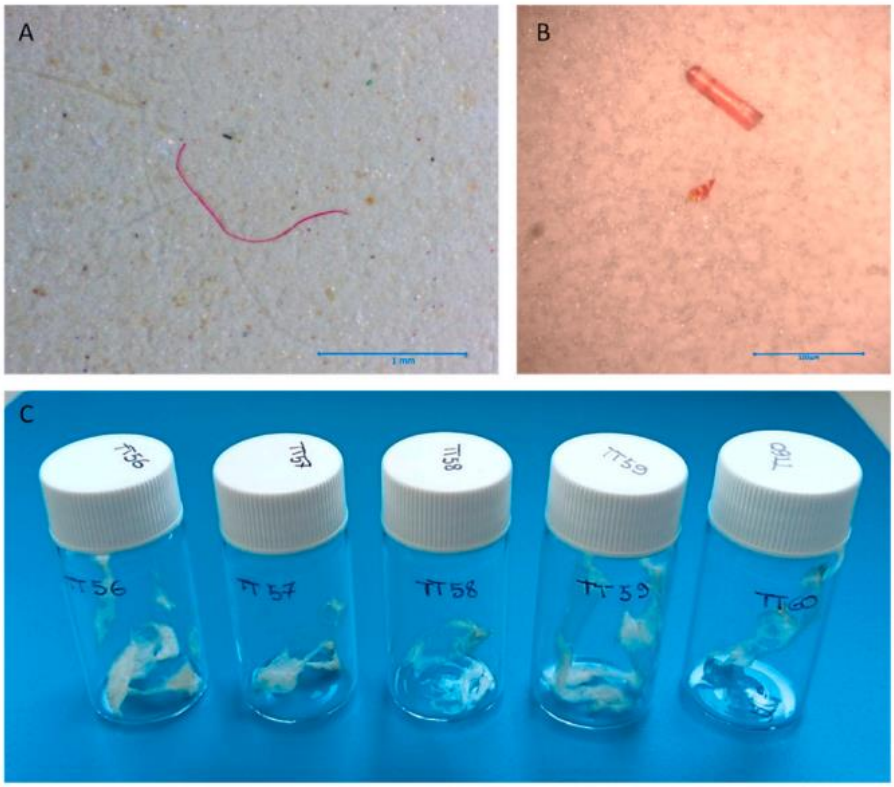

Figure 1) Microscope images of plastic particles found in different samples. A) MP filament; B) MP fragments; C) Image of undigested MaP from 5 different samples.

Table 3
Number of Microplastics (MP) composed by filaments and fragments, Macroplastics (MaP), average ingested by individual fish and frequency of ingestion.

Catch area	N. specimens	Total number MP	Filaments	Fragments	Average/fish	Frequency of ingestion
GSA 16 MDV	25	99	71	28	3.96	84%
GSA 13 LMP	28	236	108	128	8.43	96.4%
Catch area	**N. specimens**	**Total number MaP**	**Filaments**	**Fragments**	**Average/fish**	**Frequency of ingestion**
GSA 16 MDV	25	0	–	0	0	0%
GSA 13 LMP	28	52	–	52	1.86*	17.8%

*MaP were found in 5 specimen.

Table 3) Number of Microplastics (MP) composed by filaments and fragments, Macroplastics (MaP), average ingested by individual fish and frequency of ingestion.

MPs, as filaments and fragments, were found in 90.6% of all the specimens; in particular, MP were present in 84% of the fish from MDV and in 96.4% from LMP. Filaments were found in 84.9% of the specimen, in 84% of those from MDV and in 85.7% from LMP; fragments were detected in 73.6% of all fish analysed, with higher frequency in Lampedusa (82.1%) compared to MDV (64.0%). (Table 3). Filaments, most of which were dark coloured (black and blue), were characterised by different length and diameter, the latter from 10 to 30 µm. As regards MP fragments, most of them (about 80%) had dimensions in the range from 10 to 20 µm, about 15% were in the range from 20 to 50 µm, while 5% had the maximum dimension lower than 10 µm. The predominant colours were still black and blue, as for filaments, but percentages up to 15% were represented by green, white, and red fragments (Fig. 2C and D).

The presence of additives in the fibres, such as pigments, or the adhesion of organic residues made the determination of the fibres composition by Raman spectroscopy very difficult most of the time. Due to the partial identification of fibres constituents that can induce a bias, we limited the composition data only to MP filaments and MP fragments. MP fragments were composed of polystyrene, polyethylene and polypropylene, whereas MP filaments were identified mostly as nylon 6, acrylic and polyester (Fig. 2A and B).

MaP were found exclusively in the specimen collected in LMP (Fig. 1C); in particular, in five fish (TT56 – TT60) belonging to a subset (TT51 – TT60, Table 1) characterised by a higher body weight and longer total length. The MaP were found in large quantities in each fish, with a total number of 52 pieces, with average dimensions of 3 × 1 cm. The spectra obtained were similar for all the samples which, after comparison with library spectra and literature data, resulted to be composed most probably of oxidised PE or PP (see SI-1, Fig. S2).

In each spectrum, the bands caused by CH2 stretching, bending and rocking (in the 3000–2800 cm− 1 , 1470–1370 cm^{-1} and 730–715 cm^{-1} regions, respectively) characteristic of PE and PP are always present (Jung et al., 2018), moreover, the appearance of an intense band at 1715 cm^{-1} is an indication of the presence of carbonyl groups which are found in oxidised PE (Oelichmann, 1989; Rocha et al., 2010; Gardette et al., 2013). Plastic debris usually undergo photodegradation which involves modifications of the polymer matrix (such as oxidation with the formation of carbonyl groups, chain scission, radical recombination and cross-linking) and morphological alterations (Rowenczyk et al., 2020). The large quantity of MaP found exclusively in the GIT of the larger specimen subset can be an indication that bigger animals that usually feed on small fish may indeed mistake plastic debris for prey.

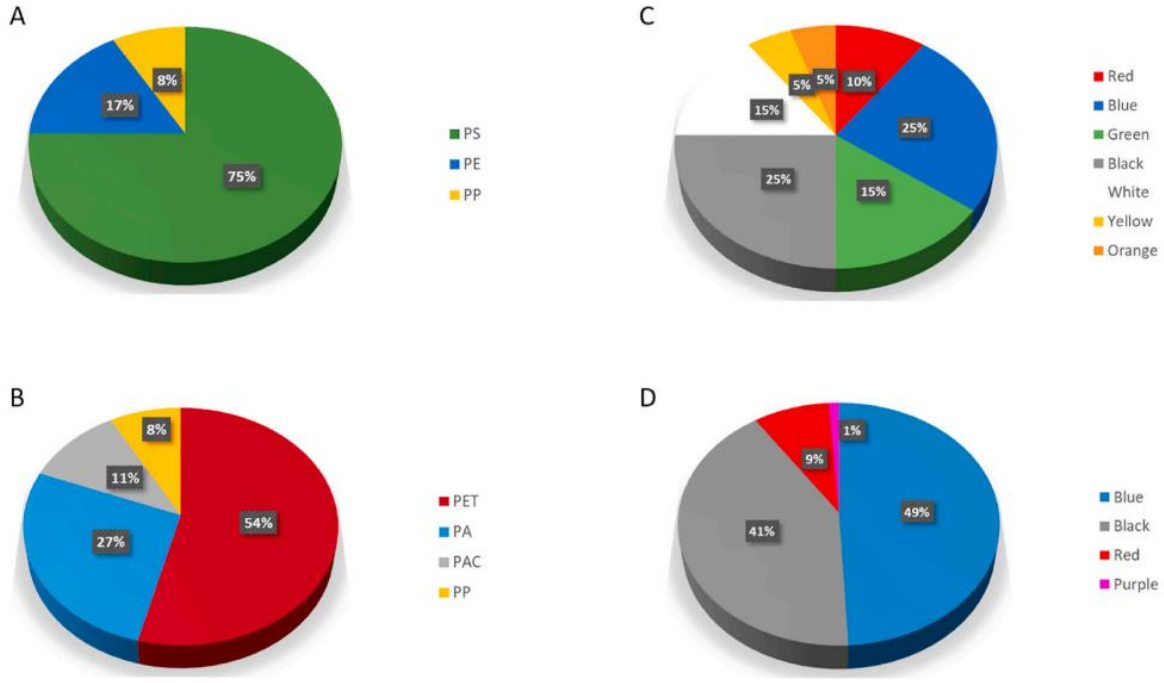

Figure 2) Plastic debris composition A, B, and colors C, D. A) MP fragments composition; B) MP filaments composition; C) MP fragments colors; D) MP filaments colors. PS polystyrene, PE polyethylene, PP polypropylene, PET polyethylene terephthalate, PA polyamide, PAC polyacrylate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

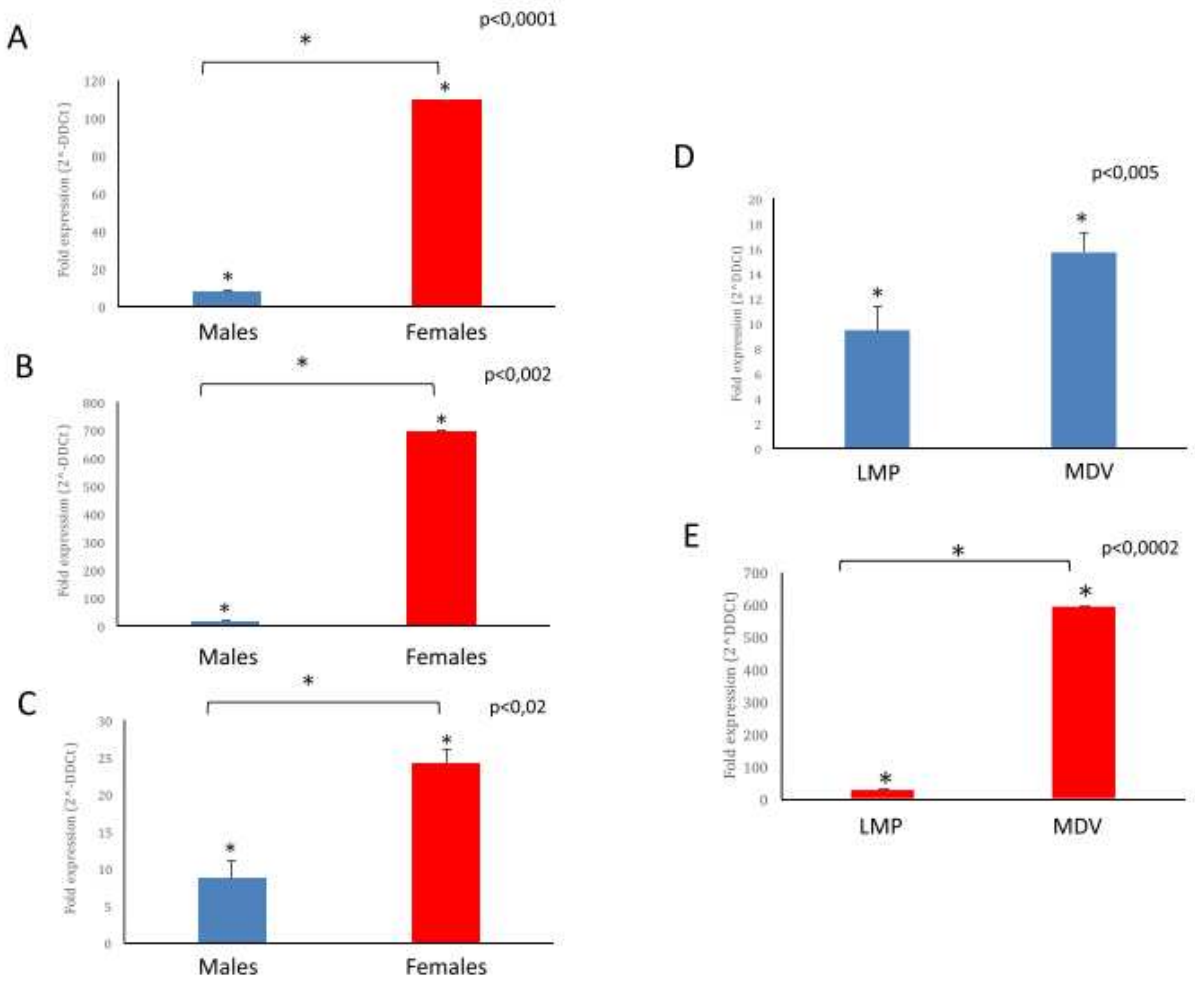

Figure 3) Expression of vitellogenin genes in T. trachurus liver samples. A) Males and females samples from both locations; B) males and females from MDV; C) males and females from LMP; D) males from LMP and MDV; E) females from LMP and MDV.

Gene expression

Almost all plastic products have been found to leach endocrine-disrupting chemicals, EDCs (Yang et al., 2011). EDCs have the potential to alter fish reproduction at various levels of organisation. The aim of this part of the study was to assess the impact of a natural environment with heavily anthropogenic influence on the physiological processes involved in reproduction in T. trachurus using the vitellogenin (VTG) gene, a specific biomarker. In fact, the expression of the egg yolk protein precursor VTG is a well-established indicator of oestrogen-mediated endocrine disruption in males (Moncaut et al., 2003; Tolussi et al., 2018). Here, hepatic VTG gene expression was evaluated in specimens of TT from both genders, as an indicator of xenoestrogen exposure that could be caused or exacerbated by the presence of plastic.

RNA from the liver of 44 specimens was retrotranscribed and used as template in the real time qPCR of VTG. There were 19 MDV and 25 LMP samples; three of these were removed from further analysis (TT14, TT61, TT74) because the amplification data of the housekeeping control were poor (Filby and Tyler, 2007). Samples were analysed within and between location and gender: A) all samples, M + F from MDV + LMP; B)

M + F from MDV; C) M + F from LMP; D) only M from MDV + LMP; E) only F from MDV + LMP). There were no samples without plastic detection, although some of the samples had higher loads (see SI-2).

A) As expected, when pooling all the samples and comparing the VTG expression of all females (16 F, 8 MDV, 8 LMP) vs all males (10 M, 5 MDV, 5 LMP) from both locations, TT female's VTG expression was about 100 times more than the male's ($p < 0,0001$) (Fig. 3A).

ID	Gender	TL (cm)	BW (g)	CF	CW (g)	CSI	VW (g)	VSI	SPL W (g)	SSI	LIV W (g)	HSI	GIT W (g)	GITI	Mat
TT52	F	36	414.60	0.889	374.38	0.903	40.22	0.097	0.48	0.001	4.56	0.011	15.42	0.037	4
TT54	F	37.5	450.90	0.855	410.20	0.910	40.70	0.090	0.56	0.001	4.09	0.009	23.68	0.053	4
TT57	F	38	479.97	0.875	428.22	0.892	51.75	0.108	0.31	0.001	5.61	0.012	18.35	0.038	5
TT61	F	39	506.77	0.854	457.35	0.902	49.42	0.098	0.57	0.001	6.15	0.012	16.16	0.032	4
TT51	M	38	466.70	0.851	423.16	0.907	43.54	0.093	1.30	0.003	4.00	0.009	23.19	0.050	4
TT56	M	36	429.60	0.921	386.07	0.899	43.53	0.101	0.50	0.001	4.66	0.011	26.94	0.063	4
TT59	M	37	427.24	0.843	390.48	0.914	36.76	0.086	0.44	0.001	3.69	0.009	21.88	0.051	4

Table 4) Morphometric data of the largest specimens collected in LMP.

Both females and males VTG expression were compared to control (males not expressing VTG, details in methods). It was interesting to notice that there is expression of VTG in males and it is statistically significant ($p < 0,0001$) (Fig. 3). Another interesting detail was that in the female group there is one specimen (TT54) that does not express VTG. The specimen was in gonadal stage 4 (maturing) so production of VTG was expected. This is one of the large TT from LMP, with all morphometric data similar to other big size females (F: TT52, TT54, TT57, TT61). However, the GIT W and the GITI values are comparable to those of the largest males, which are also males that do not express VTG and are in the control group (M: TT51, TT56, TT59) (Table 4).

B) In MDV (5 M, 8 F) the expression of VTG of both males and females is statistically significant ($p < 0,002$). The VTG expression in males is 36 times less than in females, but nevertheless, there is VTG expression in males (Fig. 3B). C) In LMP (5 M, 7 F) there is a 16-fold difference in VTG expression between males and females ($p < 0,02$) (Fig. 3C). Removing the specimen TT54 increase the difference between male and female but not the male VTG expression result. D) VTG is significantly expressed in males from both locations (5 LMP, 5 MDV, $p < 0,005$) and although in MDV specimens the expression is almost double than in LMP specimens, this difference is not significant (Fig. 3D). E) VTG is significantly expressed in females from both locations (8 LMP, 7 MDV, $p < 0,0002$) compared to control, as expected. The expression of VTG in MDV females is 22 times that of LMP females, even though there is no difference in maturity stage among specimens of the two groups (Fig. 3E).

To summarise, even though it is clear that the vitellogenin is highly expressed in TT females as expected, there is also a significant expression of the VTG gene in 60% of the TT males analysed, from both MDV and LMP. Moreover, females in LMP showed a lower expression of vitellogenin than in MDV (with one female sample, TT54, not expressing VTG at all).

The endocrine disruption represented by the alteration of VTG expression in TT specimens observed in this work can be caused by microplastic ingestion, as well as by the interactions between the marine organisms and the wide variety of endocrine-disrupting chemicals possibly present in seawater.

Conclusions

The southern region of the central Mediterranean Sea is strongly affected by the presence of anthropogenic debris, since microplastics, in the form of fibres and fragments, were detected in the GIT of all the specimens analysed. If textile fibres are excluded, and only filaments and fragments made of synthetic polymers are considered, the ingestion frequency is still high, 84% south of Mazara del Vallo and 96% south of Lampedusa. On the other hand, macroplastics were detected only in the bigger specimen collected in Lampedusa, this can be explained by the fact that only the larger fish feed also on teleosts, besides euphausiid crustaceans, and larger plastic debris could resemble fish prey.

To the best of our knowledge, this study provides the first report on anomalies in the production of vitellogenin in both males and females of T. trachurus in the Mediterranean Sea that could be caused by microplastic ingestion, besides endocrine-disrupting chemicals present in seawater. 60% of the male specimen from both locations showed a significant expression of vitellogenin, whereas females specimen showed a high expression, as expected, in south of Mazara del Vallo, but a low expression (in one specimen no expression at all) in south of Lampedusa. From this first examination, it can be hypothesised that microplastics and the potential sorbed chemicals could be a major cause for endocrine disruption leading to variations in physiological processes, in this case oogenesis process and reproduction, of T. trachurus. Overall, our study suggests that the ingestion of plastic debris in the environment may alter endocrine system function in economically important fish species and warrants further research.

Credit author statement

Tatiana Chenet, Writing, Investigation, Data curation; Annalaura Mancia, Writing, Investigation, Formal analysis; Gioacchino Bono, Fabio Falsone, Danilo Scannella, Resources, Data curation; Carmela Vaccaro, Formal analysis; Andrea Baldi, Investigation; Martina Catani, Investigation; Alberto Cavazzini, Funding acquisition; Luisa Pasti, Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

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Conclusions

The main contribution of this work of thesis to the scientific panorama is, hopefully, to promote an interdisciplinar mindset to favor sustainability, in each of its meaning (environmental-social-economic), when tackling complex contexts of interrelations between the environment and human. The concept that is highlighted regards the effective action toward endangered areas, consequences of anthropic activities and pollution, with a Circular Tutelage approach. Circular Tutelage consists in the idea of deploying critical measures of tutelage for threatened environments, with the purpose to manifest the principles of efficiency and circularity practices, among which: “reduce, reuse, and recycle” for example, aiming for both economic and social wellbeing with mutual benefits to the environment. Especially, given the number of challenges that humanity must deal with in the mid-short term, such as the global, and equal, food security, the ecological transition from a paradigm based on fossils toward one with carbon neutral / negative technologies, pollution, and, more broadly, the goals within the UN 2030 Agenda.

The Goro’s lagoon case study has been suitable to develop a holistic approach regarding both sustainability and wellbeing aspects, since the lagoon’s ecosystem quality and its services are more and more depleted by a poor handling of “harmful algal blooms” waste biomasses, bulky piles of waste seashells, and pollution.

The research promotes the reuse of these waste materials directly, or as raw resources for further productions. The improvements resulting from this valorisation mutually concern multiple sectors: environmental, economic, and social: for example, the environmental gains are correlated to the preservation, restoration, and improvement of the ecosystems’ services, the enhancement in the circularity of resources, lesser use of landfills, the economic incomes from the restored mussel’s productivity, and from the cohesive exploitation of these secondary raw materials. Along with the possibilities for innovative markets to rise, the improvement for the already existing activities, and more tourism; eventually leading to a higher degree of employment, a correct use of the available resources, and the general valorisation of the area.

The stress sources toward marine life and ecosystems coming from anthropogenic pollution have been discussed through the article reported in the third section, regarding bioaccumulation of plastic pollutant and the correlated endocrine damages as an exemplification of the complex drawbacks on biological processes in environments affected by xenobiotic substances. Problems related to pollution and stress toward the organisms were analyzed in order to highlight how biological stress can manifest within a living being when plastic litter is ingested and how the genic expression changes; also given the “adsorbed pollutants” side issue of plastic ingestion, which is an additional dangerous route for pollutants to interact with the biosphere and the trophic network.

The tutelage was conceptualized on the case study of the Goro’s lagoon, through the means of the evaluation of various exploitative and circular processes, tailored on the waste algal biomasses peculiarities and the local socio-economic context, in addition to an empiric pollutant monitoring, which includes the determination of various pollutant classes, fundamental to better discriminate the biomasses’ exploitation routes. The purpose is to halt the ecosystem’s quality depletion by removing the stress sources, excessive algal biomass, thereby restoring and enhancing the general productivity of local aquaculture activities.

Thus, a cost benefit analysis was performed, with the production of biodiesel as the most remunerative scenario and a biorefinery multiproduct as the second choice; however, the foresight analyses suggest that

the pollutant distribution could rewrite the CBA's outputs, also the intrinsic flexibility of the Biorefinery multiproduct scenario may be more suitable to handle exogenous and unpredictable variables such as wars, bio-markets consistency (in terms of times and volumes), and new policies discouraging common fuels. Also, social inclusion is a fundamental detail, emerged from the foresight, for a successful project that aims for the enhancement and restoration of the common good: the quality of the lagoon. Hence, strong communicative measures are to be adopted in order to achieve true social sustainability by the means of acceptance and trust. Eventually, the pollution monitoring seems to confirm the CBA's output, given the number of metals and organic pollutants within, even bioaccumulated, in algal biomass. Seaweeds, then, could be suitable for the production of bioethanol, biogas, soil conditioner, fodder, diet integrators, cosmetics, agar, and metals if recovered from the exhausted biomass.

Along with seaweed biomasses, the area is also affected by bulky wastes of seashells, which are by products and wastes of said aquaculture activities. Therefore, in the second section, seashells were characterized and the relation with heavy metals, among which Cadmium, was determined in order to discriminate effective usages for this waste material, introducing circularity of resources and improving the sustainability of aquaculture activities. Indeed, a Cadmium bioaccumulation was detected in mussels, which has been investigated through the determination of the interactions between the heavy metal and the biogenic carbonate constituent of the seashell. Eventually, it was determined that waste seashells can be suitable for the use as bio-remediator, biosensor, and bio-filter for cadmium (and possibly other heavy metals) given the high affinity of seashells for Cadmium, especially the darker ones. The use as soil conditioner could be also a valuable route against landfilling.

In general, given the local inhabitants' interest and protective instinct towards the lagoon basin, and the economic volumes that it provides, social aspects are fundamental to avoid NIMBY syndrome and other forms of opposition toward innovative and progressive, but "unknown" projects. Then, one of the main stakeholder, the locals, should benefit from the ex-ante pollution monitoring activities and from the participation in the circular tutelage project. Also, the decision making process for the biomass handling itself could benefit from the simplification of the variables into play, and the optimization of the possibilities' array.

In fact, in order to make the valorisation of said waste biomasses concrete, a Decision-Making process (DM) must occur. The DM process implies the agreement between stakeholders and decision makers, especially when broad-impact operations, involving several entities, public resources, and the territory, are planned. Therefore, making use of sea wastes as resource in industrial processes is indeed a complex matter, considering the legal aspects, the characters in play belonging both to private and public sector, the waste collection and products distribution, and also upon the plethora of exploitation possibilities. Considering this complex framework, highlighting the best choices among the main exploitation possibilities, with a holistic perspective, could be decisive for the simplification of the whole DM process, leading to sustainable and efficient implementation of tutelage measures.

In conclusion, the circular tutelage is a form to implement the sustainability message when facing complex situations in endangered areas. In other words, the adaptation to the environmental fabric that must be recovered and enhanced for achieving the best possible results with the favor and participation of the local population, paving the way for the valorization of the area, wealth generation, higher employment degree, and fostering bio-markets to rise, with the overall acceptance and support from the local population. Given the complexity and interdisciplinarity of our world it is fundamental to foster dialogue and participation

among disciplines, allowing for more understanding, cohesion, and therefore effective actions toward global, and human, wellbeing.

Appendix 1) supplementary information for chapter 1, section 1: "Exploitation of Waste Algal biomass in Northern Italy. A Cost Benefit Analysis"

Supplementary material

Scopus Research by Year	2012	2021	2022
"Algae and biodiesel"	794	4,114	4,226
"Algae and bioethanol"	112	765	793
"Algae and biogas"	110	1,112	1,164
"Algae and biohydrogen"	66	375	393
"Algae and biobutanol"	9	76	77

Table SM1) Publications related to algae as feed for biofuels based on Scopus.

Additional references for Ulva exploitations

(Park *et al.*, 2009b; Cruz-Suárez *et al.*, 2010; Suganya and Renganathan, 2012; Kanno *et al.*, 2014; Yaich *et al.*, 2017; el Harchi, Fakihi Kachkach and el Mtili, 2018; Koçer and Özçimen, 2018b; Tran *et al.*, 2018; Khoo *et al.*, 2019)

Landfill building costs and demolition expenditure

It is to be noted that landfill and composter costs are not completely belonging to the real situation of case zero, since those structures are not built for algal biomass handling purpose only but needed to "solve" the problem; that is also the reason why the running costs are not reported, while the capital and fixed costs, instead, are.

LANDFILL, Typical Construction Costs		
Task	Minimum	Maximum
Clear and Grub	397	1190
Site Survey	1983	3173
Excavation	39659	130875
Perimeter Berm	3966	6345
Clay Liner	12691	64248
Geomembrane	9518	13881
Geocomposite	13088	17450
Granular Soil	19036	25382
Leachate System	3173	40452
QA/QC	29744	39659
TOTAL	133255	306962

Table SM2) Typical costs Ranges for a Landfill Construction; €/Hectare

LANDFILL, Closure Care and Maintenance Costs		
Task	Minimum	Maximum
Final grades survey	1190	2380
Gas management layer	9518	12691
Compacted caly cap	10311	20226
Geomembrane cap	7139	9122
Geocomposite	13088	17450
Cover and vegetative soil	5156	10311
Seed, much, fertilize	397	793
Gas management system	11501	13881
Run-off control system	1983	2776
QA/QC	29744	39659
Total	90026	129289

Table SM3) Typical costs Ranges for a Landfill Closure Care and Maintenance Costs; €/Hectare

LANDFILL, Post-Closure Maintenance Costs		
Task	Minimum	Maximum
Security and fencing	35693	71387
Final cap and cover	3569	6742
Leachate mechanicals	10708	14277
Landfill gas mechanicals	5354	6782
Wells/probes	237955	356933
Environmental monitoring	5354	6841
Total	25382	34900

Table SM4) Typical costs Ranges for a Landfill Post-Closure Maintenance Costs; €/Hectare

Data are from (Homes & Communities Agency, 2015), and below there are reported the demolitions costs for industrial plants with ranges to better intercept the complexity, yielding a proper evaluation, or at least an idea, for the demolition costs per square meter.

Demolition Costs for industrial Plants					
		industrial			
		PHASE I		PHASE II	
Removal of redundant services	Fixed: tot € per site	35,40	112,10	112,10	188,80
Site clearance	Variable: tot € per m^2	17,70	53,10	53,10	88,50
Demolitions	Variable: tot € per m^2	37,76	56,05	56,05	74,34
Site investigation	Fixed: tot € per site	47,20	177,00	177,00	306,80
Fees	Fixed: tot € per site	224,20	507,40	507,40	790,60

Table SM5) Costs Ranges guideline for demolition of industrial sites with various complexities.

Appendix 2) supplementary information for chapter 1, section 2: "Explorative Investigations for Waste Recycling Suitability"

METHODICS OF ANALYSIS

Acidi Aloacetici e Dalapon nel Biota (EPA 552,2)

METILAZIONE: Cl-CH2-C(=O)-OH + MeOH + H+→Cl-CH2-C(=O)-Ome + H2O

1. 1 g campione (umido) in vial da 60 ml
2. + 40 ml H_2O
3. +20 uL di surrogato acido 2,3-Dibromopropionico 10ug/mL in MTBE
4. +2mL H_2SO_4 conc (pH <0.5)
5. agitare, 50°C x 1h (criostato)
6. lasciare raffreddare +18g Na_2SO_4 e agitare fino a scioglierlo
7. + 4mL MTBE, agito vigorosamente 3', riposa 5', lasciare separare fase acquosa/organica,
8. centrifugo fase superiore e schiuma, trasferire 3 ml dell'estratto organico in vial di vetro da 20 ml
9. +3mL H_2SO_4 10% in MeOH (5mL H_2SO_4 conc in 50mL di MeOH), 50°C x 2h (criostato)
10. +7mL Na_2SO_4 150g/L, agito, separo la fase organica
11. rimuovere la fase acquosa (sotto)
12. +1mL $NaHCO_3$ sat, agito, elimino CO_2 (vigorosamente per almeno 4 volte)
13. trasferire 1mL dell'estratto (*forse in mtbe*) in vial per GC (conservare gli estratti a -10°C per massimo 15-21 gg)
14. Aggiungere 10uL di IS a 25 ug/mL

Composti	CAS	ug/mL	DB-5	DB-1701
ACIDI ALOACETICI [Restek 31646 1mL in MTBE]				
Ac. Monocloroacetico (MCAA)	79-11-8	600	1	1
Ac. Monobromoacetico (MBAA)	79-08-3	400	2	2
Ac. Dicloroacetico (DCAA)	79-43-6	600	3	4
Ac. Tricloroacetico (TCAA)	76-03-9	200	5	5
Ac. Bromocloroacetico (BCAA)	5589-96-8	400	6	7
Ac. Dibromoacetico (DBAA)	631-64-1	200	8	9
Ac. Bromodicloroacetico (BDCAA)	71133-14-7	400	9	8
Ac Clorodibromoacetico (CDBAA)	5278-95-5	1000	10	10
Ac. Tribromoacetico (TBAA)	75-96-7	2000	12	12
DALAPON [Restek 32432 in ACN]				
Dalapon	75-98-0	1000	4	3
STANDARD INTERNO [Restek 30429 in MeOH]				
1,2,3 Tricloro propano (IS)	96-18-4	2000	7	6
STANDARD SURROGATO [Restek 31655 in MTBE]				
Acido 2,3-Dibromopropionico (SURR)	1729-67-5	1000	11	11

ANALISI GC-ECD

- Colonna: DB-5 (30m, 0.25mm, 0.25µm) → Colonna di misura
- Colonna: DB-1701 (30m, 0.25mm, 0.25µm) → Colonna di conferma
- Carrier gas: He, 1 mL/min
- Iniettore: 200°C, Splitless
- Volume di iniezione: 2 µl
- Programma di temperatura

Rampa (°C / min)	Temperatura (°C)	"Hold time" (min)
-	35	10
5	75	15
5	100	5
5	135	2

- Detector µ-ECD: 300°C; make-up gas N_2, 30 mL/min

	Ordine Eluizione	
	DB-5	**DB-1701**
1	MCAA	MCAA
2	MBAA	MBAA
3	DCAA	Dalapon
4	Dalapon	DCAA
5	TCAA	TCAA
6	BCAA	IS
7	IS	BCAA
8	DBAA	BDCAA
9	BDCAA	DBAA
10	CDBAA	CDBAA
11	SURR	SURR
12	TBAA	TBAA

Alofenoli nel Biota (Metodo EPA 1653)

PROCEDIMENTO:

- Pesare 1 g di campione non liofilizzato [in vial da 40 mL];
- Aggiungo 20 mL di MeOH → Estrazione: bagno ultrasuoni per 15 minuti;
- Centrifugo per 5 minuti 3000rpm usando falcon da 15 mL;
- Mettere il surnatante in un matraccio da 250 mL con ancoretta magnetica;
- Aggiungo 180 mL di H_2O;
- Aggiungo 1mL di 2,4 Dibromofenolo (prelevo dalla soluzione 12.5 ug/mL in MeOH).
- Aggiungo poi 5 mL di K_2CO_3 (600g/L) [ne preparo 50 ml -> pesata 30 g] e 5 mL di Anidride Acetica e derivatizzo per 5 minuti;
- Aggiungo 10 mL di ESANO e agito per 5 minuti;
- Prelevo la fase organica e la concentro a 1 mL con N_2;
- Aggiungo 20 uL di 2,5-Dibromotoluene dalla soluzione **25 ug/mL** in ESANO

GC-MS:

- Colonna DB-5MS
- Carrier gas (He) flow rate: 1.2mL/min
- Initial temperature: 40°C x 2min
- Temperature program:40°-100°@40°/min, 100°x0.5min, 100°-140°@2°/min, 140°-340°@30°/min
- Injector temperature: 200°C, Splitless
- **iniezione tripla**

Determino composti fenolici clorati come derivati acetati dopo acetilizzazione in sito.

Composti	CAS	Primary Ion	Secondary Ions	
ALOFENOLI [31617]				
2,4,6 Triclorofenolo	88-06-2	196	198	97
2,4 Diclorofenolo	120-83-2	162	164	63
4-Cloro-3metilfenolo	59-50-7	107	142	77
Pentaclorofenolo	87-86-5	266	268	264
STANDARD INTERNO [A19418]				
2,5-Dibromotoluene	615-59-8	250	169	171
STANDARD SURROGATO [CLM-661-S-0+B23394]				
2,4-Dibromofenolo	615-58-7	252	250	254

Alometani e Aloacetonitrili nel Biota (EPA 5021 + EPA 8260)

Preparazione:

- 5 g campione sminuzzato
- +10mL H_2O satura di NaCl
- +10uL Standard interno 20mg/L + +10uL surrogato 20mg/L
- 70°Cper 30min
- inj 1mL

Cromatografia

- Colonna HP-VOC
- Carrier gas (He) flow rate: 1 mL/min
- Initial temperature: 35°C, hold for 2 minutes
- Temperature program: 35°C to 80°C @ 8°C/min; 80°C to 150°C @ 15°C/min; 150°C to 230°C @ 6°C/min, final hold of 10 min
- Final temperature: 230°C, hold until all expected compounds have eluted
- Injector temperature: 250°C
- Transfer line temperature: 280°C

Matrix-modifying solution - Add 180 g of ACS-grade sodium chloride (NaCl) to 500 mL of reagent water.

Composti	CAS	Primary Ion	Secondary Ions	IS
ALOMETANI [Restek 30225,30259,30211 2000ppm in MeOH]				
Bromoclorometano	74-97-5	128	49, 130	
Bromodiclorometano	75-27-4	83	85, 127	1,4-Diclorobenzene-d4
Bromoformio	75-25-2	173	175, 254	1,4-Diclorobenzene-d4
Carbonio Tetracloruro	56-23-5	117	119	1,4-Diclorobenzene-d4
Clorodibromometano	124-48-1	129	208, 206	1,4-Diclorobenzene-d4
Cloroformio	67-66-3	83	85	
ALOACETONITRILI [VWR: A16994.14, A10612.14, A10565.18, tutti con purezza >98%)				
Dibromoacetonitrile	3252-43-5	120	118, 199	
Dicloroacetonitrile	3018-12-0	74	82, 76	
Tricloroacetonitrile	545-06-2	108	110, 73	
STANDARD INTERNO [2500 µg/mL in Metanolo. Restek 30241]				
Fluorobenzene	462-06-6	96	77	
Clorobenzene-d5	3114-55-4	117	82, 52	
1,4-Diclorobenzene-d4	3885-82-1	152	115, 150	
STANDARD SURROGATO [2500 µg/mL in Metanolo. Restek 30004]				
Toluene-d8	2073-26-5	98		Clorobenzene-d5
4-Bromofluorobenzene	460-00-4	95	174, 176	Clorobenzene-d5
1,2-Dicloroetano-d4	17060-07-0	102		

ANALISI IPA TRAMITE HPLC-FLUORESCENZA (EPA 8310)

- Tipo di colonna: colonne per analisi IPA, ad esempio HC-ODS Sil-X (250mm x 2.6mm, 5um), oppure Agilent ZORBAX Eclipse PAH, o altre simili.
- Fase mobile: Eluizione isocratica per 5 min con acetonitrile/acqua 40/60, gradiente lineare 100% Acetonitrile in 25min.
- Flusso: 0.5mL/min
- Volume di iniezione: da 5 a 25uL
- Detector UV: 254nm
- Detector Fluorescenza: λ eccitazione 280nm, λ emissione > 389nm.
- I campioni e gli standard sono in Acetonitrile.

Composti cercati	**CAS**	**Tempo ritenzione (min)**	**Fattore di ritenzione (k')**	**Limiti deteczione metodo (ug/L)**	
				UV	**Fluorescenza**
Naphthalene	91-20-3	16.6	12.2	1.8	
Fluorene	86-73-7	21.2	15.8	0.21	
Phenanthrene	85-01-8	22.1	16.6		0.64
Anthracene	120-12-7	23.4	17.6		0.66
Fluoranthrene	206-44-0	24.5	18.5		0.21
Pyrene	129-00-0	25.4	19.1		0.27
Benzo(a)anthracene	56-55-3	28.5	21.6		0.013
Chrysene	218-01-9	29.3	22.2		0.15
Benzo(b)fluoranthene	205-99-2	31.6	24.0		0.018
Benzo(k)fluoranthene	207-08-9	32.9	25.1		0.017
Benzo(a)pyrene	50-32-8	33.9	25.9		0.023
Dibenzo(a,h)anthracene	53-70-3	35.7	27.4		0.030
Benzo(ghi)perylene	191-24-2	36.3	27.8		0.076
Indeno(1,2,3-cd)pyrene	193-39-5	37.4	28.7		0.043

CRM di riferimento: BCR-535 e CNS-391-50G

ESTRAZIONE

- 1 grammo campione liofilizzato in vial da 40 ml
- +20mL Acetone : diclorometano 1:1 (EPA 3550)
- Sonicare 10 minuti
- Centrifugare e separare fase organica
- estrarre altre 2 volte con 2 aggiunte 20mL
- concentrare il campione a circa 1-2 mL
- Nel caso di determinazione gascromatografica è necessario eseguire una purificazione dallo zolfo (mediante reazione con rame metallico, o tetrabutilammonio solfito o mercurio metallico, o attraverso una cromatografia di gel permeazione, ecc.) e una riduzione del carico di sostanze altobollenti che si depositerebbero nel liner dell'iniettore.
- Aggiungere 8 mL di acetonitrile e portare esattamente a 1mL [cambio di solvente]
- Filtro con filtro inorganico (glass)

ANALISI IPA NEL BIOTA TRAMITE HPLC-FLUORESCENZA

CRM di riferimento: NIST 1974c

Indicazioni: pesarne almeno 3g. Contiene 89.75% umidità.

ESTRAZIONE

- 1 g campione (omogeneizzato e liofilizzato) [3g per NIST]
- +8 g florisil (purificazione lipidi)
- +20 mL esano:CH2Cl2=40:60
- sonicare 10 min
- centrifugo per 5 min. a 2500 rpm
- estrarre altre 2 volte con 2 aggiunte 20mL
- concentrare i 60mL di campione raccolto a piccolo volume 3-5mL

SAPONIFICAZIONE

- 20 mL of 0.5 M KOH in metanolo e lascio reagire per 30min a 40°C (nel sonicatore)
- eventualmente Centrifugo
- Estrazione con 20 mL di esano/CH2Cl2 80/20 * 3 volte
- separare l'estratto, concentrarlo a piccolo volume 1-2mL e poi recuperare con 8mL ACN (volume finale 1mL).
- Filtro con filtro inorganico (glass)

Analisi di PCB e pesticidi nei sedimenti (EPA3550B + EPA3660 + EPA3620C + EPA8081 + EPA8082)

- 5 g campione liofilizzato
- + 20mL solvente (acetonePESTINORM:esano=1:1)
- +0.5mL surrogato (250ppb)
- sonicare 10 min
- il solido "più grossolano" decanta da solo; prelevare il surnatanate e centrifugarlo 5 minuti 3000 rpm, tenere da parte il surnatante
- ripetere l'estrazione del solido altre 2 volte con 2 aggiunte di 20mL
- ripetere la centrifugazione, riciclando le stesse provette per lo stesso campione
- alla fine avrò un volume totale di estratto di 60 ml
- far evaporare il solvente insufflando N2 in un bagno 30°C, senza farlo andare a secco, fino a volume finale circa 1 ml (non inferiore o perdo semivolatili)
  - trasferire 1mL di campione conc e 1mL di Hex di lavaggio in vial da 12 mL (vetro)
  - +1mL soluzione TBA solfito +2mL IPA--> shake x 1min
  - +5mL H2O Organ-free shake x 1min, stand x 5-10min
  - separo strato sopra (Hex)
  - 1) condizionare la cartuccia SPE (**1 g FLORISIL**) con 4 ml di esano, senza mandarla a secco
  - 2) caricare l'estratto (1 ml) ed eluire, senza mandarla a secco
  - **3A) SE PCB E PESTICIDI ELUITI IN UNICA FRAZIONE:** 9 ml acetone:esano 10:90
  - 4) portare a piccolo volume (1 ml), aggiumgere lo std interno e portare a volume noto (2 ml) con esano

Internal standard solution

PER PESTICIDI	1-bromo-2-nitrobenzene 100 ug/ml in acetone
PER PCB	Decaclorobifenile 200 ug/ml in acetone

Calculate response factors (RFs) for each target analyte relative to one of the internal standards.

Surrogate standards

PER PESTICIDI	Tetracloro-m-xilene 200 ug/ml in acetone
PER PCB	

The concentration for semivolatile/nonvolatile organic and pesticide analyses should be such that a 1-mL aliquot spiked into 1000 mL of an aqueous sample provides a concentration of 10 times the quantitation limit of a target analyte that is chemically similar to the surrogate, or near the mid-point of the calibration curve. Where volumes of less than 1000 mL are extracted, adjust the volume of surrogate standard proportionately.
For matrices other than water, 1 mL of surrogate standard is still the normal spiking volume.

ANALISI GC – ECD

Because of the low concentration of pesticide standards injected on a GC/ECD, column adsorption may be a problem when the GC has not been used for a day or more. Therefore, the GC column should be primed (or deactivated) by injecting a pesticide standard mixture approximately 20 times more concentrated than the midconcentration standard. Inject this standard mixture prior to beginning the initial calibration
or calibration verification.

EPA 8081 (PCB)

- Colonne: DB-5 (front uECD) +DB1701 (back uECD)
- Carrier gas (He) flow rate: 1 ml/min
- Temperature program: 140°C x 2min, 140° to 270° @2.8°C/min, 270°C x 2min
- Injector temperature: 250 °C, splitless
- injection volume: 2 ul
- make up gas (N2) flow rate 30 ml/min
- detector temperature: 320 °C

EPA 8082 (pesticidi)

- Colonne: DB-5 (front uECD) + DB1701 (back uECD)
- Carrier gas (He) flow rate: 1 ml/min
- Injector temperature: 250 °C, splitless
- injection volume: 2 ul
- detector temperature: 320 °C
- make up gas (N2) flow rate 30 ml/min
- oven: 140 °C x 2 min, 140°C to 270°C @ 2.8°C/min, 270°C x 2 min

PCB:

	BZ#	NOME	CAS NUMBER
ultra scientific RPCM-240-1 WHO/ISS PCB Mixture 10 µg/mL in isooctane (CONTIENE 32 COMPOSTI)	18		
	28	2,4,4’-trichlorobiphenyl	7012-37-5
	31	2,4’,5-trichlorobiphenyl	16606-02-3
	44		
	52	2,2’,5,5’-tetrachlorobiphenyl	35693-99-3
	77	3,3’,4,4’-tetrachlorobiphenyl	32598-13-3
	81	3,4,4’,5-tetrachlorobiphenyl	70362-50-4
	95		
	99		
	101	2,2’,4,5,5’-pentachlorobiphenyl	37680-73-2
	105	2,3,3’,4,4’-pentachlorobiphenyl	32598-14-4
	110		
	114		
	118	2,3’,4,4’,5-pentachlorobiphenyl	31508-00-6
	123		
	126	3,3’,4,4’,5-pentachlorobiphenyl	57465-28-8
	128	2,2',3,3',4,4'-Hexachlorobiphenyl	38380-07-3
	138	2,2',3,4,4',5'-Hexachlorobiphenyl	35065-28-2
	146		
	149		
	151		
	153	2,2',4,4',5,5'-Hexachlorobiphenyl	35065-27-1
	156	2,3,3',4,4',5-Hexachlorobiphenyl	38380-08-4
	157		

	167		
	169	3,3',4,4',5,5'-Hexachlorobiphenyl	32774-16-6
	170	2,2',3,3',4,4',5-Heptachlorobiphenyl	35065-30-6
	177		
	180	2,2',3,4,4',5,5'-Heptachlorobiphenyl	35065-29-3
	183		
	187		
	189		
ultra scientific RPC-107AS 100 µg/mL in isooctane	35	3,3’,4-trichlorobiphenyl	37680-69-6

Pesticidi organoclorurati:

ultra scientific PPM-5090-1 , IRSA 5090 Pesticides Mixture, 100 µg/ml in Acetone (CONTIENE 25 COMPOSTI)	**Alachlor**			
	Aldrin	C12H8Cl6		309-00-2
	Atrazine			
	2,4’-DDT	C14H9Cl5	1,1,1-Trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane	789-02-6
	4,4’-DDT	C14H9Cl5	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane	50-29-3
	2,4’-DDE	C14H8Cl4	2-(2-Chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethene	3424-82-6
	4,4’ DDE	C14H8Cl4	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene	72-55-9
	2,4’-DDD	ClC6H4)2CHCHCl2	2,4’-Dichlorodiphenyl)dichloroethane	53-19-0
	4,4’ DDD	C14H10Cl4	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane	72-54-8
	alpha-BHC	C6H6Cl6	α-Esaclorocicloesano	319-84-6
	beta-BHC	C6H6Cl6	β – Esaclorocicloesano	319-85-7
	delta-BHC,			
	gamma-BHC,	C6H6Cl6	γ - Esaclorocicloesano	58-89-9
	alpha-chlordane (cis)			
	gamma-chlordane			
	dieldrin	C12H8Cl6O		60-57-1
	endrin,			
	endosulfan I,			
	endosulfan II,			
	heptachlor,			
	heptachlor epoxide - isomer B,			
	methoxychlor,			
	hexachlorobenzene,	C6Cl6	Hexachlorobenzene	118-74-1
	isodrin,			
	pentachlorobenzene			

Analisi di PCB e pesticidi nel biota (EPA3550B + EPA 3620C + ICRAM + EPA8081 + EPA 8082)

- 1 g campione liofilizzato in vial da 40 mL
- + spatolata sodio solfato anidro
- + 20mL solvente (esano:diclorometano=70:30)
- +0.5mL surrogato (25ppb)
- agitare e poi sonicare 30 min a 40°C
- centrifugare e allontanare l'estratto organico
- ripetere l'estrazione altre 2 volte con 2 aggiunte di 10mL della stessa miscela
- ripetere la centrifugazione, riciclando le stesse provette per lo stesso campione
- alla fine avrò un volume totale di estratto di 40 ml
- far evaporare il solvente insufflando N2 in un bagno 30°C, senza farlo andare a secco, fino a volume finale circa 1 ml (non inferiore o perdo semivolatili)

Purificazione

- 1) condizionare la cartuccia [Florisil + sodio solfato anidro] con 5 ml di esano, senza mandarla a secco
- 2) caricare l'estratto (ripreso con 3x1 ml di esano) ed eluire, senza mandarla a secco
- 3A) SE PCB E PESTICIDI ELUITI IN UNICA FRAZIONE: 9 ml acetone:esano 10:90
- 4) portare a piccolo volume (1 ml)
- aggiumgere 20uL di std interno a 500ppb (volume finale 1020uL)

Analisi strumentale al GC-ECD condotta come per i sedimenti

Additional results.

Ulva

Halomethanes and Haloacetonitrile	ng/g
Bromochloromethane	<LOQ
Trichloroacetonitrile	<LOQ
Dichloroacetonitrile	<LOQ
Dibromoacetonitrile	<LOQ
Chloroform	<LOQ
Carbon tetrachloride	<LOQ
Bromodichloromethane	<LOQ
Chlorodibromomethane	<LOQ
Bromoform	<LOQ

Table 4) Halomethanes and haloacetonitrile investigated in Ulva

Halophenols	µg/g
2,4,6 Trichlorophenol	<LOQ
2,4 Dichlorophenol	<LOQ
4-Chloro-3methylphenol	<LOQ
Pentachlorophenol	<LOQ
2,4,6 Trichlorophenol	<LOQ

Table 5) Halophenols investigated in Ulva

Haloacetic Acids	ng/g
Monochloroacetic Acid (MCAA)	<LOQ
Monobromoacetic Acid (MBAA)	<LOQ
Dichloroacetic Acid (DCAA)	**1.74**
Trichloroacetic Acid (TCAA)	<LOQ
Bromochloroacetic acid (BCAA)	<LOQ
Dibromoacetic Acid (DBAA)	**14.28**
Bromodichloroacetic acid (BDCAA)	<LOQ
Chlorodibromoacetic acid (CDBAA)	<LOQ
Tribromoacetic Acid (TBAA)	<LOQ
Dalapon	<LOQ

Table 6) Haloacetic acids in nanograms per gram of sample (Ulva) ±0,02 ng/g

Water

	Docks		Open waters	
PAH	Value (ng/L)	error	Value (ng/L)	error
Naphthalene	<LOQ		<LOQ	
Acenaphthene	**7.14**	**±0.81**	**5.93**	**±0.81**
Fluorene	<LOQ		<LOQ	
Phenanthrene	<LOQ		<LOQ	
Anthracene	<LOQ		<LOQ	
Fluoranthene	<LOQ		<LOQ	
Pyrene	<LOQ		<LOQ	
Benzo (a) anthracene	<LOQ		<LOQ	
Crisene	<LOQ		<LOQ	
Benzo (b) fluoran	<LOQ		<LOQ	
Benzo (k) fluoran	<LOQ		<LOQ	
Benzo (a) pyrene	<LOQ		<LOQ	
Dibenzo (a, h) ant	<LOQ		<LOQ	
Benzo (g, h, i) per	<LOQ		<LOQ	
Indeno	<LOQ		<LOQ	

Table 10) PAH content in nanograms per liter of sample (seawater)

Mussels

ng/g	**Vongola S2**
Monochloroacetic Acid (MCAA)	<LOQ
Monobromoacetic Acid (MBAA)	**11,21**
Dichloroacetic Acid (DCAA)	<LOQ
Trichloroacetic Acid (TCAA)	<LOQ
Bromochloroacetic acid (BCAA)	<LOQ
Dibromoacetic Acid (DBAA)	<LOQ
Bromodichloroacetic acid (BDCAA)	<LOQ
Chlorodibromoacetic acid (CDBAA)	<LOQ
Tribromoacetic Acid (TBAA)	<LOQ
Dalapon	<LOQ

Table 24) Haloacetic acids in nanograms per gram of sample (Clam) ±0,02 ng/g

Halomethanes and Haloacetonitrile	ng/g
Bromochloromethane	<LOQ
Trichloroacetonitrile	<LOQ
Dichloroacetonitrile	<LOQ
Dibromoacetonitrile	<LOQ
Chloroform	<LOQ
Carbon tetrachloride	<LOQ
Bromodichloromethane	<LOQ
Chlorodibromomethane	<LOQ
Bromoform	<LOQ

Table 25) Halomethanes and haloacetonitrile investigated in Clams

Halophenols	µg/g
2,4,6 Trichlorophenol	<LOQ
2,4 Dichlorophenol	<LOQ
4-Chloro-3methylphenol	<LOQ
Pentachlorophenol	<LOQ
2,4,6 Trichlorophenol	<LOQ

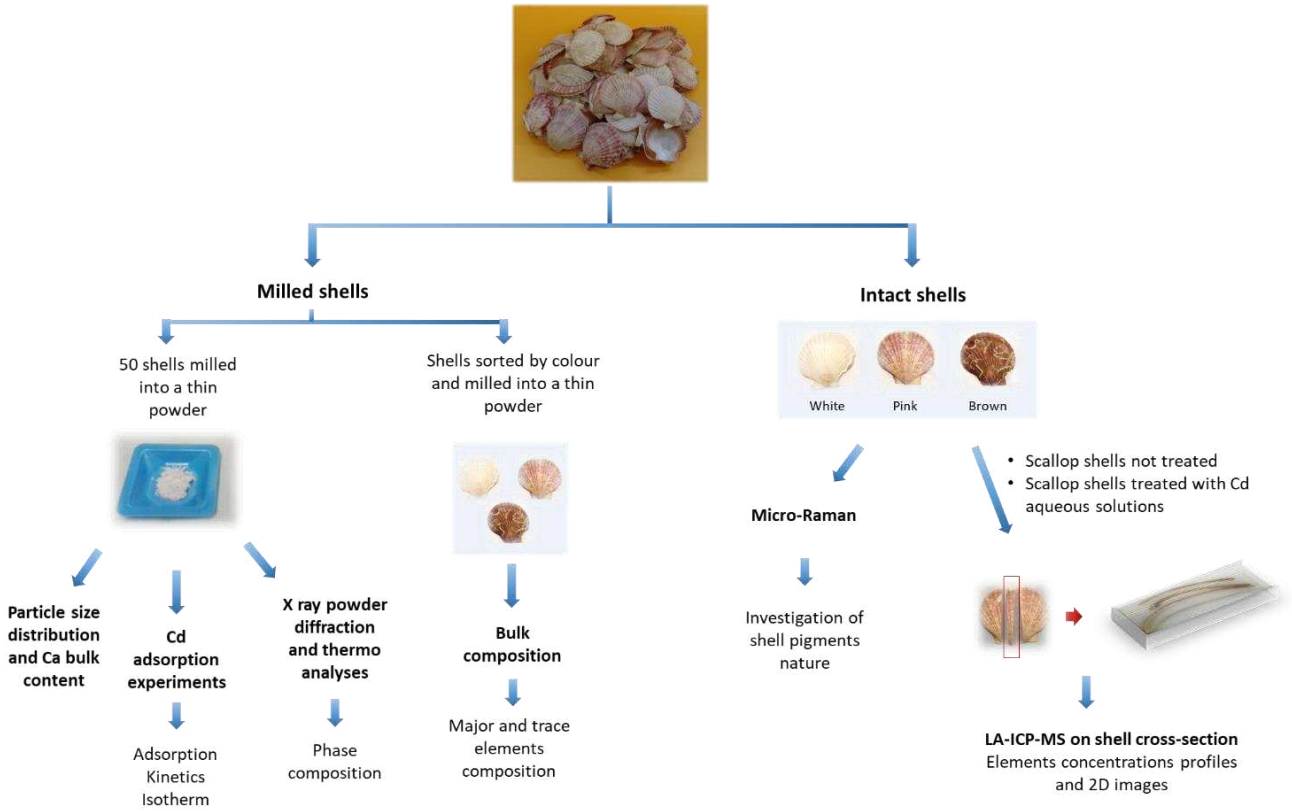
Table 26) Halophenols investigated in clams

Appendix 3) supplementary information for Chapter 2:

"Scallop Shells as Biosorbents for Water Remediation from Heavy Metals: Contributions and Mechanism of Shell Components in the Adsorption of Cadmium from Aqueous Matrix"

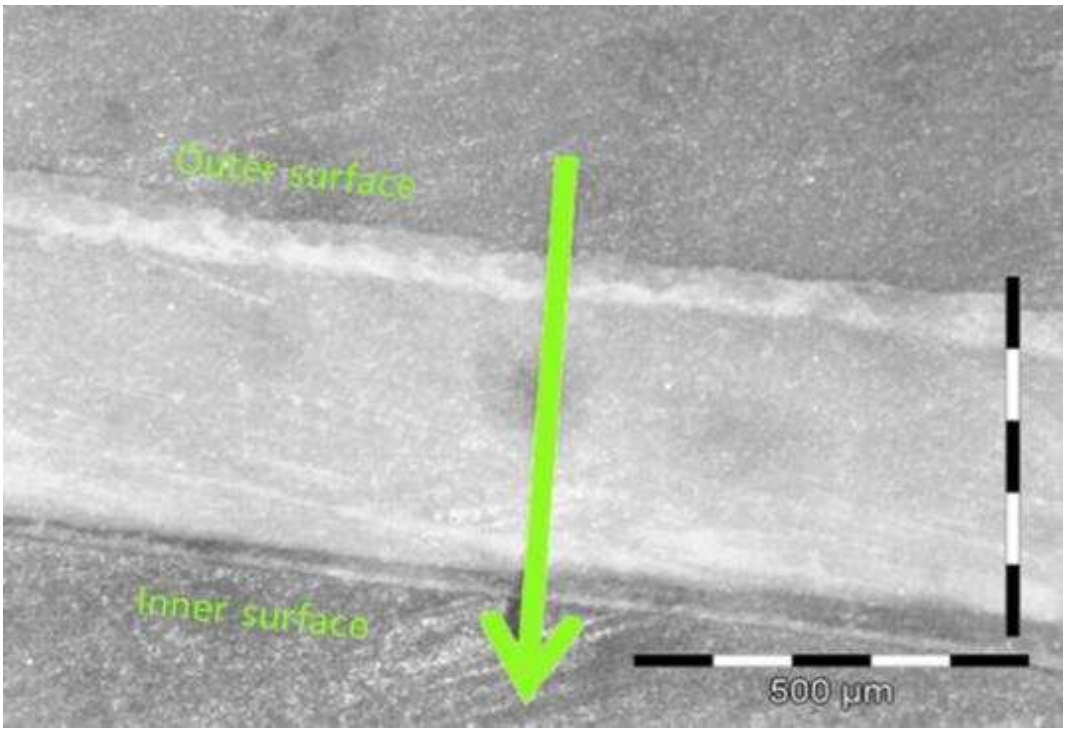
Supplementary Figure S1:

Experimental workflow



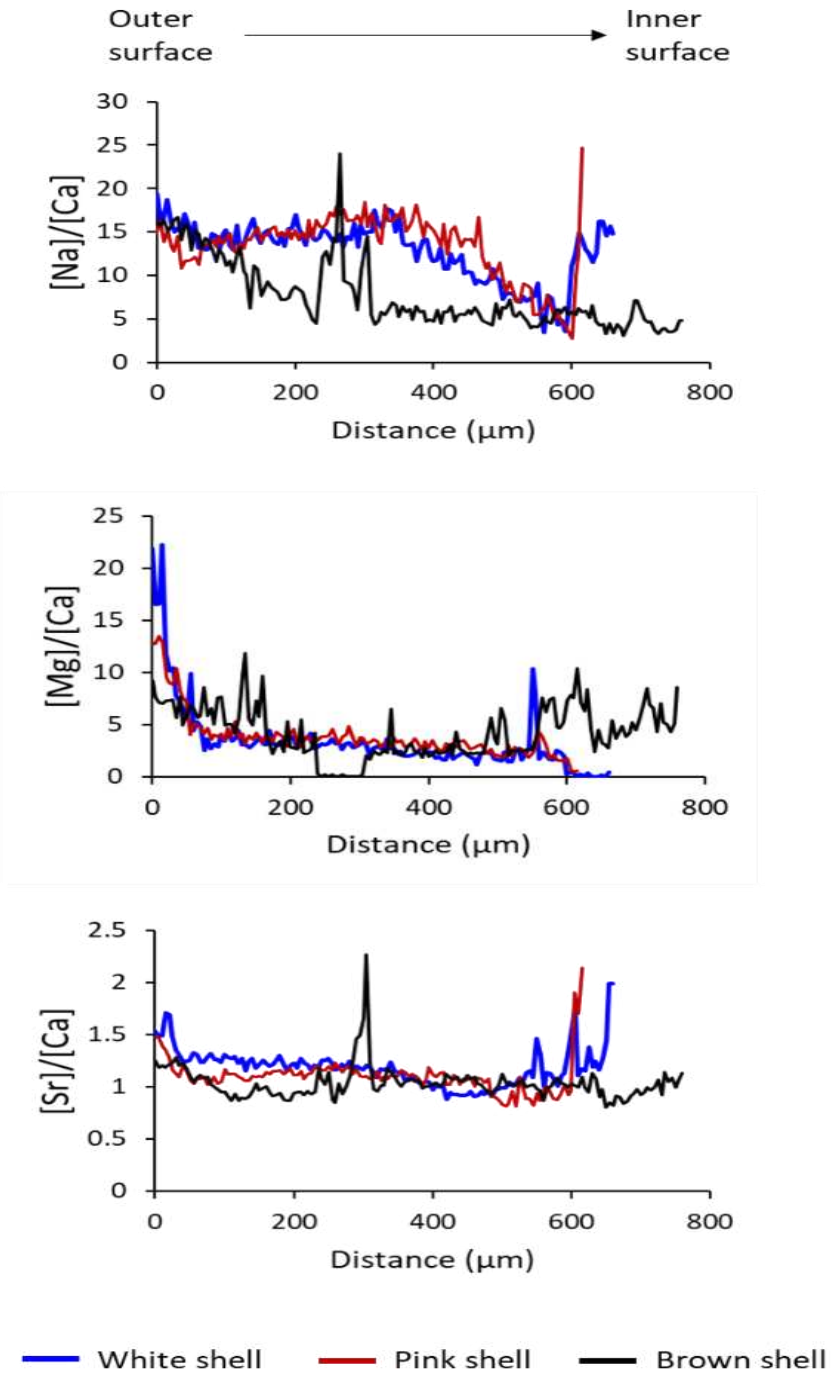
Supplementary Figure S2:

Microscope image of a scallop shell cross section with the direction, perpendicular to the direction of shell growth, in which the LA-ICP-MS line scans were performed (green arrow).



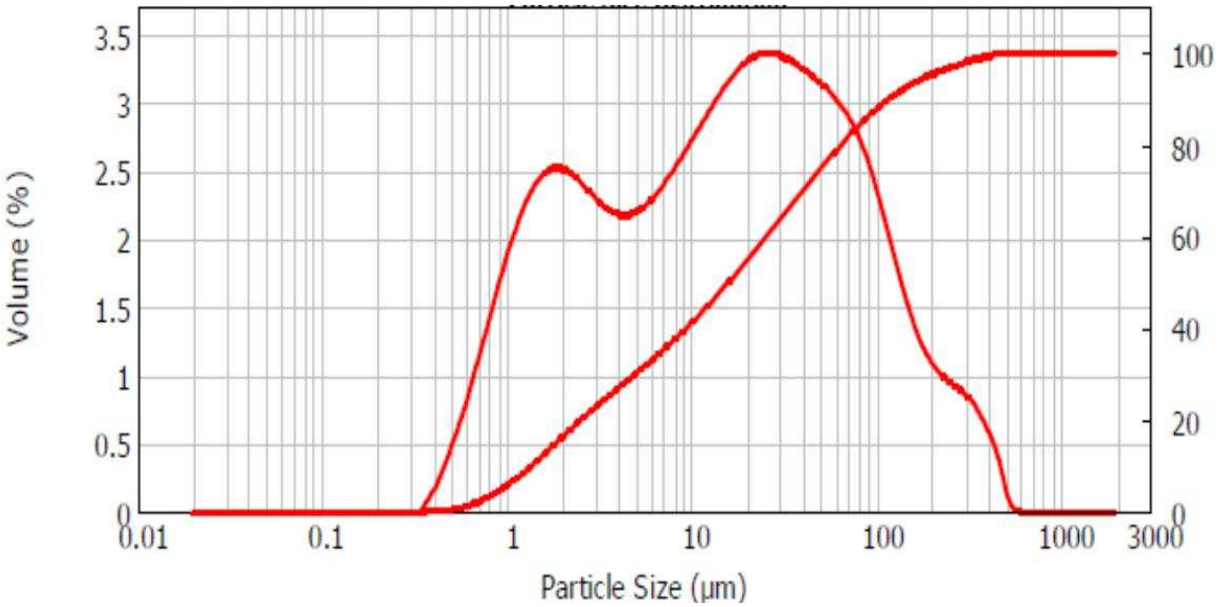
Supplementary Figure S3:

Sodium, magnesium and strontium mass fraction profiles expressed as element/Ca ratio (mmol mol^{-1}) obtained from LA-ICP-TOFMS line scans performed on three specimens (the x-axis ranges from the outer to the inner surface of the shells).



Supplementary Figure S4:

Particle size distribution of scallop shell powder.



Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %	Size (µm)	Vol Under %
0.010	0.00	0.105	0.00	1.096	7.07	11.482	43.84	120.226	90.48	1258.925	100.00
0.011	0.00	0.120	0.00	1.259	9.04	13.183	46.49	138.038	92.11	1445.440	100.00
0.013	0.00	0.138	0.00	1.445	11.17	15.136	49.26	158.489	93.48	1659.587	100.00
0.015	0.00	0.158	0.00	1.660	13.41	17.378	52.13	181.970	94.64	1905.461	100.00
0.017	0.00	0.182	0.00	1.905	15.69	19.953	55.09	208.930	95.65	2187.762	100.00
0.020	0.00	0.209	0.00	2.188	17.96	22.909	58.11	239.883	96.56	2511.886	100.00
0.023	0.00	0.240	0.00	2.512	20.17	26.303	61.15	275.423	97.41	2884.032	100.00
0.026	0.00	0.275	0.00	2.884	22.32	30.200	64.19	316.228	98.20	3311.311	100.00
0.030	0.00	0.316	0.00	3.311	24.38	34.674	67.20	363.078	98.91	3801.894	100.00
0.035	0.00	0.363	0.01	3.802	26.39	39.811	70.16	416.869	99.48	4365.158	100.00
0.040	0.00	0.417	0.14	4.365	28.36	45.709	73.07	478.630	99.89	5011.872	100.00
0.046	0.00	0.479	0.42	5.012	30.33	52.481	75.92	549.541	99.99	5754.399	100.00
0.052	0.00	0.550	0.90	5.754	32.35	60.256	78.70	630.957	100.00	6606.934	100.00
0.060	0.00	0.631	1.61	6.607	34.43	69.183	81.40	724.436	100.00	7585.776	100.00
0.069	0.00	0.724	2.58	7.586	36.61	79.433	83.97	831.764	100.00	8709.636	100.00
0.079	0.00	0.832	3.81	8.710	38.90	91.201	86.38	954.993	100.00	10000.000	100.00
0.091	0.00	0.955	5.31	10.000	41.31	104.713	88.57	1096.478	100.00		

Supplementary Discussion S1:

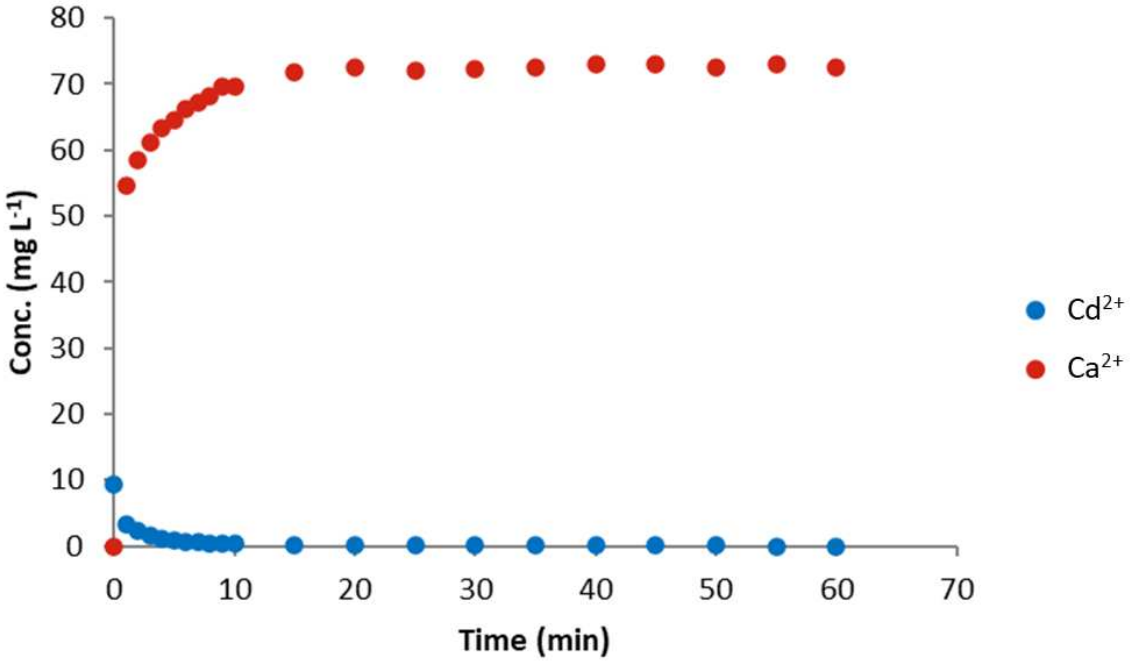
Adsorption of Cd from aqueous solution onto powdered scallop shells

Generally, the adsorption of metal ions from aqueous solutions depends on pH, since at high pH values hydroxy ions may complex and/or precipitate metal cations from aqueous solutions. On the other hand, carbonate dissolution can take place in acidic media. Dissolution equilibria for biogenic carbonate are complex phenomena since the heterogeneity of these materials on many different scales influence their solubility and kinetic behaviour [S1]. At pH lower than 7 the Cd^{2+} aqua complex is the prevailing species. At pH 7 the dissolution of biogenic carbonate is also negligible. In addition, many water bodies are characterised by pHs around neutrality and recently it has been found that in Sacca di Goro pH can vary in the range 7.2 – 8.3 depending on season and site location [S2]. Therefore, the adsorption experiments were carried out in the pH range 7.0 – 8.5.

During the kinetic experiments, the Ca concentration in the solution was also monitored; the Cd adsorption process is accompanied by an increase of calcium ions in solution and by an increase in pH due to dissolution of shell fragments. The concentration of calcium and carbonate species in the aqueous solution increases rapidly at the start of the experiment. The final pH depends on the initial concentration of Cd as already observed in Köhler et al. (2007) [S3]. This seems to be due to the formation of low-soluble Cd carbonate on the surface of the shell fragments (see Supplementary Figure S5).

Supplementary Figure S5:

Variations of the concentrations of cadmium and calcium in solution (mg L^{-1}) during adsorption of cadmium onto scallop shell powder (kinetic data).



Supplementary Table S1:

Kinetic parameters with confidence intervals at 95%, obtained using the PSO model.

C_0 (mg L^{-1})	k_2 (g mg^{-1} min^{-1})	q_e (mg g^{-1})	R^2
5	2.50 ± 0.16	0.997 ± 0.004	0.9984
10	0.870 ± 0.050	1.880 ± 0.016	0.9984
15	0.210 ± 0.013	2.880 ± 0.020	0.9968

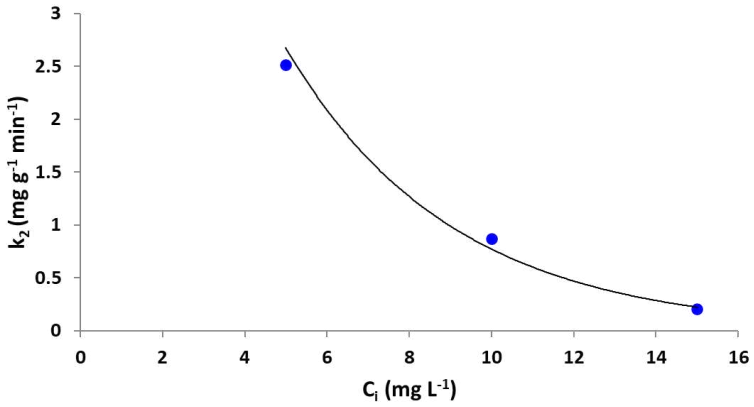
Supplementary Discussion S2:

Adsorption kinetics

The data are well fitted to a non-linear PSO, as can be seen from the high values of the coefficient of determination, R^2 (see Supplementary Table S1). The kinetic constant k_2 is a function of the initial concentration (Supplementary Figure S6), the data were fitted by using a power law equation as suggested in Ho et al. (2001) [S4], as a result the following relationship was found: $k_2 = 93.7C_0^{-2.18}$, which is similar to that obtained for the adsorption of Pb(II) on peat [S4].

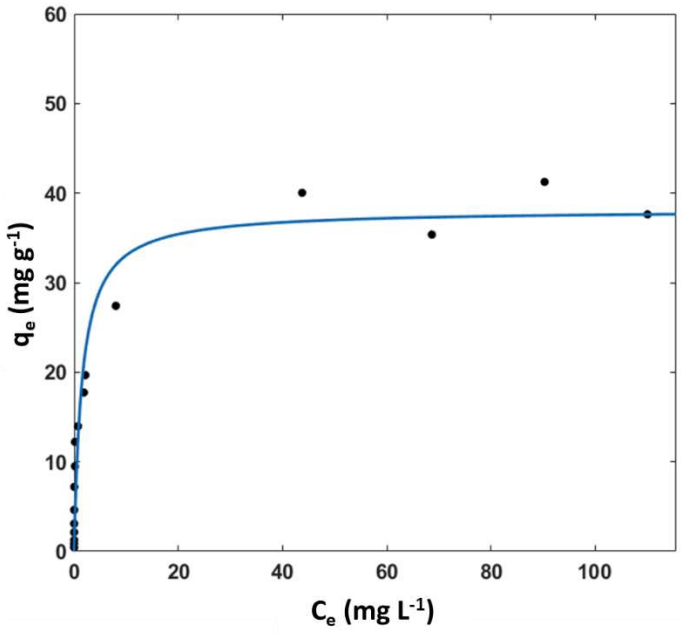
Supplementary Figure S6:

Kinetic constant as a function of initial Cd concentration.



Supplementary Figure S7:

Adsorption isotherm of Cd onto scallop shell powder at 9.0 ± 0.5°C fitted with the Langmuir model. The parameters obtained are: q_{max} = 38.1 ± 4.3 mg g^{-1}, b = 0.65 ± 0.37 L mg^{-1}, R^2 = 0.9336.



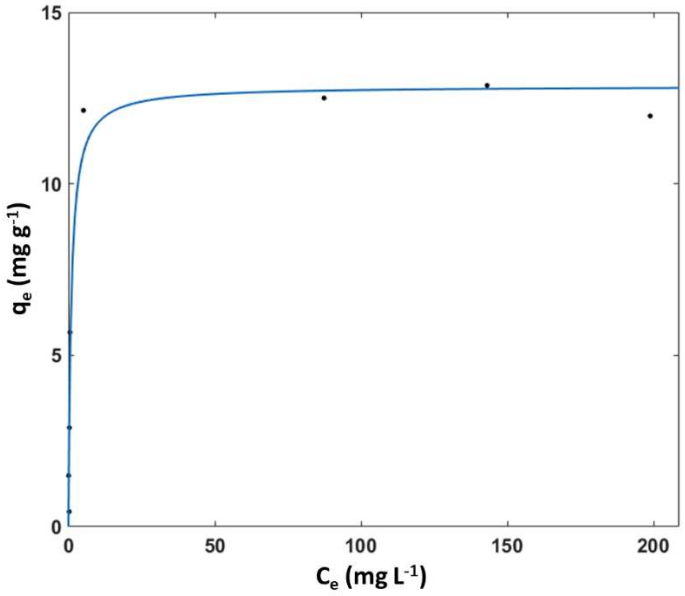
Supplementary Table S2:

Isotherm parameters with confidence intervals at 95%, for the adsorption of Cd on scallop shell powder obtained using the Langmuir model.

Q_{max} (mg g^{-1})	b (L mg^{-1})	R^2
55.3 ± 7.4	0.70 ± 0.56	0.9371

Supplementary Figure S8:

Adsorption isotherm of Cd onto pure calcium carbonate powder (21°C) fitted with the Langmuir model. The parameters obtained are: q_{max} = 12.8 ± 1.6 mg g^{-1}, b = 1.09 ± 0.74 L mg^{-1}, R^2 = 0.9515.



Supplementary References

S1. Morse, J. W., Arvidson, R. S. & Lüttge A. Calcium Carbonate Formation and Dissolution. *Chem. Rev.*, **107**(2), 342 – 381 (2007). https://doi.org/10.1021/cr050358j

S2. Pala, C. *et al*. Environmental Drivers Controlling Bacterial and Archaeal Abundance in the Sediments of a Mediterranean Lagoon Ecosystem. *Curr. Microbiol.*, **75**, 1147 – 1155 (2018). https://doi.org/10.1007/s00284-018-1503-3

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Scallop shells as biosorbents for water remediation from heavy metals: contributions and mechanism of shell components in the adsorption of cadmium from aqueous matrix

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Abstract

To ascertain their potential for heavy metal pollution remedy, we studied the adsorption mechanism of cadmium onto scallop shells and the interactions between the heavy metal and the shell matrix. Intact shells were used to investigate the uptake and diffusion of the metal contaminant into the shell carbonatic layers, as well as to evaluate the distribution of major and trace elements in the matrix. LA-ICP-MS measurements demonstrate that Cd is adsorbed on a very thin layer on the inner and outer surfaces of the shell. Structural and thermal analyses showed the presence of 9 wt.-% of $CdCO_3$ phase indicating that the adsorption is mainly a superficial process which involves different processes, including ion exchange between Ca and Cd. In addition, organic components of the shell could contribute to adsorption as highlighted by different metal uptake showed by shells with various colouration. In particular, darker shells adsorbed more contaminant than the white ones. The contribution of the organic shell components on the adsorption of heavy metals was also highlighted by the element bulk content which showed higher concentrations of different metals in the darker specimen. Raman spectroscopy allowed to identify the pigments as carotenoids, confirmed by XRD measurements which highlighted the presence of astaxanthin phases. This work indicates that the presence of organic components in the scallop shell matrix enhances the contaminant uptake. The results presented here provide new insights into the Cd adsorption mechanism highlighting the important contribution given by the organic components present in the biogenic carbonate matrix. Furthermore, the high efficiency of

Cd removal of scallop shells, supported by adsorption kinetic and isotherm studies, has been demonstrated.

Introduction

Environmental pollution caused by heavy metals is one of the major global problems leading to adverse effects on ecosystems, biodiversity and human health[1]. Among the heavy metals, cadmium (Cd) is one of the most toxic pollutants even at low concentrations. Furthermore, Cd has been classified as human carcinogen by the International Agency for Research on Cancer[2]. The main source of Cd pollution in surface waters is from anthropogenic activities. On a global scale, smelting of non-ferrous metal ores has been regarded as the largest anthropogenic source of Cd input into the aquatic environment[3]. Cd tends to accumulate in the sediments by adsorption or precipitation as insoluble salts[4]. However, under certain conditions Cd can be mobilised and its concentration in the aqueous medium can increase. This is particularly the case when the salinity of the water body increases and dissolved Cd is stabilised in solution through the formation of chloro-complexes[5].

Among the most common physical and chemical approaches for the removal of heavy metals from water, adsorption is an effective and economic technique, offering flexibility in the design and operation, and a vast variety of adsorbent materials[6]. In the last years, the use of natural or waste materials as adsorbents has been largely studied to favour eco-friendly approaches in environmental remediation applications[7]. Among the waste products generated by food industry, mollusc shells have composition and structure characteristics suitable for the removal of heavy metals dissolved in water bodies.

Indeed, many studies had reported the capability of molluscan and crustacean shell powder to adsorb heavy metals from water aqueous matrices considering the effect of the adsorbent grain size[8–10], different $CaCO_3$ shell structure[11], or after the adsorbent material had been acid-pretreated[12] or calcined[13–15]. It has been demonstrated by Tudor et al. (2006)[16] that some seashells are more efficient in the uptake of Pb and Cd compared to non-biogenic calcareous materials. Mollusc shells can also be employed as marine sediment amendments for immobilisation of potentially toxic elements, in this way the water and sediment quality would be guaranteed without introducing extraneous materials in the lagoon system, since they are native of the area of interest. Furthermore, adding biogenic carbonate in marine sediments could possibly mitigate the effect of sea acidification, as reported in Drylie et al. (2019)[17]. The use of mollusc shells for environmental remediation can contribute to improve the sustainability of shellfish farms since they are classified as waste material, and as such they require adherence to applicable policies and procedures to be correctly disposed[18]. In addition, mollusc shells are important environmental indicators. Indeed, they can accumulate heavy metals. Their concentrations in shells provide a time-integrated degree of metal availability, over long periods of time,

thus providing an archive of past seawater. Therefore, the chemistry of shells can provide useful information on environmental conditions; shells can provide a more precise symptom of pollution and environmental change than soft tissue, due to the possibility of investigating the incorporation of elements over the entire period of shell formation, higher preservation potential even after the organism's demise, and relatively cheap and easy storage. Besides, the lower metal concentrations in the shells with respect to soft tissue can be overcome by the development of sensitive analytical techniques[19,20].

The evaluation of the interactions of dissolved metal contaminants with the mollusc shell matrix and its components would provide additional information on the mechanism of the metal uptake process and can contribute to the development of the use of shells as bioadsorbents and bioindicators in environmental remediation and monitoring fields, respectively.

Herein, we present a study on the investigation of the mechanism of Cd adsorption and diffusion in the scallop shell carbonatic layers employing different analytical techniques, considering the interaction of heavy metals not only with the carbonate phase, but also with organic components present in the matrix. To detect trace elements concentrations, high spatial resolution techniques such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been employed. LA-ICP-MS allows to determine the element distribution across heterogeneous and multiphase solid samples with low μm spatial resolution. In this work, LA was applied in combination with ICP-TOFMS, which allows fast and quasi-simultaneous detection of most elements across the elemental mass spectrum[21,22], obtaining distributions of selected areas on the cross section of mollusc shells. Furthermore, it provides short analysis times with minimal sample preparation required[23] and by using LA-ICP-TOFMS in the line scan mode, continuous elemental profiles of the sample can be achieved. Many studies employed this technique to obtain natural trace elements patterns along the shell direction of growth in order to use them as proxies to retrace variations of temperature and salinity of the water body[24–28]. Despite the proven applicability of LA-ICP-MS in line scan mode for retrospective natural trace elements monitoring, only few works applied this technique to investigate metal contaminants in mollusc shells[29–31] and, to the best of our knowledge, there is no study relating to the diffusion of the metal through the shell layers in mollusc shell treated with the contaminant.

Since it has been demonstrated that the adsorption of metal can induce structural modification in the crystalline components of shells, thermogravimetric and X-ray powder diffraction analyses were carried out. The results indicate that scallop shells having different colourations show differences in Cd adsorption characteristics. This finding motivated us to investigate the role of pigments through micro-Raman and LA-ICP-TOFMS imaging[22].

The insights gathered from this study provide information to better understand the distribution and interactions of metal contaminants with the shell matrix when mollusc shells are used as adsorbents or environmental indicators.

Materials and methods

Adsorbent material preparation

One hundred scallop shells (Aequipecten opercularis, Linnaeus 1758) were collected from a shell deposal site in Sacca di Goro (Northern Adriatic Sea, Italy). The shells were first brushed to remove any residual mollusc tissue, cleaned thoroughly with deionised MilliQ water (Millipore, MA, USA) and then dried in an oven at 50°C over night.

A total of 50 shells were selected and milled using a grinder (Retsch GmbH, Germany) to obtain a fine powder which was used for the determination of the mean calcium (Ca) content, particle size distribution, Cd adsorption experiments for kinetics and isotherm determination, and for thermal and X-ray powder diffraction analyses.

The material characterisation regarding minor and trace elements bulk composition was performed on scallop shells with different colouration, milled separately into a fine powder.

28 shells were kept intact and sorted by colour (white, pink and brown) for the metal diffusion investigation by LA-ICP-MS and for pigment characterisation with micro-Raman spectroscopy.

For the overall experimental workflow see Supplementary Figure S1 in the Supplementary Information.

Characterisation

The particle size distribution of the scallop shell powder used for batch adsorption experiments was determined with a Malvern Mastersizer 2000 Particle Analyser (Malvern instruments, UK).

For the determination of the bulk concentration of Ca, 0.1 g of scallop shell powder was digested in 10 mL of HNO_3 10%, filtered with PVDF membrane 0.45 µm (ACRODISC, New York, USA), diluted and analysed by solution based ICP-OES (Perkin-Elmer, Waltham, MA, USA) (for the measuring parameters see below in the *Batch adsorption* section).

To determine the trace elements bulk composition, three scallop shells were sorted by colour, milled into a fine powder, digested in HNO_3 10%, then filtered using syringe filters with PVDF membrane 0.45 µm (ACRODISC, New York, USA). After dilution, the analyses were carried out using an Agilent 7500 ICP-QMS (Agilent Technologies, Santa Clara, CA, USA); the instrument operating parameters were: 1450 W RF power, 4.2 mm sampling depth, 15 L min^{-1} coolant gas, 1.00 L min^{-1} carrier gas, the isotopes measured were ^{25}Mg, ^{39}K; ^{49}Ti, ^{51}V, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{62}Ni, ^{65}Cu, ^{66}Zn, ^{88}Sr, ^{107}Ag, ^{111}Cd, ^{113}Cd, ^{137}Ba, and ^{208}Pb.

Batch adsorption

Cd solutions were prepared by dissolving $Cd(NO_3)_2 \cdot 4H_2O$ (Sigma-Aldrich, Steinheim, Germany) in ultrapure water (Millipore, MA, USA). The adsorption kinetics and isotherm were determined using the batch method, as explained below in the respective sections.

The Cd uptake (expressed as q_e for isotherm determination, and q_t for kinetic experiments) was calculated as follow:

$$q = \frac{(C_0 - C_e) \cdot V}{m} \quad (1)$$

where q (mg g^{-1}) is the quantity of Cd adsorbed, C_0 (mg L^{-1}) is the concentration of Cd in the initial solution, C_e (mg L^{-1}) the residual Cd concentration at a time t in kinetic experiments or at equilibrium for isotherm determination, V (L) the volume of the solution in the batch, and m (g) the quantity of scallop shell powder.

Adsorption kinetics

The kinetic experiments were conducted at three different initial Cd concentrations (5, 10 and 15 mg L^{-1}), 1 L of solution was prepared in a polypropylene bottle where 5 g of shell powder was added. The batches were kept under stirring and at a temperature of 21.0 ± 0.5°C using a refrigerating bath circulator (Jeio Tech, Daejeon, Republic of Korea). Samples (0.5 mL) were collected at different time intervals: every minute for the first 10 minutes, every 5 min up to 1 h, every 30 min up to 3 h, and every 60 min up until 5h. The samples were filtered with 25 mm syringe filters with PVDF membrane 0.45 µm (Agilent Technologies, Santa Clara, CA, USA) and the Ca and Cd residual concentration in the solution was evaluated by ICP-OES Optima 3100XL (Perkin-Elmer, Waltham, MA, USA) (axial view) equipped with a solid-state charge-coupled device detector (CCD), a peristaltic pump and a low-flow GemCone nebuliser coupled to a cyclonic spray chamber. Analytical lines 317.933 nm and 226.502 nm were selected for quantitative determination of Ca and Cd, respectively. The ICP-OES measuring parameters were: 15 L min^{-1} plasma flow, 0.5 L min^{-1} auxiliary flow, 0.65 L min^{-1} nebuliser flow, 1350 W RF power.

Adsorption isotherm

20 mL of Cd^{2+} solution were added to crimp top reaction glass flasks sealed with PTFE septa (Supelco, Bellefonte, PA, USA) with 40 mg of shell powder. The initial concentrations of Cd^{2+} in the solutions were in the range of 1-350 mg L^{-1}.

Adsorption experiments were performed with a contact time of 20 h, which is considered sufficiently longer than the equilibration time (30 min) as determined in the adsorption kinetics experiments.

After 20 h equilibration under stirring (600 rpm) and at a controlled temperature of 21.0 ± 0.5°C, the adsorbent was separated from the solution by filtration using 25 mm syringe filters with PVDF membrane

0.45 µm (Agilent Technologies, Santa Clara, CA, USA), the pH of the solution was recorded for each solution after equilibration.

The concentration of Ca^{2+} and Cd^{2+} in the solution, before and after the contact with the adsorbent material, was determined by ICP-OES, as described before.

The same procedure was followed to obtain the adsorption isotherm of Cd onto commercial pure calcium carbonate powder (Sigma-Aldrich, Steinheim, Germany).

Cd uptake across shell layers

For the study of the metal diffusion through the shell layers, the intact mollusc valves were put in contact with solutions containing Cd^{2+}, the concentrations of the metal ions were 1.0 and 5.0 mg L^{-1}. After a contact time of 20 h to ensure that the equilibrium had been reached, the shells were separated from the solution, rinsed twice with ultrapure water, air-dried and conserved for the LA-ICP-MS experiments. The Cd concentration in the solution before and after the contact with the shells was measured by ICP-OES (for the measuring parameters see *Adsorption kinetics* section) and the Cd uptake was calculated (equation 1).

To investigate the diffusion of Cd through the sample cross section, the shells were cut along the axis of maximum growth with a low-speed saw (Isomet 11-1180 Low Speed Saw, Buehler LTD, CH), embedded into an epoxy resin (Epofix, Struers, Birmensdorf, CH), ground to the desired thickness with a polishing machine (Forcipol 1V Grinder Polisher, Metkon, TR) equipped with a 120 grit diamond disc, then polished with 600 and 800 grit SiC powder. In order to obtain the trace elements distribution through the shell layers, laser ablation was conducted on the cross-sections of the samples, with the ablation direction oriented perpendicular to the direction of shell growth, from the outer to the inner surface layer (Supplementary Figure S2).

The high-resolution line scans and element imaging analyses were conducted with a 193 nm ArF Eximer laser system (GeoLas C, Lambda Physik, DE) equipped with the parallel flow ablation cell[32] and was coupled to an ICP-TOFMS (icpTOF2R, Tofwerk AG, CH). Ablation spots with 5 µm diameter were used in an edge to edge arrangement. The imaging mode “hole drilling with cleaning pulse” was applied using the imaging control system according to Neff et al.[22]. The acquisition of one sampling position was conducted using one cleaning pulse which was not acquired, then 25 laser pulses at 100 Hz repetition rate which were acquired and a fluence of 15-20 J cm^{-2}. The ICP-TOFMS measuring parameters were: 16 L min^{-1} plasma flow (Ar), 0.8 L min^{-1} auxiliary flow (Ar), 0.6-0.7 L min^{-1} make-up gas flow (Ar), 1.4-1.6 L min^{-1} carrier gas flow (He), 1400 W RF power. The carbonate reference material MACS-3 (U.S. Geological Survey, USA) was used as external standard and a 100% mass normalisation approach[33] was applied for the quantification assuming all metals are present as carbonates. The isotopes ^{23}Na,

^{24}Mg, ^{44}Ca, ^{55}Mn, ^{56}Fe, ^{66}Zn ^{88}Sr, ^{114}Cd and ^{208}Pb were evaluated. The mass resolving power of 4500-5000 achieved by the icpTOF2R in the lower mass range (24-56 m/z)[34] allows to separate $^{24}Mg^+$ from $^{12}C_2^+$, $^{44}Ca^+$ from $^{12}C^{16}O_2^+$, $^{55}Mn^+$ from $^{40}Ar^{14}NH^+$, and $^{56}Fe^+$ from $^{40}Ar^{16}O^+$ and $^{40}Ca^{16}O^+$. No significant interference of $^{114}Cd^+$ with $^{114}Sn^+$ was observed. This allowed to evaluate higher abundance isotopes to increase the sensitivity with low background intensities and therefore to lower the limits of detection.

Micro-Raman analyses

Raman spectra were recorded with a LabRam HR800 micro-Raman instrument (Horiba Scientific, FR) equipped with an air-cooled CCD detector at -70°C, an Olympus BXFM microscope, a 600 groove/mm grating and a 50 X objective to collect the Raman scattering signals. The excitation source was a He-Ne laser (632.8 nm line) with a maximum laser power of 20 mW. A minimum spectrum accumulation of 10 times per second was used; if a high background was recorded, the accumulations were increased to a maximum of 100 times per second to improve the signal-to-noise ratio.

Thermo analyses

Thermogravimetric analyses (TG) and differential thermal analysis (DTA) of both raw scallop shell powder and Cd-loaded scallop shell powder were performed on a STA 409 PC LUXX (Netzsch, DE). The measurements were carried out in air flow with a heating rate of 10°C min^{-1} from room temperature to 1000°C.

X-ray powder diffraction

The X-ray powder diffraction patterns of untreated and treated with Cd solution scallop shell powder were recorded on a D8 Advance diffractometer (Bruker, USA) equipped with a Si (Li) solid-state detector, (Cu $Ka_{1,2}$ radiation, 3–110 2θ range, counting time of 12 s per 0.02 2θ step). The unit cell parameters were refined together with the coefficients of the pseudo-Voigt function modelling the profile function of the Bragg peaks and of the function modelling the background (a six-term cosine Fourier series). Refinements were carried out by the Rietveld method using the GSAS[35] and EXPGUI[36] packages. Structural models for all the phases were taken from the ICSD database[37]. Optimised parameters in final refinement were: background coefficients, cell parameters, zero shift error, peak shape parameters, preferred orientation, and phase fractions.

Results and discussion

Adsorbent material characterisation

The bulk composition of the shells was determined to evaluate the total cation contents of the untreated shells. The mass fractions determined by ICP-MS after acid digestion of ground shells are reported in Table 1[38]. Many metals, in particular V, Mn, Fe, Co, Cu, Zn, Ba, and Pb were detected in the samples. Among these, Fe was the most abundant element. The mass fractions of Cd were between the limit of detection and quantification of the employed method, thus indicating that the environmental habitat of these seashell was not severely polluted by Cd[39], or that the replacement of Ca^{2+} in the calcium carbonate structure during the shell formation occurs to a lesser extent. Indeed, bioaccumulation rate of ions from seawater seems to be a function of many environmental and biological factors[40–42], and different habitats, species, or even individual specimens at different stages of development, may present different patterns of metal uptake. Furthermore, only few published investigations[43–46] deal with chemical composition of scallop shells and none of these in a habitat similar to that of the samples herein investigated (i.e. a lagoon on the Po River Delta). In this aspect, the present study on one hand can provide new data that can contribute to the knowledge on metal distribution in biota, On the other hand, however, the data cannot be compared with literature data.

Recently, the concentration of metals in sediments of Laguna di Goro have been investigated[47], and it has been found that trace-metals, such as Ni, Co, V and Cu, show concentration levels associated to the geological nature of the Po River alluvial sediments, whereas Pb and Zn show an enrichment in Sacca di Goro, when compared to the background alluvial sediments, probably due to anthropogenic sources. Indeed, these trace-metals present in the lagoon sediments were found in all the scallop shell samples analysed.

A particular feature that can be observed from the elements bulk composition is the fact that whereas for heavy metals such as Pb, Fe and Zn there is a higher concentration in the more coloured shell, the content of elements found naturally in the shells, such as Sr and Mg which are incorporated during the mollusc growth, does not vary significantly between shells of different colouration. These findings suggest that the components responsible for the shell colouration contribute significantly to the uptake of heavy metals from the aqueous environment surrounding the mollusc.

Table 1. Bulk composition (μg g^{-1}) with standard deviation (n = 5) and limit of detection (LOD) obtained from solution based ICP-MS analysis of powdered shells.

	White shell	Pink shell	Brown shell	LOD
^{25}Mg	92.8 ± 1.3	76.85 ± 0.75	106.82 ± 0.85	0.17
^{39}K	100.7 ± 3.6	118.6 ± 6.2	185.7 ± 4.5	9.80

^{49}Ti	<LOD	<LOD	0.91 ± 0.11	0.46
^{51}V	0.050 ± 0.040	0.0542 ± 0.0062	0.971 ± 0.047	0.054
^{55}Mn	24.45 ± 0.53	24.05 ± 0.65	178.4 ± 4.1	0.080
^{56}Fe	8.18 ± 0.39	10.60 ± 0.36	476.5 ± 8.7	0.22
^{59}Co	0.164 ± 0.021	0.0994 ± 0.0092	1.225 ± 0.018	0.0030
^{62}Ni	0.546 ± 0.089	2.20 ± 0.32	2.56 ± 0.25	0.37
^{65}Cu	1.270 ± 0.042	1.84 ± 0.10	17.40 ± 0.34	0.11
^{66}Zn	16.56 ± 0.30	16.30 ± 0.63	90.1 ± 2.8	0.60
^{88}Sr	1013.6 ± 5.8	947.2 ± 8.6	1067.7 ± 8.7	0.012
^{107}Ag	<LOD	<LOD	<LOD	0.036
^{111}Cd	<LOD	0.38 ± 0.13	0.54 ± 0.22	0.30
^{113}Cd	0.24 ± 0.11	0.304 ± 0.089	0.54 ± 0.17	0.17
^{137}Ba	1.74 ± 0.11	1.897 ± 0.069	8.40 ± 0.36	0.080
^{208}Pb	0.776 ± 0.041	0.685 ± 0.055	3.564 ± 0.080	0.030

To further investigate on the scallop shell composition, specimens of shells were analysed before the contact with solution of Cd^{2+}. Compositional trace elemental variability in biogenic carbonates can be assessed by static layer by layer removal of material to obtain a depth-compositional profile. Alternatively, it can be carried out by LA along a defined section oriented perpendicular to the accretionary growth direction[31]. The latter method was applied in the present study.

The investigation of the causes of the variability in the distribution of shell elemental components lays beyond the aim of this study, any correlation to seasonal environmental variations or pollution events cannot be made since the history of the shells, collected from a disposal site, is unknown. Nevertheless, information on the distribution of major and trace components of the shells used in this study can be obtained, highlighting the capability of the technique employed.

In Figure 1 the profile of the ratios of trace elements (i.e., Fe, Zn, Pb and Cd) with respect to Ca[30,31,48] of three scallop shell specimens are shown.

Lead was detected in every shell analysed, showing different distribution patterns across the shells, with peaks of higher abundance suggesting that during the formation of those layers, the organism was surrounded by a polluted environment.

Natali and Bianchini (2018)[47] reported that the sediments of Sacca di Goro are particularly enriched in Pb, with enrichment factors up to three with respect to the natural composition of alluvial sediments of the Padanian plain[49], taken as representative of the geogenic local background. The high Pb enrichment factor found in the lagoon sediments suggests anthropogenic contributions, possibly related to

atmospheric emissions[47]. Similar behaviours were found for Fe and Zn in the shells analysed, possibly related to the composition of the alluvial sediments[50]

Regarding the metal contaminant considered in the present work, Cd/Ca concentration profiles for the three shells before treatment show that there is no appreciable Cd distribution across the shell layers; therefore, the results on the contaminant distribution obtained after the treatment of the shells with Cd-containing solution (see below) are entirely ascribable to the treatment performed.

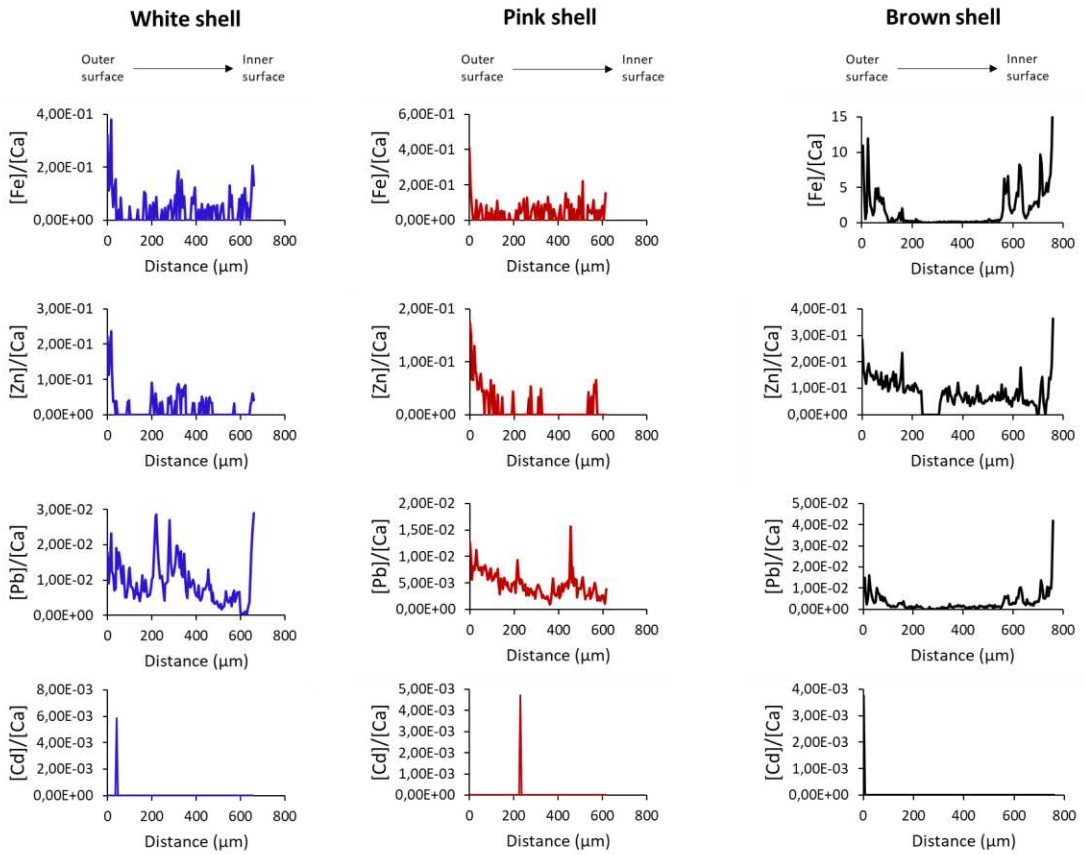

Figure 1. Trace element concentration profiles expressed as element/Ca ratio (mmol mol^{-1}) obtained from LA-ICPTOFMS line scan carried out on not treated scallop shells; (the x-axis ranges from the outer to the inner surface of the shells).

In Supplementary Figure S3 the profile of the ratios of Na, Mg and Sr with respect to Ca of three different specimens is reported. Variations in compositions could originate from shells growth[51]. These ratios, generally reported as mmol mol^{-1} [26,52], are biogeochemical parameters reflecting the Earth–ocean–atmosphere dynamic exchange of elements[53] and are commonly employed to investigate trace elemental and isotopic inventor in successively secreted carbonate layers related to past environmental conditions of seashells[53].

Generally, there is variability for all three elements in the element/Ca between the scallop shells, even though they belong to the same species. In addition, slight variations were observed within the same shell across the different regions (see the profiles of Na/Ca of all the three shells and Mg/Ca of the brown one). Regarding strontium, the Sr/Ca profiles were characterised by distinct variations, especially in the white and brown specimens, suggesting an alteration in the incorporation rate of this element during the shell layers deposition, possibly related to seasonal temperature variations which most likely influence the shell calcification and hence the Sr/Ca ratio[43,54].

Moreover, the brown shell shows a significant variation of the content of all three elements in a region between 230 and 315 μm; in particular, Mg decreases while Na and Sr increase. The investigation of the causes that led to this particular variation is beyond the aim of the present study, and correlations with seasonal variations cannot be made since the history of the shell is unknown. Nevertheless, such alteration in the distribution of these major components suggests that the mollusc was subjected to a stress condition that possibly led to an alteration in the metabolic processes or of the growth rate, resulting in a different incorporation of these elements during the formation of the shell[43,54].

Adsorption of Cd from aqueous solution onto powdered scallop shells

The effectiveness of seashells in the adsorption of several heavy metals cations has already been demonstrated[8–15].

In this work, we focus on the interaction of Cd on surface scallop shells, however, to quantify the adsorption of this material for the comparison with literature data, adsorption experiments on powdered scallop shells were carried out as well. The aim of these experiments was to investigate the capability of the powdered shell to adsorb Cd from solution. The kinetics and thermodynamics of adsorption can depend on particle dimensions, however in this study we were mainly focusing on the mechanisms of the adsorption instead of in maximising the adsorption capability.

In order to evaluate the adsorption properties of powdered scallop shells employed in the present study, the kinetics and thermodynamics of the adsorption process was investigated, details of the obtained results are reported in the Supplementary Information.

The experiments were carried out with powdered scallop shells characterised by particle dimensions spanning over a broad range, with an average value of 15 μm (see Supplementary Figure S4).

The adsorption properties of shells towards Cd^{2+} were evaluated at pH 7.0-8.5, measured at the end of the equilibration time of the batch experiments to account for shells dissolution (see Supplementary Discussion S1). The experiments were conducted at two constant temperatures, 21.0 ± 0.5°C and 9.0 ± 0.5°C, the latter to mimic seawater temperature in the region more closely.

The adsorption kinetics was investigated in a series of experiments for which the uptake was measured at different contact times.

The adsorption kinetics obtained at three different initial concentrations are reported in Figure 2a.

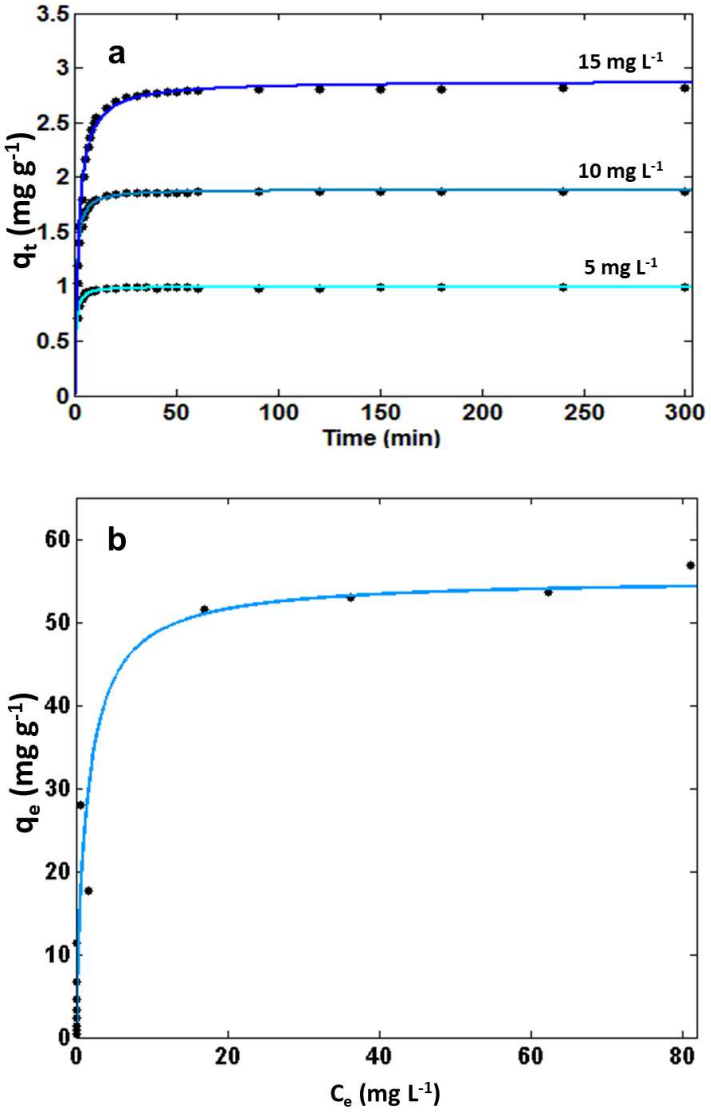

Figure 2. a) Effect of contact time on the adsorption of Cd onto scallop shell powder at three different initial metal concentrations; b) Adsorption isotherm of Cd onto scallop shell powder (21°C).

The adsorption was very fast, with the equilibrium reached within 30 min, and the majority of Cd removed in the first 15 min, for all the initial concentrations considered. These results are comparable with those found by Jeon[55] for the adsorption of Cd onto grinded pen shells.

The very fast Cd uptake highlights the applicability of scallop shells as adsorbents in water remediation technologies; moreover, the short time needed for reaching equilibrium makes this material suitable also for continuous flow systems[56].

The experimental data were fitted to the pseudo second order (PSO) kinetic model[9,14,57]:

$$q_t = \frac{k_2 \cdot q_e^2 \cdot t}{1 + k_2 \cdot q_e \cdot t} \quad (2)$$

Where q_t is the amount of Cd adsorbed at time t, q_e is the quantity adsorbed when the equilibrium is reached, and k_2 the pseudo second order rate constant. The parameters obtained from the fitting are reported in Supplementary Table S1.

The good applicability of this model is usually associated with the situation when the rate of direct adsorption/desorption process (seen as a kind of chemical reaction referred to as ‘‘surface reaction’’) controls the overall sorption kinetics[58]; for further details see Supplementary Discussion S2.

Adsorption isotherm

The data obtained from batch experiments at the constant temperatures of 21°C and 9°C were fitted using the Langmuir isotherm model[59]:

$$q_e = \frac{q_{max} \cdot b \cdot C_e}{1 + b \cdot C_e} \quad (3)$$

where q_e is the amount of Cd adsorbed at the equilibrium (mg g^{-1}), C_e is the equilibrium concentration (mg L^{-1}), q_{max} is the saturation capacity of shell powder (mg g^{-1}) and b the adsorption constant (L mg^{-1}). Fig. 2b shows the isotherm of Cd on scallop shell powder at 21°C (see Supplementary Figure S7 for the isotherm at 9°C): the curve has a concave shape, and it is characterised by a steep initial zone and a saturation plateau. The shape of the isotherm indicates a favourable adsorption of the metal onto the adsorbent material. This information, together with the relatively fast adsorption kinetics (see supra), confirms that scallop shell is a promising adsorbent for Cd. The Langmuir isotherm model refers to homogeneous adsorption without taking in consideration adsorbate-adsorbate and adsorbate-solvent interactions, moreover, the adsorption sites are energetically equivalent.

The parameters obtained from the fitting are summarised in Supplementary Table S2.

Moreover, the saturation capacity of the scallop shell powder (55.3 ± 7.4 mg g^{-1}) is much higher compared to that obtained with pure calcium carbonate powder (12.8 ± 1.6 mg g^{-1}, see Supplementary Figure S8) demonstrating that the composition of the biogenic shell matrix significantly enhances the capability of removing Cd from water matrices.

Structural and thermal analyses

The mineral phases occurring in scallop shells were identified by examination of XRD patterns. Qualitative mineralogical analysis revealed that calcite was detected in all the analysed samples. The coexistence with otavite, $CdCO_3$, in the Cd-treated scallop shells can be inferred from inspection of the 20–60° 2**θ** region (i.e. 2θ=23.56, 30.35, 36.52, 43.92, 49.60 50.02°) showing the increased complexity of the diffraction pattern of samples containing two carbonates with respect to sample with only calcite (Fig. 3). Consequently, the process involved in Cd uptake by calcite was adsorption and Cd diffusion into the calcite crystal, leading to the formation of $(Cd_xCa_{1-x})CO_3$ solid-solution. At the same time, the occurrence of otavite in the Cd-loaded Scallop shells sample indicated that shell calcite substrate immersed in aqueous solutions containing Cd^{2+} acted also as passive surface, leading to dissolution of calcite and nucleation of otavite[60]. Finally, the presence of additional and weak reflections in the 20–35° 2θ range (i.e. 2θ = 18.2, 20.5, 22.7, 25.5 and 32.2°) in Cd-scallop shells is reasonably related to polyenes (i.e. astaxanthin)[61,62], in the carbonate matrix. This result is in good agreement with the Raman spectra indicating the presence of a low fraction of pigments increasing the Cd adsorption efficiency of the biogenic $CaCO_3$ compared to geologic one[63,64].

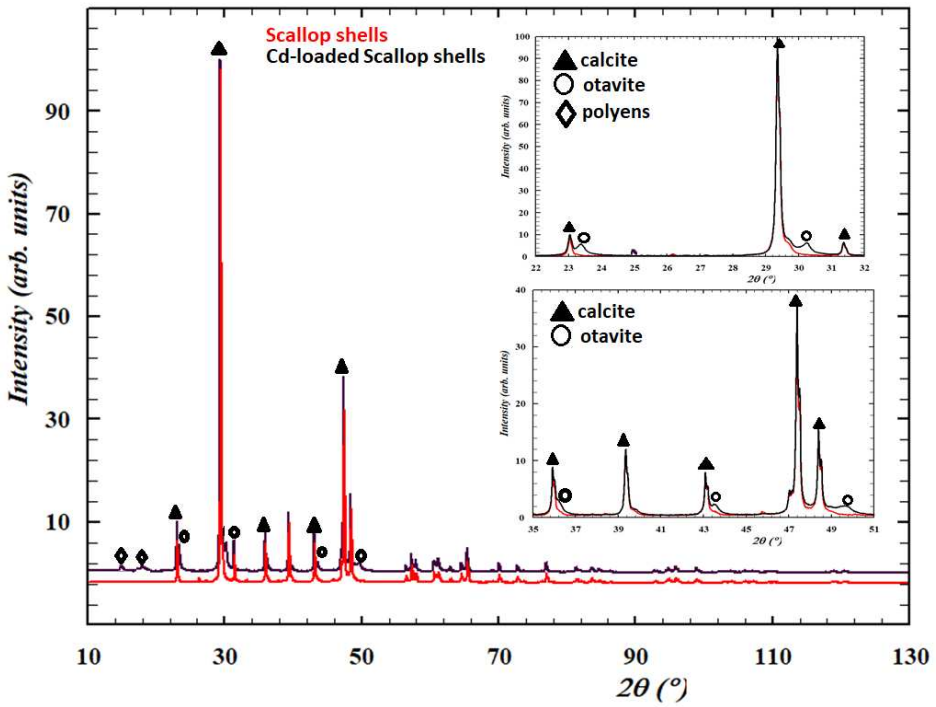

Figure 3. Comparison between XRD patterns of scallop shells (red line) and Cd- scallop shells (black line) respectively.

In order to quantify the phases content in Cd-loaded Scallop shells, quantitative phase analysis (QPA) by use of the Rietveld method was performed. It is well known that this approach gives rise to more accurate values compared to any other single technique, such as Fourier transform infrared spectroscopy (FTIR), chemical analysis and electron microscopy[65].
In this procedure, the weight fraction w*i* of each *i*th crystalline component in the multiphase system is calculated from the corresponding refined scale parameter *Si*, according to the equation:

$$wi = \frac{SiMiVi}{\sum_j SjMjVj} \quad with\ the\ normalisation\ condition \sum wi = 1.0 \qquad (4)$$

with *Mi* and *Vi*, the unit cell mass and volume, respectively[66]. Firstly, quantitative refinement was performed by assuming calcite as the only carbonate present in scallop shells. In Cd-treated samples, the quantitative refinement was including both otavite and astaxanthin; the mass fractions obtained by Rietveld refinements are 90 wt% calcite, 9 wt% otavite and 1 wt% astaxanthin.

In addition, a correlation between the refined unit cell parameters of calcite and the Cd incorporation was noted (Table 2). In order to explain these variations, we suggest that in our samples the compression of the refined unit cell volume can be mainly ascribed to an increase of the Cd content due to the smaller ionic radius of Cadmium (0.95 Å) with respect to Calcium (1.06Å)[67].

Table 2 Refined unit cell parameters for scallop shells not treated and scallop shells treated with solution containing Cd.

		Scallop shells				
Calcite	Space Group	a (Å)	c(Å)	α=β(°)	γ(°)	Volume(Å^3)
	$R\bar{3}c$	4.9987(1)	17.1099(3)	90	120	370.24(1)
		Cd-loaded Scallop shells				
	Space Group	a(Å)	c(Å)	α=β(°)	γ(°)	Volume(Å^3)
Calcite	$R\bar{3}c$	4.9981(2)	17.1109(5)	90	120	370.18(2)
Otavite	$R\bar{3}c$	4.9642(2)	16.2980(8)	90	120	347.83(3)

The trend of DTA and TG curves for both scallop shells samples are represented in Fig 4. Below 100 °C, DTA curves showed an endothermic peak weakly indicating low amounts of absorbed water. The occurrence of otavite was confirmed by a broad endothermic peak between about 370 and 460°C due to the decomposition of $CdCO_3$ into CdO under atmospheric pressure[68,69]. In the same temperature

range the polyenes decomposition also occurred[62,70]. Endothermic effects in the 720–900°C temperature range are ascribable to the CO_2 releasing by $CaCO_3$ decomposition. Lastly, the exothermic peak above 900 °C can be interpreted as the crystallisation of newly formed minerals.

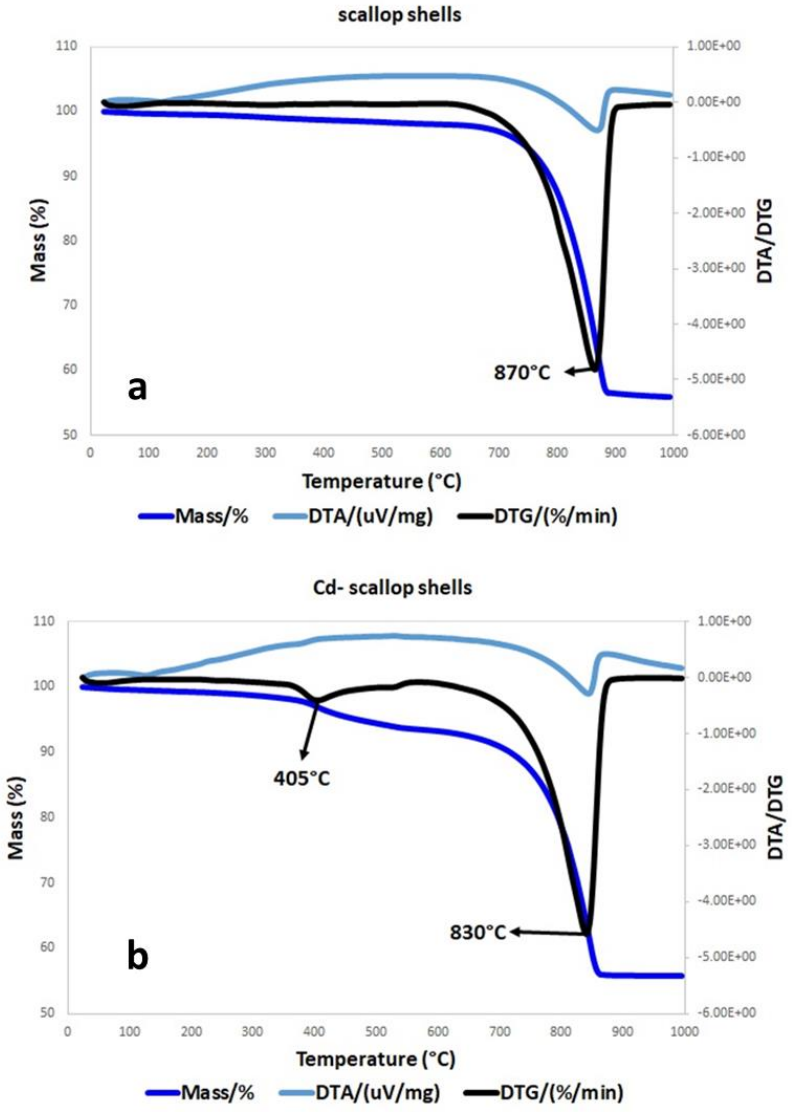

Figure 4. Comparison between TG, DTG and DTA curves of scallop shells (a) and Cd- scallop shells (b) one in the 25-1000°C temperature range, respectively.

The adsorption experiments performed using powdered scallop shells illustrated above were carried out to determine the mean values of Cd uptake obtained for a given particle distribution. To investigate the contaminant distribution and interactions with the shell matrix, intact scallop shell valves were used.

Cd profiles after adsorption

To study the distribution of the metal contaminant through the shell layers, Cd profiles were obtained from scallop shells treated with aqueous solutions containing Cd. Since scallop shells having different colours showed difference in composition, we selected scallop shells with three different colourations (white, pink and brown) to investigate potential differences in Cd adsorption characteristics. The colouration of mollusc shells is mainly due to the presence of organic pigments, mostly tetrapyrroles, carotenoids and melanins[71]. Not only the colour and pattern of shells vary between species, but different colouration can also occur among a single species, or even a single shell can present differently coloured areas[72].

LA-ICP-TOFMS Imaging

In Figure 5 the Ca, Sr and Cd distribution images of three scallop shell samples, white, pink and brown, treated with Cd solution, are reported. Calcium carbonate ($CaCO_3$) consists of 40.0% calcium. The images indicate that Ca, most probably present as calcium carbonate, is the main component of the shells. Slight variations of the Ca content were observed and have been already reported for Ca in bivalves[73]. Generally, they can be related to seashell metabolic variations or different environmental conditions. Sr is present in concentrations around 1000 mg kg^{-1} on the entire section considered and it shows more marked variations in the concentration along the shell cross section. The high-resolution images show distinct layers with different Sr concentration parallel to the inner and outer surfaces, suggesting a different incorporation rate for Sr. It could be correlated to the temperature influence on the shell growth rate[54].

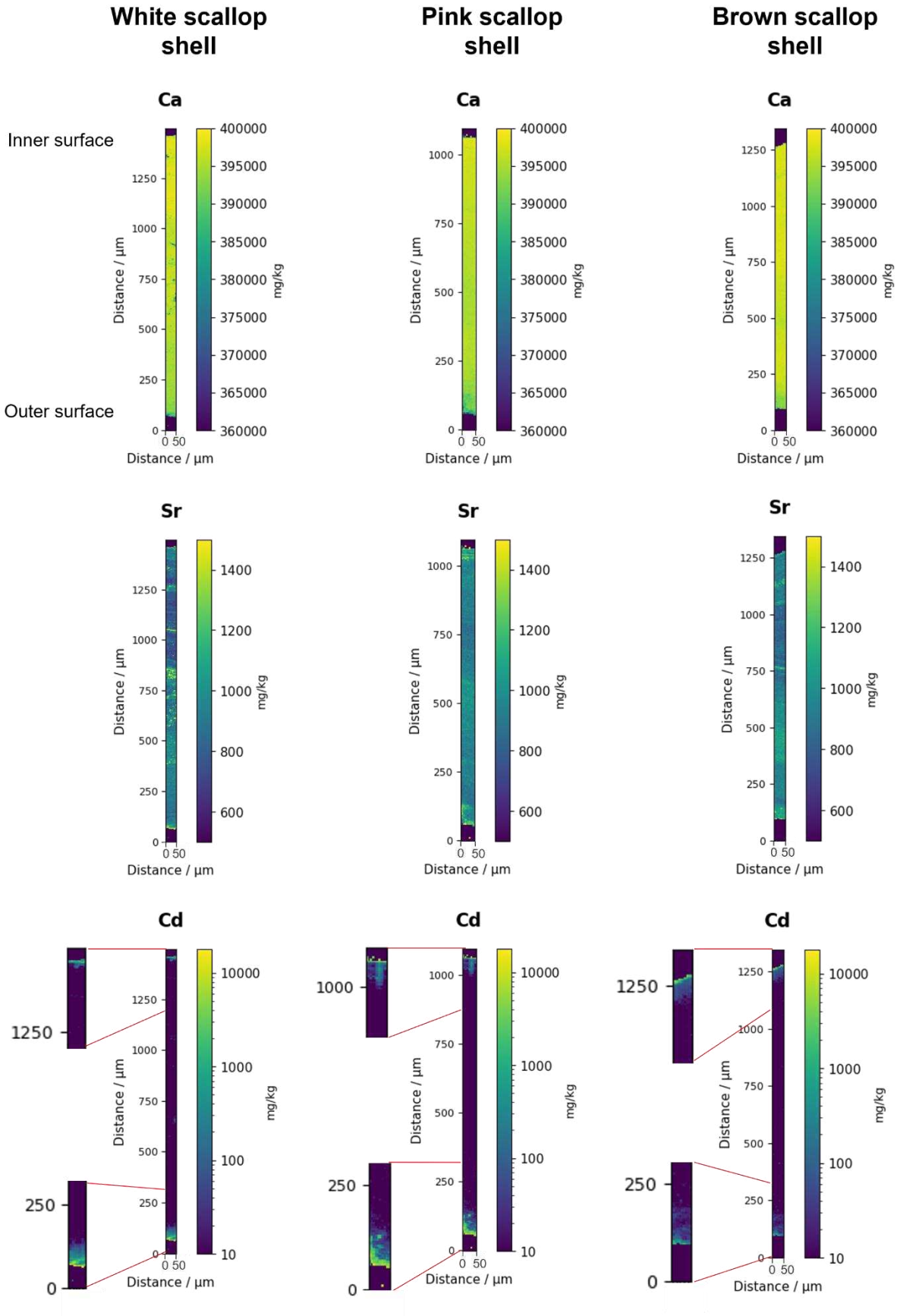
White scallop shell
Pink scallop shell
Brown scallop shell
Ca
Sr
Cd
Inner surface
Outer surface
Distance / µm
mg/kg

Figure 5. Ca, Sr and Cd images of the cross section of three scallop shells, treated with 1 mg L^{-1} Cd solution. After adsorption, Cd is located on a thin layer on the surfaces of the shells.

The Cd images of the three specimens show the metal distribution, with high concentrations on a thin layer located on the surfaces of the shell. Most of the adsorbed Cd was within the first 10 μm on both the inner and outer surface. More specifically, Cd is present in high amounts on a thin layer on both the inner and the outer surface of the shell; however, a minor concentration of Cd is found in the first 50 μm towards the internal layers on both the boundary surfaces.

Cd bulk concentration and micro-Raman

To further investigate the role of shell pigment on adsorption, batch experiments were carried out by using scallop shells with three different colourations (white, pink and brown). As reported in Fig. 6, the Cd uptake (q_e) is lower for the white shells and it increases for the pink and brown shells, with the latter showing the highest uptake values. The difference in metal adsorption, especially between white and brown shells, is more evident when the Cd concentration in the aqueous solution increases (Figure 6b). These findings suggest that the components of the shell matrix responsible for its colouration enhances the metal adsorption. Evidence of the higher uptake of metals by the coloured shells rather than the white ones is also accentuated by the bulk concentration analyses (Table 1) which showed higher content of many metals, including Fe, Cu, Zn, and Pb, in the pink and especially in the brown shells.

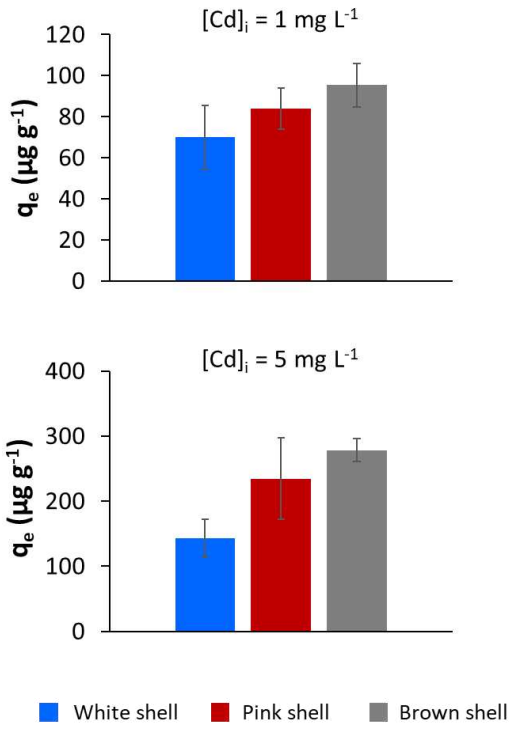

Figure 6. Cd uptake (q_e) with standard deviation (n = 3) of scallop shells with different colouration treated with 1 mg L^{-1} (a) and 5 mg L^{-1} (b) Cd aqueous solution.

To examine in depth this particular feature, we investigated the nature of the pigments contained in our samples employing the micro-Raman technique[72,74–76].

In Fig. 7 the Raman spectra acquired on the outer surface of scallop shells presenting different colours are reported. The white shell spectrum (Fig. 7a) presents only the bands at 1083, 707 and 274 cm^{-1}, belonging to the calcite matrix, while the spectra of the pink and brown samples (fig. 7b and 7c respectively) show two more bands, with higher intensities for the darker shells. The bands observed at ca. 1120 and 1500 cm^{-1} can be attributed to the stretching modes of the C=C double bond (v_1) and the C-C single bond (v_2) due to the presence of molecules characterised by a polyacetylenic chain[72,74,77]. In the Raman spectrum of the brown shell, we observed to more bands with much lower intensity at 1010 cm^{-1} and 1292 cm^{-1}, the former can be attributed to the CH=CH out-of-plane wagging mode (v_4) and the latter to the CH=CH in-plane rocking mode (v_3)[72].

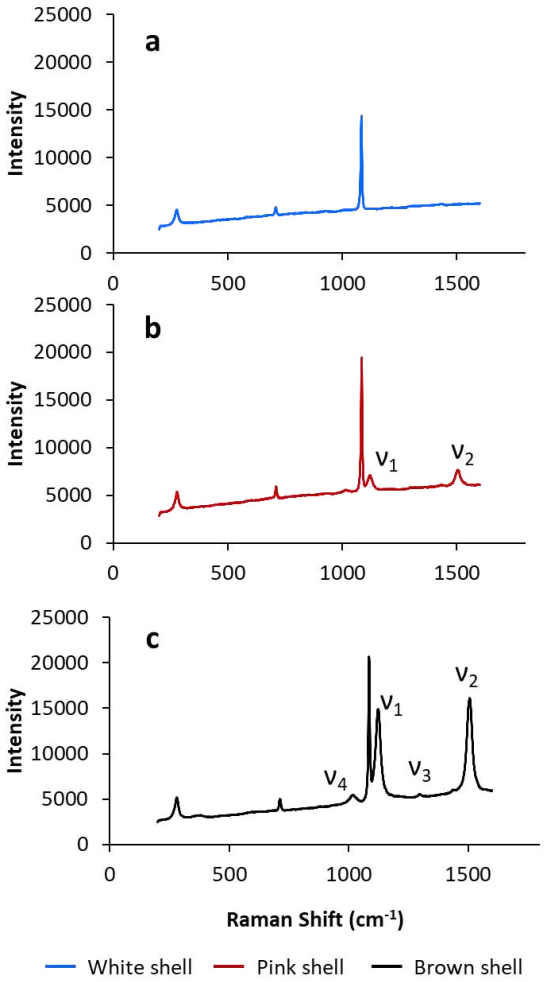

Figure 7. Micro-Raman spectra of white, pink and brown shells

The use of micro-Raman technique allowed us to identify the nature of the scallop shell pigments as polyenes, possibly belonging to the class of carotenoids by comparison with literature spectra[72,74–76]. The presence of these components in the carbonate matrix results in the enhancement of the adsorption efficiency of the biogenic $CaCO_3$ towards Cd compared to commercial $CaCO_3$[16]. This result is in good agreement with the XRD data indicating the presence of a low fraction of pigments increasing the Cd adsorption efficiency of the biogenic $CaCO_3$ compared to geologic one[63,64].

Summary

The current work focused on the study of the mechanism of adsorption of the heavy metal cadmium onto scallop shells; in particular, the interactions between the metal and the components of the shell matrix were evaluated.

The bulk composition of shells taken from a deposit site was determined with regards to their colouration. In general, the brown shell showed higher amounts of Mn, Fe, Co, Cu, Zn, Ba, and Pb than the white

and pink ones; regarding the contaminant investigated, Cd content was close to the limit of quantification of the method, indicating that the environment in which the shells were collected was not severely polluted by Cd.

LA-ICP-TOFMS was employed to investigate the distribution of major components and trace metals along the shell cross-section; Na, Mg and Sr showed minor variability between the different shells and within the layers of the same shell; in particular, Sr showed distinct layers with different Sr concentration parallel to the surface, possibly correlated to environmental factors such as temperature. Trace metals, such as Fe, Zn and Pb showed variations in their distribution across the shells cross section, especially for the brown specimen.

Powdered scallop shells, with an average particle dimension of 15 μm, were employed to investigate the capability to adsorb Cd. The kinetic experiments highlighted that the adsorption is a very fast process, reaching the equilibrium within the first 30 minutes.
The determination of the adsorption isotherm, obtained at 9 and 21°C, allowed to determine the saturation capacity of the material which resulted to be temperature dependent. In particular, at 21°C the saturation capacity resulted to be 55.3 ± 7.4 mg g^{-1}, much higher than that obtained using commercial $CaCO_3$ (12.8 ± 1.6 mg g^{-1}).

The analysis of XRD patterns of the scallop shell powder before and after Cd adsorption showed that the material is mainly composed of calcite, while, after the contact with the Cd containing solution, the presence of otavite ($CdCO_3$) phase was observed, as confirmed by DTA and TG curves. These findings, in addition with the kinetic experiments where an increase of Ca concentration in solution was observed during the adsorption, demonstrate that the adsorption process involves mainly an ion exchange between Ca and Cd.

The distribution and interactions of the Cd contaminant with the shell matrix were investigated using intact shell valves sorted by colour. High-resolution LA-ICP-TOFMS imaging allowed to assess Cd distribution after adsorption; an enrichment of Cd was found on a thin layer (10 μm) on both the inner and outer surfaces of the shell, with a minor Cd concentration found in the first 50 μm towards the internal layers.

For each shell, the total Cd uptake was evaluated: the white shells showed the lower Cd adsorption, while for the pink and brown shells the metal uptake increased, with the brown shells showing the highest adsorption values.

The nature of the pigments contained in the differently coloured shells was determined by micro-Raman spectroscopy; the Raman spectra show the bands typical of polyenes, possibly carotenoids, as also highlighted by the astaxanthin phase present in the XRD patterns.

Conclusions

The results reported provide new information on the mechanism of adsorption of Cd onto biogenic $CaCO_3$. Structural and thermal analyses highlighted the presence of cadmium carbonate phases in the scallop shells treated with aqueous solutions containing the metal contaminant, indicating that the adsorption is predominantly a superficial process involving the partial dissolution of superficial calcite and the nucleation of cadmium carbonate.

Cd is adsorbed on the inner and outer surfaces when intact scallop shells are used as adsorbent. The bulk concentration values showed that the metal adsorption depends on the mollusc species and on the quantity of organic substances (i.e. pigments) present in the carbonatic matrix. Indeed, we found higher cadmium uptake for scallop shells containing larger amounts of organic substances, even if the exchange with carbonate is the dominant adsorption mechanism. The pigments were identified as carotenoids, in particular, the XRD pattern showed the presence of astaxanthin phases which can act as complexing agent towards Cd, increasing its removal from aqueous media.

ACKNOWLEGMENTS

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Plastic ingestion by Atlantic horse mackerel (*Trachurus trachurus)* from central Mediterranean Sea: A potential cause for endocrine disruption☆

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ABSTRACT

Plastics in the oceans can break up into smaller size and shape resembling prey or particles selected for ingestion by marine organisms. Plastic polymers may contain chemical additives and contaminants, including known endocrine disruptors that may be harmful for the marine organisms, in turn posing potential risks to marine ecosystems, biodiversity and food availability.

This study assesses the presence of plastics in the contents of the gastrointestinal tract (GIT) of a commercial fish species, the Atlantic horse mackerel, *Trachurus trachurus,* sampled from two different fishing areas of central Mediterranean Sea. Adverse effect of plastics occurrence on *T. Trachurus* health were also assessed quantifying the liver expression of vitellogenin (VTG), a biomarker for endocrine disruption.

A total of 92 specimens were collected and morphometric indices were analysed. A subgroup was examined for microplastics (MP < 1 mm) and macroplastics (MaP >1 cm) accumulation in the GIT and for VTG expression.

Results indicated that specimens from the two locations are different in size and maturity but the ingestion of plastic is widespread, with microplastics (fragments and filaments) abundantly present in nearly all samples while macroplastics were found in the larger specimens, collected in one of the two locations. Spectroscopic analysis revealed that the most abundant polymers in MP fragments were polystyrene, polyethylene and polypropylene, whereas MP filaments were identified mainly as nylon 6, acrylic and polyester. MaP were composed mainly of weathered polyethylene or polypropylene.

The expression of VTG was observed in the liver of 60% of all male specimens from both locations.

The results of this study represent a first evidence that the ingestion of plastic pollution may alter endocrine system function in adult fish *T. Trachurus* and warrants further research.

1. Introduction

The occurrence and accumulation of plastic debris in the environment, especially in water, is recognised as a problem of major concern all around the world. The threat that plastics represent to the water ecosystem and, eventually, to human health, has been gaining more attention over the last few years.

Plastics in large quantities have been detected in accumulation zones (e.g. the North Atlantic and the South Pacific subtropical gyre) (Law et al., 2010; Eriksen et al., 2013), and, in less amounts, in the remote polar regions (Cincinelli et al., 2017; Morgana et al., 2018).

After the first testimony in 1980 of visible plastic in the Mediterranean Sea (Morris, 1980), many studies have described the presence of plastic debris in this semi-enclosed basin (Collignon et al., 2012; Suaria and Aliani, 2014; Cózar et al., 2015; Simon-Sánchez et al., 2019; Garofalo et al., 2020) thus identifying the Mediterranean Sea as a great accumulation zone for plastic particles. This is likely related to its densely populated coasts (Cózar et al., 2015; Vlachogianni et al., 2020) and its long water residence time, up to a century (Lacombe et al., 1981). Plastic pollution, linked to the high concentration of economic activities

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characterising the Mediterranean Sea, is expected to negatively affect the variety and abundance of the marine species populating this basin, as well as human health (Avio et al., 2017).

Plastic debris is generally sorted based on their size and their origin. According to the classification suggested by Hartmann et al. (2019), plastic particles can be divided into nanoplastics (1–1000 nm), microplastics (1–1000 μm), mesoplastics (1–10 mm) and macroplastics (1 cm and larger).

According to their origin plastic particles can be divided in primary microplastics, manufactured to be already microscopic for various industrial or domestic applications such as in toothpaste, cosmetics and in clothing in fishing nets, or in air blasting technology to remove rust and paint from machinery, and secondary microplastics, originating from the fragmentation of bigger plastic debris caused by photo-degradation, physical fragmentation, and biological and chemical processes (Auta et al., 2017).

Microplastics, both primary and secondary, occur in the marine environment in a variety of shapes and colours (Botterell et al., 2019), and the most common synthetic polymers likely to be found are polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC) and polyethylene terephthalate (PET) (Andrady, 2011).

Large plastic debris can be a threat to marine species by entanglement whereas smaller plastic particles can be ingested by fish accidently or because they resemble natural food sources due to their shapes and colours (Foekema et al., 2013). Indeed, microplastics have been identified in several marine organisms, from fish (Lusher et al., 2013; Bellas et al., 2016; Mancia et al., 2020), to seabirds (Cole et al., 2011) and mammals (Nelms et al., 2019). The macro and microplastics accumulated in the gastrointestinal tract (GIT) after ingestion can cause not only physical damage, such as blockage, reduced feeding stimuli and inhibition of gastric enzyme secretion, but also can lead to chemical effects as a consequence of the release of toxic plastic additives or waterborne persistent organic pollutants (POPs) adsorbed onto the plastic debris (Li et al., 2016). In fact, the lipophilic properties of plastic materials make them capable to adsorb and transport organic pollutants that could leach once the plastic particles have entered the organism, with possible adverse effects. The most common polymer particles, such as PE, PP, PS and PVC, have demonstrated to be efficient adsorbents for organic contaminants like polycyclic aromatic hydrocarbons (PAH), dichlorodiphenyltrichloroethane (DDT), chlorinated benzenes and pharmaceuticals (O'Donovan et al., 2018).

Adverse effects such as tissue damages, neurotoxicity and endocrine disruption produced by ingestion of plastics by marine organisms have been reported (Rochman et al., 2014; Mak et al., 2019; Wang et al., 2019; Barboza et al., 2020) but the contribution that adsorbed contaminants may have to the toxicity of ingested plastics is not yet clearly understood. A study in adult medaka fish reported the higher effects in endocrine disruption after the ingestion of marine plastics in comparison with virgin plastics, suggesting that chemicals associated with the complex matrix of marine plastic debris compete with the endogenous oestrogen for the binding to the oestrogen receptor (Rochman et al., 2014).

The Atlantic horse mackerel (*Trachurus trachurus*, Linnaeus, 1758) is a gregarious bentho-pelagic species belonging to the Carangidae family. This species is commonly distributed in the North East Atlantic, from Cape Verde Islands to the Norwegian and North Seas, as well as in the Mediterranean and Black Seas, on shelf seas reaching depths down to 200 m (Comesaña et al., 2008; Ferreri et al., 2019). The Atlantic horse mackerel is a zooplanktophagous fish feeding mainly on euphausiid crustaceans. Fish larger in size can be found at lower depths where they also feed on teleosts (Rumolo et al., 2017; Šantić et al., 2005).

Atlantic horse mackerel is abundant in many pelagic and demersal food webs (Juan-Jordá et al., 2013; Carrozzi et al., 2019). Based on this, this species is vulnerable to plastic accumulation when feeding on lower trophic levels, as well as it can be a plastic vector towards the higher trophic levels such as elasmobranches and larger Scombridae (Karakulak et al., 2009).

Most of the studies on the uptake and the outcomes of microplastics ingestion on marine species are conducted under precise laboratory conditions, using model organisms such as medaka and zebrafish, feeding the specimen with virgin or treated plastics (Rochman et al., 2014, 2017; Limonta et al., 2019; Mak et al., 2019; Wang et al., 2019).

To the best of our knowledge, only few works study the potential adverse effects of marine plastics ingested by marine biota in wild populations (Alomar et al., 2017; Barboza et al., 2020; Mancia et al., 2020).

Herein, we present a study on the presence and toxicological effects of micro- and macroplastics in *Trachurus trachurus* (TT), an important fish species both under the economic and ecological point of view, caught by the large Italian fleet engaged in trawl fishing in two important fishing areas of south-central Mediterranean.

The gastrointestinal tract (GIT) of TT was treated to isolate, characterise, and quantify the plastic debris. Additionally, a potential link between the occurrence of plastics and adverse effects on TT health status was evaluated by investigating the expression of vitellogenin (VTG), a lipoprotein produced in female fish and used as a biomarker for endocrine disruption (García-Reyero et al., 2004; Biales et al., 2007).

2. Materials and methods

2.1. Samples collection

A total of 92 specimens of TT were collected in the Strait of Sicily and caught by 2 commercial fishing trawlers and more precisely 50 (29 females and 21 males) south of Mazara del Vallo (hereinafter MDV) and 42 (13 females and 29 males) south of Lampedusa island (hereinafter LMP) between April and May 2018. According to the FAO General Fisheries Commission for the Mediterranean (GFCM), the two fishing grounds, MDV and LMP, are located in the Geographical Sub-Area GSA 16 and GSA 13, respectively. (Mancia et al., 2020; see SI-1 Fig. S1, Table S1). After collection, the samples were frozen and stored at −20 °C. Once in the laboratory, the samples were carefully washed with MilliQ water and sectioned to remove the entire GIT (from oesophagus to vent), the liver and the spleen. To avoid plastic contamination from the container, the GIT was wrapped in aluminium foil, whereas the liver and spleen were fixed in RNAlater and stored in separated containers. All the samples were kept at −20 °C until the analysis.

2.2. Biometric data

Fish evisceration was followed by biometric data recording: total length (TL), body weight (BW), liver, spleen and GIT weight (LIV W, SPL W, GIT W, respectively), gender and maturity stage. Visceral weight (VW) was calculated by subtracting the carcass weight (CW) to the BW. All the weights were measured using Sartorius balance (model: MSEE6202P-000-D0) to an accuracy of 0.01 g.

The Medits (International Bottom Trawl Survey in the Mediterranean) scale (with some modification) was used to define the sexual maturity by 7 stages of gonadic development: Stage 1 - immature; Stage 2 - virgin-developing; Stage 3 - recovering; Stage 4 - maturing; Stage 5 - mature; Stage 6 – spawner/spent; Stage 7 – resting (Anonymous, 2017).

2.3. Morphometric indices

The condition factor (CF), the spleno-somatic index (SSI), the hepato-somatic index (HSI), the GIT somatic index (GSI), the visceral somatic index (VSI) and the carcass somatic index (CSI) were calculated as previously described (Mancia et al., 2020) (see SI-1 Table S1).

2.4. Isolation and identification of plastic

Microplastics from the GIT were isolated following one of the protocols proposed by Dehaut et al. (2016). The procedure, described in detail in a previous work (Mancia et al., 2020), involved the digestion of the GIT in KOH 10% at 60 °C for 24 h, followed by a density-based separation using a NaCl hypersaline solution. The supernatant collected after the separation was filtered with 8 μm nitrocellulose filter (Whatman); filters containing undigested organic residues or minerals compromising the visualisation of MP, were further digested with a basic solution following the procedure reported in Roch and Brinker (2017) and rinsed with a HCl 0.1 M solution. The suspension was then collected and filtered.

Contamination was avoided washing and rinsing thoroughly with MilliQ water all glassware equipment before use, covering with aluminium foils all the materials, keeping filters in glass petri dishes. Personal protective equipment (gloves, lab coats and glasses) were used during the entire procedure. To account for airborne MP contamination, blank samples were prepared and analysed as controls following the same procedure as the samples (the control procedure was repeated ten times, n = 10).

The filters obtained (samples and blanks) were observed with a stereo microscope (Nikon SMZ745T stereomicroscope equipped with a Nikon Digital Sight DS-F12 camera) to visualise, count and sort the MP.

The blank filters contained only white fibres with an average value of 4.7 (±0.5) fibres per filter.

The identification of MP fragments and filaments to determine the type of plastic material was carried out via Raman spectroscopy, with a LabRam HR800 micro-Raman instrument (Horiba Scientific) as described in Mancia et al. (2020).

MaP debris composition was determined via Fourier-transform infrared spectroscopy (FT-IR) with a Bruker Vertex 70 FT-IR instrument, the FT-IR spectra were recorded between 4000 and 400 cm^{-1}, with a resolution of 4 cm^{-1} and scan number of 128.

Table 1
Sample subset of *T. trachurus* used in the morphometric, plastic quantification and identification, and gene expression analyses (samples denoted by *).

Sample ID	Gender	MAT	TL	BW	CW	VW	CF	SPL W	SSI	LIV W	HSI	GIT W	GSI	Date 2018	LS
TT1	F	5	19.50	63.10	55.30	7.80	0.85	0.05	0.0008	1.65	0.026	2.70	4.28	17-Apr	MDV
TT2	F	5	19.50	59.60	51.60	8.00	0.80	0.09	0.0015	1.12	0.019	2.10	3.52	17-Apr	MDV
TT3	M	5	19.00	55.60	50.70	4.90	0.81	0.06	0.0011	1.02	0.018	2.11	3.79	17-Apr	MDV
TT4*	F	5	20.00	64.10	56.30	7.80	0.80	0.08	0.0012	1.51	0.024	2.74	4.27	17-Apr	MDV
TT5	M	4	20.50	69.80	64.10	5.70	0.81	0.08	0.0011	0.95	0.014	2.85	4.08	17-Apr	MDV
TT6	F	6	17.50	45.40	41.80	3.60	0.85	0.03	0.0007	0.61	0.013	2.73	6.01	17-Apr	MDV
TT7*	F	5	20.00	57.30	51.60	5.70	0.72	0.03	0.0005	0.77	0.013	2.10	3.66	17-Apr	MDV
TT8*	M	4	19.00	50.80	46.60	4.20	0.74	0.08	0.0016	0.88	0.017	1.70	3.35	17-Apr	MDV
TT9*	M	5	18.50	45.50	41.53	3.97	0.72	0.03	0.0007	0.51	0.011	1.75	3.85	17-Apr	MDV
TT10*	M	4	20.00	60.10	54.50	5.60	0.75	0.08	0.0013	0.93	0.015	2.38	3.96	17-Apr	MDV
TT11*	M	5	19.00	50.79	46.90	3.89	0.74	0.04	0.0008	0.64	0.013	1.20	2.36	17-Apr	MDV
TT12*	F	6	21.00	70.10	65.80	4.30	0.76	0.05	0.0007	0.75	0.011	2.85	4.07	17-Apr	MDV
TT13*	M	5	19.00	52.70	47.38	5.32	0.77	0.02	0.0004	1.02	0.019	2.29	4.35	17-Apr	MDV
TT14*	F	4	21.00	73.80	66.30	7.50	0.80	0.07	0.0009	1.50	0.020	2.87	3.89	17-Apr	MDV
TT15*	F	5	23.00	90.90	81.64	9.26	0.75	0.03	0.0003	1.00	0.011	2.96	3.26	17-Apr	MDV
TT16*	M	5	21.50	76.70	70.40	6.30	0.77	0.03	0.0004	1.04	0.014	2.23	2.91	17-Apr	MDV
TT17*	M	5	20.50	74.10	65.80	8.30	0.86	0.08	0.0011	1.18	0.016	2.60	3.51	17-Apr	MDV
TT18*	M	4	20.00	65.70	61.10	4.60	0.82	0.11	0.0017	1.25	0.019	2.63	4.00	17-Apr	MDV
TT19*	M	4	20.00	66.19	59.25	6.94	0.83	0.03	0.0005	1.23	0.019	2.31	3.49	17-Apr	MDV
TT20*	M	4	20.00	68.20	62.40	5.80	0.85	0.10	0.0015	0.95	0.014	3.02	4.43	17-Apr	MDV
TT21*	F	4	19.50	65.40	57.70	7.70	0.88	0.07	0.0011	1.28	0.020	2.84	4.34	17-Apr	MDV
TT22*	F	4	20.50	68.26	61.80	6.46	0.79	0.04	0.0006	1.62	0.024	2.52	3.69	17-Apr	MDV
TT23*	M	5	17.50	46.30	41.21	5.09	0.86	0.05	0.0011	0.66	0.014	1.49	3.22	17-Apr	MDV
TT24*	M	4	20.00	54.80	51.71	3.09	0.69	0.05	0.0009	0.72	0.013	1.79	3.27	17-Apr	MDV
TT25*	F	4	19.00	56.40	49.37	7.03	0.82	0.06	0.0011	1.12	0.020	2.59	4.59	17-Apr	MDV
TT51*	M	4	38.00	466.70	423.16	43.54	0.85	1.30	0.00279	4.00	0.0086	23.19	4.97	21-Apr	LMP
TT52*	F	4	36.00	414.60	374.38	40.22	0.89	0.48	0.00116	4.56	0.0110	15.42	3.72	21-Apr	LMP
TT53*	M	4	37.00	395.90	366.14	29.76	0.78	1.09	0.00275	2.59	0.0065	15.81	3.99	21-Apr	LMP
TT54*	F	4	37.50	450.90	410.20	40.70	0.86	0.56	0.00124	4.09	0.0091	23.68	5.25	21-Apr	LMP
TT55*	F	4	36.00	386.70	349.73	36.97	0.83	0.40	0.00103	4.21	0.0109	13.42	3.47	21-Apr	LMP
TT56*	M	4	36.00	429.60	386.07	43.53	0.92	0.50	0.00116	4.66	0.0108	26.94	6.27	21-Apr	LMP
TT57*	F	5	38.00	479.97	428.22	51.75	0.87	0.31	0.00065	5.61	0.0117	18.35	3.82	21-Apr	LMP
TT58*	M	4	35.50	367.80	338.68	29.12	0.82	0.31	0.00084	2.81	0.0076	19.12	5.20	21-Apr	LMP
TT59*	M	4	37.00	427.24	390.48	36.76	0.84	0.44	0.00103	3.69	0.0086	21.88	5.12	21-Apr	LMP
TT60*	F	4	33.50	346.34	311.73	34.61	0.92	0.54	0.00156	3.49	0.0101	17.22	4.97	21-Apr	LMP
TT61*	F	4	39.00	506.77	457.35	49.42	0.85	0.57	0.00112	6.15	0.0121	16.16	3.19	21-Apr	LMP
TT62*	M	7	32.00	267.10	246.94	20.16	0.82	0.24	0.00090	2.66	0.0100	10.76	4.03	21-Apr	LMP
TT63*	M	6	21.50	87.80	83.45	4.35	0.88	0.05	0.00057	1.27	0.0145	2.42	2.76	07-May	LMP
TT64*	M	6	22.00	87.30	81.80	5.50	0.82	0.07	0.00080	0.68	0.0078	2.41	2.76	07-May	LMP
TT65*	M	6	21.50	79.90	75.90	4.00	0.80	0.06	0.00075	0.95	0.0119	2.56	3.20	07-May	LMP
TT66*	M	6	22.50	89.30	83.50	5.80	0.78	0.04	0.00045	0.86	0.0096	3.16	3.54	07-May	LMP
TT67*	M	6	20.50	73.90	68.80	5.10	0.86	0.06	0.00081	0.82	0.0111	1.99	2.69	07-May	LMP
TT68*	M	6	21.50	81.80	77.60	4.20	0.82	0.04	0.00049	0.84	0.0103	2.64	3.23	07-May	LMP
TT69	M	6	21.50	88.10	82.54	5.56	0.89	0.05	0.00057	0.75	0.0085	2.64	3.00	07-May	LMP
TT70*	M	6	21.00	80.95	75.96	4.99	0.87	0.02	0.00025	0.85	0.0105	2.86	3.53	07-May	LMP
TT71*	M	6	22.50	90.75	86.11	4.64	0.80	0.02	0.00022	0.85	0.0094	2.44	2.69	07-May	LMP
TT72	M	4	20.50	78.50	73.55	4.95	0.91	0.04	0.00051	1.17	0.0149	2.55	3.25	07-May	LMP
TT73	M	6	22.00	98.12	90.38	7.74	0.92	0.05	0.00051	1.50	0.0153	3.83	3.90	07-May	LMP
TT74*	M	6	21.50	79.30	75.38	3.92	0.80	0.06	0.00076	0.70	0.0088	1.61	2.03	07-May	LMP
TT75*	M	6	23.50	112.44	105.21	7.23	0.87	0.06	0.00053	1.57	0.0140	4.61	4.10	07-May	LMP
TT76*	F	6	22.00	87.96	83.11	4.85	0.83	0.03	0.00034	0.61	0.0069	2.57	2.92	07-May	LMP
TT77*	F	6	23.50	103.80	98.20	5.60	0.80	0.10	0.00096	1.15	0.0111	2.77	2.67	07-May	LMP
TT79*	F	6	23.50	102.90	95.94	6.96	0.79	0.09	0.00087	1.36	0.0132	2.77	2.69	07-May	LMP

2.5. Gene expression analysis

RNA Extraction. Total RNA from 30 mg of liver samples of 50 randomly selected samples was extracted using the Qiagen RNeasy Plus Mini Kit (Hilden, Germany) following the manufacturer's instructions. Tissue lysis, homogenization and quantification was carried as previously described (Mancia et al., 2020). RNA samples that did not meet the absorbance ratio cutoff were extracted more than once and/or excluded from further analysis. The total number of good quality RNA samples (1.8–2.0, 280/260; 2.0–2.2, 260/230) used in the following steps was N = 44 (Table 1).

Quantitative real time PCR (q-PCR). TT Vitellogenin (VTG) gene was amplified and quantified using primers designed with Primer 3 on conserved region of the sequence. Primers were designed using the sequences for the Vitellogenin gene publicly available at https://www.ncbi.nlm.nih.gov from 8 species of bony fish (KJ804266, Syacium gunteri; EF582607, *Hippoglossus hippoglossus*; JQ283442, *Dicentrarchus labrax*; MF370511, *Scophthalmus maximus*; AJ416328, Pleuronectes platessa; AJ416327, Platichthys flesus; MG934701, Sciaenops ocellatus; HQ846510, *Morone saxatilis*). The primer sequences 5'->3′ were: Fw: GGATCCCTGCAGTACGAGTT; Rev: CTTCAGAGGGGCATCTTCGT).

1 μg total RNA was retrotrascribed and 10 ng cDNA were used in qPCR as described before (Mancia et al., 2020). reaction was performed in triplicates in 96 wells plates, using the EvaGreen Dye Master mix (Bio-Rad) on CFX Connect Real-Time Detection system (Bio-Rad). qPCR efficiencies of each primer couple were calculated using a five points standard curve with serially diluted 1:5 cDNA from 4 samples (MDV: TT02, F; TT10, M - LMP: TT67, M; TT79, F) (Dhar et al., 2009). qPCR reactions were run as previously described and were run in triplicate with triplicate no-template controls. The average Ct values were normalised to the values of the housekeeping 18 S rRNA (primer sequence 5'->3': Fw: ACCACCCACAGAATCGAGAAA; Rev: GCCTGCGGCTT AATTTGACT) (Filby and Tyler, 2007). PCR amplicons were sequenced, and the identity of the products was confirmed; sequences were submitted at GenBank with Acc. Nos: MT862539 and MT862540 for VTG and 18 S, respectively.

Comparative Ct method of analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control (males not expressing VTG, with VTG Ct values $\geq$ 33; specifically, 10 M from LMP: TT70, TT64, TT51, TT59, TT62, TT56, TT65, TT53, TT58, TT67 and 5 M from MDV: TT24, TT11, TT17, TT09, TT23) and all other samples on CFX connect manager software 3.1 (Bio-Rad). The Pfaffl method based on primer efficiencies was used to calculate fold (Pfaffl, 2001).

2.6. Statistical analysis

Data were analysed within and between locations (MDV and LMP) and gender (male and females) and in relation to presence of plastic (fibres and fragments) in the GIT using Student t-test and ANOVA as previously described (Mancia et al., 2020). Differences between gonadal stage at two sites MDV and LMP in males were analysed using Student t-test with Welch's correction. Spearman correlation was used to identify significant associations ($p > 0.05$) between biometric data.

3. Results and discussion

3.1. Morphometric analysis

Samples were analysed within location (1: MDV; 2: LMP), by gender (3: M; and 4: F), as a whole (5: MDV + LMP) and in relation to high or low plastic load (6: plastic).

In the analysis 1, MDV samples (29 F and 21 M) were used. Females VSI was higher (12%), probably in relation to the liver. HSI is in fact significantly higher as well (12%). A slightly smaller CSI observed in females vs males was probably due to the cyclical effect of reproduction on females muscle mass. In fact, it is known that when the females reach the maturity stage of maturing/mature/spawner, and our MDV samples were mainly at the stage "maturing/mature", their overall body condition appears visibly suffering due to the reproductive investment that, during eggs production, uses the body muscle mass as energy reserves (Mion et al., 2018).

For analysis 2 were used LMP samples (13 F vs 29 M). Similar to the MDV samples, in the LMP females vs males comparisons, VSI was higher (23%) and CSI was slightly smaller (−2%) in females. Both males and females were in advanced gonadal maturity (stage 5 "mature", and 6 "spawner/spent") with a slight forward shift in favour of males (Table 2, A).

In analysis 3 all males of both fishing areas were analysed (50 M). Specimens sampled in the two locations were very different in weight and size. Specifically, MDV males specimens had shorter total length and lower weight, with a smaller CF, smaller organs, and a reduced gonadal maturity than males (i.e. in the stage of maturing and mature) of LMP found in the stage of spawner/spent. The correlation analysis of maturity (gonadal stage) and biometric data in all M sample set ($n = 50$) has shown a statistically significant association with VSI and HSI (VSI, rs (50) = - 0.685, $p = 0.001$; HSI, rs (50) = - 0.461, $p = 0.001$). To avoid a bias in the data related to the higher prevalence of lower maturity in MDV vs LMP site, we analysed a subgroup of individuals M from both MDV and LMP, with a weight between 60 g and 90 g and the same CF (12 M in MDV vs 11 M in LMP). We obtained two groups with larger specimens in LMP, and in the MDV vs LMP analysis, we observed that the carcass is heavier in LMP (−4%, CSI). However, the viscera weighted more in males from MDV, a difference that was not linked to gonadal maturity (−26%), but rather to the weight of liver (53%), spleen (51%) and GIT (23%).

For the analysis 4 all females were used (42 F) and, similarly to male samples, difference between MDV and LMP were observed: MDV specimens had shorter length and a minor weight, with a smaller CF, minor weight of the viscera, at an earlier gonadic stage (Table 2, A). However, there was no significant association of maturity and VSI/HSI, and maturity as observed in the 3 - all males - study ($p < 0.05$).

The analysis 5, using all the available samples (M and F from both locations MDV + LMP), highlighted the difference among the specimens in the two locations, including their maturity (Table 2, B). Females in MDV were smaller (in length and weight) than males, and the total weights and organs weight indexes reflect this difference. These results suggest that, overall, the presence of larger specimens (TL > 300 mm) in the LMP sample could be linked to shallower water that characterise this fishing ground, as well as other correlated oceanographic factors such as higher temperature and salinity of water masses, other than food availability (mainly anchovies and sardines) (Rumolo et al., 2017).

However, morphometric data from the males in MDV may show complications: here, females and males are almost at the same gonadal maturity, and females are expected to have a bigger liver and a smaller GIT than males (which is what we observed in LMP). Instead, we see MDV males with a small GIT and a big liver. A summary of the morphometric results is in Table 2, A and B (see details in SI-2). Furthermore, exposure to contaminants may impact the physiology of fish on many levels and could impact on the reproductive states of the fish populations. Plastic products may be one of the driven causes.

In analysis n. 6 (plastic load), 30 samples (15 from MDV and 15 from LMP, see SI-2) of the 53 analysed for GIT plastic (fibres and fragments) contents were divided in 2 groups: the high exposure group (H) with a total number of fibres + fragments > 90 and the low exposure group (L) with a total number of fibres + fragments < 90. Three specimens from LMP (TT61, TT62 and TT75 with a BW of 506 g, 267 g and 112 g respectively) were removed from the analysis to keep the sample set more homogeneous (BW < 100 g). The H group presents 40% more plastics in the GIT compared to the L group. In the H group, there were n = 8 samples (3 MDV, 2 F and 1 M; 5 LMP all M); in the L group there were n = 18 samples (11 MDV, 4 F and 7 M; 7 LMP all M). Animals from the H group have a bigger spleen (14%). Spleen is a lymphoid organ, and

Table 2
Percentage of statistically significant changes in the morphometric measurement of MDV samples compared to LMP samples (A) and of F vs M (A and B).

A

MDV vs LMP	CF	CSI	VSI	SSI	HSI	GISI	MAT
Females	-5%	-2%	29%	14%	54%	15%	-14%
Males	-5%	-3%	42%	17%	61%	4%	-23%
ALL	-4%	-3%	39%	14%	61%	10%	-20%
F vs M	CF	CSI	VSI	SSI	HSI	GISI	MAT
LMP	1%	-2%	23%	5%	18%	-4%	-11%
MDV	1%	-1%	12%	2%	12%	6%	-2%

B

F vs M	HSI	GISI	MAT
LMP	18%	-4%	-11%
MDV	12%	6%	-2%

its production of lymphocytes is crucial to the activation of the humoral and cellular immune response against pathogens or foreign bodies, such as plastic objects (Mancia et al., 2020).

In order to remove bias linked to the unbalance gender specimens in the H vs L group analysis, we removed the 6 females (H group, n = 6: 1 MDV, 5 LMP; L group, n = 14 samples (7 MDV; 7 LMP). In this comparison, in addition to a significant increase of the spleen in the H group samples, we also see the increase of the VSI (26%) (see SI-2).

3.2. Plastics isolation and composition

The GIT of 53 specimens (25 from MDV and 28 from LMP) were selected to evaluate the occurrence of plastic particles, their quantity and type. The plastic debris detected by visual inspection was classified as MP fibres, MP fragments or MaP according to the classification suggested by Hartmann et al. (2019). Moreover, the MP fibres characterised by a regular diameter along the entire length and with sharp ends were referred to as MP filaments (Fig. 1A) to distinguish them from textile microfibres that could be composed of natural or semi-synthetic

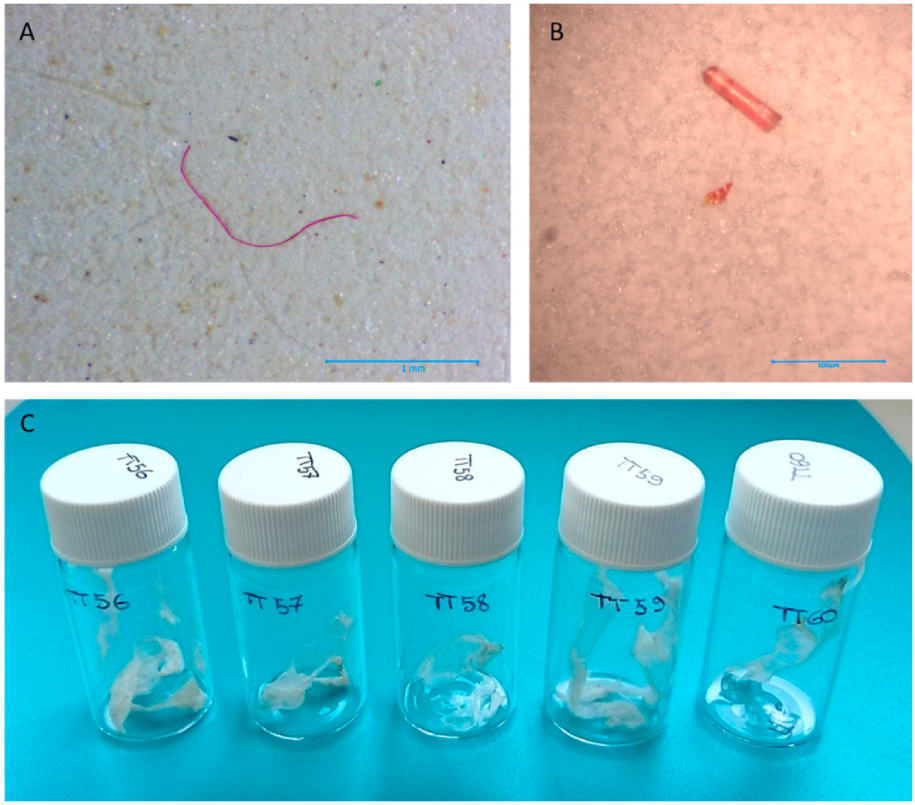

Fig. 1. Microscope images of plastic particles found in different samples. A) MP filament; B) MP fragments; C) Image of undigested MaP from 5 different samples.

Table 3
Number of Microplastics (MP) composed by filaments and fragments, Macroplastics (MaP), average ingested by individual fish and frequency of ingestion.

Catch area	N. specimens	Total number MP	Filaments	Fragments	Average/fish	Frequency of ingestion
GSA 16 MDV	25	99	71	28	3.96	84%
GSA 13 LMP	28	236	108	128	8.43	96.4%
Catch area	**N. specimens**	**Total number MaP**	**Filaments**	**Fragments**	**Average/fish**	**Frequency of ingestion**
GSA 16 MDV	25	0	–	0	0	0%
GSA 13 LMP	28	52	–	52	1.86*	17.8%

*MaP were found in 5 specimen.

materials. As MP fragments were considered that debris with an irregular shape (Fig. 1B), whereas the debris with dimensions larger than 1 cm that was not degraded by the digestion process was considered MaP (Fig. 1C). The number of ingested particles and the frequency of ingestion were employed to consider the presence of plastics (Avio et al., 2020). Microfibers characterised by irregular diameter and frayed ends were detected in all the specimen analysed from both locations, they were considered as anthropogenic particles that could cause adverse effects in the organisms, even though their basic composition may be natural (e.g. cellulose), because they underwent further processes such as dyeing (Collard et al., 2018). MPs, as filaments and fragments, were found in 90.6% of all the specimens; in particular, MP were present in 84% of the fish from MDV and in 96.4% from LMP. Filaments were found in 84.9% of the specimen, in 84% of those from MDV and in 85.7% from LMP; fragments were detected in 73.6% of all fish analysed, with higher frequency in Lampedusa (82.1%) compared to MDV (64.0%). (Table 3). Filaments, most of which were dark coloured (black and blue), were characterised by different length and diameter, the latter from 10 to 30 μm. As regards MP fragments, most of them (about 80%) had dimensions in the range from 10 to 20 μm, about 15% were in the range from 20 to 50 μm, while 5% had the maximum dimension lower than 10 μm. The predominant colours were still black and blue, as for filaments, but percentages up to 15% were represented by green, white, and red fragments (Fig. 2C and D).

The presence of additives in the fibres, such as pigments, or the adhesion of organic residues made the determination of the fibres composition by Raman spectroscopy very difficult most of the time.

Due to the partial identification of fibres constituents that can induce a bias, we limited the composition data only to MP filaments and MP fragments. MP fragments were composed of polystyrene, polyethylene and polypropylene, whereas MP filaments were identified mostly as nylon 6, acrylic and polyester (Fig. 2A and B).

MaP were found exclusively in the specimen collected in LMP (Fig. 1C); in particular, in five fish (TT56 – TT60) belonging to a subset (TT51 – TT60, Table 1) characterised by a higher body weight and longer total length. The MaP were found in large quantities in each fish, with a total number of 52 pieces, with average dimensions of 3 × 1 cm. The spectra obtained were similar for all the samples which, after comparison with library spectra and literature data, resulted to be composed most probably of oxidised PE or PP (see SI-1, Fig. S2). In each spectrum, the bands caused by CH_2 stretching, bending and rocking (in the 3000–2800 cm^{-1}, 1470–1370 cm^{-1} and 730–715 cm^{-1} regions, respectively) characteristic of PE and PP are always present (Jung et al., 2018), moreover, the appearance of an intense band at 1715 cm^{-1} is an indication of the presence of carbonyl groups which are found in oxidised PE (Oelichmann, 1989; Rocha et al., 2010; Gardette et al., 2013). Plastic debris usually undergo photodegradation which involves modifications of the polymer matrix (such as oxidation with the formation of carbonyl groups, chain scission, radical recombination and cross-linking) and morphological alterations (Rowenczyk et al., 2020).

The large quantity of MaP found exclusively in the GIT of the larger specimen subset can be an indication that bigger animals that usually feed on small fish may indeed mistake plastic debris for prey.

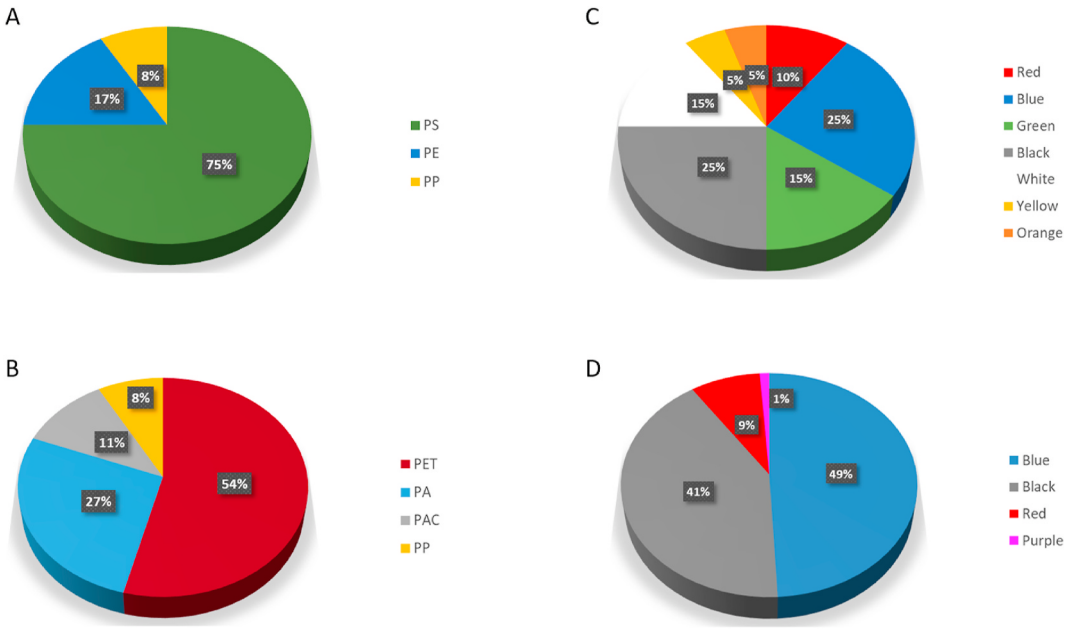

Fig. 2. Plastic debris composition A, B, and colours C, D. A) MP fragments composition; B) MP filaments composition; C) MP fragments colours; D) MP filaments colours. PS polystyrene, PE polyethylene, PP polypropylene, PET polyethylene terephtalate, PA polyamide, PAC polyacrilate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

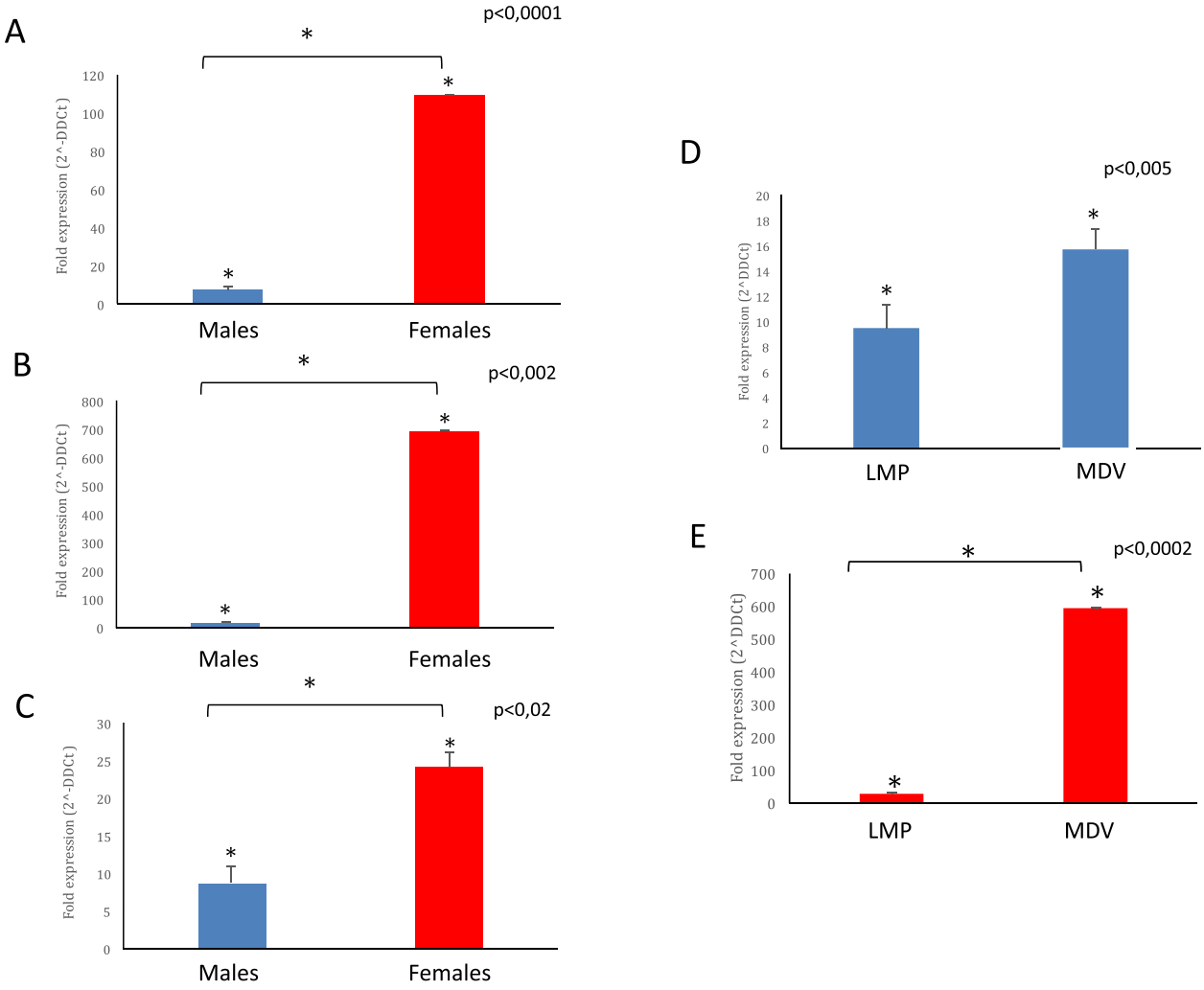

Fig. 3. Expression of vitellogenin genes in T. trachurus liver samples. A) Males and females samples from both locations; B) males and females from MDV; C) males and females from LMP; D) males from LMP and MDV; E) females from LMP and MDV.

3.3. Gene expression

Almost all plastic products have been found to leach endocrine-disrupting chemicals, EDCs (Yang et al., 2011). EDCs have the potential to alter fish reproduction at various levels of organisation. The aim of this part of the study was to assess the impact of a natural environment with heavily anthropogenic influence on the physiological processes involved in reproduction in *T. trachurus* using the vitellogenin (VTG) gene, a specific biomarker. In fact, the expression of the egg yolk protein precursor VTG is a well-established indicator of oestrogen-mediated endocrine disruption in males (Moncaut et al., 2003; Tolussi et al., 2018). Here, hepatic VTG gene expression was evaluated in specimens of TT from both genders, as an indicator of xenoestrogen exposure that could be caused or exacerbated by the presence of plastic.

RNA from the liver of 44 specimens was retrotranscribed and used as template in the real time qPCR of VTG. There were 19 MDV and 25 LMP samples; three of these were removed from further analysis (TT14, TT61, TT74) because the amplification data of the housekeeping control were poor (Filby and Tyler, 2007). Samples were analysed within and between location and gender: A) all samples, M + F from MDV + LMP; B) M + F from MDV; C) M + F from LMP; D) only M from MDV + LMP; E) only F from MDV + LMP). There were no samples without plastic detection, although some of the samples had higher loads (see SI-2).

A) As expected when pooling all the samples and comparing the VTG expression of all females (16 F, 8 MDV, 8 LMP) vs all males (10 M, 5 MDV, 5 LMP) from both locations, TT female's VTG expression was about 100 times more than the male's ($p < 0{,}0001$) (Fig. 3A). Both females and males VTG

Table 4
Morphometric data of the largest specimens collected in LMP.

ID	Gender	TL (cm)	BW (g)	CF	CW (g)	CSI	VW (g)	VSI	SPL W (g)	SSI	LIV W (g)	HSI	GIT W (g)	GITI	Mat
TT52	F	36	414.60	0.889	374.38	0.903	40.22	0.097	0.48	0.001	4.56	0.011	15.42	0.037	4
TT54	F	37.5	450.90	0.855	410.20	0.910	40.70	0.090	0.56	0.001	4.09	0.009	23.68	0.053	4
TT57	F	38	479.97	0.875	428.22	0.892	51.75	0.108	0.31	0.001	5.61	0.012	18.35	0.038	5
TT61	F	39	506.77	0.854	457.35	0.902	49.42	0.098	0.57	0.001	6.15	0.012	16.16	0.032	4
TT51	M	38	466.70	0.851	423.16	0.907	43.54	0.093	1.30	0.003	4.00	0.009	23.19	0.050	4
TT56	M	36	429.60	0.921	386.07	0.899	43.53	0.101	0.50	0.001	4.66	0.011	26.94	0.063	4
TT59	M	37	427.24	0.843	390.48	0.914	36.76	0.086	0.44	0.001	3.69	0.009	21.88	0.051	4

expression were compared to control (males not expressing VTG, details in methods). It was interesting to notice that there is expression of VTG in males and it is statistically significant ($p < 0{,}0001$) (Fig. 3). Another interesting detail was that in the female group there is one specimen (TT54) that does not express VTG. The specimen was in gonadal stage 4 (maturing) so production of VTG was expected. This is one of the large TT from LMP, with all morphometric data similar to other big size females (F: TT52, TT54, TT57, TT61). However, the GIT W and the GITI values are comparable to those of the largest males, which are also males that do not express VTG and are in the control group (M: TT51, TT56, TT59) (Table 4). B) In MDV (5 M, 8 F) the expression of VTG of both males and females is statistically significant ($p < 0{,}002$). The VTG expression in males is 36 times less than in females, but nevertheless, there is VTG expression in males (Fig. 3B). C) In LMP (5 M, 7 F) there is a 16-fold difference in VTG expression between males and females ($p < 0{,}02$) (Fig. 3C). Removing the specimen TT54 increase the difference between male and female but not the male VTG expression result. D) VTG is significantly expressed in males from both locations (5 LMP, 5 MDV, $p < 0{,}005$) and although in MDV specimens the expression is almost double than in LMP specimens, this difference is not significant (Fig. 3D). E) VTG is significantly expressed in females from both locations (8 LMP, 7 MDV, $p < 0{,}0002$) compared to control, as expected. The expression of VTG in MDV females is 22 times that of LMP females, even though there is no difference in maturity stage among specimens of the two groups (Fig. 3E).

To summarise, even though it is clear that the vitellogenin is highly expressed in TT females as expected, there is also a significant expression of the VTG gene in 60% of the TT males analysed, from both MDV and LMP. Moreover, females in LMP showed a lower expression of vitellogenin than in MDV (with one female sample, TT54, not expressing VTG at all).

The endocrine disruption represented by the alteration of VTG expression in TT specimens observed in this work can be caused by microplastic ingestion, as well as by the interactions between the marine organisms and the wide variety of endocrine-disrupting chemicals possibly present in seawater.

4. Conclusions

The southern region of the central Mediterranean Sea is strongly affected by the presence of anthropogenic debris, since microplastics, in the form of fibres and fragments, were detected in the GIT of all the specimens analysed. If textile fibres are excluded, and only filaments and fragments made of synthetic polymers are considered, the ingestion frequency is still high, 84% south of Mazara del Vallo and 96% south of Lampedusa. On the other hand, macroplastics were detected only in the bigger specimen collected in Lampedusa, this can be explained by the fact that only the larger fish feed also on teleosts, besides euphausiid crustaceans, and larger plastic debris could resemble fish prey.

To the best of our knowledge, this study provides the first report on anomalies in the production of vitellogenin in both males and females of *T. trachurus* in the Mediterranean Sea that could be caused by microplastic ingestion, besides endocrine-disrupting chemicals present in seawater. 60% of the male specimen from both locations showed a significant expression of vitellogenin, whereas females specimen showed a high expression, as expected, in south of Mazara del Vallo, but a low expression (in one specimen no expression at all) in south of Lampedusa. From this first examination, it can be hypothesised that microplastics and the potential sorbed chemicals could be a major cause for endocrine disruption leading to variations in physiological processes, in this case oogenesis process and reproduction, of *T. trachurus*. Overall, our study suggests that the ingestion of plastic debris in the environment may alter endocrine system function in economically important fish species and warrants further research.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117449.

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