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MOBILES

D2.1 - Diatoms-based biosensor for bioplastic detection in water

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Deliverable D2.1 – Diatoms-based biosensor for bioplastic detection in water

Short summary: Deliverable D2.1 reports the work performed by CUT in task 2.7. Using spectroscopic methods the research group at CUT, assisted by NTUA, realized spectra libraries for 7 diatom species as well as three different plastic typologies. The studies performed allow to identify, in 7 diatom species, spectroscopic characteristics that change when such organisms are in contact with water containing micro or nanoparticles generated by degrading plastic.

The most relevant change is found in a specific fluorescent peak of *chlorophyll a.* Indeed, when plastic particles enter the diatom cell-body they interact with chlorophyll apparatus and, as result, the absorption spectra changes.
This change can be easily detected by fluorescence spectrometry after only 4-8 hours of exposing diatoms to water containing plastic. Thus, diatoms coupled with simple light excitation and detection systems are excellent and fast biotic sensors able to estimate the presence of tiny materials generated by degrading plastic.

It must be highlighted that the method developed by CUT is not quantitative, therefore only the presence of plastic micro- and nanomaterials can be identified in water. Finally, CUT also developed a second fluorescence spectroscopic method based on diatoms and ferrocyanide to determine the presence of plastic in water that enhances the detection capability of diatoms as well as lower the detection time to less than 4 hours.

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List of participants

Participant No	Participant organisation name	Country
1 Coordinator	ETHNICON METSOVION POLYTECHNION (NTUA)	GR
2	CONSIGLIO NAZIONALE DELLE RICERCHE (CNR)	IT
3	INSTITUT NATIONAL DE RECHERCHE POUR L'AGRICULTURE, L'ALIMENTATION ET L'ENVIRONNEMENT (INRAE)	FR
4	UNIVERSITA DEGLI STUDI DI ROMA LA SAPIENZA (UR)	IT
5	EDEN TECH (EDEN)	FR
6	UNIVERSIDAD PUBLICA DE NAVARRA (UPNA)	ES
7	INSTYTUT UPRAWY NAWOZENIA I GLEBOZNAWSTWA, PANSTWOWY INSTYTUT BADAWCZY (ISSPC)	PL
8	THE AGRICULTURAL RESEARCH ORGANISATION OF ISRAEL - THE VOLCANI CENTRE (ARO)	IL
9	UNIVERSITE DE BORDEAUX (UBx)	FR
10	TECHNOLOGIKO PANEPISTIMIO KYPROU (CUT)	CY
11	HEMIJSKI FAKULTET, UNIVERZITET U BEOGRADU (UBE)	RS
12	MAT4NRG-GESELLSCHAFT FUR MATERIALIEN UND ENERGIEANWENDUNGEN MBH (Mat)	DE
13	TECHNISCHE UNIVERSITAT CLAUSTHAL (TUC)	DE
14	GRANT GARANT SRO (GG)	CZ
15	CENTRUM BADAN I INNOWACJI PRO-AKADEMIA STOWARZYSZENIE (RICPA)	PL

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Content

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SUMMARY

MOBILES project is engaged in understanding distribution, degradation, and life cycle of microplastic (MP) and nanoplastics (NP) in the marine environment. The selected tools to do so are diatoms. Some of such microscopic organisms live in marine environment using light as source of energy. Indeed, via photosynthesis they are capable to produce organic carbon that subsequently is used for all metabolic activities. Nevertheless, if MP and NP contaminate sea water they can be absorbed by diatoms cells and interfere with several biological pathways that allow such cells to survive. This phenomenon, even though detrimental for diatoms, can be used to develop biotic sensors that allow a fast MP and NP detection in marine environments.

MP and NP are produced when plastic starts to breakdown into smaller pieces. Oxo-degradable plastics are conventional plastics, like polyethylene or polypropylene, with additives designed to accelerate their breakdown into smaller pieces when exposed to oxygen, light, and/or heat. Biodegradable plastics are materials that can break down under specific conditions by the action of living organisms, like bacteria, into water, carbon dioxide, and biomass. Marine diatoms possess complex, highly regulated semipermeable membranes vital for nutrient uptake (e.g., N, P, Si, Fe) and stress response. Unfortunately, plastic polymers or monomers that are tiny enough pass through such semipermeable membranes and eventually interacts with subcellular organelles.

In the present work we studied seven different marine diatoms *(Cylindrotheca fusiformis, Phaeodactylum tricornutum, Thalassiosira pseudonana, Chaetoseros sp., Cyclotella cryptica, Fragilariopsis sp and Chaetoceros ceratosporus)* with the aim of using them as biosensor to quickly identify MP and NP in marine environments. The analysis has been performed using spectroscopic techniques such as Raman spectroscopy, UV/Vis absorbance, Laser-induced breakdown spectroscopy, Fluorescent microscopy and FTIR.

We found that once inside the diatom's cell-body MP and NP interfere with the photosynthetic systems and in particular with *Chlorophyll a* (*Chl a*) subunit. Such perturbations induce alterations in the photophysical properties of *Chl a*, ultimately resulting in reduction of fluorescence emission. When MP or NP are uptake by cells, the amplitude of the 680 nm fluorescent peak of *Chl a* reduced its amplitude in relation to the original spectrum.

To assess the suitability of marine diatoms as biosensing platforms, three selected species (*Cylindrotheca fusiformis, Phaeodactylum tricornutum, Cyclotella cryptica)* were exposed to MP and NP for incubation periods ranging from 2 to 8 hours. We found that after 4 hours of incubation the spectroscopic characteristic of diatoms change and it is possible to assess, qualitatively, the presence of MP and NP in the growth media.

Furthermore, the biosensing ability of one species (*Cylindrotheca Fusiformis*), was evaluated also using ferricyanide reagent. These experiments were not initially scheduled on MOBILES proposal, but have been performed as an addition to further improve a rapid and precise assay to identify MP and NP. By adding ferricyanide and exacting it with light at 785 nm, it was observed a more pronounced absorbance reduction of the 680 nm peak of *Chl a* after 2 hour of exposing such a diatom to MP and NP.

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1. MOBILES project short description

The National Technical University of Athens (NTUA) is working with another 15 partners from academia, research, and industry to develop prototypes of electronic and organism-based biosensors to monitor organic chemicals, antimicrobial-resistant (AMR) bacteria, and pathogens in water, soil, and air.

The MOBILES project is studying and developing biosensors for detecting heavy metals, antibiotics, pesticides, arsenic, MP, and NP. It is also developing genetically modified plants and bacteria for detecting heavy metals, antibiotics, and pesticides, and the use of marine diatoms for monitoring bioplastic degradation.

MOBILES aims are to tackle chemicals, including persistent and mobile (PMCs) pollutants and contaminants of emerging concern (CECs), that degrade the environment. Another severe global health risk is associated with increasing AMR in bacteria. Foodborne pathogens, including *Listeria*, *Salmonella*, and *Campylobacter*, pose significant public health risks and are already monitored. However, current bacterial detection methods for environmental control are slow and require specialized laboratories with trained personnel. Similarly, conventional pollutant detection methods, such as chromatography and mass spectrometry, are accurate but time-consuming and require specialized equipment. State-of-the-art detection methods are unsuitable for constant on-site and real-time monitoring. The long time between sampling and detection reduces the efficiency of public health and environmental protection authorities in implementing effective countermeasures. To tackle this problem, several electrochemical biosensors will be developed within the MOBILES project.

Biosensors are devices that combine biological elements with electronic systems to detect specific pollutants. The MOBILES project enhances these sensors with advanced nanomaterials, significantly improving their sensitivity and reliability. All biosensors will have common basic electronics and functional principles (e.g., an organic ligand able to recognize target pollutants), but they will differ in the biological element employed: (i) aptasensors based on aptamers that recognize bacterial cells or spore surfaces, (ii) electronic noses for detecting and quantifying volatile organic compounds (VOCs) produced by bacteria, (iii) genosensors for detecting genes involved in antibiotic resistance, and (iv) interdigital capacitors functionalized with aptamers for estradiol, a member of CECs family.

Continual threats (such as industrial pollution and the overuse of drugs and pesticides) to sources of drinking water require real-time solutions for wide-ranging water monitoring systems to detect toxicants such as heavy metals, pesticides, and antibiotics. Conventional methods are limited in their ability to detect sub-lethal concentrations of active antibacterial compounds. The damage caused by the activity of an antibacterial agent or pesticide may stimulate different biological mechanisms of bacterial repair. Each antibiotic and/or pesticide triggers specific cellular pathways, mechanisms, and targets within the bacterial cell. This specific biological response, enabling the detection of antibiotics and pesticides using microorganisms, is being investigated in the MOBILES project through the use of genetically modified bacteria to detect toxic pollutants in water. For detecting heavy metals (cadmium, chromium, lead, mercury) in water, MOBILES is developing a flow-through

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device for continuous monitoring using biological systems (genetically modified bacteria) combined with an optical sensor and flow unit.

Highly toxic arsenic pollution can come from various sources, including industrial activities, mining, and even natural processes. Water and food contaminated by arsenic can cause serious health problems, including cancer and heart disease. For detecting arsenic pollution in soil and groundwater, the MOBILES project is developing genetically modified plants that change colour when arsenic is present in the soil or water used to grow them. The project will conduct safety evaluations to ensure that the genetically modified organisms and developed devices have minimal environmental impact. A specific work package (WP4) is dedicated to evaluate the effects of genetically modified organisms on other organisms and the environment. Safety tests and environmental impact of MOBILES organisms are performed in laboratory using EFSA guidelines.

Microplastic and nanoplastic pollution is raising concerns about its potential impact on human health. The transfer of very small plastics through the trophic chain is a potential source of contamination at all trophic levels. Understanding the distribution, degradation, and life cycle of micro- and nanoplastics in the marine environment is limited by the intrinsic difficulties of current techniques for detecting, quantifying, and chemically identifying small particles in liquids. The MOBILES project is addressing this challenge by utilizing marine diatoms. Diatoms are known for their resilience and adaptability, making them ideal candidates for studying the biodegradation of bioplastics in marine environments. Preliminary studies have shown promising results, indicating that diatoms not only survive in environments containing bioplastics but also contribute to their biodegradation.

In addition to the development of sensors, MOBILES will undertake comprehensive metagenomic analysis, profiling the microbiota of polluted areas across Europe. This work will uncover gene clusters and reveal genetic diversity, enabling a deeper understanding of microbial functions. These insights will provide genetic markers to facilitate rapid evaluation n of soil and land health. Two annual sampling rounds are planned for at least two years, and sample collection will be conducted at different locations to target microbiota related to specific pollution types: Greece for urban wastewater contamination, Poland for heavy metal pollution, Cyprus for microplastics and plastics, France for agriculture and animal farming, Italy for arsenic, and Germany for chemicals and heavy metals from former mining activities. Genomic and transcriptomic data will be analysed, visualized, and interpreted using bioinformatic tools and soil metagenomic web-based platform specifically realized by MOBILES partners. The project's data storage, located in Spain, will be connected to other well-known genomic databases in order to provide a wide range of information.

The biosensors will be rigorously tested with real-world samples from polluted sites to validate their environmental performance.

MOBILES workplan is organized in 6 WPs listed in Table 1. Management actions and collaboration with other EU funded projects are implemented within WP6 while disseminations, exploitation and communication (DEC) activities are grouped within WP5. WP1-WP2 deal with interconnected scientific and technological activities to develop electrochemical biosensors to detect specific

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pollutants, and organism-based biosensor to monitor other typology of selected pollutants. In WP3 an extensive metagenomic analysis will be performed in order to enable searches for diverse functionalities across multiple gene clusters in polluted and not polluted areas across Europe. In WP4 the safety of all genetically modified bacteria and plants will be tested using standard EU procedures (e.g., EFSA guidelines for genetically modified organisms), and a pre-industrial design of the various biosensors will be provided along with stability (shelf-life) tests.

Table 1. MOBILES WPs list

WP	Work Package Title	Lead Name	Start Month	End month
1	Electronic biosensors for environmental monitoring	INRAE	1	36
2	Detection of pollutants via biotic sensors	UR	1	36
3	Metagenomics database and fully-sequenced polluted soil microbiota	CNR-ISAFOM	1	42
4	Environmental performance and safety of developed organisms, and packaging of sensor devices	RICPA	10	42
5	Dissemination, exploitation and communication of project outcomes	GG	1	42
6	Project Management and Coordination	NTUA	1	42

2. Introduction

2.1. Micro- and nanoplastics

Small plastic fragments, generally referred to as microplastics (MP), are unique bioaccumulative pollutants in the marine ecosystem that threaten the ability of the oceans to provide critical ecosystem services that support life on earth. MP are found in all marine environments and due to their fineness, can be readily bioavailable to most marine microorganisms. MP can enter waterways through domestic or industrial drainage systems, and even enter river systems either directly or in sewage or waste spills that will then be transported to the sea. MP are plastic pieces under 5 millimeters (mm) down to 1 micrometer (μm), while nanoplastics (NP) are even smaller, typically less than 1 μm (1000 nanometers, nm) or even down to 1 nm, making them invisible to the naked eye and capable of penetrating cells and entering the bloodstream, posing higher health risks than larger microplastics.

In addition, MP/NP pollution can derive also from bioplastic which has caused a tremendous environmental threat such as air/water pollution and human health problems. Additives, dyes, and organic pollutants in (bio)-plastics can also have negative effects on humans and nature. Biodegradation of (bio)-plastics is also affected by additives and plasticizers. To understand the seriousness of the MP/NP issue, research shows that the top 1 mm of sea microlayer surface accumulates more MP than the rest sea wate. After their release into the marine environment, they trap a variety of toxic organic pollutants, including pesticides. In MOBILES project we have

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investigated common types of plastics, PET (Polyethylene Terephthalate), PS (polystyrene) and PP (Polypropylene), and starch-based bioplastics using marine diatoms as biosensors.

The degradation of bio-plastics through biological processes is of great significance for ecological health, therefore, the feasibility of (bio)-plastic consumption by microorganisms has attracted a lot of attention.

PET is the most common thermoplastic polymer resin of the polyester family and is used in fibres for clothing, containers for liquids and foods, thermoforming for manufacturing, and in combination with glass fibre for engineering resins. NP are probably the least known field in marine litter research, but also the most dangerous. Once NP cross the cell membrane of microorganisms, they can cause the formation of toxic chemical compounds that can lead to chemical toxicity due to their large surface area. These particles have not yet been defined by their size but can be classified as NP, which have a size range from 1 to 100 nm.

Task 2.7 of MOBILES project was designed to investigate the detection/biodegradation dynamics and the roles of marine diatoms in the detection of MP/NP of PET/PP/PS. The task objective was to use diatoms as biosensor by understanding their consumption of bioplastics using a number of spectroscopic techniques such as UV/Vis spectroscopy, Raman, FTIR, Fluorescence and Laser-induced breakdown spectroscopy (LIBS). We have explored the efficient detection of MP/NP and also the consumption of starch-based bioplastics without any pretreatments. State-of-the-art methods use pretreatments, as it is usually reported in the literature in the case of microorganisms, to improve the efficiency of detection/biodegradation of (bio)-plastics.

2.2. Plastic degradation

The two main categories of this breakdown are non-biodegradation and biodegradation. Non-biodegradation usually occurs through thermal degradation, physical degradation and photodegradation. Biodegradation occurs with the help of microorganisms such as diatoms. The process starts outside the diatoms since extracellular enzymes secreted by living microorganisms can break down the polymer chains. This process produces smaller plastic particles of different structures, ultimately forming NP.

Oxo-degradable plastics are conventional plastics, like PET or PP, with additives designed to accelerate their breakdown into smaller pieces when exposed to oxygen, light, and/or heat. While often marketed as "biodegradable", the process is more accurately described as "oxo-fragmentation" because the resulting fragments (microplastics) do not fully degrade, and the plastic is not truly biodegradable. PET and PP are both versatile thermoplastic polymers, but differ in composition and properties: PET is a polyester from ethylene glycol & terephthalic acid, known for clarity, strength, and gas barrier (bottles); PP is valued for flexibility, heat resistance, and chemical resistance. PET offers rigidity and transparency, while PP provides lightness and toughness, making them suited for distinct packaging and product uses, though they can be blended for enhanced performance. Polystyrene, is a versatile, colorless, hard thermoplastic polymer made from styrene monomers, known for being

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rigid, lightweight, and easily modelled, used widely in disposable cutlery, packaging (like Styrofoam for insulation), medical devices, and model kits, existing as solid, transparent, or foamed forms. Figure 1 shows the **Oxo-degradable plastics**

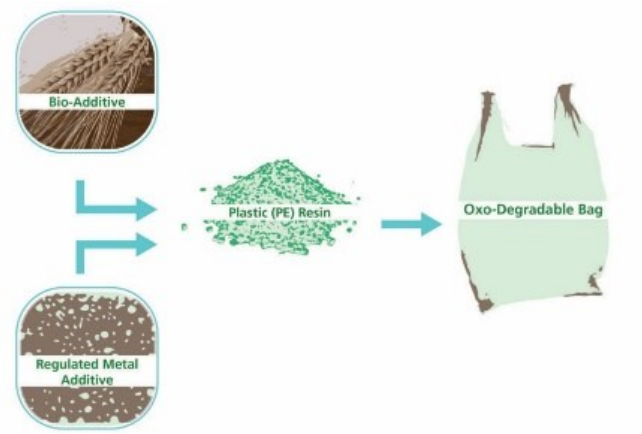

Figure 1 Synthesis pathway of oxo-degradable plastics

Biodegradable plastics (Figure 2) are materials that can break down under specific conditions by the action of living organisms, like bacteria, into water, carbon dioxide, and biomass. They are often made from renewable resources like corn starch, sugarcane, or plant-based materials, and they degrade into natural substances rather than lingering in the environment.

Diatoms perform oxygenic photosynthesis: light energy is absorbed by the pigments bound to photosystem I and II and their associated light-harvesting complexes (Lhc) and is transferred to special **chlorophyll (*Chl*) *a*** molecules in the photosystem I and II (Figure 3). Lhc are the dominant proteins in the unique antenna pigment protein complexes, which is formed by Fucoxanthin chlorophyll a/c-binding proteins (FCPs). With respect to the environmental factor light, an increase of the non-bilayer lipid Monogalactosyldiacylglycerol (MGDG) is accompanied by higher concentrations of the omega-3 eicosapentaenoic- (EPA) and docosahexaenoic -acids (DHA) in the diatoms grown under low light illumination. The high contents of EPA/DHA are responsible for membrane fluidity and the velocity of the photosynthetic electron transport. The dynamic interaction between thylakoid lipid domains and thylakoidal protein complexes in diatoms is a field, largely understudied.

Marine diatoms possess complex, highly regulated semipermeable membranes vital for nutrient uptake (e.g., N, P, Si, Fe) and stress response, featuring specialized aquaporin channels for rapid CO_2 and NH_3 transport, and flexible lipid bilayers that adjust permeability (fluidity, ion channels) to maintain homeostasis, especially under varying salinity and light, all crucial for their role as primary producers. Their silica cell walls (frustules) also contribute to selective filtration, with pores allowing smaller molecules while blocking larger particles. The polymers that are tiny enough are depolymerized into monomers that may be necessary for microbial uptake and growth. Such monomers pass through semipermeable membranes and eventually enter into cells. Then, the monomers in the cells are mineralized into CO_2, H_2O (under aerobic conditions) to produce biomass

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for energy production. In the present study, the unique dynamics of the diatoms in the presence of PET, PS, PP and (bio)-plastics have been investigated.

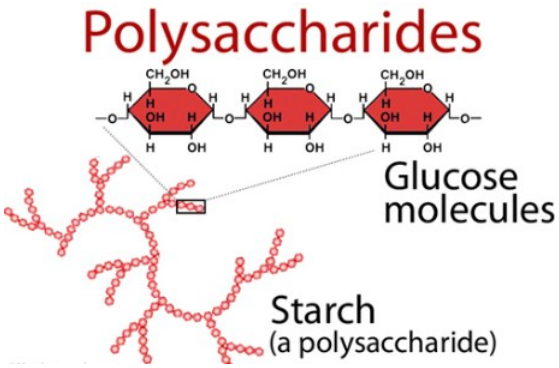

Figure 2 Building blocks of biodegradable plastics

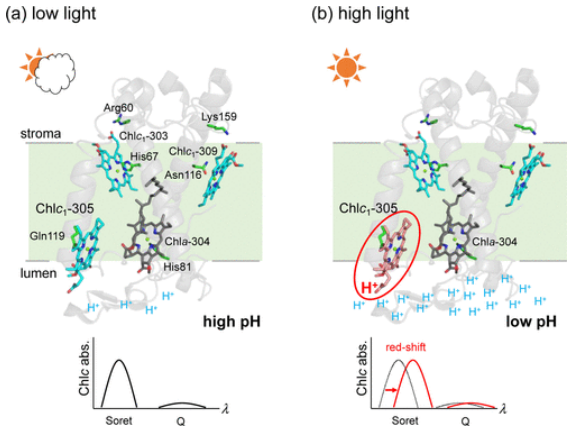

Figure 3 Photosynthesis systems in diatoms

2.3. Diatoms, materials and technologies used at CUT

We have grown under white light (Figure 4) **seven** different marine diatoms: *Phaeodactylum tricornutum, Cyclotella cryptica, Cylindrotheca fusiformis, Chaetoceros ceratosporus, Fragilariopsis sp, Thalassiosira pseudonana, and Chaetoseros sp. .*

Figure 4. Example of diatoms culture grown with withe light.

Phaeodactylum tricornutum (P. tricornutum) is a diatom with pennate symmetry and is considered the most extensively studied diatom species to date and can display different morphotypes and cell shapes which are stimulated by environmental conditions.
P. tricornutum can display different morphotypes and cell shapes which are stimulated by environmental conditions. This feature can be used to explore the molecular basis of cell shape control and morphogenesis.
Unlike most diatoms, *P. tricornutum* can grow in the absence of silicon and can survive without making silicified frustules. *P. tricornutum* is one of a handful of diatoms whose genome has been sequenced. *P. tricornutum* is the only diatom for which a telomere-to-telomere genome assembly exists. Furthermore, *P. tricornutum* is the first diatom where its crystal structure of an isolated Lhc at high resolution exist.

Cyclotella cryptica (C. cryptica) produces an exudate that has the ability to suppress the growth responses of other species, such as the diatom *Skeletonema costatum*, in environments enriched with vitamin B12. *C. cryptica* is a very important species for the growing algal biofuel industry and grown under silicon-deficient conditions has significantly higher levels of lipid production.

Cylindrotheca fusiformis (*C. fusiformis*) grows in much lower salinity than seawater, so it is expected to occur in areas with less salty water. *C. fusiformis* is a benthic pennate marine diatom, specifically identified as a motile diatom. It is used in studies focusing on diatom cell walls and biotechnological

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applications. It serves as a model organism for studies on silicification, and, due to its ability to settle to the bottom, it is considered efficient for biomass harvesting in industrial applications.

Chaetoceros ceratosporus (C. ceratosporus) is the only diatom that has the ability to use bis(p-nitrophenyl) phosphate (bis-NPP) efficiently as a sole source of phosphorus. In addition, *C. ceratosporus* can simultaneously produce both phosphodiesterase and alkaline phosphatase at almost equal levels of activity under phosphate-deficient conditions. This suggests that phosphodiester compounds probably play an important role as a source of phosphorus for phosphodiesterase-producing phytoplankton in coastal environments.

Fragilariopsis sp. is the smaller diatom (12-16 µm in length and 6-10 µm in width) showed significantly higher growth rates compared to the large species, which is related to the relatively increased light-harvesting capacity and electron transfer rates in the smaller species. The cultivation of *Fragilariopsis sp*. in dark anoxic conditions showed large increases in lipid production but also large rates of dissolved H_2 production. Overall, the small species showed significantly higher growth rates compared to the large species, which is related to the relatively increased light-harvesting capacity and electron transfer rates in the smaller species.

Thalassiosira pseudonana (T. pseudonana) is a well-known centric diatom. It is a marine phytoplankton species, noted for being the first eukaryotic marine alga to have its genome fully sequenced in 2004, which established it as a model organism for studying diatom biology, ecology, and silica biomineralization.

Chaetoceros sp is a centric marine planktonic diatom characterized by its ability to form chains with elliptical to circular valves. This circular or elliptical shape is the defining characteristic of centric diatoms, whereas pennate diatoms are generally elongated or boat-shaped.

Resonance Raman spectroscopy. Raman spectroscopy is based on the ability of the excitation source to induce a change in its electric dipole with respect to the vibrational and rotational state of the analyte. These characteristics make possible to use Raman spectroscopy for detection of known molecules and identify the unknown ones with spectra stripping combined with libraries.

PET Raman bands reveal its structure, with key peaks like the strong C=O stretch at 1726 cm^{-1}, the aromatic C=C ring modes at 1615 cm^{-1}, C-H bends at 1291cm^{-1}, making them useful as marker bands of PET.

PP Raman bands reveal its structure, with key peaks around 2961 cm^{-1} (CH_3 asymmetric stretch) bands for CH_2 at 2885 and 1460 cm^{-1}.

PS Raman bands are characteristic vibrational fingerprints, with key bands at 1002 cm^{-1} (aromatic ring breathing/CC stretch, very strong), 1031 cm^{-1} (C-H in-plane bend), and 1603 cm^{-1} (aromatic C=C stretch). Other significant bands appear >3000 cm^{-1} (aromatic C-H stretch), providing crucial data for identification and structural analysis (Figure 5).

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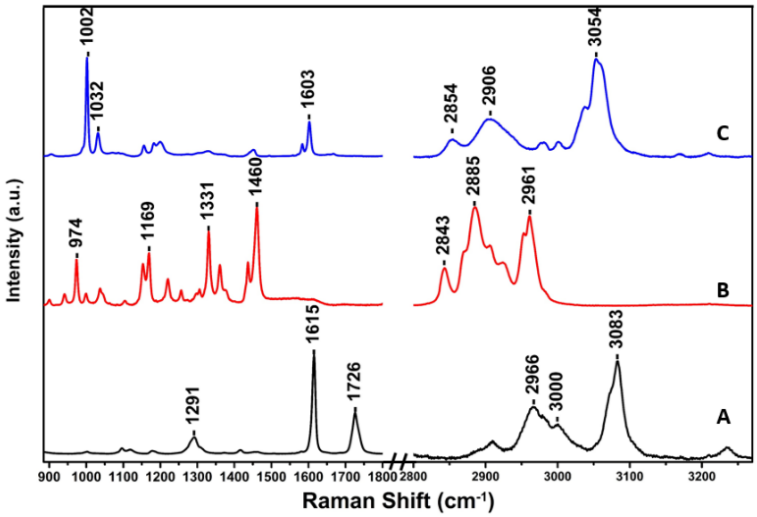

Figure 5 Representative Raman spectra of PET(A), PP(B), PS(C)

The principle of **Fourier Transform Infrared (FTIR)** spectroscopy is based on atoms vibration and rotation, and it has become a universal and widely used spectral methodology to detect the internal molecular structures in all kinds of fields.

FTIR bands for PET reveal its polyester structure, with key peaks around 1712 cm^{-1} (C=O ester stretch), 1242 cm^{-1} (C-O-C/C-O stretch), and 1097 cm^{-1} (O-C-C stretch) (Figure 6 A).

PP shows distinct FTIR bands (Figure 6 B) C-H stretching peaks at 2951, methyl bending/umbrella modes at 1451, cm^{-1}, and characteristic skeletal vibrations at 846 cm^{-1}, allowing easy differentiation from the other plastic typologies.

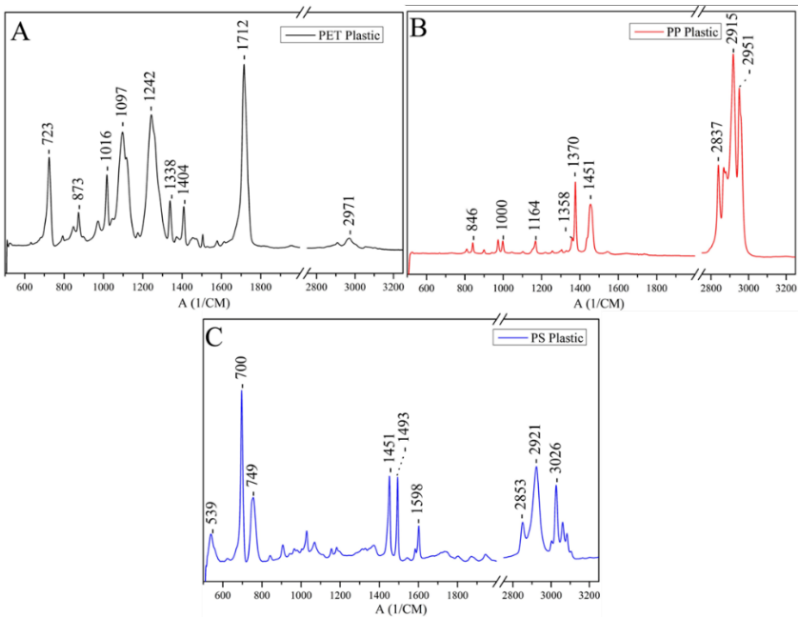

Figure 6 FTIR spectra of PET (A), PP (B) and PS (C)

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FTIR PS (Figure 6 C) shows key peaks: aromatic C-H stretches at 3026 cm^{-1}, aliphatic CH_2 stretches (2921, 2853 cm^{-1}), and aromatic ring vibrations (C=C) at 1598, 1493, 1451 cm^{-1}, plus out-of-plane C-H bending at 700 cm^{-1}.

Laser-induced breakdown spectroscopy (LIBS) is a relatively new atomic emission spectroscopic method (Figure 7). LIBS plays a key role in the elemental analysis of a wide range of samples. It is a multi-element analytical approach that is very important for laboratory systems. LIBS is easily performed by focusing a highly energetic laser pulse on the surface of a solid or liquid particle. After this state, a microplasma is created that contains excited molecular and atomic species from the sample.

These excited state species emit light at unique wavelengths which will then be collected with a spectrometer and analyzed on a computer. Since each element has a unique emission spectrum, all elements can be detected with LIBS. Here are some of the characteristics of LIBS that undoubtedly make the technique unique and superior to other analytical methods. These characteristics have been repeatedly emphasized in the literature. Suffice it to say that LIBS, in principle, can simultaneously detect all neutral and spectral features of ions of all atomic and molecular species of all elements present in any type of sample and its environment, using a single laser shot. The analytical capability of any spectroscopic (and also non-spectroscopic) method can be best appreciated if one refers to the requirements of an ideal analytical method. The two unique characteristics of LIBS are the lack of sample preparation and the ability to wait for it. **The LIBS technique will first record the structure of the plastic and the effect that the diatom will have on the plastic.** Also, thanks to the use of two lasers instead of one, the spectrum signal can be amplified and better results can be recorded in terms of sensitivity for plastic detection.

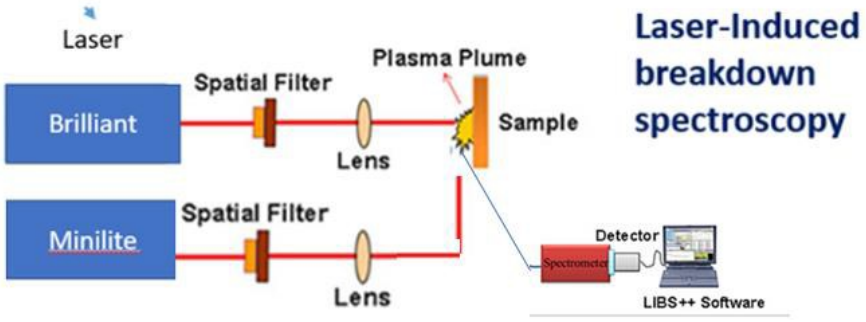

Figure7 Laser-induced breakdown spectroscopy (LIBS)

To study the biosensing properties of diatoms, CUT grounded PET plastic into powder to make the conversion to MP and NP easier. Then simple physical approaches, including stirring and ultrasonic washing, were applied to remove microplastics and nanoplastics from the surface of the PET powder. Briefly, 10 g of PET powder was placed in a 250 mL Erlenmeyer flask with 150 mL of deionized Milli-Q water (Figure 8). After sealing to avoid water evaporation, the flask was stirred at 600 rpm for 1 h under strong magnetic stirring and then sonicated for 30 min.

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The stirring and sonication processes were repeated five times to sufficiently isolate the MP and NP from the surface. This solution was subjected to two stages of filtration with filters to obtain the desired result. The filters were washed with Milli-Q water three times before the filtration experiments. Exactly 10 μm (pore size) filters were used to remove the large particles in the solution giving us the μm scale. Then, the solution was filtered through 1 μm (pore size) filters twice to ensure that the filtered particles in the filtrate were in the order of nm. The estimated concentration of NP/MP in the diatom cell cultures was 10-15 μg/L.

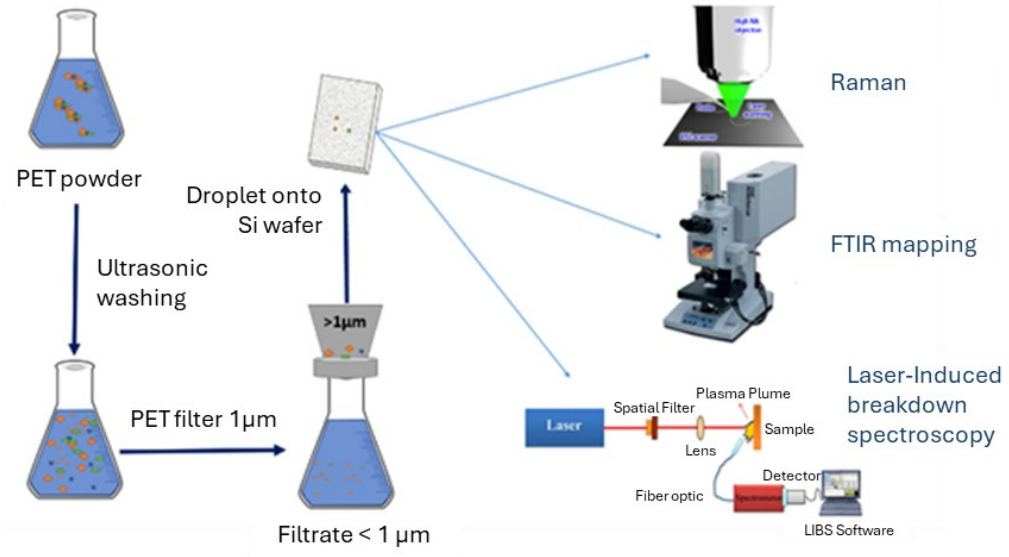

Figure 8 Protocol used by CUT to prepare NP/MP plastics

2.4. Detection of Heavy Metals in Plastics

The synthesis of plastics varies in quantity and type of materials. Large groups of plastics contain many substances toxic to the marine environment, such as heavy metals. Heavy metals are considered soluble toxic pollutants that can persist for a long time in the sea. These heavy metals are capable of accumulating in aquatic organisms. Many heavy metals, e.g. Cd, Cr and Pb, have been eliminated for the manufacture of plastics, but compounds of many metals are used as catalysts, biocides, pigments for color, and UV and heat stabilizers in plastics. MP/NP can also affect the amount of heavy metals in the sea. Not only can MP/NP affect the photosynthesis of marine microorganisms, but they can also enrich the amount of organic pollutants and heavy metals with their large specific surface area, high hydrophobicity, and high tendency to interact with diatoms. We at CUT laboratories verified that MP/NP have a high affinity for heavy metals since they absorb them from various sources of toxic metals and make microplastics carriers of increased contamination that impose a high risk for marine wildlife as potential sources of heavy metals. Therefore, the intake of MP/NP by diatoms creates a pathway for the transport of metals inside the diatom's cells.

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2.5. Rational behind the use of diatoms as MP and NP biosensor

Previous laboratory observation at CUT highlighted the fact that some diatoms have the ability to survive in marine water even if plastic is present. Close inspection at the light adsorption properties reviled that some changes in absorption spectra are due to the presence of MP and NP inside the diatom cells.

Figure 9 shows the UV/Vis absorption spectrum of *Ph. tricornutum* cells: (a) 4 months starved culture (b) 1 week culture (c) 4 months culture with starch plastic. There are a number of peaks that are characteristic of pigments found only in diatoms. In the case of *Ph. tricornutum*, in addition to the *Chl a* peaks at 438 and 675 nm, there is a peak at 460 nm caused by the presence of *Chl c* in the green region and discrete absorption peak at 492 and a broad absorption in the 500-530 nm region due to carotenoid-like pigments. In spectrum c (4 months culture with starch plastic) there are frequency shifts and intensity changes in some of these characteristic peaks. For example, **at the 675 nm characteristic peak of *Chl a* we see a shift of the order of 3 nm and the peaks of *Chls a* and *c* which are located at 440 and 460 nm respectively have been modified in relation to the original spectrum.** This indicates changes in the structure and amount of *Chls a/c* in the presence of bioplastics. It should be noted that the carotenoids have strong absorptions with similar frequencies as *Chls a/c.* The red region of the absorption of the carotenoids at 500-530 nm has lost intensity which further demonstrated the interaction of the diatom with the bioplastic.

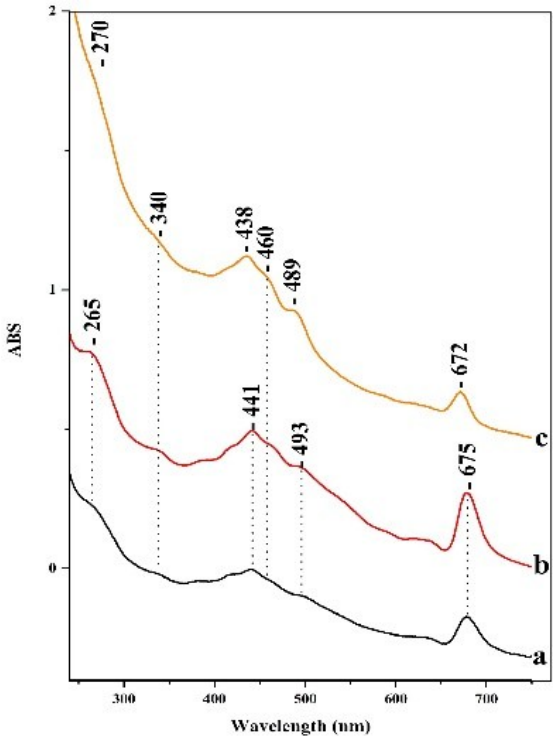

Figure 9 UV/Vis absorption spectrum of Ph. tricornutum cells (a) 4 months starved culture (b) 1 week culture (c) 4 months culture with starch plastic. The peak at 680 nm shifts when plastic is added to medium

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MOBILES

3. Diatom based biosensor for micro- and nano-plastic detection in marine environment (Task 2.7)

3.1. Full spectroscopy characterization of *Ph. tricornutum*

In order to further verify and confirm the preliminary UV/Vis spectroscopic data on *Ph. tricornutum* grown in presence of plastic we applied other light-based methods to identify potential spectra changes that can be used to assess the presence of MP/NP in diatoms cells. Thus, use such kind of organisms as biosensor to monitor plastic in marine environment. Within task 2.7 MOBILES project, *Ph. tricornutum* cells grown in presence of MP/NP have been analysed by Fluoresce microscopy, Raman and FTIR.

The emission **fluorescence spectra** (Figure 10) with excitation at 680 nm of *Ph. tricornutum* cells (a) 4 months starved culture (b) 1 week culture (c) 4 months culture with starch plastic further support the conclusions from the UV/Vis spectra.

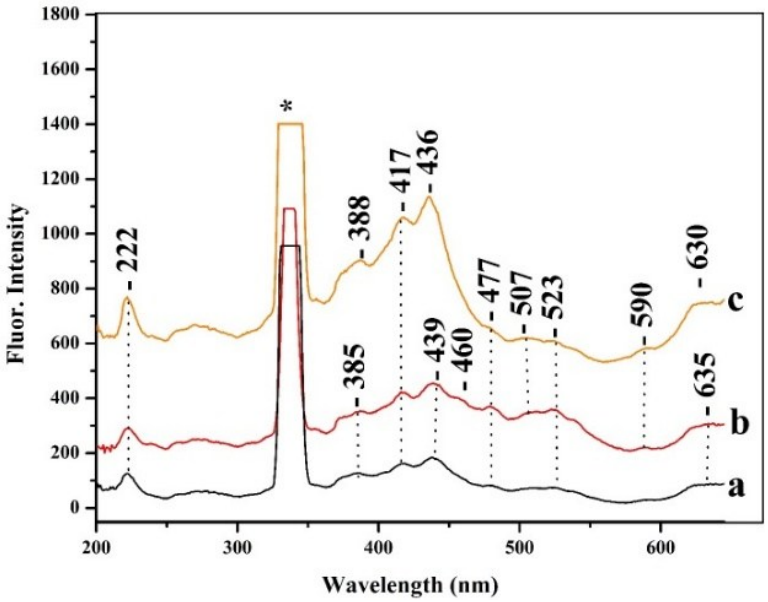

Figure 10 Fluorescence spectra emission with at excitation 680 nm of Ph. tricornutum cells (a) 4 months starved culture (b) 1 week culture (c) 4 months culture with starch plastic

The 4 months culture indicates that *Ph. tricornutum* is still active contributing to the energy transfer processes from all pigments. More specifically both *Chl a* and *c* in the 439-477 nm range contribute to the energy transfer as well the carotenoids in the 500-550 frequency range. The increased intensity in the 417-440 nm frequency range indicates that the carotenoids are highly involved in the energy transfer whereas the reduced intensity in the 500-530 nm suggests that the red carotenoids are less involved in the energy transfer pathways.

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The 442 nm excitation **Raman spectra** of *Ph. tricornutum* cells under the same conditions as the UV/vis and Fluorescence spectra were collected as well (Figure 11). Excitation within the 442 nm laser light produces a spectrum dominated by totally symmetric Frank-Condon-active modes aligned along the x-axis of the *Chls a/c* macrocycles.

In panel A of Figure 11, *trace a*, the spectral 900-1080 cm^{-1} range is characterized by peaks at 963 cm^{-1} due to vibrational modes for Fucoxanthins[1] (Fxs), 986 cm^{-1} due to Diadinoxanthins[1] (Dds), and 1004/1014 cm^{-1} due to Fx/Dd . The peaks at 918, 953 and 1048 cm^{-1} originate from *Chl a* and have similar intensities in *traces a-b*. There are minor changes in the intensity of the 964 cm^{-1} peak shown in *traces a-b* but significant changes in the intensities of the 1004/1014 cm^{-1} peaks shown in *trace c*.

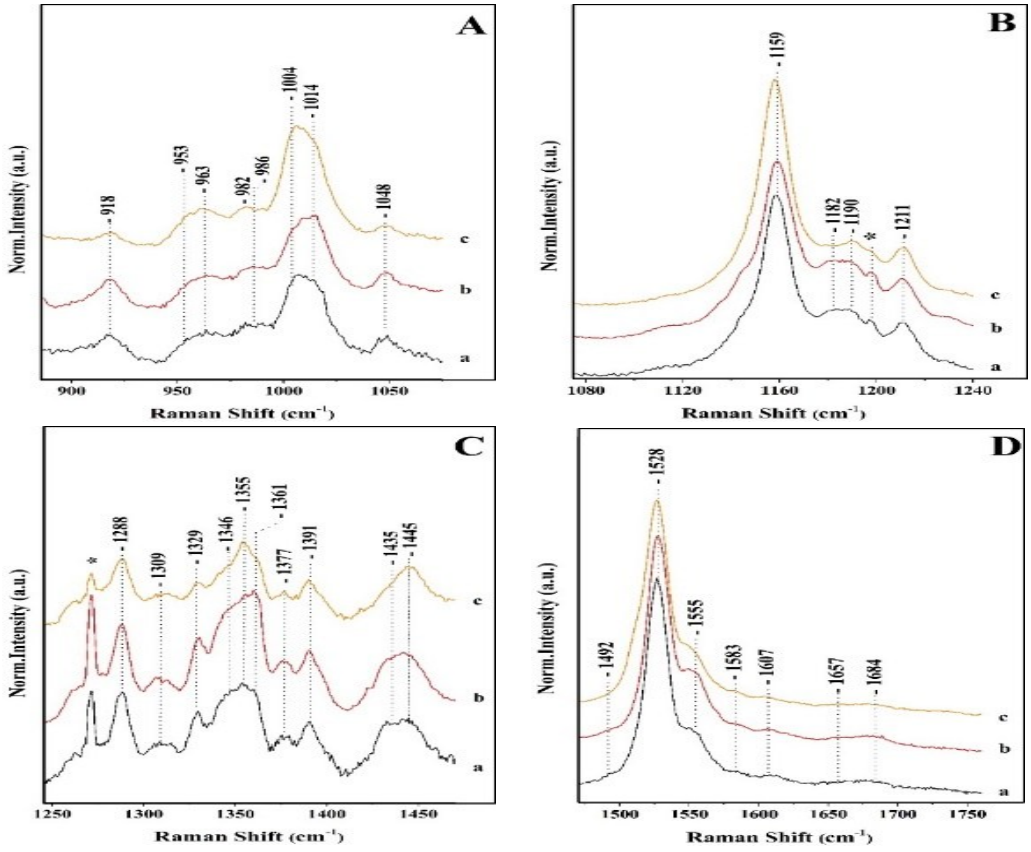

Figure 11 Raman spectra of Ph. tricornutum cells(a) 4 months starved culture (b) 1 week culture (c) 4 months culture with starch plastic. Excitation wavelength used is at 442 nm.

In panel B (*traces a-b*) of Figure 11, the spectral region contains marker bands for Fxs which are characterized by peaks at 1159, 1183, 1211 and 1230 cm^{-1} whereas the 1190 and1211 cm^{-1} are due to the presence of Dds. The increased intensity of the 1159 cm^{-1} peaks in *trace c* indicates that the C-C mode adopts a conformational distortion in *trace c*.

[1] Fucoxanthin, Diadinoxanthins and Diatoxanthins are natural carotenoids produced by diatoms

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In panel C of Figure 11, the $\nu(C_aN)$[2] bands in the 1300-1400 cm^{-1} region are highly resolved in the 442 nm excitation spectrum. In panel C, the spectra in the 1300-1420 cm^{-1} spectral region are consistent with Franck-Condon activity of in-plane ring modes in resonance with the strongly allowed Soret band (B_x) transitions of *Chl a/c* . In *trace a*, the peaks at 1329, 1342 and 1377 cm^{-1} originate from the $\nu(C_aN)$ of five- and six-coordinated *Chl a* and the peaks at 1355 and 1361 cm^{-1} from the $\nu(C_aN)$ of *Chl c* . We attribute the intensity increase of the 1355 cm^{-1} and concomitant decrease of the 1361 cm^{-1} peaks to conformational changes of the *Chl c* macrocycle resulting in the intensity/frequency changes observed in *trace c.* The conformational changes in the macrocycle of *Chl c* can be used in studies for the location and conformational dynamics of protein-bound *Chl c* in the membranes of diatoms. The observation of the 1355/1361 cm^{-1} of *Chl c* shown in panel C, *trace b*, clearly demonstrate that there is a subset of *Chl c* molecules that they experience changes in the core size.

In panel D of Figure 11, *trace a*, the ν_1 (vibration) of Fxs is observed at 1528 cm^{-1} and the peak at 1555 cm^{-1} has contributions from the $\nu(C_aC_b)$ of *Chls a/c*. There is a substantial increase in the intensity of the ν_1 that we attribute to the presence of Dds/Dts[1] (Diatoxanthins) which have an intense ν_1. Of particular interest is the change in the band shape and intensity of the *Chls a/c* peaks at 1555/1550 cm^{-1} shown in *trace c.*

The **FTIR spectra** of *Ph. tricornutum* cells are characterized by absorbance bands of well-characterized functional groups, which are observed through the frustule of the chloroplasts and the silica bands (Figure 12). The FTIR spectra are characterized by the Amide I, II and III bands at 1664, 1545 and 1459 cm^{-1} bands. Strong CH_2 bands are also observed at 2850 (symmetric), 2919 (antisymmetric) cm^{-1} and 2956 cm^{-1}. In addition, the band at 3011 cm^{-1} is characteristic of an unsaturated =C-H group from fatty acids. **In the 4-months culture with MP (*trace b*) there are significant changes in all the amide bands indicating that the MP strongly interact with the protein environment** of the diatom suggesting that the MP enter through the silicate surface and also interact with the lipids as it is evident from the changes in the frequencies of the CH_2 bands.

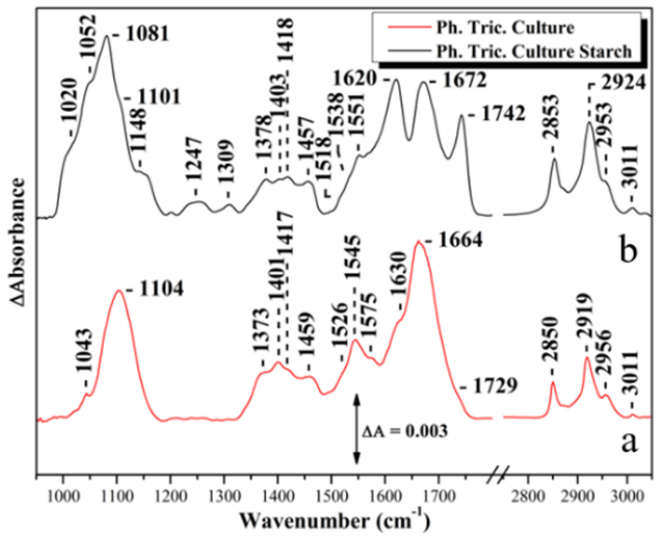

Figure 12 FTIR spectra of Ph. tricornutum cells

To further confirm that the peaks shifts are a consequence of plastic presence in marine water and that such changes permanently modify the spectra patterns, we cultivated *Ph. tricornutum* up to 7 months in presence of plastic. Figure 13 left panel shows the UV/Vis absorption spectra of *Ph.*

[2] Stretching and pyrrole breathing vibrations of the *chlorophyl a* macrocycle

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tricornutum cells (a) 7 months starved culture (b) 1 week culture (c) 1 week from starch culture and (d) 7 month culture with starch plastic.

The right panel of Figure 13 shows the emission fluorescence spectra with excitation at 680 nm of *Ph. tricornutum* cells (a) 7 months starved culture (b) 1 week culture (c) 1 week from starch culture and (d) 7 months culture with starch plastic.

The 7 months culture in presence of starch plastic demonstrate that the diatom has regain its function as it shows strong fluorescence signals in the 400-450 range originating from *Chls a/c* but there is weak contribution from the carotenoids in the 500-540 nm frequency range. The UV/Vis and the Fluorescence data in the 7 months culture strongly support the 4 months data and also demonstrate that diatoms are capable of consuming bioplastics.

The comparison with the 4-months data (Figures 10 and 11) also indicate that in the 7-months culture with starch plastic the carotenoids in the 500-550 nm frequency range although are still present their intensity has been substantially reduced. Of note is the increased fluorescence intensity of *Chl a/c* in the 400-440 nm spectral range in agreement with the results of the 4-month growth

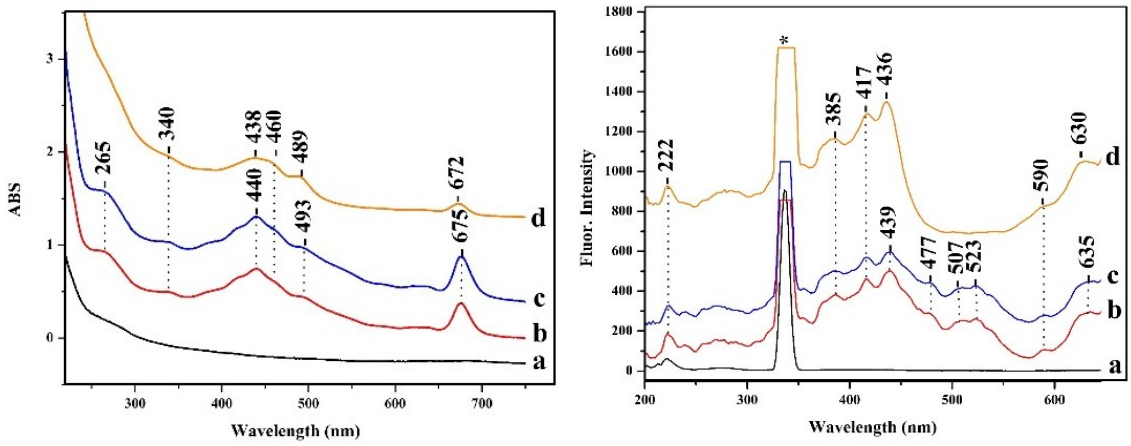

Figure 13 Left panel: UV/Vis absorption spectra of Ph. tricornutum cells (a) 7 months culture (b) 1 week culture (c) 1 week from starch culture and (d) 7 month culture with starch plastic. Right Panel: Emission fluorescence spectra with at excitation 680 nm of Ph. tricornutum cells (a) 7 months culture (b) 1 week culture (c) 1 week from starch culture and (d) 7 months culture with starch plastic.

By analysing the **Raman spectra** *trace a* (Figure 14) it is evident that the 7-months diatom shows extremely weak signals at 1159 and 1527 cm^{-1} of the carotenoids; *trace c* shows that diatom can retain its activity in the presence of bioplastic. The spectra in *trace d* demonstrates that the diatom is capable of regaining activity. However, there are differences as demonstrated in the Raman data below in the structures of the *Chl a*. The structures of carotenoids based on the frequencies of the major bands of carotenoids remain the same as those in the one week culture suggesting that the biosynthesis of carotenoids is not affected if the bioplastic is the major source of growth. On the other hand, the Raman data show that there are changes in the composition of the *Chls a/c* as shown by the intensity of the 1355/1361 cm^{-1} peaks. This observation indicates that in the absence of nitrogen the biosynthesis of *Chl a/c* is strongly affected.

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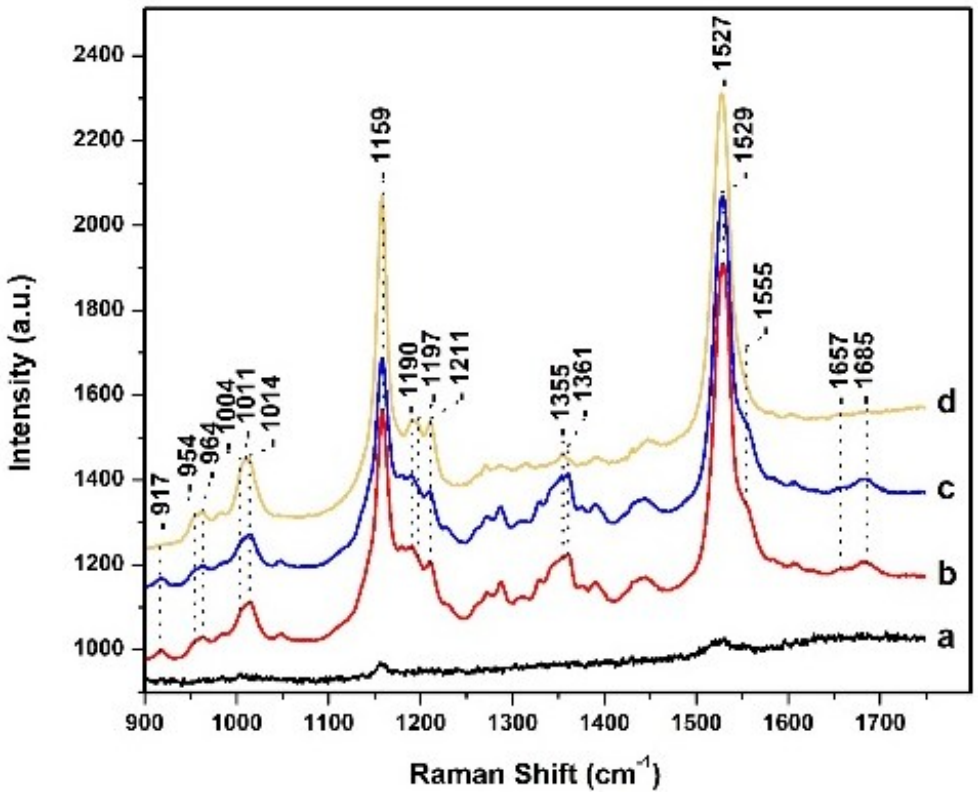

Figure 14 Raman spectra of Ph. tricornutum cells (a) 7 months culture (b) 1 week culture (c) 1 week from starch culture and (d) 7 months culture with starch plastic.

3.2. Spectra analysis of PET/PP/PS and Bioplastics

In order to accurately detect the presence of plastic in diatoms it was necessary to characterize the spectra of each element forming the different typology of plastics. For each plastic samples spectra libraries have been created using LIBS and UV/Vis methods.

3.2.1. LIBS PP plastic spectra

LIBS spectra of (A) C (natural Carbon), (B) H (Hydrogen) and (C) Zn (Zinc) elements of PP plastic (Figure 15). The different lines represent the difference in the laser's intensity and power. The black line represents the 1064nm laser, the red line is the use of 532nm laser for preheating and the 1064nm for the plasma, the blue line the preheating with the use of 1064nm and the creation of plasma from again 1064nm, and finally the orange line signifies the double pulse of 532nm and 1062nm lasers together.

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There is an alteration in the intensity of these lines especially in the use of just the 1064nm and the use of both lasers. The difference between the use of just the 1064nm line and both lasers lies at 38% at C(I), 34.8% H_a and 45% for Zn.

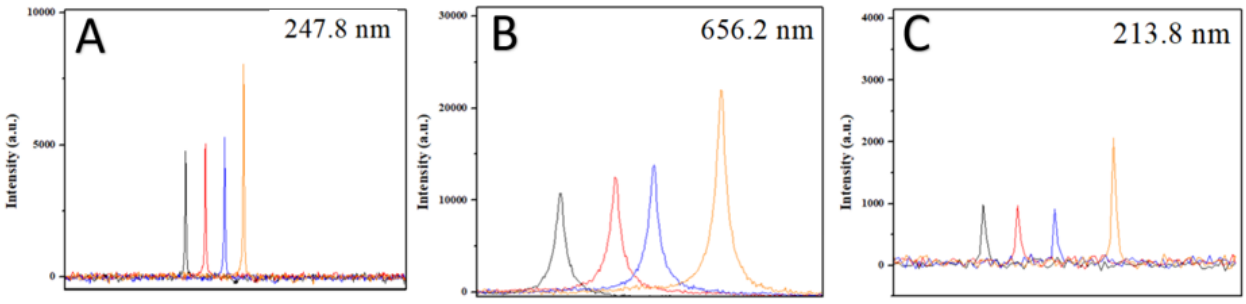

Figure 15 LIBS spectra of A) C, (B) H and (C) Zn elements of PP plastic

3.2.2. PET plastic spectra

LIBS spectra of (A) C and (B) H elements of PET plastic (Figure 16). The different lines represent the difference in the laser's intensity and power. The black line represents the 1064nm laser, the red line is the use of 532 nm line for preheating and the 1064 nm for the plasma, the blue line the preheating with the use of 1064nm and the creation of plasma from again 1064nm and finally the orange line signifies the double pulse of 532 nm and 1064 nm lasers together. There is an alteration in the intensity of these lines especially in the use of just the 1064nm and the use of both lasers. This difference lies at 32% and 41% for C(I) and H_a respectively.

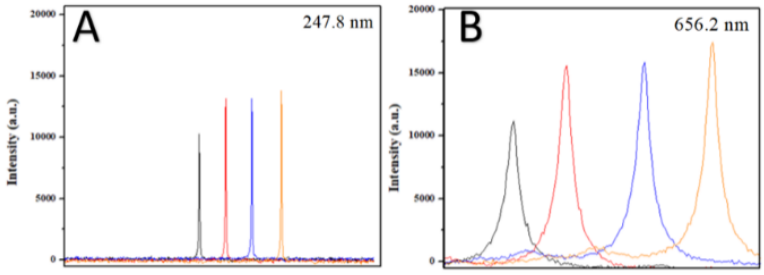

Figure 16 LIBS spectra of (A) C(I) and (B) Ha elements of PET plastic

3.2.3. LIBS PS plastic spectra

LIBS spectra of (A) C, (B) H and (C) Ca (Calcium) elements of PS (Figure 17). The different lines represent the difference in the laser's intensity and power. The black line represents the 1064nm laser, the red line is the use of 532nm line for preheating and the 1064nm for the plasma, the blue line the preheating with the use of 1064 and the creation of plasma from again 1064nm and finally the orange line signifies the double pulse of 532nm and 1064nm lasers together. There is an alteration in the intensity of these lines especially in the use of just the 1064nm and the use of both lasers. The difference between the use of just the 1064nm line and both lasers lies at 48% at C(I)

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and 56%. The use Ca which can be seen at panel (C) does not show any spectral line with the use of the 1064nm laser. When both lasers are applied the spectral line of Ca can reach 1400.

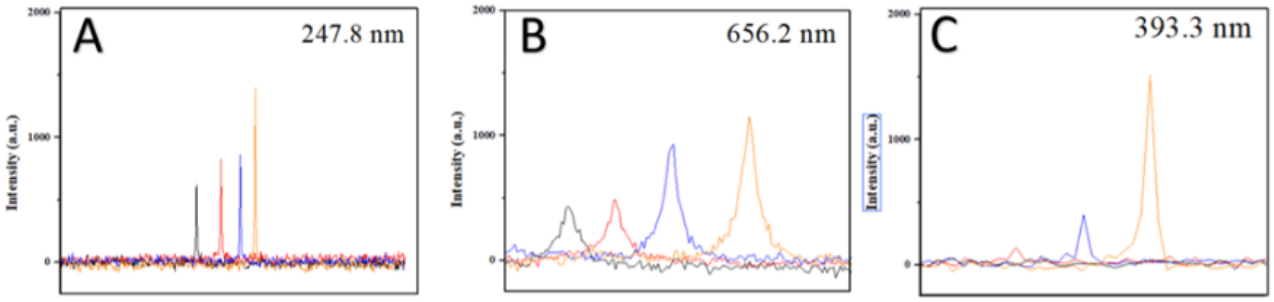

Figure 17 LIBS spectra of (A) C, (B) H and (C) Ca elements of PS

3.2.4. LIBS starch biodegradable plastic spectra

LIBS spectra of (A) C, (B) H and (C) Mg (magnesium) elements of starch biodegradable plastic (Figure 18). The different lines represent the difference in the laser's intensity and power. The black line represents the 1064nm laser, the red line is the use of 532nm line for preheating and the 1064nm for the plasma, the blue line the preheating with the use of 1064 and the creation of plasma from again 1064nm and finally the orange line signifies the double pulse of 532nm and 1064nm lasers together. There is an alteration in the intensity of these lines especially in the use of just the 1064nm and the use of both lasers. The difference between the use of just the 1064nm line and both lasers lies at 52% at C(I), 61% and a big increase of the Mg spectral line which again is almost invisible with only the 1064nm line.

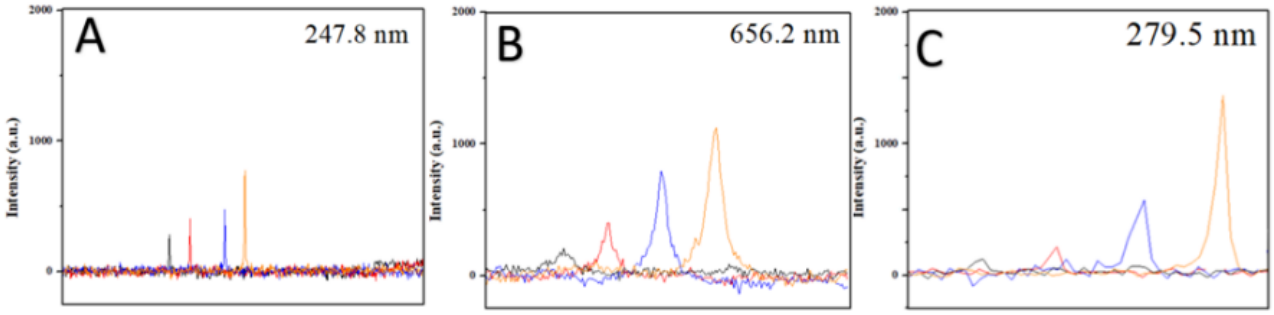

Figure 18 LIBS spectra of starch biodegradable plastic

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3.3. Spectra analysis of 7 diatom species

The comparison of the UV/Vis and fluorescent spectra of the seven diatoms (Figure 19A) revealed great similarities in the absorptivity of the pigments in the cells with small frequency alterations in the region of *Chl a* at 674-678 nm (region around 680 nm, left panel). The region of carotenoids 500-532 nm and *Chl c* is also very similar in all diatoms (Figure 19A, right panel). Therefore the subsequent spectroscopic studies in presence of MP and NP were performed in three selected species: *C. cryptica, Ph. tricornutum* and *C. fusiformis.*

Raman has been applied to monitor the presence of the silicate in all diatoms. The Raman Si-O bond is observed at 472 cm^{-1} in all diatoms we have examined (Figure 19B).

3.3.1. UV/Vis spectra of diatoms in contact with PET

In order to evaluated time-frame for the biological response of diatoms exposed to MP and NP, we performed a time course analysis exposing the three selected strains for 2, 4-6, and 8 hours. At each time-frame diatoms were collected and analysed using light-based techniques.

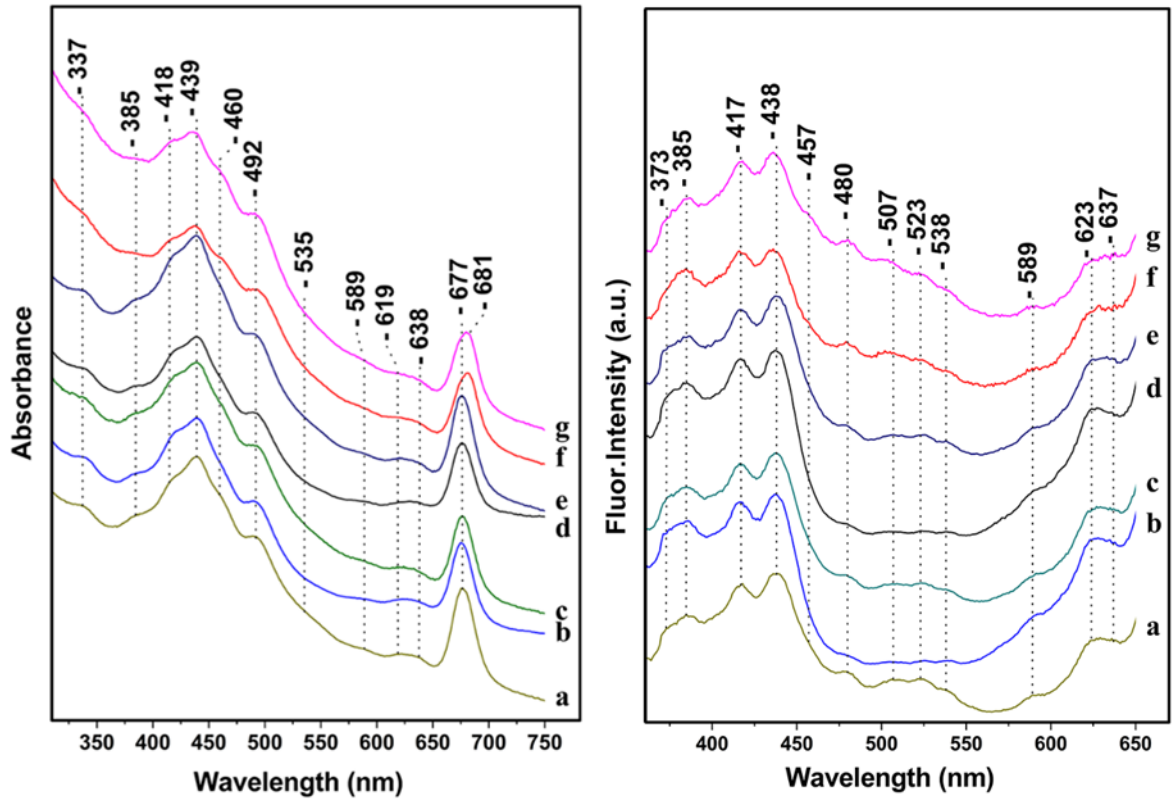

Figure 19A. Left panel: UV/Vis Absorption spectra of (a) Chaetoceros Ceratosporus (b) Fragilariopsis sp. (c) Cyclotella Cryptica (d) Chaetoceros sp. (e) Thalassiosira Pseudonana (f) Phaedactylum Tricornutum (g) Cylindrotheca Fusiformis; Right panel: fluorescence excitation spectra at 680 nm of the same diatoms

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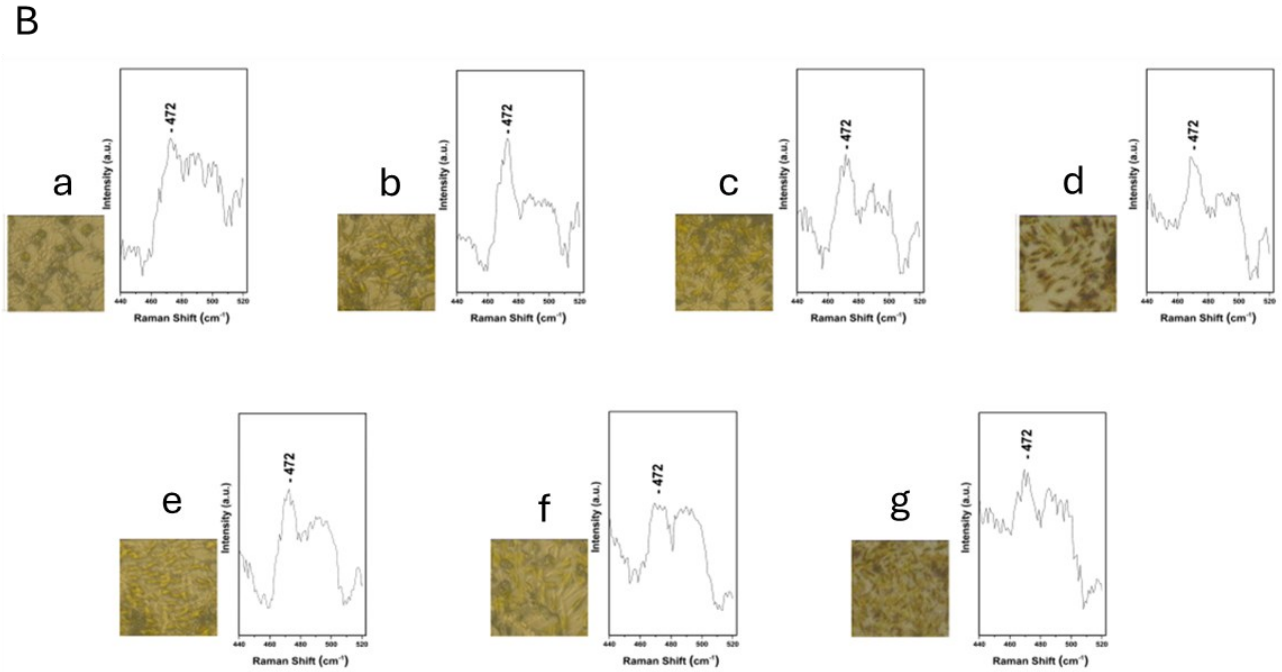

Figure 19B. Microscope images and Raman spectra of (a) Chaetoceros Ceratosporus (b) Fragilariopsis sp. (c) Cyclotella Cryptica (d) Chaetoceros sp. (e) Thalassiosira Pseudonana (f) Phaedactylum Tricornutum (g) Cylindrotheca Fusiformis cells.

Figure 20 shows the UV/Vis absorption spectra of *C. cryptica* cells (a) control, exposed to (b) MP/PET and (c) NP/PET for 2, 4 and 8 hours.

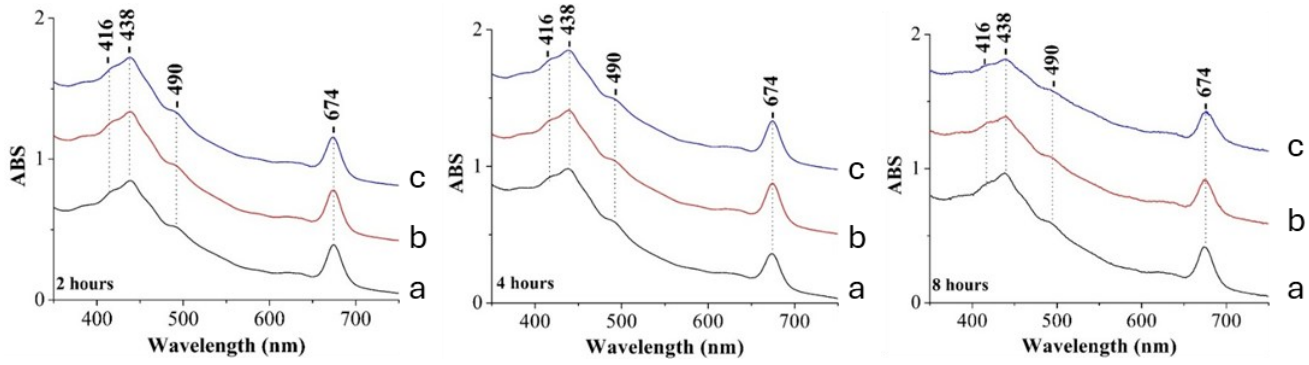

Figure 20 UV/Vis absorption spectra of C. cryptica cells exposed to PET MP and NP

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Figure 21 shows the UV/Vis absorption spectra of *C. Fusiformis* cells (a) control, exposed to (b) with MP/PET, and (c) with NP/PET for 2, 6 and 8 hours.

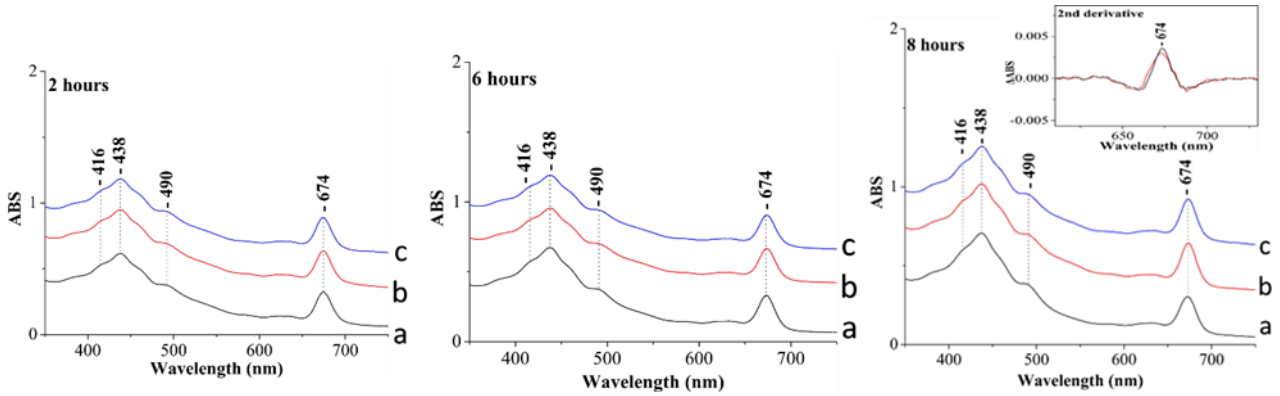

Figure 21 UV/Vis absorption spectra of C. Fusiformis cells exposed to PET MP and NP

Figure 22 shows the UV/Vis absorption spectra of *Ph. tricornutum* cells after 2 and 8 hours exposure to NP/PET and MP/PET. Line (a) represents control sample, (b) *Ph. tricornutum* cells with MP/PET and (c) *Ph. tricornutum* cells with NP/PET.

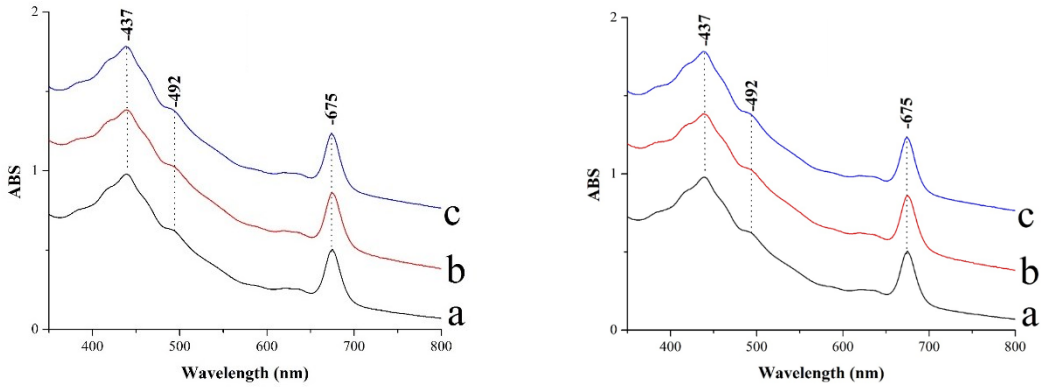

Figure 22 UV/VIS spectra of Ph. tricornutum cells after 2 hours (left) and 8 hours (right) of plastic particles exposure

A close inspection of the UV/Vis spectra of *Ph. tricornutum, C. cryptica* and *C. fusiformis* cells in contact with MP/PET and NP/PET for 2, 6 and 8 hours revealed not clear evidence for any changes in the absorption spectra suggesting that UV/Vis spectroscopy cannot identify pigments changes in the cells with MP/PET and NP/PET within 8 hours. Even the 2nd derivative spectrum showed negligible changes (Figure 21, insert in 8 hours chart). Therefore, we concluded that the change in spectral properties takes place in more than 8 hours. Thus the UV/Vis shift observed previously in *Ph. Tricornutum* after months cannot be realistic used as a marker to quickly identify NP and MP in water. However, diatoms UV/Vis spectra can be used as tools to record past contamination events since they can survive for months in presence of NP and MP.

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3.3.2. Fluorescence spectroscopy: monitoring *Chl a* every 2 hours

Figure 23 shows the fluorescence spectra of *C. fusiformis* cells with excitation at 418, 460, and 490 nm and with MP/NP PET for 2-8 hours.

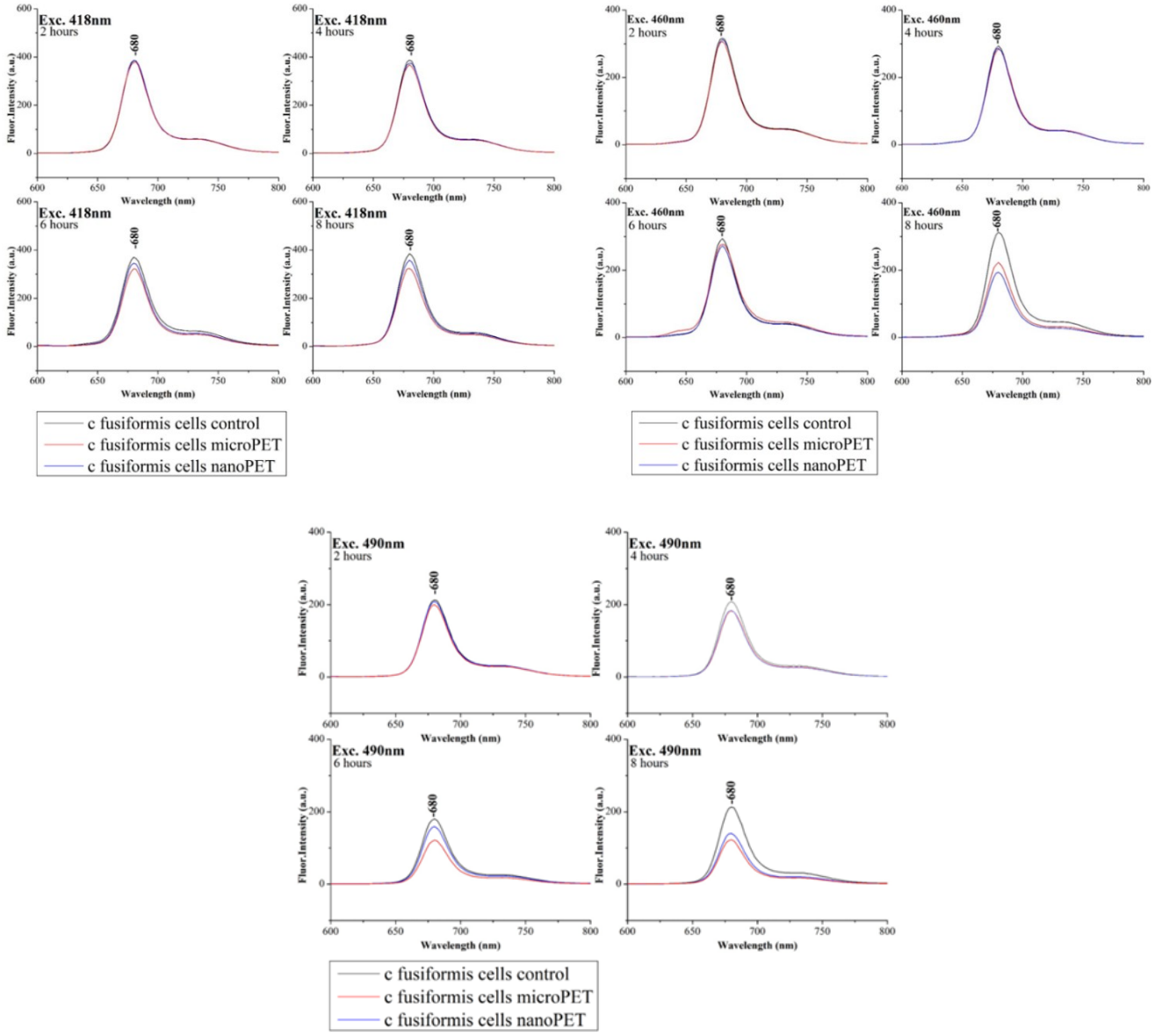

Figure 23 Fluorescence spectra of C. fusiformis cells

Figure 24. shows the fluorescence spectra of *C. fusiformis* cells with excitation at 514 and 530nm and with MP/NP PET.

The analysis of five different emission spectra (excitations at 418,460,490 524 and 530 nm) of *C. Fusiformis* cells grown in presence of MP and NP PET measured every 2 hours allowed to conclude

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that **the emission at 680 nm which is the emission peak of *Chl a* is more sensitive than those of the carotenoids at 514 and 530 nm indicating that both MP and NP interact first with C*hls a and c* and subsequently with the carotenoid molecules affect their properties. Therefore a reliable marker for the detection of MP/NP in diatoms grown in a marine environment is the monitoring of the fluorescence peak of *Chl a* within 4 hours upon interaction with both MP an NP PET**.

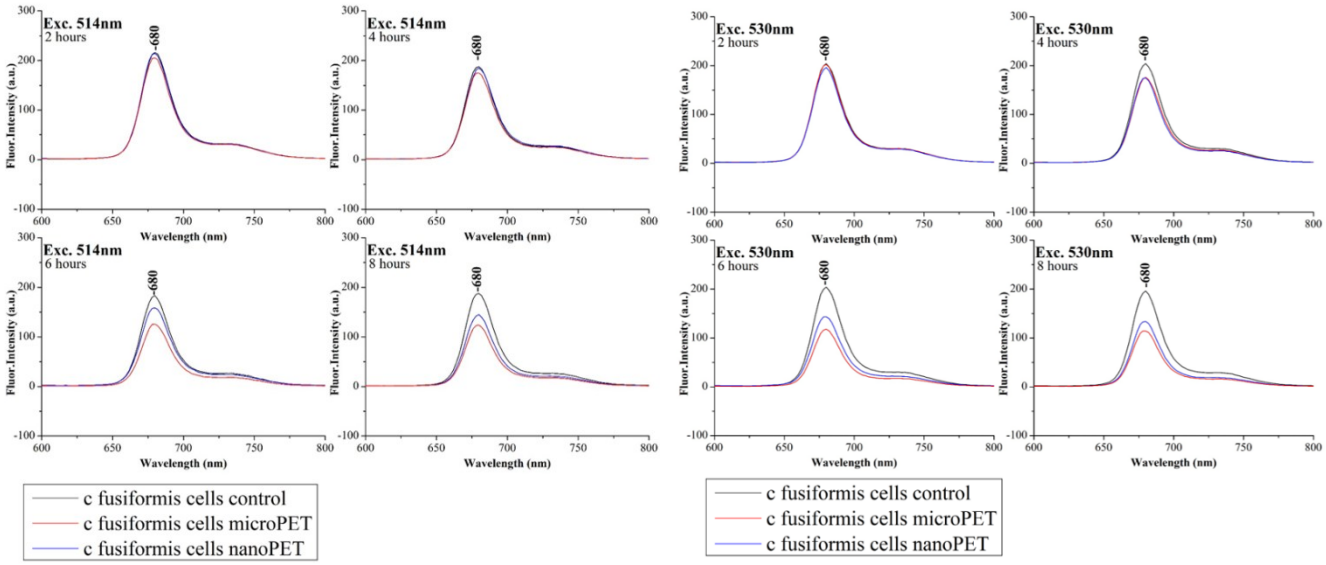

Figure 24 Fluorescence spectra of C. fusiformis cells

To confirm such data, *Ph. tricornutum* and *C. cryptica* have been exposed to MP and NP PET for 4 hours and subsequently analysed with fluorescence microscope using as excitation wavelengths 418, 460 and 530 nm and detecting the emission wavelength in the region around 680 nm.

Figure 25 shows the fluorescence spectra of *Ph. tricornutum* cells excitation 418, 460 and 530 nm with MP/NP PET for 4 hours.

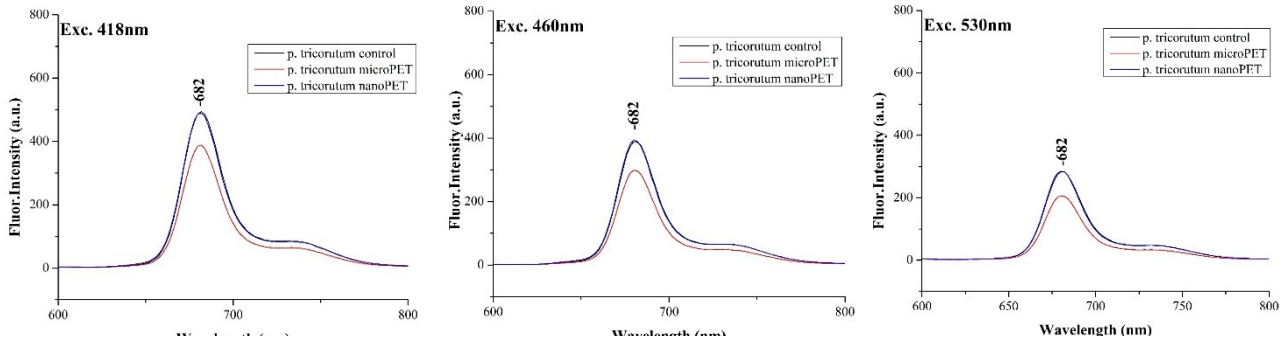

Figure 25 Fluorescence spectra of Ph. tricornutum cells at 4 hours

Figure 26 shows the fluorescence spectra of *C. Cryptica* cells excitation 418, 460 and 530 nm with MP/NP PET for 4 hours

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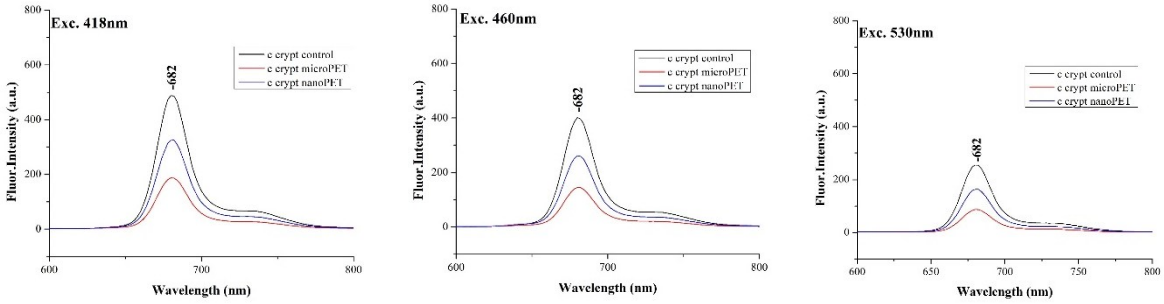

Figure 26 Fluorescence spectra at 418, 460 and 530 nm of C. cryptica with MP and NP PET for 4 hours

In both cases it was observed that, **similarly to *C. fusiformis,* when *Ph. tricornutum* and *C. cryptica* cells are exposed to MP/NP PET for a few (4-8) hours the emission spectra in the region around 680 nm drastically reduces its intensity after exacting diatoms cells with laser light at wavelengths of 418, 460 and 530 nm.**

These results allowed the MOBILES researchers to demonstrate that diatoms coupled with fluorescent microscopy can be used as biosensor to qualitatively detect MP and NP generated by PET degradation.

4. Development of a fast method for detection of MP and NP

During the research work performed at CUT laboratories we observed that diatoms interact with ferricyanide ($[Fe(CN)_6]^{3-}$ through their plasma membrane redox systems, which allow them to reduce this external electron acceptor. Key observations regarding diatoms in the presence of ferricyanide include:

- **Plasma Membrane Redox Activity:** Diatoms, including model species *C. Fusiformis*, possess membrane oxidoreductase enzymes (ferric reductase) that can use potassium ferricyanide as an artificial electron acceptor.
- **Interaction with Photosynthesis:** The presence of external ferricyanide can affect diatom metabolism by competing with photosynthesis for electrons. It has been shown to cause a marked inhibition of carbon fixation at high irradiance.
- **Irradiance Influence:** Light has been shown to stimulate the reduction of ferricyanide by increasing plasma membrane redox activity.

Based on these key observations, we decide to test the possibility to further improve the detection ability of diatoms, in particular reducing the exposure time to MP and NP. Thus, *C. Fusiformis* cells have been exposed to MP and NP of PET and then analysed with fluorescent microscope with the addition of ferricyanide in the sample.

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The presence of ferricyanide within 2 hours induces strong reduction of the 680 nm peak which is further reduced when along the 416 nm a second excitation wavelength at 785 nm is used, which activates ferricyanide, in both MP and NP of PET in contact with diatoms (Figure 27).

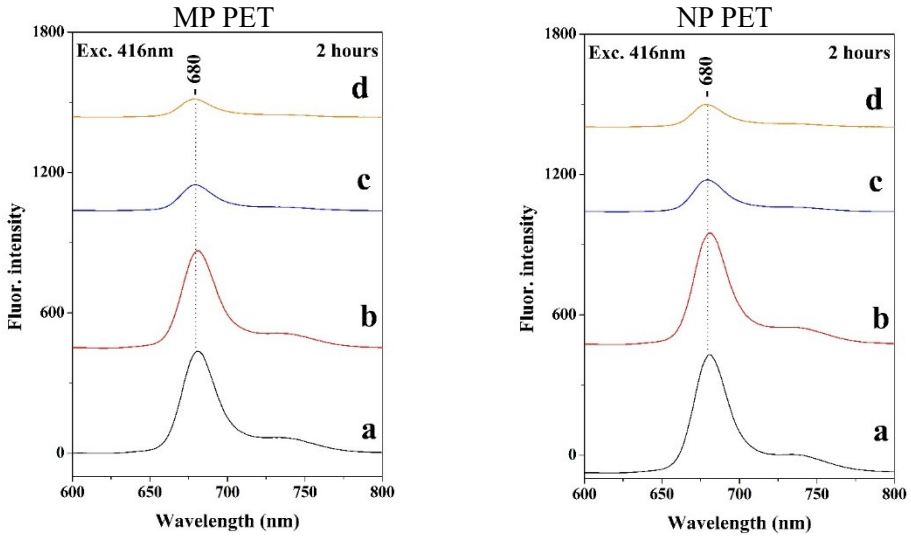

Figure 27 Fluorescence spectra of C. fusiformis cells 2 hours with MP (left panel) and NP sample (right panel) (a) C. Fusiformis control, (b) C. Fusiformis in the presence of 785nm laser (c) C. fusiformis with ferricyanide and (d) C. Fusiformis with 785nm laser and ferricyanide.

Therefore, by coupling ferricyanide and diatoms the detection time is reduced to 2 hours.

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5. Conclusion

We have used a combination of spectroscopic techniques to check the interactions of NP and MP with marine diatoms and take advantage of this interactions to evaluate the feasibility of using marine diatoms as biotic sensors. This document describes the work performed in task 2.7 primarily by CUT in collaboration with NTUA. The workflow was organized as following:

1. Previously gathered data and observations at CUT laboratory showed that *Ph. tricornutum* has the ability to survive for months in water containing degrading plastic. A subsequent UV/Vis spectral analysis revealed a change in the spectral pattern of cells grown in presence of plastic compared to those grown in regular uncontaminated sea water.

2. In MOBILES project a full spectroscopic study on *Ph. Tricornutum* was performed using fluoresce microscopy, FTIR, and Raman with the aim to identify spectral features that can be used as marker to assess the presence of NP and MP materials in marine water.

3. To complement the study on living organisms a full spectroscopic study was performed on PET/PP/PS materials in order to verify possible spectral overlapping in adsorption and emission patterns.

4. Subsequently, for another 6 diatoms species grown in uncontaminated sea water a full spectroscopic characterization was performed using UV/Vis, Fluorescence and Raman spectrometry.

5. Since the spectral patterns in all diatoms were very similar, the focus for further studies was put on three of them. *C. cryptica, Ph. tricornutum* and *C. fusiformis* were grown in sea water containing NP and MP and studied using spectroscopic techniques.

6. It was discovered that specific spectroscopic features of *Chl a* can be used as marker to assess the presence of MP and NP inside diatoms within 8 hours.

7. A rapid protocol based on the addition of ferricyanide was developed to quick (2 hours) evaluate the presence of NP and MP in water using as sensors living diatoms cells.

Furthermore, the spectroscopic techniques allow us to understand the biological mechanisms behind the interaction of plastics materials and diatoms cells. Specifically:

UV/Vis spectroscopy. The interaction of photosynthetic membranes (precisely thylakoids) with exogenous substances involves complex biochemical and physical processes. Exogenous compounds like MP, NP and heavy metals generated by oil-based as well as bioplastics and their stabilizers can bind to or cross the membrane, modulating photosynthetic efficiency, structural integrity, and pigment levels. We could not rely on the UV/Vis experiments to provide information within 2-8 hours on the interactions of diatoms with NP/MP and/or bioplastics.

Fluorescence microscopy. Using concentration of approximately 10 to 15 µg/L of MP/NP, fluorescence techniques provided useful information within two hours of initiating the experiment. Reliable changes in vivo without any exogenous treatment of the peak at 680 nm of *Chl a*

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fluorescence signal where observed. Furthermore, the fluorescence excitation spectra provided information on the specific pigments that are associated with the interactions. The outer photosynthetic membrane, acts as a crucial, porous barrier regulating traffic between the cytoplasm and the chloroplast. It is fundamentally different from the inner, strictly selective, as it facilitates transport, structural maintenance, and environmental response. **The ability of *Chl a* to sense exogenous substances is crucial for its incorporation into chlorophyll-binding proteins in the thylakoid membrane.** The coordination bond between the central Mg atom and ligand molecules can be affected by the surrounding environment in the membrane, influencing the stability of light-harvesting complexes. This sensitivity allows for the regulation of chlorophyll function and turnover but also makes the pigment vulnerable to degradation if not properly bound within protein complexes. Treatment of the cells with **ferricyanide** and 785 nm laser beam can facilitate the interaction of the PET plastic with the cells as monitored by the 680 nm *Chl a* fluorescence signal.

FTIR. We have used FTIR spectroscopy to monitor the interactions of the bioplastics with the protein environment and the lipids in the diatoms. Significant interactions have been detected in both the region of the amide I and II vibrations as well in the region 2800 cm^{-1} of the CH_2/CH_3 of the lipids in the cell membrane providing additional and area specific sensitivity.

Laser-Induced spectroscopy (LIBS) can be used for detection of transition metals and Heavy Metals used in (bio)-plastic stabilizers.

Resonance Raman spectroscopy can be used for monitoring the interactions of any exogenous substances related to MP/NP plastics with the components of the diatoms.

A scientific paper is in preparation by CUT on this topic.

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6. List of Figures

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7. List of Tables

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8. List of Abbreviations

Abbreviation	Abbreviation for
Chl	Chlorophyll
Dds	Diadinoxanthins
Dts	Diatoxanthins
FTIR	Fourier transform Infrared
Fxs	Fucoxanthins
Lhc	Light-harvesting complexes
MP	Microplastics
PET	Polyethylene Terephthalate
PP	Polypropylene
PS	Polystyrene
UV/Vis	Ultraviolet/Visible
NP	Nanoplastics
LIBS	Laser-induced breakdown spectroscopy

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9. Project Consortium

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Italian National Research Council	www.cnr.it/en
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