



**Inês Alexandra Mariano Lourenço**

Licenciada em Ciências da Engenharia Química e Bioquímica

**Utilization of FucoPol for commercial inocula  
immobilization for bioremediation  
applications**

Dissertação para obtenção do Grau de Mestre em Engenharia  
Química e Bioquímica

Orientador: Doutora Filomena Freitas, FCT-UNL

Coorientador: Doutor Pedro Teixeira, Biotask Biotecnologia Lda.

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*Aos meus pais, por serem a minha maior e melhor inspiração.*



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*"Nothing great was ever achieved without enthusiasm." - Ralph Waldo Emerson*



## Resumo

O objetivo desta tese foi produzir estruturas de FucoPol e estudar a sua estabilidade, sob diversas condições, com o propósito de determinar se estas estruturas poderiam ser implementadas em aplicações de biorremediação. Foi realizada, pela primeira vez e com sucesso, uma tentativa de imobilizar células na matriz destas estruturas.

Neste trabalho produziu-se FucoPol. Este exopolissacárido foi sintetizado pela bactéria *Enterobacter A47* e produzido, utilizando glicerol como fonte de carbono, através de um processo de cultivo fed-batch. O FucoPol é composto por fucose, glicose, galactose e ácido glucurônico, tendo também na sua composição grupos acilo. A recentemente demonstrada capacidade de formação de gel do FucoPol foi aplicada à produção de hidrogéis, filmes e esferas de FucoPol.

O trabalho focou-se apenas nas estruturas de hidrogel de FucoPol, visto serem estas as estruturas que melhor se adaptam aos parâmetros desejados para aplicações de biorremediação. Os estudos de estabilidade revelaram que os hidrogéis de FucoPol foram muito estáveis e resistentes em meio aquoso (água desionizada e solução aquosa de NaCl 0,9% (p / v)) e sob o efeito dos iões  $\text{Na}^+$  e  $\text{K}^+$ . O efeito do pH nestes géis não é totalmente compreendido, mas parece que os hidrogéis de FucoPol são mais estáveis em gamas de pH mais baixas e intermediárias (~ 3,5 - 7,0). Os géis de FucoPol também se revelaram bastante estáveis e resistentes em condições de agitação, durando entre 10 a 13 semanas nessas condições. Foi também reportado neste trabalho que os hidrogéis de FucoPol destabilizam e desintegram-se rapidamente na presença de ião fosfato ( $\text{PO}_4^{3-}$ ), semelhante ao observado em hidrogéis de alginato de cálcio. A gama preferencial de concentração de ião fosfato, na qual os hidrogéis de FucoPol podem ser aplicados, também foi determinada em  $[0.01; 0.4] \text{ [PO}_4^{3-} \text{] g L}^{-1}$ ), onde as estruturas de hidrogel podem durar até cerca de 1 a 9 semanas.

A imobilização de inóculos (*P. chlororaphis* e inóculos comerciais da Biotask) nos hidrogéis de FucoPol foi muito bem-sucedida, nas condições apresentadas neste trabalho, registando-se crescimento celular nos meios que continham apenas gel de FucoPol com células imobilizadas. Apesar destes resultados, o método de imobilização deve ser otimizado por forma a obter maiores crescimentos celulares e uma degradação mais lenta das matrizes de FucoPol.

Os hidrogéis de FucoPol foram caracterizados em termos de aparência física, propriedades mecânicas e morfológicas. Os hidrogéis inoculados apresentaram algumas diferenças em relação aos não inoculados. As propriedades mecânicas dos hidrogéis de FucoPol, em geral, revelaram propriedades relativamente fracas, no entanto os hidrogéis à base de FucoPol apresentaram propriedades mecânicas melhoradas, quando comparados com os hidrogéis inoculados. Todas as estruturas revelaram comportamento elástico, evidenciado pelos valores relativamente altos de elasticidade. As imagens de SEM demonstraram que as três estruturas de hidrogel de FucoPol em estudo têm morfologia diferente, com os géis de FucoPol não inoculados apresentando uma matriz com poros de menores dimensões, o que está de acordo com as diferenças observadas na aparência física das estruturas.

Como os hidrogéis de FucoPol produzidos são não citotóxicos, biodegradáveis e bastante estáveis e resistentes em diversas condições, aliado ao fato de a imobilização celular na matriz de gel ser exequível sem afetar a viabilidade celular, estas estruturas demonstraram ter potencial para serem implementadas em aplicações de biorremediação, quando uma otimização adequada do produto final for alcançada.

**Palavras-chave:** FucoPol, hidrogéis, exopolissacáridos, imobilização celular, biorremediação.



## Abstract

The purpose of this thesis was to produce FucoPol based structures and study their stability, under several conditions, in order to determine whether these structures could be applicable for bioremediation applications. For the first time, an attempt to immobilize cells in these structures matrices was also successfully performed.

In this work FucoPol was produced. This exopolysaccharide were synthesized by the bacterium *Enterobacter A47* and produced, using glycerol as the carbon source, in a fed-batch cultivation process. FucoPol is composed of fucose, glucose, galactose and glucuronic acid, and also contains acyl groups. The recently demonstrated gel-forming capacity of FucoPol was applied to the production of FucoPol hydrogels, films and beads.

The work focused on FucoPol hydrogel structures, as they better fit the desired parameters for bioremediation applications. The stability studies revealed that FucoPol hydrogels were very stable and resistant in aqueous media (deionized water and NaCl 0.9% (w/v) aqueous solution) and under the effect of Na<sup>+</sup> and K<sup>+</sup> ions. The pH effect on these gels is not fully understood, but it appears that FucoPol hydrogels are more stable at more low and intermediate pH ranges (~3.5 – 7.0). FucoPol gels also revealed to be quite stable and resistant in agitation conditions, lasting between 10 to 13 weeks in these conditions. It was also reported that FucoPol hydrogels destabilized, and rapidly disintegrated in the presence of phosphate ion (PO<sub>4</sub><sup>3-</sup>), similar to that observed in Ca-alginate hydrogels. The preferential range of phosphate ion concentration, in which FucoPol hydrogels can be applied, it was also determined in [0.01; 0.4] ([PO<sub>4</sub><sup>3-</sup>] gL<sup>-1</sup>), where the hydrogel structures could last for about 1 to 9 weeks.

Immobilization of inocula (*P. chlororaphis* and Biotask commercial inocula) in FucoPol hydrogels was very successful in the conditions presented in this work, with cellular growth being recorded in media that only contained FucoPol gel with immobilized cells. Despite these results, immobilization method should be optimized in future work in order to obtain greater cellular growth and a slower degradation of the FucoPol matrices.

FucoPol hydrogels were characterized in terms of physical appearance, mechanical and morphologic properties. Inoculated hydrogels presented some differences from the non-inoculated ones. The mechanical properties of FucoPol hydrogels in general revealed relatively weak properties, but FucoPol based hydrogels presented improved mechanical properties compared with inoculated hydrogels. All the structures revealed an elastic behavior, evidenced by their high springiness. SEM images demonstrated that the three FucoPol hydrogel structures in study have different morphology, with non-inoculated FucoPol gels presenting a matrix with the smallest pores, which is in accordance with the observed differences in physical appearance of these structures.

Since FucoPol hydrogels produced are non-cytotoxic, biodegradables and quite stable and resistant under several conditions, combined with the fact that cell immobilization in the gel matrix is feasible without affecting the cell viability, these structures proved to have the potential to be implemented in bioremediation applications, when a proper optimization of the final product will be achieved.

**Keywords:** FucoPol, hydrogels, exopolysaccharides, cell immobilization, bioremediation.



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## **1. Introduction and Motivation**

## **1.1 Introduction**

### **1.1.1 Biopolymers**

Nowadays, the world faces a major problem of environmental pollution by synthetic polymers, which have become essential to our life with their innumerable applications in diverse areas such as packaging, agriculture, food, cosmetic, medical, building materials, etc. As a result of that, attempts have been made in order to reduce consumption of those materials. Namely, biodegradable polymers are an innovative emerging field, and numerous biodegradable polymers have been synthesized as some microorganisms and enzymes able of degrading them have been acknowledged. (Chandra & Rustgi, 1998) Biodegradable polymers can be divided into two major classes, synthetic or natural polymers, accordingly to their origin. Synthetic biopolymers (e.g. polylactic acid (PLA), polyglycolic acid (PGA)) presents more advantages by being more versatile with a wide range of applications, being able to achieve and adapt mechanical properties and altering the rate of degradation, as need. Otherwise, natural biopolymers are in crescent demand due to their excellent biocompatibility and their enormous potential in many applications of interest such as drug delivery, tissue engineering, gene therapy, coatings, implants, and more. (Doppalapudi et al., 2014)

Biopolymers can be defined as molecules with high molecular weight which are produced by the metabolism of a living organism. Biopolymers play an important role in formulated systems because of their functional properties as water solubility, water and oil retention capacity, swelling, porosity, foaming, emulsification, viscosity, bulk density and gelling properties. (Wang et al., 2020) Biopolymers include diverse macromolecules, such as polysaccharides, proteins, polyesters, polyamides, lipids and polyphenols, and are produced by bacteria, fungi, plants and animals. (Kaplan et al., 1998)

### **1.1.2 Polysaccharides**

Polysaccharides are natural biopolymers found in almost all living organisms. They are large molecules composed of chains of smaller monosaccharides (simple sugars, like glucose) linked by glycosidic bonds. Many of these biopolymers have biological functions such as structural functions and storage-related functions and present several other advantages such as low cost, biodegradability, polyfunctionality, renewability, chemical reactivity, chelating and absorptive capacities. (Karaki et al., 2016) From a general point of view, polysaccharides are especially important in the field of watersoluble polymers, where they play an important role as thickening, gelling, emulsifying, hydrating and suspending agents, and because of the fact that some polysaccharides give physical gels under well-defined thermodynamic conditions. (Rinaudo, 2008)

Polysaccharides can be produced by plants (e.g.: cellulose, hemicellulose, pectin, guar gum), animals (e.g.: hyaluronan, chondroitin, chitin, chitosan), seaweeds (e.g.: alginate, agar-agar, carrageenan) and microorganisms (e.g.: xanthan gum, dextran). (Karaki et al., 2016) Morphologically they can be classified in three types of polysaccharides: intracellular polysaccharides (located inside or as part of the cytoplasmatic membrane), cell-wall polysaccharides and exocellular polysaccharides (located outside/ attached to the cell wall), also known by exopolysaccharides (EPS).

EPS are divided into homopolysaccharides (made up of a single monosaccharide) and heteropolysaccharides (made of several types of monosaccharide). The bacterial polysaccharides make up a group of polymers of interest because their structural variation is

almost unlimited and rare sugars (such as D-glucose, D-galactose, D-fructose, D-xylose, D-ribose, L-arabinose, L-fucose and L-rhamnose) are often components of these polymers. (Kenne & Lindberg, 1983) Heteropolysaccharide EPS make up the majority of bacterial EPS and have complex structures. They have been the focus of many researchers for environmental applications. (Donot et al., 2012; More et al., 2014; Sandford, 1979) They are composed of repeating units of D-glucose, D-galactose, L-rhamnose and, in some cases N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or glucuronic acid (GlcA). Non-carbohydrate substituent such as phosphate, acetyl and glycerol are also present sometimes. (Nwodo et al., 2012) EPS basic functions are aggregation of bacterial cells, formation of flocs and biofilms, adherence to surfaces, structural element of biofilms, protective barrier for cells and water retention to minimize desiccation of the cell, sorption of inorganic ions, enzymatic activities, sorption of exogenous organic compounds and interaction of polysaccharides with enzymes. (Moreno et al., 1998)

Table 1.1 – Properties and applications of some bacterial EPS, part I (Nwodo et al., 2012)

Bacterial EPS	Polysaccharide Component	Molecular Weight (Da)	Properties	Applications
Dextran	Glucose	$10^6$ - $10^9$	Non-ionic, good stability Newtonian, fluid behavior	Foods, Pharmaceutical industry (Blood volume expander) and Chromatographic media
Alginate	Guluronic acid and mannuronic acid	$(0.3 - 1.3) \times 10^6$	Gelling capacity, film forming	Food hydrocolloid and medicine (surgical dressings, wound management and controlled drug release)
Xanthan	Glucose, mannose and glucuronic acid	$(2.5 - 50) \times 10^6$	High viscosity, Stable over a wide temperature, pH and salt concentrations ranges	Foods, petroleum industry, pharmaceuticals, cosmetics and personal care products
Curdlan	Glucose	$5 \times 10^4 - 2 \times 10^6$	Gel-forming ability, water insolubility, edible and non-toxic has biological activity	Foods, pharmaceutical industry, heavy metal removal and concrete additive
Cellulose	Glucose	$\sim 10^6$	Not soluble in most solvents and high tensile strength	Foods (indigestible fiber), biomedical (wound healing, tissue engineered blood vessels) and audio speaker diaphragms
Succinoglycan	Glucose and galactose	$5 \times 10^3 - 10^6$	High viscosity and acid stability	Food and oil recovery

Table 1.1 – Properties and applications of some bacterial EPS, part II (Nwodo et al., 2012)

Bacterial EPS	Polysaccharide Component	Molecular Weight (Da)	Properties	Applications
Glucuronan	Glucuronic acid	$6 \times 10^4 - 6 \times 10^5$	Gelling and thickening capacity	Food and cosmetics products
Colanic acid	Fucose, glucose, glucuronate and galactose	$2 \times 10^4 - 6 \times 10^5$	Gelling capacity	Cosmetics and personal care products

### 1.1.3 FucoPol

FucoPol is a fucose-containing EPS produced by bacterium *Enterobacter* A47 (Gram-negative bacteria), with high molecular weight ( $1.7 - 5.8 \times 10^6$  Da) and composed of glucose (28-34 %mol), galactose (25-26 %mol), fucose (32-36 %mol), glucuronic acid (9-10 %mol) and acyl substituents, namely acetate (3-5 %wt.), pyruvate (13-14 %wt.) and succinate (~3 %wt.). (Fialho et al., 2019; Torres et al., 2011) For being a fucose-containing polysaccharide it has a high market value, since fucose it's a rare sugar that is available at low quantities and a high cost, and for fucose known interesting properties such as anti-inflammatory, anti-aging, anti-cancer and positive effects on diabetes prevention and interest for the cosmetic and pharmaceutical industries. (Antunes et al., 2017; Freitas et al., 2011; Saari et al., 2009)

FucoPol production is carried out in a stirred bioreactor, under conditions of controlled pH and temperature ( $6.80 \pm 0.05$  and  $30.0 \pm 0.1$  °C, respectively), using glycerol as a carbon source, however the production of this biopolymer can be quite versatile since the physicochemical factors of the cultivation conditions (pH, temperature, concentration of oxygen, agitation and the composition of the culture medium) can be manipulated and changed. (Antunes et al., 2015; Antunes et al., 2017; Freitas et al., 2014) The effect of temperature and pH on cell growth and FucoPol production reveals that the *Enterobacter* bacterium has the ability to synthesize different heteropolysaccharides, with different sugar composition and molecular weight, although the fucose content in the different polymers keeps stable, which is valuable for the production at an industrial level. (Torres, 2012)

FucoPol has the ability to form viscous aqueous solutions with shear thinning behavior. The shear thinning behavior, as well as its viscosity, were maintained over an extended range of pH and ionic strength (3.5 – 8.0 and 0.05-0.50 M NaCl, respectively) and FucoPol solutions show good thermal stability when subjected to oscillations of temperature. (Ferreira et al., 2014) These properties give this biopolymer the ability to form and stabilize emulsions of hydrophobic compounds in water, which is particularly important in the encapsulation of hydrophobic substances. FucoPol also presents properties that foresee a good potential to be used as an encapsulation material, namely its film-forming and gel-forming abilities. (Ferreira et al., 2014; Fialho et al., 2019; Roca et al., 2015)

Most recent studies also reported that FucoPol has a good cryoprotective potential to be implemented in cryopreservation formulations, as well as the ability to gel in the presence of metal cations, like iron and copper, which has been used to prepare hydrogel beads. These studies revealed that iron mediated hydrogels were stable and not cytotoxic. (Fialho et al., 2019; Guerreiro et al., 2020)

All these properties combined make FucoPol a biopolymer of high value, with high versatility, that can be applied in several areas and applications, such as pharmaceuticals, cosmetics, food products, medical uses, drug delivery systems, bioremediation and others. (Torres, 2012)

#### 1.1.4 Bioremediation

Bioremediation is defined as the process whereby wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities. (Mueller et al., 1996) It offers the possibility to eliminate or render harmless contaminants using natural biologic activity and uses relatively low-cost, low-technology techniques that have a high public acceptance. Because it seems to be a good alternative to conventional clean-up technologies its research is rapidly increasing and it has been used at a various sites worldwide. (Head, 1998; Sharma, 2012) Bioremediation techniques have been used for decontamination of surface and subsurface soils, freshwater and marine systems, soils, groundwater and contaminated land ecosystems, however the majority of bioremediation techniques initially developed were meant to treat petroleum hydrocarbon contamination. (Juwarkar et al., 2010)

Bioremediation applies bacteria, fungi and plants to degrade or detoxify hazardous substances. The microorganisms implemented may be indigenous to a contaminated area or they may be isolated from a different place and brought to the contaminated site. The harmful contaminants are transformed by living organisms through reactions of their metabolic processes and the biodegradation of a compound is often a result of the action of multiple organisms. Bioremediation can be effective only in environments with conditions that permit microbial growth and activity and bioremediation's applications often involve the manipulation of environmental parameters to allow microbial growth and degradation to occur at a faster rate. (Vidali, 2001)

Bioremediation can be divided into *in situ* bioremediation and *ex situ* bioremediation, accordingly the site of application. *Ex situ* bioremediation techniques involve excavating pollutants from the contaminated sites and transporting them to another site for treatment. *Ex situ* bioremediation techniques are usually considered based on the cost of treatment, depth of pollution, type of pollutant, degree of pollution, geographical location and geology of the contaminated site. Windrow composting, biopiles, land-farming and bioreactors are *ex situ* bioremediation techniques. Composting consist is a technique that involves combining contaminated soil with nonhazardous organic amendments such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature (55-65 °C). Landfarming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded with the objective to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of pollutants (generally limited to the treatment of superficial 10–35 cm of soil). Biopiles *ex situ* remediation is a full-scale technique in which excavated soils are mixed with soil amendments, sited on a treatment area, and bioremediated by using forced aeration, where pollutants are reduced to carbon dioxide and water. The basic biopile system includes a treatment bed, an aeration system, an irrigation/nutrient system and a leach ate collection system. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. The reactors used for *ex situ* treatments are slurry reactors or aqueous reactors. (Shukla et al., 2010; Vidali, 2001)

*In situ* bioremediation techniques involve treating hazard substances at the site of pollution. It does not require excavation; therefore it makes little or no disturbance to soil structure. *In situ* techniques are usually less expensive compared to *ex situ* bioremediation techniques, due to no extra cost required for excavation and transportation processes. Otherwise, the cost of design and on-site installation of sophisticated equipments, made to improve microbial activities during

bioremediation, represents a major con. *In situ* bioremediation techniques have been widely and successfully implemented to treat chlorinated solvents, dyes, heavy metals, and hydrocarbons polluted sites. The status of electron acceptor, moisture content, nutrient availability, pH and temperature are vital environmental conditions that need to be suitable for a successful *in situ* bioremediation to be achieved and (unlike *ex situ* bioremediation techniques) soil porosity strongly influences the application of *in situ* bioremediation. Biosparging, bioventing, biostimulation, bioaugmentation and natural attenuation are *in situ* techniques. Natural attenuation refers to processes that naturally transform contaminants to less harmful forms or immobilize contaminants so that they are less of a threat to the environment. (Kouzuma & Watanabe, 2011)

Bioventing is the most common *in situ* treatment. It involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria to grow, which will degrade the hazardous substances. Bioventing technique uses low air flow rates and provides just the amount of oxygen necessary for the biodegradation, while minimizing volatilization and release of contaminants. It works for simple hydrocarbons and can be applied where the contamination is deep under the surface. Biosparging relies in the injection of air under pressure below the water table in order to increase groundwater oxygen concentration as well as to enhance the rate of biodegradation of contaminants by bacteria. This technique increases the mixing in the saturated zone and in so doing, it increases the contact between soil and groundwater. Biostimulation is the technique by which biodegradation is stimulated by supplying the environment with nutrients, such as nitrogen and phosphorous, with electron acceptors such as oxygen, and with substrates such as methane, phenol, and toluene. The chemical additives used as substrates, phenol and toluene, are well-known toxic chemicals. Thus, the concentrations of these chemicals during biostimulation should be carefully monitored. (Iwamoto & Nasu, 2001) Bioaugmentation concerns the addition of microorganisms in order to enhance a specific biological activity, and it has been implemented in a vast number of areas, such as agriculture, forestry and wastewater treatment. It can be defined as the technique by which the capacity of a contaminated matrix to remove pollution is improved by the introduction of specific competent stains or microorganisms consortium. This technique relies in the metabolic capacities of the indigenous microbial community (already present in the contaminated site) for cleanup that will be increased by an exogenously enhanced genetic diversity, which leads to diverse productive biodegradation reaction. Bioaugmentation's utility is supported by several studies that show the incompetence of indigenous species in some cases and the apparent enhanced bioremediation rate, after the addition of indigenous, competent microorganisms. Bioaugmentation as a viable methodology is a controversial theme, however, on balance, the recent studies are encouraging it and thus bioaugmentation might well be used as a rational methodology for site remediation and could emerge as one of eco-friendly techniques for pollution reduction, under circumstances of understanding the site's ecology and the local physicochemical constraints as well. (El Fantroussi & Agathos, 2005; Vogel, 1996)

Table 1.2 – Summary of bioremediation strategies, part I (Sharma, 2012; Shukla et al., 2010; Vidali, 2001)

Technology	Examples	Benefits	Limitations
<i>In situ</i>	<ul style="list-style-type: none"> <li>• Biosparging</li> <li>• Bioventing</li> <li>• Biostimulation</li> <li>• Bioaugmentation</li> <li>• Natural attenuation</li> </ul>	<ul style="list-style-type: none"> <li>• Most cost efficient</li> <li>• Noninvasive</li> <li>• Relatively passive</li> <li>• Treats soil and water</li> </ul>	<ul style="list-style-type: none"> <li>• Extended treatment time</li> <li>• Monitoring difficulties</li> </ul>

Table 1.2 – Summary of bioremediation strategies, part II (Sharma, 2012; Shukla et al., 2010; Vidali, 2001)

Technology	Examples	Benefits	Limitations
<i>Ex situ</i>	<ul style="list-style-type: none"> <li>• Windrow composting</li> <li>• Biopiles</li> <li>• Land-farming</li> </ul>	<ul style="list-style-type: none"> <li>• Cost efficient</li> <li>• Low cost</li> </ul>	<ul style="list-style-type: none"> <li>• Space requirements</li> <li>• Extended treatment time</li> <li>• Need to control abiotic loss</li> <li>• Mass transfer problem</li> <li>• Bioavailability limitation</li> </ul>
Bioreactors ( <i>ex situ</i> )	Slurry reactors Aqueous reactors	<ul style="list-style-type: none"> <li>• Rapid degradation kinetic</li> <li>• Optimized environmental parameters</li> <li>• Enhances mass transfer</li> <li>• Effective use of inoculants and surfactants</li> </ul>	<ul style="list-style-type: none"> <li>• Soil requires excavation</li> <li>• Relatively high cost</li> <li>• Relatively high operating cost</li> </ul>

### 1.1.5 Cell immobilization

Bioremediation processes more and more often employ immobilization methods. Cell immobilization techniques considerably reduce the cost of bioremediation processes by bringing several benefits, such as higher efficiency of pollutants degradation and multiple use of biocatalysts. The methods for cell immobilization can be classified in five main techniques: adsorption, binding on a surface (electrostatic or covalent), flocculation (natural or artificial), encapsulation and entrapment. (Klein & Ziehr, 1990)

The immobilization process by adsorption relies in the physical interactions of microbial cells and enzymes with the surface of water-insoluble carriers, achieved by the formation of weak bonds. Microbial adsorption can be divided into several steps, namely adsorption of extracellular organic macromolecules on the surface, transport of cells from the bulk phase to the surface, reversible attachment of cells and biosynthesis of polymers by the cell which leads to an irreversible attachment of the microorganism to the surface. The method of cell immobilization by adsorption is quick, simple, eco-friendly and cost-effective. (Dzionic et al., 2016)

Electrostatic binding on a surface is very similar to physical adsorption, with the difference that the probability of microorganisms leaking is much lower, and it requires washing the surface of the carrier with a buffer solution in order to obtain a hydrophilic surface that can attract the negatively charged cells or enzymes. (Bickerstaff; Dzionic et al., 2016)

Encapsulation involves immobilization of the biologically active component in hydrogel beads or microcapsules. The cells are mixed into the hydrogel or a membrane-forming material, and then dispersed dropwise. The gelation/membrane formation is obtained through physical (temperature) or chemical (cross-linker) modifications. In this process the immobilized particles are separated from the external environment with a semi-permeable membrane. The major advantage of this procedure is the considerable protection of biological material against the adverse conditions of the external environment. However this technique presents limited permeability of the applied membrane and a high probability of its damage by growing cells. (Dulieu et al., 1999) To date, several substances, including DNA, proteins, viruses and live bacteria, have been encapsulated for a wide range of applications in medicine, agriculture, biocatalysts and bioremediation. (Tong et al., 2013)

Entrapment of microorganisms is well-known and has been increasingly used in bioremediation. Entrapped cell bioaugmentation consists in adding a gel or a rubber matrix embedded with microorganisms to increase biological activities and procedures normally consist of two steps: mixing of cells and matrix, and gelation. This technology is an integration of cell entrapment and cell bioaugmentation techniques, in order to obtain a new technology to remove pollutants and hazardous substances in wastewater and contaminated sites. The microorganisms entrapped in the heterogeneous carrier are physiologically diverse and the cells located near the surface have high metabolic activity (comparatively to starved cells in the interior of the carrier). (Siripattanakul & Khan, 2009) The immobilizing matrix can protect the augmented cells against external environmental stresses and prevent their loss from the target system, so the success of cell bioaugmentation relies on the viability and retention of the cells. Entrapment is a rapid, nontoxic, inexpensive and versatile method. (Orive et al., 2003) The matrices used in this method could be divided into hydrogels (alginate,  $\kappa$ -carrageenan, chitosan), thermogels (agar, agarose, cellulose), and synthetic polymers (polyacrylamide, polyvinyl alcohol, polyurethane). (Hae, 2012)

### **1.1.6 Support materials for cell immobilization**

The matrices for cell immobilization should be insoluble, non-toxic (both for the immobilized material and the environment), easily accessible, inexpensive, stable and suitable for regeneration and the immobilization process should be simple and harmless. An important aspect is that different immobilization methods require different support materials with specific properties, and the character of performed bioremediation processes also has an impact on the selection of the carrier. The immobilizing matrices used in bioaugmentation must be readily biodegradable and in wastewater treatment processes the carrier should have a high mechanical resistance derived to exposure to different types of physical forces. (King & Goosen, 1993)

The support material can be classified as natural and synthetic or organic and inorganic. Natural organic carriers, as alginate,  $\kappa$ -carrageenan, chitosan, sawdust, straw, charcoal, plant fibres, corncob, bagasse, rice, husks of sunflower seeds, diatomite and mycelium, have many functional groups, which stabilize biocatalysts, and are hydrophilic, biodegradable, biocompatible, and inexpensive. Otherwise, the possibility of their application in bioremediation processes is limited because of low resistance to biodegradation, sensitivity to organic solvents, and stability in a narrow pH range. Synthetic organic carriers, such as polypropylene, polyvinyl chloride, polystyrene, polyurethane foam, polyacrylonitrile and polyvinyl alcohol, have numerous functional groups with diversified characters and present the advantage of having the possibility to regulate their structure at the macromolecular level, as they can also be formed into various shapes (tubes, membranes, coatings, carriers of various shapes from spherical to oval), and are relatively inexpensive and easily available. Inorganic carriers (natural and synthetic), represented by magnetite, volcanic rocks, vermiculite, porous glass, silica-based materials, ceramics and nanoparticles, have a high chemical, physical and biological resistance and they

are used in the formation of hybrid carriers, combining natural polymers and synthetic nanoparticles. (Dzionek et al., 2016)

Hydrogels are being widely investigated for cell immobilization due to their material properties, which can be engineered for biocompatibility, selective permeability, mechanical and chemical stability, and other requirements (cell distribution, thickness and mechanical strength), specific for the application purposes. (King & Goosen, 1993) For successful immobilization, the hydrogel must be favorable to cell viability and function (biocompatible), have appropriate permeability (to allow sufficient diffusion and transport of oxygen and essential nutrients), biocompatibility and chemical and mechanical stability. The hydrogels must also be nontoxic. (Goosen, 1993; Jen et al., 2000) Despite the limitations, such as the nature of the particles used that can affect the performance of a continuous reactor, substrate/product diffusion limitations, the limited life of the gels and, in the case of calcium alginate, substances with even a modest affinity for  $\text{Ca}^{2+}$  will sequester the crosslinking  $\text{Ca}^{2+}$  ions consequently destabilizing the gel, hydrogels are now being applied to a wide variety of fields (e.g.: calcium alginate and k-carrageenan). (D'Souza, 1989; Smidsrod & Skjakbrk, 1990) The high mechanical and chemical stability, swelling properties, low content of toxic contaminants, defined pore size and a pore size distribution may be achieved by selection and purification of alginates, selection and control of the gelling process, and addition of co-polymers. (Salter & Kell, 1991)

Several methods for cell immobilization are being employed, namely the entrapment in sodium alginate, entrapment in agarose or agar, as well as synthetic immobilization in polyacrylamide gel and the protocols for cell immobilization are being optimized as well as new carriers are being discovered. (Moreno-Garrido, 2008; Stormo & Crawford, 1992; Trelles & Rivero, 2013)

## 1.2 Motivation

Contemporaneous society is deeply linked with industrialization and technology continues to focus on the demands of industry to increase efficiency and production output. Otherwise, contemporaneous society is also facing the worst environmental issues ever, mostly derived from human activity itself. Environmental pollution is a major public health concern due to the harmful effects of pollutants to humans, other living organisms and to the ecosystems. With the urge to reverse these effects, industry must adapt and develop sustainable production systems and technologies which mitigate the environmental impact. (Campbell et al., 2019)

Bioremediation relies on biological processes that result in efficient and permanent removal of pollutants. Its eco-sustainable features, cost-effectiveness at different scales of operations and its simplicity of operations and monitoring, make this technology a sustainable process for environmental management. (Azubuike et al., 2016)

Bio-based and biodegradable products have raised great interest since sustainable development policies tend to expand as well as the growing concern for the environment. Biopolymers bring a significant contribution to the sustainable development, with minor environmental impacts, namely polysaccharides have distinct physical-chemical properties, such as water retention capacity, rheology (e.g. thickening, emulsifying and gelling agents), and film forming capacity that allows their application in several industries (e.g. cosmetic, food and pharmaceutical). (Avérous & Pollet, 2012; Moreno et al., 1998) So, the objective of this work is to use FucoPol, a fucose-containing exopolysaccharide, as an immobilization matrix for commercial inocula (Biotask) used for bioremediation applications, as well as to averiguate if the FucoPol based structures produced have the desired properties for this purpose (namely, to access their stability in different media).



## **2. Materials and Methods**

## 2.1 FucoPol production

FucoPol exopolysaccharide was produced by *Enterobacter* strain A47 (DSM 23139) in a 10 L bioreactor (BioStat B-plus, Sartorius), with controlled pH ( $6.98 \pm 0.05$ ) and temperature ( $30.0 \pm 0.1$  °C).

*Enterobacter* culture was previously cryopreserved in glycerol (20% v/v) at -80°C, and it was reactivated by inoculating 1 mL of the preserved culture into 100 mL (liquid) Luria Bertani (LB) medium (10.0 g L<sup>-1</sup> peptone; 5.0 g L<sup>-1</sup> yeast extract; 10.0 g L<sup>-1</sup> NaCl), in 250 mL shake flasks, and incubated for 16 h, in an orbital shaker (30 °C, 200 rpm). The inoculum was prepared by adding 40 mL of inoculum into 7192 mL of Medium E\* (K<sub>2</sub>HPO<sub>4</sub>, 5.8 g L<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub>, 3.7 g L<sup>-1</sup>; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 3.3 g L<sup>-1</sup>), prepared and autoclaved inside the bioreactor itself, which was supplemented with 80 mL MgSO<sub>4</sub> solution (100 mM), 8 mL of mineral solution (per litre of 1 N HCl: FeSO<sub>4</sub>•7H<sub>2</sub>O, 2.78 g; MnCl<sub>2</sub>•4H<sub>2</sub>O, 1.98 g; CoSO<sub>4</sub>•7H<sub>2</sub>O, 2.81 g; CaCl<sub>2</sub>•2H<sub>2</sub>O, 1.67 g; CuCl<sub>2</sub>•2H<sub>2</sub>O, 0.17 g; ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.29 g) and 320 mL of glycerol (ReagentPlus 99% w/w, Sigma-Aldrich), as carbon source. The MgSO<sub>4</sub> solution, the mineral solution and the glycerol were autoclaved separately and added to the bioreactor after cooling to room temperature, before inoculating the bacterium (to avoid precipitation of medium components).

During the first 8 h of cultivation, the bioreactor was operated in a batch mode, with an initial 40 g L<sup>-1</sup> concentration of glycerol. After this, the reactor started a fed-batch mode, for 88 h, with a feed solution (Medium E\* supplemented with 900 g L<sup>-1</sup> of glycerol) that was supplied to the reactor at a constant rate of 5 mL h<sup>-1</sup>. The aeration rate (0.125 vvm, volume of air per volume of reactor per minute) was kept constant through all the cultivation, and the dissolved oxygen concentration (DO) was controlled at 10% of air saturation, by automatic variation of the stirred speed (400-800 rpm). Foam formation was automatically controlled by the addition of Antifoam A (Sigma-Aldrich) and the pH was automatically controlled by addition of HCl (2 M) or NaOH (5 M).

## 2.2 FucoPol extraction

The extraction and purification of FucoPol was performed through several steps and techniques.

Initially, the culture broth was diluted with deionised water (1:10), for viscosity reduction, to be centrifuged (45 min, 10375xg, 4 °C) (Sigma 4-16 KS, Germany) in order to separate the biomass from the supernatant. The cell-free supernatant was submitted to thermal treatment (1 h, 70 °C) and centrifuged again, under the same conditions described above, with the purpose of removing precipitated proteins and remaining cell debris. Afterwards, purification step was performed by submitting the treated supernatant to a dialysis process, using a 30 kDa cut-off membrane (Hydrosart® SARTOCON Slice Cassette, Sartorius), with a 0.6 m<sup>2</sup> effective area of filtration, operated against water at a pressure below 1.5 bar. Removed all the impurities, FucoPol was concentrated using the same membrane module, switching to an ultrafiltration mode, and the purified polymer was then freeze dried (Scanvac, CoolSafe), for 48. The resultant FucoPol was stored at room temperature.

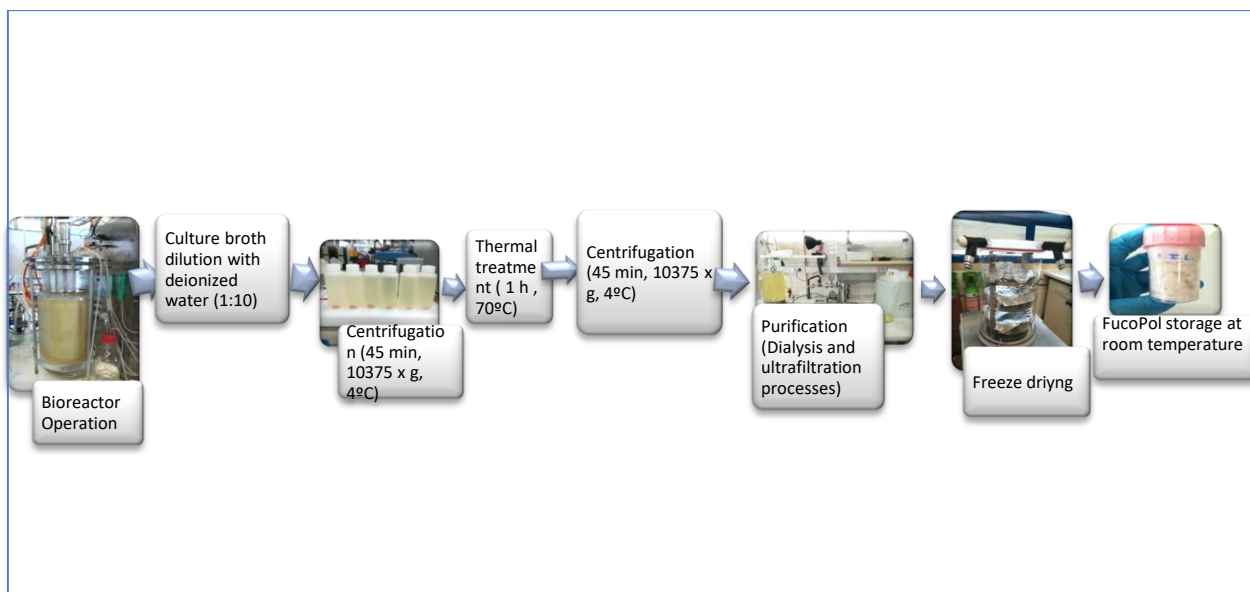


Figure 2.1 – Schematic representation of FucoPol extraction and purification

## 2.3 Development of FucoPol structures

In this work it was performed two types of FucoPol based structures: FucoPol gels production and FucoPol films production. FucoPol gel beads we're also performed with the intention to use these structures in the immobilization process.

The purpose of gelation is to enhance an insoluble character for the FucoPol structures.

### 2.3.1 FucoPol hydrogels

It is known by previous work that FucoPol has the ability to form hydrogels in the presence of divalent and trivalent cations. Therefore, hydrogels were prepared by cation gelation in the presence of iron ( $\text{Fe}^{3+}$ ) following the procedure reported by Fialho et al. (2019), with slight modifications.

An aqueous FucoPol solution (1% w/v) was prepared, under constant stirring (900 rpm) until a homogeneous solution was obtained. Afterwards, FucoPol gels were obtained by dialysis, with 5 mL FucoPol solution placed inside a 12-14 kDa MWCO membrane, against a  $1.3 \times 10^{-5}$  mM  $\text{FeCl}_3$  aqueous solution ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , Sigma- Aldrich), for 24 h, with constant stirring (200 rpm), at room temperature.

After this, the excess iron cations were removed by dialysis against deionized water, with constant stirring (200rpm), until the conductivity constant was below the value of  $10 \mu\text{S cm}^{-1}$ .

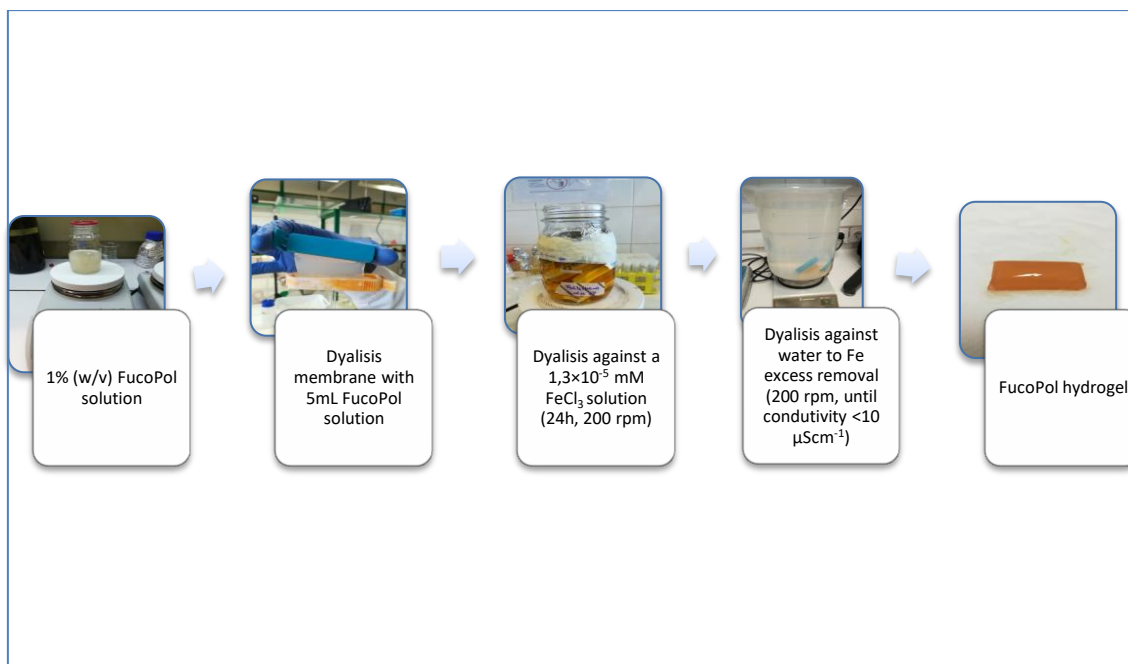


Figure 2.2 – Schematic representation of FucoPol hydrogels preparation

### 2.3.2 FucoPol and chitosan films

In this work was intended to perform FucoPol films blended with chitosan, in order to obtain a more resistant and insoluble film, to accomplish the purposes of a suitable matrix for cell immobilization to apply in bioremediation processes.

For the first approach to this procedure, a FucoPol solution (1.5%, w/v) was prepared, with deionized water, under constant stirring (900 rpm) until a homogeneous solution was obtained. Then, citric acid (Panreac Química S.L.U., Barcelona, Spain), in a proportion of 1:1 (w/w), was added to the FucoPol solution and kept under constant stirring, until a homogeneous solution was obtained. Then, a chitosan solution (1.5% w/v) was prepared, dissolving chitosan in acetic acid (1% v/v), under constant stirring (300 rpm), until a homogeneous solution is obtained. Afterwards, the two solutions were mixed, under constant stirring (300 rpm), until complete homogenization, and 20 mL of solution were transferred to Teflon petri dishes (Bola, Germany) and let dry horizontally (30°C) until the films were formed.

In the second approach, an acetic acid solution (2.5% w/v) was prepared, with deionized water, and chitosan (1.5 g) was slowly added to this solution, under constant stirring (300 rpm), until complete dissolution of chitosan is achieved. Another acetic acid solution (2.5% w/v) was prepared, with deionized water, and FucoPol (1.5 g) was slowly added to this solution, under constant stirring (300 rpm), until a homogeneous solution is achieved. Then, the two solutions were slowly and carefully mixed, under constant stirring (300 rpm), until homogeneity is obtained, and 20 mL of solution we're transferred to Teflon petri dishes (Bola, Germany) and let dry horizontally (30°C) until total evaporation of solvents and the films were formed.

The last approach to film preparation consisted in preparing a FucoPol solution (1.5% w/v), with deionized water, under constant stirring (900 rpm), and adding 30 mL of glycerol (ReagentPlus 99% w/w, Sigma-Aldrich). After the solution is homogenized, 20 mL of solution we're transferred to Teflon Petri dishes (Bola, Germany) and let dry horizontally. After drying, the films

were completely covered with a  $1.3 \times 10^{-5}$  mM  $\text{FeCl}_3$  aqueous solution ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , Sigma-Aldrich), to obtain gelation.

### 2.3.3 FucoPol gel beads

A FucoPol solution (1% w/v) was prepared, with deionized water, under constant stirring (900 rpm) until a homogeneous solution is obtained.

For gel bead preparation, the FucoPol solution was dripped through a syringe with a needle (FINE-JECT ® 23 G – 0.6 x 25 mm) into a  $\text{FeCl}_3$  aqueous solution ( $1.3 \times 10^{-5}$  mM), and kept under stirring (300 rpm, 21 °C) for 30 minutes.

For iron excess removal, the beads were washed with deionized water, with constant stirring (200rpm), until the conductivity constant was below the value of  $10 \mu\text{Scm}^{-1}$ .

## 2.4 Stability of FucoPol hydrogels

As mentioned in section 1.1.3, a good matrix for cell immobilization should be non-toxic, suitable for regeneration, insoluble and stable. Consequently, the stability of the FucoPol gel structures was evaluated, at a constant temperature (21 °C), under several conditions: aqueous media influence, pH influence, ion influence, agitation influence and phosphorous concentration influence.

### 2.4.1 Aqueous media

The stability of FucoPol hydrogels was evaluated under the influence of four aqueous media: deionized water, NaCl aqueous solution (0.9%, w/v), PBS solution ( $\text{NaCl}$ ,  $8 \text{ g L}^{-1}$ ;  $\text{KCl}$ ,  $0.2 \text{ g L}^{-1}$ ;  $\text{Na}_2\text{HPO}_4$ ,  $1.44 \text{ g L}^{-1}$ ;  $\text{KH}_2\text{PO}_4$ ,  $0.245 \text{ g L}^{-1}$ ) and hypochlorite aqueous solution (0.5%, v/v). One FucoPol gel structure was placed in 150 mL of each solution, making triplicates of each solution in study. Records of the observed changes in the hydrogels were made, and samples of 1 mL from each solution were taken periodically in order to perform a precipitation test with acetone (in the proportion acetone:water of 3:1, v/v) to evaluate if there was release of polymer to the solutions. Solution's pH was also measured periodically.

### 2.4.2 pH effect

The stability of FucoPol hydrogels were evaluated under different ranges of pH. Four buffer solutions were prepared for the study purposes: acetate buffer (pH=4.0), phosphate buffer

(pH=5.5), borate buffer (pH=8.5) and ammonium chloride buffer (pH=10.0). One FucoPol gel structure was placed in 150 mL of each buffer solution, making triplicates of each buffer solution in study. Records of the observed changes in the hydrogels were made, and samples of 1mL from each solution were taken periodically in order to perform a precipitation test with acetone (in the proportion acetone:water of 3:1, v/v) to evaluate if there was release of polymer to the solutions. Solution's pH was also measured periodically.

### **2.4.3 Stirring effect**

Wastewater effluents are not static, thus, the stability of FucoPol hydrogels was tested under the influence of stirring, to attempt simulate these effluents. NaCl (0.9% w/v) and deionized water were the media tested for this purpose. One FucoPol gel structure was placed into 150 mL of solution, under constant magnetic stirring (250 rpm). Each solution in study was made in triplicate. Records of the observed changes in the hydrogels were made, and samples of 1mL from each solution were taken periodically in order to perform a precipitation test with acetone (in the proportion acetone:water of 3:1, v/v) to evaluate if there was release of polymer to the solutions. Solution's pH was also measured periodically.

### **2.4.4 Ionic effect**

As result of the observations made in the work developed in section 2.4.1 and 2.4.2, it was necessary to study and understand if the ionic species present in solutions may have a destabilizing effect on FucoPol hydrogels.

For that purpose, solutions of NaCl (1.8%, w/v), KCl (0.7% and 1.4%, w/v) and a phosphate buffer, prepared by altering the potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) component for sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), were prepared. The intention was to evaluate the effect of the double the concentration of ions ( $\text{Na}^+$  and  $\text{K}^+$ ), as well as to averiguate if the destabilizing effects came from sodium ions or from potassium ions. One FucoPol gel structure was placed into 150 mL of solution, making triplicates of each solution in study. Records of the observed changes in the hydrogels were made, and samples of 1 mL from each solution were taken periodically in order to perform a precipitation test with acetone (in the proportion acetone:water of 3:1, v/v) to evaluate if there was release of polymer to the solutions. Solution's pH was also measured periodically

### **2.4.5 Concentration of phosphate ion ( $\text{PO}_4^{3-}$ )**

Simplified medium E\* (with diammonium hydrogen phosphate ( $(\text{NH}_4)_2\text{HPO}_4$ ) as the only component), with different concentrations ( $0.01 \text{ g L}^{-1}$ ;  $0.02 \text{ g L}^{-1}$ ;  $0.05 \text{ g L}^{-1}$ ;  $0.1 \text{ g L}^{-1}$ ;  $0.4 \text{ g L}^{-1}$ ;  $0.82 \text{ g L}^{-1}$ ;  $1.65 \text{ g L}^{-1}$ ) were prepared with deionized water, and one FucoPol gel structure was placed into 150 mL of each solution. These tests were made in duplicates Records of the observed changes in the hydrogels were made, and samples of 1 mL from each solution were taken periodically in order to perform a precipitation test with acetone (in the proportion

acetone:water of 3:1, v/v) to evaluate if there was release of polymer to the solutions. Solution's pH was also measured periodically.

## 2.5 Cell immobilization in FucoPol hydrogels

The first step to cell immobilization in FucoPol gel structures was to determine if the hydrogels were stable in the cultivation mediums, which was accomplished through the previous sections of this work (namely, in the determination of a phosphate concentration range).

### 2.5.1 *Pseudomonas chlororaphis* inocula

All the steps to cell immobilization in FucoPol hydrogels were performed in a flow chamber, aiming to work in sterile conditions (Heraeus SB 48, Germany).

A colony of *Pseudomonas chlororaphis* was inoculated in 100 mL LB medium (Tryptone, 10 g L<sup>-1</sup>; NaCl, 10 g L<sup>-1</sup>; Yeast extract, 5 g L<sup>-1</sup>), previously autoclaved (Uniclave 77, Portugal), in a 250 mL baffled shake flask, and incubated for 24 h, in an orbital shaker (30°C, 200 rpm). Then, the inoculum was harvested by centrifugation (4000 rpm, 10 min), and the resultant pellet of cells was suspended in a FucoPol solution (1%, w/v), prepared with sterilized deionized water. Afterwards, inoculated FucoPol gels were obtained by the same process described in section 2.3.1.

Modified mediums E\*((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.1 and 0.4 g L<sup>-1</sup>) were prepared, in six shake flasks (500 mL) with 150 mL of medium: 3x150 mL of 0.4 g L<sup>-1</sup> medium E\*(1A, 1B and 1C) and 3x150 mL of 0.1 g L<sup>-1</sup> medium E\*(2A, 2B and 2C). The mediums were sterilized by autoclaving (Uniclave 77, Portugal). To each medium was added 6 mL of glycerol (ReagentPlus 99% w/w, Sigma-Aldrich), 1.5 mL of mineral solution (per litre of 1 N HCl: FeSO<sub>4</sub>•7H<sub>2</sub>O, 2.78 g; MnCl<sub>2</sub>•4H<sub>2</sub>O, 1.98 g; CoSO<sub>4</sub>•7H<sub>2</sub>O, 2.81 g; CaCl<sub>2</sub>•2H<sub>2</sub>O, 1.67 g; CuCl<sub>2</sub>•2H<sub>2</sub>O, 0.17 g; ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.29 g) and 1.5 mL of MgSO<sub>4</sub> (100 mM) solution, that were previously sterilized by autoclaving (Uniclave 77, Portugal). In mediums A ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.4 g L<sup>-1</sup>) and 2A ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.1 g L<sup>-1</sup>) was added one inoculated FucoPol gel, in each. In mediums 1B ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.4 g L<sup>-1</sup>) and 2B ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.1 g L<sup>-1</sup>) was added one inoculated FucoPol gel and 10 mL of *Pseudomonas chlororaphis* liquid inoculum, previously inoculated in 50 mL LB medium (Tryptone, 10 g L<sup>-1</sup>; NaCl, 10 g L<sup>-1</sup>; Yeast extract, 5 g L<sup>-1</sup>), in a 100 mL Erlenmeyer, and incubated for 24 h, in an orbital shaker (30°C, 200 rpm). In mediums 1C ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.4 g L<sup>-1</sup>) and 2C ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.1 g L<sup>-1</sup>) was added 10 mL of *Pseudomonas chlororaphis* liquid inoculum, previously inoculated in 50 mL LB medium (Tryptone, 10 g L<sup>-1</sup>; NaCl, 10 g L<sup>-1</sup>; Yeast extract, 5 g L<sup>-1</sup>), in a 100 mL erlenmeyer, and incubated for 24 h, in an orbital shaker (30°C, 200 rpm). No FucoPol hydrogels were added in these mediums. Mediums samples (5 mL) were periodically taken to obtain pH and optical density (OD<sub>600</sub>) (VWR V-1200 spectrophotometer) measurements.

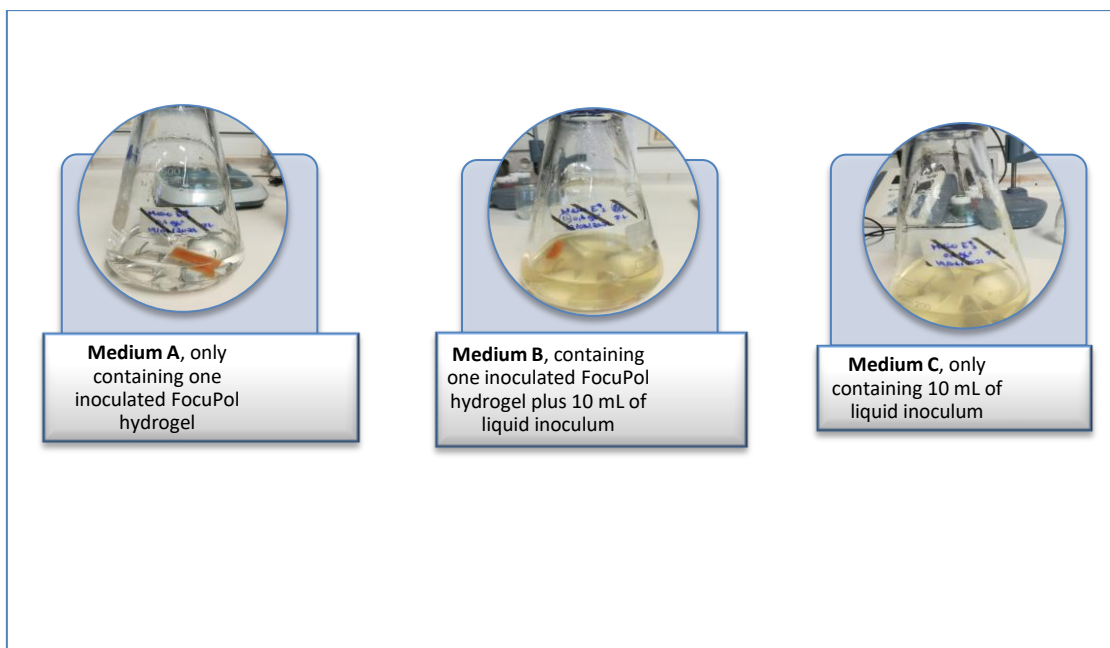


Figure 2.3 – Schematics of mediums A, B and C

## 2.5.2 Biotask commercial inocula

All the steps to cell immobilization in FucoPol hydrogels were performed in a flow chamber, aiming to work in sterile conditions (Heraeus SB 48, Germany).

A FucoPol solution (1%, w/v) was prepared with, previously sterilized (Uniclave 77, Portugal), deionized water, and the commercial inoculum ( $0.6 \text{ g L}^{-1}$ ) was added to the FucoPol solution, and mixed under constant stirring (200 rpm). Afterwards, inoculated FucoPol gels were obtained by the same process described in section 2.3.1.

Modified mediums E\* ( $(\text{NH}_4)_2\text{HPO}_4$ , 0.1 and  $0.2 \text{ g L}^{-1}$ ) were prepared, in six shake flasks (500 mL) with 150 mL of medium: 3x150 mL of  $0.1 \text{ g L}^{-1}$  medium E\* (3A, 3B and 3C) and 3x150 mL of  $0.2 \text{ g L}^{-1}$  medium E\* (4A, 4B and 4C). The mediums were sterilized by autoclaving (Uniclave 77, Portugal). To each medium was added 6 mL of glycerol (ReagentPlus 99% w/w, Sigma-Aldrich), 1.5 mL of mineral solution (per litre of 1 N HCl:  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.78 g;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1.98 g;  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.81 g;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.67 g;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.17 g;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.29 g) and 1.5 mL of  $\text{MgSO}_4$  (100 mM) solution, that were previously sterilized by autoclaving (Uniclave 77, Portugal). In mediums 3A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ ) and 4A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.2 \text{ g L}^{-1}$ ) was added one inoculated FucoPol gel, in each. In mediums 3B ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ ) and 4B ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.2 \text{ g L}^{-1}$ ) was added one inoculated FucoPol gel and 5 mL of liquid Biotask inoculum ( $0.6 \text{ g L}^{-1}$ ), diluted in deionized water. In mediums 3C ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ ) and 4C ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.2 \text{ g L}^{-1}$ ) was added 5 mL of liquid Biotask inoculum ( $0.6 \text{ g L}^{-1}$ ). No FucoPol hydrogels were added in these mediums. Mediums samples (5 mL) were periodically taken to obtain pH and optical density (OD600) (VWR V-1200 spectrophotometer) measurements.

## 2.6 Characterization of FucoPol hydrogels

### 2.6.1 Mechanical properties

The texture properties of hydrogels were assessed with a TMS-Pro texture analyzer (Food Technology Corporation, England) equipped with a 50 N load cell. The assays were performed applying a double compression cycle up to 50 % strain of the sample original height, at a speed rate of 60 mm/min, using a 60 mm aluminum plunger. The carried-out load (N) was converted to stress (Pa) according to equation:

$$\text{Stress (Pa or Nm}^{-2}\text{)} = \text{Load applied (N)} / \text{Cross-section area of hydrogel (m}^2\text{)}$$

The maximum tension of the first compression corresponds to hardness and the springiness was defined by the ratio between the second and the first compression distances until maximum forces. Cohesiveness was defined as the ratio between the positive force area of the second cycle of compression and the positive force area in the first cycle.

### 2.6.2 Morphology

Scanning electron microscopy (SEM) of FucoPol hydrogels, inoculated FucoPol hydrogels with Biotask inocula and inoculated FucoPol hydrogels with *Pseudomonas chlororaphis* were performed with Tabletop Microscope TM3030 (Hitachi in high technologies, America). Samples were observed at low temperature (-4 °C) and a magnification in the range of 150x and 500x.



### **3. Results and Discussion**

### 3.1 FucoPol hydrogels

In this work, three types of FucoPol based structures were executed: FucoPol hydrogels, FucoPol films and FucoPol gel beads. The prepared FucoPol based films and beads weren't able to match the desired characteristics, so the work was carried out focusing only in FucoPol hydrogels.

FucoPol films with chitosan blending (Figure 3.1 A) presented a non-homogeneous morphology, with the presence of granules, due to the chitosan precipitation when mixed with the FucoPol solution (Figure 3.1 B).

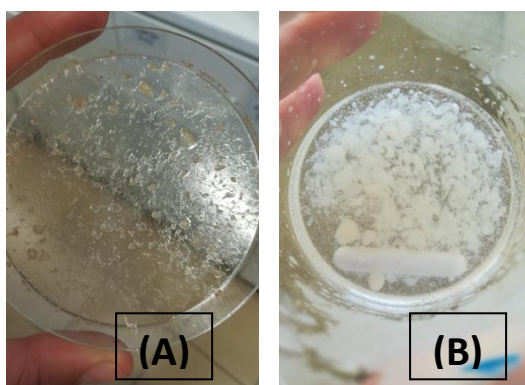


Figure 3.1 – FucoPol film with chitosan blending

FucoPol films plasticized with glycerol, and gelified with  $\text{FeCl}_3$  solution, presented a rough, wrinkled appearance, with a slightly orange color (Figure 3.2 A), and were kept in deionized water where, in a few weeks, quickly disintegrated (Figure 3.2 B).

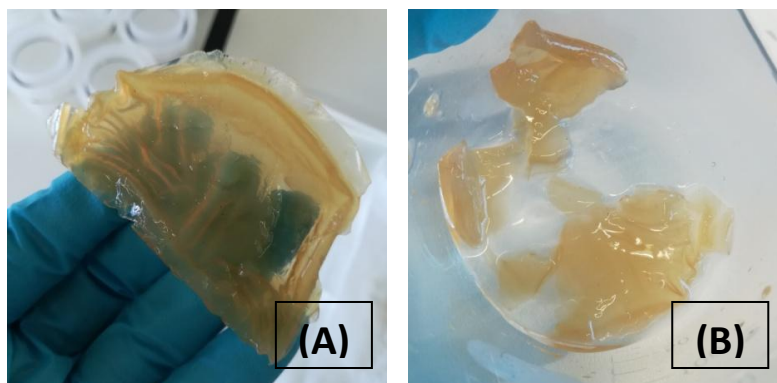


Figure 3.2 – FucoPol film plasticized with glycerol and gelified with  $\text{FeCl}_3$

The ability of FucoPol to gel in the presence of metal cation, recently demonstrated in previous work (Fialho et al., 2019) was used for the preparation of hydrogel beads, performed by ionotropic gelation with dropwise addition of polymer solution through a syringe into the cation solution. FucoPol gel beads resulted in fragile and not perfectly spherical shape beads, with a yellowish color. The beads were an average diameter of  $4.0 \pm 1.0$  mm and an average mass of

$28.3 \pm 0.05$  mg (Figure 3.3). When washed with deionized water, most of FucoPol gel beads disintegrated or resulted into more misshapen and flattened structures.



Figure 3.3 – FucoPol gel beads

FucoPol hydrogels were obtained by a new technique recently used and optimized to produce hydrogels (Rodrigues, 2020), which involves the diffusion of  $\text{Fe}^{3+}$  into FucoPol solution, allowing the cross linking among iron and FucoPol polymer within a dialysis tube, promoting its spontaneous gelation, in a vertical position. FucoPol hydrogels presented an orange color and an average mass, length and width of  $3.2533 \pm 0.0001$  g,  $3.9 \pm 0.1$  cm and  $1.5 \pm 0.1$  cm, respectively (Figure 3.4).



Figure 3.4 – FucoPol hydrogel

## 3.2 Stability of FucoPol hydrogels




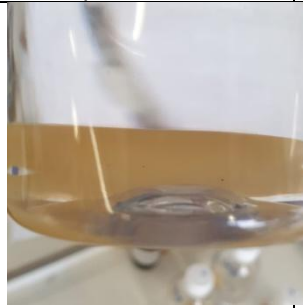

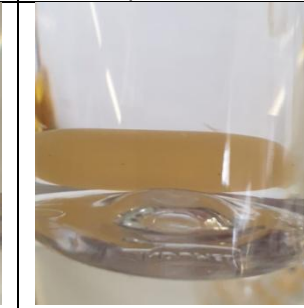

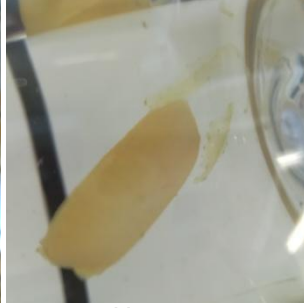

### 3.2.1 Aqueous medium

The stability of FucoPol hydrogels was evaluated, at constant temperature ( $21\text{ }^{\circ}\text{C}$ ), under the effect of four aqueous media: deionized water, NaCl aqueous solution (0.9%, w/v), PBS solution ( $\text{NaCl}$ ,  $8\text{ g L}^{-1}$ ;  $\text{KCl}$ ,  $0.2\text{ g L}^{-1}$ ;  $\text{Na}_2\text{HPO}_4$ ,  $1.44\text{ g L}^{-1}$ ;  $\text{KH}_2\text{PO}_4$ ,  $0.245\text{ g L}^{-1}$ ) and hypochlorite aqueous solution (0.5%, v/v). These solutions were selected in order to simulate the content of some effluents to be treated by bioremediation and to determine the concentration ranges in where FucoPol hydrogels can be applied.

FucoPol hydrogels placed in deionized water (structures 1, 2 and 3) proved to be very resistant and stable, having lasted for about 17 weeks with no significant changes and without reaching a total disintegration. These structures revealed a slight loss of color, increasing swelling and a

slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. Solution's pH has slightly decreased over time. Precipitation test with acetone (in the proportion 3:1) did not show any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the triplicate structures revealed the same observations, with slightly and not relevant differences. The progress of FucoPol hydrogels in deionized water is shown in the photographs presented in Table A1 (Appendices section) and summarized below, in Table 3.1.









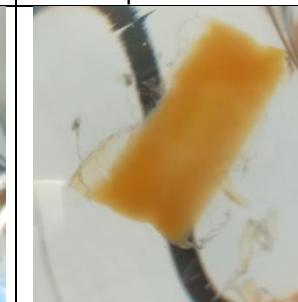
Table 3.1 – FucoPol hydrogels stability in deionized water

Structure nº	1	2	3
<b>Mass (g)</b>	3,61145	4,09851	3,42129
<b>Length (cm)</b>	3,5	3,5	3,6
<b>Width (cm)</b>	1,5	1,5	1,5
<b>Start</b> (20-01-21)	 pH = 5.28	 pH = 5.26	 pH = 5.25
<b>1<sup>st</sup> week</b>	 pH = 5.13	 pH = 5.13	 pH = 5.12
<b>17<sup>th</sup> week</b>	 pH = 4.39	 pH = 4.54	 pH = 4.57

FucoPol hydrogels placed in NaCl 0.9% (w/v) solution (structures 4, 5 and 6) also proved to be very resistant and stable, having lasted for about 17 weeks with no significant alterations. These structures revealed increasing swelling and a slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. The color in these structures has remained almost unchanged until the end of the trials. Solution's pH has slightly decreased over time. Precipitation test with acetone (in the proportion 3:1) did

not show any precipitate, which indicates that there was no significant release of polymer to the solution. All the triplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in NaCl 0.9% (w/v) is shown in the photographs presented in Table A2 (Appendices section) and summarized below in Table 3.2.


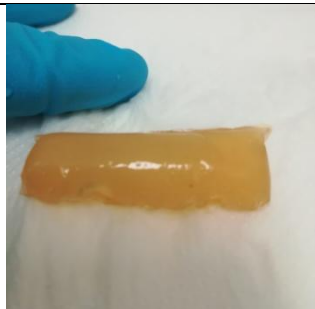

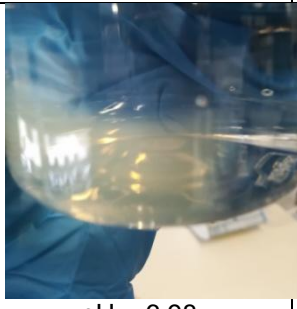
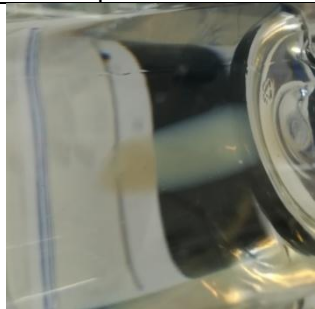
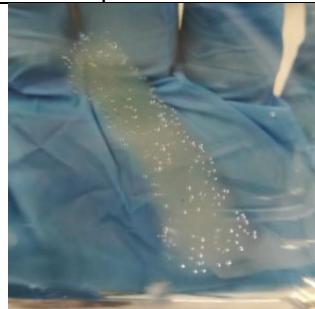

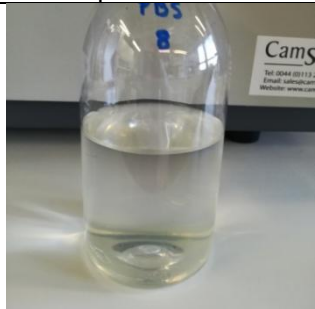
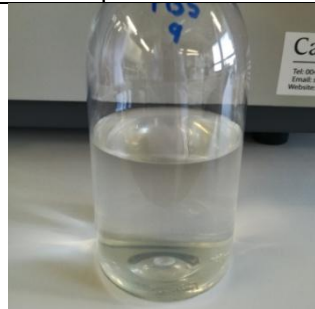
Table 3.2 – FucoPol hydrogels stability in 0.9% (p/v) NaCl solution

Structure n <sup>o</sup>	4	5	6
Mass (g)	3.2283	3.8921	3.7226
Length (cm)	3.9	3.9	3.5
Width (cm)	1.3	1.4	1.5
<b>Start</b> (20-01-21)	 pH = 5.64	 pH = 5.66	 pH = 5.64
<b>1<sup>st</sup> week</b>	 pH = 4.63	 pH = 4.63	 pH = 4.62
<b>17<sup>th</sup> week</b>	 pH = 3.79	 pH = 3.73	 pH = 3.71

FucoPol hydrogels placed in PBS solution (structures 7, 8 and 9) lost their color almost entirely after a few minutes in the solution, only revealing the outline structure contour. A fast degradation of the gel is observed, with a total disintegration reached after about 1 week of trial, only with the presence of remaining traces of gel structure in the bottom of the flask at that time. Solution's pH has increased with FucoPol hydrogel degradation. Precipitation test with acetone (in the proportion 3:1) revealed a precipitate, in the 3<sup>rd</sup> week of trials, which indicates that there was polymer release into the solution. All the triplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in







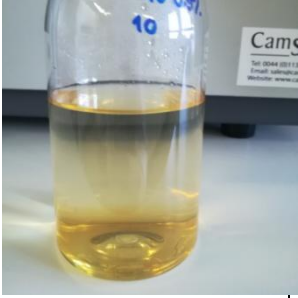
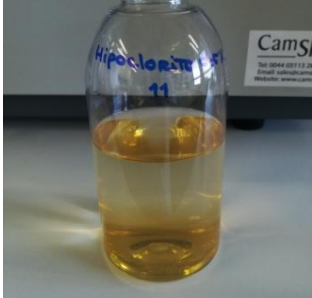
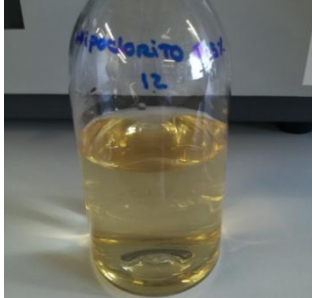
PBS solution is shown in the photographs presented in Table A3 (Appendices section) and summarized below in Table 3.3.

Table 3.3 – FucoPol hydrogels stability in PBS solution

Structure nº	7	8	9
<b>Mass (g)</b>	3.3189	3.9599	2.4216
<b>Length (cm)</b>	3.7	4.0	3.7
<b>Width (cm)</b>	1.4	1.3	1.3
<b>Start</b> (20-01-21)	 pH = 7.10	 pH = 7.10	 pH = 7.11
<b>24 H</b>	 pH = 6.98	 pH = 6.99	 pH = 6.94
<b>1<sup>st</sup> week</b>	 pH = 6.69	 pH = 6.63	 pH = 6.68

FucoPol hydrogels placed in hypochlorite solution (0.5% v/v) (structures 10, 11 and 12) showed a fast degradation, with a total disintegration reached after about 3 weeks of trial, only with the presence of remaining traces of gel structure in the bottom of the flask, similar to observed results with PBS solution. Structure presented itself with an amorphous and soft appearance, like a “floating mass” in the solution, since first week of trials. Solution’s pH has increased with FucoPol hydrogel degradation, and presented a slightly orange color. Precipitation test with acetone (in the proportion 3:1) revealed a light precipitate, in the 2<sup>nd</sup> week of trials, which indicates that there was polymer release from the structure to the solution. All the triplicate structures revealed the same observations, with slightly and not relevant differences. The progress of FucoPol hydrogels in hypochlorite solution is shown in the photographs presented in Table A4 (Appendices section) and summarized below in Table 3.4.

Table 3.4 – FucoPol hydrogels stability in 0.5% (v/v) hypochlorite solution

Structure n <sup>o</sup>	10	11	12
<b>Mass (g)</b>	4.1012	4.3288	3.3724
<b>Length (cm)</b>	4.0	3.8	3.9
<b>Width (cm)</b>	1.5	1.7	1.5
<b>Start</b> (21-01-21)	 pH = 11.48	 pH = 11.42	 pH = 11.48
<b>1<sup>st</sup> week</b>	 pH = 11.32	 pH = 11.32	 pH = 11.38
<b>3<sup>rd</sup> week</b>	 pH = 10.97	 pH = 10.97	 pH = 10.98

The data obtained show that the hydrogel structures were very stable and resistant in deionized water and in 0.9% (w/v) NaCl solutions. Otherwise, the structures placed in PBS and 0.5% (v/v) hypochlorite solutions shown faster and complete degradations. A global summary, with the results obtained for these tests, is presented Table 3.5 and in Figure 3.5.

Table 3.5 – Global summary of FucoPol hydrogels stability in aqueous media

Aqueous solution	Hydrogels Results	Hydrogels Observations
Deionized water	Stable for 17 weeks	Loss of color, swelling and slow degradation in the gel surface
NaCl 0.9% (w/v)	Stable for 17 weeks	Swelling and slow degradation in the gel surface
PBS	Degraded in 24 h; Total disintegration in 1 week	Total loss of color and total disintegration
Hypochlorite 0.5% (v/v)	Degraded in 1 week; Total disintegration in 3 weeks	Total loss of color and total disintegration

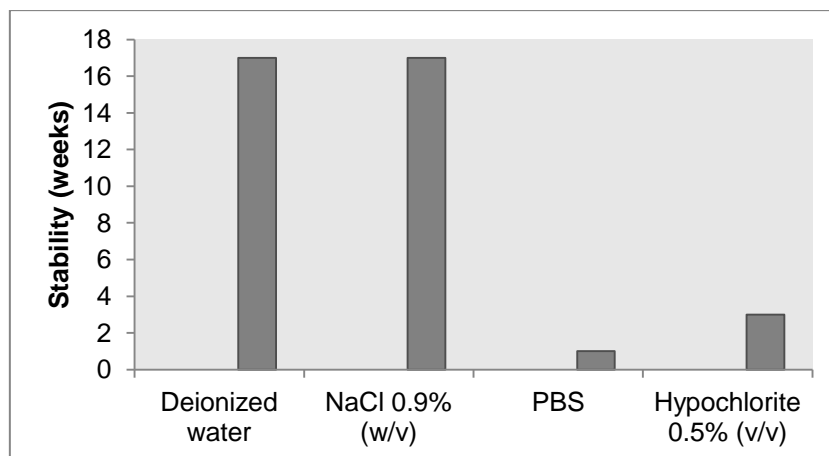


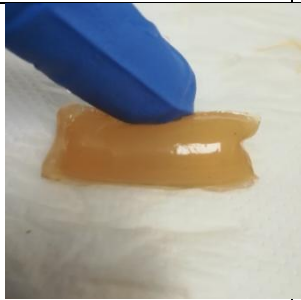

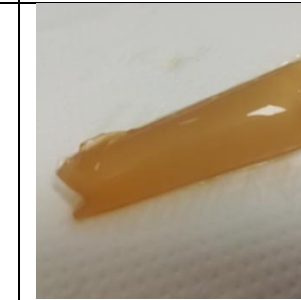


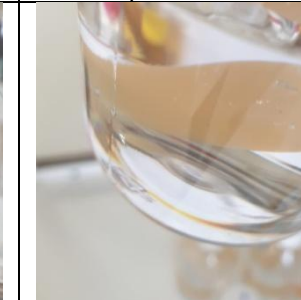



Figure 3.5 – FucoPol hydrogels stability in aqueous media

### 3.2.2 pH effect

The stability of FucoPol hydrogels were evaluated under different ranges of pH. Four buffer solutions were prepared for the study purposes: acetate buffer (pH=4.0), phosphate buffer (pH=5.5), borate buffer (pH=8.5) and ammonium chloride buffer (pH=10.0). These buffer solutions were selected because it was intended to evaluate the effect of different pH ranges on the hydrogels stability, in order to define the range in which these gels could be applied in the final application.





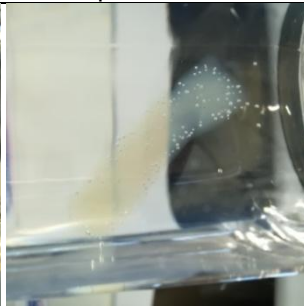
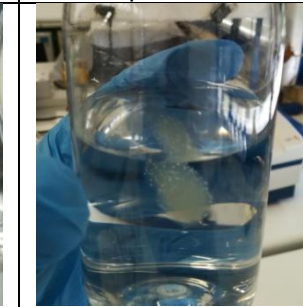
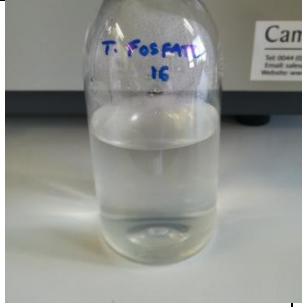
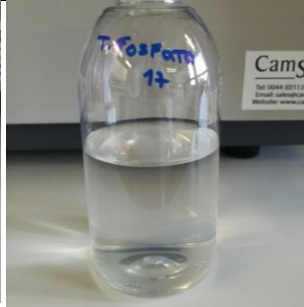
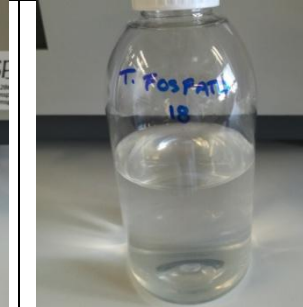
FucoPol hydrogels placed in solution of pH=4.0 (structures 13, 14 and 15) had a durability of about 4 weeks of trials but revealed a rapid disintegration, evidenced by the swollen and broken appearance of the structures, after just one week of trials. Despite this, the hydrogels did not completely disintegrate, as observed in previous solutions (PBS, hypochlorite). Precipitation tests with acetone (in the proportion 3:1) didn't reveal any precipitate, for any sample of the triplicate, which indicates that there was no significant release of polymer from the structure to the solution. The solution's pH slightly decreased over time. Structure 15 remained almost unchanged (just revealing a little swollen) for about two more weeks when compared to the other two structures in the triplicate, which may be derived from the production process of hydrogels, which may have unintentionally resulted in a more consistent gel. The progress of FucoPol hydrogels in acetate buffer solution is shown in the photographs presented in Table A5 (Appendices section) and summarized below in Table 3.6.

Table 3.6 – FucoPol hydrogels stability in acetate buffer solution (pH = 4)

Structure n <sup>o</sup>	13	14	15
Mass (g)	3.3742	2.9644	3.1720
Length (cm)	3.7	3.5	4.0
Width (cm)	1.5	1.5	1.3
<b>Start</b> (21-01-21)	 pH = 3.97	 pH = 3.99	 pH = 3.97
<b>1<sup>st</sup> week</b>	 pH = 3.71	 pH = 3.74	 pH = 3.74
<b>4<sup>th</sup> week</b>	 pH = 3.74	 pH = 3.69	 pH = 3.74





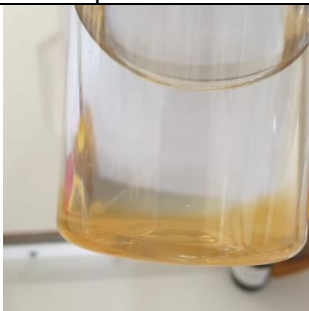
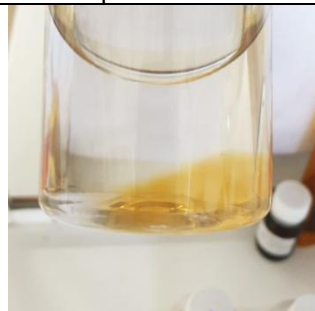
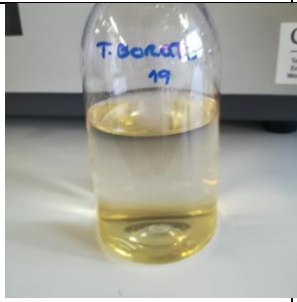
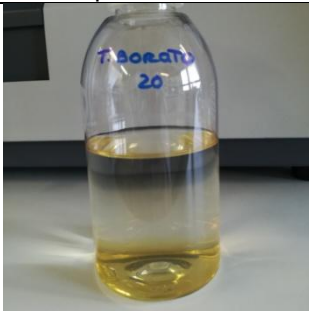
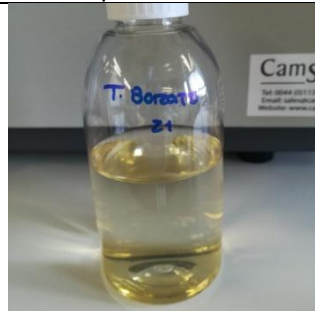
FucoPol hydrogels placed in solution of pH=5.5 (structures 16, 17 and 18) lost their color almost entirely after a few minutes in the solution, only revealing the outline structure contour. A fast and total degradation of the gel is also observed, similar to that observed in hydrogels placed in PBS solution. Structure's total disintegration was reached after about 3 weeks of trials, only with the presence of remaining traces of gel structure in the bottom of the flask at that time. Solution's pH has slightly decreased over time. Precipitation test with acetone (in the proportion 3:1) revealed an accentuated precipitate, just after one week of trials, which indicates that there was polymer release from the structure to the solution. All the triplicate structures revealed the same observations with slightly and not relevant differences. The presence of phosphate ion in solution proved to be a major destabilizing factor for FucoPol hydrogel structures. The progress of FucoPol hydrogels in phosphate buffer solution is shown in the photographs presented in Table A6 (Appendices section) and summarized below in Table 3.7.

Table 3.7 – FucoPol hydrogels stability in phosphate buffer solution (pH =5.5)

Structure n <sup>o</sup>	16	17	18
<b>Mass (g)</b>	4.5651	1.9458	4.2942
<b>Length (cm)</b>	3.9	3.6	3.8
<b>Width (cm)</b>	1.6	1.3	1.8
<b>Start</b> (21-01-21)			
	pH = 5.50	pH = 5.50	pH = 5.51
<b>24 H</b>			
<b>1<sup>st</sup> week</b>			
	pH = 4.81	pH = 4.78	pH = 4.88




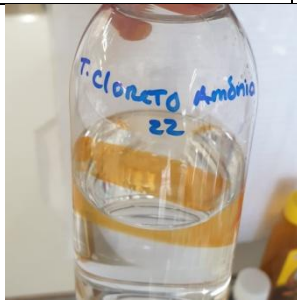





FucoPol hydrogels placed in solution of pH=8.5 (structures 19, 20 and 21) revealed a fast and total degradation of the gel, reached after just one week of trials, only with the presence of remaining traces of gel structure in the bottom of the flask. Structures were destabilized after just a few hours of trials. Solutions presented a slightly yellow color and its pH remained almost unchanged over time. Precipitation test with acetone (in the proportion 3:1) revealed a precipitate, only in the third and last week of trials (for this triplicate), which indicates that there was polymer release from the structure to the solution. All the triplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in borate buffer solution is shown in the photographs presented in Table A7 (Appendices section) and summarized below in Table 3.8.

Table 3.8 – FucoPol hydrogels stability in borate buffer solution (pH = 8.5)

Structure n <sup>o</sup>	19	20	21
Mass (g)	3.3318	3.0071	2.0689
Length (cm)	4.1	3.9	3.7
Width (cm)	1.3	1.5	1.4
<b>Start</b> (21-01-21)	 pH = 8.48	 pH = 8.49	 pH = 8.49
<b>1<sup>st</sup> week</b>	 pH = 8.07	 pH = 8.11	 pH = 8.13
<b>3<sup>rd</sup> week</b>	 pH = 8.11	 pH = 8.17	 pH = 8.21

FucoPol hydrogels placed in solution of pH=10.0 (structures 22, 23 and 24) showed a fast degradation with the structures showing themselves swollen and disintegrated, with a softened and amorphous appearance, after just 1 week of trials. Despite this, the hydrogels didn't completely disintegrate in ammonium chloride buffer solution, reaching a durability of about 4 weeks. Solution's pH has slightly decreased over time. Precipitation test with acetone (in the proportion 3:1) didn't reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. Structures revealed the same observations, except for structure 22, which remained more stable and almost structurally unaltered, showing it just slightly swollen and "open", for about three more weeks, when compared to the others. The progress of FucoPol hydrogels in phosphate buffer solution is shown in the photographs presented in Table A8 (Appendices section) and summarized below in Table 3.9.

Table 3.9 – FucoPol hydrogels stability in ammonium chloride buffer solution (pH =10)

Structure nº	22	23	24
Mass (g)	4.8514	4,6099	2.5165
Length (cm)	3.9	4.0	3.5
Width (cm)	1.7	1.6	1.7
<b>Start</b> (21-01-21)	 pH = 10.22	 pH = 10.22	 pH = 10.22
<b>1<sup>st</sup> week</b>	 pH = 10.17	 pH = 10.20	 pH = 10.15
<b>4<sup>th</sup> week</b>	 pH = 10.97	 pH = 10.97	 pH = 10.98

The data obtained shown different results for the different ranges of pH, which does not allow drawing definite conclusions about the pH range in which FucoPol hydrogels can apply in the final application. However, it seems that FucoPol gels will be more stable in more low and intermediate pH ranges. A global summary, with the results obtained for these tests, is presented Table 3.10 and in Figure 3.6.

Table 3.10 – Global summary of FucoPol hydrogels stability under pH effect

pH	Buffer solution	Hydrogels Results	Hydrogels Observations
4.0	Acetate	Disintegrated in 4 weeks	Loss of color, swelling and breaking
5.5	Phosphate	Degraded in 24 h; Total disintegration in 1 week	Total loss of color and total disintegration
8.5	Borate	Degraded in 1 week; Total disintegration in 3 weeks	Total disintegration
10.0	Ammonium chloride	Disintegrated in 4 weeks	Swelling, softening, amorphism

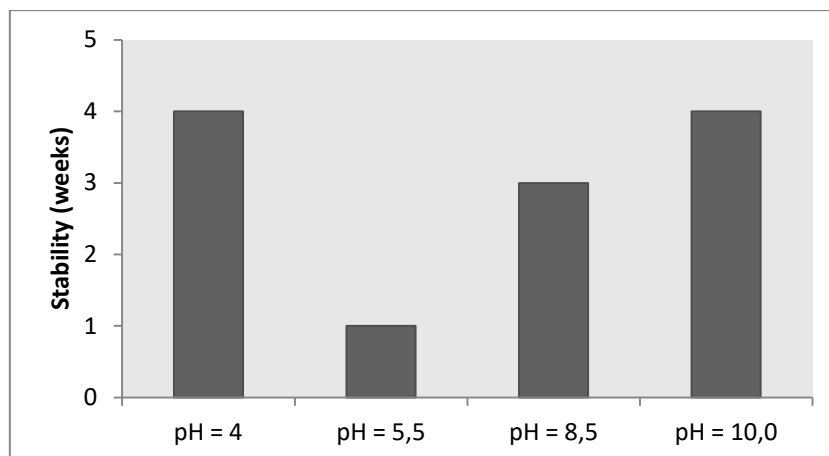


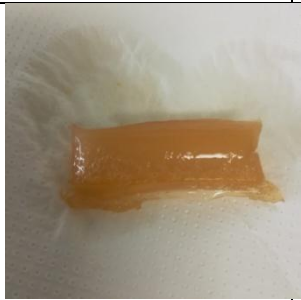


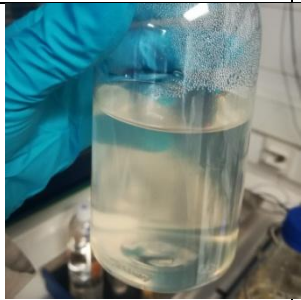



Figure 3.6 – FucoPol hydrogels stability under pH effect

### 3.2.3 Stirring effect

The stability of FucoPol hydrogels was tested under the influence of constant stirring (200 rpm), in a NaCl (0.9% w/v) solution and deionized water. The stability of FucoPol hydrogels was tested under the influence of stirring, to attempt simulates the non-static behavior of wastewater effluents.






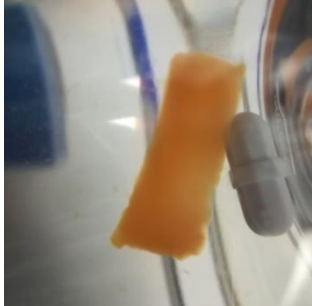


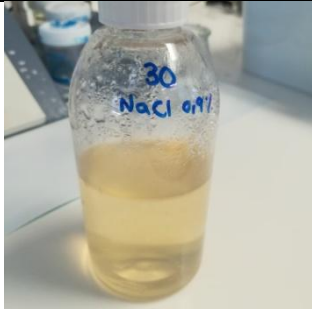
FucoPol hydrogels placed in deionized water under constant stirring (structures 25, 26 and 27) showed different results. Structures 25 and 26 quickly disintegrated, just after one week of trial. In the first 24h of trials, structure 25 was broken and with reduced dimensions, while hydrogel 26 had completely disintegrated and its solution presented an orange color. After one week of trials the structure 25 had also completely disintegrated, but in turn, its solution presented a whitish color. Otherwise, hydrogel 27 lasted for about 10 weeks. This structure also presented itself broken and with reduced dimensions since first 24h of trials, remaining practically unchanged until the 7<sup>th</sup> week, after which it showed bigger degradation and reduction in dimensions, evidenced by the existence of hydrogel particles suspended in the solution. The differences observed in this triplicate may possibly be explained by the fact that the degradation of the gels is due to the magnet impact on them, which may not have been exactly the same in the three structures, or due to some contamination in solutions 25 and 26. Solution's pH has slightly decreased over time, except solution 26 which presented a very high value (pH=9.87), that must be due to a possible contamination of the solution. Precipitation test with acetone (in the proportion 3:1) didn't reveal any precipitate, in the sample 27, which indicates that there was no significant release of polymer from the structure to the solution. Otherwise, samples 25 and 26 revealed a light precipitate at the end of their experiences. This test should be repeated since the results for the three replicates were very different, although it seems that sample 27 corresponds to the correct results, since the two other samples seem to have suffered some kind of contamination. The progress of FucoPol hydrogels in deionized water under constant stirring (200rpm) is shown in the photographs presented in Table A9 (Appendices section) and summarized below in Table 3.11.

Table 3.11 – FucoPol hydrogels stability in deionized water under constant stirring (200 rpm)

Structure n <sup>o</sup>	25	26	27
<b>Mass (g)</b>	3.2031	3.3224	2.1708
<b>Length (cm)</b>	4.0	4.1	3.5
<b>Width (cm)</b>	1.6	1.6	1.5
<b>Start</b> (18-02-21)	 pH = 5.28	 pH = 5.20	 pH = 5.28
<b>1<sup>st</sup> week</b>	 pH = 4.70	 pH = 9.87	 pH = 5.39
<b>10<sup>th</sup> week</b>	Finished trial	Finished trial	 pH = 4.76

FucoPol hydrogels placed in a 0.9 % ( w/v) NaCl solution under constant stirring (structures 28, 29 and 30) proved to be very resistant and stable, having lasted for about 13 weeks with no significant changes. These structures revealed increasing swelling and a slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. Hydrogel 29 presented itself broken at 5<sup>th</sup> week of trials, and its dimensions progressively reduced over the remaining weeks, but it didn't completely degraded, contrary to hydrogel 30, which remained intact until the 12<sup>th</sup> week, time when it was totally disintegrated and its solution presented a yellowish color. Solution's pH has decreased in the first week of trials and remained stable (with slight fluctuations, probably derived from the calibrations of pH device) since then. Precipitation test with acetone (in the proportion 3:1) did not show any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. The progress of FucoPol hydrogels in 0.9% NaCl solution under constant stirring (200rpm) is shown in the photographs presented in Table A10 (Appendices section) and summarized below in Table 3.12.

Table 3.12 – FucoPol hydrogels stability in 0.9% (w/v) NaCl solution under constant stirring (200 rpm)

Structure n <sup>o</sup>	28	29	30
Mass (g)	2.9490	3.3941	2.8486
Length (cm)	4.3	3.9	3.8
Width (cm)	1.4	1.6	1.6
<b>Start</b> (18-02-21)	 pH = 5.63	 pH = 5.63	 pH = 5.63
<b>1<sup>st</sup> week</b>	 pH = 3.69	 pH = 3.65	 pH = 3.66
<b>12<sup>th</sup> week</b>	 pH = 3.85	 pH = 3.80	 pH = 3.83

The data obtained show that the effect of agitation on the hydrogel structures accelerates their degradation process, when compared to the structures subjected to the same solution without agitation. A global summary, with the results obtained for these tests, is presented in Table 3.13 and in Figure 3.7.

Table 3.13 – Global summary of FucoPol hydrogels stability under constant stirring effect (200 rpm)

Solution	Hydrogels Results	Hydrogels Observations
Deionized water	Degraded in 24 h; Disintegrated in 11 weeks	Loss of color, swelling, breaking and loss of dimensions
NaCl 0.9% (w/v)	Stable for 4 weeks; Disintegrated in 13 weeks	Swelling, breaking and loss of dimensions

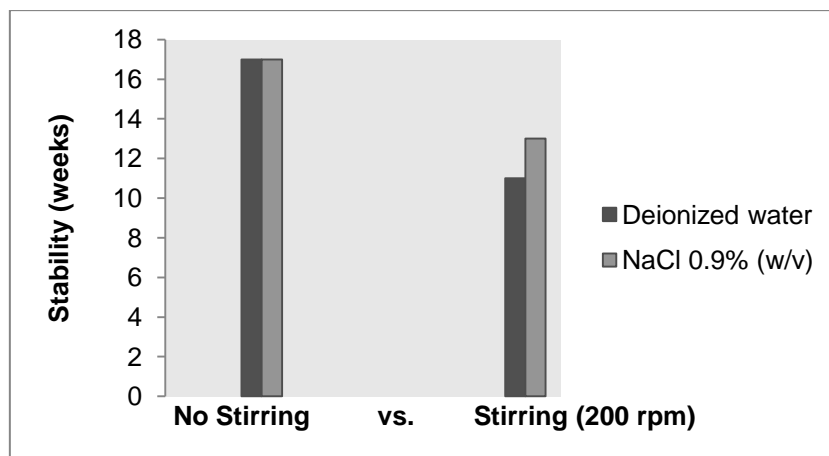


Figure 3.7 – FucoPol hydrogels stability under stirring effect






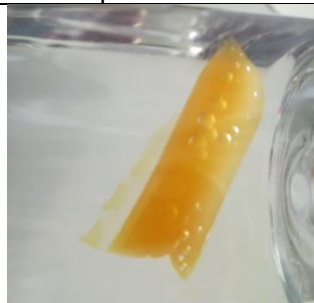





### 3.2.4 Ionic effect

As result of the observations made in the work developed in section 2.4.1 and 2.4.2, which revealed that the hydrogels structures destabilized in media containing different ions, it was necessary to study the influence of the ionic species present in solutions. The solutions were selected with the purpose of evaluating the effect of different salts, and different salt concentrations, on the gels stability.

The stability of FucoPol hydrogels was tested under the effect of different ionic solutions: NaCl (1.8% w/v), KCl (0.7% and 1.4% w/v) and an altered phosphate buffer, prepared by altering the potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) component for sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ). These solutions were prepared in order to evaluate the effect of the doubling the concentration of ions ( $\text{Na}^+$  and  $\text{K}^+$ ), as well as to averiguate if the destabilizing effects came from sodium ions or from potassium ions, or else.

FucoPol hydrogels placed in a 1.8 % ( w/v) NaCl solution (structures 31, 32 and 33) proved to be very resistant and stable, having lasted for about 13 weeks with no significant changes. These structures revealed increasing swelling and a slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. Hydrogel 32 was the only in this triplicate to show different results. This hydrogel presented itself with a softened and amorphous appearance, since the first week of trials, and it showed a faster degradation until it reached its completely disintegration, at 4<sup>th</sup> week. This result may be explained by the fact that a contamination occurred in this exemplar. Solution's pH has decreased in the first week of trials and remained stable (with slight fluctuations, probably derived from the calibrations of pH device) since then, with the exception of solution 32, whose pH values were very high (8.38 – 9.66). Precipitation test with acetone (in the proportion 3:1) did not show any precipitate, which indicates that there was no significant release of polymer from the structure to the solution (with the exception of solution 32, which revealed a strong precipitate, at 3<sup>rd</sup> week of trials). The progress of FucoPol hydrogels in 1.8% NaCl solution is shown in the photographs presented in Table A11 (Appendices section) and summarized below in Table 3.14.

Table 3.14– FucoPol hydrogels stability in 1.8% (w/v) NaCl solution

Structure n <sup>o</sup>	31	32	33
Mass (g)	2.7523	3.4182	3.1889
Length (cm)	3.5	4.0	4.2
Width (cm)	1.5	1.5	1.6
<b>Start</b> (18-02-21)	 pH = 5.54	 pH = 5.54	 pH = 5.54
<b>1<sup>st</sup> week</b>	 pH = 3.60	 pH = 9.66	 pH = 3.66
<b>3<sup>rd</sup> week</b>	 pH = 3.42	 pH = 8.38	 pH = 3.40
<b>13<sup>th</sup> week</b>	 pH = 3.69	Finished trial	 pH = 3.68

FucoPol hydrogels placed in a 0.7 % ( w/v) KCl solution (structures 34, 35 and 36) proved to be very resistant and stable, having lasted for about 13 weeks with no significant changes. These structures revealed increasing swelling and a slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. Hydrogel 35 was the only in this triplicate to show different results. This hydrogel presented itself with a softened and amorphous appearance, since the first week, and it showed a faster degradation until it almost completely disintegrated, at 3<sup>rd</sup> week of trials. This result may be explained by the

fact that a contamination occurred in this exemplar. Solution's pH has decreased in the first week of trials and remained stable (with slight fluctuations, probably derived from the calibrations of pH device) since then, with the exception of solution 35, whose pH values revealed to be very high (9.03 – 9.78). Structure 36 showed the presence of small black dots in week 7 that increased in the followed weeks which indicated a possible contamination of the gel. Precipitation test with acetone (in the proportion 3:1) did not show any precipitate, which indicates that there was no significant release of polymer from the structure to the solution (with the exception of solution 35, which revealed a precipitate, at 3<sup>rd</sup> week of trials). The progress of FucoPol hydrogels in 0.7% KCl solution is shown in the photographs presented in Table A12 (Appendices section) and summarized below in Table 3.15.

Table 3.15 – FucoPol hydrogels stability in 0.7% (w/v) KCl solution, part I







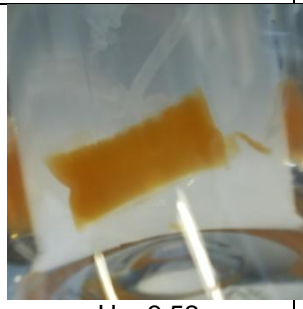

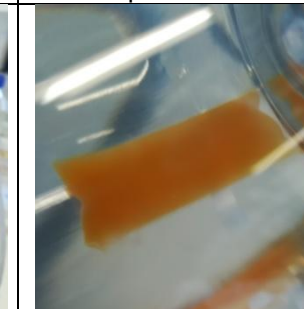
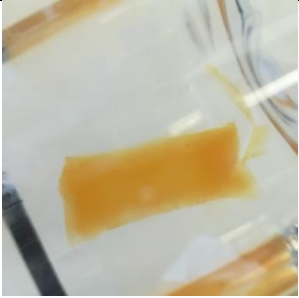

Structure n <sup>o</sup>	34	35	36
<b>Mass (g)</b>	3.1721	2.9217	3.0823
<b>Length (cm)</b>	3.8	4.0	3.7
<b>Width (cm)</b>	1.6	1.4	1.3
<b>Start</b> (18-02-21)	 pH = 5.71	 pH = 5.71	 pH = 5.71
<b>1<sup>st</sup> week</b>	 pH = 3.76	 pH = 9.78	 pH = 3.79
<b>3<sup>rd</sup> week</b>	 pH = 3.52	 pH = 9.14	 pH = 3.50

Table 3.15 – FucoPol hydrogels stability in 0.7% (w/v) KCl solution, part II

Structure n <sup>o</sup>	34	35	36
Mass (g)	3.1721	2.9217	3.0823
Length (cm)	3.8	4.0	3.7
Width (cm)	1.6	1.4	1.3
<u>13<sup>th</sup> week</u>	 <p>pH = 4.67</p>	<p>Finished trial</p>	 <p>pH = 4.78</p>

FucoPol hydrogels placed in a 1.4 % ( w/v) KCl solution (structures 37, 38 and 39) proved to be very resistant and stable, having lasted for about 13 weeks with no significant changes. These structures revealed increasing swelling and a slow, progressive degradation (essentially on structure surface), with the presence of suspended particles of the gel in the solutions. Solution's pH has decreased in the first week of trials and remained stable (with slight fluctuations, probably derived from the calibrations of pH device) since then.). Precipitation test with acetone (in the proportion 3:1) did not show any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. The structures revealed the same observations, with the exception of structure 38 which presented itself a bit broken since 3<sup>rd</sup> week of trials, which may be derived from the production process of hydrogels, which may have unintentionally resulted in a less consistent gel. The progress of FucoPol hydrogels in 1.4% (w/v) KCl solution is shown in the photographs presented in Table A13 (Appendices section) and summarized below in Table 3.16.

Table 3.16 – FucoPol hydrogels stability in 1.4% (w/v) KCl solution, part I








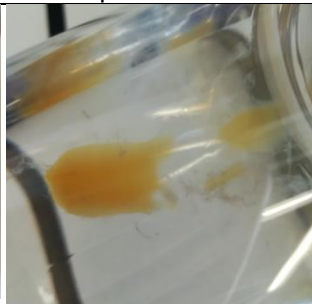





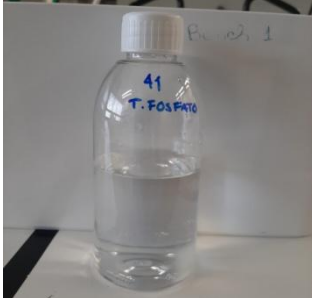
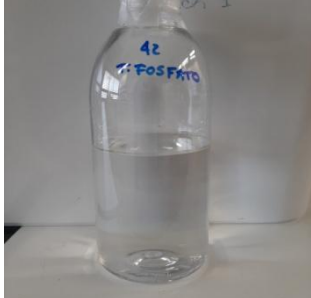



Structure n <sup>o</sup>	37	38	39
Mass (g)	3.0303	2.4905	3.2481
Length (cm)	3.9	3.1	4.0
Width (cm)	1.4	1.2	1.5
<u>Start</u> (18-02-21)	 <p>pH = 5.87</p>	 <p>pH = 5.87</p>	 <p>pH = 5.87</p>

Table 3.16 – FucoPol hydrogels stability in 1.4% (w/v) KCl solution, part II

Structure nº	37	38	39
Mass (g)	3.0303	2.4905	3.2481
Length (cm)	3.9	3.1	4.0
Width (cm)	1.4	1.2	1.5
<b>1<sup>st</sup> week</b>	 pH = 3.74	 pH = 3.89	 pH = 3.71
<b>13<sup>th</sup> week</b>	 pH = 3.76	 pH = 3.92	 pH = 3.75

FucoPol hydrogels placed in an altered phosphate buffer solution (structures 40, 41 and 42) lost their color almost entirely after a few minutes in the solution, only revealing the outline structure contour. A fast and total degradation of the gel is also observed, similar to that observed in hydrogels placed in the original phosphate buffer solution. At the first week of trials the structure already reached its almost total disintegration, only with remaining traces of the gel in the bottom of the flask. Solution's pH has slightly decreased over time. In this triplicate, precipitation test with acetone (in the proportion 3:1) didn't reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the triplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in altered phosphate buffer solution is shown in the photographs presented in Table A14 (Appendices section) and summarized below in Table 3.17.

Table 3.17 – FucoPol hydrogels stability in altered phosphate buffer solution (NaH<sub>2</sub>PO<sub>4</sub>)

Structure n <sup>o</sup>	40	41	42
Mass (g)	3.3921	2.6118	3.1098
Length (cm)	4.1	3.8	3.8
Width (cm)	1.4	1.4	1.5
<b>Start</b> (18-02-21)	 pH = 5.42	 pH = 5.39	 pH = 5.39
<b>24 H</b>	 pH = 5.15	 pH = 5.14	 pH = 5.14
<b>1<sup>st</sup> week</b>	 pH = 4.86	 pH = 4.88	 pH = 4.87

The data obtained show that the effects of K<sup>+</sup> and Na<sup>+</sup> ions, in the studied concentrations, were practically the same and didn't cause major alterations in the FucoPol structures, which revealed to be very resistant in these solutions, lasting for about 13 weeks. The hydrogels placed in altered phosphate buffer solution shown a very fast degradation, due to the destabilizing effect of PO<sub>4</sub><sup>3-</sup> ion on the hydrogels, not the Na<sup>+</sup>. A global summary with the results obtained for these tests is presented in Table 3.18 and in Figure 3.8.

Table 3.18 – Global summary of FucoPol hydrogels stability under ionic effect

Ion	Solution	Hydrogels Results	Hydrogels Observations
Na <sup>+</sup>	NaCl 1.8% (w/v)	Stable for 13 weeks	Swelling and slow degradation in the gel surface
K <sup>+</sup>	KCl 0.7% (w/v)	Stable for 13 weeks	Swelling and slow degradation in the gel surface
K <sup>+</sup>	KCl 1.4% (w/v)	Stable for 13 weeks	Swelling and slow degradation in the gel surface
Na <sup>+</sup>	Phosphate buffer (NaH <sub>2</sub> PO <sub>4</sub> )	Degraded in 24 h; Total disintegration in 1 week	Total disintegration

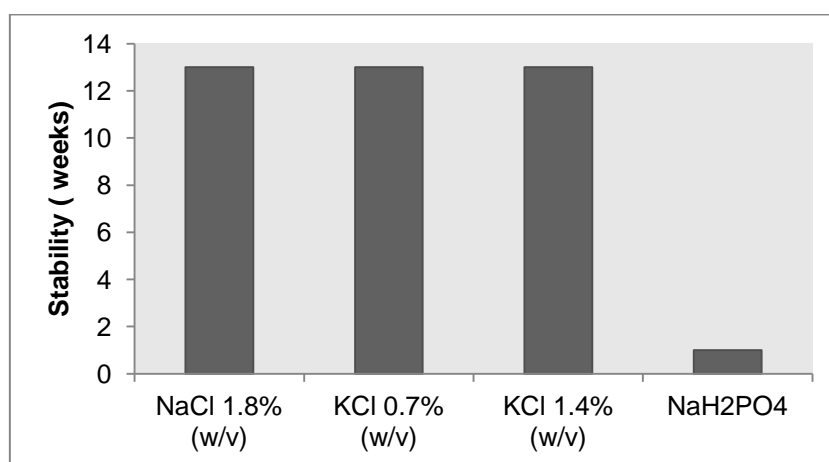


Figure 3.8 – FucoPol hydrogels stability under ionic effect

### 3.2.5 Concentration of phosphate ion (PO<sub>4</sub><sup>3-</sup>)

The observations made in the work developed in the previous sections shows that FucoPol hydrogels quickly disintegrate in the presence of phosphate ion (PO<sub>4</sub><sup>3-</sup>). These results are similar to that observed in previous work about alginate as immobilizing matrix, where alginate gel beads, gelled with a CaCl<sub>2</sub> solution, rapidly destabilized in the presence of phosphate. This is explained by the fact that ion phosphate has a high affinity for Ca<sup>2+</sup> and it will sequester the cross-linking calcium ions, consequently destabilizing the gel. (Fraser & Bickerstaff; Palmieri et al., 1994; Smidsrod & Skjakbrk, 1990)

Since phosphate is essential for cell growth to occur, it was necessary to determine the range of phosphate concentration in which FucoPol gels can be applied, aiming the cell immobilization.

The stability of FucoPol hydrogels was tested under the effect of different phosphate concentrations (0.01 g L<sup>-1</sup>; 0.02 g L<sup>-1</sup>; 0.05 g L<sup>-1</sup>; 0.1 g L<sup>-1</sup>; 0.4 g L<sup>-1</sup>; 0.82 g L<sup>-1</sup>; 1.65 g L<sup>-1</sup>) of simplified mediums E\* (with diammonium hydrogen phosphate ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) as the only component).

FucoPol hydrogels placed in a  $0.01 \text{ g L}^{-1}$  altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ) solutions (43, 44) lasted for about 9 weeks (hydrogel 43 lasted only until 6<sup>th</sup> week). Structure 44 revealed a softened and broken appearance, since the first week of trials, but has remained almost unaltered since that, with a slight and very slow degradation. With this phosphate concentration the hydrogel didn't disintegrate completely. Structure 43 registered almost the same observations, but it completely disintegrated at week 6. The pH of solution 43 has slightly decreased over time, but it registered a higher value at the end of trial. Otherwise, pH of solution 44 considerably decreased over time. In this triplicate, precipitation test with acetone (in the proportion 3:1) didn't reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution.

The progress of FucoPol hydrogels in  $0.01 \text{ g L}^{-1}$  altered Medium E\* solution is shown in the photographs presented in Table A15 (Appendices section) and summarized below in Table 3.19.

Table 3.19 – FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.01 \text{ g L}^{-1}$ ), part I



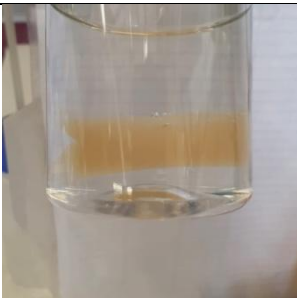
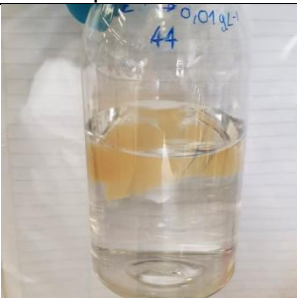
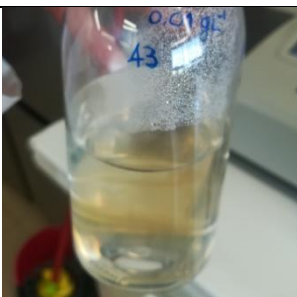





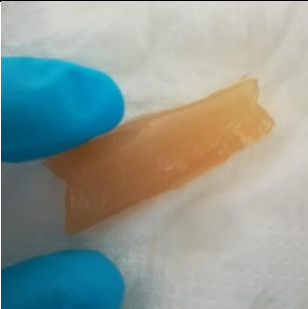
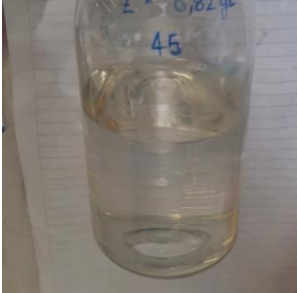
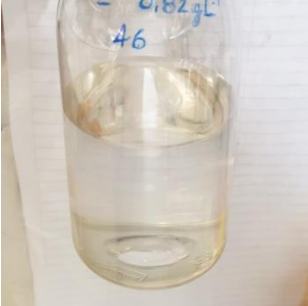


Structure n <sup>o</sup>	43	44
Mass (g)	3.3476	3.5261
Length (cm)	4.0	3.9
Width (cm)	1.4	1.6
<b>Start</b> (18-03-21)	 pH = 7.97	 pH = 7.79
<b>24 H</b>	 pH =	 pH =
<b>1<sup>st</sup> week</b>	 pH = 6.07	 pH = 5.22

Table 3.19 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.01 g L<sup>-1</sup>), part II

Structure n°	43	44
Mass (g)	3.3476	3.5261
Length (cm)	4.0	3.9
Width (cm)	1.4	1.6
<u>6<sup>th</sup> week</u>	 <p>pH = 7.05</p>	 <p>pH = 4.88</p>
<u>9<sup>th</sup> week</u>	<p>Finished trial</p>	 <p>pH = 4.48</p>



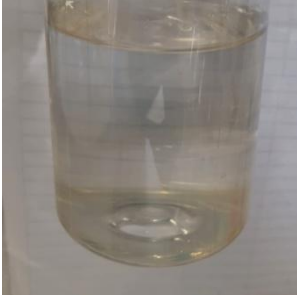
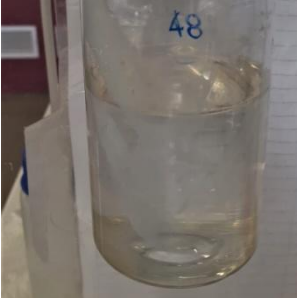

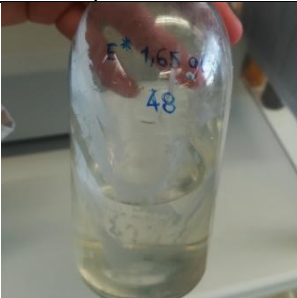
FucoPol hydrogels placed in an in a 0.82 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (45, 46) lost their color almost entirely after a few minutes in the solution, only revealing the outline structure contour. A fast and almost total degradation of the gel is also observed. In the first week of trials the structure already reached its almost total disintegration, only with remaining traces of the gel in the bottom of the flask. Solution's pH has slightly decreased over time. Precipitation tests with acetone (in the proportion 3:1) revealed a slight precipitate, which indicates that there was polymer release from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in 0.82 g L<sup>-1</sup> altered Medium E\* solution is shown in the photographs presented in Table A16 (Appendices section) and summarized below in Table 3.20.

Table 3.20 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.82 g L<sup>-1</sup>)

Structure n <sup>o</sup>	45	46
Mass (g)	2.7042	2.5681
Length (cm)	4.1	3.9
Width (cm)	1.4	1.1
<b>Start</b> (18-03-21)	 pH = 8.09	 pH = 8.11
<b>24 H</b>	 pH = 7.97	 pH = 8.03
<b>1<sup>st</sup> week</b>	 pH = 7.87	 pH = 7.93

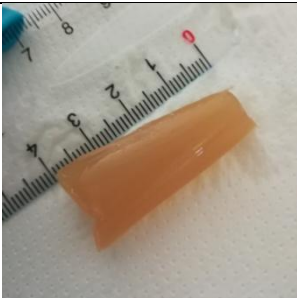

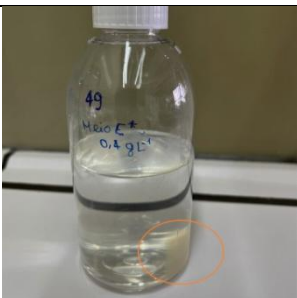
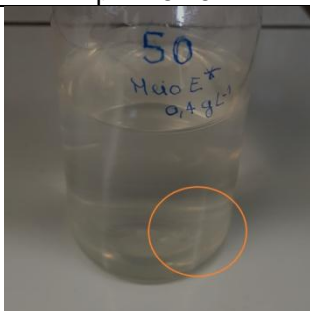
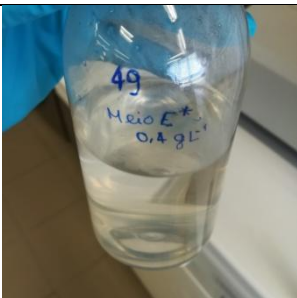
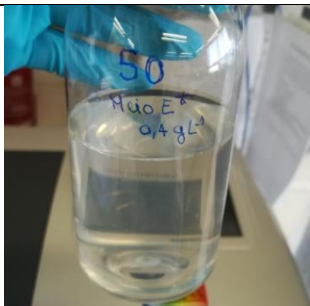
FucoPol hydrogels placed in an in a 1.65 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (47, 48) lost their color almost entirely after a few minutes in the solution. A fast and almost total degradation of the gel is also observed. In the first week of trials the structure already reached its almost total disintegration, only with remaining traces of the gel in the bottom of the flask. Solution's pH has slightly decreased over time. Precipitation tests with acetone (in the proportion 3:1) revealed a slight precipitate, which indicates that there was polymer release from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in 1.65 g L<sup>-1</sup> altered Medium E\* solution is shown in the photographs presented in Table A17 (Appendices section) and summarized below in Table 3.21.

Table 3.21 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 1.65 g L<sup>-1</sup>)

Structure n <sup>o</sup>	47	48
Mass (g)	3.8425	3.4955
Length (cm)	4.1	4.0
Width (cm)	1.3	1.7
<b>Start</b> (18-03-21)	 pH = 8.08	 pH = 8.10
<b>24 H</b>	 pH = 8.07	 pH = 8.06
<b>1<sup>st</sup> week</b>	 pH = 7.99	 pH = 8.01


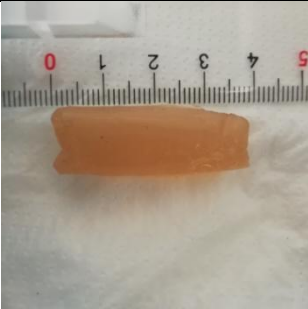
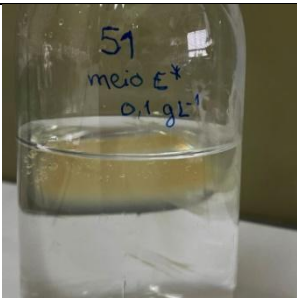

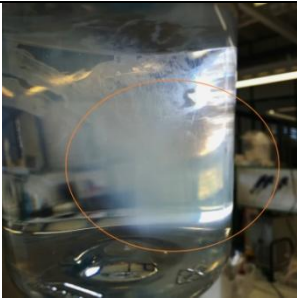

FucoPol hydrogels placed in an in a 0.4 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (49, 50) lost their color almost entirely after 24 h in the solution. A fast and almost total degradation of the gel is also observed. In the first week of trials the structure already reached its almost total disintegration, only with remaining traces of the gel in the bottom of the flask. These structures remained almost intact in solution for about 4 days. Solution's pH has slightly decreased over time. Precipitation tests with acetone (in the proportion 3:1) did not reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in 0.4 g L<sup>-1</sup> altered Medium E\* solution is shown in the photographs presented in Table A18 (Appendices section) and summarized below in Table 3.22.

Table 3.22 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.4 g L<sup>-1</sup>)

Structure n <sup>o</sup>	49	50
Mass (g)	3.5850	3.9366
Length (cm)	3.8	4.0
Width (cm)	1.6	1.7
<b>Start</b> (01-04-21)	 pH = 8.20	 pH = 8.16
<b>4<sup>th</sup> day</b>		
<b>1<sup>st</sup> week</b>	 pH = 7.48	 pH = 7.46





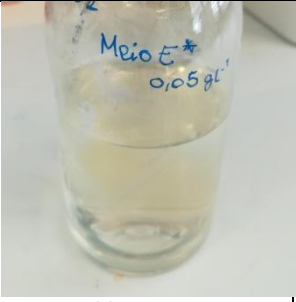
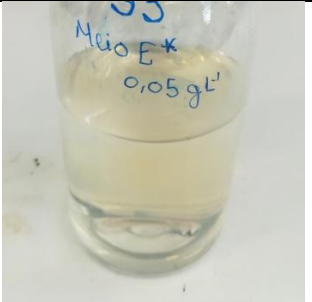
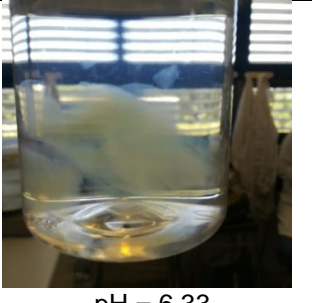
FucoPol hydrogels placed in an in a 0.1 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (51, 52) showed a slower degradation. The structures revealed a softened and broken appearance, since the first week of trials. In the end of 1<sup>st</sup> week of trials the structures were almost disintegrated, although with this phosphate concentration the hydrogel didn't completely disintegrate. Solution's pH has slightly decreased over time. Precipitation tests with acetone (in the proportion 3:1) did not reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in 0.1 g L<sup>-1</sup> altered Medium E\* solution is shown in the photographs presented in Table A19 (Appendices section) and summarized below in Table 3.23.

Table 3.23 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.1 g L<sup>-1</sup>)

Structure n <sup>o</sup>	51	52
Mass (g)	3.0202	3.0440
Length (cm)	3.7	3.9
Width (cm)	1.4	1.2
<b>Start</b> (01-04-21)	 pH = 7.94	 pH = 7.94
<b>24 H</b>	 pH = 7.83	 pH = 7.58
<b>1<sup>st</sup> week</b>	 pH = 6.86	 pH = 6.44

FucoPol hydrogels placed in a 0.05 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (53, 54) lasted for about 3 weeks (hydrogel 53 lasted only until 2<sup>nd</sup> week). Structures revealed a softened and broken appearance, since the first week of trials. With this phosphate concentration the hydrogels didn't completely disintegrate. Solution's pH has decreased over time. Precipitation tests with acetone (in the proportion 3:1) did not reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in a 0.05 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solution is shown in the photographs presented in Table A20 (Appendices section) and summarized below in Table 3.24.

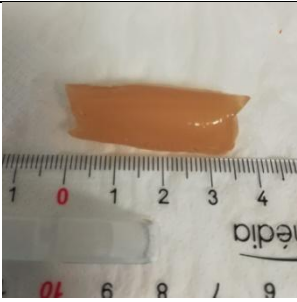
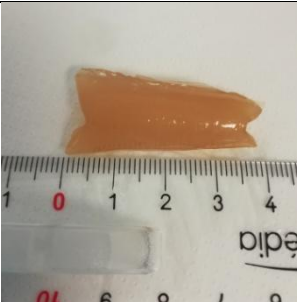



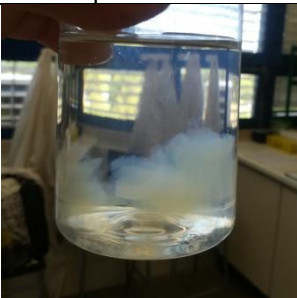
Table 3.24 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>), 0.05 g L<sup>-1</sup>)

Structure n <sup>o</sup>	53	54
Mass (g)	3.2826	3.0808
Length (cm)	3.7	3.9
Width (cm)	1.7	1.6
<b>Start</b> (15-04-21)	 pH = 8.10	 pH = 8.09
<b>1<sup>st</sup> week</b>	 pH = 6.99	 pH = 6.94
<b>2<sup>nd</sup> week</b>	 pH = 6.41	 pH = 6.37
<b>3<sup>rd</sup> week</b>	Finished trial	 pH = 6.33

FucoPol hydrogels placed in a 0.02 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solutions (55, 56) lasted for about 4 weeks. Structures revealed a softened and broken appearance, since the first week of trials. With this phosphate concentration the hydrogels did not completely

disintegrate. Solution's pH has decreased over time. Precipitation tests with acetone (in the proportion 3:1) did not reveal any precipitate, which indicates that there was no significant release of polymer from the structure to the solution. All the duplicate structures revealed the same observations with slightly and not relevant differences. The progress of FucoPol hydrogels in a 0.02 g L<sup>-1</sup> altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>) solution is shown in the photographs presented in Table A21 (Appendices section) and summarized below in Table 3.25.

Table 3.25 – FucoPol hydrogels stability in altered Medium E\* ((NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.02 g L<sup>-1</sup>)

Structure nº	55	56
Mass (g)	3.1631	3.2586
Length (cm)	3.6	3.8
Width (cm)	1.6	1.7
<b>Start</b> (15-04-21)	 pH = 7.79	 pH = 7.82
<b>2<sup>nd</sup> week</b>	 pH = 5.95	 pH = 5.07
<b>4<sup>th</sup> week</b>	 pH = 6.05	 pH = 5.18

The preferential range of phosphate ion, in which FucoPol hydrogels can be applied, it was determined in [0.4; 0.01] ([PO<sub>4</sub><sup>3-</sup>] g L<sup>-1</sup>), where the hydrogel structures could last for about 1 to 9 weeks. At higher concentrations, FucoPol structures quickly destabilize, in just a few days. A global summary with the results obtained for these tests is presented in Table 3.26 and in Figure 3.9.

Table 3.26 – Global summary of FucoPol hydrogels stability under different concentrations of ion phosphate ( $\text{PO}_4^{3-}$ )

$[\text{PO}_4^{3-}]$ ( $\text{g L}^{-1}$ )	Hydrogels Results	Hydrogels Observations
0.01	Disintegrated in 9 weeks	Loss of color, swelling, softening, amorphism and breaking
0.02	Disintegrated in 4 weeks	Loss of color, swelling, softening, amorphism and breaking
0.05	Disintegrated in 3 weeks	Loss of color, swelling, softening, amorphism and breaking
0.1	Degraded and disintegrated in 1 week	Loss of color, swelling, softening, amorphism and breaking
0.4	Degraded in 4 days; Disintegrated in 1 weeks	Total loss of color and total disintegration
0.82	Disintegrate in 24 h; Total disintegration in 1 week	Total loss of color and total disintegration
1.65	Disintegrate in 24 h; Total disintegration in 1 week	Total loss of color and total disintegration

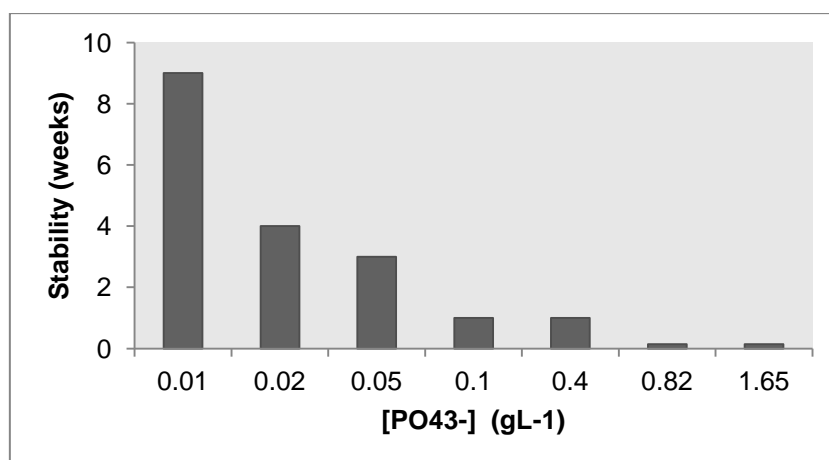


Figure 3.9 – FucoPol hydrogels stability under phosphate ion concentration effect

### 3.3 Cell immobilization in FucoPol hydrogels

Cell immobilization is a common technique for increasing the over-all cell concentration and productivity. Also, it is a strategy for protecting cells from shear forces and presents some advantages over suspended cells systems ( e.g.: easier separation of products). (Usama, 2003)

The results obtained in the immobilization tests on FucoPol gel matrices can be related to data obtained in previous literature. Namely, the microscopic visualization of cells in the gel matrix was possible, as reported by Callone et al., whose microscopic images of the immobilized cells are similar to those presented in this work (although it was used a Ca-alginate matrix and yeast and *Oenococcus oeni* cells). The reported success of the immobilization process and the fact that immobilized cells remained viable in this work is also similar to that reported in *Lactobacillus rhamnosus* immobilization in polyvinyl alcohol/calcium alginate matrix. (Radosavljevic et al., 2019)

### 3.3.1 *Pseudomonas chlororaphis* inocula

FucoPol hydrogels with immobilized *P.chlororaphis* cells showed a more intense and brighter orange color (Figure 3.10), when compared to non-inoculated gels. These hydrogels, visually, shown to have a more compact structure, resulting in hydrogels with smaller dimensions, although they were executed with the same volume of solution as the non-inoculated hydrogels. However, these gels revealed to have a non-homogeneous gelation, mainly inside the gel itself, being even less homogeneous than non-inoculated hydrogels of FucoPol.

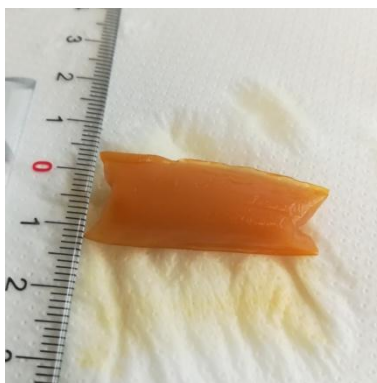


Figure 3.10 – FucoPol hydrogel with immobilized *P.chlororaphis* cells

The cell immobilization of *P.chlororaphis* in FucoPol hydrogels was sustained by the observation of the inoculated FucoPol gels at the microscope (Axio Imager 2 , Zeiss), with Nile Blue fluorescent dye (due to PHA production characteristic of this strain) (Mohan et al., 2019), which has showed the presence of cells in the gel matrix (Figure 3.11).

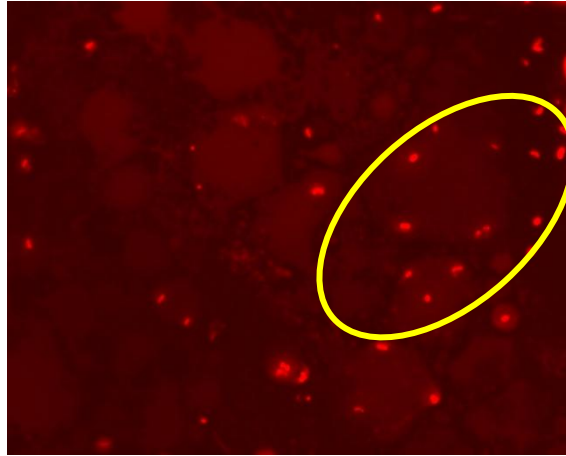


Figure 3.11 – Microscopic photograph (bright field and fluorescence visualization, 100x) of *P. chlororaphis* cells immobilized in FucoPol hydrogel

### 3.3.2 Biotask commercial inocula

FucoPol hydrogels with immobilized Biotask commercial inocula showed a yellowish color, with the presence of visible particles, which were inert fillers, constituents of the cell granulate (Figure 3.12). These hydrogels, visually, shown to have the most fragile and less compact structure, when compared with the others hydrogels produced. These gels also revealed to have a non-homogeneous gelation, mainly inside the gel itself, being the less homogeneous hydrogels of them all, due to the difficulty in obtaining a homogeneous distribution of the product (cell granulate) throughout de gel.

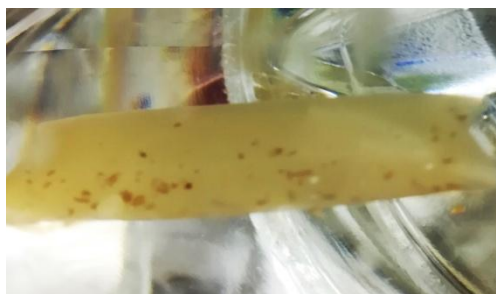


Figure 3.12 – FucoPol hydrogel with Biotask commercial cells immobilized

The cell immobilization in FucoPol hydrogels was sustained by the observation of the inoculated FucoPol gels at the microscope (Axio Imager 2 , Zeiss), which revealed, what appears to be, the existence of Biotask commercial cells in the gel matrix (Figure 3.13).

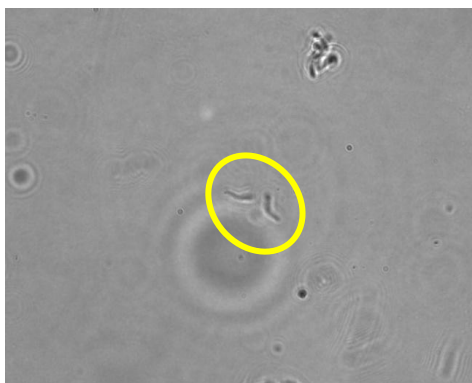


Figure 3.13 – Microscopic photograph (bright field visualization, 100x) of Biotask commercial cells immobilized in FucoPol hydrogel

### 3.4 Characterization of FucoPol hydrogels

#### 3.4.1 Mechanical Properties

The texture profile parameters, hardness, springiness, cohesiveness, toughness and compressive Young's modulus, are presented in Figure 3.14 (parts I and II).

Hardness is correlated to the maximum force necessary to cause the selected deformation in the hydrogel. As evidenced by Figure 3.14 (part I), non-inoculated FucoPol hydrogels showed higher hardness ( $9200 \pm 3244$  Pa), followed by FucoPol hydrogels immobilized with *P.chlororaphis* ( $5475 \pm 5106$  Pa). FucoPol hydrogels with immobilized Biotask commercial inocula showed to have the less hardness value ( $4196 \pm 1442$  Pa). These results are consistent with the observations made of these hydrogels and the fact that they didn't achieve a homogenous gelation.

Cohesiveness reflects the aptitude of the hydrogel to withstand to compression. A high cohesive value indicates that the product can maintain its internal structure without failures, when submitted to compression. All the hydrogels presented a relatively high cohesiveness (0.31-0.45). This fact suggests that the hydrogels had relatively high strength of polymer molecular chain's interactions.

Springiness expresses the ability of the hydrogels to physically spring back after it has been deformed in the first compression, allowing a time lag before the second compression. Springiness results are shown in Figure 3.14 (part I). As it can be seen, the springiness for FucoPol hydrogels immobilized with Biotask commercial inocula, non-inoculated FucoPol hydrogels and FucoPol hydrogels with *P.chlororaphis* cells, were quite high (0.78, 0.76 and 0.70, respectively), which is indicative of an elastic behavior. These values are in concordance with the properties described in the literature for FucoPol. (Fialho, 2017)

Young's modulus is a measure of the ability of a material to withstand changes in length when under lengthwise tension or compression. FucoPol hydrogels with no cells showed increased Young's modulus ( $192 \pm 70$  Pa) compared to inoculated FucoPol hydrogels, and gels with Biotask cells immobilized showed the lowest Young's modulus ( $62 \pm 42$  Pa) (Image 3.14 - part I). These results are in accordance with the hardness properties of these gels and also with the structural differences between them.

Toughness is an indication of how well the material can resist fracturing when force is applied. Toughness requires strength as well as ductility, which allows a material to deform before fracturing. The toughness of FucoPol hydrogels and FucoPol hydrogels containing *P.chlororaphis* were quite superior ( $20.0 \pm 7.7$  ;  $18.6 \pm 6.8$  kJ m<sup>-3</sup>, respectively) when compared to FucoPol hydrogels containing Biotask inocula, which is consistent with the fact that FucoPol hydrogels with Biotask inocula immobilized were much more fragile and had a superior non-homogeneity. These results can be seen in Figure 3.14 (part II).

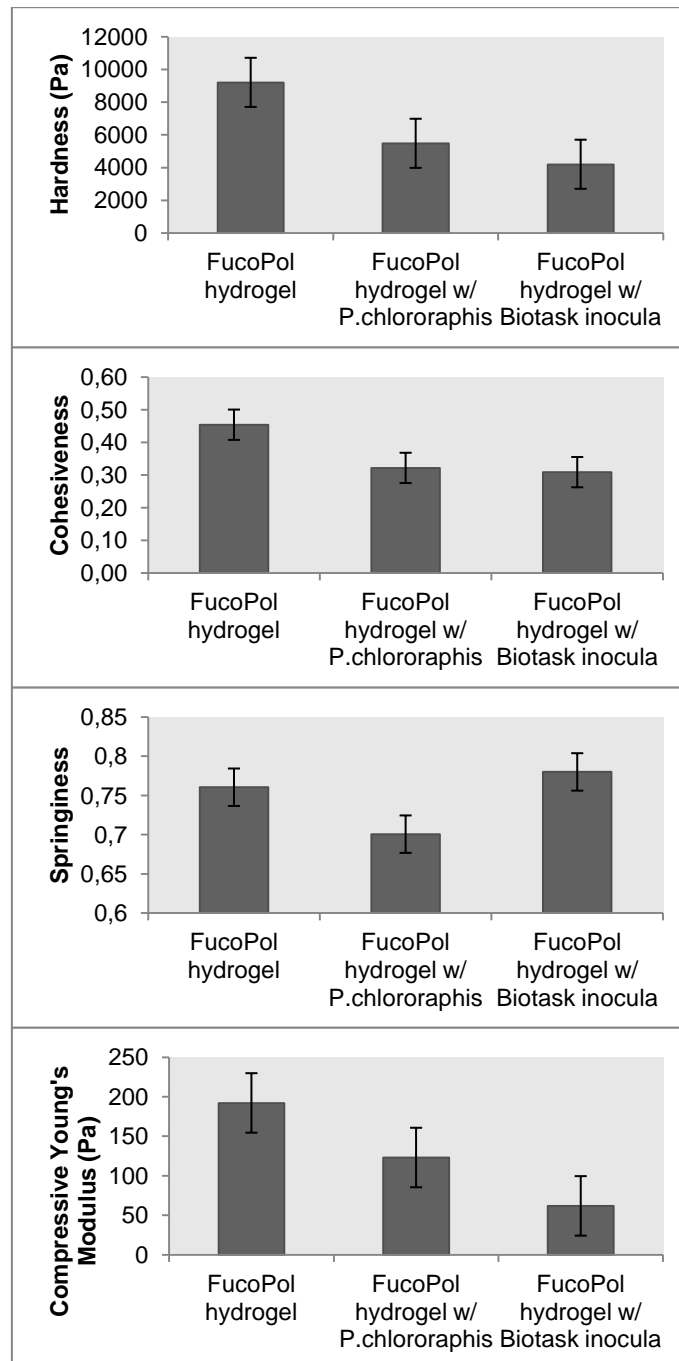


Figure 3.14 – Mechanical properties of FucoPol hydrogels, part I

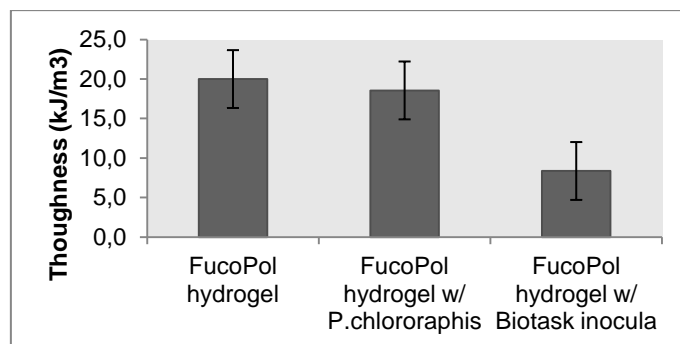


Figure 3.14 – Mechanical properties of FucoPol hydrogels, part II

### 3.4.2 Morphology

Small sections cut from FucoPol hydrogels (FucoPol gel, FucoPol with *P.chlororaphis* inocula gel and FucoPol with Biotask inocula gel) were observed by SEM to analyze structure morphology.

In the photograph taken at FucoPol gel structure (Figure 13.5\_A) it is possible to observe a structure with a smooth surface, presenting some fractures, which is consistent with that presented in previous work. (Fialho, 2019)

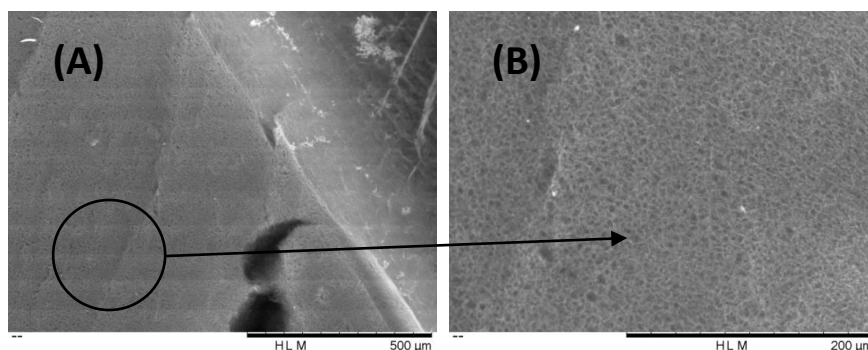


Figure 3.15 – SEM images of FucoPol hydrogel at 150x (A) and 500x (B) magnification at a temperature of -4°C

Some differences were observed when cells are present in gel structures, and it is seen that the FucoPol gel has the smallest pores (Figure 3.15\_B).

FucoPol with *P.chlororaphis* inocula (Figures 3.16\_A and 3.16\_B) also presented a smooth surface but it has slightly larger pores (perhaps due to the presence of cells).

Major alterations in gel structure were observed in FucoPol with Biotask inocula gel (Figures 3.16\_C and 3.16\_D) which seems to be less compact and presented the largest pores, which is in accordance with the mechanical properties of these gels and the fact that the product is a granule that has not dispersed well in the FucoPol solution.

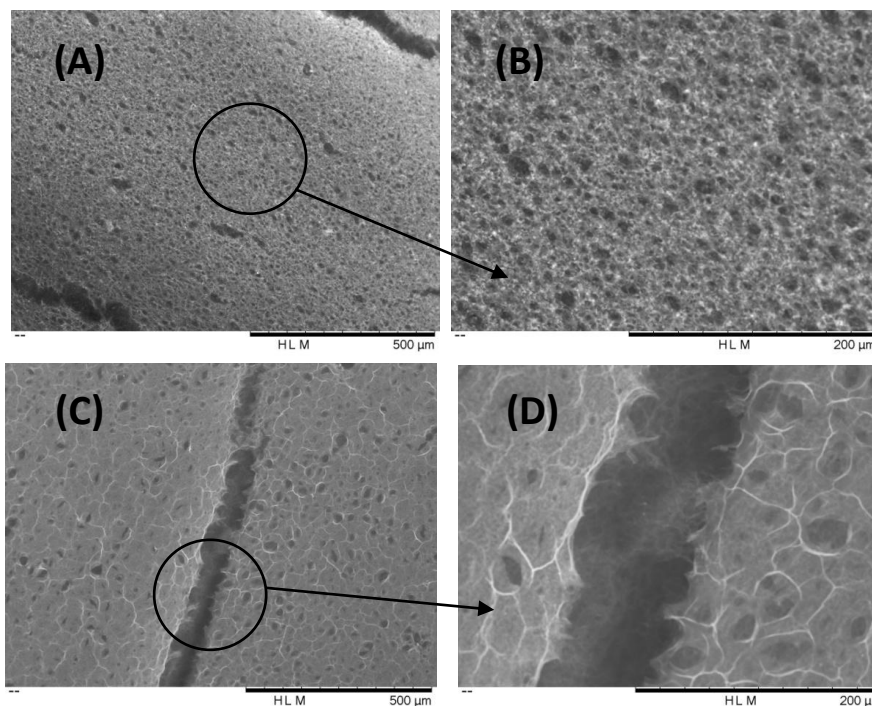


Figure 3.16 - SEM images of FucoPol hydrogel with *P.chlororaphis* at 150x (A) and 500x (B) magnification, and Biotask inocula at 150x (C) and 500x (D) magnification, at a temperature of  $-4^{\circ}\text{C}$

### 3.5 Shake flask assays with immobilized cells and cell growth

In order to evaluate if the immobilization of microbial inocula in FucoPol gels process would be feasible, altered media  $E^*$  (Flasks 1,  $0.4 \text{ g L}^{-1} (\text{NH}_4)_2\text{HPO}_4$ ; Flaks 2,  $0.1 \text{ g L}^{-1} (\text{NH}_4)_2\text{HPO}_4$ , Flasks 3,  $0.1 \text{ g L}^{-1} (\text{NH}_4)_2\text{HPO}_4$ ; Flasks 4,  $0.2 \text{ g L}^{-1} (\text{NH}_4)_2\text{HPO}_4$ ) were prepared, from which three replicates were made (replicates A, only containing one inoculated FucoPol gel; replicates B, containing one inoculated FucoPol gel and liquid inoculum; replicates C, only containing liquid inoculum).

#### 3.5.1 *Pseudomonas chlororaphis* inocula

Immobilization process with *P.chlororaphis* proved to be successful, namely in flasks that only contained inoculated hydrogels - Flask 1A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.4 \text{ g L}^{-1}$ ) (Figure 3.17\_A) and Flask 2A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ ) (Figure 3.17\_B). These results were supported by the increasing OD values registered (indicative of cell growth) along the cultivation time, as well as through the increasing intensity of the medium's orange color, characteristic of the *P.chlororaphis* strain (Figure 3.18).

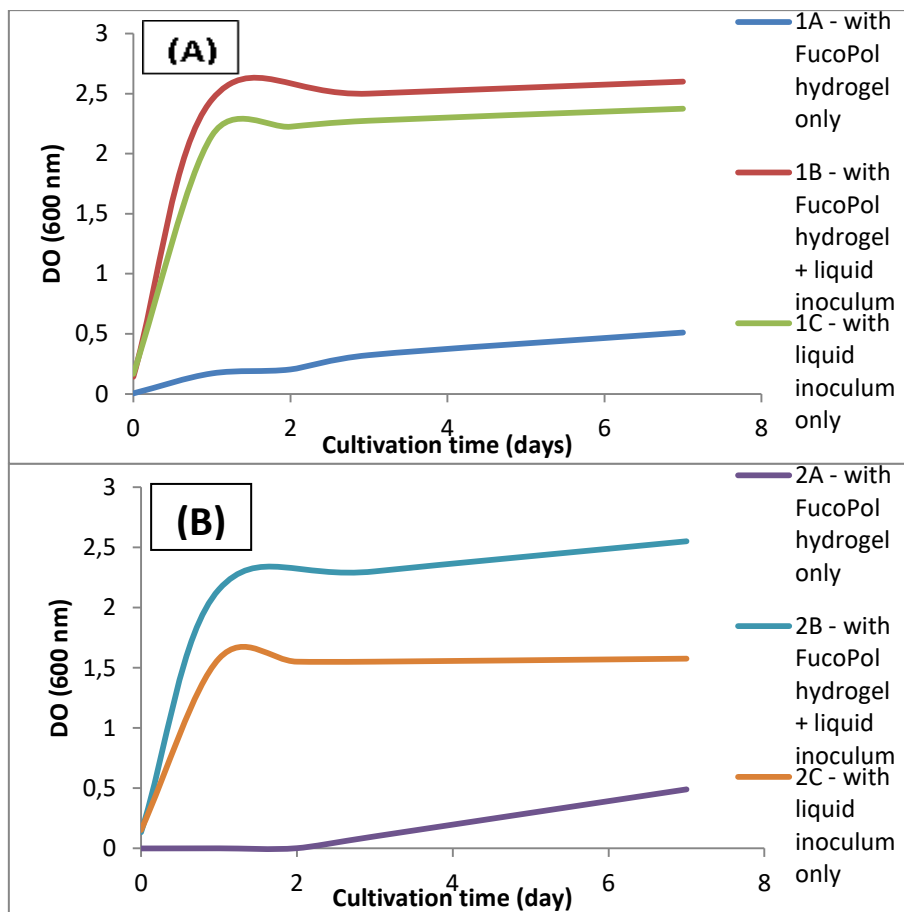


Figure 3.17 – DO<sub>600</sub> vs. Time for FucoPol hydrogels with *P.chlororaphis* inocula in medium E\*, 0.4 g L<sup>-1</sup> (A) and in medium E\*, 0.1 g L<sup>-1</sup> (B)

As expected, Flasks B recorded higher OD (grew the most) as well as the most intense orange color of all media, which is consistent with the fact that they contained two different inoculums (cells immobilized in FucoPol gel and the liquid inoculum). There was greater growth in Flask 1B (Figure 3.17\_A) than in Flask 2B (Figure 3.17\_B) because there was more phosphate available for cell growth to occur.

When compared to Flasks C, Flasks B grew more since they had cells released from the hydrogels, which proves that, effectively, there was release of cells from the gel and the cells were viable.

Flasks A, which only had immobilized cells, registered lower OD (lower growth) as well as the least intense orange color of all media, as expected. However we can conclude that encapsulation was successful due to the fact that cell growth occurred. These results show that the cells immobilized in the gel structure were viable and were not significantly affected by the immobilization procedure.

Flask 1A, which contained (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (0.4 g L<sup>-1</sup>) and a gel structure with immobilized *P.chlororaphis* cells (Figure 3.17\_A) registered higher OD, when compared with Flask 2A that contained (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> at a lower concentration (0.1 g L<sup>-1</sup>) (Figure 3.17\_B). These results are consistent with the fact that at a higher phosphate concentration (0.4 g L<sup>-1</sup>) FucoPol hydrogels disintegrate more quickly, as well as the fact that there is more phosphate available, which is essential for cell growth. So, the cells were released from the gel faster and grew more.

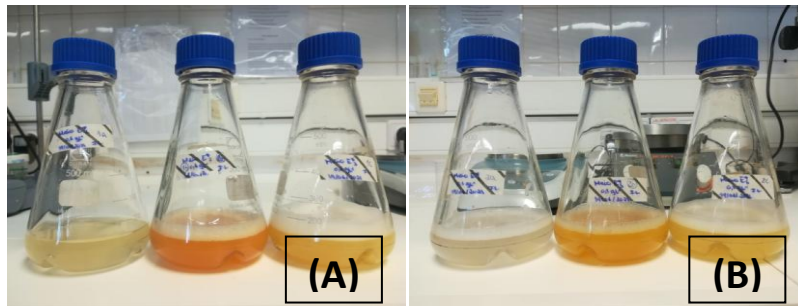


Figure 3.18 – Mediums 1(A, B and C) and 2(A, B and C) on the 7th day of *P.chlororaphis* cultivation

### 3.5.2 Biotask commercial inocula

Immobilization process with Biotask commercial inocula proved to be successful, which was shown through the increasing OD values registered (indicative of cell growth) along the cultivation time, namely in Flask 3A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ ) (Figure 3.19\_A) and in Flask 4A ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.2 \text{ g L}^{-1}$ ) (Figure 3.19\_B).

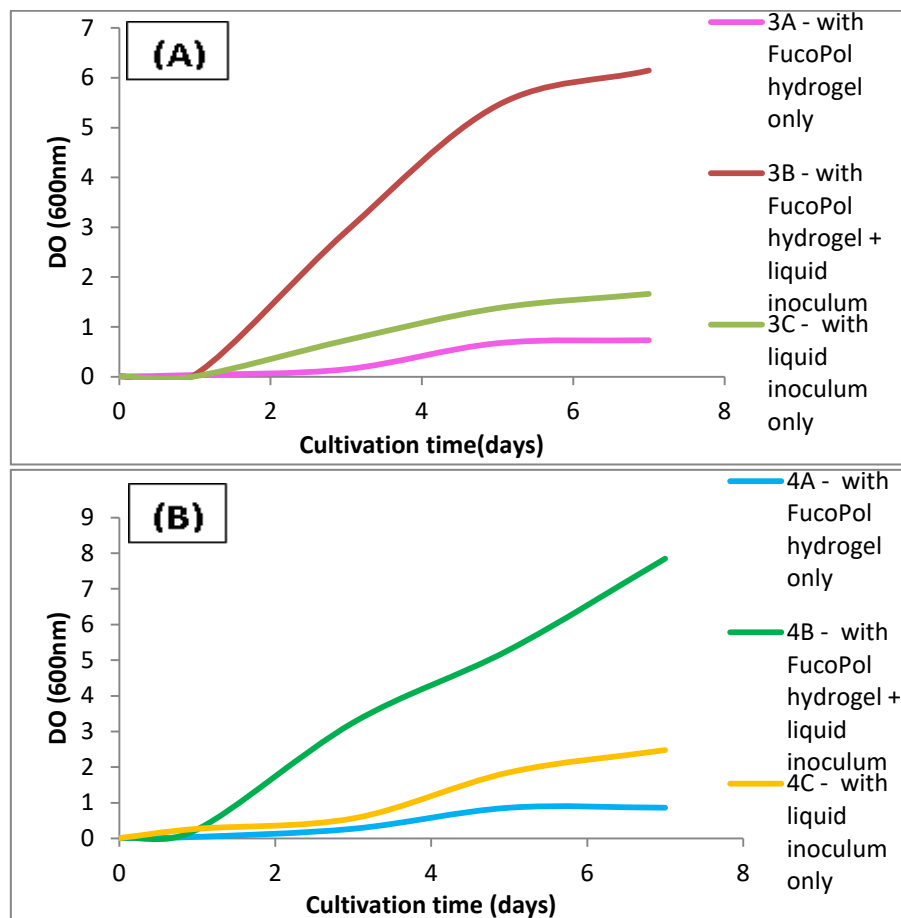


Figure 3.19 –  $\text{DO}_{600}$  vs. Time for FucoPol hydrogels with Biotask commercial inocula in medium E\*,  $0.1 \text{ g L}^{-1}$  (A) and in medium E\*,  $0.2 \text{ g L}^{-1}$  (B)

Flasks 4, which contained  $(\text{NH}_4)_2\text{HPO}_4$  ( $0.2 \text{ g L}^{-1}$ ) registered higher OD (Figure 3.19\_B), when compared with Flasks 3, which contained  $(\text{NH}_4)_2\text{HPO}_4$  ( $0.1 \text{ g L}^{-1}$ ) (Figure 3.19\_A). These results are consistent with the fact that there is more phosphate available, which is essential for cell growth.

Flask 4A, which contained  $(\text{NH}_4)_2\text{HPO}_4$  ( $0.2 \text{ g L}^{-1}$ ) and a gel structure with immobilized Biotask commercial cells registered higher OD (Figure 3.19\_B), when compared with Flask 3A (Figure 3.19\_A) that contained  $(\text{NH}_4)_2\text{HPO}_4$  at a lower concentration ( $0.1 \text{ g L}^{-1}$ ). These data also evidence the fact that the cells were released from the gel faster and grew more. These results also show that the cells immobilized in the gel structure were viable and were not significantly affected by the immobilization procedure.

As expected, Flasks B recorded higher OD values, which is consistent with the fact that they contained two different inoculums (cells immobilized in FucoPol gel and the liquid inoculum). Flasks A registered the lowest OD values, however we can conclude that encapsulation was successful due to the fact that cell growth occurred.

Flaks C recorded intermediate OD values. These observations show the fact that, despite the successful immobilization on FucoPol hydrogels, the cell growth obtained on Flasks A was lower than the growth registered on Flasks C, which contained the same initial cell concentration ( $0.6 \text{ g L}^{-1}$ ).

It is important to notice that Flask 3B registered higher OD and an intense and orange color (Figure 3.20\_A), at the 7<sup>th</sup> day of cultivation, which is not consistent with the work results, which may probably be derived from a potential medium contamination.

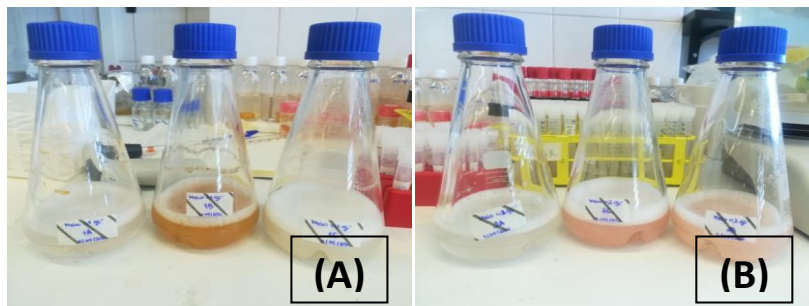


Figure 3.20 – Mediums 3(A, B and C) and 4(A, B and C) on the 7th day of Biotask commercial inocula cultivation

## **4. Conclusions and future work**

FucoPol hydrogels were produced by diffusion through dialysis tubing. These hydrogels revealed to be very stable and resistant in aqueous media (namely in water and 0.9% (w/v) NaCl aqueous solution), lasting for about 17 weeks, with no greater alterations. The pH effect on these gels is not fully understood since the observations registered a similar behavior of the structures in the presence of different ranges of pH, but it appears that FucoPol hydrogels are more stable at more low and intermediate pH ranges (~ 3.5 – 7.0). The major alterations of FucoPol hydrogels under the pH effect were registered when different ionic species were present in the solutions. For future studies, the effect of pH should be evaluated, with different buffer solutions than those evaluated in this work, with similar salt compositions, in order to be able to determine with greater precision the pH range where FucoPol hydrogels are stable, without the interference of the influence of different species present in the solutions. FucoPol gels also revealed to be quite stable and resistant in agitation conditions, lasting between 10 to 13 weeks in these conditions. The ionic effect study proved that Na<sup>+</sup> and K<sup>+</sup> ions didn't destabilize the gels, since the structures lasted until about 13 weeks without major alterations under the effect of these ions (at the concentrations applied). For future work, different ionic species should be evaluated, in order to determine which chemical species could destabilize the FucoPol gels, as well as to determine the concentration ranges that FucoPol gels could be applied, specifically, under the influence of chemical substances commonly present in wastewater effluents. In this work is also reported that FucoPol hydrogels destabilized, and rapidly disintegrated in the presence of phosphate ion (PO<sub>4</sub><sup>3-</sup>), which is explained by the fact that ion phosphate (PO<sub>4</sub><sup>3-</sup>) may have a high affinity for Fe<sup>3+</sup> and it will sequester the cross-linking iron ions from the gel structure, consequently destabilizing the gel, similar to that observed in Ca-alginate hydrogels. The preferential range of phosphate ion, in which FucoPol hydrogels can be applied, it was determined in [0.4; 0.01] ([PO<sub>4</sub><sup>3-</sup>] g L<sup>-1</sup>), where the hydrogel structures could last for about 1 to 9 weeks.

Immobilization of inocula (*P. chlororaphis* and Biotask commercial inocula) in FucoPol hydrogels was very successful in the presented conditions, with cellular growth being recorded in media that only contained FucoPol gel with immobilized cells. Despite these results, immobilization method should be optimized in future work in order to obtain greater cellular growth and a slower degradation of the FucoPol matrix. For these purposes it is necessary to improve the gelling process of the structures, in order to obtain more homogeneous hydrogels, and increasing the initial concentration of cells encapsulated in the gel matrices.

Hydrogels with immobilized cells showed different characteristics. FucoPol hydrogels with *P. chlororaphis* cells presented a more intense and brighter orange color, when compared to non-inoculated gels, and visually, they shown to have a more compact structure and smaller dimensions. These gels also revealed to have a non-homogeneous gelation. FucoPol hydrogels with Biotask commercial inocula showed a yellowish color, with the presence of visible particles, which were inert fillers, constituents of the cell granulate. These hydrogels, visually, shown to have the most fragile and less compact structure, when compared with the others hydrogels produced. These gels also revealed to have a non-homogeneous gelation.

The mechanical properties of FucoPol hydrogels in general revealed relatively weak properties, which may be derived from the non-homogeneous gelation of FucoPol gels, so future studies should focus on finding a procedure that enables a homogenous gelation. FucoPol based hydrogels presented improved mechanical properties compared with inoculated hydrogels. FucoPol hydrogels with Biotask commercial inocula registered the worst mechanical properties, which may be due to the inert fillers present in the Biotask cell granule which did not homogenize perfectly with the FucoPol solution. The non-inoculated FucoPol hydrogels showed higher hardness (9200 ± 3244 Pa), followed by FucoPol hydrogels immobilized with *P. chlororaphis* (5475 ± 5106 Pa). FucoPol hydrogels with immobilized Biotask commercial inocula showed to have the less hardness value (4196 ± 1442 Pa). All the structures revealed an elastic behavior, evidenced by their high springiness (~0.75).

SEM images demonstrated that the three FucoPol hydrogel structures in study (FucoPol hydrogel, inoculated FucoPol hydrogel with *P.chlororaphis* and FucoPol hydrogel inoculated with Biotask commercial inocula) have different morphology, with non-inoculated FucoPol gels presenting a matrix with the smallest pores. FucoPol inoculated with *P.chlororaphis* presented a similar surface but it has slightly larger pores and FucoPol hydrogels inoculated with Biotask inocula showed to be less compact and presented the biggest pores, which is in accordance with the mechanical properties of these gels.

To summarize, general conclusions are that FucoPol hydrogels are quite stable and resistant under several conditions, and the cell immobilization in the gel matrix is possible. The gelation process also didn't affect the cell viability. However, to implement FucoPol hydrogels in bioremediation applications further studies must be carried out in order to optimize and obtain a final product with all the characteristics desired for this purpose. For example, further studies should be carried out in order to optimize the FucoPol gel beads, making them more resistant and stable, in order to obtain a product in granulated form, to be applied in effluents to be treated by bioremediation.



## References

Antunes, S.; Freitas, F.; Alves, V. D.; Grandfils, C.; Reis, M. A. M. (2015). Conversion of Cheese Whey into a Fucose- and Glucuronic Acid-Rich Extracellular Polysaccharide by *Enterobacter* A47. *J. Biotechnol.* 210, 1–7. DOI: 10.1016/j.jbiotec.2015.05.013.

Antunes, S.; Freitas, F.; Sevrin, C.; Grandfils, C.; Reis, M. A. M. (2017). Production of FucoPol by *Enterobacter* A47 Using Waste Tomato Paste By-Product as Sole Carbon Source. *Bioresour. Technol.*, 227, 66–73. DOI: 10.1016/j.biortech.2016.12.018.

Avérous, L., Pollet, E. (2012). Biodegradable Polymers. *Green Energy and Technology*, 13–39. DOI:10.1007/978-1-4471-4108-2\_2

Azubuike, C.C.; Chikere, C.B.; Okpokwasili, G.C. (2016). Bioremediation techniques-classification based on site of application: principles, advantages, limitations and prospects. *World J Microbiol Biotechnol*, 32, 180. DOI:10.1007/s11274-016-2137-x

Bickerstaff, G. F. (n.d.). Immobilization of Enzymes and Cells: Some Practical Considerations. *Immobilization of Enzymes and Cells*, 1–12. DOI: 10.1385/0-89603-386-4:1

Callone, E.; Campostrini, R.; Carturan, G.; Cavazza, A.; Guzzon, R. (2008). Immobilization of yeast and bacteria cells in alginate microbeads coated with silica membranes: procedures, physico-chemical features and bioactivity. *Journal of Materials Chemistry*, 18(40), 4839. DOI:10.1039/B807301E

Campbell, F.J.; Rhodes, E.R.; Naser, H. (2019). Human Factors: The Impact of industry and the Environment Natural Resources Management and Biological Sciences. *Intech Open*. DOI: 10.5772/intechopen.90419.

Chandra, R.; Rustgi R. (1998). Biodegradable Polymers. *Prog. Polym. Sci.*, 23, 1273–1335.

Donot, F., Fontana, A., Baccou, J. C., & Schorr-Galindo, S. (2012). Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers*, 87(2), 951–962. DOI: 10.1016/j.carbpol.2011.08.083

Doppalapudi, S., Jain, A., Khan, W., & Domb, A. J. (2014). Biodegradable polymers-an overview. *Polymers for Advanced Technologies*, 25(5), 427–435. DOI: 10.1002/pat.3305

D'Souza, SF. (1989). Immobilized Cells: Techniques and Applications. *Indian J Microbiol*, 29, 83-117

Dulieu, C., Poncelet, D., & Neufeld, R. J. (1999). Encapsulation and Immobilization Techniques. *Cell Encapsulation Technology and Therapeutics*, 3–17. DOI: 10.1007/978-1-4612-1586-8\_1

Dziona, A., Wojcieszńska, D., & Guzik, U. (2016). Natural carriers in bioremediation: A review. *Electronic Journal of Biotechnology*, 23, 28–36. DOI: 10.1016/j.ejbt.2016.07.003

El Fantroussi, S., & Agathos, S. N. (2005). Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Current Opinion in Microbiology*, 8(3), 268–275. DOI: 10.1016/j.mib.2005.04.011

Ferreira, A. R. V., Torres, C. A. V., Freitas, F., Reis, M. A.; Alves, V. D., Coelho, I. M. (2014). Biodegradable films produced from the bacterial polysaccharide FucoPol. *International Journal of Biological Macromolecules*, 71, 111-116. DOI: 10.1016/j.ijbiomac.2014.04.022

Fialho, L. M. R. (2017). Preparation of biopolymer structures based on FucoPol, FCT-UNL

Fialho, L.; Araújo, D.; Alves, V. D.; Roma-Rodrigues, C.; Baptista, P. V.; Fernandes, A. R.; Freitas, F.; Reis, M. A. M. (2019). Cation-Mediated Gelation of the Fucose-Rich Polysaccharide FucoPol: Preparation and Characterization of Hydrogel Beads and Their Cytotoxicity Assessment. *Int. J. Polym. Mater. Polym. Biomater*, 70 (2), 90–99. DOI:10.1080/00914037.2019.1695205.

Fraser, J. E., & Bickerstaff, G. F. (n.d.). Entrapment in Calcium Alginate. *Immobilization of Enzymes and Cells*, 61–66. DOI:10.1385/0-89603-386-4:61

Freitas, F.; Alves, V. D.; Torres, C. A. V.; Cruz, M.; Sousa, I.; Melo, M. J.; Ramos, A. M.; Reis, M. A. M. (2011). Fucose-Containing Exopolysaccharide Produced by the Newly Isolated *Enterobacter* Strain A47 DSM 23139. *Carbohydr. Polym.*, 83 (1), 159–165. DOI: 10.1016/j.carbpol.2010.07.034

Freitas, F.; Alves, V. D.; Gouveia, A. R.; Pinheiro, C.; Torres, C. A. V.; Grandfils, C.; Reis, M. A. M. (2014). Controlled Production of Exopolysaccharides from *Enterobacter* A47 as a Function of Carbon Source with Demonstration of Their Film and Emulsifying Abilities. *Appl. Biochem. Biotechnol.* 172 (2), 641–657. DOI: 10.1007/s12010-013-0560-0.

Guerreiro, B. M., Freitas, F., Lima, J. C., Silva, J. C., Dionísio, M., & Reis, M. A. M. (2020). Demonstration of the cryoprotective properties of the fucose-containing polysaccharide FucoPol. *Carbohydrate Polymers*, 116500. DOI: 10.1016/j.carbpol.2020.116500

Goosen, M. F. A. (1993). Toxicity, biocompatibility, and mass transfer effects in immobilized cell systems. *Fundamentals of animal cell encapsulation and immobilization*, 43-54

Hae, S. (2012) Agarose-gel-immobilized recombinant bacterial biosensors for simple and disposable on-site detection of phenolic compounds. *Appl Microbiol Biotechnol* 93, 1895–1904. DOI: 10.1007/s00253-011-3700-x

Head, I. M. (1998). Bioremediation: towards a credible technology. *Microbiology*, 144(3), 599–608. DOI: 10.1099/00221287-144-3-599

Iwamoto, T.; Nasu, M. (2001). Current bioremediation practice and perspective. *Journal of Bioscience and Bioengineering*, 92(1), 1–8. DOI: 10.1016/s1389-1723(01)80190-0

Jen, A. C., Wake, M. C., & Mikos, A. G. (2000). Review: Hydrogels for cell immobilization. *Biotechnology and Bioengineering*, 50(4), 357–364. DOI: 10.1002/(sici)1097-0290(19960520)50:4<357::aid-bit2>3.0.co;2-k

Juwarkar, A. A., Singh, S. K., & Mudhoo, A. (2010). A comprehensive overview of elements in bioremediation. *Reviews in Environmental Science and Bio/Technology*, 9(3), 215–288. DOI: 10.1007/s11157-010-9215-6

Kaplan, D. L. (1998). Introduction to Biopolymers from Renewable Resources. *Biopolymers from Renewable Resources*, 1–29. DOI: 10.1007/978-3-662-03680-8\_1

Karaki, N., Aljawish, A., Humeau, C., Muniglia, L., & Jasniewski, J. (2016). Enzymatic modification of polysaccharides: Mechanisms, properties, and potential applications: A review. *Enzyme and Microbial Technology*, 90, 1–18. DOI: 10.1016/j.enzmictec.2016.04.004

Kenne, L., & Lindberg, B. (1983). Bacterial Polysaccharides. *The Polysaccharides*, 287–363. DOI: 10.1016/b978-0-12-065602-8.50010-8

King, G., Goosen, M. F. A. (1993). Cell Immobilization technology: An overview. *Fundamentals of animal cell encapsulation and immobilization*, 1-6.

Klein, J., & Ziehr, H. (1990). Immobilization of microbial cells by adsorption. *Journal of Biotechnology*, 16(1-2), 1–15. DOI: 10.1016/0168-1656(90)90061-f

Kouzuma, A.; Watanabe, K. (2011). Environmental and Related Biotechnologies. *Comprehensive Biotechnology* (Third Edition).

Mohan, N., Gislason, A., Sharma, P. K., Ghergab, A., Plouffe, J., Levin, D. B., & de Kievit, T. (2019). Quorum sensing and the anaerobic regulator (ANR) control Polyhydroxyalkanoate (PHA) production in *Pseudomonas chlororaphis* PA23. *FEMS Microbiology Letters*. DOI:10.1093/femsle/fnz223

More, T. T., Yadav, J. S. S., Yan, S., Tyagi, R. D., & Surampalli, R. Y. (2014). Extracellular polymeric substances of bacteria and their potential environmental applications. *Journal of Environmental Management*, 144, 1–25. DOI: 10.1016/j.jenvman.2014.05.010

Moreno, J., Vargas, M. A., Olivares, H., Rivas, J., & Guerrero, M. G. (1998). Exopolysaccharide production by the cyanobacterium *Anabaena* sp. ATCC 33047 in batch and continuous culture. *Journal of Biotechnology*, 60(3), 175–182. DOI:10.1016/s0168-1656(98)00003-0

Moreno-Garrido, I. (2008) Microalgae immobilization: current techniques and uses. *Bioresour Technol*, 99, 3949–3964.

Mueller, J. G.; Cerniglia, C. E.; Pritchard, P. H. (1996). Bioremediation of Environments Contaminated by Polycyclic Aromatic Hydrocarbons. In *Bioremediation: Principles and Applications*, 125–194.

Nwodo, U., Green, E., & Okoh, A. (2012). Bacterial Exopolysaccharides: Functionality and Prospects. *International Journal of Molecular Sciences*, 13(12), 14002–14015. DOI: 10.3390/ijms131114002~

Orive, G., Hernández, R. M., Gascón, A. R., Calafiore, R., Chang, T. M. S., Vos, P. D., ... Pedraz, J. L. (2003). Cell encapsulation: Promise and progress. *Nature Medicine*, 9(1), 104–107. DOI: 10.1038/nm0103-104

Palmieri, G., Giardina, P., Desiderio, B., Marzullo, L., Giamberini, M., & Sannia, G. (1994). A new enzyme immobilization procedure using copper alginate gel: Application to a fungal phenol oxidase. *Enzyme and Microbial Technology*, 16(2), 151–158. DOI:10.1016/0141-0229(94)90078-7

Radosavljević, Miloš; Lević, Steva; Belović, Miona; Pejtin, Jelena; Djukić-Vuković, Aleksandra; Mojović, Ljiljana; Nedović, Viktor (2019). Immobilization of *Lactobacillus rhamnosus* in polyvinyl alcohol/calcium alginate matrix for production of lactic acid. *Bioprocess and Biosystems Engineering*. DOI:10.1007/s00449-019-02228-0

Rinaudo, M. (2008). Main properties and current applications of some polysaccharides as biomaterials. *Polymer International*, 57(3), 397–430. DOI: 10.1002/pi.2378

Roca, C.; Alves, V. D.; Freitas, F.; Reis, M. A. M. (2015). Exopolysaccharides Enriched in Rare Sugars: Bacterial Sources, Production, and Applications. *Front. Microbiol.*, 6:288, 1–7. DOI: 10.3389/fmicb.2015.00288.

Rodrigues, T. A. (2020). Preparation and characterization of hydrogels based on biopolymers: FucoPol and Chitin-glucan complex (CGC), FCT-UNL

Saari, P., Häkkä, K., Heijilä, H., Jumppanen, J., Hurme, M. (2009). A novel chromatographic production scale separation process for L-Fucose. *Journal of Liquid Chromatography and Related Technologies*, 32:14, 2050-2064.




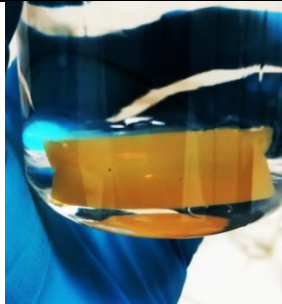








- Salter, G. J., & Kell, D. B. (1991). New materials and technology for cell immobilization. *Current Opinion in Biotechnology*, 2(3), 385–389. DOI: 10.1016/s0958-1669(05)80143-0
- Sandford, P. A. (1979). Exocellular, Microbial Polysaccharides. *Advances in Carbohydrate Chemistry and Biochemistry*, 36, 265–313. DOI: 10.1016/s0065-2318(08)60238-3
- Sharma, S. (2012). Bioremediation: Features, Strategies and applications. *Asian Journal of Pharmacy and Life Science ISSN*, 2, 2231–4423.
- Shukla, K. P.; Singh, N. K.; Sharma, S. (2010). Bioremediation: Developments, Current Practices and Perspectives. *Genetic Engineering and Biotechnology Journal*.
- Siripattanakul, S., & Khan, E. (2009). Fundamentals and Applications of Entrapped Cell Bioaugmentation for Contaminant Removal. *Emerging Environmental Technologies*, 2, 147–169. DOI: 10.1007/978-90-481-3352-9\_7
- Smidsrod, O.; Skjakbrk, G. (1990). Alginate as immobilization matrix for cells. *Trends in Biotechnology*, 8, 71–78. DOI:10.1016/0167-7799(90)90139-o
- Stormo, K. E.; Crawford, R. L. (1992). Preparation of Encapsulated Microbial Cells for Environmental Applications. *Appl Environ Microbiol.*, 58(2), 727
- Torres, C. A. V; Marques, R.; Antunes, S.; Alves, V. D.; Sousa, I.; Ramos, A. M.; Oliveira, R.; Freitas, F.; Reis, M. A. M. (2011). Kinetics of Production and Characterization of the Fucose-Containing Exopolysaccharide from *Enterobacter A47*. *J. Biotechnol.*, 156 (4), 261–267. DOI: 10.1016/j.jbiotec.2011.06.024
- Torres, C. (2012). Engineering of Bacterial Exopolysaccharides: From Synthesis to Properties. FCT-UNL.
- Trelles, J. A., & Rivero, C. W. (2013). Whole Cell Entrapment Techniques. *Immobilization of Enzymes and Cells*, 365–374. DOI: 10.1007/978-1-62703-550-7\_24
- Usama, B. (2003). Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads. *African Journal of Biotechnology*, 2(3), 3. DOI:10.5897/AJB2003.000-1012
- Vidali, M. (2001). Bioremediation. An overview. *Pure and Applied Chemistry*, 73(7), 1163–1172. DOI: 10.1351/pac200173071163
- Vogel., T. M. (1996). Bioaugmentation as a soil bioremediation approach.


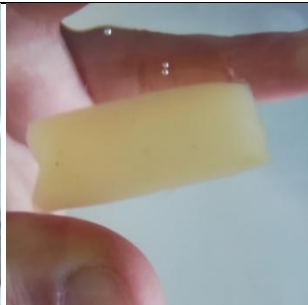


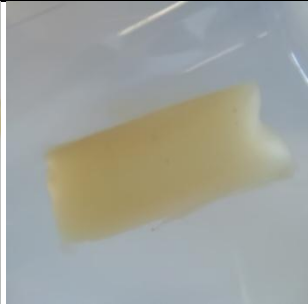





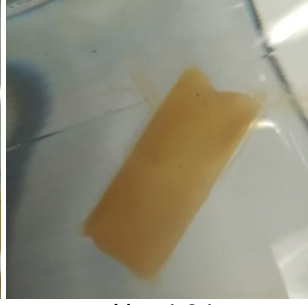
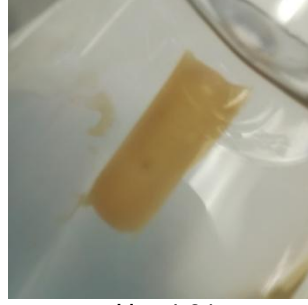

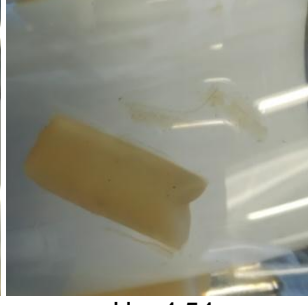

Wang, X., Majzoobi, M., Fanahnaky, A. (2020). Ultrasound-assisted modification of functional properties and biological activity of biopolymers: A review. *Ultrasonics Sonochemistry*, 105057. DOI: 10.1016/j.ultsonch.2020.105057



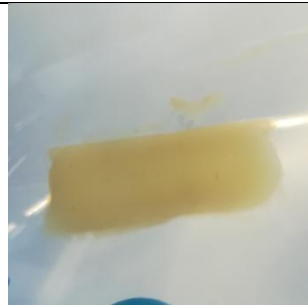


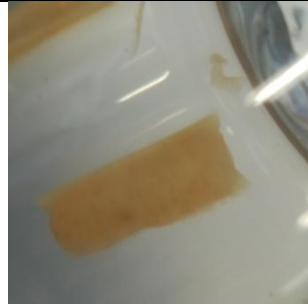

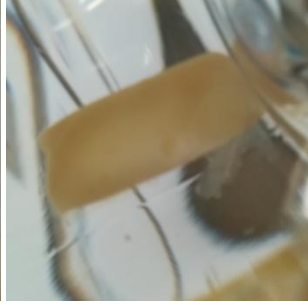







Tong, H. W., Mutlu, B. R., Wackett, L. P., Aksan, A. (2013). Silica/PVA biocatalytic nanofibers. *Materials Letters*, 111, 234-237. DOI: 10.1016/j.matlet.2013.08.102

## Appendices

Table A1 - FucoPol hydrogels stability in deionized water

Structure nº	1	2	3
Mass (g)	3,61145	4,09851	3,42129
Length (cm)	3,5	3,5	3,6
Width (cm)	1,5	1,5	1,5
<b>Start</b> (20-01-21)	 pH = 5.28	 pH = 5.26	 pH = 5.25
<b>24 H</b>	 pH = 5.16	 pH = 5.16	 pH = 5.14
<b>1<sup>st</sup> week</b>	 pH = 5.13	 pH = 5.13	 pH = 5.12
<b>2<sup>nd</sup> week</b>	 pH = 5.11	 pH = 5.13	 pH = 5.11

Structure nº	1	2	3
Mass (g)	3,61145	4,09851	3,42129
Length (cm)	3,5	3,5	3,6
Width (cm)	1,5	1,5	1,5
<u>3<sup>rd</sup> week</u>	 pH = 5.09	 pH = 5.11	 pH = 5.12
<u>4<sup>th</sup> week</u>	 pH = 5.09	 pH = 5.09	 pH = 5.03
<u>5<sup>th</sup> week</u>	 pH = 5.06	 pH = 4.94	 pH = 4.90
<u>6<sup>th</sup> week</u>	 pH = 4.62	 pH = 4.64	 pH = 4.61
<u>7<sup>th</sup> week</u>	 pH = 4.56	 pH = 4.54	 pH = 4.59

Structure n°	1	2	3
Mass (g)	3,61145	4,09851	3,42129
Length (cm)	3,5	3,5	3,6
Width (cm)	1,5	1,5	1,5
<u>8<sup>th</sup> week</u>	 pH = 4.70	 pH = 4.78	 pH = 4.81
<u>9<sup>th</sup> week</u>	 pH = 4.73	 pH = 4.83	 pH = 4.88
<u>10<sup>th</sup> week</u>	 pH = 4.81	 pH = 4.86	 pH = 4.88
<u>11<sup>th</sup> week</u>	 pH = 4.54	 pH = 4.66	 pH = 4.69
<u>12<sup>th</sup> week</u>	 pH = 4.74	 pH = 4.75	 pH = 4.79


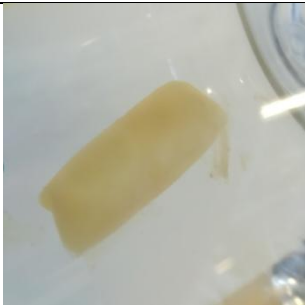
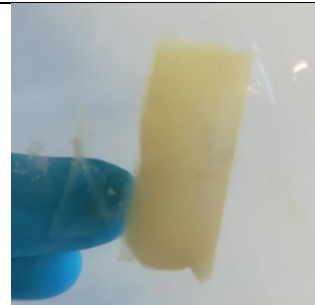


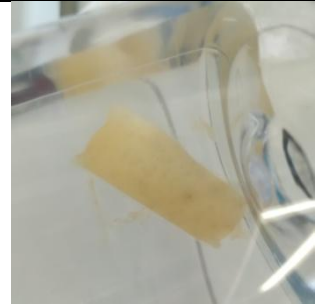

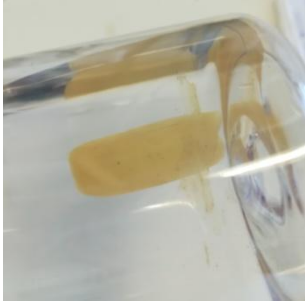



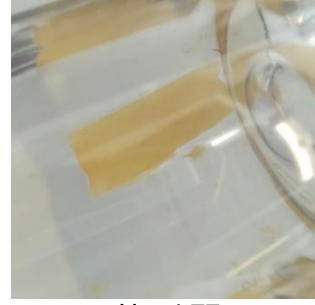

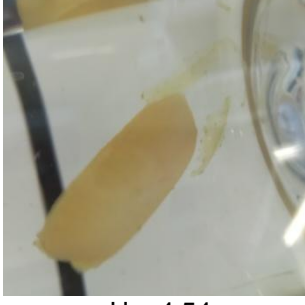




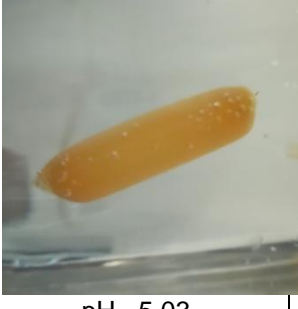


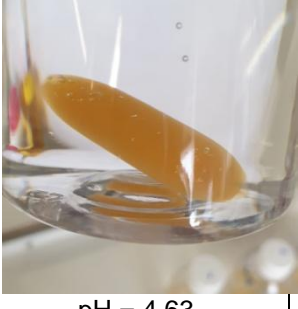


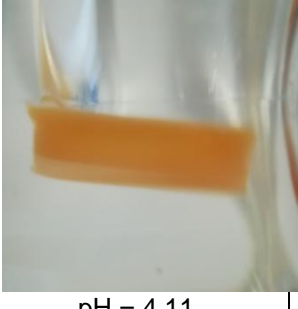

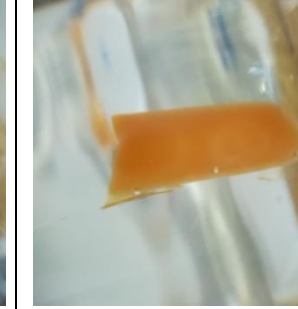



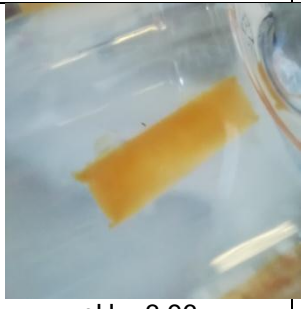
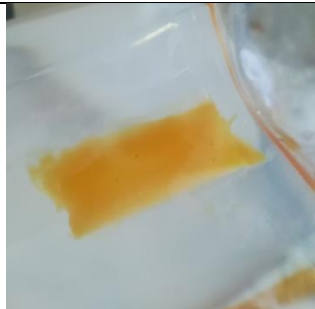
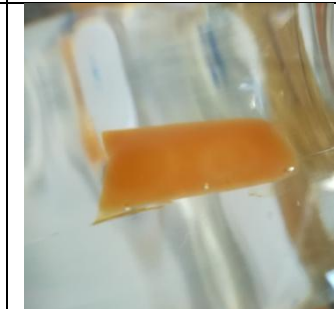


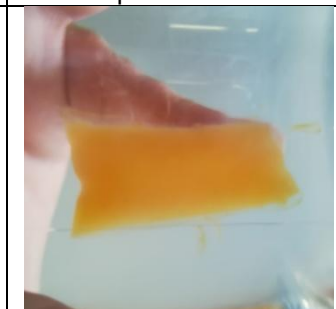
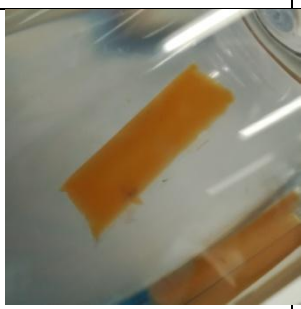




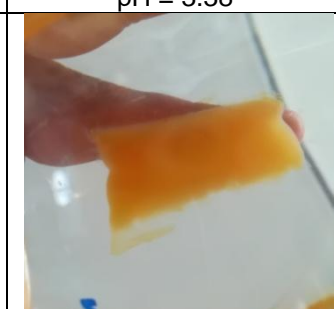

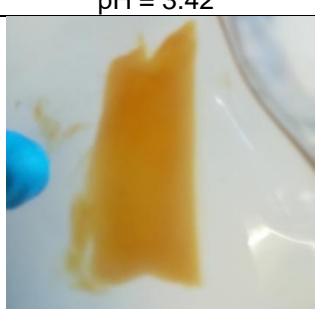
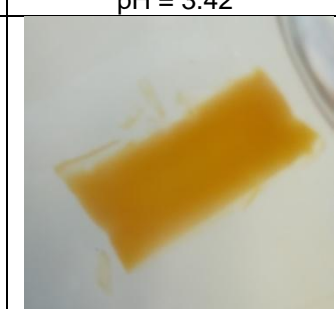






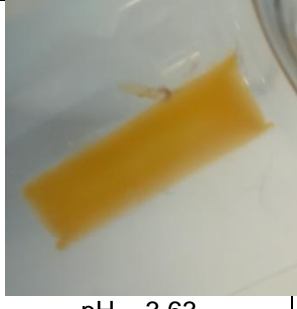





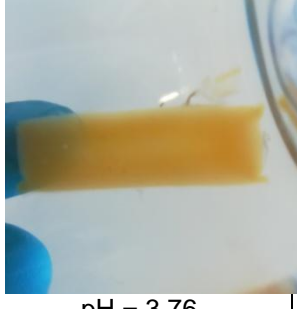

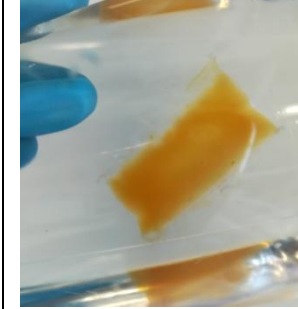
Structure n°	1	2	3
Mass (g)	3,61145	4,09851	3,42129
Length (cm)	3,5	3,5	3,6
Width (cm)	1,5	1,5	1,5
<u>13<sup>th</sup> week</u>	 pH = 4.67	 pH = 4.71	 pH = 4.78
<u>14<sup>th</sup> week</u>	 pH = 4.80	 pH = 4.80	 pH = 4.77
<u>15<sup>th</sup> week</u>	 pH = 4.63	 pH = 4.69	 pH = 4.68
<u>16<sup>th</sup> week</u>	 pH = 4.56	 pH = 4.75	 pH = 4.77
<u>17<sup>th</sup> week</u>	 pH = 4.39	 pH = 4.54	 pH = 4.57

Table A2 - FucoPol hydrogels stability in NaCl (0.9%, w/v) solution

Structure n <sup>o</sup>	4	5	6
<b>Mass (g)</b>	3.2283	3.8921	3.7226
<b>Length (cm)</b>	3.9	3.9	3.5
<b>Width (cm)</b>	1.3	1.4	1.5
<b>Start</b> (20-01-21)	 pH = 5.64	 pH = 5.66	 pH = 5.64
<b>24 H</b>	 pH = 5.03	 pH = 5.06	 pH = 5.04
<b>1<sup>st</sup> week</b>	 pH = 4.63	 pH = 4.63	 pH = 4.62
<b>2<sup>nd</sup> week</b>	 pH = 4.11	 pH = 4.13	 pH = 4.11
<b>3<sup>rd</sup> week</b>	 pH = 3.99	 pH = 3.98	 pH = 3.98

Structure n°	4	5	6
Mass (g)	3.2283	3.8921	3.7226
Length (cm)	3.9	3.9	3.5
Width (cm)	1.3	1.4	1.5
<u>4<sup>th</sup> week</u>	 pH = 3.96	 pH = 3.98	 pH = 3.96
<u>5<sup>th</sup> week</u>	 pH = 3.71	 pH = 3.57	 pH = 3.54
<u>6<sup>th</sup> week</u>	 pH = 3.46	 pH = 3.40	 pH = 3.38
<u>7<sup>th</sup> week</u>	 pH = 3.51	 pH = 3.42	 pH = 3.42
<u>8<sup>th</sup> week</u>	 pH = 3.64	 pH = 3.55	 pH = 3.55

Structure n <sup>o</sup>	4	5	6
Mass (g)	3.2283	3.8921	3.7226
Length (cm)	3.9	3.9	3.5
Width (cm)	1.3	1.4	1.5
<u>9<sup>th</sup> week</u>	 pH = 3.71	 pH = 3.64	 pH = 3.63
<u>10<sup>th</sup> week</u>	 pH = 3.71	 pH = 3.64	 pH = 3.62
<u>11<sup>th</sup> week</u>	 pH = 3.63	 pH = 3.54	 pH = 3.53
<u>12<sup>th</sup> week</u>	 pH = 3.67	 pH = 3.58	 pH = 3.57
<u>13<sup>th</sup> week</u>	 pH = 3.76	 pH = 3.65	 pH = 3.67




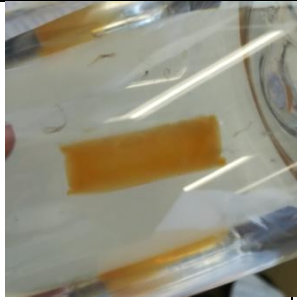




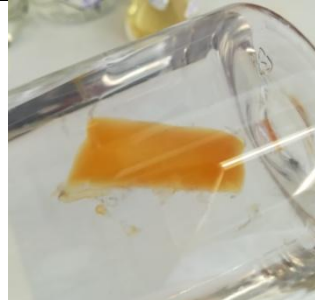
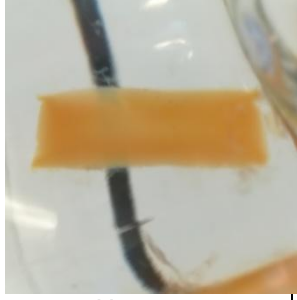


Structure n°	4	5	6
Mass (g)	3.2283	3.8921	3.7226
Length (cm)	3.9	3.9	3.5
Width (cm)	1.3	1.4	1.5
<u>14<sup>th</sup> week</u>	 pH = 3.82	 pH = 3.76	 pH = 3.74
<u>15<sup>th</sup> week</u>	 pH = 3.92	 pH = 3.86	 pH = 3.86
<u>16<sup>th</sup> week</u>	 pH = 3.80	 pH = 3.72	 pH = 3.71
<u>17<sup>th</sup> week</u>	 pH = 3.79	 pH = 3.73	 pH = 3.71

Table A3 - FucoPol hydrogels stability in PBS solution


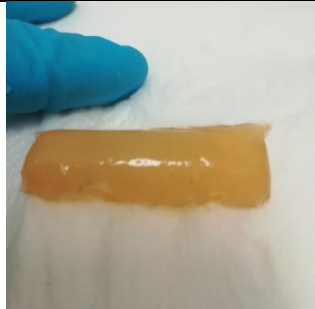

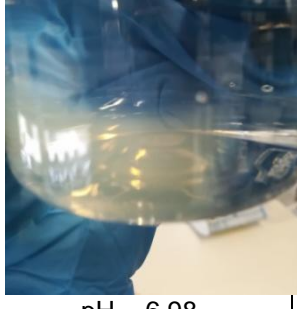

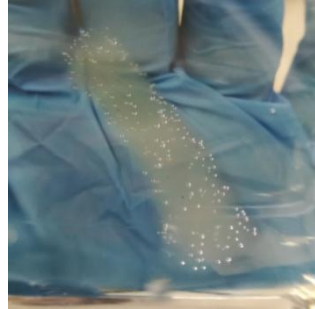

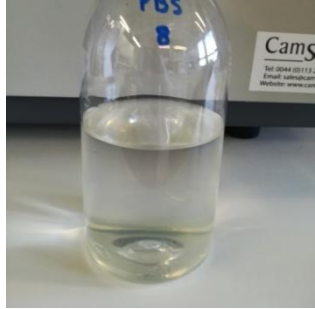
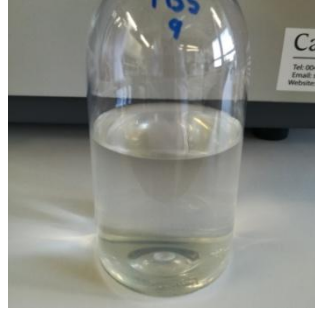
Structure n <sup>o</sup>	7	8	9
<b>Mass (g)</b>	3.3189	3.9599	2.4216
<b>Length (cm)</b>	3.7	4.0	3.7
<b>Width (cm)</b>	1.4	1.3	1.3
<b>Start</b> (20-01-21)	 pH = 7.10	 pH = 7.10	 pH = 7.11
<b>24 H</b>	 pH = 6.98	 pH = 6.99	 pH = 6.94
<b>1<sup>st</sup> week</b>	 pH = 6.69	 pH = 6.63	 pH = 6.68

Table A4 - FucoPol hydrogels stability in 0.5% (v/v) hypochlorite solution












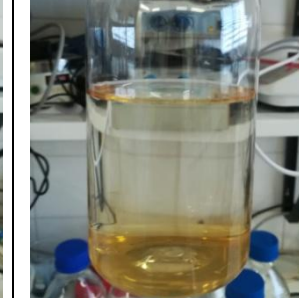
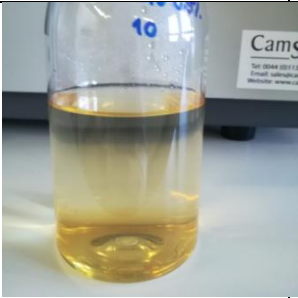

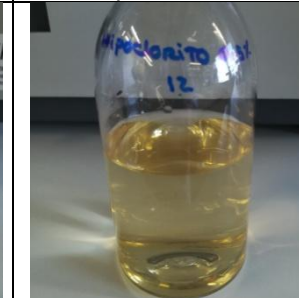




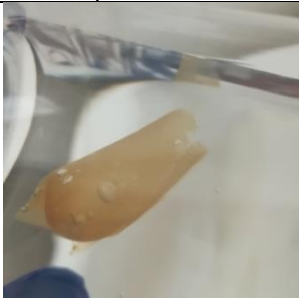


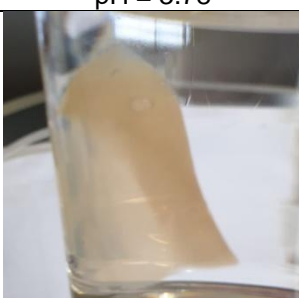
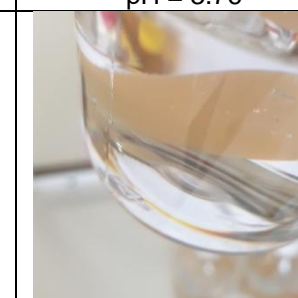

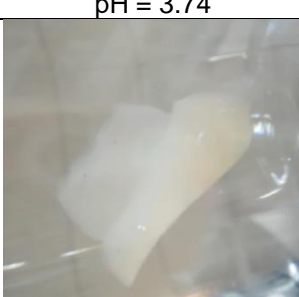
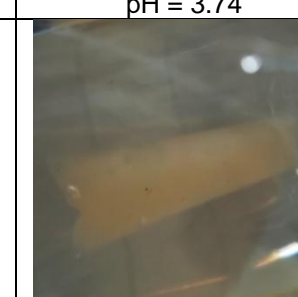
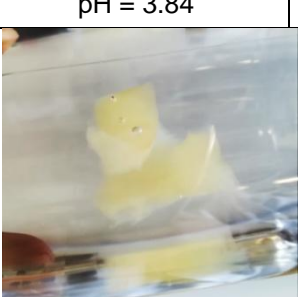
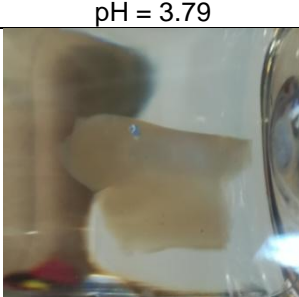
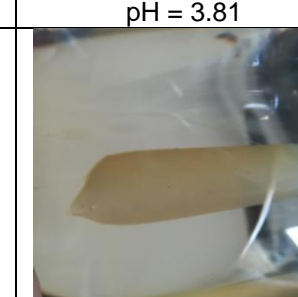
Structure nº	10	11	12
Mass (g)	4.1012	4.3288	3.3724
Length (cm)	4.0	3.8	3.9
Width (cm)	1.5	1.7	1.5
<b>Start</b> (21-01-21)	 pH = 11.48	 pH = 11.42	 pH = 11.48
<b>24 H</b>			
<b>1<sup>st</sup> week</b>	 pH = 11.32	 pH = 11.32	 pH = 11.38
<b>2<sup>nd</sup> week</b>	 pH = 11.12	 pH = 11.11	 pH = 11.19
<b>3<sup>rd</sup> week</b>	 pH = 10.97	 pH = 10.97	 pH = 10.98

Table A5 - FucoPol hydrogels stability in acetate buffer solution (pH=4.0)

Structure n°	13	14	15
Mass (g)	3.3742	2.9644	3.1720
Length (cm)	3.7	3.5	4.0
Width (cm)	1.5	1.5	1.3
<b>Start</b> (21-01-21)	 pH = 3.97	 pH = 3.99	 pH = 3.97
<b>24 H</b>	 pH = 3.74	 pH = 3.78	 pH = 3.76
<b>1<sup>st</sup> week</b>	 pH = 3.71	 pH = 3.74	 pH = 3.74
<b>2<sup>nd</sup> week</b>	 pH = 3.84	 pH = 3.79	 pH = 3.81
<b>3<sup>rd</sup> week</b>	 pH = 3.77	 pH = 3.72	 pH = 3.78



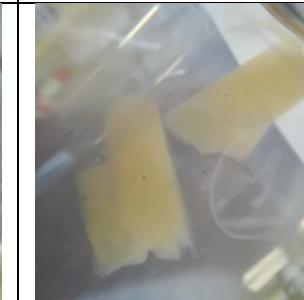
Structure n°	13	14	15
Mass (g)	3.3742	2.9644	3.1720
Length (cm)	3.7	3.5	4.0
Width (cm)	1.5	1.5	1.3
<u>4<sup>th</sup> week</u>	 <p>pH = 3.74</p>	 <p>pH = 3.69</p>	 <p>pH = 3.74</p>

Table A6 - FucoPol hydrogels stability in phosphate buffer solution (pH=5.5)





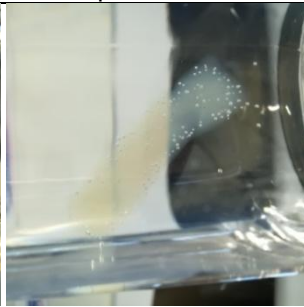
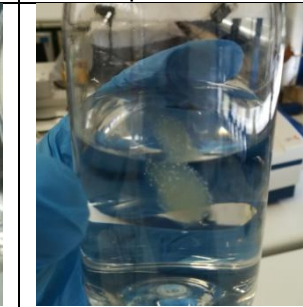
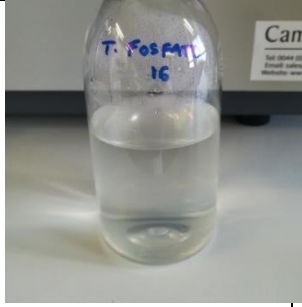
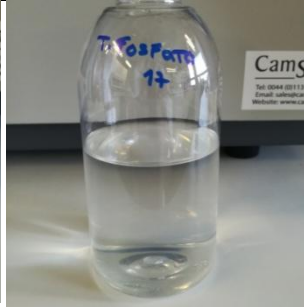
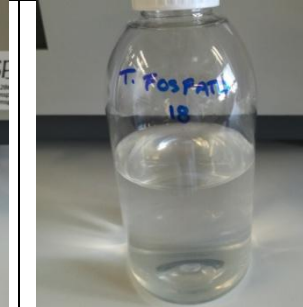
Structure n°	16	17	18
Mass (g)	4.5651	1.9458	4.2942
Length (cm)	3.9	3.6	3.8
Width (cm)	1.6	1.3	1.8
<b>Start</b> (21-01-21)	 pH = 5.50	 pH = 5.50	 pH = 5.51
<b>24 H</b>			
<b>1<sup>st</sup> week</b>	 pH = 4.81	 pH = 4.78	 pH = 4.88

Table A7 - FucoPol hydrogels stability in borate buffer solution (pH=8.5)





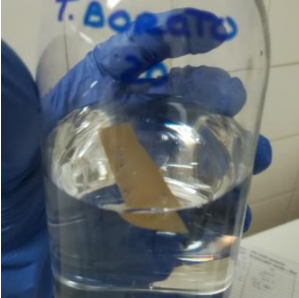


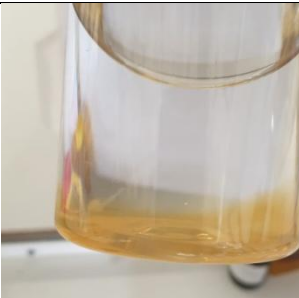




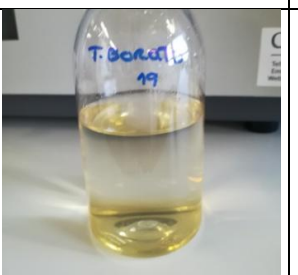
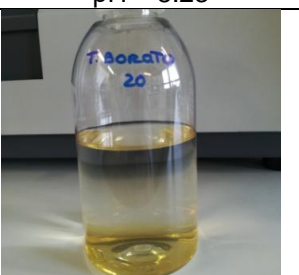
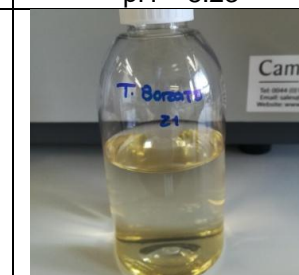






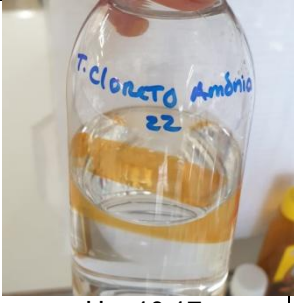


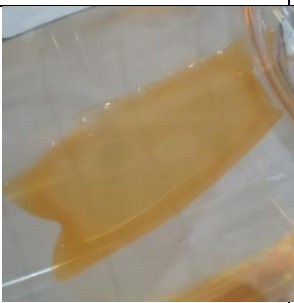

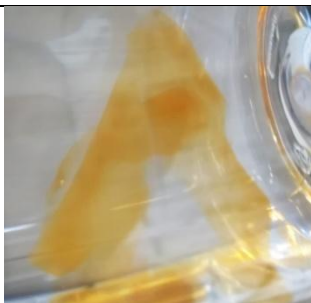
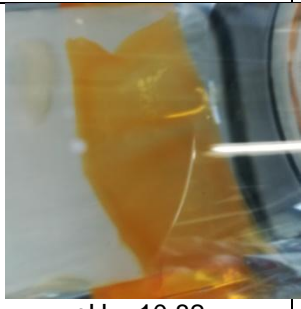















Structure n°	19	20	21
Mass (g)	3.3318	3.0071	2.0689
Length (cm)	4.1	3.9	3.7
Width (cm)	1.3	1.5	1.4
<b>Start</b> (21-01-21)	 pH = 8.48	 pH = 8.49	 pH = 8.49
<b>24 H</b>			
<b>1<sup>st</sup> week</b>	 pH = 8.07	 pH = 8.11	 pH = 8.13
<b>2<sup>nd</sup> week</b>	 pH = 8.19	 pH = 8.23	 pH = 8.23
<b>3<sup>rd</sup> week</b>	 pH = 8.11	 pH = 8.17	 pH = 8.21

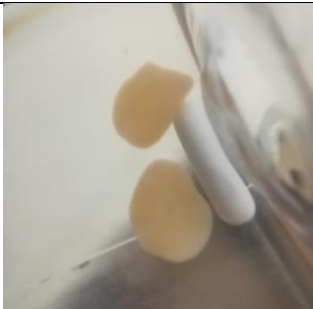



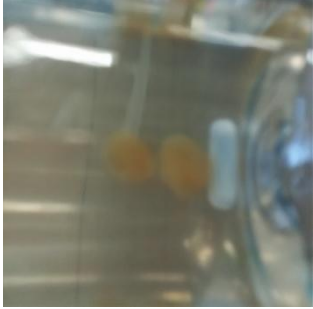
Table A8 - FucoPol hydrogels stability in ammonium chloride buffer solution (pH=10)



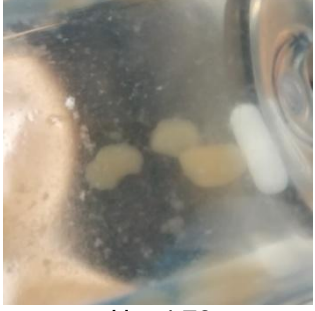
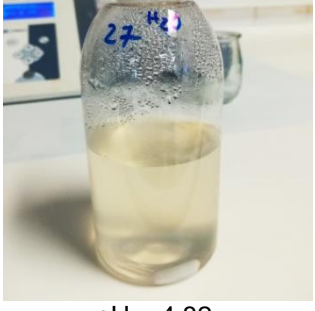
Structure nº	22	23	24
Mass (g)	4.8514	4,6099	2.5165
Length (cm)	3.9	4.0	3.5
Width (cm)	1.7	1.6	1.7
<b>Start</b> (21-01-21)	 pH = 10.22	 pH = 10.22	 pH = 10.22
<b>24 H</b>			
<b>1<sup>st</sup> week</b>	 pH = 10.17	 pH = 10.20	 pH = 10.15
<b>2<sup>nd</sup> week</b>	 pH = 10.39	 pH = 10.21	 pH = 10.29

Structure n°	22	23	24
Mass (g)	4.8514	4,6099	2.5165
Length (cm)	3.9	4.0	3.5
Width (cm)	1.7	1.6	1.7
<u>3<sup>rd</sup> week</u>	 <p>pH = 10.82</p>	 <p>pH = 10.76</p>	 <p>pH = 10.78</p>
<u>4<sup>th</sup> week</u>	 <p>pH = 10.97</p>	 <p>pH = 10.97</p>	 <p>pH = 10.98</p>





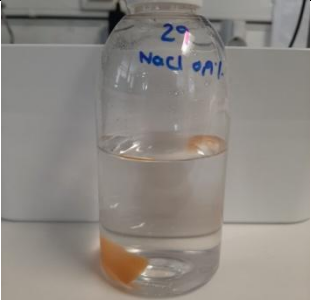




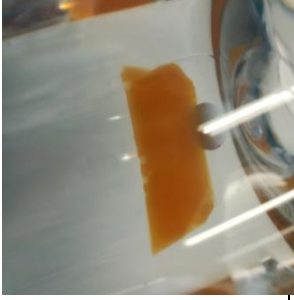


**Table A9 - FucoPol hydrogels stability in deionized water under constant stirring (200 rpm)**



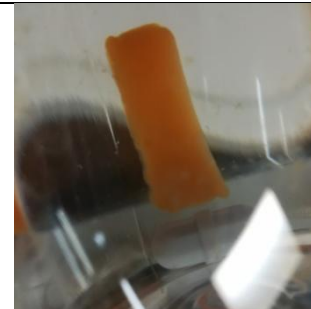



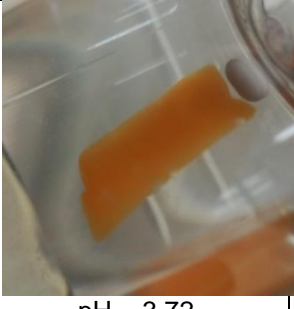

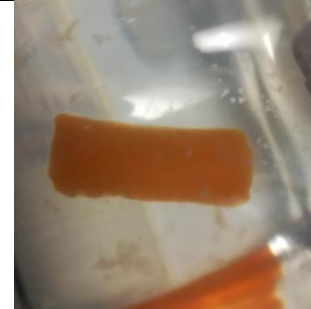
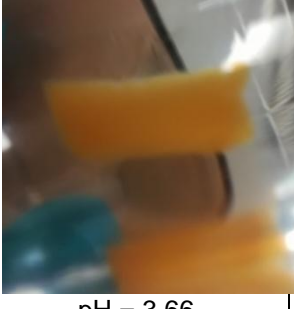


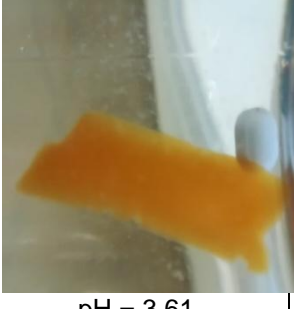


Structure n°	25	26	27
<b>Mass (g)</b>	3.2031	3.3224	2.1708
<b>Length (cm)</b>	4.0	4.1	3.5
<b>Width (cm)</b>	1.6	1.6	1.5
<b>Start</b> (18-02-21)	 pH = 5.28	 pH = 5.20	 pH = 5.28
<b>24 H</b>	 pH = 5.16	 pH = 9.23	 pH = 5.38
<b>1<sup>st</sup> week</b>	 pH = 4.70	 pH = 9.87	 pH = 5.39
<b>2<sup>nd</sup> week</b>	-	-	 pH = 4.69

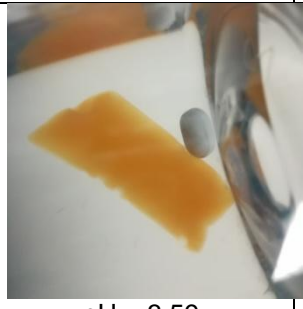

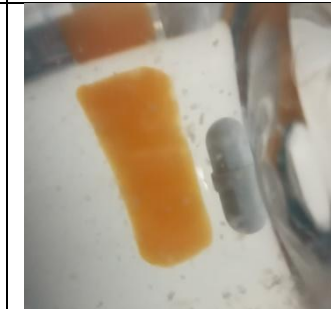


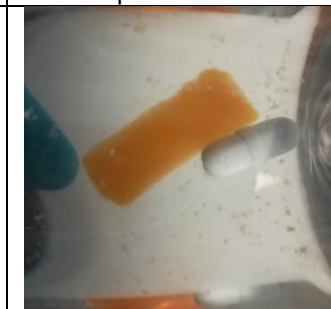
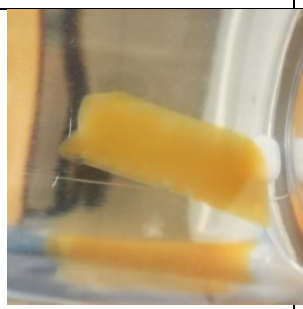




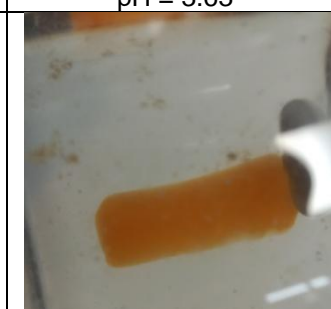


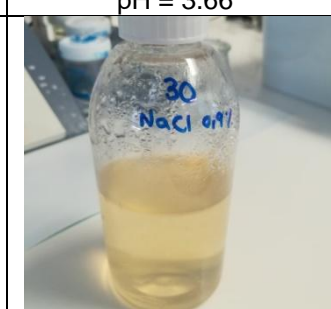
Structure n°	25	26	27
<b>Mass (g)</b>	3.2031	3.3224	2.1708
<b>Length (cm)</b>	4.0	4.1	3.5
<b>Width (cm)</b>	1.6	1.6	1.5
<b><u>3<sup>rd</sup> week</u></b>	-	-	 <p>pH = 4.83</p>
<b><u>4<sup>th</sup> week</u></b>	-	-	 <p>pH = 4.81</p>
<b><u>5<sup>th</sup> week</u></b>	-	-	 <p>pH = 4.86</p>
<b><u>6<sup>th</sup> week</u></b>	-	-	 <p>pH = 4.89</p>
<b><u>7<sup>th</sup> week</u></b>	-	-	 <p>pH = 4.80</p>

Structure n°	25	26	27
Mass (g)	3.2031	3.3224	2.1708
Length (cm)	4.0	4.1	3.5
Width (cm)	1.6	1.6	1.5
<u>8<sup>th</sup> week</u>	-	-	 <p>pH = 4.81</p>
<u>9<sup>th</sup> week</u>	-	-	 <p>pH = 4.82</p>
<u>10<sup>th</sup> week</u>	-	-	 <p>pH = 4.76</p>
<u>11<sup>th</sup> week</u>	-	-	 <p>pH = 4.82</p>

**Table A10 - FucoPol hydrogels stability in 0.9% (w/v) NaCl solution under constant stirring (200 rpm)**

Structure n°	28	29	30
Mass (g)	2.9490	3.3941	2.8486
Length (cm)	4.3	3.9	3.8
Width (cm)	1.4	1.6	1.6
<b>Start</b> (18-02-21)	 pH = 5.63	 pH = 5.63	 pH = 5.63
<b>24 H</b>	 pH = 5.32	 pH = 5.39	 pH = 5.33
<b>1<sup>st</sup> week</b>	 pH = 3.69	 pH = 3.65	 pH = 3.66
<b>2<sup>nd</sup> week</b>	 pH = 3.45	 pH = 3.45	 pH = 3.42

Structure n°	28	29	30
Mass (g)	2.9490	3.3941	2.8486
Length (cm)	4.3	3.9	3.8
Width (cm)	1.4	1.6	1.6
<u>3<sup>rd</sup> week</u>	 pH = 3.49	 pH = 3.45	 pH = 3.46
<u>4<sup>th</sup> week</u>	 pH = 3.58	 pH = 3.56	 pH = 3.55
<u>5<sup>th</sup> week</u>	 pH = 3.72	 pH = 3.66	 pH = 3.67
<u>6<sup>th</sup> week</u>	 pH = 3.66	 pH = 3.61	 pH = 3.62
<u>7<sup>th</sup> week</u>	 pH = 3.61	 pH = 3.54	 pH = 3.55

Structure n°	28	29	30
Mass (g)	2.9490	3.3941	2.8486
Length (cm)	4.3	3.9	3.8
Width (cm)	1.4	1.6	1.6
<u>8<sup>th</sup> week</u>	 pH = 3.59	 pH = 3.58	 pH = 3.57
<u>9<sup>th</sup> week</u>	 pH = 3.62	 pH = 3.59	 pH = 3.61
<u>10<sup>th</sup> week</u>	 pH = 3.64	 pH = 3.60	 pH = 3.63
<u>11<sup>th</sup> week</u>	 pH = 3.70	 pH = 3.67	 pH = 3.66
<u>12<sup>th</sup> week</u>	 pH = 3.85	 pH = 3.80	 pH = 3.83

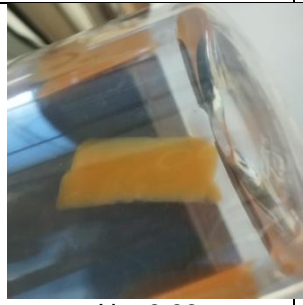





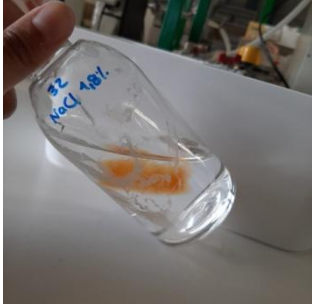
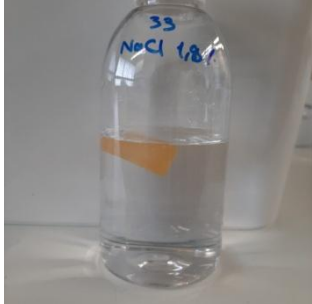



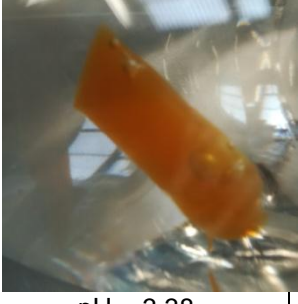


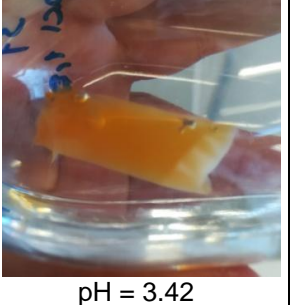
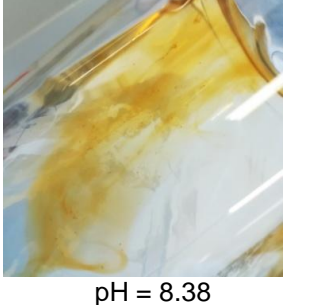
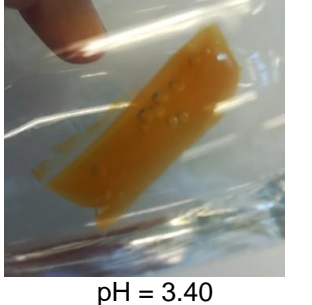





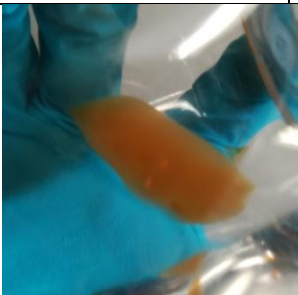
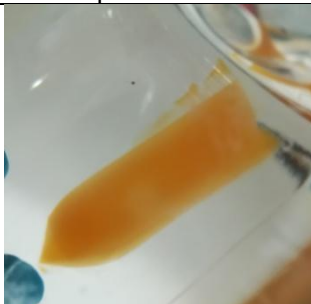


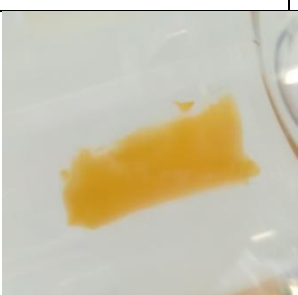
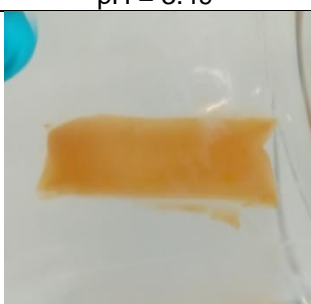
Structure n°	28	29	30
Mass (g)	2.9490	3.3941	2.8486
Length (cm)	4.3	3.9	3.8
Width (cm)	1.4	1.6	1.6
<u>13<sup>th</sup> week</u>	 <p>pH = 3.69</p>	 <p>pH = 3.67</p>	-

Table A11 - FucoPol hydrogels stability in 1.8% (w/v) NaCl solution

Structure n°	31	32	33
Mass (g)	2.7523	3.4182	3.1889
Length (cm)	3.5	4.0	4.2
Width (cm)	1.5	1.5	1.6
<b>Start</b> (18-02-21)	 pH = 5.54	 pH = 5.54	 pH = 5.54
<b>24 H</b>			
<b>1<sup>st</sup> week</b>	 pH = 3.60	 pH = 9.66	 pH = 3.66
<b>2<sup>nd</sup> week</b>	 pH = 3.38	 pH = 8.75	 pH = 3.41
<b>3<sup>rd</sup> week</b>	 pH = 3.42	 pH = 8.38	 pH = 3.40

Structure n°	31	32	33
Mass (g)	2.7523	3.4182	3.1889
Length (cm)	3.5	4.0	4.2
Width (cm)	1.5	1.5	1.6
<u>4<sup>th</sup> week</u>	 pH = 3.52	 pH = 8.86	 pH = 3.49
<u>5<sup>th</sup> week</u>	 pH = 3.63	-	 pH = 3.60
<u>6<sup>th</sup> week</u>	 pH = 3.58	-	 pH = 3.59
<u>7<sup>th</sup> week</u>	 pH = 3.51	-	 pH = 3.49
<u>8<sup>th</sup> week</u>	 pH = 3.57	-	 pH = 3.55


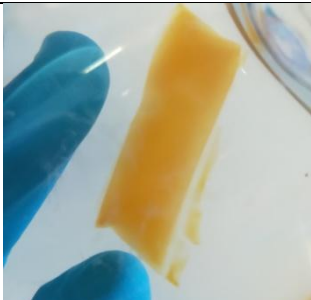

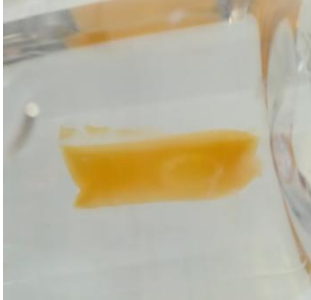




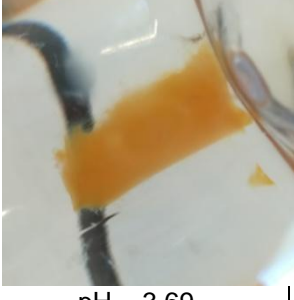




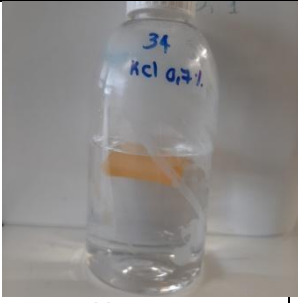
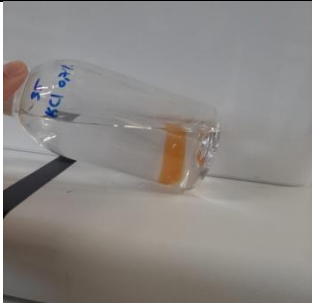






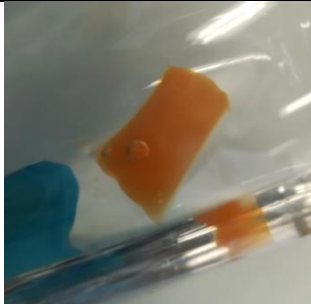
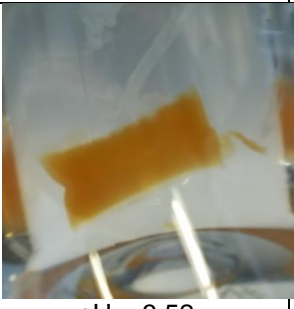
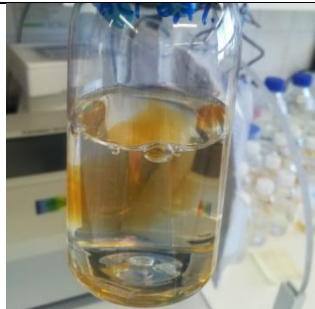
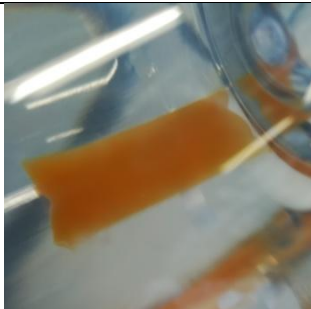
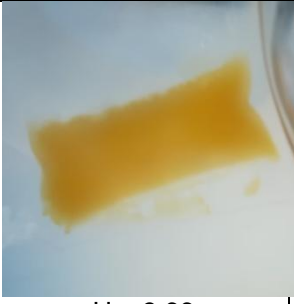
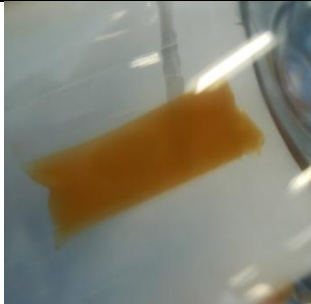

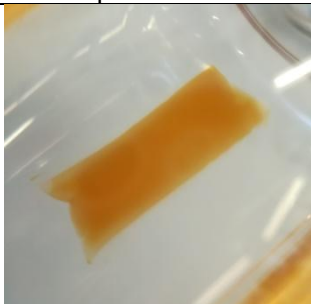
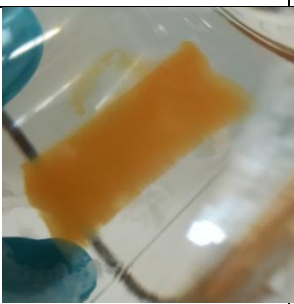
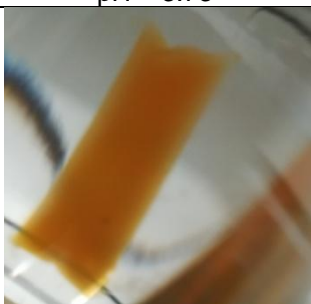








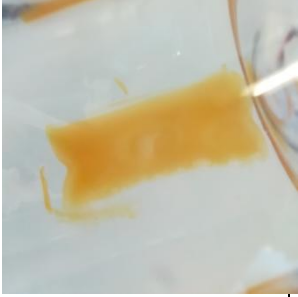

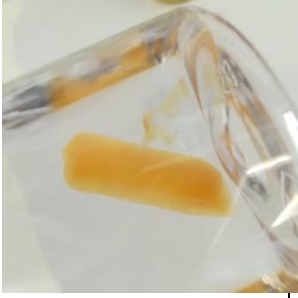

Structure n°	31	32	33
Mass (g)	2.7523	3.4182	3.1889
Length (cm)	3.5	4.0	4.2
Width (cm)	1.5	1.5	1.6
<u>9<sup>th</sup> week</u>	 pH = 3.55	-	 pH = 3.56
<u>10<sup>th</sup> week</u>	 pH = 3.61	-	 pH = 3.60
<u>11<sup>th</sup> week</u>	 pH = 3.69	-	 pH = 3.68
<u>12<sup>th</sup> week</u>	 pH = 3.67	-	 pH = 3.65
<u>13<sup>th</sup> week</u>	 pH = 3.69	-	 pH = 3.68

Table A12 - FucoPol hydrogels stability in 0.7% (w/v) KCl solution

Structure n°	34	35	36
Mass (g)	3.1721	2.9217	3.0823
Length (cm)	3.8	4.0	3.7
Width (cm)	1.6	1.4	1.3
<b>Start</b> (18-02-21)	 pH = 5.71	 pH = 5.71	 pH = 5.71
<b>24 H</b>	 pH = 5.03	 pH = 5,21	 pH = 5,18
<b>1<sup>st</sup> week</b>	 pH = 3.76	 pH = 9.78	 pH = 3.79
<b>2<sup>nd</sup> week</b>	 pH = 3.48	 pH = 9.03	 pH = 3.49

Structure n <sup>o</sup>	34	35	36
Mass (g)	3.1721	2.9217	3.0823
Length (cm)	3.8	4.0	3.7
Width (cm)	1.6	1.4	1.3
<u>3<sup>rd</sup> week</u>	 pH = 3.52	 pH = 9.14	 pH = 3.50
<u>4<sup>th</sup> week</u>	 pH = 3.66	-	 pH = 3.61
<u>5<sup>th</sup> week</u>	 pH = 3.78	-	 pH = 3.73
<u>6<sup>th</sup> week</u>	 pH = 3.76	-	 pH = 3.71
<u>7<sup>th</sup> week</u>	 pH = 3.69	-	 pH = 3.64

Structure n°	34	35	36
Mass (g)	3.1721	2.9217	3.0823
Length (cm)	3.8	4.0	3.7
Width (cm)	1.6	1.4	1.3
<u>8<sup>th</sup> week</u>	 pH = 3.75	-	 pH = 3.68
<u>9<sup>th</sup> week</u>	 pH = 3.77	-	 pH = 3.72
<u>10<sup>th</sup> week</u>	 pH = 3.78	-	 pH = 3.72
<u>11<sup>th</sup> week</u>	 pH = 3.90	-	 pH = 3.85
<u>12<sup>th</sup> week</u>	 pH = 3.85	-	 pH = 3.81

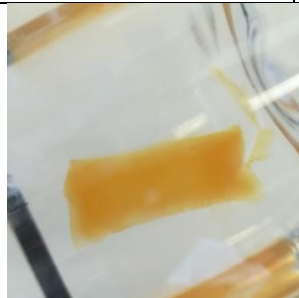




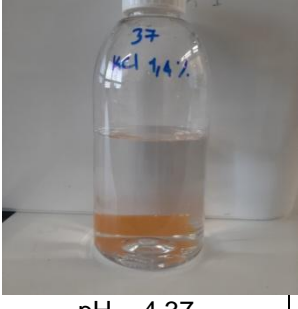
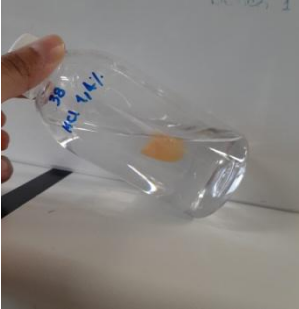
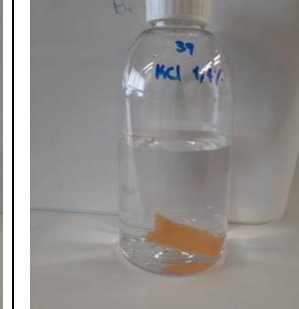



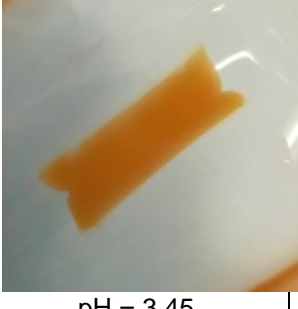


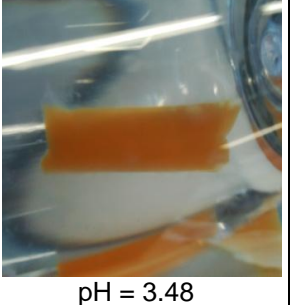
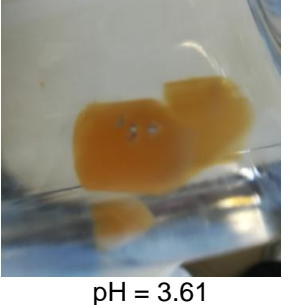
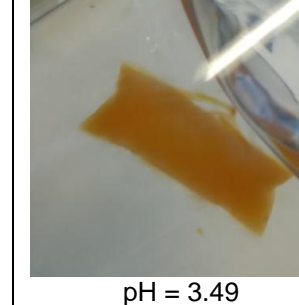
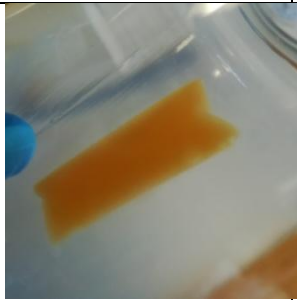





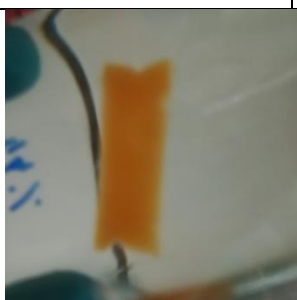



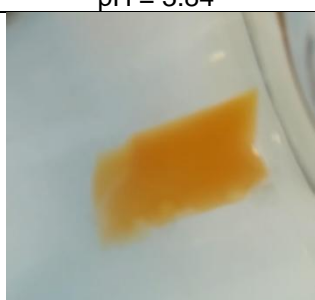



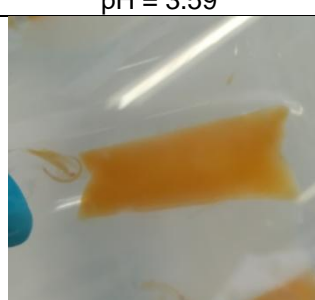
Structure n°	34	35	36
Mass (g)	3.1721	2.9217	3.0823
Length (cm)	3.8	4.0	3.7
Width (cm)	1.6	1.4	1.3
<u>13<sup>th</sup> week</u>	 <p>pH = 4.67</p>	-	 <p>pH = 4.78</p>

Table A13 - FucoPol hydrogels stability in 1.4% (w/v) KCl solution

Structure n <sup>o</sup>	37	38	39
Mass (g)	3.0303	2.4905	3.2481
Length (cm)	3.9	3.1	4.0
Width (cm)	1.4	1.2	1.5
<b>Start</b> (18-02-21)	 pH = 5.87	 pH = 5.87	 pH = 5.87
<b>24 H</b>	 pH = 4.37	 pH = 4.52	 pH = 4.39
<b>1<sup>st</sup> week</b>	 pH = 3.74	 pH = 3.89	 pH = 3.71
<b>2<sup>nd</sup> week</b>	 pH = 3.45	 pH = 3.58	 pH = 3.48
<b>3<sup>rd</sup> week</b>	 pH = 3.48	 pH = 3.61	 pH = 3.49

Structure n°	37	38	39
Mass (g)	3.0303	2.4905	3.2481
Length (cm)	3.9	3.1	4.0
Width (cm)	1.4	1.2	1.5
<u>4<sup>th</sup> week</u>	 pH = 3.59	 pH = 3.74	 pH = 3.59
<u>5<sup>th</sup> week</u>	 pH = 3.72	 pH = 3.87	 pH = 3.68
<u>6<sup>th</sup> week</u>	 pH = 3.69	 pH = 3.84	 pH = 3.66
<u>7<sup>th</sup> week</u>	 pH = 3.61	 pH = 3.76	 pH = 3.59
<u>8<sup>th</sup> week</u>	 pH = 3.64	 pH = 3.81	 pH = 3.63

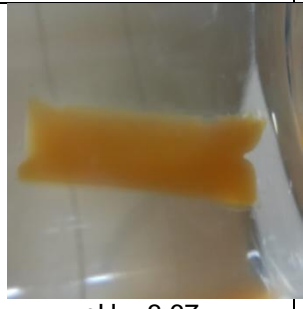

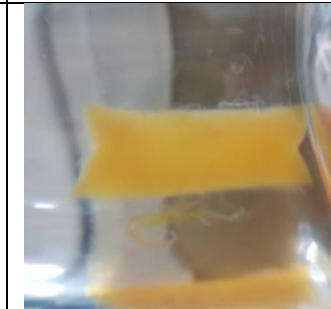
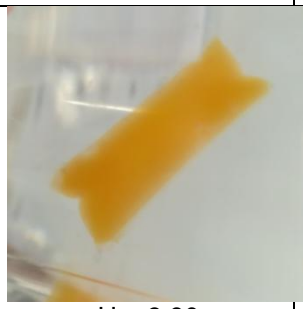
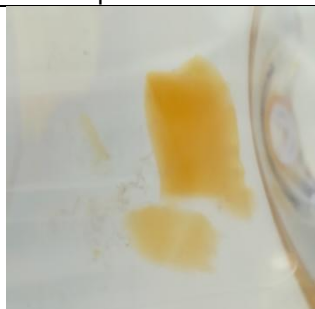



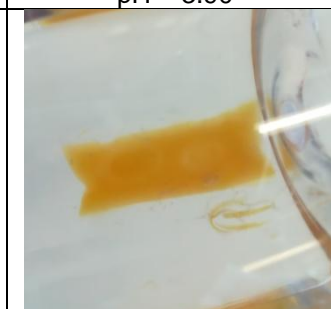


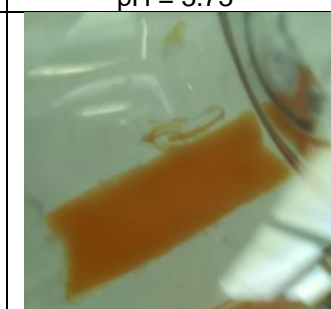

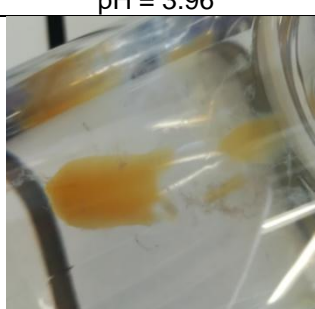
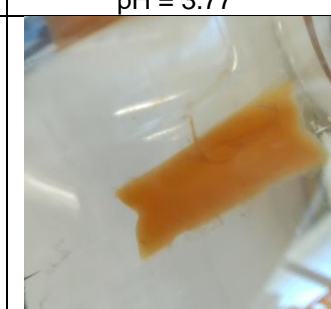
Structure n <sup>o</sup>	37	38	39
Mass (g)	3.0303	2.4905	3.2481
Length (cm)	3.9	3.1	4.0
Width (cm)	1.4	1.2	1.5
<u>9<sup>th</sup> week</u>	 pH = 3.67	 pH = 3.86	 pH = 3.67
<u>10<sup>th</sup> week</u>	 pH = 3.90	 pH = 4.11	 pH = 3.90
<u>11<sup>th</sup> week</u>	 pH = 3.75	 pH = 3.91	 pH = 3.73
<u>12<sup>th</sup> week</u>	 pH = 3.78	 pH = 3.96	 pH = 3.77
<u>13<sup>th</sup> week</u>	 pH = 3.76	 pH = 3.92	 pH = 3.75

Table A14 - FucoPol hydrogels stability in altered phosphate buffer solution ( $\text{NaH}_2\text{PO}_4$ )





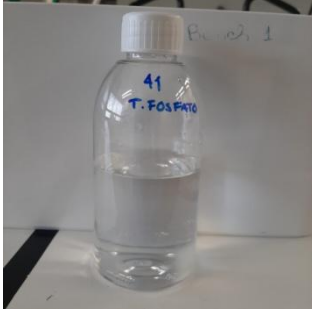
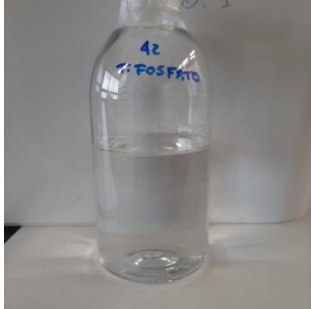
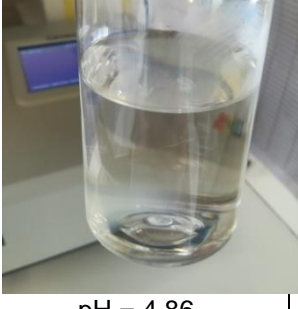
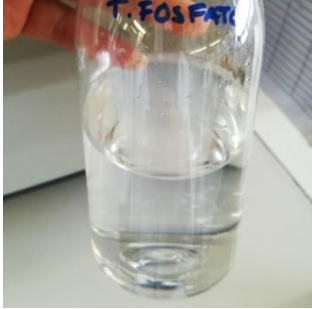



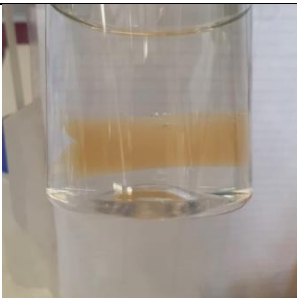
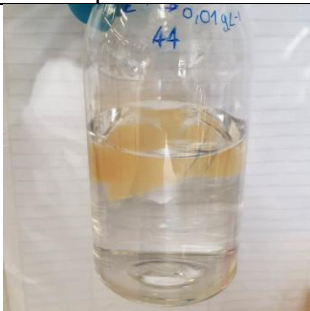
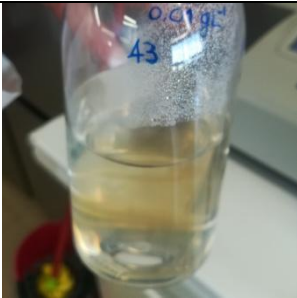


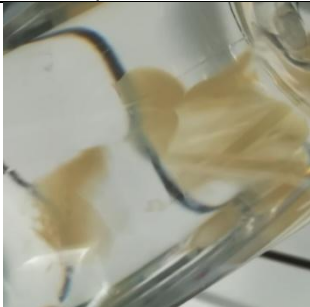

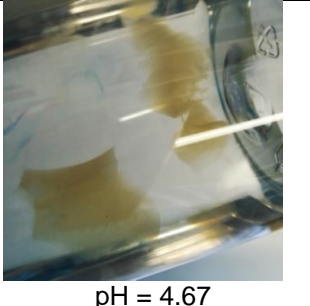
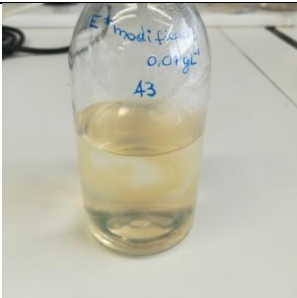
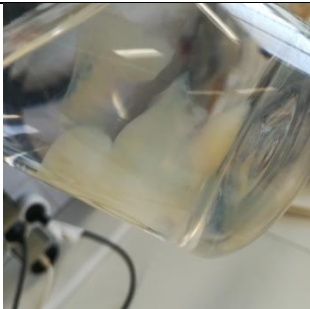
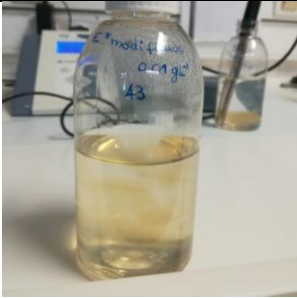

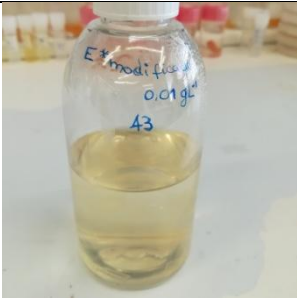



Structure nº	40	41	42
Mass (g)	3.3921	2.6118	3.1098
Length (cm)	4.1	3.8	3.8
Width (cm)	1.4	1.4	1.5
<b>Start</b> (18-02-21)	 pH = 5.42	 pH = 5.39	 pH = 5.39
<b>24 H</b>	 pH = 5.15	 pH = 5.14	 pH = 5.14
<b>1<sup>st</sup> week</b>	 pH = 4.86	 pH = 4.88	 pH = 4.87

Table A15 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.01 \text{ g L}^{-1}$ )

Structure n <sup>o</sup>	43	44
Mass (g)	3.3476	3.5261
Length (cm)	4.0	3.9
Width (cm)	1.4	1.6
<b>Start</b> (18-03-21)	 pH = 7.97	 pH = 7.79
<b>24 H</b>	 pH =	 pH =
<b>1<sup>st</sup> week</b>	 pH = 6.07	 pH = 5.22
<b>2<sup>nd</sup> week</b>	 pH = 6.37	 pH = 5.15
<b>3<sup>rd</sup> week</b>	 pH = 6.69	 pH = 4.67

Structure n°	43	44
Mass (g)	3.3476	3.5261
Length (cm)	4.0	3.9
Width (cm)	1.4	1.6
<u>4<sup>th</sup> week</u>	 <p>pH = 6.88</p>	 <p>pH = 4.91</p>
<u>5<sup>th</sup> week</u>	 <p>pH = 6.95</p>	 <p>pH = 4.96</p>
<u>6<sup>th</sup> week</u>	 <p>pH = 7.05</p>	 <p>pH = 4.88</p>
<u>7<sup>th</sup> week</u>	-	 <p>pH = 4.86</p>
<u>8<sup>th</sup> week</u>	-	 <p>pH = 4.62</p>

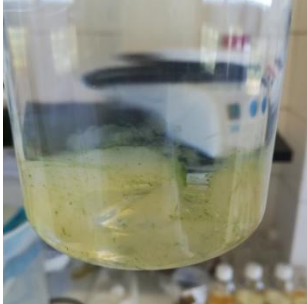
Structure n°	43	44
Mass (g)	3.3476	3.5261
Length (cm)	4.0	3.9
Width (cm)	1.4	1.6
<u>9<sup>th</sup> week</u>	-	 <p>pH = 4.48</p>

Table A16 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.82 \text{ g L}^{-1}$ )


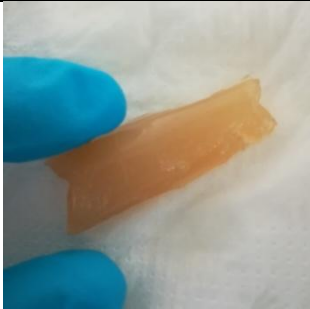
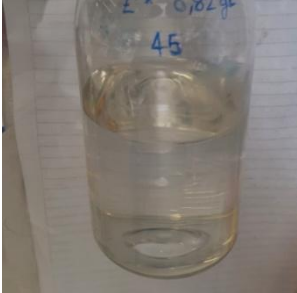
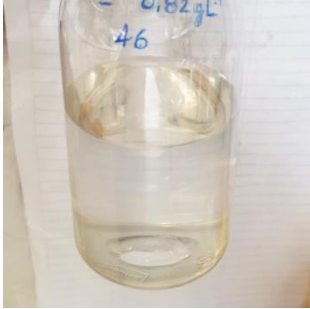


Structure n°	45	46
Mass (g)	2.7042	2.5681
Length (cm)	4.1	3.9
Width (cm)	1.4	1.1
<b>Start</b> (18-03-21)	 pH = 8.09	 pH = 8.11
<b>24 H</b>	 pH = 7.97	 pH = 8.03
<b>1<sup>st</sup> week</b>	 pH = 7.87	 pH = 7.93

Table A17 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $1.65 \text{ g L}^{-1}$ )



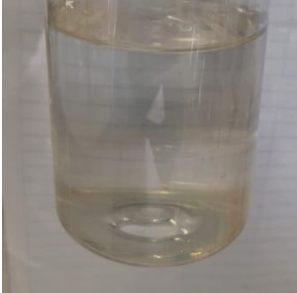
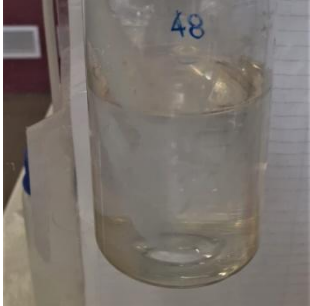
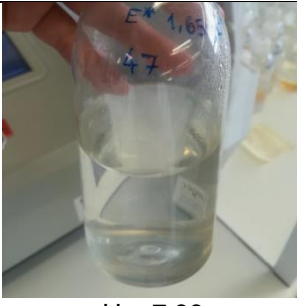
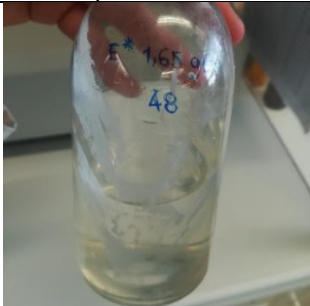
Structure n°	47	48
Mass (g)	3.8425	3.4955
Length (cm)	4.1	4.0
Width (cm)	1.3	1.7
<b>Start</b> (18-03-21)	 pH = 8.08	 pH = 8.10
<b>24 H</b>	 pH = 8.07	 pH = 8.06
<b>1<sup>st</sup> week</b>	 pH = 7.99	 pH = 8.01

Table A18 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.4 \text{ g L}^{-1}$ )

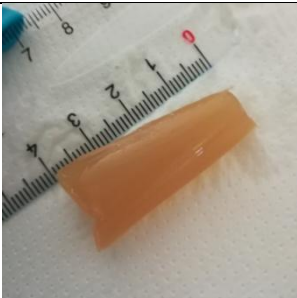

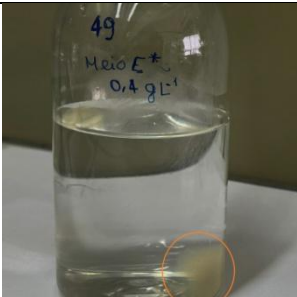
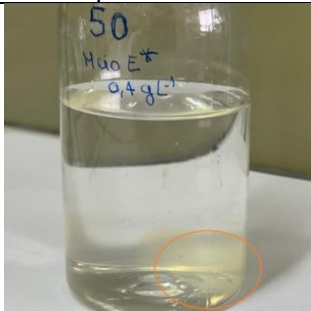
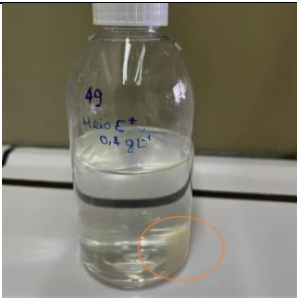
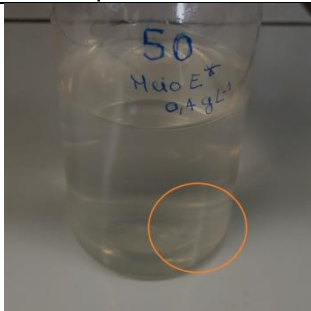
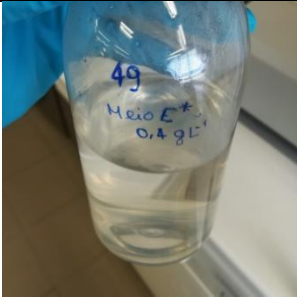
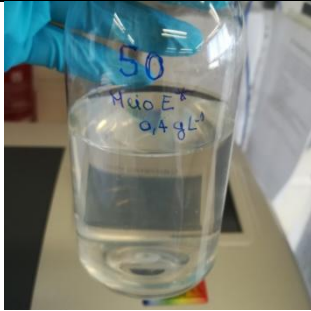
Structure n <sup>o</sup>	49	50
Mass (g)	3.5850	3.9366
Length (cm)	3.8	4.0
Width (cm)	1.6	1.7
<b>Start</b> (01-04-21)	 pH = 8.20	 pH = 8.16
<b>24 H</b>	 pH = 8.07	 pH = 7.99
<b>4<sup>th</sup> day</b>	 pH = 7.48	 pH = 7.46
<b>1<sup>st</sup> week</b>	 pH = 7.48	 pH = 7.46

Table A19 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.1 \text{ g L}^{-1}$ )


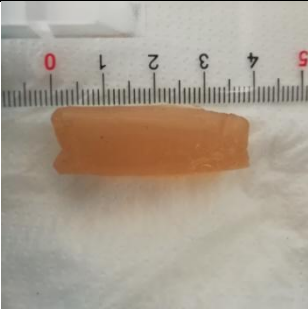
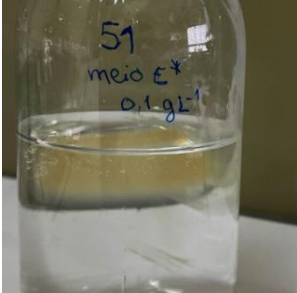

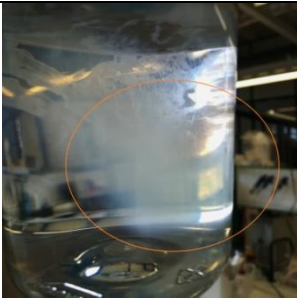

Structure n°	51	52
Mass (g)	3.0202	3.0440
Length (cm)	3.7	3.9
Width (cm)	1.4	1.2
<b>Start</b> (01-04-21)	 pH = 7.94	 pH = 7.94
<b>24 H</b>	 pH = 7.83	 pH = 7.58
<b>1<sup>st</sup> week</b>	 pH = 6.86	 pH = 6.44

Table A20 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.05 \text{ g L}^{-1}$ )







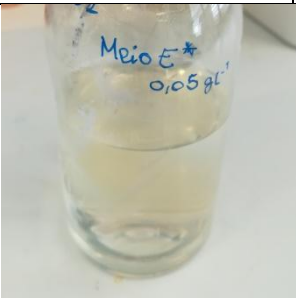
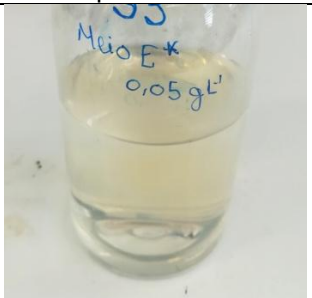

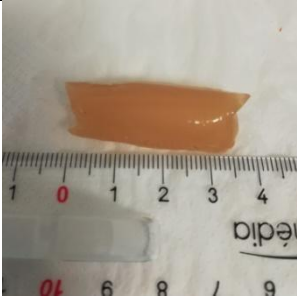



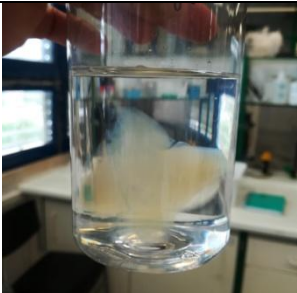



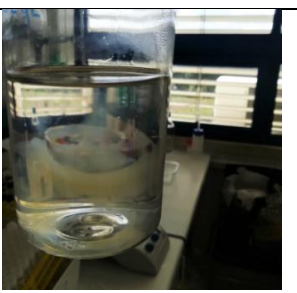
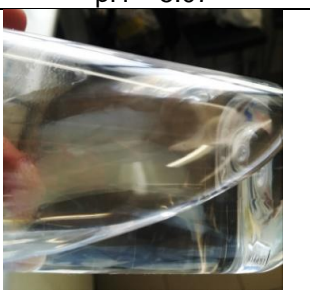


Structure n°	53	54
Mass (g)	3.2826	3.0808
Length (cm)	3.7	3.9
Width (cm)	1.7	1.6
<b>Start</b> (15-04-21)	 pH = 8.10	 pH = 8.09
<b>24 H</b>	 pH = 7.87	 pH = 7.43
<b>1<sup>st</sup> week</b>	 pH = 6.99	 pH = 6.94
<b>2<sup>nd</sup> week</b>	 pH = 6.41	 pH = 6.37
<b>3<sup>rd</sup> week</b>	-	 pH = 6.33

Table A21 - FucoPol hydrogels stability in altered Medium E\* ( $(\text{NH}_4)_2\text{HPO}_4$ ,  $0.02 \text{ g L}^{-1}$ )

Structure n°	55	56
Mass (g)	3.1631	3.2586
Length (cm)	3.6	3.8
Width (cm)	1.6	1.7
<b>Start</b> (15-04-21)	 pH = 7.79	 pH = 7.82
<b>24 H</b>	 pH = 7.12	 pH = 7.22
<b>1<sup>st</sup> week</b>	 pH = 6.11	 pH = 5.99
<b>2<sup>nd</sup> week</b>	 pH = 5.95	 pH = 5.07
<b>3<sup>rd</sup> week</b>	 pH = 6.09	 pH = 5.37

Structure n°	55	56
Mass (g)	3.1631	3.2586
Length (cm)	3.6	3.8
Width (cm)	1.6	1.7
<u>4<sup>th</sup> week</u>	 <p>pH = 6.05</p>	 <p>pH = 5.18</p>