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Sinergismos entre a indústria e a investigação científica ambicionando um desenvolvimento sustentável da Nanotecnologia

Synergies between industry and scientific research for a sustainable development of Nanotechnology

Universidade de Aveiro Departamento de Biologia

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Isabel Maria da Cunha Antunes Lopes (Investigadora Principal do CESAM e Departamento de Biologia da Universidade de Aveiro), Professor Doutor Filipe João Cotovio Eufrásio Antunes (Professor Auxiliar do Departamento de Química da Universidade de Coimbra) e do Doutor Luís Carlos Henriques Alves (Investigador Auxiliar do Departamento de Engenharia Química da Universidade de Coimbra).

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palavras-chave

Polímeros derivados de celulose, substituição catiónica e hidrofóbica, toxicidade aquática, distribuição de espécies sensíveis, design seguro, efeito de envelhecimento.

resumo

Os produtos de cuidados pessoais (PCP) são largamente utilizados nas rotinas diárias, incluindo produtos de limpeza facial, maquilhagem, champôs, amaciadores, produtos de beleza, entre outros. Na composição dos PCPs são usualmente incorporados compostos que podem representar perigos para o ambiente aquático, como por exemplo polímeros catiónicos. Os efluentes das estações de tratamento de águas residuais (ETAR) são considerados como fontes principais de PCP no ambiente aquático, uma vez que muitos destes compostos são resistentes aos tratamentos implementados nas ETAR. Uma vez libertados nos ecossistemas aquáticos, os PCPs podem causar vários efeitos adversos nos recetores ecológicos. Neste contexto e considerando a dependência da sociedade destes produtos, o desenvolvimento de produtos amigos do ambiente, ou seja, que não apresentem ou apresentem baixos riscos para a biota, mantendo a sua elevada eficiência funcional, começou a ser uma prioridade para várias indústrias. Isto pode ser conseguido através do desenho direcionado de partes específicas da estrutura dos compostos químicos. Um exemplo são os polímeros SoftCAT^{TM}, que são utilizados como espessantes pela indústria de PCPs em formulações de produtos de higiene. Estes polímeros conferem propriedades específicas a, por exemplo, champôs e condicionadores de cabelo que são essenciais para o seu condicionamento, interação, e desempenho de deposição no cabelo. Os polímeros SoftCAT^{TM} consistem num esqueleto de hidroxietilcelulose modificada com grupos quaternizados, em que as suas cargas catiónicas e substituições hidrofóbicas (HS) podem ser ajustadas. Estas modificações alteram a sua capacidade de formar soluções viscosas, o seu desempenho condicionante, e as suas propriedades antimicrobianas. No entanto, há pouca informação sobre como estas alterações na estrutura química destes polímeros podem influenciar a sua toxicidade no biota aquático. Apesar da sua ampla utilização em PCP, ainda existe pouco conhecimento acerca dos riscos que podem apresentar para os ecossistemas aquáticos, pelo que é pertinente compreender qual a variante (densidade de carga ou HS) é mais amiga do ambiente, mantendo a sua funcionalidade, de modo a orientar a indústria a investir no desenvolvimento de novas formulações utilizando essas variações mais amigas do ambiente. Neste contexto, o presente trabalho visou avaliar a influência de modificações no número de substituições hidrofóbicas e da densidade catiónica, na ecotoxicidade dos polímeros de hidroxietilcelulose quaternizada (SoftCAT^{TM}) no ambiente aquático, a fim de identificar a variante mais amiga do ambiente. Numa primeira fase, para compreender a influência da arquitetura do polímero no comportamento reológico dos polímeros de hidroxietilcelulose quaternizada, os polímeros SoftCAT^{M} com modificações na sua densidade de carga (variantes SK) e HS (variantes SL) foram caracterizados em termos de tamanho de partícula, potencial zeta e propriedades reológicas. A partir destes resultados, observou-se que o aumento do grau de substituição catiónica (variantes SK) deu origem a uma diminuição da viscosidade da solução. Por outro lado, o aumento do HS (variantes SL) resultou num aumento da viscosidade. Posteriormente, a caracterização ecotoxicológica das variantes SK e SL de polímeros SoftCAT^{TM} foi investigada através da realização de ensaios ecotoxicológicos utilizando organismos aquáticos de diferentes níveis tróficos

(bactérias, microalgas, rotíferos, cladocera, ostracodes e peixes). Para cada espécie foi determinada a concentração mediana efetiva que foi utilizada para derivar concentrações de perigo, através do método das curvas de distribuição de sensibilidades das espécies. Além disso, foram determinadas concentrações máximas aceitáveis no ambiente para cada variante dos polímeros estudados, a fim de identificar qual a arquitetura menos prejudicial. Relativamente aos polímeros com substituições catiónicas (SK), as espécies mais sensíveis a estes tipos de polímeros foram a microalga *Chlorella vulgaris* e o rotífero *Brachionus calyciflorus*. As variantes SK-M e SK-MH, apresentando níveis intermédios de substituição catiónica, revelaram-se como as menos tóxicas comparativamente às variantes com substituições catiónicas inferiores ou superiores. Entre estas duas variantes, a SK-M apresentou o valor mais baixo das concentrações máximas aceitáveis (0,00354 mg/L), sendo assim indicada como a variante SK mais amiga do ambiente. Quanto às variantes com substituição diferentes HS (SL), as duas microalgas *Raphidocelis subcapitata* e *C. vulgaris* e o rotífero *Brachionus calyciflorus* foram as espécies mais sensíveis. Foi também observado que a substituição hidrofóbica influenciou a toxicidade destes polímeros. A variante SL-5 (com menos HS) revelou ser a menos tóxica para as espécies testadas, com uma concentração máxima aceitável de 14,0 mg/L, sendo assim, considerada a variante SL mais amiga do ambiente. Finalmente, uma vez que se espera que estes contaminantes persistam no ambiente aquático durante algum tempo, a exposição dos polímeros SoftCAT™ após um processo de envelhecimento de um mês a três temperaturas diferentes (15, 20, e 25°C) foi também avaliada para as mesmas seis espécies-chave de cada nível trófico. Os resultados obtidos revelaram que as condições de temperatura do envelhecimento influenciaram de forma diferente a toxicidade das variantes SK e SL. Os processos de envelhecimento através dos quais as variantes SK e SL foram submetidas nas temperaturas mais elevadas causaram uma diminuição da sua toxicidade para a biota aquática. Contudo, de um modo geral a temperatura de 15°C revelou aumentar a toxicidade destes polímeros, comparativamente às outras temperaturas testadas e às variantes correspondentes não envelhecidas. Por exemplo, a maioria das variantes SL expostas a 15°C causaram a mortalidade da maioria dos organismos expostos das espécies *B. calyciflorus* e *Danio rerio*, ao contrário das variantes não envelhecidas. Das variantes testadas com diferentes densidades de carga, de um modo geral a SK-L revelou menor toxicidade após o processo de envelhecimento nas três temperaturas. No que respeita às variantes em que se variou a HS, a variante SL-30 foi a que revelou ser menos tóxica. Deste modo, a longo prazo estas variantes são consideradas como a melhor opção a incluir nas formulações de PCPs, pois constituem um menor risco para os ecossistemas dulçaquícolas tendo em consideração cenários de exposição ecologicamente relevantes e reais.

keywords

Hydroxyethyl cellulose polymers, cationic and hydrophobic substitution, aquatic toxicity, species sensitive distribution, safe by design, ageing effect.

abstract

Personal care products (PCPs) are highly used in daily routines since they comprise the formulations of facewashes, makeup cosmetics, shampoos, conditioners, beauty products, among others. The wastewater treatment plant (WWTP) effluents are considered as major sources of PCPs in the aquatic environment, since many of these compounds are resistant to the treatments implemented in WWTP. Once released in aquatic ecosystems PCPs may cause several adverse effects in the ecological receptors. In this context and considering the dependency of the society on these products, the development of environmentally friendly products, i.e., that exhibit no to low toxicity to the biota, while keeping their high functional efficiency, started to be a priority to several industries. This may be achieved by a directional tunning of specific parts of the structure of chemical compounds. One example is the SoftCAT^{TM} polymers that are used as thickeners by PCPs industry in formulations of hygiene products. These polymers confer specific properties to, for example, shampoos and hair conditioners that are essential for their conditioning, interaction, and deposition performance on the hair. SoftCAT^{TM} consist of a hydroxyethyl cellulose backbone modified with quaternized groups, that may be tuned for its cationic charge (SK variations) and hydrophobic substitutions (HS; SL variations). These modifications alter their capacity to form viscous solutions, their conditioning performance, and antimicrobial properties. However, there is poor information on how such changes in the chemical structure of these polymers may influence their toxicity to the aquatic biota. Though, given their wide use in PCPs, understanding which variant (cationic or HS) is more environmentally friendly, while maintaining its functionally, is of most relevance to guide industry to invest in the development of new formulations using those eco-friendly variations. In this context, the present work aimed at assessing the influence of HS and cationic density modifications in the ecotoxicity of quaternized hydroxyethyl cellulose polymers (SoftCAT^{TM}) on aquatic biota, as pristine and after ageing, to identify the most ecofriendly variation. In a first stage, to understand the influence of the polymer's architecture on the rheological behavior of quaternized hydroxyethyl cellulose polymers, SoftCAT^{TM} polymers with modifications on their charge density (SK variants) and HS (SL variants) were characterized in terms of particle size, zeta potential and rheological properties. From these results, it was observed that the increase of the degree of cationic substitution (SK variants) originated a decrease in the solution viscosity. On the other hand, the increase of HS (SL variants) resulted in an increase in viscosity. Afterwards, the ecotoxicological characterization of SK and SL variants of SoftCAT^{TM} polymers was investigated through a battery of assays using aquatic organisms from different trophic levels (bacteria, microalgae, rotifer, cladocera, ostracod, and fish). For each species the median effective concentration was computed and used to derive hazard concentrations, through the species sensitive distribution curves method.

Furthermore, maximum acceptable concentrations were estimated for each variant of the studied polymers to identify the most eco-friendlier. Concerning the polymers with cationic substitutions (SK), the most sensitive species to these types of polymers were the microalga *Chlorella vulgaris* and the rotifer *Brachionus calyciflorus*. The variants SK-M and SK-MH, presenting intermediate levels of cationic substitution, were revealed to be the least toxic ones comparatively to the variants with lower or higher cationic substitutions. Between these two variants, the SK-M presented the lowest value of the maximum acceptable concentrations (0.00354 mg/L), thus being indicated as the greenest and eco-friendlier SK variant. As for the variants with HS substitution (SL), the two microalgae *Raphidocelis subcapitata* and *C. vulgaris* and the rotifer *B. calyciflorus* were the most sensitive species. It was also observed that the HS influenced the toxicity of these polymers. The variant SL-5 (with less HS) was the least toxic to the species tested, with a maximum acceptable concentration of 14.0 mg/L, thus, being considered the most eco-friendlier SL polymer. Finally, since these contaminants are expected to persist in the aquatic environment for some time, the exposure of the SoftCAT™ polymers after an ageing process of one month at three different temperatures (15, 20, and 25°C) was also assessed to the same six key trophic level species. The obtained results revealed that temperature conditions of ageing influenced differently the toxicity of SK and SL variants. The ageing processes through which SK and SL variants went at the highest temperatures caused a decreased on their toxicity to aquatic biota. However, the temperature of 15°C revealed to increase the toxicity of these polymers, comparatively to the other tested temperatures and to the non-aged variants, for most tested species. For example, at this temperature they caused the mortality of all exposed organisms of *B. calyciflorus* and *Danio rerio* species, contrary to the non-aged variants. Of the variants tested with different charge densities, in general, SK-L showed less toxicity after the ageing process at the three temperatures. As for the variants of HS, the SL-30 variant was found to be the least toxic. Thus, in the long term these two variants are considered as a better option to include in PCP formulations as they represent a lower risk for freshwater ecosystems when considering more ecologically relevant and realistic exposure scenarios.

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Chapter I

General Introduction

1. Introduction

1.1 Personal care products

In the past few decades, consumer demand and ageing demographics have increased the main sources of contaminants of emerging concern (CEC) as, for example, pharmaceuticals and personal care products (PPCPs), plasticizers, endocrine-disrupting chemicals, flame-retardants, fuel additives and other industrial organic products (Al-Mashaqbeh et al., 2019). Besides the increase in the production and consumption of these products, it was also observed an increase of parent compounds, metabolites, degradation products in soil, sediment, and water matrices. PPCPs have been detected at concentrations ranging from ng/L to µg/L in all environmental compartments (water, air, biota, water, and wastewater) (Al-Mashaqbeh et al., 2019). These PPCPs are considered a group of chemical substances used every day but only a few years ago their presence in the environment has been confirmed in soil, sediment, and water compartments (Ortiz de García et al., 2014). Personal care products are highly demanded products in the market nowadays. They can enter the ecosystem through different ways namely the wastewater effluents from sewage treatment plants into the surrounding water bodies. They are used in daily routines as facewashes, beauty products, makeup cosmetics, etc. Despite the treatments processes present in wastewater treatment plants, the removal of PPCPs is usually incomplete (Heberer, 2002) and the presence of these compounds in the environment has been shown to result in adverse environmental and health risks for the exposed biota and humans. They can cause adverse effects to aquatic living organisms due to their potency to act biologically and through the disruption of the metabolism or even the normal functioning of the organisms but since they are not yet routinely monitored, their levels of contamination are still not known (Richmond et al., 2017). The potential environmental risks provoked by these pollutants must be investigated and not ignored.

The PPCPs possess large and complex molecular structures with some differences since these compounds contain functional groups as carboxyl, ketone, amines, and hydroxyl (Jin et al., 2020). The behavior of these compounds can also be very different, since the occurrence of small changes in their chemical structure may provoke significant effects on polarity and solubility and in other properties, defining their environmental distribution in sediments, water, soils, biota, plants, bacteria, fungi, and algae (Kümmerer, 2009). For example, synthetic musk is strongly used in the manufacture of fragrances, home cleaning products, soap scents, fabric softeners, detergents, shaving lotions, shampoo, and herbicides (Osemwengie and Gerstenberger, 2004). It was observed that this chemical can bio-concentrate and bio-accumulate in the soil and in the adipose tissue of aquatic organisms due to their chemical structure and physicochemical properties (Daughton and Ternes, 1999). The more hydrophobic is a contaminant, the more likely it bioaccumulates, increasing the inhibition of specific sets of enzymatic systems as, for example, the mixed-function oxidases present in the oxidative metabolism of lipid endogenous compounds (DelValls et al., 2007; Zhang et al., 2013).

Personal care products are used in daily routines as facewashes, beauty products, makeup cosmetics, shampoo, etc. They normally contain natural products which are further refined to be used and stored for a larger time. The main purpose of personal care products is cleaning and treat hair and skin, and they become a necessity for every skin since they are used to treat dryness by plasticizing and softening the hard, rough, tight, scaly of damaged skin (Wilmott et al., 2005). Even some care products, like shampoos, have also been produced for pet animals to keep them well-sanitized and for esthetic appearance. The shampoo for human hair has the property to build a protective chemical layer on the surface to avoid the hair top damage and spilt ends (Dias, 2015).

Personal care products, namely the shampoos, possess in their composition water-soluble cationic polymers (Gawade et al., 2020). These are formed by the reaction of a cationic base polymer with a molecule containing at least one functional group, such as the compound formed because of the polymerization of cationic vinyl polymer, acrylamide, and di-functional vinyl monomer, that can be reacted with the amino group present in the base polymer (US Patent Number 4,86,345). The product thus formed is used in personal care products, mainly in shampoos and hair conditioners. The composition of these products contains cationic vinyl addition polymer and water (Dias, 2015). The reactive substance with the amino group also acts as the thickening agent. The self-association property is also required in a personal care product. The most used personal care products that are gradually gaining more and more importance are shampoos and shower gels. These usually contain alkyl ethoxylated sulfate anionic surfactant, and amphoteric surfactants, selected from a wide group of surfactants including N-acyl-amino acid surfactant and cocoamidopropyl betaine (Cornwell, 2018). Additionally, can contain nonionic surfactants, such as fatty alcohol ethoxylated molecules and/or alkyl polyglycosides.

Despite all the above-mentioned applications and advantages of personal care products, there are many ways how they may adversely affect the environment. Personal care products may enter the aquatic and soil environment through many activities, actions and behavior of industries and individuals. These products belong to a sector of manufacturing that provides a variety of hygiene products for everyday consumption of the consumer (Vlachogianni et al., 2013) and they may be dump into the environment directly (e.g., sunscreen while people swim in the river, lakes, or sea) or indirectly (are found in municipal wastewater, as they are recalcitrant to the applied treatments), in marine litter and soil matrices, among others (Prasad et al., 2019).

There are chemicals used in these products that have been shown to cause damage to the environment. For instance, diethanolamine (DEA) is a product used in almost every personal care product, when it reaches the atmosphere reacts with the nitrogenous compounds, present in a large proportion, to form carcinogens (N-nitrosodiethanolamine, N-Nitroso dimethylamine, and N-nitrosobis (2-hydroxypropyl) amine) (NTP, 2016; SCCS, 2012). Also, compounds such as P-phenylenediamine, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), used in many personal care products for antioxidant purposes, are known to cause adverse effects to biota. The BHA and BHT were shown to cause a genetic deformation and permanent damage to the fish behavior (Lundebye et al., 2010). These results were observed in farmed

fish, namely, Atlantic salmon, Atlantic halibut (Hippoglossus hippoglossus), cod (Gadus morhua), and rainbow trout (Oncorhynchus mykiss). The highest levels of BHT (7.6 mg/kg) were found in salmon fillets. Triclosan is an antimicrobial agent widely used in personal care products. Lin et al. (2014) studied the effect of triclosan on earthworm *Eisenia fetida* and observed that that triclosan (concentration ranges from 50 to 300 mg kg^{-1}) induces oxidative stress and affects the activities of antioxidant enzymes (superoxide dismutase (SOD), malondialdehyde (MDA), aminopyrine N-demethylase (APND), catalase (CAT) and glutathione S-transferase (GST)). It also affects the cytochrome oxidases of the organism and may influence the expression of Hsp70 (Lin et al., 2014). In 2018, Sahu et al., observed that triclosan increased the activity of CAT, SOD and GST in gill and liver tissues of *Pangasianodon hypophhalmus* (Sahu et. al., 2018).

Cellulose, among different explored bio sorbents, is a handy sense unfathomable polymeric foul material with spellbinding structure and properties (Abdel-Halim, 2014). Cellulose is the main ingredient of the plant cell walls and is considered the most abundant organic, renewable, biodegradable and environmentally friendly polymer (Klemm et al., 1998, 2005; O'sullivan, 1997). It is composed by the repeated units of D-glucose building squares. Cellulose is a natural polymer composed of connected β-d-glucopyranose units through β-1,4-glycosidic linkages to originate a long-chain polysaccharide. The repeating unit of this polysaccharide is composed mainly of methoxy and hydroxyl groups and many hydroxyl groups on the outer surface of the polymer chain increases the hydrophilic nature of the cellulose polymer backbone (Godage and Gionfriddo, 2020).

This molecular structure gives to cellulose trademark properties of amphiphilicity, chirality, reactivity, and biodegradability (Peng et al. 2011; Habibi, 2014; Lindman et al., 2017; Lindman et al., 2021). Cellulose consists of a semi-crystalline polymer, containing several OH groups, which open many opportunities for chemical modification, turning this polymer into one of the most used biopolymers around the world. Also, its biocompatible character gives to this material an important role in many applications, such as personal care products. Hydrogels having cellulosic structures are biodegradable and biocompatible (Sannino et al., 2009). They are must-to-having for various present-day uses, for example, sterile materials, particularly in conditions where trademark problems are kept in view.

Nowadays, cellulose derivatives have been considered very useful in different applications as the pharmaceutical industry and industrialized products since they can influence the dimensional, thermal, mechanical stability, and functional features of composite materials and enhance the composite material due to its eco-friendly nature (Dai, 2019; Arfin, 2020). The hydrogels that are based on cellulosic compounds are extreme water absorbents and make three-dimensional lattices; the polymers SoftCAT SK and SoftCAT SL form cationic hydrogels that derivate from cellulose. These hydrogels can be obtained through physical or chemical interactions of stabilized aqueous solutions of cationic cellulose derivatives (Hasan and Abdel-Raouf, 2018). Chemical stimuli (pH, ionic factors, and chemical agents) can change the interactions between polymer chains or between polymer chains and solvent at the molecular level and change or destroy the gel network. Physical stimuli (temperature, mechanical stress and electric or magnetic fields) can influence the molecular interactions at critical onset points.

1.2 Cationic polymers

As mentioned above, polymers are commonly used in many personal care and cosmetics products. Silicones and natural, synthetic, and organic polymers are used in a great variety of personal care products as film-formers, thickeners, modifiers, emulsifiers, aesthetic enhancers, and protective barriers (Lochhead, 2007; Patil and Ferrito, 2013).

The personal care industry commonly uses polymers as thickeners in its formulations. The polymers have the capability to affect the rheological profile of the formulation, to influence the application of the product, water sensitivity of the formulation, and delivery of the active ingredients. Since the personal care products are prepared with water-based formulations and have a low viscosity, the addition of the polymers is used to thicken and, in many cases, jellify the formulation. The polymers normally used to increase the viscosity of these water-based systems are natural polymers, such as starch, polysaccharides, guar gum, xanthan gum, alginates, gelatin, agar, pectin's, among others (Gawade, 2020). Often, natural polymers such as cellulose derivatives are modified to be included in personal care products (cellulose derivatives such as hydroxyethyl cellulose, methylcellulose, hydroxypropyl cellulose). Normally, these are amphiphilic polymers, soluble in water that undergo some alterations through the addition of hydrophobic groups to their structure (Arfin, 2020).

The shampoo and hair conditioning products are composed of formulations containing cationic polymers, e.g., cationic cellulose derivatives and anionic surfactants (Hössel, et al., 2000). Since the hair is negatively charged, the cationic polymers show affinity towards negatively charged keratin. On the other hand, they can form complexes with anionic surfactants, during the rising step of the hair, and deposited on the hair surface, giving a smooth touch to the hair (Piculell and Lindman, 1992; Kronberg et al., 2014; Kronberg et al., 2014). Cationic polymers, such as the Soft CAT^{TM} have some properties, as high molecular weight, ethylene oxide substitution, charge density, and hydrophobicity that are determinant in the conditioning, deposition performance, and interaction with hair.

The polyquaternium polymers are cationic polymers with quaternary ammonium functional groups, which are known to be effective conditioning polymers in shampoos (Hössel et al., 2000). The polymers based on quaternary ammonium salts of hydroxyethyl cellulose are cations since they possess positive charges at the nitrogen atoms and chloride counterions. The polymers studied in this work are also cationic polymers, used primarily in hair and skin cleansing formulations to condition hair and skin. They have also the capability to deposit beneficial ingredients onto hair and skin, being used as film-formers and moisturizers.

The Soft CAT™ polymers (INCI: polyquaternium-67) are cationic quaternized hydroxyethyl cellulose (HEC) polymers, which form highly viscous solutions, with cationic substitution of the trimethylammonium and dimethyl dodecyl ammonium (Ballarin et al., 2011). The substituted ammonium salts are classified according to the substitution degree of the nitrogen atoms (number of hydrogen atoms replaced by an organic group), being the ones used to synthesize the Soft CAT series quaternary ammonium compounds, three hydrogens have been replaced by organic groups. The polymers Soft CAT™ SL and Soft CAT™ SK belong

to the newest generation of cationic polymers used in formulations for hair care and facial cleansing lotions. They include four hydroxyethyl cellulose polymers (HEC) quaternized, with low levels of hydrophobic substitution, and can modify the rheology of the formulations, due to their great ability to increase the viscosity of the solutions. These polymers are highly soluble in water and tend to remain in water due to their high solubility and the capacity to bounding to oppositely charged particles in the environment (Ballarin et al., 2011).

The Soft CAT™ SL polymers can provide superior performance to conventional conditioners due to their hydrophobic substitution. In these polymers, the degree of cationic substitution is approximately set to 0.2 (mol/mol of AGU), possess a fixed ethylene oxide (EO) group and the difference between the variants relies on the incorporation of different levels of substitution of hydrophobic dodecyl-dimethyl-ammonium, originating four types of variants: SL-5, SL-30, SL-60, and SL-100. The number in this nomenclature indicates the degree of hydrophobic substitution. For SoftCAT SL-5 the degree of substitution is 5×10^{-4}, for SL-30 is 5×10^{-3}, for SL-60 is 7×10^{-3} and for SL-100 is 1×10^{-2} (Drovetskay et al., 2005; Company, 2008; Milcovich et al., 2016).

The Soft CAT™ SK polymers are more used in shower gels formulations, improving skin hydration and reducing the dryness of the skin. These polymers possess a variable cationic substitution of trimethyl ammonium and dimethyl-dodecyl-ammonium (0.2 to 0.3 M), presenting the same low degree of hydrophobic substitution of dimethyl-dodecyl-ammonium (Ballarin et al. 2011). The first letter after the SK nomenclature refers to the degree of cationic substitution. They are available in four variants, SK-H, SL-L, SK-M, and SK-MH, possessing this last one six times more hydrophobic substitution relatively to the SK-M variant.

Soft CAT™ polymers also present antimicrobial properties, since they are cationic polymers and consist of two functional components: the cationic charge and hydrophobic groups (Yang et al., 2017). These polymers have the capability of being first absorbed onto the membrane of pathogenic microbes with the aid of their cationic groups and the hydrophobic groups mainly insert into the membrane, leading to the death of pathogenic microbes through the cytoplasm leakage (Ganewatta and Tang, 2015). The antimicrobial cationic polymers with quaternary ammonium groups have intrinsic charges and the pH dependence is negligible.

1.3 Wastewater discharges and treatment

Water is essential for all life forms, but its quality is deteriorating fast mainly due to chemical pollution and the destruction of freshwater ecosystems associated with industrial activity and an increase in living standards. The continuous exponential growth of the human population, associated with high levels of industrialization, highly urbanized societies, warfare combined with the increased wealth and more luxurious lifestyles, culminates in the production of huge amounts of residuals that are discarded into the water system (UN-Water, 2015). Since billions of gallons of wastewater and sewage are produced every day, one of the biggest challenges for society is the proper management and safe disposal of this contaminated water and

solid wastes. The wastewater composition varies according to the surrounding activities, but most wastewater possesses inorganic, organic, solid wastes, toxins, and biological contaminants. Industrial wastewater is one of the major sources of continuous input of contaminants into the aquatic ecosystems, for this reason, it is important to develop efficient treatments to remove contaminants (biological, inorganic, and organic) from wastewater before they are discharged into the aquatic systems (Ado et al., 2015). As said before, wastewater can contain diverse types of contaminants, such as phenolic compounds, pharmaceuticals, pesticides, surfactants, dyes, metals, organic solvents, polymers, and microorganisms (Ujang and Henze, 2006). Domestic and industrial wastes carry a range of naturally occurring and xenobiotics organic compounds (Lindqvist et al., 2005), and lipids, proteins, bacterial cells, and carbohydrates (Gray, 2005). Various several different processes that can be used for the treatment of wastewater, according to the nature and extent of contamination (Templeton and Butler, 2011). Wastewater consists of more than 95% of pure water with less than 5% of impurities. Despite the extensive treatment processes that wastewater goes through, some of these compounds are recalcitrant and remain in the effluent that is released into the aquatic systems, where they can promote the deterioration of ecosystems by both promoting changes in abiotic (e.g., increasing total dissolved solids and chemical oxygen demand) and biotic parameters (e.g., changing communities' structure) (Ado et al., 2015; Weber et al., 2010). The wastewater treatment plants use sequential processes for the removal or conversion of the harmful compounds present in wastewater. These processes include the following stages: preliminary treatment, primary treatment, secondary treatment, tertiary treatment, and solids treatment (Tempelton and Butler, 2011). The insurance of a clean environment is a global concern faced by several countries due to the increase of environmental contamination every day.

In many countries, the European Union regulations require a biological and chemical analysis of the effluents before discharging the water to sewage treatment plants or the environmental compartments. In the European Union, the Water Framework Directive (2000) is to maintain and improve the quality of the aquatic environment, being mostly concerned with the protection of receiving waters from polluted sources. The chemicals constituting the wastewater may impair the sewage treatment, as they can also have an inhibitory effect on biodegradation, which may result in a longer treatment time or a reduction of the efficiency of wastewater treatment. Furthermore, the Annex 1 of the EU Urban Waste Water Treatment Directive (1991) states that "Industrial wastewater entering collecting systems and urban wastewater treatment plants shall be subject to such pre-treatment as is required, to ensure that the operation of the wastewater treatment plant and the treatment of sludge are not impeded, ensure that discharges from the treatment plants do not adversely affect the environment, or to prevent receiving water from sources not complying with other Community Directives, ensure that sludge can be safely disposed in an environmentally acceptable manner".

The elimination process of these compounds passes through several stages such as sorption, volatilization, photo-transformation, and biodegradation. Biodegradation is probably the most effective in the removal of these compounds (Zhang et al., 2008). The degradation pathways of PPCPs in the environment

are still very poorly known as also about their intermediates of degradation (Ia Farré et al., 2008). The biodegradability of a compound may determine its hazards to the environment either discharged to recipient surface water or treated in a wastewater treatment plant (Tisler et al., 1999). The guideline for ready biodegradability (OECD, 1992) describes some tests that enable to predict if a chemical has potential to be easily biodegraded in the environment. However, these tests do not include the behavior of compounds present in the wastewater in the receiving aquatic ecosystems, and they do not give an indication about the toxicity change of the chemical due to the formation of intermediate products.

There are several factors that can affect the biodegradation of a compound in the environment, for example, the exposure and the composition of microbial communities and the concentration of the chemical (Joutey et al., 2013). For this reason, the incubation time must be long enough to simulate the real degradation conditions. Some authors defined a minimum incubation time of eight weeks (Shelton and Tiedge, 1984) and others consider this incubation time is not enough and reported that some chemicals require an incubation time of 100 days (Strevett et al., 2002) for the anaerobic degradation of compounds in sewage treatment plants. During this process, some biodegradable substances can be modified over time giving rise to breakdown products that may exhibit higher toxicity to the biota, changing the concentration in which they are present in the environmental matrices.

1.4 Sewage treatment processes

The sewage treatment process, commonly designated by wastewater treatment, is the process involving the treatment of wastewater from homes and trade facilities composed of nutrient-rich organic materials (Abdel-Raouf et al., 2019). Domestic wastewater is composed of organic and inorganic matter resultant from cleaning the clothes and cookware, food preparation and human waste.

The wastewater treatment techniques are constantly changing to be more effective to accomplish the removal of the chemicals being produced in a world that is in continuous change due to the increase of industrial development and subsequent development of new compounds and as well to meet the more stringent regulations. Also, the adequate treatment must be changed according to the local conditions, the source of wastewater and the maximum allowed contaminant level standards. Some studies have reported that inorganic contaminants from wastes applied to agriculture can accumulate in the soils at toxic levels, being contaminated with those contaminants (Karanja et al., 2010).

The first unit process in any treatment plant is the preliminary treatment unit that consists of the removal of wastewater constituents (Tchobanoglous et al., 2004). During this treatment occurs the screening and removal of solids, removal of grit and sand, and finally the flow measurement.

This treatment step is followed by the primary treatment that is mainly a physical removal process. In this treatment, occurs the removal of suspended and floating material. This primary treatment allows the separation of the solid and liquid phase fraction in the wastewater, reducing the suspended solids content of the influent wastewater (Boyd and Mbelu, 2009). In this step, most organic and suspended solids separate

from the liquid and settle at the bottom of the settling tank are transferred to the sludge digester tank.

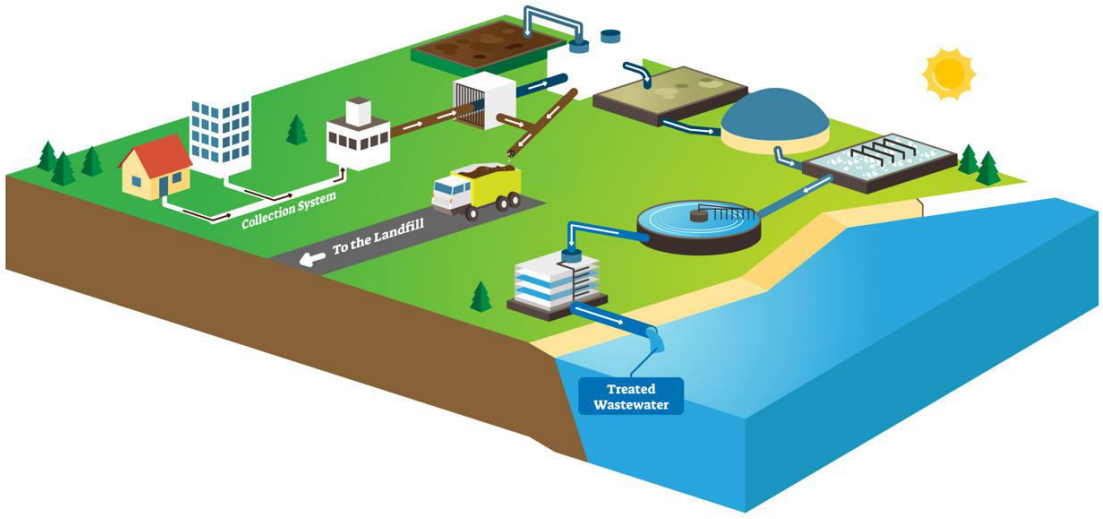

Figure 1: Sewage treatment plant.

The next step in the treatment plant is the secondary treatment that occurs predominantly by biological means to remove dissolved and suspended organic compounds. In wastewater treatment plants with secondary water treatment techniques, it is possible to remove organic and inorganic pollutants through flotation, coagulation, flocculation and membrane filtration and adsorption (Spellman, 2013). These wastewater treatments also include the pH adjusters that are used to remove the acidity from water and increases its pH to neutral values (pH 7). Some of these pH adjusters are chemicals compounds such as CaO, $Mg(OH)_2$, $Ca(OH)_2$, among others.

The next step is the formation of agglomerates with lower density through the attachment of the suspended particles to the dissolved air or gas. This method causes the flock to rise to the surface of the suspension, enabling their recovery through a skimmer (Gupta and Tripathi, 2011). This method is designated by flotation. Then, coagulation takes place, which also adjusts the pH of the effluent. In wastewater treatment, coagulation is the conversion of harmful chemicals present in the contaminated water into insoluble particles through coagulation molecules as ferric chloride ($FeCl_3$) and aluminum sulfate ($Al_2(SO_4)_3$), etc. The next step, flocculation, occur in a flocculation tank that slowly rotates a shaft in the water to combine the coagulation chemicals with the suspended materials. Further, the water is filtered through membrane processes such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis. This filtration processes enable the removal of metals from inorganic effluents, organic compounds, and suspended solids with a significant reduction of the costs of installation and operation (Fu and Wang, 2011). The final step of the treatment of wastewater is adsorption. This method has increased the research on possible adsorbents with a low cost to benefit all the processes since it has several steps and can turn to be very expensive (Barakat, 2011). The adsorption process through solid absorbents has been shown to be one of the most efficient

methods for the treatment and removal of organic contaminants in wastewater treatment plants. It is a simple design and involves a low investment, initial cost and land required. It is widely used for the treatment of industrial wastewater from organic and inorganic contaminants, and it has increased the attention of several researchers due to the search for low-cost adsorbents.

The adsorbents can be of several types, such as natural and synthetic adsorbents. The natural ones include clays, clay minerals, charcoal, ores, and zeolites (Rashed, 2013). These are considered a good option since they are relatively cheap, abundant in supply chains and possess a significant potential for modification, enhancing their adsorption capabilities. On the other hand, synthetic adsorbents are the ones prepared from agricultural products and wastes (household wastes, industrial wastes, sewage sludge) and polymeric adsorbents. Each one of these shas its specific characteristics (porosity, pore structure and adsorbing surfaces). The activated carbons are also used as adsorbents for organic pollutants.

This is a complex process due to the large number of variables involved as electrostatic, chemical, and dispersive interactions, intrinsic properties of the solute (solubility and ionization constant) and the adsorbent (pore size, distribution), solution properties (namely, pH) and temperature of the system. These compounds are strongly used for the removal of undesirable odor, taste, color, and other organic and inorganic impurities from domestic and industrial wastewater.

Finally, after secondary treatment, occurs the chemical treatment that consists in the tertiary treatment. This step provides a final treatment to improve the effluent quality before being discharged to the receiving environment and can include nutrient removal processes, and disinfection. Ideally, water destined for human consumption should be free from microorganisms (Gray, 2005). Biological effluents from domestic wastewater treatment plants must be disinfected before reuse since they still contain microorganisms of intestinal origin (Liberti et al., 2000). These disinfection techniques can include chlorination, UV irradiation and ozonation (Ahuja, 2009). After all these processes, the treated water that is now discharged to a water body (lake, river, stream, or groundwater).

Personal care products like shampoos, toothpaste and body washes are washed down the drain after their application. After entering the wastewater system, these chemicals have multiple routes to enter the environment. These wastewater treatment plants have processes to increase the breakdown of many chemicals and microbes in the water (Baird and Cann, 2012) where many of the volatile chemicals are released, entering as air pollutants in the environment. On the other hand, the non-volatile chemicals left in the wastewater can undergo many transformation steps. They can also be biologically or chemically breakdown with the addition of microbes and chemicals to the water. Each wastewater treatment plants do this process differently, so each step is unique to each one.

The chemical, physical and biological processes applied in wastewater treatment plants and the abiotic and biotic characteristics of the environmental compartments can induce several structural changes in a substance, resulting in a complete or partial transformation of the original compound, namely in the physicochemical properties, affecting their toxicity and behavior. After being used, the PPCPs are discharged and introduced into the wastewater treatment plants (Chen et al., 2015). The primary treatment in

wastewater treatment plants is not effective to remove PCCPs, and the cationic polymers are identified as toxicants in the primary effluent (Ankley and Burkhard, 1992). The removal of PPCPs in the entire wastewater treatment process is very low, because most of the used treatment systems in the secondary process of wastewater treatment plants is designed to remove the organic matter and suspended solids (Yang et al., 2017).

Since these personal care products can be persistent in the environment and the unknown toxic effects in the environment and human health, is crucial to fully understand the health risks associated with them as well as their fate in the environment.

1.5 Environmental impacts of cationic polymers

Cationic polymers are known for their solubility and cleaning properties, securing a place among detergents, other cleaning products, and cosmetic products (Ivanković and Hrenović, 2010). The quantities being used by industry increase every day, and most of them end up dispersed in different natural resources (soil, water, sediments). After being used, these compounds are discharged into sewage systems or directly on surface waters. This provokes their accumulation in excessive amounts in wastewater treatment plants, affecting the ecosystems, being toxic to organisms from mammals to bacteria (Yang et al., 2017).

When these polymers are present in water at low concentrations, they can aggregate. The threshold concentration at which this can occur is designated by overlap concentration (c*) (Kronberg et al., 2014). Surface active polymers, usually constituted by a hydrophilic backbone and hydrophobic side grafted groups (Soft CAT are surface-active polymers) have the capability of interact with other species, such as surfactants, give to the cationic polymers and the complexes formed some properties as detergency and solubilization.

The most common type of surface-active cationic polymers is the quaternary ammonium compounds. These molecules possess at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom. They are largely used in hair conditioners, detergents, and fabric softeners. Since the ones with long-chain possess better antibacterial properties, they can be also used as disinfectants (Petrocci, 1983).

In the environment, cationic polymers show a marked biological activity. They can sorb to negatively charged particulate or dissolved matter as humic acids, algae, clay, silica, and microorganisms. These sorption properties can significantly affect the apparent toxicity of cationic polymers to aquatic organism, for example, clogging of respiratory organs such as gills. These polymers are not expected to pass the biological membranes and are rather expected to affect the outer membranes of aquatic organisms or their near environment (Cumming, 2007; Cumming, 2008). It has been known for several years that these polymers are membrane-active agents. It was observed by McDonell and Russell in 1999 that the primary target site of these polymers is the cytoplasmic (inner) membrane of bacteria or the plasma membrane in yeasts and posterior disorganization through their long alkyl chain (McDonell and Russel, 1999). This increases the permeability of the membranes, originating the leakage of low molecular mass compounds, resulting in cell

death or damage by ions or amino acids loss (Cserháti, 1995). It was also observed that the cationic polymers are acutely toxic to fathead minnows and several Cladocera species, with daphnids being the most sensitive ones. These types of polymers alter the transport through the integument and/or inhibits appendage movement, hindering nutrient uptake in daphnid species (Rowland et. al., 2000).

Since these polymers are used every day, concerns about their ecotoxicity have increased massively. As mentioned in the previous sections, a major proportion of these polymers are degraded in wastewater treatment plants, but there is a small amount that ends up in surface waters, sediment, or soil. Of course, that their persistence in the sewage sludge treatment is also a concern (Holt et al., 1995). When the concentration of this type of polymers is high, can inhibit the activity of sewage sludge microorganisms and compromise the way that the wastewater treatment plants remove contaminants and breaks down sewage. Augustin et al. (1992) have reported that the cationic polymers can exhibit a detrimental effect on wastewater treatment through a biocidal effect on bacteria inhibiting the activated sludge. Furthermore, since these cationic polymers are widely used as disinfectants in healthcare due to their antimicrobial properties, there is also a growing concern about the bacterial resistance towards these polymers (Kõljalg et al., 2002; Walsh et al., 2003).

The continuous use of these polymers and their disposal into the aquatic environment may affect the ecosystems (Table 1). In general, the concentrations of cationic polymers are below the effective toxicity concentrations to aquatic organisms (Table 2) (Ivanković and Hrenović, 2010).

Table 1: Concentrations of quaternary ammonium compounds in the environment (sewage effluents, WWTP effluents, and sediment).

	Location	**Level**	**Reference**
Quaternary ammonium compounds	Sewage effluent	0.062 mg. L^{-1}	Versteeg et al., 1997
	Treated sludge	5870 mg.kg^{-1}	Fernandez et al., 1996
	Sediment	0.022 to 0.2069 mg. L^{-1}	Ferrer and Furlong, 2002

Table 2: Toxicity of quaternary ammonium compounds (QAC) against various organisms.

Organism		Endpoint	Concentration mg. L^{-1}	Reference
Bacteria	*Vibrio fischeri*	EC_{50} - Luminescence 30 min	0.5	Sütterlin et al., 2008
	Pseudomonas putida	EC_{50} - Growth inhibition 16 h	6.9	
Algae	*Dunaliella sp.*	EC_{50} - 24 h	0.79	Ying, 2006
Crustaceans	*Daphnia magna*	EC_{50} - Immobilization 24 h	0.38	Garcia et al., 2007
Fish	*Salmo gairdneri*	EC_{50} - Immobilization 48 h	1.21	Ying, 2006

The algae are the first trophic level and are the basic suppliers of oxygen in the water. Utsunomiya et al. (1997) studied the toxic effect of three QAC such as alkyl trimethylammonium chloride, dialkyldimethylammonium chloride and alkyldimethylbenzylammonium chloride on unicellular green alga *Dunaliella sp*. The 24-hour median effective concentrations were 0.79 mg. L^{-1}, 1.3 mg. L^{-1} and 18 mg. L^{-1} for the three polymers, demonstrating intra-species response variability to the same type of compound. Hrenović and Ivanković in 2007 and Hrenović et al. in 2008 evaluated the toxicity of three cationic QAC-based surfactants to phosphate-accumulating bacterium *Acinetobacter junii* and yeast *Saccharomyces cerevisiae*. They observed that the EC_{50} values of different QACs to the same organism were very different (differed up to tenfold to *A. junii* and hundredfold to *S. cerevisiae*). Herović et al. in 2008 observed that dodecyl pyridinium chloride (12 carbons atoms in the alkyl chain) was less toxic than cetylpyridinium chloride (16 carbons atoms in the alkyl chain). Also, Garcia et al in 2007 showed that the toxicity of QAC with the substitution of a benzyl group for a methyl group increased in *D. magna* and *Photobacterium phosphoreum*.

The cationic polymers are biologically biodegradable under aerobic conditions at variable rates. Some studies have reported that some organisms like *Micrococcus luteus, Pseudomonas putida, Rhodococcus rhodochrous* and *Arthrobacter globiformis* were able to use quaternary ammonium compounds as a sole carbon and energy source (Nye et al., 1994). The physicochemical properties of these polymers can have a decisive role in biodegradation in the environment. For example, biodegradability under aerobic conditions normally decreases with the number of non-methyl alkyl groups (Ying, 2006). The substitution of a methyl group with a benzyl group can further decrease the biodegradability of the cationic polymer (Garcia et al., 2001).

1.6 Eco-friendly products and rational design

The idea of eco-friendly products has launched the use of renewable materials resulting in green polymers. One of the principles of green chemistry is the design of environmentally friendly products, including for example pharmaceutical products (Kümmerer, 2007) readily degradable after their use, exhibit low toxicity to the biota while keeping a high functional efficiency for the application for which they are

designed. To achieve this purpose, during the early steps of the industrial innovation process to produce new compounds, it is necessary to consider the properties necessary for the product's successful applications but also those that will confer them a low environmental risk (Bustamante-Torres et al., 2021). In this context, the structure of a chemical and its properties are extremely important, even a small change in the structure of a chemical may deeply alter its chemical properties. Adding to this, it is also important to consider the stability of chemicals as an indispensable property for their application (Hayles, 2015). The stability of a chemical is the result of the interaction of a molecule with its environment. For example, changes in the temperature or moisture may induce the molecule to react in a different way or with a different speed. Additionally, light conditions, pH value or redox potential may also influence the behavior of the chemicals according to the environment (Mignon et al. 2019).

Specifically, hydrogels have the capacity to retain large amounts of water due to their cross-linked three-dimensional hydrophilic networks (Zhang and Huang, 2021). These possess several excellent properties, such as high biodegradability and biocompatibility, and high mechanical strength (Palmese et al., 2019; Huang et al., 2017). This group of compounds can be divided into two categories: natural and synthetic polymers. The first ones (cellulose, chitosan, alginate, DNA, and agarose that are common in the natural environment) keep their biochemical and biocompatible properties with relatively weak mechanical strength (Isobe et al., 2018; Ouyang et al., 2018; Hao et al., 2017; Bilal et al., 2019). The synthetic polymers as poly (ethylene glycol), poly (vinyl alcohol, peptide, possess high water absorption capacity, wide varieties of raw chemical resources and well-defined structures (Zhang Y. et al., 2019; Mondal et al., 2020; Gačanin et al., 2020).

The bio-based polymers are an advance that looks out for all needs and holds a tiny fraction of the total global market (Babu et al., 2013). Then again, keeping as the main concern in perspective, the broadening thought for environmental affirmation issues, such as biodegradable hydrogels, as in cellulose gels create bottomless fervor for powerful business application in restorative organizations. The initiative cellulose built gigantic obligations in lowering the reliance inclining towards oil-based products and boosting related positive normal effects, for example, diminished carbon dioxide emissions.

The water-soluble cellulose derivatives are the most studied compounds among the chemically modified polysaccharides. In industries, these are found in diverse areas with different applications (oilfield treatments, protective colloids, medical products, hair conditioners, adhesives, etc.). According to the unique structure and reactivity of this biomacromolecule, is possible to obtain diverse possibilities for the design and development of water-soluble polymers. The number of functional groups positioning along the cellulose backbone is determinant in the water solubility of the cellulose. Hydroxyethyl cellulose (HEC) is one example of a water-soluble cellulose derivative.

To face the industry and life standards increase, there is a great search for new water-soluble cellulosic polymers, mainly hydrophobically modified water-soluble cellulose derivatives with quaternary ammonium groups.

These modified polymers have some properties that turn them very wanted, such as enhanced viscosity efficiency, improved shear, and salt stability as well as shear-thickening rheological behavior (Zhang, 2001). These properties are derived from intermolecular hydrophobic aggregation. But these properties can be affected by external factors as added salts, surfactants, and polymer concentration (Regalado et al., 2012). It was observed that a determined concentration of polymer increases the solution viscosity and thickens the solution due to the network structure formed through the association of the hydrophobic moieties along the polymer backbones. The addition of salts and surfactants also can result in an increase in viscosity; depending on the ratio between polymer and surfactant the viscosity of the solution can be increased or decreased (Kronberg et al., 2014).

There is a strong research topic for the industry to develop new products, maintaining their functionality but exhibiting reduced or no toxicity to the environment. It is also important to the industry that these products do not persist in the environment and neither originate toxic products, not affecting the environment.

In this context, despite all the potential benefits that can arise from the use of cellulose derivatives for personal care products, little is known about the toxicity of these polymers to the aquatic environment. These polymers possess unique properties making them so appealing to the personal care products market but can also be responsible for ecotoxicological effects. There is a considerable gap between the available data on toxicity evaluation and the large use of these polymers worldwide. Another factor that needs to be considered is the physicochemical characteristics of the medium surrounding and the long-term presence of these polymers in the aquatic environment. That is the reason that is important to access the ecotoxicity of cationically modified hydroxyethyl cellulose polymers.

2. Goals and Thesis Structure

Over the last few years, personal care products (PCPs), widely used all over the world, have been considered as contaminants of emerging concern responsible for adverse effects on aquatic and terrestrial living systems. Therefore, there is an increasing concern about the environmental occurrence and possible harmful impact of PCPs.

Since the PCPs are continually released through rinse from human bodies and washed down drains and sewer systems, they are detected with a higher frequency mainly in the aquatic environments. In soils, sediments, surface waters, drinking and groundwater, seawater, sewage and wastewater treatment plants, the PCPs are among the most frequently detected chemicals, with concentrations ranging mainly from µg/L to ng/L.

The main goal of this thesis was to assess the influence of hydrophobic substitution and cationic density modifications in the ecotoxicity of quaternized hydroxyethyl cellulose polymers on aquatic biota. To attain this major goal, specific objectives were set and were addressed in different chapters of this thesis. The description of each chapter of the present thesis is described below.

[Chapter I-General Introduction]

Aimed to provide general information on the applications and uses of cationic polymers and their possible impacts on the environment.

[Chapter II - Effect of cationic cellulose derivatives architecture on the properties of its solutions.]

This chapter aimed to improve the understanding of the influence of the polymer architecture on the rheological behavior of cationic polymers with different cationic and hydrophobic substitution indexes. The polymers used in this work were SoftCAT™ polymers – SL and SK series and were characterized for particle size, zeta potential and rheological properties in distilled water.

[Chapter III - Hydrophobic modifications of hydroxyethyl cellulose polymers: Their influence on the acute toxicity to aquatic biota.]

This chapter aimed at studying the influence of hydrophobic substitutions on the acute ecotoxicity of quaternized hydroxyethyl cellulose polymers (SoftCAT™ polymers - SL) to aquatic biota. The ecotoxicity of four variants of SL, with different HS (SL-5, SL-30, SL-60, SL-100) was assessed for seven species, that represent different trophic and fictional levels: the bacterium *Vibrio fischeri*, the microalgae *Raphidocelis subcapitata* and *Chlorella vulgaris*, the cladoceran *Daphnia magna*, the rotifer *Brachionus calyciflorus*, the ostracod *Heterocypris incongruens*, and fish *Danio rerio*. In parallel, the SL tested suspensions were characterized for particle size, zeta potential and rheological properties, both in distilled water and in the media used to perform the ecotoxicity assay. The computed median effective concentrations computed for each ecotoxicity assay were used to derive hazard concentrations, by using species sensitive distribution curves, which enable the identification of the more environmentally friendly variant of SL. This chapter also provided crucial knowledge about the physicochemical behavior of the SoftCAT™ polymers in media with different chemical compositions.

[Chapter IV - Ecotoxicity of cationic cellulose polymers to aquatic biota: the influence of charge density.]

In this chapter, the influence of cationic modifications on the ecotoxicity of four quaternized hydroxyethyl cellulose polymers (SK-H, SK-L, SK-M and SK-MH) was accessed. The ecotoxicity characterization was developed with seven key trophic level species: *Vibrio fischeri*, *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Daphnia magna*, *Brachionus calyciflorus*, *Heterocypris incongruens,* and *Danio rerio*. To complete the characterization, these SK-variants were characterized in terms of particle size, zeta potential, rheological properties, and solubility analysis.

[Chapter V – Role of temperature on the ecotoxicity of aged, modified hydroxyethyl cellulose polymers to freshwater biota.]

The objective of this chapter was to investigate the influence of the temperature on the ecotoxicity of aged, modified hydroxyethyl cellulose polymers solutions to aquatic biota. Since temperature is always changing according to the global climate changes, the effect of this parameter in aged SK-H, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100 polymers were assessed to the ecotoxicity of six freshwater species *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Daphnia magna*, *Brachionus calyciflorus*, *Heterocypris incongruens,* and *Danio rerio*.

[Chapter VI -Final remarks]

In this chapter, a general discussion and conclusions of the major results obtained in the thesis are provided.

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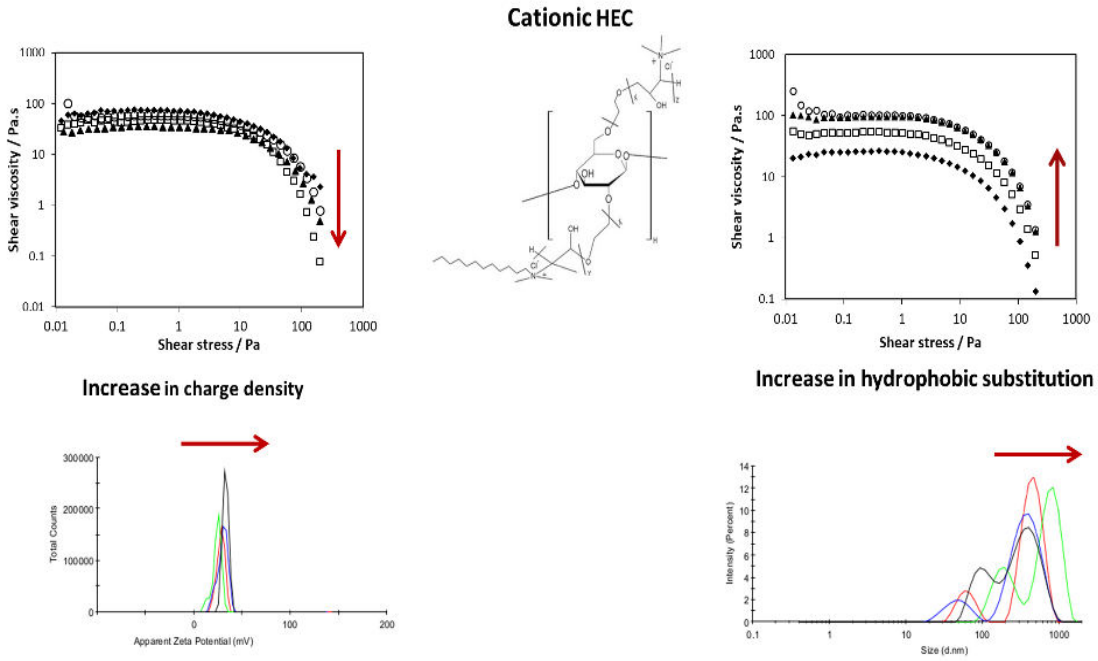
Chapter II

Effect of cationic cellulose derivatives architecture on its solutions properties.

Effect of cationic cellulose derivatives architecture on its solutions properties.

Anabela M. Simões, Luís Alves, Isabel Lopes, Filipe E. Antunes

Graphical abstract



Abstract

Cationic hydroxyethyl cellulose polymers (Polyquaternium 67) are widely used in cosmetic applications. These polymers improve the conditioner deposition efficiency of high interest compounds, as silicones, oils or other care compounds on the hair surface. Although based on a biopolymer, i.e. cellulose, these polymers are chemically modified to increase their conditioning capacity. The understanding of the way the cationic and hydrophobic modifications affects the physicochemical properties of the solutions is a key question to develop novel and efficient systems.

It was found that by increasing the degree of cationic modification leads to a slight decrease in solution viscosity, while an increase in hydrophobic modification results in a reinforced polymer network and consequently in solutions of higher viscosity. Similarly, the hydrophobic substitution (HS) degree also affects other polymer solution properties: an increase in viscosity was found with HS increment and an increase in deposition efficiency is reported in literature. This can be attributed to a better tendency of the more

hydrophobic polymers to adsorb on surfaces. The obtained results suggest that some selected polymers are excellent rheology modifiers.

Keywords: Hydrophobic modification, cationic substitution, rheology, architecture, physicochemical properties.

1. Introduction

Industrial ecology, eco-efficiency, sustainability, and green chemistry are the focus in the development of the next generation of products, materials, and processes (Patil, 2014). In this context, the production of environmentally friendly polymers based on "green" methodologies have been the great focus all over the world in last year's (He, et al., 2014). Today, there is a strong search by industry for biopolymers with specific characteristics like biocompatibility, eco-friendliness, environmental acceptability, originated from renewable sources and finally biodegradability (Mohanty, et al., 2002). Since the knowledge in nanoscience and industrial applications is also continuously growing, it is crucial to ensure the efficiency and sustainability of the developed technologies. Consequently, great concern arose regarding the production of efficient environmentally friendly polymers and national and international policy strategies have been proposed and established to promote the sustainable development.

Since most of personal care products possess organic or inorganic polymers in their formulations, it is important to fully understand their physical and chemical properties and it is important to develop studies to understand how the molecular design interferes with physicochemical efficiency (Kostal, et al., 2015).

Polymers are a class of compounds widely used in many different applications (Sau and Landoll, 1989) and because of their properties that make them very useful for rheological control among other features (Winnik and Yektaf, 1997) combined with low cost. The use of hydrophobically modified (HM) water soluble polymers is considered a useful strategy on rheological control because of the formed transient polymer association. The polymer architecture, type and degree of hydrophobic substituent and polymer composition have high impact on the formed solutions properties (Kastner, et al., 1996), also affecting the deposition capacity.

Cellulose is a natural polysaccharide, and it has been used in many different areas (Saxen and Brown, 2005; Cannon and Anderson, 1991). For this reason, the use of water-soluble cellulose derivatives like hydroxyethyl cellulose (HEC) has dramatically increased. The cationic modified HEC polymers correspond to the industry requirements since they are relatively inexpensive, highly compatible and possess antimicrobial activity (Gao, et al., 2009; Drovetskaya, et al., 2007; Ballarin, et al., 2011; Ballarin, et al., 2008; Drovetskaya, et al., 2005). The aqueous solutions of cationic hydroxyethyl cellulose (HEC) are highly viscous, and this characteristic make them widely used as thickeners of various water-based formulations (Sau and Landoll, 1989; Kastner, et al., 1996). Several studies have showed that thickening properties of HEC can be improved through hydrophobic modification (Kastner and Hoffmann, 1995). This behavior occurs due to intra- and intermolecular physical crosslinking of the hydrophobic side chains of the polymers; however, the degree of

hydrophobic substitution must be low, otherwise the polymers become water insoluble (Tanaka, et al., 1992). Hydrophobic side groups have important influence in the rheological and adsorption properties of the polymer molecules.

The aim of the present work is to understand the compromise between the polymer architecture and some physicochemical properties. The solution properties were accessed using different techniques as rheology, dynamic light scattering and zeta potential to characterize the polymer solutions.

To achieve a better understanding of the influence of the polymer architecture on rheological behavior, this work focuses on a pool of polymers with different cationic substitution (CS) and hydrophobic substitution index (HS). These polymers are considered suitable for personal care products due to their unique characteristics. The present polymers are more efficient than traditional cationic polymers, improve skin hydration and deliver surfactant-soluble ingredients (for example, fragrances or moisturizing agents) to skin. Their chemical design can be very diverse and include different types of hydrophobic modifications, different degrees of cationic substitution, chemical attachment to different blocks, etc.

Likewise, the polymer architecture can be a key factor in ecotoxicity. Ecotoxicity of polymers is an unexplored area being of the major interest the understanding of this parameter and the impact of these ingredients in aquatic life. The few studies involving cationic polymers in literature demonstrate that polymers with high charge density are potentially highly toxic to aquatic organisms comparatively to anionic or non-ionic polymers due to the expected enhanced interactions with cell wall of the organisms (Bolto and Gregory, 2007; Renault, et al., 2009; Hamilton, et al., 1996; Timofeeva, et al., 1994). It was reported that cationic polymers are toxic to fish since can block the gills, blocking the respiration of the organisms provoking suffocation (Biesinger and Stokes, 1986; Cary, et al., 1987). On the other hand, polymers with significant hydrophobicity (high HS) can preferentially adsorb on the organism's surface leading to elevated ecotoxicity. Previous works demonstrated that the increase of hydrophobic modification in a cationic polymer drastically disrupt yeast cells (Bolto and Gregory, 2007; Gosteva, et al., 2015). The present work intends to relate the polymer architecture with physico-chemical properties of the solutions enabling us to choose the more promising ones to be studied in ecotoxicity studies and enhance the understanding of the influence of the polymer architecture in aqueous life toxicity.

2. Materials and methods

2.1 Studied polymers

SoftCAT™ Polymers (INCI name: Polyquaternium-67) were supplied by Amerchol Corporation, subsidiary of Dow Chemical Company, Greensburg, LA. A schematic chemical structure of SoftCAT™ is shown in Figure 1.

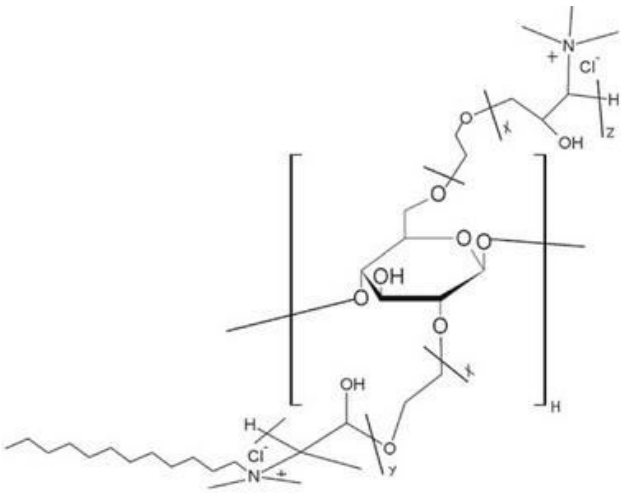


Figure 1: Molecular structure of SoftCAT™ [24].

SoftCAT™ polymers consist of quaternized hydroxyethyl cellulose, incorporating variations in charge level and hydrophobic modification (Figure 1) (Milcovich, et al., 2016). In these polymers, the polymeric quaternary ammonium salt of HEC reacted with a trimethyl ammonium-substituted epoxide and a lauryl dimethyl ammonium-substituted epoxide. Are chloride salts of N, N, N – trimethylammonium derivatives of hydroxyethyl cellulose with some dodecyl trimethylammonium residues as hydrophobic substituents and molecular weights between 200,000 – 800,000 $g.mol^{-1}$. These polymers are included in the family of cationic conditioning polymers because they combine the trimethyl ammonium functionality of polyquaternium-10 with the different levels of hydrophobic functionality (dimethyl-dodecyl-ammonium). This category includes the SoftCAT™ SK and SL variations that were used in this work.

SoftCAT™ SK are polymers that includes four high-viscosity quaternized hydroxyethyl cellulose (HEC) polymers with cationic substitution of trimethyl ammonium and dimethyl dodecyl ammonium containing low levels of hydrophobic modification. The cationic level of molar cationic substitution ranges from 0.2 to 0.3 M (Table 1). A SK variation, designed SK-MH, presents six times more hydrophobic substitution that SK-M but the same cationic substitution of 0.25 M.

SoftCAT™ SL polymers have a cationic substitution 0.2 M and a hydrophobic substitution (dimethyl-dodecyl ammonium group) that is variable (Table 1) (Milcovich, et al., 2016).

Table 1: Characteristics of SoftCAT™ series polymers.

	Viscosity (mPas) (aqueous solution 1%) (Company 2008)	**Molar cationic substitution**	**Hydrophobic substitution index**
SoftCAT™ SL			
SL-5 (Amerchol. lot: SK1050GR51)	2500	0.25	5
SL-30 (Amerchol. lot: SK1050GRS2)	2600	0.25	30
SL-60 (Amerchol. lot: SK1050GR51)	2700	0.25	60
SL-100 (Amerchol. lot: SK1050GR54)	2800	0.25	100
SoftCAT™ SK			
SK-H (Amerchol. lot: TC2450GRA2)	2100	0.3	5
SK-L (Amerchol. lot: TC2650GRA1)	2400	0.2	5
SK-M (Amerchol. lot: TC2550GRA1)	2200	0.25	5
SK-MH (Amerchol. lot: TC2550GRA2)	2300	0.25	30

2.2 Sample preparation

All the solutions were prepared after weighting the desired amounts of polymer, ranging from 1 to 50 g. $L^{-1,}$ and dispersing the polymer in Ultrapure Water (Millipore©). The solutions were kept under stirring until complete dissolution and were equilibrated prior to use to remove the presence of air bubbles.

2.3 Rheology

A Thermo Scientific HAAKE MARS III rheometer equipped with an automatic gap function was used to perform the rheological measurements of the formulations prepared. It was used a peltier system to control the temperature during the experiments and a solvent trap system to minimize water evaporation.

The rheological determinations of the SoftCAT™ formulations were done under oscillatory and steady shear conditions with a gap fixed at 1 mm. For the frequency sweep and flow curves (viscosity vs shear stress) at a fixed temperature (25°C), a plate-plate geometry (diameter 35 mm) was used. To assess the linear viscoelastic regions, we performed stress sweep determinations at a constant frequency ƒ = 1 Hz and a stress (τ) ranging from 0.01220 – 20 Pa. For the frequency sweep tests, a constant stress τ = 1 Pa was used (within the linear viscoelastic range) and a frequency ƒ range 0.01-20 Hz. The adjustment to Newtonian plateau in flow curves, to obtain the Newtonian viscosity values, was made using the software RheoWin® 4 Data Manager.

2.4 Dynamic light scattering (DLS)

The samples of SoftCAT™ were analysed through dynamic light scattering (DLS) measurements using a Zetasizer NanoZS (ZN 3500, Malvern Instruments, UK) to obtain the mean particle size of the aggregates in solution. A volume of 1.5 mL of SoftCAT™ solutions was gently transferred to a DTS 0012 polystyrene cell. The presence of bubbles was checked before starting the measurements. For the average particle size, it was used the Zetasizer Nano Software (version 6.01) where the time-averaged correlation functions were transformed into intensity-weighted distributions of the apparent hydrodynamic diameter. All the measurements were performed at 25°C and a backscatter angle of 173°. Each sample was determined in a total of 3 scans.

2.5 Zeta potential

To the determination of zeta potential samples of SoftCAT™ with concentrations of 2 g. L^{-1} were used; the electrophoretic scattering was measured in a Zetasizer NanoZS (ZN 3500, Malvern Instruments, UK) using a Zetasizer Nano Software (version 6.01)

3. Results and discussion

The prepared polymer solutions are stable during a large period, and this can be attributed to the presence of positive charges, characteristic of PQ-67 polymers. Milcovich et al., 2016 estimate a positive charges concentration of 7.1 x 10^{-3} mol kg^{-1} to a solution containing 1 wt % of PQ-67 (Milcovich et al., 2016). By increasing the polymer concentration, solutions are fluidlike at low concentrations and highly viscous at high concentrations.

Figure 2 shows the flow curves of the different variations of SoftCAT™ at a fixed concentration of 2.0 wt %. As it can be seen, the viscosity depends on the chemical structure.

The SoftCAT™ solutions exhibit a well-defined Newtonian region at low shear stress values, that is a common behavior of polymeric systems. Also, in these polymeric solutions is possible to observe a shear-thinning region that occurs due to the alignment of the polymer chains in solution, promoted by the applied shear stress. This results from the disentanglement of polymer chains and destruction of inter-polymer hydrophobic interactions.

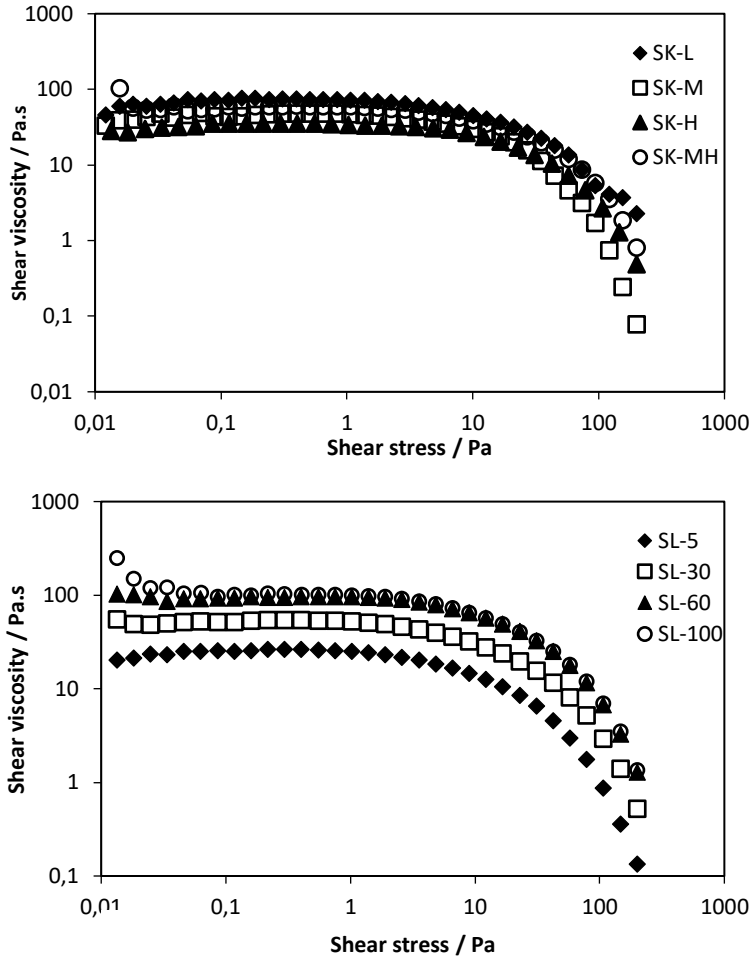


Figure 2: Flow curves, viscosity (η) as function of shear stress (τ), of the different SoftCAT™ variations at a fixed concentration of 20 g. L^{-1} at a temperature of 25°C.

It was found that an increase in charge density (from SK-L to SK-H) lead to a slight decrease in the solution viscosity; also, an increase in hydrophobic substitution degree (from SK-M to SK-MH) lead to an increase in solution viscosity.

The flow behavior of polysaccharide polycations is governed by the overall conformation and the degree of hydrogen bonding or electrostatic repulsion between neighboring segments (Muzzarelli, et al., 1989). The conformation of the three variations of SoftCAT™ SK (SK-L, SK-M and SK-H) does not change drastically due to the relatively high charge density present in the different variations of these polymers,

being adopted an extended conformation in aqueous solution due to the counterions entropy. However, the increase of charge, above a certain value, can lead to a slight decrease in viscosity, as observed to SK-H. On the other hand, an increase in HS, from 5 (SK-M) to 30 (SK-MH), leads to a small increase in the solution viscosity. This can be attributed to an extra physical crosslinking provided by the aggregation of the side hydrophobic groups, that improves the polymer network and as result the viscosity of the solution raises. Similarly, the SL series also presented a tendency of viscosity increment to higher hydrophobic substitution degrees.

The differences in viscosity are more pronounced to SL series where the viscosity values vary by one order of magnitude as the HS is improved. As mentioned before, an increase in HS lead to higher hydrophobic aggregation of the side chains of the polymers and at same time the polymer become less water soluble. The polymer concentration effect was studied and in Figure 3 is presented the Viscosity of SoftCAT™ solutions as function of polymer concentration.

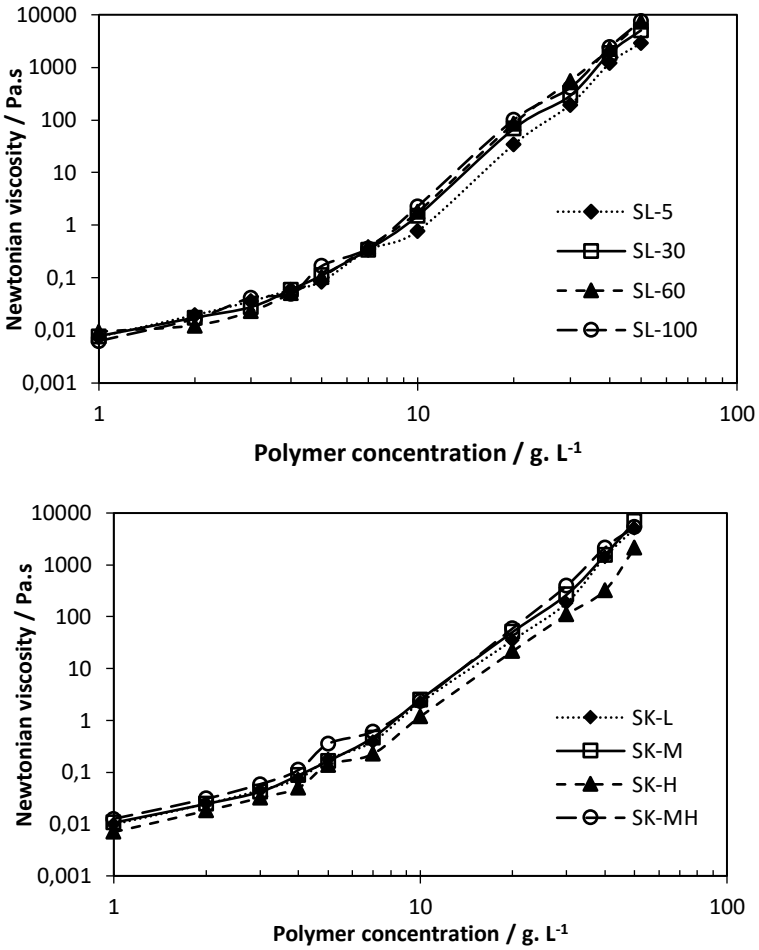


Figure 3: Dependence of Newtonian viscosity on the polymer concentration for the different SoftCAT™ solutions, at 25°C.

As expected, the solution viscosity increases with polymer concentration. However, the increase is not linear, being more pronounced above the overlap concentration (c*), due to the interactions between different polymer segments creating a 3D polymeric network.

The values obtained for Newtonian viscosity, on the studied concentration range of SoftCAT™ SK polymers, indicate that higher charge densities result in a viscosity decrease. Additionally, an improved hydrophobic association, as result of the increase of HS of the polymer, leads to superior viscosity values. A similar trend is observed to SL series being the more viscous solutions obtained for SL-100 polymer, due to the large HS index of the polymer and consequently an increased hydrophobic association. The polymer architecture has a deep influence on its overlap concentration in aqueous solution. It was found that an increase in charge density led to a slight decrease on overlap concentration of SK series polymers, from 4.8 g. L^{-1} to SK-L to 4.0 g. L^{-1} to SK-H, estimated as the breakpoint of the plot of the solution viscosity as function of polymer concentration, characterized by an abrupt change in slope. This behavior can be attributed to a better expansion of the polymer chain due to the higher charge density and consequently higher counterion entropy; other parameter affected by the higher charge density is the stiffness of the chain polymer giving the polymer a higher radius of gyration and consequently the overlap of the polymer chains is increased. The c* of the different polymer solutions is presented in table 2.

Table 2: Overlap concentration values (c*) of SoftCAT™ polymers in aqueous solution.

SoftCAT™	**Overlap concentration (c*) / g. L^{-1}**
SK-L	4.8
SK-M	4.3
SK-H	4.0
SK-MH	4.2
SL-5	5.2
SL-30	4.4
SL-60	3.3
SL-100	3.0

On the other hand, the addition of higher number of HS, lead to a decrease of the c* value of the polymer; in SK series, the increment of six times HS (SK-MH) reduces the c* from 4.3 g. L^{-1} to 4.2 g. L^{-1}. The same trend is observed in SL series with a gradual decrease of the overlap concentration from 5.2 g. L^{-1}, SL-5,

to 3.0 g. L^{-1} SL-100, induced by the raise of HS in 20 times. The increased HS degree slightly reduces the solubility of the polymer and facilitates the side hydrophobic chains aggregation, resulting in a decrease of the overlap concentration value of the polymers. This behavior is somehow opposite to the observed in other polymeric systems, as HM-PAA systems (Alves, et al., 2015) or HEC/HM-HEC systems (Lochhead and Rulison, 1994). This difference in overlap concentration trend can be attributed an increased hydrophobicity presented by the HM-PAA polymers, being composed of ca. 60-70% ester monomers, containing some hydrophobic character. The authors found that an extra hydrophobicity, induced by addition of long side chains, lead to a delay in polymer expansion and consequent higher overlap concentration compared with the polymer without side hydrophobic chains. Similarly, the system made with non-ionic HEC also presents an increase in c* value when HS is introduced, from 1.6 g. L^{-1} to 1.9 g. L^{-1}. As the solubility of non-ionic polymers is lower when compared with ionic polymers, the introduction of HS in polymer architecture lead to an enhanced compaction of the polymer chain and the overlap concentration is shifted to higher values; oppositely the authors found that in PAA systems, the introduction of HS decreases the c* value, being this attributed to the combination of the polymer expansion induced by polymer charges and the better physical crosslinking promoted by the presence of hydrophobic modification (Lochhead and Rulison, 1994). From the results obtained in present study is clear that the HS degree has a major impact on the overlap concentration of the polymers compared with the cationic substitution degree. However, both parameters are important and affect the polymer behavior. Additionally, the viscoelastic properties of the different polymer solutions were accessed by oscillatory tests, first stress sweep to evaluate the linearity range, followed by frequency sweep measurements, Figure 4.

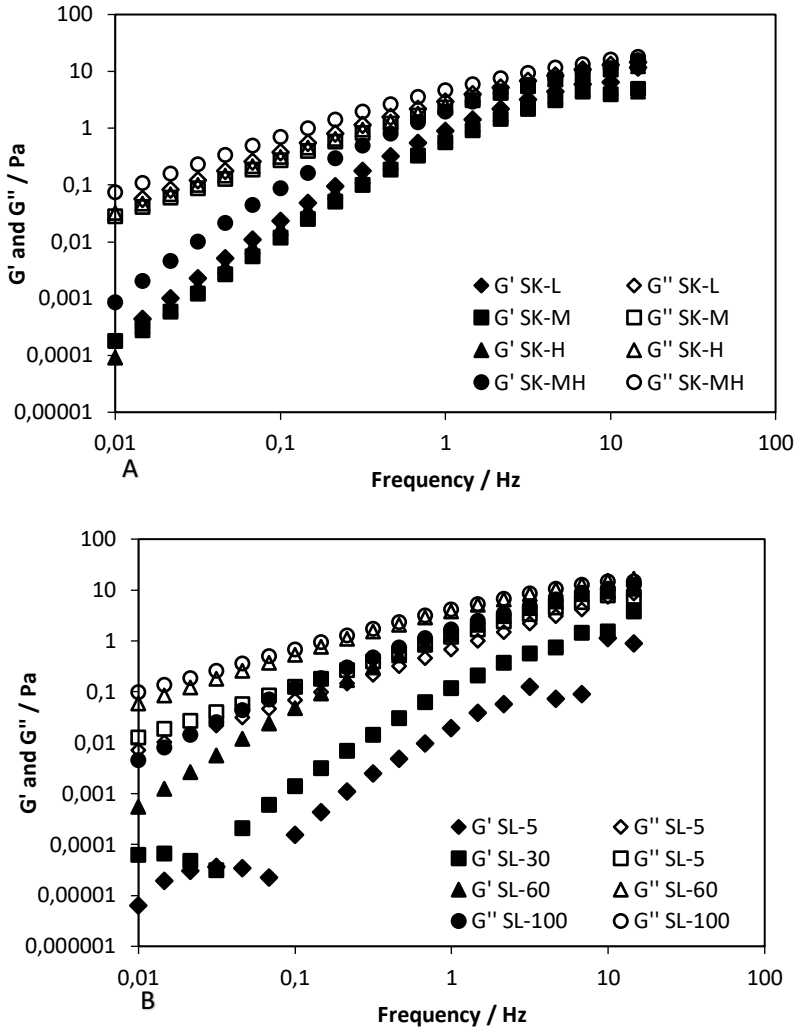


Figure 4. Frequency sweep tests for 10 g. L^{-1} SoftCAT™ polymer in water (G' elastic modulus, G'' viscous modulus), at 25°C. (A – SoftCAT™ SK and B - SoftCAT™ SL).

From Figure 4 is possible to conclude that all the samples containing 10 g. L^{-1} present a liquid like behavior in the entire frequency range studied (G'' > G'). However, an increase in G' and G'' values is observed with the number of HS, both for SK (SK-M higher modulus) and SL (SL-100 higher modulus) series. Therefore, it seems clear that the hydrophobic character of the polymer plays an important role on the polymer solutions properties.

In addition, the average particle size in solution was determined using dynamic light scattering as well the zeta potential of the solutions containing SoftCAT™ polymers (table 3).

Table 3. Average particle size and zeta potential obtained for the different polymers studied.

SoftCAT™	Average Particle Size (nm)	Zeta Potential (mV)
SK-L	327.3 ± 146.6	24.1 ± 5.0
SK-M	327.4 ± 137.0	29.8 ± 4.7
SK-H	328.0 ± 198.1	34.8 ± 4.5
SK-MH	347.6 ± 164.3	27.9 ± 4.4
SL-5	316.2 ± 149.8	32.0 ± 4.0
SL-30	328.1 ± 154.7	26.6 ± 4.8
SL-60	337.0 ± 194.1	22.5 ± 5.1
SL-100	361.6 ± 202.8	20.1 ± 3.7

The charge density of the polymer did not affect considerably the average particle size, being the obtained values nearly constant for the SK series, except for SK-MH. The introduction of more HS in SK polymer leads to an increase in the average particle size in solution, induced by the aggregation of the hydrophobic side chains of SK-MH in aqueous solution. A similar trend was observed for SL series with a clear increase of the average particle size from SL-5 to SL-100 polymers; this increase in particle size is due to the higher tendency of the more hydrophobic polymer (SL-100) to aggregate in solution because the lower aqueous solubility of this polymer.

The colloidal stability of SoftCAT™ polymers solutions is intimately related with the zeta potential of solutions and high stability is attained with elevated zeta potential values.

Zeta potential of the SK solutions is deeply related with charge density of the polymers. It was observed a clear increase in zeta potential value of the solutions with the increase of cationic modification of the polymer, being the higher value of zeta potential obtained to SK-H polymer solution. The zeta potential measures the electric potential in the interfacial double layer and is expected that an increase in the charged groups number lead to an increase in this parameter. To the SL series a different scenario is observed; a constant decrease in zeta potential value with the increase of HS amount occurs from SL-5 to SL-100. The SoftCAT™ SL-100 has higher hydrophobic substitution and the lower zeta potential value is obtained to its solutions, which can be attributed to a higher polymer aggregation in solution. As can be seen in Figure 1 the side hydrophobic chain is connected to a cationic substitution, and therefore the number of positive charges along the SL series is constant. However, the increased number of hydrophobic C_{12} (dodecyl trimethylammonium) chains lead to an enhanced aggregation, to prevent the contact with water, and thus

some of the charged groups are incorporated inside the polymer particles resulting in a lower zeta potential value. As expected, a lower zeta potential (typically bellow ± 30.0 mV) is a driving force to flocculation and instability of the particles in solution (Ostolska and Wisniewska, 2014). As result the higher values of average particle size are obtained to the polymer possessing higher hydrophobicity that can be attributed to inter-polymer aggregation of side C_{12} chains and lower zeta potential driven by intra-polymer C_{12} chains aggregation.

Likewise, the polymer architecture can be a key factor in ecotoxicity. Polymers with high charge density are potentially highly toxic to aquatic organisms comparatively to anionic or non-ionic polymers due to the expected enhanced interactions with cell wall of the organisms (Bolto and Gregory, 2007; Renault, et al., 2009; Hamilton, et al., 1996; Timofeeva, et al., 1994). It was reported that cationic polymers are toxic to fish since they can block the gills, blocking the respiration of the organisms provoking suffocation (Biesinger and Stokes, 1986; Cary, et al., 1987). On the other hand, polymers with significant hydrophobicity (high HS) can preferentially adsorb on the organism's surface leading to elevated ecotoxicity. Previous works demonstrated that the increase of hydrophobic modification in a cationic polymer drastically disrupt yeast cells (Bolto and Gregory, 2007; Gosteva, et al., 2015).

4. Conclusions

This work represents an effort to link the relevant physiochemical properties of cationic cellulose derivatives solutions and the development of eco-friendly compounds.

It was found that the polymer architecture plays a key role on the solutions properties. All the samples share a non-Newtonian behavior above overlap concentration, being dependent on the shear stress applied and/or on the shear rate experienced by the solution. To stresses higher than the yield stress of the solution a shear thinning region is observed to all the solutions containing 20 g. L^{-1} cationic HEC, due to an alignment of the polymer chains and destruction of three-dimensional polymer network.

The HS introduced on the polymer chain led to higher shear viscosity values of the solutions, being observed the highest value to the solution containing SoftCAT™ SL-100; contrary the increase in charge density along the polymer chain leads to a slight viscosity decrease. The first effect is attributed to the reinforcement of the polymer network by hydrophobic association of the side hydrophobic chains, being the late effect attributed to the reduced capacity of the polymer to form hydrogen bonds with neighbor polymer chains.

The overlap concentration and the viscoelastic properties of the solutions are affected by the HS degree and charge density of the polymers. Polymers with higher HS or charge density present lower values of overlap concentration. HS induces polymer aggregation and therefore a polymer network can be obtained at lower concentrations. Similarly, changes in HS and charge density results in different particle size and zeta potential in solution. An increase in zeta potential is observed to polymers containing higher charge density

and lower zeta potential is observed to polymers with higher HS index. This trend is opposite to the observed in average particle size.

The results obtained for the average size for SoftCAT™ polymers revealed that the SL-100 and SK-MH variations are the ones with higher size. Since they are the ones that possess higher number of hydrophobic groups, occur an increase in the aggregation of the particles, increasing the average size of the polymers.

By the exposed, for enhanced physico-chemical properties of the solutions, a balanced relation between charge density and HS should be addressed to achieve an eco-friendly polymer.

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Chapter III

Hydrophobic modifications of hydroxyethyl cellulose polymers: Their influence on the acute toxicity to aquatic biota

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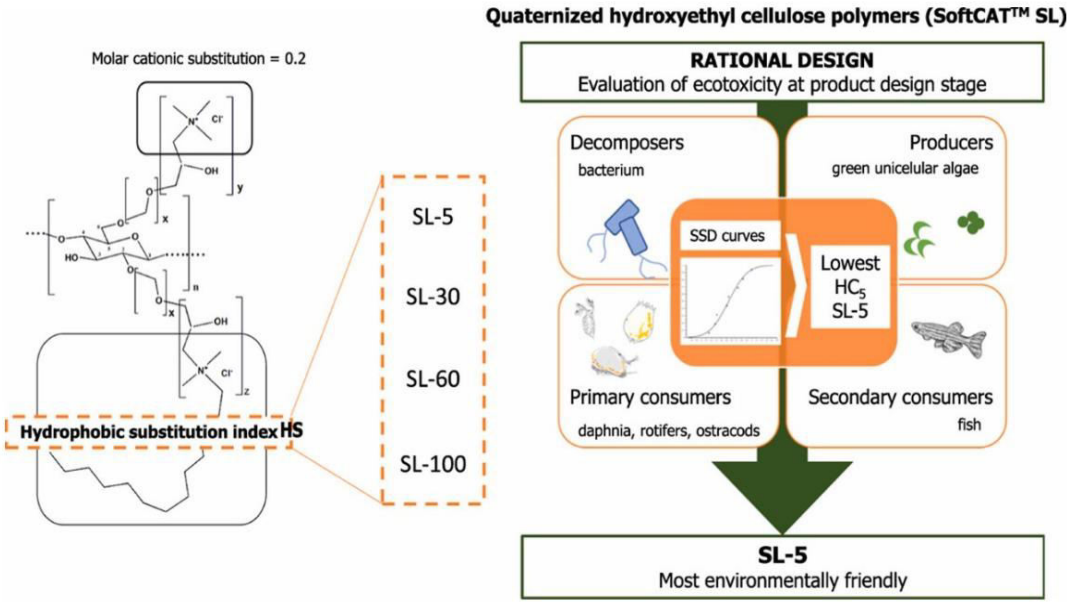
Hydrophobic modifications of hydroxyethyl cellulose polymers: their influence on the acute toxicity to aquatic biota.

Anabela M. Simões, C. Venâncio, Luís Alves, Filipe E. Antunes, Isabel Lopes

Graphical abstract



Abstract

The hydrophobic substitution (HS) of cationic cellulose derivatives may be tuned, promoting their efficiency. This work studied the influence of HS on the acute ecotoxicity of quaternized hydroxyethyl cellulose polymers (SL) to aquatic biota. The ecotoxicity of four SL with different HS (SL-5, SL-30, SL-60, SL-100) was assessed for seven species: *Vibrio fischeri*, *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Daphnia magna*, *Brachionus calyciflorus*, *Heterocypris incongruens,* and *Danio rerio*. The computed median effective concentrations were used to derive hazard concentrations, by using species sensitive distribution curves. All SL suspensions were characterized for particle size, zeta potential and rheological properties. Results indicated instability of the SL in suspension due to their relatively low zeta potential. *Raphidocelis subcapitata*, *C. vulgaris* and *B. calyciflorus* were the most sensitive to the four SL, suggesting that exposure to these compounds may imbalance the lowest trophic levels. Also, HS influenced the toxicity of SL, with the lowest HS (SL-5) revealing lower ecotoxicity. The maximum acceptable concentrations were 14.0, 2.9, 3.9 and 1.4 mg. L^{-1} for SL-5, SL-30, SL-60, and SL-100, respectively. Accordingly, SL-5 is suggested as the eco-friendliest

and is recommended to be used in the production of care products, in detriment of the other three tested variants.

Keywords: Cationic cellulose derivatives, aquatic toxicity, rational design, hydrophobic substitution.

1. Introduction

In current times, the society, as a whole, claims for new environmentally friendly compounds as most of the commercially available ones have reported unintended biological activity that provoke several adverse effects in the environment (Kostal et al., 2015). Such fact has prompted worldwide political strategies to establish a new paradigm that promotes the development of Smarter and Cleaner technologies, aiming a more sustainable development (e.g., European Commission, 2008, 2010). This poses new challenges to industry since it demands for the development of new products that, while keeping their functionally, exhibit reduced or no toxicity to the environment and that after exerting their function, breakdown into other innocuous products that do not persist in the environment (Kümmerer, 2007; Anastas and Eghbali, 2010). The development of such environmentally friendly chemicals requires a deep knowledge on the association between functionality, structure, and toxicity (Kümmerer, 2007). Therefore, it is important to include ecotoxicological assessments at early stages of the innovation process to design new products capable of exhibiting the desired functionality while presenting none or a minimum risk to the environment (Papa and Gramatica, 2010; Devito, 2012; Crawford et al., 2017). Some scientific works have already applied ecotoxicological approaches to understand the influence of the chemical characteristics of diverse compounds on their toxicity to biota, aiming to identify the structure presenting the lowest risk to the environment (e.g., Beach et al., 2009; Hernandez-Fernández et al., 2015; Pereira et al., 2018). As an example, Martins et al. (2018) studied the influence of the number of ethylene oxide (EO) groups in the toxicity of mixed micelles of sodium lauryl ether sulphates and alkylbenzene sulfonic acid. These authors reported a higher number of EO groups is associated with a lower toxicity of these micelles to the bacterium *Vibrio fischeri*, suggesting these micelles to be as the most ecologically safe variants.

The application of this approach is especially relevant when considering chemicals that are extensively used worldwide, such as the case of personal care products (PCP), as its release into wastewater treatment plants (WWTP) is expected to occur in large quantities. Furthermore, the deficient removal of these chemicals by WWTP processes, enables their entrance into the aquatic compartment (Kosma et al., 2014). Some of the PCP currently in use incorporate in their composition cationic cellulose polymers that confers them the desired properties to be effective and attractive to consumers. The chemical modifications introduced in cellulose backbone correspond to the requirements for the different industrial applications, by optimizing their physicochemical properties (Kästner, et al. 1996). The SoftCAT™ SL constitute a family of high viscosity quaternized hydroxyethyl cellulose (HEC) polymers, with low cationic substitution, of trimethyl ammonium and dimethyl dodecyl ammonium. The composition of these polymers delivers higher conditioning performance in hair care applications through the benefits of their amphiphilic and cationic

properties. For example, Drovetskaya et al. (2005) showed that SoftCAT™ SL with low levels of hydrophobes (molecules that do not interact with water), promoted a higher performance regarding the conditioning properties of shampoo formulations and also permitted to retain other good qualities such as crystal-clear formulations and volume-down effects on hair. These authors also stated that the low levels of hydrophobes assured a good compatibility without the complications of associative thickening with surfactant systems. Ballarin et al. (2011) showed that the hydrophobic substitution (HS) of SoftCAT™ SL had influence on the dye uptake by hair fibres and on anti-fading effects. A higher HS, up to SL 30, resulted in a higher quality dyeing process and slowed the fading effect during washing cycles. However, HS above 30 did not show any further improvement on dye uptake or anti-fading properties. And Jordan et al. (2007) reported that shampoos formulated with SoftCAT™ SL with HS of SL 5 showed an increase in the wet performance, but such significant improvement was not further enhanced by increasing the number of HS. For an adequate conditioning performance, a balanced amount of hydrophobes in the polymer architecture is crucial, due to the desired formation of water insoluble polymer/surfactant complexes, which deposit on the hair-surface repairing the hair damages (Fernandez-Pénã and Guzmán, 2020). Excessive number of hydrophobes can lead to polymer aggregation and the need of higher concentrations of cationic polymer to obtain the desired performance (Roos et al., 2004). Adding to the changes that HS may promote in the properties of SoftCAT™ SL polymers, they may also influence their toxicity to biota. So far, the ecotoxicity of these polymers is poorly understood, being thus very important to understand the potential impact of those modified cellulose polymers on the aquatic environment, predicted to be its ultimate fate after being released to the environment. The few ecotoxicological information that is available in the scientific literature reports that cationic polymers, with a high charge density, are the most toxic to aquatic organisms comparatively to the anionic and non-ionic polymers (Bolto and Gregory, 2007; Hamilton, et al. 1996). Furthermore, since no information is available regarding the estimated concentrations under realistic environmental scenarios, it is necessary to define maximum acceptable threshold to meet environmental quality standards (MAC-EQS). At early stages of product innovation, ecotoxicology may provide comprehensive knowledge on the influence of chemical structure and composition on the toxicity of this type of polymers, thus, promoting the developmental of more ecologically friendlier PCP products.

Within the above context, the present study aimed to clarify the influence of modifications made in the polymer architecture on its toxicity to aquatic species. For this, the toxicity of four SoftCAT™ SL polymers, containing different hydrophobic substitution degrees, was assessed in aquatic species representative of different taxonomic, trophic, and functional groups. Alongside the ecotoxicity evaluation, the polymeric suspensions were characterized in terms of particle size, zeta potential and rheological properties.

2. Materials and methods

2.1. Studied polymers

This work focused on the study of highly viscous quaternized hydroxyethyl cellulose cationic polymers with a molecular cationic substitution of trimethyl ammonium and dimethyl-dodecyl ammonium set to 0.2 M, corresponding to a percentage of about 1% of nitrogen by weight (Fig. 1; Table 1) (SoftCAT™ SL, INCI name: Polyquaternium67) (Ballarin et al., 2011). These polymers are obtained through the reaction of quaternary ammonium salt of hydroxyethyl cellulose with a trimethyl ammonium-substituted epoxide and with a lauryl dimethyl ammonium-substituted epoxide (Company, 2008). SoftCAT™ SL polymers are characterized by a fixed ethylene oxide (EO) group, molecular weight (MW), and different low levels of hydrophobic substitutions (HS) (Ballarin et al., 2011). Four SL-variants, with different levels of dimethyl dodecylammonium hydrophobic substitution, are commercialized and were used in the present study: SL-5, SL-30, SL-60 and SL-100 (please see Table 1 for detailed information on each polymer provided by the manufacturer) (Dow, 2013). The hydrophobic substitutions correspond to the average number of moles of hydrophobic residues for anhydroglucose repeat unit; for SL-5 the degree of substitution is 5×10^{-4}, for SL-30 is 5×10^{-3}, for SL-60 is 7×10^{-3} and for SL-100 is 1×10^{-2} (Drovetskaya et al., 2005; Company, 2008; Milcovich et al., 2016). All the four SL-variants were supplied by Amerchol Corporation, subsidiary of Dow Chemical Company, Greensburg, LA.

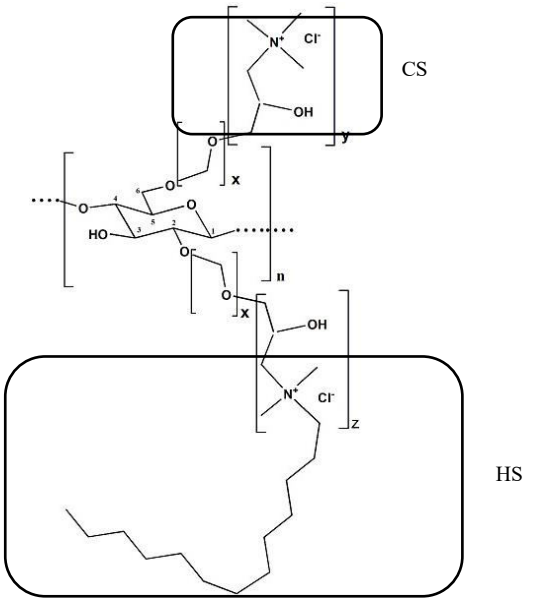


Fig. 1. Molecular structure of SoftCAT™ polymers (Company, 2008). The CS and HS abbreviation stand for the molar cationic substitution and hydrophobic substitution indexes, respectively.

Table 1: Viscosity m(Pas), molar cationic substitution (CS), hydrophobic substitution index (HS) and overlap concentration values (c*) of the four SoftCAT™ SL polymers (Company, 2008).

Variants-SL	Viscosity (mPas) (aqueous solution 1%)	Molar cationic substitution (CS)	Hydrophobic substitution index (HS)	Overlap concentration (c*) (g L^{-1})
SL-5 (Amerchol.lot:SK1050GR51)	2500	0.2	5	5.2
SL-30 (Amerchol.lot: SK1050GRS2)	2600	0.2	30	4.4
SL-60 (Amerchol. lot: SK1050GR51)	2700	0.2	60	3.3
SL-100 (Amerchol. lot: SK1050GR54)	2800	0.2	100	3.0

2.2. Characterization of tested aqueous suspensions

To complete the polymers characterization, it was important to test the polymers in the different suspension media (distilled water and test media used for each assay). For each assay three aqueous suspensions of SL-variants (lowest, intermediate, and highest tested concentrations) were characterized in terms of particle size, zeta potential, rheological properties, and solubility analysis. For that, each SL-variant was suspended in the culture media of each species used for the ecotoxicity assays and also in distilled water (to evaluate the influence of media composition in the polymeric suspension properties; comparisons among the different test media could not be done as different concentrations were tested per assay, accordingly to the species sensitivity to the compounds). All suspensions were prepared by vigorously mixing after polymers addition in the corresponding test medium or distilled water, through magnetic stirring, to obtain homogeneous suspensions. The particle size and zeta potential measurements (n = 3) were performed through dynamic light scattering (DLS) and electrophoretic light scattering measurements using a Zetasizer Nano ZS (ZN 3500, Malvern Instruments, UK) coupled with the Zetasizer Nano Software, version 6.01 (Nogueira et al., 2012). For particle size determination, samples of 1.5 mL, of each SL-variant in the culture media or distilled water, were placed in a DTS 0012 polystyrene cell and checked for the presence of bubbles. All the measurements were made at 25°C. The backscatter angle used for the average particle size was 173° with a 532 nm laser. For zeta potential measurements, the measuring cells (DTS1070) were carefully filled with the polymeric suspensions using a syringe and avoiding the air bubbles formation inside the cell. The zeta potential value was determined based in three repetitions. The rheological measurements were

performed in a Thermo Scientific HAAKE MARS III (Thermo Fisher Scientific, Germany) rheometer equipped with an automatic gap function. The control of the temperature was made by a peltier system, and it was used a solvent trap system to minimize water evaporation. The rheological measurements were done under steady shear and oscillatory conditions with a gap fixed at 1 mm, for all SL samples. It was used a plate-plate geometry (diameter 35 mm) for the measurements of frequency sweep and flow curves (viscosity vs shear stress) at 25°C. It was performed stress sweep determinations to assess the linear viscoelastic regions, with a stress (τ) ranging from 0.01220 to 20 Pa, at a constant frequency ƒ = 1 Hz. The solubility of each SL-variant was assessed at the same concentrations that were analyzed in dynamic light scattering and electrophoretic mobility. The samples for the studies were prepared by using the same methodology as described above. After sample preparation, each sample, at the different concentrations, was centrifuged at 17,968 g for 10 min (Cai et al., 2014; Melro et al., 2020). Afterwards, the solubility/insolubility of each polymer was evaluated by visual inspection of the samples; the solubility limit was then estimated based on the ratio between the amount of polymer settled at the bottom of the flask and the total amount of polymer added. The solubility evaluation of the four SL-variants was performed at time zero (just after preparing the samples) and at the time corresponding to the duration of the respective assay, for each concentration (the lowest, intermediate and highest tested): for *V. fischeri* assay after 15 min; for *R. subcapitata* assay at 24, 48 and 72 h; for *D. magna* and *H. incongruens* assays at 24 and 48 h; for *B. calyciflorus* assay at 24 h, and for *D. rerio* at 24, 48, 72 and 96 h.

2.3. Ecotoxicological assays

To evaluate the influence of the hydrophobic modifications on the ecotoxicity of the four SL-variants, assays were performed with seven aquatic species belonging to different taxonomic, trophic and functional levels: the bacterium *Vibrio fischeri* (decomposer), the microalgae *Raphidocelis subcapitata* and *Chlorella vulgaris* (producers), the cladoceran *Daphnia magna* (planktonic primary consumer), the rotifer *Brachionus calyciflorus* (planktonic primary consumer), the ostracod *Heterocypris incongruens* (epibenthonic primary consumer), and the fish *Danio rerio* (secondary consumer) (Table 1S). Stock solutions used in all assays were freshly made by direct dissolution of each SL-variant in the respective medium of the species to be tested. The concentration of each stock solution took into consideration the range of concentrations to be tested.

2.3.1. Bioluminescence inhibition assay with *V. fischeri*

The ecotoxicity of the four SL-variants was firstly determined for the bacterium *V. fischeri* by carrying out the 81.9% Basic Test protocol of bioluminescence inhibition assay (Azur Environmental, 1998). The Microtox® assay was used as a preliminary ecotoxicological indicator for the later establishment of concentration ranges for the remaining species. This type of assay is advised whenever (eco)toxicity data on the chemical compounds is lacking. It is very reliable, sensitive and in a very short time period allows to screen a wide range of concentrations and compounds. The bacterium *V. fischeri* was reconstituted according to the Microtox® protocol supplied by Microbics Inc and the 81.9% Basic Test protocol of bioluminescence inhibition assay was performed once for all four SL-variants (Azur Environmental, 1998). The bacterium was exposed to 9 concentrations from a stock solution of 10,000 mg. L^{-1} (32, 64, 128, 256, 512, 1024, 2048, 4095, and 8190 mg. L^{-1}; Table 1S) of each SL-variant and to a control (consisting in diluent solution) through a Microtox Model 500 Analyser (Azur Environmental, CA, USA) with automatic record of luminescence. Bioluminescence was measured after 15 min of exposure.

2.3.2. 72 h growth inhibition assay with *R. subcapitata* and *C. vulgaris*

The stock cultures of *R. subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) and *C. vulgaris*, were maintained at 20 ± 2°C, under continuous illumination (100 µE/m^2/s) and aeration, in Woods Hole MBL growth medium (Stein, 1973). An inoculum was obtained from these cultures, when at exponential growth, to initiate the toxicity tests. The 72 h-growth inhibition tests were performed according to the OECD Guideline 201 (OECD, 2004) adapted to 24-well microplates (Geis et al., 2000; Moreira-Santos et al., 2004). Each algal species was exposed, for 72 h, to six concentrations of each SL-variant (0.68, 1.03, 1.54, 2.31, 3.47, and 5.20 mg. L^{-1}; Table 1S), which were obtained by diluting a stock concentration of 10.0 mg. L^{-1} with MBL medium, plus a control (MBL medium; Nichols, 1973). For each concentration and control three replicates were carried out, and each replicate was filled with 100 µL of microalgae inoculum (initial cell concentration of 10^5 cell mL^{-1}, to start the assay with a cell density of 10^4 cell mL^{-1}) and 900 µL of SL-variant solution prepared in MBL medium. To exclude any potential interference of the presence of the polymers in the final absorbance measurements, wells with each concentration of SL-variant tested only in MBL medium without the algae were exposed under the same conditions for the 72-h period. Exposure occurred at 23 ± 1°C, with continuous illumination (100 µE/m^2/s) and continuous stirring using an automatic stirrer to promote active gas exchange and prevent cell clumping. At the end of the assay, a wide-spectrum microplate reader (Thermo Scientific mod. Multiskan Spectrum) was used to estimate the cell density (cells mL^{-1}) through the measure of the absorbance (ABS) at 440 nm after 72 h, using the following equations:

For *R. subcapitata*: Cells ml^{-1}

$= -17107.5 + (ABS * 7925350)$ ($R^2 = 0.99$)

For *C. vulgaris*: Cells ml^{-1}

$= -155820 + (ABS * 13144324)$ ($R^2 = 0.98$)

Growth rate (day^{-1}) was determined according to OECD (2006) for each SL-variant concentration and control:

$$\mu = \frac{\ln D_b - \ln D_a}{t_b - t_a},$$

where Da is the initial cell density, Db is the cell density at the end of the assay and tb – ta is the exposure time interval (72 h).

2.3.3. 48 h acute immobilization assay with *D. magna*

The acute immobilization assay with *D. magna* was performed according to the OECD guideline 202 (OCDE, 2004). Neonates (6–24 h-old) from the 3rd or 4th broods were obtained from monoclonal *D. magna* BEAK cultures maintained in synthetic hard water American Standards for Testing and Materials medium (ASTM, 2002) (pH 7.3 ± 0.3, 20 ± 1°C and 16 h^L: 8 h^D photoperiod). These were exposed to several concentrations of each SL-variant plus to a control (consisting of ASTM medium). The concentrations tested for SL-5 were 841, 1176, 1646, 2305, and 3227 mg. L^{-1} (Table 1S) and for SL-30, SL-60 and SL-100 were 600, 841, 1176, 1646, 2305, 3227, and 4518 mg. L^{-1} (Table 1S). All concentrations were obtained by diluting a stock solution of 5000 mg. L^{-1} with ASTM medium. Four replicates were carried out for each concentration and control, each containing 30 mL of test solution and five neonates of *D. magna*. Exposure occurred for 48 h under a temperature of 20 ± 1°C and 16 h^L: 8 h^D photoperiod, without food addition or medium renewal. At the end of the test (48 h), the number of immobilized neonates in each replicate was counted. An organism was considered immobile if no movement was observed for 15 s following gentle prodding.

2.3.4. 24 h immobilization assay with *B. calyciflorus*

This assay was performed according to the standard procedure of Rotoxkit F® (MicroBioTests, Ghent, Belgium). Newly hatched individuals of the rotifer species *B. calyciflorus* were obtained through hatching from cysts at 25°C and a constant light intensity of 3000–4000 lux for 24 h. For SL-5, SL-30 and SL-100 the concentrations tested were 306, 429, 600, 840, 1176, 1646, 2305, and 3227 mg. L^{-1} (Table 1S), all prepared by diluting a stock solution of 4000 mg. L^{-1} with standard freshwater medium (Microbiotest Rotoxkit F

protocol). The SL-60 concentrations used in this assay were 28, 39.3, 55, 76.9, 112, 156, 219, and 306 mg. L^{-1} (Table 1S), obtained by diluting a stock solution of 400 mg. L^{-1} also with standard freshwater medium. For all assays, a control consisting of rotifer standard freshwater medium was carried out. The tests were performed in 24 well plates with a total volume of 1 mL per well. Five replicates were assigned per concentration and control, and 5 newly hatched rotifers were introduced per replicate. Exposure occurred for 24 h, at 23°C in total darkness. At the end of exposure, the number of dead rotifers (organisms exhibiting no movement within five seconds of observation after gentle agitation of the medium) was quantified.

2.3.5. 48 h immobilization assay with *H. incongruens*

The mortality assay with ostracod followed the standard operation procedure for the Ostracodtoxkit F chronic with minor adaptations. Neonates of *H. incongruens* were obtained after the hatching of cysts, obtained from a commercial kit (MicroBioTest, Ghent, Belgium), at 25°C and a constant light intensity of 3000–4000 lux, for 52 h. Then, neonates (< 24 h-old) were exposed to the following concentrations of each SL-variant and to a control (standard freshwater medium; Microbiotest Ostracodtoxkit F protocol): 1646, 2305, 3227, 4518, 6325, 8855, and 12,400 mg. L^{-1} (Table 1S). These concentrations were prepared by diluting a stock solution of 12,500 mg. L^{-1} with standard freshwater medium. Three replicates in a volume of 10 mL, each with ten neonates, were carried out per treatment. Exposure occurred for 48 h at 25°C and total darkness, without food and without standard reference sand (Venâncio et al., 2019). The number of dead organisms was determined at the end of the assay.

2.3.6. 96 h fish embryo acute toxicity test with *D. rerio*

The Fish Embryo Acute Toxicity (FET) test with zebrafish (*D. rerio*) was performed according to the OECD test guideline 236 (OECD, 2013). To perform the assay, the eggs were obtained through natural crossbreeding of fish in aquaria with marbles in the bottom. After 2 h of natural mating, the eggs were carefully collected, rinse in carbon-filtered water used to rear the zebrafish (ZW) and checked the fertilized eggs with a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon, Nikon Corporation, Japan). The unfertilized eggs, with irregularities during cleavage or with injuries or other malformations, were discarded. After 6 h of fertilization, embryos were exposed to eight concentrations of each SL-variant and a control (ZW water): 647, 841, 1093, 1421, 1848, 2402, 3123, and 4058 mg. L^{-1} (Table 1S) prepared in ZW by diluting a stock solution of each SL-variant of 4100 mg. L^{-1}. Per treatment three replicates were carried out, each with ten embryos. Exposure occurred for 96 h in 24-wells microplates filled with 2 mL of test suspension per well and with four internal controls. At 24, 48, 72, and 96 h (26 ± 1°C and 16 h^{L}: 8 h^{D} photoperiod), the eggs were observed and recorded as indicators of lethality up to four apical observations and development abnormalities. Pericardial oedema, yolk sac absorption, lack of equilibrium and tail deformation were the

parameters evaluated. The developmental delay was staged according to Kimmel et al. (1995).

2.4. Statistical analysis

For each studied SL-variant the concentration causing 50% of effect at lethal and sublethal levels [L(E)C_{50}], and the respective 95% confidence limits, was computed for the seven tested species. For *V. fischeri* the EC_{50}s for bioluminescence inhibition were determined by the MicrotoxOmni® Azur software version V1.18 with a linear model (Azur Environmental, 1998). The EC_{50}s for the growth inhibition of *R. subcapitata* and *C. vulgaris*, were determined by adjusting a non-linear logistic model to the data, this was done by using the software package Statistica 8.0 (StatSoft, Inc., Tulsa, USA). The LC_{50}s of *D. magna*, *H. incongruens*, *B. calyciflorus* and *D. rerio* were computed using Probit analysis in the PriProbit software (Sakuma, 1998). The responses obtained by exposing the organisms to the SL-variants concentrations were compared with responses observed in the respective controls with a one-way analysis of variance (ANOVA unifactorial) followed by the multi-comparison Dunnett's test. The assumptions of the ANOVA were checked with the Kolmogorov-Smirnov test for normality of data and Levene's test for homoscedasticity (Zar, 1996). These analyses were done by using the software Sigmaplot version 12.5 (Systat Software, Inc., 2012). Finally, the $L(EC)_{50}$ obtained with the ecotoxicity assays performed for each SL-variant were integrated to construct species sensitivity distribution curves (SSD) and estimate the hazard concentration for 5 of the species (HC_5 safety values protecting 95 the species from an ecosystem), with the respective 95% confidence limits (CL 95%). Both SSD and HCx were generated using the US Environmental Protection Agency (USEPA) spread sheets (SSD Generator V1). It was determined the maximum acceptable concentration environmental quality standard (MAC-EQS) that was computed for each SL-variant using two methodologies: (i) the deterministic, based on the application of an assessment factor of 10 to the lowest L(E)C_{50} computed for the tested species and (ii) the probabilistic, by using the HC_5 values, which were divided by a factor of 1 (European Commission, 2011). The MAC-EQS values obtained for each one of the SL-variant were then further evaluated to be assigned to one of the categories within the classification of the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS; United Nations, 2011). Briefly, in the GHS classification system substances may be assigned to one of three major categories according to their short-term aquatic toxicity: category I if acute toxicity ≤ 1.00 mg. L^{-1}; category II if acute toxicity > 1.00 and ≤ 10.0 mg. L^{-1}; and category III if acute toxicity > 10.0 but < 100 mg. L^{-1} (United Nations, 2011).

3. Results

3.1. Characterization of tested aqueous suspensions

The results obtained for the characterization of SL-variant suspensions, prepared at lowest,

intermediate, and highest tested concentrations for each assay, are displayed in Table 2 and in Table 2S. At Table 2 are presented the results obtained for the particle size (Di0.5), zeta potential and conductivity of the different suspensions. For the higher concentrations, above the overlap concentration of the polymers (please see Table 1), the particle size determination was not performed due to the 3D network formed.

Table 2: Characterization of each variant SL (SL-5, SL-30, SL-60, SL-100) regarding particle size (nm), the zeta potential (ζ-Potential mV) and conductivity (Cond, mS cm^{-1}) at the three tested concentrations (lowest, intermediate and highest; mg. L^{-1}). Characterization was performed in each species respective medium as follows: MTox –*Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*. Values are presented as average values ± standard deviation of n = 3. n.d no data (concentration above the overlap concentration).

	SoftCAT™ SL 5			
	[SL 5] mg. L^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**
MTox	32.0	136 ± 20.5	8.70 ± 1.00	17.8 ± 0.59
	1024	864 ± 78.5	12.6 ± 2.30	4.93 ± 0.70
	10000		n.d.	
MBL	0.70	705 ± 21.9	13.6 ± 0.80	0.51 ± 0.01
	2.30	643 ± 90.8	13.7 ± 0.60	0.56 ± 0.01
	5.20	617 ± 27.3	13.9 ± 0.80	0.57 ± 0.01
ASTM	841	90.1 ± 7.50	20.5 ± 2.80	0.76 ± 0.02
	1646	29.6 ± 4.74	18.5 ± 2.00	0.77 ± 0.02
	3227	17.9 ± 0.17	20.3 ± 1.00	0.85 ± 0.03
RTox	306	48.7 ± 15.7	18.7 ± 4.40	0.23 ± 0.10
	1176	55.7 ± 9.8	19.4 ± 0.50	0.36 ± 0.01
	3227	640 ± 0.71	21.4 ± 2.20	0.52 ± 0.01
HTox	1650	98 ± 27.1	14.8 ± 0.50	0.67 ± 0.02
	4520	10.3 ± 0.21	25.2 ± 2.10	0.78 ± 0.02
	12400		n.d.	
ZF	647	218 ± 69.3	16.0 ± 1.10	0.96 ± 0.03
	1848	23.9 ± 2.97	15.7 ± 2.80	1.10 ± 0.04
	4058	7.57 ± 0.26	22.6 ± 2.00	1.26 ± 0.06

	SoftCAT™ SL 30			
	[SL 30] mg. L^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**
MTox	32.0	791 ± 146.0	13.1 ± 1.40	11.7 ± 0.33
	1024	1200 ± 84.9	12.7 ± 1.80	5.09 ± 0.76
	10000		n.d.	
MBL	0.70	541.0 ± 29.8	12.2 ± 1.10	0.56 ± 0.01
	2.30	757 ± 49.5	12.8 ± 0.50	0.56 ± 0.01
	5.20	616 ± 92.2	13.6 ± 0.40	0.57 ± 0.01
ASTM	600	981 ± 168	13.9 ± 0.70	0.68 ± 0.02
	1646	1200 ± 14.1	16.0 ± 1.30	0.74 ± 0.02
	4518		n.d.	
RTox	306	570 ± 60.8	12.1 ± 1.80	0.32 ± 0.00
	1176	421 ± 72.8	19.3 ± 0.00	0.37 ± 0.01
	3227	786 ± 107	24.4 ± 1.60	0.50 ± 0.01
HTox	1650	9.98 ± 0.45	26.8 ± 4.60	0.60 ± 0.02
	4520		n.d.	
	12400		n.d.	
ZF	647	705 ± 82.0	15.5 ± 0.90	1.03 ± 0.04
	1848	59.7 ± 2.6	16.8 ± 1.60	1.11 ± 0.04
	4058	8.4 ± 0.13	24.2 ± 2.00	1.27 ± 0.06

	SoftCAT™ SL 60					SoftCAT™ SL 100			
	[SL 60] mg. L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})		[SL 100] mg. L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})
MTox	32.0	847 ± 94.1	9.70 ± 1.00	9.31 ± 0.16	MTox	32.0	686 ± 90.5	6.80 ± 0.50	8.13 ± 0.14
	1024	485 ± 26.2	13.3 ± 1.50	4.82 ± 0.68		1024	511 ± 33.9	13.2 ± 2.30	4.75 ± 0.70
	10000		n.d.			10000		n.d.	
MBL	0.70	849 ± 27.1	13.9 ± 1.10	0.56 ± 0.01	MBL	0.70	834 ± 134	13.6 ± 0.50	0.54 ± 0.01
	2.30	697 ± 48.6	14.6 ± 0.50	0.57 ± 0.01		2.30	778 ± 56.4	14.2 ± 0.80	0.56 ± 0.01
	5.20	861 ± 96.1	15.0 ± 1.50	0.53 ± 0.01		5.20	851 ± 124	15.0 ± 0.60	0.57 ± 0.01
ASTM	600	888 ± 84.9	13.6 ± 1.30	0.70 ± 0.02	ASTM	600	194 ± 43.8	11.7 ± 2.00	0.63 ± 0.02
	1646	73.1 ± 1.6	14.3 ± 1.80	0.75 ± 0.02		1646	116 ± 47.4	14.9 ± 1.10	0.72 ± 0.02
	4518		n.d.			4518		n.d.	
RTox	28.0	349 ± 46.3	13.3 ± 1.20	4.87 ± 0.55	RTox	306	440 ± 29.0	12.3 ± 1.10	0.31 ± 0.00
	108	277 ± 54.0	12.8 ± 1.40	5.29 ± 0.50		1176	604 ± 64.1	12.3 ± 1.50	0.34 ± 0.01
	260	343 ± 37.1	15.9 ± 2.20	5.34 ± 0.49		3227		n.d.	
HTox	1650	7.22 ± 0.04	28.7 ± 2.10	0.59 ± 0.01	HTox	1650	193 ± 210	22.5 ± 1.10	0.50 ± 0.01
	4520		n.d.			4520		n.d.	
	12400		n.d.			12400		n.d.	
ZF	647	1146 ± 63.6	15.1 ± 0.70	1.01 ± 0.04	ZF	647	477 ± 87.0	14.7 ± 1.50	1.02 ± 0.04
	1848	47.7 ± 4.17	15.6 ± 1.00	1.11 ± 0.04		1848	43.6 ± 8.8	16.6 ± 1.10	1.09 ± 0.05
	4058		n.d.			4058		n.d.	

As expected, the zeta potential of the suspensions prepared in test media were lower than the obtained in distilled water, due to the presence of several salts in the test media. It is known that the excess of salts results in changes in the particle double layer reducing the overall effective net charge and introducing some instability in the suspensions. The presence of salts in the test media also induces some changes on the particle size of the suspensions. Different effects can be expected depending on the media composition and the polymer architecture. Comparing the results obtained for particle size of the four polymer architectures in distilled water and test media is possible to observe, tendentiously, an increase when the MBL test medium is used. This can be attributed to the presence of divalent anions in the composition of the medium (sulphate and EDTA), which strongly interact with the cationic groups of the polymer. It is known that divalent ions present higher affinity to interact with polyelectrolytes than monovalent ions (Ghimici and Dragan, 2002). It was reported that this kind of interactions can result in a decrease of the reduced viscosity due to the strong interaction of divalent anions with cationic groups and consequent intra and intermolecular bridging effect. Thus, depending on the polyelectrolyte architecture and concentration, opposite trends in particle size evolution can be expected. For low SL-variants concentrations, a reduction in particle size can be observed due to the formation of intra-molecular bridging and consequent contraction of the polymer particle when the polymer is dispersed in the MBL test medium. On the other hand, an increase in particle size is observed for the higher concentration of SL-5, when suspended in MBL test medium, due to the formation of intermolecular bridging. Moreover, the polymer architecture also plays an important role on the particle size of the polymers in the suspensions. For SL-variants with lower HS, SL-5 the increase of particle size due to the presence of salts (comparing the results obtained in distilled water and test media) is smaller than the observed for SL-variants with higher HS, as SL-100. This can be attributed not only to the inter- and intra-molecular bridging induced by the salts, but also to the hydrophobic interaction, which results in higher aggregation and consequent bigger particles are formed. For example, the Di0.5 of the polymer SL-5, for the concentration of 5.20 mg. L^{-1}, in distilled water, was 510 nm and for the same concentration in MBL test medium was 617 nm. Conversely, for the polymer SL-100 the Di0.5 in distilled water (for the concentration of 5.20 mg. L^{-1}) was 345 nm and in the MBL test medium was 851 nm. For the SL-5, the particle size increase was about 20%, but for the case of SL-100 the size increment was ca. 250%.

The conductivity of the different SL-variants increased with the increase of the concentrations. For example, SL-100 in *B. calyciflorus* species varied from 0.314 to 0.34 mS cm^{-1} from the lowest to the highest concentration (Table 2). This was an expected result, since at higher concentrations more ions will be present in the solutions. As expected, conductivity values were higher in SL-variants concentrations suspended in test media comparatively to those suspended in distilled water, which is mainly due to the higher conductivity of the test media.

The SL-variants showed high solubility in the tested media, as no precipitates were observed for any

tested concentration (lowest, intermediate and highest) after subjected to high gravitational acceleration (c.a. 18,000 g) and at all exposure periods, matching the duration of the ecotoxicological assays. Thus, the tested polymers were considered as fully soluble in the tested media, for the studied concentrations, as the ratio between the amount of precipitated polymer and the amount of polymer added to the test medium was closed to zero.

3.2. Rheological measurements

The flow curves of the tested concentrations of the four SL-variants, in the corresponding medium and in distilled water, are displayed in Figs. 2 and 1S. All the SL-variants exhibited a well-defined Newtonian region at low shear stress values, except when the different polymers were dissolved in MBL medium (*R. subcapitata* and *C. vulgaris* assays) (Fig. 2). In Figs. 2 and 1S, no significant differences were observed among the four SL-variants. However, when suspended in distilled water (Fig. 1S) and depending on the concentration tested for *V. fischeri*, it was possible to observe significant differences between the lowest (around 0.001 Pa. s) and highest tested concentration (around 1 Pa.s), higher than one order of magnitude. In relation to the other concentrations tested from the other species, the values of shear viscosity obtained were very similar between them.

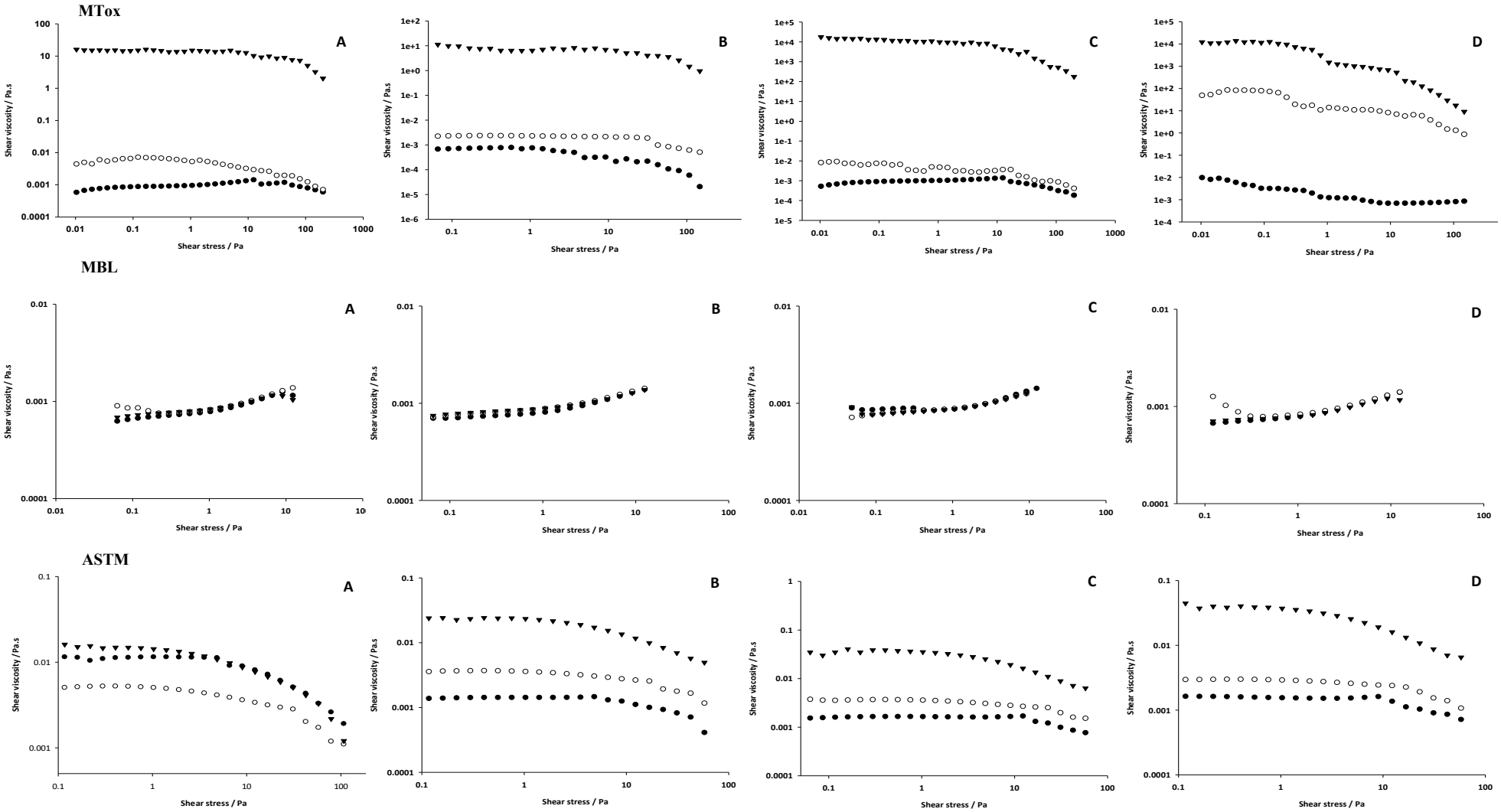
In relation to the results obtained for the shear viscosity in SL variants suspended in the different media tested, these were very similar to the ones obtained for distilled water (Fig. 2), except for the tested concentrations for the *V. fischeri* medium where an increase in viscosity from ca. 1.0 Pa.s (in distilled water) to ca. 10.0 Pa.s for SL-5 and SL-30, and to ca. 10.000 Pa.s for SL-60 and SL-100 (Figs. 2 and 1S) was observed. For the variant SL-100 also the intermediate polymer concentration presented a significant increase in viscosity when suspended in MBL test medium. These results clearly put in evidence that polymer architecture is crucial on the rheological behavior of the tested polymers. Also, it is important to highlight that these two-test media, *V. fischeri* medium and MBL medium, are the ones with the higher ionic strength of all the tested media; since the polymer chain conformation and the associated rheological behavior are related with the counterion entropy, the physicochemical properties of the suspensions are expected to be sensitive to electrolyte addition. For the SL variants with higher HS, the presence of salts in solution lead to a decrease in the charge effect (screening of charges), induced by the salt, and consequently to polymer aggregation, being more pronounced for the SL-variants with higher HS, where it was observed a larger increase in the viscosity values. Similar results were obtained in other systems with the increase of hydrophobicity of the used polymers, at different ionic strengths (Alves et al., 2015). It was also possible to observe that the variant SL-5 suspended in *V. fischeri* medium was the one that presented lowest values of shear viscosity in relation to the other SL-variants suspended in the same medium.

3.3. Acute toxicity assays

All the ecotoxicological assays that were performed accomplished the validity criteria established by the respective guidelines or protocols (Azur Environment, 1998; OECD, 2004, 2006, 2013; Rotoxkit F™ Acute Microbiotests and OstracodToxKit F™ chronic). The results obtained with the acute toxicity assays revealed that the rate of hydrophobic substitutions influenced the toxicity of the tested SL-variants, and that, it was species dependent.

For the bacterium *V. fischeri*, SL-5 exerted the highest toxicity ($EC_{50,15min}$: 9645 mg. L^{-1}). For other the three variants, SL-30 exhibited the highest $EC_{50,15min}$ (977,619 mg. L^{-1}), suggesting being the least toxic variant. However, as the 95% confidence limits overlapped with the ones of $EC_{50,15min}$ computed for SL-60 and SL-100, it is assumed that these three SL-variants exhibited similar toxicity to *V. fischeri* (Table 3S).

Regarding the results obtained for the two species of microalgae, all the tested concentrations of the four SL-variants induced a significant reduction in the growth rate of *R. subcapitata* and *C. vulgaris* (Fig. 2S). Though, the two species revealed different sensitivities to the SL variants. For *R. subcapitata* the variant SL-100, with the highest level of hydrophobic substitutions, with the highest resistance shear forces (i. e., higher viscosity), exerted the highest toxicity ($EC_{50,72h}$: 13.9 mg. L^{-1}). The other three SL-variants, though exhibiting different $EC_{50,72h}$, their 95% confidence limits overlap, suggesting similar toxicities. For both algae, the variant SL-5 exhibited the highest $EC_{50,72h}$, but, for *C. vulgaris*, the confidence intervals of the ECs of all SL-variants overlapped, indicating similar toxicities (Table 3S). The differential sensitivity between these two species, that belong to the same functional level (microalgae), might be partially explained by their physiological characteristics. Usually, *C. vulgaris* besides having the ability of producing extracellular compounds (that may buffer against external stresses), can form colonies which enables it to protect younger cells and continue to growth under adverse conditions (Watanabe et al., 2005; Luo et al., 2010), whilst *R. subcapitata* cells appear to be solitary and thus, more exposed to the presence of higher viscosity compounds that deaccelerate their growth rates. Furthermore, the higher viscosity shown by SL-100 could have a higher influence in the toxicity of this chemical to *R. subcapitata* (solitary), by promoting the aggregation of the algal cells. While, for *C. vulgaris*, being a species that form colonies, this effect would not be as critical for their performance.


MTox
A
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Shear viscosity / Pa.s
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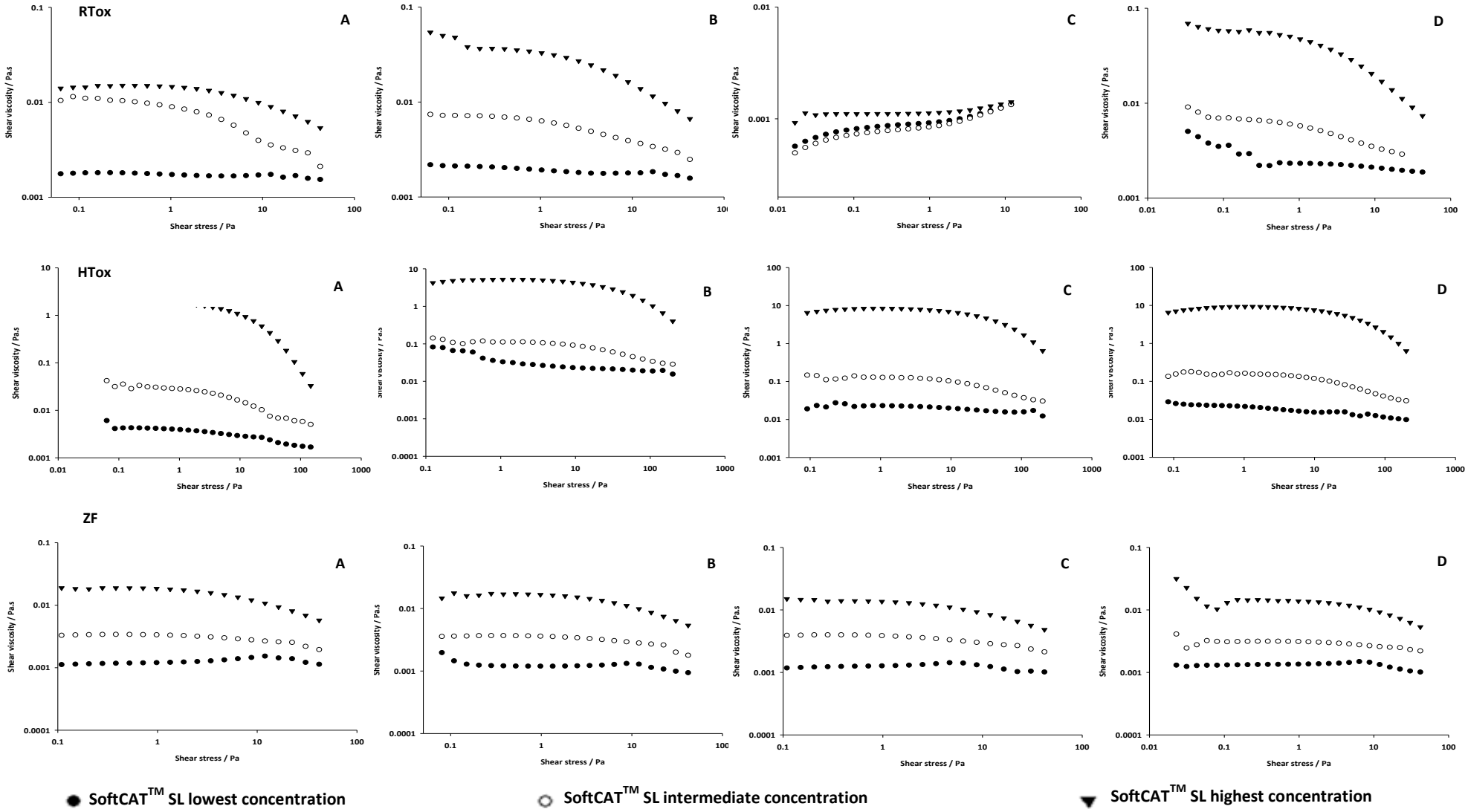

Fig. 2. Flow curves, viscosity (η) as function of shear stress (τ), of the concentrations used in the different species assays of SL-variants at 25°C (A- variant SL-5, B – variant SL-30, C – variant SL-60 and D – variant SL-100). Shear viscosity was evaluated in each species respective medium as follows: MTox – *Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*.

Regarding the primary consumers *D. magna* and *H. incongruens*, the four SL-variants showed similar lethal toxicity, with $EC_{50,48h}$ ranging from 685 to 1173 mg. L^{-1} for *D. magna* and ranging from 5465 to 8437 mg. L^{-1} for *H. incongruens* (Table 3S; Figs. 3S and 5S, respectively). For the rotifer *B. calyciflorus*, the variant SL-60 exerted the highest toxicity ($LC_{50,24h}$ 39.1 mg. L^{-1}), while the variant SL-5 showed to be the least toxic ($LC_{50,24h}$ 484.9 mg. L^{-1}) (Table 3S; Fig. 4S).

In the assays with *D. rerio* fish, estimated $LC_{50,96\ h}$ indicated that variants SL-30 and SL-60 were the most toxic variations with values of $LC_{50,96h}$ of 263.6 and 425.9 mg. L^{-1}, respectively (Table 3S; Fig. 6S). Almost all the concentrations tested for the SL-variants originated deformations on the embryos relatively to control conditions (Fig. 7S). Namely, they induced several developmental malformations in the tail (Fig. 7Sa). For instance, variants SL-5 and SL-60 induced almost 50% of tail malformations at 2401 mg. L^{-1}, and SL-5 almost 70% at 3123 mg. L^{-1} (Figs. 7Sa and 8S). The variants SL-60 and SL-100 induced malformations in almost all concentrations tested in what concerns the yolk sac adsorption (no indication of reduction of the yolk sac) and caused equilibrium loss (Fig. 7Sb and 7Sc, respectively). Finally, most of pericardial oedemas observed were registered on fish larvae exposed to variants SL-5 and SL-30 at the lowest tested concentrations (Figs. 7Sd and 8S).

3.4. Species sensitivity distribution curves and MAC-EQS derivation

The toxicity for each SL-variant for each of the seven species, estimated as the $L(E)C_{50}$, was integrated into species sensitivity distribution curves (SSD; Fig. 3), from which the respective hazard concentrations (HCx), aiming to protect 5% of the species, were computed (Table 3; Fig. 3). The estimated HC_5 indicated that the variant SL-5 was the least toxic variation with an HC_5 value of 95.5 mg. L^{-1} (CL 95%: 38.5–237.1); however, CL 95% overlapped with those of HC_5 (CL 95%) for variant-SL 30: 2.9 mg. L^{-1} (0.02–366.3). Overall, the unicellular microalga (*R. subcapitata* and *C. vulgaris*) together with the rotifer *B. calyciflorus*, formed the group of the most sensitive species (Fig. 3); whilst *V. fischeri* and *H. incongruens* the group of the most tolerant species to all SL variants (Fig. 3). In Table 3 are summarized the maximum acceptable concentration environmental quality standards (MAC-EQS) computed for each SL variant through two methodologies. The deterministic methodology - determination of MAC-EQS by lowest $L(E)C_{50}/10$ – was the most conservative for variants-SL-5, SL-60 and SL-100, as it presented the lowest values (Table 3). The probabilistic methodology – using the HC_5 estimated from SSD curves - proved to be the most conservative approach only for SL-30 (Table 3). Considering this, the predicted concentrations putting at risk the aquatic compartment would be higher than 14.0 mg. L^{-1} for variant SL-5, 2.9 mg. L^{-1} for variant SL-30, 3.9 mg. L^{-1} for variant SL-60, and 1.4 mg. L^{-1} for variant SL-100.

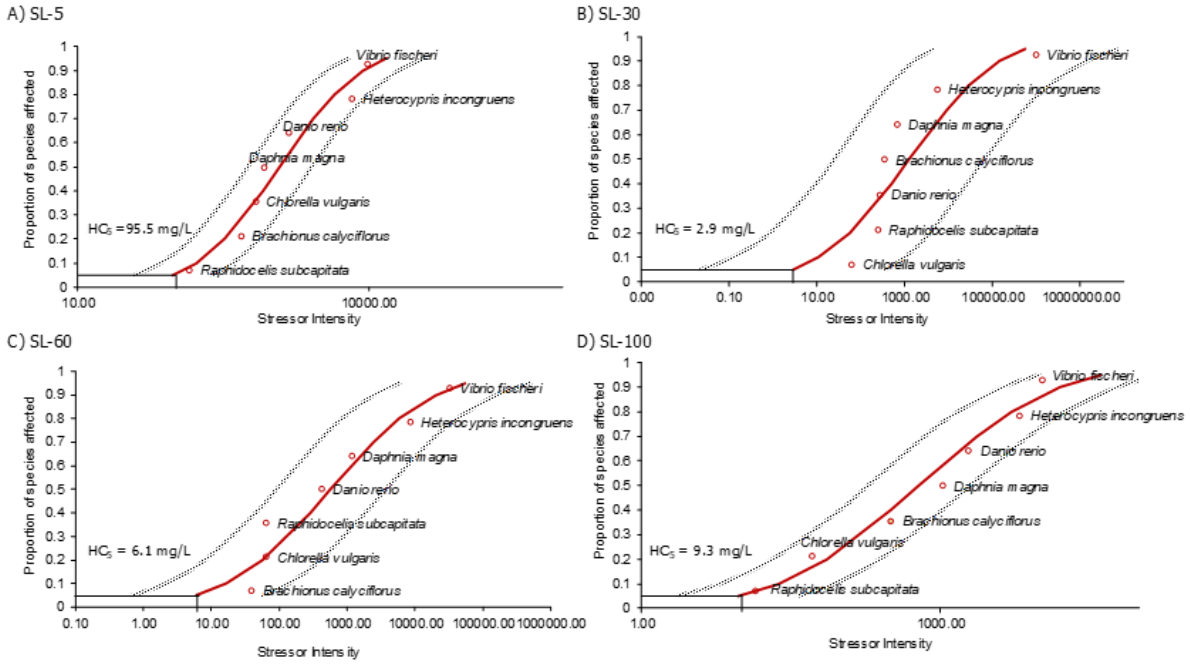

Fig. 3. Species sensitivity distribution curves (SSD) constructed for the four SL-variants, where HC_X denotes the hazard concentration that affect X % of the species, R^2 refers to the coefficient of determination of the curve, N is the number of data points. A – SSD for variant SL-5, B – SSD for variant SL-30, C – SSD for variant SL-60, and D - for variant SL-100.

Table 3. Maximum acceptable concentrations environmental quality standard (MACEQS), for the four SL-variants, computed by applying a safety factor of 10 to the lowest median lethal or effective concentration [$L(E)C_{50}$, mg. L^{-1}] and by determination of hazard concentrations that protect 95% of the species (HC_5) through species sensitivity distribution curves.

	SoftCAT™ SL variation			
	SL-5	**SL-30**	**SL-60**	**SL-100**
Lowest $L(E)C_{50}$/10 (mg. L^{-1})	14.0	6.2	3.9	1.4
HC_5 (mg. L^{-1})	95.5	2.9	6.1	9.3

4. Discussion

The present work aimed to study the influence of polymer architecture, namely hydrophobic substitutions on the ecotoxicity of quaternized hydroxyethyl cellulose polymers (SoftCAT™ SL) to several aquatic species. Complementarily, a thorough physicochemical characterization of the same polymers SoftCAT™ SL was performed giving a more comprehensive and structured conclusion regarding their potential hazard to the environmental compartment.

Due to their hydrophobic nature, it was expected a higher tendency for polymer association with the increase of polymer concentration (i.e., induce the formation of larger aggregates), as already stated by other authors (Carneiro-da-Cunha et al., 2011). Moreover, considering that the variant SL-100 presented more hydrophobic groups, it was also expected for it to aggregate more, comparatively to the other polymers tested due to their lower aqueous solubility, i.e., variant SL-100 was expected to have a higher average particle size, whilst variant SL-5 the lowest one. Although, a pattern could not be fully established between average size and the number of hydrophobic groups, and/or average size and concentrations. However, a clear trend was observed for the particle size increment of SL-variants with high HS when suspended in distilled water and test media. It was found that the increase in particle size was much more pronounced for polymers with high HS (variant SL-100) than for the polymers with low HS (variant SL-5). This can be attributed not only to bridging effects induced by the presence of salts, but also to the hydrophobic association among hydrophobic side substitutions present along the polymer chain. Yet, results here obtained showed that, overall, the size of all SL-variants particles followed these tendencies: i) when the analysis was performed in distilled water, the size of the particles was higher at the lowest tested concentrations; ii) the same tendency was observed when polymers size was evaluated in each tested media. It is known that the stability of the polymers depends on many factors, namely on the composition of the tested media. One might hypothesize that, at lower concentrations, particles tend to aggregate bearing in their core the hydrophobic structures (nonpolar), while polar (charged) regions are left on the outside (Moelbert et al., 2004). Thus, at low concentrations, hydrophobicity might be the main driver leading to particles aggregation (larger sized particles). However, as concentration increased, two scenarios might be influencing the presence of smaller aggregates. On one hand, the probability of contact between polar regions also increases, thus, other forces rather than hydrophobicity might have a preponderant role at this stage, namely repulsive forces. For instance, Song et al. (2009) attributed the high homogenous dispersion of quaternized cellulose (QC) nanoparticles to the electrostatic repulsion forces induced by the positive charges at the surface of those same QC nanoparticles (Song et al., 2009). On the other hand, the complexity of media composition might have a parallel preponderant role in the formation of smaller aggregates with increasing concentration of the variants-SL particles. This behavior, particle size decrease with increasing concentrations, has already been reported for instance by Martins et al. (2018) when studying seven variations of ether sulphate-based surfactants. The addition of salts (such as KCl, $CaSO_4$ or $MgCl_2$) into water to form the media (MBL, ASTM, others), immediately leads to

their dissociation in ions. Those ions may interact with the hydroxyl groups of the polymeric chain of the SL-variants. The presence of free ions of chloride (Cl^-), sulphate (SO_4^{2-}) or nitrate (NO_3^-) might have a significant role as sinks for hydroxyl groups (Anastasio and Newberg, 2007). Jonassen et al. (2012) evaluated the effect of the ionic strength of the solvent on the average size of chitosan nanoparticles. Chitosan and cellulose (variants-SL are derived from cellulose) for instance possess a similar molecular structure, sharing the same beta-glycoside linkage, whilst differing in their functional groups, amino (chitosan) and hydroxyl (cellulose). The ionic strength of the media was simulated by the addition of a salt at two levels (NaCl, 0.05 and 0.15 M), using as reference water, and two polymer concentrations were tested (0.05% and 0.10% chitosan). Results obtained by Jonassen et al. (2012) clearly showed that the apparent hydrodynamic size of chitosan nanoparticles was decreased in the 0.05 M NaCl solution than when in water. However, when comparing both NaCl levels, the particles obtained in the highest ionic solvent (0.15 M NaCl) were bigger than at 0.05 M NaCl, suggesting that ionic strength of the media only exert effect until a certain threshold.

Moreover, zeta potential of the SL-variants particles in distilled water remained practically unchanged (with some minor exceptions), following the same tendency in all test media. However, it decreased in the test media comparatively to distilled water. Despite, the surface charge of SL-variants remained positive. As so, the probability for the variants-SL polymers to bind to negatively charge surfaces is high, thus, meaning that their potential ecotoxicity is also high. As negatively charged surfaces, the cell membranes are a prone anchorage point to these positively charged polymers (SoftCAT™ SL). Several studies have already stated the greater toxicity of cationic compounds in relation to anionic ones (e.g., Goodman et al., 2004; El Badawy et al., 2010; Calienni et al., 2017; Nolte et al., 2017). Cationic structures, such as SL-variants, might therefore destabilize cell membranes by interfering with key signalling pathways, later leading to irreversible effects such as cell death and/or lysis (Goodman et al., 2004; Calienni et al., 2017). The solubility results showed that the SL-variants were extremely soluble in each medium tested. This is conferred by the cationic charges imparted along the hydrophobic backbone. It was reported by Mu et al. (2017), that the solubilizing efficiency depended on the polymer architecture.

The ecotoxicological evaluation of the SL-variants did not allowed to infer on any pattern relating superficial charge, average size, and toxicity. In this study, the main factor for the determination of the toxicity of SL-variants might probably be due to the routes of exposure or ingestion of these polymers or their degradation or metabolization by the organisms. Overall, the two freshwater algae species (*R. subcapitata* and *C. vulgaris*) formed consistently (with the rotifer *B. calyciflorus*) the most sensitive group of organisms to the SL-variants, as shown by the SSD curves. Other research works have also reported unicellular microalgae as the most sensitive organisms. For instance, Pereira et al. (2018) reported *R. subcapitata* as one of the most sensitive eco-receptors (out of a total of 4) to five variations of cationic acrylamide-based polyelectrolytes (cPAM), whilst Cumming (2008) study comprising 22 polyquaterniums polymers reported that *C. vulgaris* was the most sensitive species (out of a total of 3). Furthermore, the

increment on the number of hydrophobic groups (variant SL-100) provoked a greater toxicity to these unicellular organisms (*R. subcapitata* and *C. vulgaris*) comparatively to the other SL-variants. On one hand, it is possible that the size of the polymers is a restriction on their entry into the cell (Pereira et al., 2018); on the other hand, the viscosity increment (shown by rheological measurements as the increase resistance to shear forces) must be also considered. It is likely that these polymers are adsorbed in large quantities on the surface of cells (negatively charged) due to their positive charge (Nolte et al., 2017), thus preventing photosynthesis and later impair algae growth rates. It was already observed that algae cell wall is permeable to hydrophobic polymers, being absorbed or disrupting the thylakoid membrane of algae (Nolte et al., 2017). Due to the particle sizes of the polymers studied in this work, it seems that they exert their toxicity in the algae outer cell wall or through inhibition of the photosynthesis, as already reported by Nolte et al. (2017). In addition, the higher viscosity associated with increasing HS substitution, might influence nutrient and respiration circulation around the cells, and lately, intracellular metabolic mechanisms, as studied for other groups of organisms (e.g., Rombough, 1998; Serra et al., 2019).

Hydrophobic compounds possess the ability to accumulate in organisms due to their lipophilicity, mainly through membrane absorption through species gills and skin and by intestinal wall absorption after predation/ingestion of these compounds through gastrointestinal tract (Xia et al., 2015). It has been observed that the hydrophobic substitution plays a key role on the binding to the biological lipid membranes and further induce subsequent endocytosis (Liu et al., 2010). The hydrophobic modifications of polymers can enhance their absorption to the cell membrane and facilitate the absorptive endocytosis. According to Tripathy et. al. (2018), the toxicity of cationic surfactants is related to the tendency to disrupt the integral membrane through the ionic interaction of the surfactant at the cell-water interface. Since the increase of the length of a hydrophobic chain lowers the cmc of the surfactants and increase their absorption onto interfaces, this may result into an increase of the toxicity of these compounds.

For pluricellular organisms, it was observed a decrease of toxicity of the SL-variants with the highest number of hydrophobic groups (variant SL-100). In the assay with *D. rerio*, *D. magna* and *H. incongruens*, the variants-SL with higher number of hydrophobic groups (variants SL-60 and SL-100) were the least toxic. Despite, increasing HS substitution from 5 to 30 increased the compound toxicity, which might be explained by rheological properties of the polymers that showed that with HS, the size of the aggregates also increased as well as their viscosity. The contact and adhesion of the cationic SL-variants polymers with respiratory structures (gills), swimming and/or feeding appendages of the organisms might be a primary action mode of the particles due again to the interaction between opposed charges, as above discussed for algae and confirmed for other species exposed to cationic polymers (e.g., Muir et al., 1997). Yet, as discussed above for algae, the increased viscosity of the media (from SL-5 to SL-30) might also influence the polymer ecotoxicity. *Daphnia's* swimming behavior, for instance, is characterized by a hopping movement that might be hampered by medium flow. Serra et al. (2019) has demonstrated that medium shear stress above 1 $cm^2\ s^{-3}$ lead to suppression of *D. magna* feeding, and, later, mobility of the

individuals. *Danio rerio*, despite the inactivity or very low movement during the egg and embryos' stage, respectively, might be also affected by increase thickness of the medium, since viscosity might reduce respiration rates around the egg jelly or embryo (Rombough, 1998). Though, other routes of exposure, such as ingestion and/or internalization in cells, should also be considered. As filter-feeders (*D. magna* and *H. incongruens*) the possibility of ingestion of aggregates up to 50 µm in size is a possibility (e.g., Martín-de-Lucía et al., 2019). For instance, Martín-de-Lucía et al. (2019), through fluorescent tracking techniques, followed the ingestion and internalization of a commercial hyperbranched nano polymer (designated as Helux-3316), using *D. magna* as model. As soon as daphnids became in contact with the Helux-3316 nano polymer, it adsorbed immediately at the surface of the organisms; after 48 h of exposure to Helux-3316, the entire digestive tract and some surrounding tissue of the daphnids was intense blue, indicative of the presence of the nano polymer (Martín-de-Lucía et al., 2019). Once inside the tissues and/or cells, these cationic polymers might induce the formation of reactive oxygen species, that in turn may lead to the inflammation of tissues, growing of tumors or other malformations. The results here presented regarding the model fish *D. rerio* are example of such sublethal alterations. For instance, at a concentration of 600 mg. L^{-1} of variant SL-5 no mortality was registered for daphnids even after 48 h of exposure. A slightly higher concentration of the same polymer (647 mg. L^{-1} of variant SL-5) was able to induce several malformations in *D. rerio* larvae after 96 h of exposure, namely more than 60% of the larvae presented loss of equilibrium and almost 20% presented oedemas surrounding the pericardial region. Even if mortality is not registered, these polymers might induce effects that decrease organisms' performance and can certainly reduce their probabilities of reaching adulthood.

Despite the high demand for these products in daily routines and their disposal "down the drain", no regulatory structure has been developed so far to assess their ecotoxicity. Along with, there appears to be a considerable flaw with respect to measured environmental concentrations (MEC), with none found in the literature regarding the SL-variants polymers here studied. Furthermore, and according to the GHS classification and labelling of chemicals, all these variants would fall into category II which indicates that they all are acutely toxic to aquatic biota (United Nations, 2011). But, considering the highest estimated MAC-EQS, the variant SL-5 showed to be the least toxic variant and, thus, suggested as a greener alternative to the other variants. Regardless of that, and comparatively to the sparse and very fragmented works found relatively to other polymers, it must be highlighted the need for industry to ally itself with science in order to minimize downstream risks. Martínez-Carballo et al. (2007) reports MEC values for the cationic surfactant BAG-12 (dodecyl dimethyl benzyl ammonium chloride) up to 2.1 and 2.8 mg. L^{-1} in laundries and hospitals effluents of Austria, respectively; whilst (Cumming, 2008) estimations point to predicted environmental concentrations (PEC) of polyquaterniums polymers in Australian waters to be between 0.7 and 40 µg L^{-1}. Looking at the MAC-EQS derived for variant SL-5 of 14.0 mg. L^{-1} (the greener alternative), it is possible to understand that the potential to induce effects in the aquatic ecosystems to which they are discharged is still present. As an example, Tamura et al. (2017) studied the toxicity of water

samples from an urban-dominated stream effluent of Kyoto on daphnids and fish. The alkylbenzene sulfonic acid (LAS), as an indicator of the presence of PCP products, accounted for only 5.3% of the whole effluent. However, when testing the LAS only, their toxicity to daphnids went up to 86% and to zebra went up to 27%. This means that PCP products might contribute substantially to the toxicity of the effluents.

The Directive 2013/39/EU, amending the Directive 2008/105/EC highlights that the collection of supporting quality data as well as their (eco)toxicological effects is of utmost importance in order to justify the inclusion of these products in the new priority substances list. Altogether, the physical-chemical and ecotoxicological characterization presented here for the variants-SL in the context of "safety-by-design" represents a step forward in the development of more comprehensive regulatory frameworks for PCP products.

5. Conclusions

The fine-tuning of the characteristics of the polymers aiming at their reduced or no toxicity to the environment, but without loss of functionality, must go through the joint analysis of their physic-chemical characteristics and their ecotoxicity. No patterns between polymers surface charge or size and toxicity could be found. From the several taxonomic and functional groups evaluated, the most sensitive ecological receptors to the SL-variants were the producers *R. subcapitata* and *C. vulgaris* and the primary consumer *B. calyciflorus*, being suggested to be used in toxicity assaying at initial stages of the development process of more environmentally friendly cellulose base cationic polymers. Adding to the high sensitivity, these assays are also rapid and require low volumes of test solutions, which constitute extra advantages when performing toxicity assays at early stages of the development process. Overall, the variant SL-5 (with lower number of hydrophobic substitutions) revealed to be the least toxic variation according to the derived MAC-EQS of 14.0 mg. L^{-1}, whilst variant SL-100 (the one with highest number of hydrophobic substitutions) presented the lowest MAC-EQS. Thus, it might be suggested that the toxicity differed with the architecture of the polymers and variant SL-5 is the most eco-friendly variant. Considering that the scientific literature reports that SL compounds with low HS may significantly improve the desired conditioning performance of PCP, it is suggested that industry should invest in the development of SL compounds with HS lower than 30, aiming a high efficiency while minimizing the ecological risks.

Considering the deep gap in the literature on the environmentally measured concentrations of cationic polymers to which we could compare the MAC-EQS obtained for variant SL-5 (most eco-friendly variant), it should not be overlooked that effects on aquatic ecosystems might still be detected. This work provides valuable information on these contaminants of emerging concern and may be instrumental in establishing regulatory guidelines aimed at protecting aquatic ecosystems. Further studies should be encouraged and are necessary to explore the fate of these polymers in natural matrices as well as possible cumulative effects since they are continuously used and release through human daily routines.

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Supplementary information

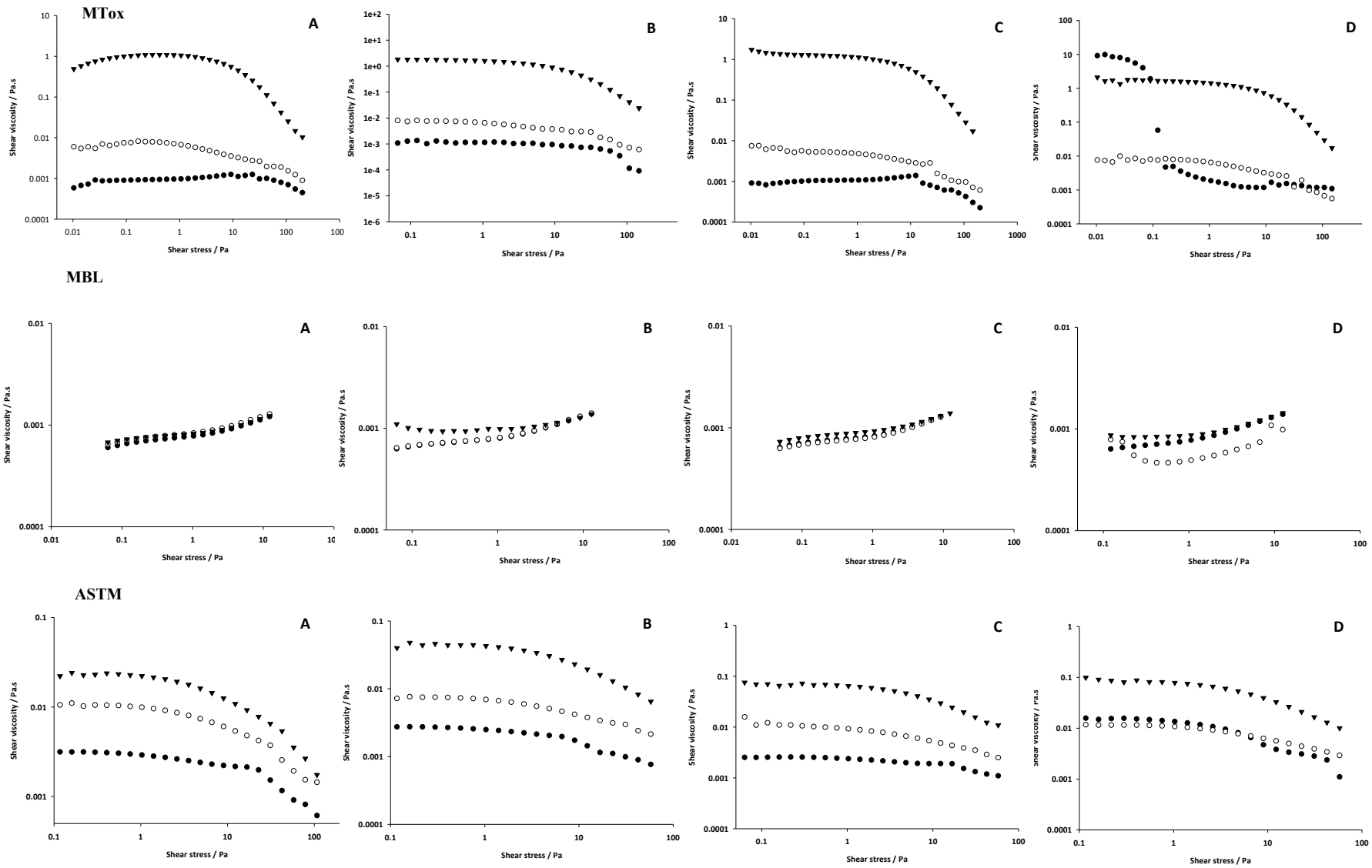
Table A.1: Summary of the procedures and conditions used to perform the ecotoxicity assays.

Test species	Exposure time	Endpoints	Dilution water	Light:Dark (L:D hours) Temperature (°C)	Concentrations tested mg. L^{-1}	Reference
Vibrio fischeri	15 min	Production of luminescence	Diluent	4 ± 1°C	32.0, 64.0, 128, 256, 512, 1024, 2048, 4095, and 8190	Microtox® protocol; Microbics Inc., USA
Chlorella vulgaris ***Raphidocelis subcapitata***	72 h	Growth rate	MBL medium	24^{L}:0^{D} h 23 ± 1°C	0.68, 1.03, 1.54, 2.31, 3.47, and 5.20	OECD, 2004: Guideline 201
Daphnia magna	48 h	Mortality	ASTM medium	16^{L}: 8^{D} h 20 ± 1˚C	For variant SL 5: 841, 1176, 1646, 2305, and 3227. For variants-SL 30, SL 60, SL 100: 600, 841, 1176, 1646, 2305, 3227, and 4518.	OECD, 2004: Guideline 202
Brachionus calyciflorus	24 h	Mortality	Standard Freshwater medium	0^{L}:24^{D} h 23 ± 1˚C	For variant-SL 60: 28.0, 39.3, 55.0, 76.9, 112, 156, 219, and 306.For variants-SL 5, SL 30, SL 100: 306, 429, 600, 840, 1176, 1646. 2305, and 3227	Rotoxkit F®, MicroBioTests, Ghent, Belgium
Heterocypris incongruens	48 h	Mortality	Standard Freshwater medium	0^{L}:24^{D} h 25 ± 1˚C	1176, 1646, 2305, 3227, 4518, 6325, 8855, and 12400	Ostracodtoxkit F chronic adapted in Venâncio et al., 2019; MicroBioTests Inc.
Danio rerio	96 h	Mortality, malformations, behaviour	zebrafish water	16^{L}:8^{D} h 26 ± 1˚C	647, 841, 1093, 1421, 1848, 2402, 3123, and 4058	OECD, 2013: Guideline 236

Table A.2: Characterization of each variants-SL (SL 5, SL 30, SL 60, SL 100) regarding particle size (nm), the zeta potential (ζ-Potential, mV) and conductivity (Cond, mS cm^{-1}) at the three tested concentrations (lowest, intermediate and highest; mg. L^{-1}). Characterization was performed in distilled water (dH2O) as follows: MTox –*Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*. Values are presented as average values ± standard deviation of n=3. n.d. – no data (concentration above the overlap concentration).

	SoftCAT™ SL 5					SoftCAT™ SL 30			
	[SL 5] mgL^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity ($mS.cm^{-1}$)**		**[SL 30] mgL^{-1}**	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity ($mS.cm^{-1}$)**
MTox	32.0	810 ± 39.6	26.6 ± 1.6	0.198 ± 0.041	MTox	32.0	827 ± 2.8	31.2 ± 2.0	0.200 ± 0.002
	1024	822 ± 48.1	30.2 ± 1.1	0.116 ± 5.77e-4		1024	595 ± 24.7	26.4 ± 4.1	0.150 ± 0.002
	10000		n.d.			10000		n.d.	
MBL	0.70	869 ± 55.5	25.7 ± 4.6	0.0151 ± 0.005	MBL	0.70	752 ± 17.7	16.0 ± 3.5	0.0464 ± 0.002
	2.30	703 ± 62.6	27.1 ± 5.6	0.0134 ± 0.007		2.30	742 ± 32.9	19.9 ± 2.1	0.166 ± 0.005
	5.20	510 ± 16.6	28.3 ± 1.1	0.0113 ± 2.83e-4		5.20	543 ± 28.6	6.5 ± 1.0	0.363 ± 0.008
ASTM	841	4.34 ± 0.3	29.8 ± 1.6	0.165 ± 0.002	ASTM	600	587 ± 43.8	26.9 ± 2.6	0.0838 ± 4.40e-4
	1646	339 ± 46.1	28.8 ± 3.1	0.223 ± 0.002		1646	487 ± 66.0	25.9 ± 4.2	0.152 ± 7.53e-4
	3227	4.41 ± 0.15	33.8 ± 3.0	0.324 ± 0.004		4518		n.d.	
RTox	306	922 ± 9.54	27.9 ± 1.4	0.060 ± 0.003	RTox	306	845 ± 31.1	23.8 ± 3.3	0.089 ± 7.2e-4
	1176	1030 ± 89.4	24.4 ± 1.7	0.123 ± 0.001		1176	890 ± 141	24.4 ± 1.1	0.116 ± 0.002
	3227	6.86 ± 0.28	30.2 ± 3.2	0.293 ± 0.003		3227	866 ± 1.4	26.0 ± 1.3	0.286 ± 0.004
HTox	1650	370 ± 64.7	28.0 ± 1.8	0.185 ± 0.002	HTox	1650	3.46 ± 0.7	29.0 ± 4.1	0.429 ± 0.007
	4520	3.66 ± 0.06	31.9 ± 1.1	0.376 ± 0.006		4520		n.d.	
	12400		n.d.			12400		n.d.	
ZF	647	925 ± 103	26.2 ± 0.5	0.169 ± 0.002	ZF	647	348 ± 40.6	23.5 ± 1.0	0.09 ± 6.03e-4
	1848	204 ± 14.8	23.9 ± 5.1	0.198 ± 0.002		1848	53.3 ± 51.5	25.6 ± 4.3	0.161 ± 0.001
	4058	3.86 ± 0.3	31.5 ± 2.9	0.340 ± 0.005		4058	4.88 ± 2.13	26.9 ± 5.2	0.321 ± 0.005

	SoftCAT™ SL 60					SoftCAT™ SL 100			
	[SL 60] mgL^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity ($mS.cm^{-1}$)		[SL 100] mgL^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity ($mS.cm^{-1}$)
MTox	32.0	3.88 ± 0.2	33.5 ± 4.1	0.228 ± 0.003	MTox	32.0	442 ± 10.6	33.7 ± 3.7	0.203 ± 0.002
	1024	351 ± 5.0	29.0 ± 1.5	0.178 ± 0.001		1024	646 ± 80.6	25.8 ± 2.2	0.138 ± 0.001
	10000		n.d.			10000		n.d.	
MBL	0.70	584 ± 38.9	20.2 ± 4.6	0.015 ± 0.001	MBL	0.70	439 ± 12.3	26.0 ± 4.2	0.0112 ± 0.007
	2.30	1340 ± 62.4	13.9 ± 2.1	0.0164 ± 2.94e-4		2.30	412 ± 9.9	14.5 ± 5.9	0.0079 ± 0.003
	5.20	483 ± 48.1	22.2 ± 1.7	0.0323 ± 0.002		5.20	345 ± 19.0	19.3 ± 1.7	0.0111 ± 5.16e-5
ASTM	600	707 ± 20.8	25.4 ± 2.0	0.0866 ± 6.25e-4	ASTM	600	247 ± 43.6	26.0 ± 1.3	0.105 ± 0.002
	1646	692 ± 46.5	28.8 ± 1.0	0.152 ± 0.001		1646	286 ± 16.3	26.1 ± 1.3	0.155 ± 0.001
	4518		n.d.			4518		n.d.	
RTox	28.0	322 ± 35.7	11.8 ± 1.2	0.530 ± 0.011	RTox	306	651 ± 17.0	19.6 ± 2.7	0.089 ± 8.9e-4
	108	605 ± 67.4	21.3 ± 1.1	0.135 ± 0.002		1176	23.6 ± 9.8	23.6 ± 3.8	0.112 ± 0.001
	260	569 ± 32.1	26.4 ± 1.5	0.0440 ± 0.002		3227		n.d.	
HTox	1650	326 ± 34.6	29.7 ± 2.5	0.354 ± 0.005	HTox	1650	506 ± 69.6	34.4 ± 3.2	0.230 ± 0.002
	4520		n.d.			4520		n.d.	
	12400		n.d.			12400		n.d.	
ZF	647	1080 ± 85.0	25.3 ± 2.4	0.102 ± 0.031	ZF	647	407 ± 22.8	23.2 ± 1.9	0.080 ± 0.001
	1848	363 ± 0.7	25.5 ± 2.9	0.179 ± 0.001		1848	62.7 ± 1.6	37.8 ± 1.5	0.194 ± 0.002
	4058		n.d.			4058		n.d.	


MTox
A
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Shear viscosity / Pa.s
Shear stress / Pa

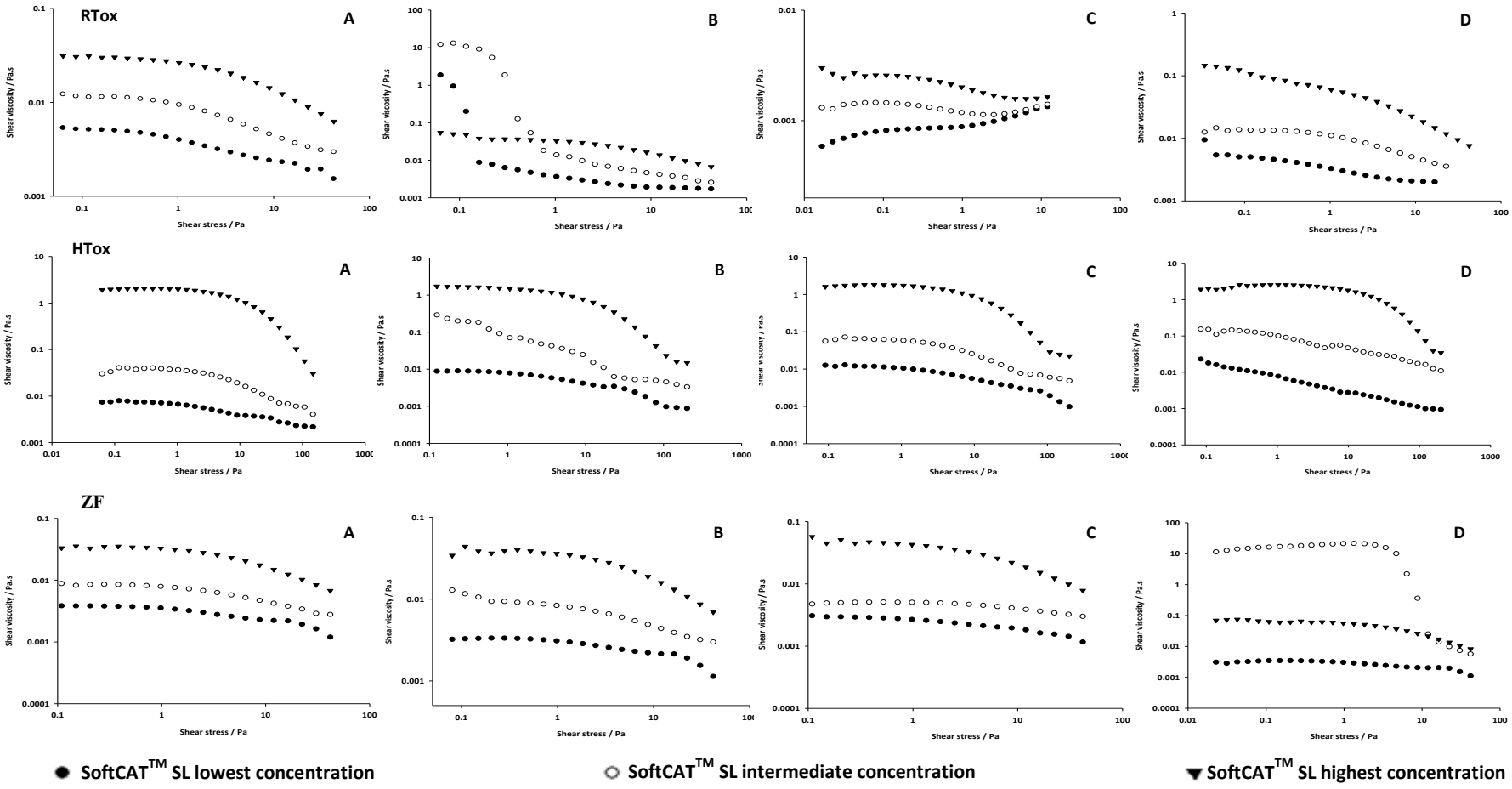

Fig A.1: Flow curves, viscosity (η) as function of shear stress (τ), of the concentrations used in the different specie assays of variants-SL at 25°C prepared in distilled water (A – variant-SL 5, B – variant-SL 30, C – variant-SL 60 and D – variant-SL 100). C1, C2 and C3 – lowest, medium and highest concentration., respectively.

Table A.3: Lethal or effective concentrations of the variants-SL causing 50% of effect (LC_{50} or EC_{50}; mg. L^{-1}) with the respective 95% confidence limits depicted within brackets. NC – could not be computed.

		LC_{50} or EC_{50} (mg. L^{-1})			
Species	**Endpoint**	**SL 5**	**SL 30**	**SL 60**	**SL 100**
Vibrio fischeri	Bioluminescence inhibition 15 min	9645 (2087 - 17203)	977619 (-11273740-13228978)	31991 (-72233-136215)	10683 (8122-13243)
Raphidocelis subcapitata	Growth inhibition 72 h	139.9 (-22.9 – 302.8)	243.6 (118.9 – 368.2)	64.0 (33.6 – 94.4)	13.9 (12.0 – 15.8)
Chlorella vulgaris	Growth inhibition 72 h	682.8 (-352.3 – 1717.9)	62.2 (29.2 – 95.2)	63.8 (7.5 – 120.2)	51.5 (17.9 – 85.1)
Daphnia magna	Immobilization 48 h	836.6 (NC)	685.0 (268.7-1003)	1173 (952.1-1393)	1059 (906.6-1215)
Brachionus calyciflorus	Mortality 24 h	484.9 (433.6-539.7)	343.9 (275.9-396.6)	39.1 (35.0-43.1)	320.3 (276.3-352.4)
Heterocypris incongruens	Mortality 48 h	6610 (6203-7082)	5465 (4882-6160)	8437 (7778-9151)	6309 (4526-10155)
Danio rerio	Mortality 96 h	1499 (792.2-2545)	263.6 (35.2-486.3)	425.9 (NC)	1937 (1028-10007)

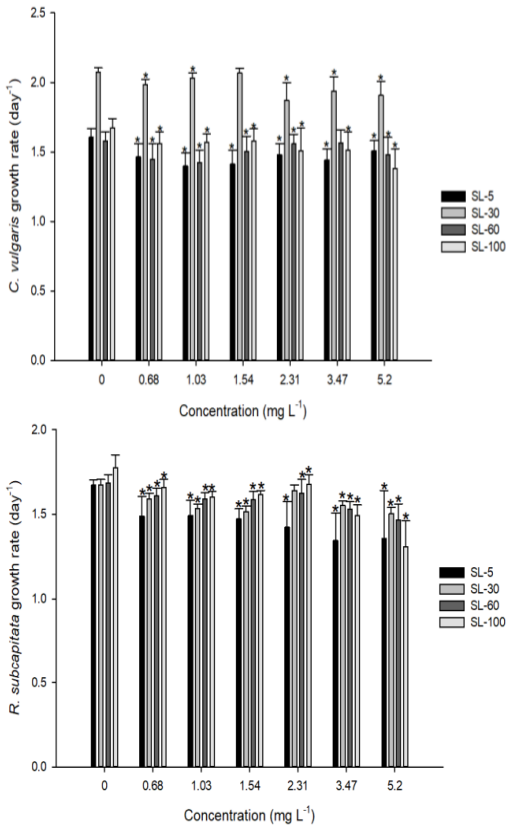

Fig A.2: Growth rate (day^{-1}) of *Chlorella vulgaris* (top figure) and *Raphidocelis subcapitata* (down figure) after exposure, for 72 h, to increased concentrations of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}). Vertical bars correspond to standard error. *denotes statistical differences between SL concentrations and the respective control, within each variant-SL ($p<0.05$).

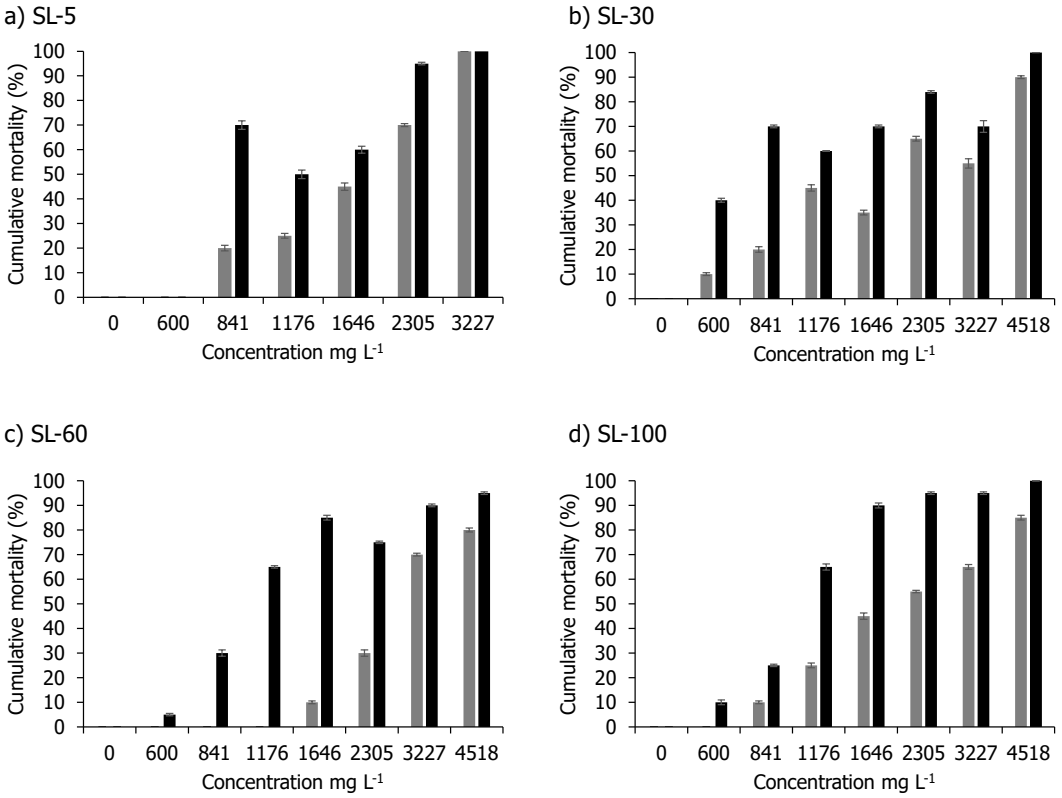

Fig A.3: Average cumulative mortality at 24 hours (light grey bars) and 48 hours (black bars) of neonates of *Daphnia magna* after being exposed to seven concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}): SL 5, SL 30, SL 60 and SL 100. Vertical bars correspond to standard deviation.

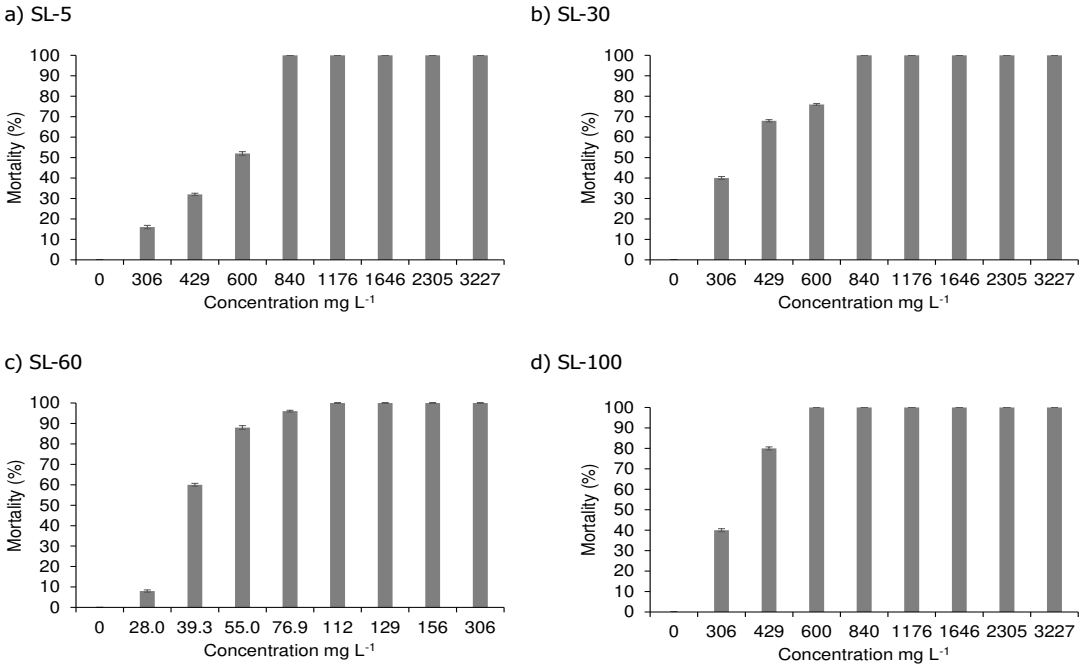

Fig A.4: Average mortality of neonates of *Brachionus calyciflorus* after being exposed, for 24h, to eight concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}): SL 5, SL 30, SL 60 and SL 100. Vertical bars correspond to standard deviation.

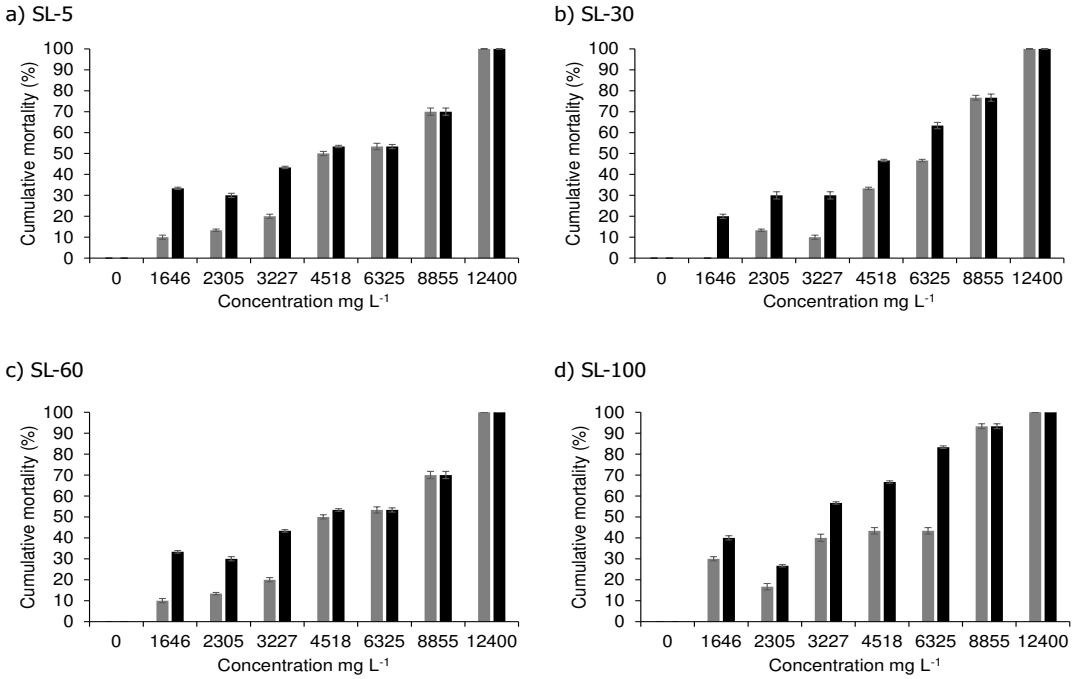

Fig A.5: Average cumulative mortality at 24 hours (light grey bars) and 48 hours (black bars) of *Heterocypris incongruens* after being exposed to seven concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}): SL 5, SL 30, SL 60 and SL 100. Vertical bars correspond to standard deviation.

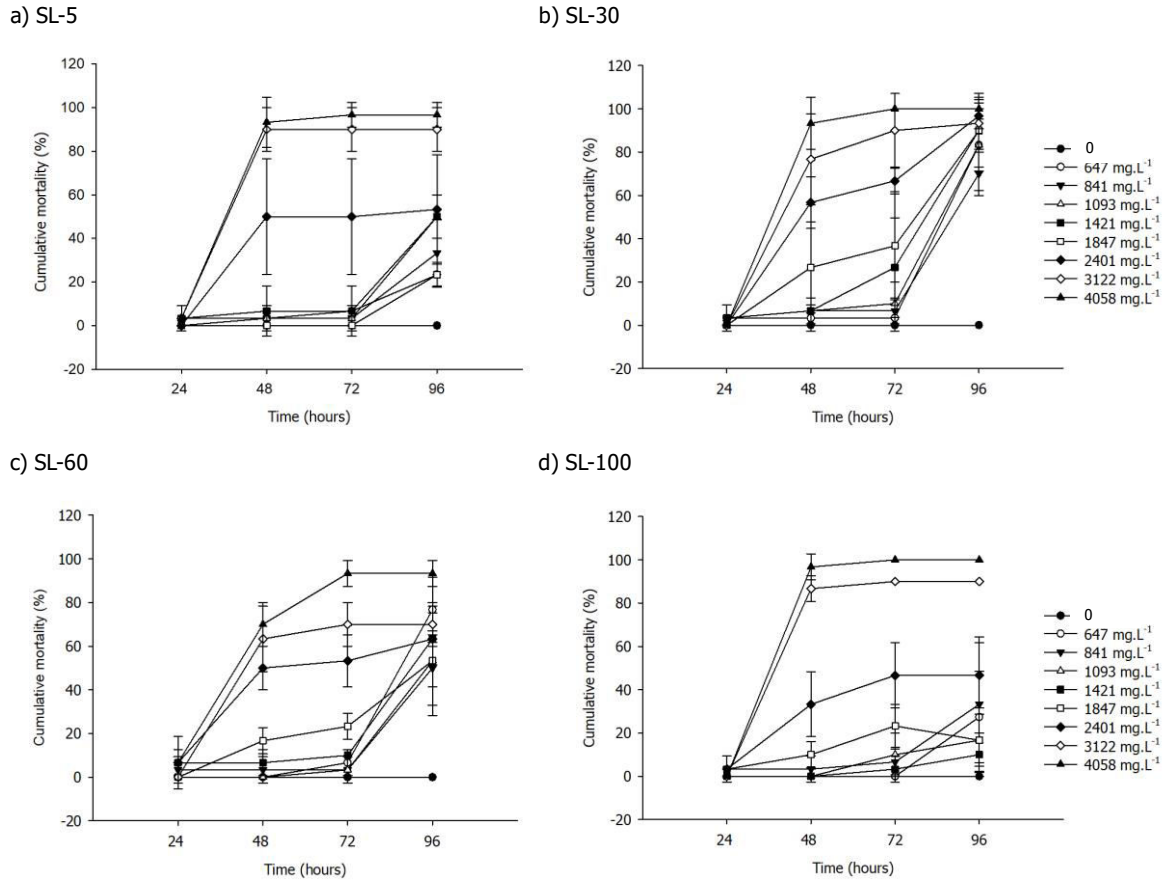

Fig A.6: Average cumulative mortality curves for *Danio rerio* exposed during 96 h to seven concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}): SL 5, SL 30, SL 60 and SL 100. Vertical bars correspond to the standard deviation.

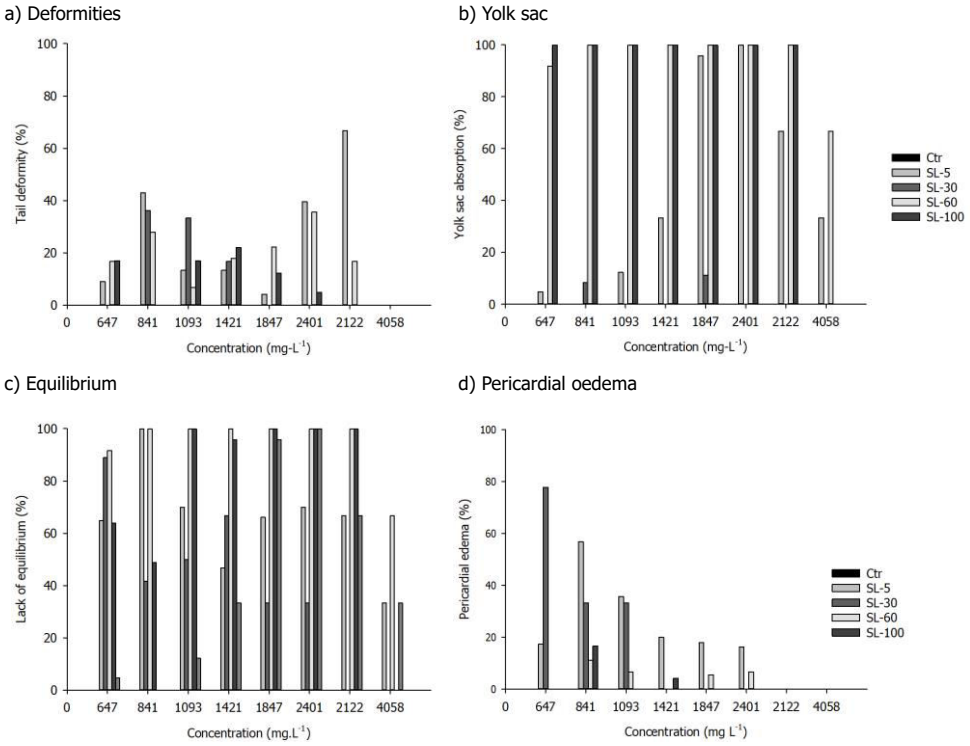

Fig A.7: Percentages (relatively to control conditions) of the different registered malformations and of altered behavior in larvae of *Danio rerio* after being exposed to eight concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}): SL 5, SL 30, SL 60 and SL 100.

Figure A.8: Development abnormalities of *Danio rerio* larvae exposed to eight concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (variants-SL; mg. L^{-1}). At 96 hours: (A) control embryos, (B) lack of equilibrium (exposure to variant-SL 60 1421 mg. L^{-1}), (C) tail deformity (exposure to variant-SL 5 4058 mg. L^{-1}), (D) pericardial oedema (exposure to variant-SL 5 3123 mg. L^{-1}) and (E) delay on yolk sac absorption (exposure to variant-SL 100 1421 mg. L^{-1}).

Chapter IV

Ecotoxicity of cationic cellulose polymers to aquatic biota: The influence of charge density

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Ecotoxicity of cationic cellulose polymers to aquatic biota: The influence of charge density

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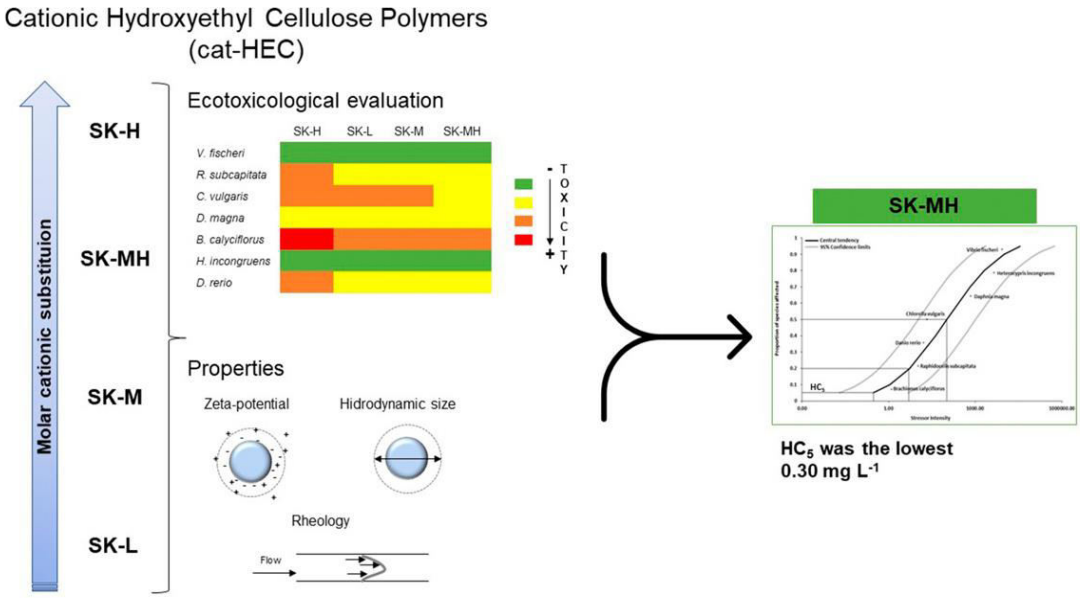
Graphical abstract



Abstract

Better performances of cellulose-based polymers can be achieved by adjust their architecture including the density of cationic modifications. In this study, the influence of cationic substitution on the ecotoxicity of four quaternized hydroxyethyl cellulose polymers (SK-H, SK-L, SK-M, SK-MH) was studied, using an aquatic biota acute ecotoxicity classification, and rheological and physicochemical characterization. The ecotoxicity characterization was achieved by performing standard ecotoxicity assays with seven key trophic level species: *Vibrio fischeri*, *Raphidocelis subcapitata*, *Chlorella vulgaris*, *Daphnia magna*, *Brachionus calyciflorus*, *Heterocypris incongruens*, and *Danio rerio*. Median effective concentrations were used to compute hazard concentrations, through the species sensitive distribution curves method. The microalga *C. vulgaris* and rotifer *B. calyciflorus* were the most sensitive species to the studied polymers. The SK-H variant was highly toxic to the rotifer. Overall, variants with intermediate levels of cationic charge (SK-M, SK-MH) presented the lowest toxicity. The SK-M variant showed the lowest value of maximum acceptable concentration (0.00354 mg/L), thus being indicated as the least toxic variant. Therefore, the obtained results suggest that industry

could direct the development of this type of polymers by tailoring its cationic substitution to moderate levels, in such a way that both functionality and environmental toxicity could be maximized.

Keywords: Hydroxyethyl cellulose polymers, cationic substitution, aquatic maximum acceptable concentrations, green toxicology

1. Introduction

The personal care products (PCP) industry is a consumer-driven trade. To satisfy and meet the market demands/requirements, PCPs production has grown considerably, producing a large variety of compounds at a worldwide scale for cosmetics, hygiene, and sanitization products (Montes-Grajales et al., 2017; Roy, 2020). Hence, their discharge to sewer/wastewaters is most certainly continuous (Montes Grajales et al., 2017; Biel-Maeso et al., 2019). Consumers' increasing awareness and consciousness towards the choice of healthier and environmentally greener products urged industry to search for products as natural and sustainable as possible. Cellulose is an abundant renewable and biodegradable polymeric material (Klemm et al., 2005), therefore, cellulose-based polymers besides exhibiting excellent biocompatibility, are expected to be eco-friendly presenting no to low-toxicity, characteristics that granted them a place as ingredients in a wide range of PCPs (Prabaharan and Mano, 2006; Shang et al., 2008; Wang et al., 2008). Hydroxyethyl cellulose polymers (HEC) are cellulose derivatives commonly used in PCPs, since they exhibit high chemical resistance, good solubility, their hydrophilicity/lipophilicity may be tuned by chemical modification and may form gels in water (Kusumocahyo et al., 2005; Malhotra et al., 2015; Lindman et al., 2021). However, their environmental fate and (eco)toxicological burden has been poorly addressed. Despite once considered to cause low to no harm to aquatic biota (Daughton and Ternes, 1999; Caliman and Gavrilescu, 2009), one must not disregard that: i) PCP consumption rates are most likely continuous due to their presence in a large number of daily routine products; and ii) conventional methods for wastewater treatment (WWT) are inefficient for removing PCP (e.g., reviewed by Yang et al., 2017). Considering last year's COVID-19 pandemic, news reported daily runs to supermarkets in search for hygiene products (namely alcohol-based and incorporating HEC) (Berardi et al., 2020). So, though being previously considered to present no to low environmental risk, their inadequate removal by conventional methods of WWT and increased daily use potentiates their released to the environment at higher concentrations, becoming pseudo-persistent and potentially presenting environmental risks (Cumming et al., 2011). PCP toxicity is assessed by different regulatory agencies (Berardi et al., 2020); according to the Registration, Evaluation, Authorization, and Restriction of Chemicals authority, HEC are considered toxic in the long-term, though no data is available for the immediate or acute toxicity (ECHA, 2021).

Through integrating industry and environmental safety, green chemistry targets alternative synthetic polymers, by tunning physical-chemical properties, which are simultaneously functional and exhibit no to low environmental risks (Roy, 2020). HEC polymers are an excellent example of such compounds as they may be

tailored through hydrophobic and/or cationic modifications. These cationic polymers are commonly used in hair care products due to their capacity to protect the hair, possess excellent affinity for damaged hair, and improve its wet-combability, the polymers adsorb irreversibly to the hair surface, that is negatively charged in the pH range of the product, thus not being effectively removed in the rinsing process (Rhein, 2007; Aparecida da França et al.,2015). Jordan et al. (2008) reported that an increase of charge density and/or molecular weight of polyquaternium polymers increases wet-combability reduction. These cationic polymers can also act with film-forming properties and electrostatic attractions originating higher volumizing and conditioning efficacy in hair care products (Shokri et al., 2017). Shokri et al. (2017) reported molecular weight and positive charge density as the main parameters influencing the deposition efficacy; polyquaternium polymers PQ-68 (with highest molecular weight) exhibited the greatest hair deposition efficacy at pH 9. In the present work, the influence of cationic density substitution on the ecotoxicity of four HEC polymers was studied, aiming to identify safer chemicals to the environment whilst maintaining their functionality. To attain this, the four HEC polymers were studied regarding their rheological and physico-chemical properties in distilled water and in different ionic media, and their ecotoxicity characterized for aquatic key species from different trophic levels. This approach allowed to provide maximum acceptable concentrations to be considered in future regulatory frameworks regarding these compounds.

2. Materials and methods

2.1. Studied polymers

The studied cationic-modified hydroxyethyl cellulose polymers (SoftCAT™SK, INCI name-Polyquaternium-67) were supplied by Amerchol Corporation (Dow Chemical Company, Greensburg, LA; Company, 2008). These are polycationic polymers consisting of quaternized hydroxyethyl cellulose, incorporating variations in charge and hydrophobic modification level (Fig. 1S; Table 1S). SoftCAT™ polymers are chloride salts of N, N, N–trimethylammonium derivatives of hydroxyethyl cellulose with some dodecyl trimethylammonium residues as hydrophobic substituents, and a molecular weight between 200,000–800,000 g mol−1 (Dow Chemical Company, 2008). They are composed by 90–100% of cationic hydroxyethyl cellulose, 0–5.5% of water, 0–4.9% sodium acetate, 0–3.5% of sodium chloride and less than 1% of isopropanol. The SoftCAT™ SK polymers are available in four variants with different cationic substitution: SK-H, SK-L, SK-M and SK-MH (please see Table 1S for detailed information; Dow, 2013). Their degree of cationic substitution of trimethyl ammonium and dimethyl-dodecyl ammonium varies from 0.2 to 0.3 M corresponding to a percentage of about 1% of nitrogen by weight (Table 1S; Fig. 1S) (Ballarin et al., 2011). Three of the four variants have the same low degree of dimethyl-dodecyl-ammonium hydrophobic substitution (Ballarin et al., 2011). The SK-MH variant presents 6-fold more hydrophobic substitution comparatively to SK-M. They have the ability to modify the rheology of a solution and also the stability of a

dispersion, according to the requirements of cosmetics and personal care products industries (Karlson et al., 2002).

2.2. Characterization of tested aqueous suspensions

Each SK variant was characterized in terms of hydrodynamic size, zeta potential, solubility, and rheological properties. This characterization was always performed in two different media: distilled water and the test media used for each species. Three concentrations were characterized: low, intermediate, and high concentration tested in each assay (please see Table 1). Dynamic light scattering (DLS) was used for the determination of the particle size (Di0.5) whilst zeta potential was determined by electrophoretic light scattering (measured through a Zetasizer Nano ZS, ZN 3500, Malvern Instruments, UK) coupled with the Zetasizer NanoSoftware, version 6.01 (Nogueira et al., 2012) using a DTS 0012 polystyrene cell with a sample volume of 1.5 mL for each SK variant solvent (culture media or water) combination, without the presence of bubbles. The measurements were performed at 25°C with a backscatter angle of 173°. The zeta potential provided information about the stability of the polymeric solutions, the stability is high at values above |± 30 mV| (Malvern Instruments, Malvern, UK).

The solubility of each SK variant was assessed at the same concentrations prepared as above described. Briefly, after preparing the desired concentrations (low, intermediate, and high) they were centrifuged at 17.968 ×g for 10 min (Cai et al., 2014; Melro et al., 2020). Then, the solubility of each polymer was assessed based on the visual inspection of the amount of polymer settled at the bottom of the flask and calculated based on the portion of the polymer deposited at the bottom of the flask and the total amount added, for each sample. The solubility evaluation was performed at time zero (just after preparing the concentrations) and at the time corresponding to the duration of the respective assay, as follows: 15 min for *V. fischeri*; 24, 48, and 72 h for *R. subcapitata* and *C. vulgaris*; 24 and 48 h for *D. magna* and *H. incongruens*; 24 h for *B. calyciflorus*; and 24, 48, 72, and 96 h for *D. rerio*.

The viscosity and rheological behavior of the polymeric solutions was evaluated using a Thermo Scientific HAAKE MARS III rheometer equipped with an automatic gap function. In these determinations, a strict control of temperature was done through a Peltier system. A solvent trap system was used to minimize the water evaporation. All measurements were performed under steady shear with a gap fixed at 1 mm. The flow curves (viscosity vs shear stress) were determined at 25°C with a plate-plate geometry with a diameter of 35 mm.

2.3. Species cultures maintenance

The influence of cationic substitution of the four SK-variants on their toxicity to aquatic biota was explored through the employment of a battery of standard assays. The used species are representative of key

trophic and functional levels (Table 2S): the gram-negative bacterium *Vibrio fischeri*, the green microalgae *Raphidocelis subcapitata* and *Chlorella Vulgaris* (stock cultures were maintained at 20 ± 2°C, under continuous illumination 100 µE/m^2/s, aeration, in Woods Hole MBL growth medium), the cladocera *Daphnia magna* (monoclonal D. magna BEAK cultures were maintained in synthetic hard water American Standards for Testing and Materials medium with pH 7.3 ± 0.3, 20 ± 1°C and 16h^L:8^D photoperiod), the rotifer *Brachionus calyciflorus*, the ostracod *Heterocypris incongruens*, and the fish *Danio rerio* (adult zebrafish were maintained under standard controlled conditions 26 ± 1°C, 750 ± 50 µS/cm, pH 7.5 ± 0.5, dissolved oxygen saturation ≥ 95%, 80% humidity, photoperiod cycle of 16 h^L:8 h^D in tanks with recirculating systems, maintained in carbon-filtered water: 0.34 mg/L of Instant Ocean® synthetic sea salt).

2.4. Standard ecotoxicity assays

2.4.1. Microtox® assay

The first step on the ecotoxicity characterization of the four SK variants was performed with *V. fischeri* through the 81.9% Basic Test protocol of bioluminescence inhibition assay (Azur Environmental, 1998). This assay was carried out because it is commonly used as a preliminary ecotoxicological indicator and has the advantage of providing a fast assessment. Nine concentrations of each SK-variant plus a control (consisting in the diluent solution) were tested (Table 2S). The bioluminescence was evaluated after 15 min of exposure using a Microtox Model 500 Analyser (Azur Environmental, CA, USA) equipped with an automatic record of luminescence.

2.4.2. 72-H growth inhibition assay with *R. subcapitata* and *C. vulgaris*

The 72-h growth inhibition assay was performed using an inoculum of *R. subcapitata* and of *C. vulgaris* algae at the exponential growth. The OECD Guideline 201 (OECD, 2004b) adapted to 24-well microplates was used in this assay (Geis et al., 2000; Moreira-Santos et al., 2004). The algae were exposed to six concentrations of each SK-variant (Table 2S) plus a control (MBL medium, Nichols, 1973) for 72 h. The SK-variant concentrations used in this assay were prepared from a stock concentration of 10.0 mg/L prepared in MBL medium. Three replicates were performed for each concentration and for the control, with 1 mL of test solution and a cell density of 104 cell mL^{-1}. Adding to these, wells with each concentration of SK-variants (or MBL medium only for control conditions) without microalgae were exposed under the same conditions for 72 h, to exclude any potential interference of the presence of the SK-variants in the final absorbance measurement (for instance, potential flocculation of the polymers in the test medium). The assays were performed under continuous illumination (100µE/m2/s), at 23 ± 1°C and using an automatic stirrer to promote active gas exchange and prevent cell clumping through a continuous stirring. To measure the absorbance (ABS) at 440 nm after the 72 h of exposure, it was used a wide-spectrum microplate reader

(Thermo Scientific mod. Multiskan Spectrum). The cell density (C) was determined according to the following equations based on absorbance linear regressions showing the correlation coefficients, the regression constant, and the regression coefficient:

For *R. subcapitata*:

C (cells ml−1) =−17,107.5 + (ABS∗7925350) (R^2= 0.99)

For *C. vulgaris*:

C (cells ml−1) =−155,820 + (ABS∗13144324) (R^2= 0.98)

Growth rate (day −1) was determined according to OECD (2004b) for each SK-variant concentration and control:

$$\mu = \frac{\ln D_b - \ln D_a}{t_b - t_a},$$

where D_a is the initial cell density, D_b is the cell density at the end of the assay and tb–ta is the exposure time interval (72 h).

2.4.3. 48-H acute immobilization assay with *D. magna*

According to the OECD guideline 202 (OECD, 2004a), it was used neonates (6 h to 24 h-old) from the 3rd or 4th broods from monoclonal *D. magna* BEAK cultures to perform the acute immobilization assay. Neonates were exposed to seven concentrations of each SK-variant, as described at Table 2S, and a control (ASTM medium; ASTM, 2002). The concentrations of SK-variant tested were diluted from a stock solution of 5000 mg/L with ASTM medium. Four replicates were performed per treatment, each containing 30 mL of test solution and five neonates of *D. magna*. The assay was performed at 20 ± 1°C and $16h^L$:$8h^D$ photoperiod, with no food addition or medium renewal. After 48 h, the number of immobilized organisms in each replicate was determined.

2.4.4. 24-H immobilization assay with *B. calyciflorus* (Rotoxkit F®)

To perform this assay, organisms were exposed to at least seven concentrations of each SK-variant and a control (Standard Freshwater medium composed by sodium bicarbonate ($NaHCO_3$), calcium sulfate dihydrate ($CaSO_4{\cdot}2H_2O$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and potassium chloride (KCl)). The concentrations tested in this assay are presented at Table 2S. These concentrations were prepared through dilution of a stock solution of 40.0 mg/L in Standard Freshwater medium. It was used 24-well plates in this

assay, per well five newly hatched rotifers were introduced in 1 mL of test solution. Five replicates were carried out per treatment. Exposure took place for 24 h, at 23°C in the dark. At the end of the assay the number of immobilized rotifers was counted.

2.4.5. 48-H immobilization assay with *H. incongruens*

In this assay, neonates of ostracod *H. incongruens* were used. The immobilization assay was performed according to Ostracodtoxkit F protocol (MicroBioTests Inc., Belgium). After the hatching of cysts, the neonates of *H. incongruens* were exposed to seven or eight concentrations of SK-variant and to a control consisting of Standard Freshwater medium (Table 2S). The concentrations used in this assay were prepared by dilution from a stock solution of 12,500 mg/L. For each SK variant treatment, three replicates were performed, each with ten neonates. Exposure occurred for a period of 48 h, under total darkness and at 25°C. The number of immobilized organisms was determined at the end of the assay.

2.4.6. 96-H fish embryo acute toxicity test with *D. rerio*

This assay was performed with embryos of zebrafish according to the corresponding OECD test guideline 236 (OECD, 2013). The eggs were obtained through natural crossbreeding of *D. rerio*. The fertilized eggs were identified under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon, Nikon Corporation, Japan) and selected to perform the assay. The embryos were exposed to eight SK-variant concentrations and a control (Table 2S), after reaching 6 h of fertilization. Dilutions of each polymer were prepared in Zebrafish Water (ZF), from a stock solution of 150 mg/L. Ten embryos were used per replicate in a total of three replicates per treatment. Exposure occurred for 96 h in 24-wells microplates filled with 2 mL of test suspension per well and with four internal controls. The assay was performed at 26 ± 1°C and 16 hL:8hD photoperiod. Indicators of lethality up to four apical observations (coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and lack of heartbeat), malformations (head, spinal and tail), and development delay were the parameters evaluated at 24, 48, 72 and 96 h (Kimmel et al., 1995).

2.5. Statistical analysis

For the seven tested species and for each SK-variant, the concentration causing 50% of effect L(E)C_{50} and the corresponding 95% confidence limits were determined. The EC_{50s} for bioluminescence inhibition in *V. fisheri* were determined through MicrotoxOmni® Azur software version V1.18 with a linear model (Azur Environmental, 1998). For the mortality results recorded for *D. magna*, *H. incongruens*, *B. calyciflorus* and *D. rerio*, the LC_{50}s were determined through the PriProbit software (Sakuma, 1998). The software package

Statistica 8.0 (StatSoft, Inc., Tulsa, USA) was used to adjust a non-linear logistic model to the data for the determination of EC_{50}s for the growth inhibition of *R. subcapitata* and *C. vulgaris*.

One-way analysis of variance (ANOVA), followed by the multicomparison Dunnett's test, were performed to compare the responses of algae exposed to the different SK-variant concentrations with those exposed to the control. To verify the ANOVA assumptions of normality of data and homoscedasticity of variances, the Kolmogorov-Smirnov test and the Levene test were run, respectively (Zar, 1996). These analyses were done by using the software Sigmaplot version 12.5 (Systat Software, Inc., 2008).

The species sensitivity distribution curves (SSD) were constructed to determine hazard concentration for 5, 20 and 50% of the species (HC_5, HC_{20}, and HC_{50}, respectively) with their 95% confidence limits (Boeckman and Layton, 2016), by using the US Environmental Protection Agency spread sheets (SSD Generator V1).

The maximum acceptable concentration environmental quality standard (MAC-EQS) was computed for each SK-variant using two methodologies: (i) the deterministic approach, where an assessment factor of 1000 was applied to the lowest EC50computed for the tested species and (ii) the probabilistic approach, where an assessment factor of 1 was applied to the computed HC5(European Commission, 2011).

3.Results

3.1. Characterization of tested aqueous suspensions

In Table 1 and in Table 3S are presented the results obtained for hydrodynamic size (Di0.5), zeta potential and conductivity of the lowest, intermediate, and highest concentrations tested for each SK-variant suspension. Due to the formation of a 3D network in these polymers, the determination of particle size could not be evaluated for the concentrations above the overlap concentration of SK-variants, estimated by rheological measurements, indicated in Table 1.

In general, zeta potential values were lower in test media comparatively to those of distilled water, with some exceptions (e.g., lowest concentration of SK-H and SK-L in MBL medium, SK-H concentrations prepared in RTox medium; Tables 1, 3S). This pattern was expected due to the presence of salts on the test media that induces changes in the particle double layer, resulting in the reduction of the overall effective net charge and causing instability in the suspensions. Nevertheless, in all media most values of zeta potential were below |± 30 mV|, indicating some instability of the suspensions. Regarding hydrodynamic diameter, a uniform pattern, of increase or decrease in size of the SK-variants suspended in distilled water versus in test media, was not observed. Tendentiously, hydrodynamic sizes decreased (in test media) for the lowest tested concentrations and increased for the highest ones. The presence of salts in test media may have led to a charge screening of the polymer causing a compaction of polymer chains; at low concentrations, this compaction results in a decrease of particle size; conversely, if the polymer concentration is high enough, due to the reduced repulsion among polymer chains and counterions entropy, the polymer aggregates and an increase in particle

size is observed. Since these polymers can also form flocs when dissolved in water, by adsorption/interaction with organic and inorganic anions, this can also have an impact in the particle size (Anderson et al., 2019). As well, is important note that even being these polymers highly water soluble, this does not mean that all the polymer molecules are dissolved at a molecular level. In literature is possible to find works reporting the extraction and characterization of cellulose nanocrystals from cellulose derivatives; these nanocrystals were found to be a cellulose I polymorph, the native form of cellulose (Alves et al., 2015). Thus, the fully molecularly dispersed state is highly difficult to obtain, even using highly soluble cellulose derivatives. In fact, the particle size measurements performed in the test media, without addition of polymers, revealed the presence of some particles of, tendentiously, small dimensions; the presence of cationic cellulose derivatives led to an increase in particle size and substantial changes in zeta potential, showing the impact of the polymers in the particle size and zeta potential of the suspensions. An exception occurred for SK-M and SK-MH, for which the size increased when suspended in the test media used for the ostracod and fish assays, comparatively to the size measured in the respective suspensions made in distilled water (Tables 1, 3S). The increased size of SK at the lowest tested concentration, could be explained by the formation of intermolecular bridging, induced by the presence of divalent anions (sulphate). For the highest concentrations, SK size tends to increase as a result from the formation of intra-molecular and intermolecular bridging and predominantly induced by polymer aggregation. Since SK-MH has a higher hydrophobic substitution index (HS) in relation to the other variants (Table 1S), the increase in size due to salts presence is higher comparatively to the increase for SK-M, with lower HS. This outcomes from the higher aggregation and formation of bigger particles, a consequence of the hydrophobic interactions and of the inter- and intramolecular bridging as mentioned earlier. In Tables 1and 3S, is possible to observe that for SKMH, the Di50 in distilled water for the concentration 2.31 mg/L was 399 nm and for the same concentration in MBL medium was 1100 nm. In relation to the remaining test media (ASTM, HTox and ZF), it was observed an increase of particle size for all SK-variants, with few exceptions for some concentrations (SK-H, SK-L and SK-MH; Tables 1,3S). For RTox media, the particle size decreased for all SK-variants concentrations, except for the highest concentration of SK-MH (Tables 1,3S).

In terms of the conductivity, it was higher in concentrations prepared in the test media comparatively to those prepared in distilled water, which was expected since higher concentrations of ions are present in the former (Tables 1,3S).

Concerning solubility, all SK-variants showed high solubility in the tested media, attested by the absence of precipitates in any tested concentration (low, intermediate, and high), at all exposure periods matching the duration of the ecotoxicological assays. These results agree with the information supplied by the manufacturer that these cationic polymers are soluble in water and readily disperse into solution (Dow, 2013).

Table 1: Characterization of each variant SK (SK-H, SK-L, SK-M and SK-MH) regarding particle size (nm), the zeta potential (ζ-Potential, mV) and conductivity (Cond, mS cm^{-1}) at the three tested concentrations (lowest, intermediate and highest; mg L^{-1}). Characterization was performed in each species respective medium as follows: MTox –*Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*. Values are presented as average values ± standard deviation of n=3. n.d. – no data (concentration above the overlap concentration).

	SoftCAT™ SK-H			
	[SK-H] mg L^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**
MTox	32.0	1260 ± 21.2	2.2 ± 0.2	33.5 ± 0.07
	1024	1220 ± 247	4.9 ± 1.0	37.1 ± 0.42
	10000		n.d.	
MBL	0.70	742 ± 27.5	25.0 ± 1.7	0.57 ± 0.01
	2.30	488 ± 36.2	21.4 ± 1.2	0.63 ± 0.01
	5.20	603 ± 13.5	18.2 ± 1.6	0.61 ± 0.01
ASTM	220.0	562 ± 56.3	17.8 ± 2.2	0.62 ± 0.01
	600.0	559 ± 15.3	15.9 ± 1.6	0.64 ± 0.01
	1646.0	23.6 ± 0.27	19.2 ± 1.6	0.83 ± 0.02
RTox	0.07	150 ± 15.0	9.3 ± 1.7	2.6 ± 0.34
	0.18	300 ± 30.2	10.9 ± 2.0	3.6 ± 0.23
	0.50	615 ± 36.0	7.9 ± 2.3	3.0 ± 0.28
HTox	429.0	934 ± 20.5	28.4 ± 5.1	1.93 ± 1.00
	1646.0	726 ± 61.5	29.3 ± 2.9	3.24 ± 0.24
	4518.0		n.d.	
ZF	3.0	545 ± 22.4	16.2 ± 1.2	0.89 ± 0.03
	8.6	1270 ± 165	16.7 ± 1.1	0.9 ± 0.03
	19.0	654 ± 70.0	17.0 ± 1.2	1.0 ± 0.03

	SoftCAT™ SK-L			
	[SK-L] mg L^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**
MTox	32.0	520 ± 17.8	3.3 ± 1.1	36.5 ± 0.44
	1024	1620 ± 49.5	4.2 ± 1.6	38.3 ± 0.42
	10000		n.d.	
MBL	0.70	909 ± 22.5	15.0 ± 2.1	0.56 ± 0.01
	2.30	1100 ± 55.1	9.7 ± 4.0	0.60 ± 0.01
	5.20	842 ± 4.36	12.1 ± 1.1	0.57 ± 0.03
ASTM	600.0	685 ± 151	16.1 ± 1.2	0.62 ± 0.01
	1646.0	29.2 ± 0.23	17.9 ± 2.2	0.74 ± 0.02
	4518.0	10.3 ± 0.83	30.3 ± 1.2	0.95 ± 0.04
RTox	1.9	277 ± 1.73	21.0 ± 1.5	5.17 ± 0.47
	7.3	288 ± 8.74	11.3 ± 1.5	4.84 ± 0.44
	28.0	417 ± 15.0	16.0 ± 2.5	5.22 ± 0.45
HTox	1176.0	654 ± 7.8	36.8 ± 3.7	3.01 ± 0.22
	4520.0	10.3 ± 0.14	32.1 ± 3.0	3.05 ± 0.24
	12400		n.d.	
ZF	21.0	60.3 ± 12.7	21.0 ± 1.6	0.89 ± 0.03
	60.0	681 ± 98.8	16.4 ± 1.6	1.00 ± 0.04
	130.0	419 ± 46.3	17.1 ± 0.9	1.17 ± 0.05

	SoftCAT™ SK-M			
	[SK-M] mg L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})
MTox	32.0	451 ± 31.5	2.6 ± 1.4	37.2 ± 0.41
	1024	1300 ± 80.8	3.0 ± 1.6	37.3 ± 0.46
	10000		n.d.	
MBL	0.70	1020 ± 46.4	7.2 ± 1.4	0.58 ± 0.01
	2.30	626 ± 61.4	9.9 ± 0.9	0.63 ± 0.01
	5.20	524 ± 62.4	11.2 ± 1.6	0.62 ± 0.01
ASTM	600.0	445 ± 9.2	16.4 ± 0.6	0.57 ± 0.01
	1646.0	53.2 ± 3.61	18.2 ± 1.4	0.74 ± 0.02
	4518.0	n.d.		
RTox	2.7	367.0 ± 14.4	14.3 ± 1.7	4.9 ± 0.40
	10.2	316 ± 23.5	15.4 ± 1.9	5.5 ± 0.50
	39.3	331 ± 17.0	15.3 ± 1.8	5.5 ± 0.48
HTox	1650.0	899 ± 34.6	34.2 ± 3.0	1.86 ± 0.11
	4520.0	n.d.		
	12400.0		n.d.	
ZF	19.0	910 ± 16.3	12.9 ± 0.9	0.93 ± 0.03
	54.0	660 ± 79.1	13.9 ± 2.2	0.98 ± 0.03
	120.0	753 ± 41.0	19.6 ± 1.9	1.0 ± 0.04

	SoftCAT™ SK-MH			
	[SK-MH] mg L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})
MTox	32.0	490 ± 19.1	1.5 ± 0.3	37.8 ± 0.64
	1024	844 ± 28.3	2.1 ± 1.0	38.0 ± 0.37
	10000		n.d.	
MBL	0.70	1200 ± 62.4	7.0 ± 0.6	0.58 ± 0.01
	2.30	1100 ± 28.3	3.4 ± 0.5	0.61 ± 0.01
	5.20	842 ± 86.6	13.4 ± 0.8	0.62 ± 0.01
ASTM	600.0	16.8 ± 1.01	21.5 ± 2.3	0.62 ± 0.01
	1646.0	28.6 ± 0.91	21.0 ± 1.5	0.73 ± 0.02
	4518.0	n. d		
RTox	1.9	169 ± 10.6	22.5 ± 2.2	4.31 ± 0.38
	7.3	258 ± 5.7	19.6 ± 1.4	4.58 ± 0.39
	28.0	1240 ± 107	19.9 ± 1.8	5.25 ± 0.44
HTox	1176.0	729 ± 36.3	22.6 ± 2.3	3.15 ± 0.22
	4520.0	n.d.		
	12400		n.d.	
ZF	19.0	705 ± 70.0	14.9 ± 1.7	0.9 ± 0.03
	54.0	1070 ± 32.1	9.3 ± 1.8	1.0 ± 0.03
	120.0	634 ± 126	16.1 ± 1.1	1.0 ± 0.03

3.2. Rheological measurements

In Figs. 1 and 2S are represented the flow curves of the SK-variants suspended in the different test medium and distilled water, respectively. The SK-variants suspended in test media showed a Newtonian behavior, except for SK-variants suspended in RTox and HTox media, where a pseudoplastic behavior was observed. Overall, the obtained results show two tendencies: (i) polymers with lower charge density tended to face higher aggregation (at the same test medium) resulting in higher viscosities and changes in rheological behavior. For example, for SK-H and SK-L suspended in the RTox and HTox media, is possible to observe a clear increase in the viscosity at low shear rates for SK-L, contrary to the SK-H that kept the viscosity almost constant and at low values, in the entire range of the studied stresses. (ii) higher density of HS, results in a viscosity increase due to the extensive aggregation induced by the HS and the reduction in charge density. Similar results were observed in other polymeric systems (Alves et al., 2015). The SK-MH and SK-L showed a pseudoplastic behavior when suspended in RTox medium, contrary to SK-M.

Differences in the viscosity of SK-variants were observed when suspended in tested media of *V. fisheri* and distilled water (Figs. 1, 2S). For this species, the SK-variants showed a shear viscosity of 0.01 Pa·s and 1 Pa·s for the lowest and highest tested concentrations.

The viscosity of SK-variants suspended in test media was dependent on the medium composition. For example, the viscosity for the highest tested concentration of SK-L ranged from 0.01 Pa·s up to 10 Pa·s, on the different test media, meaning that viscosity can vary in four orders of magnitude depending on the medium composition. Similar results were obtained for SK-MH. Also, it was observed that SK-H showed lower shear viscosity values comparatively to the same concentrations suspended in distilled water (Figs. 1and 2S).

3.3. Acute toxicity assays

The validity criteria established by each guideline (Azur Environmental, 1998; OECD, 2004 a,b, 2013; RotoxkitF™ Acute Microbiotests, Ostracodtoxkit F™ Microbiotests; Table 2S) for controls were accomplished for all the performed assays.

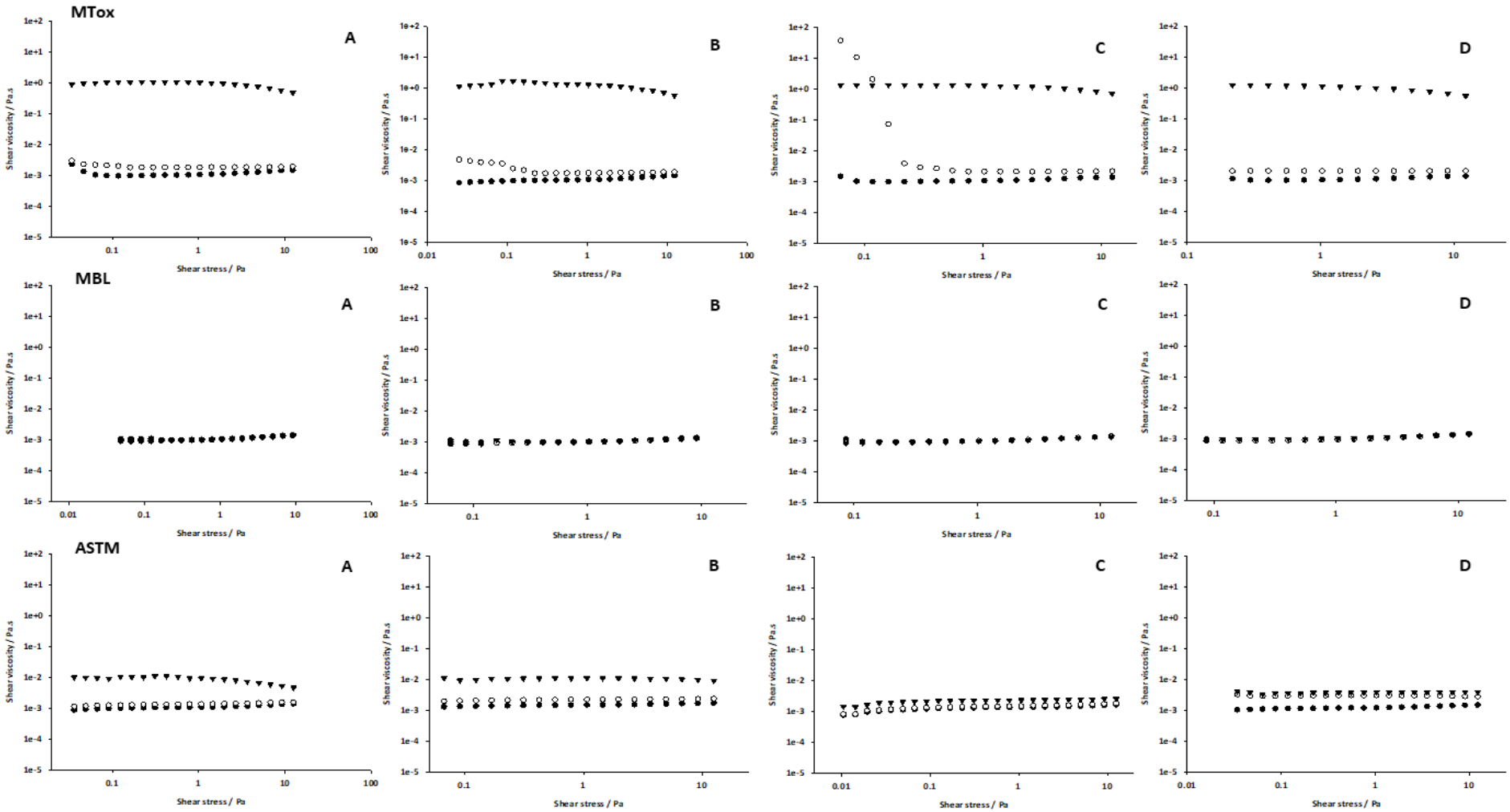
The toxicity ranking of the four SK-variants was species dependent. For *V. fischeri* a similar toxicity was observed among SK-H, SK-L and SK-M. The $EC_{50,15min}$ for SK-MH was approximately 2-fold lower than the EC50,15min of the other three variants, nevertheless, all were above 100 mg/L [maximum limit range of United Nations, 2011 for chemicals with acute toxicity] (Table 2).

The two microalgae presented distinct patterns of sensitivity to the SK-variants (Table 2; Fig. 3S). All SK-variants exerted similar toxicities to *R. subcapitata* with $EC_{50,72h}$ ranging from 9.51 to 17.2 mg/L (95% confidence limits overlapped for all SK-variants; Table 2). Significant statistical differences in relation to control growth rates were detected for all variants from the concentration 0.68 mg/L onwards (Fig. 3S). For *C. vulgaris*, SK-L showed to be the most toxic with an $EC_{50,72h}$ (CL 95%) of 2.16 (2.03–2.30) mg/L and SK-MH the least toxic with an $EC_{50,72h}$

(CL 95%) of 21.6 (12.0–31.3) mg/L (Table 2). All growth rates of *C. vulgaris* were statistical lower than the control except for SK-L and SK-M at 1.03 mg/L (Fig. 3S). Furthermore, according to the GHS classification (United Nations, 2011) for *R. subcapitata* three of the SK variants presented low toxicity (SK-L, SK-M and, SK-MH), with SK-H presenting intermediate toxicity (Table 2), whilst for *C. vulgaris* only SK-MH presented low toxicity and the remaining variants were intermediately toxic (Table 2).

For *D. magna*, *B. calyciflorus*, and *H. incongruens*, SK-H was the most toxic variant (Table 2; Figs. 4S, 5S, 6S). The LC_{50} (95% CL) values for SK-H differed in one order of magnitude between *B. calyciflorus* and the two other primary consumers (Table 2, Figs. 4S, 5S, 6S): for *B. calyciflorus* the $LC_{50,24h}$ was 0.17 (0.16–0.19) mg/L, whilst for daphnids and ostracods the $LC_{50,48hs}$ were 209 (122–268) mg/L and 664 (398–898) mg/L, respectively. All SK-variants presented no to low toxicity to *H. incongruens* and *D. magna*, according to the GHS classification (Table 2). Though, SK-H was the variant starting to induce significant mortality in D. magna at lower concentrations (≥220 mg/L; Fig. 4S). For *B. calyciflorus* all SK-variants presented intermediate toxicity, except for SK-H that revealed to be severely toxic (Table 2); SK-H started to induce significant mortality at 0.36 mg/L, whilst the remaining variants started to induce significant mortality from 2.66 mg/L onwards (Fig. 5S).

For *D. rerio*, cumulative mortality rates showed a similar trend in all tested polymers, never surpassing 20% of effect until the 48 h of exposure and increasing greatly until the end of the assay at 96 h. The variant SK-H showed to be the most toxic, with an $LC_{50,96h}$ of 8.69 mg/L (Table 2; Fig. 7S). The main malformations observed in organisms exposed to SK-H, were related to pericardial oedemas and tail bending. The final percentages of pericardial oedemas went up to 75.6% at 11.2 mg/L of SK-H (Fig. 8S). Embryos exposed to the other SK-variants also presented high percentage of malformations, namely, development of pericardial oedemas (SK-M at 41.0 mg/L and always above 25% in all concentrations of SK-MH; Fig. 7S), lack of equilibrium (presented by more than half of the larvae exposed to all SK-MH concentrations; Fig. 8S) and tail deformities (over 40% at 24.0, 32.0, 70.0, and 90.0 mg/L of SK-MH).


MTox
A
B
C
D
MBL
A
B
C
D
ASTM
A
B
C
D
Shear viscosity / Pa.s
Shear stress / Pa

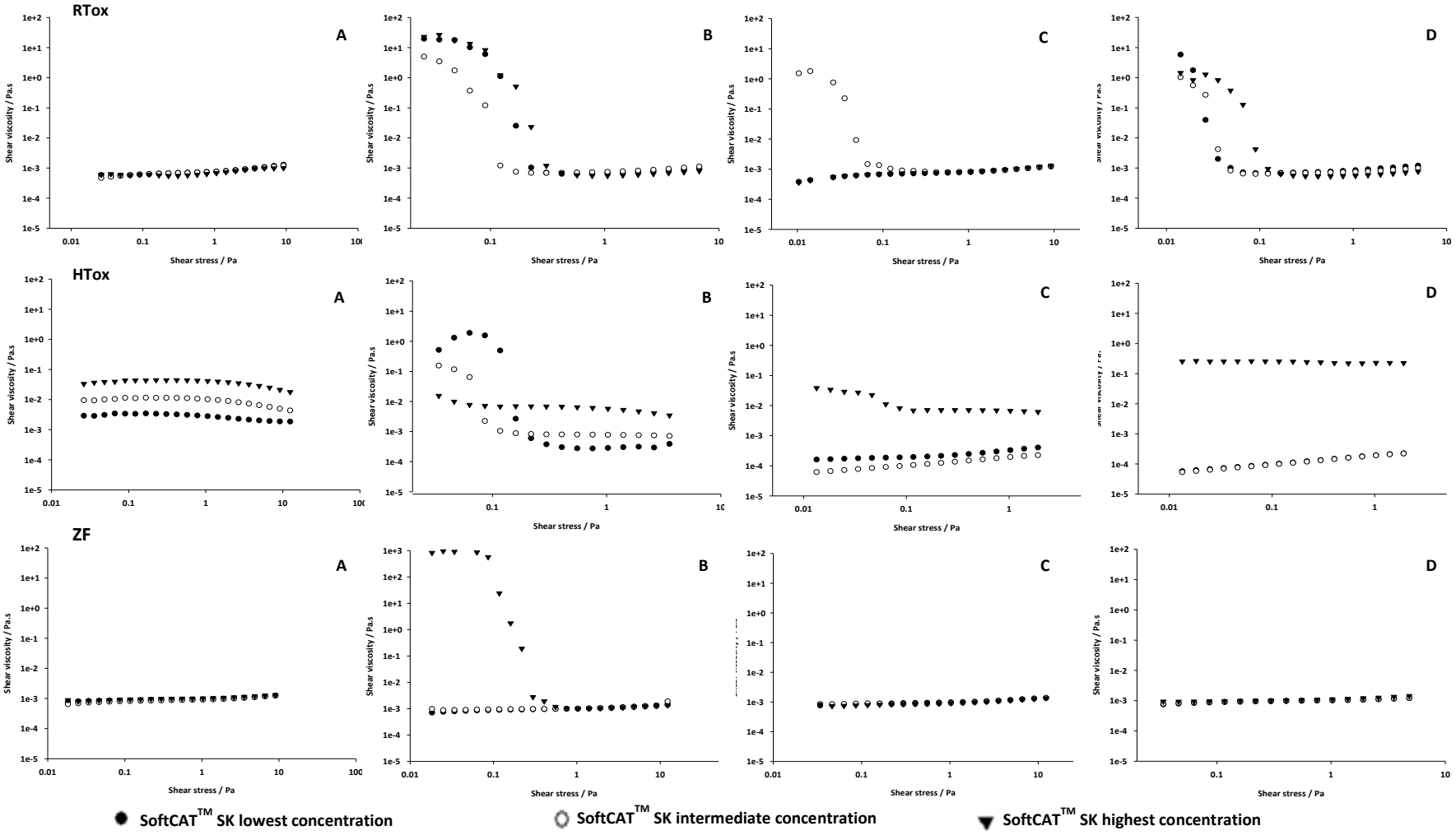


Fig. 1. Flow curves, viscosity (η) as function of shear stress(τ), for three concentrations of SK-variants at 25 °C (A: SK-H, B: SK-L, C: SK-M and D: SK-MH) suspended in the different test medium: MTox – *Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*.

Table 2: Computed lethal or effective concentrations of the SK-variants causing 50% of effect (L(E)C_{50}; mgL^{-1}), with the respective 95% confidence limits depicted within parenthesis, of the seven studied species. Color code indicates higher or lower toxicity according to the Globally Harmonized System of the United Nations (2011). RED-category I if acute toxicity ≤1.00 mg/L; ORANGE-category II if acute toxicity >1.00 and≤10.0 mg/L; YELLOW - Category III if acute toxicity >10.0 but≤100 mg/L; and WHITE–category IV if acute toxicity >100 mg/L. n.d.–no data.

		LC_{50} or EC_{50} (mg. L^{-1})			
Species	**Endpoint (exposure period)**	**SK-H**	**SK-L**	**SK-M**	**SK-MH**
Vibrio fischeri	Bioluminescence inhibition (15 min)	18973 n.d.	182532 n.d.	15059 n.d.	8545 (6619 – 10471)
Raphidocelis subcapitata	Growth inhibition (72 h)	9.51 (8.41 – 10.6)	17.2 (0.65 – 33.7)	16.2 (7.31 – 24.1)	10.6 (8.72 – 12.6)
Chlorella vulgaris	Growth inhibition (72 h)	4.73 (4.66 – 4.81)	2.16 (2.03 – 2.30)	5.07 (1.97 – 5.18)	21.6 (12.0 – 31.3)
Daphnia magna	Immobilization (48 h)	209 (122 – 268)	686 (434 – 881)	759 (617 – 876)	706 (493 – 871)
Brachionus calyciflorus	Mortality (24 h)	0.17 (0.16 – 0.19)	3.84 (2.69 – 4.97)	3.54 (3.23 – 3.86)	1.27 (0.51 – 1.93)
Heterocypris incongruens	Mortality (48 h)	664 (398 – 898)	5356 (3868 – 8281)	6392 (5515 – 7603)	4478 (3316 – 6313)
Danio rerio	Mortality (96 h)	8.69 (7.94 – 9.54)	61.2 (55.3 – 68.1)	24.7 (18.9 – 29.5)	16.4 (8.51 – 22.6)

3.4. Species sensitivity distribution curves and MAC-EQS derivation for the aquatic compartment

The L(E)C_{50} obtained for the seven species and for each SK-variant were integrated into SSD to derive the respective hazard concentrations protecting 5, 20 and 50% of the species (Fig. 2). Though an overlap in the 95% confidence limits occurred, SK-H exhibited the lowest HCx values (Fig. 2). Overall, *B. calyciflorus* and the microalgae were the species showing the highest sensitivity to SK-variants (Fig. 2).

The MAC-EQS computed for each SK-variant for the aquatic compartment are shown in Table 4S. The values of MAC-EQS obtained with the determinist approach were lower than those obtained with the mechanistic approach, with a difference equal to or greater than 60-fold. Thus, the former methodology revealed to be more conservative than the latter. Furthermore, MAC-EQS for the aquatic compartment estimated for SK-H was consistently the lowest one, therefore, suggesting this variant as the most toxic to aquatic biota. From these results is also possible to predict that, considering the goal of protecting 95% of the species, the aquatic ecosystems would be at risk at concentrations above 0.00017 mg/L for the SK-H, 0.00216 mg/L for SK-L, 0.00354 mg/L for SK-M and 0.00127 mg/L for SK-MH.

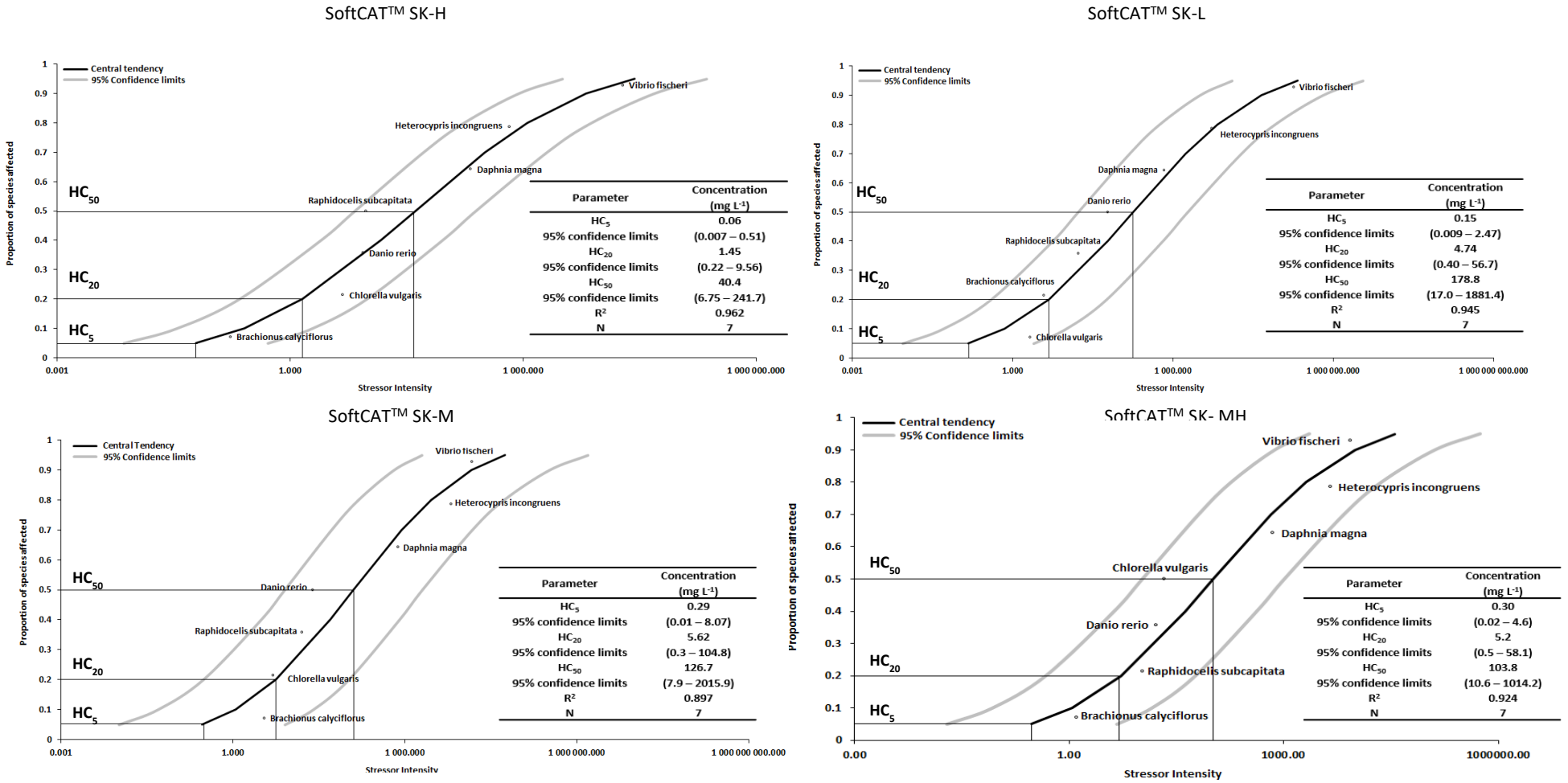


Fig. 2. Species sensitivity distribution curves constructed for the four SK-variants, where HC_X denotes hazard concentration that affect X % of the species, R^2 refers to the coefficient of determination, N is the number of data points.

4. Discussion

In this work it was intended to assess the influence of cationic substitution in the physico-chemical properties and ecotoxicity of SK cellulose-derived polycationic polymers, aiming to identify the least toxic variant. A clear association between the ecotoxicity of SK variants and their physical-chemical properties was not found. Despite, the integration of both sets of information allowed to establish some overall patterns. For instance, SK-H, with the highest molar cationic substitution (0.3) was the most toxic variant. According to the GHS of the United Nations (2011) the toxicity of SK-H is classified in the highest toxicity categories (I and II) in five out the seven tested species. None of the other variants was included in category I. Such result might be related to the architecture of the polymer itself. Increasing the cationic charge density correlates with increased toxicity (to cells and/or organisms), most probably because cell membranes are negatively charged. Variants with a larger number of cationic groups are more prone to establish electrostatic interactions with cell membranes (Narita et al., 2001; Roy, 2020). Goodrich et al. (1991) studying the effects of two acrylamide-derived cationic polymers on rainbow trout has concluded that toxicity was charge density-dependent (polymers were denominated as B-1 and B-2 with 10% and 39% of charge density, respectively). More specifically, the derived 24 to 96 h LC_{50} ratios were of 1 and 1.1 for B-1 and B-2, respectively. Moreover, B-1 did not present acute toxicity to the fish in a flow-through system (Goodrich et al., 1991). As well, Fischer et al. (2003) results with eight different polymers, verified that PLL and PEI [poly (L-lysine hydro bromide) and poly(ethylenimine), respectively]–the two polymers with the highest ratio of cationic charges per molecular weight–were the ones inducing the most damage in mouse fibroblasts cell membranes (Fischer et al., 2003). Likewise, Narita et al. (2001) using protoplasts verified that increasing charge density of cationic polymers would lead to a higher number of disrupted yeast cells (Narita et al., 2001). Despite agreeing with the results here presented, one must highlight that the results obtained from Fischer et al. (2003) are from single cells, whilst those of Narita et al. (2001) used protoplasts (meaning that yeast cell wall was firstly removed) and thus may not accurately reflect what happens in more complex or intact structures/organisms. Narita et al. (2001) also fundament that despite charge density is an important factor, solely was not sufficient to induce yeast cell disruption, but when considering the hydrophobicity index (HS), both the number of disrupted cells and HS were well correlated. However, the same observation could not be made in here, since the SK-H shared the same HS as two of the others less toxic polymers.

Furthermore, the hydrodynamic size of the SK-variants may have also influenced their toxicity. Here, two aspects should be considered: the influence that the ionic composition of the media may exert on polymers' conformation, and that the toxicity of each polymer may be species-specific. In general, the hydrodynamic size of SK-variants suspended in test media was higher than when suspended in distilled water, with few exceptions. The more complex ionic composition of the media relatively to distilled water may in part explain such phenomenon because the salts present in the media might induce changes in the particle double layer, resulting in the reduction of the overall effective net charge and consequently introducing some instability in the suspensions. In one hand, the presence of salts in test media may lead to a charge screening

of the polymer, resulting in the shrinking or compaction of the polymer chains explaining the lower size particles in some cases; although in other cases, due to the characteristics of the polymer itself and/or the media, this behavior might result not in compaction but in a dispersion or dilation of the polymer, possibly due to the reduced repulsion among polymer chains and counterions entropy, resulting in higher sized particles (Bolto and Gregory, 2007). Still, it must be highlighted that these behaviors are hypothesized to vary according to the composition of the media and to the characteristics of the polymers and concentrations (e.g., rotifers and ostracods shared the same culture media, although tested concentrations varied, leading to different behaviors of the polymers). The variability of the medium (with regard to hardness, total composition, pH) has been pointed as one of the main drawbacks for the further understanding of the toxicity of cationic polymers (e.g., Salinas et al., 2020). There has been a growing driving force so that there is harmonization about the type of media used, not only for the purpose of proceeding with more realistic exposure scenarios, but also to eliminate potential sources of variability that can cloud the results and therefore make it difficult to compare with each other and even with other published studies. Since, in the present study each species was tested with its optimal medium, one should pinpoint that even slight variations in tested water media might account for the different toxicity outcomes (Salinas et al., 2020). As an example, SK-H was toxic to zebrafish, whilst not presenting harm to daphnia's and ostracods. In this case, water used for the fish is much more soft (lower calcium content) than ASTM medium, and therefore being in agreement with Salinas et al. (2020) results that clearly showed that increasing water hardness decreases the toxicity of cationic polymers. Notwithstanding, for rotifers, that share with ostracods the same test medium, it was found that the same variant SK-H was very toxic. Thus, here it is hypothesized that other factors might have a preponderant role rather than the composition of the media itself. Along with zeta-potential and size of the polymers, the viscosity of the SK-variants might have also accounted for their toxicity. The SKMH exhibited the lowest viscosity index (approximately ten times lower than the other SK-variants), whilst being the polymer posing the least toxicity to all the studied species as well. Previous studies have clearly shown that the viscosity of a fluid can change the sensorial and mechanical sensitivity of cell membranes, and therefore change the behavior of organisms and induce metabolic changes to counterbalance the potential energy costs related to the resistance that the fluid causes to the movement of the organisms. Sohn et al. (2013) and Orchard et al. (2016) showed that when medium viscosity increased, dinoflagellates swimming speed decreased as well as flagella movement frequency, migration patterns in the water column were more erratic, whilst turning rates were sometimes increased, suggesting that organisms were under stressful conditions. Serra et al. (2019) results also corroborate this hypothesizes; by using *D. magna*, these authors found that increasing shear stress led to feeding inhibition and later to organisms' death.

Finally, the species specificities might have also played a major role regarding the toxicity of the polymers. For instance, considering the two algae species that shared the same tested medium and *B. calyciflorus* and *H. incongruens* (also shared the same test medium), the pattern of toxicity as very distinct. Looking firstly at *C. vulgaris*, previous studies aiming on the ecotoxicity of other polyquaternium polymers

identified this species was very sensitive (e.g., Cumming, 2008; Simões et al., 2021), stating that the size of the polymer was the main toxicity driver by determining its entrance in the cells. But other characteristics of *C. vulgaris* may contribute to the high sensitivity of this species to such polymers. *Chlorella vulgaris* can excrete exopolymeric substances under stressful conditions, which are basically polysaccharide-rich anionic polymers (e.g., Chen et al., 2015), thus probably with high affinity to these cationic SK-variants. Regarding the ostracod *H. incongruens*, none of the tested SK-variants induced toxicity. This species holds a hard, thick, external exoskeleton, which can be closed during stressful situations (Karanovic, 2012). Inside the shell, the organisms might hold some water and food stored and wait until better conditions arrive (Karanovic, 2012), which may explain their higher tolerance to the SK-variants. These differences between species of the same ecological groups (algae as producers, and rotifers and ostracod as primary consumers), highlight the importance of performing extensive ecotoxicological reviews of these new polymers, since even though they present high degree of similarities, they might exert very distinct toxicities. Although it is not possible to make a comparison with another species of the same ecological group, the zebrafish also responded in a peculiar way when exposed to these polymers. It should be remembered that during the first 48 h of exposure to the polymers, the cumulative mortality registered was less than 20%, rising from this moment until the end of the tests (96 h). This observation may be related to hatching moment of the larvae, with the chorion playing a protective barrier against the entry of the polymers and interaction with the organisms, as already stated for other xenobiotics (e.g., Busquet et al., 2014; Kim and Tanguay, 2014). Moreover, after hatching, ionic regulation and respiratory function may be severely compromised since, for instance, gills have already been reported as toxicity action sites for other cationic polymers (e.g., Muir et al., 1997). Also, the bacterium here evaluated stood out from other organisms: it was the only species for which none of the polymers tested showed toxicity. These organisms are very versatile and can thrive in very unfavorable environments. For example, this bacterium can form associations with several marine organisms (which, in exchange for a niche and nutrients, confer light properties to avoid predators). It is now known and may be one of the reasons that may explain their tolerance here, that they manage to do this not by adjusting themselves to the host, but actively shaping the surrounding environment according to their needs (e.g., secreting molecules, making physiological adjustments, or even altering the gene expression of the host; reviewed by Norsworthy and Visick, 2013). Another explanation is that large cationic polymer molecules would most likely bind to the outer surface of the bacteria and therefore cannot be internalized and likely to exert internal effects at the level of the bioluminescence pathway.

As stated above, cationic polymers are designed to interact with dirt (negatively charged), with the main purpose of removing it. In the same way, when released via wastewater they become in contact with a panoply of other environmentally relevant contaminants and adsorb to them (anionic surfactants, organic matter, debris). Such aspect might result in confounding factors that interfere with analytical methods impairing the detection of these compounds in environmental matrices. According to the study performed by Bolto and Gregory in 2007, the cationic polymers are the more toxic ones especially to aquatic organisms

(Bolto and Gregory, 2007). They also observed that the chain shortening that can result from the interactions established with negatively charged particles, originate a loss of polymer charge, resulting in a reduction in the efficiency to clarify the effluent in wastewater treatment due to a faster filter time. The impossibility of comparing the MAC-EQS here estimated with environmentally measured concentrations was also highlighted as a gap in literature when studying SL-variants (also cellulose-derived; Simões et al., 2021). Coming across it, solutions developed upstream in the industry, such as the projection of polymers whose properties (rheological and physicochemical) and ecotoxicity have been firstly explored before their commercialization might be of added value for regulatory agencies. This information is liable to be complementary of that already existing (e.g., REACH, USEPA), thus consolidating the available databases.

This aspect appears even more relevant when the world is under a pandemic and the use of this type of products has increased exponentially (Berardi et al., 2020). Safeguarding environmental matrices from poorly or non-explored polymers must be a priority. All SK-variants here explored presented low MAC-EQS. Other widely used surfactants such as those LAS-based (lauryl ammonium sulphate; anionic) or DTDMAC-based (ditallow dimethyl ammonium chloride; cationic) have been detected in discharge effluents in the µg/L range (up to 1090µg/L and 62µg/L, respectively; Jardak et al., 2016); yet, when considering the world consumption rates and that no proper removal is applied to these polymers, they might start to pose a threat to the aquatic compartment (Cumming et al., 2011; Berardi et al., 2020). Still, considering those same MAC-EQS and that all formulations are described as possessing similar foam enhancement and emollient deposition efficiencies (Dow, 2013), one might suggest SK-M (presenting the highest MAC-EQS value) as the least toxic SK-variant, and therefore encourage its application in formulations at the expense of others more environmentally toxic.

5. Conclusions

From the results obtained in this work, no relation could be drawn between rheological and/or physicochemical properties and ecotoxicological data. Still, some patterns could be detected. Media composition influenced polymers charge with higher ionic strength and the presence of divalent anions in the media leading to a decrease in zeta-potential. Moreover, other polymer properties such as viscosity might have played a major role in their toxicity, with highly viscous fluids inducing higher toxicity to the studied species. Ecotoxicity data allowed to compute MAC-EQS values ranging from 0.00017 mg/L in SK-H to 0.00354 mg/L in SK-M. Considering the demands for these products today (Berardi et al., 2020), it is hypothesized that environmental concentrations can easily exceed these values, although no value has been reported so far in the literature. Still, considering MAC-EQS, it is suggested that SK-M is the least toxic SK-variant that should be further explored by PCP industry.

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Supplementary information

Table 1S - Viscosity m(Pas), Molar cationic substitution (CS), hydrophobic substitution index (HS) and overlap concentration (c*) of the four SoftCAT™ SK polymers (Company, 2008).

SoftCAT™ SK	Viscosity (mPas) (aqueous solution 1%)	Molar cationic substitution (CS)	Hydrophobic substitution index (HS)	Overlap concentration (c*) ($g\ L^{-1}$)
SK-H *(Amerchol. lot: TC2450GRA2)*	2100	0.3	5	4.0
SK-L (Amerchol.lot: TC2650GRA1)	2400	0.2	5	4.8
SK-M (Amerchol.lot: TC2550GRA1)	2200	0.25	5	4.3
SK-MH (Amerchol.lot: TC2550GRA2)	200	0.25	30	4.2

Table 2S - Summary of the procedures used to perform the ecotoxicity assays.

Test species	Exposure time Endpoint	Light:Dark (hours) Temperature (°C)	Validity criteria	Dilution water	Replicates Organisms/replicate	Concentrations (mg. L^{-1})	Protocol
Decomposer							
Vibrio fischeri	15 min Bioluminescence	- 4 ± 1°C	Bioluminescence loss in control: 0.6 < x < 1.8	Diluent	1	**[SK-H]**, **[SK-L]**, **[SK-M]**, **[SK-MH]**: 32.0, 64.0, 128, 255, 512, 1024, 2048, 4095, 8190.	Microtox®; Microbics Inc., USA
Producers							
Chlorella vulgaris ***Raphidocelis subcapitata***	72 h Growth rate	24:0 h 23 ± 1°C	16-fold increase	MBL	3 10^4 cells/mL	**[SK-H]**, **[SK-L]**, **[SK-M]**, **[SK-MH]**: 0.68, 1.03, 1.54, 2.31, 3.47, 5.20.	OECD, 2004: Guideline 201
Primary consumers							
Daphnia magna	48 h Mortality	16: 8 h 20 ± 1°C	Mortality in control < 20%	ASTM	4 5 org/rep	**[SK-H]**: 220, 310, 430, 600, 841, 1176, 1646. **[SK-L]**, **[SK-M]**, **[SK-MH]**: 600, 841, 1176, 1646, 2305, 3227, 4518.	OECD, 2004: Guideline 202
Brachionus calyciflorus	24 h Mortality	0:24 h 23 ± 1°C	Mortality in control < 20%	ASTM	5 5 org/rep	**[SK-H]**: 0.07, 0.09, 0.13, 0.18, 0.30, 0.40, 0.50. **[SK-M]**: 2.67, 3.72, 5.21, 7.30, 10.2, 14.3, 20.3, 28.0, 39.3. **[SK-L]**, **[SK-MH]**: 1.90, 2.67, 3.72, 5.21, 7.30, 10.2, 14.3, 20.3, 28.0.	Rotoxkit F®, MicroBioTests, Ghent, Belgium
Heterocypris incongruens	48 h Mortality	0:24 h 25 ± 1°C	Mortality in control < 20%	ASTM	3 10 org/rep	**[SK-H]**: 429, 601, 841, 1176, 1646, 2305, 3227, 4518. **[SK-M]**: 1650, 2310, 3230, 4520, 6320, 8850, 12400. **[SK-L]**, **[SK-MH]**: 1176, 1650, 2310, 3230, 4520, 6325, 8850, 12400.	Ostracodtoxkit F® chronic; MicroBioTests Inc.
Secondary consumer							
Danio rerio	96 h Mortality	16:8 h 26 ± 1°C	Mortality in control < 10%	zebrafish water	3 10 embryos/rep	**[SK-H]**: 3.00, 3.90, 5.10, 6.60, 8.60, 11.2, 14.2, 19.0. **[SK-L]**: 21.0, 27.0, 35.0, 45.0, 60.0, 80.0, 100, 130. **[SK-M]**, **[SK-MH]**: 19.0, 24.0, 32.0, 41.0, 54.0, 70.0, 90.0, 120.	OECD, 2013: Guideline 236

Table 3S - Characterization of each variant SK (SK-H, SK-L, SK-M and SK-MH) regarding particle size (nm), the zeta potential (ζ-Potential, mV) and conductivity (Cond, mS cm^{-1}) at the three tested concentrations (lowest, intermediate and highest; mg. L^{-1}). Characterization was performed in distilled water (dH2O) as follows: MTox –*Vibrio fischeri*; MBL – *Raphidocelis subcapitata* and *Chlorella vulgaris*; ASTM – *Daphnia magna*; RTox – *Brachionus calyciflorus*; HTox – *Heterocypris incongruens*; ZF – *Danio rerio*. Values are presented as average values ± standard deviation of n=3. n.d. – no data (concentration above the overlap concentration).

	SoftCAT™ SK-H					**SoftCAT™ SK-L**			
	[SK-H] mg. L^{-1}	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**		**[SK-L] mg. L^{-1}**	**Particle size (Di0.5, nm)**	**ζ-potential (mV)**	**Conductivity (mS cm^{-1})**
MTox	32.0	460 ± 19.8	7.61 ± 1.42	9.86 ± 0.27	MTox	32.0	922 ± 60.1	5.94 ± 0.52	9.07 ± 0.23
	1024	1410 ± 28.3	18.4 ± 2.03	2.41 ± 0.20		1024	1090 ± 127	10.6 ± 0.83	1.60 ± 0.09
	8190		n.d.			8190		n.d.	
MBL	0.68	481 ± 32.5	17.5 ± 3.42	0.13 ± 0.002	MBL	0.68	721 ± 55.2	5.02 ± 1.71	0.05 ± 0.002
	2.31	569 ± 4.36	23.0 ± 1.85	0.02 ± 0.004		2.31	860 ± 74.8	26.8 ± 3.74	0.05 ± 9.83 e-4
	5.20	845 ± 51.9	22.4 ± 0.90	0.01 ± 1.83 e-4		5.20	488 ± 28.4	24.5 ± 4.03	0.02 ± 0.003
ASTM	220	744 ± 86.3	20.4 ± 1.41	0.14 ± 0.002	ASTM	600	57.4 ± 4.48	18.3 ± 2.33	0.25 ± 0.004
	600	290 ± 17.7	39.2 ± 2.23	0.11 ± 8.16 e-4		1646	4.15 ± 0.49	32.6 ± 4.40	0.16 ± 8.66 e-4
	1646	5.96 ± 1.44	34.7 ± 2.32	0.25 ± 0.003		4518	6.22 ± 0.04	36.5 ± 4.52	0.41 ± 0.007
RTox	0.07	516 ± 22.5	5.22 ± 1.83	0.48 ± 0.007	RTox	1.90	703 ± 28.9	22.9 ± 3.89	0.04 ± 1.47 e-4
	0.18	775 ± 29.4	5.25 ± 1.30	0.06 ± 0.002		7.30	548 ± 42.4	23.0 ± 3.23	0.17 ± 0.003
	0.50	221 ± 12.7	6.6 ± 2.7	0.05 ± 0.002		28.0	515 ± 35.0	14.1 ± 2.71	0.04 ± 0.003
HTox	429	794 ± 96.6	30.3 ± 1.71	0.36 ± 0.004	HTox	1176	861 ± 8.50	27.1 ± 6.75	0.49 ± 0.009
	1646	565 ± 50.5	42.5 ± 1.94	0.23 ± 0.002		4520	4.25 ± 0.44	42.3 ± 2.81	0.75 ± 0.02
	4518		n.d.			12400		n.d.	
ZF	3.00	335 ± 20.5	17.6 ± 7.62	0.02 ± 0.005	ZF	21.0	398 ± 65.5	17.3 ± 1.02	0.09 ± 0.002
	8.60	305 ± 10.4	24.9 ± 1.93	0.01 ± 0.002		60.0	976 ± 124	24.8 ± 1.40	0.04 ± 0.002
	19.0	506 ± 10.6	28.9 ± 2.70	0.01 ± 4.5 e-4		130	474 ± 22.6	26.8 ± 1.08	0.03 ± 0.03

	SoftCAT™ SK-M					SoftCAT™ SK-MH			
	[SK-M] mg. L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})		[SK-MH] mg. L^{-1}	Particle size (Di0.5, nm)	ζ-potential (mV)	Conductivity (mS cm^{-1})
MTox	32.0	1100 ± 35.4	2.91 ± 1.12	8.75 ± 0.26	MTox	32.0	761 ± 152	1.91 ± 0.73	8.25 ± 0.40
	1024	116 ± 5.51	12.3 ± 1.69	1.65 ± 0.11		1024	1470 ± 70.0	13.5 ± 1.02	1.56 ± 0.10
	8190		n.d.			8190		n.d.	
MBL	0.68	685 ± 24.2	14.9 ± 4.01	0.05 ± 0.003	MBL	0.68	1820 ± 77.7	10.6 ± 2.21	0.08 ± 0.001
	2.31	523 ± 58.4	21.0 ± 3.46	0.02 ± 0.002		2.31	399 ± 15.5	20.7 ± 3.13	0.02 ± 0.006
	5.20	975 ± 62.2	23.1 ± 6.23	0.01 ± 0.007		5.20	237 ± 6.51	26.3 ± 4.85	0.01 ± 0.004
ASTM	600	409 ± 0.71	21.3 ± 2.65	0.42 ± 0.01	ASTM	600	378 ± 30.2	30.4 ± 3.65	0.12 ± 0.001
	1646	21.0 ± 1.13	36.0 ± 2.51	0.20 ± 0.001		1646	225 ± 25.7	40.2 ± 2.23	0.17 ± 0.002
	4518		n.d.			4518		n. d	
RTox	2.67	621 ± 14.1	20.5 ± 2.21	0.06 ± 0.001	RTox	1.90	520 ± 43.1	15.6 ± 1.28	0.35 ± 0.013
	10.2	604 ± 187	25.9 ± 6.37	0.02 ± 0.001		7.30	302 ± 21.2	17.8 ± 3.21	0.04 ± 0.003
	39.3	727 ± 43.1	26.3 ± 3.41	0.02 ± 0.004		28.0	446 ± 3.54	20.2 ± 3.03	0.08 ± 0.002
HTox	1650	187 ± 17.6	27.1 ± 4.74	0.16 ± 0.002	HTox	1176	644 ± 52.3	40.8 ± 3.23	0.94 ± 0.03
	4520		n.d.			4520		n.d.	
	12400		n.d.			12400		n.d.	
ZF	19.0	790 ± 82.4	24.0 ± 3.01	0.05 ± 6.24 e-4	ZF	19.0	615 ± 14.1	16.2 ± 2.21	0.13 ± 0.002
	54.0	553 ± 20.1	22.8 ± 3.74	0.02 ± 0.003		54.0	652 ± 24.0	30.0 ± 3.74	0.03 ± 0.004
	120	531 ± 26.1	22.1 ± 2.76	0.06 ± 0.001		120	525.5 ± 24.9	33.9 ± 6.47	0.03 ± 0.003

Table 4S - Maximum acceptable concentrations environmental quality standard (MAC-EQS), for the four SK variants, computed by applying a safety factor of 1000 to the lowest median lethal or effective concentration [$L(E)C_{50}$, mg. L^{-1}] or by determination of Hazard Concentrations that protect 95% of the species (HC_5) through Species Sensitivity Distribution Curves (SSD).

	SK-H	**SK-L**	**SK-M**	**SK-MH**
Lowest $L(E)C_{50}$/1000 (mg. L^{-1})	0.00017	0.00216	0.00354	0.00127
HC_5 (mg. L^{-1})	0.06	0.15	0.29	0.30

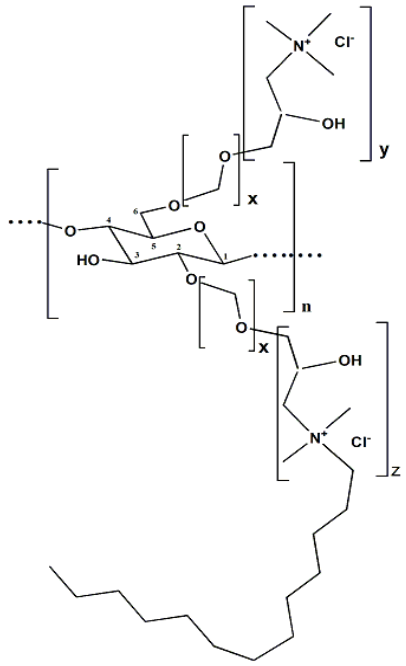


Figure 1S - Molecular structure of SoftCAT™ SK polymers (Company, 2008). CS refers to cationic substitution.

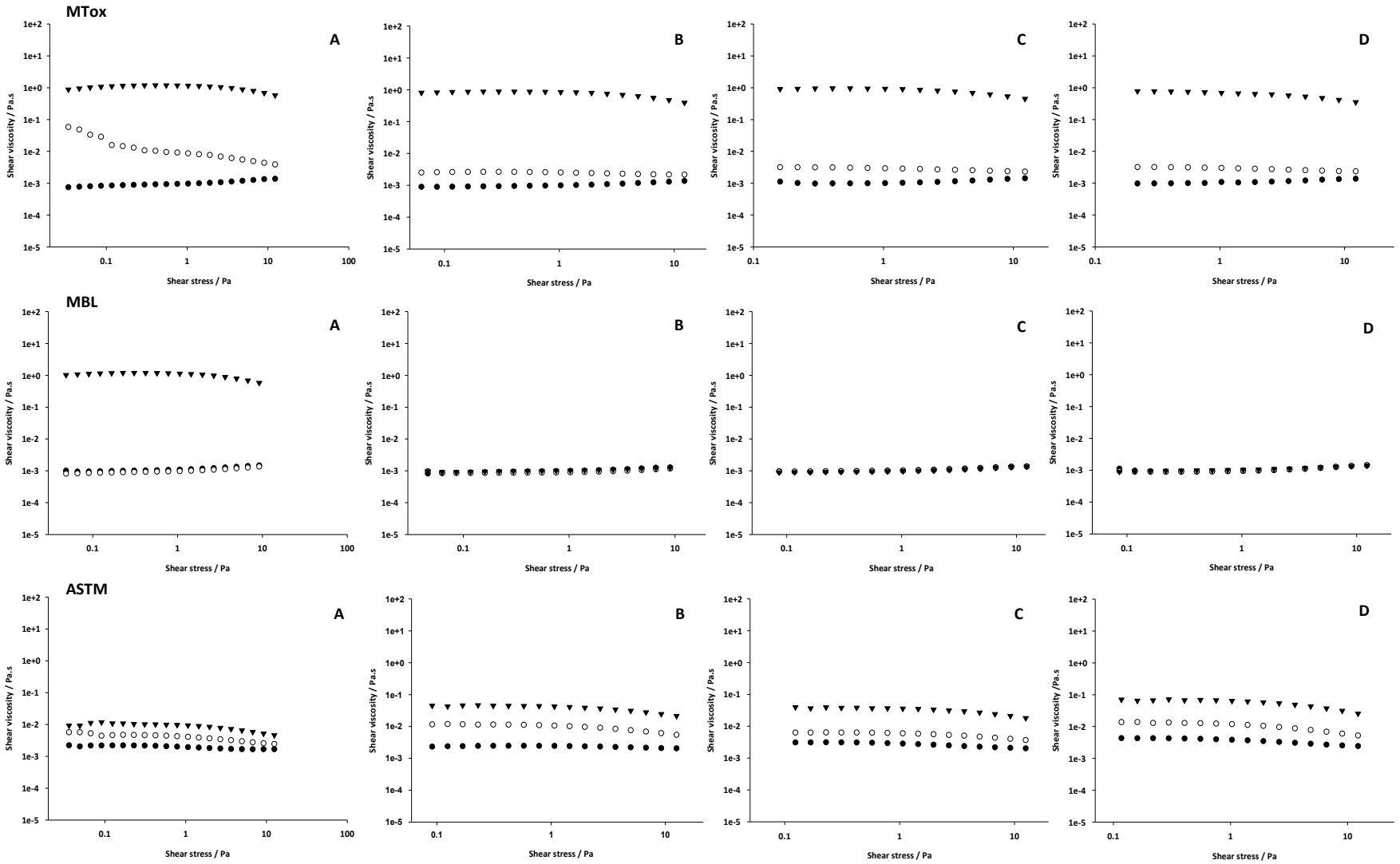


Figure 2S - Flow curves, viscosity (η) as function of shear stress (τ), of the concentrations used in the different specie assays of SoftCAT™ SK variants at 25°C prepared in distilled water (A- SoftCAT™ SK-H, B - SoftCAT™ SK-L, C - SoftCAT™ SK-M and D - SoftCAT™ SK-MH).

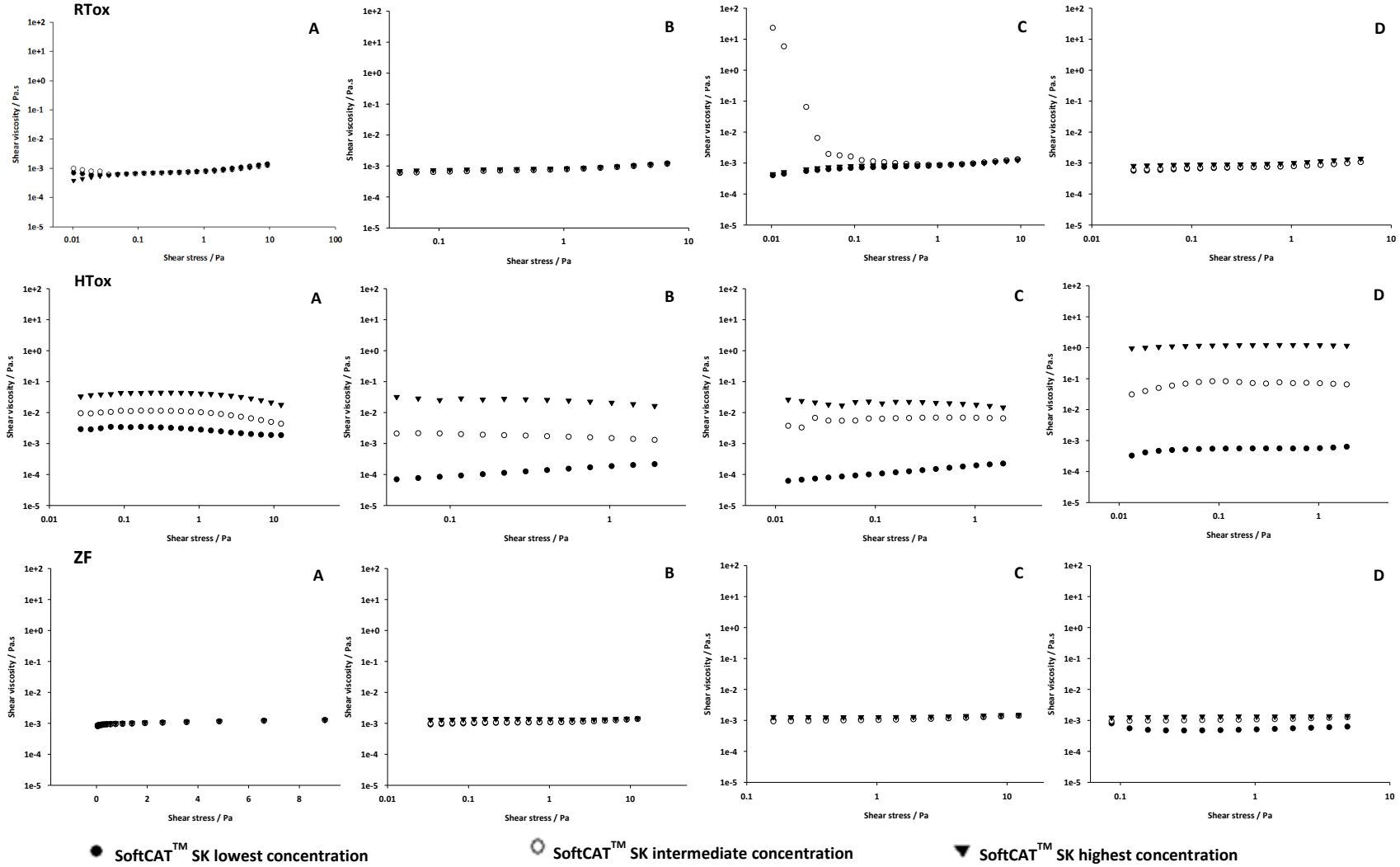


Figure 2S (Cont.) - Flow curves, viscosity (η) as function of shear stress (τ), of the concentrations used in the different specie assays of SoftCAT™ SK variants at 25°C prepared in distilled water (A- SoftCAT™ SK-H, B - SoftCAT™ SK-L, C - SoftCAT™ SK-M and D - SoftCAT™ SK-MH).

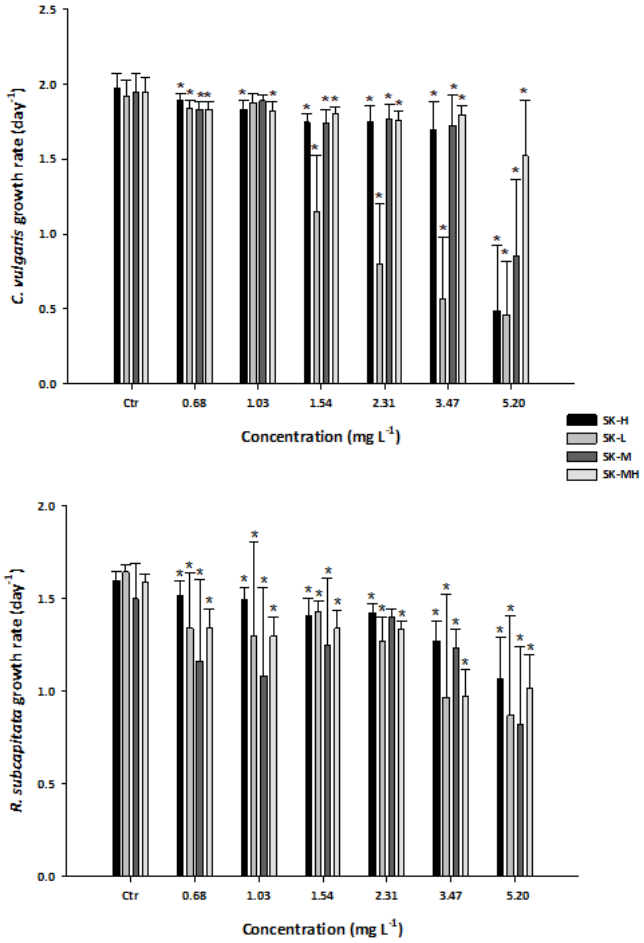


Figure 3S: Growth rate (day^{-1}) of *Chlorella vulgaris* (top figure) and *Raphidocelis subcapitata* (down figure) after exposure, for 72 h, to increased concentrations (mg/L) of the four hydrophobically modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SK variants; mg. L^{-1}). Vertical bars correspond to standard error. *denotes statistical differences between SK concentrations and the respective control, within each SK variant ($p<0.05$). n=3.

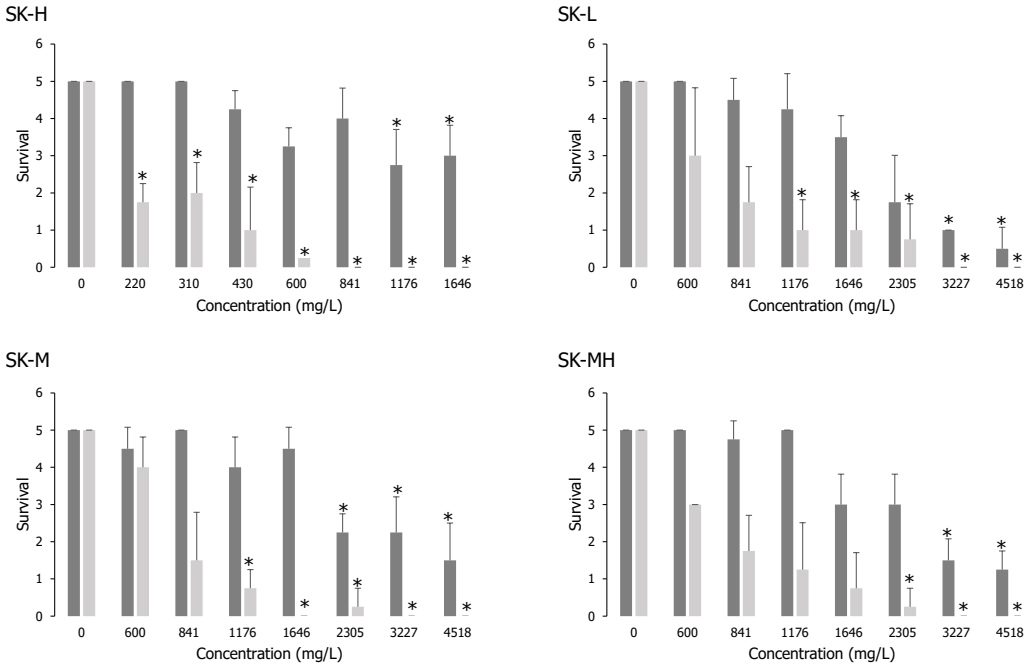


Figure 4S: Average survival of *Daphnia magna* after 24 hours (light grey bars) and 48 hours (black bars) of exposure to increased concentrations (mg/L) of four different cationically modified hydroxyethyl cellulose polymers (SoftCAT™ SK variants; mg/L): SK-H, SK-L, SK-M, and SK-MH. Vertical bars correspond to standard deviation. *denotes statistical differences between SL-K treatments and the respective control within each time (24h or 48h; $p<0.05$). n=4.

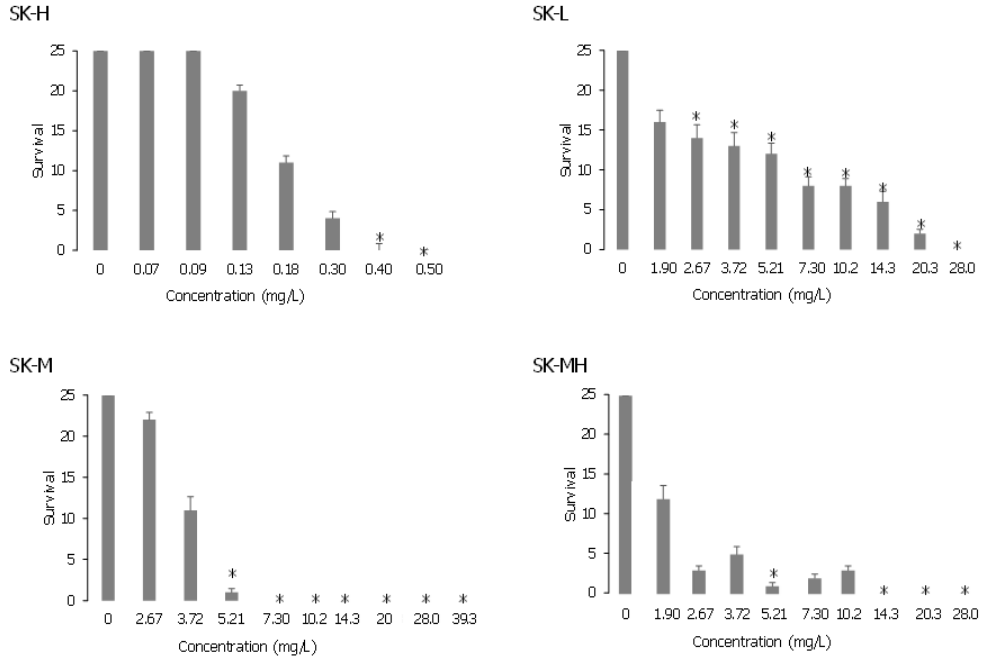


Figure 5S: Average survival of *Brachionus calyciflorus* after 24 hours of exposure to increased concentrations (mg/L) of four different cationically modified hydroxyethyl cellulose polymers (SoftCAT™ SK variants; mg/L): SK-H, SK-L, SK-M, and SK-MH. * indicates statistical differences in relation to control ($p<0.05$). n=5.

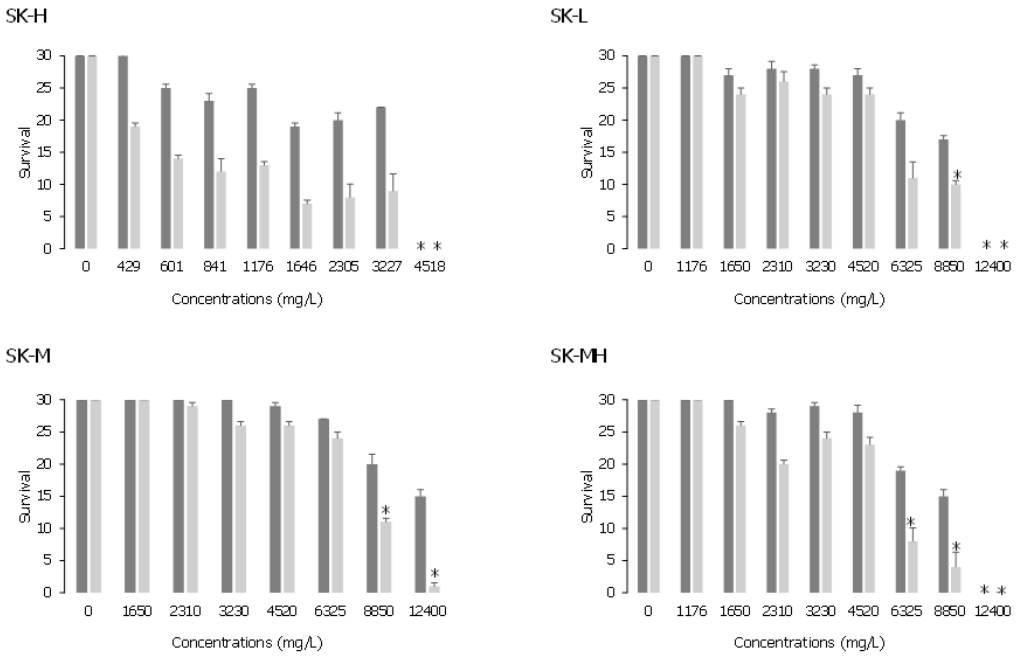


Figure 6S: Average survival of *Heterocypris incongruens* after 24 (dark grey) and 48 (light grey) hours of exposure to increased concentrations (mg/L) of four different cationically modified hydroxyethyl cellulose polymers (SoftCAT™ SK variants; mg/L): SK-H, SK-L, SK-M, and SK-MH. * indicates statistical differences in relation to control within each time (24h or 48h; $p<0.05$). n=3.

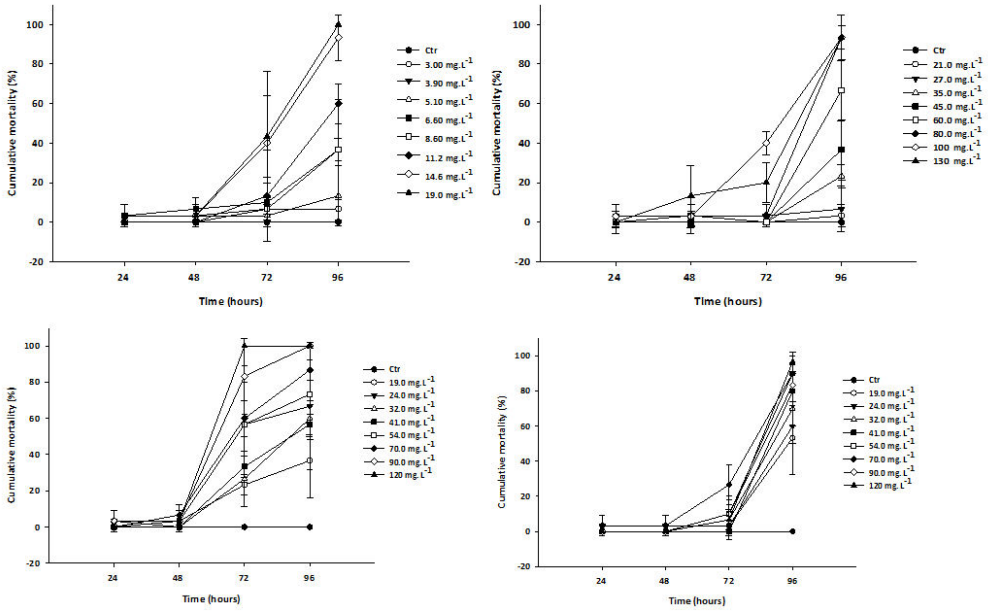


Figure 7S: Average cumulative mortality curves for *Danio rerio* exposed during 96 h to seven concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (SoftCAT™ SK variants; mg. L^{-1}): SK-H, SK-L, SK-M and SK-MH. Vertical bars correspond to the standard deviation. n=30.

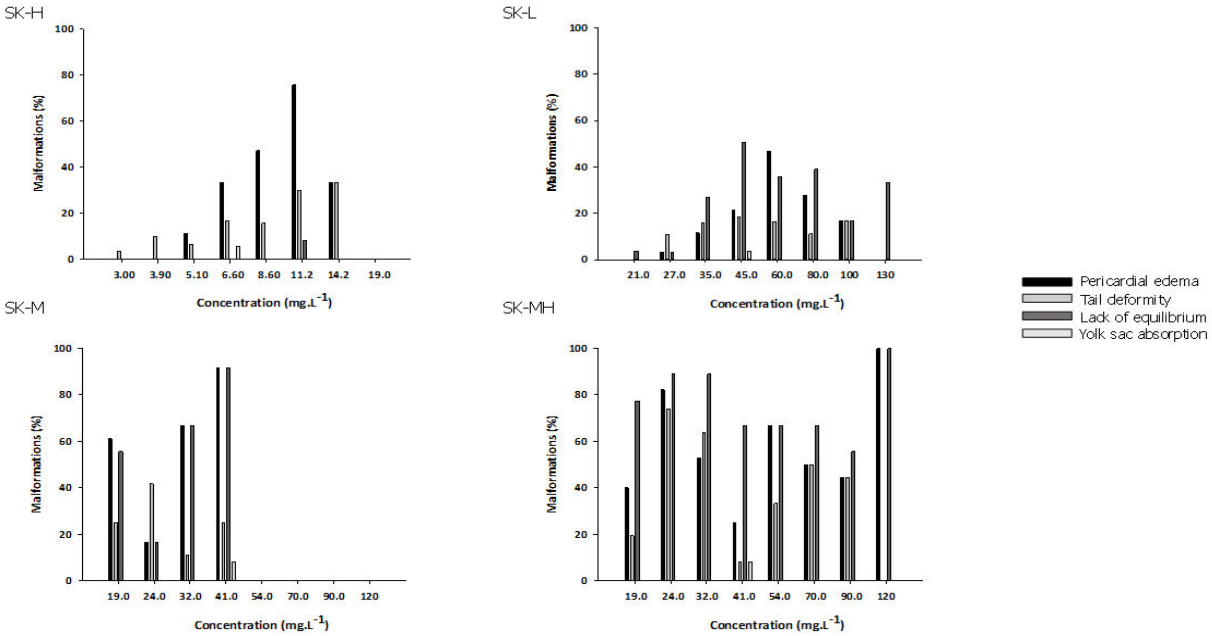


Figure 8S: Percentages (relatively to control conditions) of the different registered malformations in larvae of *Danio rerio* after being exposed to eight concentrations of each of the four hydrophobically modified hydroxyethyl cellulose polymers (SoftCAT™ SK variants; mg. L^{-1}): SK-M, SK-L, SK-M and SK-MH.

Chapter V

Role of temperature on the ecotoxicity of aged hydroxyethyl cellulose polymers to freshwater biota

Role of temperature on the ecotoxicity of aged hydroxyethyl cellulose polymers to freshwater biota

Anabela M. Simões, Luís Alves, Filipe E. Antunes, Isabel Lopes

Abstract

The ageing process of cationic cellulose derivatives, following their release into the environment, may be influenced by several physical, chemical, and biological factors. Temperature is a physical parameter that has been shown to play an important role in such ageing processes. This parameter acquires further relevancy on the ecotoxicity of these chemical compounds as it is predicted to suffer a significant increase within the context of global climate changes. Considering this framework, the present study intended to evaluate the influence of temperature on the acute ecotoxicity of aged quaternized hydroxyethyl cellulose polymers to freshwater biota. For this, four variants of SoftCAT™ polymers of SL with different HS (SL-5, SL-30, SL-60, SL-100) and of SK with different cationic substitution (CS) (SK-H, SK-L, SK-M, SK-MH) were aged for one month, in the dark and at 15, 20 and 25°C. The short-term ecotoxicity of these variants was assessed then for six species: *Raphidocelis subcapitata, Chlorella vulgaris, Daphnia magna, Brachionus calyciflorus, Heterocypris incongruens,* and *Danio rerio*. *Raphidocelis subcapitata, C. vulgaris* and *B. calyciflorus* were the most sensitive to the SL and SK variants. The computed median effective concentrations were used to derive hazard concentrations, by using species sensitive distribution curves, and the SL-30 variant showed to be the least toxic variant with the higher value of maximum acceptable concentration (3.17 mg/L). According to the obtained results, the temperature of 15°C was the one that induced higher toxicity of the aged polymers to the species studied, since it provoked the total mortality of the *B. calyciflorus* and *D. rerio* organisms after exposure to these polymers.

Keywords: Aquatic ecotoxicity, cationic cellulose polymers, temperature, ageing

1. Introduction

Personal care products (PCPs) are a group of compositions designated for external use being used in soaps, toothpaste, lotions, sunscreens, fragrances, etc. Disinfectants, insect repellents, fragrances, UV filters and preservatives are included in the primary classes of these compounds and their discharge to sewer/wastewater is most certainly continuous (Montes-Grajales et al., 2017; Biel-Maeso et al., 2019). They are not subjected to metabolic alterations; however, they enter the environment in large quantities, remaining unaltered due to their regular use by humans and industry (Ternes et al., 2004). Recent studies reported that many of these compounds are environmentally bioactive, persistent, and potentially bioaccumulate in the biota (Peck, 2006; Mackay and Barnthouse, 2010).

The persistence of a large variety of personal care products in natural freshwater resources is a constant concern all over the world (Kaspryk-Hordern et al., 2009; Bahlmann et al., 2014; Petrie et al., 2014;

Blair et al., 2015). Their occurrence and persistence in the environmental compartments can be mainly attributed to human use, sewage treatment plants and discharge from industries, etc (Ebele et al., 2017). Some of these compounds are newly synthesized chemical agents and due to the lack of enough information about their fate and persistence in the environment, and on its potential ecotoxicological effects, their ecological risks remain largely unknown (Ebele et al., 2017).

Some of the personal care products are not easily removed from the aquatic environment by conventional water treatment due to their physicochemical properties, posing a potential risk to aquatic organisms and public health (Jin-Lim and Wong, 2013; Ebele et al., 2017). The choice of healthier personal care products together with the introduction of new chemical compounds to the market contributes to the presence of these chemicals and their active metabolites in the aquatic environment and biota (Juliano and Magrini, 2017). Cellulose is a biodegradable polymeric and renewable material (Motloung et al., 2019) and polymers based in cellulose like cationic polymers exhibiting excellent biocompatibility are widely used in several consumer products and industrial processes as hair care products and fabric softeners (Kierkegaard et al., 2020) increasing the discharged during or after their use and the extent of their impacts on humans and aquatic life (Whang et al., 2008; Malhotra et al., 2015). The hydroxyethyl cellulose polymers (HEC) are cellulose derivatives used in PCPs, amphiphilic in nature, possessing the ability to tune their hydrophilicity/lipophilicity through chemical and hydrophobic modification. These have also the capability to form gels in the presence of water (Lindman et al., 2021). However, the toxicity of these polymers present in the PCPs remains unknown, regarding the modifications in their physicochemical properties when in contact with water but also the effect of temperature on these polymers. In the municipal wastewater treatment plant, these polymers were detected in a range of $\mu g\ L^{-1}$. Cationic polymers sorb very strongly to phospholipid membranes (Timmer et al., 2017) and possess the ability to disrupt cell membranes in organisms (Xia and Onyuksel, 2000; Groothuis et al., 2019). Thus, it seems like that these polymers, due to their increased use every day and the inadequate removal by conventional methods in effluent treatment plants, will be released to environmental matrices at higher concentrations and potentially present environmental risks, even though that they were previously considered to possess low environmental risk due to their low persistence. However, they might become pseudo-persistent and provoke toxicity as other persistent compounds (Cummings et al., 2011). It is of extreme importance to propose and develop alternative synthetic polymers through the change of specific physicochemical properties to minimize the ecotoxicological impacts on the aquatic environment. The cationic polymers are widely used in hair care products produced by the industry since they have the capability to protect the hair through the reversible adsorption to the air surface that possesses a negative charge that avoids being effectively removed during the rinsing process (Rhein, 2007). These polymers have excellent performance properties, capable of thickening, binding, emulsifying, stabilizing, capability to retain water and form films providing good protective action (Abdel-Halim, 2014). When dissolved in water, the electrostatic repulsion of the cationic groups of these hydroxyethyl cellulose polymers originates the expansion of their chain and a sudden increase in their viscosity (Wang and Ye, 2010).

Since these polymers are used in several applications that can result in the release to the aquatic environment, and due to the lack of studies on their persistence, bioactivity and/or potential accumulation in the environment, it is important to identify the effect of temperature on these compounds and the potential environmental effects. In 2010, Wand and Ye observed that an increase of temperature in cationic hydroxyethyl cellulose polymers induced a sharp decrease in their viscosity (Wang and YE, 2010). The study of the influence of temperature on the ageing process and subsequent toxicity of these compounds is important in the context of the climate change we are experiencing since these polymers are not easily removed from the aquatic environment by conventional water treatment, remaining in the aquatic environment. According to the last IPCC report, the global surface temperature was 1.09 °C (0.95-1.20) higher in 2011-2020 than in 1850-1900 (IPCC, 2021). This increase in temperature results mainly from total human influence, greenhouse gas concentrations, aerosols, ozone and land-use change, solar and volcanic drivers, and internal climate variability. In the present work, eight hydroxyethyl cellulose polymers with cationic and hydrophobic modifications were studied, aiming to identify the effect of temperature on the aquatic ecotoxicity after an ageing process of these polymers. Four cationically modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SK, INCI name – Polyquaternium-67) and four hydrophobically modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SL, INCI name – Polyquaternium-67) were studied after an ageing process of one month at three different temperatures, 15, 20, and 25 °C, to an array of assays with key species from different trophic levels. With this study, it was possible to provide maximum acceptable concentrations to be considered in future regulatory frameworks regarding these polymers.

2. Materials and methods

2.1 Studied polymers

A series of eight variants of cationically and hydrophobically modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SK and SL, INCI name - Polyquaternium-67), supplied by Amerchol Corporation, subsidiary of Dow Chemical Company, Greensburg, LA (Company, 2008) were studied in this work. These are cationic polymers composed of quaternized hydroxyethyl cellulose, with hydrophobic substitution (HS) and molecular cationic substitution (CS) of trimethyl ammonium and dimethyl-dodecylammonium (Figure 1; Table 1; Baillarin et al., 2011). SoftCAT^{TM} polymers derived from a reaction of a quaternary ammonium salt of hydroxyethyl cellulose (HEC), trimethyl ammonium-substituted epoxide and lauryl dimethyl ammonium-substituted epoxide (Company, 2008). SoftCAT^{TM} SK polymers are constituted by a fixed ethylene oxide (EO) group, molecular weight, a variable CS with the same low degree of dimethyl-dodecyl-ammonium hydrophobic substitution (Baillarin et al., 2011). These are chloride salts of N, N, N – trimethylammonium derivatives of hydroxyethyl cellulose with some dodecyl trimethylammonium residues as hydrophobic substituents with a molecular weight between 200,000 – 800,000 g mol^{-1} and are included in the family of cationic conditioning polymers.

Four commercial variants, SK-H, SK-L, SK-M and SK-MH, possessing different degrees of CS of trimethyl ammonium and dimethyl-dodecyl ammonium, varying from 0.2 to 0.3 M, corresponding to a

percentage of about 1% of nitrogen by weight, were studied in this work. The SK-MH is an exception since it has the same cationic modification as SK-M and a hydrophobic substitution of 30 in relation to the other SK variants.

On the other hand, the SoftCAT™ SL are characterized by a fixed ethylene oxide (EO) group number and MW, and different low levels of HS (Baillarin et al., 2011). In this work, were studied four SL-variants presenting different levels of HS that correspond to the average number of moles of hydrophobic residues for anhydro glucose repeat unit; for SL-5 the degree of substitution is 5 X 10^{-4}, for SL-30 is 5 X 10^{-3}, for SL-60 is 7 X 10^{-3} and for SL-100 is 1 X 10^{-2} (Drovetskaya et al., 2005; Company, 2008; Milcovich et al., 2016).

These eight variants of SoftCAT™ polymers have the ability to modify the rheology and the stability of a suspension, corresponding to the requirements of personal care products (Karlson et al. 2002).

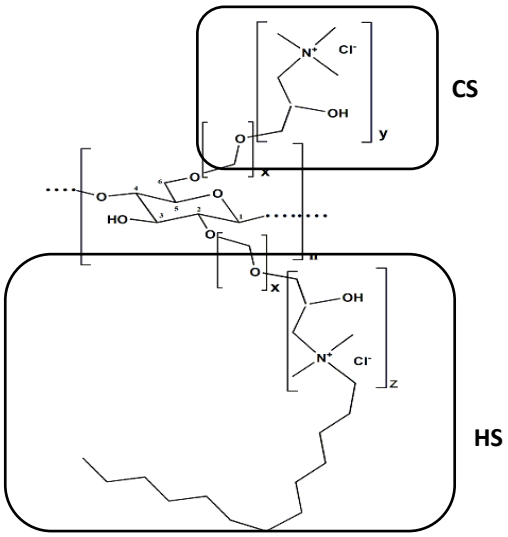


Figure 1: Molecular structure of SoftCAT™ SK polymers (Company 2008). The abbreviations CS and HS correspond to the cationic and hydrophobic substitution indexes, respectively

2.2. Ageing of SK variants

In this study, the influence of temperature on the ecotoxicity of the eight SoftCAT™ variants were assessed. For this, stock suspensions of each variant were aged, for one month, under controlled conditions of total darkness and at three different temperatures (15, 20 and 25°C). to simulate the degradation process of these polymers in the aquatic environment (Table 1S). The aged stock concentrations for each variant of SL and SK were as follows: (i) 10.0 mg. L^{-1} prepared in MBL medium (Nichols, 1973) for the assays with algae (*R. subcapitata and C. vulgaris*); (ii) 5000 mg. L^{-1} prepared in ASTM medium (ASTM, 2002) for the assay with *D. magna*; (iii) 4000 and 12500 mg. L^{-1} prepared in standard freshwater medium (Microbiotest Rotoxkit F protocol) for the assay with *B. calyciflorus*; (iv) 12500 mg. L^{-1} prepared in standard freshwater medium for the assay with *H. incongruens*, and (v) 150 and 4100 mg. L^{-1} prepared in ZW water for the assay with *D. rerio*.

Table 1: Viscosity (m(Pas)), molar cationic substitution (CS), hydrophobic substitution index (HS) and overlap concentration values (c*) of the eight SoftCAT™ polymers (Company, 2008).

SoftCAT™	Viscosity (mPas) (aqueous suspension 1%)	Molar cationic substitution (CS)	Hydrophobic substitution index (HS)	Overlap concentration (c*) (g L^{-1})
SK-H (Amerchol. lot: TC2450GRA2)	2100	0.3	5	4.0
SK-L (Amerchol. lot: TC2650GRA1)	2400	0.2	5	4.8
SK-M (Amerchol. lot: TC2550GRA1)	2200	0.25	5	4.3
SK-MH (Amerchol.lot: TC2550GRA2)	2300	0.25	30	4.2
SL-5 (Amerchol. lot: SK1050GR51)	2500	0.2	5	5.2
SL-30 (Amerchol. lot: SK1050GRS2)	2600	0.2	30	4.4
SL-60 (Amerchol. lot: SK1050GR51)	2700	0.2	60	3.3
SL-100 (Amerchol. lot: SK1050GR54)	2800	0.2	100	3.0

2.2 Ecotoxicological assays

After the ageing process for one month, the ecotoxicity of each variant was assessed by performing a set of ecotoxicity assays with aquatic species belonging to different taxonomic, trophic, and functional

levels: the microalgae *Raphidocelis subcapitata* and *Chlorella vulgaris* (producers), the cladoceran *Daphnia magna* (planktonic primary consumer), the rotifer *Brachyonus calyciflorus* (planktonic primary consumer), the ostracoda *Heterocypris incongruens* (epibenthonic primary consumer), and the fish *Danio rerio* (secondary consumer) (Table 2).

2.2.1 72-h growth inhibition assay with *Raphidocelis subcapitata* and *Chlorella vulgaris*

The stock cultures of algae *R. subcapitata* and *C. vulgaris* used to perform the growth inhibition assay, were maintained in Woods Hole MBL growth medium under continuous illumination (100 µE/m^2 /s) with aeration, at 20 ± 2°C (Stein, 1973). To initiate these assays, an inoculum of each species in an exponential growth phase was collected from the lab cultures. The 72h-growth inhibition assays were adapted to 24-well microplates and performed according to the OECD Guideline 201 (OECD, 2004) (Geis et al., 2000; Moreira-Santos et al., 2004). The 72-h growth inhibition assay was initiated with the exposure of each algal specie to six concentrations of each SK and SL variant (Table 2), plus a control (MBL medium; Nichols, 1973). Each concentration and control had three replicates with a cell density of 10^4 cell mL^{-1} and 1 mL of test suspension. It was also performed wells with each concentration of SK and SL variants or only with MBL medium and no microalgae exposed to the same conditions for 72-h that enabled to exclude any potential interference of the presence of SK and SL variants in the final absorbance measurement. The conditions that these assays were performed included an automatic stirrer with a continuous stirring (promote active gas exchange and prevent cell clumping), continuous illumination of 100 µE/m^2/s, and a temperature of 23 ± 1°C. After the 72-h of exposure, the absorbance (ABS) was determined at 400 nm with a wide-spectrum microplate reader (Thermo Scientific mod. Multiskan Spectrum). The following equations were used to determine the cell density (C):

For *R. subcapitata*:

conc (cells ml^{-1}) = - 17107.5 + (ABS * 7925350) (R^2 = 0.99)

For *C. vulgaris*:

conc (cells ml^{-1}) = - 155820 + (ABS * 13144324) (R^2 = 0.98)

Growth rate (day^{-1}) was determined according to OECD (2006) for each polymer concentration and control:

$$\mu = \frac{\ln D_b - \ln D_a}{t_b - t_a},$$

where D_a is the initial cell density, D_b is the cell density at the end of the assay and $t_b - t_a$ is the exposure time interval (72 hours).

2.2.2 48-h acute immobilization assay with *Daphnia magna*

For the 48-h acute immobilization assay with *Daphnia magna,* it was used neonates (6h to 24h-old) from the 3rd or 4th broods from monoclonal *D. magna* BEAK cultures, maintained in synthetic hard water American Standards for Testing and Materials medium (pH 7.8 ± 0.2, 20 ± 2°C and 16 h^L: 8 h^D photoperiod) (ASTM, 2002), according to the OECD guideline 202 (OECD, 2004). These were exposed to several concentrations of each SK and SL variant (Table 2), plus a control (ASTM medium), prepared from the aged stock suspension of 5000 mg. L^{-1} with ASTM medium. To perform this assay, it was prepared four replicates with 30 mL of each test suspension and a control with five neonates of *D. magna*. This assay was performed under a temperature of 20 ± 1˚C and 16 h^L: 8 h^D photoperiod, with no food addition or medium renewal, for 48 hours of exposure. After 48 hours, the number of immobilized neonates in each replicate was determined.

2.2.3 24-h immobilization assay with *Brachionus calyciflorus*

To perform this assay, it was used newly hatched individuals of the rotifer species *B. calyciflorus*, according to the standard procedure of Rotoxkit F® (MicroBioTests, Ghent, Belgium). These were obtained through hatching from cysts at 25°C, under a constant light intensity of 3000 – 4000 lux for 24 h. The concentrations tested of SK and SL variants were diluted from the aged stock suspensions of 12500 and 400 mg. L^{-1}, respectively (Table 2). This assay was carried out in 24 well plates with five replicates with a volume of 1 mL per well and five newly hatched rotifers, plus a control, for 24 hours, at 23°C in total darkness. After 24 hours, it was determined the number of dead rotifers.

2.2.4 48-h immobilization assay with *Heterocypris incongruens*

The 48-hours immobilization assay was carried out with the neonates of ostracod *H. incongruens*, obtained after the hatching of cysts available in a commercial kit (MicroBioTest, Ghent, Belgium), according to the standard operation procedure for the Ostracodtoxkit F chronic with some adaptations. The hatching of these neonates was performed over a period of 52 hours, at 25°C with a constant light intensity of 3000 – 4000 lux. After the hatching, the neonates (< 24-h-old) were exposed to several concentrations of SK and SL variants plus a control consisting of Standard Freshwater medium (Table 2). The test concentrations were prepared through the dilution of the aged stock suspension of 12500 mg. L^{-1}. Each treatment was performed with three replicates, each containing a total volume of 10 mL and ten neonates. The duration of the assay was 48-h, at 25 ºC in total darkness and, in the end, the number of dead organisms was determined.

2.2.4 96-h fish embryo acute toxicity test with *D. rerio*

This assay was performed according to OECD test guideline 236 (OECD, 2013) and the eggs were obtained through natural crossbreeding of zebrafish (*D. rerio*) in aquaria with marbles in the bottom. After two hours, the fertilized eggs were identified and collected under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon, Nikon Corporation, Japan).

After this selection and six hours of fertilization, the eggs were exposed to eight concentrations of each SK and SL variant plus a control (Table 2). The several concentrations were prepared through dilution from the aged stock suspension of 150 mg. L^{-1} (SK variant) and 4100 mg. L^{-1} (SL variant) with Zebrafish Water (ZF). The assay was carried out in 24-wells microplates with three replicates and ten embryos per treatment, with a total volume of 2 mL, for 96 hours. Four internal controls were also prepared, and the eggs were observed at 24, 48, 72 and 96 hours (26 ± 1° C and 16 h^L: 8 h^D photoperiod). The parameters evaluated during the assay were pericardial oedema, yolk sac absorption, lack of equilibrium and tail deformation, according to Kimmel et al., 1995.

Table 2: Summary of the procedures used to perform the ecotoxicity assays.

Test species	Exposure time	Endpoint	Dilution water	Test conditions	Concentrations (mg. L^{-1}) (temperature 15°, 20°, 25°C, one month)	Reference
Chlorella vulgaris; Raphidocelis subcapitata	72 hours	Growth rate	MBL[1]	23 ± 1°C, continuous light (100 µE m^{-2} s^{-1})	0.68, 1.03, 1.54, 2.31, 3.47 and 5.20	OECD, 2004: Guideline 201
Daphnia magna	48 hours	Mortality	ASTM[3]	20 ± 1° C, 16 h^{L}: 8 h^{D} photoperiod	For SK-H – 220.0, 310.0, 430.0, 600.0, 841.0, 1176.0 and 1646.0. For SK-L, SK-M, SK-MH – 600.0, 841.0, 1176.0, 1646.0, 2305.0, 3227.0 and 4518.0. For variant SL 5: 841, 1176, 1646, 2305, and 3227. For SL 30, SL 60, SL 100 variants: 600, 841, 1176, 1646, 2305, 3227, and 4518.	OECD, 2004: Guideline 202
Brachionus calyciflorus	24 hours	Mortality	Standard Freshwater[4]	23 °C ± 1°C, total darkness	For SK-H – 0.07, 0.09, 0.13, 0.18, 0.025, 0.036 and 0.50. For SK-M – 2.67, 3.72, 5.21, 7.30, 10.22, 14.31, 2.03, 28.04 and 39.26. For SK-L and SK-MH – 1.9, 2.67, 3.72, 5.21, 7.30, 10.22, 14.31, 2.03 and 28.04. For SL 60 variant: 28.0, 39.3, 55.0, 76.9, 112, 156, 219, and 306. For SL 5, SL 30, SL 100 variants: 306, 429, 600, 840, 1176, 1646. 2305, and 3227	Rotoxkit F®, MicroBioTests, Ghent, Belgium
Heterocypris incongruens	48 hours	Mortality	Standard Freshwater[4]	25°C ± 1°C, total darkness	For SK-H – 429.0, 601.0, 841.0, 1176.0, 1646.0, 2305.0, 3227.0 and 4518.0. For SK-M – 1650.0, 2310.0, 3230.0, 4520.0, 6320.0, 8850.0 and 12400.0. For SK-L and SK-MH – 1176.0, 1650.0, 2310.0, 3230.0, 4520.0, 6325.0, 8855.0 and 12400.0. For SL variants: 1176, 1646, 2305, 3227, 4518, 6325, 8855, and 12400	Ostracodtoxkit F chronic adapted in Venâncio et al., 2019; MicroBioTests Inc.
Danio rerio	96 hours	Mortality	ZF water[5]	26 ± 1°C, 16 h^{L}: 8 h^{D} photoperiod	For SK-H – 3.0, 3.9, 5.1, 6.6, 8.6, 11.2, 14.2 and 19.0. For SK-L – 21.0, 27.0, 35.0, 45.0, 60.0, 80.0, 100.0 and 130.0. For SK-M and SK-MH – 19.0, 24.0, 32.0, 41.0, 54.0, 70.0, 90.0 and 120.0. For SL variants: 647, 841, 1093, 1421, 1848, 2402, 3123, and 4058	OECD, 2013: Guideline 236

2.3 Statistical analysis

All the concentrations of SK and SL variants that caused 50% of effect at lethal and sublethal levels [L(E)C_{50}], and the respective 95% confidence limits, were determined for the six tested species. The EC_{50}s for the growth inhibition of *R. subcapitata* and *C. vulgaris* were computed by the software package Statistica 8.0 (StatSoft, Inc., Tulsa, USA) through the adjustment of a non-linear logistic model to the data. The LC_{50}s for the mortality assays carried out with *D. magna*, *B. calyciflorus*, *H. incongruens* and *D. rerio* were determined by using Probit analysis in the PriProbit software (Sakuma, 1998).

The results obtained for the algae exposure to several concentrations of SK and SL variants were compared through the one-way analysis of variance (ANOVA unifactorial) followed by the multi-comparison Dunnett's test. The Kolmogorov-Smirnov and the Levene tests were carried out to verify the ANOVA assumptions of normality of data and homoscedasticity of variances, respectively (Zar, 1996). The software Sigmaplot version 12.5 (Systat Software, Inc., 2008) was used to perform this analysis.

The $L(EC)_{50}$ obtained for each SK and SL variant in the ecotoxicity assays were integrated to construct species sensitivity distribution curves (SSD) and estimate the hazard concentration for 5 of the species (HC_5), which were generated through the US Environmental Protection Agency (USEPA) spreadsheets (SSD Generator V1).

The maximum acceptable concentration environmental quality standard (MAC-EQS) was determined for each SK and SL variant using two methodologies: (i) deterministic: based on the application of an assessment factor of 1000 to the lowest L(E)C_{50} computed for the tested species and (ii) probabilistic: based on the HC_5 value, which was divided by a factor of 1 (European Commission, 2011).

3. Results

3.1 Acute toxicity assays

The validity criteria established by the respective guidelines or protocols (OECD, 2004; OECD, 2006a; OECD, 2013; Rotoxkit F™ Acute Microbiotests and OstracodToxKit F chronic) were accomplished for all the ecotoxicological assays performed in this work. The results obtained with the acute toxicity assays concluded that the temperature had an impact on the toxicity of the tested SK and SL-variants and was dependent on the species.

According to the results obtained for the SL variants, it was observed an increase of toxicity for all the species tested with the increase of temperature, except for the two species of microalgae and *D. rerio* (Fig. 2). For the *C. Vulgaris* species, all the SL variants, except the SL-100, exhibited lower toxicity at a temperature of 20 ºC (Table 1S, Fig. 1S). The SL-30 and SL-60 aged at the temperature of 15°C induced a significant reduction in the growth rate of *R. subcapitata* and *C. vulgaris* (Fig. 1S). The SL-100 was the variant with the highest toxicity for C. vulgaris ($EC_{50,72h}$ 0.74 mg/L), and the SL-30 was the one with highest toxicity for *R. subcapitata* ($EC_{50,72h}$ 0.47 mg/L). The SL-30 variant aged at the temperature of 25°C was the least toxic with $EC_{50,72h}$ of 10.7 for the C. vulgaris (Table 1S). For the *D. rerio* species, the increase of temperature

provoked a decrease of toxicity for all the SL variants tested, except for the SL-5 and SL-100 variants that induced, respectively, a higher and lower toxicity at the temperature of 20°C (Table 1S, Fig. 5S). For this species, the highest toxic variant was SL-30 aged at the temperature of 15°C ($LC_{50,96h}$ of 0.728 mg/L) and the least toxic was the SL-5 variant aged at the temperature of 25°C ($LC_{50,96h}$ of 3292.5 mg/L). The SL-5 variant aged at the temperature of 15°C, induced almost 30% of lack of equilibrium at 1848 mg/L, and the SL-60 variant induced 100% at the same concentration (Fig. 8S). In relation to the SL-100 variant aged at the temperature of 20ºC, no developmental malformations were observed (Fig. 9S). For the *D. magna*, *B. calyciflorus* and *H. incongruens* species, the variant that exhibited lower toxicity was the SL-30 aged at the temperatures of 15 and 25 ºC (Table 1S, Fig. 2S, 3S, and 4S). The SL-60 variant aged at the temperature of 15 ºC induced more than 80% of mortality above a concentration of 28 mg/L to the *B. calyciflorus* species (Fig. 3S). For the rotifer *B. calyciflorus*, the SL-5 variant aged at the three temperatures and the SL-100 variant aged at the temperatures of 15 ºC and 25 ºC induced total mortality to all concentrations tested (Table 1S; Fig. 3S). For the SL-30 variant, it was observed a total mortality at concentrations of 306 and ≥ 600 mg/L at the temperature of 20°C and ≥ 429 mg/L at the temperature of 25°C.

About the results obtained for the SK variants, the effect of temperature on the toxicity of these variants was species dependent (Table 1S). For instance, for two microalgae species, it was observed an increase of toxicity with the increase of temperature for the SK-H and SK-MH variants and a decrease of toxicity for the SK-L and SK-M variants (Table 1S, Fig 1S). The SK-M variant showed to be the most toxic with an $EC_{50,72h}$ (CL 95%) of 0.64 (0.35 – 0.94) mg/L and 0.58 (0.28 – 0.87) at the temperature of 15ºC and the SK-MH variant the least toxic with an $EC_{50,72h}$ of 10.22 mg/L at the temperature of 25 ºC (Table 1S). The temperature of 25 ºC caused 100% of reduction in the growth rate for the SK-H and SK-MH variants in *C. vulgaris* (Fig. 1S). Regarding the primary consumers *D. magna*, the LC_{50} (95% CL) value for SK-H aged at the temperature of 25 ºC was 175.9 (74.6 - 227) mg/L (Table 1S). The other SK variants showed similar lethal toxicity over the three different temperatures, with $LC_{50,\ 48h}$ ranging from 506.8 to 1275.2 mg/L for *D. magna* (Table 1S; Fig. 2S). For *H. incongruens*, the LC_{50} (95% CL) value for SK-H aged at the temperature of 25 ºC was 30.3 (1.08E-07-166.6) mg/L (Table 1S; Fig. 4S). All the SK variants presented similar toxicity in *B. calyciflorus* compared to control conditions ranging from 0.035 to 14.3 mg/L (Table 1S). In the assays with *D. rerio* fish, it was observed that the SK-L and SK-MH aged at the temperature of 15 ºC induced total mortality to all the concentrations tested for this species (Figure 3; Table 1S). Almost all the concentrations tested for SK variants aged at the three temperatures (15, 20, and 25 °C) provoked deformations on the embryos relatively to control conditions (Fig. 8S, 9S, and 10S), with the exception for SK-MH at the temperature of 15 ºC (total mortality) (Fig. 8S). The lack of equilibrium was the developmental malformations more prominent at all the temperatures and the SK variants tested. The three temperatures used in this work induced a lack of equilibrium malformations in all the concentrations tested for the SK-L, SK-M, and SK-MH (Fig. 8S, 9S, and 10S). Most of the pericardial oedemas were observed on fish larvae exposed to all SK variants aged at the temperature of 25 ºC (Fig. 10S).

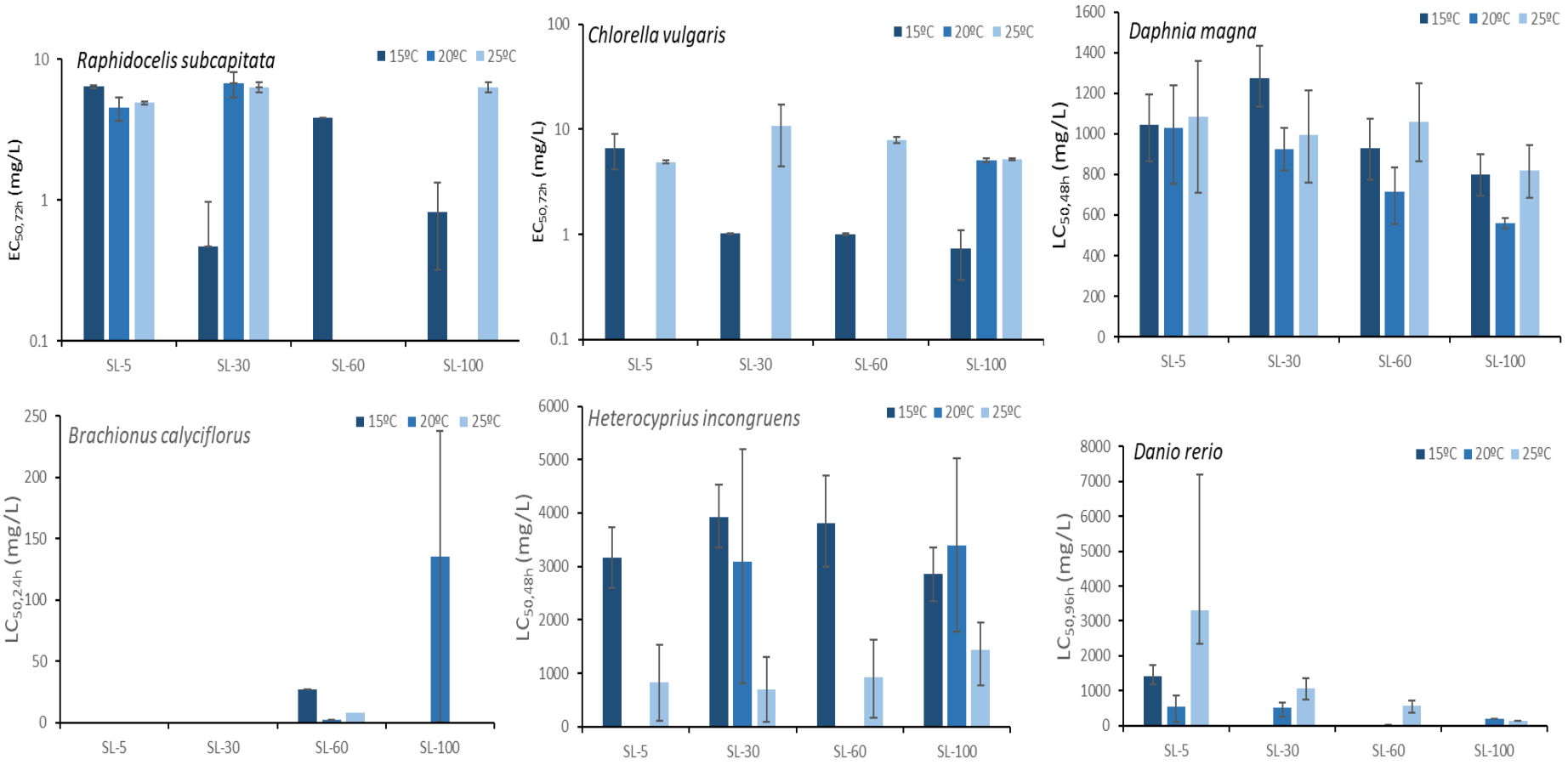


Figure 2 –Lethal or effective concentrations (LC_{50} or EC_{50}) of the SL variants after being aged at 15, 20 and 25°C, causing 50% of effect in the six test species Error bars represent the 95% confidence limits. For the *B. calyciflorus* species, SL-5 variant provoked a total mortality of the organisms for the three temperatures tested, and SL-30 and SL-60 at the temperature of 15°C. The SL-30 variant provoked a total mortality at concentration 306 and ≥ 600 mg/L at the temperature of 20°C and at concentration ≥ 429 mg/L at the temperature of 25°C. For the *H. incongruens* species, SL-5 and SL-30 variant provoked a total mortality of the organisms for the temperature of 20°C. For the *D. rerio* species, SL-60 and SL-100 variants provoked a total mortality of the organisms for the temperature of 15°C.

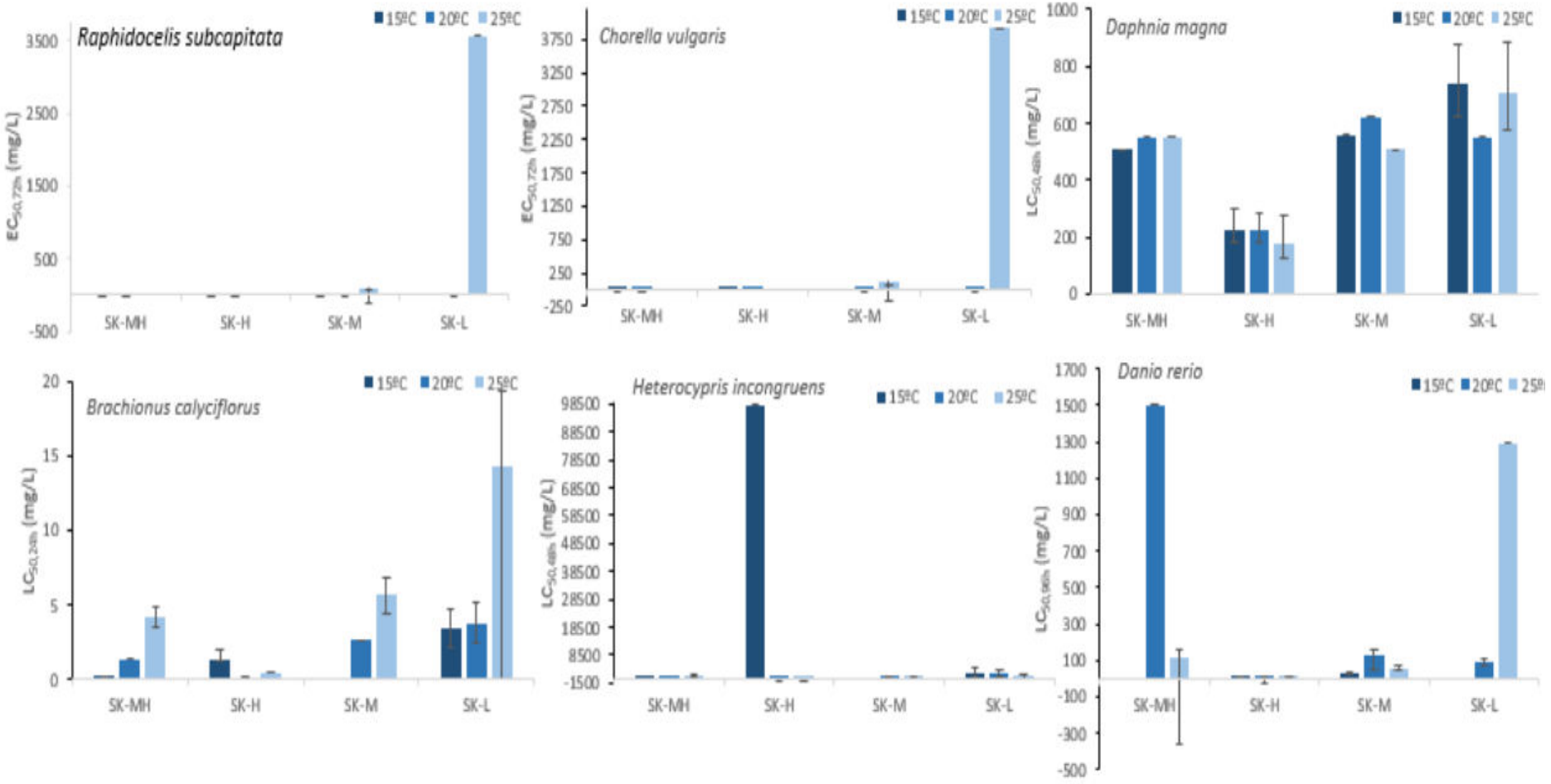
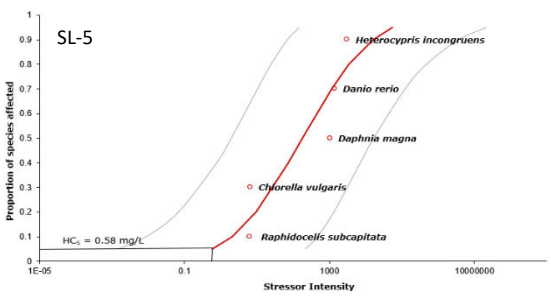


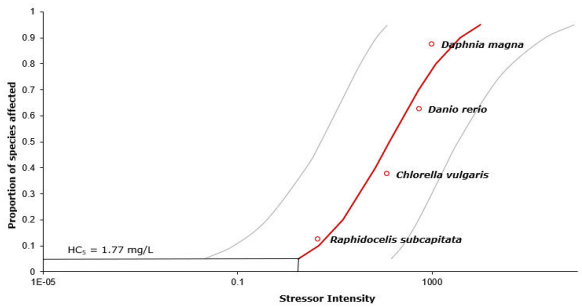
Figure 3 –Lethal or effective concentrations (LC_{50} or EC_{50}) of the SK variants after being aged at 15, 20 and 25°C, causing 50% of effect in the six test species Error bars represent the 95% confidence limits. For the *C. vulgaris* species, the SK-H and SK-MH variants provoked a total mortality of algae for the temperature of 15°C. For the *D. rerio* species, SK-L and SK-MH variants provoked a total mortality of the organisms for the temperature of 15°C.

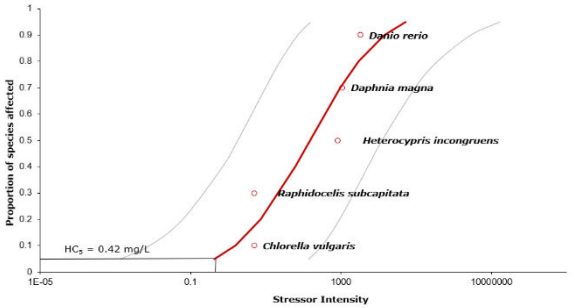
3.2 Species sensitivity distribution curves and MAC-EQS derivation for the aquatic compartment

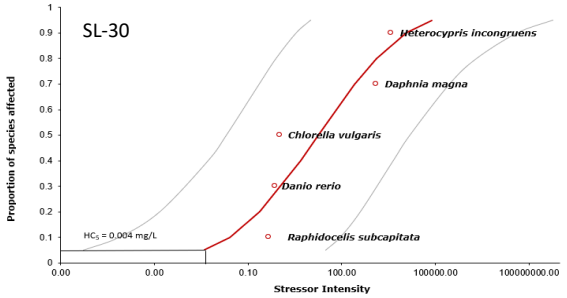
The toxicity for each temperature and each variant, estimated as the $L(E)C_{50}$, was integrated into species sensitivity distribution curves, the SSD (Fig. 12), and the respective hazard concentrations (HCx) were also computed (Table 4, Fig. 12). The temperature that induced the lowest HCx values was 15°C for the SK-M variant (Fig. 12). The least toxic variant, according to the estimated HC_5, was the SL-30 variant at the temperature of 20°C. Overall, the microalgae (*R. subcapitata* and *C. vulgaris*) together with *B. calyciflorus*, were the species that showed the highest sensitivity to the effect of the temperature (Fig. 12) for the SL variants. For the SK variant, it was the microalgae *R.subcapitata* and *B. calyciflorus* (Fig. 12).

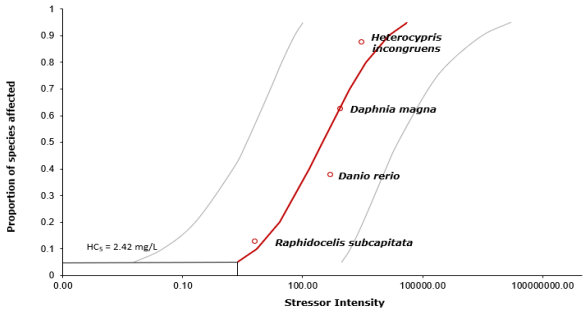
In Table 4, are the results obtained for the MAC-EQS computed for each temperature and each SoftCAT polymer for the aquatic compartment. The values of MAC-EQS obtained with the determinist approach were lower than the ones obtained with the mechanistic approach, (Table 4). The predicted concentrations putting at risk the aquatic compartment would be greater than 0.0045 mg/L for the SL-5 variant, 0.0007 mg/L for the SL-30 variant, 0.001 for the SL-60 variant, 0.0007 for the SL-100 variant, 0.0004 for the SK-H variant, 0.003 for the SK-L, 0.00003 for the SK-M variant, and 0.0002 for the SK-MH variant (Table 4).

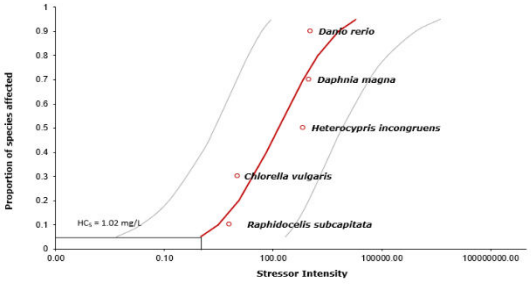

SL-5
Heterocypris incongruens
Danio rerio
Daphnia magna
Chlorella vulgaris
Raphidocelis subcapitata
HC5 = 0.58 mg/L
Proportion of species affected
Stressor Intensity

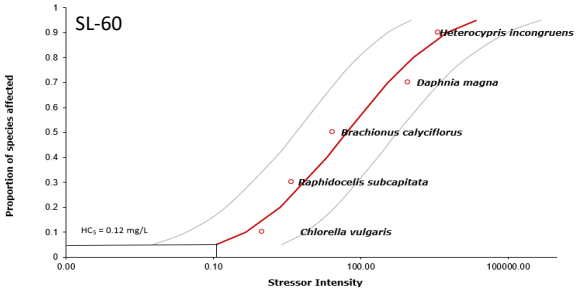

Daphnia magna
Danio rerio
Chlorella vulgaris
Raphidocelis subcapitata
HC5 = 1.77 mg/L
Proportion of species affected
Stressor Intensity

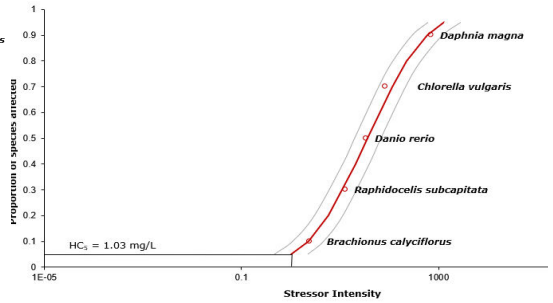

Danio rerio
Daphnia magna
Heterocypris incongruens
Raphidocelis subcapitata
Chlorella vulgaris
HC5 = 0.42 mg/L
Proportion of species affected
Stressor Intensity

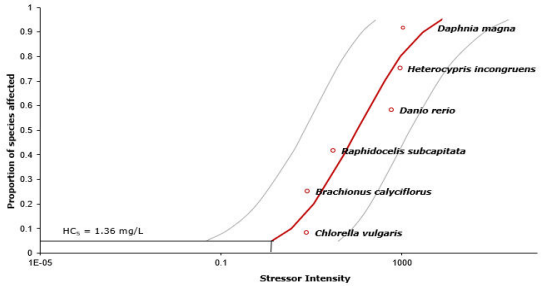

SL-30
Heterocypris incongruens
Daphnia magna
Chlorella vulgaris
Danio rerio
Raphidocelis subcapitata
HC5 = 0.004 mg/L
Proportion of species affected
Stressor Intensity

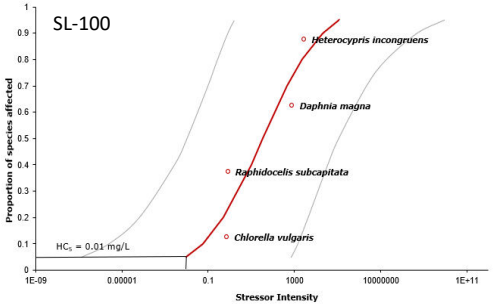

Heterocypris incongruens
Daphnia magna
Danio rerio
Raphidocelis subcapitata
HC5 = 2.42 mg/L
Proportion of species affected
Stressor Intensity

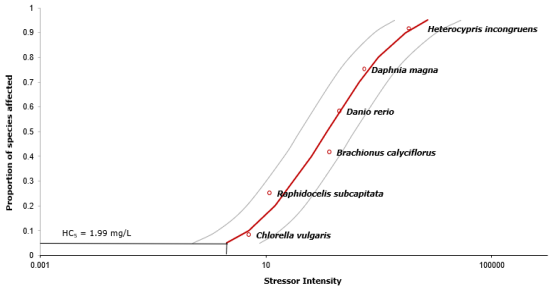

Danio rerio
Daphnia magna
Heterocypris incongruens
Chlorella vulgaris
Raphidocelis subcapitata
HC5 = 1.02 mg/L
Proportion of species affected
Stressor Intensity

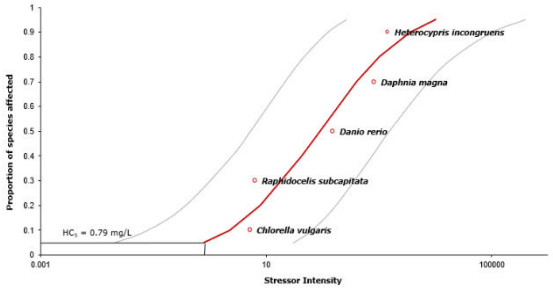

SL-60
Heterocypris incongruens
Daphnia magna
Brachionus calyciflorus
Raphidocelis subcapitata
Chlorella vulgaris
HC5 = 0.12 mg/L
Proportion of species affected
Stressor Intensity


Daphnia magna
Chlorella vulgaris
Danio rerio
Raphidocelis subcapitata
Brachionus calyciflorus
HC5 = 1.03 mg/L
Proportion of species affected
Stressor Intensity


Daphnia magna
Heterocypris incongruens
Danio rerio
Raphidocelis subcapitata
Brachionus calyciflorus
Chlorella vulgaris
HC5 = 1.36 mg/L
Proportion of species affected
Stressor Intensity


SL-100
Heterocypris incongruens
Daphnia magna
Raphidocelis subcapitata
Chlorella vulgaris
HC5 = 0.01 mg/L
Proportion of species affected
Stressor Intensity


Heterocypris incongruens
Daphnia magna
Danio rerio
Brachionus calyciflorus
Raphidocelis subcapitata
Chlorella vulgaris
HC5 = 1.99 mg/L
Proportion of species affected
Stressor Intensity


Heterocypris incongruens
Daphnia magna
Danio rerio
Raphidocelis subcapitata
Chlorella vulgaris
HC5 = 0.79 mg/L
Proportion of species affected
Stressor Intensity

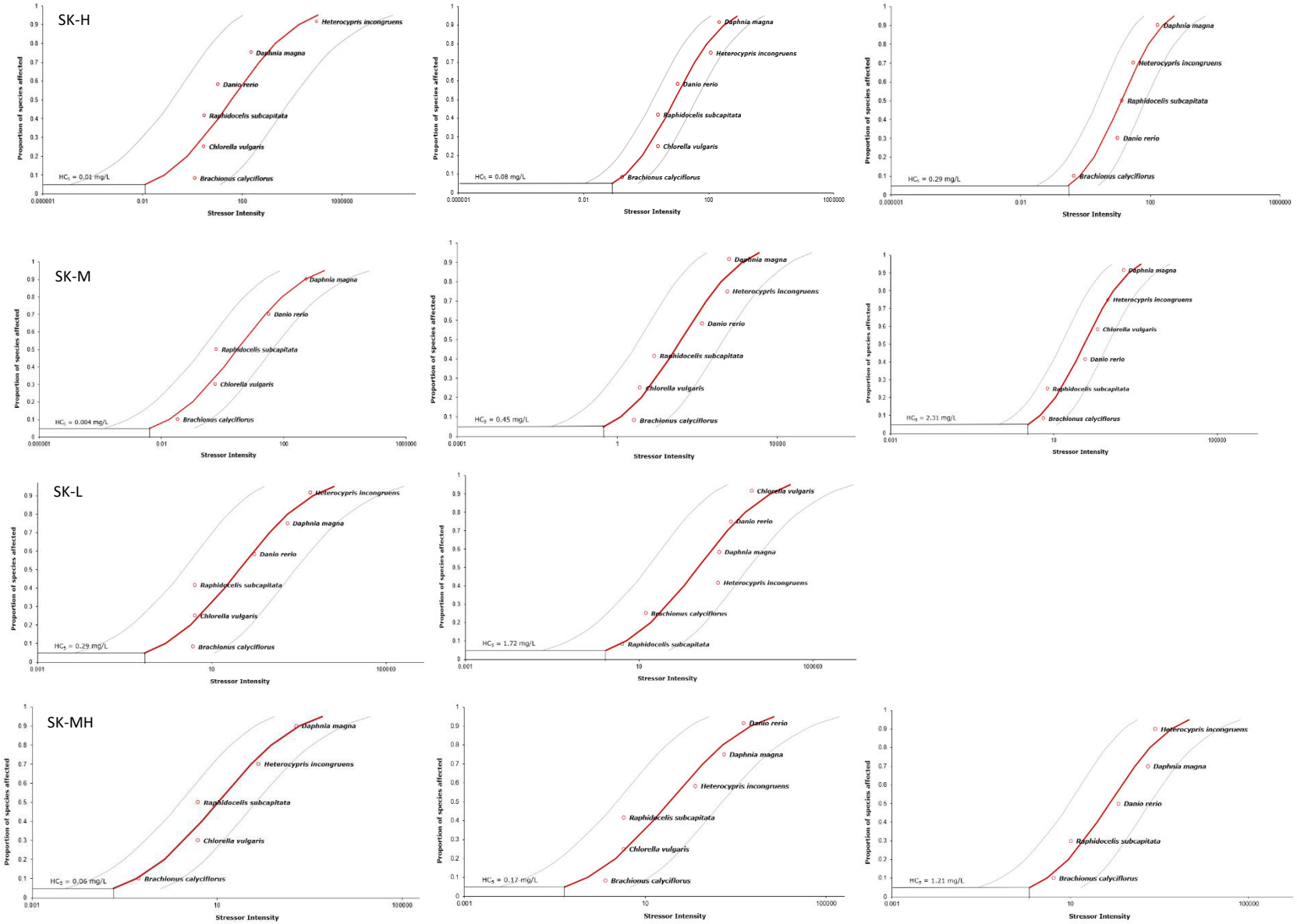


Figure 12: Species Sensitivity Distribution curves (SSD) of the four SL and four SK variants, where HCx denotes the hazard concentration that affect X % of the species. The first, second and third grafts correspond to the temperatures of 15, 20, and 25°C, respectively. For the SL- 30 and SK-L variants was not possible to construct the SSD for the temperature.

Table 4: Maximum acceptable concentrations environmental quality standard (MAC-EQS), for the eight SoftCAT polymers, computed by applying a safety factor of 10 to the lowest median lethal or effective concentration [$L(E)C_{50}$, mg. L^{-1}] or by determination of Hazard Concentrations that protect 95% of the species (HC_5) through Species Sensitivity Distribution Curves (SSD).

	SoftCAT™ variant							
	SL-5	**SL-30**	**SL-60**	**SL-100**	**SK-H**	**SK-M**	**SK-L**	**SK-MH**
Lowest $L(E)C_{50}$/1000 (mg. L^{-1})	0.0045	0.0007	0.001	0.0007	0.0004	0.00003	0.003	0.0002
HC_5 (mg. L^{-1}) Temp 15°C	0.58	0.004	0.12	0.01	0.01	0.004	-	0.06
HC_5 (mg. L^{-1}) Temp 20°C	1.77	2.42	1.03	1.99	0.08	0.45	0.29	0.17
HC_5 (mg. L^{-1}) Temp 25°C	0.42	1.02	1.36	0.79	0.29	2.31	1.72	1.21

4. Discussion

This work aimed to identify the temperature that induced the least toxic variant through the ecotoxicity of SK and SL cellulose derived polycationic polymers. The ecotoxicological evaluation of the temperature on the SK and SL variants allowed us to establish some overall patterns. For instance, the temperature of 15ºC was the one that provoked more toxic effects on the SK and SL variants for several species, inducing the total mortality during the exposure time of ecotoxicological assays. The temperature of 15ºC provoked 100% mortality to *B. calyciflorus* during the exposure to SL-5, SL-30, and SL-100 variants. The two freshwater algae species *C. vulgaris* and *R. subcapitata* formed consistently with the rotifer *B. calyciflorus* the most sensitive group of organisms to the SL variants as it is possible to observe in the SSD curves. The same effect was already demonstrated by Simões et al. (2021) that reported the two algae and rotifer as the most sensitive group of organisms to the SL variants. Also, Pereira et al. (2018) reported that during the exposure of algae *R. subcapitata* to five variations of cationic acrylamide-based polyelectrolytes (cPAM), this alga was the most sensitive eco-receptor. The increment in the number of hydrophobic groups presents in the SL-100 variant induced greater toxicity to the algae *C. vulgaris* relatively to the other SL-variants. Their positive charges in the surface enable them to be absorbed in large quantities on the surface of cells that are negatively charged (Nolte et al., 2017), and this way can avoid the photosynthesis of the cells and consequently, can lead to the growth rate inhibition. Nolte et al. (2017) also observed that the wall of the cells of this unicellular organism

is permeable to hydrophobic polymers through their absorption or disrupt the thylakoid membrane of algae. According to the work developed by Hong et al (2015), the viscosity of hydroxyethyl cellulose polymers is influenced by their concentration and temperature. As the temperature is increased, the viscosity of these type of polymers decreases. Since these polymers are less viscous because of the effect provoked on them by the temperature, the particle sizes of the polymers have also decreased, and this way they can exert their toxicity in the algae outer cell wall (Wang and Ye, 2015; Nolte et al., 2017). Since the SL variants are amphiphilic, they have the capability to be absorbed through the membrane of species gills, skin, intestinal wall after ingestion of these compounds, and they can accumulate in the organisms (Xia et al., 2015). The hydrophobic substitution present in these polymers can induce endocytosis after binding to the biological lipid membranes (Liu et al., 2010). It was reported by Tripathy et al (2018) that the increase of the length of a hydrophobic chain lowers the cmc of surfactants, increasing their consequent absorption in the interfaces through ionic interaction between the surfactant and the cell-water interface, resulting in an increase of the toxicity of these compounds. The present cationic polymers possess long alkyl side chains, dodecyl, which can act similarly to the long chain surfactants and induce high toxicity to these compounds.

For pluricellular organisms, it was observed an increase of toxicity of the SL variants. In the assay with *D. rerio*, *D. magna* and *B. calyciflorus*, the variants with a higher number of hydrophobic groups (SL-60 and SL-100 variants) were the highest toxic ones. This can be explained by the presence of higher HS that originates the increase of the size of the aggregates and their viscosity. Besides the temperature had an effect of lowering the viscosity in these polymers, they continue to be more viscous than the other two variants tested. The respiratory structures (gills), swimming and/or the feeding appendages of the organisms mentioned may contribute to the higher toxicity of these polymers due to the interaction between opposed charges. For instance, the swimming behaviour of *D. magna* might be impaired by the medium viscosity (Serra et al., 2019). For *D. rerio*, the increase of the viscosity may reduce the respiratory rates around the egg jelly or embryo (Rombough, 1998). The ingestion and/or internalization in cells can be the action mode of these SL variants to provoke toxicity to *D. magna* and *B. calyciflorus* (Martín-de-Lucía et al., 2019).

Concerning, the SK variants, the most sensitive group of organisms was the *B. calyciflorus* as reported by the SSD curves. According to the results obtained for the $L(E)C_{50}$, the temperatures of 15 and 25°C were the ones that induced the highest toxicity to the SK variants, since provoked the total mortality of the organisms for the freshwater algae species *C. vulgaris* and *D. rerio*. According to previous works developed with other polyquaternium polymers and the algae *C. vulgaris*, this species was identified as a very sensitive one (Simões et al., 2021) due to the size of the polymer that determines its entrance into the cells. However, the high sensitivity of *C. vulgaris* to these polymers may also be influenced by other characteristics of this species, since this species can excrete exopolymeric substances with high affinity to these SK variants under stress conditions (Chen et al., 2015). From the results obtained, it was not possible to make a correlation between the toxicity of these polymers and the species tested. The toxicity observed for the *D. rerio* species was also interesting during the exposure to these SK variants. During the first 48 hours of exposure to these polymers, the cumulative mortality was less than 20%. This can be related to the hatching of the larvae that have the

chorion layer possessing a protective barrier against the entry of these polymers and further interaction with the organisms (Busquet et al., 2014).

According to the results obtained for the L(E)C_{50}, the SK-M variant, with a molar cationic substitution of 0.25 was the most toxic variant. This can be related to the architecture of the polymer since the charge density of this polymer promotes a higher binding through electrostatic binding to the cell membranes of the species that are negatively charged (Narita et al., 2001; Roy, 2020). According to the study developed by Narita et al (2001), the increase of the charge density of cationic polymers leads to a higher number of disrupted yeast cells. Since the temperature can decrease the viscosity of hydroxyethyl cellulose polymers, this can also influence their toxicity, once the hydrodynamic size of the SK variants also decreases due to the compaction of the polymer chains (Hong et al., 2015). Since the species have different media compositions, this can also have an impact on the toxicity of these polymers. For instance, SK-L was toxic to *D. rerio* at the temperature of 15ºC, but not present harm to daphnia and ostracods and the water used for the zebrafish is much soft than the ASTM medium, correlating the results observed by Salinas et al. (2020) that concluded that the increase of the water hardness decreases the toxicity of cationic polymers. However, the SK-L had different toxicity for rotifers and ostracod, which share the same test medium, being very toxic for the rotifer species to the ostracods.

Despite the presence of these polymers in several personal care products, their ecotoxicity is still poorly evaluated and known. There is a strong lack of knowledge about their toxicity to the aquatic environment since these polymers are continuously released in the aquatic compartment during the wastewater treatment plants process. According to the results obtained in the MAQ-EQS, the SL-60 variant showed to be the least toxic one, suggesting a “safer” option over the other variants. All the SL and SK variants tested presented low MAC-EQS, except for the SL-60 variant where the MAC-EQS was 0.025 mg. L^{-1}. Consider these results and the world consumption rates of personal care products that are composed of these polymers, they can have a toxic impact on the aquatic compartment, since these are not completely removed during the process of the wastewater treatment plants (Cummings et al., 2011; Berardi et al., 2020). Considering these results, the SL-60 variant can be considered as the better option to include in the formulations of personal care products to reduce the toxic impact on the aquatic environment.

5. Conclusions

In this work, the effect of temperature in the toxicity of hydroxyethyl cellulose modified polymers was investigated to provide valuable information on these polymers widely used in personal care products. Due to the lack of information about the aquatic impact of these polymers, the indication about the most eco—friendly variant is crucial for the industry as an option to consider in the formulation of personal care products, minimizing the ecological risks. From the several taxonomic and functional groups evaluated, the most sensitive ecological receptors to the SK and SL variants were the producers *R. subcapitata* and *C. vulgaris* and the primary consumer *B. calyciflorus*. Overall, the SL-30 variant was revealed to be the least toxic

according to the derived MAC-EQS of 2.42 mg. L^{-1}, whilst the SK-M variant presented the lowest MAC-EQS. The temperature of 15°C seems to have an impact on the toxicity of the hydroxyethyl cellulose polymers since it induced the total mortality of the *B. calyciflorus* and *D. rerio* organisms after exposure to these polymers.

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Supplementary information

Table 1S: Lethal or effective concentrations of the SK and SL variants causing 50% of effect (LC_{50} or EC_{50}; mg. L^{-1}) with the respective 95% confidence limits depicted within brackets. NC – could not be computed, no progressive dose-response curve was obtained. 100% mortality denotes total mortality for all the concentrations tested. For some species it was not possible to determine the CL 95%.

		LC_{50} or EC_{50}(mg. L^{-1})							
	Temperature (°C)	**SL-5**	**SL-30**	**SL-60**	**SL-100**	**SK-H**	**SK-M**	**SK-L**	**SK-MH**
R. subcapitata Growth inhibition (72 h)	15	6.39 (4.13-8.66)	0.47 (0.445-0.496)	3.88 (2.31-5.45)	0.83 (0.46-1.19)	3.01 (2.73-3.29)	0.64 (0.35-0.94)	NC	3.86 (3.58-4.14)
	20	4.53 (3.69 – 5.38)	6.72 (5.32 – 8.13)	> 5.2	> 5.2	2.45 (2.33-2.57)	8.40 (7.23 – 9.58)	4.16 (4.06-4.26)	3.36 (3.16-3.55)
	25	4.92 (4.80-5.04)	6.33 (5.82 – 6.84)	> 5.2	6.33 (5.82 – 6.84)	> 5.2	7.03 (5.70 – 8.36)	4.18 (3.75 – 4.61)	10.22 (8.41 – 12.02)
C. vulgaris Growth inhibition (72 h)	15	6.61 (4.16-9.05)	1.02 (1.00-1.03)	0.999 (0.98-1.02)	0.74 (0.37-1.10)	2.97 (2.70-3.25)	0.58 (0.28 – 0.87)	NC	3.84 (3.56-4.11)
	20	> 5.2	> 5.2	> 5.2	5.09 (4.89-5.29)	2.42 (2.31-2.54)	3.72 (3.61-3.83)	4.13 (1.03-4.23)	3.34 (3.15-3.54)
	25	4.89 (4.77-5.01)	10.7 (4.42-17.05)	7.91 (7.36-8.45)	5.14 (5.02-5.27)	100% mortality	> 5.2	> 5.2	100% mortality
Daphnia magna Immobilization 48 h	15	1045.2 (863.5 - 1194.5)	1275.2 (1131.3 - 1435.2)	928.0 (776.4 - 1072.4)	799.6 (696.5 - 896.8)	227.1 (157.1 - 273.2)	560.1	737.8 (600.9 - 848)	506.8
	20	1031 (753.6 - 1239.0)	922.1 (818.1 - 1028.9)	713.5 (556.9 - 833.7)	560.0 (534.7 - 585.2)	220.5 (156 - 259.2)	621.3	549.4	549.4
	25	1085.9 (711.7 - 1357.4)	995.7 (760.8 - 1212.9)	1057.9 (865.6 - 1246.9)	820.4 (684.5 - 941.6)	175.9 (74.6 - 227)	506.8	707.3 (532.7 - 840.8)	549.4
Brachionus calyciflorus Mortality (24 h)	15	100% mortality	100% mortality	26.6	100% mortality	1.29	0.035	3.35	0.203
	20	100% mortality	NC	2.48	134.9	0.175	2.663	3.74	1.322
	25	100% mortality	NC	8.17	100% mortality	0.45	5.64	14.3	4.23
Heterocypris incongruens Mortality (48 h)	15	3165.4 (2592.5-3733.9)	3917.1 (3350.0-4537.7)	3805.0 (2987.9-4695.8)	2855.5 (2341.6-3347.5)	> 12400	NC	1870.5 (149.6-3301.4)	78.73
	20	100% mortality	3091.5 (808.6-5201.9)	100% mortality	3382.2 (1768.6-5033.0)	119.8 (4.57-292.3)	555.8	1812.5 (643.9-2775.2)	127.9
	25	825.9 (111.1-1535.2)	689.7 (91.1-1297.6)	925.1 (168.0-1632.6)	1433.4 (777.6-1955.8)	30.3 (0.11^-7-166.6)	214.1 (0.13^-7-873.4)	666.5 (151.4-1184.0)	809 (368.2 - 1180.0)
Danio rerio Mortality 96 h	15	1408.0 (1197.5-1635.6)	0.728	100% mortality	100% mortality	10.8 (8.97-13.9)	33.4 (29.4-37.5)	100% mortality	100% mortality
	20	553.4 (118.2-878.9)	520.6 (252.1-664.8)	33.9	202.0	10.5 (4.96-52.8)	132.0 (108.9-212.9)	96.4 (82.1-121.3)	> 120
	25	3292.5 (2357.4-7199.2)	1078 (741.6-1369.6)	586.1 (362.5-719.1)	152.4	10.1 (8.53-12.4)	59.2 (52.6-67.1)	> 130	120.1 (76.2-600.4)

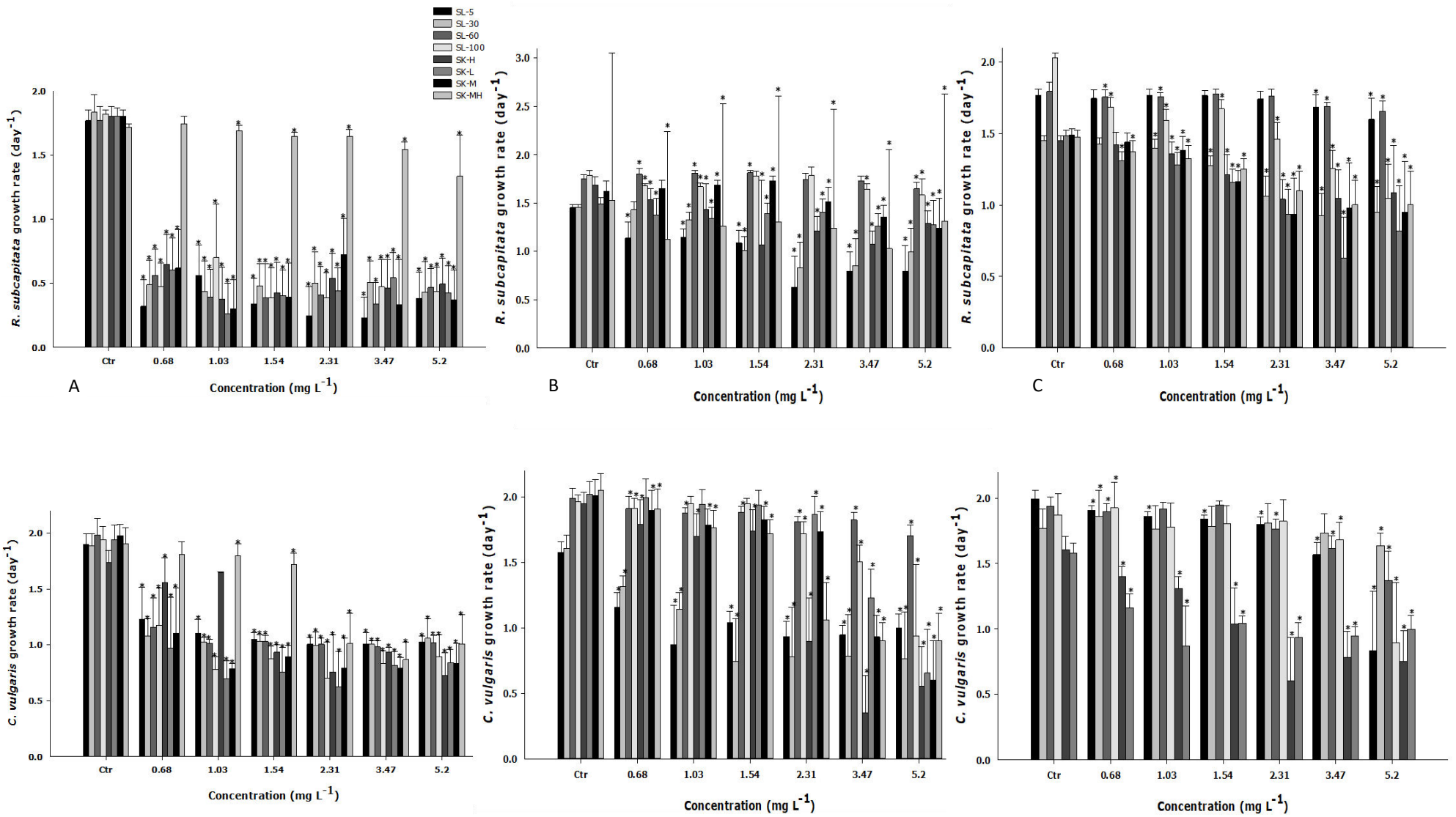

Fig. 1S: Growth rate (day-1) of *Raphidocellis subcapitata* (top figure) and *Chrorella vulgaris* (down figure) after 72 h of exposure to increased concentrations of the eight hydrophobically and cationically modified hydroxyethyl cellulose polymers (SL and SK variants; mg. L^{-1}). Vertical bars correspond to standard error. * denotes statistical differences between SK and SL concentrations and the respective control, within each SK and SL variants ($p<0.05$). The letters A, B, and C correspond to the temperatures of 15, 20, and 25 °C.

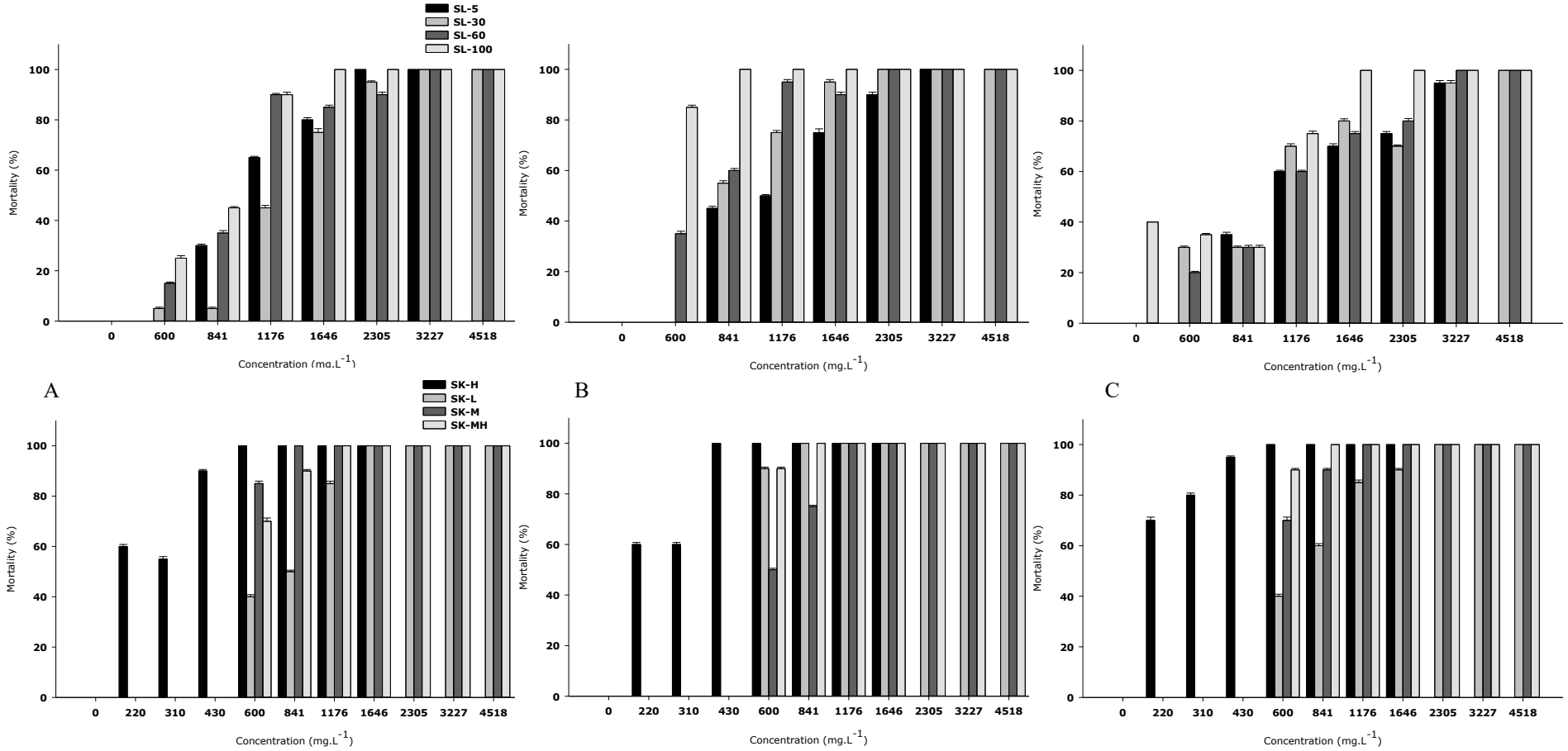

Figure 2S: Average survival of *Daphnia magna* after 48 hours of exposure to increased concentrations (mg/L) of four different cationically modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg/L): SK-H, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100. Vertical bars correspond to standard deviation. The letters A, B, and C correspond to the temperatures of 15, 20, and 25 °C, respectively.

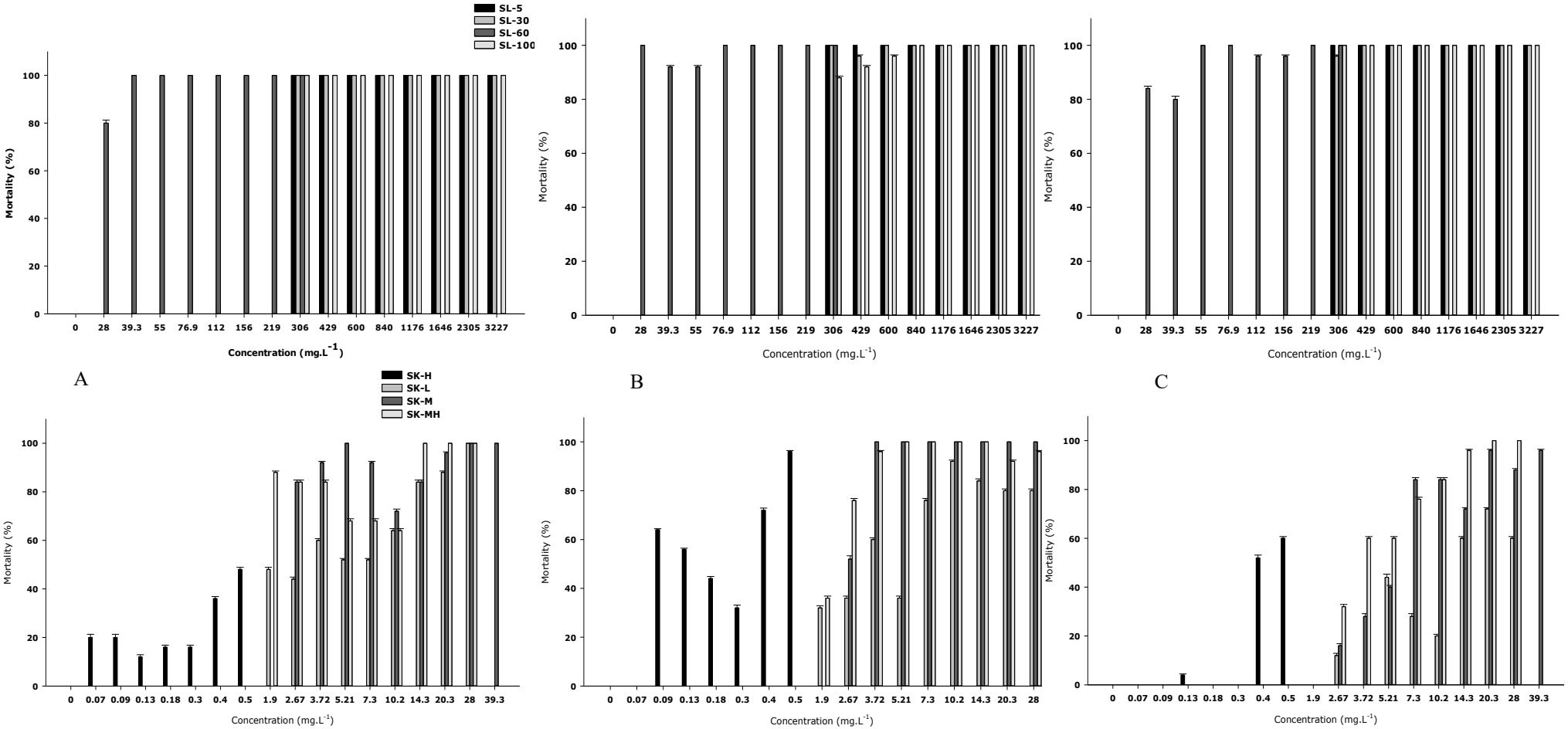

Fig 3S: Average mortality of neonates of *Brachionus calyciflorus* after being exposed, for 24h, to eight concentrations of each of the eight hydrophobically modified hydroxyethyl cellulose polymers (SK and SL variants; mg. L^{-1}). Vertical bars correspond to standard deviation and A, B, and C correspond to the temperatures of 15, 20, and 25°C, respectively. The letters A, B, and C correspond to the temperatures of 15, 20, and 25°C, respectively.

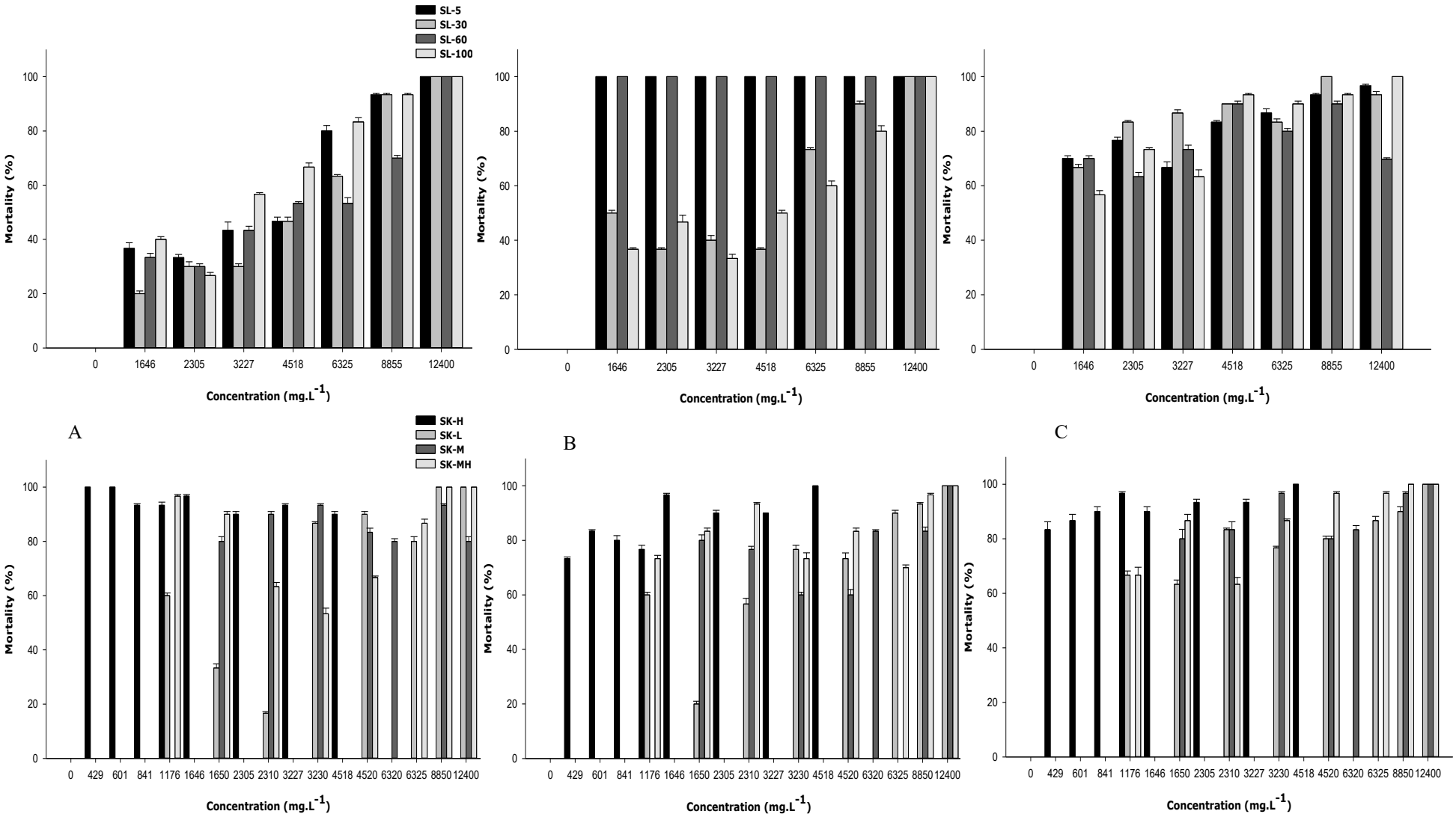

Figure 4S: Average survival of *Heterocypris incongruens* after 48 hours of exposure to increased concentrations (mg/L) of four different cationically modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg/L): SK-H, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100. The letters A, B, and C correspond to the temperatures of 15, 20, and 25 °C, respectively.

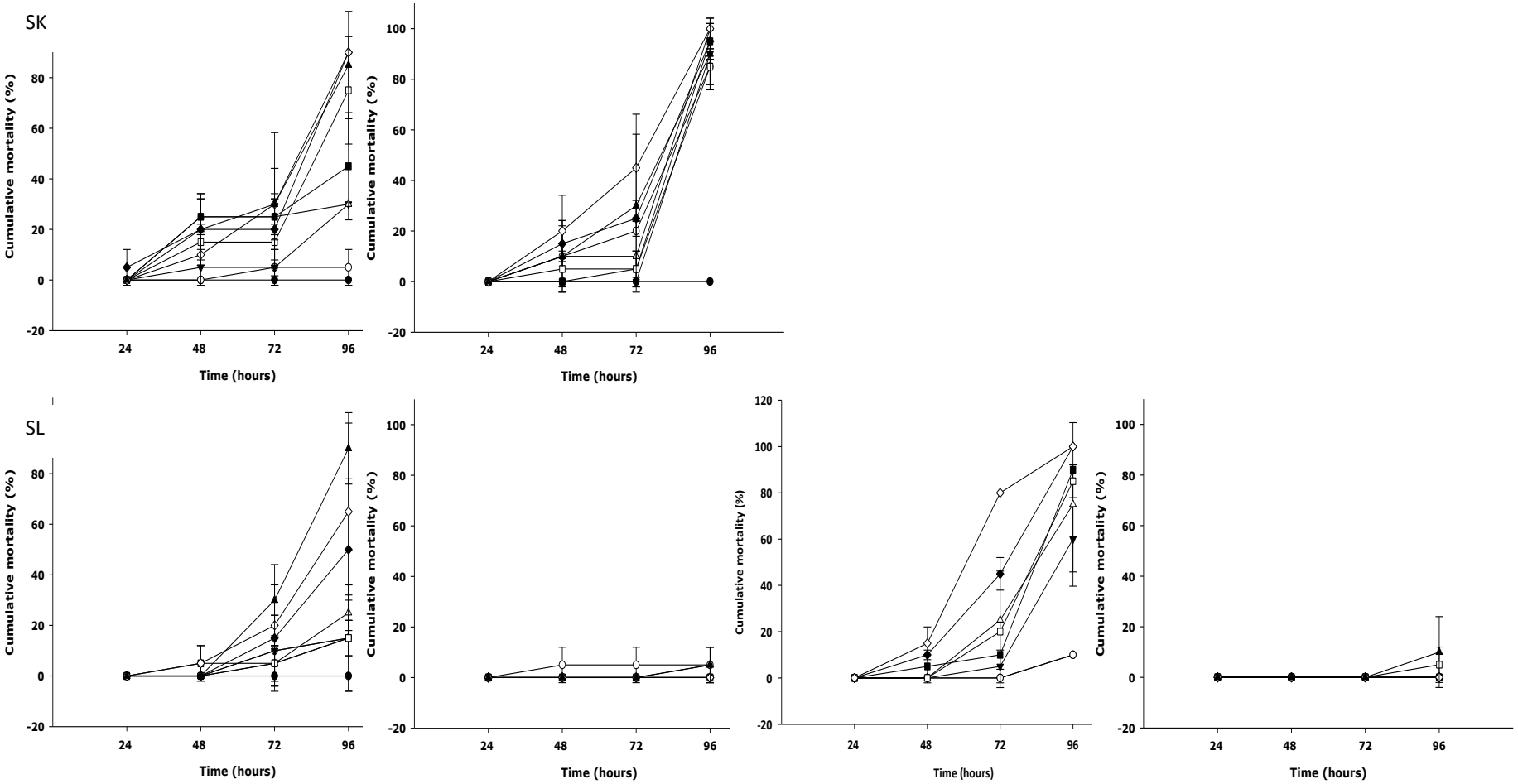

Figure 5S: Average cumulative mortality curves for *Danio rerio* exposed during 96 h to seven concentrations of each eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg. L^{-1}): SK-H, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 15°C. Vertical bars correspond to the standard deviation. n=30.

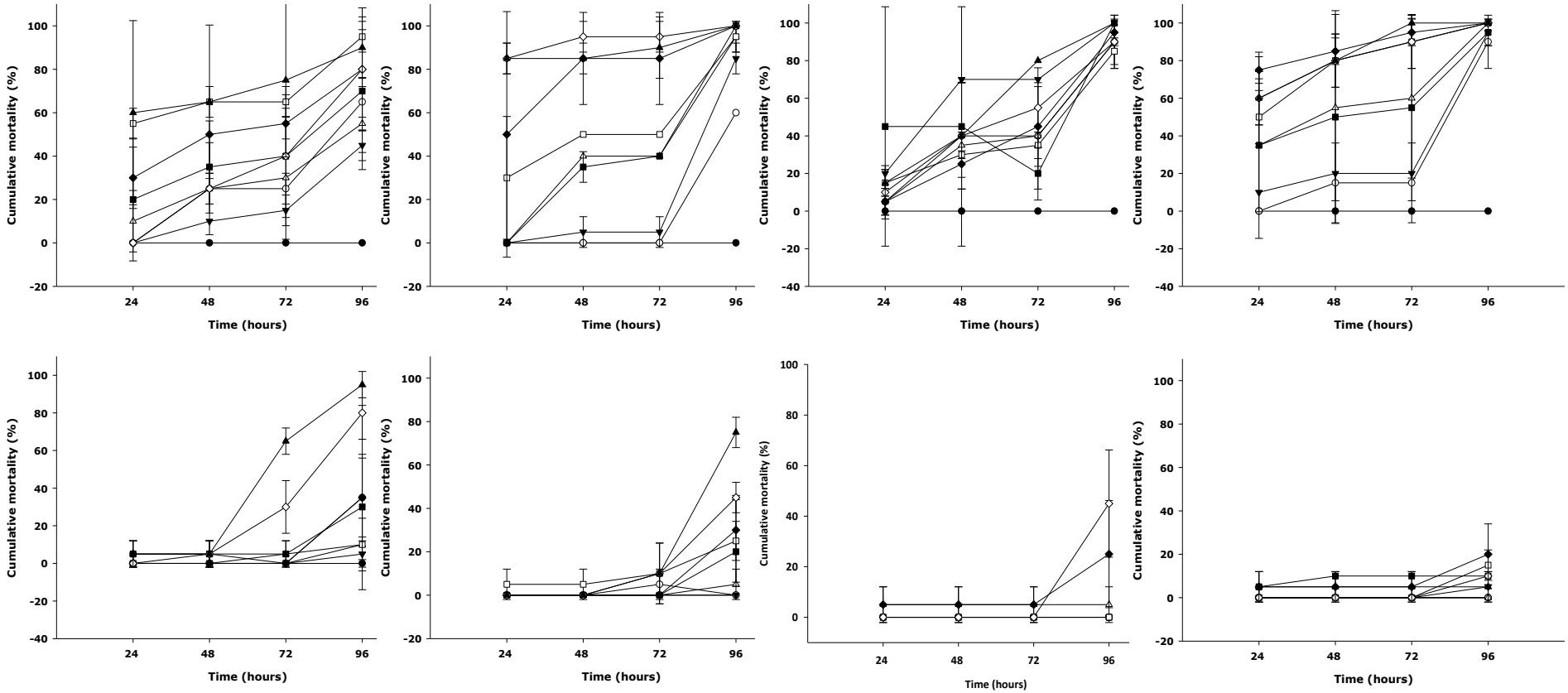

Figure 7S: Average cumulative mortality curves for *Danio rerio* exposed during 96 h to seven concentrations of each eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg. L^{-1}): SK-M, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 25°C. Vertical bars correspond to the standard deviation. n=30.

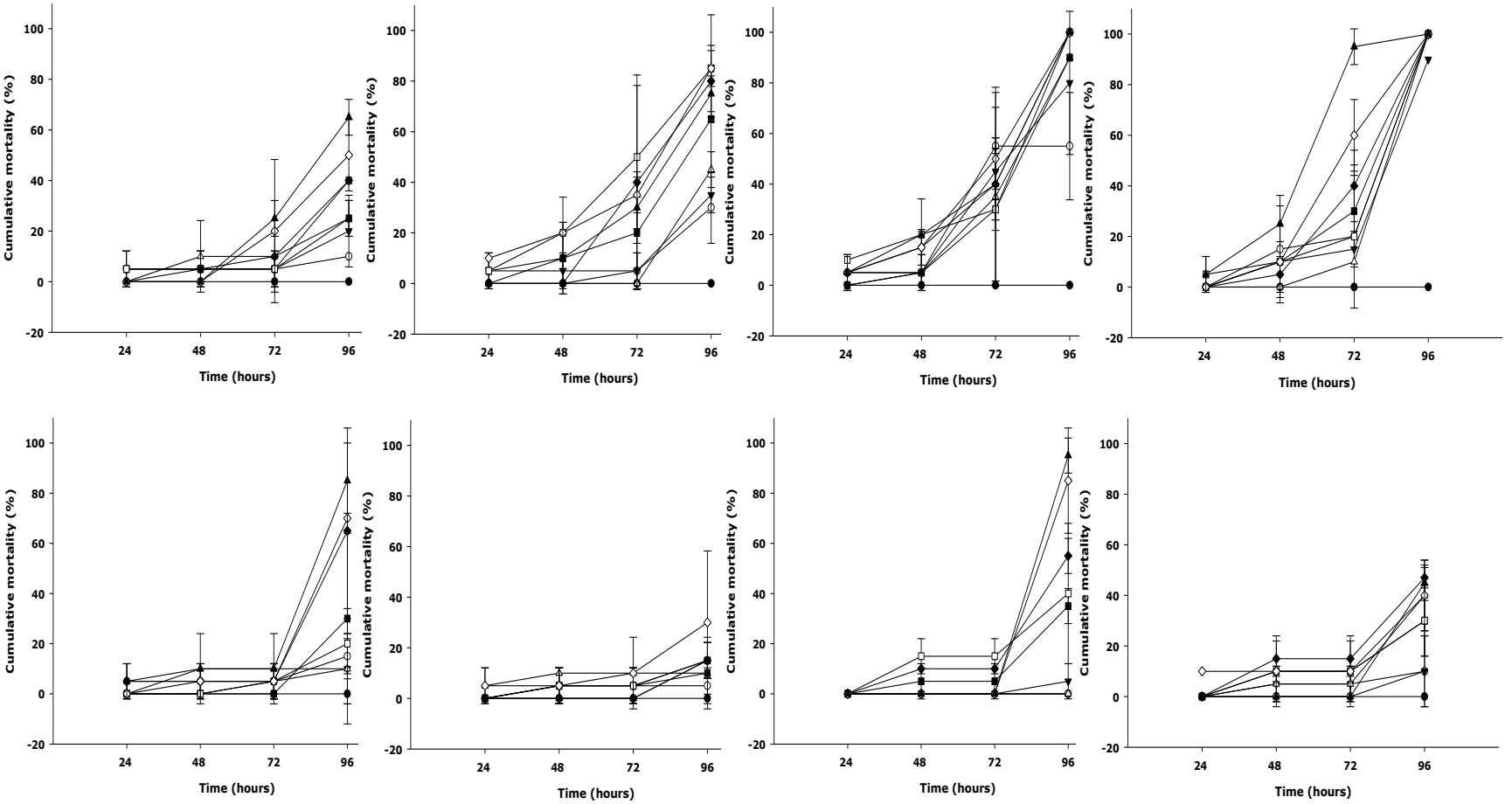

Figure 7S: Average cumulative mortality curves for *Danio rerio* exposed during 96 h to seven concentrations of each eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg. L^{-1}): SK-M, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 25°C. Vertical bars correspond to the standard deviation. n=30.

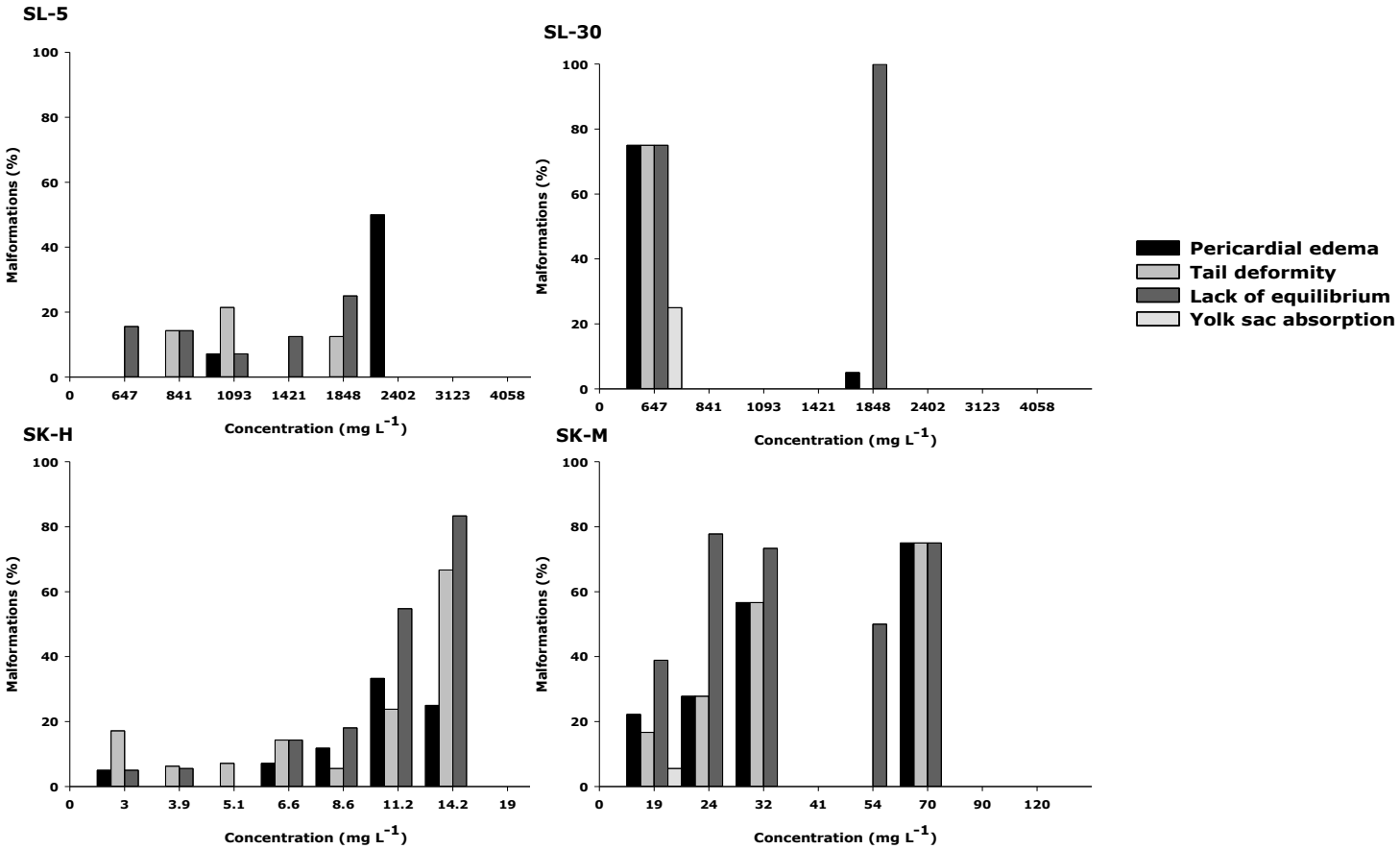

Figure 8S: Percentages (relatively to control conditions) of the different registered malformations in larvae of *Danio rerio* after being exposed to eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SK and SL variants; mg. L^{-1}): SK-M, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 15°C.

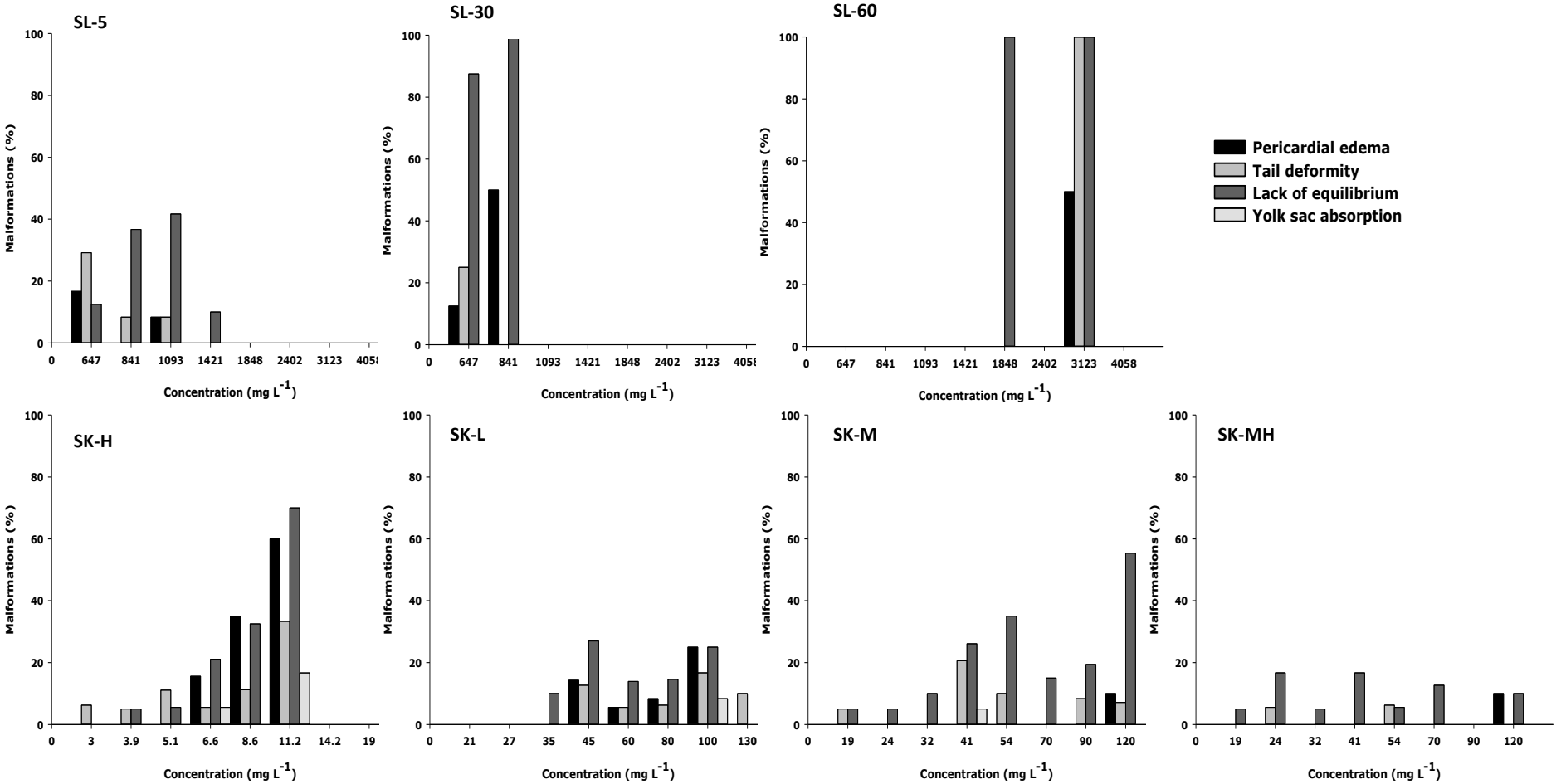

Figure 9S: Percentages (relatively to control conditions) of the different registered malformations in larvae of *Danio rerio* after being exposed to eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT™ SK and SL variants; mg. L^{-1}): SK-M, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 20 °C.

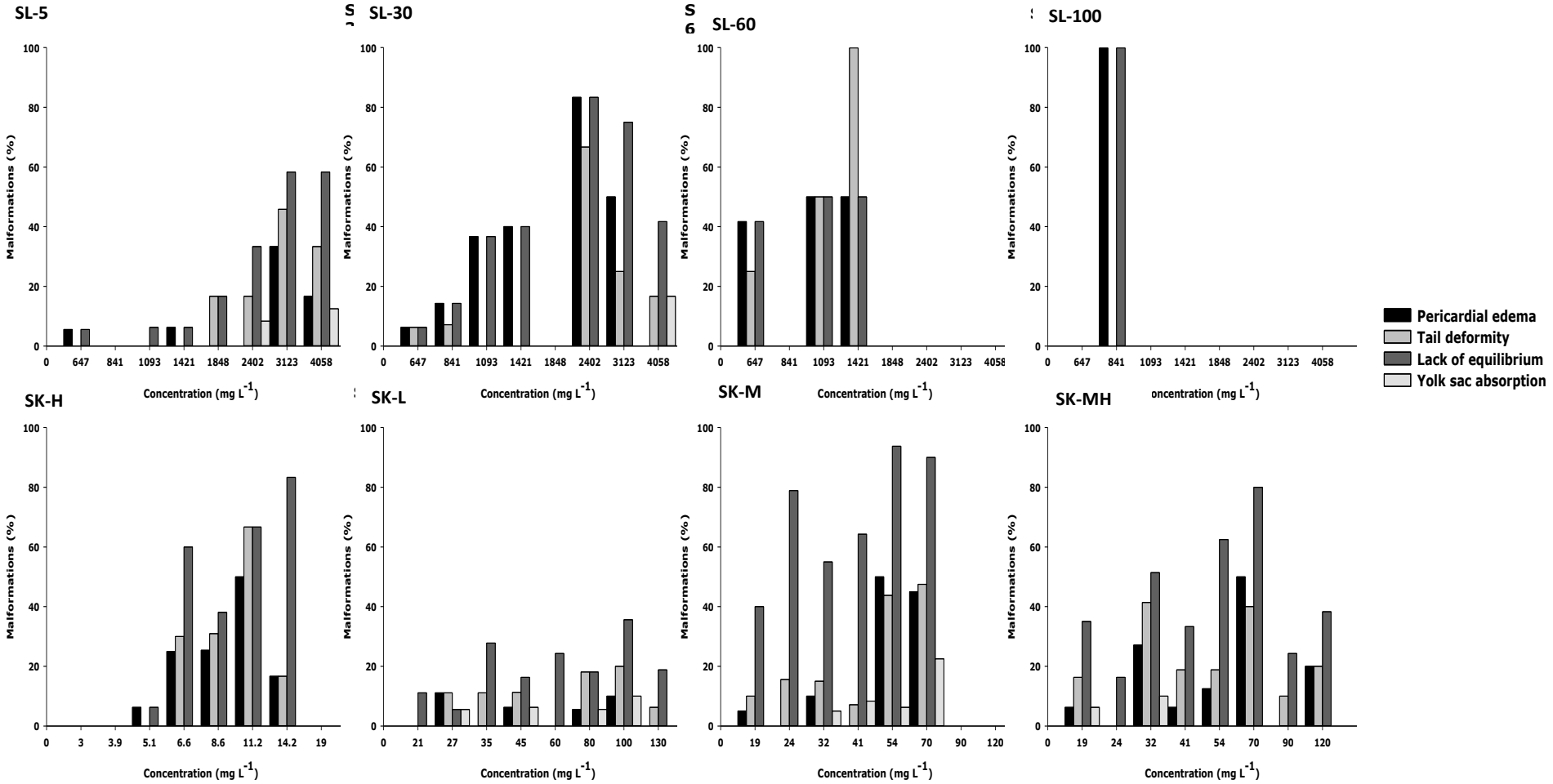

Figure 10S: Percentages (relatively to control conditions) of the different registered malformations in larvae of *Danio rerio* after being exposed to eight concentrations of each of the eight hydrophobically and cationicallly modified hydroxyethyl cellulose polymers (SoftCAT^{TM} SK and SL variants; mg. L^{-1}): SK-M, SK-L, SK-M, SK-MH, SL-5, SL-30, SL-60, and SL-100, aged at the temperature of 25 °C.

Chapter V

General Discussion and Conclusions

Over the past 15 years, has been a growing interest in the occurrence, fate and effects of compounds included in personal care products in the environment (Gogoi et al., 2018). The concentrations of these products in the environment have increased over time due to their continuous use on an everyday basis. Besides some amount of knowledge is available on the levels of personal care products in aquatic and terrestrial systems, and their uptake and effects in the organisms from these systems, there is still a lack of knowledge about some constituents of these products. Some studies have been developed to explore the uptake, depuration, and metabolism of surfactants and fragrances present in PCPs products in aquatic and terrestrial organisms including plants (Dolliver et al., 2007; Kumar et al., 2005), and in fish and aquatic invertebrates (Dussault et al., 2009; Ramirez et al., 2009). Nowadays, the main concern of society is the search for environmentally friendly compounds for personal care products, since most of the ones that are being commercialized pose several adverse effects to the environment (Kostal et al., 2015). Due to the increasing use and high requirements of the public for safe, stable, and long-lasting personal care products, this issue is very relevant since the contamination of the environment, in particular watercourses, does not only pose threat to the aquatic wildlife, flora, and fauna, but also drinking water. Hence, it is imperative to develop innovative products. For this reason, the industry faces new challenges to develop new products with reduced or no toxicity to the environment, while maintaining the same functionally (Kümmerer, 2007; Crawford et al., 2017; Cinteza et al., 2018; Jamieson et al., 2021). The advance of the rational design of chemicals with minimal unintended biological activity has been widely supported through the knowledge that has been acquired from the advances in computational chemistry and mechanistic toxicology.

Despite the mentioned above, there is a lack of understanding of what concerns possible effects of personal care products under different levels of biological organization and realistic exposure scenarios. The cellulose-based polymers are expected to be eco-friendly polymers with low toxicity, since they are commonly used in PCPs due to their high chemical resistance, possess the capability of forming gels in water, present hydrophilicity/lipophilicity that can be modified through chemical and/or hydrophobic modification, and have a great solubility (Malhotra et al., 2015; Lindman et al., 2021). These polymers are ingredients present in hair care products since they can protect the hair from different external factors capable of damage it, improve the hair wet-combability, absorb irreversibly to the hair surface, not being removed during the rinsing process (Aparecida da França et al., 2015). The studies described in this thesis focused on generating ecotoxicological data that can be used for regulation and for a better evaluation of ecological effects of high viscosity quaternized hydroxyethyl cellulose polymers with different cationic density and hydrophobic substitution, aiming to identify the safer chemicals to the environment without compromising their functionality. In detail, the objectives of the work were: i) identify the more promising HEC polymers to be studied in ecotoxicological studies through the relation of the polymer architecture with physicochemical properties; ii) assessing the ecotoxicological effects of SoftCAT™ SL polymers, containing different hydrophobic substitution degrees, to aquatic species from different taxonomic, trophic and functional groups; iii) assessing the influence of cationic density substitution on the ecotoxicity of SoftCAT™ SK polymers for aquatic key species from different trophic levels; and iv) assessing the effects of temperature on the

ecotoxicity of aged modified SoftCAT™ polymers to freshwater biota. Furthermore, understanding the effects that cationic density and hydrophobic substitution may provoke on freshwater biota can be helpful to the personal care products industry for the development of safer polymers and commercial formulations, maximizing the functionality and environmental friendliness.

Taking this into consideration, our first approach aimed to identify the more promising HEC polymers to be studied in ecotoxicological studies through the relation of the polymer architecture with physicochemical properties. All the SoftCAT™ polymers proved to be stable for a long time, showing a fluidlike behaviour at low concentrations, forming highly viscous solutions at high concentrations (Chapter II). These results are in line with the few data already available in the scientific literature (Ballarin et al., 2011; Drovetskaya et al., 2007; Milcovich et al., 2016; Milcovich et al., 2017). The viscosity degree depends on the chemical structure of these polymers. The formulations composed of SoftCAT™ SK polymers (SK-L, SK-M, and SK-H) bearing a relatively high charge density adopt an extended conformation in aqueous solution due to the counterions entropy. However, the increase in the hydrophobic substitutions as present in SoftCAT™ SL polymers lead to a slight increase of the solution viscosity, due to the aggregation of the side hydrophobic groups and consequent improvement of the polymer network. Concerning the average particle size of the SoftCAT™ polymers, the variants with higher hydrophobic substitution as SL-100 and SK-MH were the ones with higher size, due to an increase of the aggregation of the polymer molecules.

Considering the previous results, this study proceeded to obtain a comprehensive effect assessment of four SoftCAT™ SL polymers with different degrees of hydrophobic substitution for aquatic organisms. The scarcity of studies assessing the effects of SoftCAT™ SL polymers on aquatic organisms together with their unknown specific mode of action in these organisms led us to assess the effects of these compounds through the identification of the most sensitive ecological receptors to SoftCAT™ SL polymers in the aquatic environment. The results obtained in the present work showed that the two freshwater algae species, *R. subcapitata* and *C. vulgaris*, along with rotifer *B. calyciflorus*, formed the most sensitive group of organisms to lethal and sublethal levels of these polymers. The identification of the two freshwater algae species as the most sensitive group has already been reported by several studies developed (Pereira et al., 2018; Cumming, 2008). Due to their higher viscosity (greater number of hydrophobic groups), the SL-100 was the SoftCAT™ SL variant that provoked greater toxicity to these two algae species. The higher viscosity presented by this polymer enhanced the absorption to the surface of the cell membrane, resulting in an increase in the toxicity of this compound through the prevention of photosynthesis and inhibiting the cells to grow (Nolte et al., 2017; Serra et al., 2019). In relation to the pluricellular organisms like *D. rerio*, *D. magna* and *H. incongruens*, the SL variants with the higher number of hydrophobic groups like SL- 60 and SL-100, were the least toxic ones. The same occurred in these species as for the algae, the interaction between opposite charges was the primary action mode of the particles (Nolte et al., 2017). Since the viscosity of the media might also influence the polymer ecotoxicity, this can explain the effects of these polymers to *D. magna*, once the swimming behaviour of this species could be affected by the medium viscosity (Serra et al., 2019), and to *D. rerio* due to a reduction of respiratory rates around the egg jelly or embryos (Rombough, 1998). However, other routes

of exposure like the ingestion of aggregates (Martín-de-Lúcia et al., 2019) must also be considered. These cationic polymers can induce the formation of reactive oxygen species and consequently to the development of malformations, as observed for the species *D. rerio*, where the SL-5 variant induced several malformations after 96h of exposure. Considering the highest estimated MAC-EQS obtained for these polymers, the concentrations that are considered as a risk to the aquatic compartment are higher than 14.0 mg. L^{-1} for the SL-5 variant, 2.9 mg. L^{-1} for the SL-30 variant, 3.9 mg. L^{-1} for the SL-60 variant and, finally, 1.4 mg. L^{-1} for the SL-100 variant. From these results, in addition to the GHS classification and labelling chemicals, all the SL variants are included in category II, being considered as acutely toxic to aquatic biota (United Nations, 2011). However, from the results obtained in the highest estimated MAC-EQS, the SL-5 variant showed to be the least toxic one and the SL-100 the highest toxic one. Since these polymers have different architectures, SL-5 presenting the lowest hydrophobic substitution and the SL-100 with the highest substitution, we can conclude that this influences the toxicity of these polymers to the aquatic biota.

Given the previous results obtained for the ecotoxicity of SoftCAT™ SL polymers, the influence of cationic substitution of SoftCAT™ SK polymers to aquatic biota acute ecotoxicity was also addressed, to identify the least toxic variant. According to the results obtained in this work, the SK-H variant was the most toxic, since it is the one with the highest cationic charge density. As mentioned before, the increase of the cationic charge can be related to the increase of toxicity, since the cell membranes of the organisms are negatively charged where the cationic groups of SoftCAT™ SK polymers can establish electrostatic interactions (Narita et al., 2001; Roy, 2020). The viscosity of these polymers can also influence their toxicity to the aquatic environment. The SK-MH, variant with the lowest viscosity index, showed to be the least toxic variant to all the species studied in this work. It was already showed that the viscosity can have some influence on the behaviour of organisms since it can decrease the movement and feeding of the organisms and provoke alterations in the sensorial and mechanical sensitivity of cell membranes (Sohn et al., 2013; Orchard et al., 2016; Serra et al., 2019). According to the results obtained in this work, the microalgae and *B. calyciflorus* were the species that showed the highest sensitivity to SK variants (the same most sensitive species for SL variants) (Chen et al., 2015). Considering the estimated MAC-EQS obtained for these polymers, the MAC-EQS obtained with the determinist approach were 60-fold or lower than the ones obtained with the mechanistic approach. For instance, all the SK variants used in this work revealed low MAC-EQS values. According to the GSH classification, the SK-H is included in the category with the highest toxicity, category I and II, resulting from the results obtained to five of the species studied (United Nations, 2011). On the other hand, the other variants evaluated in this work were not included in the same category of this variant. The SK-H variant (0.00017 mg. L^{-1}) was the one that presented the lowest MAC-EQS value for the aquatic compartment, indicating that this is the variant most toxic to the aquatic biota. Thus, the SK-M variant (0.00354 mg. L^{-1}) showed to be the least toxic and should be considered by the personal care products industry.

Since some of the personal care products are not easily removed from the aquatic environment by the wastewater treatment plants, the effect of temperature after an ageing process of one month was also addressed (Ebele et al., 2017). In the context of the climate changes that the world is facing and since these

polymers are not easily removed from the aquatic environment through conventional water treatment, the evaluation of the effect of different temperatures on the ageing process is crucial according to the increase of the global surface temperature reported in the last IPCC report. The toxicity of the SoftCAT ™ was previously evaluated, and the hydrophobic and cationic substitutions had an impact on the toxicity of these polymers, so it is important to evaluate the influence of temperature on the toxicity of these several variants. According to the results obtained in this work, the temperature of 15˚C was the one that induced the highest toxicity of the SK and SL variants, provoking the total mortality of *B. calyciflorus* after the exposure to SL-5, SL-30, and SL-100 variants. The two freshwater algae species and the rotifer *B. calyciflorus* continue to be the most sensitive group of organisms for SK and SL variants (Pereira et al., 2018; Simões et al., 2021). It was already observed that the temperature influences the viscosity of polymers, for instance, the increase of the temperature, turn the polymer less viscous (Hong et al., 2015), being more easily absorbed by the organisms and facilitate their entrance into the cells (Wang and Ye, 2015; Xia et al., 2015; Nolte et al., 2017). Considering the highest estimated MAC-EQS obtained for these polymers, the SL-5 variant showed to be the least toxic one, all the other SL and SK variants tested presented low MAC-EQS. According to the results of the predicted concentrations putting at risk the aquatic compartment would be greater than 0.0009 mg/L for the SL-5 variant, 0.0007 mg/L for the SL-30 variant, 0.025 for the SL-60 variant, 0.0007 for the SL-100 variant, 0.0002 for the SK-H variant, 0.033 for the SK-L, 0.00003 for the SK-M variant, and 0.0002 for the SK-MH variant. Relating to the GSH classification, the temperature of 15 ºC included several SL and SK variants in the category with the highest toxicity, category I and II (United Nations, 2011). All the SK variants after being aged at the temperature of 20 ºC were included in category II, only the SK-H variant was also included in category I, at this temperature. All the SL variants after being aged at the temperature of 15 ºC were included in the category I and II. Only the SK-H variant aged at the temperature of 25 ºC was included in category I, all the other SL and SK variants at this temperature were included in category II, with the exception of the SL-30 and SK-L variants that were not included in either these two categories.

Preliminary results showed that the cationic polymers had a toxic effect on the aquatic environment, however, the results were promising for the SL-5 variant and should be taken into consideration during the development process of more environmentally friendly cellulose-based cationic polymers. The personal care product industry should consider the SL compounds with a hydrophobic substitution lower than 30 as an environmentally friendly option to be included in these products formulations since they also maintain their high efficiency, besides minimizing the ecological risks. All findings reported in this thesis demonstrate the toxicity and potential deleterious effects of hydroxyethyl cellulose polymers, providing valuable information that should be considered in the information related to the environmental concentrations of cationic polymers.

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