RENATO GRILLO

CHITOSAN/TRYPOLYPHOSPHATE NANOPARTICLES AS A MODIFIED RELEASE SYSTEM FOR PARAQUAT HERBICIDE: PREPARATION, CHARACTERIZATION, INTERACTION WITH HUMIC SUBSTANCES AND EVALUATION OF BIOLOGICAL ACTIVITY

NANOPARTÍCULAS DE QUITOSANA/TRIPOLIFOSFATO COMO SISTEMA DE LIBERAÇÃO PARA O HERBICIDA PARAQUAT: PREPARO, CARACTERIZAÇÃO, INTERAÇÃO COM SUBSTÂNCIAS HÚMICAS E AVALIAÇÃO DA ATIVIDADE BIOLÓGICA

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Supervisor/ *Orientador*: Prof. Dr. Leonardo Fernandes Fraceto

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ABSTRACT

Agrochemicals have become one of the most important compounds used in the world and are the main responsible for the increase in agricultural production. Paraquat, a non-selective herbicide used in several crops in the world, has high toxicity to living beings and considerable persistence in the soil, which led to its banishment in some countries. Although agrochemicals have an important function in increasing food production by minimizing pests, they can cause serious environmental impacts through contamination of soil and water resources. With the nanotechnology growth, several mechanisms have been studied in order to minimize these damages, such as the development of modified release systems using biodegradable polymers, which allows an increase in performance/efficiency of the product. Studies indicate that the natural organic matter present in the soil, sediment and water can interact with agrochemicals and nanoparticles, and therefore, modify the destination of these substances in the environment. The purpose of the present Thesis is to show results of the development and characterization of chitosan/trypolyphosphate polymeric nanoparticles for modified release of the Paraquat herbicide, as well as to study the stability and toxicity of this nanoparticles with the presence or absence of natural organic matter (mainly in the form of humic substances). Herbicide activity and sorption of the nanoparticulate herbicide in the soil were also evaluated. Chitosan/trypolyphosphate nanoparticles (with or without Paraquat) have been prepared by ionic gelation method and presented average size, polydispersity index and zeta potential of ~ 300 nm, 0.250 and 45 mV, respectively. The Paraquat encapsulation efficiency was 62.6 ± 0.7 % and the nanoparticles showed themselves spherical and good colloidal stability. The release kinetics and sorption test in the soil showed that the nanoparticles modified the diffusion and release of herbicide and the mathematical model that best described the sorption kinetics was pseudo-second order. Cytotoxicity, genotoxicity and ecotoxicity tests indicated that the association of Paraquat with the nanoparticles reduced the toxic effect, since less herbicide was released into the environment as function of time, but even so, the herbicidal activity was preserved or enhanced. The presence of humic substances in the medium altered the dynamic equilibrium of the herbicide in solution, and diminished the toxicity of the nanoparticle/paraquat system. This study has innovative and promising results that open new discussions and perspectives for the development of modified release systems for agrochemicals.

Keywords: Controlled release, Polymeric nanoparticles, Paraquat, Organic Matter, Nanotoxicology.

RESUMO

Os defensivos agrícolas tornaram-se alguns dos mais importantes compostos utilizados no mundo e são os principais responsáveis pelo aumento da produção agrícola. O Paraquat, um herbicida não seletivo de contato utilizado em diversas culturas no mundo, apresenta alta toxicidade para os seres vivos e considerável persistência no solo, o que fez com que ele fosse banido em alguns países. Embora os defensivos agrícolas possuam uma importante função no aumento da produção de alimentos, através do controle de pragas, eles podem gerar sérios impactos ambientais, através da contaminação dos recursos hídricos e do solo. Com o crescimento da nanotecnologia, diversos mecanismos estão sendo estudados a fim de minimizar estes danos, tais como o desenvolvimento de sistemas de liberação modificada, utilizando polímeros biodegradáveis, o qual permite um aumento no desempenho/eficiência do produto. Estudos indicam que a matéria orgânica natural presente no solo, sedimento e água podem interagir com os defensivos agrícolas e nanopartículas e consequentemente modificar o destino destas substâncias no ambiente. A presente Tese tem como objetivo principal mostrar resultados do desenvolvimento e caracterização de nanopartículas poliméricas de quitosana/tripolifosfato para liberação modificada do herbicida Paraquat, bem como, estudar a estabilidade e a toxicidade deste sistema nanoparticulado com a presença ou ausência de matéria orgânica natural (na forma de substâncias húmicas). A atividade herbicida e a sorção do herbicida nanoparticulado no solo também foram avaliados. Nanopartículas de quitosana/tripolifosfato (com ou sem Paraquat) foram preparadas pelo método de gelificação iônica e apresentaram tamanho médio, índice de polidispersão e potencial zeta de ~ 300 nm, 0,250 e 45 mV, respectivamente. A eficiência de encapsulação do Paraquat foi de 62,6 ± 0,7 % e as nanopartículas apresentaram-se esféricas e com boa estabilidade coloidal. A cinética de liberação e o ensaio de sorção no solo mostraram que as nanopartículas retardaram a difusão e a liberação do herbicida e o modelo matemático que melhor descreveu a cinética de sorção foi o de pseudo-segunda ordem. Ensaios de citotoxicidade, genotoxicidade e ecotoxicidade indicaram que a associação do Paraquat às nanopartículas reduziram o efeito tóxico, uma vez que menos herbicida foi liberado para o ambiente em função do tempo, e ainda assim a atividade herbicida foi preservada ou aumentada. A presença de substâncias húmicas naturais não alterou os parâmetros físico-químicos estudados; entretanto, mostrou que pode reduzir a genotoxicidade e a ecotoxicidade do herbicida quando associado às nanopartículas, através de modificações na dinâmica do sistema. Este trabalho possui resultados inovadores e promissores que abrem novas discussões e perspectivas para o desenvolvimento de sistemas de liberação modificada para defensivos agrícolas.

Palavras chaves: Liberação modificada, Nanopartículas poliméricas, Paraquat, Matéria Orgânica, Nanotoxicologia.

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“*O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas*”. *(Claude Lévi-Strauss)*

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LISTA DE ABREVIATURAS E SIGLAS

AIDS	Síndrome da Imunodeficiência Adquirida
AHS	Substâncias Húmicas Aquáticas
ANOVA	Análise de Variância
ANVISA	Agência Nacional de Vigilância Sanitária
C_{60}	Fulerenos
CHO	Ovário de Hamster Chinês
CNPEM	Centro Nacional de Pesquisa em Energia e Materiais
CNTs	Nanotubos de Carbono
CS	Quitosana
CS/TPP	Nanopartículas de Quitosana/tripolifosfato
DLS	Espalhamento Dinâmico de Luz
ENPs	Nanopartículas Engenheiradas
EPA	Agência de Proteção Ambiental dos Estados Unidos
ESHA	Ácidos Húmicos Extraídos do Solo Elliott
FCS	Espectroscopia de Fluorescência
FFF	Flow Field-Flow Fractionation
FTIR	Espectroscopia na Região do Infravermelho por Transformada de Fourier
GSH	Glutationa
HA	Ácidos Húmicos
HPLC	Cromatografia Líquida de Alta Eficiência
HPSEC	Cromatografia por Exclusão Molecular
HS	Substâncias Húmicas
ICP	Espectrometria de Emissão Atômica por Plasma Acoplado Indutivamente
IHSS	Sociedade Internacional de Substâncias Húmicas
ISO	Organização Internacional de Padronização
KDa	Quilodaltons
LHA	Ácidos Húmicos Leonardite
MTT	3-(4,5-Dimetiltiazol-2yl)-2,5-Difenil Brometo de Tetrazolina)

MW	Massa Molar
MWNTs	Nanotubos de Carbono Multicamadas
NADP	Nicotinamida Adenina Dinucleótido Fosfato
NLFA	Ácidos Fúlvicos Extraídos do Lago Nordic
NLHA	Ácidos Húmicos Extraídos do Lago Nordic
NP	Nanopartículas
NP:PQ	Nanopartículas Contendo Paraquat
NPs	Nanopartículas Poliméricas
NTA	Nanoparticle Tracking Analysis
PH_{PZC}	pH = Ponto de Zero Carga
PQ	Paraquat
SEM	Microscopia Eletrônica de Varredura
SRFA	Ácidos Fúlvicos Extraídas do Rio Suwannee
SRHA	Ácidos Húmicos Extraídas do Rio Suwannee
SRNOM	Matéria Orgânica Extraída do Rio Suwannee
STM	Microscopia de Tunelamento
TA	Ácidos Tânicos
TEM	Microscopia Eletrônica de Transmissão
TPP	Tripolifosfato de Sódio
UV	Espectroscopia de UV-Visível
WPFA	Ácidos Fúlvicos Extraídos de Turfa Waskish
WPHA	Ácidos Húmicos Extraídos de Turfa Waskish
XRD	Difração de Raio-X
XAD7	Resina Amberlite

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GENERAL INTRODUCTION

INTRODUÇÃO GERAL

A população mundial cresceu de 2,5 bilhões em 1950 para 7 bilhões em 2014 e, de acordo com a Organização das Nações Unidas, a tendência é aumentar para 9,6 bilhões em 2050 (United Nations, 2014). Este crescimento populacional tem intensificado expressivamente a demanda por alimentos provenientes de atividades agrícolas, uma vez que as plantas constituem uma das principais fontes de nutrientes para os seres vivos (Sopena et al., 2009). No entanto, aproximadamente 842 milhões de pessoas foram relatadas com desnutrição entre 2011-2013, o que representa 12% da população global (FAO, 2013). A maioria destas pessoas desnutridas vive em regiões em desenvolvimento do planeta e estima-se que a escassez de alimento pode matar mais pessoas anualmente que doenças como a AIDS, malária e tuberculose juntas (WFP, 2014), o que implica na necessidade de desenvolver medidas mais eficientes para minimizar o desperdício de alimentos e aumentar a produtividade agrícola.

O aumento da produção de alimentos per capita mundial pode ser obtido por vários meios, tais como expandindo as áreas agrícolas e aumentando o rendimento das culturas, através do uso de defensivos agrícolas, adubos orgânicos, controles biológicos, melhor gestão do solo e da água e através da mecanização (Carvalho, 2006). Além disso, o uso de variedades de plantas mais produtivas e variedades resistentes a pragas também poderiam ajudar no aumento da produtividade agrícola (Powles and Yu, 2010; Bala et al., 2013). Entretanto, muitas destas alternativas têm sido desenvolvidas e utilizadas em todo o mundo, apresentando resultados positivos ou até mesmo controversos (Carvalho, 2006). Sabe-se que a escassez de água é um sério problema em muitas regiões do planeta e que, além disso, a expansão de áreas agrícolas não é tarefa fácil de se realizar; desta forma, o uso de defensivos agrícolas tem sido escolhido para aumentar a produção agrícola, por se tratar de um método simples e acessível aos produtores agrícolas (Pingali, 2012; Ghimire and Woodward, 2013), todavia sabe-se que o manejo e o uso destes produtos podem apresentar impactos negativos para o ambiente e a saúde humana.

Os defensivos agrícolas tornaram-se um dos mais importantes compostos responsáveis pelo aumento da produção agrícola, especialmente após 1960, com a "Revolução Verde" na agricultura (Ghimire and Woodward, 2013). Estes compostos químicos são utilizados para prevenir, controlar e/ou eliminar pragas, tais como fungos, insetos, plantas, bactérias e vírus que comprometem a produtividade das plantas no campo (Oliveira Jr, 2011). Estudos mostram que os danos causados pelas pragas podem representar 42 % na perda mundial da produção agrícola (Raileanu et al., 2010; Ghimire and Woodward, 2013), o que pode fundamentar o uso destes produtos químicos no campo.

Dentre os defensivos agrícolas mais utilizados no mundo, estão os herbicidas, que são compostos orgânicos de massa molar pequena (geralmente < 700 Da), utilizados para matar ou inibir o crescimento das plantas indesejáveis que se desenvolvem junto à plantação (Javaroni et al., 1999; Duke and Dayan, 2011). Há centenas de tipos de herbicidas formulados, em milhares de produtos comerciais em todo o mundo, porém muitos são restritos em diferentes países por causa de leis regulatórias, econômicas e agrícolas (Duke and Dayan, 2011). Segundo a Agência Nacional de Vigilância Sanitária (Anvisa), órgão responsável pela liberação do uso comercial de defensivos agrícolas no Brasil, o mercado de defensivos agrícolas cresceu 190 % na última década no país, em um ritmo de expansão duas vezes maior que o apresentado pelo mercado mundial no mesmo período (Anvisa, 2013). Em

2013, o setor de defensivos agrícolas movimentou US$ 11,454 bilhões o que corresponde a 40% aplicado em inseticidas, 33% para herbicidas, 23% para fungicidas e 4% para acaricidas e, para o ano de 2014, a expectativa é que finalize o ano com um avanço entre 6% e 9% (Sindiveg, 2014).

Dentre os herbicidas mais empregados estão o Glifosato (N-(fosfonometil) glicina), seguidos pela Atrazina (2-cloro 4-etilamina 6-isopropilamina 1,3,5-triazina) e pelo 2,4D (2,4-diclorofenoxiacético), entre outros. No entanto, o herbicida Paraquat (1,1'-dimetil 4,4'-bipiridina dicloreto) é uma alternativa em culturas resistentes ao herbicida Glifosato (Donaldson, 2013) e sua utilização iniciou-se a partir da década de 60, sendo hoje largamente utilizado em mais de 100 países em culturas de fumo, algodão, arroz, café, cana-de-açúcar, feijão, soja, dentre outras (Almeida et al., 2007). Este herbicida de contato não seletivo pode agir interferindo no sistema intracelular de transferência de elétrons das plantas, inibindo a redução de coenzimas ($NADP^+$ a NADPH) durante a fotossíntese e levando à formação dos radicais superóxido (O_2^-), hidroxila ($OH^\bullet$) e peróxido de hidrogênio (H_2O_2), cujo mecanismo é bastante semelhante ao que causa toxicidade nos animais e no corpo humano (Figura 1).

Embora os herbicidas apresentem diversas vantagens, sabe-se que, dependendo do método de aplicação e das condições climáticas, cerca de 60 a 70 % dos herbicidas convencionalmente aplicados no campo nunca atingem seus alvos, ou seja, não produzem a resposta biológica desejada no momento exato e nas quantidades específicas requeridas (Kumar et al., 2014). Estes problemas ocorrem muitas vezes devido à baixa especificidade destes compostos com a planta, devido a fenômenos de sorção ou lixiviação do composto no solo e pela baixa estabilidade físico-química que estas moléculas apresentam (Boehm et al., 2000; Kumar et al., 2014). Além disso, herbicidas podem ser tóxicos e proporcionar intoxicações agudas ou crônicas nos seres humanos (Roberts et al., 2010; Seok et al., 2012).

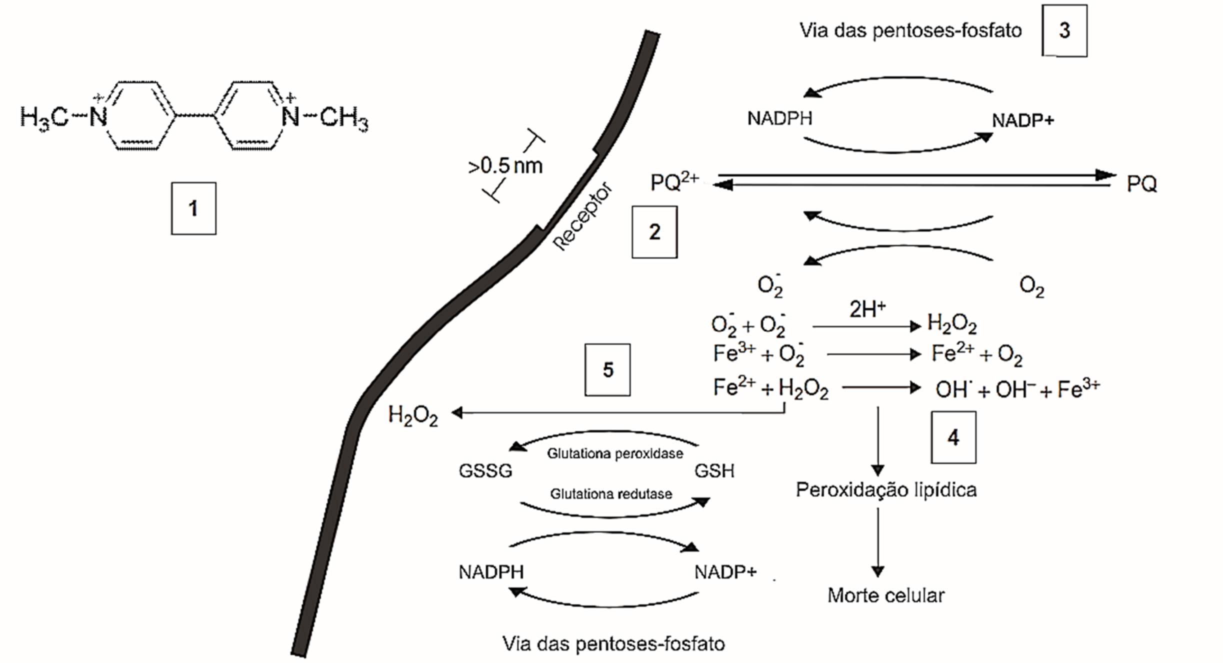

Figura 1. Representação esquemática do mecanismo de toxicidade do PQ: 1) Estrutura do PQ; 2) Entrada do herbicida na célula; 3) Ciclo redox do PQ utilizando NADPH; 4) formação de radicais hidroxilas ($OH^{\bullet}$) gerando a peroxidação; e 5) desintoxicação de H_2O_2 via GSH redutase/peroxidase utilizando NADPH (modificado de Gupta, 2012).

A intoxicação por herbicidas nos animais e seres humanos pode acontecer pela ingestão e absorção pela pele ou pelas vias aéreas e, dependendo da toxicidade do composto, pode aumentar os riscos de desenvolvimento de câncer (Morrison et al., 1992; Ji et al., 2001; Turner et al., 2010; Sathiakumar et al., 2011), gerar má formação fetal (Gupta, 2012), infertilidade feminina (Greenlee et al., 2003), além de desencadear problemas endócrinos, imunes e no sistema nervoso (Porter et al., 1999). Sendo assim, existe a necessidade do desenvolvimento de novas alternativas para aplicação de herbicidas mais eficazes e seguros, uma vez que a contaminação por estes compostos vem aumentando em águas pluviais e subterrâneas (de Armas et al., 2007). Neste contexto, os Estados Unidos popularizaram a expressão LISA (Low Input Sustainable Agriculture), que consiste na busca de uma agricultura sustentável, buscando um aumento na produção agrícola com reduzida quantidade de produtos químicos (Alves Filho, 2002).

Com o crescimento acelerado da nanociência e nanotecnologia, muitos nanomateriais estão sendo desenvolvidos com propósito de melhorar a eficácia e a segurança destes defensivos agrícolas (Khot et al., 2012; Kah et al., 2013; Kah and Hofmann, 2014). Herbicidas, quando associados a nanomateriais, passam a ter superfície de contato e reatividade química maior (Jefferson, 2000; Elsaesser and Howard, 2012), o que pode favorecer sua interação e ação com as plantas. Diversos tipos de nanoformulações foram desenvolvidas para a área agrícola atualmente (Campos et al., 2014), entre elas, nanodispersões, nanoemulsões, nanopartículas inorgânicas e orgânicas (Vemmer and Patel, 2013; Kah and Hofmann, 2014). De acordo com Kah e colaboradores (2014), as nanopartículas orgânicas, principalmente as nanopartículas poliméricas (NPs), são as que possuem maior potencial de desenvolvimento e aplicação prática para a agricultura, no entanto, alguns herbicidas já podem ser encontrados associados a este sistema, os quais podem promover a liberação modificada do ativo no ambiente (Silva et al., 2011; Grillo et al., 2012; Pereira et al., 2014), diminuir sua genotoxicidade (Grillo et al., 2012; Pereira et al., 2014), modificar sua sorção e lixiviação no solo (Silva et al., 2011), bem como aumentar a solubilidade destes bioativos (Boehm et al., 2000).

Os polímeros biodegradáveis utilizados para preparar estas NPs são similares àqueles utilizados para produtos farmacêuticos e cosméticos, como os poliésteres (p. ex., poli (ε-caprolactona), poli (lactídeo) e poli (lactídeo-*co*-glicolídeo)) e os polissacarídeos (quitosana, alginato e amido) (Kah and Hofmann, 2014; Lopez-Serrano et al., 2014). A quitosana tem sido um polímero extensivamente utilizado como carreador de sistemas de liberação modificada para bioativos, devido a sua biocompatibilidade, baixa toxicidade, baixo custo e alta biodegradabilidade (Muzzarelli, 2010; Harris et al., 2011; Luo et al., 2011; Fan et al., 2012; Hosseini et al., 2013). Entre vários métodos desenvolvidos para preparar nanopartículas de quitosana, a técnica de gelificação iônica atraiu considerável atenção por ser um processo não-tóxico (livre de solvente orgânico), prático e controlável (Agnihotri et al., 2004; Fan et al., 2012). Esta técnica é baseada na interação eletrostática entre os grupamentos amino (NH_3^+) da quitosana com poliânions, tais como, o tripolifosfato de sódio (TPP), como representado esquematicamente na Figura 2 (Dyer et al., 2002; Yang et al., 2011).

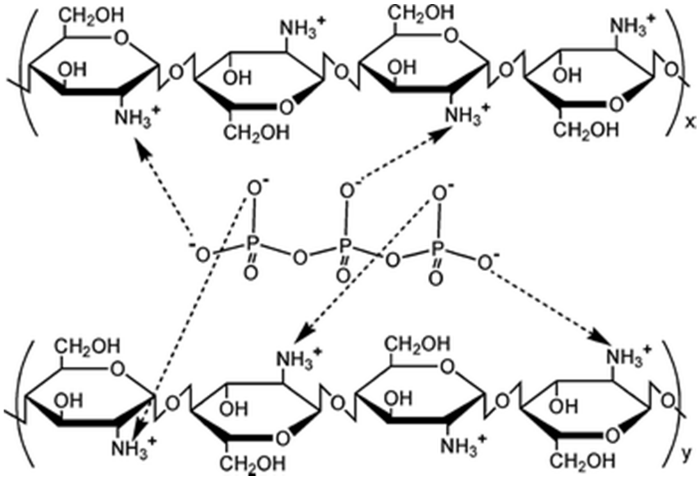

Figura 2. Mecanismo de síntese das nanopartículas é baseado no fato de que em pH baixo, grupamentos das moléculas quitosana são ionizadas e interagem com o ânion TPP formando nanopartículas (Yang et al., 2009a).

Enquanto a nanotecnologia e a produção de nanopartículas crescem exponencialmente, as pesquisas voltadas para os impactos ambientais e os riscos destes nanomateriais para a saúde humana ainda são bastante escassas e lentas (Elsaesser and Howard, 2012; Kah et al., 2013; Maurer-Jones et al., 2013; Kah and Hofmann, 2014; Lopez-Serrano et al., 2014). Neste contexto, estudos toxicológicos são importantes, uma vez que NPs podem modificar o transporte, a biodisponibilidade e o destino dos defensivos agrícolas no ambiente (Remédios et al., 2012; Kah and Hofmann, 2014). Alguns estudos sobre toxicidade de NPs são realizados através de ensaios de fitotoxicidade (Faisal et al., 2013; Gao et al., 2013; Josko and Oleszczuk, 2013; Qian et al., 2013; Kumar et al., 2014; Pereira et al., 2014), citotoxicidade (Kim et al., 2010; Dalai et al., 2013; Hamzeh and Sunahara, 2013; Lima et al., 2013; Zhang et al., 2013a; Hashem et al., 2014), genotoxicidade (Dalai et al., 2013; Lima et al., 2013; Dobrzynska et al., 2014; Doktorovova et al., 2014; Magdolenova et al., 2014) e ecotoxicidade (Barrena et al., 2009; Clemente et al., 2012; Clemente et al., 2013; Ma et al., 2013; Park et al., 2014). Entretanto, a maioria deles são para nanopartículas inorgânicas utilizadas para outras aplicações, que não seja a agricultura (Klaine et al., 2008; Sharma et al., 2014).

Estudos de citotoxicidade relacionados a nanopartículas poliméricas em linhagens de células humanas e de mamíferos são encontrados na literatura; no entanto, vários protocolos e resultados ainda são contrastantes (Lima and Fraceto, 2014). Poucos são os estudos que envolvem ensaios de ecotoxicidade, citotoxicidade e genotoxicidade para NPs contendo defensivos agrícolas; todavia, alguns mostram que NPs de alginato de sódio contendo o inseticida Imidacloprida apresentaram menor citotoxicidade em células isoladas de epitélio renal de macacos (Kumar et al., 2014) e nanopartículas de poli(ε-caprolactona) contendo herbicidas triazínicos apresentaram menor genotoxicidade através de ensaios cometa (Grillo et al., 2012) e *Allium cepa* (Grillo et al., 2012; Pereira et al., 2014). Entretanto, sabe-se que muitos fatores influenciam na toxicidade das NPs, entre eles, o tamanho médio, a matéria prima que as compõem, a superfície de contato, porosidade, carga de superfície e principalmente sua estabilidade no ambiente em que se encontram (ar, solo, sedimento ou água) (Dietz and Herth, 2011; Elsaesser and Howard, 2012). Pesquisas mostram que substâncias húmicas presentes na matéria orgânica do ambiente podem formar complexos com NPs e modificar sua estabilidade e biodisponibilidade no ambiente (Yang et al., 2009b; Delay et al., 2011). No entanto, além de avaliar a toxicidade das NPs em protocolos laboratoriais convencionais, também se faz necessário considerar o ambiente em que elas se encontram. A carência de textos de revisão nesta área fez com que fosse elaborado um artigo, tratando da interação de NPs com a matéria orgânica, e assim mais detalhes podem ser encontrados a seguir no Capítulo I.

O conhecimento atual sobre nanopartículas associadas a herbicidas ainda é bastante escasso, especialmente considerando que há pouca regulamentação e as que existem não são específicas para todos os nanomateriais (Kah and Hofmann, 2014; Kookana et al., 2014). Algumas entidades governamentais, como EPA (United States Environmental Protection Agency), têm realizado esforços no sentido não só de compreender as propriedades das nanopartículas e seus potenciais riscos para a saúde humana e ao ambiente, mas também para regulamentar o uso, armazenamento e descarte desses materiais para permitir, assim, um uso seguro e sustentável destes produtos (Remédios et al., 2012).

A presente Tese tem como objetivo principal mostrar resultados do desenvolvimento e caracterização de nanopartículas poliméricas para liberação modificada do herbicida

Paraquat, bem como estudar a estabilidade deste sistema com a matéria orgânica natural (na forma de substâncias húmicas) e avaliar a atividade herbicida em plantas, bem como investigar sua citotoxicidade, genotoxicidade e ecotoxicidade. A Tese foi estruturada em capítulos, cada um correspondendo a um artigo já publicado e/ou submetido para periódico científico internacional. Esta introdução contextualiza a temática do estudo e como parte dela surgiu o primeiro capítulo que apresenta uma revisão bibliográfica da interação entre nanopartículas com substâncias húmicas e algumas das possíveis aplicações desta associação. Como síntese do manuscrito, temos a matéria orgânica influenciando na estabilidade e na toxicidade de vários tipos de NPs, além da combinação destes compostos para o desenvolvimento de biosensores e removedores de moléculas orgânicas e inorgânicas do ambiente. O segundo capítulo discorre sobre o desenvolvimento (preparo e caracterização) de um sistema de liberação modificada para o herbicida Paraquat utilizando nanopartículas de quitosana/tripolifosfato; neste estudo avaliou-se também a sorção do herbicida livre e associado às NPs com o solo, bem como a citotoxicidade, a genotoxicidade e o efeito da atividade herbicida em culturas de milho e mostarda. Os resultados apresentados mostraram que o sistema de liberação modificada para o herbicida Paraquat é estável e apresenta menor toxicidade para células de ovário de hamster Chinês (CHO) e *Allium cepa* em comparação com o herbicida não associado às NPs e ainda, proporcionou o mesmo efeito na atividade herbicida. O terceiro capítulo apresenta um estudo da estabilidade e toxicidade das NPs de quitosana/tripolifosfato contendo Paraquat com substâncias húmicas aquáticas e algumas técnicas espectroscópicas foram utilizadas para caracterizar o sistema, bem como ensaios de genotoxicidade em A*llium cepa* e ecotoxicológicos em *Pseudokirchneriella subcapitata*. A partir dos resultados obtidos, foi possível observar que as substâncias húmicas aquáticas não alteram a estabilidade destas nanopartículas poliméricas; no entanto, podem interagir no equilíbrio dinâmico do herbicida PQ no meio, diminuindo sua toxicidade no ambiente. O quarto capítulo trata-se de uma discussão de todo o trabalho, mostrando um cenário geral do uso destas nanopartículas para o agronegócio, bem como quais são as perspectivas para esta área em expansão. Por fim, são apresentadas as considerações finais da Tese.

CHAPTER I

ENGINEERED NANOPARTICLES AND ORGANIC MATTER: A REVIEW OF THE STATE-OF-THE-ART

NANOPARTÍCULAS ENGENHEIRADAS E MATÉRIA ORGÂNICA: UM REVISÃO DO ESTADO DA ARTE

Artigo publicado em Chemosphere (Fator de impacto 3,49):

Grillo R., Rosa A.H., Fraceto L.F. “*Engineered Nanoparticles and organic matter: a state of the art review*”.

ABSTRACT

Growth in the development and production of engineered nanoparticles (ENPs) in recent years has increased the potential for interactions of these nanomaterials with aquatic and terrestrial environments. Carefully designed studies are therefore required in order to understand the fate, transport, stability, and toxicity of nanoparticles. Natural organic matter (NOM), such as the humic substances found in water, sediment, and soil, is one of the substances capable of interacting with ENPs. This review presents the findings of studies of the interaction between ENPs and NOM, and the possible effects on nanoparticle stability and the toxicity of these materials in the environment. In addition, ENPs and NOM are utilized for many different purposes, including the removal of metals and organic compounds from effluents, and the development of new electronic sensors and other devices for the detection of active substances. Discussion is therefore provided of some of the ways in which NOM can be used in the production of nanoparticles. Although there has been an increase in the number of studies in this area, further progress is needed to improve understanding of the dynamic interactions between ENPs and NOM.

Keywords: Nanoparticles, Organic matter, Humic/fuvic substances, Nanotoxicology.

1. INTRODUCTION

The rapid development of nanotechnology has provided a basis for innovation in a wide range of fields, including health, agriculture, food, transport, energy, electronics, and communications, resulting in a significant increase in the development of novel nanomaterials (Roco, 2011; Mangematin and Walsh, 2012). According to the European Committee for Standardization, nanomaterials are defined as materials with any external dimension at the nanoscale, or that possess nanoscale internal or surface structures. Nanoscale describes the size range from approximately 1 to 100 nm (ISO/TS 27687:2008) (Lövestam et al., 2010). Other definitions of nanomaterials can also be found in the literature (Lövestam et al., 2010), and in some cases nanomaterials have not been defined directly in terms of a size range, but rather as nanometric-scale materials whose characteristics differ from those of the corresponding bulk material (Maynard, 2011).

Nanoparticles (NP), which are now amongst the most widely studied nanomaterials, possess external dimensions on the order of 100 nm or less (Lövestam et al., 2010). NP can be spherical, tubular, or irregularly shaped, and can exist in fused, aggregated, or agglomerated forms (Nowack and Bucheli, 2007). They are of two main types: non-engineered and engineered. Non-engineered nanoparticles present in the environment derive from natural events such as terrestrial dust storms, erosion, volcanic eruptions, and forest fires (Nowack and Bucheli, 2007; Cupaioli et al., 2014). Engineered nanoparticles (ENPs) are intentionally produced by man using many different materials, such as metals (including Ag, Zn, Au, Ni, Fe, and Cu) (Xu et al., 2012), metal oxides (TiO_2, Fe_3O_4, SiO_2, CeO_2, and Al_2O_3) (Bozon-Verduraz et al., 2009), nonmetals (silica and quantum dots) (Probst et al., 2013), carbon (nanotubes and fullerene) (Isaacson et al., 2009; Ma et al., 2010), polymers (alginate, chitosan, hydroxymethylcellulose, polyhydroxyalkanoates, and poly-ε-caprolactone) (Rao and Geckeler, 2011; Paques et al., 2014), and lipids (soybean lecithin and stearic acid) (Wang et al., 2012; Kumar and Sawant, 2013).

Many ENPs have been developed for use in the area of medicine (Irache et al., 2011; Etheridge et al., 2013), as well as in agriculture (Campos et al., 2014), where they can increase the efficiency and productivity of cultivations (Grillo et al., 2012; Khot et al., 2012; Kah et al., 2013; Grillo et al., 2014a; Kah and Hofmann, 2014; Pereira et al., 2014).

Although the use of ENPs can be beneficial in diverse areas including industry, medicine, and electronics, it is still necessary to evaluate the environmental risks of these nanomaterials, considering their behavior and possible toxicity in different environmental systems (Handy et al., 2008; Bystrzejewska-Piotrowska et al., 2009; Baalousha and Lead, 2011; Dickson et al., 2012). Factors such as pH, ionic strength, light, temperature, water hardness, microorganisms, and natural organic matter (NOM) can influence the chemical and biological transformation of the nanostructures and affect microbiota and other organisms, including humans (Mohd Omar et al., 2014). Environmental exposure models have shown that soils can provide important reservoirs of these nanomaterials, especially in the presence of high concentrations of NOM (Schwabe et al., 2013).

Natural organic matter is a complex matrix formed during the microbial decomposition of plant and animal residues present in soil, groundwater, and rivers, and plays a vital role in the global carbon cycle (Matilainen et al., 2010; Derenne and Tu, 2014). NOM can be divided into two fractions. The hydrophobic (non-humic) portion contains aliphatic carbon and nitrogen, including carboxylic acids, carbon hydrates, tannic acids (TA), and proteins. The hydrophilic portion, composed of humic substances (HS), is rich in aromatic carbon, phenolic structures, and conjugated double bonds. Humic substances can be operationally classified in terms of their solubility at different pH: humic acids (HA) comprise the fraction that is insoluble at low pH; fulvic acids (FA) are low molecular weight compounds that are soluble over a wide pH range; and humin is the fraction that is insoluble at any pH (Wandruszka, 2000). The oxygen atoms in HS are mainly present in the form of carboxylic and phenolic groups (Senesi et al., 1995; de Oliveira et al., 2007; Rocha et al., 2009) that can play an important role in creating binding sites for certain inorganic species (such as soft and polarizable metal cations) and trace organic compounds (including pesticides and endocrine disruptors) present in the environment (Martin-Neto et al., 1994; Rosa et al., 2007; De Moraes and Rezende, 2008). Due to the many different origins of NOM, the composition of this material shows substantial geographical and seasonal variability (van den Berg et al., 2012; Ritson et al., 2014).

Many studies have demonstrated the interaction of metals and organic compounds with NOM, although there has been considerable debate concerning the mechanisms involved, largely because the molecular structures of the components of NOM remain poorly understood (Santschi et al., 1999; Ellerbrock and Gerke, 2013). Senesi and Loffredo (2005) showed that metal ions can form two main types of complexes with HS: inner-sphere complexes result from the formation of covalent-type bonds between the ligand and the metal, while outer-sphere complexes are produced by electrostatic interaction between the ligands (Senesi and Loffredo, 2005). It has been shown that the interaction of HA with organic compounds such as s-triazine and bipyridylium occurs due to ionic bonding, charge transfer, and hydrogen bonds (Senesi et al., 1995). Pignatello (2012) showed that the interactions (adsorption) between different molecules and NOM can be described in three ways, based on: i) the affinity for specific microdomains within the NOM, with attraction due to different functional groups; ii) strong interactions of functional groups, such as hydrogen bonding; and iii) the thermodynamic physical state of the NOM. The interactions of NOM with ENPs can differ from those involving soil minerals and organic compounds, due to the differences in structural and surface features (Tang et al., 2014).

The numbers of studies that have investigated the interaction between ENPs and NOM are illustrated in Figure 1. The data employed were derived from ISI Web of Knowledge searches using different combinations of keywords. It can be seen that a growing number of papers have been published (especially using the keywords “nano” and “natural organic matter” as search parameters). This growth offers the opportunity for new discussions to improve understanding of the interactions between ENPs and the environment. The purpose of the present review is therefore to describe studies that have reported on the interaction between ENPs and NOM (mainly in the form of humic substances), as well as to suggest possible applications and future developments in this promising field of research. Studies considered were those that involved the use of the interaction of humic materials (humic acids and fulvic acids) with ENPs in order to control the stability and toxicity of the particles, as well as to remove toxic substances from effluents, amongst other applications.

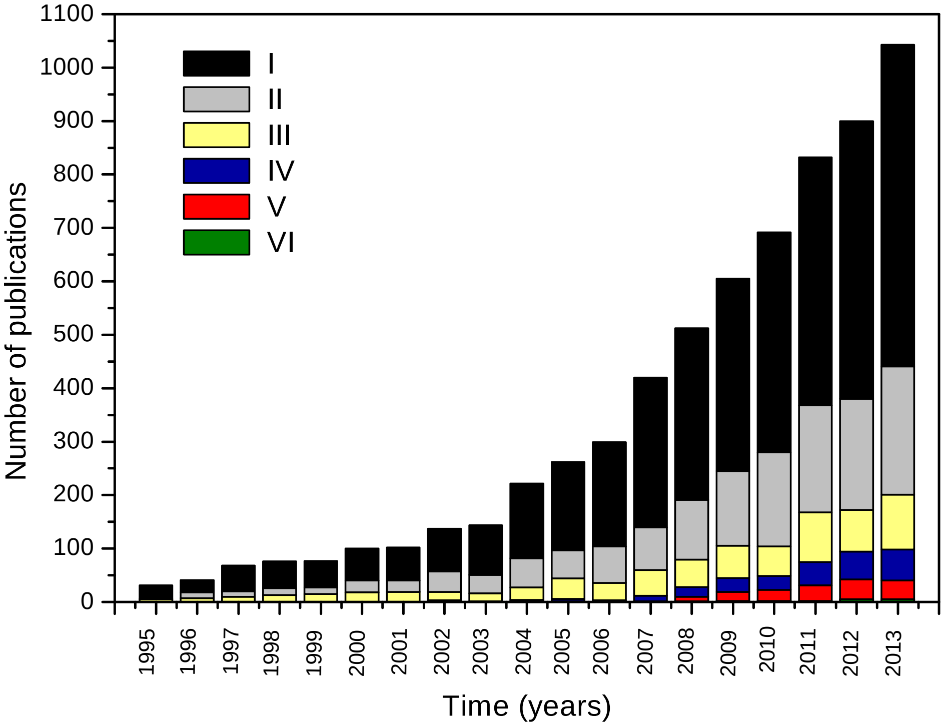

Figure 1. Numbers of papers published annually concerning the interaction between nanoparticles and humic substances. The Roman numerals indicate the different search terms used in the ISI Web of Knowledge database: I - Nano* and Organic Matter; II - Nano* and Natural Organic Matter; III - Nano* and Humic Substances; IV - Engineered Nano* and Organic Matter; V - Engineered Nano* and Natural Organic Matter; VI - Engineered Nano* and Humic Substances.

2. Types of studies concerning NPs and NOM

The interaction between ENPs and NOM (mainly in the form of humic substances) has been investigated for a variety of different purposes. NOM has been used as a stabilizer during nanoparticle production (Akaighe et al., 2011; Akaighe et al., 2012; Litvin et al., 2012; Litvin and Minaev, 2013; Cunha et al., 2014), for control of the stability of nanoparticles in the environment (Baalousha et al., 2008; Baalousha, 2009; Baalousha et al., 2013; Mohd Omar et al., 2014), for control of toxicity (Van Hoecke et al., 2011a; Van Hoecke et al., 2011b; Gao et al., 2012; Kim et al., 2013), and for remediation of media contaminated with heavy metals (Liu et al., 2008; Wang et al., 2011; Mert et al., 2013; Tang et al., 2014) or organic compounds (Lu et al., 2009; Zhang et al., 2009b; Niu et al., 2011; Peng et al., 2012; Zhang et al., 2013c). The interaction between NPs and HS can also be used in the detection of herbicides (Dubas and Pimpan, 2008) and organic matter (Gatselou et al., 2014), in the production of more efficient optical sensors (dos Santos et al., 2005; Alvarez-Puebla et al., 2007), and in the photocatalysis of drugs (Haroune et al., 2014). These different applications will be considered in greater depth in the subsequent sections of this review. An important point to note is that the studies published to date only report the interaction of organic matter with inorganic NPs.

2.1. Preparation of nanoparticles in the presence of NOM

ENPs can be synthesized using many materials and routes, employing a variety of physical or chemical methods, depending on the application and the properties desired (Rao and Geckeler, 2011; Ladj et al., 2013). Metallic nanoparticles are generally produced chemically by the reduction of metal ions, which requires the use of stabilization agents such as anionic (citrate and tannic acid), neutral (2-[2-(2-mercaptoethoxy)ethoxy]ethanol and polyvinylpyrrolidone), and cationic species (mercaptopentyl(trimethylammonium)) in order to control aggregation (Chen and Liu, 2011). Several studies have used HS to prepare metallic NP; this procedure is especially attractive because the HS can act as both reducing agents as well as stabilizers of the colloidal system (Akaighe et al., 2011; Akaighe et al., 2012; Litvin et al., 2012; Litvin and Minaev, 2013; Cunha et al., 2014). HS have been used as stabilization agents for NP of silver (Litvin et al., 2012; Litvin and Minaev, 2013; Akaighe et al., 2011; Akaighe et al., 2012), alpha-alumina (Al_2O_3) (Cunha et al., 2014), iron oxide (Zhang et al., 2013c), and gold (dos Santos et al., 2005; Alvarez-Puebla et al., 2007; Baigorri et al., 2008; Litvin and Minaev, 2014). Synthesized ENPs normally present spherical morphology, good colloidal stability, and average diameters between 1 and 60 nm when analyzed by scanning or transmission electron microscopy (Akaighe et al., 2011; Akaighe et al., 2012; Litvin et al., 2012; Litvin and Minaev, 2013; Cunha et al., 2014).

Litvin and colleagues (2012) demonstrated the use of three synthetic fulvic acids (synthesized from pyrocatechol, hematoxylin, and urotropin) as reducing agents and stabilizers during the preparation of silver nanoparticles. The formation mechanism was proposed to depend on: i) the presence of carbonyl, carboxyl, and phenolic groups in HS residues adsorbed on the surfaces of silver nanoparticles under formation, enabling the silver atoms to be reduced from Ag^+ to Ag^o and ii) an alkaline pH, with the hydroxyl and carboxyl groups of the HS adsorbed on the particles being ionized so that the negative charges on the surface increased repulsion between the particles, resulting in greater stability of the systems in an aqueous medium. These metallic Ag NPs showed antimicrobial and antifungal activity, probably due to the release of Ag^+ ions to the medium (Lapresta-Fernandez et al., 2012).

The use of HS as reducing agents and stabilizers during the preparation of nanoparticles is attractive due to their ability to enhance colloidal stability (see Section 2.2). Use of HS also avoids the need for the toxic reagents used in other procedures for the preparation of ENPs (Cunha et al., 2014). Nonetheless, there are a number of problems that need to be considered when using NOM (such as HA, HS, and FA) during nanoparticle synthesis. These include the difficulty and cost of extracting large quantities of NOM for use on an industrial scale (Bagheri et al., 2007); a lack of reproducibility in the production of NOM, given the disparate origins of the material and consequent variations in physicochemical characteristics associated with different source materials, locations, seasonal factors, and so on (van den Berg et al., 2012; Ritson et al., 2014); and difficulty in characterizing NOM (Frazier et al., 2003; Derenne and Tu, 2014). There are also issues related to the likelihood of environmental impacts resulting from the extraction of NOM in sufficient quantities for use on an industrial scale.

2.2. Stability of the nanoparticles

The majority of the inorganic nanoparticles synthesized are employed in catalysis, electronic applications, textile treatment, sensors, cosmetics, pharmaceutical equipment, and effluent remediation, amongst other uses (Mohd Omar et al., 2014; Sharma et al., 2014). Due to the substantial growth in nanotechnology, these NPs increasingly find their way into aquatic and terrestrial environments. Here, the possibility of environmental impacts is affected by the extent of particle aggregation, which determines the sedimentation rate and the mobility of the nanomaterials in the environment (Baalousha, 2009; Maurer-Jones et al., 2013). In the case of sedimentation of NPs, transport in the water column is reduced, while local exposure to nanomaterials can increase. Conversely, when the mobility of nanoparticles is increased, prolonged suspension in aquatic systems can lead to the transport of these nanomaterials over considerable distances (Baalousha et al., 2008).

Studies have shown that inorganic NPs have a strong tendency to agglomerate, and that to increase the stability of the dispersions it is essential to modify the surfaces of the particles using surfactants or other compounds that act to increase repulsion between the particles (Kango et al., 2013). In addition to the effect of ionic strength, the presence of NOM (~1-30 mg of C per liter) (Maurer-Jones et al., 2013) provides a means of controlling the state of aggregation of the nanoparticles by forming a charged external interaction with the nanoparticle surface that improves the steric stability of the system, hence influencing the mobility of the particles in the environment (Johnson et al., 2009).

Table 1 lists studies that have reported the stabilization of ENPs by the presence of NOM and its components (HS, HA, and FA), together with the main findings concerning the interactions responsible for the stabilization. NOM can stabilize ENPs by the formation of a coating that is often patchy (Gibson et al., 2007) due to variability in the concentrations of NOM and NPs in the medium, which can be reflected in different types of coating (Baalousha and Lead, 2011). The coating mechanism can involve a complex combination of electrostatic forces, van der Waals forces, and steric effects between the NOM and the surfaces of the ENPs (Baalousha et al., 2008; Diegoli et al., 2008; Baalousha, 2009; Baalousha and Lead, 2011; Li and Chen, 2012). However, few studies have provided an in-depth discussion of the mechanisms of the interactions at the surfaces of nanoparticles. The existence of patchy coatings has been speculatively attributed to interactions due to anisotropic effects (dipolar or quadrupolar) or spatial differences in the chemical composition of the particle surface (Hermans et al., 2009; Tartaglia and Sciortino, 2010).

Table 1. Studies describing the interaction between ENPs and NOM for different types of nanoparticles (silver, gold, zinc, titanium dioxide, iron oxide, cerium oxide, aluminum oxide, magnetite, fullerene, and carbon nanotubes). The types of organic materials employed are described, together with the ranges of concentrations, pH, and the main results obtained in each study, including interactions observed between the nanoparticles and the organic matter.

Nanoparticles	NOM types	[NOM] and pH	Effect of NOM on nanoparticles	Type of interaction / effect	References
Silver (Ag)	SRFA, SRHA, and PHA (IHSS, Atlanta, GA)	10 mg/L pH = 7	NOM diminished aggregation and sedimentation of NPs in the presence of sodium and low concentrations of calcium.	Steric and charge stability	Cumberland and Lead, 2009
	Fresh waters (NIST, Gaithersburg, MD)	10.0 mg/L pH = n.a.	NPs exposed to a low salinity solution and the presence of NOM showed greater colloidal stability, which modified the behavior of NPs in different locations.	Steric stability	Chinnapongse et al., 2011
	Lake Hohloh, (Black Forest, Germany)	17.8 mg C/L pH = 7.8-8.7	NOM influenced the stability and the surface properties of NPs in the aqueous phase. NPs exposed to high ionic strength (Na^+ and Ca^{2+}) were more stable in the presence of NOM.	Steric stability	Delay et al., 2011
	SRFA (IHSS, Atlanta, GA)	5 mg/L pH = 7.0	SRFA increased the stability of NPs in the aqueous phase, and stability was maintained in the presence of different electrolytes (NaCl, $NaNO_3$, Na_2SO_4, $CaCl_2$, $Ca(NO_3)_2$, $CaSO_4$, $MgCl_2$, and $MgSO_4$).	Steric stability	Baalousha et al., 2013
Gold (Au)	HA (Sigma-Aldrich, MO, USA)	2-8 mg/L pH = n.a.	Interaction between HA and NPs coated with two stabilization agents (β-D-glucose and citrate) indicated two different morphologies of the complexation with the NPs.	n.a.	Pallem et al., 2009
	FA (Acros Organics, Geel, Belgium)	25, 100, and 250 mg/L pH = 5-11	The size and morphology of NPs could be controlled by varying the pH and the concentration of FA in colloidal solution. The addition of FA increased the size of the NPs.	Interactions between FA and gold NPs favored thermodynamically	dos Santos et al., 2005
	SRHA (IHSS, Atlanta, GA)	10 mg/L pH = 1.5-13	High concentrations of ions (0.1 M) caused aggregation of the NPs. SRHA improved the stability of the NPs at extreme pH and at concentrations of ions <0.01 M. The results showed that the NPs could present different behaviors in surface and subterranean waters.	Steric stability	Diegoli et al., 2008
	SRHA (IHSS, Atlanta, GA)	5 mg C/L pH = 5-6	Use of different capping agents to prepare NPs did not affect their mobility in water containing NOM. No alteration in the colloidal stability of the NPs was observed in the presence of SRHA. However, high concentrations of divalent cations destabilized the NPs, even in the presence of NOM.	Steric stability	Stankus et al., 2011
Zinc (Zn)	SRNOM (IHSS, Atlanta, GA)	0-100 mg/L pH = 8-11	Two types of NPs with different morphologies (spherical and irregular) were submitted to different conditions of ionic strength, pH, and NOM. The presence of NOM stabilized the colloidal system (above 10 mg/L for spherical NPs and above 1 mg/L for irregular NPs), showing that stability was dependent on morphology.	Steric repulsion for [NOM] >10 mg/L Bridging effect for [NOM] = 1 mg/L	Zhou and Keller, 2010
	SRHA (IHSS, Atlanta, GA)	1 mg C/L pH = 8	SRHA reduced the amount of NPs retained on a porous medium (at concentrations below 1 mg/L) and reduced deposition of NPs on the collection surface of the column.	Electrostatic force and steric repulsion	Jiang et al., 2012
	SRHA (IHSS, Atlanta, GA)	0.02-0.5 mg/L. pH = 3, 4, and 12	Stabilization of NPs was dependent on the pH of the solution, the concentration of SRHA, and the physicochemical properties of the SRHA. The system was stabilized by SRHA, and instability was only observed in two circumstances: when the SRHA concentration was low (0.05 mg/L) and when the NPs were at their isoelectric point.	Electrostatic attractive and repulsive interactions, van der Waals and steric stability	Mohd Omar et al., 2014
Titanium oxide (TiO_2)	SRHA (IHSS, Atlanta, GA)	10 mg/L pH = 3, 5, and 9	The presence of SRHA increased the stability of the NPs and hindered their deposition onto a silica surface. The findings had important implications for the mobility of these NPs.	Electrostatic force and steric repulsion	Thio et al., 2011
	SRHA (IHSS, Atlanta, GA) and alginate (Sigma-Aldrich)	100 mg/L pH = 2-11	The stability of the NPs was dependent on the pH of the medium, the concentration of environmental NOM compounds, and the equilibrium time. The findings indicated that typical environmental concentrations of NOM are sufficient to stabilize NPs, even at elevated concentrations of NOM (50 mg/L).	Electrostatic force, steric repulsion, van der Waals and steric stability	Loosli et al., 2013
	SRFA (IHSS, Atlanta, GA)	0.2-5 mg/L pH = 2-8	FA altered the size and zeta potential of the NPs, and caused the disaggregation of small aggregates of NPs, suggesting that the dispersion of TiO_2 in the natural environment could be greater than expected.	Steric repulsion	Domingos et al., 2009

Titanium oxide (TiO_2)	SRHA, SRFA, and SRNOM (IHSS, Atlanta, GA)	10 mg/L pH = 4.5-9.5	Sedimentation of NPs decreased at higher concentrations of HA, following the order soil > freshwater > sediments.	Steric repulsion	Erhayem and Sohn, 2014
	SRHA (IHSS, Atlanta, GA)	30 mg/L pH = n.a.	HA improved the stability of NPs, and in the presence of light underwent photocatalytic degradation, causing a reduction of suspension stability. This demonstrated that light was an important factor affecting aggregation of these NPs.	n.a.	Yang et al., 2013a
Iron oxide (Fe_2O_3)	SRHA (IHSS, Atlanta, GA)	5 mg/L pH = 2-6	The presence of SRHA shifted the aggregation of NPs to lower pH. SRHA could coat the NPs to a thickness of up to 0.8 nm, at pH 2, depending on the SRHA concentration (0-25 mg/L).	Electrosteric stability	Baalousha et al., 2008
	SRHA (IHSS, Atlanta, GA)	10-100 mg/L pH = 1-12	The interaction of SRHA at the surfaces of NPs could increase the surface charge and stability, as well as cause shifts in the isoelectric point and aggregation to lower pH values. The SRHA also contributed to disaggregation of particles sized 5-10 μm.	Electrosteric stability	Baalousha, 2009
	HA (Acros Organics)	100 mg/L pH = 6-8	The NPs exhibited aggregation when exposed to a low ionic concentration (0.1 mM of NaCl), and remained dispersed for 168 h at the same ionic concentration when HA (100 mg/L) was added.	Steric and electrostatic repulsion	Dickson et al., 2012
	HA (Sigma-Aldrich) and SRNOM (IHSS, Atlanta, GA)	19.1 mg C/L (HA) 18.9 mg C/L (SRNOM) pH = 3-10	Carboxylate anions interacted with the surfaces of the NPs and increase the stability of the system, although after 14 days the NPs agglomerated, with greatest aggregation when SRNOM was used, after which it was difficult to disintegrate the particles.	Electrosteric and steric stability, hydrogen bonding	Chekli et al., 2013
Cerium oxide (CeO_2)	SRNOM (IHSS, Atlanta, GA) and NOM (Bihain, Belgium)	0-40 mg C/L pH = 4-10	The presence of NOM stabilized the NPs in suspension and showed that more than 88% and 41% of the initial concentration of NPs remained suspended in deionized water and algal growth medium, respectively, after 12 days.	Steric hindrance	Quik et al., 2010
	NOM (Bihain, Belgium)	0-10 mg C/L pH = 6.0-9.0	Increase in the concentration of NOM diminished the tendency for aggregation of larger NPs, but was unable to overcome strong van der Waals bonds between small NPs, which showed sedimentation.	Electrostatic repulsion	Van Hoecke et al., 2011b
	SRHA (IHSS, Atlanta, GA)	1 and 10 mg/L pH = 5.7	SRHA stabilized the NPs at concentrations of KCl up to 500 mM. Destabilization occurred at concentrations greater than 0.08 mol/L of calcium ions, due to cross-linking between Ca^{2+} and the SRHA.	Steric and bridging forces, van der Waals attraction	Li and Chen, 2012
Aluminum oxide (Al_2O_3)	HA (Amherst peat soil)	20 mg/L pH = 5 and 9	Variation in the structure of NOM strongly influenced the colloidal stability of the NPs. High polarity NOM showed a strong influence on particle aggregation, while NOM possessing aliphatic chains and low polarity reduced aggregation in the presence of Ca^{2+}, due to greater interaction with the NPs, which increased stability.	Steric stability	Ghosh et al., 2010
Magnetite (Fe_3O_4)	ESHA (IHSS, Atlanta, GA)	0-20 mg/L pH = 3-10	The presence of HA could both stabilize and destabilize the NPs, due to changes in the surface charge. HA acted in a similar way as molecules of surfactant, causing the formation of micelles. Interaction with the magnetic surfaces resulted in surface charge modifications that increased stability.	Electrostatic repulsion and steric repulsion	Hu et al., 2010
Fullerene (C_{60})	SRHA (IHSS, Atlanta, GA)	1 mg C/L pH = 7.5 and 8.5	SRHA increased the stability of C_{60} in the presence of NaCl, $MgCl_2$, and low concentrations of $CaCl_2$. An increase in electrophoretic mobility showed that the SRHA interacted rapidly with the C_{60} particles.	Steric repulsion	Chen and Elimelech, 2007
	TA (Fisher Scientific), SRFA (IHSS, Atlanta, GA), and HA (soil from Amherst, MA)	2 mg/L pH = 7	NOM with greater molar mass, lower polarity, and more hydrophobic chains showed greater interaction with C_{60}, and acted to increase the stability of the system. In the presence of $CaCl_2$, stability followed the order HA > FA > TA.	Steric stability	Mashayekhi et al., 2012
	DOM (municipal wastewater treatment plant, Japan)	0-30 mg/L pH = 3-11	Fullerenes were stable in the presence of dissolved organic matter (DOM), and stability increased in the presence of NaCl.	Steric and electrostatic stability	Yang et al., 2013b
	SRHA (IHSS, Atlanta, GA)	1 and 10 mg/L pH = 6	SRHA showed different influences on the stability of fullerenes, with or without UV irradiation in the presence of NaCl or $CaCl_2$. Photochemical transformation was strongly influenced by the HA.	Steric hindrance	Qu et al., 2010
Fullerene (C_{60})	SRHA and SRFA (IHSS, Atlanta, GA)	5-10 mg/L pH = 6	The presence of NOM had clear effects on the dispersivity and physicochemical properties of C_{60}, and the dispersion process was accelerated in the presence of light.	Steric hindrance	Li, 2009

	HA and FA (Sahan River, Ukraine)	100 mg/L pH = 6	The solubility of fullerenes in water increased between 8 and 540 times in the presence of HS, due to a reduction in surface tension that enabled the formation of micellar aggregates.	Cross-linked aromatic networks	Terashima et al., 2007
	SRHA and SRFA (IHSS, St. Paul, MN)	4-11 mg C/L pH = 3-10	SRHA was more effective than SRFA for the stabilization of fullerenes, due to its more hydrophobic structure. The effect of the NOM on stabilization was less pronounced in the presence of NaCl than in the presence of $MgSO_4$, probably due to greater aggregation in the presence of $MgSO_4$.	Steric repulsion	Zhang et al., 2013a
Nanotubes (CNTs)	SRNOM (IHSS, St. Paul, MN)	10-100 mg/L pH = 3.4	SRNOM provided better stabilization of the CNTs, compared to sodium dodecyl sulfate (SDS). This was due to the presence of aromatic fractions in the NOM, while SDS is aliphatic.	Steric and electrostatic stability	Hyung et al., 2007
	SRNOM, SRHA, SRFA, LHA, ESHA, NLHA, NLFA, WPHA, and WPFA (IHSS, St. Paul, MN)	2.5-50 mg C/L pH = 5-9	The degree of interaction of the NOM with the CNTs varied depending on the type of NOM and the proportion of aromatic carbons, and increased at lower pH and higher ionic strength.	Steric and electrostatic stability	Hyung and Kim, 2008
	SRHA (IHSS, Atlanta, GA)	2.5 mg C/L pH = 6	The presence of SRHA and other biomacromolecules significantly retarded aggregation of the CNTs in the presence of both monovalent and divalent salts.	Steric repulsion	Saleh et al., 2010
	HA (soil collected from Amherst, MA)	10 mg/L pH = 6.57-6.96	The introduction of hydrophilic moieties into the CNTs, due to interaction with the NOM, increased the affinity of the particle surfaces for water by means of hydrogen bonds.	Electrostatic repulsion and hydrophilic interactions with water molecules	Zhou et al., 2012
	SRHA (IHSS, Atlanta, GA)	1−200 mg/L pH = 7.06	The presence of HA decreased the sorption of pyrene by the CNTs. Interaction between the CNTs and pyrene occurred even in the presence of coatings on the particles. This interaction was suppressed when the addition of HA was combined with sonication.	n.a.	Zhang et al., 2012b

n.a.: not available.

The majority of studies have shown that the presence of NOM can increase the stability of ENPs, even in the presence of high concentrations of salts such as NaCl and KCl. However, this has not often been observed for divalent ions (such as Ca^{2+} and Mg^{2+}) present at levels exceeding the critical coagulation concentration (Mashayekhi et al., 2012). Divalent species can assist the formation of complexes between HA and ENPs, resulting in the production of aggregates, as shown in the Figure 2 (Chen and Elimelech, 2007).

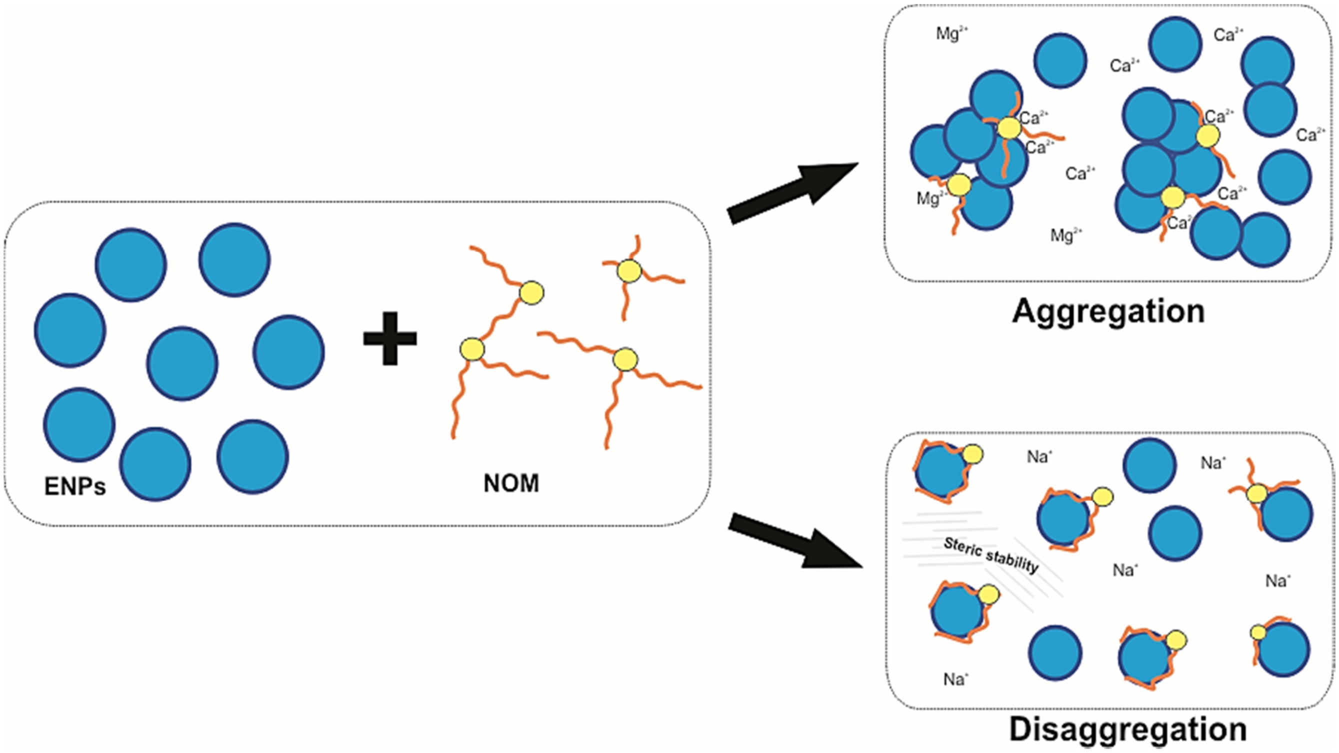

Figure 2. Schematic illustration representing the phenomena that affect the stability of NOM/ENP systems, showing the influence of bonding with mono- and divalent electrolytes.

Complex formation depends on the type of NP and the composition of the NOM (Baalousha et al., 2013). Mashayekhi (2012) suggested that calcium ions could act to diminish the extent of the diffuse double layer around aggregates of fullerene, hence reducing the energy barrier and causing rapid aggregation of the particles, even in the presence of Suwannee River fulvic acid (SRFA, Standard I, IHSS), TA (Fisher Scientific), and HA (extracted organic soil from Amherst, MA). In other words, the exposed negative charges of NOM bound to the NPs can be neutralized by divalent cations, which diminishes the electrophoretic mobility and leads to aggregation of the NPs (Zhang et al., 2009a). Other studies have reported similar aggregation of nanoparticles of boron (Liu et al., 2010), cerium oxide (Li and Chen, 2012), fullerene (Chen and Elimelech, 2007; Chae et al., 2012), multiwalled carbon nanotubes (MWNTs) (Saleh et al., 2008), and silver (Akaighe et al., 2012; Gao et al., 2012; Zhang et al., 2012a). In all these cases, aggregation of the NPs was attributed to the same mechanisms described above.

The stability of NPs can also be affected by characteristics of the NOM including concentration, hydrophobicity, molecular mass, and polarity (Hyung and Kim, 2008; Ghosh et al., 2010; Mashayekhi et al., 2012). NPs of Al_2O_3 and fullerene were found to be more stable when they were associated with HS possessing hydrophobic chemical groups, high molar mass, and low polarity (Ghosh et al., 2010; Mashayekhi et al., 2012; Zhang et al., 2013b). Mohd Omar and colleagues (2014) studied the effect of different concentrations (between 0.002 and 0.5 mg/L) of Suwannee River humic acid (SRHA, IHSS) on the aggregation and disaggregation of NPs of ZnO, as a function of pH. It was observed that particle aggregation occurred in only two cases: i) when the NPs were not associated with HS and were not charged (pH = point of zero charge, pH_{PZC}), and ii) at $pH < pH_{PZC}$ with low concentrations of HS (0.05 mg/L). Similar results have been reported for other metallic NPs (Fe_3O_4) (Hu et al., 2010). These findings suggest that even at typical environmental concentrations of humic acids (< 10 mg/L) (Buffle et al., 1998), it is still possible to stabilize the particles by electrostatic and steric interactions (Mohd Omar et al., 2014).

The structure and conformation of NOM can also be influenced by the solution pH, since electrostatic repulsion of the ionizable functional groups of the NOM (carboxylic and phenolic groups) can result in the particles becoming more flexible and expanded at high pH or low ionic concentrations, and more rigid and compact at low pH or high ionic concentrations (dos Santos et al., 2005; Zhang et al., 2013b). Diegoli and colleagues (2008) prepared gold nanoparticles stabilized with citrate and acrylate and studied their interaction with SRHA (IHSS) at different pH values. A schematic representation of the interaction showed that at first the NPs were electrostatically stabilized by the negative charges of the citrate anions, while a decrease in the pH of the suspension resulted in the protonation of citrate and aggregation of the particles. When SRHA was added to the system, there was partial substitution of the citrate anions by the SRHA polyanions at low pH, with protonation of the phenolic and carboxylic groups of the SRHA, which hindered aggregation and improved the steric stability of the particles (Diegoli et al., 2008).

The parameters used to describe the stability of NPs are measured using techniques to evaluate particle size (microscopy), hydrodynamic diameter (DLS, FIFF, FCS), surface charge or zeta potential (electrophoretic mobility analysis), aggregation/disaggregation (microscopy, DLS, and spectrophotometry), and chemical interactions (DSC, FTIR, XRD, and separation techniques such as SFS, ICP, HPSEC, and HPLC) (Baalousha and Lead, 2011; Chekli et al., 2013). Figure 3 provides a schematic illustration of some of the main techniques used to characterize the interactions of ENPs with NOM.

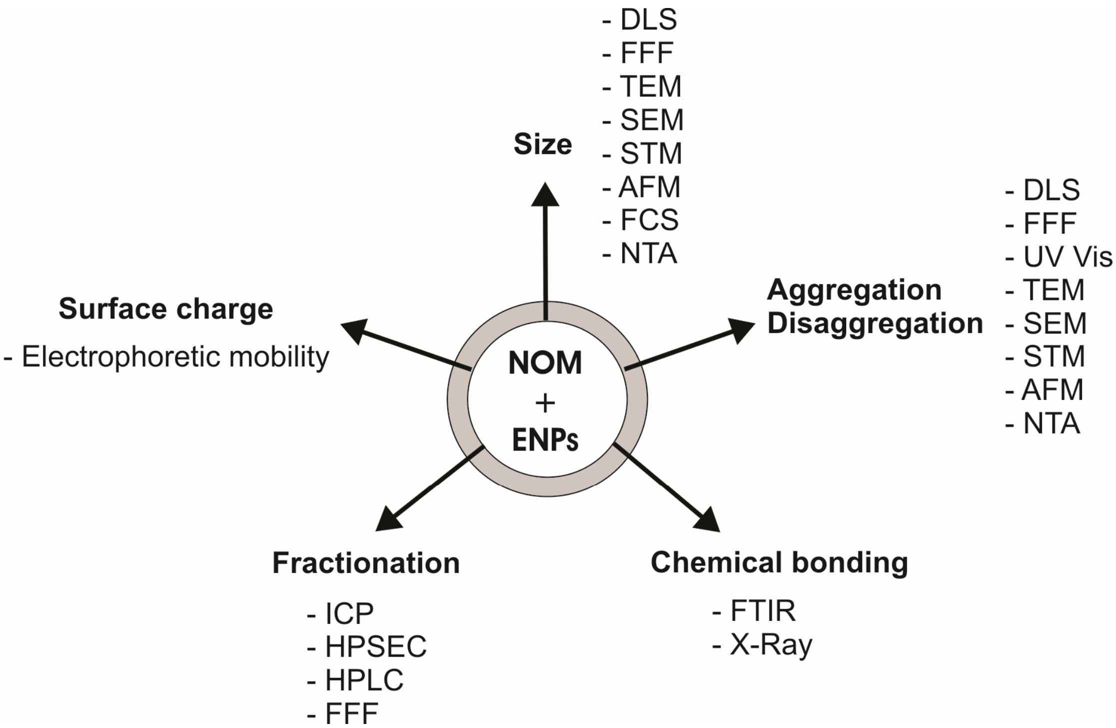

Figure 3. Principal methods used to characterize the interactions between NOM and ENPs, including techniques to study nanoparticle size, surface charge, and aggregation and disaggregation, as well as methods to determine fractionation between species and alterations in chemical bonding due to the interaction between ENPs and NOM.

2.3. Toxicity of nanoparticles in the presence of humic substances

The numbers of studies concerning the toxicity of ENPs have increased exponentially in recent years and nanomaterials have been identified as potential causative agents of toxicity in microorganisms, invertebrates, and vertebrates (Handy et al., 2008; Kahru and Dubourguier, 2010; Clemente et al., 2012; Levard et al., 2012; Bondarenko et al., 2013; Maurer-Jones et al., 2013). As described above, NOM can increase the stability of nanoparticle suspensions, hence extending the residence times of these nanomaterials in the water column, increasing their transfer to aquatic environments in runoff, and increasing the exposure of aquatic biota, including benthic organisms (Yang et al., 2013b; Mohd Omar et al., 2014). Several studies have reported on the eco-toxicological effects of the addition of NOM to ENPs. A summary of the observed impacts is provided in Table 2.

Table 2. Toxicity of different nanoparticles (silver, copper, fullerene, titanium dioxide, silicon dioxide, cerium oxide, zinc oxide, zerovalent iron, and carbon nanotubes) and NOM in different organisms (bacteria, algae, microcrustaceans, and fish).

Nanoparticle	Organism	Findings	References
Silver	*Ceriodaphnia dubia*	Higher NOM concentrations reduced the toxicity to the micro-crustaceans.	Gao et al., 2009; Gao et al., 2012
	MetPLATE bioassay	Higher NOM concentrations reduced the toxicity to the organisms tested.	Gao et al., 2009
	Daphnia	Nanoparticles (50 mg/L and pH 7) caused increased toxicity to *Daphnia* in the presence of greater quantities of NOM.	Gao et al., 2012
	Japanese medaka embryos	A higher concentration of NOM reduced toxicity to these embryos.	Kim et al., 2013
	Natural aquatic bacteria	The presence of HS reduced the cellular viability of the bacteria, and the effect was more pronounced with terrestrial HS than with aquatic HS.	Dasari and Hwang, 2010
	Escherichia coli and Bacillus subtilis	The addition of NOM had no significant effect on bacterial activity. Bacterial activity was reduced after addition of calcium (Ca^{2+}).	Liu et al., 2013b
	Pseudomonas putida	NP interfered in the production of biofilms by the bacteria. When FA was added together with the NPs, there was increased absorption and bioaccumulation of the nanomaterial in the biofilms, causing a long-term effect.	Fabrega et al., 2009b
	Pseudomonas fluorescens	NOM increased the bactericidal activity of silver nanoparticles.	Fabrega et al., 2009a

Copper	*Ceriodaphnia dubia*	A higher NOM concentration reduced toxicity to the microcrustaceans.	Gao et al., 2009
	MetPLATE bioassay	A higher concentration of NOM increased the toxicity to the organisms tested.	Gao et al., 2009
Copper	*Escherichia coli*	The toxicity of copper nanoparticles was reduced in the presence of 5-40 mg/L of fulvic acids.	Zhao et al., 2013
Fullerene	*Ceriodaphnia dubia and* MetPLATE bioassay	For both organisms, fullerene showed no toxicity when the concentration of NOM exceeded 3 mg/L.	Gao et al., 2009
	Escherichia coli	The antibacterial activity of fullerene was reduced at higher NOM concentrations.	Li et al., 2008
TiO_2	*Chlorella sp.*	The presence of NOM attenuated the toxicity to the algae.	Lin et al., 2012
	Zebrafish (*Danio rerio*)	The toxicity of TiO_2 nanoparticles increased in the presence of humic acids.	Yang et al., 2013a
SiO_2	*Pseudokirchneriella subcapitata*	The addition of NOM in assays employing SiO_2 nanoparticles strongly reduced toxicity.	Van Hoecke et al., 2011a
CeO_2	Algae medium	The presence of NOM in the medium increased the stability of the nanoparticles, resulting in reduced sedimentation.	Quik et al., 2010
	Pseudokirchneriella subcapitata	A reduction in the toxicity of the nanoparticles was observed. This was due to their reduced bioavailability following interaction with NOM.	Van Hoecke et al., 2011b
ZnO	*Bacillus subtilis, Escherichia coli and Pseudomonas putida*	HS reduced the antibacterial activity of NPs of ZnO.	Li et al., 2011; Li et al., 2013
Zerovalent iron	*Escherichia coli*	The presence of NOM hindered the adhesion of NZVI and reduced its toxicity to the bacteria.	Li et al., 2010; Chen et al., 2011
	Bacillus subtilis	Concentrations of 20 mg/L of humic acids increased the toxicity to the bacteria.	Chen et al., 2011
Carbon Nanotubes	*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus epidermis*	HA/FA in wastewater decreased the attachment of bacteria on SWCNTs, but did not reduce the toxicity.	Kang et al., 2009

It is clear that the addition of NOM to the different ENPs tested resulted in lower toxicity to the majority of the organisms. Although the reasons for this reduction have not been fully explained, several hypotheses have been proposed: i) NOM could form complexes with the ions, hence diminishing their bioavailability in the environment (Gao et al., 2009;

Li et al., 2011; Van Hoecke et al., 2011a; Gao et al., 2012; Kim et al., 2013); ii) changes in the surface charges of the ENPs due to bonding with the NOM could modify the interactions with other compounds and/or pollutants (Romanello and de Cortalezzi, 2013); iii) NOM could act as antioxidants by reacting with reactive oxygen species (ROS), hence reducing oxidative stress, as well as provide a source of carbon for some species (Carlos et al., 2012; Lin et al., 2012); iv) NOM could provide passivation of the particle surface (Gao et al., 2012). Nonetheless, the addition of NOM to nanoparticles of TiO_2 resulted in toxicity in zebrafish (*Danio rerio*), an aquatic vertebrate that inhabits the water/sediment interface (Yang et al., 2013a), while the addition of NOM to nanoparticles of Cu caused toxicity in the MetPLATE bioassay (Gao et al., 2009). Yang and colleagues (2013) studied the effect of combining SRHA (IHSS) with TiO_2 nanoparticles on the toxicity to zebrafish, and found that the HS acted to stabilize the NPs and therefore increased the toxicity to the fish following ingestion. It was found that exposure of the organisms was altered in both the water column (affecting free-swimming fish) and at the sediment/water interface (inhabited by fish embryos).

3. OTHER APPLICATIONS

Although most of the reported studies involving ENPs and NOM concern the physicochemical stability and toxicology of these nanomaterials, several new perspectives are emerging. The affinity of NOM for ENPs can be used in the production of more sensitive optical sensors (dos Santos et al., 2005; Alvarez-Puebla et al., 2007), in the removal of heavy metals (Liu et al., 2008; Wang et al., 2011; Mert et al., 2013; Tang et al., 2014) and organic compounds (Lu et al., 2009; Zhang et al., 2009b; Niu et al., 2011; Peng et al., 2012; Zhang et al., 2013c), in the photocatalysis of pharmaceuticals (Haroune et al., 2014), and in the detection of herbicides (Dubas and Pimpan, 2008) and organic matter (Gatselou et al., 2014).

The use of nanomaterials to remove heavy metals and organic compounds from water and effluents is attractive because these materials offer a high surface area and high reactivity. NOM usually influences the bioavailability of metals due to mechanisms involving complexation, adsorption, electrostatic forces, and oxidation/reduction, all of which act to reduce the quantity of metal ions available in the environment (Tang et al., 2014). Several studies have shown the benefits of combining HS with NPs for this purpose (Zhang et al., 2009a; Tang et al., 2014). Liu and colleagues (2008) studied the efficiency of removal of heavy metals (Hg^{2+}, Pb^{2+}, Cd^{2+}, and Cu^{2+}) using metallic NPs (Fe_3O_4) coated with HA (Acros Organics, Morris Plains, NJ; Sigma-Aldrich, Steinheim, Germany), and found greater adsorption of the metals in the presence of the humic material (Liu et al., 2008). Similar findings have been reported using SiO_2 nanoparticles and FA/HA (extracted from a forest soil collected in the suburbs of Beijing, China) (Liang et al., 2011).

Haroune and colleagues (2014) investigated the effects of pH, ionic strength, and NOM (FA, TA, and HA; Sigma-Aldrich, Winston, ON, Canada) on nanoparticles of TiO_2 and ZnO used in the photocatalytic removal of the drug carbamazepine and some of its derivatives (carbamazepine epoxide, acridine, and acridone) present in aquatic environments. It was found that the presence of NOM affected drug photodegradation, depending on the compound and the type of HS used (Haroune et al., 2014). It has also been found that magnetic NPs (Fe_3O_4) coated with HS (Acros Organics, Morris Plains, NJ, USA) were able to degrade 3.4 times more of the drug sulfathiazole, compared to a conventional method using hydrogen peroxide alone (Niu et al., 2011). This system was also able to remove 98.5% of Rhodamin B present in water (Peng et al., 2012).

The association of NPs with HA can be used in the development of sensors for the detection of organic compounds. Dubas and colleagues (2008) used the association of silver nanoparticles with HA in the development of a sensor for the colorimetric detection of herbicides by UV-Vis spectroscopy. The HA (Sigma Aldrich, St. Louis, MO, USA) was used as an agent to protect and stabilize the silver nanoparticles, and as a receptor for the herbicides. The ideal HA concentration for stabilization of the silver nanoparticles was 100 mg/mL, and the biosensor was sensitive to herbicides in the concentration range 100-500 mg/L (Dubas and Pimpan, 2008). Gatselou et al. (2014) developed a simple, rapid, and highly sensitive (<1 mg/L) method for the quantification of dissolved organic matter in aquatic ecosystems, based on the degree of photoreduction of silver ions to metallic NPs at different concentrations of HA (Sigma–Aldrich, Steinheim, Germany), FA (extracted from eucalyptus leaves), and SRFA (1S101F, IHSS).

4. CONCLUSIONS

The association between ENPs and NOM results in modification of the interactions, transport, fate, and toxicity of nanomaterials. NOM (and the HS, HA, and FA fractions) acts to diminish aggregation of the NPs, which increases their stability and under some circumstances can increase or reduce their toxicity, depending on the characteristics of the environment. These modified systems can also be used in the development of sensors, as well as for the remediation of aqueous media contaminated with metals and organic compounds.

Although recent studies have provided valuable information concerning the interaction of NPs with NOM, further work in this promising area of research should consider different types of NOM (including HA, HS, and FA) and ENPs (inorganic and organic), as well as a wider range of organisms used in toxicity evaluations. New investigations are needed to elucidate the dynamics of the interactions between ENPs and NOM, as well as the mechanisms of stabilization and aggregation of these particles in the environment, taking into account their toxic effects. These studies should include consideration of the sources, composition, and concentrations of NOM. Studies of the use of NOM in the synthesis of ENPs are needed in order to demonstrate the advantages of such synthesis routes, and should provide evidence of the environmental benefits that could be derived from the use of NOM for this purpose.

5. ACKNOWLEDGMENTS

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CHAPTER II

CHITOSAN/TRIPOLYPHOSPHATE NANOPARTICLES LOADED WITH PARAQUAT HERBICIDE: AN ENVIRONMENTALLY SAFER ALTERNATIVE FOR WEED CONTROL

NANOPARTÍCULAS DE QUITOSANA/TRIPOLIFOSFATO CONTENDO O HERBICIDA PARAQUAT: UMA ALTERNATIVA SEGURA PARA O CONTROLE DE PRAGAS

Artigo publicado em Journal of Hazardous Materials (Fator de impacto 4,33):

Grillo R, Pereira E.S.P., Nishisaka C.S., Lima R., Rosa A.H., Oehlke K., Greiner R., Fraceto L.F. "*Chitosan/tripolyphosphate nanoparticles loading paraquat herbicide as safe alternative to the environment*". Journal of Hazardous Materials 278, 163-171, 2014.

ABSTRACT

Paraquat is a fast acting nonselective contact herbicide that is extensively used worldwide. However, the aqueous solubility and soil sorption of this compound can cause problems of toxicity in nontarget organisms. This work investigates the preparation and characterization of nanoparticles composed of chitosan and sodium tripolyphosphate (TPP) to produce an efficient herbicidal formulation that was less toxic and could be used for safer control of weeds in agriculture. The toxicities of the formulations were evaluated using cell culture viability assays and the *Allium cepa* chromosome aberration test. The herbicidal activity was investigated in cultivations of maize (*Zea mays*) and mustard (*Brassica* sp), and soil sorption of the nanoencapsulated herbicide was measured. The efficiency association of Paraquat with the nanoparticles was 62.6 ± 0.7%. Encapsulation of the herbicide resulted in changes in its diffusion and release as well as its sorption by soil. Cytotoxicity and genotoxicity assays showed that the nanoencapsulated herbicide was less toxic than the pure compound, indicating its potential to control weeds while at the same time reducing environmental impacts. Measurements of herbicidal activity showed that the effectiveness of Paraquat was preserved after encapsulation. It was concluded that the encapsulation of Paraquat in nanoparticles can provide a useful means of reducing adverse impacts on human health and the environment, and that the formulation therefore has potential for use in agriculture.

Keywords: Environmentally safe formulations, Herbicides, Nanoparticles, Genotoxicity.

1. INTRODUCTION

Paraquat (PQ) (1,1'-dimethyl-4,4'-bipyridinium dichloride), a bi-quaternary ammonium salt, is a fast acting nonselective contact herbicide that is normally synthesized in the form of the dichloride salt (Lock and Wilks, 2010; Tsai, 2013). It is one of the most common herbicides used worldwide. It has defoliating and desiccating properties, and can control the growth of weeds that are resistant to other herbicides such as glyphosate (Santos et al., 2013). Its mode of action is based on the inhibition of Photosystem I, by interfering with intracellular transfer of electrons (Donaldson, 2013). Paraquat is commercially in solid, liquid, and granular forms, and it can also be combined with the herbicide diquat (Donaldson, 2013).

In many countries, notably within the European Union, the use of Paraquat is restricted due to its potential to contaminate the environment and interfere with human health. In the latter case, the lung is the main organ affected and treatment is difficult (Lock and Wilks, 2010). Furthermore, the high aqueous solubility of this herbicide increases the risk of contamination of rivers and groundwater, while its affinity for clays and soil organic matter can diminish its herbicidal action as well as its biodegradation (hence increasing its persistence) (Ait Sidhouma et al., 2013). Half-lives of 16 months and up to 13 years have been reported for Paraquat adsorbed to soil under laboratory and field conditions, respectively (Rao and Davidson, 1980).

It has to be emphasized that less than 0.1% of the pesticides applied in the field actually reach their target, with the remainder being available to cause adverse effects in nontarget organisms (Kumar et al., 2014). Different materials have therefore been investigated for use with Paraquat, including hydrogels (Alemzadeh and Vossoughi, 2002; Aouada et al., 2010), clays (Zhang et al., 2006; Han et al., 2010), and polymeric nanoparticles (Silva et al., 2011), in order to reduce its soil sorption, to protect the Paraquat molecule from external factors (especially photodegradation and hydrolysis), and to minimize some of its toxic effects.

Among those materials, polymeric nanoparticles (NPs) for the modified release of pesticides has shown promising results (Silva et al., 2011; Grillo et al., 2012; Kumar et al., 2014). Chitosan is a linear copolymer that is widely used for the encapsulation of bioactive compounds due to its biocompatibility, low toxicity, low cost, and biodegradability (Muzzarelli, 2010). Amongst the various methods developed to prepare chitosan nanoparticles, ionic gelification using sodium tripolyphosphate (TPP) is attractive because the process is relatively easy to control and does not require the use of organic solvents (Agnihotri et al., 2004; Fan et al., 2012). The main objective of this research was to characterize a carrier system for Paraquat using polymeric nanoparticles composed of chitosan/TPP. The herbicide (free or encapsulated in the nanoparticles) was evaluated in terms of its soil sorption, cytotoxicity, and genotoxicity. The activity of the herbicide was investigated using cultivations of maize (*Zea mays*), and mustard (*Brassica* sp) in order to determine whether the encapsulation of Paraquat altered its herbicidal effectiveness. The overall objective was to produce an efficient herbicidal formulation that was less toxic and could be used for safer control of weeds in agriculture as well as, improving knowledge in this field.

2. EXPERIMENTAL

2.1 Materials

Paraquat dichloride, chitosan (MW: 27 kDa, degree of deacetylation: 75% - 85 %), sodium 1-hexanesulfonic acid (chemical used in HPLC mobile phase), acetic carmine and Schiff reagents were obtained from Sigma-Aldrich. Membrane filters (0.22 and 0.45 µm) and Microcon regenerated cellulose filtration units (30 kDa) were purchased from Millipore. The maize and mustard seeds were acquired from Agroceres and Topseed, respectively, and planted in Orgam Biomix substrate. The deionized water used to prepare the solutions was obtained from a Milli-Q system (Millipore). All other analytical grade reagents were obtained from local suppliers.

2.2 Preparation of the chitosan/tripolyphosphate nanoparticles

The nanoparticles were prepared by the ionic gelification technique first described by (Calvo et al., 1997a). A solution of 0.1 % chitosan (MW: 27 kDa, degree of deacetylation: 75-85 %), in 0.2 % acetic acid was maintained under magnetic agitation for 12 h at pH 4.7. A separate aqueous solution of 0.1 % TPP was prepared and refrigerated at 4 ºC. Both solutions were filtered through a membrane (0.45 µm, Millipore) to remove any aggregated or insoluble material. After preparation of these solutions, 5 mL of the TPP solution was added to 20 mL of the chitosan solution, under magnetic stirring. Thereafter the mixture was stirred for another 10 min. The resulting chitosan/TPP nanoparticles (CS/TPP) were stored in amber flasks at ambient temperature (25 ºC). Paraquat (12 mg) was incorporated into the chitosan solution prior to preparation of the nanoparticles, and the final concentration of the herbicide in the solution containing the NP was 0.48 mg/mL.

2.3 Efficiency of encapsulation of Paraquat in the nanoparticles

The amount of herbicide encapsulated in the nanoparticles was determined by the ultrafiltration/centrifugation method. The suspension of nanoparticles was centrifuged in Microcon 30 kDa regenerated cellulose ultrafiltration units, and the Paraquat in the filtrate was then quantified using high performance liquid chromatography (HPLC) (Gamisans et al., 1999; Schaffazick et al., 2003; Kilic et al., 2005). The total amounts of herbicide in the NP suspensions were determined after dilution in methanol to disintegrate the particles, followed by filtration through a 0.22 µm membrane. Paraquat was then quantified using HPLC. Paraquat quantification was performed using a Varian ProStar instrument fitted with a PS 210 pump, a PS 325 UV-VIS detector, a Metatherm oven, and an autosampler under the following analytical conditions: Gemini RP-18 column (5 µm, 150 mm x 4.6 mm, Phenomenex), mobile phase consisting of 3.5 mL of diethanolamine and 1 g of sodium 1-hexanesulfonic acid in 1 L of water (pH 2.5), flow rate of 2.3 mL/min, temperature of 35 ºC, and detector wavelength of 257 nm. The chromatograms were processes using the Galaxy Workstation software.

2.4 Physicochemical characterization of the nanoparticles

2.4.1 Physicochemical stability

The stabilities of the CS/TPP nanoparticle formulations (with and without PQ) were determined using measurements of particle size (hydrodynamic diameter), polydispersion index, and zeta potential. Data was acquired using a Zetasizer Nano ZS90 particle analyzer (Malvern Instruments, UK), operated with a fixed angle of 90º. The pH of the formulations was measured using a pH meter (Tecnal) that had been previously calibrated with pH 7 and pH 4 buffer solutions. All the samples were analyzed in triplicate, at 25 °C, after 0, 15, 30, and 60 days.

2.4.2 Atomic force microscopy (AFM) analysis

Images were acquired in tapping mode (intermittent contact mode) using a Nanoscope III AFM (Digital Instruments, CA, USA), at MTA/LNNano (Scanning Probe Microscopy Platform of the LNNano, CNPEM, Campinas, Brazil). The nanoparticle suspensions were diluted (1:200, v/v) and then deposited onto a silicon surface that had been previously cleaned and dried. Topographic images and phase images were captured at 512 × 512 pixels resolution, with a scan size of 1.5 μm × 1.5 μm, at 25 ºC and low humidity. All imaging was carried out at a scan rate of 0.5 Hz, with a resonant frequency of 300 kHz, using cantilever aluminum probes (Otespaw; spring constant ∼10 N/m^2, Bruker, CA, USA). All the images were analyzed using the Gwyddion 2.31 program.

2.5 *In vitro* assays of Paraquat release kinetics

The release kinetics assays were performed using a system containing a donor compartment separated from an acceptor compartment by a cellulose membrane with a molecular exclusion pore size of 1 kDa (Spectrapore) as showed in Figure 1. The sample Paraquat (PQ) or Paraquat loaded nanoparticles (NP:PQ) was placed in the donor compartment, and the acceptor compartment contained an aqueous solution of TPP (0.1 %). Aliquots were periodically collected from the acceptor compartment and analyzed by HPLC (Paavola et al., 1995). The experiments were performed under "sink" dilution conditions and the pH value in the sample compartment and acceptor compartment were 4.5 and 9.2 (pH from the TPP solution) respectively.

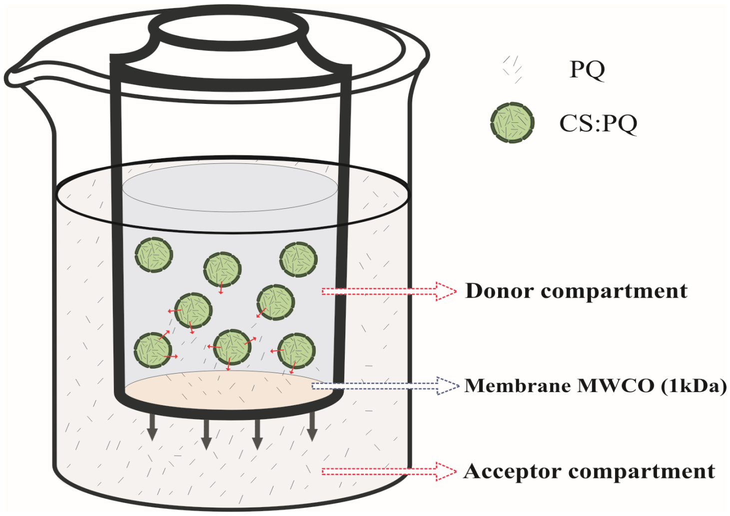


Figure 1. Schematic representation of the kinetic release experiments. This system has two compartments a donor and an acceptor separated by a membrane with a molecular weight cut-off 1 kDa. In the donor compartment was placed the active compound (free, PQ or encapsulated, CS:PQ, pH = 4,5) and in acceptor compartment was composed by 0.1% of tripolyphosphate solution (pH = 9.2) in enough amount to guarantee sink dilution condition.

As function of time 1 mL of acceptor solution was collected and quantified by HPLC and 1 mL of 0.1% of tripolyphosphate solution was replaced.

The semi-empirical Korsmeyer-Peppas model (Equation 1) was applied to the herbicide release curves in order to identify the type of mechanism involved (Costa et al., 2001):

$$\frac{M_t}{M_\infty} = Kt^n + b \qquad (1)$$

where M_t is the herbicide release at time t, M_∞ is the quantity of herbicide release at infinite time, K is a constant that considers structural and geometrical aspects of the system, and the value of the exponent (n) indicates the release mechanism. The model analysis employed the values obtained from the linear regressions for the different treatments.

2.6 Herbicide soil sorption assays

The soil sorption assays were performed using the standard equilibrium test, with the herbicide present in 0.01 mol/L $CaCl_2$. A 15 mL aliquot of solution containing the herbicide (Ho and McKay, 1999) was placed in an Erlenmeyer flask together with 1 g of soil, and maintained under constant agitation at 25 ºC. After predetermined time intervals, 1 mL aliquots were removed and centrifuged for 5 min at 2000 rpm. The supernatant was then filtered through a 0.22 μm membrane and the herbicide concentration was determined by HPLC.

Soil sorption experiments were performed in triplicate using herbicide-free soil collected in the municipality of Sorocaba, Brazil (-23.432628 ^{o}S, -47.369862 ^{o}W, 601 m altitude). The sorption at equilibrium was calculated as the difference between the concentrations measured in the initial solution and the solution in equilibrium with the soil. The sorption kinetics was evaluated using the pseudo-first order and pseudo-second order mathematical models (Ho and McKay, 1999). The model that best described the sorption was selected based on the value obtained for the linear correlation coefficient (r^2).

2.7 Toxicity assays

2.7.1 Tetrazolium reduction (MTT test)

The MTT test was used to determine the cytotoxicity of the free herbicide (PQ), the nanoparticles (NP), and the nanoparticles containing the herbicide (NP:PQ). Firstly, a 96-well culture plate was prepared with approximately 10^6 *Chinese Hamster Ovary* cells (CHO) per well and placed in a cabinet heated at 37 ºC until adherence of the cells was observed. The cultures were then treated with the formulations for 24 h, using seven different concentrations between 0.0048 and 0.12 mg/mL. Each well was then treated with 100 µL of a solution containing 5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The plate was maintained under CO_2 at 37 °C for 3 h, after which 100 µL of ethanol was added to each well in order to fix the cells. The ELISA reading was performed at a wavelength of 570 nm.

2.7.2 Allium cepa chromosome aberration assays

Allium cepa seeds were germinated in the presence of 0.38 mg/mL of PQ, either free or encapsulated in the nanoparticles, as well as in the presence of the nanoparticles alone. Water was used as the control medium. After germination, when the roots had reached a length of 2 cm they were collected and fixed using methanol/acetic acid (3:1, v/v). After fixing, the samples were immersed in 1 mol/L HCl for 9 min and then washed three times in distilled water. The roots were then incubated in Schiff reagent in the dark for 2 h. Slides were prepared by placing the root on the slide and cutting the meristematic region, which was colored purple by addition of 100 µL of acetic acid/carmine dye. A cover slip was used to gently squash and spread the cells (Pereira et al., 2014).

The slides were analyzed to determine the damage index, which considers the changes that occur during the different stages of cell growth (prophase, metaphase, anaphase, and telophase) in relation to the total number of cells in division. Each treatment was performed in triplicate, with 500 cells counted for each replicate. Statistical analysis of the results employed analysis of variance (ANOVA) and the Tukey-Kramer post-test (GraphPad InStat v.3 software).

2.8 Herbicidal activity assays

In order to evaluate the activity of the herbicide (free or associated with the NP), seeds of maize (*Zea mays*) and mustard (*Brassica* sp) were planted in plastic vases (9.3 cm high, with an upper diameter of 12.5 cm and a lower diameter of 9.3 cm) containing 600 g of plant substrate (Orgam Biomix). Ten seeds of maize or mustard were sown in each pot. A fully randomized 4 x 4 experimental design was adopted.

Twenty days after sowing, PQ solutions and the NP formulations (with and without PQ) were applied using a concentration of the active principle equivalent to 2 kg/ha (a typical concentration used in the field). The plants were photographed and removed from the vases after 48 h. Thereafter, they were washed, dried, and weighted. The results were expressed in terms of the mean and standard deviation of the mass. Statistical analysis employed analysis of variance (ANOVA) and the Tukey-Kramer post-test.

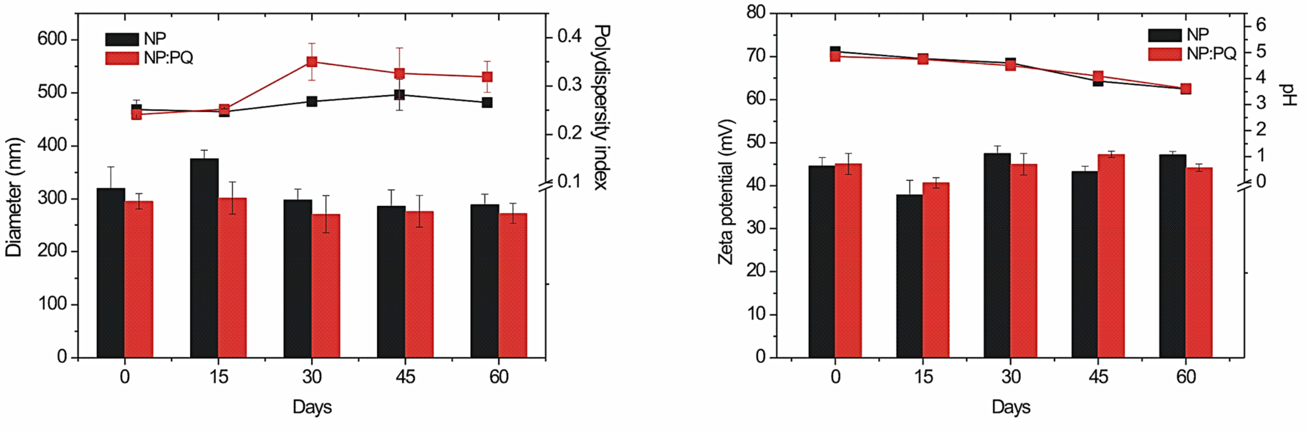
3. RESULTS AND DISCUSSION

3.1 Characterization of the nanoparticles

3.1.1 Encapsulation efficiency and physicochemical stability of the system

The encapsulation efficiency obtained for the CS/TPP nanoparticle formulation containing PQ was 62.66 ± 0.77%, indicating good affinity between the components. This interaction is the result of electrostatic forces, since PQ carries a positive charge and the TPP anion is negatively charged, which favors capture of the active principle by the reticulated network of chitosan and TPP (Fan et al., 2012). In agricultural applications, it is often desirable that a fraction of the active compound should remain unassociated with the polymeric nanoparticles, because the free agent can then provide an initial eradication of weeds, with subsequent control provided by slower release from the nanoparticles. The encapsulation efficiency obtained here indicated that these effects could be achieved using the proposed system. In previous work, Silva et al. (2011) prepared nanoparticles of alginate and chitosan, and obtained values similar to those observed in the present study.

The physicochemical stability of the CS/TPP nanoparticles was evaluated over a period of 60 days. The values obtained for the hydrodynamic diameter, polydispersion index, zeta potential, and pH are illustrated in Figure 2.



A) B)

Figure 2. Physicochemical stability of the CS/TPP nanoparticles, unloaded (NP) or containing Paraquat (NP:PQ), as a function of time (0, 15, 30 and 60 days), at 25 ºC (n = 3): A) Average diameter (bars) and polydispersion index (lines); B) Zeta potential (bars) and pH (lines).

No significant differences were observed over time, and the hydrodynamic diameter remained at around 300 nm (Figure 2 A). There was a small increase in the size of the nanoparticles without herbicide after 15 days, followed by a return to the initial value. The polydispersion index values were in the range 0.20-0.35, indicating that the nanoparticles were monodisperse and homogeneous in terms of size. The particles without herbicide showed no significant changes over time, while the particles with herbicide showed a small increase in polydispersion, which was probably due the interaction of PQ with the negative charge of the TPP. Polydispersion indices similar to those found here have been reported previously for CS/TPP nanoparticles used with different active compounds (Bulmer et al., 2012; Fan et al., 2012; Hansson et al., 2012).

The values of the zeta potential were positive due to the presence of the cationic chitosan polymer, and were around 40 mV (Figure 2 B). Nanoparticles with zeta potential greater than (±) 30 mV are considered to be stable in suspension (Mohanraj and Chen, 2006) because of the charge repulsion between the particles, which can be sufficient to inhibit aggregation and coalescence (Couvreur et al., 1995).

Another stability parameters analyzed was pH value (Guterres et al., 2010), which showed that the concentration of H^+ in the formulation increased approximately 10-fold during the course of 60 days. Some reports on literature describe the hydrolysis of chitosan to glucosamine and oligochitosans in acid medium and in presence of ions (Novikov, 2004; Zhang et al., 2009c). In our case, the decrease in pH could be due the acid hydrolysis of chitosan polymer (present in the nanoparticles) and in this way, the glucosamine and oligochitosans were released to the medium decreasing the pH.

Overall, the results of the physicochemical analysis indicated that the system was stable and could be successfully stored for 60 days at room temperature (25 ºC).

3.1.2 Atomic force microscopy (AFM)

AFM analysis of nanoparticle morphology revealed the presence of homogeneous particle populations, in agreement with the data illustrated in Figure 2. The particles were spherical (Figure 3 A) and in the phase contrast images regular halos could be seen around the particles (Figure 3 B), together with small grooves that were probably formed by the chitosan during the sample drying process. The average sizes of the nanoparticles were ~390 nm (without PQ) and ~ 420 nm (with PQ) (Figure 3 C). Observed a small increase in the average sizes of the nanoparticles measured by AFM, which can be explained for the deformation (spread) during the nanoparticles drying process.

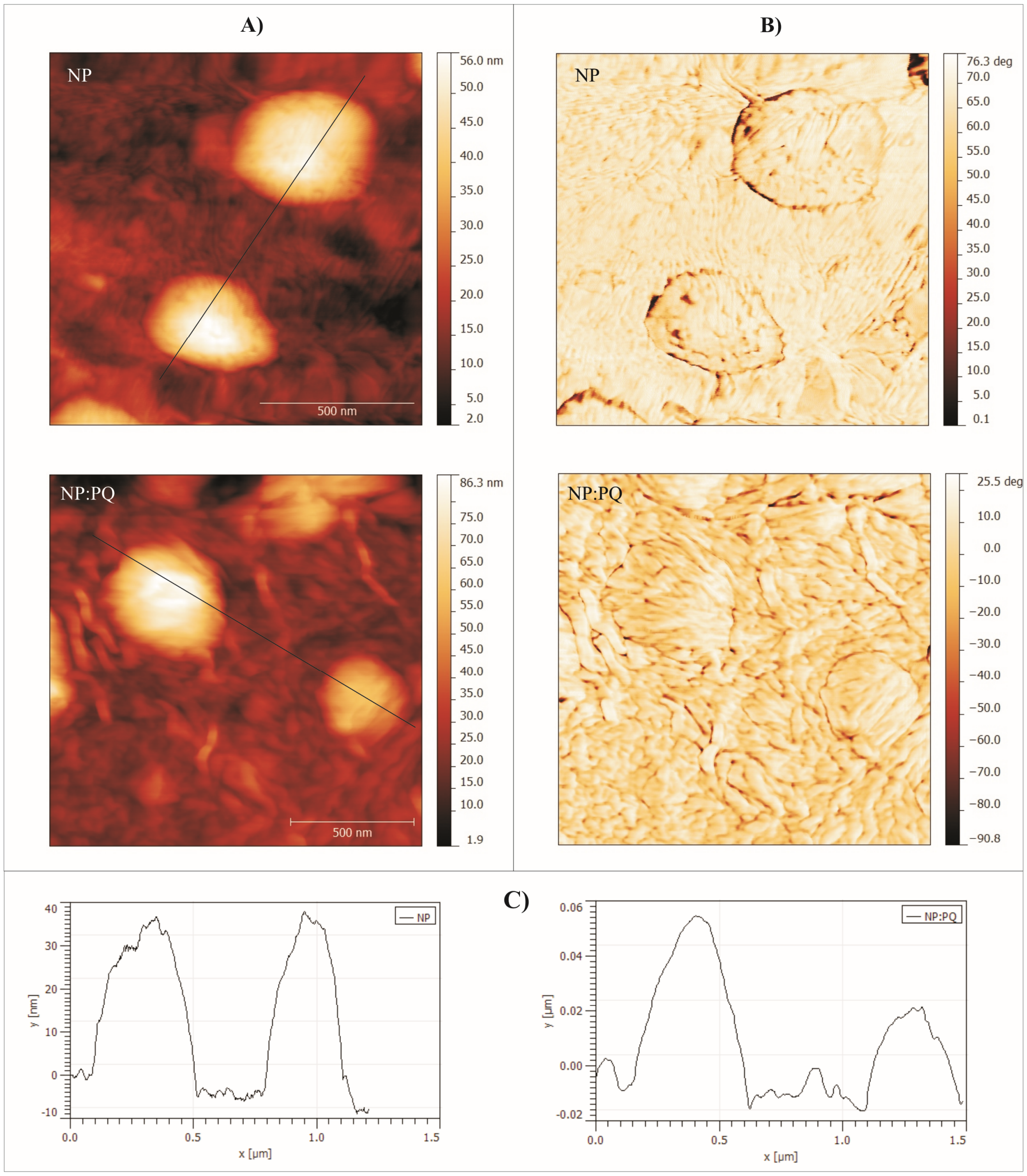
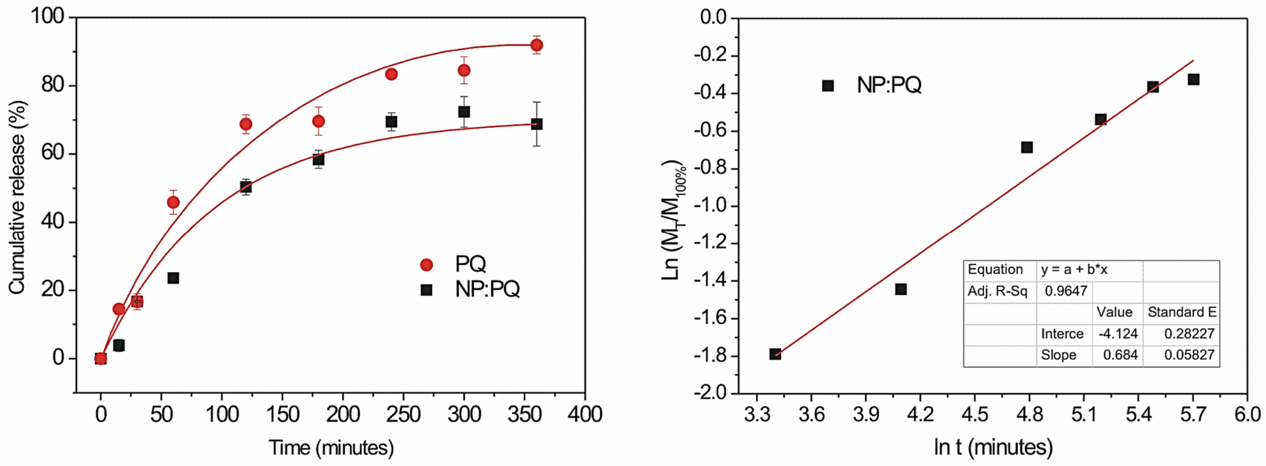


Figure 3. Atomic force microscopy analysis (1.5 μm x 1.5 μm) of CS/TPP nanoparticles without PQ (NP) and with PQ (NP:PQ): A) Topography images; B) Phase contrast images; C) Size distribution graph (bar = 500 nm). Images were acquired in tapping mode (intermittent contact mode) and captured at 512 × 512 pixels resolution, with a scan size of 1.5 μm × 1.5 μm, at 25 °C and low humidity.

3.2 Herbicide release kinetics

The release of an active agent from nanoparticles is determined by factors including the size of the particles, the molecular weight of the polymer, the nature of the reagents used, and the type of interaction (Siepmann and Peppas, 2012). Here, the release profile was evaluated by comparing the kinetics of release of PQ from the nanoparticle polymeric matrix with that of the free herbicide (Figure 4).



A) B)

Figure 4. Release kinetics of Paraquat, unloaded (PQ) or associated with the CS/TPP nanoparticles (NP:PQ), at 25 ºC and pH 4.5 (donor compartment) and pH 9.2 (acceptor compartment): A) Release profile; B) Fitting of the data using the Korsmeyer-Peppas mathematical model. The values represent the mean of three experiments (n = 3).

Under the laboratory conditions employed, 92 % of the free herbicide was accessible in the acceptor compartment after 350 min, while during the same time period 70 % of the herbicide associated with the nanoparticles was released (Figure 4 A). Thus, the release of encapsulated PQ is delayed compared to its free form. Under field conditions, accessibility of PQ is likely to be lower due to the influence of factors such as low humidity and interaction of the nanoparticles with soil organic matter, amongst others. In previous work, PQ has been encapsulated using other kind of nanoparticles, such as, alginate/chitosan nanoparticles (Silva et al., 2011) and hydrogels of polyacrylamide and methylcellulose (Aouada et al., 2010). In both cases, significant differences were observed between the diffusion and release of the free or encapsulated herbicide.

Application of the Korsmeyer-Peppas mathematical model to the release kinetics data (Figure 4 B) gave values of the release constant (*k*), the release exponent (*n*), and the linear correlation coefficient (r) of 1.99 min^{-1}, 0.68, and 0.984, respectively. The value of the release exponent, which provides an indication of the type of mechanism involved in the release process, was within the range $0.43 < n < 0.85$, indicating that the kinetics was governed by anomalous transport (a combination of diffusion and Case II transport), in other words, the diffusion and relaxation of polymeric chain might be a factor of PQ release. The same mechanism was previously identified for the release of atrazine from nanoparticles of poly(epsilon)caprolactone coated with chitosan (Grillo et al., 2013).

3.3 Soil sorption kinetics assays

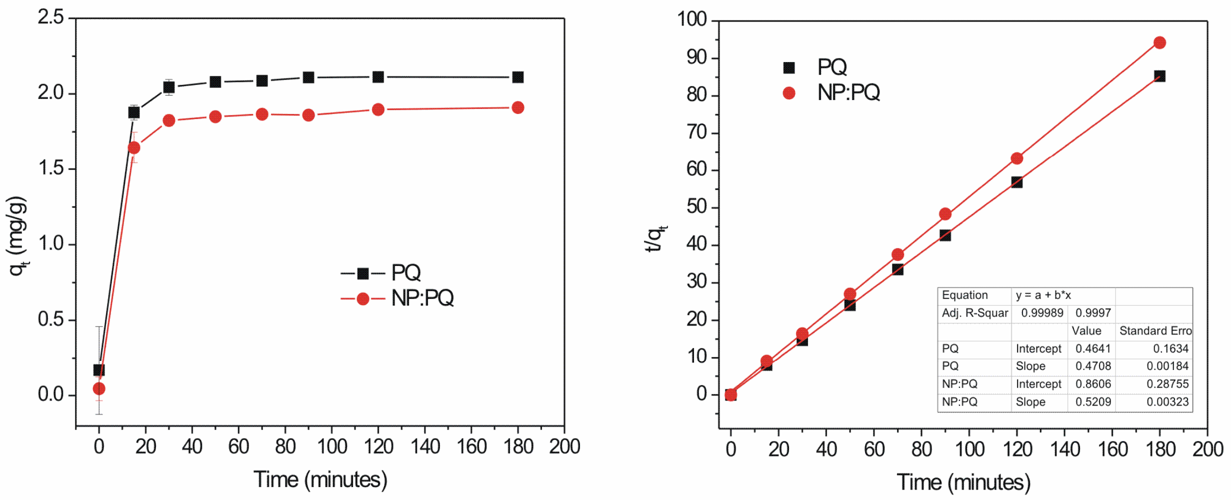
Curves of the soil sorption kinetics at 25 °C were constructed using free PQ or PQ associated with the nanoparticles. The soil used consisted of silt (3.9 %), clay (11.3 %), sand (84.8 %), and organic matter (3.2 %).

The sorption process was fast, with an equilibrium reached in ~ 30 min (Figure 5 A). This is in agreement with previous reports (Tsai et al., 2004; Muhamad et al., 2011; Leite et al., 2013; Santos et al., 2013). Comparison of the kinetics profiles obtained with and without the nanoparticles revealed a small difference, with less sorption of the herbicide when it was associated with the nanoparticles. This is an important finding because it indicates that use of the nanoparticles could help to mitigate the problem of soil sorption of PQ, leaving more of the herbicide available to target the plant.

The data were fitted using the pseudo-first order and pseudo-second order kinetic models (Figure 5 B). Table 1 lists the values obtained for the model parameters, including the linear correlation coefficient (r^2), the adsorption constant (k), the initial rate of PQ adsorption (*h*), and the maximum quantity adsorbed (q_e max).

Table 1. Use of the pseudo-first order and pseudo-second order models to fit the data for soil sorption of PQ, either free or associated with the CS/TPP nanoparticles, at 25 °C and pH 4.5.

Sample	Kinetic model	k (mg/g.min)	q_e max. (mg/g)	h (mg/g.min)	r^2
PQ	Pseudo-first order	0.066	3.942	--	0.905
	Pseudo-second order	0.477	2.124	2.155	0.999
NP:PQ	Pseudo-first order	0.035	0.724	--	0.648
	Pseudo-second order	0.315	1.919	1.161	0.999



Equation	y = a + b*x		
Adj. R-Squar	0.99989	0.9997	
		Value	Standard Erro
PQ	Intercept	0.4641	0.1634
PQ	Slope	0.4708	0.00184
NP:PQ	Intercept	0.8606	0.28755
NP:PQ	Slope	0.5209	0.00323

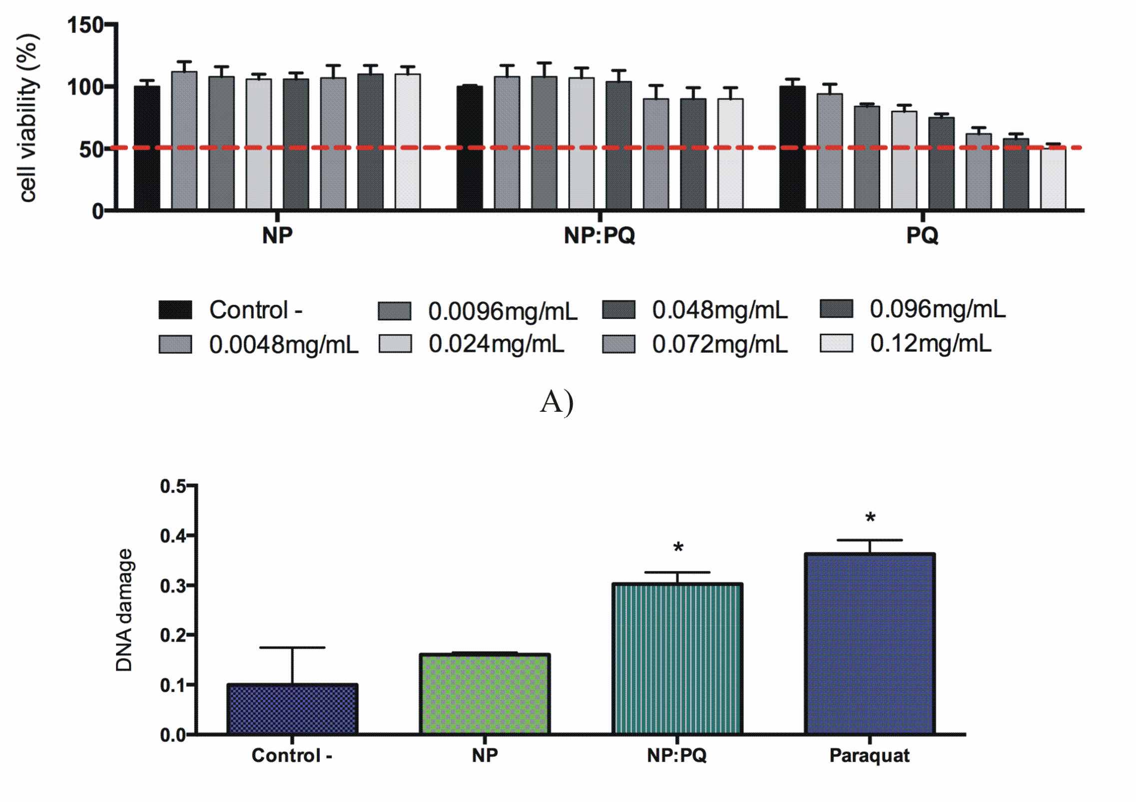
A) B)

Figure 5. Kinetics of soil sorption of Paraquat, unloaded (PQ) or associated with the CS/TPP nanoparticles (NP:PQ), at 25 ºC and pH 4.5 (n = 3): A) Adsorption profile; B) Application of the pseudo-second order mathematical model, where q_t is amount of soluted sorbate on the surface of the sorbent at any time *t* (mg/g).

The results shown in Table 1 and Figure 5 indicated that the best fit to the experimental data was provided by the pseudo-second order model. The linear correlation coefficient (r^2) values were close to 1 and the calculated maximum q_e values were close to the values obtained experimentally. This model is widely used for the fitting of sorption kinetics curves, because it is able to describe sorption by heterogeneous surfaces such as those of soil (Plazinski et al., 2009). The type of kinetics, the kinetic constant, and the PQ sorption capacity of the soil were similar to those reported previously for diatomaceous earth and modified clays (Tsai et al., 2004; Tsai and Lai, 2006). The PQ sorption process could be explained by the transfer of sorbate present in aqueous solution to active sites on the sorbent and/or by complexation and ion exchange at these sites (Silva et al., 2011).

3.4 Cytotoxicity and genotoxicity assays

For nanoparticulate systems, it is not sufficient to characterize the particles and show that they have a good association with the active compound. It is also necessary to evaluate their toxicity in order to suggest ways of reducing impacts on human health and the environment. The results of the cytotoxicity and genotoxicity assays with CHO cells and *Allium cepa* seeds are shown in Figure 6, respectively.



A)

B)

Figure 6. Assays of the toxicity of paraquat (PQ), CS/TPP nanoparticles without the herbicide (NP), and nanoparticles containing Paraquat (NP:PQ): A) Results of cytotoxicity

assays of cellular viability (MTT reduction test) using CHO cell in different concentrations of Paraquat; B) Results of *Allium cepa* genotoxicity assays using treatment for 24 h with 0.38 mg/mL of Paraquat. Statistical analysis employed analysis of variance (ANOVA) and the Tukey-Kramer post-test, ($p < 0.05$). * indicates a statistically significant difference between NP:PQ in relation to PQ.

The IC_{50} value for free PQ obtained from the cytotoxity assays was approximately 0.12 mg/mL, while the nanoparticles (with or without PQ) showed virtually no toxicity at the concentrations tested for 24 h. Comparison of NP with NP:PQ revealed a small decrease in cellular viability when PQ was associated with the nanoparticles, although the viability values remained close to 100 %, indicating a good ability of the nanoparticles to reduce the toxicity of herbicide in this CHO cell (Figure 6 A).

The genotoxicity data showed that there was increased DNA damage for all treatments, compared to the negative control (Figure 6 B). However, the alterations were not significant when unloaded nanoparticles were used. Greater damage was caused when free PQ or PQ associate with the particles was used. Nonetheless, association of the herbicide with the nanoparticles reduced the degree of damage, compared to the use of free PQ, suggesting that the particles provided a degree of protection against toxic effects. Similar results have been reported in previous studies involving the encapsulation of active compounds in microparticles and nanoparticles for use in agriculture (Grillo et al., 2012; Clemente et al., 2014).

Overall, these findings indicated that the association of PQ with the nanoparticles diminished the cytotoxicity and genotoxicity of the herbicide. The next step was to determine whether encapsulation might act to reduce the availability of the active agent and therefore be detrimental to the herbicidal activity.

3.5 Evaluation of herbicidal activity

The tests described above showed that association of PQ with the CS/TPP nanoparticles resulted in a formulation that was stable, and helped to reduce the soil sorption and toxicity of the herbicide. The herbicidal activity of the formulation was investigated using two different types of plants, one a monocotyledon (*Zea mays*) and the other a dicotyledon (*Brassica* sp). The application rate of PQ (equivalent to 2 kg/ha) was the same as that employed commercially in field crops (Norsworthy et al., 2011).

Both free and encapsulated forms of PQ showed activity in both cultivations (Figure 7), and within 48 h caused limp leaf necrosis, a characteristic effect of this herbicide (Lock and Wilks, 2010). The greatest effect was on the *Brassica* sp, with the plants showing faster necrosis. In the case of *Zea mays*, use of the nanoparticulate herbicide (NP:PQ) caused greater necrosis, probably due to better adhesion of the nanoparticles to the leaf or reduced decomposition of the active principle, which increased the effective concentration available to act on the plants. This effect was less pronounced in the case of *Brassica* sp, probably due to the greater sensitivity of the plants to the herbicide. Other work has shown that the use of chitosan can help to increase the adhesion of particles to surfaces, hence increasing the effectiveness of the associated active agent (Calvo et al., 1997b; Mazzarino et al., 2012).

The unloaded CS/TPP nanoparticles showed no effects on the plants studied, and no differences in dry plant mass were observed between plants treated with free or nanoparticulate PQ (Figure 7). The differences in mass between the plants that received the free or nanoparticulate herbicide were not pronounced. This can be explained by the fact that the tests were performed using the concentration normally used commercially (2 kg/ha), which is sufficient for complete elimination of plants, and because the action of the herbicide is extremely fast.

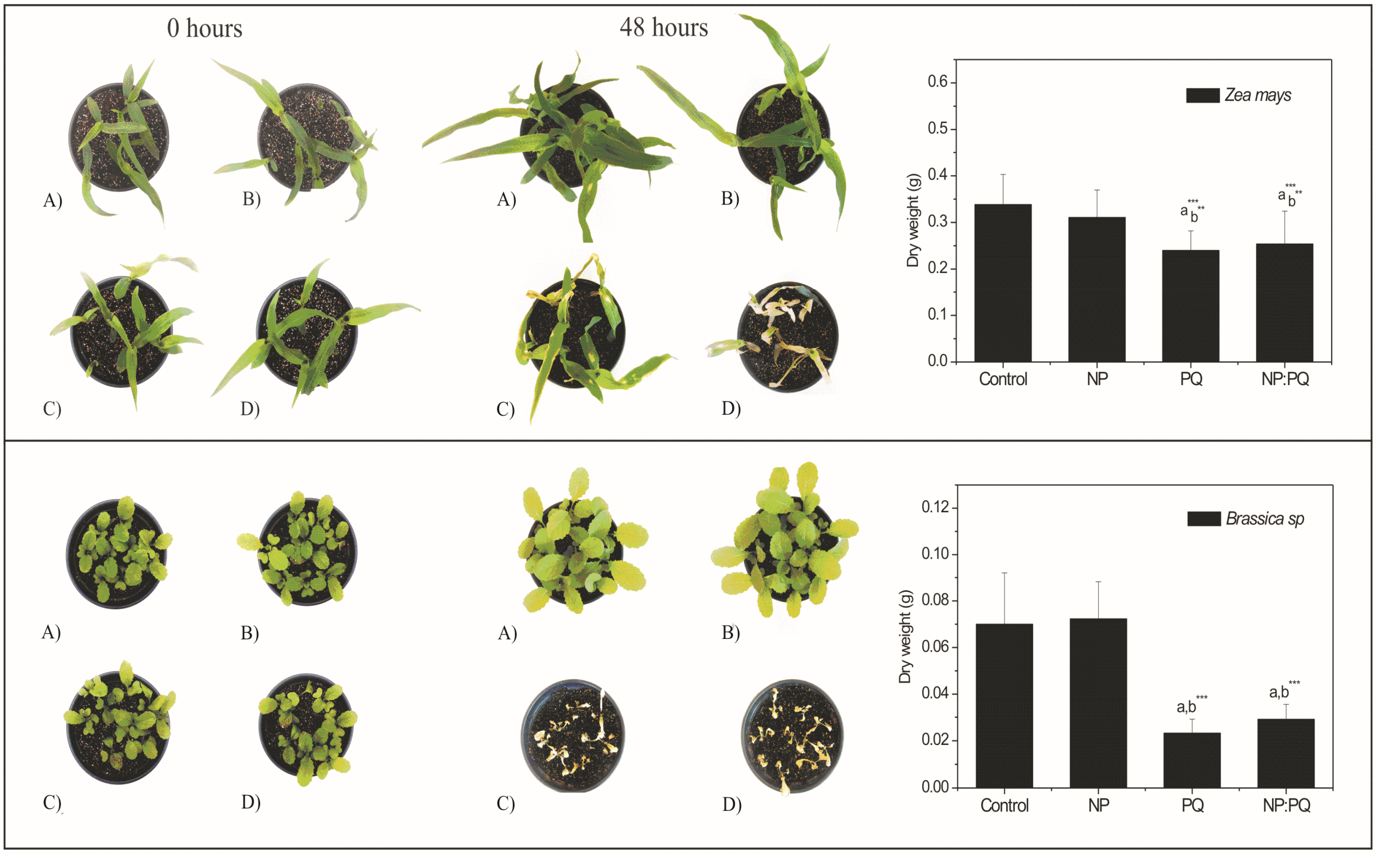


Figure 7. Evaluation of the herbicidal activity of Paraquat in cultivations of maize (*Zea mays*) and mustard (*Brassica* sp) after 48 h, and dry mass of the plants after treatment and drying: A) Control, B) unloaded CS/TPP nanoparticles (NP), C) free Paraquat (PQ), and D) nanoparticles of CS/TPP containing Paraquat (NP:PQ). Data expressed as mean ± s.d. Statistical differences refer to a – control and b – NP, with *** $p < 0.001$ and ** $p < 0.01$ (one-way ANOVA with the Tukey-Kramer post-hoc test).

Further studies are underway to investigate the use of lower herbicide concentrations in CS/TPP formulations. This should reduce the rate of action and therefore enable detailed observation of the interaction of PQ with the plant.

4. CONCLUSIONS

The results reported in this work provide important information concerning the use of nanostructured systems with the herbicide Paraquat. The CS/TPP nanoparticles showed good encapsulation efficiency (62 %) and were stable for at least 60 days. Diffusion and release of the herbicide was modified when it was associated with the nanoparticles, and soil sorption was reduced. Use of the MTT test showed that association of the herbicide with the particles reduced its cytotoxicity towards CHO cells, while *Allium cepa* assays demonstrated that the particle-bound Paraquat caused less chromosome damage, compared to the free herbicide. Herbicidal activity tests showed that the herbicide remained available following encapsulation. The findings provide insight into the ways in which this type of bioactive agent can be optimized in terms of its toxicity, stability, and application in agriculture.

5. ACKNOWLEDGMENTS

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CHAPTER III

INVESTIGATION OF THE EFFECT OF THE PRESENCE OF AQUATIC HUMIC SUBSTANCES ON THE STABILITY AND TOXICITY OF CHITOSAN/TPP NANOPARTICLES CONTAINING PARAQUAT

INVESTIGAÇÃO DO EFEITO DA PRESENÇA DE SUBSTÂNCIAS HÚMICAS AQUÁTICAS NA ESTABILIDADE E TOXICIDADE DE NANOPARTÍCULAS DE QUITOSANA/TPP CONTENDO PARAQUAT

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Grillo R., Clemente Z., Chulupe V.C., Jonsson C.M., Lima R., Sanches G., Nishisaka C.S., Oehlke K., Greiner R., Rosa, A.H., Fraceto L.F. “*Investigation of the effect of the presence of aquatic humic substances on the stability and toxicity of chitosan/tripolyphosphate nanoparticles containing paraquat*”.

ABSTRACT

There is increasing production and use of nanoparticles, which can then be released into the environment. The knowledge on stability of nanoparticles in environmental media containing humic substances is limited. Humic substances are known to interact among others with metals and organic compounds resulting in an alteration of their behavior in the environment. The objective of this work was the investigation of the effect of the presence of aquatic humic substances (AHS) on the stability and toxicity of polymeric chitosan/tripolyphosphate nanoparticles containing the herbicide Paraquat. Evaluation of the stability of the nanoparticles showed that there were no changes in hydrodynamic diameter or zeta potential in the presence of AHS, although the results of infrared was indicative of small alterations over time. Association of the nanoparticles with the AHS resulted in decreased *Allium cepa* genotoxicity, due to competition and/or binding of Paraquat with free binding sites in the humic substance molecules. The ecotoxicity assays with the alga *Pseudokirchneriella subcapitata* also showed a decreased toxicity of the herbicide when associated with the AHS. The findings of this work indicated that AHS did not alter the stability of these polymeric nanoparticles, but nevertheless affected the dynamic equilibrium of the herbicide in the medium, hence altering the toxicity of the formulation. The findings presented here provide a basis for the establishment of protocols for the commercial use of these systems.

Keywords: Polymeric nanoparticles, Natural organic matter, Herbicides, Paraquat, Toxicity.

1. INTRODUCTION

Advances in the development and application of engineered nanoparticles (ENPs) have resulted in many new nanomaterials. These substances possess unique properties due to their high surface area and chemical reactivity (Kim et al., 2012; Zanker and Schierz, 2012). ENPs can enter soil and water systems, but uncertainty remains concerning their fate and behavior in the environment (Bystrzejewska-Piotrowska et al., 2009; Kim et al., 2012; Maurer-Jones et al., 2013). There are many factors that can affect the dynamics of nanoparticles in different media, and it is necessary to understand both the physicochemical properties of the NPs as well as the modifications that can occur under environmental conditions (Kim et al., 2012; Grillo et al., 2014b).

At present, inorganic nanoparticles are the most commonly produced ENPs, and are therefore those most frequently encountered in aquatic and terrestrial environments (Bystrzejewska-Piotrowska et al., 2009; Tang et al., 2014). However, many different polymeric nanoparticles are under development, and are likely to appear in increasing quantities in the environment. Potential application of polymeric nanoparticles include the use as carriers for a variety of active compounds (Rao and Geckeler, 2011; Lopez-Serrano et al., 2014), such as drugs (Wu et al., 2011; Panyam and Labhasetwar, 2012) and agricultural chemicals (Boehm et al., 2000; Silva et al., 2011; Grillo et al., 2012; Kah et al., 2013; Kah and Hofmann, 2014; Grillo et al., 2014a). Depending on the concentration of organic matter, pH, and the ionic strength of the medium (Baalousha, 2009; Delay et al., 2011; Kim et al., 2012; Yang et al., 2013b), ENPs can undergo physicochemical alterations and the organic compounds associated with them can follow unexpected pathways in the environment

(Klaine et al., 2008). Further research in this area is therefore essential, especially considering that only a few nano-specific regulations for some specific applications of nanomaterial exist in some countries (Lövestam et al., 2010).

Humic substances (HS) are complex natural macromolecules produced in the environment from the decomposition and transformation of organic matter in soil, peat, sediments, and natural waters (Theng, 2012). They are the main natural agents responsible for complexation with metals (Senesi, 1994; dos Santos et al., 2004; Rosa et al., 2007) and organic compounds (Senesi, 1992; Botero et al., 2011). In surface waters, aquatic humic substances (AHS) account for between 30 and 50% of dissolved organic carbon (DOC). They can reach concentrations of around 20 µg L^{-1} in subterranean water and as high as 30 mg L^{-1} in surface waters (Thurman and Malcolm, 1981; Senesi, 1993; Rocha and Rosa, 2003; Rodriguez and Nunez, 2011). Some studies have found that the interaction between inorganic nanoparticles and HS can improve colloidal stability and diminish the toxicity of certain types of nanoparticles (Gao et al., 2012; Kim et al., 2013), while others have shown decreases in stability (Chen and Elimelech, 2007; Zhang et al., 2012a) and increased toxicity (Yang et al., 2013a).

In agriculture, polymeric nanoparticles can be used to produce more effective agrochemical formulations that are less toxic to the environment (Silva et al., 2011; Grillo et al., 2012; Khot et al., 2012; Kah et al., 2013; Kah and Hofmann, 2014, Grillo et al., 2014a). The purpose of the present study was the investigation of the effect of the presence of aquatic humic substances (AHS) on the stability and toxicity of polymeric chitosan/tripolyphosphate nanoparticles containing the herbicide paraquat. This study can be important to obtain information concerning the way by which AHS could interact with polymeric nanoparticles and herbicides, contributing to the development of safer systems for use in agriculture that pose less risk to the wider environment.

2. METHODOLOGY

2.1 Materials

Paraquat dichloride (PQ), chitosan (MW: 27 kDa, degree of deacetylation: 75% - 85 %), sodium 1-hexanesulfonic acid (chemical used in HPLC mobile phase), acetic carmine and Schiff reagents were obtained from Sigma-Aldrich. The deionized water used to prepare the solutions was obtained from a Milli-Q system (Millipore). All other reagents were obtained from national suppliers and were of analytical grade.

2.2 Extraction of the aquatic humic substances

The aquatic humic substances were extracted from water collected from the Sorocabinha River (24º43’31.3’’S, 47º35’42.6’’W), a tributary in the basin of the Ribeira do Iguape River, in the southeast of the State of São Paulo, Brazil in a bare land area. The pH, temperature, conductivity, and dissolved oxygen content of the water were determined *in situ*, and the total carbon content was measured using a Multi N/C 3100 instrument (Analytik Jena). Extraction of the AHS was performed by chromatographic adsorption on a gravity-fed column containing macroporous XAD7 resin (Thurman and Malcolm, 1981). A stock solution of AHS (200 mg L^{-1}) was prepared in ultrapure water and stored at 4 °C for up to 24 h prior to use.

2.3 Preparation of the chitosan/tripolyphosphate (CS/TPP) nanoparticles

The CS/TPP nanoparticles were prepared from chitosan and sodium tripolyphosphate using the ionic gelification method described by (Grillo et al., 2014a). A 0.1 % solution of chitosan in acetic acid (0.2 %), pH 4.7 was prepared and maintained under agitation for 12 h and other aqueous solution of 0.1 % TPP was prepared and kept at 4 °C. Thereafter, 5 mL of the TPP solution was added to 20 mL of chitosan solution, under magnetic agitation for 10 min, in order to obtain the nanoparticles. The CS/TPP nanoparticles were stored in amber flasks kept at room temperature (25 °C). Paraquat (12 mg) was incorporated in the chitosan solution prior to mixing the solutions, and the final concentration of the herbicide in the NPs was 0.48 mg mL^{-1}. A physicochemical characterization of these NPs has been reported previously (Grillo et al., 2014a).

2.4 Physicochemical stability of the NPs and AHS

The stability of the CS/TPP nanoparticles (with or without PQ) was evaluated in the presence of AHS. The samples containing the NPs and AHS were maintained under agitation for 12 h prior to any treatment or analysis to enable the system to reach equilibrium (Mohd Omar et al., 2014).

2.4.1 *Size and Zeta potential measurements*

Initially the pH of the nanoparticles samples was adjusted using sodium hydroxide in a range of pH values (4, 5, 6, 7, and 10), in the presence and absence of AHS (20 mg L^{-1}). In a second study, the NPs were kept at a constant pH 5, and the concentration of AHS was varied (0, 2, 5, 10, 15, and 20 mg L^{-1}). Colloidal stability was determined by size distribution and zeta potential measurements, using a Model ZS90 particle analyzer (Malvern Instruments). The data were analyzed using Origin Pro v.8 software. All the experiments were performed in triplicate (n=3).

2.4.2 Fourier Transform Infrared Spectroscopy (FTIR)

The CS/TPP nanoparticles (with and without paraquat and AHS (20 mg L^{-1}) were dried in a ventilated drying cabinet (DeLeo) at 35 °C during 6 h and then kept in a desiccator prior to analysis. An Agilent Series 660 FTIR spectrometer equipped with an attenuated total reflectance (ATR) accessory was used, with a frequency range of 400-4000 cm^{-1}, resolution of 8 cm^{-1}, and 32 scans per sample.

2.5 Toxicity evaluation of the nanoparticles combined with AHS

2.5.1 Determination of genotoxicity using the Allium cepa chromosome aberration test

Seeds of *Allium cepa* were germinated in the presence of free PQ, Paraquat associated with the nanoparticles (NP:PQ), nanoparticles (NP), aquatic humic substances (AHS), nanoparticles with humic substances (NP-AHS), and nanoparticles with Paraquat and humic substances (NP:PQ-AHS). The concentrations of PQ and AHS used were 0.38 mg mL^{-1} and 20 mg L^{-1}, respectively. Deionized water was used as a negative control. After the seeds had germinated, the roots were retrieved and fixed with methanol/acetic acid (3:1, v/v). After fixing, the samples were immersed in 1 mol L^{-1} HCl for 9 min and then washed three times with distilled water. The roots were then incubated in Schiff reagent in the dark for 2 h. The meristematic regions of the roots were placed on slides and dyed (purple color) with acetic carmine solution. Cover slips were used to carefully squash and spread the cells. After preparation, the slides were analyzed to determine the damage index. Each treatment was performed in triplicate, with 700 cells counted for each repetition. Statistical treatment of the data employed analysis of variance (ANOVA) using GraphPad Prism v. 6.0c software.

2.5.2 Determination of ecotoxicity using Pseudokirchneriella subcapitata

The alga *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum*) was obtained from cultures maintained in liquid culture medium prepared as described by Blaise (2000).

The growth inhibition assays were performed using the procedure described previously by OECD (OECD201), with modifications for 96-well microplates. A 250 µL aliquot of algal inoculum (culture medium containing 10^5 cells mL^{-1}) was added to each well together with 50 µL of the test solution (PQ, AHS, PQ-AHS, NP:PQ and NP:PQ-AHS diluted in culture medium). The cell density in the inoculum was determined by absorbance measurements at 684 nm (A_{684}) (Rodrigues et al., 2011), using a UV-Vis 1650PC spectrophotometer (Shimadzu). The following formula was used to calculate the cell density:

$$Cell\ Density = e^{\frac{(\ln(A_{684})+16.439)}{1.0219}} \quad (1)$$

A minimum of five different concentrations were tested for each sample with and without the humic substances (10 mg L^{-1}), in a gradient with a factor of 2. Control groups employed 250 µL of inoculum and 50 µL of culture, or humic substances alone (10 mg L^{-1}). The suspensions were kept for 96 h at a temperature of 24 ± 2 °C and an illumination intensity of approximately 3000 lux (provided from standard 20 W fluorescent lamps). The suspensions were agitated daily for 60 s and algal growth was monitored by measurements of the absorbance of the suspensions at 684 nm, using a microplate reader (Sunrise, Tecan), after 0, 24, 48, 72, and 96 h.

The rate of algal growth was given by the angular coefficient of the fitted linear regression model, expressed as log[absorbance] per unit time. The values obtained for growth inhibition at each test concentration were analyzed using simple regression in order to construct the concentration-response curve and calculate the 96-h EC_{50} (effective concentration to inhibit algal growth by 50 %), with a confidence interval of 95 %. Statistical analysis of the data was performed with Statgraphics Plus v. 5.1 software.

3. RESULTS AND DISCUSSION

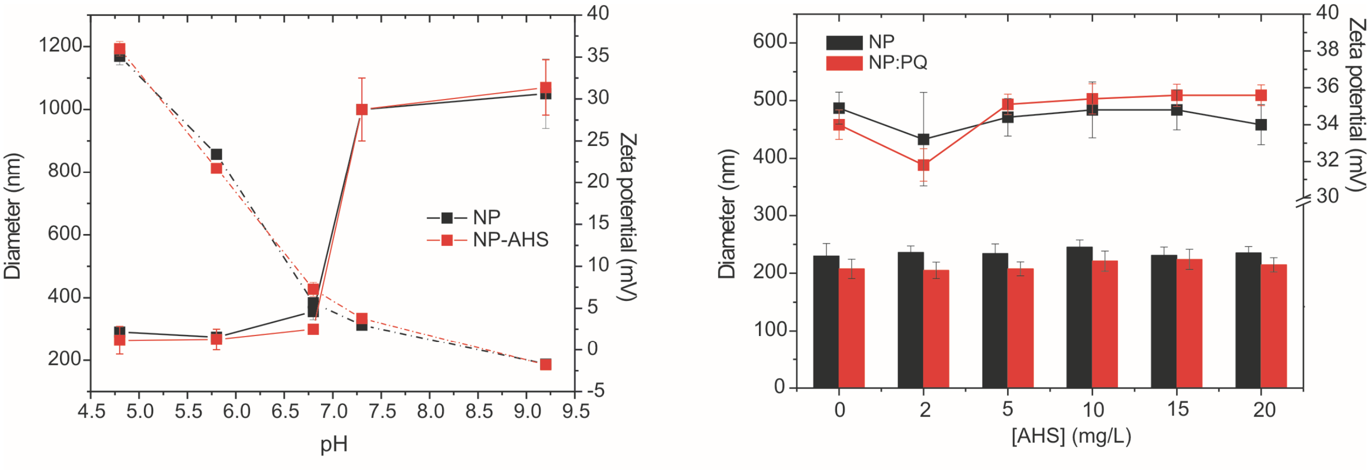
3.1 Characterization of the CS/TPP nanoparticles and humic substances

Previous characterization of the CS/TPP nanoparticles containing PQ (Grillo et al., 2014a) showed that the system presented good colloidal stability, a herbicide encapsulation efficiency of 62.66 ± 0.77 %, slow release, low toxicity, and effective herbicidal activity. The water collected from the Sorocabinha River had a pH of 6.2, temperature of 28 °C and a conductivity of 148 µS cm^{-1}. The content of dissolved oxygen was determined to be 1.8 mg L^{-1} and a total organic carbon content of 1.53 g L^{-1} has been observed. The humic substances were purified and dried prior to mass determination and used in the stability tests with the nanoparticles.

3.2 Physicochemical stability of the system containing AHS

3.2.1 Hydrodynamic diameter and zeta potential

The colloidal stability of the CS/TPP nanoparticles (with and without PQ) was assessed in the presence of AHS, and measurements of size and zeta potential were performed using different pH values and concentrations of humic substances (Figure 1).



A) B)

Figure 1. Stability of the CS/TPP nanoparticles with AHS: A) Values of the hydrodynamic diameter (---) and zeta potential (–) obtained for the nanoparticles as a function of 20 mg L^{-1} AHS, pH (4.5 to 9.5) without PQ ; B) Values of the hydrodynamic diameter (columns) and zeta potential (lines) obtained for the nanoparticles with and without PQ in different concentrations of humic substances (0 to 20 mg L^{-1}, at pH = 5.0). The values represent the average of three experiments performed at 25 °C.

Figure 1 A shows that the CS/TPP nanoparticles showed greater colloidal stability at acid pH (between 4.5 and 6.5), average diameters of 282 ± 13 nm (NP) and 265 ± 38 (NP-AHS), and zeta potentials greater than 5 mV. A sharp increase in the average diameter of the nanoparticles was observed at pH values around pH 7.0, This observation is due to the fact that the nanoparticle surface charges became zero (pH_{PZC}, isoelectric point between pH 6.5 and 7.0) and aggregation was favored (Fan et al., 2012; Grillo et al., 2013).

The stability of the CS/TPP nanoparticles was affected by the pH of the medium because the polymer must be protonated (pK_a 6.5) in order to bind to the TPP anion by cross-linking in order to form stable particles (Fan et al., 2012). It has been found that humic substances can influence the stability of nanoparticles of silver (Dubas and Pimpan, 2008; Cumberland and Lead, 2009; Chinnapongse et al., 2011; Baalousha et al., 2013), gold (dos Santos et al., 2005; Diegoli et al., 2008; Stankus et al., 2011), and zinc (Zhou and Keller, 2010; Jiang et al., 2012; Mohd Omar et al., 2014) in aquatic environments. However, here addition of AHS at a concentration of 20 mg L^{-1} to the NPs did not result in any observable alteration of the colloidal stability profile. The same effects were found for NPs with PQ and AHS (data not shown). Thus, it could be concluded that the humic substances present in the medium did not interact with the surfaces of the nanoparticles under these conditions. This conclusion was further confirmed by a missing observable alteration in the size distribution or zeta potential values of the nanoparticles in the presences of AHS.

The concentrations of humic substances in natural watercourses is in general lower than those used in the studies described (20 mg L^{-1}) (Rodriguez and Nunez, 2011), and the pH of the water in unpolluted Brazilian rivers was reported to be between 4.7 and 5.7 (Botero et al., 2011). Therefore, a further study in respect to nanoparticle stability was performed maintaining the pH of the formulations constant (at pH 5) and varying only the concentration of AHS (0, 2, 5, 10, 15, and 20 mg L^{-1}), in order to detect any possible alterations in the physicochemical stability of these NPs in the environment. In all cases, the hydrodynamic diameter values remained constant at 235 ± 5.4 (NP) and 213 ± 7.8 nm (NP:PQ), and the zeta potentials remained at around 35 mV (Figure 1 B), showing that under these conditions, the AHS did not affect the physicochemical stability of the NPs in either the presence or absence of PQ. Figure 2 shows that in addition to the fact that the diameters of the NPs were similar, with and without the AHS, the size distribution profile also remained unchanged.

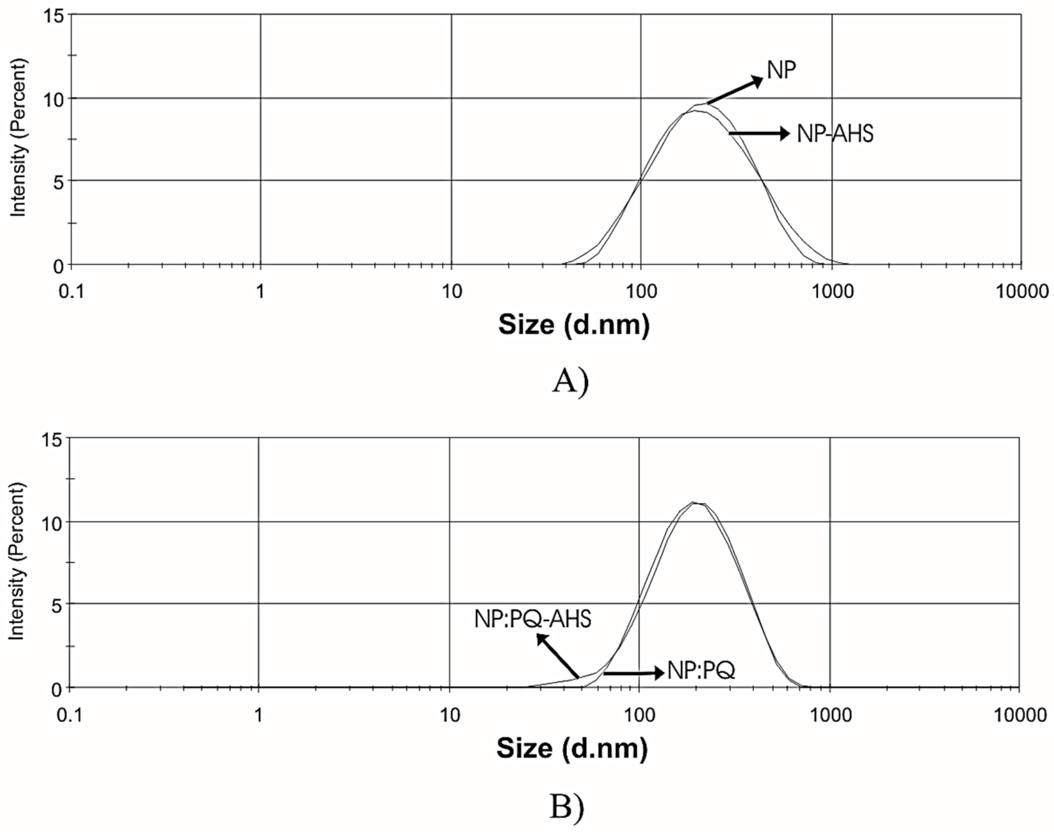


Figure 2. Size distributions of the CS/TPP nanoparticles, with and without AHS (20 mg L^{-1}), at pH 5: A) Nanoparticles without AHS (NP) and with AHS (NP-AHS); B) nanoparticles associated with PQ in the absence of AHS (NP:PQ) and with AHS (NP:PQ-AHS). The values represent the average of three experiments performed at 25 °C.

The inability of the AHS to alter the stability of the nanoparticles in the medium could be due to the molecular size of HS, which is in the range of 1 to 100 kDa (Rocha and Rosa, 2003). Even though the concentration of CS used to prepare the nanoparticles was in the region of 1 mg L^{-1}, and the range of AHS concentrations tested was greater than that of the polymer, no alterations were observed by this technique in the properties of the nanoparticles.

Furthermore, many of the carboxylic groups present in AHS (pKa ~ 4.2) can be ionized (with negative charge) at pH 5 (Shin et al., 1999), and may therefore interact with the positive charges of the ionized amino groups present in the polymeric chains that were not cross-linked with TPP. In the present case, this could be indicative of strong electrostatic interactions between the TPP and the ionized groups of chitosan (CS), which prevented interaction with the AHS. In addition, the ionized groups of the AHS may also interact with metal species (divalent cations) and organic compounds and those interactions may prevent their binding to the nanoparticles.

3.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to investigate the chemical structures of the species composing the CS/TPP nanoparticles (with and without PQ), in the presence and absence of AHS. The spectra obtained are illustrated in Figure 3.

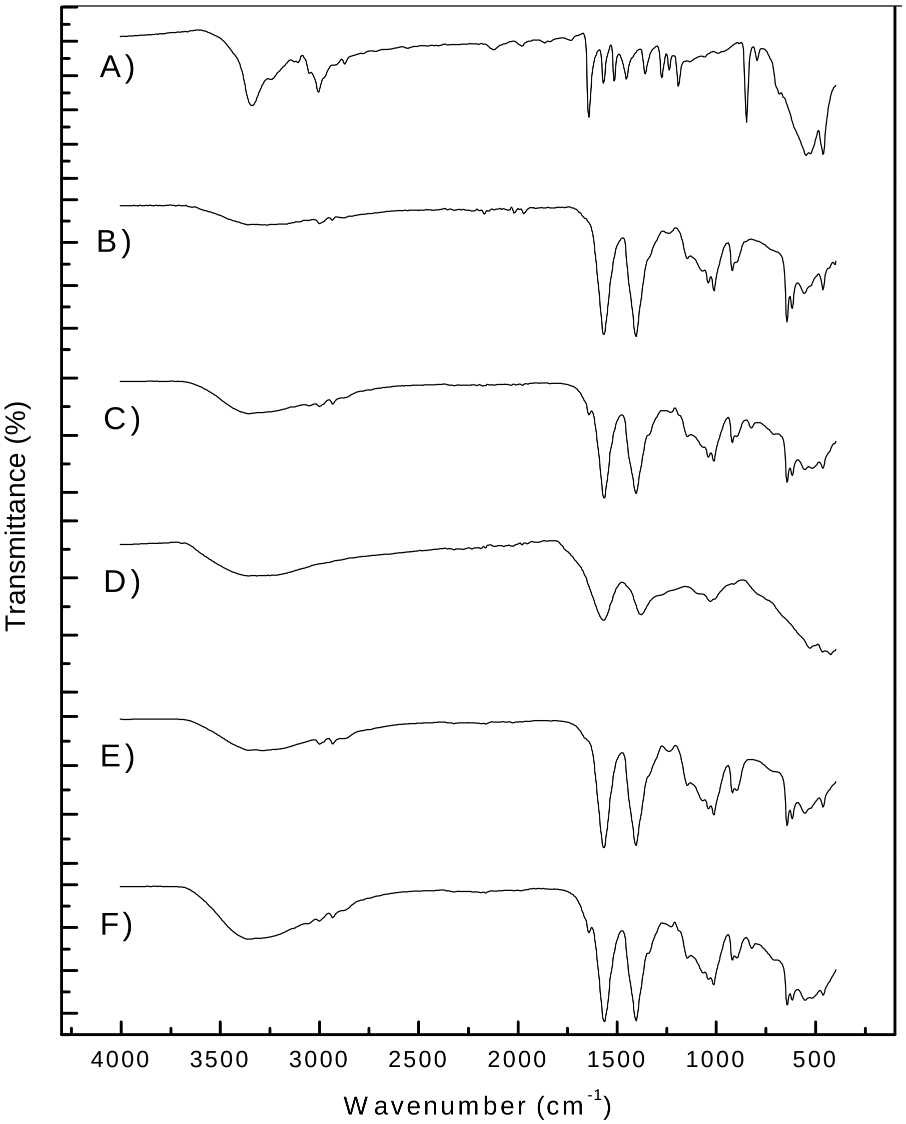


Figure 3. Analysis of the samples using Fourier Transform Infrared Spectroscopy: (A) Paraquat (PQ), B) nanoparticles (NP), C) nanoparticles containing Paraquat (NP:PQ), D), aquatic humic substances (AHS), E) nanoparticles with AHS (NP-AHS), and F) nanoparticles containing Paraquat with AHS (NP:PQ-AHS). The numbers indicate the wavenumbers of the main groups present in the components of the samples.

In the case of PQ (Figure 3 A), the main bands were observed at 3345 cm^{-1} (stretching vibration of aromatic amines, N-H), 2940 cm^{-1} (stretching of the C-H of aromatic rings), 1643 cm^{-1} (stretching of C=N and C–N bonds), 1508 cm^{-1} (weak deformation of CH_3–N^+ bonds), and 840 cm^{-1} (deformation of *para*-substituted aromatic rings of the herbicide) (Silva et al., 2011; Hsu and Pan, 2007; Martins et al. 2012; Cocenza et al., 2012).

The CS/TPP nanoparticles (Figure 3 B) showed the main bands at 3670-3080 cm^{-1} (stretching vibrations of amine groups (N-H) and hydroxyls) (Garrido-Herrera et al., 2006), 2940 cm^{-1} (stretching of C-H bonds), 1565 and 1400 cm^{-1} (symmetrical angular deformation of N-H), 1151 cm^{-1} (P-O groups of the TPP polyanion) (Bhumkar and Pokharkar, 2006; Keawchaoon and Yoksan, 2011; Martins et al., 2012), 1043 cm^{-1} (stretching of C-O-C), and 650 cm^{-1} (stretching of the pyranoside ring) (Tonhi and Plepis, 2002; Hebeish et al., 2013).

The bands found for the nanoparticles were also observed for the combinations of the NPs with PQ and/or the AHS (Figures 3 C, 3 E, and 3 F). However, in the case of the NPs containing the herbicide (Figure 3 C), there was a reduction in the intensity of the peak at 1643 cm^{-1} (C=N and C–N) and an absence of the bands at 1508 cm^{-1} (CH_3–N^+) and 840 cm^{-1}, present in the herbicide spectrum. This was indicative of the interaction of PQ with the NPs due to electrostatic attraction between the (CH_3–N^+) groups and the polyphosphate ($P_3O_{10}^{5-}$) groups of the TPP molecules present in the NPs (Keawchaoon and Yoksan, 2011).

Although the spectrum obtained for the AHS (Figure 3 D) was very similar to that of the NPs, a band at 1380 cm^{-1} (due to the COO^- group) could be easily mixed up with the band at 1400 cm^{-1} present in the spectrum for the NPs (Rodriguez and Nunez, 2011). The spectrum for the NPs and AHS revealed substantial increases in the intensities of the vibrational bands of O-H (3670-3080 cm^{-1}) and N-H (1565 and 1400 cm^{-1}), reflecting possible interaction between some AHS molecules and the NPs (Keawchaoon and Yoksan, 2011). Similar increases in the intensities of these bands were reported in the literature (Keawchaoon and Yoksan, 2011). In that case, the FTIR technique was able to detect some effects on the vibrational bands of the molecules present in NPs.

3.3 Toxicity evaluation of the nanoparticles with AHS

3.3.1 Allium cepa chromosomal aberration assays

Amongst the different tests available to measure chromosomal aberrations in cells, the *Allium cepa* assay can be conveniently used to evaluate the genotoxicity of nanoparticles (Kumari et al., 2009; Kumari et al., 2011; Grillo et al., 2012; Pesnya, 2013; Pakrashi et al., 2014, Grillo et al., 2014a). Figure 4 shows the results obtained for the different samples.

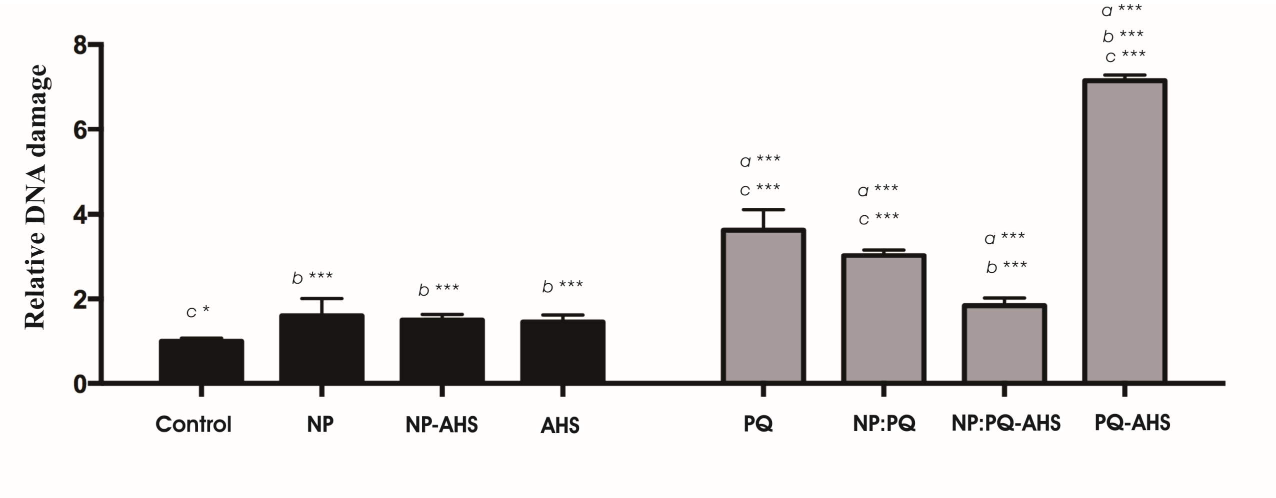


Figure 4. Evaluation of chromosomal damage using the *Allium cepa* test. The cells were exposed in the presence of Paraquat (PQ), nanoparticles (NP), nanoparticles containing Paraquat (NP:PQ), aquatic humic substances (AHS), nanoparticles with humic substances

(NP-AHS), and nanoparticles with Paraquat and humic substances (NP:PQ-AHS). The results for PQ, NP:PQ and NP without AHS were obtained from reference Grillo et al., 2014a. The concentrations of PQ and AHS were 0.38 mg mL^{-1} and 20 mg L^{-1}, respectively. The negative control was deionized water. Data expressed as mean ± s.d. Statistical differences refer to a – control and b – PQ and c – NP:PQ-AHS, with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ (one-way ANOVA with the Tukey-Kramer post-hoc test).

No statistically significant differences relative to the control, were observed for the nanoparticles (NP), the AHS, and the nanoparticles with AHS, indicating that these materials caused no chromosomal damage (relative to the control). However, increased levels of chromosomal damage were observed for free PQ, Paraquat with AHS (PQ-AHS), and Paraquat combined with the NPs (NP:PQ) and with the NPs and AHS (NP:PQ-AHS), as shown in the diagram of Figure 5. The combination of Paraquat and the humic substances (PQ-AHS) caused the greatest chromosomal damage. A possible explanation for this is competition between the positively charged herbicide molecules and potentially toxic substances (such as metal ions and/or organic compounds) adsorbed by the AHS. This competition for the active sites of the AHS (ionized carboxylic and phenolic groups) could have resulted in the release of toxic species into the medium, causing an increase in toxicity, relative to the toxicity of PQ alone (Figure 5-II). Many studies have shown the ability of AHS to form complexes and exchange ionic species with the surrounding environment (Zhou et al., 2005; Lippold et al., 2007; Furukawa and Takahashi, 2008; Stern et al., 2014; Grillo et al., 2014a), and in some cases, AHS have been employed for the removal of pollutants (Burlakovs et al., 2013; Tang et al., 2014).

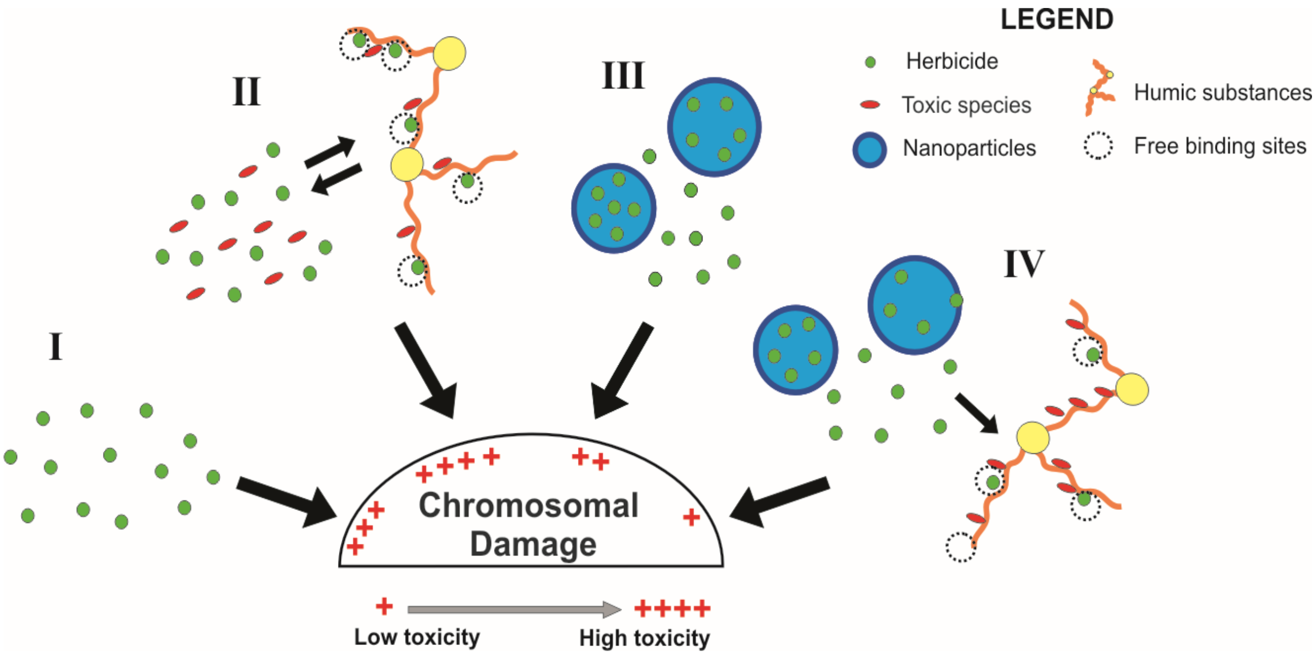


Figure 5. Schematic representation of the chromosomal damage observed for: I) free PQ, II) Paraquat with AHS (PQ-AHS); III) Paraquat combined with the NPs (NP:PQ) and IV) NPs with PQ and AHS (NP:PQ-AHS). Symbol (+) represent increased of the chromosomal damage to *Allium cepa* cells.

There was a significant reduction in the toxicity of PQ when the herbicide was associated with the NPs as showed in Grillo et al., 2014a, and the inclusion of AHS resulted in further improvement, showing that the AHS were able to reduce genotoxicity of the herbicide in the case of the model used here. This can be explained by the fact that the herbicide was encapsulated in the CS/TPP nanoparticles with an efficiency of 62 %, leaving 38 % of the PQ free in solution (Figure 5-III). The free herbicide could therefore interact with free functional groups of the AHS (Figure 5-IV), as a result of which the system became less toxic, compared to PQ alone (Figure 5-I) or the nanoparticles containing PQ (Figure 5-III).

In this case, it appears that the amount of free PQ in the medium was insufficient to cause any substantial shift in the equilibrium of other potentially toxic species that could have been adsorbed on the AHS (which was observed for PQ:AHS).

The findings therefore demonstrate that the presence of humic substances in the medium with nanoparticles containing PQ was able to reduce the genotoxicity of the herbicide, which is similar to results reported for some other nanoparticles in different organisms (Grillo et al., 2014b). An important point is that even though no major interactions were observed between the AHS and the CS/TPP nanoparticles (with or without herbicide), humic substances can have a large impact on the dynamics of the interaction between the herbicide and the aquatic environment.

3.3.2 Ecotoxicity tests using algae

Algae are found in many aquatic environments, and are therefore suitable organisms for the monitoring of toxicity in systems containing polymeric nanoparticles and aquatic humic substances. In the control group, the algae showed a growth rate of 0.005 ± 0.001 log $A_{684}.h^{-1}$ (mean ± standard deviation), while for the control group with humic substances the growth rate was 0.007 ± 0.001 log $A_{684}.h^{-1}$. The 96-h CE_{50} values for the different test groups, together with the corresponding confidence intervals, are given in Table 2.

Table 2. Toxicity to *Pseudokirchneriella subcapitata* of CS/TPP nanoparticles (NP), Paraquat (PQ), nanoparticles containing Paraquat (NP:PQ), paraquat with humic substances (PQ-AHS), and nanoparticles containing Paraquat with humic substances (NP:PQ-AHS).

Statistical analysis employed analysis of variance (ANOVA) and different letters indicate statistically significant between the groups ($p < 0.05$).

	96-h CE_{50} (mg L^{-1})*	**Lower limit**	**Upper limit**
NP	>50.0 [a]	-	-
PQ	0.48 [b]	0.24	0.77
NP:PQ	1.15 [c]	1.04	1.32
PQ-AHS	0.78 [b]	0.66	0.91
NP:PQ-AHS	1.65 [c]	1.28	2.29

Exposure of the algae to the medium containing humic substances resulted in an increased growth rate (relative to the control), as expected because these organisms use organic matter to obtain energy. The formulation containing the nanoparticles alone showed no toxicity to this species of alga (96-h CE_{50} >50 mg L^{-1}). The 96-h CE_{50} found here for PQ is similar to values in the range 0.3-0.6 mg L^{-1} reported previously using *P. subcapitata* (PAN, 2014).

The data indicated that the association of PQ with the nanoparticles significantly reduced its toxicity to algae. According to the classification proposed by the United States Environmental Protection Agency (USEPA, 1985), the 96-h CE_{50} value obtained for PQ alone was within the range considered highly toxic (0.1-1 mg L^{-1}), while the value for PQ in association with the nanoparticles was within the moderately toxic range (1-10 mg L^{-1}). These findings confirmed that the formulation was able to reduce toxicity in this organism. The inclusion of humic substances in the medium did not cause any statistically significant changes in the 96-h CE_{50} values of the formulations tested (using free or encapsulated PQ), although the data were indicative of a tendency towards lower toxicity when the organisms were co-exposed to the AHS, suggesting that there was interaction between PQ and the humic substances. It is known that AHS are able to form complexes with organic compounds (Zhou et al., 2005; Lippold et al., 2007; Furukawa and Takahashi, 2008; Stern et al., 2014). The results obtained in these tests were very similar to the findings of the genotoxicity assays, despite the fact that the two techniques are used for different purposes, and again demonstrate the ability of AHS to interfere in the dynamics of nanoparticles and PQ in aquatic systems.

4. CONCLUSIONS

The results indicated that AHS molecules did not cause any substantial changes in the physicochemical stability of the CS/TPP nanoparticles (with or without Paraquat). The hydrodynamic diameter and the zeta potential of the NPs were not influenced by the addition of the AHS. Small alterations were observed in the infrared spectra, indicative of a dynamic interaction between the AHS and the NPs, although further elucidation was not possible using the selected techniques and time-frame. The presence of AHS influenced the genotoxicity of Paraquat associated with the NPs. The results of ecotoxicity assays showed that the encapsulation of Paraquat acted to reduce its toxicity, and that further reduction could be achieve by the inclusion of AHS. The presence of humic substances in the medium altered the dynamic equilibrium of the herbicide in solution, and diminished the toxicity of the nanoparticle/paraquat system. The findings contribute to a better understanding of the environmental stability and toxicity of agrochemicals encapsulated in polymeric nanoparticles, indicating one possible way in which the risks of nanotechnology used in agriculture might be mitigated. Further work will be needed to further improve knowledge in this area, considering the diversity of available polymeric nanoparticles and agrochemicals.

5. ACKNOWLEDGMENTS

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CHAPTER IV

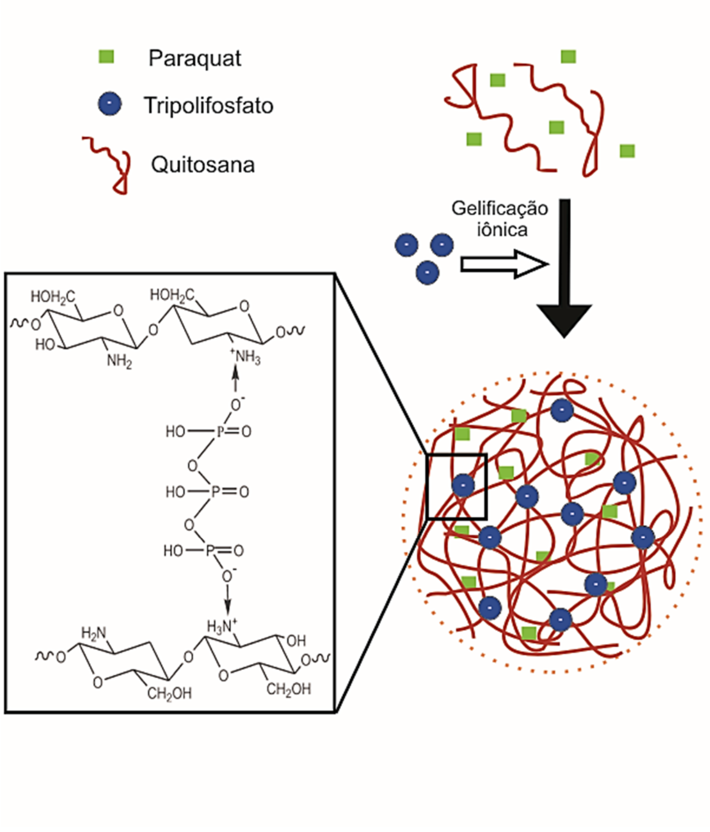
DISCUSSION AND PROSPECTS

DISCUSSÃO E PERSPECTIVAS

DISCUSSÃO DOS RESULTADOS E PERSPECTIVAS

O desenvolvimento de novas tecnologias agrícolas tem sido um importante fator para o crescimento da agricultura moderna. Entre as últimas inovações tecnológicas, a nanotecnologia ocupa uma posição de destaque na transformação da agricultura e na produção de alimentos (Nair et al., 2010). O surgimento de novos produtos responsáveis por aumentar a produção e a proteção das plantas (Bin Hussein et al., 2005; Liu et al., 2006; Cifuentes et al., 2010; Grillo et al., 2010; Silva et al., 2010; Grillo et al., 2011; Silva et al., 2011; Grillo et al., 2012; Perez-de-Luque et al., 2012; Grillo et al., 2014a; Pereira et al., 2014), melhorar a detecção de resíduos de pesticidas no ambiente (Kang et al., 2010; Parham and Rahbar, 2010; Khan and Akhtar, 2011; Kuang et al., 2011; Kumaravel and Chandrasekaran, 2011; Zhao et al., 2011) bem como a detecção de patógenos (Baac et al., 2006; Boonham et al., 2008; Yao et al., 2009) vem sendo objeto de discussão e estudo na última década (Khot et al., 2012; Kah et al., 2013; Kah and Hofmann, 2014; Kookana et al., 2014).

A presente Tese, como já descrito nos capítulos anteriores, mostra o desenvolvimento de um novo sistema de liberação modificada para o herbicida Paraquat (PQ), um herbicida não seletivo de contato, bastante utilizado no Brasil e em mais de 100 países em culturas de milho, arroz, soja, trigo, batatas, maçãs, laranjas, bananas, café, chá, cacau, algodão, entre outras (Donaldson, 2013). O uso deste herbicida é proibido em alguns países, principalmente na União Europeia desde 2007, devido a sua toxicidade para os seres vivos e pela alta persistência no solo (Han et al., 2010). Assim sendo, os resultados discutidos ao longo desta Tese mostraram que o sistema de liberação modificada desenvolvido com nanopartículas poliméricas (NPs) de quitosana/tripolifosfato (CS/TPP) para o herbicida PQ foi capaz de reduzir a toxicidade deste bioativo, além de diminuir a liberação e a sorção deste composto no ambiente, da mesma maneira, que continuou agindo no combate das plantas indesejáveis. Além disso, pensando na exposição destes compostos no ambiente, as NPs (sem e contendo o herbicida) também foram colocadas em presença de matéria orgânica natural (na forma de substância húmica aquática, AHS) e observou-se que a genotoxicidade e a ecotoxicidade do herbicida reduziram ainda mais. A Figura 1 mostra um esquema das interações entre o polímero quitosana (CS), o herbicida PQ e o ânion tripolifosfato (TPP) e um resumo de todos os dados experimentais de caracterização físico-química, cinética de liberação, sorção, toxicidade e atividade herbicida deste sistema.



Caracterização

Diâmetro médio em solução: ~ 300 nm
Índice de polidispersão: < 0,3
Potencial zeta: + 45 mV
Tempo de estabilidade: > 60 dias
Eficiência de encapsulação: 62,6 ± 0,7%
Morfologia: Esféricas

Cinética de Liberação

$t_{50\%}$ = 140 min e n= 0,68
Constante (k): 1,99 min^{-1}
Modelo: transporte anômalo
Conclusão: liberação do PQ > NP:PQ

Sorção no solo

q_t= 1,8 mg/g e k = 0,315 mg/g.min
Modelo: pseudo-segunda ordem
Conclusão: sorção do PQ > NP:PQ

Toxicidade

Allium cepa: PQ livre > NP:PQ
CHO: PQ livre > NP:PQ

Atividade Herbicida

Zea mays: NP:PQ mais eficiente que PQ
Brassica sp: NP:PQ igual eficiência que PQ

Figura 1. Representação esquemática das interações entre os componentes da formulação e um breve resumo sobre os resultados encontrados para nanopartículas de CS/TPP contendo o herbicida PQ.

Como observado na Figura 1, o polímero catiônico quitosana pode reagir com íons polivalentes, tais como os tripolifosfatos (TPP), que na presença de água, se dissociam e liberam íons OH^- e $P_3O_{10}^{5-}$, os quais podem interagir com os grupamentos protonados da quitosana (NH_3^+). Estas interações intermoleculares e/ou intramoleculares formam uma espécie de rede entre os compostos e assim a molécula de herbicida consegue se associar (Yang et al., 2009a; Fan et al., 2012; Tripathy et al., 2012). Alguns estudos utilizam NPs de CS/TPP para sistemas de liberação de fármaco (Hu et al., 2008; Hasanovic et al., 2009; Tripathy et al., 2012), como gene delivery (Csaba et al., 2009; Peymani et al., 2009; Jiang et al., 2011; Vimal et al., 2012; Vimal et al., 2013; Vimal et al., 2014), como sistema antimicrobiano associado com metais (Du et al., 2009), entre outros (de Moura et al., 2009; Fabregas et al., 2013; Wang et al., 2014). No entanto, este é o primeiro trabalho na literatura que utiliza nanopartículas de quitosana/tripolifosfato para fins de liberação modificada para defensivos agrícolas.

Os dados de caracterização físico-química aqui apresentados corroboram com os encontrados na literatura, uma vez que estudos descrevem que estas nanopartículas (NPs) podem apresentar diâmetro hidrodinâmico entre 200 a 1000 nm, polidispersão < 0,5, potencial zeta entre 20 a 60 mV e possuem a capacidade de modificar a liberação de bioativos (Tsai et al., 2008; Fan et al., 2012; Tripathy et al., 2012; Shukla et al., 2013). Morris e colaboradores (2011) avaliaram a estabilidade de NPs de CS/TPP preparadas por gelificação iônica (mesmo método proposto neste trabalho) durante 12 meses em três diferentes temperaturas (4 °C, 25 °C ou 40 °C) e observaram que as NPs são estáveis durante 12 meses quando armazenadas em baixas temperaturas (4 °C) e temperatura ambiente (25 °C), o que demonstra que a estabilidade destas NPs pode ser maior que o tempo relatado para os nosso estudos (~ 60 dias). Além da estabilidade coloidal das NPs, a eficiência de encapsulação do herbicida também foi calculada (~ 62 %). No entanto, estes valores dificilmente são semelhantes com os descritos na literatura, uma vez que a encapsulação depende de uma série de fatores, tais como, a massa molar, a proporção entre os componentes, a estrutura química, o pH, entre outros (Stoica et al., 2013). Alguns trabalhos mostram porcentagem de eficiência de associação para NPs de CS/TPP entre 14 a 99 % para genes (Vimal et al., 2012; Vimal et al., 2013; Vimal et al., 2014), proteínas (Shukla et al., 2013; Koppolu et al., 2014) e fármacos (Shu and Zhu, 2000; Tripathy et al., 2012; Stoica et al., 2013).

Um outro fator estudado foi a cinética de liberação do PQ e observou-se que a associação do herbicida nas NPs proporcionou uma liberação mais lenta, um resultado relevante, uma vez que se espera que um sistema de liberação diminua a quantidade de herbicida livre no ambiente, pois assim pode reduzir a toxicidade e os efeitos de degradação físico-química, através da luz, oxidação e calor, que tanto afetam estes compostos (Costa et al., 2013). Alguns sistemas de liberação modificada podem ser encontrados na literatura para o herbicida PQ, utilizando minerais, tais como, montmorillonite (Han et al., 2010) e zeólitos (Zhang et al., 2006); hidrogéis de álcool polivinílico (PVA) (Alemzadeh and Vossoughi, 2002) e nanopartículas poliméricas (Silva et al., 2011). No entanto, nenhum destes trabalhos discutem os efeitos de toxicidade desta associação (herbicida + carreador) no ambiente e nem o efeito da atividade herbicida nas plantas, o que comprova a importância dos resultados aqui apresentados. Silva e colaboradores (2011) também observaram uma liberação mais lenta para o herbicida PQ em NPs de alginato/quitosana, apresentando um $t_{50\%}$ de ~ 80 minutos em comparação com um $t_{50\%}$ de 25 minutos do ativo livre. Estes resultados são bastante semelhantes com os encontrados neste trabalho ($t_{50\%}$ = 85 minutos para o PQ livre e $t_{50\%}$ =

175 minutos para o PQ nanoencapsulado), mostrando que a diferença de liberação entre o ativo livre e o ativo encapsulado para ambos os trabalhos pode ser de aproximadamente 90 minutos nas condições testadas. Apesar disto, cabe ressaltar que as condições experimentais testadas para ambos os estudos foi acelerada, uma vez que o herbicida é forçado a passar de um compartimento doador para o compartimento aceptor por difusão, como mostra a Figura 1, capítulo II.

Ensaios de sorção mostraram que a adsorção do PQ no solo é muito rápida e o modelo que melhor descreve este fenômeno é o de pseudo-segunda ordem (Ho and McKay, 1999), como descrito também para PQ adsorvido em biochar (Tsai and Chen, 2013), solo diatomito (Tsai et al., 2005) e solos iara e carvoeiro (Silva et al., 2011). A rápida redução da concentração do herbicida no ambiente ocorre devido a fortes interações entre a superfície carregada de íons do solo com o PQ (Tsai and Chen, 2013). Os nossos estudos mostraram que o herbicida quando associado às NPs apresentou uma menor sorção no solo, o que possivelmente contribuiu para o aumento da biodisponibilidade deste ativo no ambiente, melhorando sua ação herbicida, como observado nos ensaios de atividade herbicida propostos para culturas de *Zea mays*. Parâmetros tais como a atividade herbicida e os mecanismos de sorção e degradação de herbicidas nanoencapsulado em solo ainda são pouco estudados e pode ser uma área bastante promissora. Kah e colaboradores (2014) testaram e discutiram a aplicabilidade de protocolos de regulação para avaliar a sorção e a degradação de herbicidas em formulações de liberação modificada e observaram que nanocápsulas contendo o herbicida Atrazina, preparadas pelo nosso grupo de pesquisa (Grillo et al., 2012), possuem efeito sobre o destino do ativo no ambiente. No entanto, investigações mais detalhadas e novos protocolos experimentais devem ser realizados para melhor entender estas interações com o solo (Kah et al., 2014).

O estudo de toxicidade para NPs tem crescido bastante nos últimos tempos; no entanto, muitas dificuldades são encontradas para comparar os resultados obtidos, uma vez que, muitos parâmetros podem se alterar nestes sistemas, tais como, o tamanho, a rugosidade, a morfologia, carga, entre outros (Elsaesser and Howard, 2012). Nossos estudos mostraram que os efeitos de citotoxicidade, genotoxicidade e ecotoxicidade do herbicida foram reduzidos quando associados às NPs de CS/TPP para as células e algas utilizadas. A redução da toxicidade provavelmente ocorreu devido à liberação modificada que este sistema apresentou, uma vez que menos herbicida foi liberado em função do tempo. Resultados semelhantes de redução de toxicidade são observados para micropartículas (Grillo et al., 2010; Lima et al., 2012) e nanopartículas (Grillo et al., 2012; Kumar et al., 2014; Pereira et al., 2014) associadas com defensivos agrícolas.

A maioria dos estudos toxicológicos para NPs é realizado com culturas de células e organismos específicos. No entanto, sabe-se que diferentes fatores ambientais, tais como, pH, luz, temperatura, microrganismos, força iônica e matéria orgânica, também podem modificar e interferir na estabilidade, toxicidade e persistência destes nanomateriais no ambiente (Baalousha and Lead, 2011; Maurer-Jones et al., 2013). Portanto, com o objetivo de avaliar modificações na estabilidade e toxicidade das NPs de CS/TPP contendo PQ, foi avaliada a influência da matéria orgânica (na forma de substâncias húmicas aquáticas, AHS) e observou-se que a AHS realmente pode interferir na toxicidade do herbicida, como descrito na Figura 2.

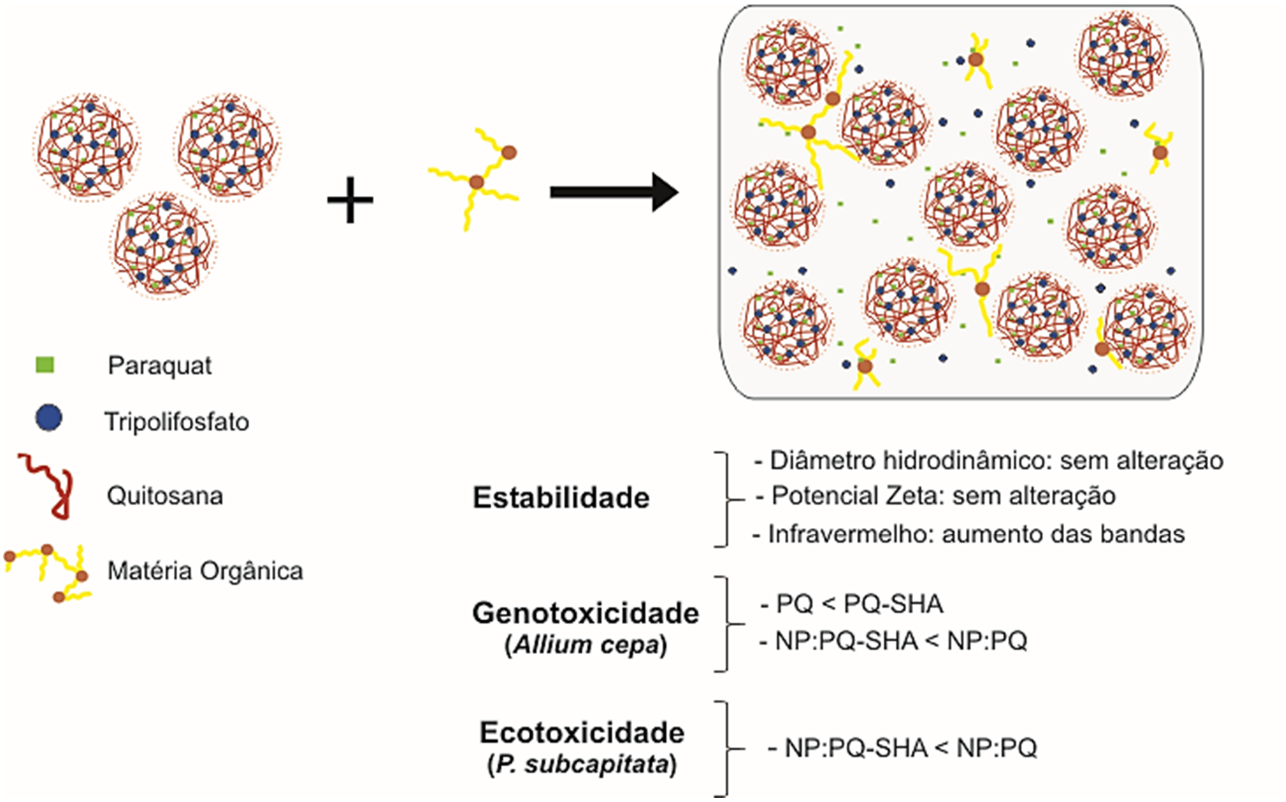


Figura 2. Representação esquemática das interações entre a matéria orgânica e as nanopartículas de CS/TPP contendo PQ e um breve resumo dos resultados encontrados com esta associação.

Nossos dados reportaram uma diminuição da toxicidade deste herbicida quando associado à SH, dada provavelmente as diferentes interações ocorridas entre NPs, herbicida e SH, que necessitam ser mais estudadas em trabalhos futuros.

Levando-se em conta a boa estabilidade destas NPs, a redução da toxicidade do herbicida apresentada pelos experimentos de citotoxicidade, genotoxicidade e ecotoxicidade, em conjunto com a redução do perfil de liberação, da sorção no solo e da boa ação herbicida, conclui-se que esta pode ser uma formulação promissora para o agronegócio, embora muitos estudos ainda sejam necessários, a fim de compreender melhor os mecanismos que favorecem a interação destes compostos com o ambiente, bem como avaliar os riscos para os seres humanos e a interação/translocação destas NPs com as plantas e outros organismos. Desta forma, a presente Tese mostra resultados promissores da associação entre nanotecnologia e agroquímicos visando aplicações em agricultura.

GENERAL CONCLUSIONS

CONCLUSÕES GERAIS

Muitos sistemas de liberação para defensivos agrícolas estão sendo criados e várias indústrias têm reunido esforços para desenvolvimento de novas estratégias para melhorar a ação destes produtos no campo. Diversos artigos e patentes são publicados sobre este tema constantemente, mas poucos são os produtos comerciais disponíveis no mercado. A maioria dos estudos de liberação modificada mostra apenas o desenvolvimento (preparo e caracterização) destes sistemas e não estuda a fundo a sua eficácia, bem como, a segurança para os seres vivos e o ambiente. Assim neste contexto reside a importância deste trabalho, uma vez que foi possível desenvolver um sistema de liberação para herbicidas e avaliar alguns aspectos de toxicidade, estabilidade com a matéria orgânica e atividade herbicida.

Os resultados aqui apresentados e discutidos mostram que as nanopartículas poliméricas podem ser bons carreadores para o herbicida PQ. Este é o primeiro trabalho na literatura que estuda a estabilidade de nanopartículas poliméricas (ordem de tamanho de 100 a 500 nm) na presença de substâncias húmicas aquáticas, e observa-se aparentemente que as moléculas de AHS não causam grandes alterações na estabilidade das nanopartículas de quitosana/tripolifosfato (contendo ou não herbicida). No entanto, nota-se redução na toxicidade do herbicida quando as nanopartículas estão associadas as AHS, mostrando que a interação entre as AHS e as NPs podem ser um fenômeno dinâmico e que na escala de tempo dos métodos utilizados não foram possíveis medir certas interações.

Embora os resultados apresentados sejam bastante satisfatórios sabemos que muitos estudos ainda são necessários para aprimoramento deste sistema de liberação para uso na área agrícola, uma vez que devem ser levados em consideração requisitos legais de toxicidade, aspectos regulatórios, de registro e de escalonamento. Estima-se que o valor de investimento mundial para defensivos agrícolas e fertilizantes poderá chegar a 200 bilhões de dólares neste ano de 2014 (Campos et al., 2014), o que deverá fornecer novo impulso para o desenvolvimentos de sistemas de liberação modificado e assim beneficiar o setor agrícola, além de abrir novas perspectivas sobre esse sistema para futuras aplicações.

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