Modified tannins and their application in wastewater treatment

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ABSTRACT

The bio-flocculants used in this study were synthesised by the Mannich reaction, which includes three reagents: a substrate (tannin extracts of Acacia, Quebracho, and Castanea), formaldehyde, and an amine derivative (ethanolamine, diethanolamine, ammonium chloride). Nine natural flocculants were prepared by combining extracts and amines; these products were evaluated in three different wastewater samples in two experimental phases. In phase I, five physicochemical parameters were analysed. From the data obtained, a multivariate, completely randomised design (CRD-Manava) was used, with a factorial arrangement and mean plots. In phase II, the three bio-flocculants with the most statistically significant responses and their mixtures were examined, evaluating 14 biological and physicochemical parameters. Statistical analysis was guided in this phase by CRD blocks, finding a significant removal in the physicochemical parameters analysed in the different types of wastewater and obtaining removal rates between 50 and 90%, depending on the parameter. At the end of both phases, the bio-flocculants acacia-ammonium chloride and quebracho-diethanolamine were the most efficient in the removal of turbidity (34–99%), true colour (93–100%) and total solids (12–99%). In addition, the natural flocculants showed low mutagenicity index (MI: 0.33–0.93) compared to aluminium sulphate (MI: 4.87–8.81).

Key words | bio-flocculants, coagulation/flocculation, Mannich reaction, tannins, wastewater

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INTRODUCTION

Inorganic salts such as aluminium sulphate $Al_2(SO_4)_3$ and ferric chloride ($FeCl_3$) have been used for coagulation-flocculation processes in wastewater treatment. Scientific studies around the world have shown that these compounds generate the adverse effect of high acidity in the treated water (Cooke *et al.* 1986) and may become precursors in the etiopathogenesis of neuronal disorders such as Alzheimer's disease (Teh *et al.* 2016). The design of organic flocculants from polysaccharides or natural polymers such as tannins, cellulose, and chitosan is one of the most promising natural methods (Lee *et al.* 2014); these compounds generate waste sludge that can be degraded by microbes (Teh *et al.* 2016). Tannins are water-soluble polyphenolic secondary metabolites of high molecular weight, produced in various plant tissues such as bark, fruits, leaves, and roots depending on the genus considered. Traditionally, these compounds were used for leather tanning because of the strong structures generated between leather proteins and tannins (Pizzi 2008). These metabolites can be divided according to their structure into gallotannins, ellagitannins, complex tannins, and condensed tannins (Khanbabaee & van Ree 2001); the latter can be used as polymeric additives with inorganic salts or strengthened with cationic regions by the Mannich reaction for direct flocculation (Beltrán *et al.* 2010). This condensation reaction requires three main components: a compound containing an active hydrogen atom (substrate), formaldehyde, and a primary or secondary amine. These compounds cause aminomethylation of the substrate (Subramaniapillai 2013) and form a Mannich base

doi: 10.2166/wst.2018.336

(Tramontini & Angiolini 1994). This chemical modification process in an acidic medium imparts a cationic character to the structure of condensed tannins (Beltrán & Sánchez 2009). In addition, these modified structures acquire an amphoteric character facilitating the removal capacity of ionised heavy metals and the decrease of various physicochemical parameters (Beltrán & Sánchez 2009a, 2009b). On the other hand, wastewater pollution is mainly caused by colloids and suspended particles characterised by a negative surface charge, which causes electrostatic repulsion between them, preventing their sedimentation and removal. These particles are destabilised when a cationic substance such as a natural flocculant derived from modified tannins is added (Beltrán *et al.* 2012). Moreover, these flocculation and removal proprieties of the modified tannins have been evidenced in pilot WWTPs (Beltrán & Sánchez 2009a, 2009b; Hameed *et al.* 2018). Therefore, the aim of this research was to compare and evaluate the coagulant–flocculant activity of tannin extracts from Acacia, Quebracho, and Castanea, chemically modified by the Mannich reaction using three nitrogen compounds (ethanolamine, diethanolamine, and ammonium chloride) in a completely randomised design.

MATERIALS AND METHODS

To design and evaluate the bio-flocculants used in this research, the reagents and commercial kits detailed below were used. The positive control reagent was aluminium sulphate (type B), supplied by Farmavicola SA Colombia; and chemical modification of tannins with:

(1) tannin extracts from *Acacia mearnsii* (Acacia), *Schinopsis balansae* (red Quebracho), and *Castanea sativa* (Castanea) were supplied by Uniproquim SA Bogotá, Colombia;
(2) ethanolamine (ETA), diethanolamine (DEA), ammonium chloride (NH_4Cl), formaldehyde, and hydrochloric acid (HCl) were supplied by Sigma Aldrich, USA.

To analyse colour, nitrates, nitrites, chemical oxygen demand (COD), and phosphorus were used a HI 83099 multiparameter photometer (Hanna Instruments, Inc., USA) and its specialised commercial kits.

Chemical modification of tannins

Chemical modifications with ethanolamine and diethanolamine were performed using procedure A (Beltrán *et al.* 2010). This consisted of gradually adding 12.58 g of tannin extract to 13 mL of distilled water (65 and 70 °C) with continuous stirring. 4.73 mL of ethanolamine or 7.62 mL of diethanolamine was gradually added (75 and 80 °C), after that adding 5.5 mL of HCl (32%) (pH 6.4 to 6.7). To this solution was added 16.78 mL of formaldehyde. When the viscosity was in the range of 40 to 100 cps, the solution was inactivated with 4.52 mL of distilled water and HCl (32%) to pH of 3–2. Procedure B was used for the chemical modifications with NH_4Cl to generate an adequate reaction of Mannich. In this procedure, two solutions were prepared (Quamme & Kemp 1985). For the first solution, 8.66 g of tannin extract was dissolved in 10.5 mL of distilled water (65 and 70 °C). For the second solution, 30 mL of formaldehyde was gradually added to 3.8 g of NH_4Cl, and this solution was then refluxed for 2 h. At the end of this time, both solutions were mixed and stirred continuously until the viscosity reached 40–100 cps. The reaction was then stopped like in procedure A. In addition, the equimolar proportions of tannin, amines, and formaldehyde were 1:1.8:1.8 for each method. Table 1 lists the chemical modification of the nine natural flocculants and their conventions, specifying the procedure used in each case.

Wastewater samples

For phases I and II of the experiments, three wastewater samples with different parameters were used (Table 2). The first sample was obtained from domestic textile-washing wastewater, containing dyes and surfactants from detergents and fabrics, and collected in the city of Bogotá, Colombia (4°44′23.1″N 74°01′52.5″W). The second sample was collected the most contaminated area from Salitre River (Pérez & Zamora 2015) at the coordinates 4°41′06.2″N 74°04′35.5″W (Bogotá, Colombia). The third sample was synthetic wastewater designed at Pontificia Universidad Javeriana, simulating toxic waters polluted with diazo dyes

Table 1 | Conventions of the chemical modification procedures of the nine bio-flocculants

		Tannin		
		Quebracho	**Acacia**	**Castanea**
Amine	Ethanolamine	Q-ETA[a]	A-ETA[a]	C-ETA[a]
	Diethanolamine	Q-DEA[a]	A-DEA[a]	C-DEA[a]
	Ammonium chloride	Q-NH_4Cl[b]	A-NH_4Cl[b]	C-NH_4Cl[b]

[a] Procedure A.
[b] Procedure B (Q = Quebracho, A = Acacia, C = Castanea, ETA = ethanolamine, DEA = diethanolamine).

Table 2 | Initial physicochemical and microbiological parameters of the three wastewater samples in the two phases (results in triplicate). TC: total coliforms, TH: total heterotrophs, BH: heterotrophic bacteria, NR: not recorded

		Washing-machine wastewater		**Salitre River**		**Simulated wastewater**	
Parameter	**UNIT**	**Phase I**	**Phase II**	**Phase I**	**Phase II**	**Phase I**	**Phase II**
Turbidity	NTU	134 ± 6.7	177.0 ± 4.0	150.0 ± 7.5	293.7 ± 1.5	366.0 ± 18.3	246.7 ± 1.5
Colour	PCU	320.0 ± 16	238.7 ± 1.5	280.0 ± 14	298.3 ± 1.5	1,620.0 ± 8.1	633.3 ± 0.57
pH	–	7.55 ± 0.10	7.42 ± 0.0173	7.21 ± 0.205	8.11 ± 0.0153	6.37 ± 0.57	5.30 ± 0.0200
ORP	mV	− 23.90 ± 5.71	− 24.60 ± 0.10	5.70 ± 0.285	− 61.53 ± 1.19	5.70 ± 0.285	9.53 ± 0.152
Conductivity	mS/cm	NR	0.60 ± .010	NR	0.65 ± 0.0058	NR	0.84 ± 0.010
COD	mg/L	979.0 ± 49.0	517.0 ± 3.00	163.0 ± 8.15	913.7 ± 18.50	1,405.0 ± 70.25	500.7 ± 2.52
Nitrates (NO_3^-)	mg/L	160.3 ± 8	21.8 ± 0.200	18.90 ± 0.945	52.70 ± 0.900	47.82 ± 2.39	31.60 ± 0.100
Nitrites (NO_2^-)	mg/L	0.33 ± 0.020	0.11 ± 0.010	0.030 ± 0.0015	0.17 ± 0.010	0.100 ± 0.005	0.080 ± 0.010
Phosphorus (P)	mg/L	2.70 ± 0.15	2.60 ± 0.00	1.10 ± 0.055	8.63 ± 0.35	11.60 ± 0.58	15.00 ± 0.60
Total solids (TS)	mg/L	910.0 ± 45.5	400.0 ± 10.0	1,000.0 ± 50	920.0 ± 26.46	1,930.0 ± 96.5	1,110.0 ± 95.4
E. coli	CFU/mL	NR	$1.02 \times 10^{+3}$ ± 72.29	NR	$2.19 \times 10^{+3}$ ± 100.79	NR	0.00
TC	CFU/mL	NR	$5.81 \times 10^{+5} \pm 3.38 \times 10^{+4}$	NR	$2.29 \times 10^{+4}$ ± 1,055.45	NR	0.00
TH (nutritive agar)	CFU/mL	NR	$4.65 \times 10^{+5}$ ± 26,762.89	NR	$3.98 \times 10^{+4}$ ± 1,834.16	NR	$6.03 \times 10^{+12} \pm 3.93 \times 10^{+11}$
HB (nutritive agar + ketoconazole)	CFU/mL	NR	$8.32 \times 10^{+3}$ ± 383.21	NR	$2.00 \times 10^{+4}$ ± 72.29	NR	$7.95 \times 10^{+10} \pm 5.18 \times 10^{+9}$

(Glucose 2,000 mg/L, yeast extract 500 mg/L, $MgSO_4 \cdot 7H_2O$ 500 mg/L, KH_2PO_4 mg/L, $Fe_2(SO_4)_3$ 13 mg/L, Congo Red $C_{32}H_{22}N_6Na_2O_6S_2$ 5 mg/L) (Do-Hun *et al.* 2013).

Method for wastewater clarification

The jar test was used to evaluate the coagulant–flocculant activity of the nine natural treatments and the positive control [$Al_2(SO_4)_3$ type B]. This process consists of three steps. The first begins with high-speed mixing at 125 rpm ($G = 133\ s^{-1}$) for 2 min. In the second step, the speed is reduced to 55 rpm ($G = 46\ s^{-1}$) for 15 min; and in the last step, sedimentation can occur for 30 min. Additionally, 400 mL of the wastewater sample and four concentrations or application doses of the treatments were used, which varied according to the type of wastewater (500, 750, 1,000, and 1,250 mg/L for the washing machine and river samples and 32.5, 35, 37.5, and 40 mg/L for the simulated sample). The concentrations doses most statistically significant were also used for phase II.

Analysis of physicochemical parameters

Phase I

In the first experimental phase, the turbidity was measured with an HI 88713 turbidity meter. The pH and ORP were analysed with an HI 98190 pH/ORP meter. To measure the true colour, each sample was centrifuged at 4,000 rpm for 20 min and then read in an HI 83099 multiparameter photometer (Hanna Instruments, Inc., USA). Finally, TS values were obtained by drying 10 mL of each sample in a muffle furnace for 1 h (2540B Standard Methods 1998).

Phase II

In the second phase, an analysis of the effect of the three most statistically significant treatments or bio-flocculants and their mixtures on COD, conductivity, nitrates, nitrites, phosphorus, total heterotrophs, heterotrophic bacteria, toxicity, mutagenicity, and the five parameters evaluated in the first phase was performed. An HI 83099 multiparameter photometer and specialised commercial kits for wastewater analyses were used to read nitrate, nitrite, and phosphorus.

Analysis of biological parameters (Phase II)

To determine total heterotrophs and bacteria, an agar plate count was used (ISO 6222:1999). To evaluate only bacterial growth, ketoconazole was added to the medium. In contrast, to analyse total coliforms and *Escherichia coli*, a plate count was performed on Chromocult agar (Merck), with each experiment performed in triplicate.

Toxicity bioassays performed with seeds of *Lactuca sativa* L. var. *crispa*, cv. *milanese* (lettuce), which were obtained from the technical warehouse of plants in the city of Bogotá DC. During the test, 20 seeds of similar size, shape, and colour were collected. They were evenly distributed on Whatman #3 paper impregnated with 5 mL of each flocculant in a Petri dish and incubated in the dark at 22 ± 2 °C for 5 days. After this incubation period, the average lengths of the radicles and hypocotyls were measured and recorded for each flocculant concentration (Sobrero & Ronco 2008). Finally, mutagenicity tests were performed using the Ames test with two types of *Salmonella typhimurium* strains (TA 98 and TA 100).

Statistical procedure

Phase I

To analyse the various responses in phase I, a statistical model based on a multivariate CRD was developed, fulfilling all the assumptions of parametric statistics (Supplementary Figure S1, available with the online version of this paper). The three most effective flocculants were selected by post hoc contrast and mean plots because these tests show factor interactions better than conventional *a posteriori* tests.

Phase II

In phase II, the interactions between the three most representative bio-flocculants and their mixtures (1:1 ratio) were examined using a completely randomised block design (Supplementary Figure S2, available online). The plots of means were maintained as post hoc tests because they show interactions between the factors and responses. In both phases, the data were analysed with the statistical program IBM SPSS Version 22 (IBM Corp.).

RESULTS AND DISCUSSION

Analysis of FTIR spectra

Figure 1 shows the characteristic signals of the tannin extracts used in this research; the region between 1,900 and 400 cm^{-1} is highlighted because it contains signals

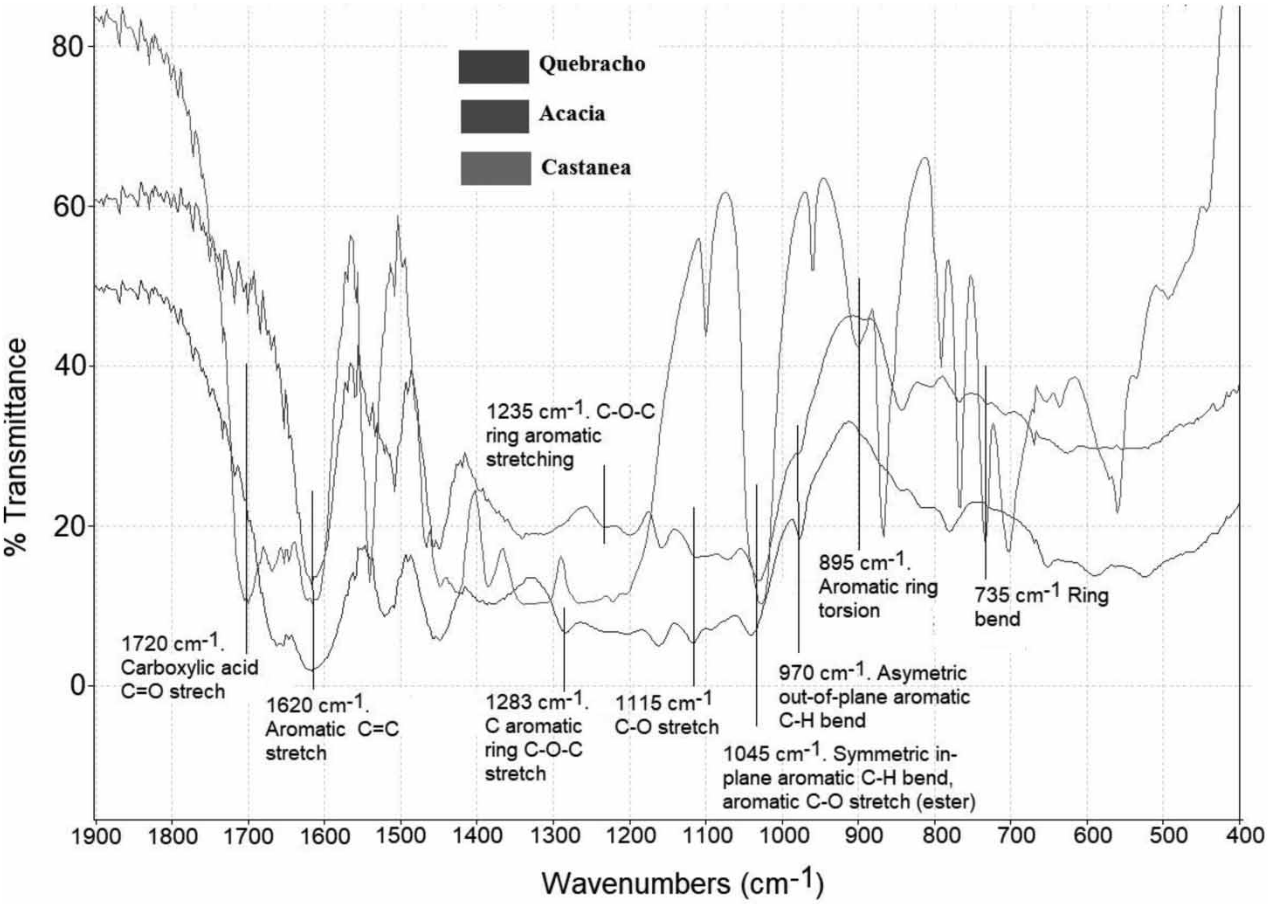

Figure 1 | FTIR spectra of Quebracho, Acacia, and Castanea extracts in the region of 1,500 cm^{-1} to 400 cm^{-1}.

important for the comparison of the extracts used, rather than the region between 3,600 and 2,400 cm^{-1}.

Quebracho and Acacia have a high content of condensed tannins; therefore, the spectra and bands of these extracts are very similar. For example, at 1,115 cm^{-1}, a peak appears, probably due to the C-O stretch of the various hydroxyl groups. At 970 cm^{-1}, bands assigned to the asymmetric bending of C-H aromatic interactions are observed; and in the region of 1,283 cm^{-1}, there is a peak pertaining to the C-O-C stretch of the C ring of the flavonoids that make up the polymer structure of condensed tannins. Unlike the two previous extracts, Castanea is identified as containing a large proportion of ellagitannins such as vescalagin and castalagin (Pash & Pizzi 2002), characterised by having ester functional groups in their structure, which is confirmed by the 1,045 cm^{-1} and 1,720 cm^{-1} bands; these bands relate to the presence of C-O groups and signals generated by the $C=O$ stretch of the carboxyl group, respectively (Tondi & Petutschnigg 2015). Despite the differences discussed above regarding these three extracts, there are signals among them proving their properties as polyphenolic compounds, such as the $C=C$ aromatic stretch vibrations (1,620 cm^{-1}) and asymmetric in-plane C-O bending (1,045 cm^{-1}).

Effects of the bio-flocculants on physicochemical parameters in phase I

During phase I, the effects of nine bio-flocculants on five physicochemical parameters were evaluated. Three of the parameters were used as exclusion variables (turbidity, colour, and pH) because there was a high correlation between some variables (turbidity-TS, and pH-ORP, PEARSON $\alpha = 0.05$, $p = 0.001$). Each parameter was examined in the three wastewater samples described above. Figure 2 shows the turbidity removal rate by each treatment in each application dose. The use of the bio-flocculants resulted in removal efficiency values of 20–90% for washing-machine wastewater, 40–99% for the river sample, and 80–99% for simulated wastewater. Likewise,

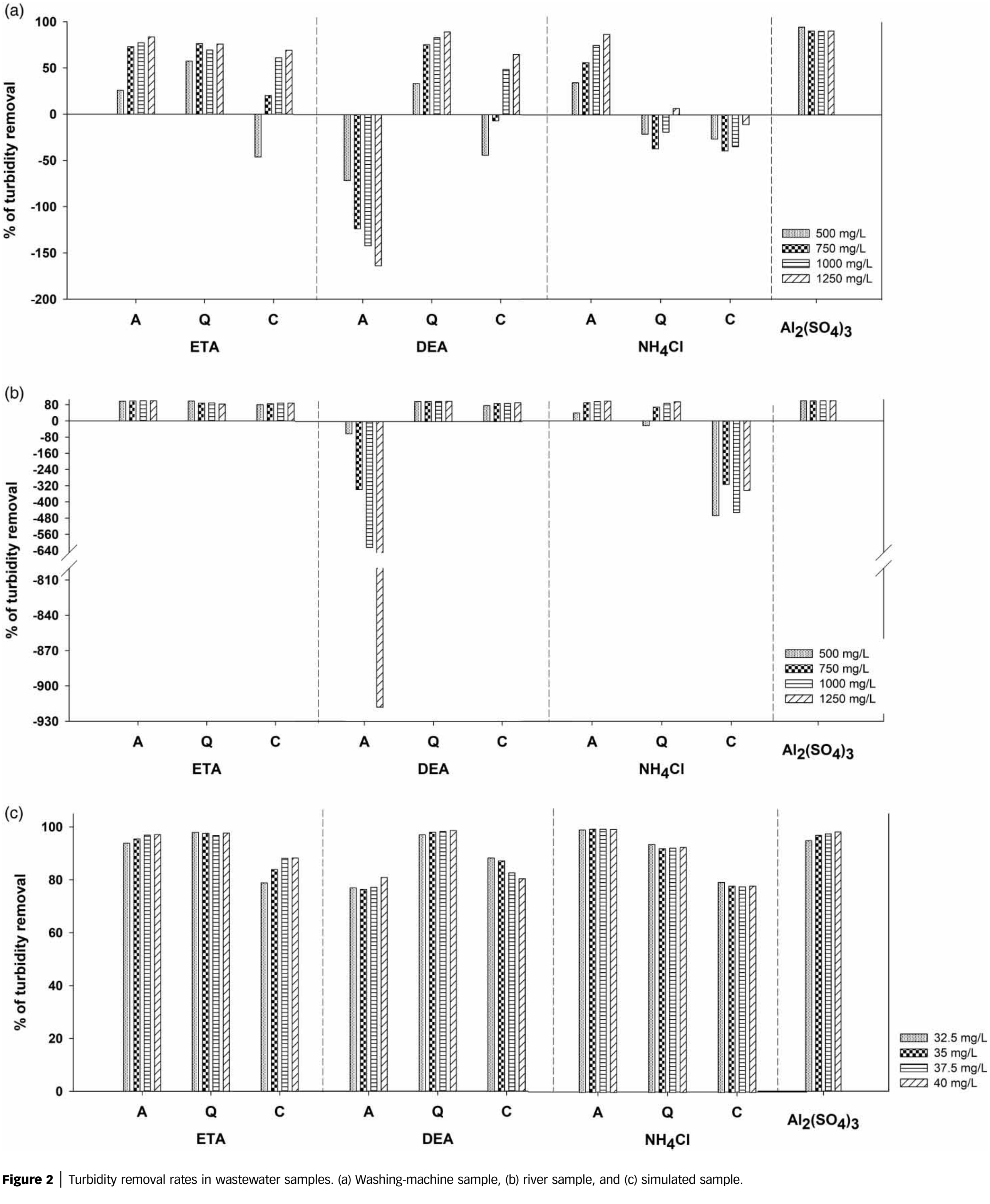

Figure 2 | Turbidity removal rates in wastewater samples. (a) Washing-machine sample, (b) river sample, and (c) simulated sample.

settling velocities were obtained of 1.12–1.76 cm/min (washing machine), 1.15–1.79 cm/min (Salitre River) and 9.5–10.2 cm/min (wastewater simulated). These settling velocities and the elimination of turbidity in natural experiments is due to the cationic centres of modified tannins, which are obtained by the Mannich reaction.

During the first step of the Mannich reaction, the amine and formaldehyde react to generate the iminium ion. This species is added to any phenolic ring (phenoxide ion) of the tannins by replacing a hydrogen in substituting a hydrogen of this aromatic structure. After aminomethylation, acidic conditions must be maintained to generate the protonation of the amine bound to the tannin (Supplementary Figure S3, available with the online version of this paper); this positive nitrogen destabilise the colloidal particles present in the various wastewaters, obtaining very significant turbidity and true colour removal rates.

Apparently aminomethylation does not proceed in the same way or is not as efficient in Castanea extracts, as each treatment has a negative turbidity (between −26 and −470%) removal rate (Figure 2(a) and (b)) because of the increase in the initial conditions of the wastewater samples, as shown in treatments with C-ETA (500 mg/L), C-DEA (500 mg/L), and C-NH_4Cl (all dosages). These also show low true colour removal rates compared with other natural treatments (Figure 3).

The low activity of Castanea is possibly caused by two chemical properties of hydrolysable tannins (ellagitannins): first, the presence of carboxyl or ester groups, which have deactivating electrophilic properties in the aromatic ring that prevent or slow down the reaction of the iminium ion with tannin phenols; and second, the low availability of active hydrogens in polyphenols for their substitution by iminium ions because these 'sites' are occupied by hydrolysable ester bonds in tannins, preventing the electrophilic attack

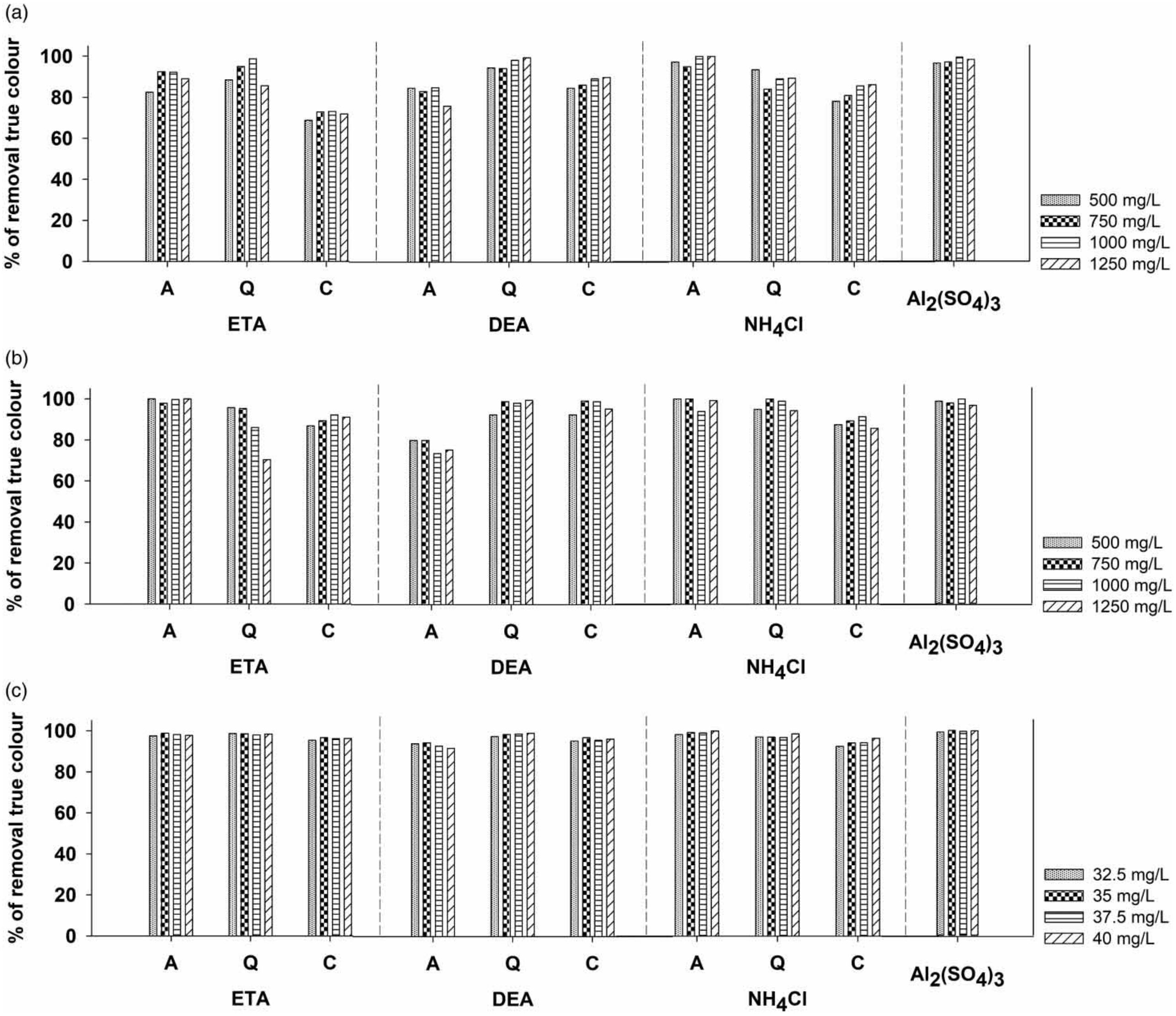

Figure 3 | True colour removal rates in wastewater samples. (a) Washing-machine sample, (b) river sample, and (c) simulated sample.

that would achieve aminoalkylation and protonation (Supplementary Figure S4, available online).

In contrast to the type of tannin content present in Castanea (Chestnut), Acacia and Quebracho have a high percentage of condensed tannins characterised by their polymeric flavonoids. Flavonoid monomers have more active hydrogens available for a possible substitution reaction and do not have electrophilic groups. Possibly because of these reasons, the bio-flocculants Q-ETA, A-ETA, A-NH4Cl, and Q-DEA generated statistically significant results in the removal of turbidity, TS, and true colour in the three samples of wastewater (Tukey and Scheffe $\alpha = 0.05$; $p = 0.001$).

In addition, the last three treatments show statistically similar results compared with aluminium sulphate, as shown in Figure 2(b) and 2(c). The high efficiency of the A-NH_4Cl and Q-DEA modifications are also reported by Beltrán *et al.* (2010), who also ascribe their high potential for destabilisation of colloidal particles to protonation by the Mannich reaction. Although significant removal rates were obtained from extracts containing condensed tannins (Acacia and Quebracho), there were some exceptions in the various treatments evaluated, such as Q-NH_4Cl (dosages: 500, 750 and 1,000 mg/L) and, above all, A-DEA (all dosages). These irregularities and negative percentages of Q-NH_4Cl (between −37 and 19%) and A-DEA (between −920 and −61%) treatments are probably due to the following reasons:

1. Generally, catechin is present as a flavonoid component of the Quebracho and Acacia extracts (Quamme & Kemp 1985; Beltrán *et al.* 2010), but these two plant genera have condensed tannins with different conformations and chemical structures (Venter *et al.* 2012), and these properties could interfere with the aminomethylation reaction. Acacia has units of prorobinetidin-type angular flavonoids, which have more hydroxyl groups compared to the linear profisetinidin present in Quebracho (Supplementary Figure S3); these additional -OH groups in Acacia replace active spaces in the aromatic ring, causing some type of steric hindrance for some larger amines such as diethanolamine, diminishing their action in the addition process.
2. To carry out the Mannich reaction, there are two commonly used sequences in the addition of reagents. The first is the substrate-amine-formaldehyde sequence (procedure A), and the second involves a previous reaction between the amine and formaldehyde and a later interaction with the substrate (procedure B) (Tramontini & Angiolini 1994). Their implementation would ensure that the formation of preformed substances such as imines, which have important electrophilic properties, would generate a high probability of a nucleophilic substitution (S_N2) in tannins and would even prevent the formation of byproducts that can be obtained by using primary and secondary amines (Arend *et al.* 1998). One of these undesirable compounds is the formation of rigid structures or resins generated between condensed tannins and formaldehyde. This happens because the active hydrogens of tannins needed for the aminomethylation reaction are replaced by methylene groups from formaldehyde, preventing a proper Mannich reaction. Additionally, the speed of formation of this byproduct depends on the type of tannin extract used. For example, Acacia generates a more stable resin in less time compared to Quebracho because Acacia has a strongly branched structure and contains angular units (Pizzi 2008). This rapid reaction between Acacia and formaldehyde could prevent aminomethylation and cause low removal rates by the A-DEA bio-flocculant.
3. Kleinman (1991) states that the conditions of a protic solvent (water) and an acid medium (pH 4–2) favour the production of reactive imines from ammonium salts compared with other amines, which would also explain the significant results of the A-NH_4Cl treatment synthesised by procedure B, in which ammonium chloride and formaldehyde were first combined, generating methyleneimine hydrochloride, a compound that is later added to the phenolic rings of tannins (Supplementary Equations 1 and 2, available online).

The decrease in pH caused by the use of aluminium sulphate as a coagulating-flocculating agent is frequently cited in the literature (Cooke *et al.* 1986). This characteristic is due to the production of hydronium ions by the interaction between aluminium sulphate [$Al_2(SO_4)_3$] and water molecules, which can generate sulfuric acid by the presence of additional sulphate ions (Supplementary Equation 3, available online).

Figure 4 shows the decrease in pH caused by an increase in the dose of the positive control, in some cases approaching pH 4. These conditions can cause cellular stress in humans and a high mortality of fish that are in these waters (Boyd 1979). To prevent this, it would be necessary to implement neutralisation and regulation processes of this parameter by the addition of calcium oxide, magnesium oxide, or sodium hydroxide. The contrary happens with natural treatments, in which the pH remained constant or decreased only slightly, staying within the pH range allowed by the respective environmental authorities.

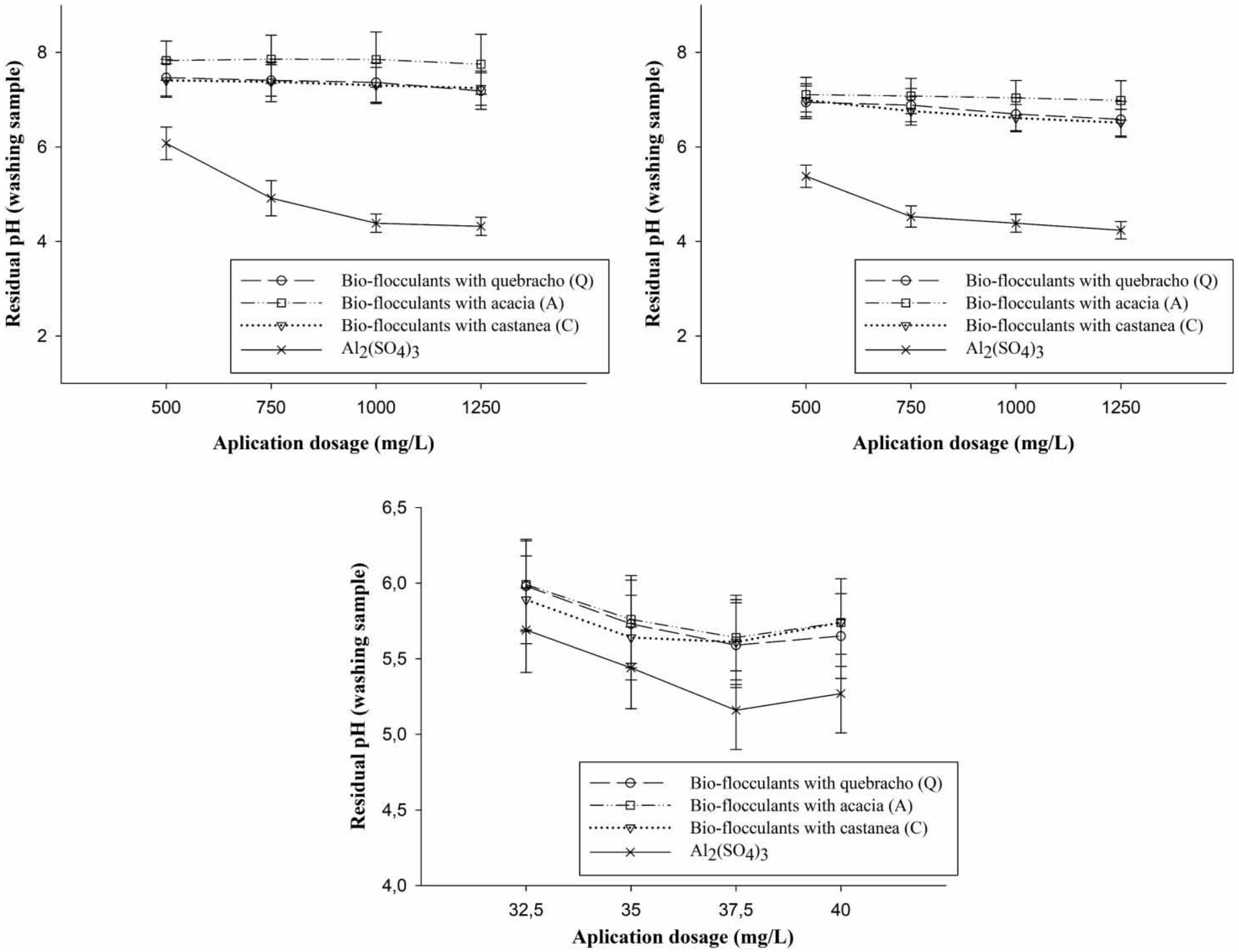

Figure 4 | Mean pH of treatments (modified tannins and positive control) in the different wastewater samples. There are statistically significant differences in the pH change with $Al_2(SO_4)_3$; while tannins are in the same subset (Scheffe $p = 1.000$; $\alpha = 0.01$), showing similar behaviour.

Although hydrochloric acid was used in the chemical modification of tannins, these methods did not affect the final acidity of the treated wastewater because most of the hydronium ions produced reacted or were absorbed by these secondary metabolites. This involved two main routes. The first route produces favourable conditions for the generation of the iminium ion and, later, aminomethylation; the second route imparts a cationic character to the nitrogen attached by the chemical modification process.

Finally, the bio-flocculants A-ETA, Q-DEA and A-NH4Cl and the doses of 1,250 mg/L (washing-machine wastewater and Salitre River) and 40 mg/L (simulated wastewater) were the statistically most significant treatments in Phase I (Tukey and Scheffe $\alpha = 0.05$, $p = 0.000$). From them and their mixtures, a second phase was carried out in which other physicochemical, microbiological and toxic parameters were analyzed.

Evaluation of physicochemical and microbiological parameters (phase II)

Table 3 shows the elimination rates of each test performed in phase II, the mixture of bio-flocculants Q-DEA + A-NH_4Cl showed good results in the elimination of turbidity (9.93 UNT-94.39%), true colour (58 PCU-75.70%) and nitrates (0.00 mg/L – 99.99%) in the sample of washing-machine wastewater. On the other hand, the mixture of A-ETA + A-NH_4Cl remove TS (125 mg/L-68.8%) and the mixture A-ETA+ Q-DEA decreases nitrites (0.027 mg/L-75.7%) in this same wastewater. Regarding the decrease of physicochemical parameters in the water of the Salitre River, treatments that include the bio-flocculant A-NH_4Cl achieved relevant removals in turbidity (98–99%), colour (77–90%), COD (62–72%) and TS (90–95%). At the same time, the treatments that contain the chemical modification

Table 3 | Physicochemical and microbiological residual parameters of the three wastewater samples. For this stage (phase II) the bio-flocculants (A-ETA, Q-DEA, A-NH$_4$Cl), mixtures and statistically significant dosages (1,250 mg/L and 40 mg/L) of the previous phase were used

Treatments	Turbidity (NTU)	Colour (PCU)	COD (mg/L)	Nitrates (mg/L NO$_3^-$)	Nitrites (mg/L NO$_2^-$)	Phosphorus (mg/L P)	ST (mg/L)	*E. coli* (CFU/mL)	TC (CFU/mL)	TH (CFU/mL)	HB (CFU/mL)
	Washing-machine wastewater										
A-ETA	29.87 ± 1.55	117.67 ± 0.58	342.67 ± 0.58	0.00 ± 0.00	0.120 ± 0.026	2.93 ± 0.15	301.00 ± 87.48	1.00 ± 0.00	$8.92 \times 10^{+3} \pm 4.10 \times 10^{+2}$	$9.49 \times 10^{+4} \pm 5.48 \times 10^{+3}$	$7.95 \times 10^{+3} \pm 3.65 \times 10^{+2}$
Q-DEA	14.60 ± 0.70	58.67 ± 3.51	500.33 ± 4.62	6.67 ± 1.55	0.077 ± 0.035	3.27 ± 0.15	350.00 ± 10.00	1.00 ± 0.00	$1.26 \times 10^{+4} \pm 6.00 \times 10^{+2}$	$4.58 \times 10^{+4} \pm 2.10 \times 10^{+3}$	$5.02 \times 10^{+3} \pm 2.30 \times 10^{+2}$
A-NH$_4$Cl	21.27 ± 0.85	78.00 ± 7.00	289.67 ± 5.51	6.40 ± 0.30	0.057 ± 0.006	2.73 ± 0.058	218.33 ± 17.56	1.00 ± 0.00	$1.02 \times 10^{+4} \pm 7.42 \times 10^{+2}$	$1.02 \times 10^{+5} \pm 7.42 \times 10^{+3}$	$6.46 \times 10^{+3} \pm 2.95 \times 10^{+2}$
A-ETA + Q-DEA	21.10 ± 0.20	104.67 ± 6.51	305.67 ± 1.53	0.00 ± 0.00	0.027 ± 0.006	3.07 ± 0.12	160.00 ± 20.00	1.00 ± 0.00	$1.02 \times 10^{+4} \pm 7.42 \times 10^{+2}$	$1.30 \times 10^{+5} \pm 1.05 \times 10^{+4}$	$5.29 \times 10^{+3} \pm 3.07 \times 10^{+2}$
Q-DEA + A-NH$_4$Cl	9.49 ± 1.02	58.00 ± 3.00	257.67 ± 6.51	0.00 ± 0.00	0.043 ± 0.006	3.20 ± 0.20	231.67 ± 3.51	1.00 ± 0.00	$1.30 \times 10^{+4} \pm 1.05 \times 10^{+3}$	$5.59 \times 10^{+4} \pm 3.21 \times 10^{+3}$	$7.20 \times 10^{+3} \pm 4.17 \times 10^{+2}$
A-ETA + A-NH$_4$Cl	9.93 ± 0.15	83.00 ± 3.00	178.00 ± 2.00	0.00 ± 0.00	0.060 ± 0.010	2.87 ± 0.12	125.00 ± 5.00	1.00 ± 0.00	$6.03 \times 10^{+4} \pm 2.80 \times 10^{+3}$	$2.00 \times 10^{+4} \pm 9.00 \times 10^{+2}$	$6.92 \times 10^{+3} \pm 3.15 \times 10^{+2}$
Al$_2$(SO4)$_3$	13.77 ± 0.15	2.33 ± 0.58	93.00 ± 2.00	10.47 ± 0.65	0.063 ± 0.006	0.23 ± 0.058	180.00 ± 10.00	1.00 ± 0.00	$2.00 \times 10^{+3} \pm 9.00 \times 10^{+1}$	$1.50 \times 10^{+4} \pm 8.54 \times 10^{+2}$	$1.02 \times 10^{+3} \pm 7.42 \times 10^{+1}$
	Salitre River										
A-ETA	13.80 ± 0.10	833.33 ± 150.11	476.67 ± 1.16	0.00 ± 0.00	0.013 ± 0.0058	4.70 ± 0.10	130.00 ± 20.0	20.00 ± 0.90	$1.06 \times 10^{+5} \pm 5.13 \times 10^{+3}$	$3.05 \times 10^{+6} \pm 1.81 \times 10^{+5}$	$3.05 \times 10^{+4} \pm 1.81 \times 10^{+3}$
Q-DEA	9.00 ± 0.29	141.67 ± 3.79	498.33 ± 1.53	0.00 ± 0.00	0.033 ± 0.0115	5.23 ± 0.15	90.00 ± 10.0	1.00 ± 0.00	$3.05 \times 10^{+2} \pm 1.81 \times 10^{+1}$	$1.00 \times 10^{+0} \pm 0.00 \times 10^{+0}$	$1.00 \times 10^{+0} \pm 0.00 \times 10^{+0}$
A-NH$_4$Cl	3.15 ± 0.24	29.00 ± 1.00	256.00 ± 6.00	15.20 ± 1.40	0.073 ± 0.0058	5.03 ± 0.15	100.00 ± 10.0	10.02 ± 0.48	$3.42 \times 10^{+2} \pm 1.97 \times 10^{+1}$	$1.05 \times 10^{+6} \pm 5.00 \times 10^{+4}$	$3.98 \times 10^{+5} \pm 1.85 \times 10^{+4}$
A-ETA + Q-DEA	11.40 ± 0.20	399.00 ± 13.00	508.67 ± 24.50	0.00 ± 0.00	0.043 ± 0.0058	4.10 ± 0.40	54.33 ± 4.51	127.67 ± 170.03	$4.58 \times 10^{+2} \pm 2.10 \times 10^{+1}$	$3.05 \times 10^{+6} \pm 1.81 \times 10^{+5}$	$2.61 \times 10^{+5} \pm 9.17 \times 10^{+3}$
Q-DEA + A-NH$_4$Cl	5.41 ± 0.070	69.33 ± 5.51	343.00 ± 5.00	24.20 ± 1.20	0.063 ± 0.0058	5.50 ± 0.50	67.33 ± 10.21	1.00 ± 0.00	$1.81 \times 10^{+2} \pm 1.05 \times 10^{+1}$	$1.14 \times 10^{+6} \pm 5.29 \times 10^{+4}$	$1.50 \times 10^{+4} \pm 8.54 \times 10^{+2}$
A-ETA + A-NH$_4$Cl	2.57 ± 0.15	187.67 ± 11.1	326.67 ± 3.51	0.17 ± 0.15	0.100 ± 0.010	5.07 ± 0.15	44.33 ± 5.13	1.00 ± 0.00	$1.00 \times 10^{+0} \pm 0.00 \times 10^{+0}$	$1.00 \times 10^{+0} \pm 0.00 \times 10^{+0}$	$1.00 \times 10^{+0} \pm 0.00 \times 10^{+0}$
Al$_2$(SO4)$_3$	3.49 ± 0.12	61.33 ± 1.53	50.67 ± 1.53	10.53 ± 0.21	0.073 ± 0.0058	0.12 ± 0.029	80.00 ± 10.0	127.67 ± 170.03	$2.00 \times 10^{+2} \pm 9.00 \times 10^{+0}$	$1.26 \times 10^{+5} \pm 5.20 \times 10^{+3}$	$2.29 \times 10^{+4} \pm 1.05 \times 10^{+3}$
Treatments	**Turbidity (NTU)**	**Colour (PCU)**	**COD (mg/L)**	**Nitrates (mg/L NO$_3$)**	**Nitrites (mg/L NO$_2$)**	**Phosphorus (mg/L P)**	**ST (mg/L)**				
	Simulated wastewater										
A-ETA	30.20 ± 4.30	70.00 ± 1.00	274.67 ± 2.52	0.00 ± 0.00	0.020 ± 0.00	13.40 ± 0.17	185.00 ± 15.00				
Q-DEA	6.81 ± 0.02	29.33 ± 6.43	215.00 ± 1.00	0.00 ± 0.00	0.020 ± 0.01	13.57 ± 0.15	125.00 ± 35.00				
A-NH$_4$Cl	4.12 ± 0.17	23.00 ± 5.00	172.00 ± 2.00	11.07 ± 1.75	0.037 ± 0.0058	13.17 ± 0.15	40.00 ± 10.00				
A-ETA + Q-DEA	4.53 ± 0.32	43.00 ± 1.00	153.00 ± 5.00	4.43 ± 0.00	0.030 ± 0.00	13.13 ± 0.058	161.67 ± 55.30				
Q-DEA + A-NH$_4$Cl	2.48 ± 0.40	24.00 ± 2.00	147.67 ± 6.51	6.87 ± 0.67	0.043 ± 0.0058	12.90 ± 0.10	130.00 ± 10.00				
A-ETA + A-NH$_4$Cl	6.41 ± 3.89	25.33 ± 3.51	184.67 ± 8.51	0.00 ± 0.00	0.040 ± 0.010	13.07 ± 0.058	100.00 ± 20.00				
Al$_2$(SO4)$_3$	9.01 ± 0.26	15.67 ± 1.53	160.00 ± 3.00	0.00 ± 0.00	0.040 ± 0.010	8.70 ± 0.20	25.67 ± 16.92				

Q-DEA (A-ETA + Q-DEA) excelled in the decrease of analytes such as nitrates (99.99%), nitrites (75–80%), and phosphorus (52.5%). Finally, in the sample of simulated wastewater, the bio-flocculant A-NH_4Cl and the mixture Q-DEA + A-NH_4Cl generated decreases in turbidity (6.41 NTU-99%), colour (96%), COD (172 mg/L-70.5%) and total solids (40 mg/L-96.4%). The previous removals of COD and true colour through the bio-flocculants is mainly due to ion-dipole interactions and Yoshida forces between tannins and some dyes or surfactants present in wastewater (Blackburn & Burkinshaw 2002). The first mechanism occurs when there is an interaction between the partially negative oxygen (δ^-) of the -OH groups of tannins and the cationic regions of some surfactants, causing the removal of these pollutants, which would cause a removal in COD because these substances are a source of organic matter. In the second mechanism, a bond is generated between the partially positive hydrogens (δ^+) of the -OH groups of tannins and either the electrons or π bonds found in the aromatic structures of the dyes and surfactants, promoting the flocculation of these substances.

Other substances that contribute significantly to the increase in COD are proteins. These macromolecules represent approximately 60% of the biodegradable organic matter in municipal wastewater (Riffat 2012). These biomolecules can form a complex structure and later be precipitated by interacting with condensed tannins through hydrogen bonds established between the hydroxyl groups of the phenols and the organic groups present in proteins (carbonyl and amide).

For the removal of nitrates and nitrites, other mechanisms could be proposed involving tannin aminomethylation. These polyphenols undergo a structural modification by the Mannich reaction, thus obtaining cationic regions, which subsequently interact with the negative surface charge of suspended particles, causing their precipitation. Nitrates and nitrites present in the three wastewater samples are probably closely related to these suspended particles. In addition, another mechanism that could decrease concentration of nitrates and nitrites is electrostatic interaction that could occur between the negative charge (-1) with the cationic centers that presents the chemical modifications of the tannins (Mannich reaction). Another possible mechanism that could occur in the removal of nitrates and nitrites is ion sweeping or trapping at the time of colloidal agglomeration, precipitating them simultaneously with these larger bodies.

The positive control showed an important role in COD removal and more so in the removal of phosphorus compared with the tannin extracts. This situation is due to the large difference in charge between the phosphate ion and the cationic regions of the bio-flocculants. Although modified tannins are polyelectrolytes, each charge is $+1$ compared to the phosphate ion (a species present at a high concentration in municipal wastewater), which has a charge of -3. To destabilise this anion, similar or stronger electrostatic forces are required (the Schulze-Hardy rule), as is the case of other salts with these same properties, such as aluminium sulphate and iron chloride, which produce Al^{3+} and Fe^{3+} ions.

The antimicrobial and fungicidal properties of tannins have been well studied (Haslam 1996), and as is clear in this study, these properties are still maintained after undergoing a chemical modification and a protonation process. However, some compounds (complex tannins) or amine residues can be a source of food for some heterotrophs and generate rates of growth (between -30 and -40%) or proliferation (Table 3). On the other hand, treatments such as Q-DEA and A-ETA + A-NH_4Cl had good rates of removal of *E. coli* (45–99%), coliforms (43–99%), and heterotrophs (12–26%). This result is mainly due to the elimination mechanisms caused by the electrostatic destabilisation between the cationic centers of the bio-flocculants and the negative charge of the cell surface of most microorganisms. This characteristic is caused by the presence of carboxyl and phosphate groups on cell surfaces (Dworniczek *et al.* 2011; Zarin *et al.* 2016). The previous mechanism can also be presented with the positive control (aluminium sulphate), achieving percentages of decrease of *E. coli* (45–99%), total coliform (42–47%) and heterotrophs (26%).

Toxicity and mutagenicity of bio-flocculants

To determine the level of toxicity and the lethal and sublethal inhibitory concentration (IC_{50}) of coagulants–flocculants, seeds of *Lactuca sativa* were exposed to different concentrations of the treatments. The results of these bioassays showed that aluminium sulphate has a higher sublethal rate (85.14%) from the first concentration analysed (500 mg/L) (Figure 5(a)), compared with the other treatments or bio-flocculants, predicting a possible mutagenic behaviour of the positive control. Regarding the lethal inhibitory concentration (IC_{50}), very similar values were found in most treatments (between 3,000 mg/L and 5,385 mg/l), except for the Q-DEA flocculant, which was 8,830 mg/L (Figure 5(b)).

Although the determination of these inhibitory concentrations is important to establish treatment toxicity, the doses used (32.5–1,250 mg/L) for coagulation-flocculation

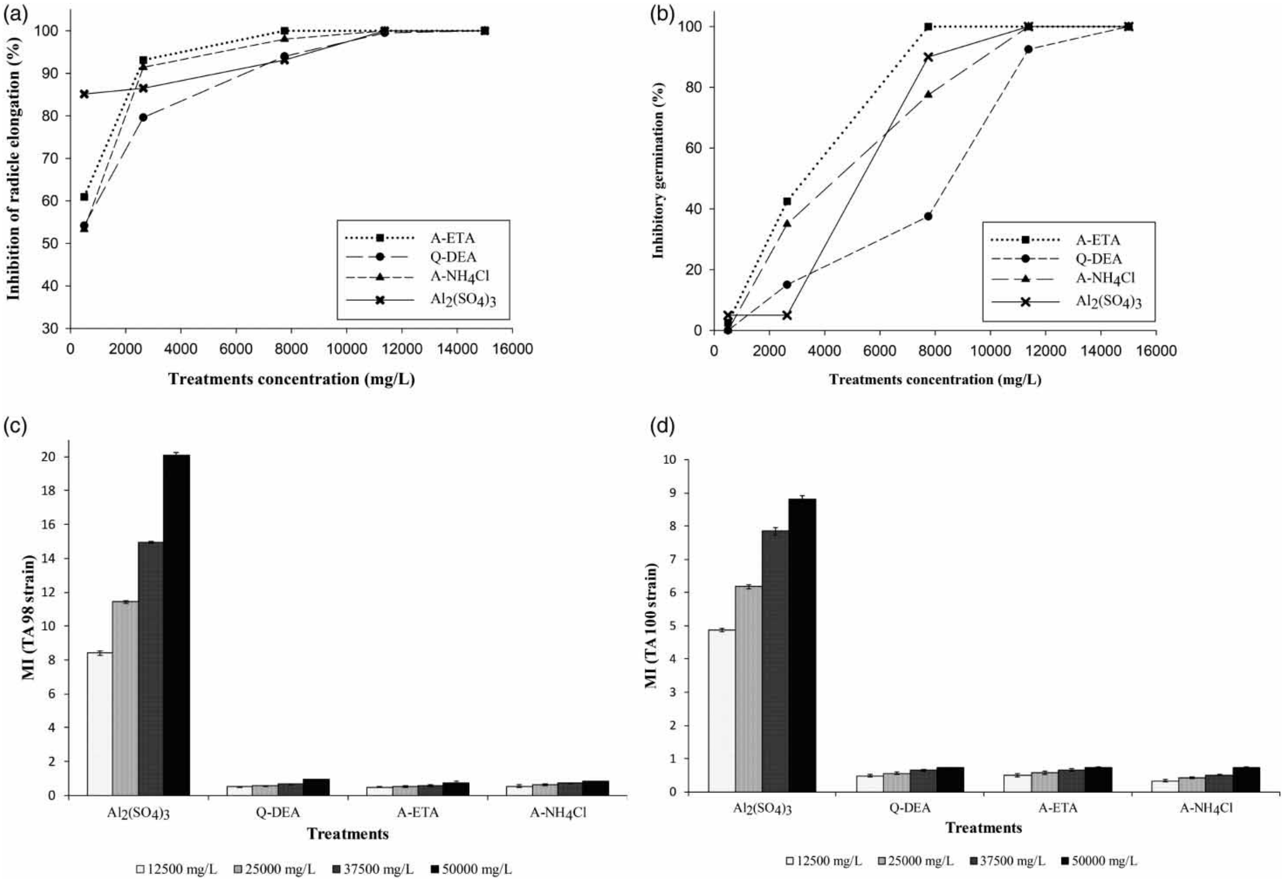

Figure 5 | Sublethal (a) and lethal (b) toxicity tests of the different natural treatments and positive control in seeds of *Lactuca sativa*. Mutagenicity indices obtained from the Ames tests with Salmonella typhimurium strains TA 98 (c) and TA 100 (d). In this test the bio-flocculants are statistically most relevant.

processes in different wastewaters are very low in comparison with those necessary to inhibit germination completely. Radicle elongation processes were delayed or inhibited as a sublethal effect, but this characteristic is more evident in the positive control.

With regard to the mutagenicity of the different treatments, aluminium is one of these mutagenic substances. Al^{3+} is a highly bioactive ion that is easily absorbed by cells and even interacts with DNA, inhibiting the proliferation and repair of this nucleic acid by its interaction with phosphate groups (Lansdown 2013). Several research studies show that some breast cancers could be related to the aluminium content (4–437 nmol g^{-1}) in certain antiperspirants (WHO 1998; Lansdown 2013). A relationship between aluminium concentrations (98 µg/L) in drinking water and long-term cognitive impairment has also been reported (WHO 1998).

Mena *et al.* (2016) propose several categories for the classification of different carcinogenic substances by their mutagenicity index (MI), where an index ≥ 2 represents a mutagenic compound and an MI less than 1.25 characterises a non-mutagenic substance. Figure 5(c) and 5(d) show the MI of aluminium sulphate, which greatly exceeds the MI of 2 from the first concentration, compared to the bio-flocculants, which achieve a maximum MI value of 0.70 in the TA 98 strain and 0.80 in the *Salmonella typhimurium* TA 100 strain, values that are below the minimum MI.

In all the literature analysed and consulted in this research, until now there are no studies evaluating the mutagenicity of bio-flocculants or modified tannins used for the treatment of wastewater. With these results and the removal of different physicochemical parameters, the viability of this new flocculant alternative for the treatment of wastewater is confirmed.

CONCLUSIONS

From the methods of tannin modification used in this study, it was established that the preliminary reaction between formaldehyde and amine (procedure B) is the most suitable because of the production of highly active electrophiles, thus having greater reactivity to generate more aminoalkylation, according to the physicochemical results evaluated with the A-NH_4Cl bio-flocculant.

The treatments carried out with the A-NH_4Cl and Q-DEA modified tannins and 1:1 combinations of A-ETA + A-NH_4Cl and Q-DEA + A-NH_4Cl showed the highest removal rates of the quality parameters evaluated, suggesting that tannins from Acacia (*Acacia mearnsii*) and Quebracho (*Schinopsis balansae*) are the most suitable to reach a pilot scale study and, later, to be applied as primary treatment in wastewater treatment plants (WWTPs).

Bio-flocculants made from tannins, compared to the aluminium sulphate commonly used in WWTPs, showed low levels of mutagenicity and toxicity, ability to stabilise pH, and removal in TS and other physicochemical factors such as turbidity, colour, COD, etc.

ACKNOWLEDGEMENTS

We thank Pontificia Universidad Javeriana, Vice-Rectory of Research for financing the projects, ID 00005874 and 00007223. We also thank Professor Ricardo Vera, Claudia Campos and Alix Loaiza for their support in this project.

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First received 23 March 2018; accepted in revised form 17 July 2018. Available online 30 August 2018