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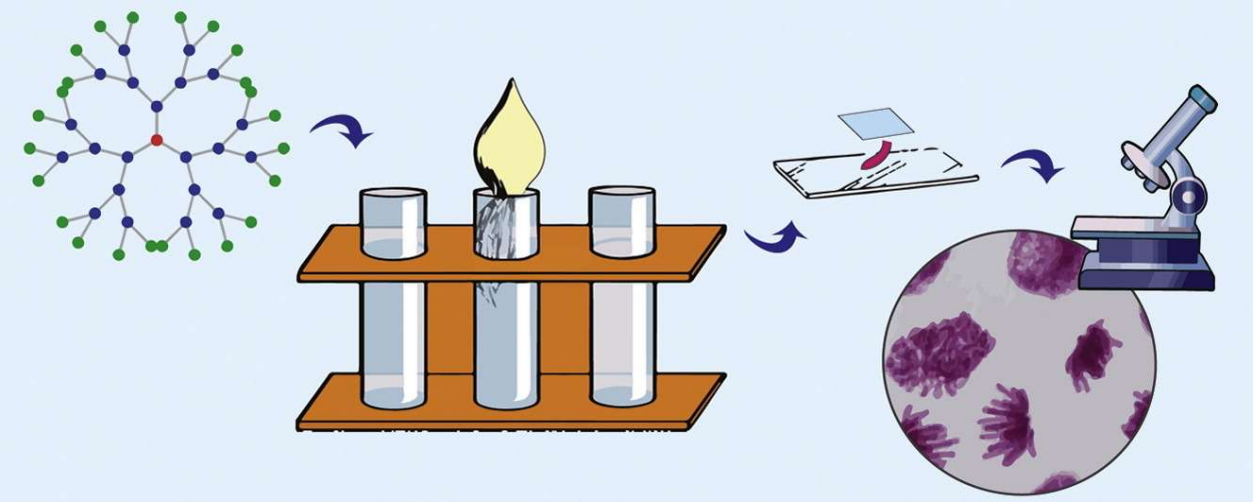

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Toxicological assessment of third generation (G3) poly (amidoamine) dendrimers using the *Allium cepa* test

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Abstract

Despite the expected increase of nanotechnology applications, limited information is currently available on the occurrence, fate and negative impact of engineered nanosized particles in the environment. Plants are an integral and essential part of the ecosystems and their response to nanomaterials exposure is therefore of great interest. In this work, different parameters including root growth, mitotic index and chromosome aberrations were used to estimate the potential ecotoxicity of low generation (G3) hydroxyl- and amine-terminated poly(amidoamine) dendrimers using the *Allium cepa* test. The findings of the present study indicate that both tested dendrimers produce toxic effects in a higher plant system. The analysis of macroscopic parameters, used in testing for general toxicity, revealed reduction of mean root length in bulbs exposed to high concentrations. In parallel, we observed a decrease in the mitotic activity of root meristems which was associated with severe defects in chromosome segregation. Our results may greatly contribute to characterize the toxicological profile and risk of these potentially emerging pollutants in the environment.

Keywords: Poly(amidoamine) dendrimers; Environmental risk assessment; *Allium cepa* test; Phytotoxic effects; Genotoxicity

1. Introduction

The increasing production and use of engineering nanoscale materials in the last few years is raising serious concerns about their safety for human and environmental health. Poly(amidoamine) (PAMAM) dendrimers are nanosized polymers consisting of an alkyl-diamine core, an amidoamine repeat branching structure (generations) and different terminal functional groups (Tomalia, 2005). PAMAM dendrimers with amino ($–NH_2$), carboxyl (–COOH) and hydroxyl (–OH) end groups are emerging as promising nanostructures with a variety of biomedical applications such as drug or gene delivery and diagnostic imaging (Daneshvar et al., 2013, Menjoge et al., 2010). Consequently, the potential adverse effects of these synthetic macromolecules have been studied extensively in recent years (for review, see Jain et al., 2010), although the underlying mechanisms of toxicity and their relevance to human risk assessment are still a matter of debate. There is, however, a general consensus that dendrimers exhibit a concentration and usually generation-dependent cytotoxicity in mammalian cell lines, primarily associated with the increased production of reactive oxygen species (ROS) (Kunzmann et al., 2011). In addition, the cytotoxic effects of PAMAM dendrimers have been linked to their ability to interact with cell membranes and thus, cationic dendrimers have been shown to be more toxic to mammalian cells than neutral or anionic PAMAM dendrimers (Mecke et al., 2005).

In addition to potential hazards to humans, the emerging use of engineering nanomaterials in consumer and industrial products may result in significant negative effects on the aquatic, terrestrial and atmospheric environments (Nowack and Bucheli, 2007). However, limited information is currently available about their environmental occurrence, fate and conceivable ecotoxicity (Kahru and Dubourguier, 2010, Maurer-Jones et al., 2013). In particular, only a few recent studies have evaluated the adverse effects of PAMAM dendrimers in model organisms representing different trophic levels of aquatic ecosystems (Gonzalo et al., 2014, Ulaszewska et al., 2012). Much less consideration has been given to the toxicological evaluation of these polymeric nanostructures on terrestrial plant systems, except for a recent study by Santiago-Morales et al. (2014) showing that amine-terminated G3 PAMAM dendrimer affects normal seed germination of monocotyledonous and dicotyledonous species, such as *Lolium perenne* (ryegrass), *Lycopersicon esculentum* (tomato) and *Lactuca sativa* (lettuce). Nevertheless, higher plants may represent a potential transport pathway of nanomolecules in the environment, through uptake and

bioaccumulation (Ma et al., 2010, Monica and Cremonini, 2009), and therefore phytotoxicity data will be required for a comprehensive environmental risk assessment of nanomaterials (USEPA, 2007).

In this study, we examined the ecotoxic potential of two commercially available PAMAM dendrimers of third-generation (G3) with hydroxyl (OH) or amino (NH_2) surface groups.G3-OH and G3-NH_2 are representative of neutral (hydroxyl-terminated) and cationic (amine-terminated) molecules due to the lack of ionizable moieties and the partial protonation of the amino surface groups respectively. To investigate the toxic effects of both dendritic polymers, we used the *Allium cepa* test which is considered one of the most efficient approaches to evaluate the toxicity of chemicals and complex mixtures in the environment (Leme and Marin-Morales, 2009). Moreover, recent studies have established the reliability of this short-term plant bioassay for assessing the environmental impact of a variety of engineered nanomaterials, including metallic nanoparticles (De Lima and Fernandes Fraceto, 2014) and multiwalled carbon nanotubes (De Andrade et al., 2014, Ghosh et al., 2011). This work provides new data concerning the toxic effects of PAMAM dendrimers on a higher plant system, that may greatly contribute to characterize the toxicological profile and risk of these potentially emerging pollutants in the environment.

2. Materials and methods

2.1. Characterization of dendrimers

Hydroxyl- and amine-terminated G3 PAMAM ethylenediamine core dendrimers were purchased from Sigma-Aldrich (St. Louis, MO, USA, 20 wt.% in methanol). The test solutions, ranging from 0.9–9 mg/L (0.13–1.30 μM), were prepared fresh in filtered tap water (pH 7.5). Nanoparticle size distributions were obtained using dynamic light scattering in a Malvern Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., Worcestershire, UK). Zeta-potential (ξ) measurements were conducted at 25 °C in pure water and in tap water at the prescribed pH by means of electrophoretic light scattering combined with phase analysis light scattering in the same Zetasizer Nano ZS instrument.

2.2. *Allium cepa* test

The *A.cepa* test that included the analysis of macroscopic and microscopic parameters was performed according to Fiskesjo (1985) with slight modifications. Onion bulbs (*A.cepa* L.; weight, 15–30 g), free from agricultural pesticides and growth inhibitors (kindly provided by Hnos. Aparici y Rosa S.L., Valencia, Spain), were grown in the dark at a constant temperature of 25 ± 0.5 °C in a refrigerated incubator IRE 160 (Raypa, Spain). The bulbs were placed onto cylindrical glass receptacles filled with filtered tap water, which was renewed every 24 h and aerated continuously by bubbling air at a rate of 10–20 mL/min using an aquarium pump (Rena, France). The experiments started when newly emerging roots had reached 15–20 mm in length, using a series of five bulbs for each concentration and control group.

To evaluate root growth inhibition as an index of general toxicity, the bulbs were exposed for 72 h to increasing concentrations of G3 PAMAM dendrimers. Thereafter, the length of the whole root bundle was measured and the results were expressed as a percentage of the control (tap water). Other signs of toxicity such as changes in root consistency and colour and the presence of tumours, hook roots and twisted roots were also examined.

The microscopic analysis included the determination of the mitotic index (MI) and the scoring of chromosome aberrations in ana-telophase cells. Root tips of *A.cepa* bulbs treated with the test solutions for 48 h were excised, fixed in ethanol/glacial acetic acid (3:1, v/v) and kept at 4 °C overnight. After hydrolysis during 15 min in 5 N HCl at room temperature, root tips were stained by the Feulgen reaction and the apical 2 mm were squashed in a drop of 50% acetic acid. One slide was prepared per bulb and MI was calculated as the ratio between the number of cells in mitosis and the total number of cells, by counting 1000 cells per slide. The frequencies of abnormal ana-telophases were characterized in 100 mitotic cells per slide and classified as bridges, laggard or vagrant chromosomes, chromosome missegration and multipolar spindles. The slides were coded and scored blind under bright-field microscopy by at least two observers, using a Leica DMI 3000B epifluorescence microscope (Germany) equipped with an EL6000 compact light source. The images were acquired with a CCD camera Leica DFC310FX and processed using the software Leica Application Suite 3.5.0 and Adobe Photoshop 9.0 (Adobe Systems Inc., USA).

2.3. Data analysis

Statistical analysis, carried out with GraphPad Prism 4.0 for Windows (GraphPad Software Inc., USA), included analysis of variance (ANOVA) with the appropriate post hoc test (Bonferroni), and nonlinear regression using a sigmoidal dose-response model for the determination of the 50% effective concentrations (EC50 values). The level of statistical significance was in all cases $p \leq 0.05$. Each data point represents the arithmetic mean ± standard deviation of at least three independent experiments.

3. Results

3.1. Physicochemical characteristics of G3 PAMAM dendrimers

Particle characterization in terms of hydrodynamic diameter and zeta- potential of G3-OH and G3-NH_2 PAMAM dendrimers is shown in Table 1. Dynamic light scattering (DLS) measurements were performed at 14 mg/L (2.0 μM) and at a higher concentration of 70 mg/L (20 μM). For lower

concentrations, measurements displayed low reproducibility because the refractive index of dendrimers is not different enough to that of water, which limits the sensitivity of DLS for PAMAM dendrimers (Chu, 2008). At 2.0 μM a peak was clearly observed near 3.5 nm, which represented a low percentage of the intensity signal, but dominant in Mie's number distributions. These signals corresponded to the expected size of the dendrimer molecule which is 36 Å according to the manufacturer's information. At higher concentrations, this signal was not observed and the distribution was monodisperse with a diameter of aggregates of about 500 nm. These results are essentially coincident with those reported before (Gonzalo et al., 2014) and are consistent with the presence of a large number of individual dendrimers, which co-occur with larger aggregates.

Table 1. Dynamic light scattering molecular size and ζ-potential determined by electrophoretic light scattering with 95% confidence intervals (pH 7.5).

Physicochemical characteristics of G3 PAMAM dendrimers			
Dendrimer	Mol. weight (g/mol)	DLS size (nm)	ζ-potential (mV)
G3-OH	6940.35	538 ± 97 (at 10 μM)	-3.2 ± 0.4 (pure water)
		3.44 ± 0.36 (at 2.0 μM)	-5.8 ± 0.7 (tap water)
G3-NH_2	6908.84	486 ± 68 (at 10 μM)	+12.6 ± 0.7 (pure water)
		3.87 ± 0.51 (at 2.0 μM)	+10.3 ± 0.5 (tap water)

Zeta-potential measurements were recorded at 10 μM, the lowest concentration which allowed obtaining reproducible data. Amine-terminated dendrimers showed a positive charge due to the 32 tertiary amines forming the dendrimer surface groups, the pK_a of which is near 7 (Tomalia et al., 1990). Hydroxyl-terminated dendrimers bore a slight negative charge in water at pH 7.5, consistent with the absence of protonable amino groups. The salts present in water did not significantly alter the zeta-potential of nanoparticle suspensions, which displayed only slightly lower values with respect to pure water. This is in good agreement with the low content of dissolved salts of local tap water, which correspond to granitic lands.

3.2. Toxicity of PAMAM dendrimers in *Allium cepa* bulbs

The assessment of phytotoxicity was performed macroscopically by measuring the root length after exposure for 72 h to increasing concentrations of PAMAM dendrimers. It should be mentioned that *A.cepa* roots growing in tap water for 72 h (untreated controls) had an average length of 6.8 cm and exhibited normal morphology. As shown in Fig. 1, the mean root length was reduced in the presence of dendrimers compared with the control group, although differences were only statistically significant in bulbs exposed to the highest G3-OH concentration (9 mg/L). The EC50 value for G3-OH was 8.43 mg/L and greater than 9 mg/L for G3-NH_2. Other signs of toxicity, such as changes in form and consistency, were not evident in the growing roots in any of the experimental conditions.

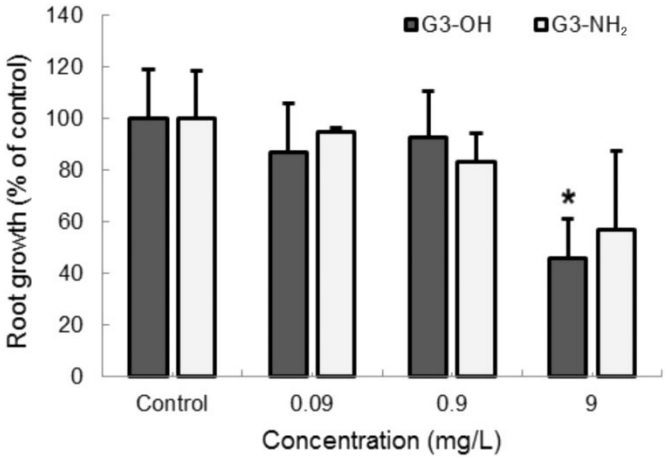

Figure 1. Root growth of *Allium cepa* bulbs exposed to different concentrations of the two tested G3 PAMAM dendrimers for 72 h. Data are expressed as percentage of control values. Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

With the objective of investigating the possible mechanism involved in the reduction of growth rate, we evaluated the mitotic activity that reflects the frequency of cell division. As summarized in Table 2, the mitotic indices of root tip meristematic cells of *A.cepa* declined significantly following a 48 h exposure to the highest concentration of G3 dendrimers compared to the controls. EC50 values for G3-OH and G3-NH_2 were 7.76 and 8.66 mg/L respectively.

Table 2. Mitotic index values in *Allium cepa* root tip cells exposed to increasing concentrations of the two tested dendrimers for 48 h.

Mitotic index (mean ± SD)[a,b]		
Treatment (mg/L)	G3-OH	G3-NH_2
0	100 ± 18.79	100 ± 20.90
0.09	98.98 ± 19.19	97.20 ± 18.95
0.9	100.64 ± 17.90	90.17 ± 21.58
9	38.83 ± 15.22*	45.72 ± 13.62*

[a] Data are expressed as percentage of control values.
[b] Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

3.3. Cytogenetic effects of PAMAM dendrimers on *Allium cepa* meristematic cells

To complete our analysis, chromosome aberrations in anaphase and telophase cells of *Allium cepa* root meristems were scored following a 48 h exposure to the hydroxyl- and amine-terminated G3 PAMAM dendrimers. According to recommendations by Rank and Nielsen (1997), the mitotic index should never be below 50% of the control value in order to obtain a reliable genotoxicity analysis. We thus selected for

subsequent studies the concentrations of polymers that meet this criterion. As depicted in Table 3, G3 dendrimers induced significant chromosome missegregation at the highest concentration tested (0.9 mg/L), being particularly pronounced after exposure to G3-OH which also caused vagrant chromosomes and bridges. Representative micrographs of the most common chromosome aberrations found in *A.cepa* meristematic cells are shown in Fig. 2.

Table 3. Abnormal ana-telophases in meristematic cells of *Allium cepa* following treatments with different concentrations of the G3 PAMAM dendrimers for 48h.

	Abnormal ana–telophases (AT) (mean ± DS)[a, b]					
	Treatment (mg/L)	Bridges	Vagrants	Chromosome missegregation	Multipolar	Total abnormal AT
G3-OH	0	0.99 ± 0.74	0.92 ± 0.60	2.07 ± 0.98	0.06 ± 0.16	3.38 ± 1.75
	0.09	3.44 ± 1.31*	1.84 ± 1.72	3.51 ± 1.78	0.39 ± 0.55	9.18 ± 2.99*
	0.9	3.27 ± 1.50*	4.24 ± 1.75*	12.30 ± 4.23*	0.72 ± 1.01	20.53 ± 5.48*
G3-NH_2	0	1.48 ± 1.09	0.87 ± 0.81	1.17 ± 0.80	0.10 ± 0.24	3.62 ± 1.26
	0.09	2.21 ± 0.65	1.40 ± 0.73	0.92 ± 0.78	n.d.	4.52 ± 0.71
	0.9	2.98 ± 1.42	2.30 ± 2.03	3.75 ± 2.22*	0.22 ± 0.49	9.25 ± 3.17*

n.d.: not detected.
a The frequency of each aberration type is expressed in terms of percentage.
b Asterisks indicate statistically significant differences between treated and control groups ($p \leq 0.05$).

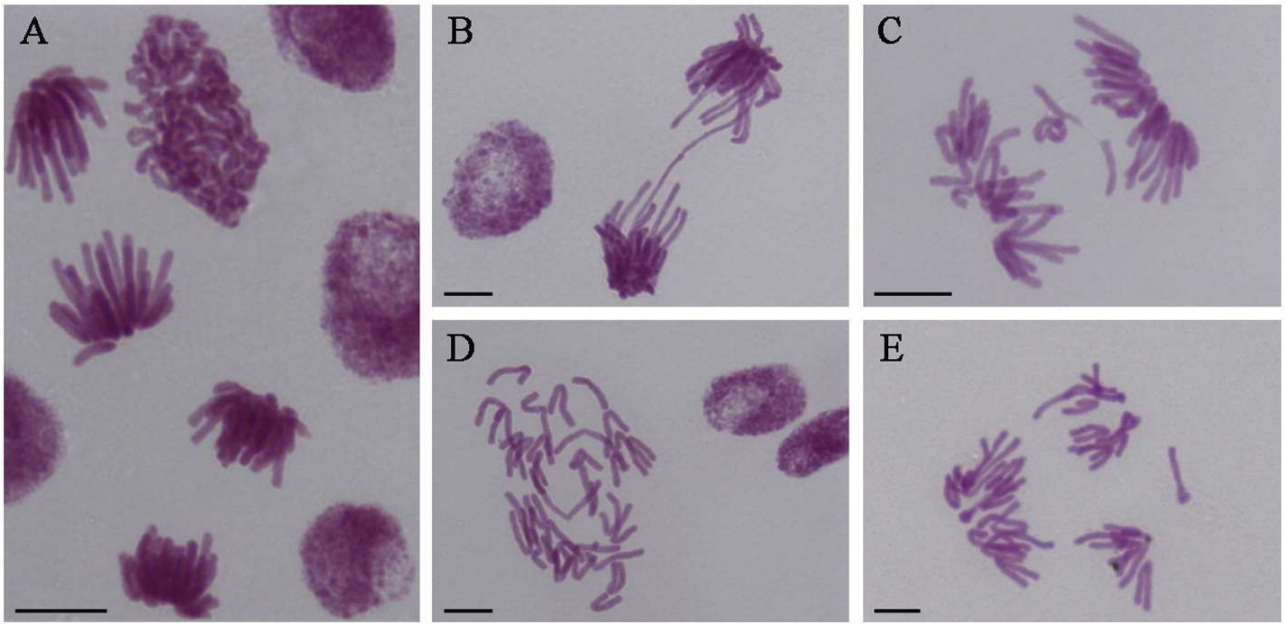

Figure 2. Representative images of different types of chromosome aberrations observed in meristematic cells of *Allium cepa* after a 48 h exposure to G3 PAMAM dendrimers. (A) Normal mitotic stages in untreated root meristems. (B) Chromosome bridge in telophase after treatment with 0.09 mg/L G3-OH. (C) Abnormal anaphase with vagrant chromosomes caused by 0.9 mg/L G3-OH. (D) Chromosome missegregation and (E) multipolar anaphase following exposure to 0.9 mg/L G3-NH_2 and G3-OH respectively. Bar, 10 μm.

4. Discussion

Plant bioassays are essential components of test batteries used for environmental monitoring of unregulated substances and chemical mixtures. However, very limited information is currently available concerning the adverse effects of engineered nanomaterials on terrestrial plant systems. The results presented in this paper provide the first evidence that low generation (G3) hydroxyl- and amine-terminated PAMAM dendrimers can cause adverse effects in the root meristems of *A.cepa*. The root growth inhibition test, commonly used as a quantitative parameter to evaluate toxicity in higher plants, indicated that mean root length was reduced in bulbs exposed to the tested dendrimers. Although the progressive decline in root length is reported frequently in response to various environmental stresses, the underlying mechanisms remain partly elusive. However, it is well established that sustained root growth is mainly regulated by the combined activities of cell division in the meristematic

zone and cell elongation that occurs subsequently in the more proximal regions of the root tip (Shishkova et al., 2008). Based on our results, it is reasonable to suggest that toxicity of G3 dendrimers in *Allium cepa* bulbs is caused mainly from impaired cell proliferation since mitotic activity in the meristems decreased in parallel with root growth. In support of this assumption, the cytogenetic analysis indicated that the reduction in mitotic index values was associated with severe defects in chromosome segregation.

Progression through mitosis is largely dependent on spindle assembly and function which in turn requires regulated microtubule dynamics and the activity of microtubule-associated motor proteins. The chromosome segregation failure in anaphase reflects mitotic spindle disturbances that may result in aneuploidy, while chromatin bridges at telophase may result from sticky chromosomes or clastogenic events (Gisselsson, 2008). On the basis of our results, it appears likely that ana-telophase alterations could be a consequence of mitotic spindle disturbances rather than a result of direct DNA damage, since no chromosome fragments or micronuclei were observed in any experimental condition. Interestingly, emerging evidence indicates that different types of nanomaterials can interact with tubulin and cause spindle dysfunction not only in rodent and human cell lines (Gonzalez et al., 2010) but also in *A.cepa* root-tip cells (De Andrade et al., 2014, Ghodake et al., 2011, Ghosh et al., 2012, Kumari et al., 2011, Pakrashi et al., 2014). The aberrant mitotic figures found under our experimental conditions would therefore be in agreement with these previous findings and strongly suggest that G3 PAMAM dendrimers, like other engineered nanomaterials, may interfere with the proper function of the mitotic spindle. Further studies are underway to confirm this assumption because, to our knowledge, no data are available concerning the influence of these nanosized polymers on the tubulin cytoskeleton, except from a study by Cline et al. (2013) showing that G5 PAMAM dendrimers are able to bind and bundle pre-formed microtubules *in vitro*. In addition, it should be noted that ROS-mediated oxidative stress is now regarded as the primary toxic mechanism of PAMAM dendrimers in both mammalian (Mukherjee and Byrne, 2013, Naha et al., 2010) and ecotoxicity models (Gonzalo et al., 2014, Naha and Byrne, 2013, Petit et al., 2012, Pryor et al., 2014). The possible contribution of ROS to G3 dendrimers-induced toxicity in *Allium cepa* bulbs cannot be dismissed, since redox imbalance in root meristems is commonly associated with reduced cell division and/or cell elongation (Tsukagoshi, 2012, Wolf et al., 2012) as well as with failed chromosome segregation (Livanos et al., 2012).

On the other hand, it has been established that toxicity of PAMAM dendrimers is influenced, among other factors, by the nature of their functional groups (Jain et al., 2010). In most cases, amine-terminated dendrimers exhibit higher toxicity than their hydroxyl-terminated analogues (Greish et al., 2012, Suarez et al., 2011). However, it has been consistently reported that organism or cell specific characteristics as well as toxicity endpoints considered might account for the diverse responses observed in both animal and plant models (Chen et al., 2010, Rico et al., 2011). The results of this study indicate that the two tested dendrimers produce similar toxic effects in a higher plant system and that G3-OH appears even slightly more toxic than G3-NH2, despite being almost neutral at the working pH. Notably, Gonzalo et al. (2014) showed that not only cationic dendrimers but also G4-OH PAMAM dendrimer may cause stressful effects in the green alga *Chlamydomonas reinhardtii*. These results, together with those obtained in the present work, suggest that both cationic and noncationic dendrimers exhibit dose-dependent ecotoxic properties.

In conclusion, our findings provide complementary information regarding the ecotoxicity of PAMAM dendrimers and highlight the importance of assessing the specific modes of action of nanomaterials in higher plant systems, to avoid underestimation of their environmental risks.

Conflict of interest statement

The authors declare no competing financial interest.

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