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Title:

Root-level exposure reveals multiple physiological toxicity of triazine xenobiotics in *Arabidopsis thaliana*

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Summary

Herbicides are pollutants of great concern due to environmental ubiquity resulting from extensive use in modern agriculture and persistence in soil and water. Studies at various spatial scales have also highlighted frequent occurrences of major herbicide breakdown products in the environment. Analysis of plant behavior toward such molecules and their metabolites under conditions of transient or persistent soil pollution is important for toxicity evaluation in the context of environmental risk assessment. In order to understand the mechanisms underlying the action of such environmental contaminants, the model plant *Arabidopsis thaliana*, which has been shown to be highly responsive to pesticides and other xenobiotics, was confronted with varying levels of the widely-used herbicide atrazine and of two of its metabolites, desethylatrazine and hydroxyatrazine, which are both frequently detected in water streams of agriculturally-intensive areas. After 24 h of exposure to varying concentrations covering the range of triazine concentrations detected in the environment, root-level contaminations of atrazine, desethylatrazine and hydroxyatrazine were found to affect early growth and development in various dose-dependent and differential manners. Moreover, these differential effects of atrazine, desethylatrazine and hydroxyatrazine pointed to the involvement of distinct mechanisms directly affecting respiration and root development. The consequences of the identification of additional targets, in addition to the canonical photosystem II target, are discussed in relation with the ecotoxicological assessment of environmental xenobiotic contamination.

Abbreviations

ATZ, atrazine; d, day; DEA, desethylatrazine; F_0 , minimum fluorescence; F_m , maximum fluorescence in dark conditions; F'_m , maximum fluorescence in light conditions; F_v , variable fluorescence; h, hour; HA, hydroxyatrazine; L, length of the primary root; NPQ, non-photochemical quenching; POPs, persistent organic pollutants; ppm, parts per million; PSII, photosystem II; PSI, photosystem I; ROS, reactive oxygen species; RRG, relative root growth.

Keywords

atrazine/desethylatrazine/hydroxyatrazine; chemical injury; chemical priming; herbicide pollution; photosystem II; xenobiotic stress

Introduction

Conventional agriculture uses large amounts of pesticides to enable large-scale crop protection, increase of crop yields and economic viability of agriculture. However, pesticides, including herbicides, also induce chemical stresses that can affect crop growth and development. In general, phytosanitary products, through drift, run-off and leaching, significantly contribute to soil and water pollutions that can affect natural plant communities (Dévier et al., 2011; Helander et al., 2012). Given their dissemination, persistence and accumulation in the environment, pesticides behave as Persistent Organic Pollutants (POPs), which are a cause of concern because of their physico-chemical properties that favor their cellular uptake, consequent intracellular transport, storage in the tissue of organisms, and bioaccumulation through the food chain (Jones and de Voogt, 1999; Nadal et al., 2015). Environmental compartments act as transfer intermediates or as sinks for agricultural POPs, starting from soils surrounding application sites in agriculturally intensive areas, moving through field

margins and ending, *via* airborne or soil processes, in ground waters and streams (Lohmann et al., 2007). These agricultural POPs are indeed detected as micropollutants in surface or ground waters in concentrations ranging from ng/L to µg/L (Luo et al., 2014; Vandermaesen et al., 2016).

Under such conditions of environmental contamination, many different organisms are exposed to variable concentrations of such agricultural POPs, from terrestrial non-target plants to aquatic micro- and macro-flora and fauna (Coutris et al., 2011; Graymore et al., 2001). Given the diversity of pesticide applications, agricultural soil, field margin soil and surface water are commonly contaminated with mixtures of pesticides at various levels, from residual to very high. Besides the chemical complexity of such mixtures, their impact on organisms, and particularly on plants, depends on contamination pathways (spray drift, run-off, leaching), on the plant organ that is initially exposed, as well as on the frequency and duration of the exposure. Plant exposure in field margins often occurs at the root system level. It can also be transient and dynamic, as the composition and the single-component concentrations in contaminating mixtures vary as a consequence of application rate, raining event and geomorphological properties of the field.

Understanding the mechanisms underlying such complex environmental impact requires well-defined experimental procedures focusing on model xenobiotics and model organisms. Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] (ATZ) is one of the most widely applied herbicides worldwide, especially in the USA, Brazil, China and India (Pathak and Dikshit, 2012). The persistence of atrazine in soils and waters has been demonstrated by numerous studies. Its presence is detected in the environment (Table 1) several years after end of use occurs, as in the European Union where it has been banned in 2004. Because of its intensive use, its persistence in the environment and its toxicological effects, atrazine is a typical POP at the global level (Loos et al., 2010). Atrazine pollution can hinder the growth of succeeding crops and poses significant threats to non-target organism and ecosystem health (Sun et al., 2016). Moreover, abiotic chemical reactions, microorganism-mediated processes or plant metabolism reactions lead to the production of several atrazine degradation products, such as desethylatrazine (DEA) and hydroxyatrazine (HA) (Barriuso

and Houot, 1996; Blumhorst and Weber, 1994; Tan et al., 2015). Although these compounds are also detected in the environment (Table 1), few dedicated ecotoxicological studies are available (Ralston-Hooper et al., 2009). In plants, atrazine is known to inactivate the D1 subunit of Photosystem II (PSII), thus leading to interruption of the photosynthetic electron transport chain, photosynthesis inhibition and production of Reactive Oxygen Species (ROS) (Rutherford and Krieger-Liszkay, 2001). Conversely, plants can tolerate herbicide and chemical stress through the induction of antioxidative, repair, or detoxification pathways (Ramel et al., 2007; Sulmon et al., 2006; Tan et al., 2015). Moreover, atrazine degradation products, at low environmental concentrations, can alter plant metabolism and gene expression in the absence of any visible effect on photosynthesis and growth (Serra et al., 2013).

It is therefore essential to understand the processes involved in plant responses to herbicide-related pollution under conditions that take into account the characteristics of environmental exposure: (i) variety of herbicide-related pollutants including degradation products, (ii) range of contamination, from low to high levels, (iii) root system primary exposure, (iv) transient dynamics. For that purpose, we chose to study a coherent chemical series consisting of ATZ, DEA and HA, which, respectively, differ from the parent molecule by the absence of an ethyl group and of chlorine (Fig. 1). Plant responses to increasing doses of ATZ, DEA and HA applied at the root level were comparatively analyzed in seedlings of the model plant *Arabidopsis thaliana*, which has been shown to be highly responsive to pesticides and other xenobiotics (Ramel et al., 2007, 2009, 2012). Plants were exposed during 24 h to concentrations covering the range of triazine contamination detected in the environment, and especially in field margins of agriculturally-intensive areas (Table 1). Analysis of physiological traits aimed to characterize early responses induced by low doses of active ingredient and theoretically non-active metabolites. We found that root-level contaminations of ATZ, DEA and HA could affect early growth and development in a dose-dependent and differential manner. Moreover, the differential effects of ATZ, DEA and HA pointed to the involvement of distinct

mechanisms that remain to be characterized and that may contribute to contrasting consequences on environmental impact.

Material and methods

Plant material and growth conditions

Seeds of *A. thaliana* (Columbia ecotype, Col-0) were surface sterilized in sodium hypochlorite (2.63%):ethanol (1:1, v/v), rinsed in absolute ethanol, and dried overnight. Germination and growth were carried out under axenic conditions in square Petri dishes. Growth medium consisted of 0.8% (w/v) agar in 1X Hoagland basal salt mix (Caisson laboratories, Smithfield, UT, USA) adjusted to pH 6. After seeds were sown, Petri dishes were placed in the dark at 4°C for 48 h in order to break dormancy and homogenize germination, and were then transferred to a control growth chamber at 22°C/20°C under a 16 h light (6000 lux)/8 h dark regime. Alternatively, seedlings were transferred on vermiculite, an inert and hydroponic culture substrate, and grown for a month with 1X Hoagland watering.

Xenobiotic treatments

ATZ, DEA and HA were purchased from Sigma (St. Louis, MO, USA) as analytical standards and used to prepare 10 mM stock solutions. They were added axenically at various concentrations to melted agar-Hoagland medium or to liquid 1X Hoagland watering solution. After growth on xenobiotic-free medium, seedlings were transferred at the end of the night period to fresh agar-Hoagland medium in the absence or presence of various concentrations of xenobiotics. Alternatively, young plants grown on vermiculite were exposed to xenobiotics by watering with xenobiotic-containing Hoagland solution.

Growth parameters

Length of the primary root (L) was measured on vertical plates and Relative Root Growth (RRG) was calculated as $[(L_x - L_0)/L_0]$ with X as days after transfer (T_0). Fresh weight of whole plantlets was determined for individual biomass analysis after measuring all the other parameters.

Chlorophyll fluorescence

Maximum PSII efficiency (F_v/F_m) and Stern–Volmer Non-Photochemical Quenching (NPQ) capacity were measured using a PAM chlorophyll fluorometer system (Heinz Walz, Effeltrich, Germany) in saturating pulse mode. After 30 min of dark adaptation, minimum fluorescence (F_0) of leaves was determined under weak red light, a subsequent saturating pulse of red light was applied, and maximum fluorescence was then measured (F_m). The photochemical yield of open PSII reaction centers, commonly known as PSII efficiency, was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Alternatively, plants were dark-adapted for 30 min, maximum fluorescence (F_m) of leaves was then measured under dark conditions after the saturating pulse was applied, and a further 1 min exposure to actinic light was applied to measure maximum fluorescence under light conditions (F'_m). NPQ was calculated as $NPQ = (F_m - F'_m)/F'_m$ (Bilger and Björkman, 1990).

CO₂ exchange

After 20 days of growth on xenobiotic-free agar-Hoagland medium under 16 h-light photoperiod, seedlings were transferred for 24 h to fresh growth medium in airtight sterile plastic boxes in the absence or presence of varying concentrations of xenobiotics. Day and night CO₂ levels (ppm) were measured with a Micro-GC Agilent 3000 (SRA) equipped with a 6m Poraplot U column and a katharometer.

CO₂ maximal variation was calculated as the difference between the highest and the lowest CO₂ concentration during the day. CO₂ assimilation rate was obtained as $\Delta CO_2/(\Delta t)$, in which $\Delta CO_2 = ([CO_2]_{\text{end of night}} - [CO_2]_{\text{beginning of day}})$, which corresponds to the variation between CO₂ concentration 1 h before light photoperiod start and CO₂ concentration 1 h after daybreak. Night CO₂ production rate was calculated as the ratio between $\Delta CO_2/(\Delta t)$, in which ΔCO_2 corresponds to the variation between CO₂ concentration 1 h before nightfall and CO₂ concentration after 7 h in the dark.

Statistical analysis

Physiological parameters were measured on five independent biological replicates of 10 plantlets for RRG, PSII efficiency, whole plant biomass, and on 25 plantlets for CO₂ measures. Results were given as the mean (\pm SEM) of these determinations. Statistical analysis was carried out with the R version 3.1.3 software. Pairwise comparisons of means used Student's t-test. Correlation analysis was performed with the Spearman correlation test.

Results

Effects of triazine xenobiotics on plant growth

The herbicide ATZ and its metabolites DEA and HA (Fig. 1) were used for a comparative dose–response analysis of triazine impacts on plant growth [relative root growth, biomass], with concentrations from 1 μ M (200 μ g/L) to 20 μ M (4 mg/L), thus covering a wide range of agricultural and environmental situations of triazine presence in soil and water compartments (Table 1). Twenty-four-hour exposures of seedlings at root level were found to affect both root growth and plant biomass with significant differences among the impacts of ATZ, DEA and HA (Fig. 2). Both ATZ and DEA caused a marked inhibition of root growth (Fig. 2A), with IC₅₀ values of between 1 and 5 μ M for ATZ and of 10 μ M for DEA. This inhibition led to complete or nearly-complete root growth arrest at 20 μ M ATZ and DEA (Fig. 2A). In contrast, HA in the 1-20 μ M concentration range showed a weak and partial inhibition of root growth (Fig. 2A). However, whereas there was no observable adverse effect at 1 μ M ATZ or DEA, a partial inhibition of root growth by HA was observed at 1 μ M (Fig. 2A).

Plant biomass was affected by ATZ and DEA in the higher range of concentrations (\geq 10 μ M), whereas HA showed no observable adverse effects up to 20 μ M (Fig. 2B). The contrasted effects of 5 μ M ATZ and 10 μ M DEA on root growth and plant biomass (Fig. 2) indicated that root growth, and therefore probably root meristem activity, was particularly sensitive to root-level exposure to triazines. The absence of HA observable effect on plant biomass showed that partial root growth

inhibition (Fig. 2A) did not affect global plant growth (Fig. 2B) under condition of short-term exposure.

Analysis of relative root growth under long-term exposure confirmed the differential impacts of ATZ, DEA and HA (Fig. 3). Both ATZ and DEA showed a strong effect on root growth over 21 days of growth, with complete root growth arrest after 24 or 48 hours of treatment for both concentrations of ATZ and after approximately 5 days of treatment for 10 μM DEA. The IC₅₀ for root growth at the end of the treatment was lower than 1 μM for ATZ (approximately 90% inhibition at 1 μM ATZ) and 1 μM for DEA. A comparison of Fig. 2 and Fig. 3 clearly shows that the IC₅₀ impact of ATZ and DEA was strongly dependent on the duration of xenobiotic exposure, with for instance a 10-fold increase in sensitivity of root growth to DEA exposure. The slope dynamics of the growth curve showed that, even under conditions of no observable adverse effects in the short term (Fig. 2) and partial inhibition in the long term (Fig. 3), 1 μM DEA progressively affected relative root growth with a very strong decrease from day 0 to day 21 (Fig. 3), thus indicating that long-term or chronic root exposure to DEA can have deleterious effects on plant growth. In contrast, partial inhibition of root growth by HA under conditions of short-term exposure (Fig. 2A) was found to be progressively lifted over the 21-d treatment (Fig. 3), leading to no observable adverse effects of HA on root growth at day 21. Whereas ATZ treatment and DEA treatment showed a progressive decrease of relative root growth, the presence of HA, whether at 1 μM or 10 μM , thus seemed to sustain root growth and therefore probably root meristem activity.

Effects of triazine xenobiotics on photosystem functioning

The impact of ATZ, DEA and HA on photosystem functioning was analyzed in terms of PSII efficiency (F_v/F_m) and non-photochemical quenching (NPQ), which were measured at the end of short-term (24 h) root-level exposure to triazines (Fig. 4), using the same treatments as those of Fig. 2 and 3. PSII efficiency showed, under such conditions of exposure, significant sensitivity to 1 μM ATZ, with however an apparent IC₅₀ greater than 20 μM .

In contrast, DEA and HA showed a positive impact on PSII efficiency, with a slight but significant increase in F_v/F_m values. In particular, DEA seemed to induce a hormetic effect on PSII efficiency in the range 1-15 μM , with a subsequent decrease at 20 μM . Analysis at low (1 μM) and high (10 μM) concentrations revealed that ATZ and DEA, but not HA, had negative effects on NPQ (Fig. 4B). However, ATZ showed a stronger effect, with nearly complete inhibition at 10 μM , whereas 10 μM DEA caused an inhibition of approximately 50%. The different triazines showed specific interactions with photosystem functioning, since ATZ decreased both F_v/F_m and NPQ, DEA slightly increased F_v/F_m and decreased NPQ, and finally, HA slightly increased F_v/F_m without any effect on NPQ.

Root growth and PSII efficiency varied in different ways, depending on the nature of the triazine and on its concentration. Indeed, Spearman correlation analysis of PSII-root growth relationships showed that the inhibitory effect of ATZ on root growth was strongly correlated with inhibition of PSII efficiency (Fig. 5). The significant sensitivity of PSII efficiency to 1 μM ATZ (Fig. 4A) in the absence of an effect on relative root growth (Fig. 2A) further suggested that PSII-root growth correlation of ATZ impact may be ascribed to primary effects on PSII, in accordance with the canonical effect of ATZ on the D1 protein of PSII (Rutherford and Krieger-Liszkay, 2001). In contrast, Spearman correlation analysis showed that, in the case of DEA and HA, the effect on root growth was not significantly correlated with PSII efficiency effects (Fig. 5). Moreover, there was a sharp contrast between the positive impacts of 5 or 10 μM DEA on PSII efficiency (Fig. 4A) and its significant inhibition of root growth at these concentrations (Fig. 2A), thus highlighting that the root-level effects of DEA were disconnected from its action on PSII efficiency. Similarly, the negative effects of HA on root growth (Fig. 2A) contrasted with its positive effects on PSII efficiency (Fig. 4A). Such disconnection may suggest that DEA and HA affect root growth through interaction with non-PSII targets.

Effects of triazine xenobiotics on CO_2 exchange and carbon metabolism

The impacts of ATZ, DEA and HA on CO₂ exchange and carbon metabolism parameters were analyzed in terms of CO₂ consumption and rate of consumption during successive day-night cycles (Fig. 6, Fig. 7), under conditions of short-term (24 h) root-level exposure. Daytime CO₂ maximum variations (Fig. 6) were significantly affected by ATZ and DEA at the 10 μM concentration. In the case of ATZ, this impact on net CO₂ assimilation could be related to direct photosynthesis perturbation highlighted by F_v/F_m and NPQ parameters (Fig. 4). Similarly, the slight effect of low (1 μM) levels of ATZ on PSII efficiency (Fig. 4A) did not result in significant perturbation of CO₂ assimilation (Fig. 6). This relationship was less obvious in the case of DEA, where the effect on CO₂ net assimilation was disconnected from any effect on F_v/F_m (Fig. 4A), even though a decrease of NPQ was observed in the presence of 10 μM of the metabolite (Fig. 4B). Finally, in accordance with the absence of any negative effect on photosystem functioning for both F_v/F_m and NPQ (Fig. 4), HA treatment did not affect CO₂ net assimilation (Fig. 6).

Analysis of the rate of CO₂ net exchanges during successive day-night cycles (Fig. 7) showed that not only photosynthesis, but also respiration, was perturbed by triazines. The IC₅₀ of ATZ on CO₂ assimilation rate was lower than 10 μM, in agreement with NPQ data (Fig. 4). Ten μM DEA resulted in 40% inhibition of CO₂ assimilation rate, in accordance with its effects on NPQ (Fig. 4B), but in contrast with its positive impact on F_v/F_m (Fig. 4A). Moreover, surprisingly, for both ATZ and DEA at 10 μM, parallel and drastic inhibition of night-time respiration was observed, suggesting that mitochondrial processes were affected under such conditions. The negative effects of 10 μM DEA on root growth (Fig. 2A) in parallel with positive effects on PSII efficiency (Fig. 4A) could thus be related with perturbation of night-time respiration, in agreement with the above hypothesis of non-PSII targets for DEA. However, this was not the case for HA, since 1 μM and 10 μM HA treatments showed no significant effects neither on daytime CO₂ assimilation rate, nor on night-time respiration rate (Fig. 7). In contrast with the absence of effects of 1 μM DEA or HA, 1 μM ATZ completely inhibited night-time respiration rate, in the absence of any drastic variation of CO₂ daytime assimilation rate (Fig. 7). This complete inhibition of night-time respiration was therefore much

stronger than the 10% inhibition of PSII efficiency described above (Fig. 2A). This higher sensitivity of night-time respiration to ATZ therefore strongly suggested that there may also be an additional non-PSII ATZ target related to respiratory and mitochondrial processes. Given the effects of DEA described above and the absence of HA effects, the interaction of triazines with such a respiration-related target is likely to require the chloride moiety that is found in ATZ and DEA, but not in HA.

Discussion

Transient root exposure reveals short-term multi-level effects of triazine xenobiotics

The use of atrazine as foliar spray and its direct effects through its PSII target have been well described (Rutherford and Krieger-Liszkay, 2001; El-Lithy et al., 2005). However, the consequences of ATZ exposure at root level may follow distinct dynamics, especially under conditions of short-term or transient exposure. In this study, transient root exposure to variable levels of ATZ, DEA and HA revealed the complexity of triazine impact on several physiological traits of *Arabidopsis thaliana* seedlings. The originality of the present short-term conditions of exposure was reflected by a higher ATZ IC50 for inhibition of root growth, compared to longer-term experiments (Sulmon et al., 2004). These conditions revealed that, after short-term exposure, ATZ had a profound and multiple impact on several physiological parameters leading to a rapid and global effect (Fig. 2) and drastic long-term effects (Fig. 3) on plant growth.

Photosystem functioning was strongly affected by ATZ, with a dramatic decrease of PSII efficiency and NPQ (Fig. 4), and with a high sensitivity of PSII efficiency, thus entailing a rapid translocation from roots to shoots, in agreement with direct radiolabeling experiments (Sulmon et al., 2007). These effects can be directly ascribed to inhibition of electron transport at the PSII level, in agreement with the impact of ATZ on D1 protein (Rutherford and Krieger-Liszkay, 2001), subsequent accumulation of reducing power, and, as outlined by NPQ decrease, possible energy transfer from PSII to PSI

(Frankart et al., 2003). The relationship between F_v/F_m and NPQ variations is complex in the case of xenobiotic stress. Under conditions of paraquat exposure, PSII efficiency decreases, while NPQ greatly increases in relation with energy dissipation (Frankart et al., 2003; Moustaka and Moustakas, 2014). The contrasting effect of ATZ, which leads to NPQ decrease (Fig. 4), as also described in *Lemna minor* (Frankart et al., 2003), may be reminiscent of senescence processes, where both F_v/F_m and NPQ are found to decrease (Nath et al., 2013). Transcriptomic analysis has also shown that ATZ responses involved apoptosis, cell death and senescence mechanisms (Ramel et al., 2007).

Given that D1 protein is the canonical target of ATZ (Rutherford and Krieger-Liszkay, 2001), the high sensitivity of PSII functioning to ATZ (Fig. 4) and the correlation between PSII functioning and root growth (Fig. 5) indicate that the global perturbation of carbon assimilation and plant growth result from the initial impact on PSII. Similarly, in rice, long-term effects of root-level exposure to ATZ on photosynthesis and growth have been ascribed to root uptake from the hydroponic medium and subsequent re-distribution towards the shoots (Su et al., 2005, 2007; Zhang et al., 2014; Tan et al., 2015). However, surprisingly, night-time respiration was also found to be highly sensitive to ATZ (Fig. 7), thus suggesting that interference with mitochondrial processes was also involved. Moreover, DEA caused a significant inhibition of root growth (Fig. 2) in the absence of any decrease of PSII efficiency, thus suggesting that triazine action could entail effects that are independent from PSII efficiency inhibition.

The differential effects of ATZ, DEA and HA reveal novel potential targets related to respiration and root growth

DEA had a profound and varied impact on several physiological parameters leading to rapid and global effects (Fig. 2) and drastic long-term effects (Fig. 3) on plant growth, with however no negative impact on PSII efficiency. In other words, DEA treatment led to the whole range of ATZ-related symptoms, including NPQ decrease, except PSII efficiency inhibition. In contrast, HA treatment led to a very distinct pattern of responses, only consisting of a weak increase of PSII efficiency (Fig. 4)

and a slight decrease of root growth (Fig. 2), thus demonstrating the importance of the chloride moiety in the mechanism of action of triazines.

The negative effects of DEA on NPQ with the parallel increase of PSII efficiency highlighted that, although DEA may interact with PSII, this interaction significantly differed from that of ATZ. Moreover, in parallel with its negative effects on NPQ, DEA was shown to inhibit night-time respiration, as it is also the case for ATZ (Fig. 7). The negative effects of DEA on root growth and plant biomass (Fig. 2) could therefore be related to PSII to PSI energy transfer (Fig. 4) and/or inhibition of night-time respiration (Fig. 7). In the case of ATZ, the lowest-concentration treatment (1 μ M) completely abolished night-time respiration (Fig. 7), while PSII efficiency inhibition was approximately 10% and NPQ was not affected (Fig. 2). This differential impact between respiratory effects and PSII effects could be related to the modality of root-to-shoot exposure. Moreover, direct effects of triazines on mitochondria and energy metabolism have been described in the context of ATZ treatment of mammalian cells (Lim et al., 2009) and of *Chlamydomonas* cells (Esperanza et al., 2015). Transcriptomic analysis of plant-atrazine interactions in *Arabidopsis thaliana* has also shown important changes of energy-related and mitochondrial dysfunction molecular markers, such as the over-expression of fermentation genes (Ramel et al., 2007) and the activation of protein and amino acid catabolism genes (Ramel et al., 2013). The two most highly-induced genes in the presence of ATZ and sucrose (Ramel et al., 2007) were the anoxia-related (Mustroph et al., 2010) gene *Atlg19530* and the carbon-limitation-related (Lam et al., 1998) *At3g47340 ASN1* gene. Similarly, in *Chlamydomonas* cells, the impact of ATZ on mitochondria was associated with induction of amino acid catabolism genes (Esperanza et al., 2015). Finally, mitochondrial dysfunction and energy deficit are often characterized by ROS production (Couée et al., 2006), and indeed, increase of H₂O₂ results from ATZ treatment, even under conditions of absence of effects on PSII functioning (Ramel et al., 2009). It could therefore be hypothesized that the negative effects of ATZ and DEA on night-time respiration could result from an intrinsic action of triazines on mitochondrial respiration rather than from indirect effects of PSII inhibition.

The specific and unique negative effect of HA on root growth was found to occur in the absence of PSII efficiency inhibition, of PSII to PSI energy transfer and of night-time respiration inhibition (Fig. 2, 4, 7). Interestingly, Zhang et al. (2014), using ATZ-resistant transgenic rice plants, where ATZ is actively metabolized to HA, showed that inhibition of root growth was more important in ATZ-resistant plants with high levels of HA than in ATZ-sensitive plants with low levels of HA. Serra et al. (2013) have also described the cryptic effects of low levels of HA on the expression of development-related genes, in the absence of PSII efficiency inhibition. It was therefore reasonable to speculate that HA must act through an alternative mode of action related to root development, such as root meristem activity.

DEA and HA can be considered to be partial types of the complete ATZ structure, with the absence of the ethyl moiety and the absence of the chloride moiety, respectively (Fig. 1). The absence of PSII efficiency inhibition was useful to show distinct perturbations which entailed the potential involvement of other targets, such as mitochondrial respiration for DEA and root development processes for HA. Removal of the ethyl moiety (DEA) and of the chloride moiety (HA) resulted in a sequential simplification of triazine impact highlighting the importance of the chloride moiety and in the disclosure of effects that were otherwise concealed by the inhibition of PSII functioning. Conversely, the complete ATZ structure was likely to interfere with the full range of potential targets, which may explain the intensity and multiplicity of ATZ effects, and the efficiency of its action on plant growth and development. Further work is in progress to clarify this complex situation of multiple triazine targets. An exhaustive transcriptomic analysis should contribute to determine how the distinct patterns of early physiological responses shown in this study may be reflected in differential gene expression patterns and activation of regulatory pathways linked to the initial inhibitory events.

Environmental relevance and risk assessment of short-term multi-level effects of triazine xenobiotics

Pesticide pollutants, including herbicides, are detected in the environment as complex mixtures, whose toxicity on plant communities can result from synergism or antagonism depending on the physicochemical conditions of the mixtures, the combinations of single components and the interactions with the plant compartment (Serra et al., 2013). Several types of plant communities are confronted to root-mediated exposure to herbicide contaminants. In field margins, natural non-target plant populations are exposed to a flux of herbicides or pesticides by direct run-off following field application (Saunders et al., 2013). Moreover, repeated herbicide application results in persistent accumulation not only of active ingredients, but also of metabolites, such as DEA and HA (Jablonowski et al., 2010). The same kind of transient and persistent exposures of roots affect plant communities of the vegetated filter strips that are used at the edge of agricultural fields to attenuate the potential environmental risk of diffuse complex mixtures of pesticides (Marshall and Moonen, 2002; Reichenberger et al., 2007). Finally, sensitive crops that are adjacent to herbicide-treated fields or cultivated in rotational cropping systems that follow herbicide-treated crops can also be subjected to herbicide injury resulting from root exposure (Zhang et al., 2016; Bohnenblust et al., 2016).

Given the diversity of triazine contamination conditions in soil and water environments (concentration range, temporal variations, multiple contamination), the existence of several triazine targets in plants is likely to change the ecotoxicological assessment of environmental pesticide contamination. The existence of three main potential targets of triazines and the differential effects of ATZ, DEA and HA entail that their varying proportions in the soils or waters of contaminated environments may result in contrasted perturbations of either photosynthesis, respiration or root growth. Such complexity of targets for a single family of herbicides must be integrated in the context of ecotoxicological studies of actual soil and water pollutions. Thus, ATZ and HA concentrations that can be found in the environment (Table 1) cover the range of effective levels that can affect multiple physiological targets (Fig. 2; Fig. 3; Fig. 4; Fig. 7). Although environmental concentrations of DEA are generally low (Table 1), specific conditions of ATZ treatment can yield higher DEA accumulation up to 1430 $\mu\text{g}/\text{kg}$ (Albright et al., 2013) and, similarly to HA, DEA has been reported to be a major

metabolite of ATZ *in vivo* biotransformation (Tan et al., 2015). Moreover, the characteristics of microbial metabolization (Jablonowski et al., 2010) and of physico-chemical properties of soils result in varying proportions of ATZ, DEA and HA (Ngigi et al., 2013).

The potential effects of the different situations of environmental ATZ contaminations (Table 1) can range from a major impact on night-time respiration at low environmental concentration to multiple impacts on respiration, photosynthesis, root growth and plant growth at high environmental concentration. The characterization of a significant impact on night-time respiration (Fig. 7) indicates that lower concentrations of ATZ contamination are not innocuous for plant communities, even when the impact on PSII efficiency remains low (Fig. 4). HA environmental range (Table 1) is likely to cause minor physiological effects on root growth and on PSII efficiency. Given the concomitant presence of ATZ and HA contaminants in the environment (Table 1) and in ATZ-treated plants (Tan et al., 2015), further work would be required to investigate potential interferences of ATZ and HA effects on plant communities in the environment. Moreover, under peculiar environmental conditions (Albright et al., 2013; Ngigi et al., 2013), the additional presence of DEA, with its effects on root growth, NPQ and night-time respiration, should also be taken into account as another level of potential interference. Finally, the relationships of triazine impacts with low energy stress, apoptosis and senescence indicate that modulation of environmental stress responses by short-term exposure to triazines could play a role in plant community dynamics, with potential repercussions at higher levels of ecosystem functioning (Alberto et al., 2016).

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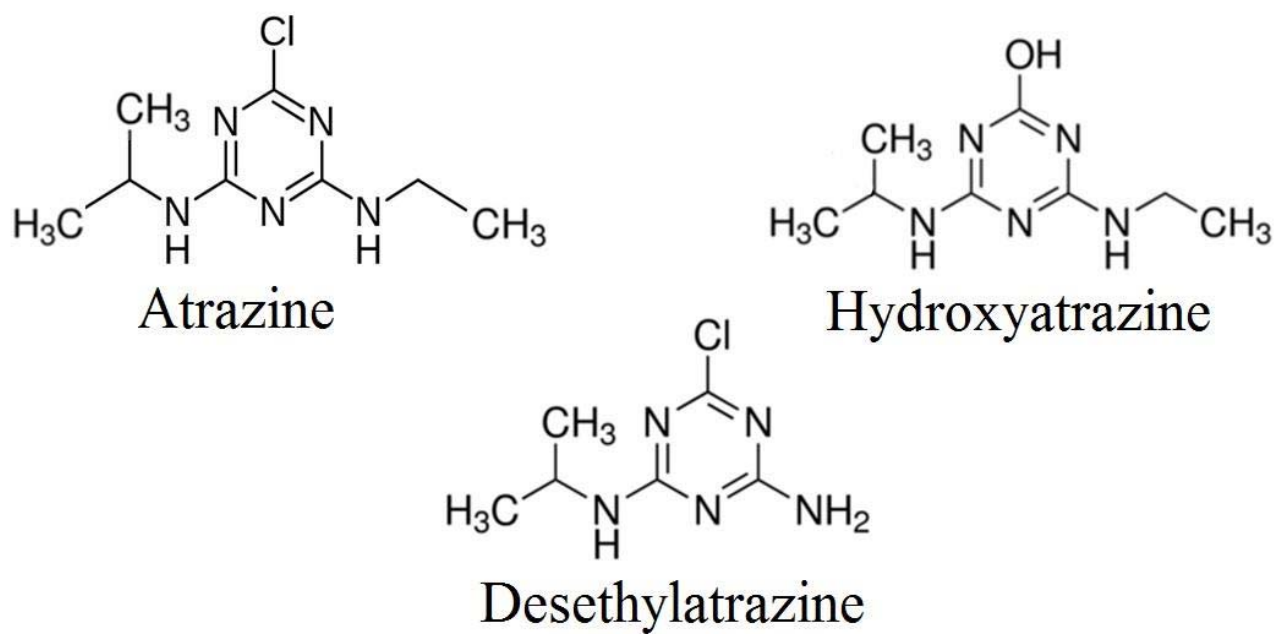


Figure 1. Chemical structure of atrazine (ATZ), desethylatrazine (DEA) and hydroxyatrazine (HA).

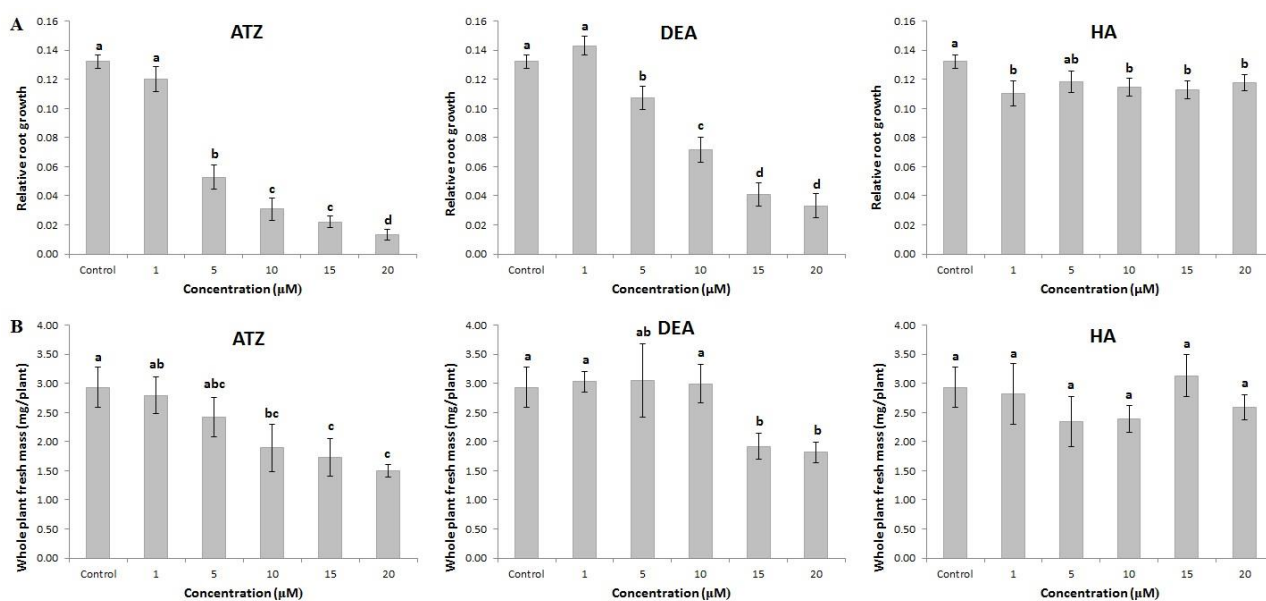


Figure 2. Short-term effects of ATZ, DEA and HA on relative root growth and plant biomass. *Arabidopsis thaliana* seedlings were grown for 10 days in the absence of xenobiotics and then transferred to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Values (mean \pm SEM) of the parameters (A: relative root growth; B: plant biomass) measured after 24 h transfer are shown. Statistical analysis between means was carried out using Student t-test. Different letters above bars indicate statistical significance of differences ($P \leq 0.05$) between concentrations.

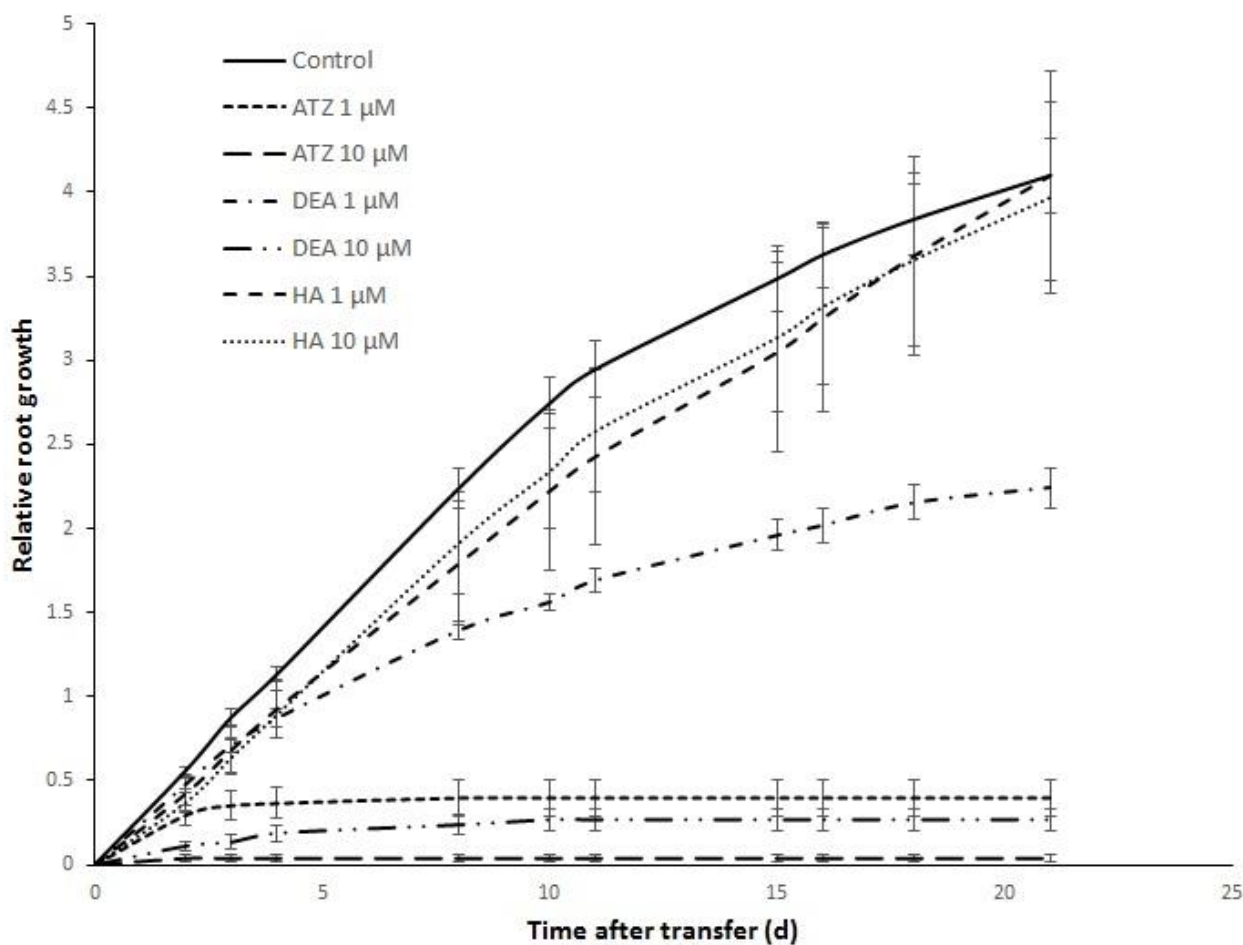


Figure 3. Long-term effects of ATZ, DEA and HA on root growth. *Arabidopsis thaliana* seedlings were grown during 10 days in the absence of xenobiotics and transferred to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Values (mean \pm SEM) of root growth have been measured during a 21-day follow-up.

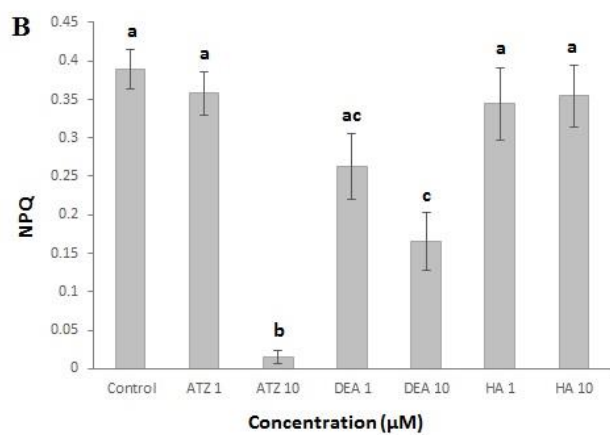
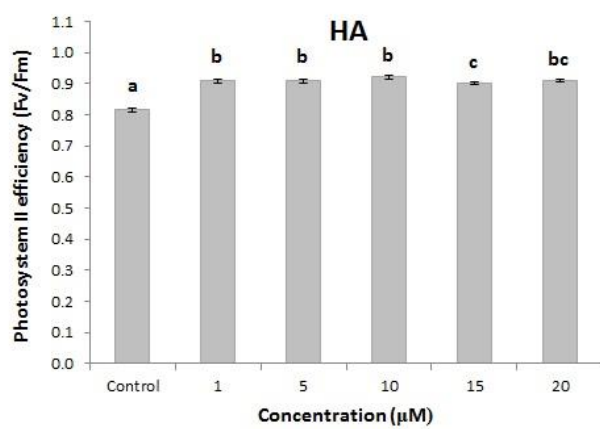
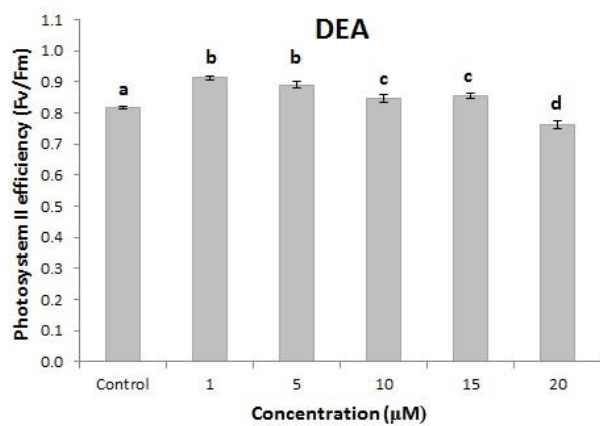
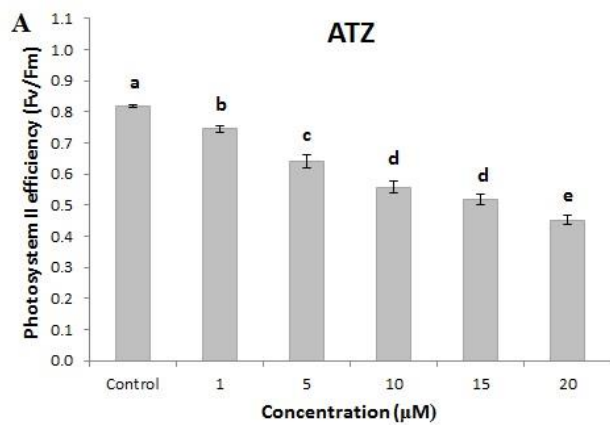


Figure 4. Short-term effects of ATZ, DEA and HA on PSII efficiency (F_v/F_m) and NPQ. *Arabidopsis thaliana* seedlings were grown for 10 days in the absence of xenobiotics and then transferred to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Values (mean \pm SEM) of F_v/F_m measured after 24 h transfer are shown (A). NPQ was measured on leaves of *Arabidopsis thaliana* plantlets grown on vermiculite in the absence of xenobiotics during 40 days and then watered with solutions containing varying concentrations of ATZ, DEA and HA. Values (mean \pm SEM) of NPQ measured 24 h after xenobiotic exposure are shown (B). Statistical analysis between means was carried out using Student's *t*-test. Different letters above bars indicate statistical significance of differences ($P \leq 0.05$) between treatments.

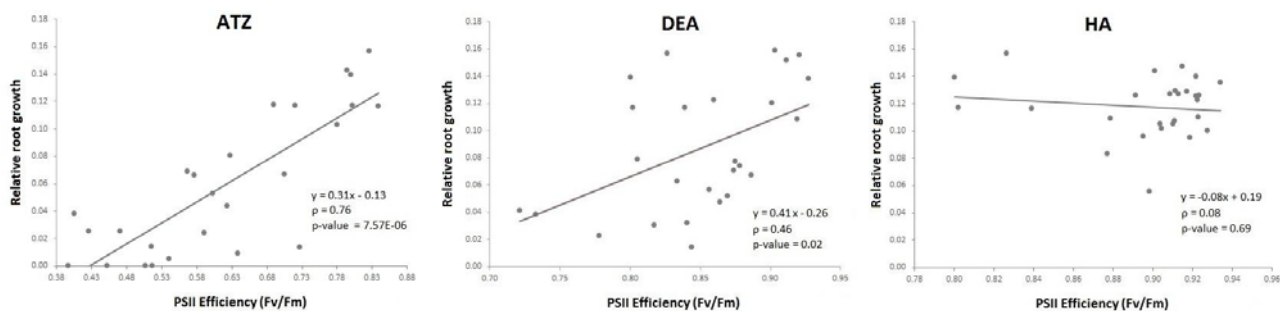


Figure 5. Correlation analysis of Relative Root Growth (RRG) and PSII efficiency (Fv/Fm). *Arabidopsis thaliana* seedlings were grown during 10 days in the absence of xenobiotics and then transferred for 24 h to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Regression equation, Spearman correlation coefficient (ρ) and p-value are shown.

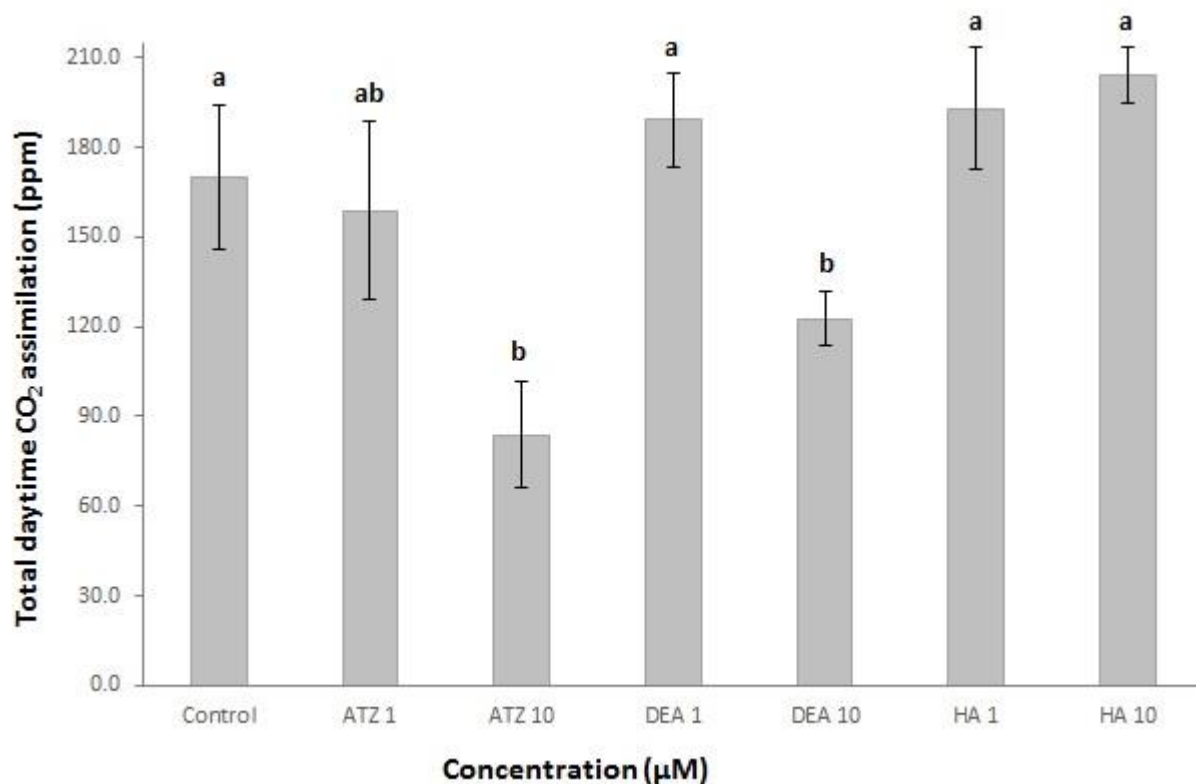


Figure 6. Effects of ATZ, DEA and HA on CO₂ daytime variations. *Arabidopsis thaliana* 20-day-old seedlings were transferred to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Values (mean ± SEM) of daytime CO₂ maximal variations are shown. Statistical analysis between means was carried out using Student's *t*-test. Different letters above bars indicate statistical significance of differences ($P \leq 0.05$) between treatments.

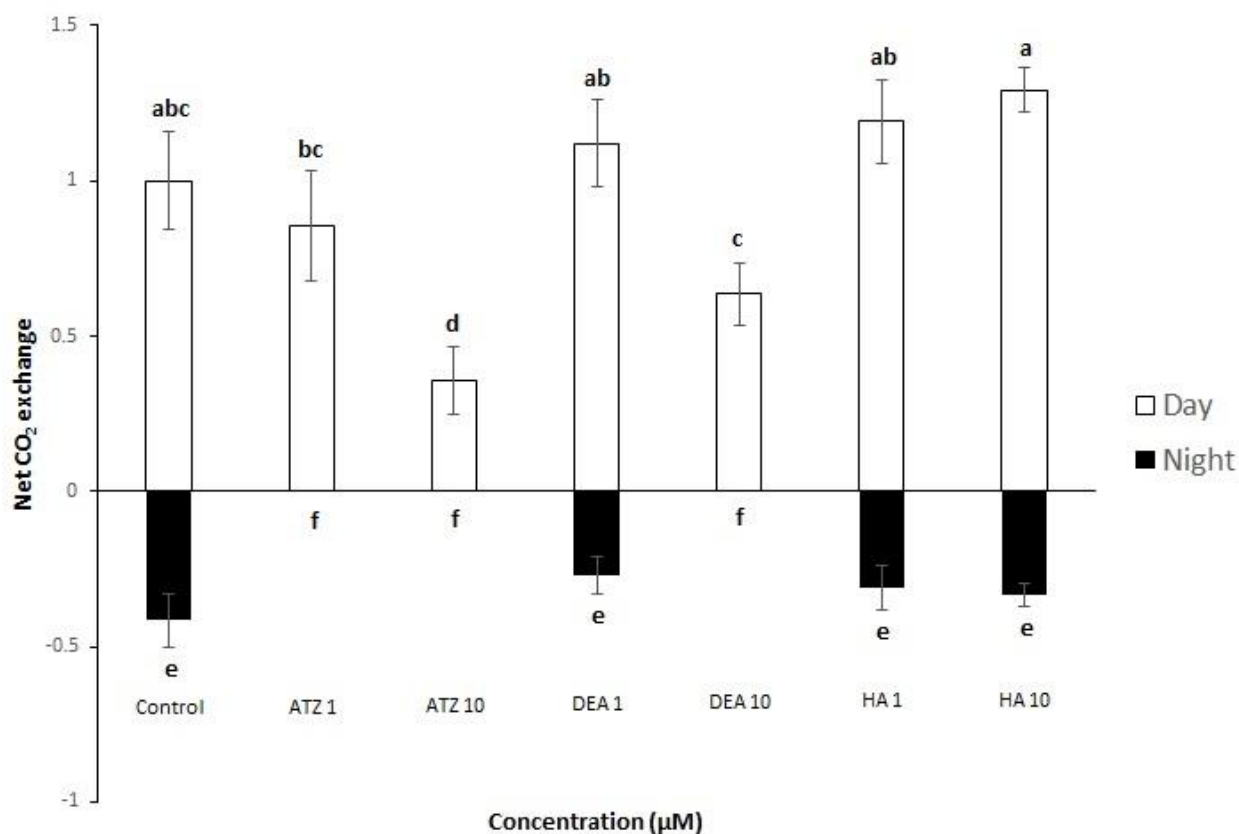


Figure 7. Effects of ATZ, DEA and HA on net CO₂ exchanges. *Arabidopsis thaliana* 20-day-old seedlings were transferred to fresh growth medium in the presence of varying concentrations of ATZ, DEA and HA. Values (mean ± SEM) of daytime CO₂ assimilation rate (in white) and night-time CO₂ production rate (in black) were determined as described in Material and methods. Statistical analysis between means was carried out using Student's *t*-test. Different letters above bars indicate statistical significance of differences ($P \leq 0.05$) between treatments.

Table 1. Environmental concentrations of ATZ, DEA and HA in soils, waters and sediments.

	ATZ	DEA	HA	References
Expected environmental concentration in water or soil	2667 µg/L 2667 µg/kg			Peterson et al., 1994
Atrazine spray drift-related expected environmental concentration in water or soil	266.7 µg/L 266.7 µg/kg			Peterson et al., 1994
Post-atrazine-treatment agricultural soils (dry weight)	9.9 - 1320 µg/kg	3.4 µg/kg	14 - 640 µg/kg	Lerch et al., 1999; Jablonowski et al., 2010
Post-atrazine-treatment agricultural soil water	Up to 1795 µg/L	Up to 41 µg/L		Vryzas et al., 2012
Soils in agricultural margins (fresh weight)			140 - 160 µg/kg	Serra et al., 2013
Post-atrazine-treatment agricultural sediments (dry weight)	0.25 - 5.6 µg/kg	0.83 µg/kg	11 - 96 µg/kg	Lerch et al., 1999
Surface waters in agriculture-intensive areas	Up to 1795 µg/L	Up to 41 µg/L	0.2 - 5.7 µg/L	Lerch et al., 1998, 1999; Vryzas et al., 2012
Streams	3.97 - 224 µg/L	7.50 µg/L	Up to 3.72 µg/L	Lerch et al., 1998; Battaglin et al., 2000