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Impacts of agricultural irrigation on nearby freshwater ecosystems: The seasonal influence of triazine herbicides in benthic algal communities

Carmen Lorente a,b, Jesús Causapé c, Ronnie N. Glud b, Kasper Hancke b, Daniel Merchán c, Selene Muñiz a, Jonatan Val a, Enrique Navarro a,b

HIGHLIGHTS

• Agricultural impacts via run off impacted on nearby freshwater algal communities.
• Triazine herbicides impacts changed through the agricultural year.
• Traditional methods do not capture the seasonality of triazine concentrations.
• Algal tolerance informed about the source of herbicides: application vs. background.
• Assessment of herbicide tolerance is a promising monitoring tool.

ABSTRACT

A small hydrological basin (Lerma, NE Spain), transformed from its natural state (steppe) to rain-fed agriculture and recently to irrigation agriculture, has been monitored across four seasons of an agricultural year. The goal of this study was to assess how and whether agricultural activities impacted the nearby freshwater ecosystems via runoff. Specifically, we assessed the toxicity of three triazine herbicides, terbuthylazine, atrazine and simazine on the photosynthetic efficiency and structure of algal benthic biofilms (i.e., phototropic periphyton) in the small creek draining the basin. It was expected that the seasonal runoff of the herbicides in the creek affected the sensitivity of the periphyton in accord with the rationale of the Pollution Induced Community Tolerance (PICT): the exposure of the community to pollutants result in the replacement of sensitive species by more tolerant ones. In this way, PICT can serve to establish causal linkages between pollutants and the observed biological impacts. The periphyton presented significantly different sensitivities against terbuthylazine through the year in accord with the seasonal application of this herbicide in the crops nowadays. The sensitivity of already banned herbicides, atrazine and simazine does not display a clear seasonality. The different sensitivities to herbicides were in agreement with the expected exposures scenarios, according to the agricultural calendar, but not with the concentrations measured in water, which altogether indicates that the use of PICT approach may serve for long-term monitoring purposes. That will provide not only causal links between the occurrence of chemicals and their impacts on natural communities, but also information about the occurrence of chemicals that may escape from traditional sampling methods (water analysis). In addition, the EC50 and EC10 of periphyton for terbuthylazine or simazine are the first to be published and can be used for impact assessments.

1. Introduction

Agriculture uses half of the total land in Europe (Stoate et al., 2009). Traditional agriculture has been replaced by intensive works which maximize the impact on the soil and nearby freshwater ecosystems (De Almeida Azevedo et al., 2000; Loos et al., 2009; Arroita et al., 2013). One way to increase crop production is by implementing irrigation, which affects both physical (altering water flow) and chemical (altering nutrient and pollutant concentrations) conditions in rivers by the runoff of excess waters (Abrahao et al., 2011a, 2011b; Merchán et al., 2013). In the Mediterranean climate irrigation is more intense during spring and summer, in those seasons natural rivers have lower flows so the impacts of the runoff waters from irrigation may be maximized.

This study is focused on the impacts of the triazine family of herbicides which is widely used in Europe. Due to environmental concerns, some triazines have been banned (such as atrazine, simazine and propazine European Commission (SANCO/10496/2003-final), 2003;
European Commission (SANCO/10495/2003-final), 2003). However, these herbicides are still detected in the environment together with the triazines in use nowadays as terbuthylazine. This is due to their long retention time in the soil and aquifers, which leads to perma-
turable leaching and long-lasting levels in different ecosystems even years after their prohibition.

The Lerma basin (within Ebro depression in Spain), recently trans-
formed from its natural state (gypsum soils covered by scrubland and steppe-like vegetation) to rain-fed agriculture, has been monitored during a whole agricultural season (one year). The goal of this study was to assess the impacts of triazines from agricultural runoff on the function and structure of algal benthic communities present in the small creek draining the basin (periphyton). In previous years, the creek contained noticeable concentrations of both atrazine and simazine (banned) and terbuthylazine (still in use). These data are available at the website of the Ebro Hydrological Confederation (www.chebro.es).

Among the various methods and tools available to assess the impact of pollutants, the Pollution Induced Community Tolerance (PICT) approach offers the means to partially isolate and identify the effects of individual toxicants within an ecosystem subjected to multiple stressors. The ration-
ale behind the PICT is that the exposure to a toxicant will eliminate or hinder the success of sensitive species and favor the development of the more tolerant ones (Blanck et al., 1988), and this will be measured as an increase of the community tolerance against such toxicant.

The hypothesis of this work was that the different exposures to the herbicides through the year due to the seasonal agriculture practices would result in changes in the algal community. The sensitivity of the algal communities to the same herbicide in different seasons would depend on the exposure during the growing period. A previous similar seasonal study showed that the structural and functional responses of algal communities to pesticides are likely to reflect past selection pressures (Dorigo et al., 2004).

The sources of herbicides were expected to be: a) the background released from soils (for the two banned herbicides, atrazine and sima-
zine), or b) the direct application on irrigated crops (for terbuthy-
latine). Triazine herbicide main application takes place at the beginning of spring just after the seeding of the summer cereals. The applications can be extended until the plants reach a certain growth stage, about 30 cm tall in the case of corn, which is the main crop of the region. As a result, a peak of herbicide discharge is usually registered in spring and summer in the Ebro basin yearly (www.chebro.es). In the period of study these applications were between the end of April to mid May (information provided by local farmers).

2. Materials and methods

2.1. Study site

The study was carried out in a small agricultural basin, Lerma (7.3 km²) located in the Ebro hydrological basin (north-east of Spain). Here 49% of the area has been transformed from natural steppe to crops dominated by corn (40%) and winter cereals (18%). Agriculture works are the only anthropogenic activity in the watershed, which offers an exceptional opportunity to study the impact of agricultural use of soil in the water quality (Pesce et al., 2008).

2.2. Physicochemical water analysis

Water flow (L/s), temperature (°C) and nitrate concentration (NO₃⁻, mg/L) of the creek were measured in-situ by a water quality sta-
tion (from Geological Survey of Spain, IGME). Water pH was measured in situ periodically with a multi-parameter probe (model Pro-Plus from YSI, USA). The concentration of the six main macro nutrients applied in soil fertilization were analyzed from periodical water samplings (4–6 per season), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S).

2.3. Algal biofilm sampling and analysis

Benthic algal communities grew on artificial substrates placed on a creek downstream of the basin at different agricultural time periods: pre-herbicide application (autumn and winter), mid-herbicide applica-
tion (spring) and post application (summer). Communities grew on art-
ificial substrata (small pieces of methacrylate 7.55 × 2 × 0.15 cm, similar to microscopy glass slides) that were fixed in plastic holders. These were anchored to rocks on the middle of the creek bed at ca. 15 cm depth. The substrates were placed at the same point every season. They were re-
moved when the algal biofilm reached an average thickness of around 0.75 mm and a steady-state fluorescence of photosystem II of 1000 (Fs PAM quantification at constant light, measured by a Mini-PAM of Walz©). This ensured that the toxicity tests were performed on algal bio-
film communities of similar biomass and physical dimensions.

Chlorophyll a content was analyzed with Jeffrey and Humphrey's (1975) method and calculated with the adjusted formula of Ritchie (2006). Taxonomic identification was done in three replicate samples using a light microscope. Cells were counted according to Utermöhl (1958) technique. Diversity indices, Shannon and Weaver (1963) and the inverse of Simpson index, D or 1 − (Lande, 1996) were calculated. The abundance of species in each community was calculated based on the number of individuals of each species and the total number of indi-
viduals in the community. Species representing less than 1% of total abundance were not included in the analyses.

2.4. Herbicide analysis

Two different methods were used to assess the herbicide levels in the water: passive sampling with Chemcatcher® devices and discrete water samples. 1 L of water was collected at the end of each sampling period to analyze the concentration of triazines and 10 other pesticides by chromatography (SBSE/GC/MS/HPLC). Moreover, three passive sam-
ples of Chemcatcher (Kingston et al., 2000) were placed in the creek close to the algal biofilms on the creek (Fig. 1). Each Chemcatcher device consists of a sampler disk (Empore® 3M, SDB-RP, styrene-divinyl-
benzene-reverse phase sulfonated; 47 mm Ø, 145 μm thick, 8 mm pore size) covered by a protective membrane (Supor® 200, PALL, 47 mm Ø, 145 μm thick, 0.2 μm pore size) fitted into a methacrylate holder by grabbing its edges. The disks integrated the pesticides present in the water into its matrix over a certain exposure time, whereas the protective membrane acted as a diffusion-limiting media and minimized bio-fouling due to its low-protein binding properties (Schäfer et al., 2008; Vrana and lan, 2009). The disks and protective membranes were conditioned follow-
ing the procedure of Vermeirssen et al. (2009). The sampling window during winter, spring and summer period was the last 18 days of algal bio-
film incubation in the creek (Fig. 1), while the sampling window during autumn was 60 days (due to problems of access to the sampling site).

At the end of the sampling period, the disks were removed from the holder with forceps and submerged into 7 mL of acetone. The presence of tri-
azines and 10 other pesticides was analyzed following Vermeirssen et al. (2009) at Labagua Laboratories (Alicante-Spain).

2.5. Dose–response test in flow-through artificial channels

The tolerance of periphyton (measured as the effect of triazines on the photosynthetic efficiency) against each herbicide was measured in mesocosm (i.e., flow-through artificial channels) by dose–response test. The concentration of herbicide required to reduce 50% and 10% of the photosynthetic performance of the benthic algal community (EC₅₀, EC₁₀) was used to compare the community tolerance between the dif-
ferent seasons. The three herbicides were provided by Sigma-Aldrich in powder form. The stocks were freshly prepared two days before ex-
perimentation and stored at −20 °C. Terbuthylazine and atrazine stock solutions were prepared with acetone (32 mM) whereas simazine was dissolved in methanol (1 mM) due to its poor solubility in acetone.

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The toxicity experiments were carried out in flow through methacrylate channels (90 cm long and 10 cm wide) connected to separate water reservoirs. Those reservoirs were submerged in a thermostatic bath where the temperature was adjusted to that of the creek (10, 7, 13 and 20 °C for autumn, winter, spring and summer, respectively). Aquarium pumps re-circulated the water from the reservoirs through every channel at 1.3 cm s⁻¹. Every reservoir had the same volume (4 L) of buffer solution MOPS 0.01 nM (3-morpholinopropane-1-sulfonic acid) adjusted to a pH of 7.5 using KOH. Light was provided by fluorescence lamps (Blau aquaristic T5HO, 39 w/10,000°K, 80 μmol photon m⁻² s⁻¹ at the channel surface).

Every experimental setup for terbuthylazine, atrazine or simazine had 6 channels including one control (herbicide free), and 5 different herbicide doses ranging from 20 nM to 8000 nM. Concentration range had 6 channels including one control (herbicide free), and 5 different surface).

Exposure of passive sampling discs on the creek

18 days

- In situ measurements: water T*, conductivity, pH.
- Punctual water sampling for nutrient analysis in the laboratory and pH in situ measurements.
- Punctual water sample to analyze the herbicide concentration.

Fig. 1. Experimental and sampling design.

Table 1
Average dissolved nutrients along the sampling periods (from 4-6 discrete water samples). Temperature (T*) and pH were registered hourly with a probe connected to a data logger. Phosphorus and nitrogen levels were measured as total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN).

<table>
<thead>
<tr>
<th>Season</th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na (mg/L)</td>
<td>439.43 ± 45.17</td>
<td>471.01 ± 18.79</td>
<td>466.33 ± 52.83</td>
<td>465.83 ± 12.39</td>
</tr>
<tr>
<td>K (μg/L)</td>
<td>670.86 ± 167.43</td>
<td>604.21 ± 71.44</td>
<td>647.82 ± 45.77</td>
<td>844.18 ± 128.88</td>
</tr>
<tr>
<td>Ca (mg/L)</td>
<td>11482 ± 31.79</td>
<td>150.71 ± 8.96</td>
<td>142.23 ± 10.78</td>
<td>158.52 ± 5.02</td>
</tr>
<tr>
<td>Mg (mg/L)</td>
<td>101.84 ± 0.97</td>
<td>115.51 ± 5.80</td>
<td>117.70 ± 12.89</td>
<td>127.93 ± 2.47</td>
</tr>
<tr>
<td>S (mg/L)</td>
<td>210.54 ± 27.11</td>
<td>237.99 ± 18.39</td>
<td>240.62 ± 36.54</td>
<td>243.01 ± 18.51</td>
</tr>
<tr>
<td>TDP (μg/L)</td>
<td>7.74 ± 1.54</td>
<td>8.44 ± 1.32</td>
<td>43.81 ± 84.21</td>
<td>115.06 ± 60.86</td>
</tr>
<tr>
<td>TDN (mg/L)</td>
<td>26.80 ± 2.53</td>
<td>26.82 ± 5.01</td>
<td>21.41 ± 2.67</td>
<td>30.99 ± 2.50</td>
</tr>
<tr>
<td>pH</td>
<td>8.50 ± 0.22</td>
<td>8.50 ± 0.28</td>
<td>8.40 ± 0.33</td>
<td>8.20 ± 0.22</td>
</tr>
</tbody>
</table>

2.6. Data analysis

The dose–response curves at 1 h and 2 h were fitted with R software using a specific developed dose response curve (drc package). This package fits the data to a two parameter log-logistic regression model (Ritz and Streibig, 2005). The upper limit of the curve was normalized to 1, and the lower limit to 0. From the curves we estimated the effective concentrations which reduced the photosynthetic yield of the periphyton by 10% and 50% (EC10 and EC50 respectively). The seasonal EC50 values were compared using the CompParm function, included in package drc. This function performs t-test between EC50 values. Ratios (EC50 case A/EC50 case B) were compared with 1 (i.e. the case of no differences between parameters). Those ratios different from 1, with p-values below 0.05 and adjusted using Bonferroni correction for multiple tests, were considered significantly different. The canonical correspondence analysis – CCA – was performed using ade4 package of R software.

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to autumn or winter (total dissolved phosphorus, TDP, Table 1). The concentrations showed an acute increase during summer (×14) compared together with the low retention capacity of the soils temporarily increased and crop irrigation (mainly between April and August). These events to- 
creek had distinct herbicides in the water samples were in disks were calculated as ng/day of exposure while the concentration of 3.2. Herbicide level in the creek 
3. Results  
3.1. Physicochemical water analysis  
The annual water temperature ranged from 4.5 °C to 22 °C while pH remained constant and slightly basic. Most of the nutrient levels were similar during the four studied periods (Table 1). Only phosphorus concentrations showed an acute increase during summer (×14) compared to autumn or winter (total dissolved phosphorus, TDP, Table 1). The creek had distinct flow-peaks during periods of intense rain (autumn) and crop irrigation (mainly between April and August). These events together with the low retention capacity of the soils temporarily increased the base flow from 20 L/s to 60 L/s. The flow peaks provoked the dilution of dissolved substances (i.e. a drop in concentrations) as was the case of nitrate (Fig. 2).

2. Herbicide level in the creek  
The averaged integrated values of herbicides in the passive sampler disks were calculated as ng/day of exposure while the concentration of herbicides in the water samples were in μg/L (Table 2). The total herbicides included other herbicides that appear in the basin as metholachlor. They belong to other chemical families with a different mode of action than triazines and are out of the scope of this study.

3.3. Algal biofilm analysis  
The algal community was mostly composed of pennate diatoms with negligible presence of other groups (Fig. 3). Generally, two species were dominant: *Gomphonema olivaceum* (sp1) during autumn and winter (46 and 38% of total individuals, respectively) and *Achnanthes minutissima* (sp2) during spring and summer (65 and 68%, respectively, Fig. 3). 

Chl a concentrations (a proxy of biomass) of the communities sampled were similar during all seasons. According to diversity indexes, *H* and *T*, algal communities were most diverse during autumn and winter (Table 3). Moreover spring and summer communities were less diverse as more than half of the individuals belonged to only one species (*A. minutissima*) and most of the other species had a small number of individuals (<0.01%).

3.4. Toxicity tests  
According to the EC50 values, benthic algal communities were most sensitive to terbuthylazine followed by atrazine and finally simazine (Table 4). The periphyton tolerance to terbuthylazine changed significantly through the seasons (p < 0.05). The summer algal community was 3 times more tolerant than the winter community and two times more than the spring one, based on the EC50 values (Table 4 and Fig. 4). The EC50 values for atrazine and simazine were more constant through the year, i.e., the community tolerance did not present a clear seasonality. Only the EC50 values for atrazine were significantly higher in summer and in the case of simazine significantly lower in spring. The EC50 values in general are shown, aligned with the trends of EC50 (Table 4).

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Passive samplers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terbutylazine (ng/day)</td>
<td>0.09 ± 0.00</td>
<td>7.79 ± 0.41</td>
<td>0.60 ± 0.25</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>Atrazine (ng/day)</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Simazine (ng/day)</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td>Total triazines (ng/day)</td>
<td>0.09 ± 0.00</td>
<td>14.29 ± 1.41</td>
<td>1.19 ± 0.37</td>
<td>1.58 ± 0.30</td>
</tr>
<tr>
<td>Total herbicides (ng/day)</td>
<td>0.15 ± 0.02</td>
<td>15.39 ± 1.83</td>
<td>82.59 ± 19.54</td>
<td>52.49 ± 15.60</td>
</tr>
<tr>
<td><strong>Water samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terbutylazine (μg/L)</td>
<td>0.011</td>
<td>0.11</td>
<td>0.08</td>
<td>0.095</td>
</tr>
<tr>
<td>Atrazine (μg/L)</td>
<td>0.008</td>
<td>&lt;</td>
<td>0.020</td>
<td>0.012</td>
</tr>
<tr>
<td>Simazine (μg/L)</td>
<td>0.022</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Total triazines (μg/L)</td>
<td>0.041</td>
<td>0.190</td>
<td>0.170</td>
<td>0.198</td>
</tr>
<tr>
<td>Total herbicides (μg/L)</td>
<td>0.190</td>
<td>0.185</td>
<td>0.398</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Fig. 2. Flow and nitrate concentration were registered with an in-situ monitoring system; data plotted are daily averages provided by IGME Institute. The gray-shadowed blocks are the colonization periods of the algal biofilm on the artificial substrata.

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To explore the effect of herbicides on shaping the structure of the benthic algal communities, a multivariate analysis was done. This canonical correspondence analysis (CCA) distributed the species abundance in a multidimensional space according to herbicide tolerance (EC50 values at 1 h and 2 h herbicide exposition) during each season (Fig. 5). Together the first and second axes explained up to 94% of the total variability, meaning that the tolerance level expressed by the communities can be mostly explained by differences in species composition.

4. Discussion

4.1. Seasonal trends in the ecotoxicology of triazines

The sensitivity of benthic algal communities growing downstream of an agricultural basin to an herbicide can change seasonally. Based on the EC50 values the algal community had higher tolerance to terbuthylazine in summer may be due to a greater exposure period (likely due to the application of fertilizers) due to an extra photosynthesis (Hancke et al., 2008) helping the community to overcome the herbicide stress. The N/P ratios were lower during the summer period (likely due to the application of fertilizers) due to an extra input of P. In laboratory conditions high P concentrations seem not to modulate the sensitivity of the periphyton (Pesce et al., 2008). High temperatures and the higher phosphorus concentrations (Table 1) correlate with the highest EC50 values for terbuthylazine at the summer sampling. High temperatures may have stimulated the photosynthesis (Hancke et al., 2008) helping the community to overcome the herbicide stress. The algal communities presented significantly different tolerances (EC50) for terbuthylazine between all seasons with the summer algal community being the more tolerant (Table 4). Higher concentrations of terbuthylazine were expected to be found in the creek that season as it is the post-application period of this herbicide. The herbicide applications in the field would lead to a greater exposure of the algal community via run off. In accord with the PICT rationale, the high community tolerance to terbuthylazine in summer may be due to a greater exposure during that season. Therefore EC50 values suggest that there were differential exposures to terbuthylazine through the seasons, even if the methods used for measuring its concentrations in water failed in catching that seasonality.

The already banned herbicides, atrazine and simazine appeared in low concentrations in the creek during the whole year (Table 2). The exposure of the algal biofilm to these herbicides seems to be constant and low along the year, in agreement with the similar EC50 values measured in the algal communities during all seasons. It has been estimated (based on information gathered from farmers and the total surface of the basin used in the basin during the studied year (1 kg/ha year).

Some natural and anthropogenic variables co-varying with the herbicide exposure may modulate the sensitivity of the periphyton (Pesce et al., 2008). High temperatures and the higher phosphorus concentrations (Table 1) correlate with the highest EC50 values for terbuthylazine at the summer sampling. High temperatures may have stimulated the photosynthesis (Hancke et al., 2008) helping the community to overcome the herbicide stress. The N/P ratios were lower during the summer period (likely due to the application of fertilizers) due to an extra input of P. In laboratory conditions high P concentrations seem not to...
The numerous rain events during spring and irrigation peaks as were registered by nitrate levels (Fig. 2). Water quality monitoring data in www.chebro.es, not shown), support the hypothesis of the occurrence of herbicide pollution at these seasons as indicated by the EC50, even if the measured herbicide concentrations do not correlate with the calculated tolerance values of EC50 for terbuthylazine. As an example, the higher concentration was measured in winter while the higher tolerance was measured in summer, i.e., higher EC50.

We ascribe this discrepancy to the fact that both measuring approaches poorly reflect variability in pollutant water concentrations because of their intrinsic limitations. There are reasons to suspect that herbicide applications in small basins may result in short-time (hours) herbicide discharges, phosphorus inputs (from fertilizers) and high temperatures. The EC50 and EC10 concentrations for terbuthylazine, atrazine and simazine at 2 hour exposure. Error terms are the standard error. The response of the herbicide dose was the yield inhibition on photosystem II measured with fluorescence techniques (PAM). Values significantly different are shown with a different letter in the superscript.

### 4.2. Herbicide exposure vs. measured effects

To assess the in situ herbicide concentrations during the study period we applied a combination of direct water analysis and passive sampling (Table 2). However the expected differences in terbuthylazine concentrations through the year were not registered by any method. The measured herbicide concentrations do not correlate with the calculated tolerance values of EC50 for terbuthylazine. As an example, the higher concentration was measured in winter while the higher tolerance was measured in summer, i.e., higher EC50.

We ascribe this discrepancy to the fact that both measuring approaches poorly reflect variability in pollutant water concentrations because of their intrinsic limitations. There are reasons to suspect that herbicide applications in small basins may result in short-time (hours) herbicide discharges, phosphorus inputs (from fertilizers) and high temperatures. The EC50 and EC10 concentrations for terbuthylazine, atrazine and simazine at 2 hour exposure. Error terms are the standard error. The response of the herbicide dose was the yield inhibition on photosystem II measured with fluorescence techniques (PAM). Values significantly different are shown with a different letter in the superscript.

### 4.3. Impact of herbicides on algal biofilm

The seasonal herbicide physiological changes (i.e. in tolerance to herbicides) were related to changes in the community structure (i.e. changes in algal life forms dominant through the year). The algal species can be classified into: (1) the low-profile guild, consisting of species of short stature, including prostrate, small erect, solitary centric and slow-moving species; (2) the high-profile guild, consisting of species of tall stature including large erect, filamentous, branched, chain-forming, tube-forming, stalked and colonial centric and (3) the motile guild, consisting of fast-moving species (Rimet and Bouchez, 2012).

Species from the high guild disappeared during summer (Gomphonema group, sp1, 18 and 19) together with C. affinis (sp4) and F. capucina (sp19). In fact the abundance of these species was negatively correlated with the EC50 to terbuthylazine and atrazine, i.e. high community tolerances (Fig. 5). Previous studies suggest that each guild responds differently to stress situations; therefore the dominance/absence of a guild can be used to distinguish between different levels and source of disturbance (Berthon et al., 2011). Conversely, the dominant species during spring and summer belonged to the low-profile and motile guild, A. minitissima (sp2), N. lanceolata (sp8), C. microcephala (sp10), Nitzschia palea (sp15), A. pediculus (sp20) and S. brebissonii (sp21). The abundance of these species was positively correlated to the EC50 of terbuthylazine and atrazine i.e. high community tolerances (Fig. 5). The fact that two of these species are known as pioneers, i.e. appearing after chemical disturbances (A. minitissima and N. palea) support the hypothesis of the occurrence of herbicide pollution at these seasons as indicated by the EC50 even if the measured herbicide concentrations do not show it.

### 4.4. Environmental relevance and application of the results

The EC50 calculated for atrazine is within the range of values previously reported for benthic algal communities (measured as EC50 using the same PAM protocol) which range from 400 to 2500 nM (Guasch and Sabater, 1998; Navarro et al., 2002; Ryan and Prosser, 2013). The EC50 and EC10 values for terbuthylazine or simazine are the first to be published. The EC50 concentrations are between 3 and 20 times higher than those of herbicide levels normally found in agricultural watersheds, i.e. from 2 nM to 40 nM (Louchart et al., 2001). The EC10 are close to peak concentration values measured in similar agricultural basins (Louchart et al., 2001). All these findings, together with the methodological limitations of the traditional continuous monitoring programs, support the use of PICT approaches as a useful and powerful monitoring tool for herbicides.

### 5. Conclusions

The periphyton presented seasonal variances in its tolerance against terbuthylazine; higher in summer and lower in winter with intermediate values during spring and autumn. The changes in the tolerance may have been influenced by the combination of factors as: short-time (hours) herbicide discharges, phosphorus inputs (from field fertilization) and high temperatures.

![Fig. 4. EC50 values for each tested herbicide in the different seasons. The EC50 were normalized by the winter values (set as 1).](image-url)
The herbicide levels detected in the creek did not correlate with the seasonality of the community tolerance (EC50), as expected based on the PICT concept. However, they were in agreement with the different potential sources of each herbicide: the punctual use during the agricultural year (for terbutylazine) and the gradual and constant background release from soils in which banned herbicides have been stored upon past use (atrazine and simazine). The presence of pioneer species and absence of species from the high-guild during the period of herbicide application (spring and summer), also supported these findings.

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