Impacts of agricultural irrigation on nearby freshwater ecosystems: the impacts of triazine herbicides in algal biofilms during an agricultural year

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Abstract

A natural basin (Lerma, NE Spain), recently transformed from its natural state (steppe) 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 on the nearby freshwater ecosystems via runoff. Specifically, we assessed the toxicity of three triazine herbicides, terbuthylazine, triazine 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 concentration of the different herbicides in the creek affected the sensitivity of the periphyton in accordance with the rationale of the Pollution Induced Community Tolerance. The effective concentrations values for terbuthylazine and simazine are the first published; in addition their EC\textsubscript{10} are close to concentrations measured in EU rivers. The periphyton presented significantly different sensitivities against terbuthylazine along the year in accordance with the seasonal application in the crops. The tolerance of banned herbicides, atrazine and simazine do not presented 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 the water sampling. Altogether indicates that the use of PICT approaches 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 information about the occurrence of chemicals that may escape from traditional sampling methods as the direct water analysis.
Highlights

1. It has been assessed the agricultural impacts on nearby freshwater ecosystems.

2. Freshwater benthic algal communities have been exposed to herbicides present in runoff waters.

3. Herbicide’s EC_{50} vary according the source: recent application or background release.

4. Traditional water sampling methods (discrete and passive sampling) seem not catch the year variability of such concentrations in small basins.

5. Herbicide tolerance of natural communities complement environmental monitoring.
Agriculture uses half of the total land in Europe (Stoate et al., 2009). The traditional agriculture has been replaced by intensive works which have an impact on the soil and nearby freshwater ecosystem (de Almeida Azevedo et al., 2000; Loos et al., 2009; Arroita et al., 2013). Specifically, artificial crop irrigation 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 artificial irrigation is more intense during spring and summer, at that periods natural rivers have lower flows so the impacts of the runoff waters from artificial irrigation transporting agrochemicals, may be maximized.

This study is focused on the impacts of the triazine family of herbicides which are widely used in Europe. Due to environmental concerns, some triazines have been banned (as atrazine, simazine and propazine,(European commission SANCO/10496/2003-final;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 a perdurable leaching and long-lasting levels in different ecosystems even years after its prohibition.

A natural basin (Lerma, NE Spain), recently transformed from its natural state (gypsum soils covered by scrubland and steppe-like vegetation) to irrigation 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 periphyton communities present in the small creek draining the basin (algal benthic biofilms). In previous years, the creek contained noticeable concentrations of both atrazine and simazine (banned) and the terbuthylazine (still in use).

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 rationale behind the PICT is the increase of the community tolerance; as the species in the exposed community present differences in their sensitivity against a certain chemical, the toxicant will eliminate or hinder the success of sensitive species and favor the development of the more tolerant ones (Blanck et al., 1988).

The hypothesis of work was that the different exposure to the herbicides along the year due to the seasonal agriculture practices would result on changes in algal community tolerance. The sensitivity of the algal communities against the same herbicide in different seasons would...
depend on the exposure during the growing period. The sources of herbicides were expected to be a) the background released from soils (for the two banned herbicides, atrazine and simazine), or b) the direct application on irrigated crops (for therbutylazine).

The benthic algal communities grew on artificial substrates placed on a creek downstream the basin at different agricultural time periods: pre-herbicide application (autumn and winter), mid-herbicide application (spring) and post application (summer). 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.

2. Materials and Methods

Study site. The study was carried out in a small agricultural basin, Lerma (7.5 km²) located in the Ebro hidrological basin (North-East of Spain). Here 85% of the area has been transformed from natural steppe to crops dominated by corn (40%) and winter cereals (18%). Agriculture work is 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).

Physicochemical water analysis. Water flow (L/s), temperature (°C) and nitrate concentration (NO₃⁻ mg/L) of the creek were measured in-situ by a data-logger station (from Geological and Mining Institute of Spain, IGME). The pH was measured in situ 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 sulphur (S).

Periphyton sampling and analysis. Standard microscope slides of methacrylate (7.55 x 2 x 0.15 cm) fixed in plastic holders were placed in the creek for algae colonization at the beginning of each sampling period. They were removed when the algae biofilm reached an average thickness of ≈ 0.75mm and a steady-state fluorescence of photosystem II around 1000 units (measured at constant natural midday light, using a mini-PAM of Walz ©). This ensured that the toxicity tests were performed on algae biofilm communities of similar biomass and physical dimensions.

Chlorophyll a content was analyzed with Jeffrey and Humphrey (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 using a Utermöhl chamber.

Diversity index were calculated Shannon and Weaver and the inverse of Simpson index (Lande, 1996). The abundance of species in each community (Fig 2) was calculated based on the...
number of individuals of each species and the total number of individuals in the community.
Species representing less than - 1% of total abundance were not included in the analyses.

Herbicide analysis. Two different methods were used to assess the herbicide levels in the water: passive sampling with Chemcatcher® devices and discrete water samples. During each of the 4 sampling periods, 1L of water was collected at the last day of algae biofilm incubation.

For passive sampling we applied Chemcatcher® (Kingston et al., 2000). This system uses passive sampler disks (Empore® 3M, SDB-RPD, styrene-divinyl-benzene-reverse phase sulfonated; 47mm Ø, 145 µm thick, 8nm pore size) and a protective membrane (Supor® 200, PALL, 47 mm Ø, 145 µm thick, 0,2 µm pore size). This last acts as a diffusion-limiting media, having a low-protein binding properties which minimize bio-fouling and the degradation of the disk surface (Schäfer, et al., 2008; Vrana et al., 2009). The disks and protective membranes were conditioned in methanol and nanopure water (30 minutes of stirring in each) following Vermeirssen, 2009 procedure. Finally they were fitted into the Chemcatcher® holder by grabbing its edges with methacrylate pieces (containing three disks). The Chemcatchers were placed in the creek close to the substrates for biofilm growth and the automatic monitoring station. The sampling window during winter, spring and summer period was the last 18 days of algae biofilm incubation (Fig. 1), while the sampling window during autumn was 60 days (due to problems on the access to the sampling site).

After exposure, the disks were removed from the holder with forceps and submerged into 7 mL of acetone. The concentration of the terbuthilazine, atrazine and simazine together with other 30 common pesticides in Chemcatchers and in the discrete water samples were analyzed following Vermeirssen et al., 2009 procedure at Labaqua Laboratories (Alicante-Spain).

Dose-response test in flow-through artificial channels. The tolerance was assessed as the toxicity of the different herbicides to the photosynthetic yield of the algal community. The concentration of herbicide required to reduce 50% and 10% of the photosynthetic performance of the benthic algae community (EC50,EC10) was used to compare the community tolerance between the different time periods. The three herbicides were provided by Sigma-Aldrich in powder form. Acetone was used for terbuthylazine and atrazine (5ml stock solution 32mM) whereas simazine was dissolved in methanol, (50ml stock solution 1mM) due to its poor solubility in acetone. Appropriate control experiments were done using the same biofilms, to discard toxicological effects of the trace concentrations of solvents remaining during the dose-response. The stocks were freshly prepared two days before experimentation and stored in a freezer.
The toxicology experiments were carried out in flow through methacrylate channels (90 cm long and 10 cm width) connected to separate water reservoirs. Those reservoirs were submersed in a thermostatic bath. Every reservoir had the same volume (4 L) of a buffer solution MOPS 0.01 nM (3-morpholinopropane-1-sulfonic acid). An aquarium pump re-circulated the water from every reservoir through every channel at 1.3 cm s⁻¹. Light was provided by fluorescence lamps (Blau aquaristic T5HO, 39 w/10000 °K, 80 µmol photon m⁻² s⁻¹ at the channel surface). The water temperature of the thermostatic bath was set to the average in situ temperature (10, 7, 13 and 20 °C for autumn, winter, spring and summer, respectively).

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. The herbicide doses applied in the channels were prepared according to OCDE (Aquatic toxicology test, nº23). After 20 h acclimation, two algae biofilms in each channel were tested.

Concentration range for dose-response curves were decided based on previous short-term experiments to be sure that EC50 were be within the range (data not shown).

The photosynthetic response of the biofilms to the herbicide exposure was estimated with the Pulse Amplitude Modulated protocol using a Photosynthesis Yield Analyzer Fluorometer (mini-PAM, Waltz ©). The effective quantum yield was calculated from the steady-state fluorescence (Fs) and the maximum fluorescence (Fm’) after a saturation light pulse, as (Fm’ – Fs)/Fm’ (Genty et al. 1989). The fluorescence measurements (n=6) were taken non-invasively from below the channels. The first measurement was done before inoculating the herbicide (0 h measurement) and at 1 and 2 hours of exposure. The measurements were done at the same biofilm positions holding similar biomass (similar thickness and Fs estimated by the PAM), to avoid the biases of difference in biofilm thickness. At the end of each experiment water samples from the channels were sent for analyses as described above.

**Data analysis.** The dose-response curve was 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. The effective concentration which reduced the photosynthetic efficiency by 10% and 50% of the yield (EC10 and EC50 respectively), were estimated. 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. that is 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 were performed using ade4 package of R software (Braak and Šmilauer, 1998; Braak and Verdonschot, 1995; ter Braak and Smilauer, 2002).

3. Results

Physicochemical water analysis. The annual water temperature ranged from 4.5°C to 22°C while pH remains constant and slightly basic. Most of the nutrient levels were similar during the four studied periods (Table 1). Only phosphorus (total dissolved phosphorus, TDP) shown levels 14 times higher during summer than during autumn or winter (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 on dissolved substances (i.e. a drop on concentrations) as nitrate (Fig. 1).

<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>114.82 ±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 ±9.97</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>Tª (°C)</td>
<td>10.70 ±3.53</td>
<td>7.20 ±1.71</td>
<td>13.07 ±1.46</td>
<td>19.56 ±1.24</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>

Table 1. Average dissolved nutrients along the sampling periods (from 4-6 discrete water samples). Tª was registered continuously with a data logger and pH with probe. Phosphorus and nitrogen levels were measured as total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN).
Figure 1. Flow and nitrate concentration were registered with an in-situ monitoring system; data plotted are daily averages provided by IGME institute. The grey-shadowed blocks are the periods of the algae biofilm on the artificial substrata.

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 (Table2). The total herbicides included other herbicides that appear in the basin as methalaclor. They belong to other chemical families with a different mode of action than triazines.

<table>
<thead>
<tr>
<th></th>
<th>Autumn</th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<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>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>

Table 2. In the upper part of the table, average ng of herbicide accumulated in a passive sampler disk per day (SDB-RPD 3M Empore™ disk in a Chamcather device). Error terms are SD of three replicate disks. For water only one sample was analyzed. Detection limit was 0.001 ng/day for the passive samplers and 0.01 µg/L for the water samples.
**Algae biofilm analysis.** The algal community was mostly composed by pennate diatoms with negligible presence of other groups (Table 6). Generally, two species were dominant: *Gomphonema olivaceum* (sp1) during autumn and winter (46 and 38% of total individuals respectively) and *Achnanthes minutissima* Kütz. (sp.2) during spring and summer (65 and 68% respectively, see (Fig. 2).

Chl a concentrations (i.e. biomass) of the communities sampled were similar during all seasons.

Both diversity indexes, *H* and *1-ʎ*, showed that algae communities were most diverse during autumn and winter (Table 3). The spring and summer communities were less diverse as more than half of the individuals belonged to only one specie (*A. minutissima*) and most of the other species had a few number of individuals (<0.01%).

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**Figure 2.** Specie composition as % of total individuals of periphyton at the four seasons. The species and their respective codes are: sp1 *Gomphonema olivaceum* (Horn.) Bréb., sp13 *Nitzschia dissipata* (Kütz.) Grun., sp2, *Achnanthes minutissima* Kütz., sp14 *Gomphonema micropus* Kütz., sp3 *Navicula radiosa* Kütz., sp15 *Nitzschia palea* (Kütz.) W.Smith, sp4 *Cymbella affinis* Kütz., sp16 *Nitzschia linearis* (Ag.) W.Smith, sp5 *Navicula tripunctata* (O.F.Müller) Bory, sp17 *Gomphonema stauroneiforme* Grun., sp6, *Fragilaria pulchella* (Ralfs)Lange-Bertalot, sp18 *Gomphonema angustatum* (Kütz.) Rabenh., sp7 *Rhicosphenia abbreviata* (Ag.) Lange-Bertalot, sp19 *Fragilaria capucina* Desm., sp8 *Navicula lanceolata* (Ag.) Ehr., sp20 *Amphora pediculus* (Kütz.) Grun., sp9 *Nitzschia fonticola* Grun., sp21 *Surirella brebissonii* Krammer&Lange-Bertalot, sp10 *Cymbella microcephala* Grun., sp22 *Jaaginema subtilissimum* (Kütz.ex De Toni) Anagn, sp11 *Navicula veneta* Kütz., sp23 *Melosira varians* Ag.
Table 3. Biomass (chlorophyll a, b), density, and diversity of periphyton from different seasons. Chlorophyll content in 90% acetone extraction (Ritchie, 2006). Density and diversity index based on diatoms.

<table>
<thead>
<tr>
<th>Season</th>
<th>Chlorophyll a (µg cm⁻²)</th>
<th>Florescence (F)</th>
<th>Density (cells.cm⁻²)</th>
<th>Shannon-Wiener index (H)</th>
<th>Simpson index inverse (1-ʎ)</th>
<th>Richness (n° total species)</th>
<th>Non diatom species n°(group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>13.61 ±2.79</td>
<td>949 ±127</td>
<td>1.64 10⁶ ±3.8510⁵</td>
<td>2.05</td>
<td>0.76</td>
<td>23</td>
<td>1 (Cyano)</td>
</tr>
<tr>
<td>Winter</td>
<td>12.32 ±2.30</td>
<td>936 ±123</td>
<td>3.59 10⁶ ± 6.85 10⁵</td>
<td>1.56</td>
<td>0.68</td>
<td>28</td>
<td>1 (Cyano)</td>
</tr>
<tr>
<td>Spring</td>
<td>11.79 ±2.77</td>
<td>1152 ±174</td>
<td>5.17 10⁶ ±2.06 10⁵</td>
<td>1.19</td>
<td>0.54</td>
<td>34</td>
<td>1 (Cyano) 1 (Chloro)</td>
</tr>
<tr>
<td>Summer</td>
<td>13.78 ±3.43</td>
<td>1060 ±174</td>
<td>4.51 10⁶ ±7.28 10⁵</td>
<td>1.32</td>
<td>0.52</td>
<td>42</td>
<td>2 (Cyano) 3 (Chloro)</td>
</tr>
</tbody>
</table>

Toxicity tests. According to the EC₅₀ values, periphyton communities were most sensitive to terbutylazine followed by atrazine and finally simazine (Table 4). The tolerance to terbutylazine changed significantly through seasons (p< 0.05). The summer community was 3 times more tolerant than the winter community and two times more than the spring one, based on EC₅₀ values (Table 4 and Fig. 3). EC₅₀ for atrazine and simazine were more constant along the year i.e. the community tolerance did not presented a clear seasonality. The EC₅₀ for atrazine only were significantly higher in summer and in the case of simazine significantly lower in spring. The EC₁₀ values in general, aligned with the trends of EC₅₀ values.

<table>
<thead>
<tr>
<th>Season</th>
<th>Terbutylazine nM</th>
<th>Atrazine nM</th>
<th>Simazine nM</th>
<th>Terbutylazine nM</th>
<th>Atrazine nM</th>
<th>Simazine nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>196 ±22°</td>
<td>640 ±51°</td>
<td>1968 ±172°</td>
<td>24 ±7</td>
<td>117 ±19</td>
<td>168 ±39</td>
</tr>
<tr>
<td>Winter</td>
<td>109 ±10°</td>
<td>560 ±33°</td>
<td>2101 ±113°</td>
<td>25 ±5</td>
<td>127 ±16</td>
<td>554 ±79</td>
</tr>
<tr>
<td>Spring</td>
<td>144 ±9°</td>
<td>730 ±48°</td>
<td>1441 ±73°</td>
<td>35 ±6</td>
<td>148 ±24</td>
<td>307 ±49</td>
</tr>
<tr>
<td>Summer</td>
<td>321 ±21°</td>
<td>993 ±46°</td>
<td>1925 ±127°</td>
<td>69 ±11</td>
<td>145 ±23</td>
<td>206 ±39</td>
</tr>
</tbody>
</table>

Table 4. The EC₅₀ and EC₁₀ concentrations for terbutylazine, atrazine and simazine. 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.

The sensitivity of the algal biofilm against terbutylazine was most variable (Fig.3). Because its discontinuous application during the year, it was expected that the algae were exposed to a wide range of terbutylazine concentrations as compared to the two banned herbicides, which only remained at residual soil levels from past applications (Fig 3).
Figure 3. EC50 for each tested herbicide at the different seasons. The EC50 values are normalized by the winter value (set as 1).

To explore the effect of herbicides on shaping the structure of the benthic algal communities, a multivariate analysis was done. The canonical correspondence analysis (CCA) distributed the species abundance in a multidimensional space according to the herbicide tolerance (EC$_{50}$ values at 1h and 2h herbicide exposition) during each season (Fig. 4). Both axes explained up to 94% of the total variance, meaning that the tolerance level expressed by the communities can be mostly explained by differences in species composition.
Figure 4. Biplot presenting the values of EC50 for each herbicide atrazine – atr-, terbuthylazine – trb- and simazine - smz- (at 1 hour “ec1” and at 2 hours “ec2” of exposure) in the space defined by the species. Code number of algal species has been detailed in the results section.
4. Discussion

4.1 Seasonal trends on the ecotoxicology of triazines. The benthic algal communities growing downstream an agricultural basin have a seasonal herbicide tolerance. The algal community had higher tolerance to terbuthylazine and atrazine during summer period, and lower during the coldest season, winter. The seasonal differences in tolerance are more significant for the herbicide that is currently apply in the crops (terbuthylazine), than for those that remain in the environment from past applications (atrazine and simazine).

Based on the EC$_{50}$ values for terbuthylazine, the alga e communities presented significantly different tolerances between all seasons being the summer community the more tolerant (Table 4). These results were expected as the biggest application of terbutylazine in the crops take place in spring and summer, therefore the algae exposure via runoff was expected to be higher. Only the algae community from summer (for atrazine) or winter (for simazine) presented significantly different tolerance from any other season (Table 4). That, and according the PICT rationale, indicates that a differential exposure to terbuthylazine happened through the year, even if the methods used for measuring its concentrations failed in catching that process.

The already banned herbicides, atrazine and simazine appeared in low concentrations in the creek during the whole year (Table 2). The exposure of the algae biofilm to these herbicides seems to be constant and low along the year, in agreement with the similar EC$_{50}$ values measured in the algae biofilm along the seasons.

Some natural and anthropogenic variables co-varing 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 EC$_{50}$ 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); that extra input of P may have enhanced the energetic metabolism of the periphyton. However, the contradictory results found in previous studies (Pesce et al., 2008).

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 along the year were not registered by any method. The measured herbicide concentrations do not correlate with the calculated tolerance values of EC$_{50}$ for terbuthylazine. As an example, the higher
concentration was measured in winter while the higher tolerance is measured at summer, i.e. higher EC$_{50}$.

We describe this discrepancy to the fact that both measuring approaches poorly reflect variability in pollutants water concentrations because their intrinsic limitations. There are reasons to suspect that herbicide applications in small basins may result in short-time (hours) pollution events after the first rain or irrigation, that may escape from passive samplers and discrete water sampling (Stoeckel et al., 2012; Ulrich et al., 2013). The numerous rain events during spring and irrigation in summer, together with the detection of a terbuthylazine peak in summer, 20 km downstream from the study site (publicly available water quality monitoring data, not shown), supports this rationale. Moreover, the fast mobility of herbicides and the lower concentrations reached in the runoff waters after intense rain events, would hamper the “capture” of those high pollution events using discrete or passive water sampling. As example, atrazine reached the corresponding creek during the first hours of the first rainfall events that followed its application (Leu et al., 2004). After two hours of the raining event the concentrations were quickly diluted from 35 to 4 nM, which hinder its detection (Leu et al., 2004). In a similar manner, the flow peaks in Lerma basin may quickly dilute the herbicide pollution peaks as it was registered for nitrate levels (Fig. 1).

Impact of herbicides on algae biofilm. The seasonal herbicide physiological changes (i.e. in tolerance to herbicides) were related with changes the community structure (i.e. changes on algal life forms dominate along the year). The algal species can be classified into: (1) the low-profile guild, i.e. species of short stature, including prostrate, small erect, solitary centric and slow-moving species; (2) the high-profile guild, i.e. species of tall stature including large erect, filamentous, branched, chain-forming, tube-forming, stalked and colonial centric and (3) the motile guild, i.e. 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 EC$_{50}$ to terbuthylazine and atrazine i.e. high community tolerances (Fig. 4). Previous studies suggest that each guild respond 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. minutissima (sp2), N. lanceolata (sp8), C. microcephala (sp10), N. palea (sp15), A. pediculus (sp20) and S. brebissonii (sp21). The abundance of these species was positively correlated to the EC$_{50}$ of terbuthylazine and atrazine i.e. high community tolerances (Fig. 4). The fact that two of these species are known as pioneers, i.e. appearing after chemical disturbances (Achnantes...
minutissima and Nitzia palea) support the hypothesis of the occurrence of herbicide pollution at these seasons as indicated by the EC50, even if the measure herbicide concentrations does not shown it.

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 et al., 1998; Navarro et al., 2002; Ryan S Prosser, 2013). The EC50 and EC10 values for terbuthylazine or simazine are the first published. The EC50 concentrations are between 3-20 times higher than background herbicide levels normally found in agricultural watersheds, i.e. from 2 nM to 40 nM (Louchart et al. 2001). However, the EC10 values indicate that even these low concentrations may significantly affect the photosynthetic yield of algal communities. Moreover, 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, supports the use of PICT approaches as a useful and powerful monitoring tool for herbicides.

5. Conclusions

The periphyton presented seasonal variance on 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. 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, it was in agreement with the different potential sources of each herbicide: the punctual use during the agricultural year (for terbuthylazine) 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.

5. Acknowledgements

To Dr. Marta Luścińska for the algal taxonomic analysis. This work was supported by Spanish Ministry of Economy and Competitiveness (National Research Plan, ref. BFU2010-22053).
6. References


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

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Keywords: Terbutylazine, atrazine, simazine, herbicides, algal biofilm, periphyton, community tolerance, photosynthetic efficiency.

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, terbutylazine, atrazine and simazine on the photosynthetic efficiency and structure of algal benthic biofilms (i.e., phototrophic 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 accordance 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 terbutylazine along the year in accordance with the seasonal application of this herbicide in the crops nowadays. The sensitivity of already banned herbicides, atrazine and simazine do 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. 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 EC$_{50}$ and EC$_{10}$ of periphyton for terbuthylazine or simazine are the first published and can be used for impact assessments.

Highlights

1. It has been assessed the agricultural impacts via run off on nearby freshwater benthic algal communities.

2. The seasonal impact of triazine herbicides on the structure and photosynthetic efficiency of algal benthic communities have been studied seasonally.

3. Traditional direct water sampling methods seem not to capture the seasonal variability of herbicide concentrations in small basins.

4. Herbicide’s tolerance (EC$_{50}$) varies according to source: recent application or background release.

5. Assessment of herbicide tolerance of natural communities complements environmental water monitoring.
1. Introduction

Agriculture uses half of the total land in Europe (Stoate et al., 2009). The traditional agriculture has been replaced by intensive works which maximize the impact on the soil and nearby freshwater ecosystem (de Almeida Azevedo et al., 2000; Loos et al., 2009; Arroita et al., 2013).

One way to increase the crop production is 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, at that 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 triazines family of herbicides which are 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; 10495/2003-final, 2003). However, these herbicides are still detected in the environment together with the triazines in use nowadays as terbutylazine. This is due to their long retention time in the soil and aquifers, which leads to a pernurable leaching and long-lasting levels in different ecosystems even years after its prohibition.

The Lerma basin, (within Ebro depression in Spain), transformed from its natural state (gypsum soils covered by scrubland and steppe-like vegetation) to rain-fed agriculture and recently to, 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 the terbutylazine (still in use). These data are available at the web 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 rationale 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 work was that the different exposure to the herbicides along the year due to the seasonal agriculture practices would result on changes in algal community. The sensitivity
of the algal communities against the same herbicide at different seasons would depend on the
exposure during the growing period. A previous similar seasonal study shown that the structural
and functional response of algal communities to pesticides is 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 simazine), or b) the direct application on irrigated crops (for
terbutylazine). Triazine herbicides main application takes place at the beginning of spring just
after the seeding of the summer cereals. The applications can be extended until the plant 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 at 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

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 is 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).

Physicochemical water analysis. Water flow (L/s), temperature (°C) and nitrate concentration
\( \text{(NO}_3^-\text{, mg/L)} \) of the creek were measured in-situ by a water quality station (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 sulphur (S).
Figure 1. Experimental and sampling design.

Algal biofilm sampling and analysis. Benthic algal communities grew on artificial substrates placed on a creek downstream the basin at different agricultural time periods: pre-herbicide application (autumn and winter), mid-herbicide application (spring) and post application (summer). Communities grew on artificial substrates (small pieces of methacrylate 7.55 x 2 x 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 c.a. 15 cm depth. The substrates were placed in the same point every season. They were removed when the algal biofilm reached an average thickness around 0.75 mm and a steady-state fluorescence of photosystem II of 1000 (Fv PAM quantification at constant light, measured by a Mini-PAM of Walz ©). This ensured that the toxicity tests were performed on algal biofilm communities of similar biomass and physical dimensions.

Chlorophyll $a$ content was analyzed with Jeffrey and Humphrey (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 et al. (1958) technique. Diversity indices, Shannon and Weaver (1963) and the inverse of Simpson index, D or $1-\lambda$ (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 individuals in the community. Species representing less than –1% of total abundance were not included in the analyses.
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 other 10 pesticides by chromatography (SBSE/GC/MS/HPLC). Moreover, three passive samplers Chemcatcher (Kingston et al., 2000) were placed in the creek close to the algal biofilms on the creek (Fig. 1). Chemcatcher device consist of a sampler disc (Empore ® 3M, SDB-RPD, styrene-divinyl-benzene-reverse phase sulfonated; 47mm Ø, 145 µm thick, 8nm 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 discs integrated the pesticides present in the water into its matrix over a certain exposure time, whereas the protective membrane acts as a diffusion-limiting media and minimize bio-fouling due to its low-protein binding properties (Schäfer, et al., 2008, Vrana et al., 2009). The discs and protective membranes were conditioned following the procedure by Vermeirssen (2009). The sampling window during winter, spring and summer period was the last 18 days of algal biomass incubation in the creek (Fig. 1), while the sampling window during autumn was 60 days (due to problems on the access to the sampling site). At the end of the sampling period, the discs were removed from the holder with forceps and submerged into 7 mL of acetone. The presence of triazines and other 10 pesticides was analyzed following Vermeirssen et al. (2009) at Labaqua Laboratories (Alicante-Spain).

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 different seasons. The three herbicides were provided by Sigma-Aldrich in powder form. The stocks were freshly prepared two days before experimentation 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. The toxicology experiments were carried out in flow through methacrylate channels (90 cm long and 10 cm width) connected to separate water reservoirs. Those reservoirs were submerged in a thermostatic bath where the temperature was adjusted to the one on 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 a 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/10000 °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 for dose-response curves were decided based on previous short-term experiments to ensure that EC$_{50}$ were within that range (data not shown). The herbicide doses applied in the channels were prepared according to OCDE (Aquatic toxicology test, n²23). After 20h acclimation in the channels (i.e. same flowing, temperature and light conditions than during the tests), biofilms were transferred to the experimental channels with the herbicide doses during 2 hours. The photosynthetic response of the biofilms to the herbicide exposure was estimate with the Pulse Amplitude Modulated protocol using a Photosynthesis Yield Analyzer Fluorometer (mini-PAM, Waltz ®). The effective quantum yield was calculated from the steady-state fluorescence (Fs) and the maximum fluorescence (Fm’) after a saturation light pulse, as (Fm’ – Fs)/Fm’ (Genty et al., 1989). The fluorescence measurements (n=6) were taken below the channels through the methacylate in a non-invasively from. The first measurement was done before inoculating the herbicide in the channel (0 h measurement) and after 1 and 2 h of exposure. The measurements were done at the same biofilm positions the three times, with similar thickness and Fs estimated by the PAM, to avoid the biases of difference in biofilm thickness. At the end of each experiment water samples from the channels were send for analyses as described above.

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 it were estimated the effective concentrations which reduced the photosynthetic yield of the periphyton by 10% and 50% (EC10 and EC50 respectively). The seasonal EC$_{50}$ values were compared using the CompParm function, included in package drc. This function performs t-test between EC$_{50}$ values. Ratios (EC$_{50}$ case A/ EC$_{50}$ 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 were performed using ade4 package of R software (Braak and Šmilauer, 1998; Braak and Verdonchot, 1995; ter Braak and Šmilauer, 2002).

3. Results

Physicochemical water analysis. The annual water temperature ranged from 4.5 ºC to 22 ºC while pH remains constant and slightly basic. Most of the nutrient levels were similar during the four studied periods (Table 1). Only phosphorus concentrations shown an acute increase during
summer (x 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 on dissolved substances (i.e. a drop on concentrations) as was the case of nitrate (Fig.
2).

Table 1. Average dissolved nutrients along the sampling periods (from 4-6 discrete water samples). Temperature (Tº) 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>114.82 ±31.79</td>
<td>150.71 ±68.96</td>
<td>142.23 ±10.78</td>
<td>158.52 ±5.02</td>
</tr>
<tr>
<td>Mg (mg/L)</td>
<td>101.84 ±9.97</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>243.01 ±18.51</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>

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

Herbicide level in the creek. The averaged integrated values of herbicides in the passive
 sampler discs 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 methoalclor. They belong to other chemical families with a different mode of action than triazines and are out of the scope of this study.

<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>
</tbody>
</table>

Table 2. Upper part: average of herbicide accumulated in a passive sampler disc per day in ng (SDB-RPD 3M Empore ™ disc in a Chamcather device). Error terms are SD of three replicate discs. Lower part: concentration in water samples (only one sample was analyzed). Some measurements were under the detection limit (0.001 ng/day for passive samplers and 0.01 µg/L for water samples).

Table 2. Upper part: average of herbicide accumulated in a passive sampler disc per day in ng (SDB-RPD 3M Empore ™ disc in a Chamcather device). Error terms are SD of three replicate discs. Lower part: concentration in water samples (only one sample was analyzed). Some measurements were under the detection limit (0.001 ng/day for passive samplers and 0.01 µg/L for water samples).

Algal biofilm analysis. The algal community was mostly composed by pennate diatoms with negligible presence of other groups (Figure 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 *J*-*x*, 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 specie (*A. minutissima*) and most of the other species had a few number of individuals (<0.01%).
Figure 3. Species composition as % of total individuals of benthic algal communities at the four seasons. The 241 species and their respective codes are: sp1 Gomphonema olivaceum, sp13 Nitzschia dissipata, sp2, Achnanthes minutissima, sp14, Gomphonema micropus, sp3 Navicula radiosa, sp15 Nitzschia palea, sp4 Cymbella affinis, sp16 Nitzschia linearis, sp5 Navicula tripunctata, sp17 Gomphonema stauroneiforme, sp6, Fragilaria pulchella, sp18 Gomphonema angustatum, sp7 Rhoicosphenia abbreviata, sp19 Fragilaria capucina, sp8 Navicula lanceolata, sp20 Amphora pediculus, sp9 Nitzschia fonticola, sp21 Surirella brebissonii, sp10 Cymbella microcephala, sp22 Jaaginema subtilissimum, sp11 Navicula veneta, sp23 Melosira varians.

<table>
<thead>
<tr>
<th></th>
<th>Chlorophyll a (µg cm⁻²)</th>
<th>Fluorescence (F)</th>
<th>Density (cells cm⁻²)</th>
<th>Shannon–Wiener index (H)</th>
<th>Simpson index inverse (1-λ)</th>
<th>Richness (nº total species)</th>
<th>Non diatom species n°(group)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Autumn biofilm</strong></td>
<td>13.61 ±2.79</td>
<td>949 ±127</td>
<td>1.64·10⁸ ± 3.85·10⁷</td>
<td>2.05</td>
<td>0.76</td>
<td>23</td>
<td>1 (Cyano)</td>
</tr>
<tr>
<td><strong>Winter biofilm</strong></td>
<td>12.32 ±2.30</td>
<td>936 ±123</td>
<td>3.59·10⁸ ± 6.85·10⁷</td>
<td>1.56</td>
<td>0.68</td>
<td>28</td>
<td>1 (Cyano)</td>
</tr>
<tr>
<td><strong>Spring biofilm</strong></td>
<td>11.79 ±2.77</td>
<td>1152 ±174</td>
<td>5.17·10⁸ ± 2.06·10⁷</td>
<td>1.19</td>
<td>0.54</td>
<td>34</td>
<td>1 (Cyano)</td>
</tr>
<tr>
<td></td>
<td>13.78 ±3.43</td>
<td>1060 ±174</td>
<td>4.51·10⁸ ± 7.28·10⁷</td>
<td>1.32</td>
<td>0.52</td>
<td>42</td>
<td>2 (Cyano)</td>
</tr>
</tbody>
</table>

Table 3. Biomass (chlorophyll a, b), density, and diversity of periphyton from different seasons. Chlorophyll content in 90% acetone extraction (Ritchie, 2006). Density and diversity index based on diatoms.

Toxicity tests. According to the EC₅₀ values, benthic algal communities were most sensitive to terbutylazine followed by atrazine and finally simazine (Table 4). The periphyton tolerance to terbutylazine changed significantly through 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 EC₅₀ values (Table 4 and Fig. 4). EC₅₀ for atrazine and simazine were more constant along the year, i.e., the community tolerance did not present a clear seasonality. The EC₅₀ for
atrazine only were significantly higher in summer and in the case of simazine significantly lower in spring. The EC\textsubscript{10} values in general, aligned with the trends of EC\textsubscript{50} (Table 4).

<table>
<thead>
<tr>
<th>Season</th>
<th>Terbuthylazine (\pm\text{SEM})</th>
<th>Atrazine (\pm\text{SEM})</th>
<th>Simazine (\pm\text{SEM})</th>
<th>Terbuthylazine (\pm\text{SEM})</th>
<th>Atrazine (\pm\text{SEM})</th>
<th>Simazine (\pm\text{SEM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>196 (\pm)22\textsuperscript{a}</td>
<td>640 (\pm)51\textsuperscript{ef}</td>
<td>1968 (\pm)172\textsuperscript{h}</td>
<td>24 (\pm)7</td>
<td>117 (\pm)19</td>
<td>168 (\pm)39</td>
</tr>
<tr>
<td>Winter</td>
<td>109 (\pm)10\textsuperscript{b}</td>
<td>560 (\pm)33\textsuperscript{a}</td>
<td>2101 (\pm)113\textsuperscript{b}</td>
<td>25 (\pm)5</td>
<td>127 (\pm)16</td>
<td>554 (\pm)79</td>
</tr>
<tr>
<td>Spring</td>
<td>144 (\pm)9\textsuperscript{c}</td>
<td>730 (\pm)48\textsuperscript{d}</td>
<td>1441 (\pm)73\textsuperscript{c}</td>
<td>35 (\pm)6</td>
<td>148 (\pm)24</td>
<td>307 (\pm)49</td>
</tr>
<tr>
<td>Summer</td>
<td>321 (\pm)21\textsuperscript{e}</td>
<td>993 (\pm)46\textsuperscript{d}</td>
<td>1925 (\pm)127\textsuperscript{h}</td>
<td>69 (\pm)11</td>
<td>145 (\pm)23</td>
<td>206 (\pm)39</td>
</tr>
</tbody>
</table>

Table 4. The EC\textsubscript{50} and EC\textsubscript{10} concentrations for terbuthylazine, atrazine and Simazine at 2 hours 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.

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 the herbicide tolerance (EC\textsubscript{50} 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.

Figure 4. EC\textsubscript{50} for each tested herbicide at the different seasons. The EC\textsubscript{50} were normalized by the winter value (set as 1).
Figure 5. Biplot presenting the values of EC_{50} for each herbicide atrazine – atr, terbuthylazine – trb, and simazine – smz (at 1 hour “ec1” and at 2 hours “ec2” of exposure) in the space defined by the species. Code number of algal species has been detailed in the legend of Fig. 3, while “othsp” represents other species.
4. Discussion

Seasonal trends on the ecotoxicology of triazines. The sensitivity of benthic algal communities growing downstream an agricultural against an herbicide can change seasonally. Based on the EC$_{50}$ values the algal community had higher tolerance to terbuthylazine and atrazine during summer period, and lower during the coldest season, winter. The seasonal differences in tolerance are more significant for the herbicide that is currently used in the crops (terbuthylazine), than for those that remain in the environment from past applications (atrazine and simazine).

The algal communities presented significantly different tolerances (EC$_{50}$) for terbuthylazine between all seasons being the summer algal community 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 accordance with the PICT rationale, the high community tolerance to terbuthylazine in summer may be due to a greater exposure during that season. Therefore EC$_{50}$ values suggest that there were differential exposure to terbuthylazine along 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 EC$_{50}$ values measured in the algal communities during all seasons. It has been estimated (based on information gathered from farmers and the total surface of corn), that a maximum of 126 kg of terbuthylazine could have been used in the basin 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 EC$_{50}$ 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 mask the effect of a triazine herbicide on the periphyton (Guasch et al., 2007), whereas opposite results where concluded in field experiments (Pesce et al., 2008).

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 terbutylazine concentrations along the year were not registered by any method. The measured herbicide concentrations do not correlate with the calculated tolerance values of EC\textsubscript{50} for terbutylazine. As an example, the higher concentration was measured in winter while the higher tolerance is measured at summer, i.e., higher EC\textsubscript{50}.

We describe this discrepancy to the fact that both measuring approaches poorly reflect variability in pollutants water concentrations because their intrinsic limitations. There are reasons to suspect that herbicide applications in small basins may result in short-time (hours) pollution events after the first rain or irrigation, that may escape from passive samplers and discrete water sampling (Stoeckel et al., 2012; Ulrich et al., 2013). The numerous rain events during spring and irrigation in summer, together with the detection of a terbutylazine peak in summer, 20 km downstream from the study site (publicly available water quality monitoring data in www.chebro.es, not shown), supports this rationale. Moreover, the fast mobility of herbicides and the lower concentrations reached in the runoff waters after intense rain events, would hamper the “capture” of those high pollution events using discrete or passive water sampling. As example, atrazine reached the corresponding creek during the first hours of the first rainfall events that followed its application (Leu et al., 2004). After two hours of the raining event the concentrations were quickly diluted from 35 to 4 nM, which hinder its detection (Leu et al., 2004). In a similar manner, the flow peaks in Lerma basin may quickly dilute the herbicide pollution peaks as it was registered for nitrate levels (Fig. 2).

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 on algal life forms dominate along the year). The algal species can be classified into: (1) the low-profile guild, consisting on species of short stature, including prostrate, small erect, solitary centric and slow-moving species; (2) the high-profile guild, consists of species of tall stature including large erect, filamentous, branched, chain-forming, tube-forming, stalked and colonial centric and (3) the motile guild, consists 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 t he EC\textsubscript{50} to terbutylazine and atrazine, i.e. high community tolerances (Fig. 5). Previous studies suggest that each guild respond 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. minutissima (sp2), N. lanceolata (sp8), C. microcephala (sp10), N. palea (sp15), A. pediculus (sp20) and S. brebissonii (sp21). The abundance of these species was positively correlated to the EC\textsubscript{50} 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 (Achnantes
minutissima and Nitzia palea) support the hypothesis of the occurrence of herbicide pollution at
these seasons as indicated by the EC₅₀, even if the measure herbicide concentrations does not
shown it.

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 S Prosser, 2013). The EC₅₀ and EC₁₀ values for terbuthylazine or
simazine are the first published. The EC₅₀ concentrations are between 3–20 times higher than
background 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, supports the use of PICT
approaches as a useful and powerful monitoring tool for herbicides.

5. Conclusions

The periphyton presented seasonal variance on 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.
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, it was in
agreement with the different potential sources of each herbicide: the punctual use during the
agricultural year (for terbuthylazine) 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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