# Synergistic Effects of Contaminants in Lombardy Waters

**Caterina La Porta** (caterina.laporta@unimi.it)
University of Milan
**Maria Fumagalli**
University of Milan
**Stefano Gomarasca**
University of Milan
**Maria Lionetti**
University of Milan
**Stefano Zapperi**
University of Milan
**Stefano Bocchi**
University of Milan

---

**Research Article**

**Keywords:** water pollution, herbicides, AMPA, Glyphosate, pesticide, Lombardy, synergistic effect, biosensor, Chlamydomonas, geolocalization

**Posted Date:** March 3rd, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-257357/v1



---

**Version of Record:** A version of this preprint was published at Scientific Reports on July 6th, 2021. See the published version at https://doi.org/10.1038/s41598-021-93321-6.

# Synergistic effects of contaminants in Lombardy waters

Caterina AM La Porta[a,b,c,1], Maria Rita Fumagalli[a,b,c], Stefano Gomarasca[b], Maria Chiara Lionetti[a,b], Stefano Zapperi[d,e,f], Stefano Bocchi[b]

[a] *Center for Complexity and Biosystems, University of Milan, via Celoria 16, 20133 Milano, Italy*
[b] *Department of Environmental Science and Policy, University of Milan, via Celoria 26, 20133 Milano, Italy*
[c] *CNR - Consiglio Nazionale delle Ricerche, Istituto di Biofisica, via Via De Marini 6,16149 Genova, Italy*
[d] *Center for Complexity and Biosystems, University of Milan, Via Celoria 16, 20133 Milano, Italy*
[e] *Department of Physics, University of Milan, Via Celoria 16, 20133 Milano, Italy*
[f] *CNR - Consiglio Nazionale delle Ricerche, Istituto di Chimica della Materia Condensata e di Tecnologie per l'Energia, Via R. Cozzi 53, 20125 Milano, Italy*

---

## Abstract

Quantifying synergistic environmental effects in water contamination is still an open issue. Here, we have analyzed geolocalized data of pollutants recorded in 2018 in surface and groundwater of Lombardia, one of the areas with the highest agricultural production rates, not only in Italy, but also in Europe. Both herbicides and insecticides are present at concentration levels above the legal limit, mainly in surface waters. Geolocalizated analysis allows us to identify interesting areas particularly affected by a combination of multiple pesticides. We thus investigated possible synergistic effects of these compounds on the environment, using the alga *C. reinhardtii* as a biosensor. Our results show that exposure for 7 days to four compounds, that we found present together at high concentration in surface waters, was able to induce a stress in the algae, as indicated by the presence of palmelloids. Our work results in a pipeline that could easily be exported to monitor other territories in Italy and abroad.

*Keywords:* water pollution, herbicides, AMPA, Glyphosate, pesticide, Lombardy, synergistic effect, biosensor, Chlamydomonas, geolocalization

[*] Corresponding author: caterina.laporta@unimi.it

## 1. Introduction

The increasing intensification in cropping system management carried out during the last decades led to a massive use of agrochemicals, particularly pesticides. Their intensive use is not always accompanied by increased yield, leading to important negative consequences for the environment and for the economic balance of the farm. In fact, on the one side, the massive use of agrochemicals creates the conditions for soil and water contamination and, on the other hand, it represents a high economic costs in terms of product purchase, distribution and management for the farm [1]. In October 2009, the European Union published the directive 128/2009 which establishes a framework to achieve the sustainable use of pesticides. Nowadays, the broad category of products used for plant/crop protection from stresses (Plant protection products PPP) is mainly regulated by framework Regulation /(EC) No 1107/2009.

Evidence that some of these chemical substances pose a potential risk to humans and other life forms and unwanted side effects to the environment are not new [2, 3]. In the last years, water contamination is becoming an increasingly serious problem worldwide and the regulatory jurisdiction specifies the maximum concentration level (MCL) that may occur for each pesticide as well as the impact on human health of the maximum concentration pesticide levels [4]. During the crop management cycle, farmers usually apply different pesticides resulting in a possible multiple contamination of the environment, including water. Inside the broad category of pesticides, herbicides are the most extensively used, accounting for up to 50% of the global plant protection market [5].

Even if a single pesticide could be at the same time effective and safe at a sufficiently low concentration, nowadays the main question is the impact of the accumulation of multiple pesticides in the environment, considering the entire ecosystem including humans. In this connection, the presence of chemical mixtures existing in samples of groundwater used for public supply was recently investigated by [6]. In this study the authors showed that the combined exposure to different contaminants can be a potential concern for more than half of the samples studied and that, even though the water devoted to public supply is treated to reduce contamination according to the current legislation, it can still contain mixtures of the chemicals at worryingly high concentrations [6].

Herein our studies want to create a pipeline for the geolocalization of water contaminants using a big data approach to identify the most abundant compounds present in waters, their co-presence and the hotspot sites where they are found. This pipeline allows us to monitor the territories and helps possible interventions to correct possible contamination. We focus our studies on the Lombardia region since here the agrofood sector is characterized by a very intensive model with one of the largest use of agrochemicals in Italy [7]. Moreover, Lombardia has an interesting hydrography with many lakes, rivers, channels, and springs. In Lombardia there is also a high population density with about 10 million people, representing more than one-sixth of the entire Italian population.

In addition to the geolocalized statistical analysis, we investigated possible synergic effects of multiple compounds, detected at concentration above the legal limit, using a biosensor, the algae *C. reinhardtii*. Algae and microalgae are well known as biological indicators for the evaluation of the water and soil quality. In fact, they quickly respond to changes of the environmental quality allowing us to monitor synergistic and antagonistic effects of various pollutants [8]. Moreover, it is known that algae respond to environmental stress through phenotypic plasticity by forming reversible colonies, defined as palmelloids [9, 10, 11, 9].

## 2. Materials and Methods

*Data*

Data relating pollutants in Lombardia in the year 2018 were provided by Regional Environment Protection Agency (ARPA). Lombardia ARPA monitors 492 groundwater stations and 350 points placed along the main river network monthly (temporally and small water bodies, often linked to the irrigation system, are excluded).The list of pollutants reported in the database are those provided by the European and Italian Water Directives (Dir. 2000/60/EC, Dir. 2006/118/EC, Dir. 2008/105/EC, Dir. 2009/90/EC, M.D. 56/2009, M.D. 260/2010, Leg. D. 15,/2006, Leg. D. 30/2009, Leg. D. 219/2010). Databases contain 160160 and 269858 records for groundwater and surface waters, respectively. We started from a total of 304 parameters for groundwater and of 435 for surface waters. These pollutants were subdivided into two subgroups: the first relating to herbicides and the second to insecticides. We identified 48 insecticides and 51 herbicides for groundwater and 48 insecticides and 51 herbicides for surface water (Table S1). In the data analysis, only the parameters with a concentration of $c > c_0 = 0.1\mu$g/l were considered since this value is reported to be the legal limit value detected. Data relating the routes of the main river network, as well as those of the land use and cover classes (DUSAF 2018) were download from the Geoportale of the Lombardy Region (http://www.geoportale.regione.lombardia.it/).

*Data analysis*

Data were analyzed using a custom made python 3.7 jupyter notebook using the pandas package. Cluster of substances where obtained using the Apriori algorithm [12] as implemented in the mlxtend package [13]. Apriori is an algorithm to determine association rules in databases. It is based on the identification of frequent individual items in the database and extending them to item sets that appear frequently together in the database. In the context of our analysis, the Apriori algorithm is used to find clusters of substances that were found together in the same location. We restrict our analysis to clusters containing 2,3 and 4 substances.

*Data visualization*

Geolocalized shape files were obtained from the geoportal of Regione Lombardia (http://www.geoportale.regione.lombardia.it) and plotted using the geopandas, matplotlib and seaborn packages. The rivers displayed in the maps are only those classified as "Reticolo idrico principale (RIP)". Boxplots, barplots and heatmaps were plotted using the seaborn package.

*2.1. Chlamydomonas culture growth and exposure to stress conditions.*

*C. reinhardtii* cells were growth in TAP medium (cod. A1379801, Invitrogen) as batch cultures until they reached a concentration of $\approx 10^6$ cells/ml (corresponding to mid-exponential phase of growth). The cells were cultured under continuous cool-white fluorescent lamps ($\approx 100\mu$mol photons $m^{-2}s^{-1}$) within a 110rpm shaking incubator, at 25° C [10]. 5ml of cells were spun at 1100g/5min/25°C and resuspended in 15 ml of fresh TAP medium containing Glyphosate (10.3 $\mu g$/L, cod. 45521, Sigma-Merk), (aminomethyl)phosphonic acid (AMPA) (37.2 $\mu g$/L, cod. 324817, Sigma-Aldrich), Terbuthylazine ( 3.6 $\mu g$/L, cod. 45678, Merk-Millipore), Bentazon ( 6.4 $\mu g$/L, cod. 2052, Sigma-Aldrich) separately or in combination at the same concentrations detected in shallow water in 2018. Cells grown in TAP medium without contaminants have been used as control.

### *2.2. Chlamydomonas growth and palmelloids quantification*

The growth of *C. reinhardtii* was monitored by measuring optical density at 680 nm of 100 $\mu$L cell culture using a microplate reader (Ensight, Perkin Elmer), immediately after the seeding the cells and after the exposure to the compounds, alone or in combination [10].

Palmelloids quantification. For each experimental condition, 100 $\mu$L of the cultured cells was harvested, washed in 1X Phosphate buffered saline (PBS, pH 7.0, cod. P4417, Sigma), and fixed overnight at 4° C in 1% paraformaldehyde in PBS. The next day, cells were washed again, resuspended in 200 $\mu$L of 1X PBS and spotted onto a coverslip and images were acquired with DMi8 (Leica) using brightfield objective at 20x. To quantify the presence of palmelloids a custom pipeline including Trainable Weka Segmentation (TWEKA) Fiji plugin [14, 15] and ICY (V. 1.9.5 [16]) has been used for image pre-processing to optimize background recognition and area calculation. At least 500 cells or aggregates were considered for each condition. Particles with size below $30 \mu m^2$ or above 250 $\mu m^2$ were discarded. Seaborn Python library [17] was used to plot size distributions.

## Results

We investigated the quantity of herbicides and insecticides in surface and deep waters recorded by ARPA in Lombardia in the year 2018. Herbicides and insecticides represent 33% of the total parameters analyzed for surface waters and 36% for groundwater (see Table S2). The dataset includes a total of 160160 records for groundwater and 269858 records for surface waters.

We first analyze the concentration and localization of individual substances. Fig 1a and 1c display the geographycal distribution and the number of herbicides and insecticides detected in surface waters with a concentration larger than $c > c0 = 0.1 \mu$g/l (see Materials and Methods for details). The points relative to the sampling stations have different sizes and colors: size and color gradient of each point increments with the grow of the relative pesticide concentration and corresponds to the maximum concentration found for that particular point (for more details see Materials and Method section). The figure clearly shows that herbicides are detected less in the alpine area while insecticides are equally distributed all over the region. However, insecticides are clearly detected close to valleys (such as Valtellina) where fruit trees and vineyards are cropped. We then report in Fig. 1b and 1d box plots of the concentrations — in units of $c_0$ — of the substances with concentration exceeding $c_0$. The graphs show that a wide variety of pesticides with predominant presence in terms of concentrations of AMPA and Glyphosate among the herbicides, and Imidacloprin among insecticides. In particular AMPA can reach concentrations up to 20$\mu$g/l which is 200 times the value of $c_0$. Similarly, Imidacloprin is found in concentrations that are up to 40 times larger than $c_0$.

The massive presence of AMPA and Glyphosate in surface waters is confirmed in Fig. 2 where we show the geolocalization and relative concentration of Glyphosate (Fig. 2a) and its metabolite AMPA (Fig. 2b). We observed nine records relating to Glyphosate exceeding the concentration of 10mg/l, those of AMPA are 134. Figure. 2 shows that these substances are both very abundant and widespread most in connection with agricolture region. In particular it seems interesting the high levels of Glyphosate detected in the area of Roggia Vignola, an important irrigation channel near the town of Trevglio (see red dot in Fig. 2a). AMPA is accumulated in the North West area of Lombardy, which corresponds to the territory of the Varese Province, where there are Olona, Seveso, Bozzente and Lura , four known polluted rivers [18, 19].

In order to identify which of the substances are co-present at high concentration in the same place, we constructed a cross-correlation matrix showing the number of times two substances, herbicides or insecticides, are recorded in the same location and at the same time both with $c > c0$ (Fig. 3a). The matrix highlights that several substances are co-present, in particular AMPA and Glyphosate that are often found together but also in combination with other substances. To quantify multiple combinations of substances, we use the Apriori algorithm [12] which is able to find association rules in a database. In Fig. 3b and Fig. 3c, we show the probabilities of find clusters of three and four substances in the same location with $c > c_0$. We thus obtain a ranked list of the most likely clusters of substances. In particular, Glyphosate and AMPA are often found in combination with Terbuthylazine, Bentazon, 2,4-Dichlorophenol and Metolachlor.

Finally we analysed the groundwater with the same approach. As shown in Fig. 4a, the map shows the sites where herbicides and insecticides have been found with $c > c0 = 0.1\mu$g/l and the color code shows the number of substances found in the same location at the same time. The boxplots with the recorded concentrations are reported in Fig. 4b, showing that Glyphosate and AMPA are found at high concentrations, often two to four times the value of $c > c0 = 0.1\mu$g/l. We also find again the substances found in surface waters such as Terbuthylazine and Bentazon.

We then perform again the clustering analysis with the apriori algorithm to identify groups of substances occurring together in the same location. Fig. 4c and 4d report the clusters of two and three substances, showing again that similar patterns than those observed in surface waters. The difference is that the abundance of substances is smaller in groundwater than in surface waters, but despite this clusters of substances are still observed.

While the number of substances found in large concentration is large, there is a net prevalence of AMPA and Glyphosate both in surface and groundwater. Twelve herbicides and four stable metabolites are present in combination with Glyphosate and/or AMPA (Fig. 3, Fig. 4 and Table S3. Among these substances DNCO, Metolachlor, Molinate, Oxadiazon, Atrazine (Desethyl Atrazine), Dichlobenil (2,6 Dichlorobenzamide) were no longer in the market. We then chose for further analysis Bentazone and Terbuthylazine since they are always present in combination with Glyphosate and AMPA (Fig. 4b).

To investigate the possible synergic impact of these substances on the environment, we used *Chlamydomonas reinhardtii* as a biological sensor. Using *Chlamydomonas reinhardtii* we quantified the possible effect of AMPA, Glyphosate, Bentazon and Terbuthylazine, individually or in combination, on the growth rate of the algae after 7 days and quantifying the presence of palmelloids. As shown in (Fig. S1), no significant effects on *Chlamydomonas reinhardtii* growth rate have been observed. However, we found that the exposure to AMPA and Glyphosate, alone or in combination with Terbuthylazine and Bentazon as well as the mix of the four compounds, leads to a broad size distribution with the presence of peaks in the larger sizes distribution, that is the typical dimension of cells aggregates (palmelloid shape) (Fig. 5).

## Discussion

Lombardia is the richest region of Italy and one of the area with highest agricultural production rates not only in Italy but in Europe. In fact, the conventional agriculture spreads over 94.5% of Lombardia covering about one million hectares, while organic farming currently 5.5% only. The intense exploitation of agricultural soils is also accompanied to a large use of pesticides. In all Europe about 37 million tons of insecticides, 127 of herbicides and 168 of fungicides have been used, according to the Europaean Environmental Agency Environmental (EAE), in 2018. Eurostat data in 2020 also reported that Italy is the third European country, after Spain and France, for the consumption of pesticides. The analysis of data collected by the Regional Environmental Protection Agency (ARPA) for 2018 of surface and groundwater goes in this direction showing how herbicides and insecticides are important pollutants in Lombardia. In the groundwater less pollutants above the legal threshold are present in comparison to the surface water. In addition, comparing the concentration found in the surface water with respect to the groundwater for the same compounds a lower concentration is present. Another interesting aspect is that the geolocalization of the pollutants detected above the legal threshold both in surface and groundwater shows that herbicides are detected less in the alpine area while insecticides are present all over the region. Interestingly, insecticides have been detected closed to valleys such as Valtellina which is rich of fruit trees and vineyards. Therefore, intense agricolture area like Valtellina appears important to monitor during the time.

Between the herbicides present in both surface and groundwater above the legal threshold there are compounds that are no longer in the market such as Molinate, Oxadiazon, Atrazine (Desethyl Atrazine), Dichlobenil (2,6 Dichlorobenzamide) but others that are still in the market such as AMPA, Glyphosate, Bentazon and Terbuthylazine.

Glyphosate is the worlds most widely used herbicide in agriculture since more than four decades. It is intensively applied to agricultural fields, before planting the crop, pre- or post-harvest, in both conventional and reduced/no-till farming, to control the growth of annual and perennial weeds. It is marketed as having no effect in animals since it is designed to specifically inhibit an enzymatic pathway required for protein synthesis unique to plants. In particular, Glyphosate is reported to be able to block plant growth by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [20, 21]. The EPSPS is a particular enzyme presents in plants, bacteria and fungi, but not in metazoans. Recently, Glyphosate was shown in Daphnia, a small planktonic crustaceans, to bind toxic metals present in the soil becoming more toxic on the crustaceous [22].

Aminomethylphosphonic acid (AMPA) is one of the primary degradation products of Glyphosate. AMPA is normally present in soils and waters due to its chemical stability and it was found to be 3-6 times more resistant than Glyphosate against degradation [23]. There is no specific toxicity reported in the literature with the exception of a recent study showing the synergistic interaction between AMPA and an other surfactant polyethoxylated tallow amine (POEA) affecting the development of fish embryo of zebrafish (Danio rerio) [24]. The geolocalization analysis of AMPA showed that this is present in the agricolture area of Lombardia. In particular, it is visible an accumulation of AMPA in the North West area of Lombardy, which corresponds to the territory of the Varese Province, a well known polluted area citepazzellino2013,bocchi2012. Even till now is uncertain the toxicity of AMPA this pipeline offer a useful approach to monitor the possible impact on the territory of this pollutant during the time, in particular when is present with others pollutants at high concentration.

Bentazon and Terbuthylazine, are important herbicides applied to maize and other crops to control pre-emergence or early post-emergence broadleaf and grass weeds. In environment they form metabolites such as N-methyl-bentazone, desethyl-Terbuthylazine, 2-hydroxy-Terbuthylazine [25, 26]. Bentazon and Terbuthylazine have a comparable mechanism of action. These two pesticides act as an inhibitors of photosynthesis by blocking the electron transfer flow in photosystem II (PSII) [27], the latter in turn may inducessecondary effects in several metabolic pathways [28, 29]. These energized compounds can promote oxidative damage in chloroplasts proteins and membranes of photosynthetic cells [30] causing cell death.

it is clear from the geolocalization analysis that the environment is exposed to multiple pollutants each at concentration above the legal threshold. The possible synergyic effect of the exposure to multiple pollutant is almost neglected in the literature where it is often reported the effect of single pollutants. To investigate this point, suggesting a simple and easy pipeline, we exposed *C. reinhardtii* to a mix containing the higher concentration of AMPA, Glyphosate, Bentazone or Terbuthylazine found in the surface water. While the impact on the growth of *C. reinhardtii* was not significant, [9, 10, 11, 9], we found a significant increase of palmelloids, which represents a stress response of algae to environmental factors after 7 days of exposure. *C. reinhardtii* plays an important role in the equilibrium of aquatic ecosystems, representing the first level of the trophic chain. Due to their highly sensitive nature, microalgae are claimed as early indicator of deteriorating environmental conditions [31].

All together our findings based on a combination of data analysis and experiments highlight the potential environmental relevance of synergistic effects of multiple pollutants accumulating in surface and groundwater. The same pipeline could easily be exported to monitor other territories in Italy and abroad.


[1] M. Lechenet, F. Dessaint, G. Py, D. Makowski, N. Munier-Jolain, Reducing pesticide use while preserving crop productivity and profitability on arable farms, Nat Plants 3 (2017) 17008. doi:10.1038/nplants.2017.8.

[2] S. O. Igbedioh, Effects of agricultural pesticides on humans, animals, and higher plants in developing countries, Arch Environ Health 46 (4) (1991) 218–24. doi:10.1080/00039896.1991.9937452.

[3] J. Jeyaratnam, Health problems of pesticide usage in the third world, Br J Ind Med 42 (8) (1985) 505–6. doi:10.1136/oem.42.8.505.

[4] Z. Li, A. Jennings, Global variations in pesticide regulations and health risk assessment of maximum concentration levels in drinking water, J Environ Manage 212 (2018) 384–394. doi:10.1016/j.jenvman.2017.12.083.

[5] D. Massa, Y. I. Kaiser, D. Andújar-Sánchez, R. Carmona-Alférez, J. Mehrtens, R. Gerhards, Development of a geo-referenced database for weed mapping and analysis of agronomic factors affecting herbicide resistance in apera spica-venti l. beauv.(silky windgrass), Agronomy 3 (1) (2013) 13–27.

[6] P. L. Toccalino, J. E. Norman, J. C. Scott, Chemical mixtures in untreated water from public-supply wells in the u.s.–occurrence, composition, and potential toxicity, Sci Total Environ 431 (2012) 262–70. doi:10.1016/j.scitotenv.2012.05.044.

[7] C. Rega, C. Short, M. Pérez-Soba, M. L. Paracchini, A classification of european agricultural land using an energy-based intensity indicator and detailed crop description, Landscape and Urban Planning 198 (2020) 103793.

[8] T. K. Parmar, D. Rawtani, Y. Agrawal, Bioindicators: the natural indicator of environmental pollution, Frontiers in life science 9 (2) (2016) 110–118.

[9] S. Sasso, H. Stibor, M. Mittag, A. R. Grossman, From molecular manipulation of domesticated chlamydomonas reinhardtii to survival in nature, Elife 7. doi:10.7554/eLife.39233.

[10] D. K. Khona, S. M. Shirolikar, K. K. Gawde, E. Hom, M. A. Deodhar, J. S. D'Souza, Characterization of salt stress-induced palmelloids in the green alga, chlamydomonas reinhardtii, Algal Research 16 (2016) 434 – 448. doi:https://doi.org/10.1016/j.algal.2016.03.035.
URL `http://www.sciencedirect.com/science/article/pii/S2211926416301175`

[11] F. de Carpentier, S. D. Lemaire, A. Danon, When unity is strength: The strategies used by chlamydomonas to survive environmental stresses, Cells 8 (11). doi:10.3390/cells8111307.
URL `https://www.mdpi.com/2073-4409/8/11/1307`

[12] R. Agrawal, R. Srikant, et al., Fast algorithms for mining association rules, in: Proc. 20th int. conf. very large data bases, VLDB, Vol. 1215, 1994, pp. 487–499.

[13] S. Raschka, Mlxtend: Providing machine learning and data science utilities and extensions to python's scientific computing stack, The Journal of Open Source Software 3 (24). doi:10.21105/joss.00638.
URL `http://joss.theoj.org/papers/10.21105/joss.00638`

[14] I. Arganda-Carreras, V. Kaynig, C. Rueden, K. W. Eliceiri, J. Schindelin, A. Cardona, H. Sebastian Seung, Trainable weka segmentation: a machine learning tool for microscopy pixel classification, Bioinformatics 33 (15) (2017) 2424–2426.

[15] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, et al., Fiji: an open-source platform for biological-image analysis, Nature methods 9 (7) (2012) 676–682.

[16] F. De Chaumont, S. Dallongeville, N. Chenouard, N. Hervé, S. Pop, T. Provoost, V. Meas-Yedid, P. Pankajakshan, T. Lecomte, Y. Le Montagner, et al., Icy: an open bioimage informatics platform for extended reproducible research, Nature methods 9 (7) (2012) 690–696.

[17] M. Waskom, the seaborn development team, mwaskom/seaborn (Jan. 2020). doi:10.5281/zenodo.592845.
URL `https://doi.org/10.5281/zenodo.592845`

[18] A. Azzellino, M. Antonelli, S. Canobbio, S. Cevirgen, V. Mezzanotte, A. Piana, R. Salvetti, Searching for a compromise between ecological quality targets, and social and ecosystem costs for heavily modified water bodies (hmwbs): the lambro-seveso-olona system case study, Water Sci Technol 68 (3) (2013) 681–8. doi:10.2166/wst.2013.277.

[19] S. Bocchi, D. La Rosa, P. Pileri, Agro-ecological analysis for the eu water framework directive: An applied case study for the river contract of the seveso basin (italy), Environmental Management 50 (4) (2012) 514–529. doi:10.1007/s00267-012-9925-3.
URL `https://doi.org/10.1007/s00267-012-9925-3`

[20] H. C. Steinrücken, N. Amrhein, 5-enolpyruvylshikimate-3-phosphate synthase of klebsiella pneumoniae 2. inhibition by glyphosate [n-(phosphonomethyl)glycine], Eur J Biochem 143 (2) (1984) 351–7. doi:10.1111/j.1432-1033.1984.tb08379.x.

[21] C. M. Benbrook, Trends in glyphosate herbicide use in the united states and globally, Environ Sci Eur 28 (1) (2016) 3. doi:10.1186/s12302-016-0070-0.

[22] L. R. Hansen, P. Roslev, Behavioral responses of juvenile daphnia magna after exposure to glyphosate and glyphosate-copper complexes, Aquat Toxicol 179 (2016) 36–43. doi:10.1016/j.aquatox.2016.08.010.

[23] M. Sun, H. Li, D. P. Jaisi, Degradation of glyphosate and bioavailability of phosphorus derived from glyphosate in a soil-water system, Water Res 163 (2019) 114840. doi:10.1016/j.watres.2019.07.007.

[24] L. de Brito Rodrigues, G. Gonçalves Costa, E. Lundgren Thá, L. R. da Silva, R. de Oliveira, D. Morais Leme, M. M. Cestari, C. Koppe Grisolia, M. Campos Valadares, G. A. R. de Oliveira, Impact of the glyphosate-based commercial herbicide, its components and its metabolite ampa on non-target aquatic organisms, Mutat Res 842 (2019) 94–101. doi:10.1016/j.mrgentox.2019.05.002.

[25] L. Guzzella, S. Rullo, F. Pozzoni, G. Giuliano, Studies on mobility and degradation pathways of terbuthylazine using lysimeters on a field scale, J Environ Qual 32 (3) (2003) 1089–98. doi:10.2134/jeq2003.1089.

[26] R. Loos, G. Locoro, S. Comero, S. Contini, D. Schwesig, F. Werres, P. Balsaa, O. Gans, S. Weiss, L. Blaha, M. Bolchi, B. M. Gawlik, Pan-european survey on the occurrence of selected polar organic persistent pollutants in ground water, Water Res 44 (14) (2010) 4115–26. doi:10.1016/j.watres.2010.05.032.

[27] S. Matsunaka, S. Kuwatsuka, Environmental problems related to herbicidal use in japan, Environ Qual Saf 4 (1975) 149–59.

[28] P. Müller, X. P. Li, K. K. Niyogi, Non-photochemical quenching. a response to excess light energy, Plant Physiol 125 (4) (2001) 1558–66. doi:10.1104/pp.125.4.1558.

[29] K. Asada, Production and scavenging of reactive oxygen species in chloroplasts and their functions, Plant Physiol 141 (2) (2006) 391–6. doi:10.1104/pp.106.082040.

[30] B. Demmig, K. Winter, A. Krüger, F. C. Czygan, Photoinhibition and zeaxanthin formation in intact leaves : a possible role of the xanthophyll cycle in the dissipation of excess light energy, Plant Physiol 84 (2) (1987) 218–24. doi:10.1104/pp.84.2.218.

[31] P. V. McCormick, J. Cairns, Algae as indicators of environmental change, Journal of applied phycology 6 (5) (1994) 509–526.

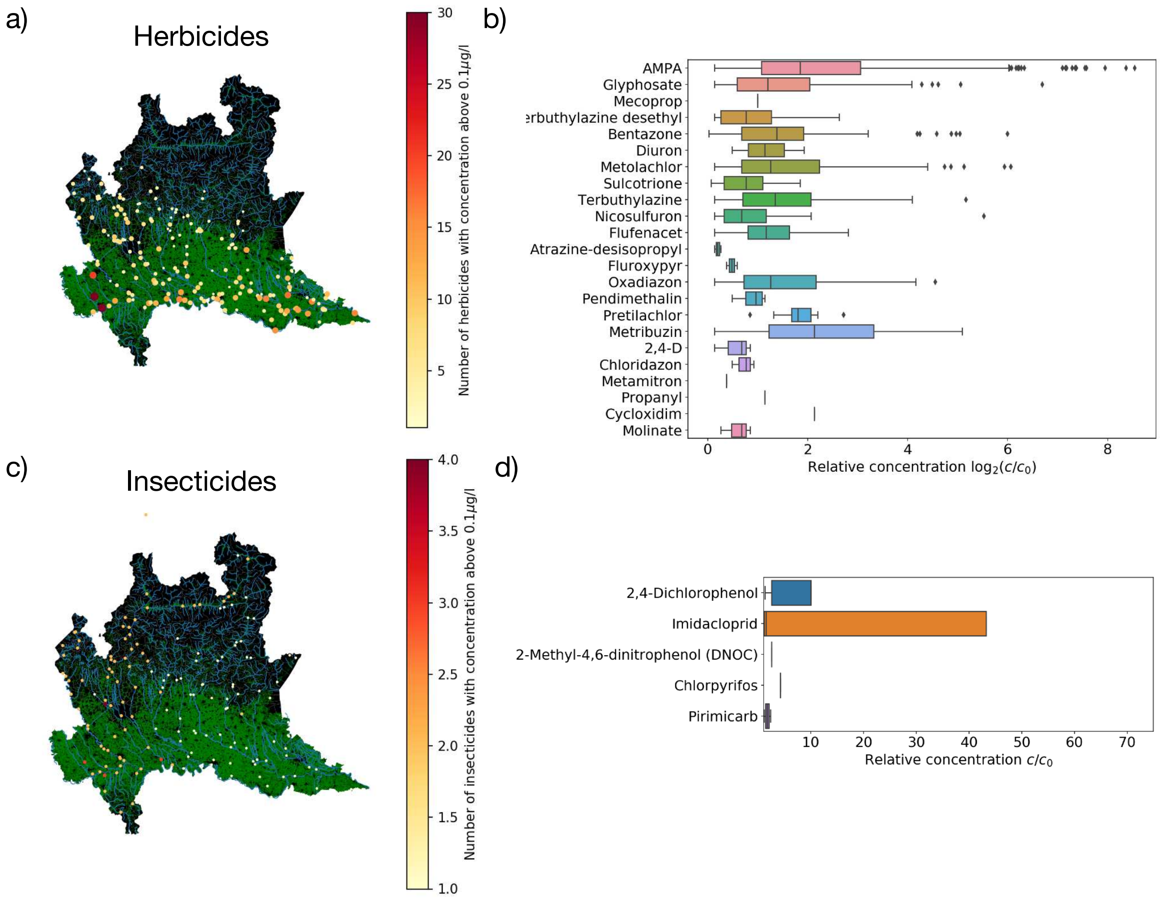


Figure 1: **Distribution of herbicides and insecticides in surface waters.** a) Map showing where herbicides with $c > c_0 = 0.1\mu$g/l have been recorded. The color code represents the number of substances found in the same location at the same time. b) The distribution of relative concentrations $c/c_0$ of herbicides (only when $c > c_0$) Error bars in the boxplot are 1.5 of the inter-quartile range. c) Same as panel a) but for insecticides. d) Same as panel b) but for insecticides.

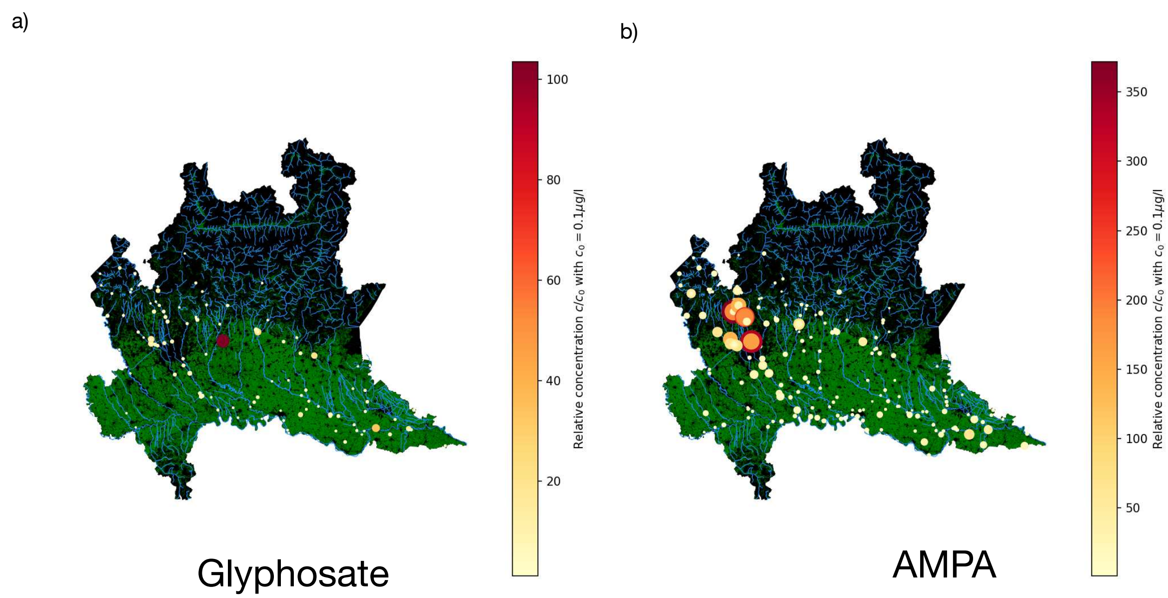


Figure 2: **Glyphosate and AMPA in surface waters.** Relative concentration of a) Glyphosate and b) AMPA. We only report data when $c > 0.1\mu$g/l. Colorbar and marker size both indicate the relative concentration.

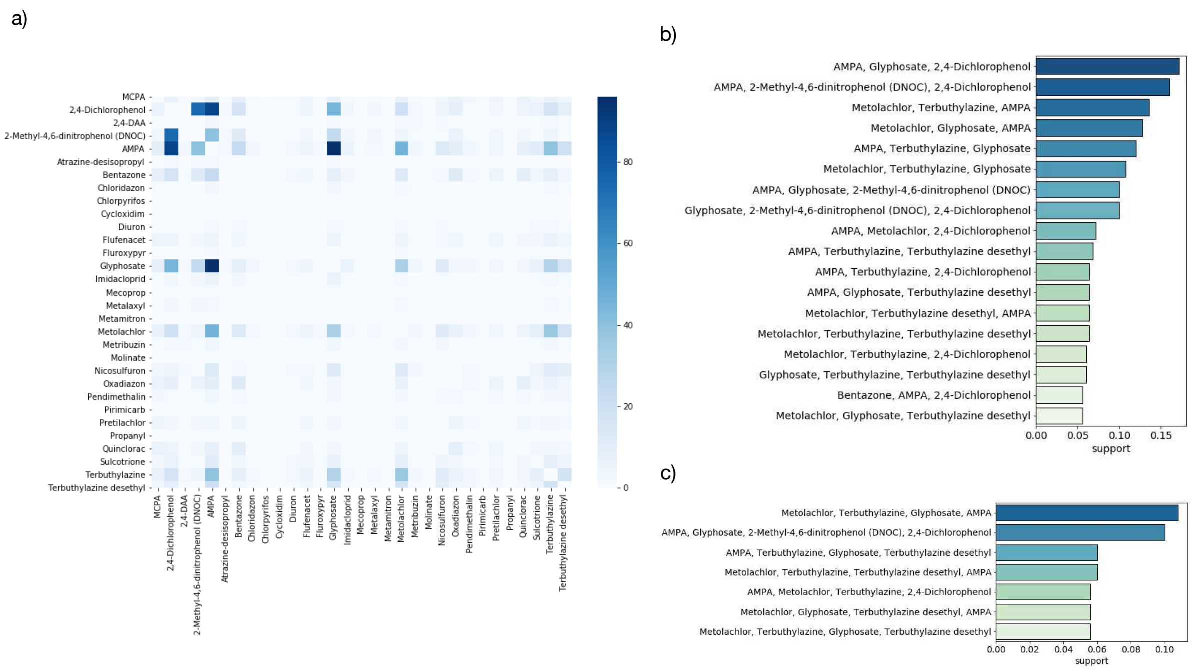


Figure 3: **Clusters of substances in surface waters.** a) Substance cross-correlation matrix showing the number of times two substances (herbicides or insecticeds) have been found together in the same location and at the same time both with $c > c_0 = 0.1\mu$g/l. b) Fraction of instances in which three substances have been recorded (all with $c > c_0$) in the same location at the same time. c) Fraction of instances in which four substances have been recorded (all with $c > c_0$) in the same location at the same time.

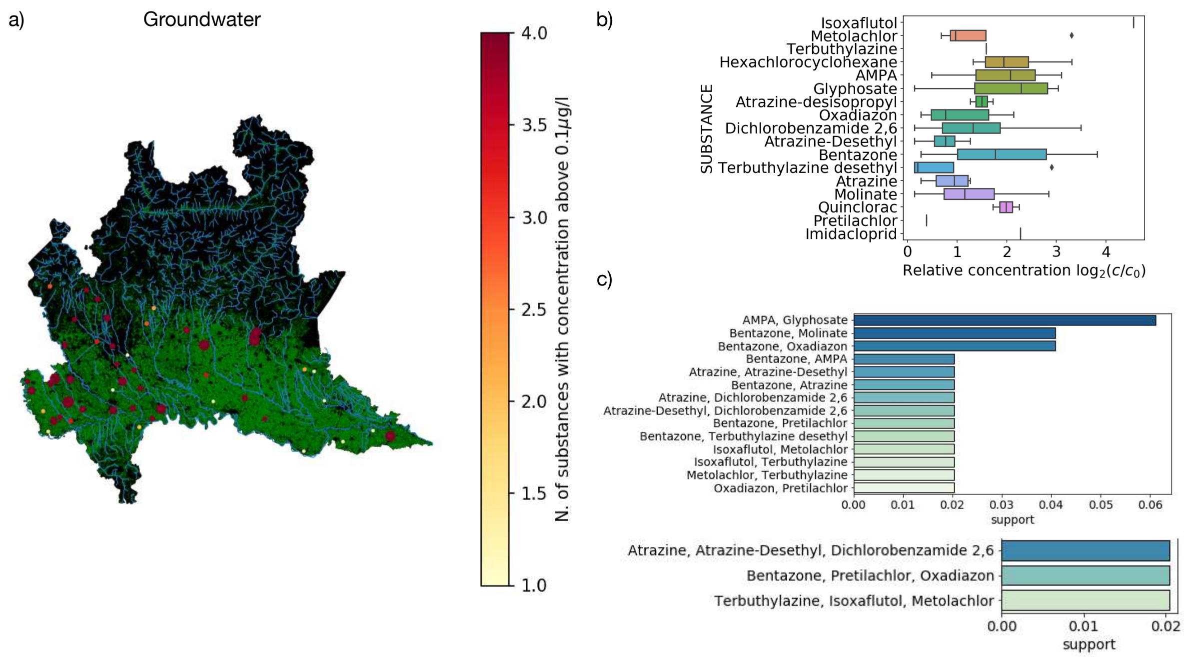


Figure 4: **Clusters of substances in groundwater.** a) Map showing the sites where herbicides and insecticides have been found with $c > c_0 = 0.1\mu$g/l. The color code represents the number of substances found in the same location at the same time. b) The distribution of relative concentrations $c/c_0$ (only when $c > c_0$). Error bars in the boxplot are 1.5 of the inter-quartile range. c) Fraction of instances in which three substances have been recorded (all with $c > c_0$) in the same location at the same time. d) Fraction of instances in which three substances have been recorded (all with $c > c_0$ in the same location at the same time.)

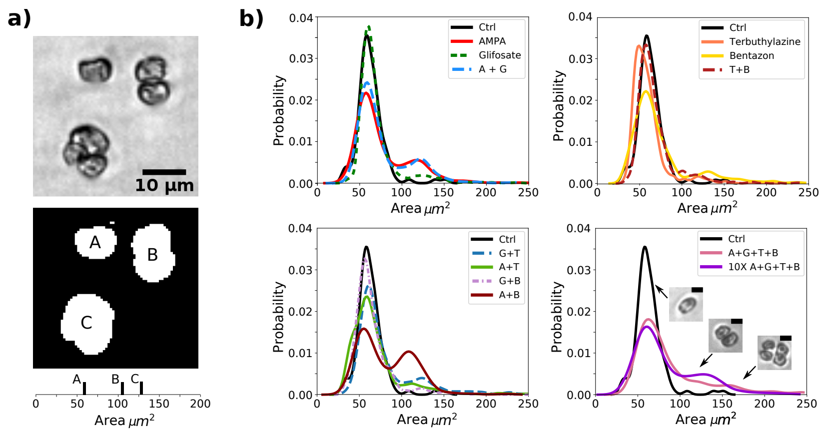


Figure 5: a) Figure shows an exemplificative image of *C. reinhardtii* single cell and aggregates of two and more cells. Binary masking was created in order to calculate the area of each cell/aggregate. Bottom panel shows the values obtained for the three particles shown. Note that area of the aggregate does not scale linearly with the number of particles due to three-dimensional conformation of the aggregates. b) Size particle distribution obtained as described in panel a) and in MM section for all the experimental conditions, as in legend. The presence of multiple peaks in the distributions suggests the presence of aggregates, expecially in presence of AMPA and AMPA in combination with Bentazon (A+B) compared to control condition. The presence of all the four substances at concentration equal to the one detected in swallow waters (MIX) or ten times higher (10 X MIX) gives a broad distribution of sizes.

## Supplementary Information

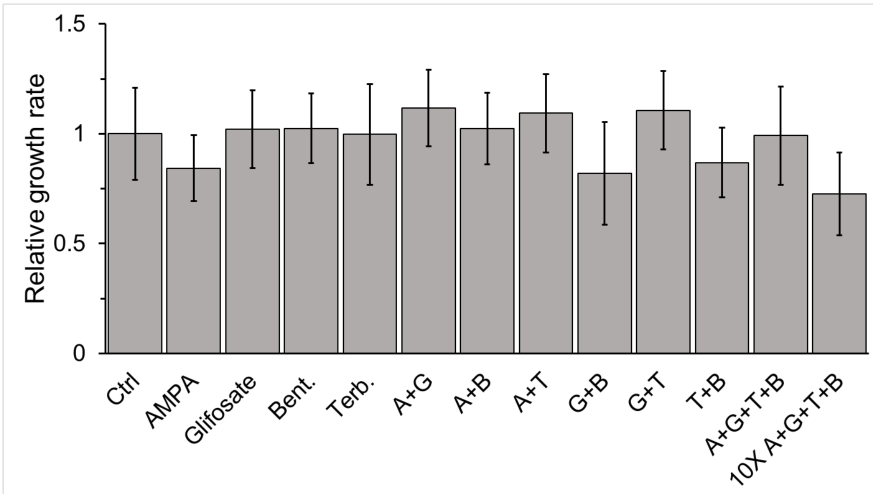


Figure S1: *C.reinhardtii* **relative growth rate.** *C. reinhardtii* cells were cultured in TAP medium containing Glyphosate, AMPA, Bentazon (Bent.), Terbuthylazine (Terb.) separately and in combination at the same concentrations detected in shallow water. Additionally, we also considered a concentration ten times higher for the mix of all the four substances (10X A+G+B+T), and cells grown in TAP medium without any other contaminant were used as control (Ctrl) as described in Materials and Methods section. *C. reinhardtii* growth was monitored by measuring optical density at 680 nm of 100 $\mu$L cell culture using a microplate reader (Ensight, Perkin Elmer). Growth rate was calculated for every experimental condition considered as the log2 ratio between optical density immediately after the seeding and after seven days. Plot shows the average growth rate relative to control condition over three experimental replica. For each experiment and condition, two independent measurements were performed. Error bars are standard deviation over replica. There are no statistically significant differences.

*Supplementary table captions*

Table S1: List of substances (herbicides and insecticides) analyzed in the paper for surface and groundwater.

Table S2: Classes of substances and quantities of products measured by ARPA in the Lombardy surface and groundwater in 2018.

Table S3: W-T: water tipology; RE-LI: revoked license ; YE-RE: year of last revoked; AU-PR: authorized products; RE-PR: re-registered products; EX PR: expired license; surface*, under*: stable products derived from degradation of Glyphosate (AMPA), Dichlobenil (2,6,Dichlorobenzammide) Atrazine (Desethyl Atrazine), Terbuthylazine (Desethyl Terbuthylazine)

# Figures

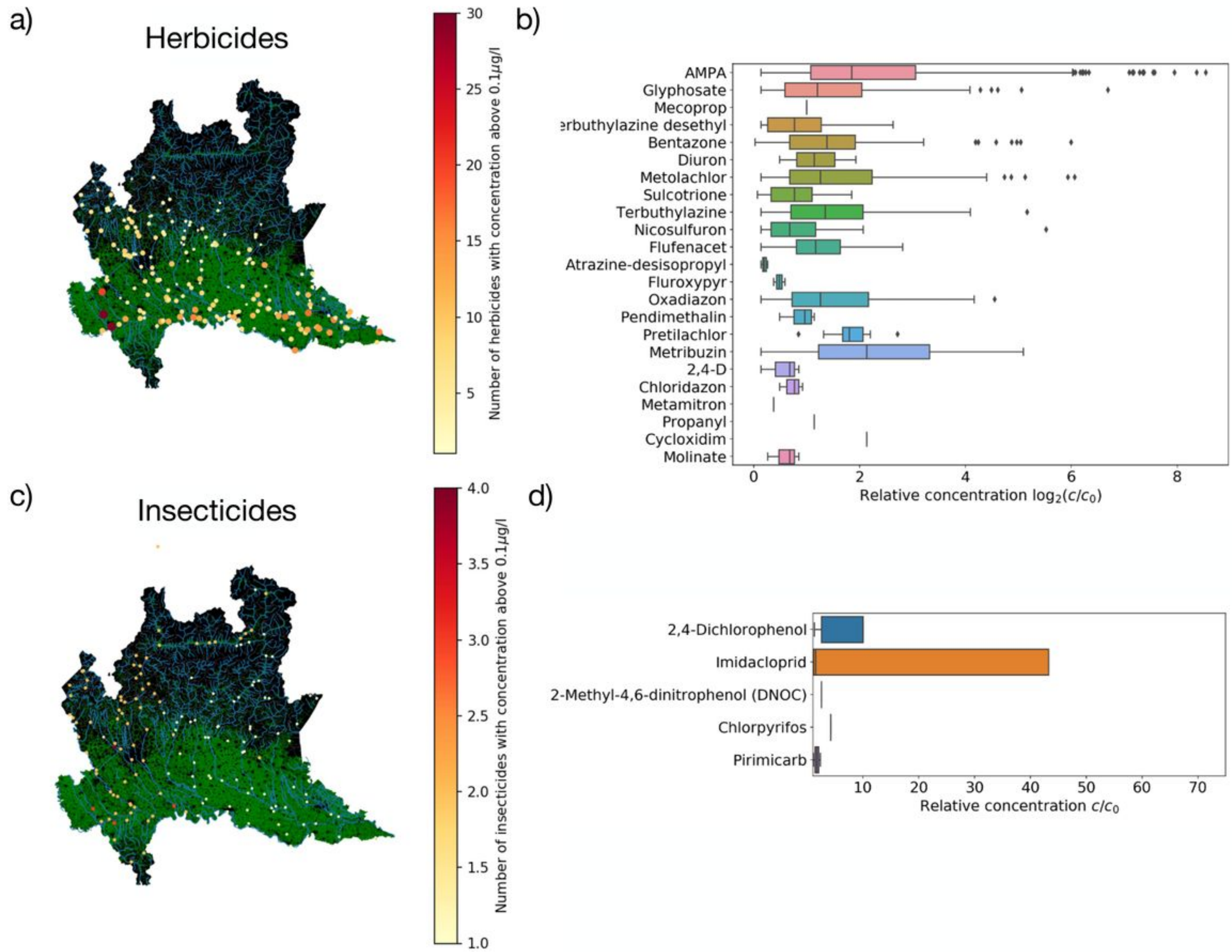


**Figure 1**

Distribution of herbicides and insecticides in surface waters. a) Map showing where herbicides with c > c0 = 0.1µg/l have been recorded. The color code represents the number of substances found in the same location at the same time. b) The distribution of relative concentrations c/c0 of herbicides (only when c > c0) Error bars in the boxplot are 1.5 of the inter-quartile range. c) Same as panel a) but for insecticides. d) Same as panel b) but for insecticides. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

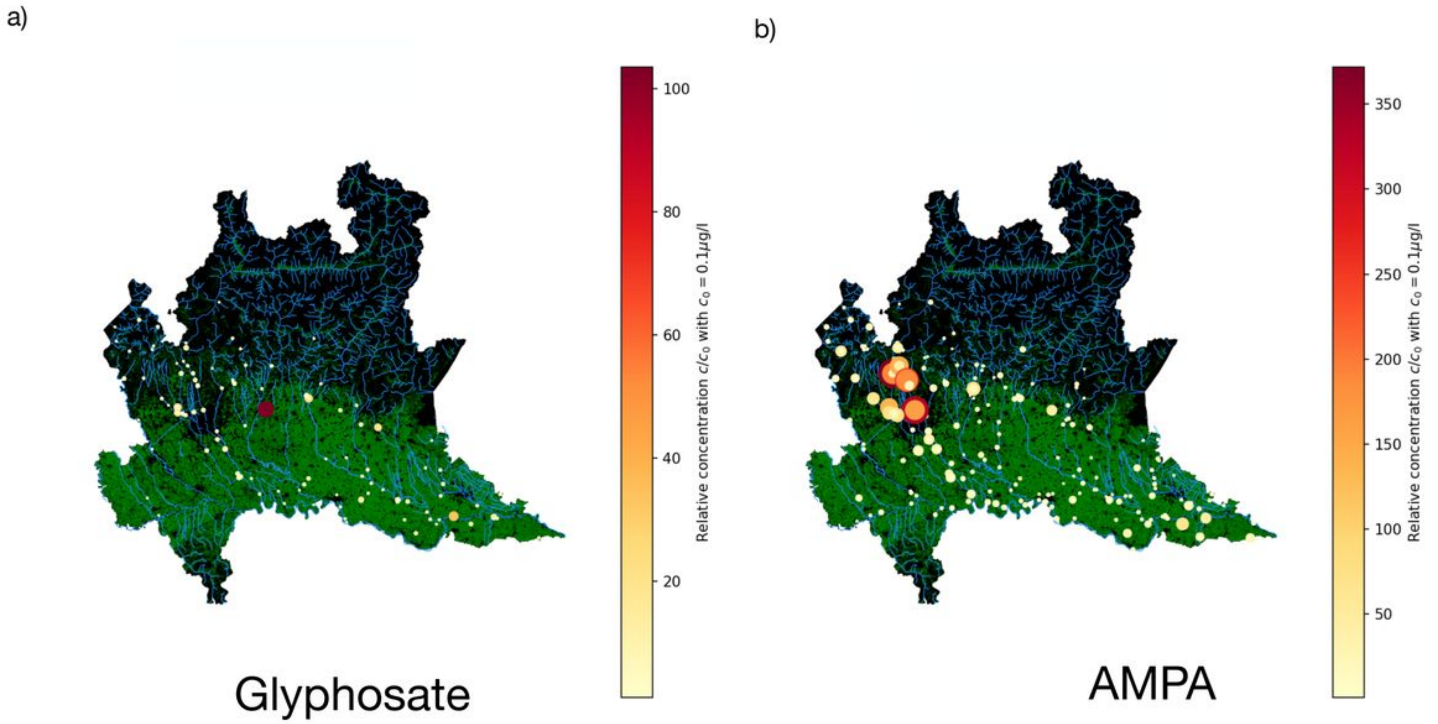


**Figure 2**

Glyphosate and AMPA in surface waters. Relative concentration of a) Glyphosate and b) AMPA. We only report data when c > 0.1μg/l. Colorbar and marker size both indicate the relative concentration. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

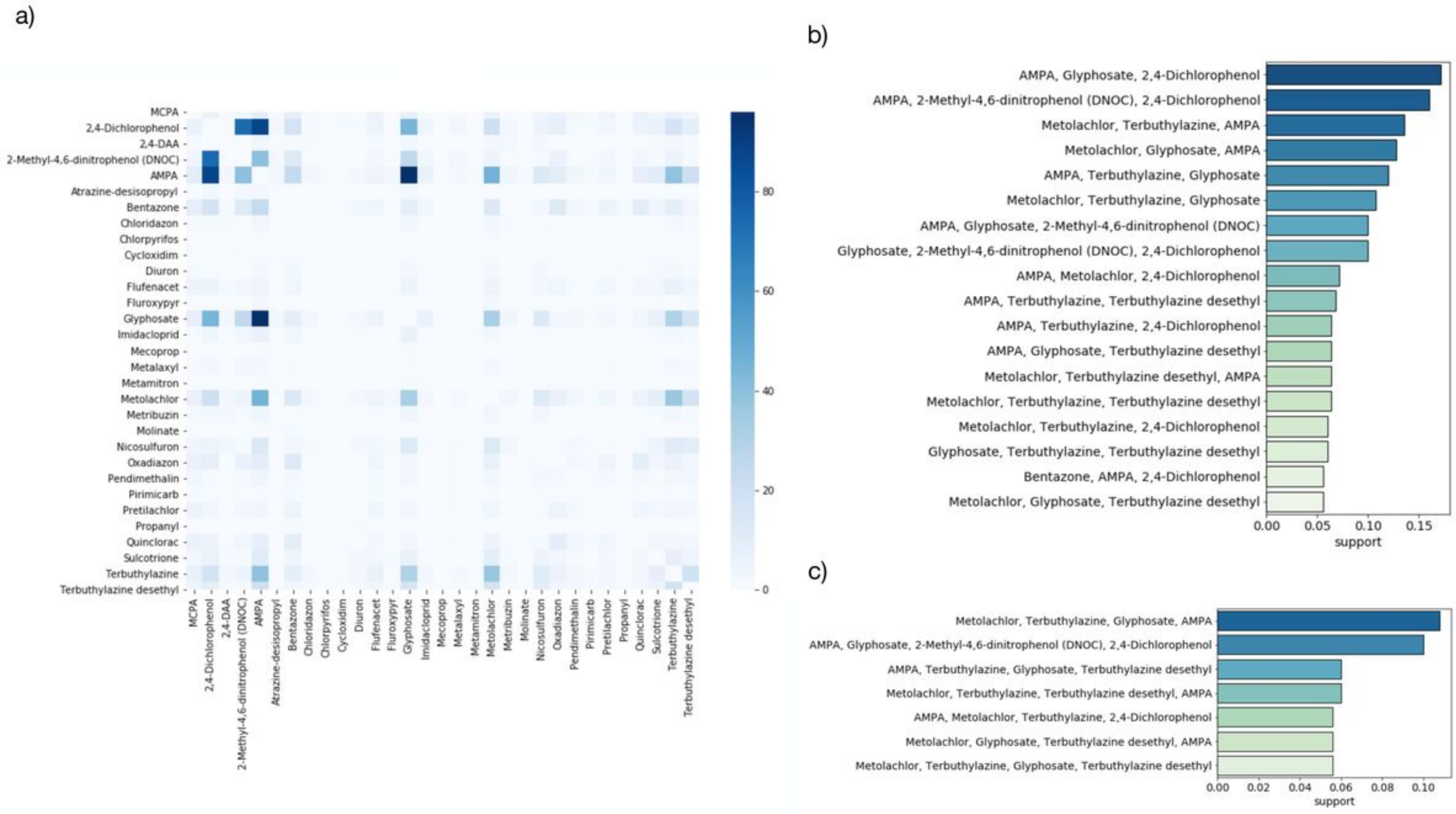


**Figure 3**

Clusters of substances in surface waters. a) Substance cross-correlation matrix showing the number of times two substances (herbicides or insecticeds) have been found together in the same location and at the same time both with c > c0 = 0.1µg/l. b) Fraction of instances in which three substances have been recorded (all with c > c0) in the same location at the same time. c) Fraction of instances in which four substances have been recorded (all with c > c0) in the same location at the same time.

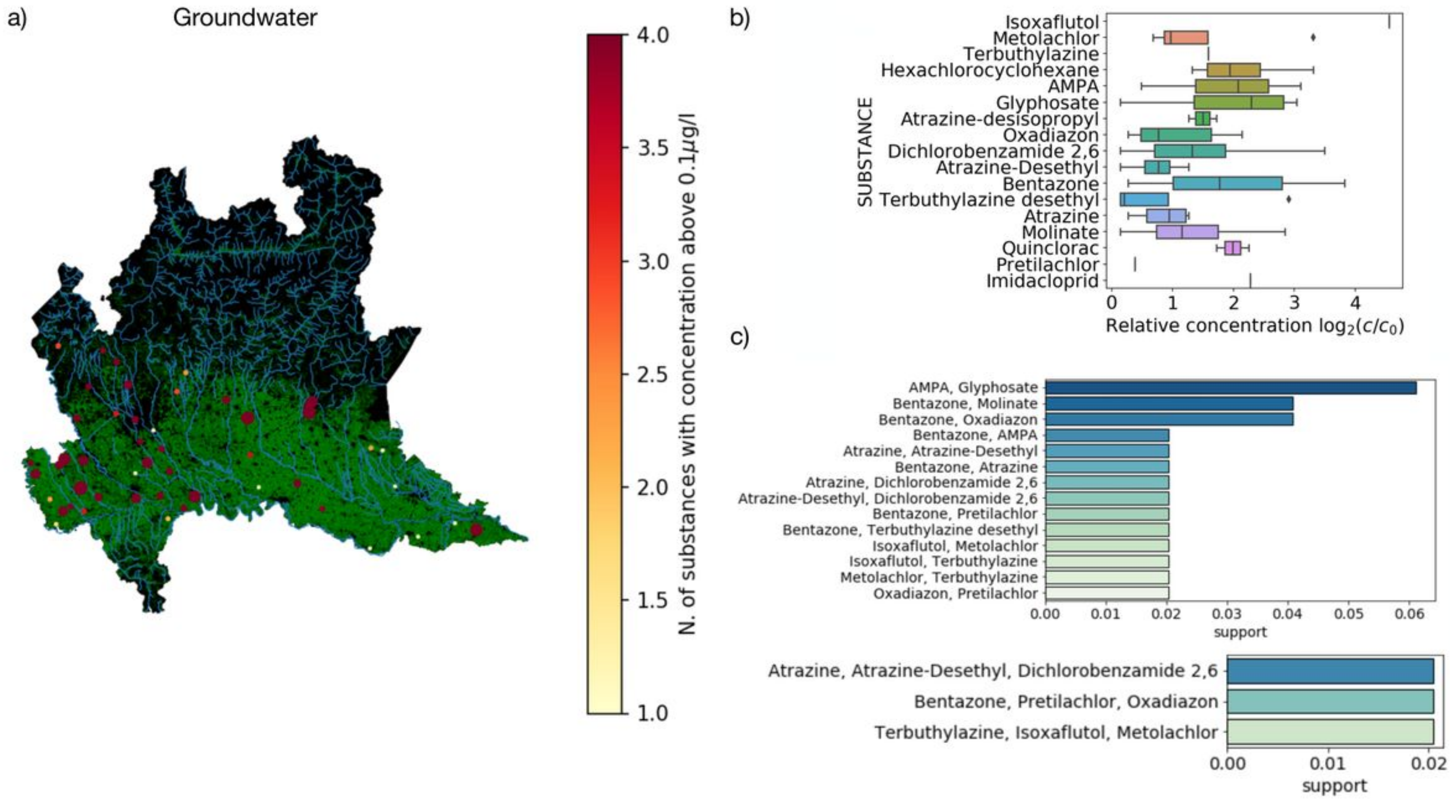


**Figure 4**

Clusters of substances in groundwater. a) Map showing the sites where herbicides and insecticides have been found with c > c0 = 0.1µg/l. The color code represents the number of substances found in the same location at the same time. b) The distribution of relative concentrations c/c0 (only when c > c0). Error bars in the boxplot are 1.5 of the inter-quartile range. c) Fraction of instances in which three substances have been recorded (all with c > c0) in the same location at the same time. d) Fraction of instances in which three substances have been recorded (all with c > c0 in the same location at the same time.) Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

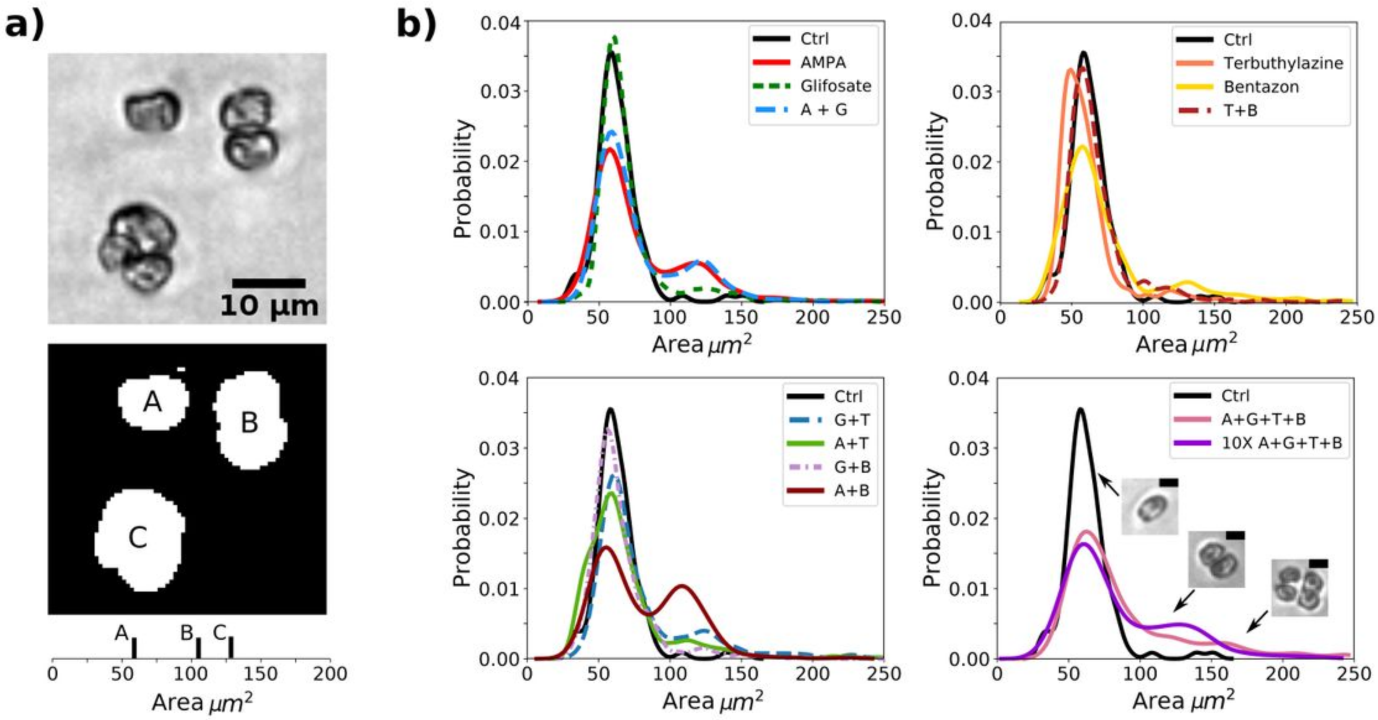


**Figure 5**

a) Figure shows an exemplificative image of C. reinhardtii single cell and aggregates of two and more cells. Binary masking was created in order to calculate the area of each cell/aggregate. Bottom panel shows the values obtained for the three particles shown. Note that area of the aggregate does not scale linearly with the number of particles due to three-dimensional conformation of the aggregates. b) Size particle distribution obtained as described in panel a) and in MM section for all the experimental conditions, as in legend. The presence of multiple peaks in the distributions suggests the presence of aggregates, expecially in presence of AMPA and AMPA in combination with Bentazon (A+B) compared to control condition. The presence of all the four substances at concentration equal to the one detected in swallow waters (MIX) or ten times higher (10 X MIX) gives a broad distribution of sizes.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.pdf
- SI.pdf
- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx