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Environmental risk assessment of neonicotinoids in surface water

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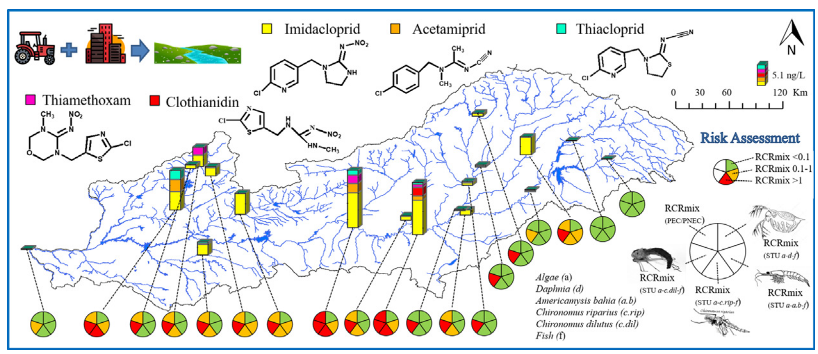
HIGHLIGHTS

- Banned neonicotinoids are still present on the Tagus River basin.
- Imidacloprid and acetamiprid surface water levels were related to irrigated crops.
- Daphnia based risk assessments understated the neonicotinoids risk in river waters.
- Neonicotinoid water levels showed risk for *Chironomus sp* and *Americamysis bahia*.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoids (NNIs) are active substances used as insecticides mainly in plant protection products (PPPs) but also in veterinary applications. The increasing evidence of affecting non-targeted organisms led the European Commission to severely restrict or even ban outdoor uses. To evaluate their current use and their influence in the ecological status of freshwater ecosystem, a total of 19 river water samples were collected to determine the presence of 5 NNIs (acetamiprid, clothianidin, imidacloprid, thiamethoxam and thiacloprid) in the Tagus basin. At least one target analyte was quantified by HPLC-MS/MS analysis in 17 of the 19 water samples, with $\sum$NNIs ranging from <MDL to 16.8 ng/L. Imidacloprid (2.75 ng/L; mean) and acetamiprid (0.47 ng/L) were quantified in most of the samples. Source identification evidences imidacloprid agricultural use. Risk assessment for different trophic levels was conducted with the data obtained calculating Risk Characterization Ratios (RCR) by two approaches, predicted non effect concentrations (PEC/PNEC) and Toxic Units (TU). RCRs were derived for each NNI and for the mixture of all (RCRmix). Results showed risk for imidacloprid in freshwater organism (RCRfw>1) and for the mix of NNIs (RCRmix (PEC/PNEC) > 1). RCRmix(PEC/PNEC) and the sum of toxic units (STU) showed a risky situation for some locations with different organisms related to agriculture practices. This data arouses concern about NNIs (legal or forbidden) use in Tagus basin, and manifest the need of monitoring their presence and effect on the aquatic ecosystem.

1. Introduction

Neonicotinoids (NNIs) are a group of active substances with systemic insecticidal properties that are principally used in agriculture in a vast variety of crops (vegetables, cereals, fruit, rice, cotton, potatoes and tobacco) (Kundoo et al., 2018) but also in urban pest control and veterinary applications (Simon-Delso et al., 2015). NNIs had experienced a rapid expansion, replacing other pesticides due to several advantages, as lower dose is required than organochlorines, carbamates, and organophosphorus insecticides (Kundoo et al., 2018) In agriculture, their systemic nature allows the translocation to all the parts of the plant, that combined with their versatile application (foliar spray, seed

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dressing, soil treatment…), and high persistence gives them the ability to efficiently target above and below ground sucking pests, assuring entire and long term crop protection (Simon-Delso et al., 2015). NNIs have been commercialized in more than 120 countries being the most widely used group of insecticides globally. Among them, imidacloprid is the largest selling insecticide worldwide used in more than 200 plant protection products (PPPs) and more than 1000 applications (Pietrzak et al., 2020; Simon-Delso et al., 2015). Imidacloprid was the first NNI marketed in the European Union (2000; EC/List no.: 604-069-3), followed by acetamiprid (2004; EC/List no.: 603-921-1), and thiacloprid (2004; EC/List no.: 601-147-9), clothianidin (2006; EC/List no.: 433-460-1) and later thiamethoxam (2007; EC/List no.: 428-650-4), were also commercialized (European Commission, 2010, 2006, 2005, 2004). Since then, NNIs use has grown exponentially, reaching 25% of global pesticide sales in 2014 (Tasman et al., 2021) Nevertheless, the increasing evidence of affecting non-targeted organisms (imidacloprid and thiamethoxam induce acute toxicity to bees: acute contact toxicity 48-h LD_{50} 0.081 and 0.024 μg/bee, respectively; EFSA, 2015a, 2015b) particularly their implication in honey bee and bumble bee mortality when used in flowering crops, led the European Commission to gradually restrict their applications. Imidacloprid, clothianidin, and thiamethoxam's use were first banned in bee-attractive crops (including maize, oilseed rape, and sunflower) with the exception of uses in greenhouses, of treatment of some crops after flowering and of winter cereals in 2013 (Reg. (EU) No 485/2013). In 2008, EC prohibit all outdoor uses (Reg. (EU) 2018/783-4-5) and finally expired the approval for clothianidin (31 January 2019), thiamethoxam (30 April 2019) and imidacloprid (1 December 2020). Similarly, the approval of thiacloprid was withdrawn on 30 April 2020, and therefore only the use of acetamiprid is currently allowed in Europe (28 February 2033; Reg. (EU) 2018/783-4-5). Nevertheless, some European governments skip these restrictions allowing emergency authorizations not only to fight insect pest in crops planted over large areas like sugar beet (Finland, Belgium, Poland, and Spain), maize (Romania) or oilseed rape (Denmark), but also in lettuce (Belgium), carrots (Latvia), broccoli (Latvia), sunflower (Romania,) or even golf courses (Denmark) (European Commission, Authorisation of Plant Protection Products Database). These authorizations reinforce the need to address the effects of those regulated pesticides in the environment. NNIs are highly water soluble (Table S1), and therefore, once applied in agricultural areas, they often move into aquatic ecosystems via runoff (Pietrzak et al., 2020). Moreover, they slowly hydrolyze at acidic or neutral pH, and are also not readily biodegradable (Pietrzak et al., 2020), being able to persist in waters (Barbieri et al., 2020a; Llorens et al., 2020; Postigo et al., 2021) Therefore, the study of NNIs presence in river waters is a reliable tool to evaluate their use and environmental distribution.

Because of the accumulation and persistence of NNIs in superficial waters, aquatic organism's health and aquatic ecological environment are threatened. These highly neurotoxic insecticides have a great impact on the structure and functionality of aquatic invertebrate communities. Mesocosm experiments conducted by Merga and Van den Brink, 2021 showed that the macroinvertebrate and zooplankton community structure changed significantly due to imidacloprid contamination in mesocosms repeatedly dosed with ≥0.1 and ≥0.01 μg/L, respectively. Consequently, any changes in emergent aquatic insect abundance and composition may impact the success of higher trophic level organisms (Cavallaro et al., 2019), disrupting food chain and reducing fish yields (Yamamuro et al., 2019). Like other contaminants, monitoring of actual distribution of neonicotinoid insecticides in soil-water systems can provide important information about the scale and magnitude of the threat posed by them (Pietrzak et al., 2020). The present study aims to complete an integrated analysis related to NNIs presence on the Tagus River evaluating the implications of each pollutant and the combined effect (additivity) of the mixture in the aquatic ecosystem. To achieve this objective NNIs were measured in superficial waters and data obtained were used to: investigate the risk derived from the exposure of aquatic organisms for each NNI, the potential harmful risk of the combination of pollutants and the trophic levels in order to estimate effects in the community. Finally, influence of NNI levels in the diversity of aquatic organisms was explored.

2. Material and methods

2.1. Reagents and chemicals

Native (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) and isotope-labeled (acetamiprid-d3, clothianidin-d3, imidacloprid-d4, thiacloprid-d4 and thiamethoxam-d3) standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Labstandard (Barcelona, Spain), respectively. Acetonitrile, methanol (HPLC grade) and ammonium acetate were purchased from Scharlab SL (Barcelona, Spain).

2.2. Study area and sample collection

Tagus River is the longest river of the Iberian Peninsula (1007 km, 80,600 km^2 basin; Fig. 1) and travels in an east-west direction, from the Central Spanish Plateau to Lisbon (Portugal) with an average annual flow of 456 m^3/s. In the upper part predominates forest and

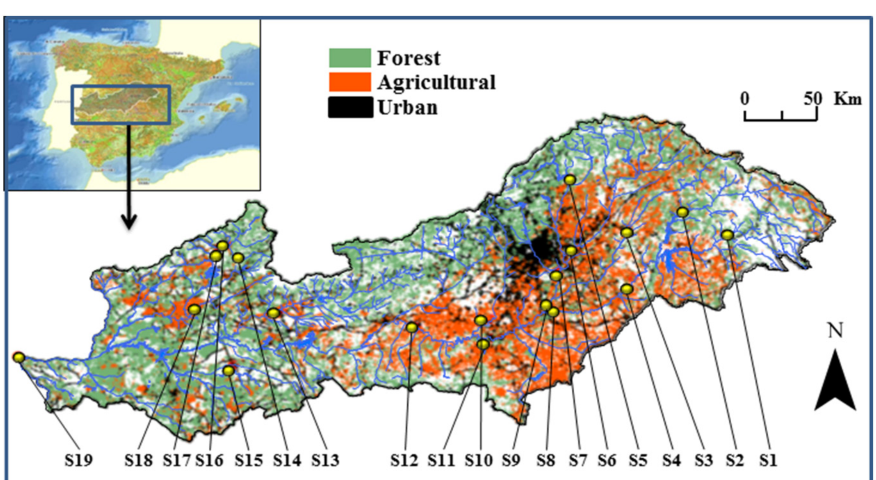

Fig. 1. Geographical sample distribution and soil uses in the studied area.

conservation areas with low anthropogenic influence. In the middle of its watershed it is affected by a marked demographic pressure, coming mainly from Madrid city (6.8 million inhabitants; (INE, 2020a) and on a lesser extent downstream from Aranjuez (60,000 habitants; INE, 2020b)) and Toledo (700,000 inhabitants; (INE, 2020c)), where agriculture and livestock production gain great importance. As a consequence of the hydrological variability, climate and extensive demographic pressure, Mediterranean watersheds are highly vulnerable to agricultural contamination (Arenas-Sánchez et al., 2019) but also the zoning of these agricultural, industrial and urban pressures in the Tagus hydrographic basin allows the identification of pollutants sources. Tagus River flows through several ecosystems with a rich flora and fauna considered as natural and conservation reserves (Navarro et al., 2020). Therefore, the selection of this study area it is also appropriate to evaluate NNIs potential risk on the aquatic ecosystem.

Nineteen water samples were collected along the Tagus River watershed (Fig. 1) during June and July 2020. This period of the agricultural cycle was selected because it is associated with a greater use of pesticides in the studied area. Sampling locations were selected to cover all potential typologies and soil uses as show in Fig. S1. Up to 4 samples were located in remote areas near the rivers source (samples S1, S2 and S5) and next to the Portuguese border (S19). Samples S6 and S7 covered high populated areas with industrial zones while the rest were located in rural and agricultural areas with herbal crops, deciduous hardwood, scrub, conifers or olives. Water samples were collected in the field into polypropylene bottles and once in the laboratory, were frozen at −20 °C until analysis. Additionally, seven field blanks were monitored at S2, S6, S8, S13, S15, S16 and S18.

2.3. Chemical analysis

Prior to extraction 1 L of filtered water samples were spiked with 20 ng of deuterated standards (acetamiprid-d3, clothianidin-d3, imidacloprid-d4 and thiamethoxam-d3). The analytical protocol was previously published by Wan et al., 2019. Briefly, the sample was loaded onto an Oasis HLB SPE (6 mL, 500 mg; Waters, Milford, MA, USA) cartridge preconditioned with 5 mL acetonitrile and 5 mL of Milli-Q water. Filtered water was pumped through the SPE and let dry for 1 h. Next, analytes were eluted from the column using 6 mL of acetonitrile, concentrated to 140 μL under a gentle nitrogen stream and reconstituted with 300 μL of acetonitrile and 200 μL of 2 mM ammonium acetate containing 20 ng of thiacloprid-d4 as internal standard. Instrumental analysis was carried out on a high-performance liquid chromatography system (Varian HPLC 212) coupled to a triple quadrupole mass spectrometer (Varian 320- MS-TQ), see Supplementary Materials (SM) for details. Aliquots of the sample extracts (20 μL) were injected into a C18 column Polaris 3 C18-A 150 × 2.0 mm (Agilent). The column flow rate was 200 μL/min, and the column temperature was set at 40 °C. The mobile phases were 2 mM ammonium acetate in water (solvent A) and methanol (solvent B). The optimized gradient program was: 5% of B for 10 s, after a linear gradient up to 98% of B in 15 min (kept 3 min) and finally came back to initial conditions (5% of B). Data were collected in the multiple-reaction-monitoring (MRM) mode (Table S2).

2.4. QA/QC and statistical analysis

Quantification of all analytes was carried out by isotopic dilution using deuterated standards. Method recoveries of deuterated surrogates were 105 ± 12%, 79 ± 15%, 82 ± 17% and 79 ± 19% (mean ± RSD) for acetamiprid-d3, clothianidin-d3, imidacloprid-d4 and thiamethoxam-d3, respectively. Field blanks, consisting in fulfilling polypropylene bottles with MilliQ waters in the field (7) and procedural blanks (2) were processed as samples. Only two field blanks presented imidacloprid concentrations (<2% of the lowest quantified concentration). These values were used to calculate imidacloprid method detection limit (MDL), defined as the average field blank level plus three times the standard deviation (Table S1). For those analytes not detected in the blanks, instrumental detection limits were used. All MDLs were above maximum limit acceptable on the detection method (8.3 μg/L) defined for NNIs (Reg (UE) 2018/840). Statistical analyses were performed with Statgraphics Centurion XVII. For descriptive statistical analysis values <MDL were replaced by MDLs divided by the square root of 2. Clothianidin levels were below MDLs for more than 75% of the samples and they were excluded from this analysis. RSD values ranged from 0.12 to 0.33 ng/L, being 0.13 ng/L for the sum of NNIs (Table S3). Analyte concentrations were not normally distributed (Shapiro-Wilk W and Kolmogorov-Smirnov tests), hence Spearman rank correlation coefficient was derived to investigate bivariate relationship. Mann-Whitney (Wilcoxon) W-test was run for median comparison between analytes and land uses. For exploring bivariate associations (Spearman test) values <MDLs were removed. A significant level of $p < 0.05$ (two sided) was accepted.

2.5. Calculation of environmental exposure

Environmental protection targets of freshwater ecosystem generally cover freshwater organism, sediment organism and fish eating predators (ECHA, 2016). However, considering NNIs physico-chemical properties, log Koc and log Kow < 3 and bioconcentration factors (BCF) < 100 (Table S1), risk assessment for the sediment compartment and organism from secondary poisoning (fish eating predators) were discarded (Carvalho et al., 2015). Therefore, only the risk for direct toxicity to pelagic organisms from the presence of NNIs in the water column (risk characterization ratio for the freshwater organism; RCR_{fw}) were derived from the ratio between the compartmental concentrations (PEC_{fw}) and the concentration below which unacceptable effects on organisms will most likely not occur (predicted no effect concentration ($PNEC_{fw}$; Carvalho et al., 2015)). Later, considering that combined risk can be predicted from a sum of the potency concentration of chemicals with a similar mode of action (Kienzler et al., 2019), NNIs mixture risk characterization ratios (RCRmix) were obtained with two approaches as proposed by Backhaus and Faust, 2012. First, calculation based on the sum of PEC/PNEC values (Eq. (1)) which is conceptually analogous to the hazard index of a mixture, which is applied for the human health assessment of chemical mixtures using reference doses. Second approach was based on the sum of toxic units (STU) for the most sensitive trophic level (Eq. (2)) applying an assessment factor (AF) of 1000 (Backhaus and Faust, 2012).

$$RCR_{mix\left(\frac{PEC}{PNEC}\right)} = \sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} \tag{1}$$

$$\begin{aligned} RCR_{mix(STU)} &= \max\left(STU_{algae}, STU_{invert}, STU_{fish}\right) X\ AF \\ &= \max\left(\sum_{i=1}^{n} \frac{MEC_i}{EC50\ i, algae}, \sum_{i=1}^{n} \frac{MEC_i}{EC50\ i, invert}, \sum_{i=1}^{n} \frac{MEC_i}{EC50\ i, fish}\right) x\ AF \end{aligned} \tag{2}$$

In this approach, the toxic unit (TU) is defined as the ratio between the maximum environmental concentration (MEC; concentration of chemical i analyzed in a water sample) and the EC_{50} for a given trophic level and a given chemical (concentration of a compound where 50% of its maximal effect is observed). In case of missing the EC_{50} of a species, the LC_{50} has been used, corresponding with the lethal concentration for half of the studied population. Those endpoints were used with selected organisms in order to cover all significant trophic levels for aquatic ecosystem: algae species ($EC_{50,}$ based on population abundance – 5 days) fish species ($EC_{50,}$ based on multiple factors – NA), and crustacea species, concretely *Daphnia* sp. ($EC_{50,}$ based on intoxication and immobility – 48 h), *Americamysis bahia* ($LC_{50,}$ based on mortality – 4 days), *Chironomus riparius* ($EC_{50,}$

based on acute test – 48 h) and *Chironomus dilutus* ($EC_{50,}$ based on emergence – NA).

Simpson' (*SiD*) and Sannon-Weaver (*H′*) diversity indexes were calculated according to (Eq. (3)) and (Eq. (4)).

$$SiD = 1 - D_{Si}\ ;\ D_{si} = \sum_{i=1}^{S} p_i^2 = \sum_{i=1}^{S} \left(\frac{n_i}{N}\right) \tag{3}$$

$$H' = -\sum_{i=1}^{S} (p_i\ x\ \log_2 p_i) \tag{4}$$

With p_i= proportional abundance of the *i*th species; represents the probability that an individual of species *i* is present in the sample, calculated as the number of individuals of the *i* species (n) divided by the total number of individuals for all the *S* species of the community (N) (Shannon and Weaver, 1949; Simpson, 1949). Necessary data about present taxa in surface water on the selected points were obtained from www.chtajo.es to calculate diversity indexes.

3. Results and discussion

3.1. Neonicotinoid distribution in river waters. Concentrations and mass flow rates

Neonicotinoid concentrations are shown in Figs. 2, S2 and Table S3. NNIs were quantified in 17 out of 19 water samples. Acetamiprid and imidacloprid presented high quantification frequencies (>65%) while the presence of thiacloprid (37%), thiamethoxam (37%) and clothianidin (21%) in the water samples decreased significantly ($p < 0.01$; Mann-Whitney W-test). This result reflected the predominant use of imidacloprid and acetamiprid among all NNIs, partially consequence of the only current legal status of acetamiprid, and the recent expiring of imidacloprid approval (December 2020).

Imidacloprid concentrations in surface waters reported in the literature are shown in Table S4, for a comparative purpose. Levels quantified in the present study (<MDL-10.2 ng/L, 1.24 ng/L; min-max, median) are similar that those found in other Spanish rivers since 2010 (Ccanccapa et al., 2016a; Ccanccapa et al., 2016b; Quintana et al., 2019). Interestingly, imidacloprid concentrations are in the same range than those obtained in samples collected in 2018 (<MDL-21.5 ng/L, 2.68 ng/L) in the same river basin (Rico et al., 2019), evidencing that although imidacloprid outdoor use was banned in May 2018 by the European Commission, surface water levels are in the same order of magnitude two years later. The approval of all imidacloprid uses expired on 1 December of 2020 and therefore its presence in the environment it is expected to diminish in the future.

Acetamiprid concentrations (0.07 ng/L; median) in surface waters were lower ($p < 0.01$; Mann-Whitney W-test) compared to imidacloprid (Table S3). Initially, this result could suggest a higher use of the latter; nevertheless differences in terms of water solubility (Table S1) make foresee that acetamiprid would be more associated to the particulate matter. Nonetheless, it should be noted that acetamiprid lipophilicity may result in higher bioaccumulation and therefore even at lower levels its presence in surface waters may arouse high concern in the aquatic ecosystem. Interestingly, previous studies aroused lower frequency of detection for acetamiprid in Spanish rivers compared to imidacloprid (Table S4 and S5). However, in the present research, although the levels are clearly lower, the frequency of detection of both NNIs are similar 79% (15/19, imidacloprid) and 68% (13/19 acetamiprid). Acetamiprid surface water concentrations in Tagus basin are in the same range than those reported in Sweden, Canada, USA, Australia or Japan (Table S5). Acetamiprid levels were screened in samples collected in 2018 in the Tagus River by Rico et al., 2019, but this NNI was only detected in 3 of 60 samples and was discarded for quantitative analysis. Therefore, the higher quantification frequency obtained for acetamiprid in the present study (13/19), may suggest an increasing use during the last years. Today, acetamiprid is the only NNI allowed in Europe so this trend can be expected to continue in the future. Positive association was found between acetamiprid and imidacloprid concentrations ($r_s = 0.918$; $p < 0.01$, Table S6), indicating a similar origin. In this sense, sample size does not allow an unequivocal identification, however it could be observed that sampling points close to agricultural areas presented higher ($p < 0.05$) imidacloprid water concentrations, reaching maximum levels in samples obtained downstream irrigated crops (corn, tomato, cucumber, eggplant, melon and watermelon; S11, S12 and S18) which evidenced their agricultural use (Fig. S3). As expected lower levels were found in urban waters (S6 and S7), but results of special interest the presence of these NNIs in forestall sites (S14 and S15). Products like COURAZE® (FMC Agricultural Solutions, S.A.U) containing imidacloprid as active substances have been used in conifers and leafy trees (MAPA, 2021) to fight pests.

Thiacloprid and thiamethoxan were quantified above MDL in 7 samples. As happened for imidacloprid and acetamiprid, they reach maximum values for samples S11, S12 and S18 (irrigated herbal crops), but in thiamethoxan case, data obtained at sample S16 (rainfed fruit trees) were also remarkable. Mixtures of miticide and insecticide products containing thiamethoxam active ingredients (ACTARA® from Bayer, AGRI-FLEX®, PLATINUM® and CRUISER® from Syngenta), and thiacloprid (CALYPSO® from Bayer) have been recommended for fruit crops. Values found on this study remains below the maximum found in other studies, but the little bibliography available about thiacloprid

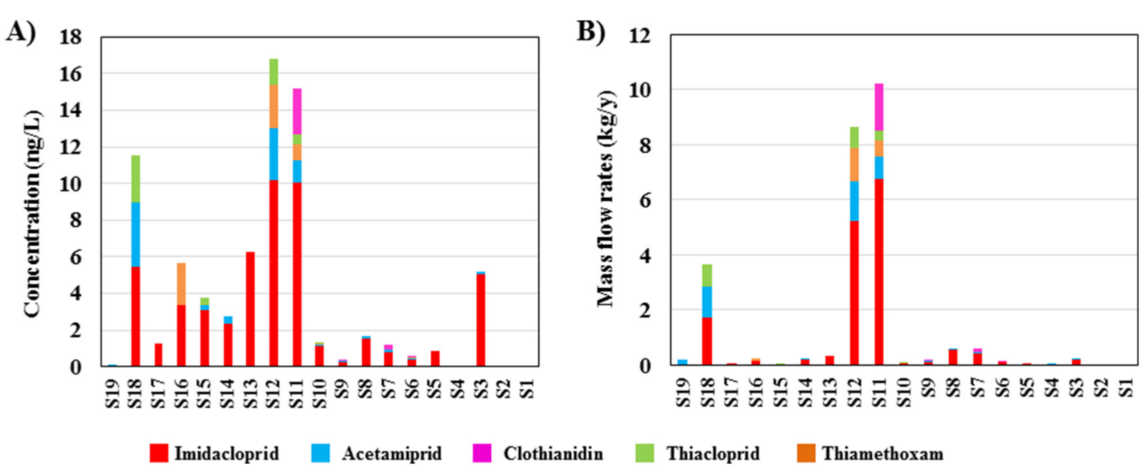

Fig. 2. Neonicotinoids concentrations (A; ng/L) and mass flow rates (B; kg/y) in Tagus river watershed.

and thiamethoxam concentrations in surface waters in Europe complicates the comparison with other studies. (Tables S7 and S8).

Finally, clothianidin was only quantified in 4 samples. Low quantification frequencies were also found in previous studies (Table S9). Barbieri et al., 2020 included clothianidin on their research, but no concentrations of this compound were quantified, which could reflect a low use of this NNI in Spain. Interestingly, two of the samples with values above MDL in the present study were urban emplacements (S6 and S7). This result may suggest its use for pest control in urban areas (TITAN ® from BASF or DANTOP® 50 WG from MASSO). Moreover, clothianidin reaches a maximum value (2.5 ng/L) in sample S11. Clothianidin was banned in 2019 (European Commission, 2018c) but until then, it was used in non-citric fruit trees (grapevine, almond tree, and crops as tomato and corn) which are present at S11 location.

Surface water is a very heterogeneous environment: there are several differences among a creek and a big river in terms of concentration and dilution of pollutants. So, in this media, results of special interest to consider the flow mass at each sampling point. Calculated concentrations were combined with mean daily flows (m^3/s) to obtain mass flow rates (kg/y) in the Tagus river basin (Fig. 2 and Table S10). After this flow normalization, mass flow pattern maintained the result obtained for surface water concentrations. Imidacloprid (0.83 kg/y; mean) and acetamiprid (0.20 kg/y) were the major contribution to total NNIs discharge (1.34 kg/y). Interestingly, samples collected at zones of the basin with little agricultural influence (S1, S2, S4, S5, S6, S7, S14 and S19) did not present a remarkable presence of NNIs. Concentrations increased with agricultural input (S3, S13 and S16) and reach maximum contributions to river water pollution in samples S11, S12, and S18 (Fig. 2b), reinforcing that the use of these pesticides in irrigated herbal crops results in an important discharge in superficial waters of Tagus basin.

3.2. Risk assessment

Data obtained in the present study on any of the evaluated sampling points are below the environmental quality standards (EQS) in surface water proposed by the Water Framework Directive: the annual average concentrations (AA-EQS) and the maximum acceptable concentration EQS (MAC-EQS; 0.2 μg/L; Van Dijk et al., 2013) or the Annual Average environmental quality standard determined for imidacloprid (AA-EQS; 0.067 μg/L; Vijver and Van Den Brink, 2014), even though some studies claim that long-term threshold value for imidacloprid should be lowered to 8.3 ng/L (Smit et al., 2015). At first, this fact could indicate a low ecological risk to aquatic organisms. However risk characterization ratios for the freshwater (RCR_{fw}) were derived to corroborate this hypothesis. In order to obtain a conservative $PEC_{fw}/PNCE_{fw}$ ratio and assuming that pesticides are applied mostly before or during the growing season, resulting in short term peaks that could affect the local biota, the quantified maximum concentration for each NNI were regarded as PEC_{fw} (Du et al., 2013; von der Ohe et al., 2011). RCR_{fw} resulted in a neglected risk situations for acetamiprid (0.01; Table S11), clothianidin (0.02), thiacloprid (0.05) and thiamethoxam (0.02). However imidacloprid RCR_{fw} clearly surpassed 1. At present, the use of imidacloprid is not allowed, but given the high risk for the aquatic environment obtained in the present study ($RCR_{fw} = 1.13$), emergency authorizations currently in force and those that may be proposed in the future arouse great concern.

As expected, $RCRmix_{(PEC/PNEC)}$ for the 5 NNIs using sum of PEC/PNEC ratios approach was above one (1.23; Table S11), but on contrary total potency of the mixture expressed as toxic units (TUs) for algae, daphnia and fish $RCRmix_{(STUa\text{-}d\text{-}f)}$ aroused a value well below 1 (0.005; Table S11). However, recent works have evidenced that D*aphnia* sp. is not that much affected by NNIs (EC50 ranging from 22.5 to 119 mg/L; Table S1) (Morrissey et al., 2015). Plant Product Regulation (Reg (EU) N° 283/2013) requirements for insecticides and substances with insecticidal activity included an acute test for a second aquatic arthropod species besides *Daphnia*, proposing as candidates the insect *Chironomus* spp. (also present in Tagus river basin) and *Americamysis bahia*. Considering that, STU calculation were also conducted with *Americamysis bahia* ($RCRmix_{(STUa\text{-}a\text{-}f)} = 0.45$), *Chironomus riparius* ($RCRmix_{(STUa\text{-}c.rip\text{-}f)} = 2.57$) and *Chironomus dilutus* ($RCRmix_{(STUa\text{-}c.dil\text{-}f)} = 64.9$), showing a medium risk ($0.1 < RQ < 1$; Sousa et al., 2018) for the saltwater crustacean *Americamysis bahia* and high risk ($RCR > 1$) for *Chironomous* freshwater species and revealing great differences among risk assessments using one specie or another. This fact becomes even more apparent when risk characterization ratios were calculated at each location (Table S12). None of the sampling points exceeded risk thresholds with *Daphnia* analysis ($RCRmix_{(STUa\text{-}d\text{-}f)}$). On the other hand $RCRmix_{(STUa\text{-}a\text{-}f)}$, $RCRmix_{(STUa\text{-}c.rip\text{-}f)}$, and $RCRmix_{(STUa\text{-}c.dil\text{-}f)}$ revealed medium and high risk in 5, 10 and 17 of the evaluated locations, respectively. Even assuming differences between species, $RCRmix_{(PEC/PNEC)}$ and RCRmix (STU) approaches showed a positive risk for samples S11, S12 and S18, which aroused high NNIs levels associated to agricultural use.

Finally, diversity indexes were calculated to evaluate the quality of the water at each sampling point (Table S13 and Excell SM) and to explore their relationship with NNI concentrations in surface waters. Simpson's Index of Diversity (SiD) represents the probability that two individuals chosen at random and independently from the population will belong to the same group. The greater the Simpson Index (SiD = 1), the higher the diversity. Something similar happens with Shannon Index's calculations (H´) that quantify the entropy within a population and computes species population diversity. In general, a homogeneous distribution was obtained for the values for both indexes (0.71 ± 0.13 and 2.40 ± 0.65; mean ± SD for SiD and H) in line with other Spanish rivers (Kuzmanović et al., 2016). Interestingly, two of the four samples that presented higher imidacloprid water concentrations, showed diversity indexes below the mean (0.45- and 1.25; SiD and H´ for sample S18; Table S13). However, no associations ($p > 0.05$; Table S6) were found between NNI water concentrations and diversity indexes. The sample size of the present study does not allow to rule out that the levels of these pollutants could have an influence in the biodiversity of the sampled points and results found should be corroborate in more habitats. Relation between physical-chemical parameters (pH, O_2 (mg/L), O_2 (%sat), ammonium, phosphates, and nitrates; Table S13) and NNIs concentrations were also evaluated. No associations were found between them, however diversity indexes (SiD and H´) increased with dissolved and saturated oxygen ($r_s > 0.684$, $p < 0.01$; Table S6) and decreased with fosfate levels ($r_s < -0.745$; $p < 0.05$).

4. Conclusions

NNIs became very important and nowadays are the most widely used group of insecticides globally, with imidacloprid being the largest selling insecticide worldwide. The use of imidacloprid and acetamiprid has been predominant, specially related to agricultural practices and probably to the legal status of acetamiprid and the recently banned for imidacloprid situation. The frequency of detection of both NNIs are similar 79% (15/19, imidacloprid) and 68% (13/19 acetamiprid), and concentration levels of those compounds are in line with previous studies on the same basin. Values found for other not allowed compounds, thiacloprid and thiamethoxam, remains below the maximum found in other surface water studies (e.g., 31 ng/L as maximum; Barbieri et al., 2020b), but the short bibliography available concentrations in surface waters in Europe complicates the comparison. Risk assessment with *Daphnia* sp. aroused little information; on the other hand, calculations with more sensitive taxa evidenced the possible underestimation of risk if using this model organism. RCR with mean values found in waters showed risk for imidacloprid (RCRfw>1) and for the mix of NNIs (RCRmix (PEC/PNEC) > 1), endangering the living organisms of the river basin. RCRmix(PEC/PNEC), the sum of toxic units (STU) for each sampling site showed a risky situation for some locations with different organisms related to agriculture practices. This data arouses concern about NNIs (legal or forbidden) use in Tagus basin. Current emergency authorizations and results found in this study highlight the need to monitor its presence in order to evaluate the implementation of these restrictions and manifest the need to evaluate their effect on the complete aquatic ecosystem.

CRediT authorship contribution statement

Casillas Nogales Alba: Investigation, Data curation, Methodology, Validation, Writing – original draft. **De la Torre Haro Adrián:** Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Project administration, Writing – original draft. **Navarro Martín Irene:** Investigation, Methodology, Writing – review & editing. **Paloma Sanz Chichón:** Investigation, Methodology, Writing – review & editing. **Martínez Calvo María Ángeles:** Conceptualization, Investigation, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.151161.

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