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Evidence of Neonicotinoid Contamination in Aquatic Invertebrates: An analysis of the current state of the river Seyon in Switzerland

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1 Abstract

Since their introduction in the 1990s, neonicotinoids have rapidly become the most widely used pesticides in the crop protection industry. Their harmful effects on natural environments and wildlife have been recognized for a long time but are still little studied. This work analyzes the incidence and prevalence of five neonicotinoids in four different taxa of aquatic invertebrates from the Seyon River, in Switzerland. To do this, two methods were combined: CHBI methods for the sampling, and an HPLC-MS analysis (coupling between liquid chromatography and mass spectrometry). All samples analyzed were contaminated with at least one neonicotinoid, on average at a concentration of 0.8 ng/g. This provides evidence of the ubiquity of these pesticides in the environment, and the chronic exposure to which invertebrates are subjected. Two of the four neonicotinoids found in the samples had been banned from use nine months prior to the sampling, proving their high persistence. An outlier value of 7.375 ng/g, nearly tenfold higher than the others, indicates the likelihood of extremely high contamination of some invertebrate populations. These results are worrisome and reveal the need for more research on the subject. Aquatic invertebrates play key roles in the food chain and in the water quality of their habitats and are particularly exposed to the great damage that neonicotinoids cause in natural environments. This work contributes to documenting this critical exposure and proposes experimental methods that can be used in future studies.

2 Introduction

Neonicotinoids have recently become a key topic in public discussion. In Switzerland, the topic is more relevant than ever with two federal popular initiatives filed in 2018 on the banning of synthetic pesticides and on the protection of the purity of food and drinking water. Also in Switzerland, after a partial ban introduced in 2012, 3 neonicotinoid molecules (imidacloprid, clothianidin and thiamethoxam) were banned from use in 2018. Despite these bans, neonicotinoids remain the most widely used pesticides today, both in Switzerland and worldwide (Simon-Delso et al. 2015). Scientific attention is also focused on the subject: these insecticides acting on the nervous system of insects are accused of being extraordinarily harmful to our environment (Pisa et al. 2017) and to human health (Cimino et al. 2017) by countless studies. Special attention has been drawn to the recent decline of pollinators linked to broad neonicotinoid use, especially for bees (EFSA 2013, Mitchell et al. 2017). Studies on that subject were the first ones to raise a substantial debate about the use of pesticides. However, this work contributes to the far less documented research on the impact of neonicotinoids on the aquatic environment. It provides a first overview of the state of the contamination of the river Seyon's invertebrate fauna.

2.1 Pesticides and neonicotinoids: a brief chronology

Along with the development of organic chemistry at the end of the First World War, pesticides such as DDT and other organochlorine molecules paved the way for the rise of synthetic pesticides in the early 1940s. Following the work of Rachel Carson and the publication of her book Silent Spring in 1962, the scientific community began to document the toxicity of synthetic pesticides. The banning of DDT because of its effects on non-target wildlife marked the beginning of public awareness regarding the great harmfulness of synthetic pesticides to the environment and human health. Despite this ban, the agrochemical industry began to develop an array of various crop protection products (MCaffee 2017).

Neonicotinoids entered the race in the late 1980s. Chemically, they are a derivative of nicotine, a natural molecule produced by tobacco to defend itself against pests. The mode of action of these insecticides affects nicotinic receptors in the central nervous system of targeted insects, which leads to overstimulation of these receptors and causes paralysis and eventual death of the organism (Tomizawa and Casida 2011). In the 1990s, the first companies to develop these formulas to a larger scale were Bayer CropScience, Syngenta, and Sumitomo Chemical. Their main active ingredients, still marketed today, were acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam (Bass et al. 2015). After their discovery, neonicotinoids became the most successful and fastest growing pesticides on the market in record time. It is estimated that by 2015 they accounted for 25% of the total pesticides sold worldwide (Bass et al. 2015).

2.2 Aquatic contamination

This work focusses on neonicotinoids present in the river Seyon, which flows between Villiers and Neuchâtel in the North-West of Switzerland (see Appendix). According to preliminary studies, the Seyon is one of the most polluted streams in Neuchâtel. This high contamination can be explained by neonicotinoid’s ease of propagation in natural environments. The propagation begins during the treatment of the crops in the fields. Neonicotinoids are widely known to be systemic, i.e., they can propagate in the vascular system of plants. This characteristic enables crop treatment by seed coating, applying the product to the surface of the seed before sowing it. As the plant germinates and grows, the neonicotinoid is distributed in the whole plant, which allows to optimize the quantity of product used and facilitates the process of treatment, all in all resulting in a more efficient way of fighting pests. This method was intended to allow a very deliberate and precise application, to spare non-targeted wildlife for example. But the seed coating method is less effective than it was thought at the time of its popularization. Studies show that only about 5% to 20% of the coating appears to be absorbed by the seed (Sur & Stork, 2003). This leaves 80% to 95% of the applied neonicotinoid in the soil and subsurface water (Goulson 2013). Because of the high solubility of neonicotinoids, water is very easily contaminated with these residues and runs off to larger streams or infiltrates to groundwater (Eichelberger 1971). In the Seyon's case, this infiltration is facilitated by the very porous and calcareous characteristics of the Val-de-Ruz region. As the Seyon's source is in a highly agricultural region, it is particularly prone to this type of contamination.

Today, because of their extensive use and their properties, neonicotinoids are everywhere in our environment, as vapors in the air, in soil, or in water (Goulson 2013, Bonmatin et al. 2019). Once they arrive in such environments, neonicotinoids can be devastating, even at small concentrations; only a few tenths of ng/g are needed to have long-lasting effects. It is known that chronic exposure to neonicotinoids at very low doses can be just as dangerous as acute exposure at high doses (Van der Brink et al. 2015). Compared to their ancestor, DDT, neonicotinoids have a toxicity estimated to be about 10,000 times higher, according to the TFSP (Task Force on Systemic Pesticides). Their effects are therefore even more harmful. While at the beginning of their widespread use neonicotinoids were known to have little influence on non-target biodiversity around crops, they have shown to have a considerable impact on non-target organisms, particularly invertebrates (Pisa et al. 2017). The chronic exposure to these substances that is mainly observed today in natural habitats results in cumulative effects for exposed invertebrates, such as respiratory (Lukancic et al. 2009), reproductive (Charpentier et al. 2014), motor (Girolami et al. 2009), and other behavioral disorders. Their persistence in our environment is quite high, their half-life is up to 1000 days in soil and 40 days in water (Goulson 2013).

2.3 This research

This work attempts to document the incidence and prevalence of neonicotinoids in the invertebrate fauna of the Seyon. To do so, I will perform an analysis of the concentrations of different neonicotinoids in benthic macroinvertebrates (aquatic invertebrates larger than 0.5 mm) which play a key role in their environment as decomposers and as food source. This research provides appropriate experimental methods to be applied in future studies. While this work's main goal is method development, I will also hypothesize about the different factors of invertebrate contamination and the impact it has on their ecosystem. Finally, I expect that the results will allow me to illustrate the current state of neonicotinoid contamination in the Seyon and in the whole of Switzerland.

3 Methods

3.1 Field methods

The following sampling method is based on the Swiss Biodiversity Index CHBI method (FOEN, 2010).

Sampling. Three benthic macroinvertebrate samplings were carried out for this work in October 2019 (2^{nd}, 7^{th}, and 16^{th} October 2019). They were carried out in the river Seyon, near the Prés Maréchaux in Vauseyon (see Appendix). Survey sites were different for each of the three sampling days to maximize the diversity of the samples. Five sampling sites were selected per sampling day to maximize the number of species and obtain the most accurate invertebrate representation and distribution in the samples. The selected sites had to be as diverse as possible: they mainly differed in substrate type, current and vegetation.

Samples were collected using the kick-sampling method, which consists of catching benthic fauna in a net by lifting the bottom of the riverbed with foot work on a square foot area. Large organic debris and rocks were removed, the water was strained, and the remaining material was placed in Falcon tubes and submerged in alcohol (70%).

Sorting. All organisms in the samples were separated from the remaining organic waste and sorted, while always being kept in alcohol to prevent desiccation. Organisms were sorted into morpho-groups, i.e., into approximate and temporary groups based on their appearance, without formal determination. The factors of a morpho-group sorting are the invertebrate's size, the symmetry, the number of legs or the presence of gills.

Identification. Preservation varied according to the organisms and their exposure to light or heat. The sampling and the determination of the organisms should be spaced up to two to three weeks, protected from direct light exposure to minimize putrefaction or other degradation. The identification was carried out with a binocular magnifying glass and a Petri dish with approximatively 5mm of alcohol. According to the different states of preservation, the identification was carried out up to the genus or the taxonomic family. Only the taxa of morpho-groups that had 30+ representatives for each sampling day were determined. After identification, the samples were sorted according to their sampling day and taxon and returned to Eppendorf tubes. A census of the collected organisms was also carried out. A precise count was made only for those organisms whose taxa were abundant (30+ representatives), whereas the other counts were rounded off to the dozen. For some samples with more than 100 individuals, the number was approximated. This rough census was crucial in determining the number of invertebrates present in the final analyzed samples. They were also used to determine a biodiversity index (CHBI) of the analyzed river reach to estimate the degree of pollution of the water in which these invertebrates live.

3.2 Sample processing

The experimental design of this work revolves around an analytical chemistry assessment based on a mixture of high-pressure liquid chromatography and mass spectrometry (HPLC-MS). The objective was to measure the incidence, prevalence, and concentration of 5 different neonicotinoids in the samples. Four taxa were selected for analysis based on their abundance in the samples and their diversity, considering factors like food web position, tolerance to neonicotinoids and morphology. The selected taxa were the Amphipods Gammaridae *Gammarus* and *Echinogammarus*, the Ephemeroptera Baetidae *Centroptilium* (mayflies), the Trichoptera Hydropsychidae *Hydropsyche* (caddisflies) and the Arynchobdellida Arhynchobdellidae *Erpobdella* (a family of leeches) (Fig.1)..

Figure 1 : the four selected taxa, Gammarus, Centroptilium, Hydropsyche and Erpobdella

The next few steps of the protocol are based on a protocol used to measure neonicotinoid levels in fish livers. It had to be adapted to be applicable to the analysis of invertebrates.

Drying. The invertebrates selected for analysis were arranged in Petri dishes separated by taxa and sampling day. These Petri dishes were placed to dry lid open for 2 days in a fume hood ventilating at low intensity. Each petri dish was labeled as follows: ABC# (ABC being the first three letters of the taxon in the dish [amp, eph, tri or arh], # being the number of the sampling day [day 1, 2 or 3]).

Grinding. The samples were grinded one by one using a mortar and pestle, with a centimeter of liquid nitrogen to facilitate the grinding. They were ground until the nitrogen had completely dissipated, and a very fine powder was obtained. The powder was then carefully transferred into 15 mL tubes and weighed. Aquilots between 0.001 and 0.1 grams were weighed for analysis. If the sample was heavier, the excess was discarded. The mass of one of the samples was smaller than 0.001 grams and it had to be excluded from the analysis process. These steps were repeated for each sample, after disinfection of all utensils.

Purification. Prior to handling, a salt tube 1 (TS1: 15 mL Falcon tube, filled with 3.25g of extraction salts) and a salt tube 2 (TS2: 15 mL Falcon tube, filled with 150 mg of $MgSO_4$, 100 mg of PSA, and 100 mg of C18) were prepared for each sample and set aside. 5.0 mL of acetonitrile and 20 µL of internal standard were pipetted into each sample tube. The tubes were then centrifuged at 4500 rpm for 5 minutes. As much supernatant as possible was then pipetted into the sample's corresponding TS1. 5 mL of mili-Q water were added, and the tube was shaken vigorously until the salts disappeared. The tubes were again centrifuged for 5 minutes. The maximum supernatant in each tube was pipetted into the corresponding TS2s. The tubes were shaken vigorously for 30 seconds and then run through the centrifuge. The supernatant from the tubes were carefully collected and deposited into one 13x100mm glass tube each. All glass tubes were then left in a Speedvac vacuum for 5 hours to evaporate. 500 µL of H2O:MeOH 75:25 were added to each tube and centrifuged for twenty seconds. The tubes were finally put in an ultrasonic bath for one minute. The contents of the glass tubes were transferred to Eppendorf tubes and centrifuged for 2 minutes at maximum speed. The suspension from each tube was recovered and filtered through PTFE filters into an HPLC flask with a 250 µl conical insert.

3.3 Analysis

The analysis of the samples was carried out by Dr. Gaétan Glauser at the Neuchâtel platform of analytical chemistry, at the Faculty of Sciences of Neuchâtel. It is based on a HPLC-MS analysis process, i.e., liquid chromatography combined with mass spectrometry. This process allows the separation of the different neonicotinoids suspended in the samples and their identification.

The non-polar liquids (C18 in the TS2 mixture added to the samples) and the polar liquids (acetonitrile and water) that were added to the samples interact in the sample tubes. They respectively are the stationary and mobile phases of the chromatography. The mobile phase is sent to a column where the fixed phase is already present. Depending on their polarity, the different neonicotinoids in the mobile phase move through the column at different speed and are separated. They are then ionized, weighed, and finally identified, using their mass/charge ratios. The internal standard solution added to the samples is used as a signal: it is made of known masses of all neonicotinoids that are to be analyzed, suspended in water. These known additional neonicotinoid concentrations help to separate the actual concentrations in the solution from the accidental noise of the analysis method. Finally, the respective concentrations of the five studied neonicotinoids in one gram of invertebrates can be calculated in ng/g.

For this work, ten samples of invertebrate pools were analyzed.

4 Preliminary analysis of the biodiversity of the Seyon

It is interesting to devote a part of this work to the river reach's biodiversity index CHBI, which is an evaluation of the water quality of a river according to the biodiversity of its aquatic macroinvertebrate wildlife. An adapted version of the CHBI was used to interpret my sampling results. The value of a CHBI depends on two factors: the faunistic group and the variety class. The faunistic group FG is evaluated based on the presence of specific taxa in the studied sample. The taxa have a varying affinity for polluted waters. Depending on the invertebrate taxa present in the samples, a grade from 1 to 9 is given to the stream. The variety class VC is evaluated based on the diversity of the invertebrates in the sample. A grade from 1 to 14 is given to the sample accordingly. The CHBI is then computed with the formula CHBI = FG + VC -1, resulting in a grade from 1 (worst) to 14 (best).

A CHBI is usually determined with an exhaustive list of each taxon represented in each sample, as well as an exact count of the number of individuals of each taxon. In this work, only a basic census was done during the sorting of samples, but a CHBI grade can still be approximated rather precisely. It allows a more comprehensive understanding of the water quality of the Seyon and puts the analysis results in perspective.

5 Results

The CHBI obtained with the preliminary analysis was of 6 on a scale of 14. This value indicates poor water quality (HWI between 5 and 8). Such a low value, while not being solely due to the presence of neonicotinoids in the water, indicates the probable fact that neonicotinoids are present in the water and that their effects on invertebrates are observable. This value is also consistent with previous results on the water quality of the Seyon and confirms the results found in this work. The HPLC-MS process resulted in an analysis of the concentrations in ng/g invertebrate of five different neonicotinoids in my ten samples. The five substances were thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid (see Table 1).

	thiaethoxam	clothianidin	imidacloprid	acetamiprid	thiacloprid
amp1	0	0.0275	0	0	0.0193
amp2	0	0	0	0	0
amp3	0	0.1396	0.1974	0.0259	0
eph1	0	0	0	0	0
eph2	0	0.1437	0.8059	0	0
eph3	0	0	0	0	0
tri2	0	0.1337	0	0	0
tri3	0	0.1330	7.3751	0	0
arh1	0	0	0	0	0.0543
arh2+3	0	0.0341	0.2568	0.0201	0.0853

Table 1 : Raw results of HPLC-MS analysis (concentrations of neonicotinoids, ng/g)

Thiamethoxam was not found in any sample. Clothianidin was detected in six samples and all four studied taxa were contaminated, with concentrations ranging from 0.0275 to 0.1396 ng/g. Imidacloprid was detected in four samples and is represented in all four different taxa. The three lowest concentrations ranged from 0.1974 to 0.806 ng/g, but one sample was well above this range, with a concentration of 7.375 ng/g. This last value will not be considered in the following graphs and statistics because it would overwhelm all other data. However, it will be given special attention during the discussion of the results. Acetamiprid is represented in two samples, with concentrations of 0.02 and 0.026 ng/g. Thiacloprid was detected in three samples, with concentrations ranging from 0.019 to 0.0853 ng/g.

6 Discussion and limitations of the methods

One of the objectives of this work is to be a methodological test for future studies on the same subject. In this light, the methods used for the sampling and analysis were also tests. Therefore, I stand by the trial-and-error aspect of my methods. However, in retrospect, some modifications would improve the accuracy and value of the results obtained.

6.1 Method adaptations

Sampling. The CHBI sampling needed little adaptation and allowed a diverse sampling of the invertebrate fauna and is adapted to a work like ours. The Seyon was an apt sampling site choice for this first study, thanks to its proximity but also regarding the results of preliminary studies which highlighted its strong contamination.

The sampling for this work was carried out during the month of October. A sampling in April would have been optimal to avoid extreme temperatures or floods that would disturb the usual wildlife. In my case, the Neuchâtel Wildlife Service forbade me to take samples between November and May.

Sample processing. To maintain the diversity of the samples, I was careful to select four taxa for analysis that were as diverse as possible, both in sensitivity and development. These differences are to be considered during the discussion of the results.

Analysis. The chemical protocol used for this work had never been used on invertebrates before. An objective of this work was to see if it was adaptable to organisms other than fish. Filtration and chemical treatments prior to analysis were very effective with invertebrates. The adaptations made were adequate, the samples obtained were pure and the analysis was accurate. The analytical method is therefore very suitable for invertebrates. The results obtained in this analysis are concentration values in organisms. These values can be used to determine the effects of the neonicotinoids on the contaminated organisms themselves, but usually, the values used to determine this are environmental concentrations like LC50s, not internal ones. However, my results could be used to determine the dynamics of neonicotinoid contamination in a specific environment, how the contamination varies following the food web, for example. This will be considered in the discussion of the results.

6.2 Limitations

The main limitation of this work is the number of samples. Only three sampling sessions were carried out due to limited time, resources, and the scope of this work being a high school project. The three samplings are very close in time, but at irregular intervals (5 and 9 days), and were all carried out in the same river stretch. The results of my analysis, while being good first indicators of the situation, are therefore not representative of the overall state of the waters and invertebrates of the Seyon. For these methods to be valuable and for the results obtained to be significant, it would first be necessary to carry out a much more exhaustive sampling. A primary focus of study such as investigating geographic, taxonomic, or seasonal factors in contamination, should also be chosen and the sampling should be tailored accordingly. This would allow for a more precise study.

One further limitation of these methods is that they only consider the initial form of neonicotinoids prior to any degradation. Metabolites of neonicotinoids derived from their degradation, particularly those of imidacloprid, can be just as dangerous to invertebrates as the original substance (Casida & Tomizawa, 2011). Although these metabolites are rarely considered by neonicotinoid analytical methods used in research, their significance must be recognized.

Another limitation is that the methods only consider dry invertebrates: the calculations do not consider the fact that at any given time invertebrates are surrounded and filled with water. It was however decided that the concentrations based on dry invertebrates were sufficiently accurate for this initial work.

7 Discussion

The analyses were carried out on sets of invertebrates, the number of which varies between 22 and 280, depending on their mass. The exact meaning of the analysis results is difficult to understand: the environment studied in this work has been little considered in previous studies and the data available in literature are only partial. Moreover, my limited sampling does not allow complete hypotheses on the contamination of the subjects studied. However, several observations on the contamination of the section of river and its invertebrates can be made by comparing the different concentrations detected in the samples. The results can ultimately be used to illustrate ground principles of neonicotinoid contamination elsewhere.

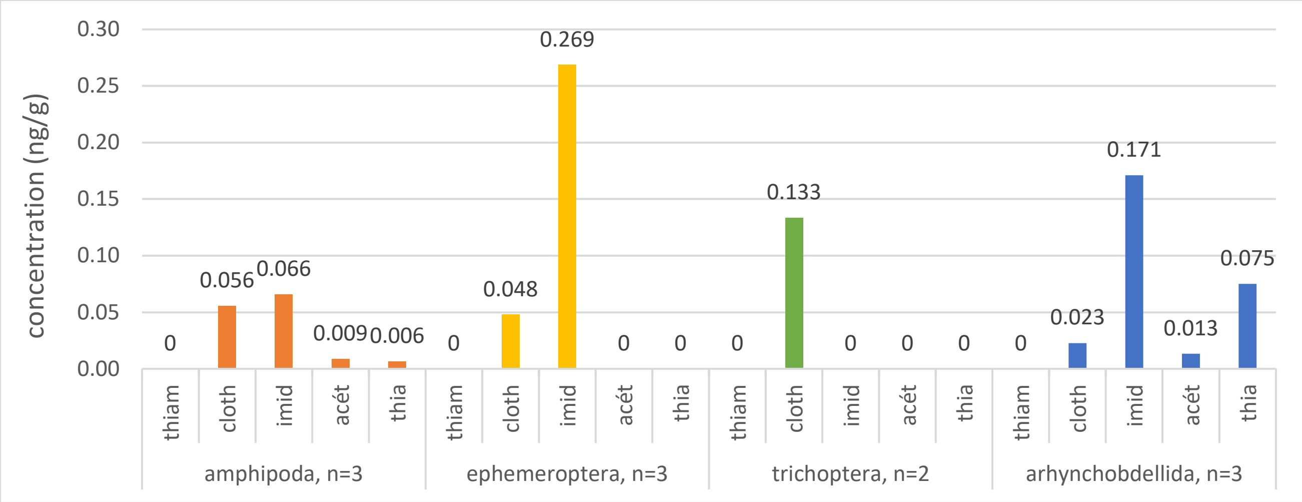

Figure 2: Average concentrations of neonicotinoids per day, all days (n) combined. The taxa are called by their order's name.

7.1 Ubiquitous neonicotinoids

Overall, each sample analyzed was contaminated by at least one substance (Fig.2), demonstrating the ubiquity of these pesticides in our environment: in one way or another, each organism is contaminated by phytosanitary products, to varying degrees but always existent. This observation highlights the ease of propagation of neonicotinoids. They flow easily from cultivated areas to various springs/streams/water bodies, from which they infiltrate the entire surface water network of the region. The Seyon is especially exposed to neonicotinoid contamination, flowing in an intensely agricultural area with calcareous soil.

7.2 Chronic exposure

Overall (outlier sample tri3 excluded) the concentrations are quite low and do not exceed 0.8 ng/g. These results match the values obtained 2016 in a study of pesticide concentrations in aquatic invertebrates in the Danube River, with insecticide concentrations ranging from 0.1 to 0.53 ng/g (Inostroza et al. 2018). However, contamination of aquatic invertebrates remains minimal compared to contaminations of pollinators, for instance. In 2016, a study conducted on honeybee contamination rates shows concentrations of 53 ng/g for imidacloprid and 32 ng/g for acetamiprid (Calatayud-Vernich et al. 2016). By comparison, the aquatic invertebrate exposure to neonicotinoids is clearly chronic. As mentioned in the introduction, such exposure can be just as devastating as acute exposure and has lasting effects on invertebrate populations.

7.3 High persistence

With the samples' analysis, the concentrations of five different neonicotinoids in each of the samples were obtained. It is interesting to compare the concentrations of these different substances.

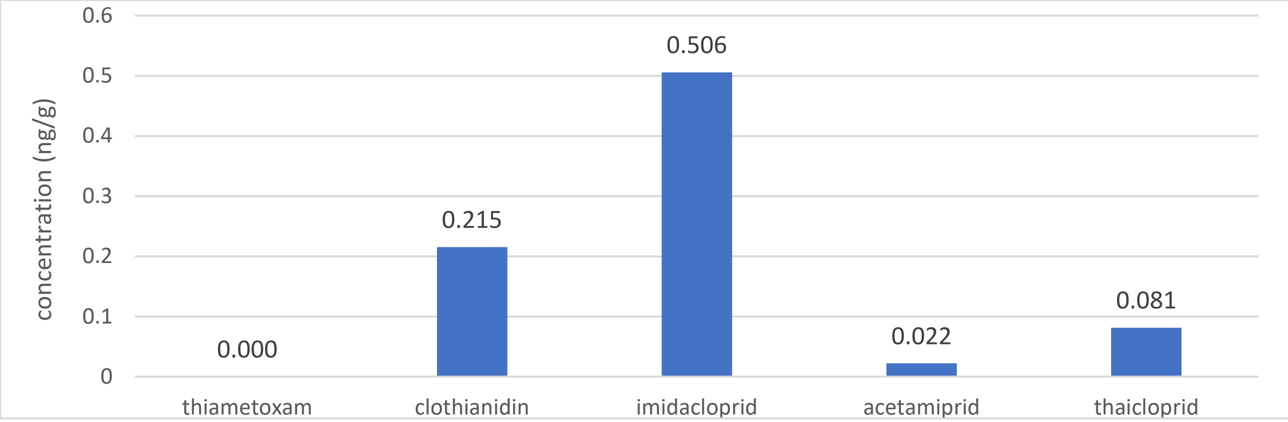

Figure 3 : : Average concentration per substance, all days and taxa combined (number of samples=10)

Of the five neonicotinoids analyzed, three substances are banned from use in Switzerland since January 2019 by the OFAG: Thiamethoxam, clothianidin and imidacloprid. Thiamethoxam is no longer present in the analyzed invertebrates, but clothianidin and imidacloprid are the two most concentrated substances in the samples (Fig.3), showing the high persistence of these two substances in our environment. However, clothianidin and imidacloprid are considered fast degrading substances in water (Pena et al. 2011): their presence in invertebrates nine months after their ban proves that neonicotinoids can be stored for a long time in soil, where their half-life is extended, before being diffused gradually into aquatic environments. Imidacloprid, for example, can have a persistence of up to 1000 days in crop soils (Bonmation et al. 2015). It may also indicate that substances have prolongated persistence once in the organisms themselves, with the persistence of neonicotinoids increasing in the absence of light among other things (Tišler et al. 2009).

7.4 Bioaccumulation and biomagnification

The results can also be compared according to the four taxa analyzed, which will be referred to with the name of their family: Amphipods, Ephemeroptera, Trichoptera and Arynchobdellida.

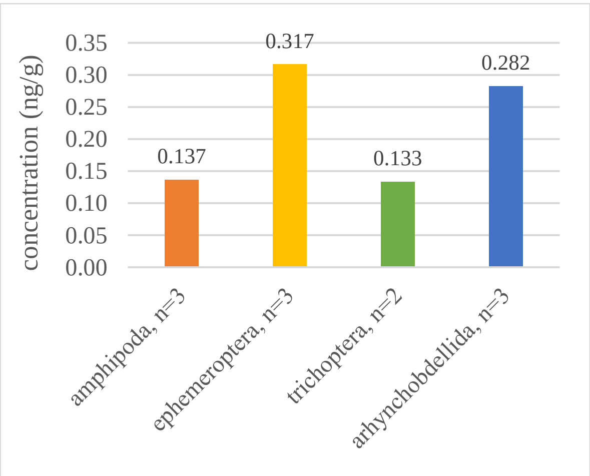

Figure 5: Average concentrations per taxa, all days (n) and substances combined.

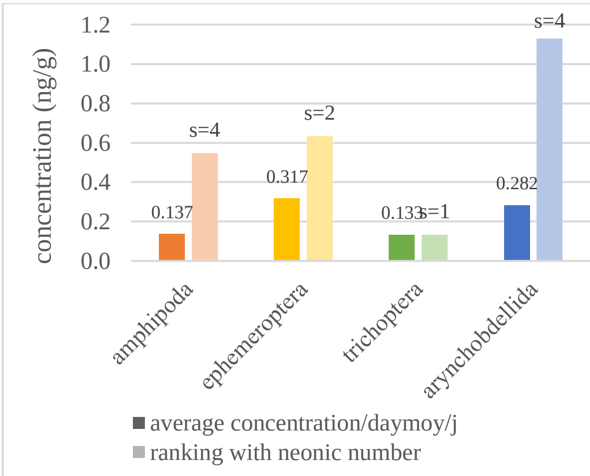

Figure 4 : Ranking of the taxa according to their average neonicotinoid concentrations and number of substances.

Figure 4 is a comparative table of the arithmetic means of the concentrations of all neonicotinoids present in the different taxa. However, this ranking is not very representative of the real situation: to discuss the effective neonicotinoid contaminations of the organisms, one also must consider the diversity of substances in the samples. Indeed, neonicotinoids are known for their synergic effects, emerging when several of these molecules are mixed. This property is recognized by scientific research (Maloney et al. 2017) and agrochemical manufacturers, for example Bayer Crop AG. It is important to take this into account in this discussion.

To consider the diversity of substances present in each of the taxa, a second table can be designed, for which each taxon's average concentration is multiplied by the number of substances found in each taxon. The table represents the net concentrations and diversity of neonicotinoids, thus the ranking of the taxa's contamination changes considerably (Fig.5). Arhynchobdellida are now the most affected by neonicotinoids, as they are contaminated by four different substances at high concentrations. They are followed by Ephemeroptera, contaminated by only two substances but with a high concentration of imidacloprid. Amphipoda are contaminated by four different substances but at low concentrations, and finally, Trichoptera are contaminated by only one substance at low dose (the extreme value tri3 is not considered in these calculations).

Bioaccumulation. One hypothesis to explain such differences in contamination is the bioaccumulation capacity of each taxon. The bioaccumulation of an organism is its capacity of progressive absorption of a substance present in its environment. This capacity can vary from taxon to taxon and even from one individual to another, depending on age, health, or genetic reasons. The different capacities of neonicotinoid absorption of the analyzed taxa could explain why the same concentrations are not found in each taxon.

Biomagnification. Another factor of contamination could be a variation in diet. The taxon most affected by neonicotinoids in the samples is carnivorous. The next two taxa are detritivores and the last one is algivorous-detritivore/carnivore. This alignment of different diets could indicate a biomagnification phenomenon. Biomagnification is a term that refers to the increase in concentrations of certain substances at each stage of the food web. A predator eating its prey also absorbs the toxins that they have accumulated over their lifetime, which gradually accumulate in the predator's fatty tissues. Neonicotinoids have been documented to show biomagnifying properties (Berlioz-Barbier et al. 2014, Tennekes et al. 2011). Arhynchobdellids eat exclusively living invertebrates, which themselves store various neonicotinoids in varying concentrations. During their digestion, neonicotinoids that were in their prey accumulate in the leeches and add to those that the leeches have absorbed environmentally. Ephemeroptera and Amphipoda are detritus feeders and consume organic waste from debris of decaying plants or other invertebrates. Remnants of accumulated neonicotinoids may still be present in the carcasses of the invertebrates they feed on. Ephemeroptera and Amphipoda are thus subject to the same biomagnification phenomenon as leeches, but on a smaller scale. Finally, the diet of Trichoptera, for the most part algivorous, exposes them less to this type of phenomenon.

7.5 Outlier value

The sample analysis shows an extreme imidacloprid concentration of 7.375 ng/g in sample tri3, a value almost ten times higher than the second highest value. Since all samples were analyzed simultaneously in the same machine and the concentrations of the other four neonicotinoids in the sample were normal, this value is experimentally valid.

For the discussion of this result, it is important to remember that the samples analyzed consist of pools of a few dozen invertebrates on average. Sample tri3 represents a pool of about 76 individuals of the taxon Hydropsychidae Hydropsyche, collected on 16.10.19. This high concentration is therefore not an isolated case of a single individual but the average of a small population of Hydropsyche, all collected on the same day within a few meters. The other Trichoptera sample obtained on 07.10.2019 had an imidacloprid concentration close to zero. In view of the time lag between the two Trichoptera samples, the extreme value in sample tri3 could have been caused by a sudden acute exposure to imidacloprid, e.g., by spilling substance from the stream. However, the other taxa analyzed on the same day did not show a comparable change in imidacloprid contamination, and even a regression in concentrations in the case of Ephemeroptera.

This value could also indicate extreme contamination at the scale of entire Trichoptera populations. The 76 Trichoptera in this sample are indicative of at least a portion of the population with very high imidacloprid concentration. This hypothesis is alarming as it would indicate a much higher population contamination than previously suspected. Neonicotinoid levels in some individuals would exceed many of the threshold concentrations allowed for aquatic ecosystem health. It is also possible that this is the case for the other taxa analyzed. My sampling would then have captured only the less contaminated part of the population (the majority), and the highly contaminated portion would have escaped my sampling (except for sample tri3).

A peak of imidacloprid concentration of this kind is also observed in the study of Louise Barbe on neonicotinoid contamination of fish in Switzerland, conducted in parallel to this work. An imidacloprid concentration of 14.184 ng/g is observed in a sample of chub liver caught in the Jura on 27.08.2019. This peak is just as extreme compared to other fish than the one for invertebrates. It must be also noted that Barbe's sampling is much more extensive than the invertebrates, with a few hundred samples. The extreme contamination of this fish is therefore more likely to be an isolated case than in the Trichoptera. These irregularities in imidacloprid concentration are observed in two distinct but linked food chain levels, algivorous-detritivore invertebrates, and the fish, a predator. Trichoptera are preyed upon by a variety of fish, and occasionally by the chub tested by Barbe. A biomagnification phenomenon is again possible to explain such a peak in fish. With the biomagnification hypothesis, one can explain the outlier fish sample by speculating that it had, some time before the sample was taken, eaten a population of aquatic invertebrates (possibly Trichoptera), which itself was contaminated due to high exposure to imidacloprid. This also explains why the concentration in fish was higher than the concentration found in insects. These two outliers in parallel studies indicate that such concentration peaks are probably observable at all levels of the food web in an aquatic environment.

7.6 Impacts on invertebrates

Too high concentrations of neonicotinoids can have disastrous effects on the contaminated environment. Generally, these effects are measured in relation to environmental concentrations of substances, not in relation to concentrations within organisms as measured in this work. This is for example achieved with the values of LC50s (half lethal concentration) and EC50s (half maximal effective concentration) for specific invertebrates.

Ephemeroptera and Trichoptera are considered highly sensitive orders to neonicotinoids (Morrissey et al. 2015), with very low LC50s. Gammarids are more resistant, and the impact of neonicotinoids on leeches is only sparsely studied. The minimum EC50 values of three of the four taxa analyzed (Trichoptera, Ephemeroptera, and Gammarids, all neonicotinoids combined) found in a 2018 study are all below the 10 µg/L threshold (Raby et al. 2018). These values are below the neonicotinoid concentration of the Seyon, estimated at 9.79 µg/L by Alex Aebi in a preliminary research. These EC50 values were furthermore calculated for acute exposures, whereas invertebrates in the Seyon are subject to mostly chronic exposures; it has been proven that chronic exposure to the same substances decreases the LC and EC50 of organisms considerably, with chronic LC50s between 3 and up to 800 times lower than acute LC50s, going from an exposure time of 24 to only 96 hours (Van der Brink et al. 2015. Thus, it is very likely that the average EC50 values for Trichoptera, Ephemeroptera, and Gammarids in the Seyon were reached and their populations were impacted by the presence of neonicotinoids in their environment.

Such contamination in aquatic invertebrates can have disastrous consequences for the whole environment. Detritus-feeding and algivorous benthic macroinvertebrates, such as Ephemeroptera, Trichoptera and Gammarids, are extremely important players in the recycling of organic matter that is deposited in waterways. This recycling is crucial for the diet of many aquatic organisms, but also to maintain the quality of the water itself (Covich et al. 1999). Macroinvertebrates also form the basis of the food web in their environment, thus are necessary for the survival of other invertebrates and fish. If the exposure to neonicotinoids is too high, whether acute or chronic, it can cause the decline of benthic invertebrate population (either by causing the death of individuals or by affecting their reproduction) (Sánchez-Bayo & Goyka 2006, Hayasaka et al. 2012). The gradual disappearance of these populations disrupts the entire food web balance of their environment and lowers the quality of that same environment (Sànchez-Bayo et al. 2016).

8 Conclusion

This work allowed to develop and test a method adapted to the analysis of neonicotinoid contamination of a river and the aquatic invertebrates living there. Using these methods, samples were collected and analyzed to study the incidence and prevalence of neonicotinoids in aquatic invertebrates. By interpreting the results obtained, I was able to draw up an overview of the situation of the Seyon and of four common taxa of its fauna.

The quality of the analytical results validated the CHBI and HPLC-MS methods used. The adapted methods proved to be efficient, accurate, and adapted to the analysis of aquatic invertebrates. These methods can therefore be used in further research. However, for such methods to be truly valuable, they must be applied to a sampling with of wider range than ours. One could consider a weekly follow-up of a particular site over the long term, or a multiplication of sampling sites, to consider the geographical factors that influence the contamination of aquatic invertebrates. Finally, the choice of taxa to be analyzed should also be modified: research should either focus on a single taxon to obtain targeted results or broaden their analyses to as many taxa as possible, to obtain overarching results that would cover the entire invertebrate fauna of the environment. In this way, meaningful and statistically supported hypotheses could be reasonably advanced.

All samples analyzed showed contamination with at least one neonicotinoid, demonstrating the ubiquity of these substances in the researched environment. Four of the five neonicotinoids analyzed were represented in the samples. Two of these four substances had been banned from use in early 2019 (imidacloprid and clothianidin): the time lag between this ban and the sampling illustrates the persistence of these substances in the Seyon as well as in the organisms living there. The concentrations obtained ranged from 0.0275 to 0. 806 ng/g, which indicate chronic exposure to neonicotinoids. Chronic exposures are still scarcely considered in pesticide toxicity research, even though they are the most common type of exposure in our environment. In addition to these low concentrations, an outlier imidacloprid concentration of 7.375 ng/g was found in sample tri3. A particularly worrisome hypothesis could be that this extreme value is a proxy for extreme contamination of entire Trichoptera populations. Evidence of extreme neonicotinoid contaminations in other populations of the ecosystem, such as Barbe's fish, suggest a systemic-scale phenomenon. To examine this further, combined studies on all different levels of the ecosystem would be appropriate: it would give a complete overview of the situation and would allow links to be made between the data found. This is in part studied at the university of Neuchâtel, where studies around neonicotinoids are carried out for various wildlife, such as invertebrates, fish, bees, and pollinators.

The aquatic environment and its fauna constitute a compartment of our ecosystem that has been little considered by scientific studies to date but deserves greater attention. The results obtained with my limited sampling confirm a need for further research on the subject, the result of which would be interesting to discuss and mobilize in future assessments of the health of our streams. In general, studies assessing invertebrate contamination in their natural environments and not in laboratories are sorely lacking. These studies, representative of the actual state of the environment, are crucial for the regulation of neonicotinoid use. A greater diversity of this kind of research would allow us to draw up a coherent and most exhaustive inventory of the presence of neonicotinoids around us.

Finally, every research about neonicotinoids allows us to question our use of these substances. In Switzerland, it is a debate at the heart of public attention, with proposals for radical measures on the issue, such as the initiative "For a Switzerland free of synthetic pesticides" launched two years ago, on 24.04.2019, which aims to ban the use of all synthetic pesticides in Switzerland.

Personally, writing this work has been an extremely rewarding experience, both from a scientific and human perspective. It opened the doors to the university of Neuchâtel's research laboratory for me, filled with motivated and brilliant specialists. I had the opportunity to conduct my experiments on the field and in the lab, which allowed me to finalize a research of my own. This work convinced me to pursue biology studies, to develop my knowledge, beliefs, and principles about the environment, and to get myself involved in the debate of neonicotinoid use and environmental protection.

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10 Appendix: Maps of the sampling sites

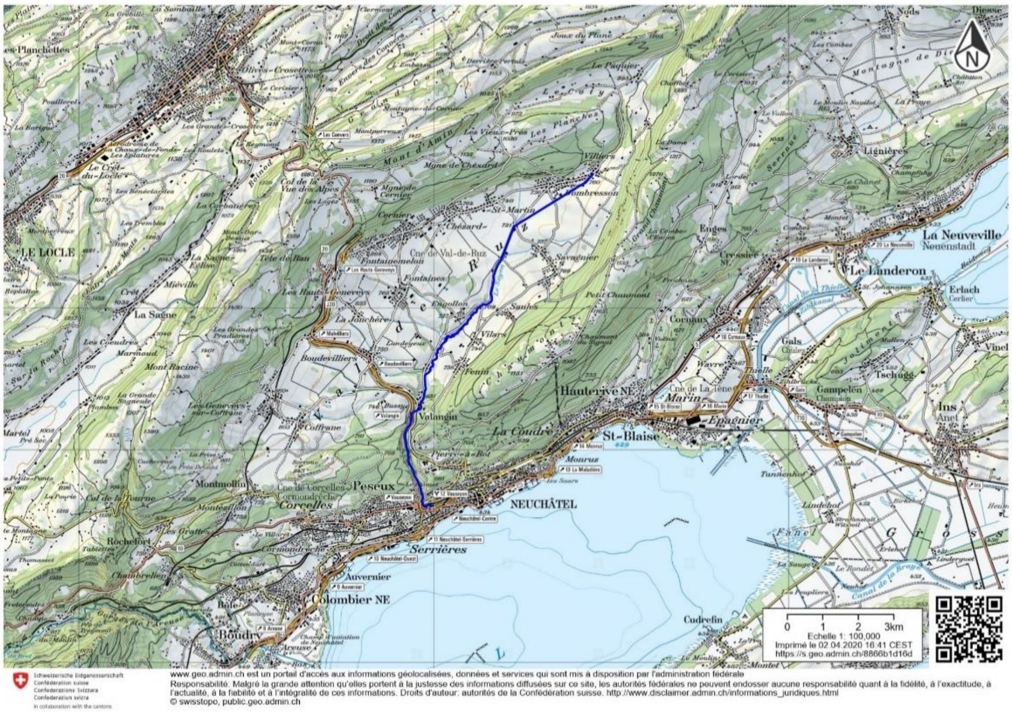

Figure 1: Map of the Seyon (blue), running across the canton of Neuchâtel

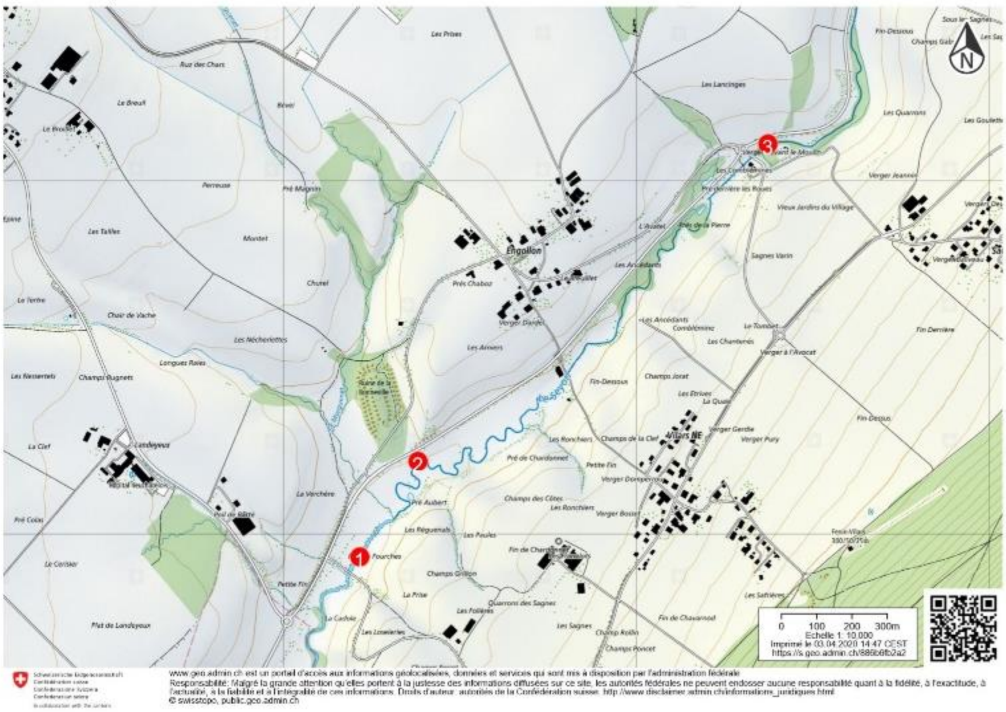

Figure 2: Map of the three sampling sites, between Engollon and Vilars
(1: sampled 10/02/2019, 2: sampled 10/07/2019, 3: sampled 10/16/2019)

Both maps come from https://map.geo.admin.ch/