This project is co-financed by the European Union

Grant Agreement No.: 824603
Call: H2020-SwafS-2018-1
Type of action: RIA
Starting date: 1/02/2019

Dragonflies and insecticides

Coordinator: [Roy van Grunsven]
Quality reviewer: [Kat Austen]

Deliverable nature	R
Dissemination level	PU
Work package and Task	D2.4
Contractual delivery date	01/2022
Actual delivery date	28/01/2022

Authors

Author name	Organization	E-Mail
Roy H.A. van Grunsven	**DBC**	**roy.vangrunsven@vlinderstichting.nl**

Abstract	This document describes the technique developed to measure neonicotinoids in water samples and the relation with natural dragonfly populations in the Dutch Monitoring Scheme.

Keywords	Neonicotinoids - Dragonflies - Sampling - Citizen Science

Disclaimer

The information, documentation and figures available in this deliverable, is written by the ACTION project consortium under EC grant agreement 824603 and does not necessarily reflect the views of the European Commission. The European Commission is not liable for any use that may be made of the information contained herein.

How to quote this document
R.H.A. van Grunsven (2022), Dragonflies and insecticides

This is a project in collaboration between Dutch Butterfly Conservation and the Leiden Centre for Applied Bioscience. Important contributions to this work have been done by Isa Onkenhout, Nanda Koopman, André van Roon and Peter Lindenburg (developing and performing the chemical analysis) and the citizen scientists of the Dutch Dragonfly Monitoring Scheme who collected the dragonfly data and the water samples.

TABLE OF CONTENTS

1. Introduction

This document describes the methods and results of an analysis performed to see whether we could detect an effect of neonicotinoids on the presence of dragonflies in the Netherlands. In order to do that we combined data of the Dutch Dragonfly Monitoring Scheme with the analysis of water samples from locations where dragonflies are monitored. For the analysis of the water samples the Leiden Centre for Applied Bioscience optimized and validated a technique based on HPLC-MS. The details of the citizen science water sampling methods have been previously reported (van Grunsven 2019).

Dutch Butterfly Conservation (https://www.vlinderstichting.nl/english) is a non-profit organisation that studies butterflies, dragonflies and moths in order to advise management organisations and better protect these species. One of the core activities is the monitoring of these groups, this is primarily done with citizen scientists that count animals according to a standardized protocol. To understand why certain species or groups of species are doing poorly additional research is done, with citizen scientists when possible. This research can be independent of the monitoring, even if it is inspired by the monitoring results, or incorporated in the monitoring. In the latter case we can use the wealth of data the monitoring program provides, as is the case in the current study.

R.H.A. van Grunsven (2019), Tutorial for water sampling and selection of transects, 10.5281/zenodo.3885721

This is a citizen science pilot study of the H2020-SwafS-2018-1-824603 EU project. More info about the project at https://actionproject.eu/

2. Insecticides and dragonflies

Insecticides and other pesticides are widespread in the environment , including in nature reserves (Brühl 2021). These compounds are all tested in laboratories but their impact under natural conditions are poorly known. In typical conditions multiple compounds can be found at the same time and may interact and the environment is far from those in laboratory studies. In the recent study by Barmentlo et al. (2019) we found that, under natural conditions, damselfly larvae of the Blue Tailed Damselfly (Fig 1) were negatively affected by low concentrations (0.1 µg/l) of the neonicotinoïd thiacloprid.

Since 1999 Dutch Butterfly Conservation has a monitoring program for dragonflies where dragonflies are counted along transects by citizen scientists following a protocol. This is done on more than 500 transects per year and allows us to calculate trends in the abundance of dragonflies in the Netherlands. We would like to build on this data to research whether there is a correlation between pesticide presence in water and dragonfly prevalence. Our transects are in nature reserves but also in parks and agricultural areas. This allows us to select locations that differ in their exposure to pesticides. Samples of water have been collected from these sites by the same citizen scientists who count the dragonflies. Testing of the samples for pesticides requires sophisticated laboratory equipment, and is carried out by students at the University of Applied Sciences in Leiden. In order to do this they had to develop a suitable method for the low concentration of insecticides you expect to find in surface waters.

Selection of locations suitable for sampling can be found in deliverable 2.3 (10.5281/zenodo.3885721). Water samples were collected by the citizen scientists that count the dragonflies on the transects. This was done twice in the flying season and each time three 50 ml containers were filled. To avoid photodegradation dark colored Greiner tubes were used. Samples were stored in a refrigerator by the citizen scientists as soon as possible after collection and at 4°C after arriving at DBC.

Brühl, C. A. et al. (2021). Direct pesticide exposure of insects in nature conservation areas in Germany. Scientific Reports 11 (2021): 24144. doi.org/10.1038/s41598-021-03366-w
Barmentlo, S. H., et al. (2019). Environmental levels of neonicotinoids reduce prey consumption, mobility and emergence of the damselfly Ischnura elegans. Journal of Applied Ecology, 56(8), 2034-2044.

Figure 1. Blue tailed damselfly is declining in the Netherlands and exposure to pesticides is a possible cause of this decline (Photo: Kim Huskens).

3. Measuring technique

The technique developed focusses on five neonicotinoids: Thiamethoxam, Clothianidine, Imidacloprid, Acetamiprid and Thiacloprid. These are the ones that are being used in agriculture recently and therefore most likely to occur in surface water. The concentrations of neonicotinoids were expected to be very low, in the order of nanograms/liter. This makes detection difficult but concentrations at this level can have biological impact and the maximum allowed concentrations for these compounds are in this order of magnitude as well (0.0083 µg/l average and 0,2 µg/l as peak concentration for imidacloprid). Therefore it is essential to be accurate enough to detect these low concentrations.

In order to be able to detect low levels of neonicotinoids a method to concentrate the compounds using Solid Phase Extraction was tested and used. This allowed for a 1000

fold concentration. The concentrated sample was than measured using Liquid chromatography–mass spectrometry HPLC-MS, a technique that uses liquid chromatography combined with mass spectroscopy to separate compounds with a triple quadrupole mass spectrometer. This combination results in a very low detection level and, in combination with the method of concentrating the neonicotinoids in the samples, allows for measurements in the range of interest (Fig 2).

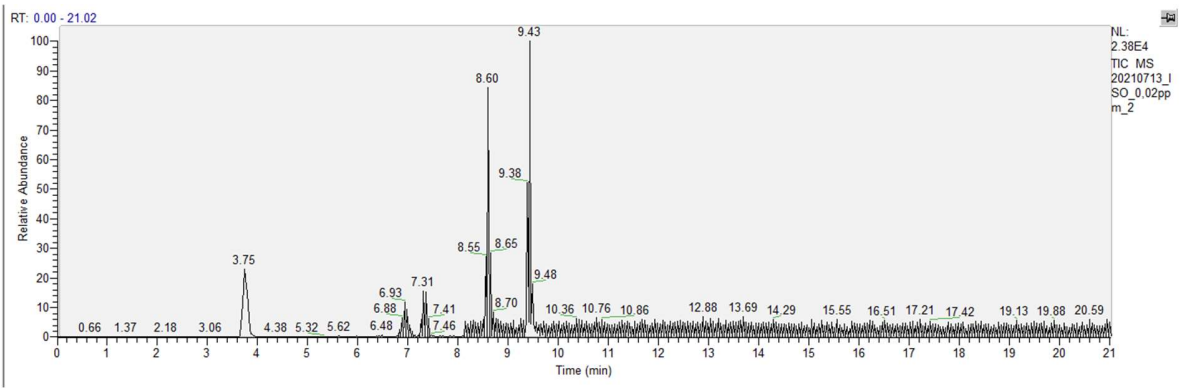

Figure 2 Chromatogram of neonicotinoids with a concentration of 20 µg/L. From left to right the peaks indicate concentrations of Thiamethoxam, Clothianidine, Imidacloprid, Acetamiprid and Thiacloprid

By measuring standards (test solutions with different, known, concentrations) the relationship between signal and concentration can be quantified for different compounds. This has been done for concentration of 10 to 200 µg/l and resulted in a linear relationship (R^2=0.999) for all compounds, indicating that the method is suitable to assess the quantity of these compounds in this range (Fig. 3).

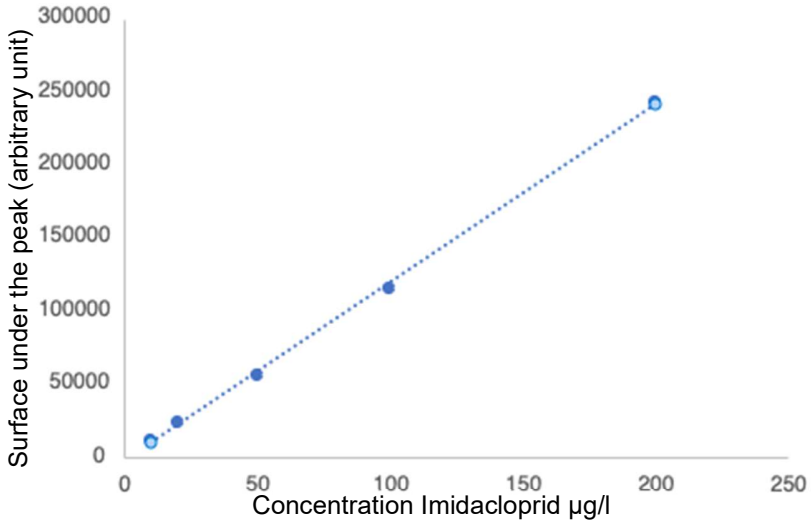

Figure 3 Calibration series for Imidacloprid showing a linear relationship between measurement and concentration. Linear trend: Y=1120,37 * x -2101,23; R^2 = 0,999.

4. Method validation

To assess whether this method is reliable a number of test were performed. The following aspects where investigated:

- Reproducibility (under different circumstances, e.g. days)
- Repeatability (under identical circumstances)
- Memory effect (cross contamination during analysis)
- Loss (loss of analytes during preparation needs to be quantified)
- Matrix effect (influence of other compounds in the samples)
- Range (at which concentration is the signal linear)
- Detection limit (what is the lowest concentration that can be measured)

4.1 Reproducibility and repeatability

Several test samples (with added neonicotinoids) have been measured on multiple days. By comparing these measurements we can gain insight in the reproducibility. Relative standard deviations were calculated as a measure for the uncertainty (Table 1).

Table 1 The relative standard deviation over multiple measurements of the different compounds.

Neonicotinoid	RSD
Thiamethoxam	9%
Clothianidine	13%
Imidacloprid	12%
Acetamidprid	13%
Thiacloprid	15%

Given the low concentrations used these are very reasonable values. Variation is caused primarily by the preparation of the samples and random noise in the measurements themselves. There was a difference between days and the calibration was done on one of those days. Recalibrating would likely further improve the accuracy. Additionally internal standards (known amounts of a compound added to each samples) can be used to scale all analytes to, that has not been done in this case.

4.2 Memory effect

Analytes can remain in the system and show up in later measurements as contaminants. A sample without neonicotinoids was measured ten times, followed by a sample with the neonicotinoid followed by the clean sample. If there is a memory effect the last measurement would be higher than those measured before the sample with neonicotinoid. This was not the case for any of the neonicotinoids studied.

4.3 Loss and Matrix effect

During the concentration and further preparation some of the analyte can be lost. It is essential to correct for this to calculate the accurate concentration in the original sample. Test solutions of known concentration can be made by adding neonicotinoids to ultra-pure water. These solutions differ from the samples collected from the dragonfly monitoring transects, as they do not contain additional compounds, including organic compound, that are often present in surface water. Therefore additional test solutions were created by diluting known amounts of neonicotinoids with surface water. These test

solutions were then concentrated using the Solid Phase Extraction method, measured with HPLC-MS and compared to the initial, known, concentration to quantify the loss of the analyte.
There is a substantial loss, probably this is largely during the concentration phase. The difference between the pure water and the surface water can be a result of the matrix but might also be a result of random variation as discussed under reproducibility.

Table 2 Recovery in pure water and surface water.

Neonicotinoïden	% measured	
	Ultra-pure water	Surface water
Thiamethoxam	64	66
Clothianidine	55	60
Imidacloprid	55	75
Acetamiprid	58	77
Thiacloprid	56	85

As recovery was less than 100% (Tabel 2) a correction for the measured concentration is needed to assess the concentration in the original sample correctly. This can be done using the values indicated here or with internal standards

4.4 Measuring range

To establish the range of concentrations over which the measurements are reliable a calibration was done with concentrations from 2.5 to 500 µg/l. The range over which there is a linear relationship between the area of the peak and the concentration in the calibration solution is the measuring range. The measurements from 2.5 to 200 µg/L are on a straight line but the measurement of 500 µg/l is below this line (Fig 4). This indicates that at these high concentration the analysis underestimates the concentration. These concentrations would be extremely high for surface waters and the linear part does include all levels that can be expected in surface waters.

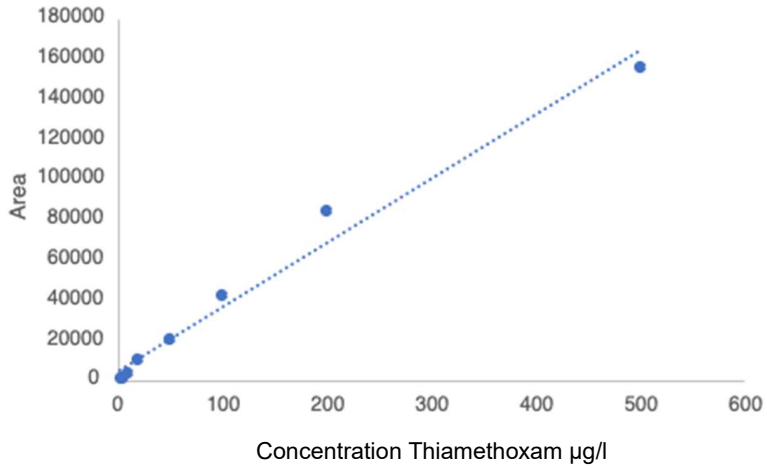

Figure 4 The relation between the concentration and area under the curve is linear up to at least 200 µg/l (the line is fitted through all points including 500 µg/l).

4.5 Detection limit

Based on the calibration curves the lowest measurable concentrations for the different compounds was calculated. The concentrations that could be measured reliably in calibration solutions were: Thiamethoxam 11 µg/l, Clothianidine 11 µg/l, Imidacloprid 3 µg/l, Acetamiprid 3 µg/l, Thiacloprid 4 µg/l. This corresponds to a detection limit of around 1000 times lower for surface water. Taking into account the additional uncertainty introduced through the analytical preparation process, we estimate the actual lower detection limit to be approximately 5 ng/l. This is in the same order as the maximum allowed concentrations for these compounds. Lower concentrations can be detected but not reliably quantified.

4.6 Daily variation

There is a possibility for differences between days when measuring with a HPLC. Therefore, a sample was measured In triplicate over four days. Difference between the three measurements on one day was very small but between days there are substantial differences, ranging from 900 ng/l to 1500 ng/l (using the same calibration curve). This emphasizes the need to calibrate daily and use of an internal standard. If this is not done the reliability of the measurements are reduced.

4.7 Fieldsample

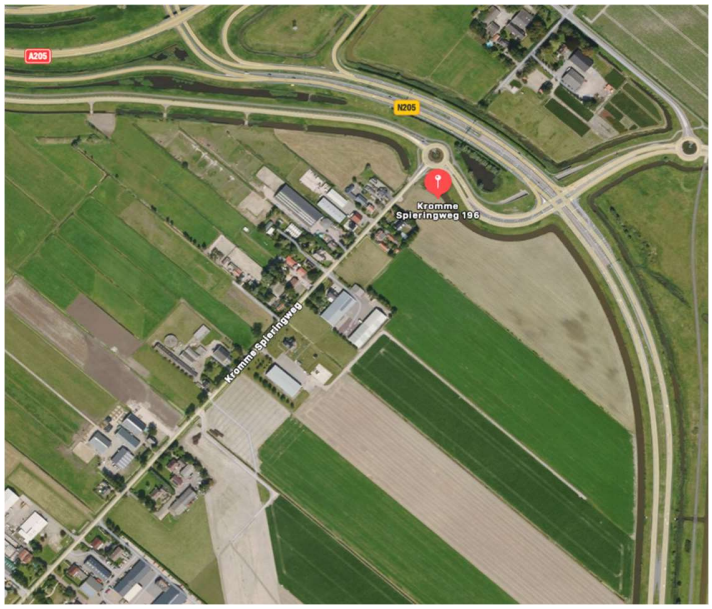

Figure 5 A field sample was collected near Vijfhuizen, at the location of the red marker. This location was selected as the presence of neonicotinoids was considered likely.

Before the actual samples from the insect monitoring sites were analyzed, a trial run was carried out with a field sample collected from a site where neonicotinoid pollution is likely to be detected (Fig 5). Thie site is a ditch near Vijfhuizen, the Netherlands. This sample was collected at 52.3683, 4.7034. This is an area with many farms and in the Dutch pesticide atlas (https://www.bestrijdingsmiddelenatlas.nl) it was indicated that at this location Imidacloprid was found.

A sample from this ditch was prepared as described above. The chromatogram is depicted in figure 6. There is a clear peak at 7.36 seconds and at 10.76 seconds. The first is Imidacloprid and the second is Acetamiprid. These peaks correspond to a concentration of 27 ng/l for Imidaclorpid and 2 ng/l for Acetamiprid. The second value is below the detection threshold and therefore this concentration is considered unreliable. It is safe to say that this compound is present in the sample but the concentration cannot be assessed with a reasonable uncertainty. As there are no natural sources of neonicotinoids the presence of a small amount of Acetamiprid does indicate it comes into this ditch from agricultural or other use.

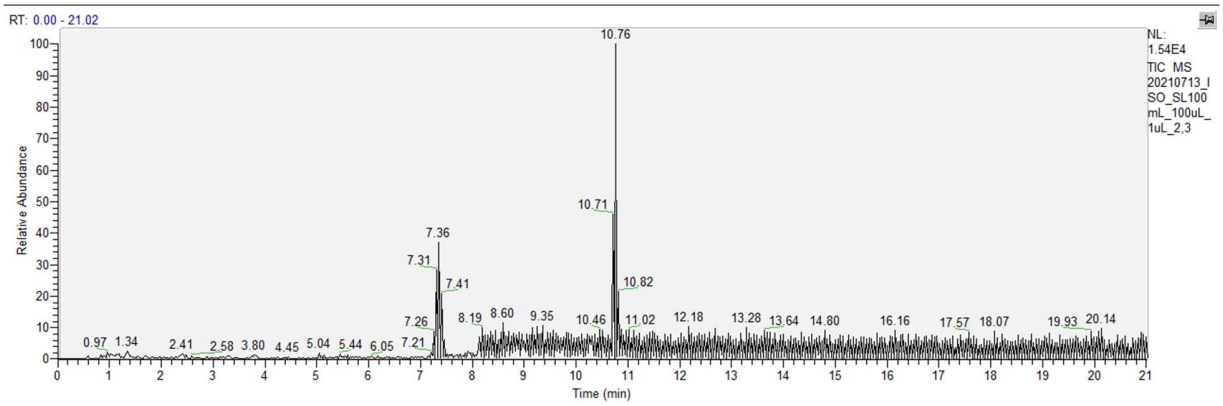

Figure 6 The chromatogram of the field sample with a peak for Imidacloprid and for Acetamiprid.

The concentration of Imidacloprid is more than 3 times the AA-EQS norm of 8 ng/l. The AA-EQS is the Annual Average Environmental Quality Standard.

4.8 Conclusions

The developed method is suitable to assess the presence and concentration of five neonicotinoids in surface water. Internal standards and frequent calibration improve data quality as there is a difference between days, when measuring the same sample. By concentrating the sample and using sensitive HPLC-MS techniques low concentrations can be quantified. Concentrations below 5 ng/l can be detected but the concentration cannot be quantified.

Figure 7. An example of a dragonfly monitoring transect with four sections. All dragonfly species are counted for each section.

5. Dragonfly data

Dragonflies are monitored by citizen scientists as part of the Dutch Dragonfly Monitoring scheme. This is coordinated by Dutch Butterfly Conservation and development of the scheme and the analysis is done in collaboration with Statistics Netherlands (www.cbs.nl) as part of the Network Ecological Monitoring (www.netwerkecologischemonitoring.nl). Dragonflies are counted fortnightly on fixed transects (Fig 7). The counts are used primarily to calculate trends in dragonfly abundance. As not all transects are counted for the entire period that the scheme is running (since 1998), but transects are stopped and other transects started, a statistical correction is needed. Additionally not all transects are counted every second week but can be counted more often or there may be gaps in the data because of e.g. holidays or bad weather. Missing data is imputed using the statistical package rtrim in R (https://cran.r-project.org/web/packages/rtrim/). This results in annual values per transect, these can be seen as an estimate of the number of individuals that would have been seen if the transect would have been counted fortnightly. These estimates for the annual values can be used to compare transects and will be used to investigate the effect of neonicotinoids on dragonflies, as sampled by the same citizen scientists.

6. Dragonflies and insecticides

For 32 transects, two water samples of 150 ml each were collected by the citizen scientist. These samples were taken at least two weeks apart to avoid measuring the same peak. In these samples the above-mentioned neonicotinoids were measured and the additional insecticides: Atraton, Metoxuron, Metolcarb and Isoxaben for which the same method of analysis is suitable. These values were compared with the statistically corrected annual numbers (divided by the length of the transect).

In all samples insecticides were detected. Thiamethoxam is found in all samples, often in low concentrations but five times in concentrations above 5 ng/l and thus quantifiable. These concentrations ranged from 31 to 66 ng/l, see table 3 for the overview and supplement 1 for all measurements.

Table 3 Number of samples were the different compounds were detected, detected but not quantified (<.5 ng/l) or were quantified (>.5 ng/l often much higher).

	Thiamethoxam	Clothianidin	Imidacloprid	Acetamiprid	Thiacloprid	Atraton	Metoxuron	Metolcarb	Isoxaben
Not detected	0	51	7	48	43	44	61	56	61
<5 ng/l	59	9	39	13	18	17	0	5	0
>5 ng/l	5	4	18	3	3	3	3	3	3
total	64	64	64	64	64	64	64	64	64

There are relatively few samples with measurable concentrations. In itself this is a good thing but it makes it hard to find a relationship with dragonfly occurrence. The number of positive analyses is however very high, on average each sample contained 3.2 insecticides of the 9 tested (see supplement for details).

As very few concentrations are quantified and the concentration quickly goes down after a release event we chose to compare the abundance of dragonflies with number of peaks detected, summed over the two samples. This can range from 0, no insecticides found, to 18, all tested insecticides detected in both samples. However, there were no locations where no insecticides were found so the value ranged from 2 to 18 (Fig 8). There is a large number of factors that can impact dragonfly abundance and are correlated with insecticide presence. Some of these are nutrient levels, as areas were insecticides are used are typically also fertilized, but insecticide use likely varies over regions, as different

crops are grown and are likely less present in nature reserves. However, a substantial number of samples is from nature reserves and still contain insecticides. Transect 945 is a small lake in the dunes, an area protected under the Habitat directive but still has 19.5 and 11.6 ng/l of imidacloprid, above the maximum allowed in surface water (AA-EQS) of 8.3 ng/l.

When plotting the dragonfly density (sum of annual values for dragonflies/length of transect in meters) the number of insecticide peaks detected we do see that the highest densities of dragonflies are found at locations with few peaks – ie: fewer different neonicotinoids - and of the locations where quantifiable concentrations of insecticides were found nearly all had low densities, irrespective of the total number of peaks detected. Linear regression did however not reveal a significant relationship between these two factors.

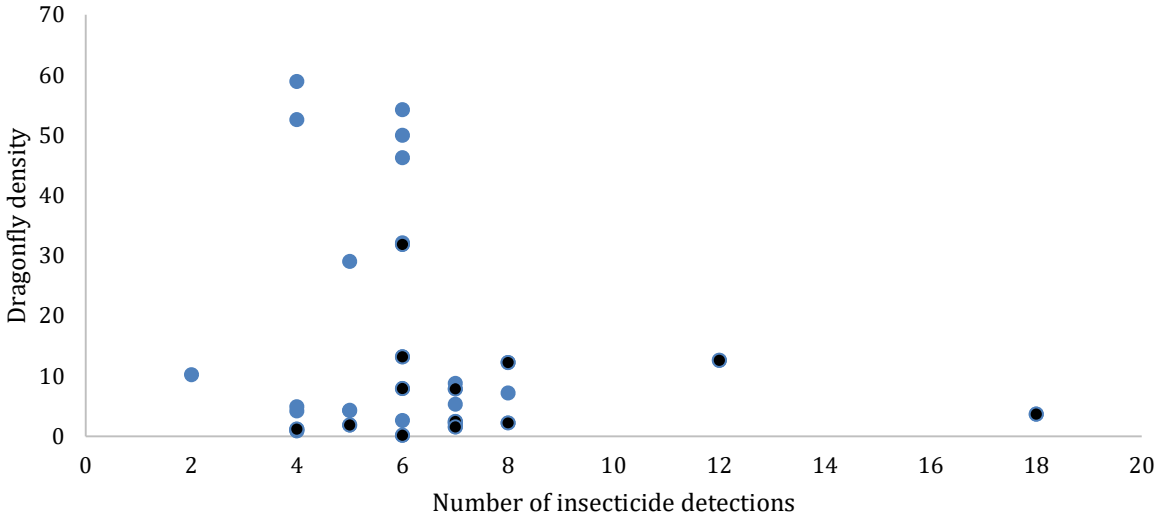

Figure 8. Number of insecticide peaks detected plotted against the density of dragonflies on each location. Black dots indicate that at those locations at least one compound was detected at >0.5 ng/l.

We can conclude that these insecticides are very common in surface waters and are likely to impact aquatic insect communities. The fact that these compounds are so common makes it hard to test for their impact. The patterns we see when looking at the number of detections and dragonfly abundance does suggest a negative effect but cannot be considered proof. Improvements can be made by more frequent or timed sampling to increase the chance to measure directly after pollution events.

7. Conclusions

We were able to develop a method to measure neonicotinoids and some other insecticides and, together with citizen scientists determine the abundance of dragonflies and the exposure to insecticides. Biodiversity monitoring, including the Dutch Dragonfly Monitoring Scheme relies heavily on citizen scientists. While normally the citizen scientists in such projects follow a protocol to quantify the biota of interest, in this case count dragonflies, we did show that there is interest in additional assessment to gain insight in the mechanism that affect the dragonflies. The combination of data and samples collected by citizen scientists and analytical techniques that are only available in well equipped laboratories allows us to study questions that would be impossible with only citizen science and prohibitively expensive with only professional researchers. This approach illustrates the complementarity of citizen science and professional research. The technique developed to measure these compounds can now also be used in other citizen science projects.

These insecticides are widespread and all samples tested did at least contain traces of neonicotinoids (that do not naturally occur) and in most cases several compounds were detected. Regularly these were above the norm (AA-EQS) including in nature reserves. It is very likely that these compounds have an impact, lethal or sublethal, on dragonflies but also other organisms. We were not able to find a clear relationship between insecticide exposure and dragonfly abundance as a result of the lack of clean locations and the large natural variation in dragonfly abundances. This does not mean that there is no impact. Given the existing literature (Barmentlo et al. 2021) and the frequency and concentration at which insecticides were detected in this study it is likely that there is an impact

This method can be used in citizen science projects but we would suggest more frequent sampling as we know that many of these compounds are quickly bound to organic matter and therefore concentration quickly go down after exposure. Doing this can give insight in the presence of insecticides and therefore shed a light on this factor that is normally hard to assess but likely very important in many aquatic ecosystems.

Barmentlo, S. Henrik, et al. "Experimental evidence for neonicotinoid driven decline in aquatic emerging insects." Proceedings of the National Academy of Sciences 118.44 (2021).

Supplement 1 Dragonflies and insecticides is available at10.5281/zenodo.5912996

This supplement contains the measured values and the sampled transects.