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Giving Beekeeping Guidance by cOmputatiOnal-assisted Decision making



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Preface

Deliverable 2.5 is a manuscript on lateral flow device based pesticide detection. This test was developed in Task 2.4 of WP2. The manuscript was submitted in Biosensors on July 28, 2022. The submitted manuscript and supplemental material are attached to this document.

Manuscript and supplemental material

Biosensing for chlorpyrifos in environmental water samples based on a carbon nanoparticle indirect lateral flow assay

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Abstract: Pesticides used in agriculture prevent pests. Chlorpyrifos (CHLP) is an insecticide with potential serious detrimental effects on humans, bees and the aquatic environment. Its effects have led to a total ban by the European Union, but outside the EU it is still produced and used. An indirect lateral flow immunoassay (LFIA) for the detection of CHLP was developed and integrated into a cassette to create a lateral flow device (LFD). Species-specific reporter antibodies were coupled to carbon nanoparticles to create a detector conjugate. Water samples were mixed with a specific CHLP monoclonal antibody and detector conjugate and applied to the sample well of the LFD. Based on dose-response curves, low concentrations of CHLP ($< 1 \mu\text{g/L}$) could be detected. This sensitivity was recorded visually and through rapid handheld digital imaging. The application to a range of European surface water samples, fortified with CHLP, revealed a general sensitivity of $2 \mu\text{g/L}$, both visually and by rapid digital imaging. To improve the simplicity of the CHLP LFIA, the assay reagents were dried in tubes, after which only water samples were added to the tube, and the LFIA strip was inserted. Thus, the CHLP LFIA is very suitable for on-site screening of surface waters.

Keywords: chlorpyrifos; lateral flow; immunoassay; surface water; on-site; environment

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1. Introduction

Pesticides are used in agriculture to avoid or fight pests. These pesticides can be divided into several subclasses such as insecticides, fungicides and herbicides. Their usage in agriculture, and their accumulation in the environment, has always been a point of discussion and their environmental impacts are critically assessed more than ever [1]. In contrast, the effect of pesticide-free urban green spaces has also been critically studied [2]. Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is an organophosphate insecticide that was introduced to the market in 1965 [3] and is also known by its trade names Dursban and Lorsban [4]. It has been effectively applied to target pest insects of citrus trees, onions, corn, and seeds amongst others [5,6]. In humans, chlorpyrifos (CHLP) can cause cardiovascular diseases, endocrine disruption, neurotoxic effects and can even be lethal at high doses [7–9]. CHLP has a strong impact on the aquatic environment, as it is genotoxic to freshwater fish and is also directly toxic by inhibiting the cholinesterase (ChE) enzyme activity [10]. Recent studies showed endocrine and neurodevelopment disrupting effect of CHLP on *Xenopus laevis* brain formation [11]. Additionally, CHLP is highly toxic to bees, through oral exposure (oral LD50 360 ng/bee), or even more so via direct contact (contact LD50 70 ng/bee) [12]. CHLP is found in pollen, honey,

beeswax, and honeybees individuals [13–16]. Adult bees exposed to sub-lethal levels of CHLP showed reduced walking; increased grooming; difficulties in righting themselves; unusual abdominal spasms [17], morphological deviations to the body and mouthparts [18], and reduced appetitive learning and specificity of memory recall [19]. Since CHLP is also found in surface waters, or dew, it may impose a threat to bees next to foraging on contaminated flowers [20–22].

A case study, based on food monitoring and human biomonitoring, showed strong reductions of CHLP usage from 2016 onwards, which can be subscribed to regulations and awareness of the potential harmful effects of CHLP [23]. In addition, the production of CHLP by Dow/Coverta was halted [9]. Nevertheless, since CHLP is not patent-protected, large quantities of it are still produced in countries like India and China [9]. But even though pesticides may be banned by global institutions, it does not mean that they are not in use anymore. Especially in developing countries, banned pesticides are still illegally used which imposes a health risk for the exposed citizens and the environment [24]. Within Europe, the illegal use of CHLP on flowering trees in Austria, led to the total extinction of more than 50 honeybee colonies [25]. In 2020, the European Union decided to impose a total ban on the use of CHLP and set a maximum residue level (MRL) of 0.01 mg/kg as the default value for all products [26,27]. This means that fast screening methods need to approach a zero-tolerance and therefore should also be able to detect CHLP at 0.01 mg/kg (10 ppb). Although the solubility of CHLP in water is rather low (1.4 mg/L) [28], it does occur in surface waters around the world [10,29–31]. For monitoring the presence of CHLP in the environment, analytical instrumental analysis is a popular technology, often applied as a multi-detection method for many pesticides, including CHLP [32,33]. Although highly reliable, and capable of detecting hundreds of pesticides in one measurement, these analytical instrumental methods are in general time-consuming, nor portable on-site, mostly need extensive sample preparation and require highly skilled personnel for machine operation and data analysis.

A range of rapid methods have been developed for the detection of CHLP. Zhu *et al.* combined surface-enhanced Raman spectroscopy and chemometrics for the rapid detection of CHLP in tea samples after QuEChERS-based sample extractions and with ppm (mg/kg) based sensitivities [34]. Even though this measurement time may be considered rapid, the extraction procedure is not suitable for a point of need approach. Sankar *et al.* developed a paper-based device for the rapid detection of CHLP in water samples based on the inhibition of lipase enzymatic activity [35]. This assay was combined with smartphone readout and had an incubation time of 15 minutes and a limit of quantification of 200 ppb ($\mu\text{g/L}$). Enzyme-linked immunosorbent assay (ELISA)-based immunoassays are a popular tool for the detection of a wide range of contaminants and these also exist in different formats for the detection of CHLP [36,37]. Despite being robust and semi-high throughput, their portability and suitability for rapid testing are rather low. A very popular immunoassay format for rapid and point-of-need testing is the lateral flow immunoassay (LFIA) [38]. Nowadays this diagnostic platform is well-known, since lateral flow tests have been substantially used by the world population for self-testing during the SARS-CoV-2 pandemic to test oneself [39]. Both viral antigen in nasopharyngeal swabs and specific antibodies in blood can be easily and rapidly detected. Besides large proteins such as viral antigens and antibodies, the LFIA also qualifies for the detection of small molecules such as phytoproducts, mycotoxins and pesticides [40–43]. An LFIA for the direct detection of CHLP, based on colloidal gold detection has been developed previously [44].

In the presented research, a novel indirect LFIA with low ppb sensitivities was developed for the detection of CHLP, implementing Goat anti-Mouse IgG antibody molecules (GAM) immobilized onto carbon nanoparticles (CNP) as the detector conjugate (GAM-CNP). Its sensitivity for the detection of CHLP in water samples was assessed by applying it to tap water, aquaponics water and six independent European surface water samples. Readout of the results was done visually with the naked eye and by rapid

intensity-based imaging applying a handheld detector (Cube). For future point-of-need applicability, the LFIA was designed for implementation into cassettes with sample and assay reagents presented in microtiter plate wells. The developed CHLP LFIA was rapid and showed high sensitivities in both visual and handheld detector readout. A simplified approach of the LFIA, implementing ready to use reagent tubes, showed good perspectives for the easy on-site detection of CHLP in water samples.

2. Materials and Methods

2.1 Instruments

For spraying and cutting the membranes we used a XYZ 3060 BioDot Dispense Platform and a CM4000 Biodot Guillotine (BioDot Inc., Irvine, CA, USA). Readout of LFIAs was performed on a Cube handheld analyzer running on confirmation software, version V1.5.062 (Chembio, Berlin, Germany). Images of the LFIA results were recorded with a Samsung A50 smartphone and adjusted for white balance with Adobe Photoshop software (23.4.1 release), using the curves option and the wicking pad as the reference. Sonication of the carbon conjugate was performed in a sonicator bath (VWR International, Radnor, Pennsylvania, USA) and the Bioruptor Plus Diagenode ultrasonicator (SA, Seraing, Belgium). Measuring antibody concentrations was performed by a DS-11 FX spectrophotometer (DeNovix, Wilmington, USA). For centrifugation steps, the Sigma 2K15 centrifuge was used (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). For drying the assay reagents in glass tubes, we used the reacti-therm™ heating and stirring modules from Thermo Fisher (Rockford, USA) combined with nitrogen gas flow.

2.2 Chemicals and materials

The CHLP monoclonal antibody (mAb) (AT279, 0.01M PBS, 5.50 mg/ml) and chlorpyrifos-BSA conjugate (CHLP-BSA) (AG279, 0.01M PBS, 5.70 mg/ml) were purchased from Ecalbio (Wuhan, China). Chlorpyrifos-ovalbumin conjugate (CHLP-OVA), 1 mg/ml, was kindly provided by Dr. Yirong Guo of Zhejiang University. Goat anti-Mouse IgG FcY (GAM) specific polyclonal antibodies (1.8 mg/ml, 115-005-071) and the donkey anti-Goat IgG (DAG) polyclonal (H+L) antibodies (1.3 mg/ml, 705-005-003) were obtained from Jackson ImmunoResearch (Sanbio, Uden, The Netherlands). The CHLP stock solution (200 µg/mL in MeOH) was kindly supplied by the pesticides department within Wageningen Food Safety Research (WFSR). LFIAs were sprayed on CN140 nitrocellulose membranes (Unisart, Sartorius, Gottingen, Germany). Plastic backing cards, sample pads and aluminum foil pouches for storage of the produced LFIAs, were purchased from Kenosha (Amstelveen, the Netherlands). Wicking pads were purchased from Whatman (GE Healthcare, Eindhoven, the Netherlands) and minipax absorbent packets from Merck (Darmstadt, Germany). Global surface water samples from rivers, ponds and brooks were previously collected by Dr. Rubing Zou [45] and were supplemented with additional samples collected by WFSR. Aquaponics water was kindly provided by the Circle (Rome, Italy) (Table S1). Boric acid, Tween-20 and sodium azide (NaN_3) were purchased from Merck (Darmstadt, Germany). The bovine serum albumin (BSA), phosphate-buffered saline (PBS), Triton X-100 and tergitol were all obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The sodium tetraborate was acquired from VWR (VWR, Leuven, Belgium). Deionized water ($>18.2 \text{ M}\Omega/\text{cm}$) was prepared fresh, by filtering distilled water through a Milli-Q direct water purification system (Millipore, Burlington, MA, USA) for the preparation of buffers and solutions. Dilutions of the CHLP stock were prepared in Methanol Ultra LC-MS from Actua Chemicals (Oss, the Netherlands). Spot-based LFIAs were developed in cellstar 96-well plates (Greiner bio-one, Alphen a/d Rijn, the Netherlands). Cassettes for the construction of the lateral flow device (LFD) were kindly supplied

by Zhejiang University. Zeba spin columns (7K MWCO), used for desalting antibody solutions, were obtained from Thermo Scientific (Rockford, USA). Conjugation of GAM to carbon nanoparticles (CNPs) was performed using special Schwartz 4 (Orion Engineered Carbons GmbH, Eschborn, Germany). The AR glass tubes 75x11.5x0.7 mm were obtained from VOS instrumenten b.v. (Zaltbommel, the Netherlands).

2.3 Conjugation of GAM antibodies to amorphous CNPs

Conjugation of the GAM polyclonal antibody (pAb) to CNPs was performed as described previously by Sharma et al. [46]. In short, a 1% (w/v) aqueous suspension of amorphous CNPs was sonicated in an ultrasonic bath for 1 h at 40 KHz at room temperature (RT). Subsequently, a 0.2% (w/v) carbon suspension was prepared in 5 mM Borate buffer (BB) (pH 8.8) containing boric acid and sodium tetraborate. This suspension was sonicated for 5 min using the ultrasonic bath. For conjugation, the purified GAM pAb was desalted/buffer exchanged against 5 mM BB (pH 8.8) using 0.5 mL spin columns. One mL of the 0.2% (w/v) carbon suspension was mixed with the purified antibody preparation to a final antibody concentration of 350 µg/mL. The content was stirred at 4°C for 12 h on a magnetic stirrer. Subsequently, 1/10 volume of washing buffer (5 mM BB, pH 8.8 containing 1% BSA) was added and incubation was prolonged for one hour. The suspension was subsequently centrifuged at 13,636 g (4°C, 15 min). The pellet was washed three times with washing buffer and finally suspended in storage buffer (100 mM BB, pH 8.8 containing 1% BSA) to a final concentration of 0.2% (w/v) CNPs. The GAM-CNP conjugate was stored at 4°C until further use.

2.4 Method setup by implementation of spot-based strips

Initial set-up and optimization of CHLP LFIA was performed by spotting the CHLP-BSA, or the CHLP-OVA conjugate, and the DAG pAb manually onto the nitrocellulose (NC). To this end, the NC membranes (30 cm width, 2.5 cm length) were attached to a plastic backing and overlaid with a 15 mm wicking pad. This assembly was then cut into 4 mm strips. The stock solution of CHLP-BSA and the CHLP-OVA were diluted in PBS (10 mM potassium phosphate buffer, pH 7.4) to obtain a concentration series of 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL. The DAG mAb was diluted to 0.25 mg/mL in 5 mM BB (pH 8.8). CHLP-BSA (or CHLP-OVA) and the DAG pAb were spotted manually by a micropipette, dispensing 0.5 µL onto the NC membrane, 5 mm part from each other, with the DAG pAb last in order of the flow direction. The strips were then dried at RT for 30 minutes and then stored at RT in a sealed package containing minipax absorbent packets, until further use. Next, dilution series of the CHLP mAb stock (1:100 – 1:512,000 times) were prepared in running buffer (RB) composed of 0.01 M PBS pH 7.4, 1% (w/v) BSA and 0.05% (v/v) tween-20. CHLP calibration standards were prepared by 10-fold stepwise dilutions, resulting in a concentration range of 0.1 to 100 µg/L (ppb). Diluted CHLP mAb (1 µL) and GAM-CNP conjugate (1 µL) were added to the diluted CHLP antigen (98 µL), mixed and then added to the well of a cellstar 96-well plate. The same procedure was repeated for the following running buffers: RB2: 100 mM BB, 1% (w/v) BSA, 0.05% (v/v) Tween-20, 0.01% (v/v) Tergitol, RB3: 100 mM BB, 1% (w/v) BSA, 0.2% (v/v) Tween-20, 0.05% (v/v) Triton X-100, RB4: 100 mM BB, 1% (w/v) BSA, 0.05% (v/v) Tween-20. The LFIA strips were placed upright in a well and incubated for 10 minutes. After incubation, the strips were placed on a white sheet of paper and photographed using daylight for exposure.

2.5 Preparation of line-based strips for sensitivity testing

For the production of line-based LFIAs, we chose to spray the test line with the CHLP-BSA conjugate at 0.125 mg/mL and the DAG pAb at 0.05 mg/mL onto the NC

membranes using 0.01M PBS (for CHLP-BSA) and 5 mM BB (for DAG pAb) as the spraying buffers. The sprayed NC was dried for 30 minutes at RT. Next, the dried NC was secured on a plastic backing overlaid with a wicking pad (as described previously in paragraph 2.4) at the top end and a sample pad at the bottom end. Subsequently, the assembly was cut into 4 mm strips by the guillotine. The cut strips were placed into a dedicated cassette and stored at RT..

2.6 Sensitivity testing of the CHLP LFIA

To test the sensitivity of the line-based LFDs, we prepared CHLP calibration standards of 100, 50, 10, 5, 1 and 0.5 ppb ($\mu\text{g/mL}$) in RB. The RB buffer without any CHLP added, was chosen as the blank standard (negative control). The CHLP mAb was diluted 1:3200 in RB and 1 μL was added to the diluted CHLP stock (98 μL). Next, 1 μL of the GAM-CNP conjugate was added. This mixture was vortexed very briefly and added to the sample zone of the LFD. The LFIA was allowed to develop for 10 minutes, which is assumed to be a rapid response time. After visual readout of the result, the strips were removed from the LFD cassettes, sample- and wicking pad were removed, and then digitally analyzed using the Cube reader. Both the LFDs and the separate strips were photographed.

2.7 Pre-validation of the LFIA's on surface water samples

To test the suitability of the CHLP LFIA for the application to environmental surface water samples, the CHLP stock was spiked to a range of water samples (Table S1) at concentrations of 200, 20 and 2 ppb, while the same water samples were also left blank as negative controls. The spiked water samples were diluted 1:1 with 2 times concentrated RB (2x RB), composed of 200 mM BB pH 8.8, 2% (w/v) BSA and 0.01% (v/v) Tween-20. The samples were supplemented with diluted CHLP mAb (1 μL , 3200x dilution, in RB) and GAM-CNP conjugate (1 μL in RB) and briefly mixed. This mixture was added to the sample zone of the LFD and allowed to develop for 10 minutes. After incubation, the strips were analyzed and documented..

2.8 Preparation of assay tubes for on-site application

To facilitate future on-site testing, assay reagents were dried in tubes, largely based on a method previously described by Koets et al. [47]. In short, glass tubes were blocked with RB (300 μL) and incubated at 37°C for 2 hours. Next, 25 μL of GAM-CNP conjugate and 25 μL of CHLP mAb (both in 2x RB) and 50 μL of 2x RB were added at the bottom of the tube and gently mixed. This 100 μL reagent solution was dried under nitrogen flow for 30 minutes at RT. CHLP calibration standards of 10 and 100 ppb were prepared in water, and 100 μL of each standard was added to the dried reagents at the bottom of the glass tube. The glass tube was gently mixed by hand for a few seconds, placed in a tube rack and the LFIA strip was inserted and incubated for 10 minutes and read out was performed as described previously.

3. Results and discussion

3.1 Choice of assay format

The indirect, competitive assay format we have chosen for the development of the CHLP-specific LFIA (Figure 1), is based on the earlier work of one of the authors, focusing on detection of sulphamethazine in urine [48]. Experimentally it was observed, that the indirect approach in which the specific antibody was diluted and mixed with the

nanoparticle-coupled anti-species antibodies resulted in a higher sensitivity compared to the direct approach, because the specific antibodies were coupled directly to the nanoparticles. In the indirect format, more precise dosing of the specific antibody molecules enables a better balance between a good signal in the case no hapten is present in the sample and a sensitive displacement of colored particles at low hapten concentration. In a recent publication, Majdinasab *et al.* [49] applied the same indirect approach and, although presented as a new format, they concluded that this approach indeed leads to a more sensitive assay for detection of the hapten. This was further substantiated by a mathematical modelling study of this competitive format by analytical and numerical approaches [50]. One of the recommendations for increasing the efficiency of analyte detection was to reduce the concentration of labelled antibodies to the minimum detectable limit. The indirect approach as applied in this study, is a perfect assay design to translate this recommendation into a practical assay format.

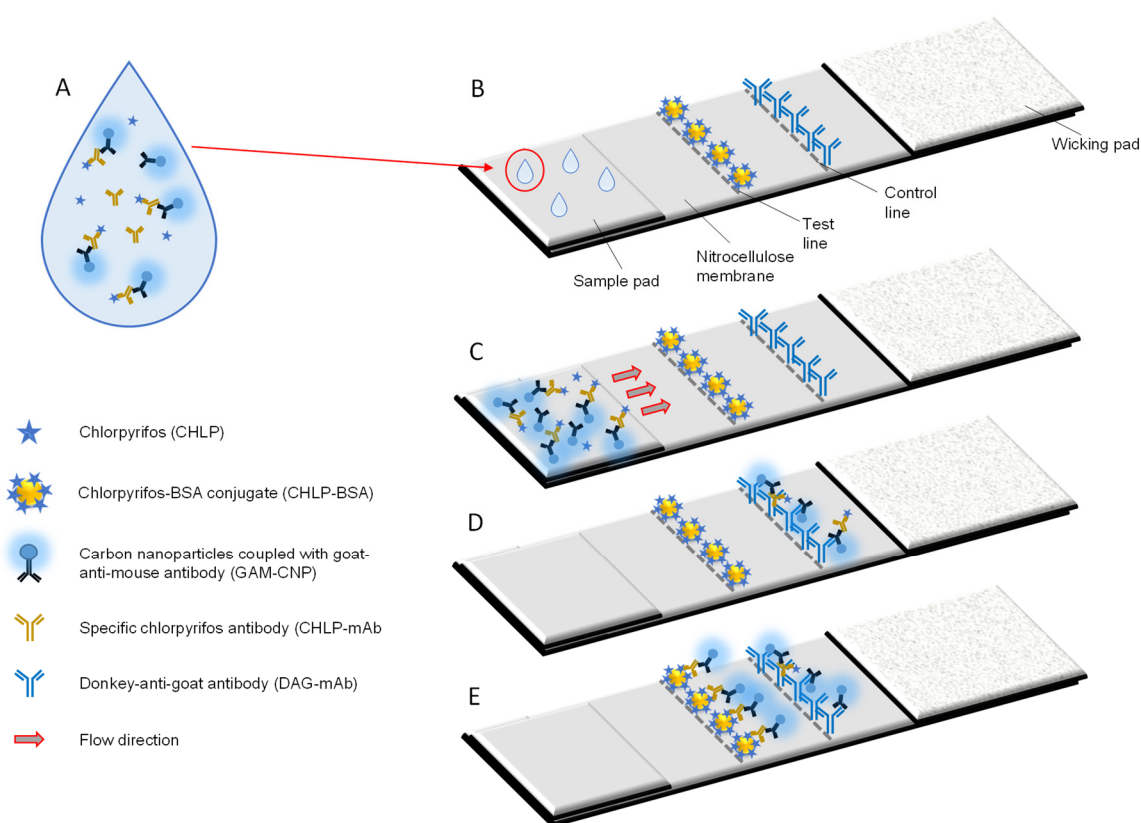


Figure 1. Principle of the chlorpyrifos lateral flow immunoassay (LFIA). Surface water samples are mixed with CHLP-mAbs and the GAM-CNP reporter (A). They are applied to the sample pad (B) and start to flow over the nitrocellulose membrane towards the wicking pad of the LFIA (C). In case a high concentration of CHLP is present, the CHLP-mAbs will not bind to the CHLP-BSA conjugate on the test line. The GAM-CNP and the GAM-CNP/CHLP-mAb complex will bind to the DAG-mAb on the control line (D). In case no CHLP is present in the sample, the CHLP-mAb and the GAM-CNP/CHLP-mAb complex will bind to the CHLP-BSA conjugate on the test line and also to the DAG-mAb on the control line (E).

3.2 Initial setup of the CHLP LFIA by spotted membranes

In the initial experiments, several membranes were tested for the CHLP LFIA. From these membranes, CN140 was chosen as the most suitable, based on initial sensitivity and

running time (results not shown). To determine the optimal sensitivity for the CHLP assay, both CHLP-OVA and CHLP-BSA conjugates were manually spotted on the membranes in several concentrations, combined with a spot of the control line DAG pAb of a fixed concentration to ensure the confirmation of test functionality. More than 500 hand-spotted strips were tested by applying several dilutions of the antibodies in four different running buffers. A selection of those 500 tests can be found in Figure S1. From all these results we determined that CHLP-OVA was the most suitable conjugate when spotted at 0.125 mg/mL, using RB as the running buffer and the antibody diluted 1:3200 times (1.7 µg/mL).

3.3 Sensitivity determination of the sprayed LFIA

Using the previously determined optimal conditions, CHLP-OVA test lines were sprayed on the NC membranes at a conjugate concentration of 0.125 mg/mL, while the control line was sprayed with DAG pAb at 0.05 mg/mL. We chose this DAG pAb concentration to reach similar intensities for both test and control lines. Roughly 30 strips were cut from each assembled card. These strips were placed in the LFD cassettes and calibration standards were applied to the sample well and the results were recorded after 10 minutes. To check for variability, this experiment was performed three times on the same day with 2 hours intervals between the experiments (Figure 2). Visual readout, by naked eye, determined the average sensitivity of the test at 1 ppb (1 µg/L). Next, the strips were removed from their cassettes and the intensities of the test lines were measured with the Cube reader. The results of those readings are plotted in Figure 3, displayed as percentages of relative response (B/B_0), calculated by dividing the responses of the calibration standards by the response of the blank sample.

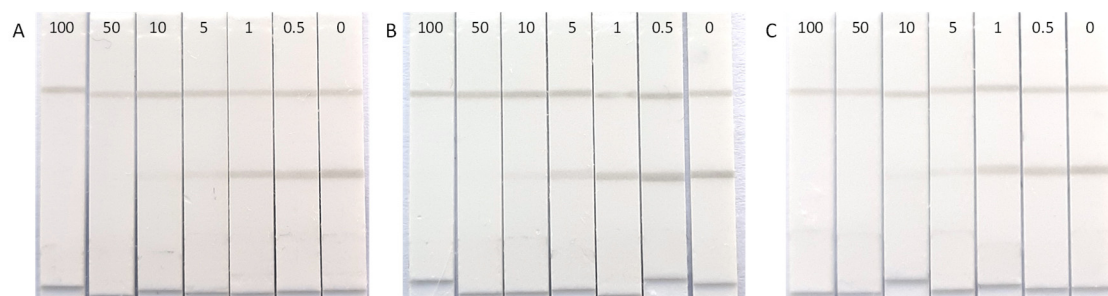


Figure 2. Sensitivity testing of the chlorpyrifos LFIA by application of calibration standards in three independent runs (A, B and C), in one day with two hour intervals between them.

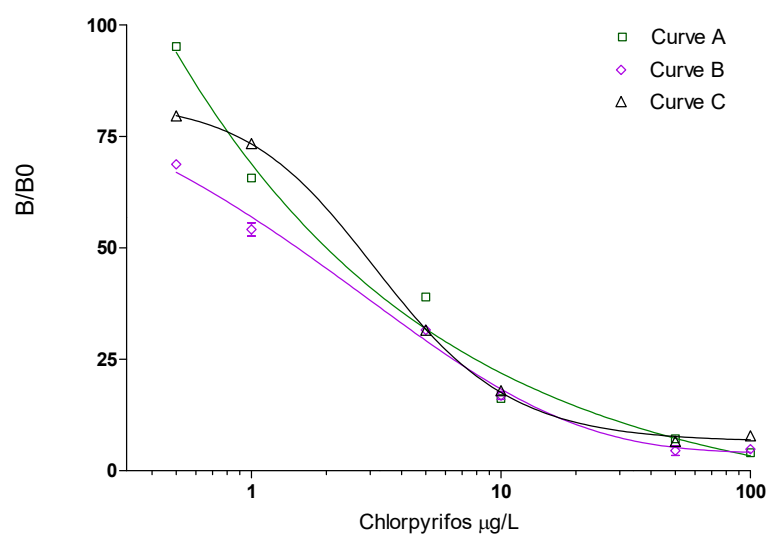


Figure 3. Dose-response curves based on duplicate cube readings of calibration standards in the CHLP LFIA, performed in 3 independent experiments in one day with two hour intervals between them, displayed as relative response (B/B_0) calculated by dividing the responses of the calibration standards by the response of the blank sample.

The constructed dose-response curves, based on four parameter logistics, show that sensitivities up to 0.5 µg/L can be read for each experiment, considering that the blank value (B/B_0) is 100%. However, the dose-response curves also show that, experiments at different times of the day, CHLP solutions with concentrations lower than 5 ppb showed similar readings, while concentrations higher than 5 ppb showed increasing variations between the curves. Serial incubations, where the strip is first inserted in a CHLP mAb solution and then into the GAM-CNP solution did not improve the assay sensitivities. Decreased sensitivities were observed when the CHLP mAb and GAM-CNP were added to the constructed LFD as pads (results not shown).

3.4 Application of the CHLP to water samples

A total of 8 water samples were fortified with CHLP at 200, 20 and 2 µg/L. Next these water samples were mixed with an equal volume of 2x RB and then applied to the LFD. After 10 minutes the LFIA results were evaluated visually to determine the sensitivities. After that, the strips were removed from the LFD and the sample- and wicking pads were removed from the strip before digital imaging (Figure 4). For each water sample the visual sensitivities were set at 2 µg/L (Figure S2), which was equal to the sensitivities obtained upon digital imaging of the LFIAs (Figure 5, Table S2). In some cases, this was visually challenging, like for the water sample from the Seine river. However, the digital imaging for this particular sample showed a clear distinction between the Test to Control ratio (T/C) for 1 µg/L (1.764) and the T/C for 0 µg/L (2.051).



Figure 4. Surface water from a brook (Wageningen, The Netherlands) fortified with chlorpyrifos at 200, 20, 2 and 0 µg/L applied to the LFIA in duplicate. Visual imaging was done in the assembled cassettes (A) and with strips removed from the cassettes for digital imaging (B).

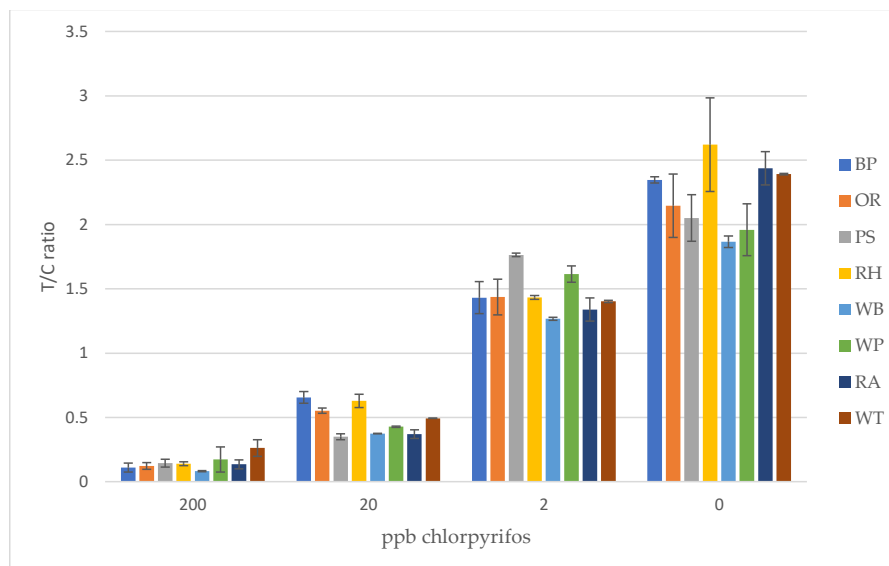


Figure 5. Average relative LFIA readings of all the fortified water samples, displayed as test-line/control line ratios. BP = Pond Brussels, OR = Rhine Oosterbeek, PS = Seine Paris, RH = Harbour Rotterdam, WB = Brooke Wageningen, WP = Pond Wageningen, AQ = aquaponics water, Rome and T = tap water.

If we want to make decisions based on handheld digital imaging without calibration before each measurement, decision levels based on previously acquired T/C readings needs to be determined. Therefore we should compare the lowest reading of the blanks (minus the SD, 1.821) to the highest values of the fortified samples. In case of the 1 ppb samples, the Seine River (PS) sample (plus the SD, 1.764) comes close to the lowest blank value. This makes installing decision levels for pre-calibrated cube readings at 1 ppb challenging, but not impossible. Additionally, assay optimization may still prove the sensitivity, e.g. different CHLP antibodies and/or conjugates. For 10 ppb, which is the set detection limit by the EU, the highest T/C reading (0.656) is highly distinctive from the lowest blank reading. Nevertheless, a trained eye can in general distinguish the 1 ppb samples from the blank sample. This high sensitivity is very relevant since CHLP is highly toxic to the environment, and moreover, banned by the EU. Therefore the developed LFIA is a rapid and sensitive CHLP detection tool to aid environmental control.

3.5 Application to apple blossom

In initial experiments, the CHLP LFIA was applied to water-based extracts of apple blossom to investigate its relevance in environmental safety monitoring for bees. To approximate a real-life scenario, concentrated CHLP standard was applied to the pollen and apple blossom and allowed to dry for 2 hours. After extraction with water, sensitivities of 10 ppb were observed for the extracts by visual readout. However, this relates to a 100 ppb sensitivity for the apple blossom. Digital imaging did not show improved detection limits for apple blossom (Figure S3). Additionally, the acquired sensitivities do not comply with the set 0.01 mg/kg (10 ppb) detection level set by the EU. Extractions containing methanol did not show improvements. This means that future work for the application

on bee-related matrices should focus on suitable extraction methods for more efficient extraction which are not detrimental to the LFIA and at the same time do not introduce unwanted interferences to the functionality of the test. Nevertheless, high concentrations of CHLP, as a consequence of illegal use, will be detected by the current LFIA. Moreover, the additives present in commercial pesticide formulae for field applications may contribute to easier extraction of CHLP from flowers or leaves. Therefore these should be considered as well.

3.6 Towards improved point of need detection

The presented LFD format could be performed at the point of need; however, it would still need three pipetting steps using laboratory pipettes for adding the sample buffer, CHLP mAb and the GAM-CNP separately. Therefore we tried to introduce simplifications based on the work of Koets et al. [47]. To this end, we added all the reagents and the 2x RB buffer into a 5 ml tube that was pre-blocked with RB and allowed the water fraction to evaporate under a liquid nitrogen flow. In this initial experiment, we manually spotted strips and dried them as described previously. To the dried reagent mix at the bottom of the tube, calibration standards of 100 and 10 ppb in tap water were added and mixed by manually shaking the tube. The LFIAs were inserted and allowed to develop. Both calibration standards showed less intense spots when compared to the blank water sample, indicating that the simplified approach has potential for on-site use (Figure S4). The introduction of simple, but accurate, disposable micropipettes would finally avoid any laboratory pipet usage [51]. Additionally, the developed LFIA strip can easily be measured at the point of need with the Cube reader, as it doesn't need to be removed from the LFD cassette when using the tube-based method. Next to that, future digital imaging of the LFIA strips, could also be performed by using a smartphones combined with freely downloadable applications [52].

Conclusion

An indirect competitive LFIA, based on a GAM-CNP detector conjugate, was developed for the detection of chlorpyrifos. The LFIA proved to be effective for detecting CHLP at low concentrations in European surface water samples. No background interference was observed even though the water samples were of very diverse sources. The visual readout of the LFIA proved to be successful, which is economically beneficial since it resolves the need for a digital imaging device. Nevertheless, readout by rapid digital imaging was able to slightly enhance the sensitivities in some cases and prevents the need of a trained, or scientific eye. Additionally, the digital imager may be programmed to decide which samples test positive or negative. Since the digital imager is handheld, it is easy to use at the point of need. Improving simplicity of the CHLP LFIA by using sample tubes with dried reagents, makes the LFIA user-friendly and very suitable for on-site screening of surface waters. Therefore, the LFIA is a perfect tool for controlling the European ban on CHLP at the point of need. Moreover, it can be implemented worldwide to screen for the use of CHLP in countries where it is still permitted for application. Besides surface water, bee related matrices are also to be considered. Honeybees (and their hive products) can serve as excellent bioindicators because of their ability to provide high resolution information on the presence of agrochemicals in the environment, as well as the repertoire of simple assays that can be performed to assess the effects of agrochemicals on their physiology and behavior [53]. To contribute to the health state of the bee's environment, improvements of extraction protocols need to be undertaken to apply the LFIA to bee-related matrices, thereby contributing to the health state indexes of the bee environment. Moreover, the current single analyte LFIA could be further developed as a multiplex LFIA by adding other substances harmful to the (bee) environment, like e.g. fipronil and pyrethroids.

Supporting Information

Table S1: Collected surface water samples, Table S2: Average relative LFIA readings of all the fortified water samples LFIAs, Figure S1: Method setup by the implementation of spot-based strips with constant concentrations of DAG pAb for the control spot (upper) and varying concentrations of CHLP-protein conjugates for the test spot (lower) on the membrane combined with serial CHLP-mAb dilutions, Figure S2: Overview of LFIA results for all the assessed environmental water samples. Each concentration was tested in duplicate, Figure S3. LFIA detection of chlorpyrifos (applied and dried) on apple blossom after water extractions by visual (A, B) readout and using a cube digital imager (C), for duplicate readings (D). The test line signals are divided by the control line signals to acquire T/C ratios, Figure S4: Simplification of the LFIA by drying assay reagents and buffer in tubes (A), after which a water sample containing chlorpyrifos is added and the LFIA inserted (B), showing 10 ppb sensitivities for spot-based LFIAs in duplicate measurements after visual readout (C, D).

Author Contributions: L.W., J.W., M.X. and R.H. performed the experiments. J.P. and L.W. wrote the initial manuscript, with substantial contributions of C.D., A.A. J.W. and M.X. while R.H., A.A., J.W. were responsible for the methodology. J.P. and C.D. were responsible for conceptualization of the research and writing and reviewing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Supporting Information

Lateral flow detection of chlorpyrifos in environmental water samples based on a carbon nanoparticle indirect assay format

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11 Tables

12 **Table S1.** Collected surface water samples

Sample No.	Code	Country of origin	Sample location	Sampling date
1	BP	Belgium	Botanic garden pond, Brussel	2019/12/30
2	OR	the Netherlands	Rhine river, Oosterbeek	2019/12/28
3	PS	France	Seine river, Paris	2019/12/23
4	RH	the Netherlands	Leuvehaven harbour, Rotterdam	2019/12/28
5	WP	the Netherlands	Campus pond, Wageningen	2022/04/15
6	WB	the Netherlands	Agricultural brook, Wageningen	2022/04/15
7	RA	Italy	Aquaponics farm, Rome	2021/04/12
8	WT	the Netherlands	Tap water, Wageningen	2022/04/15

13

14 **Table S2.** Average relative LFIA readings of all the fortified water samples LFIAs

15

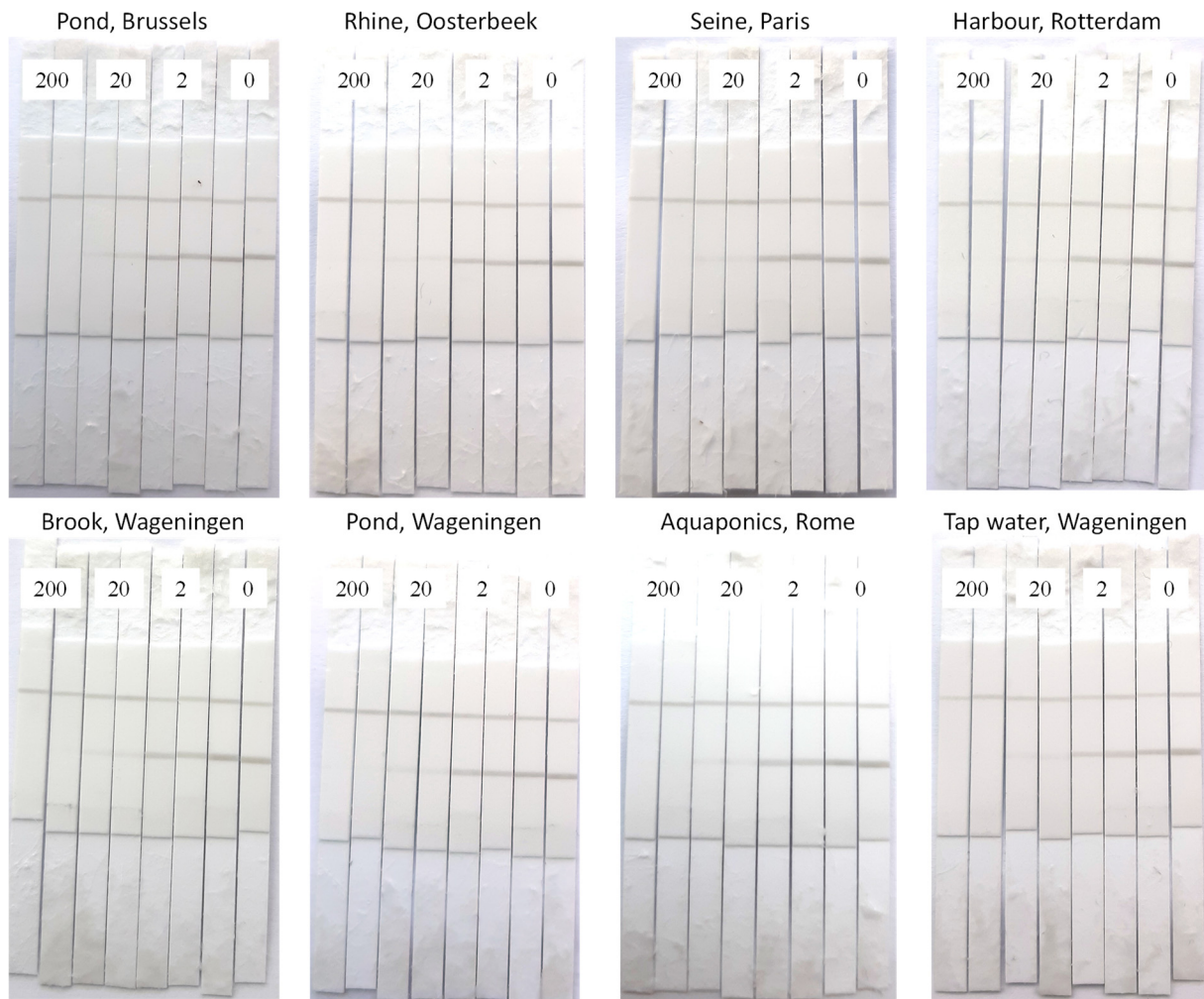
Sample ¹	200 ppb ²		20 ppb		2 ppb		0 ppb	
	Mean	St.dev. ³	Mean	St.dev.	Mean	St.dev.	Mean	St.dev.
BP	0.110	0.035	0.656	0.045	1.431	0.124	2.346	0.025
OR	0.122	0.026	0.552	0.021	1.437	0.139	2.146	0.247
PS	0.145	0.031	0.350	0.023	1.764	0.014	2.051	0.181
RH	0.140	0.015	0.629	0.052	1.433	0.015	2.621	0.363
WB	0.083	0.005	0.374	0.002	1.267	0.012	1.866	0.045
WP	0.173	0.098	0.428	0.005	1.615	0.064	1.959	0.201
RA	0.135	0.034	0.370	0.033	1.339	0.090	2.437	0.130
WT	0.263	0.064	0.493	0.001	1.402	0.008	2.392	0.005

16

17 ¹BP = Pond Brussels, OR = Rhine Oosterbeek, PS = Seine Paris, RH = Harbour Rotterdam, WB = Brook
 18 Wageningen, WP = Pond Wageningen, RA = aquaponics water, Rome and WT = tap water, Wageningen. ² ppb =
 19 parts per billion (µg/L). ³ St.dev. is standard deviation (n=2).

20

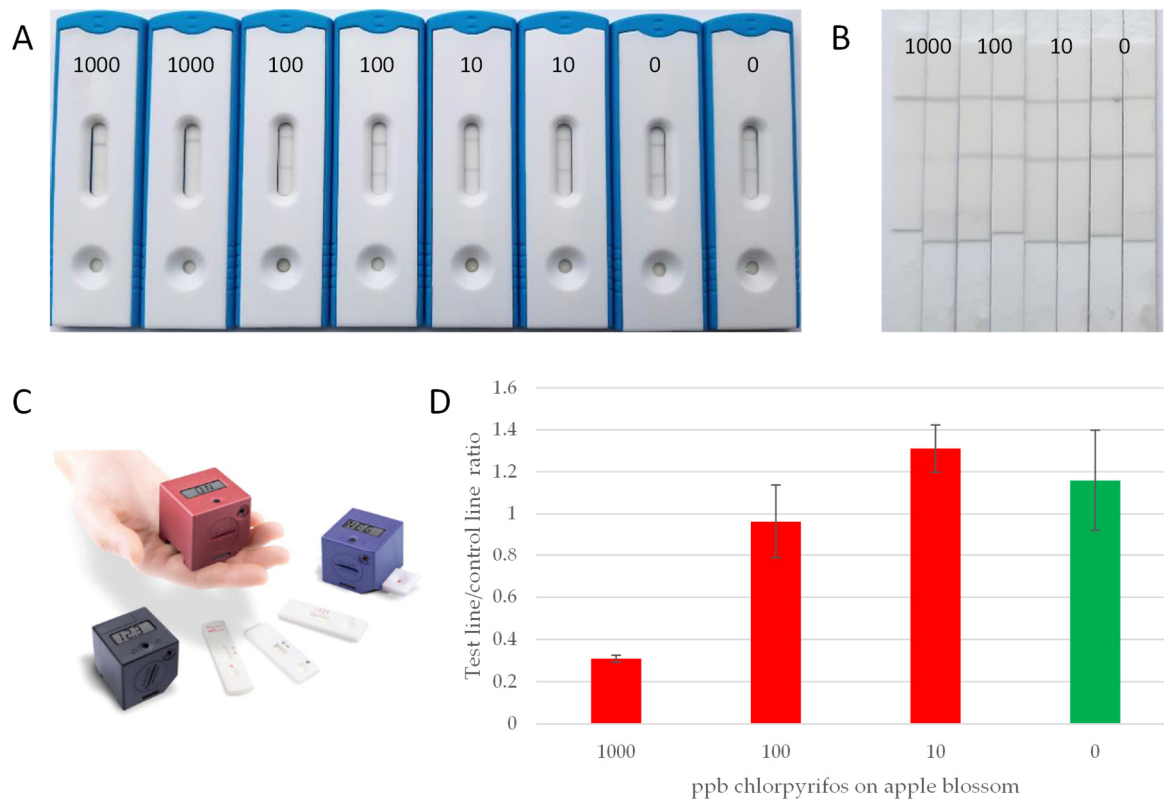
21



27

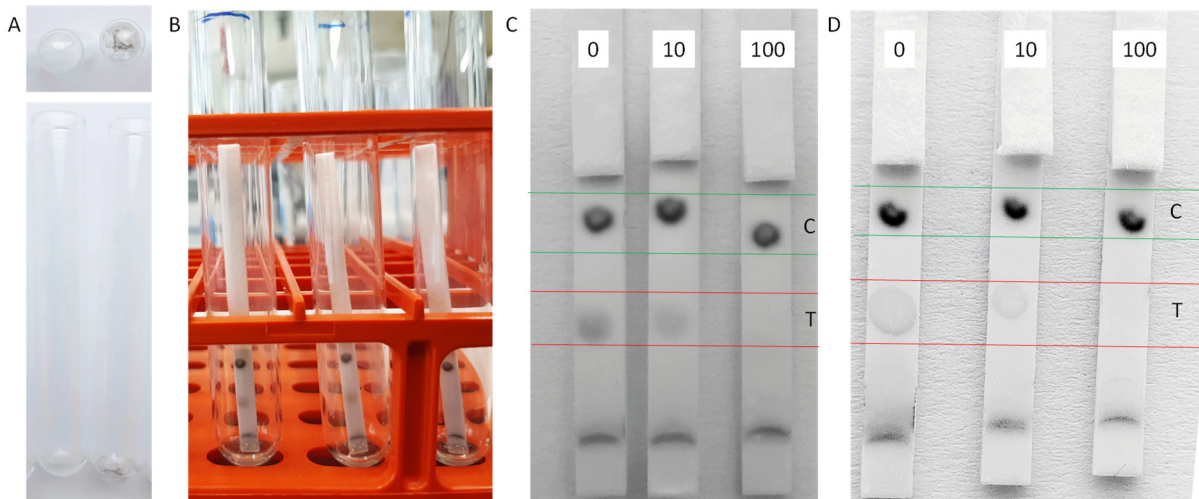
28 **Figure S2.** Overview of LFIA results for all the assessed environmental water samples. Each
29 concentration was tested in duplicate.

30



31

32 **Figure S3.** LFIA detection of chlorpyrifos (applied and dried) on apple blossom after water extractions
 33 by visual (A, B) readout and using a cube digital imager (C), for duplicate readings (D). The test line
 34 signals are divided by the control line signals to acquire T/C ratios.



35

36 **Figure S4.** Simplification of the LFIA by drying assay reagents and buffer in tubes (A), after which a
 37 water sample containing chlorpyrifos is added and the LFIA inserted (B), showing 10 ppb sensitivities
 38 for spot-based LFIAs in duplicate measurements after visual readout (C, D). See the main body of the
 39 text for more explanation.