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Labinfo

Newsletter for the approved food safety laboratories

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NRL NATIONAL REFERENCE LABORATORIES

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Newsletter for the approved food safety laboratories

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Labinfo Editorial

Dear reader,

You are about to read Labinfo n°19 for the year 2020, the second issue of Labinfo available only in English. After having been confronted with food safety-related crises or incidents in previous years, the Agency had to contend with the coronavirus crisis in 2020, as did our society as a whole. The Agency had to lend its assistance, as some of you have also done, in managing the crisis by deploying staff and equipment to cope with this pandemic as well as possible. We also had to adapt our way of working in order to respect the social distancing rules. To this end, we have, for example, made use of video conferencing and implemented teleworking on a large scale. As in previous years, the Agency could count on the support and cooperation of the NRLs and some other approved laboratories. I would, therefore, like to wholeheartedly thank all of the parties involved for their cooperation.

Following the elections of May 2019, the political situation in Belgium has become very complicated. So after several unsuccessful attempts, were finally have a new government and thus also a new minister, David Clarinval, Minister of Middle Classes, Independents, SMEs and Agriculture. We therefore look forward to hearing about their future food safety policy and to knowing what resources we will have available to accomplish our mission.

This year, we have implemented the European Regulation (EU n° 2017/625) on official food safety controls, also known as the OCR (Official Control Regulation), regarding the designation of official laboratories and NRLs (National Reference Laboratories). Our Royal Decree on "approvals" is currently under revision and we have adapted the approval conditions for foreign laboratories.

The articles published in this issue cover a wide variety of topics: the contamination of cut flowers by pesticides, the presence in food of additives in the form of nano particles, pest control agents, the presence of GE, 2- and 3-MCPD in foodstuffs and the presence of toxic cyanobacteria in flowers.
I hope you will enjoy reading this nineteenth issue of Labinfo.

Bert Matthijs
Director-general of DG Laboratories

Cut flowers contaminated by pesticide residues: what is the risk for florists?

Khaoula Toumi[(1)], Laure Joly[(2)], Christiane Vleminckx[(2)], Bruno Schiffers[(1)]

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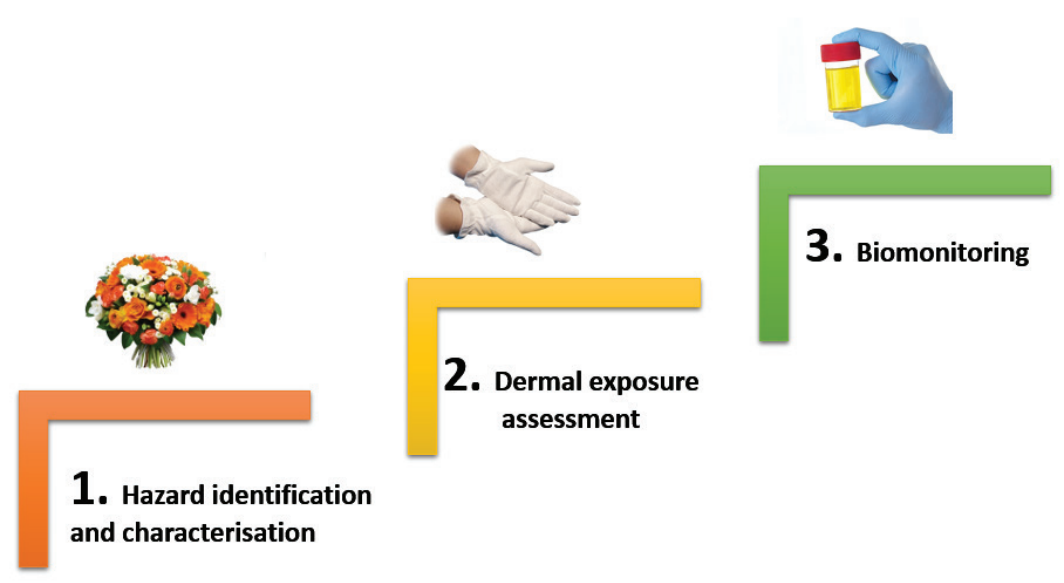
In Europe, as elsewhere in the world, flowers are closely associated with the most important and meaningful occasions in our lives. They are sold throughout the year, especially during the peak periods (New year, Valentine's Day, Mother's Day, etc.). Pesticides are deemed necessary by the great majority of flower growers to provide high crop yields, to achieve large-scale production of good quality flowers and to have competitive prices. As in any intensive culture, floriculture requires the use of a broad range of pesticides to manage various pests and diseases, which can affect production and marketability. Unlike other crops which are harvested for consumption, flowers are usually treated with a wide range of pesticides with high rates because of the weakness of local regulations and the lack of maximum residue limits (MRL) for flowers. Therefore, florists, who come in contact with flowers contaminated with pesticide residues, are exposed by different routes (mainly dermal route by direct contact with the contaminated cut flowers, respiratory by inhalation of volatile active substances and secondarily by oral route through hand-to-mouth transfer), with possible negative effects on their health. In order to evaluate the risk for florists exposed to pesticide residues during their professional tasks, for the first time in Europe, a study was conducted at Gembloux Agro Bio Tech, in collaboration with Sciensano (formerly Scientific Institute of Public Health WIV-ISP), as part of the doctoral thesis of Dr. Khaoula Toumi, under the supervision of Prof. Bruno Schiffers.



The first step of this study was to assess the average levels of contamination of the cut flowers most commonly sold in Belgium (roses, the number one flower sold annually, gerberas, and chrysanthemums). Cut flowers, whatever their origin, are highly contaminated by pesticide residues [1]. Human contact with contaminated flowers may dislodge a portion of these residues, resulting in their transfer to the skin or clothing. For the study, pairs of cotton gloves were distributed to a group of florists who wore them during their professional activities in order to evaluate their potential dermal exposure. Contact with contaminated flower samples resulted in the transfer of pesticide residues to gloves worn by the florists allowing measurement of the contaminants. All glove samples appeared to be highly contaminated by many pesticide residues [(2)].
Lipophilic pesticides are absorbed through the skin, metabolized into more polar products and excreted in urine. To assess their total exposure, urine samples were collected from florists during preparation of bouquets and handling of contaminated flowers, during the three most important commercial periods (Valentine's Day, Mother's Day and All Saints' Day) [(3)]. Results demonstrated the relationship between the levels of residues on the cut flowers, the dermal exposure and the excretion of pesticide residues in florist urines during professional activities. Belgian florists are exposed daily to both a very high number of toxic chemicals and rather high concentration levels, with a potential effect on their health.

Authors recommend that florists should be made aware of the issue of pesticide residues and trained in good practices and hygiene rules in order to limit their exposure to residues. Authors also recommend the setting of MRLs in cut flowers. Currently MRLs for pesticides exist only for food and feed but not for plants not intended for human or animal consumption. An extension of MRLs to cut flowers should be considered at the European level. To this end, authors' publications have been shared with the European Commission and other Member States.

In Belgium, pending a possible European approach on the setting of MRLs in cut flowers, two actions have been undertaken. The Federal Administration responsible for the evaluation and authorization of plant protection products has published on its website an information page for florists and tips for handling cut flowers (https://fytoweb.be/en/protect-products/usage/protection-de-protection-destination-de-fleuristes). In addition, the Royal Union of Florists of Belgium has received funding for preparation of a poster, to be distributed free of charge to all Belgian florists, informing them or recalling the good practices of handling cut flowers.

References:

(1) Toumi, K., Vleminckx, C., Van Loco, J. & Schiffers, B. (2016). Pesticide residues on three cut flower species and potential exposure of florists in Belgium. International Journal of Environmental Research and Public Health, 13(10), 943-957.

(2) Toumi, K., Joly, L., Vleminckx, C. & Schiffers, B. (2017). Risk Assessment of Florists Exposed to Pesticide Residues through Handling of Flowers and Preparing Bouquets. International Journal of Environmental Research and Public Health, 14(5), 526-544.

(3) Toumi, K., Joly, L., Vleminckx, C., & Schiffers, B. (2019). Biological monitoring of exposure to pesticide residues among Belgian florists. Human and Ecological Risk Assessment: An International Journal, 1-18.

Physicochemical characterization of the (nano)particles in the titanium dioxide food additive E171 in a regulatory context

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Introduction

E171 (titanium dioxide) is a food additive approved by the European Commission (EC) [1], authorized to be used as colorant of food. It is widely used for its refractive properties providing a shiny coating. It is frequently applied in confectionery such as candies, chewing gum, glazings, and is also applied in pastries, low fat dairy products and sauces[2]. We daily consume food products containing food colorants such as E171.

E171 is a material distributed as a white to slightly colored powder which is insoluble in water and organic solvents, and consists of titanium dioxide particles [3] (Insert 1). The application of E171 in food has become an issue of debate within the European Union. There are indications that it may alter the intestinal barrier [4] : Further, repeated oral administration of E17[1] to mice resulted in titanium dioxide deposition in the gastrointestinal tract and the liver, and is associated with molecular and cellular alterations in the inflammatory response [5]. These findings worried the public opinion and urged governmental health agencies to reassess the safety of titanium dioxide as a food additive [6]. These health issues were related to titanium dioxide in nanoparticulate form. At this moment, the specification of E171[1] does not foresee a limitation regarding its particle size.

Insert 1: The food additive E171

The food additive E171 consists of titanium dioxide and occurs in several crystalline phases.
The specification of E171 allows the application of two titanium dioxide phases: anatase or rutile.
Their reactive and photocatalytic properties are different [7] but their crystal structures are rather similar [8]. They can be differentiated by their diffraction patterns recorded in the TEM. Certain rutile grades of titanium dioxide are produced using mica (i.e. potassium aluminum silicate) as a template to form a basic platelet structure [9]. Afterwards, specifications require that all mica is removed resulting in a platelet form of rutile titanium dioxide.[1]

In this perspective, EFSA noted a need for more data, and particularly for information related to the particle size distribution of E171 together with information on the analytical methods and techniques used for detection and quantification of the nanofraction [10]. Recently, EFSA published a scientific opinion on the proposed amendment of the EU specifications for E171 with respect to the inclusion of additional parameters related to its particle size distribution [11]. It proposed to insert a specification of more than 100 nm for median minimal external dimension in the current EU specifications, which means that less than 50% of the number of constituent particles have a minimal external dimension below 100 nm. The Panel also reiterated the need for further research to decrease the level of uncertainty of the size measurement.

It is difficult to compare the results of studies characterizing E171 because often method variation and bias are not considered, because methods are not standardized and because results systematically lack measurement uncertainties obtained through validation studies. Therefore, Sciensano has taken the initiative to build an analytical capacity for analyses in a regulatory context to identify and characterize the nano-sized fraction of particles in food additives, including E171, using standardized and validated methodologies.

Samples

Fifteen food additive powders labelled as E171 were examined. Nine of these food additives were purchased from web shops specialized in bakery and confectionery products from several countries within the European Union. A selection of six E171 food additives was obtained from the producers, who claim that these are representative for the E171 materials on the European market [53]. In this study, E171 additives obtained from web shops are labeled as E171-01, 02, …, 09 and E171 additives of producers are labeled as E171-A, B, …, F.

A product, namely "silver colored sugar pearls" ("Perline Argento", Italy) containing both E171 and E174 (silver) was analyzed to show the possibilities of the proposed methods to measure silver and titanium dioxide particles separately, and to demonstrate the purity and the coating of these particles.

Methods

In the context of the new, recent legislation regarding food and food additives containing a fraction of nanoparticles, a specialized expertise and analytical capacity was built up in Sciensano. In general, the sample preparation and the developed analytical approach for identification of nano-objects in food are based on the CEN guidance on detection and identification of nano-objects in complex matrices [12]. In this work, the particles in the titanium dioxide food additive E171 are characterized in following consecutive steps summarized in Figure 1. This approach is described in more detail by Mast et al. [13] and can be extended to characterize titanium dioxide particles in food (matrices) also, provided that the sample preparation method is adapted (not shown).

Descriptive TEM

To demonstrate the presence of particles in the sample, conventional descriptive transmission electron microscopy (TEM) is applied. Before more advanced quantitative analyses are initiated, a detailed description of the material is a first and essential step to determine the basic properties of the examined nano-objects and to assess the quality of the sample and TEM specimen (grid) preparation. A descriptive EM analysis includes, at least, representative and calibrated micrographs; a description of (i) the roughly estimated

size (distribution) of the constituent and aggregated/agglomerated particles; (ii) the agglomeration and aggregation status; (iii) the general morphology; (iv) the surface topology; (vi) the structure (crystalline, amorphous, etc.); and (vii) the presence of contaminants and aberrant particles. In addition, selected micrographs can highlight unusual or rare features, such as impurities and crystal defects. When nano-objects are observed, the descriptive TEM analysis reports the specimen quality describing, the number of particles on the TEM specimen, their contrast with the background and how even they are distributed on the TEM specimen. Supporting on these parameters, the relevance and suitability of a quantitative TEM analysis, and the need for optimization of the sample and TEM specimen preparation is assessed. The proposed methodology complies with the EFSA Guidance documents [14] that foresee application of EM to describe key parameters important to assess the nanomaterial safety.

Electron diffraction

Since E171 materials are crystalline, their diffraction patterns recorded in the TEM provide information about their crystallographic structure. The recorded diffraction patterns are indexed according to the online database http://crystdb.nims.go.jp/index_en.html, which allows determining the structure and phase (anatase or rutile) of the materials.

HAADF-STEM and EDX

In this work, besides conventional TEM, high angle annular dark-field (HAADF) – scanning transmission electron microscopy (STEM) imaging with sub-nm resolution is applied combined with energy dispersive X-ray spectroscopy (EDX) spectral imaging with nm-resolution. These types of electron microscopy are explained in Insert 2.

Insert 2: Different types of electron microcopy

Conventional bright field transmission electron microscopy (TEM) illuminates a specimen using a parallel electron beam and detects the transmitted electrons. In scanning electron microscopy (SEM), a focused electron beam is scanned over the specimen and specific signals (secondary electrons and backscattered electrons) are recorded.
In high angle annular dark-field– scanning transmission electron microscopy (HAADF STEM), the TEM is operated in scanning mode: the electron beam is focused into a (sub-)nanometer-sized probe that scans the specimen. At each point, only electrons scattered at a high angle are detected using a large ring-shaped (HAADF) detector. This imaging mode is also called Z-contrast imaging because the high angle scattering signal depends strongly on the scattering of the electron beam by the atomic nuclei, and has an intensity which is more or less proportional with the squared atomic number ($I \sim Z^{1.6-2}$). It allows to qualitatively distinguish materials based on differences in elemental composition: heavier elements give brighter pixels.
During the STEM scan, the elemental composition of the particles in the sample is measured by energy dispersive X-ray spectroscopy (EDX), based on the energies of the X-rays emitted from the particles.
The X-ray signal is measured at each point the STEM beam interacts with during the scan. This allows to generate, besides a spectrum showing all elements in the analyzed region, spectral images for each detected element. By overlaying the STEM image with the spectral image of a specific element, the position of this element can be visualized. The nm-resolution of EDX, determined by the probe size of the STEM beam and the interaction volume, allows to determine the elemental composition of individual nanoparticles. A detailed method description is given by Mast et al. [13]

Quantitative TEM analysis

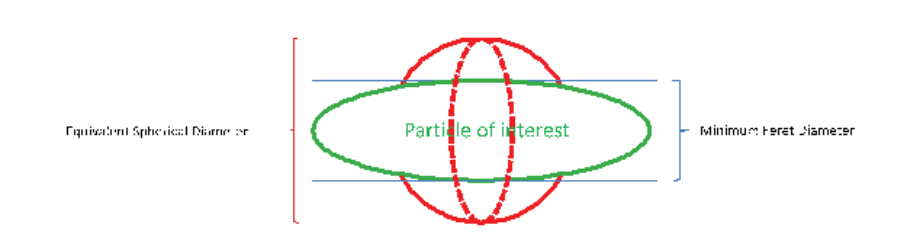
Quantitative TEM analysis combines TEM imaging with image analysis.
A set of calibrated electron micrographs showing particles that are representative for the particles deposited on the TEM specimen, are recorded. Critical factors are the optimization of the dispersion and TEM specimen preparation, the microscope alignment, the magnification selection and the application of a random and systematic imaging scheme. These aspects are described in general in the publications of Mast et al. [13]. Specific information for the quantitative TEM analysis of E171 is provided in Verleysen et al. 2020 [15].

The particles visualized in electron micrographs are detected and measured using (semi)automated image analysis approaches. Individual nano-objects of interest are detected and identified based on criteria such as mass-thickness contrast, elemental composition, size, morphology, and crystallographic structure. A semi-automatic approach for TEM based particle size and shape measurement is applied based on ISO 13322 [16]. The applied image analysis method consists of an image preparation step, the setting of the detection parameters and measurands (descriptors), the definition of the detection area, the setting and adjusting of the threshold value for particle detection, the detection of nano-objects, and the measurement of nano-objects.

A range of size and shape measurands are measured for each individual particle according to ISO 92766:2008 [17]. Here, the measurands that are relevant in the context of regulation and risk analysis, are for example an estimate of the minimal external particle dimension, measured as the minimum Feret diameter, and the aspect ratio, the ratio between the longest and the smallest dimension of particles, quantifying the particles shape (Insert 3).

Insert 3: Size measurements of particles

The minimum Feret diameter of a particle, measured from EM images, is the minimal distance between parallel tangential lines enclosing the particle. It approximates ('proxy') the minimal external diameter, a size parameter related to many physical properties of nanoparticles, such as the reactivity, the melting point and the catalytic and fluorescent properties, …). The equivalent spherical diameter (ESD) calculated from the mass of Ti per particle measured by spICP-MS is also a proxy of the minimal external dimension of the particles. It assumes that the particles are spherical. A detailed overview of concepts and terms applied in nanometrology is provided by Rausher et al.[18].



spICP-MS analysis of E171 materials

spICP-MS uses an ICP-MS device for data acquisition in time-resolved analysis mode. This method is applied to estimate the distributions of the size, assessed as the Equivalent Spherical Diameter (ESD), the particle number concentrations and the particle mass concentration in dispersions of the E171 samples. The dispersion protocol and the analysis conditions optimized for E171 particle analysis are described by Verleysen et al. [15].

Standardization, validation and measurement uncertainties of the methods

The quantitative TEM and spICP-MS analysis methods are standardized and validated, and the results are reported with estimates of the measurement uncertainty. The procedures and the results of the validation studies for quantitative TEM and spICP-MS of titanium dioxide particles, including an uncertainty balance are described in detail by Verleysen et al.[15],[19].

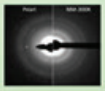
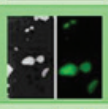
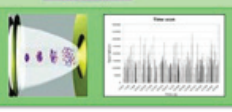
Sub-question	Methodology	Illustration
Particles present?	Descriptive (conventional) EM	
Phase?	Powder electron diffraction	
Chemical composition?	HAADF-STEM-EDX	
Nano?	Quantitative TEM	
How many?	Total: ICP-MS Nanoparticles: SP-ICP-MS	

Figure 1: Analytical approach to characterize the particles in the titanium oxide food additive E171.

Analysis results and interpretation

The developed analytical approach allowed to differentiate two different types of materials labelled as E171, as illustrated in Figure 2.

Descriptive TEM (Figure 2C) and STEM analyses (Figure 2A) show that the first type of materials contains near-spherical constituent particles with a diameter of approximately 100 nm, which were often agglomerated. No impurities were observed. Electron diffraction analysis showed that for most materials the diffraction patterns of these particles match with anatase titanium dioxide (Figure 2E). One material, E171-F, was rutile. EDX analyses showed that the particles consist of the elements titanium and oxygen, confirming that they are made of titanium dioxide (Figure 2B).

Descriptive TEM (Figure 2H) and STEM analyses (Figure 2F) showed that the second type of materials contains strongly aggregated, near spherical constituent particles measuring 20 to 30 nm. In addition, the samples contained other structures with a smooth, non-particulate surface. The particles often formed a layer on large flakes supporting the particles. Electron diffraction analysis showed that the diffraction patterns of these particles match with rutile titanium dioxide (Figure 2J). EDX analysis demonstrated structures containing potassium, aluminum, silicon, and often small quantities of iron. This elemental composition is in agreement with potassium aluminum silicate, also known as mica, which can be applied as a template for food-grade rutile titanium dioxide applied in E171 [2]. These results indicate that this second type of materials are potassium aluminum silicate-based pearlescent pigments of Type I, as specified by JECFA [20].

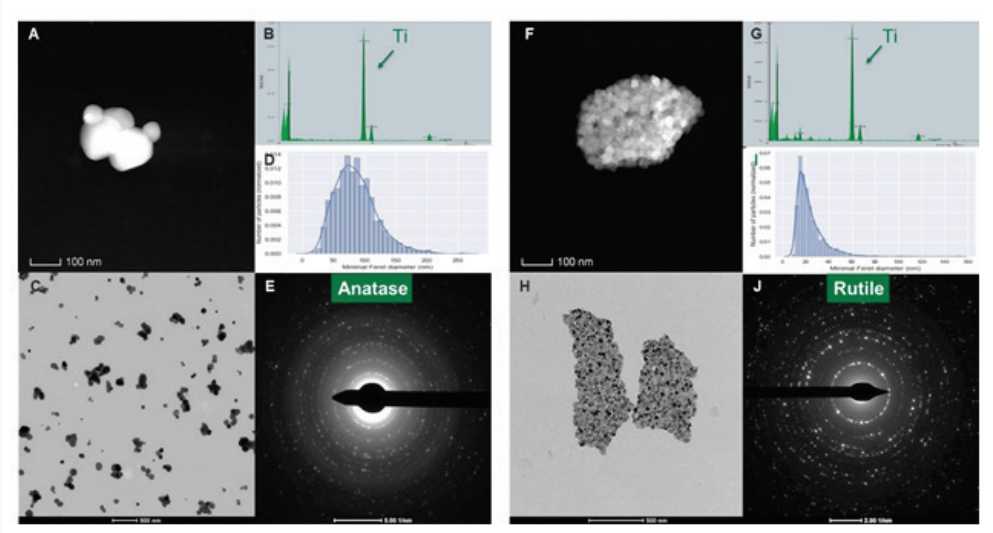


Figure 2: Illustration of the physicochemical characterization of two (A-E and F-J) pristine E171 food additives using electron microscopy. Particles are visualized by STEM (A,F) and TEM (C, H). The elemental composition of the particles is derived from EDX analysis (B,G). The constituent particle size distribution (D,I) is determined by image analysis of representative TEM micrographs, and the phase of the material is determined by electron diffraction (E,J).

Using the conditions for sample preparation and image analysis, optimized by Verleysen et al. (2020), number-based size and shape distributions of the constituent particles of the pearlescent pigments (Example shown in Figure 2I) and all other E171 materials (Example shown in Figure 2D) could be determined by quantitative TEM. spICP-MS allowed measuring all samples except the pearlescent pigments, where the constituent particles are smaller than the limit of quantification (around 40 nm) and where aggregation of particles strongly biases measurements (Figure 3). Detailed descriptive statistics of these quantitative analyses are provided by Verleysen et al. 2020 [15]. Using the optimized sample preparation protocols and image analysis settings, 12 of the 15 materials show a median minimum Feret diameter value lower than 100 nm. When the expanded measurement uncertainties (k=2) are added, 11 materials show no overlap with the 100 nm threshold, implying that the majority of the particles are nanoparticles. For the pearlescent pigments, all constituent particles were smaller than 100 nm. These materials do not meet the specifications proposed for E171 by EFSA [11].

The electron micrographs, such as Figure 2A, C, F and H, and the measurements of the aspect ratio, ranging from 1.15 to 1.30, indicate that the particles are near-spherical and strongly dispersed. Provided that the constituent particles are larger than the limit of quantification, the median ESD values determined by spICP-MS correspond well with the median values of the minimum Feret diameter determined by TEM
Our findings confirm the conclusion of Geiss et al. (2019) [21] that spICP-MS can be applied as a screening method, but not as a confirmatory method for the specification of E171 based on particle size. Nine materials have a median value below 100 nm. However, taking into account the expanded measurement uncertainties determined for spICP-MS, only one out of these nine materials had no overlap with the 100 nm threshold, and the constituent particles of pearlescent pigments could not be measured with an acceptable precision because the constituent particles are smaller than the detection limit and they were not stable in the aqueous dispersions.

Nevertheless, spICP-MS remains important since it is the only method that allows to measure the amount of particles as mass or as particle concentrations: for the tested E171 samples, the particle mass concentration varied between 0.68 and 0.87 kg/kg, while the particle number concentration ranged from 0.57 to 1.80 x 10^{17} particles/kg. In the context of risk assessment, such information is critical.

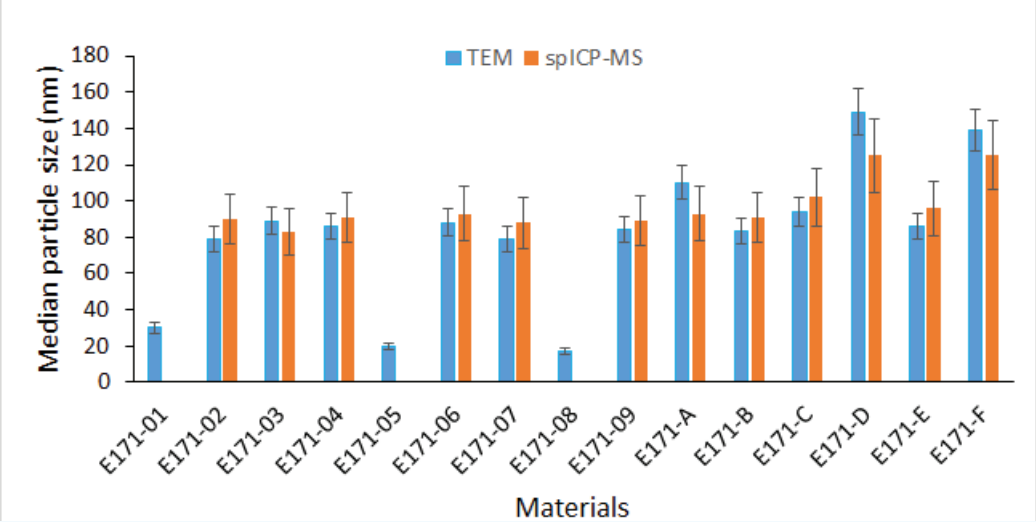


Figure 3. Medians of particle size distributions of 15 E171 materials. Sizes are expressed as minimum Feret diameter and as equivalent spherical diameter for TEM and spICP-MS, respectively. The error bars represent the expanded measurement uncertainties (k=2; 95 % level). Adapted from Verleysen et al. [15].

The possibility of STEM-EDX analysis to determine the elemental composition of particles with nanometer resolution allowed to characterize the presence of E171 in a mixture (Figure 4), and to evaluate the coating of particles (Figure 5) and its purity (Figure 6).

In a sample containing a mixture of the food additives E174 and E171, the (smaller) silver and (larger) titanium dioxide particles cannot be distinguished based on their mass-thickness contrast, seen as gray value in TEM (Figure 4A) and their Z-contrast in STEM images (Figure 4B). They can however readily be identified by their EDX spectral images of Ti and Ag (combined in Figure 4C). This finding allowed to determine the shape distributions (not shown) and the size distributions of the titanium dioxide particles (Figure 4D) and the silver particles (Figure 4E) separately.

STEM-EDX analysis further allowed to show that certain titanium dioxide particles in this E171 containing product had a silicon dioxide coating (Figure 5). In addition, it allowed to specifically identify potassium aluminum silicate (Figure 6), showing that the mica template of pearlescent pigments is not removed during production of E171, but is still present in the end product (Figure 6).

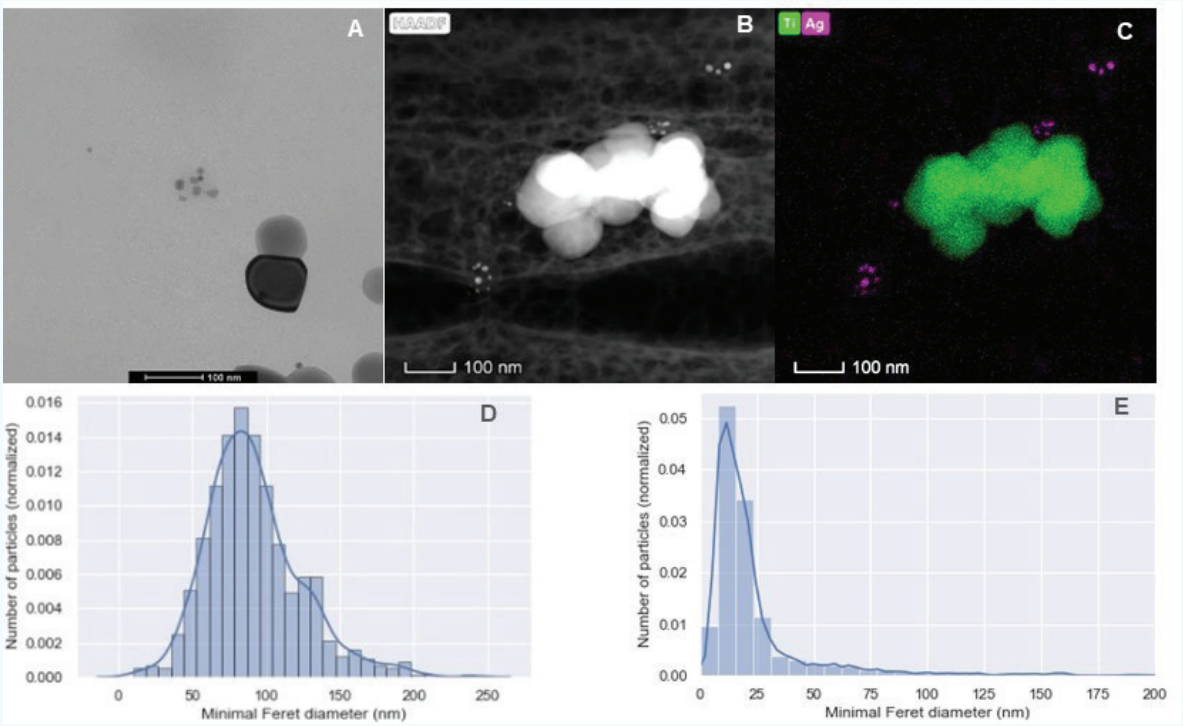


Figure 4: Identification of E171 particles in a product containing both E171 and E174 (silver) food additives. EDX spectral images of Ti (green) and Ag (pink) (C) in combination with STEM images (B) demonstrates that the titanium dioxide particles (E171) can be distinguished from much smaller silver particles (A). Separate number-based size distributions were determined by image analysis of representative TEM micrographs for E171 (D) and E174 particles (E).

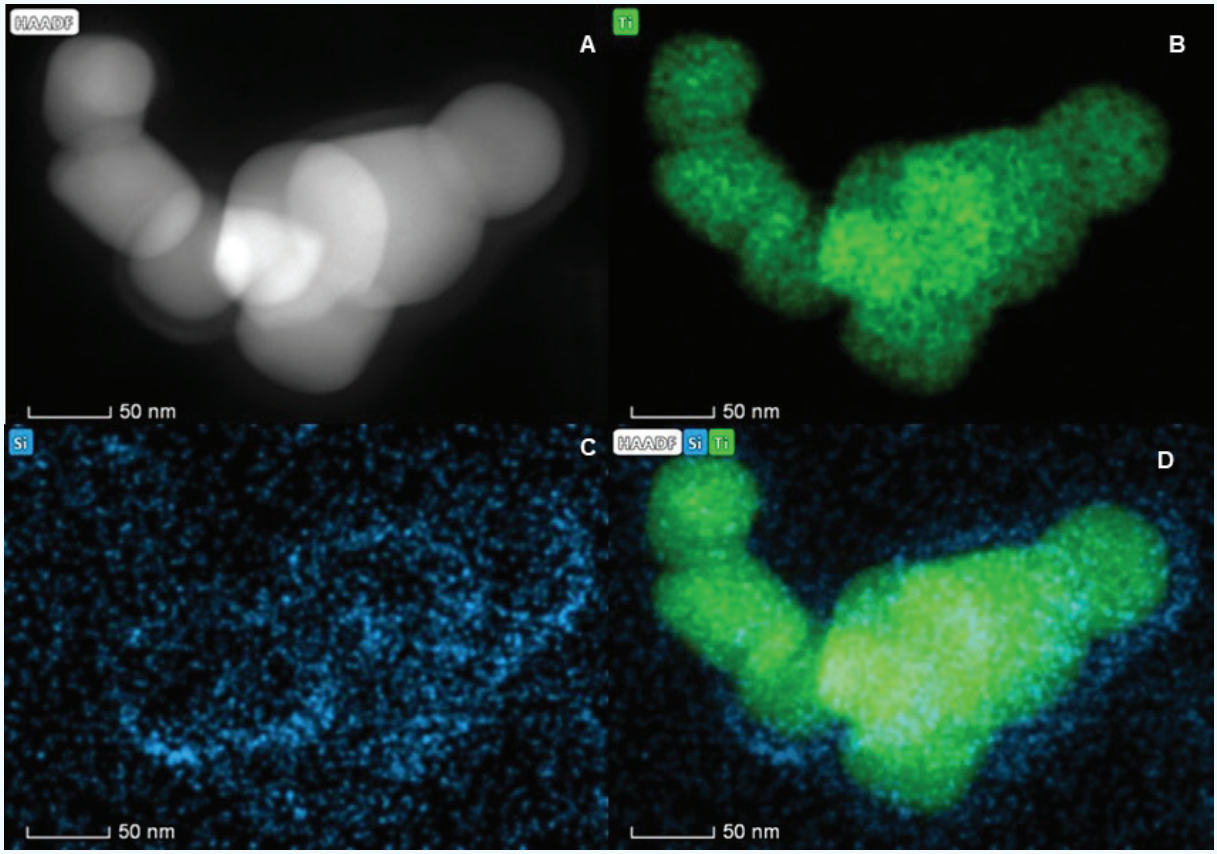


Figure 5: Identification of the silicon coating of E171 particles in a product .
Overlay (D) of EDX spectral images of silicon (Si, blue, C) and titanium (Ti, green, B) with the STEM image of E171 particles (A) indicates the presence of a Si-containing coating.

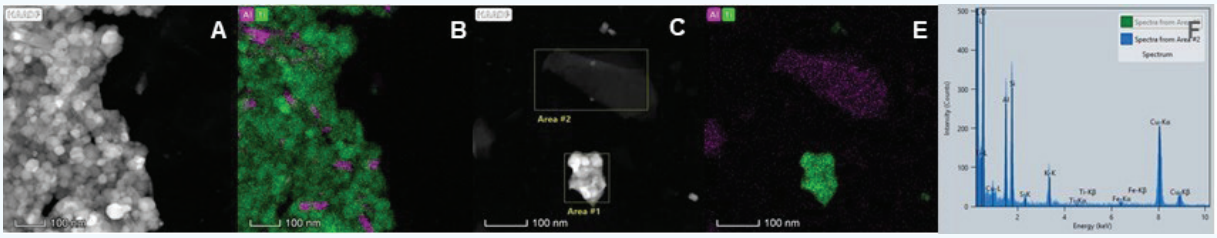


Figure 6: Identification of residual mica in a E171 food additive by HAADF-STEM combined with EDX-spectral imaging. Overlays (B, E) of the EDX spectral images of aluminum (Al, pink) and titanium (Ti, green) with the STEM images (A, C) of the « platelet form of rutile E171 » indicate incomplete removal of mica. This is confirmed in the EDX spectrum (N) of the region indicated in F.

Conclusion

- An analytical capacity based on EM and spICP-MS analyses was built to implement and control the legislation regarding food and food additives containing a fraction of nanoparticles.
- The developed approach allowed to identify and characterize the nano-sized fraction of the particles in the food additive E171 in a regulatory context using standardized and validated methodologies. It differentiated several (nano)forms of titanium dioxide, labelled as E171. It is unclear whether the risk assessment of E171 comprehends the observed variation in (nano)forms. In the E171 materials,
  - o two different phases, namely rutile and anatase were observed,
  - o two different types of materials, namely near-spherical particles of approximately 100 nm and pearlescent pigments were detected,
  - o uncoated and silicon dioxide coated particles were found,
  - o aa significant variation in constituent particles size was seen.
- TEM can be applied as a confirmatory method, spICP-MS can be applied as a screening method for the specification of E171 based on particle size, however with limitations due to particle size and type. spICP-MS measurement of E171 particles are important to estimate the mass and particle concentrations which are critical for exposure assessment.
- The possibility of STEM-EDX analysis to determine the elemental composition of particles with nanometer resolution allowed to characterize the presence of E171 in a mixture, and to evaluate its purity and the coating of particles. STEM-EDX showed that the mica template of pearlescent pigments is not removed during production of E171, but remains present in the end product. Further research could focus on the co-presence of particles in food additives and the consecutive risk assessment.
- In most of the examined E171 samples, the majority of the particles are nanoparticles. These samples do not meet the new specifications of E171 proposed by EFSA [11].
- The proposed examples demonstrate the necessity of the availability of analytical techniques for the detection of (nano) particles in food and food additives.

Acknowledgments

This publication is based on the following publications where more detailed information can be found :

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Antiparasitic agents : emerging challenges for old compounds

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Parasitic infections remain a major health threat in both humans and animals. While using the general term of parasite, a broad variety of organisms are concerned : around 70 % of parasites are not visible to the human eye, such as the malarial parasite, but some worm parasites can reach over 30 meters in length. From the evolutionary perspective, most of human parasites have emerged over the last 11,000 years, due to a shift to living in larger groups, which are able to sustain epidemic diseases, and because of close contact with animals via agriculture [Prokop and Fedor, 2013].
Regarding animals, both companion animals and livestock are impacted by parasites; however solutions are designed differently depending on the target species and living conditions.

Use of antiparasitic agents as veterinary drugs

Parasitic diseases inflict severe economic losses on the livestock industry and adversely affects the health, weight gain, feed conversion efficiency and reproduction of animals.
Veterinary antiparasitic agents play an important role to prevent and/or to treat endoparasites and/or ectoparasites infestations in farm animal's trough prophylactic or therapeutic treatment
Fleas, ticks, mosquitoes, scabies, lice, roundworms, strongles, flukes, tapeworms, coccidia, toxoplasma, giardia, or babesia: the parasites of animals are numerous. Antiparasitic agents too.

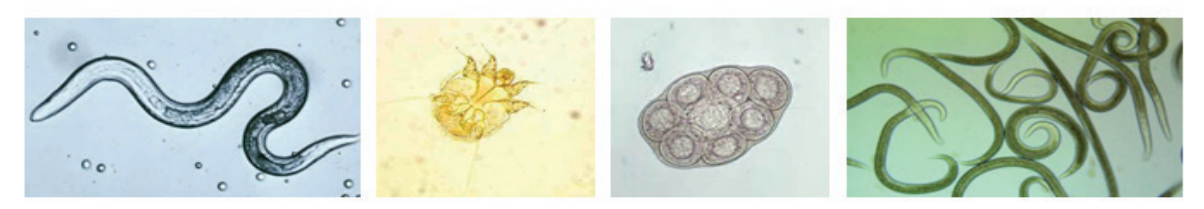

Figure 1 : Illustration of (a) Toxocora larva (b) Scabies (Sarcoptes scabiei), (c) Nematodirus, (d) Nematodes

Discovery of antiparasitic agents is a challenging process, requiring discovery of molecules with the ability to kill parasites without being toxic for their hosts. When designing a new veterinary antiparasitic drug, some criteria are important. End-user (farmers or pet owners) preference is for fewer doses and ease of application, but this is not always compatible with reduced withdrawal times, human food safety and/or user safety [Woods and Knauer, 2010]. Safety is of course the vital component of delivering a new antiparasitic drug ; its evaluation requires investigation of adverse reactions, species- and breed-specific effects, mutagenicity/ carcinogenicity, drug:drug interactions, human food safety (for food animals) and handler safety.

As described in Table 1 (https://www.vetcompendium.be/sites/default/files/rcmvet_2017.pdf), four main groups of compounds belong to the category of antiparasitic agents : anthelmintics, antiprotozoalsagents (anticoccidials), ectoparasiticides and endectocides.
Among all the veterinary medicinal products authorized in Belgium or elsewhere in Europe, only some may be used for food producing animals.
Allowed medicines, which contain pharmacologically active substances, are listed in the Table 1 of the annexe to Regulation EU 37/2010. Coccidiostats are also authorized as additives for animal feed in accordance with Regulation EU 1831/2003.

Table 1 : Classification of antiparasitic agents, their approval use as veterinary drugs for pet and livestock and Maximum Residue Limit for all authorized substances

Therapeutic class		Family	Substance name	Authorized drugs (1)		Maximum Residue Limit (2)	Authorized Additives (3)
				Pet	Livestock		
Endoparasiticide	Anthelmintic	Amino-acetonitrile derivative	Monepantel				
		Benzenensulfonamide	Clorsulon				
		Benzimidazole	Albendazole				
			Febantel				
			Fenbendazole				
			Flubendazole				
			Mebendazole				
			Netobimin				
			Oxfendazole				
			Oxibendazole				
			Thiabendazole				
			Triclabendazole				
		Depsipeptide	Emodepsid				
		Imidazothiazole	Levamisole				
		Other	Piperazine				
		Quinoline	Praziquantel				
		Salicylanilide	Closantel				
			Niclosamide				
			Oxyclozanide				
			Rafoxanide				
		Spiroindole	Derquantel				
		Substituted phenol	Nitroxinil				
		Tetrahydropyrimidine	Morantel				
			Pyrantel				
	Antiprotozoal	Carbanilide	Imidocarb				
			Dimetridazole				
		Nitro-Imidazole	Metronidazole				
			Carnidazole				
			Ronidazole				
		Aminopyrimidine	Amprolium				
	Antiprotozoal - Anticoccidial	Anticoccidial	Nicarbazine				
			Robenidine				
			Lasalocid				
		Polyether Ionophore antibiotic	Maduramicin				
			Monensin				
			Narasin				
			Salinomycin				
			Semduramicin				
		Quinazolinone	Halofuginone				
		Quinoleine	Decoquinate				
		Sulfamide	Sulfadimethoxine				
		Triazine	Diclazuril				
			Toltrazuril				
Both endo and ectoparasiticide	Endectocide	Avermectin	Abamectin				
			Doramectin				
			Emamectin				
			Eprinomectin				
			Ivermectin				
		Milbemycin	Selamectin				
			Milbemycin Oxime				
			Moxidectine				
Ectoparasiticide	Ectoparasiticide	Acyl urea derivate	Teflubenzuron				
		Benzoylphenylure	Hexaflumuron				
		Formamidine	Amitraz				
		IGR. Insect growth regulator	Cyromazine				
			Dicyclanil				
			Diflubenzuron				
			Fluazuron				
			Lufenuron				
			Methoprene				
			Pyriproxyfen				
		Isoxazoline	Afoxolaner				
			Fluralaner				
			Lotilaner				
			Sarolaner				
		Neonicotinoïd	Imidacloprid				
			Dinotefurane				
			Nitempyran				
		Organophosphate	Azamethiphos				
			Coumaphos				
			Diazinon (dimpylate)				
			Phoxim				
		Oxadiazine	Indoxacarb				
		Phenylpyrazole	Fipronil				
			Pyriprole				
			Sisapronil				
		Pyrethrinoïd	Cyfluthrin				
			Cyhalothrin				
			Cypermethrin				
			Deltamethrin				
			Fenvalerate				
			Flumethrin				
			Permethrin				
		Spinosyn	Spinosad				

(1) Allowed pharmacologically active substances in Belgium https://www.vetcompendium.be/sites/default/files/rcmvet_2017.pdf

(2) Maximum residue limits (MRL) according to Regulation (EU) 37/2010

(3) Regulation (EU) No 1831/2003 on additives for animal feed.

The European Medicines Agency's (EMA) Veterinary Medicines Division is responsible for the authorization of veterinary medicines and for the establishment of Maximum Residue Levels (MRLs) in foodstuffs of animal origin. Indeed, the use of veterinary medicines may result in the presence of drug residues in foodstuffs of animal origin such as muscle, liver, fat, kidney, milk, eggs and honey and MRLs should be established to ensure food safety. Commission Regulation 2018/782 describes the methodology to be used in the scientific risk assessment and establishment of risk management recommendations relevant to MRL applications.

Use of antiparasitic agents as pesticides

Beside their use as veterinary drugs, several antiparasitic agents are also used as plant protection products. Their use during the production of fruits, vegetables or cereals can also lead to the presence of residues in food and feed. In this frame, Maximum Residue Levels (MRLs) are also set in the European legislation in order to check the good use of plant protection products (according to good agricultural practices) and to protect the consumers.
EFSA's Pesticides Unit is responsible for the EU risk assessments of active substances used in plant protection products while all matters related to legal limits for pesticide residues in food and feed are covered by Regulation (EC) No 396/2005. This regulation also contains provisions on official controls of pesticides residues in food of plant and animal origin that may arise from their use in plant protection.

It should be emphasis that some antiparasitic agents are authorized as pesticides but not for use in food-producing animals. Other compounds are authorized as both vet drug and pesticides but with different MRLs.

Table 2 : Examplse of regulated compounds and their respective MRL in muscle.

Substance	Authorized drugs/pesticides			Maximum Residue Limit (µg/kg)	
	Pet	Livestock	Pesticides	Drugs	Pesticides
Abamectin		N	Y	20	10-20
Deltamethrin	Y	Y	Y	10	20-30
Indoxacarb	Y	N	Y		10-2000
Milbemycin Oxime	Y	N	Y		20
Spinosad	Y	N	Y		100-300
Teflubenzuron		N	Y	500	50
Thiabendazole		N	Y	100	50-100

By example, amitraz, coumaphos, flumequine, oxytetracycline, permethrin and streptomycin are substances used in veterinary medicinal products; in the past they were also used in the EU as active ingredients in plant protection products. Legal limits for residues in food resulting from the different types of uses were established in the pesticide legislation (Regulation (EC) No 396/2005) and under the legislation related to veterinary medicinal products (Regulation (EU) No 37/2010). The European Commission planned to align the pesticide legislation on MRLs with the veterinary MRLs. EFSA was therefore requested to provide an opinion on MRL harmonization for amitraz, coumaphos, flumequine, oxytetracycline, permethrin and streptomycin. Based on risk assessment, EFSA concluded that while acceptable for 4 compounds, additional risk management measures should be applied for coumaphos and that oxytetracycline required additional data [EFSA, 2016].

European Monitoring Plans results and issues

In accordance with EU legislation, residues of veterinary medicinal products are controlled in living animals and in food of animal origin through Monitoring Annual Control Plans.
As shown in Table 2, a high rate of samples analyzed for the 2017 monitoring plan were hopefully compliant. Non-compliant samples were reported for anthelmintics (B2a) in bovines, pigs, sheep and goats, milk and poultry, as well as for anticoccidials (B2b) in horses, pigs, poultry, rabbits and eggs. Some 'non classified' antiparasitic agents (B3f), were also reported for honey, eggs and poultry. The identified substances were fipronil, thiacloprid, captan/folpet and boscalid.

Table 3: Monitoring of veterinary medicines products in food of animal origin. Number of targeted samples analysed for B2 subgroups in different animal categories and frequency of non-compliant samples. Anthelmintics (B2a); anticoccidials (B2b) and carbamates and pyrethroids (B2c).

Group	B2a % NC	B2a Samples	B2b % NC	B2b Samples	B2c % NC	B2c Samples	B2d % NC	B2d Samples	B2e % NC	B2e Samples	B2f % NC	B2f Samples
Aquaculture	0	560	0	379	0	315	NA	NA	0	3	0	327
Bovines	0.10	5,856	0	3,384	0	1,791	0	1,671	0.05	5,473	0.19	12,827
Farmed game	0	252	0	143	0	107	0	8	0	62	0	36
Wild game	0	102	0	14	0	35	NA	NA	0	3	0	22
Sheep/goats	0.89	3,041	0	2,260	0	2,270	0	244	0.06	1,796	0	776
Horses	0	186	0.85	118	0	139	0	198	0.66	605	0	241
Pigs	0.04	7,610	0.01	8,495	0	2,239	0.02	5,674	0.06	6,243	0.03	8,791
Poultry	0.03	3,670	0.21	12,736	0	1,754	0	128	0	1,552	0.05	3,780
Rabbits	0	118	0.65	153	0	60	NA	NA	0	65	0	67
Milk	0.17	6,635	0	1,272	0	1,778	0	82	0.96	4,474	0	489
Honey	0	219	0	124	0	922	NA	NA	NA	NA	0	755
Eggs	0	596	0.47	4,073	0	554	0	196	NA	NA	0	629

%NC : percentage of non-compliant samples NA : not applicable
Source: EFSA (European Food Safety Authority), 2019a. Report for 2017 on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products. doi:10.2903/sp.efsa.2019.EN-1578

On the other hand, the 2017 European Union report on pesticide residues in food provides an overview of the 2017 official control activities on pesticide residues carried out in the European Union (EU). In Figure 2, the 48 pesticides found in animal products at levels at or above the LOQ are presented. The most frequently quantified substances were copper, HCB, DDT, chlordecone, thiacloprid, fipronil. Because of the fipronil incident in chicken eggs in summer 2017, fipronil was ranked in the top hazards.

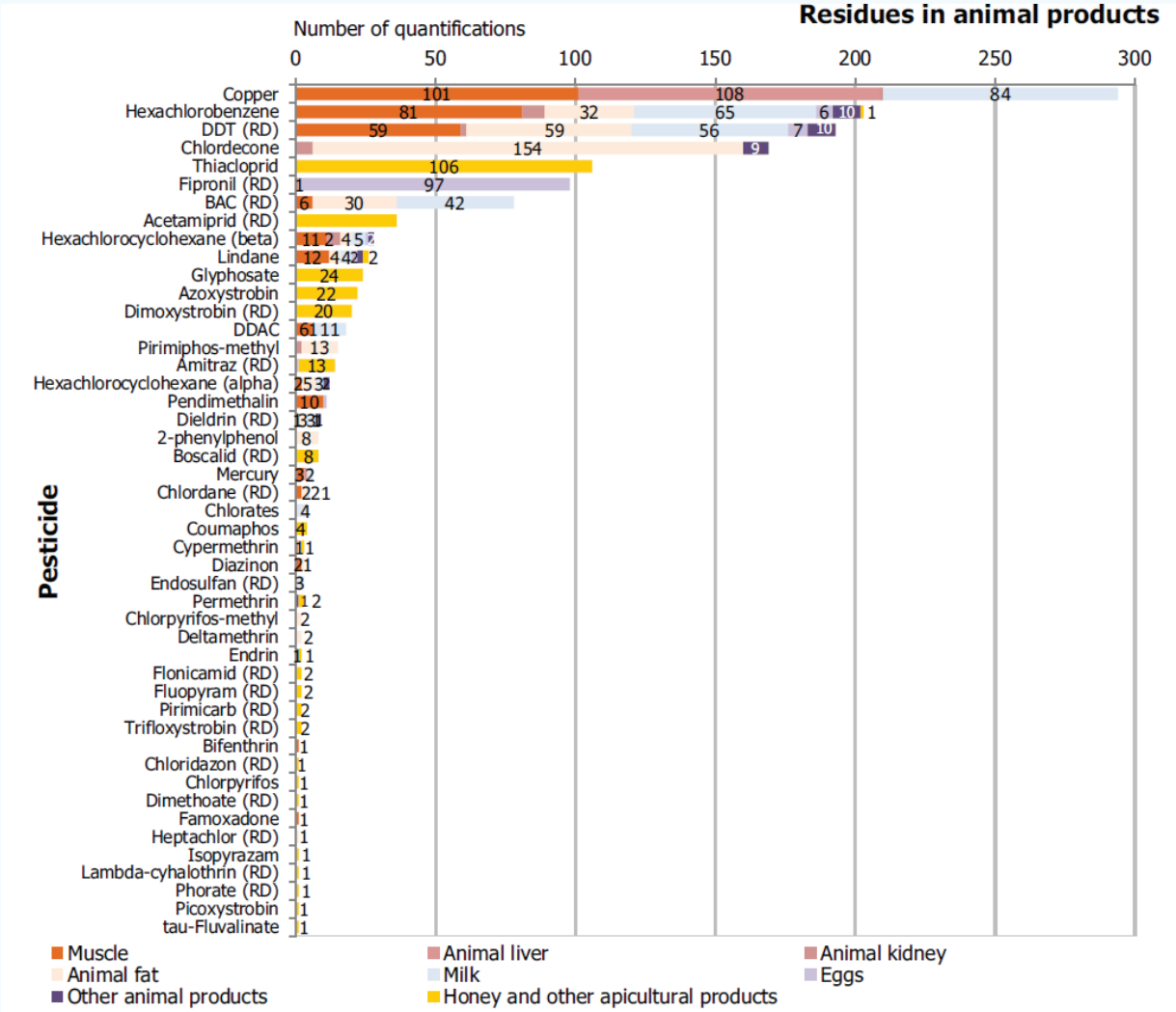

Figure 2: Pesticides most frequently quantified in animal products (in absolute numbers of detections at or above the LOQ) (From EFSA 2019b)

Monitoring Plan, as well as self-checking program from food business operators, are design to state on samples compliancy for authorized compounds, but should also be able to detect frauds. Fraudulous use of antiparasitic agents, authorized as pesticides but not allowed for food producing animals, had gained attention recently. What is known as "the fipronil incident" started in the summer 2017. A Belgian egg processing company notified to the Belgian Federal Agency for the Safety of the Food Chain (FASFC) that fipronil was detected in eggs from a Belgian farm. This finding was unexpected, since fipronil is not allowed for food-producing animals and has never been detected in this sector. What initially appeared to be an isolated contamination turned out to be, after a few weeks of intensive investigations, a much more significant incident.
Such incident underlines the importance of a robust food safety system and have led to an increase in controls of pesticides use in the meat production sector.

Analytical strategies and challenges

A robust food safety system requires adapted analytical strategies.
Screening of antiparasitic drugs in a single multi-analytes method is challenging since, despite their common pharmacological effects, they differ greatly in their chemical structures and properties.
Multi-class sample preparation protocols have been developed, targeting more than 200 vet drugs [Robert et al., 2016]. Those protocols are often based on a generic extraction solvent, avoiding any clean-up of sample extract to avoid losses. During the last decade, they have been helped greatly through the improvements of analytical instrumentation with most vet drugs being analyzed by UHPLC-MS/MS. Some volatile pesticides require however GC-MS/MS analysis like pyrethroids.

Analytical methods should be 'fit for purpose' for control, which suppose of course enough sensitivity but also 'fit for purpose' scope, method validation and results reporting according to the appropriate legislation.
The fact that antiparasitic agents belong to two different legislation make things more complicate for control laboratories. Questions are raised regarding MRLs that should be taken into account but also regarding method performance criteria (relative retention time, ion ratio, precision and trueness tolerance), validation scheme and uncertainty expression (CCα or U).

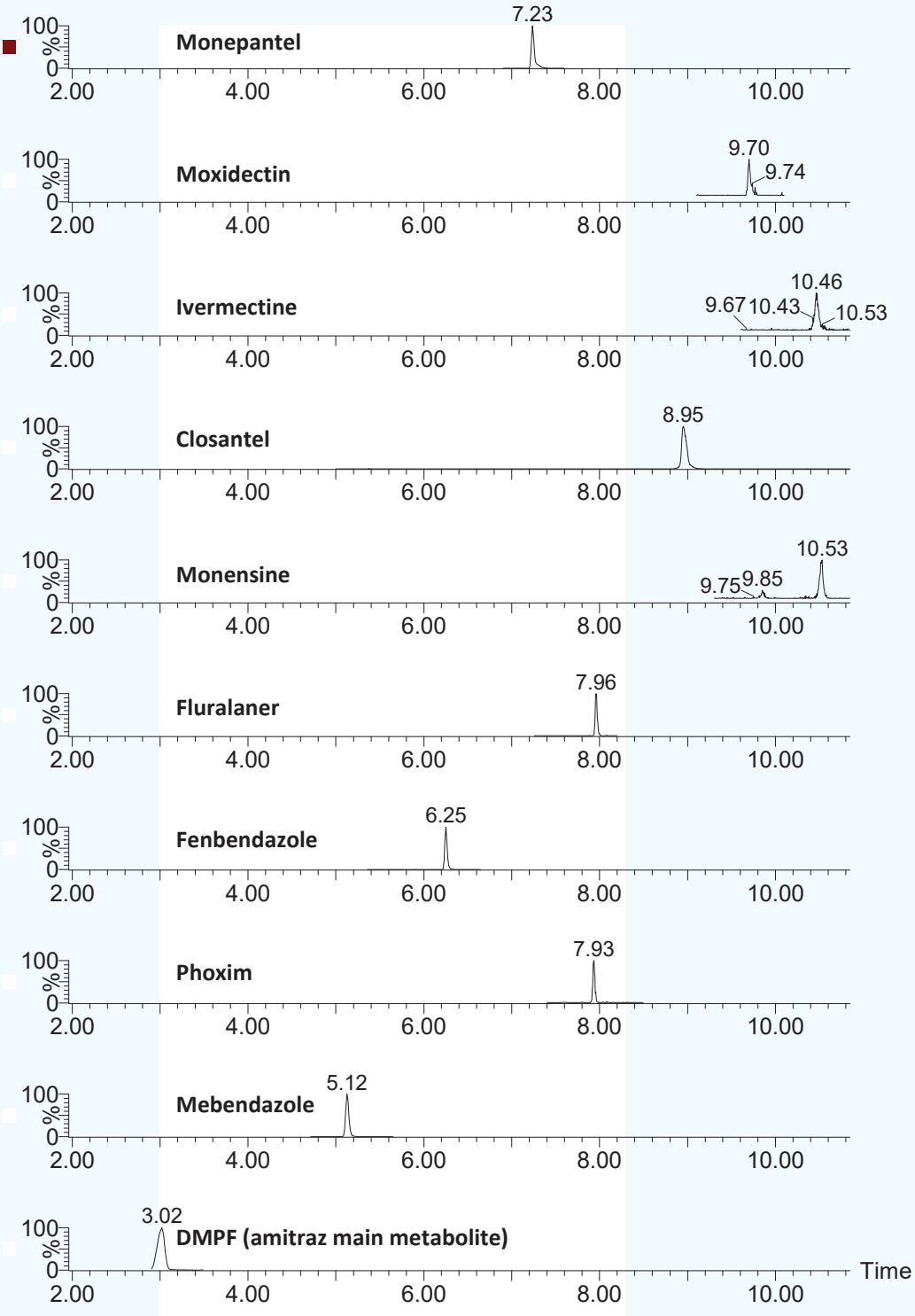

Figure 3 : UHPLC-MS/MS chromatograms of the fortified sample at the targeted level of some antiparasitic agents in muscle.

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Glycidyl fatty acid esters, 2-monochloropropanediol, 3-monochloropropanediol and their fatty acid esters

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Introduction:

Glycidyl fatty acid esters (GE), 2-monochloropropanediol (2-MCPD), 3-monochloropropanediol (3-MCPD) and their fatty acid esters (Fig. 1) are classified food process contaminants formed in edible vegetable oils during their refining processes (1,2,3).

Refining is a physicochemical process used to bland the flavour, light the colour and increase the oxidation resistance of the oils (4). It involves a chemical removal of phospholipids, a neutralisation of the free fatty acids, a bleaching with clay minerals and a deodorisation by high temperature (180-220°C) steam distillation. GE are formed in this last step by either high temperature degradation of diacylglycerol neither elimination of hydrochloric acid from MCPD monoesters (1,2,5). The MCPDs formation occur in the same refining step with a different mechanism: hydrolysis of triacyl and diacylglycerol followed by a nucleophilic ring-opening substitution by a chlorine anion (1,2,5,6). As a consequence, the amount of diacylglycerol in the crude oil and the temperature of the thermal treatment are critical for the formation of those compounds.

Crude palm oil contains a very high amount of diacylglycerol, thus after the refining step, the level of 3-MCPDs can achieve levels up to 3000 µg/kg, classifying it as the most contaminated food item. Occurrence studies (1,2,3,5,6) report that the other type of vegetable oils including margarine and fat emulsion are about 6 times less contaminated (about 500 µg/kg for 3-MCPDs) than palm oil. Those studies also show that every food item processed with or containing vegetable oils such as bakery products, snacks, crisps and even baby milk powder are contaminated (within 10 and 200 µg/kg for 3-MCPDs). It is also reported that those food items are the main contributors to the MCPDs and GE daily intake. Finally, a correlation between the concentrations of 2-, 3-MCPDs and GEs has been highlighted for every food groups: 3-MCPDs and GEs are in the same concentration range while 2-MCPDs displays concentrations about 40% lower compared to 3-MCPDs.

Analysis:

Hundreds of individual GE, 2- and 3-MCPD esters can be found in foods depending on the length of the saturated fatty acid(s) present in the molecule (1,3,5,6,7). While GE variety is limited because there is a lone ester substitution position, 2-MCPDs can be found with either 1 or 2 fatty acids derivatives of different lengths, and as 3-MCPDs is asymmetrical it has an even greater variety of individual compounds (Fig. 2).

Currently, there are two analytical approaches dedicated to the analysis of these compounds : the direct and Indirect methodologies, both are using the isotope dilution technique for quantitative purposes.

In the Direct approach, each individual GE, 2- and 3-MCPDs fatty acid ester derivatives are analysed by Liquid Chromatography coupled to Mass Spectrometry (LC-MS) after a Solid Phase Extraction (SPE) clean-up (AOCS Official Method Cd 28-10). Unfortunately, there is no labelled corresponding standard for each compound in its series of homologues, yielding the quantitative result per individual compound tricky.

In the Indirect approach the ester derivates are converted into their free forms (Glycidol, 2-MCPD and

3-MCPD) and only these 3 compounds are quantified by Gas Chromatography coupled to Mass Spectrometry (GC-MS) after derivatisation (AOCS Official Method Cd 29a-13, 29b-13, 29c-13, EU-RL PC standardised method and ISO 18363). The main issues with this approach are the matrix effect and the reproducibility of the chemical reactions involved in the preparation process.

Nevertheless, from an international point of view, the indirect approach is preferred as the toxicology has been assessed on the mixtures and the regulations are set on the equivalent free form amount present in foods.

Regulations:

3-MCPD free form has been of European concern for over 3 decades. So a maximum allowed levelled has been early enforced for soy sauce and hydrolysed vegetable protein in the first version of the Commission Regulation (EC) No 1881/2006. In addition, a set of analytical requirements for these analyses have been lay down in the Commission Regulation (EC) No 333/2007 and have been updated in 2011 (Commission Regulation (EU) No 836/2011).

It is only in 2018 (Commission Regulation (EU) 2018/290) that a maximum allowed level for GEs have been introduced in the European Regulation for vegetable oils and infant formula. A revision of the Commission Regulation (EC) No 333/2007 is in the pipe to update the criteria to the new analytes.

Up to now there is no regulation setting maximum levels for the 2- and 3-MCPDs esters and only a Commission Recommendation (2014/661/EU) with suggested analytical performances to achieve for a reliable monitoring. Nevertheless, many regulations update are in preparation based on EFSA risk assessments.

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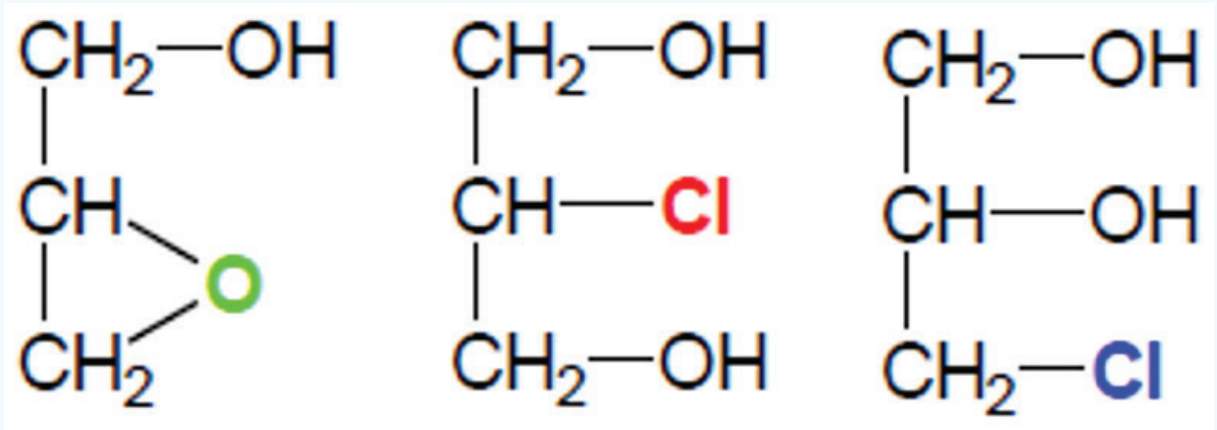

Figure 1: From left to right: Glycidol, 2-MCPD and 3-MCPD.

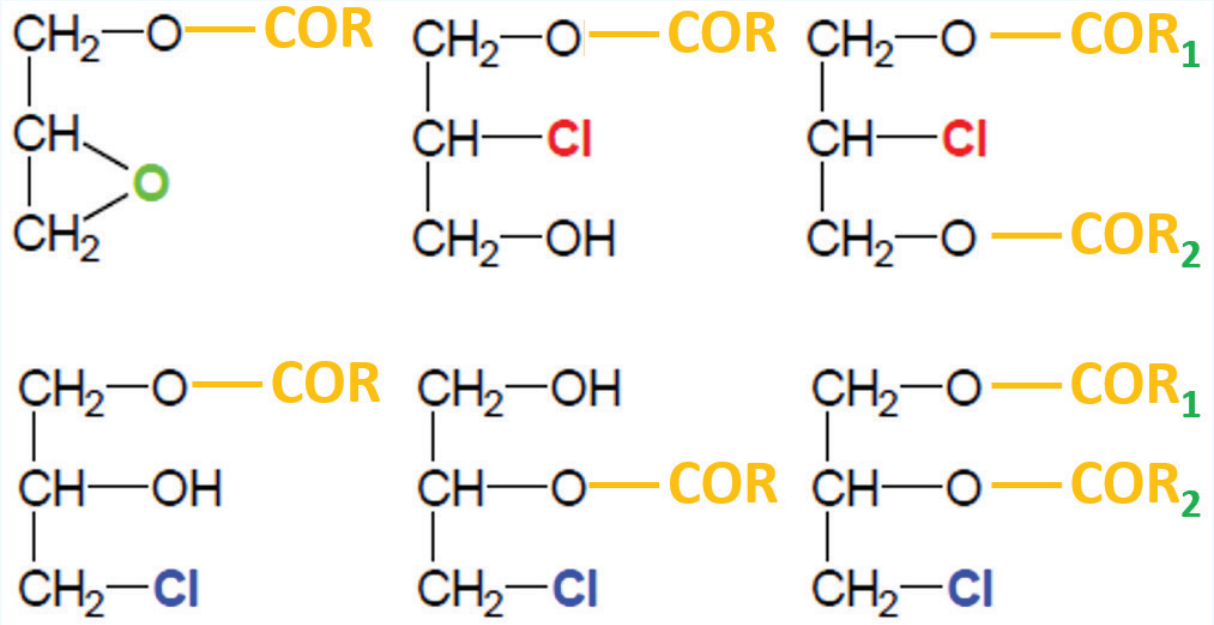

Figure 2: Esters derivatives of Glycidol, 2-MCPD and 3-MCPD with R standing for a saturated fatty acid chain.

Toxic cyanobacterial blooms in Brussel: A case study

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Abstract

Between 2006 and 2009, the presence of toxic cyanobacterial blooms in a large number of lakes and ponds all over Belgium was studied during the B-BLOOMS2 project. This BelSPO-funded project reported that cyanotoxins were detected in a number of lakes and ponds1. As a reaction, the Flemish and Walloon environmental agencies started monitoring public bathing sites and recreational lakes for blooms, but this was not the case for the region of Brussels.

To gather data and assess whether there were still toxic blooms in the Brussels region, we sampled putative blooms during September 2018 in four ponds in and around Brussels. Microscopic observations were performed and molecular identification of the main cyanobacterial taxon was performed based on 16S rRNA gene sequences. Furthermore, the presence of cyanobacterial genes involved in the toxin production was tested as well as the presence of cyanotoxins (microcystins and nodularin).

In three of the four samples, possible toxic cyanobacterial genera were observed by microscopy as well as the presence of microcystin producing genes. Simultaneously, we were able to quantify various microcystin congeners in all the samples. In two samples (BL1 and BL2), the total concentration of microcystin congeners exceeded the provisional guideline values for recreational waters. Fishing and recreational activities (excluding swimming) are common in some of the ponds and could pose a risk to public health.

Abbreviations

Microcystin	MC
Nodularin	Nod
Protein Phosphatase 1	PP1
Protein Phosphatase 2A	PP2A
World Health Organization	WHO
Tolerable daily intake	TDI
Polymerase chain reaction	PCR
Equivalent	Equiv

INTRODUCTION

Cyanobacteria (also called 'blue-green algae') are prokaryotic organisms that developed more than 2.3 billion years ago. They were the first to develop a photosystem capable of producing oxygen. Over time, cyanobacteria have diversified and colonized many environments, including extreme ones. An emerging research topic concerns the species living in eutrophic lakes, ponds or marine environments. In these environments, cyanobacteria communities can increase in cell density and form a so-called 'bloom'. These blooms can disrupt the oxygen balance in the environment, asphyxiating plants and animals. Moreover, some of these blooming species are capable of producing cyanotoxins that contaminate the water, aquatic life and other organisms that are exposed to it.

This contamination can be a potential risk for humans when it is accumulated in shellfish [2–6], fish [7–9], animals[10],[11] or plants, through irrigation [12]. Also, certain food supplements made from cyanobacteria could be at risk of contamination [13–16].

In our case study, we have focused on microcystin congers (MC's) and nodularin (Nod). MC's consist of a heptacyclic peptide ring. On the other hand, Nod possesses a pentacyclic peptide ring with a similar structure to microcystin. Both cyanotoxins have a recognizable Adda-group that has an important role in their hepatotoxic activity. This group is also commonly used for analytical detection of these hepatotoxic molecules [17]. Microcystin-LR (MC-LR) is the most common cyanotoxin found in the freshwater lakes in Europe [18].

Guidelines and regulations for microcystin-LR

For many cyanotoxins, there is not yet enough prevalence, toxicity and consumption data to properly assess the risk. MC-LR is the exception. For this toxin, abundant information is already available. Acute, sub-acute and chronic toxicity were assessed in the late 1980s and 1990s based on multiple mouse and pig models. They showed that most of the damage was inflicted in the liver. Nevertheless, other organs like the lungs, intestines and kidneys could also be affected during acute exposures at high levels of MC-LR [27–29]. Further, during chronic exposures, mice showed bronchopneumonia, chronic liver injury and increased mortality [30]. These studies corroborate earlier assessments suggesting that the presence of Microcystis blooms were causative for an outbreak of hepato-enteritis among children in Palm Island. Similarly, two British army recruits were hospitalized after experiencing nausea, vomiting, muscular pain, painful diarrhoea, pneumonia,… preceded by a full-body immersion in a bloom-containing recreational lake during a canoeing exercise. Additionally, an epidemiological study in South-China suggests that the presence of microcystin in the drinking water could be one of the major risk factors for developing hepatocellular carcinoma [31].

After careful examination of the available data, the WHO determined a tolerable daily intake (TDI) of 0.04 µg/kg body-weight. From this value, they also set a provisional guideline value of 1 µg/l for microcystin-LR in the drinking water. Since this guideline was published in 1999, many countries started adopting the same values for their monitoring programs (e.g.: Australia, New Zeeland, South-Africa,…)[32].

The value is also used as the basis for regulatory guideline concerning toxin content in recreational lakes10. It can be noted that the guidelines values are expressed as MC-LR equivalent (Equiv) what points to the lack of detailed knowledge about the toxicity of the other MC congeners. For the moment, we use the sum of all concentration of the congeners as the value for MC –LR Equiv Once more information about the toxicity is available. Concentrations of the toxins could be adjusted to MC-LR Equiv with a factor describing the relation in toxicity between MC-LR and another congener. The potential risk for intoxication calls for the development of guidelines and regulations for the cyanotoxins in Belgium or Europe as a whole.

Current status of monitoring efforts in Belgium

By now it should be clear that cyanobacterial blooms are quite abundant in surface waters and can lead to increased risk for public health through food, drinking water and contact with cyanobacterial blooms during recreational activities. Yet, large scale monitoring programs have been lacking over the last years. The last and only attempt in Belgium was organized between 2006 and 2009. The project was funded by the Belgian Science Policy Office and named "B-BLOOMS2"[(1)]. During the project, multiple water bodies were analyzed for species diversity and presence of microcystin congeners. Different universities from Belgium participated in the project as well as a laboratory from Dundee University led by Prof G. Codd [(11),(18)]. In total, 162 samples were tested from 67 water bodies. In addition to MC quantification by ELISA, the presence of gene fragments from the mcy gene cluster responsible for the synthesis of MC was determined. The mcyE and mcyA gene fragments were present in 89% and 82% of the samples, respectively. The presence of either of these genes suggests that a bloom is potentially toxic. Moreover, microcystin congeners were found in every sample that tested positive for one of the mcy genes [(1)]. However, the concentrations were very variable between samples, from 0.120 µg L^{-1} to 37500 µg L^{-1} [1]. Since that project, Flemish and Walloon local environmental agencies have been monitoring blooms in certain bathing areas during the summer period to ensure the safety of swimmers. Yet until summer 2018, only sporadic monitoring was performed in other ponds and lakes. Even though, these could also present a risk when used for other recreational activities, like fishing. Moreover, in Brussels, there is an absence of monitoring guidelines for cyanobacterial blooms. However, a number of these lakes are situated in densely populated areas and are a focal point of recreational activities during the summer.

Aim of the study

In order to obtain preliminary data about the presence of blooms in Brussels, we visited different lakes and ponds in search of blooms in summer 2018. The collected data includes the concentration of 8 microcystin congeners and nodularin, the presence of 3 genes involved in the microcystin production and the identification of the most abundant species of cyanobacteria.

METHOD

Materials

Extraction solvents and solvents used for the mobile phases were UPLC/MS grade. The microcystin congener and nodularin standards were obtained from Enzo Life Sciences (Antwerp, Belgium)®. They were received in a solid powder and dissolved in 100% methanol. Subsequently, mixed stock solutions were prepared in 50% Methanol with 1% acetic acid. Whatman GF/C grade filters were bought from Sigma Aldrich (Overijse, Belgium).

Sampling

During September 2018, 4 ponds in the Brussel area were selected based on scum presence or intense green colorations: Parc Roi Baudouin phase 1, Parc Roi Baudouin phase 2, a pond in Mellaert and pond Tercoigne in Watermael-Boitsfort (see table1). Samples were taken from the accumulated scum near the border of the ponds with a clean 500 ml PTFE bottle attached to a water sampler.

Table 1: Overview of the samples, names, locations and weight biomass per liter after filtration

Collected Samples	Location	Mass/volume of sample (g/l)
BL1	Parc Roi Baudouin Phase 1	27.67
BL2	Parc Roi Baudouin Phase 2	10.85
BL3	Mellaert's pond	6.75
BL4	Pond Tercoigne	1.22

Prior to the analysis, samples were filtered on GF/C Whatman® filters under vacuum. The weight of the filters was determined before and after filtration to assess biomass to volume ratio. Moreover, 50 ml of the filtrate was kept to determine free cyanotoxin concentrations. Both the filters and the liquid phase were kept in 50 ml plastic tubes at -20°C before analysis.

Toxin extraction from filters.

To analyses the intracellular toxin content, the toxins have to be extracted. To this effect, 80% methanol was added at a volume of 4.5 ml per 0.5 g of sample in 50 ml plastic tubes. The tubes were regularly gently swirled during 1 hour to increase the contact between the extraction solvent and the biomass.

Then the extraction solvent was removed, filtered through a Phenomenex 0,2 µm RC syringe filter and stored in a 15 ml plastic tube at -20°C.

Before injection, liquid samples were also purified using the same syringe filter to reduce debris in the sample.

Detection and quantification of toxins.

A Waters Acquity UPLC H-class (Eten-Leur, The Netherlands) in combination with a Waters XEVO TQ-S was used for the detection of the cyanotoxins. They were first separated using a 1,7 µm, 2,1 mm x 100 mm Waters Acquity BEH C18 column fitted with a Waters Acquity BEH C18 1,7 µM VANGUARD PRE-Col.

The separation of the toxins was accomplished with a gradient program in which the eluent strength was increasing the fraction of organic modifier.

The detection of the toxins was done in Multiple Reaction Monitoring (MRM). MRM is a specific and sensitive mass spectrometry technique that can selectively quantify compounds within complex samples. This technique uses a triple quadrupole MS that initially targets the ion corresponding to the toxin of interest, referred to as the 'precursor ion'. The subsequent collision-induced fragmentation produces a range of product ions. One (or more) of these fragment ions can be selected for quantitation purposes. The signal ratio between these fragment ions and the quantitation ion should be constant over known standards and unknown samples and thus is used for identification. The MS parameters were set according to the literature data and optimized to the instrument setting.

During the experiment, the recovery of the extraction method was assessed by spiking the filters with 40 ng/g toxin mix, containing 8 microcystin congeners and nodularin at equal concentrations.

Morphological Identification

A portion of each sample was fixed with 4% formaldehyde for later morphological identification of the cyanobacteria. This was performed with an Olympus BX43 microscope.

Detection of the 16S rRNA gene and the mcy genes involved in the synthesis of microcystin

DNA Extraction

First, 1.2 ml lysis buffer (40 mM EDTA, 50nM Tris-HCl, 0.75 M sucrose) was added to each filter and a bead-beating step (at 30 m/s for 30 seconds) was performed. Then, 60 µl lysozyme (20 mg/ml) was added to the samples, followed by a 30 minute incubation at 37°C. Thereafter, the samples were supplemented with 27 µl Proteinase K (22.22 mg/ml) and 100 µl SDS (100%) and incubated for 2 hours at 55°C. The lysate was recovered and transferred to a new Eppendorf tube. Subsequently, the filters were rinsed again with 1 ml lysis buffer and incubated at 55°C for 10 min. The lysate was again removed from the filter and stored in another Eppendorf tube.

A Phenol-chloroform-isoamyl alcohol solution (25:24:1, pH 8) was added in an equal volume to the extract volume (V:V) to both lysate tubes. This was followed by centrifugation at 14000 g for 15 min. Each supernatant was transferred to a new Eppendorf tube and chloroform-isoamyl alcohol (24:1, pH 8) was added V:V and again the tube was centrifuged at 14000 g for 15 min. The supernatant, containing the nucleic acids, was collected.
At last, the DNA was precipitated with 0.1 V:V of sodium acetate (3 M, pH 5.2) and 0.6 V:V of cold isopropanol. After centrifugation, the DNA was rinsed once with 300 µl ice-cold ethanol (100%) and once with 300 µl ice-

cold ethanol (70%). Finally, the supernatant was removed and the pellet was air-dried. Finally, the pellet was dissolved in 100 µl TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8) and stored at -20°C.

Gene amplification

The 16S rRNA gene was amplified with cyano-specific primers, as well as three genes involved in the production of MC's: mcyA, mcyB and mcyE. The primers and PCR programs were used during the B-BLOOMS2 project and are adequately described in the final report [(1)]. The presence of the PCR products was visualized by electrophoresis on a 1.5% agarose gel during a 95 min run at 60V.

Sequencing

After PCR, the 16S rRNA and mcyE amplicons were sent for Sanger sequencing at Giga Genomics (ULiege). Closely related 16S rRNA sequences were found using the SeqMatch tool of the Ribosomal Database Project (RDP). On the other hand, NCBI nucleotide BLAST (Basic Local Alignment Search Tool) was used to collect sequences similar to the mcyE fragments. The sequence identity (≥ 90%) and sequence coverage (≥80%) were used as selection parameters. The sequences for 16S and mcyE were separately aligned using ClustalW in BioEdit. The aligned sequences were introduced in Mega-X (10) to create a phylogenetic tree using a Maximum Likelihood with 1000 Bootstrap replicates. The Jukes-cantor substitution model was used in combination with Gamma Distributed mutation rates corrected with Invariant Sites. The data for gaps and missing data were treated by partial deletion with a site coverage cut-off of 95%. A Nearest-Neighbor-interchange Tree was automatically constructed for the Heuristic Method.

Results

Morphological Identification by light microscopy

Cyanobacteria were present In three of the four samples. Sample BL1 contained colony-forming cyanobacteria of the genus Microcystis. The typical clustering of cells within a polysaccharide layer are its most distinctive features. BL2 also contained Microcystis, but with a less firm polysaccharide layer than in BL1. This suggests that the bloom was in the early stages of collapse. In BL3, a Planktothrix species appeared dominant, with a few Microcystis colonies. Moreover, a detrital portion included cell debris where heterocysts could be recognized, indicating the presence of a heterocystous cyanobacterial taxon that had been lysed beyond recognition. In BL4, no cyanobacteria were observed. The genera observed in BL1, 2 and 3 are all potentially toxic (figure 2).

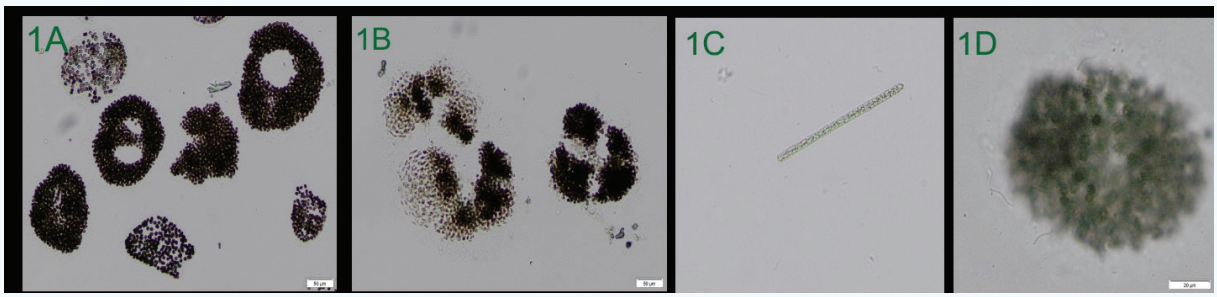

Figure 2: A. Microscopic image of sample BL1, showing Microcystis colonies. B. Microscopic image of sample BL2, showing Microcystis colonies. C. Microscopic image of sample BL3, showing a Planktothrix filament. D. Microscopic image of sample BL3, showing a Microcystis colony.

Presence of mcy genes in environmental samples

The presence of mcyA, mcyB and mcyE genes was tested by PCR in the four environmental samples. After visualizing the PCR products on a gel, we showed that mcyA was present in BL1 and BL2. We also observed a very faint band for BL3 that also seemed to have a longer sequence. For BL4, no mcyA gene was observed (see figure 3A). The same results were observed for the mcyB gene (data not shown). The mcyE gene was tested only in BL1, BL2 and BL3 as a confirmation. Again we saw the presence of the gene in all the three environmental samples, but the band in BL3 was fainter compared to the others (see figure 3B).

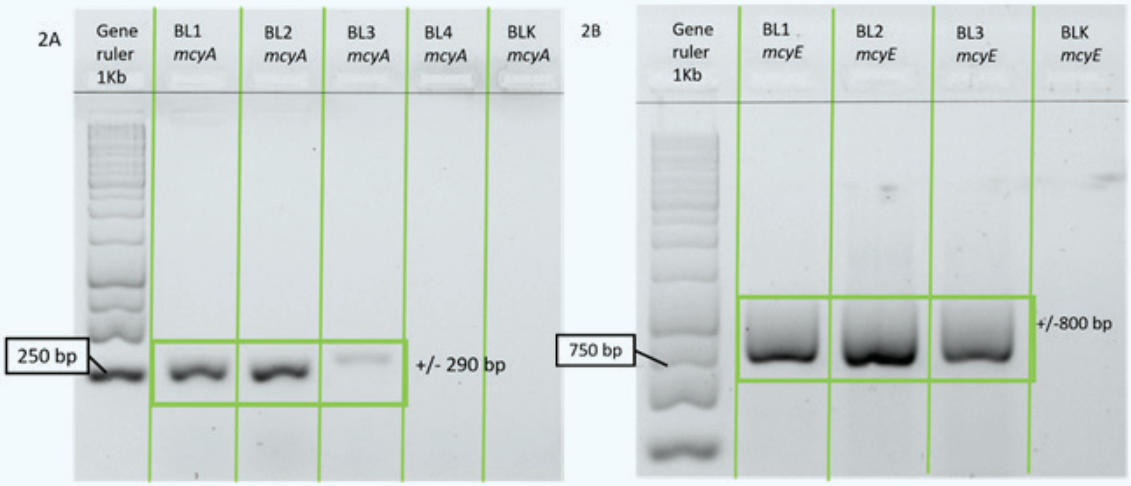

Figure 3: A. PCR products for the mcyA gene. The fragment is present for BL1 and BL2. In BL3 a faint band is present. For BL4, no fragment is present. No fragment is detected in the negative control (no DNA added). B. The PCR fragment for mcyE is visible for BL1, BL2 and BL3. Although the band is a bit fainter for BL3 compared to the other

samples. For the negative control, no fragment is present.

Taxonomic affiliation based on 16S rRNA sequencing

In addition to the morphological identification, the taxonomic affiliation of the cyanobacteria was determined by sequencing a fragment of the 16S rRNA gene. Sequences in both BL1 and BL2 clearly belonged to Microcystis aeruginosa, both with a 100% percent identity to the same Microcystis aeruginosa strain. However, the majority of the cyanobacterial DNA in BL3 corresponded to Dolichospermum compactum, with a percent identity of 100%. This result differs from the result obtained with the microscopic observations. Although no cyanobacteria were observed by microscopy in BL4 and the produced 16S rRNA sequence was of low quality, it could be affiliated to the Cyanobium genus. For BL4, the branch length was slightly longer than for the other Cyanobium sequences. This is probably due to the lower quality of the sequence compared to the other samples (see figure 4). The mcyE PCR product was also sequenced for the three samples that showed the appropriate bands on the gel electrophoresis after PCR. A clear similarity was observed with partial sequences of mcyE genes from Microcystis aeruginosa for BL1 (99.03%), BL2 (99.86%) and BL3 (98.97%) (see figure 5).

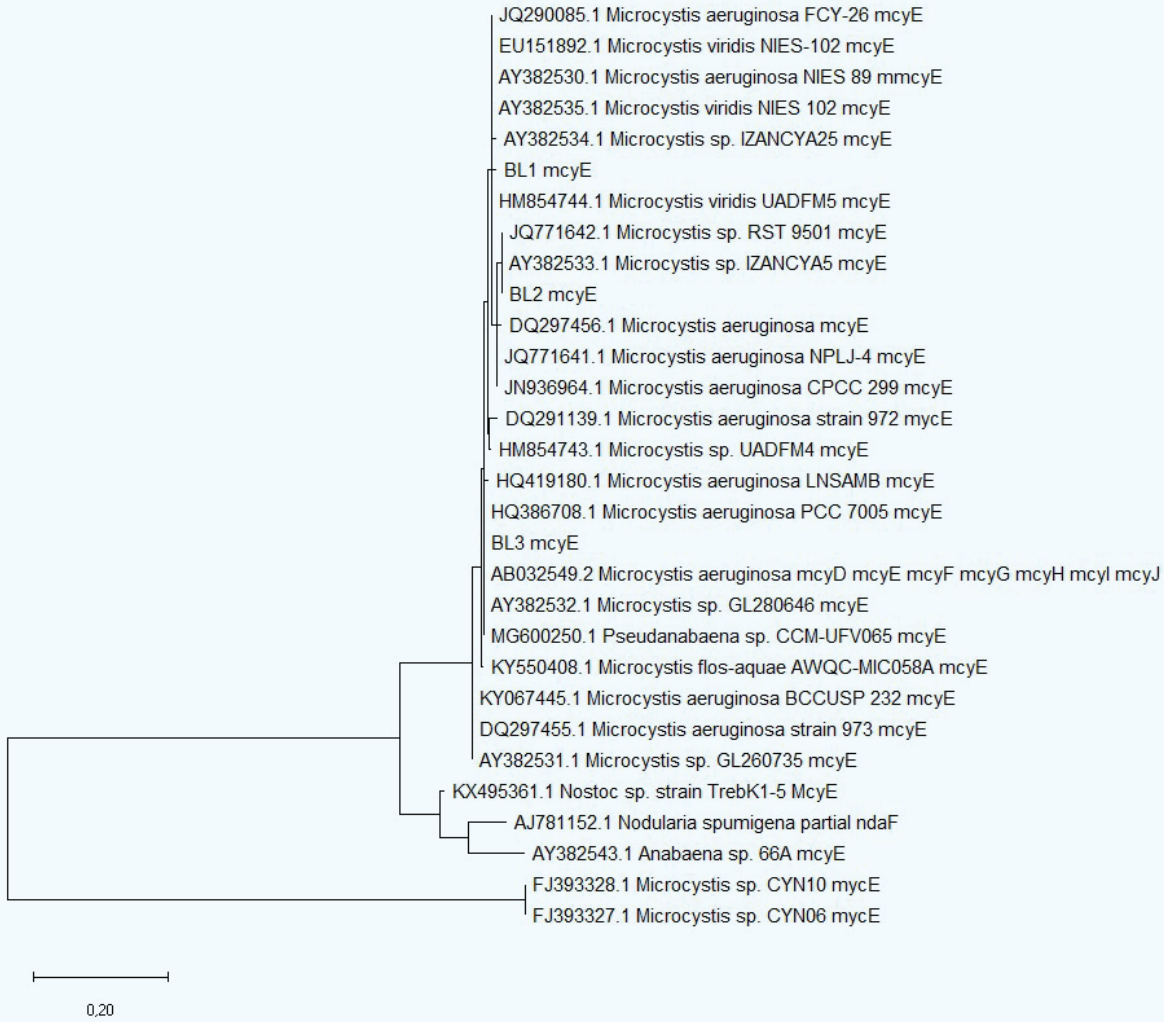

Figure 4: Maximum likelihood tree describing the relation of the mcyE genes in the different samples with earlier isolated strains in GenBank. A Maximum Likelihood test with 1000 Bootstrap replicates was used in combination with the Jukes-Cantor substitution model and Gamma Distributed mutation rate and Invariant Sites' correction. The data was treated for gaps and missing data by partial deletion with a site coverage cut-off of 95%. A Nearest-Neighbour-interchange Tree was automatically constructed for the Heuristic Method.

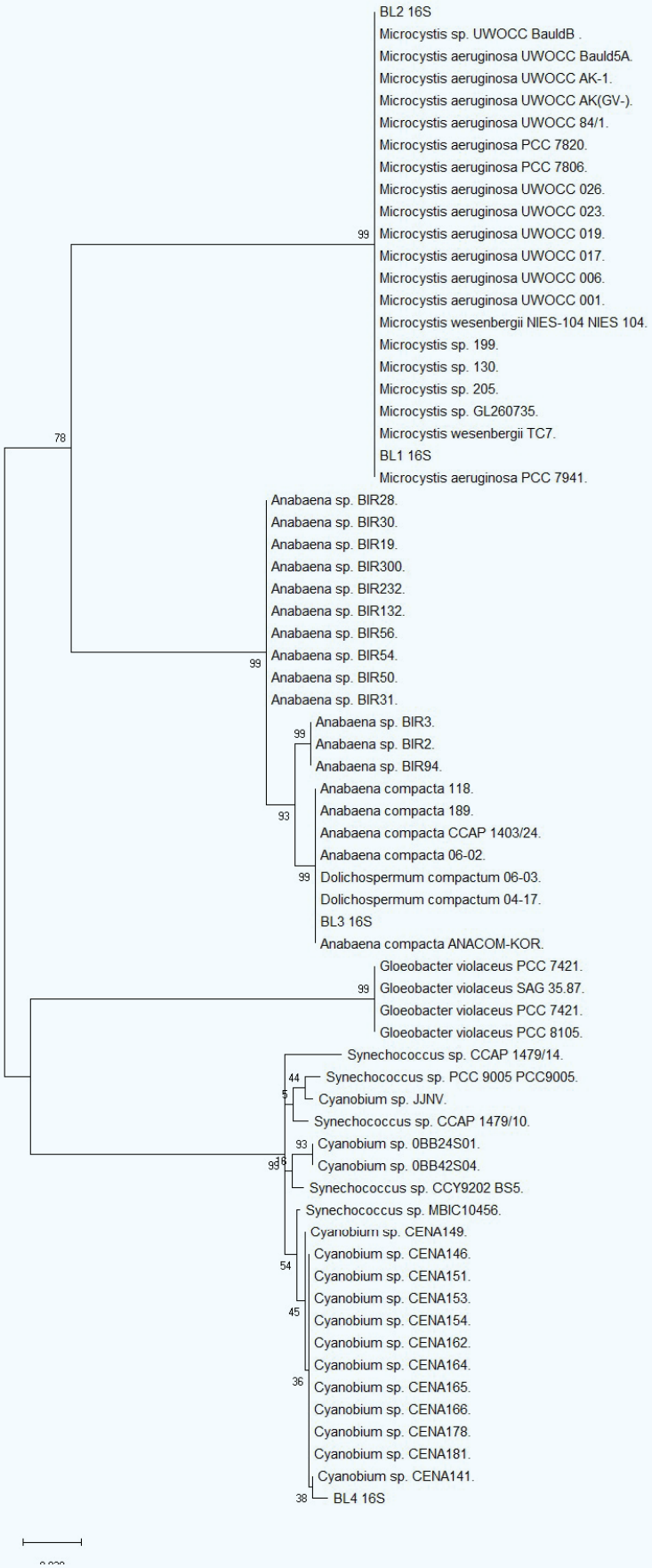

Figure 5: Maximum likelihood tree, showing the phylogenetic relation of the major species in each sample based on the 16S rRNA sequence. A Maximum Likelihood test with 1000 Bootstrap replicates was used in combination with the Jukes-Cantor substitution model and Gamma Distributed mutation rates and Invariant Sites' correction. The data was treated for gaps and missing data by partial deletion with a site coverage cut-off of 95%. A Nearest-Neighbour-interchange Tree was automatically constructed for the Heuristic Method.

Toxin content

Multiple microcystin congeners were detected when analyzing the environmental samples but nodularin was only detected in BL1 at very low levels. Additionally, we found that the major fraction of the toxins was intracellular. Sample BL4 was the exception and the highest signal was detected for extracellular toxins. The total cyanotoxin concentrations are illustrated in figure 6.

For BL1, all the 8 tested microcystin congeners were detected and quantified. The concentration did vary for the different congeners. The concentrations of MC-LF, MC-LY and MC-LW were under 1 µg/l whereas MC-LA and MC-WR were present with a concentration slightly above this value. Furthermore, the concentrations of MC-RR, MC-LR and MC-YR were close to or higher than 20 µg/l. In total, a concentration of 84.72 µg/l microcystin-LR Equiv was determined. This is well beyond the safety limit for drinking water, but also beyond the limit for recreational waters.

In comparison, BL2 seems to be more heavily contaminated. All of the toxins are detected, with MC-LF, MC-LY and MC-LW at low concentrations. MC-YR was found in lower concentrations compared to BL1, around 1 µg/l. The concentrations of MC-RR, MC-LR, MC-LA and MC-WR were around or higher than 20 µg/l. The total concentration was 206,47 µg/l MC-LR Equiv.

In the sample from BL3, most congeners were detected but at low concentrations. In this case, only MC-RR, MC-LR and MC-YR were above 1 µg/l. The total sum of the concentrations of all MC's was 9.32 µg/l MC-LR Equiv.

As briefly mentioned earlier, the concentrations in BL4 were very low. Only MC-RR, MC-LA, MC-LF, MC-LR, MC-YR and MC-WR were detected (Figure 6).

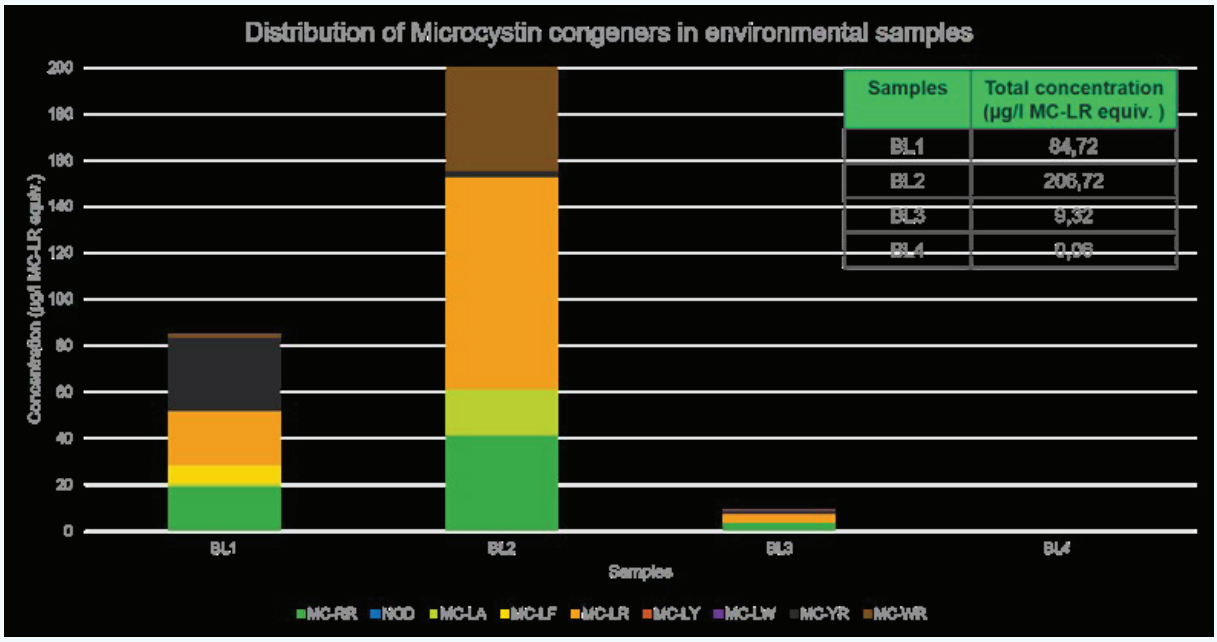

Figure 6: The total concentration of cyanotoxins (ng/l MC-LR Equi.) as well as the individual contribution of different congeners in the four pond samples. The total concentration of the toxins is also indicated in the window as MC-LR Equiv.

Discussion

Our study shows that cyanobacterial blooms are still present in the region of Brussels. In the B-BLOOMS2 project, possible toxic blooms were detected in more than 80% of the samples1. We detected potentially toxic species in 3 of the 4 sampled ponds in the Brussels Region. Our identification of the genera in the bloom samples also seems to be supported by our molecular approach based on the direct sequencing of the amplicons. This is only possible if the populations are dominated by only one taxon. If several taxa would be present in quasi equal quantities, the direct sequencing would be impossible. The morphological features, combined with the sequencing of the 16S rRNA gene, provide strong evidence that we are dealing with a toxic Microcystis species for BL1 and BL2. The sequencing of the mcyE gene also confirms this affiliation with the Microcystis family. For BL3, the results are more complex. Here the 16S rRNA sequence data suggests the dominance of Dolichospermum compactum that can only correspond to the lysed cell debris accompanied by heterocysts. Our hypothesis is that a past bloom of that taxon has left enough DNA, maybe in the detritic material, to dominate the genomic DNA extracted from the sample. Yet, we can be fairly certain that a Microcystis sp. was present because the mcyE gene sequence in BL3 is clearly affiliated to this genus. This is a quite interesting result when remembering that the concentration of microcystin congeners in BL3 is higher than the proposed value of the WHO for drinking water. This suggests that a minor population of cyanobacteria was capable of producing high amounts of toxins or that past blooms can leave a significant quantity of toxins in the water after the producers almost disappeared. Such toxic subpopulations could also exist in commercially harvested, natural blooms (like from Klamath lake, US) [22,23]. This further indicates that morphological identification and identification through sequencing might not be sufficient to ensure the safety of a product. For this purpose, the detection of different mcy genes indicates that the genetic potential for toxin production is present. However, definitive proof should come from the analytical detection of the cyanotoxins.

The total concentrations of MC's measured during the B-BLOOMS2 project varied conspicuously in different lakes and with time. The values ranged between 0.120 µg/l and 37500 µg/ml MC-LR Equiv.1 For three of our 4 samples, the cyanotoxins concentrations are within the same range. The total concentration of the MC's, expressed in MC-LR Equiv, heavily exceeded the current guideline values for drinking water and recreational use in BL1 and BL2. These guideline values were derived from the original guideline value decided by the WHO, 0.04 µg/kg body weight per day. For a 60 kg adult, who drinks 2 l water per day, the guideline value is set at 1 µg/l. The value for recreational ponds is further derived from this value. The current guideline value is 20 µg/l, assuming that 100 ml of water could be ingested involuntarily during recreational activities. It has to be mentioned that BL1 and BL 2 are not used for recreational activities causing contact with the water. On the other hand, BL3 is accessible to pedal boats and recreational fishing is also allowed. In this pond, we detected a concentration of toxins of 9.32 µg/l MC-LR Equiv. Although this is not above the guideline value for recreational ponds, it is higher than the value for drinking water. In BL4, which is a well-known fishing pond in the Brussels region, we did not find high concentrations of MC's. The trace amounts we measured, can be interpreted as an indication of past blooms. Furthermore, the 16S rRNA sequence in the sample pointed to a Cyanobium sp., which are generally not considered to be toxic.

This study was limited in its scope, by assessing only four ponds and only one-time point. Furthermore, we have not investigated the presence of MC's in fish, other animals in the pond or companion animals (e.g. dogs) for the accumulation of MC's. These domestic animals have lower body weight and thus are more susceptible to the toxins[33].

Furthermore, if the water of one of these ponds or any pond with a bloom would be used to irrigate vegetable gardens in the city, this would cause concern as it was shown that cyanotoxins can accumulate in plants [24]. In contrast, the influence of the pond contamination on the cyanobacteria based supplements available in Belgium is minimal, as most supplements are imported or grown in closed reactors.

As was mentioned in the introduction, contamination of different food types with cyanotoxins could be a risk for the population. This newly developed method to detect and quantify these eight MC's and Nod is a first step in the development of a flexible method that can detect the toxins in different matrices and that will then be utilized within the scope of the NRL Toxin group in Sciensano. Food supplements based on cyanobacteria, freshwater fish and shellfish are matrices that would be interesting to analyse for the presence of MC's and Nod. An additional extension of the method for other congeners of MC's or NOD and the inclusion of the other cyanotoxins would also be interesting.

Conclusion

With our limited sample size, we were able to show that there are still toxic blooms in the Brussels region. Molecular tools based on genetic markers allowed to identify the potentially toxigenic cyanobacterial taxa. Moreover, through quantitative toxin analysis, we unveiled microcystin congeners in three of the four ponds at concentrations above the drinking water guideline set by the WHO, and above the recreational water guideline for two ponds. Also, we showed the presence of trace amounts of toxins in the fourth pond, suggesting a past bloom. Given the fact that climatic change is foreseen to increase the frequency of bloom events [18], monitoring of surface waters in the three regions seem necessary, as well as measures for bloom prevention and control[18].

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Conflict of interest
The authors specify that there are no conflicts of interest

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Workshops & Symposia

The trainings for the approved laboratories organized by the FASFC in co-operation with the National Reference Laboratories are available on the website of the FASFC (www.favv.be > Home > Business Sectors > Laboratories > Seminars & workshops).

The schedule is updated regularly, it is therefore recommended to check the website from time to time.

Other interesting workshops and symposia are mentioned below.

Date	Subject	Place	More information (website)
25-26 November 2020	Smart Tech for Food (ST4F) Workshop	On Line	http://smarttech4food.activacongresos.com/
25-27 November 2020	Journées Francophones de Nutrition: Comment la préparation de l'aliment impacte l'allergénicité des protéines alimentaires ?	On Line	https://www.lesjfn.fr/
19-21 April 2021	Food Allergy Forum	On Line	https://foodallergyforum.org/index.php
10-12 May 2021	Euroresidue IX	Egmond aan Zee, The Netherlands	http://www.euroresidue.nl/
24-26 February 2021	7th International Symposium on Food Packaging - Scientific Developments Supporting Safety and Innovation	ILSI Europe	https://ilsi.eu/event/7th-international-symposium-on-food-packaging-scientific-developments-supporting-safety-and-innovation/

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