What is known about the import, distribution and presence of GM oilseed rape (*Brassica napus*) in the Netherlands?

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What is known about the import, distribution and presence of GM oilseed rape (*Brassica napus*) in the Netherlands?

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Preface

Most genetically modified crops that are authorized for import and processing in the EU are not able to establish themselves in the Netherlands. Oilseed rape (*Brassica napus*) is currently the only authorized GM crop that is able to do so. In addition, oilseed rape can hybridize with wild relatives such as *Brassica rapa*. Because of this special position, *B. napus* has been the topic of many research projects commissioned by COGEM in the past (Tamis and de Jong 2009; Luijten & de Jong 2010; 2011).

To assess potential environmental risks of GM crops, a worst-case scenario is used. When assessing potential environmental risks of import and processing of GM oilseed rape, it is assumed that seeds are spilled, and establishment of GM oilseed rape occurs. In addition, it cannot be excluded that GM oilseed rape hybridizes with other oilseed rape or *B. rapa* plants and that this would eventually result in GM plants with multiple transgenic traits in the rare case that both parents are GM for different traits. As it cannot be ruled out in advance that a combination of GM traits could have an adverse effect, COGEM repeatedly advised to monitor whether GM oilseed rape plants are present in handling areas or along transport routes.

COGEM's advice is based on reports on the presence of GM oilseed rape from other countries. In several countries, GM oilseed rape plants were detected close to highways or railroads, or in harbour areas. Information on the presence of GM oilseed rape in the Netherlands is, however, not available. Therefore, COGEM commissioned a research project to investigate whether GM oilseed rape plants are present at areas where oilseed rape is handled or along transport routes in the Netherlands. The project is focused on those areas where seed spillage due to import and processing is most likely to occur, i.e. transport routes and handling areas.

The researchers that carried out the project are experts in the identification and ecology of oilseed rape and in the detection of GM crops. The involvement of the Human Environment and Transport Inspectorate (Inspectie Leefomgeving en Transport) made it possible to sample oilseed rape at locations that would otherwise not be accessible. Due to their combined effort, the chance to detect GM oilseed rape was maximized. Their research is therefore of high value to COGEM's future advises on authorizations for import and processing of GM oilseed rape.

Rommie van der Weide

Chair of the Advisory Committee

English summary

Seed spillage as a consequence of the import, transport and processing of genetically modified (hereafter GM) oilseed rape (*Brassica napus* L.) can lead to subsequent establishment of feral transgenic plants in the Dutch environment. Previous research on the presence of GM oilseed rape along transport routes and at handling sites (i.e. oil mills) already showed the ability of GM *B. napus* to settle after seed spillage in Germany, Switzerland and Japan. After settlement, GM *B. napus* plants can cross with their wild relatives or other sexually compatible congeners like *Brassica rapa* L. which is a common plant species in the Netherlands. Repeated outcrossing between different GM *B. napus* events can result in the stacking of transgenes from which the effects are not yet known. Therefore, research on the presence and distribution of GM *B. napus* is a first step in assessing the possible adverse effects of seed spillage during transport.

Several GM *B. napus* events are authorized for import to the Netherlands and are allowed for processing by the food and feed industry. In order to quantify the amount of *B. napus* that is entering the country and to assess whether *B. napus* is imported from countries where GM events are cultivated, an inquiry was conducted on the origin and presence of GM *B. napus* in imported seeds (see Chapter 2). From available import data, it was difficult to estimate current inflows of GM *B. napus*. This was because the Dutch Customs do not make a distinction between *B. napus* and *B. rapa* and they currently do not register whether the shipment contains GM *B. napus*. In addition, the different data sources showed different contributions by the countries of origin. Overall, imports of canola *(B. napus* and *B. rapa*) seeds appeared to have largely originated from Australia, Ukraine and Argentina. However, previous studies from the Wageningen Food Safety Research showed that the likelihood of importing GM *B. napus* from these countries is low (Australia and Argentina) or not known (Ukraine). According to the large oil producers themselves, no GM *B. napus* is used in their products. This leads us to conclude that no large quantities of GM *B. napus* are currently imported into the Netherlands.

In order to investigate whether feral GM *B. napus* is present in the Netherlands, several areas were visited where spillage of seeds was most likely to occur (see Chapter 3). For the identification of such *B. napus* hotspots, a critical selection was made on the basis of previous observations and former research on the transport and distribution of *B. napus* in the Netherlands. This included areas along important transport routes (railways, roads and rivers), (un-)loading sites (ports) and processing facilities (oil mills). Cultivation areas were not visited in this study as spillage of conventional rather than GM *B. napus* is to be expected at these sites. At sites where the species was present, leaf material was collected for genetic analysis. In addition, leaves of family members of the Brassicaceae (*B. rapa* and *S. arvensis*) were sampled in order to detect possible gene flow of transgenes to wild relatives.

Oilseed rape plants were found at many of the preselected sites. Most plants were faced at the premises of the two large oil pressing companies ADM and Cargill and in riverbanks along the Waal between Nijmegen and Millingen aan de Rijn. Plants were also observed in road verges along highways that are presumably used for transport of oilseeds (A1, A7, A12, A28, A67) and along local roads that were in the vicinity of (former) handling sites (Farmsum, Lobith, Harlingen). In contrast, railways were often faced without *B. napus*. No oilseed rape plants were found along railways in the eastern part of the Netherlands. Here, the ballast bed was cleaned from vegetation or recently renewed. Consequently, opportunities for establishment and regeneration from seed banks were likely reduced. Plants were only found along freight railway tracks in the industrial areas of Rotterdam and Vlissingen, and at the train stations of Woerden and Ede-Wageningen. At freight railway tracks in the industrial area of Rotterdam, small populations of various sizes (5-75 plants) were found. Plants were mainly present at transit freight tracks rather than the neighbouring shunting yards. Finally, plants were observed from which the origin could not be linked to transport or processing of oilseed rape (Goor, Utrecht, Heiloo, Delft).

From the current distribution of *B. napus*, it appeared that transfer of seeds at hot-pressing companies together with transport via highway traffic and ship are the main facilitators in the spillage of seeds and thus places where *B. napus* might establish. Since no plants were observed outside the premises and plants on the premises itself were eradicated or mowed, handling activities at hot-pressing companies seem to be of minor importance for the escape of seeds into the environment. As *B. napus* is an arable species, disturbed sites and places where competition with other species is low (road verges, pavements, quays, railway beds, flower beds, groynes) are considered to be most suitable for establishment along transportation routes. Based on the observations, it was not possible to conclude whether the number of feral *B. napus* populations has increased or decreased during the last ten years. This is because different sites were visited compared to the study on the distribution of *B. napus* carried out in 2010. For the few revisited sites, no clear changes in density numbers were apparent and populations showed to be persistent for at least ten years.

To detect the possible presence of GM *B. napus*, *B. rapa* or crossings thereof, a screening strategy with five targets and an endogenous control was designed that cover all GM events that are commercially cultivated abroad (see Chapter 4). In total, 668 individual plants were screened in 160 samples. Quality of the isolated DNA was assessed with a qPCR for the endogenous control FatA, which was positive (and therefore fit for purpose) for all samples. In none of the samples, one or more targets were detected. Therefore, the 668 individual plants are not genetically modified with respect to the >25 events covered. It is not likely that other GM events are commercially grown outside of the Netherlands that are not covered by the current set. In conclusion, we did not find evidence for the occurrence of GM *B. napus* at the selected oil pressing companies and transportation routes.

Nederlandse samenvatting

Het morsen van zaad als gevolg van de import, transport en verwerking van genetisch gemodificeerd (hierna gg) koolzaad (*Brassica napus* L) kan leiden tot vestiging van wilde transgene planten in het Nederlandse milieu. Buitenlands onderzoek naar de aanwezigheid van gg-koolzaad langs transportroutes en rond plekken waar verwerking van zaden plaatsvindt (o.a. oliemolens), heeft reeds aangetoond dat gg *B. napus* zich na het morsen van zaad kan vestigen in Duitsland, Zwitserland, en Japan. Na vestiging kunnen gg-koolzaad planten kruisen met hun wilde verwanten of andere kruisbare compatibele soortgenoten zoals raapzaad (*Brassica rapa* L.), een veel voorkomende plantensoort in Nederland. Herhaaldelijke uitkruising tussen verschillende gg-koolzaad *events* kan leiden tot stapeling van transgenen waarvan de effecten nog niet bekend zijn. Daarom is onderzoek naar de aanwezigheid en verspreiding van gg-koolzaad een eerste stap in de beoordeling van de mogelijk negatieve effecten van het morsen van zaad tijdens transport.

Verschillende gg-koolzaad *events* zijn toegelaten voor import in Nederland en mogen worden gebruikt in de levensmiddelen- en diervoederindustrie. Om de hoeveelheid *B. napus* die het land binnenkomt te kwantificeren en om te beoordelen of *B. napus* wordt geïmporteerd uit landen waar gg *events* worden geteeld, is onderzoek gedaan naar de herkomst en aanwezigheid van gg *B. napus* in geïmporteerd bulkmateriaal (zie Hoofdstuk 2). Op basis van de beschikbare importgegevens was het moeilijk om de huidige toevoer van gg *B. napus* in te schatten. Dit komt omdat er door de Nederlandse Douane geen onderscheid wordt gemaakt tussen *B. napus* en *B. rapa* en er in het Douane registratiesysteem niet wordt bijgehouden of een lading gg bestanddelen bevat. Verder bleken de landen van herkomst verschillende hoeveelheden *B. napus* te leveren volgens de verschillende gegevensbronnen. Over het geheel genomen, lijkt de invoer van canola *(B. napus* en *B. rapa*) grotendeels afkomstig te zijn uit Australië, Oekraïne en Argentinië. Volgens eerder onderzoek uitgevoerd door Wageningen Food Safety Research is de kans op import van gg *B. napus* uit Australië en Argentinië echter laag. Het is niet bekend hoe groot de kans is dat gg *B. napus* uit Oekraïne geïmporteerd wordt. Volgens de grote olieproducenten zelf, wordt er geen gg *B. napus* gebruikt in hun producten. Hierdoor komen we tot de conclusie dat er momenteel geen grote hoeveelheden aan gg *B. napus* worden geïmporteerd in Nederland.

Om te onderzoeken of gg-koolzaad in Nederland voorkomt, zijn een aantal gebieden bezocht waar de kans op het morsen van zaden het meest waarschijnlijk was (zie Hoofdstuk 3). Voor de identificatie van zulke *B. napus* kerngebieden, is een kritische selectie gemaakt op basis van eerdere observaties en eerder onderzoek dat is gedaan naar het transport en de verspreiding van *B. napus* in Nederland. Hiertoe behoorden gebieden langs belangrijke transportroutes (spoorwegen, wegen en rivieren), overslaggebieden (havens) en verwerkingsbedrijven (olieperserijen). In deze studie zijn geen teeltgebieden bezocht, omdat het morsen van conventioneel in plaats van genetisch gemodificeerde *B. napus* hier te verwachten valt. Op plekken waar de soort werd aangetroffen, is bladmateriaal verzameld voor genetische analyse. Daarnaast zijn bladeren van andere soorten uit de familie van de Brassicaceae (*B. rapa* en *Sinapis arvensis*) bemonsterd om mogelijke stapeling van transgenen in nabije verwante planten op te sporen.

Koolzaadplanten werden gevonden op veel plekken die vooraf geselecteerd waren. De meeste planten werden aangetroffen op het terrein van de twee grote oliepersbedrijven ADM en Cargill en langs de Waal tussen Nijmegen en Millingen aan de Rijn. Planten werden ook waargenomen in wegbermen langs snelwegen die vermoedelijk worden gebruikt voor het transport van oliehoudende zaden (A1, A7, A12, A28, A67) en langs lokale wegen die zich in de buurt van (voormalige) overslaglocaties bevonden (Farmsum, Lobith, Harlingen). Daarentegen werd er langs spoorwegen op veel van de bezochte locaties geen *B. napus* waargenomen en dan met name in oost Nederland. Hier was het ballastbed vrijgemaakt van vegetatie of onlangs vernieuwd. Mogelijkheden voor vestiging en regeneratie uit zaadbanken waren hierdoor waarschijnlijk beperkt. Planten werden alleen gevonden langs doorgaande routes voor

goederentreinen in de industriegebieden van Rotterdam en Vlissingen, en op treinstations Woerden en Ede-Wageningen. Langs het goederenspoor in het Rotterdamse havengebied werden kleine populaties van verschillende grootte (5-75 planten) gevonden. De koolzaadplanten stonden vooral in het kiezelbed van het doorgaande goederenspoor en niet op de naastgelegen rangeerterreinen. Ten slotte werden planten waargenomen waarvan de herkomst niet in verband kon worden gebracht met het transport of de verwerking van koolzaad (Goor, Utrecht, Heiloo, Delft).

Uit de huidige verspreiding van *B. napus* blijkt dat overslag en intern transport bij *hot pressing* bedrijven samen met het transport via snelwegen en schip de belangrijkste locaties zijn waar zaden gemorst worden en dus de plaatsen waar *B. napus* zich zou kunnen vestigen. Echter, omdat er geen planten werden gevonden buiten de bedrijventerreinen en omdat planten op de terreinen waren verwijderd of gemaaid, lijkt de verwerking van zaden door *hot pressing* bedrijven een minder grote rol te spelen bij het ontsnappen van zaden in het milieu. Aangezien *B. napus* een akkersoort is, worden verstoorde plekken en plaatsen waar de concurrentie met andere soorten laag is (omgewoelde en kale bermen, perkjes, kades, ballastbed van spoorrails, kribben langs rivieren) het meest geschikt geacht voor vestiging langs transportroutes. Op basis van de observaties kan niet worden geconcludeerd of het aantal verwilderde *B. napus* populaties is toegenomen of afgenomen in de afgelopen tien jaar. Dit komt omdat er verschillende gebieden zijn onderzocht ten opzichte van het onderzoek dat is gedaan naar de verspreiding van *B. napus* in 2010. Voor de enkele opnieuw bezochte locaties waren er geen duidelijke veranderingen in populatiegrootte zichtbaar en leken de populaties persistent voor minstens tien jaar.

Om de mogelijke aanwezigheid van gg *B. napus*, *B. rapa* en hun kruisingen op te sporen, is een screeningsstrategie met vijf *targets* en een endogene controle ontworpen die alle gg-*events* omvat die commercieel geteeld worden in het buitenland (zie Hoofdstuk 4). In totaal zijn 668 individuele planten gescreend in 160 monsters. De kwaliteit van het geïsoleerde DNA werd beoordeeld met een qPCR voor de endogene controle FatA, die positief (en dus geschikt) was voor alle monsters. In geen van de monsters werden een of meerdere *targets* gevonden. Daarom zijn de 668 afzonderlijke planten niet genetisch gemodificeerd wat betreft de >25 *events* in kwestie. Het is niet waarschijnlijk dat andere gg-*events* commercieel worden geteeld buiten Nederland die niet met de gebruikte screeningsstrategie gedetecteerd kunnen worden. Concluderend hebben we geen bewijs gevonden voor de aanwezigheid van gg *B. napus* bij de bemonsterde olieperserijen en transportroutes.

1. Introduction

1.1. Background

In Europe, various genetically modified (GM) oilseed rape (*Brassica napus* L.) events are permitted for import and processing. In addition, license applications for the import of new GM oilseed rape events are submitted on a regular basis (i.e. EC 2013). Genetically modified oilseed rape is the only GM crop authorized within the EU that can establish itself in North-Western Europe via spilled seed.

Through the import and processing of transgenic seeds and bulk material, there is a significant possibility that GM *B. napus* can establish into the environment. Although exact measures of spillage of viable seeds are not available, overall loss is estimated to range from 0.1% to 3.0% (Tamis and de Jong 2010). In addition to seed spillage as a direct consequence of the import of GM *B. napus* seeds, admixture and contamination can also lead to the introduction of GM *B. napus*. For example, in Switzerland, where the import of GM *B. napus* is prohibited, four transgenic events were found in the port of Basel from which the source could be traced back to the import of Canadian wheat (Schulze *et al.* 2015).

Currently, limited information is available on the amount of GM *B. napus* entering the country and the routes by which seeds are transported. The existing data gap is partly due to trade authorities who do not make a distinction between GM and non-GM oilseeds in their declaration system. It means that data is missing on the presence (or absence) of genetically modified events in imported shipments. At the same time, the probability of admixture with GM seeds cannot be evaluated. Secondly, information is lacking on companies that use GM *B. napus* for seed oil or animal feed, which makes it difficult to track how GM oilseed rape is transported across the country. In 2010/2011, the ILT conducted a survey to investigate the presence of GM *B. napus* in non-GM canola used for the production of feed and oil. Eight shipments from Australia, one from Argentina, one from Chile and one from the Russian Federation were analysed. Two samples from Australia, one for production of feed and one for the production of oil were positive for GT73, but with amounts below the level of quantification (ILT 2012).

Oilseed rape is imported to the Netherlands as oilseed rape meal or as whole and viable seeds which are used in the food and feed industry. Oil is extracted from seeds at different plants by means of solvent-extraction with hexane, mechanical extraction of seeds that were previously conditioned by a heat treatment or cold-pressing at 60 °C (Heuzé *et al.* 2018). Waste products that remain after pressing, also called rape meal or oil cake, are largely used by the fodder industry as a component for animal feed. In addition, oilseed rape seeds are processed in bird and rodent feed, and flower seed mixtures. Frick *et al.* (2018) found high percentages of GM oilseed rape in birdfeed, suggesting that birdfeed might be a vehicle through which GM oilseed rape may spread into the environment. Some studies even showed the potential of birds to disperse viable oilseed rape seeds thereby enhancing transgenic gene flow (Twigg *et al.* 2008, 2009). During the period of 2016 to 2019, an average of 10 samples of birdfeed per year were tested by Wageningen Food Safety Research for the presence of GM material. Only trace amounts of GM *B. napus* (Rf3, GT73 and Ms8) were found in 2016. It is not known whether these events were present as single or as stacked events (EUginius). Since the samples are grinded prior to DNA analysis, no conclusion could be drawn on the presence of viable seeds.

It is known that *B. napus* is able to establish and persist in the Dutch environment (Luijten & de Jong 2010). When spillage and sequential establishment occurs, plants can cross-pollinate with conventional *B. napus* or with the closely related *B. rapa* that is commonly found in the Netherlands. In addition, *B. napus* could potentially cross with other sexually compatible Brassicaceae like *Brassica juncea* and *Sinapis arvensis*, but records on high survival rates of hybrid seedlings are scarce (Warwick *et al.* 2003). Eventual outcrossing involving GM *B. napus* could result in the gene flow of transgenes to feral populations. The effects of transgene transfer on recipient conventional *B. napus* or wild relatives are expected to be negligible (Warwick *et al.* 2008). However, mutual outcrossing between different GM *B. napus* events may result in the stacking of different transgenes thereby contributing to the spread of new combinations of transgenic traits (Knispel *et al.* 2010).

All of the genetically modified *B. napus* events that are currently authorized for import in the Netherlands are known to exhibit herbicide tolerant genes responsible for the resistance to either glyphosate or glufosinate. Interspecific gene flow from herbicide resistant *B. napus* to wild relatives can alter the

fitness of the recipient plants (Vrbničanin *et al.* 2017). However, this is only the case in areas where herbicides are applied for weed control and when there is a selection pressure for herbicide resistance.

In various countries all over the world, transgenic *B. napus* was found in road verges (Nishizawa *et al.* 2010), along transportation routes (Yoshimura *et al.* 2006) and in port areas (Aono *et al.* 2006, Saji *et al.* 2005, Kawata *et al.* 2008). Escape of GM *B. napus* as a result of the transportation and processing of seeds has also frequently been recorded in Europe (Hecht *et al.* 2014, Schulze *et al.* 2014, Franzaring *et al.* 2016). Hecht *et al.* (2014) detected the genetically modified line GT73 at three locations in Switzerland. They found significantly more GM *B. napus* along railway lines that transport goods towards oil-extracting companies. In a follow-up study, three other glufosinate resistant lines were detected in the same area (Schulze *et al.* 2014). In Germany, a field study was conducted on the presence of GM *B. napus* along the Rhine near oil mills and seed processing industries (Franzaring *et al.* 2016). Here, one plant out of 1918 was found to be transgenic in the Neuss Harbour (North Rhine-Westphalia).

Previous research on the distribution of *B. napus* in the Netherlands has shown that the species mainly occurs in disturbed arable areas and places where cultivation, transhipment and transport take place (Luijten & de Jong 2010). The species has a scattered distribution and populations are generally small although several larger populations were found. Its presence often descended from seed spillage through transport and handling activities, which included *i)* cultivation of *B. napus* as a crop, which occurs throughout the Netherlands with an emphasis on Groningen, *ii)* transport losses of seeds from trucks or freight trains, *iii)* seed losses near transhipment locations, and *iv)* sowing of bird feed (IAG, 2018). Hybridization with its wild relative *B. rapa* appeared to be rare, because very few mixed populations with both species were found. Hybrids (F1) were only found in three out of 27 sampled *B. rapa* populations (Luijten & de Jong 2011). Whether GM *B. napus* is present in the Netherlands is not yet known and will be investigated in the current study.

1.2. Project aims

Because of uncertainty about the occurrence of (unintended) spillage of GM *B. napus* in the Dutch environment, research is needed on its presence in the Netherlands. The main objective of this study is to gain insight in the appearance of GM oilseed rape plants at transfer stations and transport routes, as this is where the chance of seed spillage is highest. In addition, *B. rapa* plants will be sampled to examine whether transgenes spread to *B. rapa* plants and/or populations. The results of this study will eventually be used by COGEM to update its advice on monitoring obligations for the import of GM *B. napus*. The main objective is subdivided in three goals:

1. Clarify whether and to what extent GM *B. napus* is imported to the Netherlands and to detect transport routes along which GM *B. napus* enters the country by analysing available import data of authorized GM events on both national and international scale (see Chapter 2).
2. Determine whether *B. napus* is present in the Dutch environment due to seed spillage (persistence and population size) with emphasis on transhipment locations and transport routes. Populations were visited and sampled for genetic analysis (see Chapter 3).
3. Screen for the presence of GM *B. napus* events. *B. rapa* was screened simultaneously in order to assess whether transfer of transgenes has occurred (see Chapter 4).

1.3. Nomenclature of *B. napus* and *B. rapa*

In this report different names for *B. napus* and *B. rapa* are used. Within published papers and documents, both scientific and non-scientific, the terminology of both species is unclear and sometimes both *Brassica* species are referred to by a single name. It was therefore not possible to consequently use the terminology of *Brassica napus* or *Brassica rapa* in this report. In general, the species *Brassica napus* is named *B. napus* or oilseed rape. *Brassica rapa* is referred to as *B. rapa*. Canola is used by import authorities as a broad term for *Brassica* species that have seeds low in erucic acid. This term is adopted from import data at the appropriate places and thus only applied when no distinction was made between *B. napus* and other canola species, including *B. rapa*.

1.4. Ecology of *B. napus* and *B. rapa*

The crop *B. napus* and the wild relative *B. rapa* are difficult to distinguish and mistakes with identification are often made. In the Netherlands, wild populations of *B. rapa* are frequent along roads and canals and such populations can be large, consisting of thousands of plants. Populations of *B. napus* are much rarer, occur under very disturbed conditions and usually contain few plants. *B. napus* is less persistent than *B. rapa*. After sowing seeds from 29 accessions on disturbed plots, *B. napus* typically established, flowered and produced abundant seeds in the first year but then disappeared from the vegetation in the second year (Hesse *et al.* 2018). Under the same conditions, *B. rapa* persisted in the second and sometimes even in the third year. Domestication traits present in *B. napus* likely reduce its ability to establish and survive in the wild. Seeds and seedlings, especially of modern canola-type cultivars with low glucosinolate content, are heavily predated in the field by slugs (Moshgani *et al.* 2014). Cafetaria experiments showed that mice greatly preferred *B. napus* over *B. rapa* seeds (de Jong *et al*. 2016). Within *B. napus*, mice avoided feeding on seeds from cultivars with high glucosinolate content and, to a lesser degree, high erucic acid content. While the species flower simultaneously the time required for seeds to ripen is about two months for *B. napus*, which is longer than for *B. rapa*. Therefore, *B. rapa* is more likely to produce viable seeds before the vegetation is mown. Furthermore, the smaller seeds of *B. rapa* are more likely to become buried and develop dormancy than the seeds of *B. napus* (de Jong *et al.* 2013).

2. Import of *B. napus*

2.1. General

Globally, the area of GM canola was 10.1 million hectares in 2018 (ISAAA 2018). This is 29% of the global hectarage of canola. The largest GM canola growing countries are Canada (8.7 million Ha), the USA (0.9 million Ha), Australia and Chile. Cultivation of GM *B. napus* is not authorized in the European Union (EU 2019), but seeds, oil and oil meal can be imported and used in food and feed. Because little is known about the identity, amount and origin of GM *B. napus* events that are imported to the Netherlands, we *i)* made an inventory of the events that are currently authorized for import (see section 2.2) and *ii)* performed data analysis on import data in order to reveal exporting countries of origin (see section 2.3).

2.2. Inventory of GM *B. napus* events imported in the EU

For the inventory of commercially available GM *B. napus* events, different classes can be identified. These are *i*) events that have been approved in the EU, *ii*) those for which an authorisation procedure is pending or the authorisation of which is expired (EC 2011), *iii*) events that are subject to commission decisions on withdrawal from the market, *iv*) events that are approved elsewhere, and *v*) events that are not authorised anywhere. An overview of the different classes is given below.[1]

i. In Table 1, an overview is given of all GM *B. napus* events authorized for *a)* foods and food ingredients containing, consisting of, or produced from oilseed rape (including food additives), *b*) feed containing, consisting of, or produced from oilseed rape, and *c)* other products containing or consisting of oilseed rape with the exception of cultivation (EU 2019).

ii. There is a list of GMOs which currently fulfils the requirements of Regulation (EC) no. 619/2011 (EC 2011). This lays down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorization procedure is pending or the authorization of which is expired. This list contains Ms8 x Rf3 x GT73 rapeseed and oilseed rape 73496. Note that this is only for the presence in animal feed up to 0.1%.

iii. Currently, oilseed rapes Ms1, Rf1, Ms1 x Rf1, Rf2, Ms1 x Rf2 and Topas 19/2 are withdrawn from the market. Their transition period was extended until 31 December 2016 with a tolerance threshold of 0.1% (EC 2012). As regards the tolerance period for traces of these GM oilseed rapes, as well as their derived products, there is a supplementary transitional period until 31 December 2019 in order to provide sufficient time to allow the total removal of the GM material from the food and feed chain (EC 2016, EC 2017). Recently, this transition period was extended until 31 December 2022 with a tolerance threshold of 0.1% (EC 2019).

iv. Import is probably not restricted to GM *B. napus* events that are authorized for import in the Netherlands. Therefore, an inventory was made to identify most, if not all, known commercially grown GM *B. napus* worldwide (see Table 2). The overview only contains single-event GMOs, and not the stacks that are conventional crossings between different single-events. The rationale is that in seed batches, it is not possible to discriminate between the presence of stacks of two or more events, or the single-events. Furthermore, when quantification of the GM-content is required, this is done on the presence of the separate events. As far as authorisation is concerned, many countries do not require deregulation of the stacks once the separate events have been deregulated. This is not the case in the EU.

v. Currently, no GM *B. napus* events are known that are not (previously) authorized or deregulated anywhere in the world.

[1] For more detailed information on GM events, please consult www.EUginius.eu.

Table 1. GM canola events authorized in the EU (as of November 2019).

Type	GMO	OECD unique identifier	Company	Trait
Swede rape	Ms8	ACS-BNØØ5-8	Bayer	Male sterility, glufosinate tolerance
Swede rape	Rf3	ACS-BNØØ3-6	Bayer	Fertility restoration, glufosinate tolerance
Swede rape	Ms8 x Rf3	ACS-BNØØ5-8 x ACS-BNØØ3-6	Bayer	See single events
Swede rape	Ms1		Bayer	Male sterility, kanamycin/neomycin resistance, glufosinate tolerance
Swede rape	Rf2		Bayer	Kanamycin/neomycin resistance, fertility restoration, glufosinate tolerance
Swede rape	Ms1 x Rf2		Bayer	See single events
Swede rape	TOPAS19/2		Bayer	Kanamycin/neomycin resistance, glufosinate tolerance
Oilseed rape	GT73	MON-ØØØ73-7	Monsanto	Glyphosate tolerance
Oilseed rape	T45	ACS-BNØØ8-2	Bayer	Glufosinate tolerance
Oilseed rape	MON88302	MON-883Ø2-9	Monsanto	Glyphosate tolerance
Oilseed rape	MON88302 x Ms8 x Rf3	MON-883Ø2-9 × ACSBNØØ5-8 × ACS-BNØØ3-6	Bayer and Monsanto	See single events
Oilseed rape	MON88302 x Ms8	MON-883Ø2-9 × ACSBNØØ5-8	Bayer and Monsanto	See single events
Oilseed rape	MON88302 x Rf3	MON-883Ø2-9 × ACS-BNØØ3-6	Bayer and Monsanto	See single events

2.3. Analysis of import data

In data sets on import of canola, no distinction is made between GM and non-GM canola. Although this information may be present in the Customs Declaration or other documents accompanying the goods, it forms no part of the accumulated data on canola in the AGS declaration system. Thus, the amount of imported GM canola into the Netherlands in recent years is unknown. In addition, GM food and feed need to be labelled if GM contents exceed a threshold of 0.9% of adventitious presence of EU-approved GMOs (EC 2003). As a consequence, GM canola might be present at low levels in non-GM oilseed rape lots and therefore we performed data analysis on import of oilseed rape in the Netherlands.

2.3.1. Import data sources

There are a number of organizations collecting import data: *i*) the Observatory of Economic Complexity (OEC)*, ii*) the Food and Agriculture Organisation (FAO[2]), *iii*) the Dutch Customs and *iv)* the Dutch Central Bureau of Statistics (CBS). The OEC uses data sets to provide access to trade data for roughly 200 countries. They use data from the Center for International Data and data provided by UN COMTRADE (OEC 2019). The Dutch Customs make use of the Integrated Tariff (TARIC 2019). For canola, the code 1205 applies (rape or colza seeds, either broken or not). This product group consist of both *B. napus* and *B. rapa*, making it difficult to discriminate between separate import flows. The Central Bureau of Statistics (CBS) makes use of the same Tariff codes (CBS 2019).

The datasets contain data in different formats. The OEC set only contains data on the monetary value of imports, while the other three have both weight and value data. The weight data of the FAO is specified in tonnes, and the Dutch Customs and the CBS data are in kilogrammes. Furthermore, the value data of the OEC and the FAO are in US dollars, while the Dutch Customs and CBS are in Euro. The data set of the Dutch Customs lacks information on goods originating from EU member states.

[2] The FAO dataset was omitted from further analysis as it lacked information on 2017.

Table 2. Inventory of commercially available GM *B. napus* (previously) authorized or deregulated worldwide (November 2019).

Species	GMO	OECD unique identifier	Companies	Trait
B. napus	23-18-17	CGN-89111-8	Monsanto Company	Altered fatty acids and oils, kanamycin/neomycin resistance
B. napus	23-198	CGN-89465-2	Monsanto Company	Altered fatty acids and oils, kanamycin/neomycin resistance
B. napus	61061	DP-061061-7	Pioneer Hi-Bred Intl	Glyphosate tolerance
B. napus	73496	DP-073496-4	Pioneer Hi-Bred Intl, DuPont	Glyphosate tolerance
B. napus	DHA	NS-B50027-4	Nuseed Americas Inc.	Altered fatty acids and oils
B. napus	Falcon GS 40/90	ACS-BN010-4	Bayer CropScience	Glufosinate tolerance
B. napus	GT200	MON-89249-2	Monsanto Company	Glyphosate tolerance
B. napus	GT73	MON-00073-7	Monsanto Company	Glyphosate tolerance
B. napus	HCN10		Bayer CropScience	Kanamycin/neomycin resistance, glufosinate tolerance
B. napus	HCR-1		AgrEvo	Glufosinate tolerance
B. napus	LBFLFK	BPS-BFLFK-2	BASF	Altered fatty acids and oils, imidazolinone tolerance
B. napus	Liberator	ACS-BN009-3	Bayer CropScience	Glufosinate tolerance
B. napus	MON88302	MON-88302-9	Monsanto Company	Glyphosate tolerance
B. napus	MPS961		BASF	Phytate degradation
B. napus	MPS962		BASF	Phytate degradation
B. napus	MPS963		BASF	Phytate degradation
B. napus	MPS964		BASF	Phytate degradation
B. napus	MPS965		BASF	Kanamycin/neomycin resistance, phytate degradation
B. napus	Ms1	ACS-BN004-7	Bayer CropScience	Male sterility, Kanamycin/neomycin resistance, glufosinate tolerance
B. napus	Ms11	BCS-BN012-7	Bayer CropScience	Male sterility, glufosinate tolerance
B. napus	Ms8	ACS-BN005-8	Bayer CropScience	Male sterility, glufosinate tolerance
B. napus	OXY-235	ACS-BN011-5	Bayer CropScience	Bromoxynil tolerance
B. napus	Rf1	ACS-BN001-4	Bayer CropScience	Kanamycin/neomycin resistance, fertility restoration, glufosinate tolerance
B. napus	Rf2	ACS-BN002-5	Bayer CropScience	Kanamycin/neomycin resistance, fertility restoration, glufosinate tolerance
B. napus	Rf3	ACS-BN003-6	Bayer CropScience	Fertility restoration, glufosinate tolerance
B. napus	T45	ACS-BN008-2	Bayer CropScience	Glufosinate tolerance
B. napus	Topas 19/2	ACS-BN007-1	Bayer CropScience	Kanamycin/neomycin resistance, glufosinate tolerance
B. rapa	ZSR500		Monsanto Company	Glyphosate tolerance
B. rapa	ZSR502		Monsanto Company	Glyphosate tolerance
B. rapa	ZSR503		Monsanto Company	Glyphosate tolerance

2.3.2. Data analysis

The analysed data represent combined information on import of both *B. napus* and *B. rapa* (canola) in the Netherlands. Results could not be extracted for *B. napus* separately and are therefore referred to as canola.

The red line in

Figure 1 and

Figure 2c and depicts CBS-data on the tonnage of total import from non-EU member states. It is clear that the highest amount of canola was imported in 2014, with a total of 783,045 tonnes. This decreased with 28.4% in 2015, to 560,797 tonnes. In 2016, canola import was diminished to 18,226 tonnes. It recovered in 2017 and reached 572,535 tonnes.

Figure 2a and b show the data including the contribution of EU member states. Although the trend is similar, with a minimum in 2016, the total amounts are higher (1,584,987 in 2014, 1,197,041 in 2015, 514,092 in 2016 and 1,087,580 tonnes in 2017). This means that there is a steady import from within the EU of 500,000 to 600,000 tonnes, with a peak in 2014 of about 800,000 tonnes. However, these imports are of a lesser concern, as no GMO is expected in those countries. The member state of origin has to apply EU legislation to the contents of these deliveries.

The bar graph in

Figure 1 represents the top six non-EU countries contributing to the import of canola in percentages of the total value of each year, based on the data from the Dutch customs. The bar graphs in

Figure 2 represent the top ten countries contributing to the import in percentages of the total value of each year, based on the data from the OEC or the CBS. This covers more than 95% of the imports if only non-EU countries are considered and >87% if EU countries are included. The CBS and OEC import data of the non-EU countries are broadly in agreement with each other, except for 2016. The country, which contributes most, varies between the years, but the top three exporters of canola to the Netherlands were Australia, Ukraine and Argentina. The percentages from the CBS and the customs declaration system do not match. This is remarkable since they make use of the same tariff code. We were not able to come to a satisfactory explanation for this discrepancy.

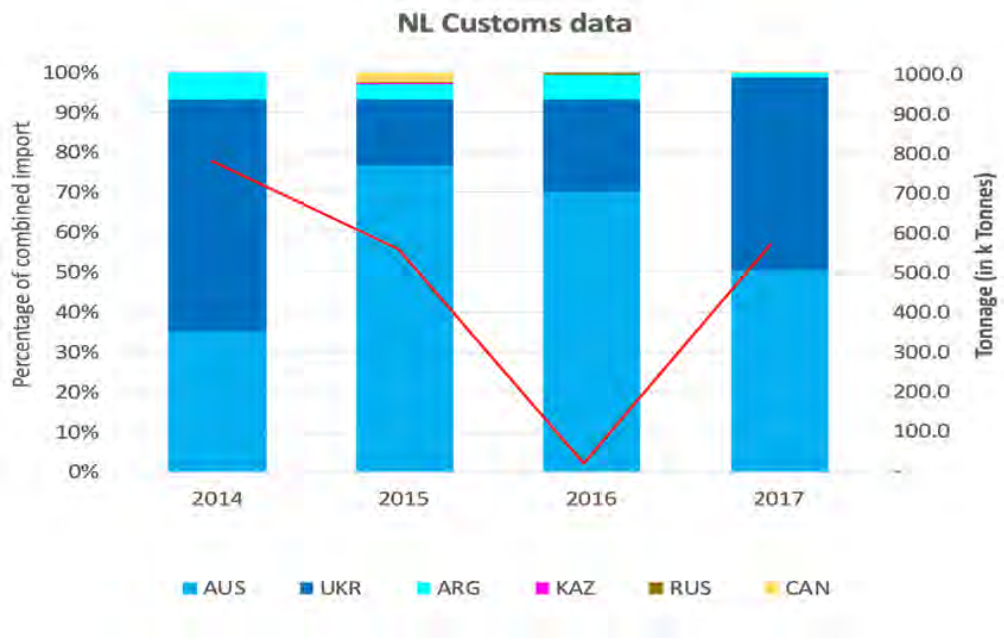

Figure 1. Import of *B. napus* and *B. rapa* to the Netherlands form outside the EU presented in percentages (bar chart) and tonnage (in kilo Tonnes) per year (line chart), based on data from Dutch Customs. These countries represent >99% of all imports from countries outside the EU as reported by the Dutch Customs. AUS = Australia, UKR = Ukraine, ARG = Argentina, KAZ = Kazakhstan, RUS = Russia, CAN = Canada.

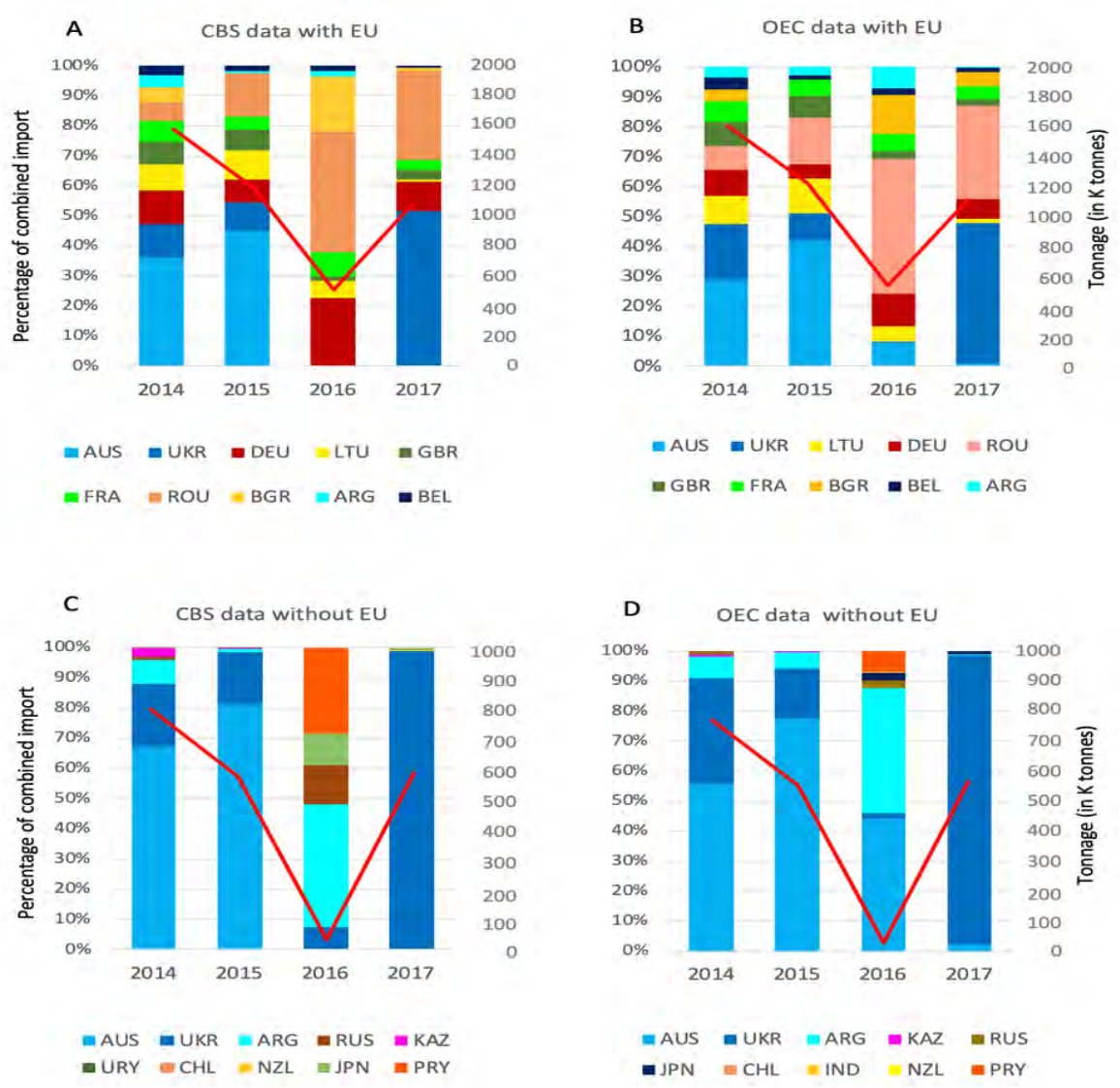

Figure 2. Import of *B. napus* and *B. rapa* to the Netherlands. The graphs depict CBS data on tonnage of total import (in kilo Tonnes) per year and correspond to the right axes. The left axes correspond to the bar-graphs, which depict the contribution of top ten countries from which NL imported *B. napus* and *B. rapa*, based on the data from CBS (A and C) and OEC (B and D). These countries represent >95% of all imports if only countries outside the EU are considered (A and B) and >87% if countries from the EU are included (C and D). AUS= Australia, UKR= Ukraine, DEU= Germany, LTU= Lithuania, GBR= United Kingdom, FRA=France, ROU=Romania, BGR=Bulgaria, ARG=Argentina, BEL=Belgium, RUS=Russia, KAZ=Kazakhstan, URY=Uruguay, CHL=Chile, NZL=New Zealand, JPN=Japan, PRY=Paraguay.

In 2014/2015, the prediction model "GMOnitor" was developed by WFSR in collaboration with ILT and the NVWA (van den Heuvel *et al.* 2019) to assess the likelihood of importing GM material from certain countries. The model is based on different criteria like the authorization/deregulation of GM crops, field trials and import data. This allows searching on a certain country/crop combination to predict the likelihood of importing, but also includes an environmental assessment. It is also possible to select a certain crop and view the countries with a relative likelihood for exporting a GM variant.

Of all the canola cultivated in Australia, 24%, of the total crop acreage is GM. The presence of trace levels of authorised GM canola in non-GM material from Australia is likely, since authorised GMOs are not subject to traceability or labelling requirements if they contain traces of these GMOs below a limit of 0.9%. No data are available for *B. napus* from Argentina or Ukraine. According to the ISAAA database, Argentina does not produce GM *B. napus.* Nevertheless, GT73 plants were found, among others, in roundup ready soybean fields (Pandolfo *et al.* 2016).

3. Sampling of *B. napus*

3.1. General

Potential sites of seed spillage and establishment of *B. napus* were selected prior to sampling. In order to identify sample locations, we used data from previous research on the transport and distribution of *B. napus* in the Netherlands, comparable studies carried out in other EU-countries and recent observations of feral *B. napus*. An important baseline was former research conducted by Tamis & de Jong (2009) and Luijten & de Jong (2010) who respectively *i)* estimated seed spillage along transport chains and *ii)* studied the presence of *B. napus* and *B. rapa* in the industrial, agricultural and rural environment in the Netherlands. The companies mentioned in the previous research reports, have been reassessed in order to create a clear picture of locations prone to leakage of seeds.

During the selection of sampling sites, we mainly focussed on transhipment locations and transport routes. Agricultural areas were not included in the study because this research was aimed to reveal sites where GM oilseed rape plants are present solely as a consequence of seed spillage during import and transport. In addition, plants in these areas originate from non-GM seeds rather than GM seeds. Spillage of oilseed rape seeds along railways was underexposed (with the exception of shunting yards and railway stations) as access to these areas is prohibited and solely possible in the absence of train traffic and under surveillance of an organisation who is entitled to enter such sites, like The Human Environment and Transport Inspectorate (ILT). Extra attention was paid to smaller inland harbours and its most likely routes to the end user, which would be small oil pressing companies.

In the following sections, some of the potential sites of seed spillage are summarized (see section 3.2) and the sampling method is explained (see section 3.3). The results on the distribution of *B. napus* are reported in section 3.4.

3.2. Selection of sampling sites

3.2.1. Oil pressing industry

Oil pressing companies are major end users of *B. napus* seeds in the production of plant oil. As a result, these companies can be considered as an important link within the transport chain of *B. napus* and may be a possible route along which (GM) seeds end up in the environment. Previous research already showed the presence of at least one GM *B. napus* plant (GT73) near a large oil mill in Germany (Franzaring *et al.* 2016). Within the oil pressing industry, loss of seeds is likely to take place during transfer to and from the processing site, during unloading of goods and possibly through waste streams. Because most oil crushing sites are in close proximity of harbours, seeds are mainly transported by ship. In addition, transport of commercial *B. napus* takes place through railway and road traffic. Feral oilseed rape can therefore be found along quays, railways and road verges that are connected with oil pressing sites.

In the Netherlands, 90% of the imported *B. napus* seeds are processed by the large oil pressing facilities ADM and Cargill (Tamis and de Jong 2009). These companies are situated in the large seaports of Rotterdam and Amsterdam respectively and are supplied by vessels. Spillage is expected to occur on the premises of the company or in the adjacent area. In addition, the Netherlands counts around ten small oil presses scattered across the country. It is unclear how these companies are supplied. However, it is very likely that the supply of seeds may take place through road traffic from a nearby harbour.

In order to select sampling sites near oil presses, we assessed companies by several criteria. First, it is important to make a distinction between the production process of large and small companies since this has a direct relation to the chance of seed spillage and the kind of transport chains involved (Tamis & de Jong 2009). Large companies, such as ADM and Cargill, use hot pressing, whereby seeds are crushed under high temperatures, high pressure and the addition of chemicals. Due to these extreme conditions, there is very little chance that viable seeds will remain in the waste product. Spillage of seeds may therefore only take place through the loading, unloading and transportation of bulk material on the premises and not necessarily through waste streams. As a result, plants are largely expected around places where transfer and transportation of goods takes place, like hoists and conveyor belts.

In contrast, small production facilities make use of cold pressing during which no chemicals are added. Therefore, waste products (i.e. oil cake) might still contain viable seeds. Furthermore, loading and unloading of seeds occur more frequently at small companies while transport takes place by train or truck. For this reason, spillage of seeds is expected to occur along the entire supply and transport routes causing plants not only to be found in the near vicinity of the oil press.

From the small oil pressing companies, it is known that the one in Farmsum is no longer in operation. Likewise, the chance of finding oilseed rape that established after recent introduction is less likely. However, it is still possible that plants have emerged from a persistent population through a yearly seed production or a soil seed bank that has formed during the period that the oil mill was still in operation. In 2010, feral oilseed rape was observed in roadside verges and along a quay (Luijten & de Jong 2010). These plants were probably remnants from the former oil pressing company. According to information on their websites, at least four companies produced their own seeds locally and were therefore not visited. It is very unlikely that GM seeds are found at the premises of these companies as the cultivation of GM *B. napus* is not allowed in the Netherlands.

Permission to access the large oil companies Cargill and ADM was obtained through ILT before entering preselected sampling sites. The oil pressing company in Nieuwe Tonge (Spack BV) did not give permission to access their premises and was therefore not visited.

During our visit, large companies Cargill and ADM were questioned about the origin of their imports, known GM contents, modes of transportation and minimalization strategies to prevent seed losses.

3.2.2. Transport

In the Netherlands, *B. napus* seeds and bulk derivatives are transported by ship (Tamis and de Jong 2009). However, distribution data also show presence of *B. napus* at train stations, railroads and shunting yards (Luijten & de Jong 2010). Spillage of *B. napus* may therefore occur during international transport when seeds are transported both by ship and train. In addition, transport from harbours to areas where *B. napus* seeds are processed mainly takes place via road traffic. Spilled (GM) *B. napus* might therefore be found around port areas, along quays and riverbeds, on and around railways and along motorways.

Detailed information on the mode and identity of transportation routes are not registered and could therefore not be used for the selection of sampling sites. This was especially the case for motorways, as truck drivers chose their own driving route and are not restricted to routes predefined by the oil processing company. Because no exact transport routes were known, we looked for *B. napus* plants around the main suggested transport routes according to their geographical orientation, the traffic density and available opportunities for *B. napus* to settle. These included riverbeds, ports, road verges and railway beds.

3.2.2.1. Ports and rivers

It is supposed that oilseed rape is largely transported by ship, causing ports and rivers to be major routes via which *B. napus* is transported. Since oil presses are mainly located near harbours, these areas serve as an important transhipment point and an influx and outflow of *B. napus*. Occurrence of the species in harbour areas has repeatedly been demonstrated in Europe. Previous research on the occurrence of GM *B. napus* in Switzerland showed high concentrations of plants at unloading sites of ships (Schulze *et al.* 2014). Consequently, the researchers suggested goods transportation by ships to be the main route for the introduction of GM *B. napus* events.

The largest quantity of all *B. napus* processed in the Netherlands is shipped to the ports of Rotterdam and Amsterdam (Tamis and de Jong 2009). In the present study we also focused on small ports that were in the proximity of oilseed processing companies. Furthermore, we selected sites along the major waterways via which bulk material is transported to those harbours.

3.2.2.2. Railroad tracks and shunting yards

Although it is presumed that seeds are not transported in large numbers by cargo train, spillage of GM seeds of *B. napus* along railways has been found abroad (Hecht *et al.* 2014, Schulze *et al.* 2014). The main railways over which goods are transported in the Netherlands are the 'Betuwelijn' (port of Rotterdam-Germany), the 'Brabantroute' (Dordrecht-Germany) and the 'IJsselroute' (port of Amsterdam-Germany) (see Figure 3A). The Betuwelijn is of particular interest because with the arrival of this track a fast and direct connection between the port of Rotterdam and Germany has been established. This connection forms the gateway to the rest of Europe and might therefore be an important inter-European route in case *B. napus* is transported by train. In addition, large parts of the Betuwelijn are solely used for freight traffic, while the other two tracks are shared with passenger transportation (see Figure 3B). Since 2016, however, a large part of the Betuwelijn (from Dordrecht to the German border) is not or partly available for freight. Trains are temporarily diverted via the Brabantroute and IJsselroute, while the Betuwelijn almost solely transports dangerous goods rather than products that can be linked to *B. napus* (pers. comm. ILT). In case *B. napus* is transported by train, these two tracks might be the most important routes along which *B. napus* may currently enter the Netherlands and might be transported through the country.

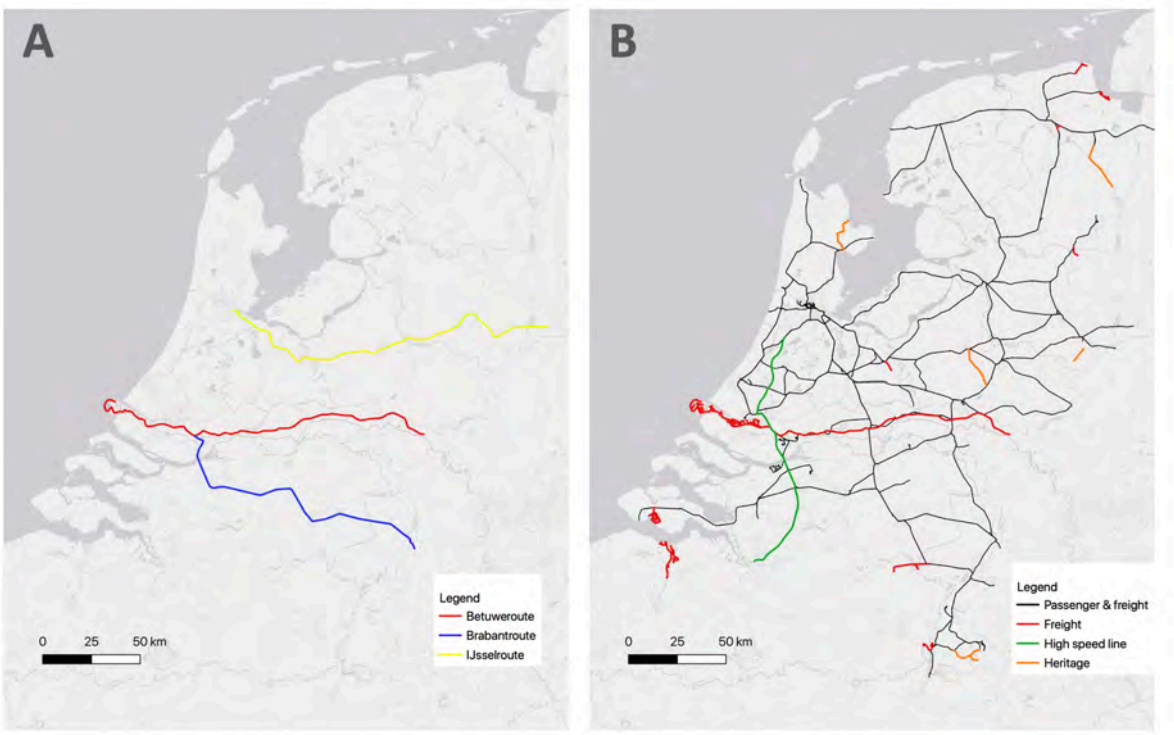

Figure 3. Railroad tracks in the Netherlands, specified as (A) main routes and (B) type of routes.

In order to check for sampling sites, the entire length of the three mentioned transportation tracks were screened for potential sites of seed spillage using Google Earth. We mainly considered shunting yards, bends, switches, stations and passageways since these are places where seed spillage is likely highest. Presumably, the cargo experiences more vibrations which means that the seeds are lost more easily. In addition, we identified sites in close vicinity of oil crushing plants as hotspots and included sites of previous observations of feral *B. napus* like the train stations in Ede-Wageningen and Woerden. Here, oilseed rape plants have been found for several consecutive years (Luijten & de Jong 2010) and have been seen more recently. This suggests the presence of recurring plants and probably a self-recruiting persistent population.

Railways are of particular interest because they could be treated with herbicides, like glyphosate[3]. All known GM *B. napus* events that are authorised for import into the Netherlands are resistant to herbicides containing glufosinate or glyphosate. Genetically modified *B. napus* has a selective advantage over conventional *B. napus* and *B. rapa* at sites where herbicides are applied for weed control. It has been shown that spraying of herbicides promotes selection of herbicide tolerant plants and thus the persistence and spread of genetically modified *B. napus* (Londo *et al.* 2010). However, this will only be the case if there is a selection pressure in the presence of the herbicide. ProRail, who is responsible for the maintenance of the Dutch Railways, was therefore asked about their use of herbicides on railway tracks. It appeared that herbicide use was not registered by ProRail as the actual maintenance was performed by different parties that make their decisions locally (BAM rail, Strukton rail, ASSET rail and Volker rail). In turn, these companies did not keep track of any eradication activities which makes sites that have been applied with herbicides untraceable. However, the use of glyphosate on railway tracks is not prohibited by the Dutch law, so there is a good possibility that glyphosate is used as a weed killer at railroad tracks.

3.2.2.3. Highways, provincial and local roads

Beside waterways and railways, motorways form another transport route along which *B. napus* can escape directly into the environment. During the process of selection, we scanned for hotspots along highways using freely accessible geographical data on Google Earth. We concentrated on highways near harbour areas, oil processing factories and motorways that are in direct connection with the German and Belgian borders. Along those highways, sites were selected depending on the direction of import as more plants are usually found along the road leading towards oilseed processing companies rather than the verge at the opposite site of the road (Crawley and Brown 2004).

In 2008 and 2009, *B. napus*, as well as a number of F1 hybrids, were found at a disturbed site close to carpool parking in Almere (Luijten & de Jong 2010). Recent observations have revealed that the population has now disappeared (pers. comm. Tom de Jong). In addition, a number of plants were observed along the highway A2 between Amsterdam and Eindhoven in 2009. Whether these plants were still present was not yet clear at the start of the sampling period.

Road verges are annually mown from July onwards and usually after the seed set of *B. napus* and *B. rapa*. It is thus very likely that seeds are spread from annual seed dispersal, creating persistent populations.

3.2.3. Other sites

Nowadays, information on species observations are intensively shared on the internet on so called citizens science platforms. As a consequence, online platforms, like waarneming.nl, can serve as a valuable source of information on recent observations of oilseed rape that is readily available. In the period of January 2018 to March 2019, a total of 172 reports of oilseed rape were recorded on waarneming.nl.

The area in which oilseed rape was observed, was concentrated in the middle of the country along the major rivers (Waal, Rijn and Maas) and in the northeast near oilseed rape fields. A number of recurring sites have been reported around Almere - De Poort (fallow land), Duffelt - Millingerwaard (riverbed) and Finsterwolde - Oostwolderpolder (along oilseed rape fields). Sometimes, several reports were made by the same person. Not all observations were equally reliable. A large part of the observations probably concerned the closely related and phenotypically resembling rapeseed, which is more common in the Netherlands and which is difficult to distinguish from oilseed rape. For this reason, the most relevant locations from all the observations of the past year have been selected on the basis of *i)* the presence of important transport routes and/or handling and processing sites such as ports and oil presses, and *ii)* the reliability of the observation on the basis of any available photographic material or local habitat characteristics. The latter included disturbed sites and places where competition with other species is low (road verges, pavements, quays, railway beds) as these are sites where the establishment of *B. napus* is largely expected.

[3] https://cdn.nufarm.com/wp-content/uploads/sites/39/2019/03/11163944/Productfolder-2019-BE-NL.pdf

3.3. Sampling procedure

Sampling of plant material on the preselected sites took place from April to June, during the main flowering period of *B. napus* and before the start of the mowing season. Plants were mainly flowering during this period but non-flowering plants that already bore fruits were also observed. Plants of all different growth and developmental stages (seedling, mature, flowering) were sampled only when they could be determined on the basis of morphological appearance. Finally, all sampling sites at railways were only entered under surveillance of ILT and after passing a restricted safety test.

From each plant, one to three leaves and/or flower buds were collected and immediately stored in plastic bags or tubes at 4 °C until further analysis. The collection of flower buds was preferable to that of leaf material, because of the larger amount of rapidly dividing tissue that is found in the buds. However, in many cases the plants were already in fruit due to the warm and dry spring in 2019, so it was not always possible to collect flower buds. When flower buds were absent, leaf material was collected. The youngest and greenest leaves were sampled since extraction of young leaves generally provides a better quality of DNA. In some cases, even leaves were absent and seed pods had to be collected. If plants were found individually, they were sampled separately. In case more plants were growing together, a maximum of 25 plants were sampled and were pooled up to a maximum of 5 individuals per sample. Where possible, additional leaf material was collected separately so that plants could be screened twice in case GM *B. napus* events would be detected.

Whenever *B. rapa* was found either on railway tracks or in the direct vicinity of *B. napus*, leaf material from *B. rapa* was also collected. At all sampling sites, the coordinates of plants were recorded, and the population size was estimated. Every observation was photographed in order to build an archive of references data on phenotypic characteristics. GPS coordinates were plotted on a map in order to visualize the current distribution of *B. napus*.

3.4. Results

In total, 710 individuals of *B. napus* were sampled at 25 sites (see Table 3). In addition, leaf material of 17 *B. rapa* and 28 *S. arvensis* plants was collected. Locations where *B. napus* was observed and sampled, included both preselected areas and sites that were discovered by accident during our survey. The latter ones were mostly situated along motorways. Table 3 shows all locations that were visited during our study.

Strikingly, most *B. napus* plants were found along rivers and highways as transportation routes rather than railways (see Table 3). At sites where *B. napus* was observed, plants were often scattered throughout the area, while massive growth was only faced on the premises of the large hot-pressing oilseed factories. This was in line with previous observations of *B. napus* in the Netherlands, where plants were found in small numbers (<25 plants) and in a linear shape (Luijten & de Jong 2010). In the following sections, presence (and absence) of *B. napus* at the different sampling sites are discussed in further detail.

3.4.1. Oil pressing companies

At the two large oil pressing companies Cargill and ADM, oilseed rape plants were found on the premises. These companies are supplied by vessels. It is an open system where seeds are grabbed by a crane from a supply vessel to release them in a weighing silo before they are transported on an open conveyor belt to the storage facility. At both companies, oilseed rape plants with an erect (flowering) stem were recently mown, which made it difficult to estimate accurate plant numbers. Nevertheless, large numbers of young rosettes plants were observed along the storage facility and at a pile of sand together with sunflowers at Cargill (see Figure 5A). At ADM non-flowering rosettes were found and sampled as well. It was not allowed to take photos at this company. In Europoort, close to ADM, flowering oilseed rape plants were also present along a private railway. This private cargo track was no longer in use or not used for an extended period of time. A small population of oilseed rape was still present here in 2019 as it was in 2008 and 2009, although the population size was larger than (Luijten & de Jong 2010).

Table 3. Overview of the number of *B. napus* plants (and other members of the Brassicaceae) that were observed and sampled near oil presses and along transportation routes.

Type	Nr	Location, description	Species	Observed	Sampled
Oil presses	1	ADM-Europoort, Rotterdam, *premises*	*B. napus*	250-500	47
	2	Cargill BV, Amsterdam, *premises*	*B. napus*	500-1000	67
	3	Noord-Nederlandse Oliemolen BV, Harlingen, *supply route*	*B. napus/B. rapa*	3/8	2/3
	4	Noord-Nederlandse Oliemolen BV, Farmsum, *supply route*	*B. napus*	35	22
	5	Oudendijk Oils BV, supply route	*B. napus*	1	1
Railways	6	Europoort, Rotterdam, *private cargo rail track*	*B. napus*	15	14
	7	Arnhem, *station and shunting yard*	*B. napus*	0	0
	8	Botlek, *rail track*	*B. napus*	15	15
	9	Kijfhoek, *shunting yard and rail track*	*B. napus*	25	24
Betuwelijn	10	Hardinxveld-Giessendam, *station* (pers. comm. L. Langbroek)	*B. napus*	0	0
	11	Pernis, *rail track*	*B. napus*	40	25
	12	Rozenburg, *rail track*	*B. rapa*	50-75	5
	13	Waalhaven, *shunting yard*	*B. rapa*	5	5
	14	Zevenaar, *railtrack*	*B. napus*	0	0
IJsselroute	15	Almelo, *station*	*B. napus*	0	0
	16	Amersfoort, *shunting yard*	*B. napus*	0	0
	17	Deventer, *station*	*B. napus*	0	0
	18	Hengelo, *station*	*B. napus*	0	0
Brabantroute	19	Blerick, *station, rail track and shunting yard*	*B. napus*	3	3
	20	Blerick, *railroad crossing*	*B. napus*	1	1
	21	Venlo, *station and shunting yard*	*S. arvensis*	80	23
	22	Venlo, border with Germany *rail track*	*S. arvensis*	5	4
Other freight tracks	23	Axelse vlakte, Terneuzen, *rail track*	*B. napus*	0	0
	24	Barneveld (animal feed company), *supply rail track*	*B. napus*	0	0
	25	Moerdijk, *rail track to industrial area*	*B. rapa*	15	4
	26	Sloe, Vlissingen, *rail tracks and shunting yard*	*B. napus*	4	4
Other combined freight & passenger	27	Dordrecht, *station*	*B. napus*	0	0
	28	Driebergen-Zeist, *station*	*B. napus*	0	0
	29	Ede-Wageningen, *station*	*B. napus*	5 (dead)	0
	30	Woerden, *station*	*B. napus*	50	10
	31	Zwolle, *station and shunting yard*	*B. napus*	0	0
Ports and waterways	32	Amsterdams Rijnkanaal (pers. comm. T. de Jong)	*B. napus*	0	0
	33	Lobith, *port*	*B. napus*	200	122
	34	Nijmegen, *port*	*B. napus*	0	0
	35	Waal, *riverside*	*B. napus*	>400	121
Highways and local roads	36	A1 (Voorthuizen - Terschuur - Hoevelaken)	*B. napus*	>200	0
	37	A2 – Utrecht	*B. napus*	10	0
	38	A4 – Delft	*B. napus*	0	0
	39	A7 (Scheemda - Marum)	*B. napus*	>100	0
	40	A12 (De Meern – Woerden)	*B. napus*	>100	0
	41	A28 (Zeist – junction A27)	*B. napus*	>50	0
	42	A32 (Barneveld)	*B. napus*	2	0
	43	N35 – Almelo	*B. napus*	>25	3
	44	A44 – (Oestgeest – Wassenaar)	*B. napus*	4	4
	45	A67 – Venlo-Eindhoven	*B. napus*	>300	15
	46	N62 – Axel	*B. napus*	>25	16
	47	N237 – Bilthoven	*B. napus*	10	9
	48	N243 - Schermerhorn	*B. napus*	1	0
	49	N254 – Sloe	*B. napus*	>15	13
	50	N307 - Hoorn-Hoogkarspel	*B. napus*	?	0
	51	N470 - Delft	*B. napus*	0	0
	52	N811 – Zevenaar	*B. napus*	>25	14
Other	53	Amsterdam, *city centre*	*B. napus*	2	0
	54	Delft, *city centre*	*B. napus/S. arvensis*	3/1	3/1
	55	Goor and surroundings	*B. napus*	250	94
	56	Heiloo, *shopping centre*	*B. napus*	1	0
	57	Leiden, *residential area*	*B. napus*	0	0
	58	Utrecht, *public park*	*B. napus*	>25	20
Total			*B. napus*	>2500	710

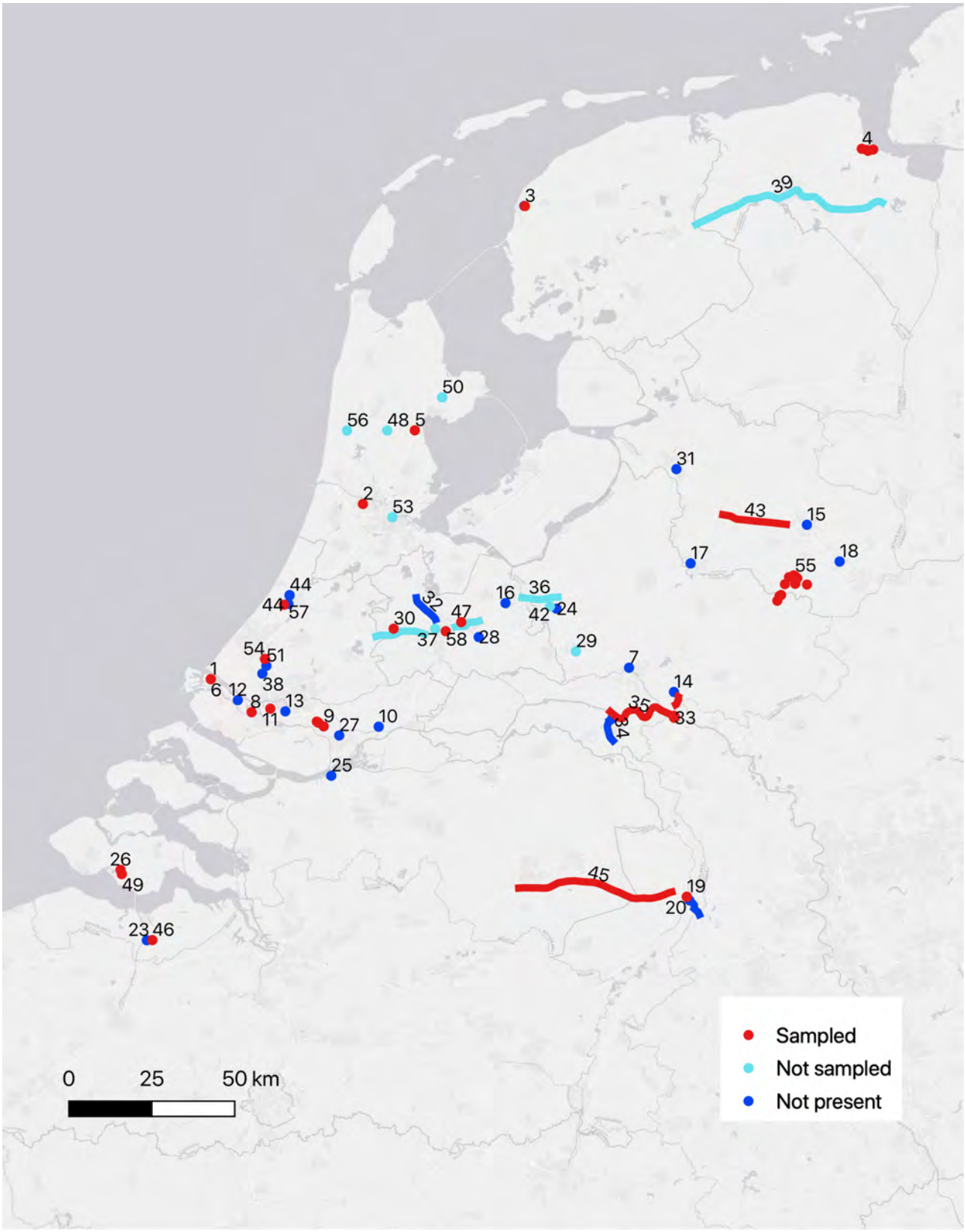

Figure 4. Presence (or absence) of *B. napus* near oil pressing companies and along transportation routes. Red dots represent sites where *B. napus* was observed and sampled, light blue dots represent sites where *B. napus* was observed but plants could not be sampled due to safety reasons and/or access restrictions, and blue dots represent sites where *B. napus* was not found. Numbers correspond with the location numbers in Table 4.

Both ADM and Cargill were asked about the origins and yearly amounts of processed *B. napus* seeds. According to both companies, the processing of oilseed rape only made up a small part of their production process (about ten percent). According to Cargill, imported bulks were imported from non-GM sources in Australia. In order to ensure the absence of (contamination with) GM seeds, the company tests incoming seed material on a regular basis.

At two small facilities, individual plants were found in verges of transport roads in the direct vicinity of the company. In Farmsum, around 40 plants were observed in road verges close to a roundabout. This road might be a former transportation route leading to the smaller Farmsumerhaven or the main port of Farmsum. Only one plant was observed along the road to the former oil press company Nieveen see Figure 5B) which belonged formerly to Noord-Nederlandse Oliemolen b.v. Ten years ago, less than five plants were seen in this area (Luijten & de Jong 2010). Only one plant was found in the vicinity of the oil press Oudendijk Oils BV which is situated along a local road. The road verges just outside the oil pressing company were recently mown. It was therefore not possible to estimate the number of feral oilseed rape plants. The observed plant was found in the vegetation in front of a fence (see Figure 5C) and probably survived the mowing activities. It is possible that the plant established from seed spillage during transportation to the nearby oil mill as it was found several tens of meters from the company.

We did not face any plants of *B. rapa* at the hot oil presses. Only at Harlingen, a few rapeseed plants were found which were collected for analysis (see Table 3).

Figure 5. *Brassica napus* in the vicinity of (former) oil pressing companies: **(A)** young rosettes in front of a storage facility at Cargill BV, **(B)** a fruiting plant along a narrow road to Oudendijk Oils BV, and **(C)** a large flowering plant nearby the abandoned oil pressing company 'Nieveen', a part of Noord-Nederlandse Oliemolen in Farmsum. Photos: Sheila Luijten

3.4.2. Railroad tracks

In the period of May-June, freight railways, shunting yards, switch points, bends and passageways were visited under the supervision of ILT security guards. The presence of *B. napus* at these localities was rather variable. Very few *B. napus* plants were found in the eastern part of the Netherlands along the Brabantroute and IJsselroute (see Table 3). Here, most railways were well maintained and cleared from weeds, i.e. train stations Arnhem, Hengelo, Almelo, Deventer, Dordrecht and Zwolle, rail tracks in Zevenaar close to the German border, and the rail track between Venlo and Blerick in the direction of Eindhoven (see Figure 6A). Railway beddings were 'clean' and freed from vegetation. In some cases, the entire ballast was renewed, which was for example the case in Driebergen-Zeist where the whole station was renovated. Ballast beds are usually washed before they are replaced (pers. comm. ILT-rail safety guard), which might be the reason that at some parts no weeds were found. In some cases, the vegetation was treated with herbicides, which was observed at train station of Venlo (see Figure 6B).

Figure 6. Impression of visited rail tracks: **(A)** clean track between Venlo and Blerick and **(B)** herbicide treated vegetation at train station Venlo. Photos: Sheila Luijten.

It was not clear whether the yellow plants at the train station in Ede-Wageningen were treated with herbicides or that they were dried because of the warm and dry spring of 2019 (see Figure 7). *Brassica napus* has been observed at this train station between platforms since 2008 (Luijten & de Jong 2010). In early Autumn of 2019, green rosettes were still present (pers. comm. Gerard Oostermeijer (IBED-S4N)).

Figure 7. *Brassica napus* plants (circled in light blue) **(A)** before and **(A)** after possible application of herbicides. *Photos*: Lilian Seip.

In contrast to the other two routes, many *B. napus* plants were observed and sampled along the Betuweroute. Plants were found in the harbour area of Rotterdam along various shunting yards and freight train tracks at Pernis, Kijfhoek, Waalhaven, Botlek (see Figure 8A-D) and Europoort (discussed in section 5.1.1.). In addition, plants were seen at train station Woerden, which might form an old relict from transport routes which were used before the arrival of the Betuwelijn. Here, plants were found close to the platform (see Figure 8E) and on the middle and main tracks which were used by high speed trains. Only plants close to the platform could therefore be sampled. Population sizes at the other sites were small and the number of plants varied from five to forty. Interestingly, plants were mainly found in

the bedding of the daily used freight tracks and not in the bedding of the shunting yards. A short visit was made to inspect a part of freight rail track in between shunting yards near Rozenburg. No *B. napus* plants were found on this track, only plants of *B. rapa* that reached the railroad from a steep slope adjacent to the highway.

Very few plants were present along freight tracks in the industrial areas of Vlissingen and Terneuzen. Only four plants were found at a very small shunting yard at Terneuzen. In the 'Sloe-area' (port and industrial area of Vlissingen), *B. napus* plants were observed along a local main road of the industrial site and a single plant at a junction with a railroad crossing. No plants were faced along freight train tracks. This raises the question if plants found at railroad crossings are lost from trains or lost from trucks. This was also the case in Blerick (see Figure 8F) and the small port and industrial area around Axel, which is also known as Axelse vlakte. Here, no plants were found in the area, but they were seen along a main road and roundabout. Oilseed rape plants grew together with other cereals in the road verges. This might suggest that transport of seeds in this area mainly goes by trucks and not by cargo trains or supply vessels, although the roundabout was close to a railroad crossing. It is also possible that spillage of seeds is more prominent with trucks than with cargo trains and vessels.

At the freight track to the industrial area of Moerdijk, no *B. napus* plants were found. Only *B. rapa* plants, which did not typically resemble *B. rapa*, were observed and sampled for analysis.

On suggestion of ILT-railway, a visit was made to an old freight railroad track to an animal feed store company in Barneveld. No oilseed rape plants were found in the vicinity of the company, roads and railroad track.

Figure 8. Impression of sites along railroad tracks with *B. napus* plants at **(A)** Pernis, **(B)** Kijfhoek, **(C)** Waalhaven, **(D)** Botlek, (**E**) railway station Woerden, and **(F)** railroad at Blerick. Photos: Sheila Luijten.

3.4.3. Inland ports and river Waal

In the beginning of May, a number of locations along the Waal near Nijmegen were visited, both on the north and the south side of the river. Within this area, about ten observations were registered at waarneming.nl in several consecutive years, some of which clearly considered *B. napus* when seen from the available photographic material. On the south side of the Waal we looked for *B. napus* along the route that leads from the quay of Nijmegen along the river banks of the Waal and ended at Millingen aan de Rijn. Several hundred plants were found in this area. The plants were mainly present on the groynes (see Figure 9A) but were also sporadically found on the 'beaches' (see Figure 9B). Most plants were vigorous but small plants were also observed in large quantities. This might indicate the presence of a rejuvenating population maintained by either self-recruitment or a continuous input of seeds. The origin of the plants could be linked to seed spillage from ships going up and down the river Waal or seeds transported from upstream the river, as the plants were mainly found along the waterside. In contrast, *B. rapa* was hardly found along the water's edge, but rather on the dikes.

On the north side of the Waal, a smaller area was visited. At this site of the river, more than 50 plants were found, mainly on the groynes. The actual population size was possibly larger than could be estimated from our observations alone, because the population supposedly extended further than the sampled area. The north side of the river, however, appeared to offer fewer suitable places for *B. napus* to establish. Consequently, no plants were observed at this side of the river, except for a small harbour at Lobith that serves as a port of refuge. Here, more than 50 *B. napus* plants were found. Plants mainly grew on the embankment and in the gravel bed along the water. The plants in the gravel bed were very large and bore fruits (see Figure 9C). Although it is not a transhipment site, there is a possibility that seeds have fallen off the ships when residing at the inland port. Finally, no *B. napus* was found in the port of Nijmegen, along the Maas-Waal canal or the Amsterdam Rijnkanaal (pers. comm. Tom de Jong).

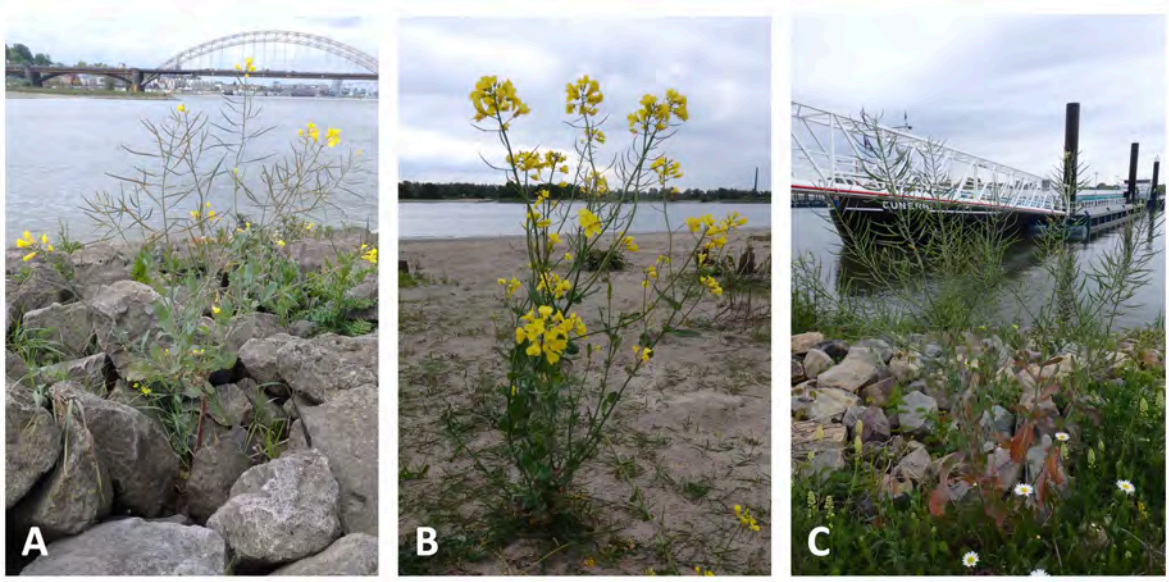

Figure 9. *Brassica napus* observed along the Waal **(A)** in the gravel bed on the groynes, **(B)** on the sandy part between groynes and **(C)** in the harbour of Lobith. Photos: Lilian Seip.

3.4.4. Highways, provincial and local roads

At many spots along various highways, oilseed rape plants grew just on the border of the asphalt, underneath the crash barrier. Here, the habitat is more open and probably more similar to an arable field than the dense vegetation behind the crash barrier where its wild relative *B. rapa* is usually found. *Brassica napus* may also be salt tolerant, because more salt-tolerant plant species were seen in the small strip along the asphalt. Plants of *B. napus* occurred scattered in small groups (1-8 plants) in stretches of dozens of kilometres and may add up to several hundreds of plants. Only at highway A67, plants could safely be sampled with the security of ILT (see Figure 10A).

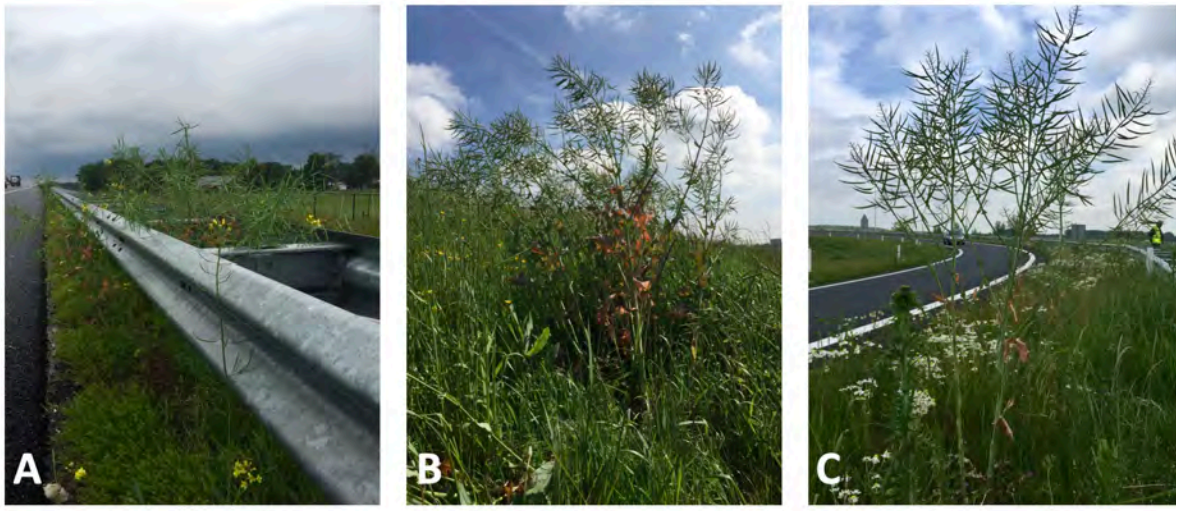

Figure 10. Brassica napus along **(A)** highway A67 (between Venlo and Eindhoven), **(B)** a local road at Sloe (port and industrial area near Vlissingen) and **(C)** provincial road N62 near Axel. Photos: Sheila Luijten.

Apart from highways, plants were found along provincial roads and local roads. Plants were often seen in curves of the road or on highway ramps, suggesting spillage of seeds transported by trucks. This was for example the case near Axel (see Figure 10C).

In the Achterhoek, in the area around Goor, at least 13 populations of various size from a single plant up to 200 plants were found in road verges (see **Error! Reference source not found.**The presence of these populations could not be explained transportation routes to oil pressing companies.

3.4.5. Other

The origin of at least three observations could not be traced back to the spillage of seeds during oilseed handling or transport. In the centre of Delft, four plants were found in a flowerbed along a canal. On the basis of their phenotypic appearance, probably at least one plant was the strong resembling field mustard (*S. arvensis*). In addition, *B. napus* plants were found in the shopping area of Heiloo and the centre of Amsterdam. In Utrecht, about 30 plants were found in a small public park. Here, *B. napus* was observed in a denser patch and the majority of plants seemed to have been here for more than one flowering season. We assumed that the origin of these plants probably lied in the sowing of a flower mixture or bird feed. The presence of these plant could also have been the result of added soil containing *B. napus* seeds. Seeds may have established from dormant seeds after the soil was disturbed.

4. Detection of GM *B. napus* events

4.1. General

Genetically modified *B. napus* can be detected with molecular methods using quantitative real-time PCR (qPCR). Wageningen Food Safety Research (WFSR), part of Wageningen University & Research, is the National Reference Laboratory for GMO detection in food and feed and is accredited for GMO screening and identification. WFSR is also member of the European Network of GMO Laboratories (ENGL). Certified Reference material (IRMM, Geel, Belgium) is available for all authorized GM *B. napus* events and those that are withdrawn from the EU-market. For *B. napus* event 73496 that is in the process of being authorized, Certified Reference Material is also available (AOCS, Urbana (IL), USA).

4.2. Screening

For the screening on presence of GM *B. napus* in feral populations in the Netherlands, an inventory was made on known *B. napus* GMOs worldwide (see Table 2). Based on the elements present in the GM *B. napus* events, a strategy was designed to cover all events with a minimum set of qPCR detection methods. With GM *B. napus*, the definition restricts here to 'classical GMOs' and not to plant products obtained by new mutagenesis techniques (ENGL 2019).

In order to design a minimum set of detection methods that cover most GM *B. napus* events, the elements for which a detection method exists were inventoried (www.EUginius.eu). In Table 4, the detectable elements are categorized by their number count.

With the five markers P-35S (promoter of the 35S of Cauliflower mosaic virus), T-nos (terminator of nopaline synthase from *Agrobacterium tumefaciens*), T-ocs (terminator of octopine synthase from *Agrobacterium tumefaciens*), T-E9 (pea ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) *E9* gene) and the construct-specific method for gat/T-pinII (junction between the *Bacillus licheniformis* glyphosate-N-acetyltransferase gene and the proteinase inhibitor II terminator from *Solanum tuberosum*), it is possible to cover 26 events. Positive signals obtained with the set of screening elements could also be derived from their donor organism; P-35S is derived from CaMV, which has a broad host range in the Brassicaceae and can cause false-positive signals. Therefore, a CaMV detection method was applied to exclude positive signals derived from the virus.

MPS961 to MPS964 (Phytaseed™ *B. napus*) cannot be detected with this screening set. According to the ISAAA [7], these are only deregulated in the USA for food and feed use, but not for cultivation and not approved elsewhere. According to the United States Department of Agriculture (USDA)[4], these are not commercialized anymore. For these reasons, it is not likely that these events are present in feral populations in the Netherlands, and it is therefore not relevant that these events are not covered in the screening. ZSR500, ZSR502, ZSR503 and HCR-1 can be detected, but are not commercialised or no longer registered according to ISAAA (ISAAA Approval Database), CFIA[5] and USDA and are therefore unlikely to occur in the Netherlands.

[4] https://www.ams.usda.gov/sites/default/files/media/BECanolaCropSummary.pdf

[5] http://inspection.gc.ca/active/netapp/plantnoveltraitpnt-vegecarnouvcn/pntvcne.aspx#table-heading

Table 4. Elements of GM *B. napus* with an available detection method. In green the selected screening elements. For events for which an event-specific method is available, the detection (x) is confirmed. For other events, the x is based on theoretical data.

GMO	Event method available?	P-35S	T-nos	T-35S	pat	P-nos	P-FMV	nptII	cp4-epsps	T-ocs	bar	T-E9	P-ta29	T-g7	barstar	barnase	gat/T-pinII
23-18-17		x						x									
23-198		x						x									
61061																	x
73496	yes																x
DHA		x	x		x												
Falcon GS 40/90		x		x	x												
GT200							x		x			x					
GT73	yes						x		x			x					
HCN10		x		x	x	x		x		x							
HCR-1		x		x	x												
LBFLFK				x						x							
Liberator		x		x	x												
MON88302	yes						x		x			x					
MPS961																	
MPS962																	
MPS963																	
MPS964																	
MPS965			x			x		x									
Ms1	yes		x			x		x		x	x		x	x		x	
Ms11			x			x					x		x		x	x	
Ms8	yes		x								x		x	x		x	
OXY-235		x	x														
Rf1	yes		x			x				x	x		x	x	x		
Rf2	yes		x			x		x		x	x		x	x	x		
Rf3	yes		x								x		x	x	x		
T45	yes	x		x	x												
Topas 19/2	yes	x		x	x	x				x							
ZSR500	yes (GT73)						x		x			x					
ZSR502	yes (GT73)						x		x			x					
ZSR503	yes (GT73)						x		x			x					
Total		**10**	**9**	**7**	**7**	**7**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**5**	**4**	**3**	**2**

4.3. Laboratory screening

In the laboratory, equal amounts of leaf material from a maximum of five individual plants were combined and isolated in duplicate. Material of individual plants was also stored separately. The samples were subjected to the endogenous control (*FatA*). The samples were subsequently screened for the presence of the five selected GM elements, including a suitable positive control for each screening element. One or more detected elements will indicate that one or more plants within the set of five individual plants is indeed GM *B. napus*. Here, the expected signal strength of one out of five GM leaves would be not more than 2.32 Cq values later as the *FatA* signal. Samples from a maximum of

five plants are pooled, so we cannot determine which of the five plants was actually genetically modified. Although identification was not part of this study, several samples were subjected to event-specific detection methods to rule-out some faint signals in the screening. When identification was required, this was performed *in silico*, for example in the analysis module of EUginius, or in the laboratory. For laboratory analyses, the *B. napus* events 73496, GT73, MON88302, Ms1, Ms8, Rf1, Rf2, Rf3, T45 and TOPAS 19/2 can be identified with event-specific qPCR methods at WFSR. The choice for one or more events is based on the detected element(s). For other combinations of detected elements, EUginius can be used to determine the responsible events(s).

4.4. qPCR theory

The quantitative real-time polymerase chain reaction (qPCR) allows amplification and detection of a specific target. The outcome is a quantification cycle (Cq) at which the signal is detected. Therefore, an early (lower) Cq value means that more target is present, while a late (higher) Cq means that less target is present.

To establish the amount of *B. napus* DNA, the endogenous *FatA* target is applied. Assume this has a Cq value of 21 for the leaf samples. Assumed that a leaf is 100% GM (heterozygous, with only one copy of each GM element and two copies of the endogene in a haploid genome according to Jacchia et al., 2018)), the Cq value for the GM element should be 1 Cq value later than the endogenous control. When only 1 out of 5 leaves are positive for one or more of the elements tested, the expected signal of the GM element will have a later Cq value. The ΔCq after a dilution is calculated as log (dilution)/log 2 under optimal conditions. This means that no inhibition is expected, and the qPCR works optimal. A 5x 'dilution' (one out of five leaves) of a signal caused by GM element compared the endogenous control will therefore be 2.32 Cqs later (log 5/log 2 = 2.32). A signal of the screening elements should be considered positive when the Cq value is around 21+1+3.32=25.32 or earlier. According to the same principle, a signal that is 10 Cq values later than the endogene would mean about 1000 times lower amount of the GM element. As proof of principle, Certified Reference Material (CRM) of *B. napus* T45 was used at different dilutions GM and screened with P-35S to confirm this calculation in practice (see Table 3). Here, the *FatA* signal in 100% T45 should be compared to the P-35S signal in 10% T45 since one out of five leaves can be heterozygous GM. In this example, the Cq value of P-35S is 3.80 Cq values later. Therefore, as a rule-of-thumb, a signal from the GM elements that is more than 5 Cq values later, cannot be derived from one or more leaves in the sample.

Table 5. Results of screening elements tested with dilutions of T45 CRM. ND = not detected.

Sample	FatA Cq	P-35S Cq
100% T45	**21.64**	22.14
80% T45	22.00	22.46
60% T45	22.32	22.99
40% T45	22.73	23.47
20% T45	23.45	24.42
10% T45	23.82	**25.44**
1% T45	24.50	28.97
0.1% T45	24.59	32.20
0% T45	24.59	ND
MQ	ND	ND

4.5. Materials and methods

4.5.1. DNA extraction

DNA was isolated from 100 mg plant material in equal amounts of maximum 5 leaves in duplicate using a CTAB/Qiagen lysis step, followed by the DNeasy Plant Mini Kit (Qiagen) isolation as described in Prins *et al.*, (2017). Optical density was measured on a Nanodrop spectrophotometer (ND-1000, Montchanin, DE, USA) in order to quantify and check the purity of the DNA. DNA was diluted with water (Gibco distilled water, DNase/RNase free, Life Technologies, Grand Island NY, USA) to 10 ng/µl final concentration and kept at 4 °C, or at −20 °C for long-term storage.

4.5.2. qPCR

Real-time qPCR analyses were performed using screening methods (Scholtens *et al.* 2013, 2017) and targeting the P-35S and T-nos (Kuribara*, et al.* 2002), T-ocs and T-E9 (Debode *et al.* 2013) elements and the *gat*/T-pinII construct (Prins *et al.* 2017). Primers and probes are described therein. Real-time PCR was performed in 96-well microtiter plates in 25 µl reaction volume, containing 1× universal mastermix (DMMLD2D600, Diagenode, Liège, Belgium), 400 nM of each primer, 200 nM probe and 50 ng DNA. PCR was performed with the CFX (Bio-Rad) with an initial UDG decontamination step at 50 °C for 2 min. After an initial denaturation at 95 °C for 10 min, 45 cycles were performed (denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min). Each of the two DNA isolations was analysed once in order to retrieve two qPCR results per sample.

4.5.3 Endogenous control

For a positive control of the amplifiability of the isolated DNA, the detection method for the *fatA* gene (QL-TAX-FatA primer1/FatA primer2: www.EUginius.eu), commonly used in the European Network of GMO Laboratories (ENGL) as endogenous control for *B. napus*, was applied. This method works well for the detection of DNA amplification in *B. napus* (Monsanto, 2004) and targets the endogenous acyl-acyl carrier protein thioesterase (*FatA*) gene. The estimated number of target copies per haploid *B. napus* genome is two (Jacchia *et al.*, 2018).

In silico analysis of the *FatA* amplicon (see Table 6) shows that *B. napus* and *B. oleracea* are detected 100%. Although there are some mismatches, the *FatA* amplification probably also works in related species like *B. rapa*, *B. juncea* and *Raphanus sativus*.

Table 6. In silico analysis of the host range of the *FatA* detection method. Underlined are the primers and probe. In bold are the mismatches. The codes refer to the GenBank entries.

Amplicon	GGTCTCTCAGCAAGTGGGTGATnATGAACCAAGACACAAGGCGGCTTCAnnnnnTTACAGATGAAGTTCGGGACGA
B. napus X87842	GGTCTCTCAGCAAGTGGGTGATGATGAACCAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGTTCGGGACGA
B. oleracea LR31873	GGTCTCTCAGCAAGTGGGTGATGATGAACCAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGTTCGGGACGA
B. juncea AJ294419	GGTCTCTCAGCAAGTGGGTGATGATGAA**T**CAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGTTCGGGACGA
B. rapa LR031576	GGTCT**TA**CAGCAAGTGGGTGATGATGAACCAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGTTCGGGACGA
R. sativus XM_018622298	**C**GT**GC**TACAGCAAGTGGGTGATGATGAACCAAGACACAAGGCGGCTTCAAAGAGTTACAGATGAAGTT**A**GGGACGA

Once the endogenous control is positive, we know that the DNA is being amplified and the outcome of the subsequent screening can be correctly interpreted with respect to 'Not Detected' (ND) results; these will be truly ND since the positive control was established.

4.6. Results

Results of the qPCR reactions are depicted in Table S1 and Table S2. Here, the Cq values of the qPCRs performed for endogene detection (*FatA*), and the screening elements (P-35S, T-nos, T-ocs, T-E9 and construct gat/T-pinII) on 160 pooled samples (representing 668 individual plants) are given. All samples tested positive for the endogenous *FatA* control (Cq~21). This means that all samples investigated contained either *B. napus* and/or *B. rapa, B. oleracea, B. juncea* and *R. sativus*. It also predicts the latest Cq value at which a sample should be considered 'detected' for one or more of the GM screening

elements. As explained in the theoretical calculation of the qPCR results, the 'detected' Cq value should be before Cq=26.

In some samples (Table S1), P-35S, T-nos and/or the T-E9 gave very late signals (Cq > 36). These signals are not relevant for the detection of GM *B. napus* since the ratio between the Cq value of the endogenous control *FatA* and the Cq value of the element are not in accordance with calculated expected value. Moreover, in the samples that show background signals of the screening elements, the difference in Cq between endogene and GM-element is at least 10 Cq values. This indicates a factor 1000 difference. Hence, the signal of the GM-element cannot be derived from one single GM *B. napus* leaf and is possibly derived from other sources than the leaves.

To test this hypothesis, two detection methods for CaMV (Cankar *et al.* 2005, Chaouachi *et al.* 2008) and other elements-specific detection methods for GM elements pat and nptII were tested on a few samples. CaMV is the control for presence of the Cauliflower mosaic virus, while pat and nptII are elements that should be positive in case a GM *B. napus* would be present, as can be seen in Table4. The CaMV methods applied were both negative for all samples tested, so presence of the virus strains that can be detected by these methods could not be confirmed as a source of the P-35S signal. This can be caused by the many variations in sequence of CaMV strains. Pat and nptII were either not detected or had a late Cq of >36. Therefore, it was concluded that no GM *B. napus* was present in the samples.

Sample 16 (Europoort) showed a very early Cq value (18.54 on average) for P-35S, which was earlier than the endogenous *FatA*. This is not likely to be derived from the leaves for several reasons: *i.* even in 100% GM material, the Cq value of the GM element cannot be 2.5 Cq values earlier than the endogene unless all 5 leaves are GM and contain more than 5 P-35S promoters. *ii.* as can be seen in **Error! Reference source not found.**, there are no GM *B. napus* events that only have a P-35S element, and lack T-nos, *pat* or *nptII*. Since these three additional elements are also not detected in sample 16, it is unlikely that the sample contains GM *B. napus*. A viral infection of CaMV on the other hand, might explain the Cq value for P-35S.

Since both detection methods for CaMV were also negative, the downstream primer of P-35S and the upstream primer of T-35S flanking the CaMV gene of which the product is 35 Svedberg units (35S) were used in a conventional PCR to generate an amplicon of 230 nt. After Sanger sequencing, it was confirmed that the P-35S and T-35S were adjacent to each other and that the sequence was nearly identical to CaMV sequences in GenBank (see Table 7). In GM *B. napus*, the promoter and terminator would flank an introduced coding sequence. This is not the case here. This strongly indicates that the early signal for P-35S is indeed derived from a wild type CaMV strain that cannot be detected by the two CaMV-specific detection methods. Therefore, it was concluded that no GM *B. napus* was present in sample 16. The positive P-35S signal resulted from a wild type CaMV that cannot be detected by the CaMV-specific detection methods.

Table 7. CLUSTAL Omega (1.2.4) multiple sequence alignment with Cauliflower mosaic virus (GenBank: NC_001497.2) and the amplicon of sample 16, generated with the downstream primer of P-35S (yellow) and the upstream primer of T-35S (blue). The probes (bold) and downstream primers (underlined) are also indicated. The * indicates where the sequence from the plant and the virus are identical.

```
CaMV        ATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAG-   60
16          ATTGATGTGATATCTCCACTGACGT-AAGGGATGACGCAC-ATCCCACTATCCATCGCAAG   59
            ************************************ ************ *******

CaMV        ACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGCTGAAATCACCAGT   120
16          ACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAAGGCACGCTGAAATCACCAGC   119
            ************************************ * ******************

CaMV        CTCTCTCTACAAATCTATCTCTCTCTA-----TAATAATGTGTGAGTAGTTCCCAGATAA   175
16          CTCTCTCTACAACTCTCTCTCTAAATTTCTCCATAATGTGTGAGTAGTTTCCCGATAA   179
            ************ *** *******  *        ***************** ** *****

CaMV        GGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCT      230
16          GGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAAC---      231
            ************ ************************************
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Samples 93-102 (sampled from ADM-Europoort, Table S2) showed an increased number of positives, albeit with a late Cq. To investigate whether GM *B. napus* was present, event-specific methods that could be present based on the detected elements (see Table 4) were tested (Ms8, Rf3, T45, Gt73 and MON88302). They were all not detected in the samples. To investigate whether the late signals could be derived from dust from transshipment of GM soy or maize, endogenous tests for these species were performed. The additional tests for the presence of soy (lectin) and maize (hmg) showed that the leaves were positive for soy and/or maize, albeit with a late Cq. The signal of the GM elements was compared with the endogenous signal for soy/maize and is in accordance with the presence of GM soy and/or maize. Since the leaves were sampled from *B. napus* plants, the trace amounts (and late-positive signals for some GM elements) could be explained by 'dust' derived from GM soy and/or maize. Therefore, we conclude that no GM oilseed rape was present in the samples.

5. Concluding final remarks

5.1. Import of *B. napus*

We performed data analysis on import of *B. napus* to the Netherlands. Although data was available on import of canola to the Netherlands (CBS, customs, OEC), it was difficult to estimate current amounts of imported GM *B. napus* and to draw conclusions on the countries of origin. The data analysed, represented combined information on import of *B. napus* and *B. rapa* (canola) and the data from the different data sets did not match. Furthermore, no distinction was made between non-GM and GM canola in the registration system of the Dutch Customs. Although not all data seemed to be consistent, it appeared that Ukraine, Australia and Argentina are the main exporting countries of canola to the Netherlands. Although 24 % of all canola cultivated in Australia was GM, GMOnitor analysis showed that the relative likelihood of importing GM canola from Australia was low. Since Argentina does not produce GM canola, the relative likelihood of importing oilseed rape from this country was also estimated to be low. Production data of GM oilseed rape in Ukraine were not available.

Based on the available information, it was not likely that large quantities of GM canola from these countries are imported to the Netherlands. This is in line with a survey carried out by Dutch competent authorities that showed the presence of trace amounts of GM *B. napus* imported from Australia in birdfeed and oilseed rape used for the production of food which were below the 0,9% threshold for labelling (ILT 2012). Moreover, the large oilseed crushers ADM and Cargill have policies to ensure that all canola is produced from non-GM seed (pers. comm.). We therefore conclude that no large quantities of GM canola are currently imported to the Netherlands.

5.2. Sampling of *B. napus*

Because detailed information on imported commodity flows, current transfer stations and transport routes, and downstream users was lacking, sites were visited where feral *B. napus* was previously observed and where an enhanced possibility of seed spillage may exist. In total, we observed more than 2500 *B. napus* plants at almost half of the visited sites (25 out of 58). Feral *B. napus* was present at premises of hot oil pressing companies, road verges of highways and local roads, and several places in the rail bed of railroad tracks and shunting yards. Sometimes *B. napus* plants were found close to a rail road crossing and a local road. In this case it is not clear if seeds are spilled by rail or road transport. In addition, several plants were found in residential areas that presumably originated from the sowing of flower or bird feeding mixtures, or established after disturbances of soil that contained seeds.

At oil pressing companies, plants were both observed on the premises and the direct surroundings along important supply routes. At the small facilities, most of the individual plants were found in road verges in the direct vicinity of the company and places where translocation of goods took place. Mass occurrences were only found at the two large facilities. We did not face any wild relatives of *B. napus* (*B. rapa* or *S. arvensis*) at any of the oil presses.

There was a clear difference in the number of observed *B. napus* plants between tracks in the eastern part and the western part of the Netherlands. Large numbers of plants were found along freight tracks in the industrial areas of Rotterdam (Europoort) and Vlissingen while no plants were found along combined freight and passenger railways, with the exception of train stations Ede-Wageningen and Woerden. Here, plants were still present since their first observation in 2010.

The presence (or absence) of feral oilseed plants and therefore the potential for GM *B. napus* to spread into the environment, partly depended on the mode by which the area was maintained. On the premises of the two hot-oil pressing companies, removal of plants was carried out prior to our visit. Flowering plants were mowed but small rosettes of non-flowering plants were still found at sites where transfer of seeds from supply vessels to storage facilities took place. This shows that possible measures taken for the eradication of oilseed rape plants were only partly effective. However, no plants were found outside of the premises and this suggests that spillage of seeds takes place at a local scale. Handling activities at large pressing companies may therefore be a vector of minor importance for the spread of *B. napus* seeds into the environment.

At highways, vegetation in road verges are mown on a yearly basis with a fixed mowing scheme that usually starts in July. By then, most fruiting *B. napus* and *B. rapa* plants have already released their seeds causing seed banks to accumulate. Presence and persistence of *B. napus* plants in road verges can therefore not only be assigned to new annual seed input but also to recruitment from the seed bank over several consecutive years. The importance of recruitment from soil seed banks in road verges has already been demonstrated in France (Pivard *et al.* 2008). Numerous other studies showed the ability of *B. napus* to persist and recruit from seed banks in and near agricultural fields for a period of up to ten years (Pessel *et al.* 2001, Lutman *et al.* 2003, 2005, Beckie and Warwick 2006, D'Hertefeldt *et al.* 2008, Knispel *et al.* 2010). In addition, *B. napus* plants might have a high chance to escape from mowing and therefore to persist in road verges as they usually grow just beside the asphalt or underneath the crash barrier and not in the dense vegetation alongside roads (like *B. rapa*). This area is usually not mown or mown later in the year providing *B. napus* a longer period to flower and to set fruit. We found at least one persistent population along the highway A7 (between Groningen and Scheemda) where plants were present for a minimum period of ten years.

Oilseed rape plants were found in large numbers along the river Waal between Nijmegen and Millingen aan de Rijn. Riverbanks are not actively managed or mown and thus are also places where *B. napus* can establish from soil seed banks. The presence of plants along the river Waal suggest that seeds are washed ashore after they have fallen from supply vessels. There is also a possibility that they have been taken with the current from upstream the river.

Removal of plants was most apparent along railways. Freight and passenger railway tracks in the eastern part of the Netherlands were free from any vegetation and plants were presumably eradicated or treated with herbicides. At train stations, and especially shunting yards, vegetation was actively and frequently removed and, in some cases, whole railway beds were renewed or washed and replaced. In this way, spilled seeds did not get the chance to accumulate into the soil and form a seed bed from which new plants could arise. At shunting yards, wild flowering plants were present giving the impression that maintenance is far less regular than along railroads. Although no information was available on the frequency of herbicide appliance at railways, it is possible that glyphosate is used as an eradication agent as its use is not permitted on Dutch railroad tracks. This has important implications for the invasiveness and therefore the persistence of *B. napus* at railways.

There are no signs that populations of *B. napus* are expanding or diminishing in number or size.

In conclusion, our results suggest that transport via highways and freight railroad tracks, together with handling activities at cold-pressing companies are the main route along which *B. napus* escapes into the environment. In addition, motor- and waterways are considered the main route through which *B. napus* is imported and transported across the country.

5.3. Detection of GM *B. napus* events

In total, 668 individual plant samples were sampled and analysed in 160 pooled samples in duplicate. All 160 samples were positive for the endogene *FatA*, confirming that the plants were either *B. napus* or *B. rapa* and that the isolated DNA was of sufficient quality for the GM screening. Five screening elements were tested. In most samples, the screening elements were not detected. In 25 samples, one or more screening elements showed a late Cq that was not in line with the expected Cq of a positive sample. Additional experiments showed that these late signals did not derive from GM *B. napus*. One sample (collected at Europoort, Rotterdam) showed a very early signal for P-35S, but this could be attributed to wild type Cauliflower mosaic virus.

Ten samples collected at the premises of the processing facility ADM showed late signals for some of the screening elements. Since the GM *B. napus* events containing these elements were not detected while soy/maize DNA traces were detected, the late signals of GM elements could be derived from dust that is commonly to occur when GM soy or maize is transported or processed. We therefore conclude that the 668 *B. napus* plants including a smaller fraction of *B. rapa* and *S. arvensis* plants that were sampled in the Netherlands, are not genetically modified.

In summary, we conclude that no spillage and spread of GM *B. napus* in the Dutch environment have occurred, at least not at the sampled locations with small population sizes. There is still a possibility

that GM *B. napus* is present at places were mass occurrences were observed (hot pressing companies, riverbanks, road verges) as samples did not include all individuals. In addition, there might be other locations where a chance of seed spillage exists that were not sampled in this study (i.e. important waterways like the IJssel and Rijn, other highways). In order to assure the absence of feral GM *B. napus*, we therefore recommend monitoring on the presence of feral GM *B. napus* on a regular basis. Furthermore, the presence of *B. napus* at hot pressing companies might be the consequence of poor measurements taken to avoid seed spillage and low efforts to mow standing vegetation. It is therefore advised to increase the frequency of mowing at premises of oil crushing facilities to avoid unnecessary establishment of oilseed rape plants and potential escape of seeds outside of the facility borders. Plants that establish from spilled seeds should be eradicated on a routine basis. Finally, a universal nomenclature for *B. napus* and *B. rapa* should be introduced and used by trade authorities to prevent ambiguity between rapeseed and oilseed rape and to be able to trace commodity flows of each species separately.

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Supplementary material

Table S1. Results of the qPCR reactions performed in duplicate on 150 samples. ND is 'not detected', green fields are 'detected', orange fields are not significant and therefore to be considered as 'not detected'.

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII
2-1	Cargill BV, Amsterdam, *premises*	6	21.43/21.76	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-1	Cargill BV, Amsterdam, *premises*	6	21.25/21.95	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-1	Cargill BV, Amsterdam, *premises*	5	22.47/22.21	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-1	Cargill BV, Amsterdam, *premises*	5	21.32/21.37	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-2	Cargill BV, Amsterdam, *premises*	5	21.32/21.29	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-2	Cargill BV, Amsterdam, *premises*	5	20.91/21.27	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-2	Cargill BV, Amsterdam, *premises*	5	21.25/21.32	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-3	Cargill BV, Amsterdam, *premises*	5	21.48/21.06	ND/ND	ND/ND	ND/ND	ND/42.04	ND/ND			
2-3	Cargill BV, Amsterdam, *premises*	5	21.24/21.42	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-3	Cargill BV, Amsterdam, *premises*	5	21.17/21.29	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-4	Cargill BV, Amsterdam, *premises*	5	21.10/21.19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-4	Cargill BV, Amsterdam, *premises*	5	21.36/21.89	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
2-4	Cargill BV, Amsterdam, *premises*	5	21.25/20.65	ND/ND	ND/ND	ND/ND	ND/43.52	ND/ND			
3-1	Noord-Nederlandse Oliemolen BV, Harlingen, *supply*	2	23.18/22.74	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
3-1	Noord-Nederlandse Oliemolen BV, Harlingen, *supply*	3	21.38/20.96	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-1	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	2	32.65/33.51	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-2	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	1	31.48/30.05	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-3	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	5	21.13/21.28	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-3	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	5	21.23/21.08	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-3	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	5	20.91/20.76	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
4-3	Noord-Nederlandse Oliemolen BV, Farmsum, *supply*	4	20.79/21.34	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
5-1	Oudendijk Oils BV, *supply route*	1	21.07/21.69	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
6-1	ADM-Europoort, Rotterdam, *supply route*	5	21.90/21.76	18.87/18.21[6]	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND

[6] The positive P-35S signal in sample 16 is derived from CaMV so this sample is also 'not detected' for GM *B. napus*.

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII
6-1	ADM-Europoort, Rotterdam, *supply route*	5	22.10/21.78	34.63/36.39	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
6-1	ADM-Europoort, Rotterdam, *supply route*	4	21.33/21.42	35.41/39.12	ND/ND	ND/ND	ND/39.68	ND/ND	ND/ND	ND/ND	38.37/ND
8-1	Botlek, *rail track*	5	21.52/21.85	37.93/38.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
8-1	Botlek, *rail track*	5	20.55/20.31	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
8-1	Botlek, *rail track*	5	21.31/21.47	38.48/35.94	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	39.11/ND
9-1	Kijfhoek, *shunting yard and rail track*	2	20.71/21.19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
9-2	Kijfhoek, *shunting yard and rail track*	5	20.85/20.82	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
9-2	Kijfhoek, *shunting yard and rail track*	5	22.49/21.90	37.76/38.18	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
9-2	Kijfhoek, *shunting yard and rail track*	5	21.02/21.22	ND/42.70	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
9-2	Kijfhoek, *shunting yard and rail track*	3	21.10/21.18	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
9-3	Kijfhoek, *shunting yard and rail track*	1	21.49/21.21	ND/43.35	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
9-4A	Kijfhoek, *shunting yard and rail track*	3	21.43/21.60	31.37/30.06	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	38.29/ND
9-4B	Kijfhoek, *shunting yard and rail track*	2	22.32/22.79	36.90/30.03	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/37.75
11-1	Pernis, *rail track*	5	21.64/21.88	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
11-1	Pernis, *rail track*	5	21.27/21.38	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
11-1	Pernis, *rail track*	5	22.06/21.99	37.08/36.35	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	37.91/38.02
11-1	Pernis, *rail track*	7	21.20/21.11	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
11-1	Pernis, *rail track*	5	20.87/21.03	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
12-1	Rozenburg, *rail track*	5	21.48/21.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
13-1	Waalhaven, *shunting yard*	4	22.07/22.18	35.78/37.65	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
19-1	Blerick, *rail track*	3	27.26/27.92	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
20-1	Blerick, *railroad crossing*	1	20.73/20.86	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
21-1	Venlo, *shunting yard*	5	21.43/21.23	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
21-1	Venlo, *shunting yard*	5	21.53/21.11	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
21-2	Venlo, *shunting yard*	5	21.06/21.10	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
21-2	Venlo, *shunting yard*	5	21.33/21.40	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
21-2	Venlo, *shunting yard*	3	20.96/21.49	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
22-1	Venlo, *rail track border with Germany*	2	20.75/21.02	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
22-2	Venlo, *rail track border with Germany*	2	23.47/24.09	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
25-1	Moerdijk, *rail track to industrial area*	4	21.15/22.71	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
26-1	Sloe, Vlissingen, *shunting yard*	4	29.34/29.29	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
30-1	Woerden, *station*	5	20.82/21.28	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
30-1	Woerden, *station*	5	20.40/20.74	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII
33-1	Lobith, *port*	4	21.24/21.43	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-2	Lobith, *port*	2	20.98/20.55	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-3	Lobith, *port*	5	20.95/21.03	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-4	Lobith, *port*	4	21.40/21.40	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-5	Lobith, *port*	5	21.82/22.14	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-6	Lobith, *port*	5	21.00/20.89	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-7	Lobith, *port*	6	21.68/21.36	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-8	Lobith, *port*	4	22.02/21.35	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-9	Lobith, *port*	5	21.47/22.16	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-10	Lobith, *port*	5	21.46/21.37	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-11	Lobith, *port*	4	21.14/21.62	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-12	Lobith, *port*	4	21.33/21.30	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-13	Lobith, *port*	3	22.00/21.91	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-14	Lobith, *port*	3	21.19/20.93	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-15	Lobith, *port*	4	21.33/21.65	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-16	Lobith, *port*	5	21.35/21.17	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-17	Lobith, *port*	5	20.99/21.20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-18	Lobith, *port*	4	20.41/20.60	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
33-19	Lobith, *port*	3	21.21/21.34	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-1	Waal, *riverside* - Ooij	6	34.92/36.75	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-2	Waal, *riverside* - Ooij	5	22.56/22.17	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-3	Waal, *riverside* - Bizonbaai	6	30.50/29.84	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-4	Waal, *riverside* - Bizonbaai	4	22.42/23.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-5	Waal, *riverside* - Bizonbaai	5	21.26/21.32	40.08/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
35-6	Waal, *riverside* - Bizonbaai	5	21.69/21.52	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-7	Waal, *riverside* - Bizonbaai	4	21.57/21.62	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-8	Waal, *riverside* - Bizonbaai	5	21.42/21.58	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-9	Waal, *riverside* - Bizonbaai	5	21.22/21.58	ND/ND	ND/ND	ND/ND	44.45/ND	ND/ND			
35-10	Waal, *riverside* - Bizonbaai	5	21.69/21.93	ND/ND	ND/ND	ND/ND	ND/44.50	ND/ND			
35-11	Waal, *riverside* - Groenlanden	5	21.82/21.75	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-12	Waal, *riverside* - Groenlanden	4	21.28/21.26	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-13	Waal, *riverside* - Groenlanden	5	22.08/22.46	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-14	Waal, *riverside* - Groenlanden	4	22.05/21.72	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII
35-15	Waal, *riverside* - Millingenwaard	5	21.10/21.21	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-16	Waal, *riverside* - Millingenwaard	5	21.73/22.03	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-17	Waal, *riverside* - Millingenwaard	3	21.40/20.81	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-18	Waal, *riverside* - Nijmegen	5	21.06/20.77	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-19	Waal, *riverside* - Nijmegen	4	20.76/21.27	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-20	Waal, *riverside* - Nijmegen	5	20.84/20.62	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-21	Waal, *riverside* - Nijmegen	3	20.63/20.69	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-22	Waal, *riverside* - Nijmegen	6	20.98/20.89	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-23	Waal, *riverside* - Nijmegen	3	20.61/20.57	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-24	Waal, *riverside* - Nijmegen	5	21.08/20.76	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
35-25	Waal, *riverside* - Nijmegen	2	20.98/21.10	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
43-1	N35 – Almelo	3	21.52/21.33	43.34/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
44-1	A44 – Leiden	4	21.61/21.56	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
45-1	A67 – Venlo-Eindhoven	5	21.00/21.19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
45-1	A67 – Venlo-Eindhoven	5	20.51/21.04	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
45-1	A67 – Venlo-Eindhoven	5	21.16/20.30	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
46-1	N62 – Axel	5	20.95/21.07	ND/43.11	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
46-1	N62 – Axel	5	21.19/21.21	39.37/39.84	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
46-1	N62 – Axel	3	21.39/21.20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
46-1	N62 – Axel	3	20.96/20.92	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
47-1	N237 – Bilthoven	4	21.06/20.44	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
47-1	N237 – Bilthoven	2	25.77/26.65	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
47-1	N237 – Bilthoven	3	21.22/21.30	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
49-1	N254 – Sloe	5	24.43/24.17	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
49-1	N254 – Sloe	5	20.71/21.00	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
49-1	N254 – Sloe	3	21.68/22.09	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
52-1	N811 – Zevenaar	5	22.18/22.18	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
52-1	N811 – Zevenaar	2	21.03/22.01	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
52-1	N811 – Zevenaar	5	21.60/21.77	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
52-1	N811 – Zevenaar	3	21.63/21.28	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
54-1	Delft, *city centre*	3	21.17/21.08	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
55-1	Goor and surroundings - Haaksbergerweg	4	21.45/21.48	40.21/41.60	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
55-2	Goor and surroundings - Haaksbergerweg	5	21.20/21.18	40.16/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII
55-2	Goor and surroundings - Haaksbergerweg	4	21.05/21.16	39.16/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-3	Goor and surroundings - Haaksbergerweg	1	21.19/21.12	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-4	Goor and surroundings – Hengeveld - Bentelosestraat	2	21.29/21.62	39.77/39.36	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
55-5	Goor and surroundings - Burcoloseweg	5	21.66/21.49	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-5	Goor and surroundings - Bentoloseweg	2	21.13/21.19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-6	Goor and surroundings - Lochemseweg - Twentekanaal	2	23.19/22.99	ND/39.63	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
55-7	Goor and surroundings - Diepenheim	2	21.42/21.48	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-8	Goor and surroundings - Diepenheim - Goorseweg	1	21.42/21.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-9	Goor and surroundings - Slotweg	2	21.93/21.22	ND/40.01	ND/ND	43.65/ND	ND/ND	ND/ND	ND/ND		
55-10	Goor and surroundings - Slotweg	5	21.29/21.59	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-10	Goor and surroundings - Slotweg	2	22.19/21.74	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-11	Goor and surroundings - Gelselaar	5	25.52/25.04	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-11	Goor and surroundings - Gelselaar	5	24.36/23.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-11	Goor and surroundings - Gelselaar	5	24.68/26.88	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-11	Goor and surroundings - Gelselaar	3	26.46/25.20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	21.36/21.40	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	21.31/21.26	ND/ND	ND/ND	43.76/43.25	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	22.02/22.57	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	21.18/20.84	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	21.10/21.36	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	5	21.32/21.27	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND		
55-12	Goor and surroundings - Gelselaar	3	20.90/20.99	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
55-13	Goor and surroundings - Gelselaar	3	22.68/22.70	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
55-13	Goor and surroundings - Gelselaar	3	28.35/29.43	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
58-1	Utrecht, *public park*	5	21.09/21.27	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
58-1	Utrecht, *public park*	5	21.09/21.04	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
58-1	Utrecht, *public park*	5	21.35/21.32	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			
58-1	Utrecht, *public park*	5	24.34/24.70	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND			

Tabel S2. Results of the qPCR reactions performed in duplicate on 10 samples from ADM-Europoort. The Cq value of the endogene *FatA* is an average of duplicates. ND is 'not detected', green fields are 'detected', orange fields are not significant and therefore to be considered as 'not detected'.

Nr	Location	# leaves	Endogene	Screening					Virus	Additional elements			Events					Endogene	
			FatA	P-35S	T-Nos	T-ocs	T-E9	gat/T-pinII	CaMV	pat	nptII	CP4epsps	Ms8	Rf3	T45	GT73	MON88302	Maize hmg	Soy lectin
1-1	ADM-Europoort, Rotterdam, *premises*	4	23.86	ND/ 34.85	ND/ND	ND/ND	ND/ 38.47	ND/ND	ND/ND	ND/ 38.62	ND/ND	ND/ 37.09	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	36.53/ 36.84
1-2	ADM-Europoort, Rotterdam, *premises*	6	21.59	34.77/ 36.48	37.34/ ND	ND/ND	36.71/ 37.89	ND/ND	ND/ND	39.49/ ND	ND/ND	37.82/ ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	38.65/ 36.06
1-3	ADM-Europoort, Rotterdam, *premises*	6	21.57	ND/ND	ND/ND	ND/ND	37.54/ ND	ND/ND		40.19/ ND	ND/ND	ND/ 38.04	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	38.34/ ND
1-3	ADM-Europoort, Rotterdam, *premises*	5	21.55	ND/ND	ND/ND	ND/ND	38.41/ 36.94	ND/ND		ND/ 39.66	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	37.28/ ND	38.79/ ND
1-4	ADM-Europoort, Rotterdam, *premises*	5	21.89	37.81/ 37.10	41.65/ 40.98	ND/ND	37.13/ 37.96	ND/ND	ND/ND	ND/ND	ND/ND	ND/ 38.25	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	36.50/ 36.15
1-4	ADM-Europoort, Rotterdam, *premises*	5	21.49	35.91/ 35.40	ND/ 39.14	ND/ND	37.05/ 33.92	ND/ND	ND/ND	ND/ND	ND/ND	ND/ 35.44	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	38.07/ ND	36.47/ 35.63
1-4	ADM-Europoort, Rotterdam, *premises*	3	21.67	37.58/ 36.71	38.39/ ND	ND/ND	ND/ 38.21	ND/ND	ND/ND	ND/ND	ND/38. 80	37.33/ 38.40	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	38.60/ 38.83
1-5	ADM-Europoort, Rotterdam, *premises*	5	21.83	35.75/ 35.91	39.02/ ND	ND/ND	36.73/ 35.77	ND/ND	ND/ND	ND/ND	ND/ND	38.25/ 37.85	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	35.31/ 37.17
1-5	ADM-Europoort, Rotterdam, *premises*	5	21.37	35.15/ ND	ND/ND	ND/ND	35.82/ 43.33	ND/ND	ND/ND	ND/ND	ND/ND	37.96/ 37.15	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	35.75/ 35.50
1-5	ADM-Europoort, Rotterdam, *premises*	3	20.97	35.46/ 36.22	ND/ 39.41	ND/ND	35.54/ 34.49	ND/ND	ND/ND	38.09/ ND	37.73/ ND	37.01/ 36.27	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	37.82/ 37.06