National Institute for Public Health
and the Environment
*Ministry of Health, Welfare and Sport*

# Potential introduction of unapproved GM animals and GM products in the Netherlands

RIVM report 609021118/2012

H.C.M. van den Akker | A.L.M. Wassenaar

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# Colophon



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# Abstract

**Potential introduction of unapproved GM animals and GM products in the Netherlands**

The RIVM has made an inventory of genetically modified (GM) organisms that could be illegally imported into the European Union, now or in the near future. In recent years, some varieties of genetically modified ornamental fish have appeared illegally on the EU market. The research in the current report focused on genetically modified animals and micro-organisms that have not yet been authorized on the EU market, especially since an inventory of genetically modified crops has already been drawn up.

It appears that besides genetically modified ornamental fish, veterinary vaccines and pesticides that contain genetically modified micro-organisms could potentially be illegally imported. Furthermore, 'medical tourism' and 'do-it-yourself biology' may lead to the undesirable introduction of genetically modified organisms into the environment. There are currently no genetically modified food/feed animals, pets, or insects on the market, but this may change in the near future, depending on the admission or rejection of current market applications.

This report was commissioned by the Human Environment and Transport Inspectorate, formerly the VROM Inspectorate. One of the report's objectives is to provide decision-making tools for the Inspectorate with regard to which genetically modified organisms will require the most attention (now and in the near future), how they can be detected and which agency is responsible for the enforcement.

The RIVM has examined which genetically modified organisms have already been admitted to the market or could be admitted soon. This was done by consulting the databases of agencies dealing with authorization of genetically modified organisms, both within and outside Europe. In addition, literature and internet resources were studied. Data were also taken from agencies involved in the inspection and enforcement of genetically modified organisms.

For each category of organisms within the inventory (ranging from genetically modified bacteria and viruses, insects, fish, and small animals to cattle) an estimation of the likelihood of import was made. Further included is whether an environmental risk assessment is available that may be helpful for assessing the potential risks to human health and the environment.

Keywords:
genetic modification, animal, vaccine, gene therapy, micro-organism, illegal import

# Rapport in het kort

**Potentiële introductie van niet toegelaten ggo dieren en producten in Nederland**

Het RIVM heeft geïnventariseerd welke genetisch gemodificeerde organismen nu en in de toekomst zouden kunnen worden geïmporteerd, zonder dat daarvoor de benodigde EU-toelating of vergunning is verleend. De afgelopen jaren zijn namelijk varianten van genetisch gemodificeerde siervissen illegaal in de handel gebracht. Het onderzoek heeft zich toegespitst op genetisch gemodificeerde dieren en micro-organismen die in de Europese Unie nog niet op de markt zijn toegelaten, aangezien een dergelijke inventarisatie voor genetische gemodificeerde gewassen al heeft plaatsgevonden.

Het blijkt dat, behalve de genetisch gemodificeerde siervissen, onder andere vaccins voor dieren en gewasbeschermingsmiddelen die genetisch gemodificeerde micro-organismen bevatten, illegaal zouden kunnen worden geïmporteerd. Verder kunnen 'medisch toerisme' en 'doe-het-zelf biologie' er mogelijk toe leiden dat genetisch gemodificeerde organismen ongewenst in het milieu terechtkomen. Er zijn op dit moment nog geen genetisch gemodificeerde dieren voor de voedselproductie, gezelschapsdieren of insecten op de markt beschikbaar, maar dit kan in de nabije toekomst veranderen, afhankelijk van de toelating of afwijzing van marktaanvragen hiervoor.

De inventarisatie is uitgevoerd op verzoek van de Inspectie Leefomgeving en Transport, voorheen de VROM-Inspectie. Hiermee krijgt deze Inspectie handvatten om te beslissen welke organismen nu en in de toekomst de meeste aandacht behoeven, hoe ze kunnen worden gedetecteerd en wie verantwoordelijk is voor de handhaving.

Het RIVM heeft onderzocht welke genetisch gemodificeerde organismen reeds op de markt zijn toegelaten of binnenkort toegelaten zouden kunnen worden. Dit is gedaan door de databases te raadplegen van instanties die zich binnen en buiten Europa bezighouden met toelating van genetisch gemodificeerde organismen. Bovendien zijn bronnen in de literatuur en op internet bestudeerd. Tevens zijn beschikbare data van toezichthoudende instanties in Europa bijeengebracht.

Voor de inventarisatie is van elk categorie organismen, variërend van genetisch gemodificeerde bacteriën en virussen, insecten, vissen, kleine huisdieren tot vee, ingeschat wat de kans op import is. Ook staat vermeld of er een milieurisicobeoordeling beschikbaar is waardoor de eventuele risico's voor mens en milieu beter kunnen worden ingeschat.

Trefwoorden:
genetische modificatie, dier, vaccin, gentherapie, micro-organisme, illegale import

# Contents

# Summary

In recent years, various countries in the European Union have reported on the illegal introduction of genetically modified (GM) zebrafish. In the Netherlands, for example, GM zebrafish were found to be on sale in various pet stores and via the internet. These fish are not approved for commercial sale in the European Union (EU) and had been illegally imported from, most probably, South-East Asia.

In the Netherlands, the enforcement of genetically modified organism (GMO) regulations, including the monitoring of unapproved GMOs, is one of the tasks of the Human Environment and Transport (ILT) Inspectorate. The GM zebrafish referred to here appeared to be the first example of the illegal import of a GMO (other than GM crops) on quite a large scale, that the ILT Inspectorate was confronted with. The illegal introduction of GM zebrafish raised the question whether other unapproved GM animals and GM products containing living GMOs could potentially be imported from outside the European Union, both now and in the near future.

The RIVM has made an inventory of genetically modified organisms that could be illegally imported into the European Union, now or in the near future. The research in the current report focused on genetically modified animals and micro-organisms that have not yet been authorized on the EU market, especially since an inventory of genetically modified crops has already been drawn up. Using a variety of resources, including resources from the internet, an inventory was compiled that provides a broad overview of the current worldwide status of the commercialization of GM animals and GM products. This inventory can be useful for prioritizing the activities of the ILT Inspectorate regarding the control of the (potential) illegal import and/or illegal use of these GMOs.

The inventory includes examples of GM ornamental fish, GM pets, GM animals for food/feed and production of substances, GM insects for disease control, recombinant live veterinary vaccines and veterinary therapeutics, GM human therapeutics and vaccines, pesticides containing GM micro-organisms and GM micro-organisms for other uses. The inventory also contains the following information where available:

a) An exact description of GMOs or products containing living GMOs on, or about to come onto the market, including the name and nature of the product, the genetic modification and the technique applied for modification. Also products that are suspected of being GMOs, but not regarded as such under European legislation are described.
b) The extent of the import and potential import of GMO products and their availability for the Dutch consumer market.
c) From which countries, by whom and by which routes introduction could take place.
d) An indication of the hazards/risks associated with the product; inclusion of an existing environmental risk assessment (if available).
e) Exploration of possibilities for the detection and control of these products. When a specific detection method is available, this is mentioned.

One conclusion of the inventory is that the commercial availability of GMOs to the general public outside the EU is currently limited to GM ornamental fish (such as the GM zebrafish), GM veterinary vaccines, a GM human influenza vaccine and a small number of pesticides consisting of GM bacteria. In all four categories, there are examples of products that are available to the general public through web stores or retailers on the internet. These products could potentially be illegally imported into the EU.

Furthermore, there are a number of other developments that may lead to the undesirable introduction of genetically modified organisms into the Netherlands. Examples are: medical tourism that involves gene therapy products and do-it-yourself biology that involves individuals performing biological experiments outside regular laboratories.

With the notable exception of the GM ornamental fish and a few GM animals approved outside the European Union for the production of substances (and which are held under contained conditions), no GM animals have so far received market approval in any country worldwide. However, in the near future, GM fish and livestock may be approved for food/feed in North America and in China since some products have supposedly reached a near final decision.

The inventory includes examples of GMOs belonging to biologically very different categories (e.g. viral vaccines, pesticides, livestock, insects, ornamental fish) that inherently will also show considerable differences in the conditions under which they will be made available on the market.

For each category of organisms within the inventory, an estimation of the likelihood of import was made. Further included is whether an environmental risk assessment is available that may be helpful for assessing the potential risks to human health and the environment. These and other characteristics of the different categories of GMOs were used to generate a relative priority or 'awareness' score that the ILT Inspectorate can apply to prioritize their current and future activities. The report provides tools for the ILT Inspectorate to decide which genetically modified organisms will require the most attention now and in the near future, and how they can be detected.

# 1 Introduction

## 1.1 Objectives and demarcation

In 2006, 2008 and 2011, genetically modified (GM) zebrafish expressing red fluorescent protein (RFP, Figure 1) were found to be on sale in various pet stores and via internet shops in the Netherlands. These fish were (and still are) not approved for commercial sale in the European Union (EU) and had been illegally imported from, most probably, South-East Asia. Also various other countries in the European Union reported on the illegal introduction of similar genetically modified zebrafish [1].

*Figure 1. RFP expressing GM zebrafish*
*Photo provided by Jan-Piet Tijssen, ILT.*

In the Netherlands, the enforcement of GMO regulations, including the monitoring of unapproved GMOs, is one of the tasks of the Human Environment and Transport Inspectorate (ILT; the previous VROM-Inspectorate of the Ministry of Infrastructure and the Environment). The GM zebrafish referred to here, appeared to be the first example of the illegal import of a GM organism (other than a GM crop) on quite a large scale, that the ILT Inspectorate was confronted with. This illegal introduction raised the question which other unapproved genetically modified organisms (other than plants) could potentially be imported from outside in the European Union, both now and in the near future. In this report, this main question will be addressed, together with a number of other questions concerning the illegal import of GMOs (see below).

The (un)intended introduction of viable plant materials and seeds in the European Union has since long been acknowledged as a potential problem. A large number of GM plants, in particular GM crops, have been approved on the market both in- and outside the European Union. In 2009, a report on the potential introduction of unapproved GM crops in the Netherlands was generated, comprising a shortlist of species that (may) require specific attention with regard to (potential) environmental dispersal. Taking into account actual trade and import data, the shortlist was subsequently translated into a priority list for the monitoring of unapproved GMOs [2].

The underlying report can be regarded as a sequel to this report in which other organisms than plants on or near the market are evaluated for their potential of unapproved introduction: in particular GM animals and GM micro-organisms

approved on the market outside the European Union. Similarly to the report on unapproved GM crops, the evaluation should result in a priority list that can be recommended to the ILT inspectorate.

For this purpose, an inventory of GM animals and micro-organisms was generated, focusing on the relevant organisms that can be expected both now or in the near future, meaning those being sold already abroad for commercial purposes and not (yet) authorized in the European Union, and those which are developed for commercial purposes and that are (supposedly) near commercialization.

This inventory is aimed at including the following information concerning the most relevant GMOs:

a) An exact description of GMOs or products containing living GMOs on or about to come onto the market, including name and nature of the product, the genetic modification and the technique applied for modification. Also products that are suspected of being GMOs, but not regarded as such under European legislation should be described.
b) The extent of the import and potential import of GM animals and GM products and of the availability of these products for the Dutch consumer market.
c) From which countries, by whom and by which routes introduction could take place.
d) An indication of the hazards/risks associated with the product; inclusion of an existing environmental risk assessment (if available).
e) Exploration of possibilities for the detection and control of these products. When a specific detection method is available, this is mentioned.

As indicated above, this study focuses on GM animals and GM micro-organisms. GM plants were left outside of this inventory, since the main category of GM plants, GM crops, have been the subject of a previous report [2].

Furthermore, it is important to note that products (e.g. substances) on the market derived from GMOs that have been removed during the production process, or that contain killed GMOs are outside the main focus of this inventory, simply because these products are not considered GMOs under European GMO legislation. Some of these products will however be mentioned to illustrate developments regarding the GM production organisms that these products are derived from. Moreover, market applications of GM products may include an extensive environmental risk assessment in which also the safety of the production chain is evaluated, particularly in cases where the GM production organism is not kept under regular, contained use conditions. In the USA there are several examples in which the safety of both GMO and the GMO derived product are addressed within the same marketing application and these cases are included in the inventory.

To generate the inventory, information on relevant GMOs was mainly gathered from the internet, especially focusing on databases and sources that supply information on the marketing status or licensing of GMOs, and by applying specialized software that searches a selection of news sources on the internet. For several subjects (e.g. GM fish, GM insects, medical tourism) we made use of recently published reports that already contain an inventory. In some cases additional information was communicated by the ILT Inspectorate, and also a number of international GMO inspectors were contacted for additional information.

Before these methods and the inventory are described in more detail we will first focus on the legislation of GMOs in the European Union and the USA. This information is provided to give an indication of the procedures that are normally needed for GMOs to receive a marketing license in the European Union and the USA, and of the involved authorities/agencies. Since these agencies are mentioned throughout this report, this will give a better understanding of their particular role. Furthermore, a short overview is provided of the provisions of the Cartagena protocol on Biosafety in relation to the trans-boundary movement of GMOs.

## 1.2 GMO regulations in the European Union

### *1.2.1 Regulatory framework and competent authorities within the European Union*

In the European Union, the deliberate release of genetically modified organisms into the environment is regulated by the EU Directive 2001/18/EC. This Directive concerns both the placing on the market of GMOs and deliberate release of GMOs into the environment for non-commercial purposes (e.g. field trials) [3]. The Directive obliges member states to ensure that all appropriate measures are taken to avoid adverse effects on human health and the environment which might arise from the deliberate release or the placing on the market of GMOs. Part C of the Directive specifically describes the general procedure for the placing of the market of GMOs as or in products. In short, an application is submitted to the competent authority of the Member State where the GMO is to be placed on the market for the first time. Based on the application, an assessment report is prepared by this competent authority that is forwarded to the European Commission and the competent authorities of other member states. The Commission or other Member States are invited to provide comments on this assessment report, to ask for further information, or to raise objections to the application. Taking into account all reasoned objections, a community decision is taken whether the Competent Authority (CA) that has prepared the assessment report is granted permission to give a license for the product to be placed on the market and this authorization is valid in all Member States. If there are no reasoned objections from the other Member States, the CA that has prepared the assessment report can issue the license directly, e.g. without a community decision.

The European Medicines Agency (EMA) is responsible for the scientific evaluation of applications for EU marketing authorizations for both human and veterinary medicines, including medicines derived from genetically modified organisms. This evaluation is carried out under a centralized procedure in tight collaboration with the member states. The establishment of the agency, its tasks and the community procedures for the authorization and supervision of medicinal products for human and veterinary use have been laid down in the Regulation (EC) 726/2004. The marketing authorization is granted by the European Commission and valid in all European Union (EU) and the EEA-EFTA states Iceland, Liechtenstein and Norway. Medicinal products containing, or consisting of genetically modified organisms should be subjected to an environmental risk-assessment procedure similar to the procedure under Directive 2001/18/EC [4].

The procedures for evaluation and authorization of genetically modified foods and feeds are laid down in Regulation EC/1829/2003 on GM food and in Directive 2001/18/EC in case the application involves deliberate release of

GMOs. The core task of the European Food Safety Authority (EFSA) with respect to GMOs is to independently assess any possible risks of GMOs to human and animal health and the environment. The GMO panel of the EFSA evaluates the safety of products containing, consisting of, or produced by (non-viable) GMOs, before a final market authorization decision is taken by the European Commission and Member States. The marketing authorization may include the import, processing, food and feed uses, and, in specific cases, also the cultivation/breeding of the GMO. It is important to note that food and feed produced under contained conditions by fermentation using a genetically modified micro-organism that is not present in the final product is also evaluated by the EFSA [5].

In all authorization procedures involving deliberate release of GM products, an environmental risk assessment has to be performed in which the potential risks for human health and the environment are evaluated. Annex II of the Directive 2001/18/EC describes in general terms the objective to be achieved, the elements to be considered, and the general principles and methodology to be followed to perform an environmental risk assessment involving the deliberate release of GMOs [3]. This secures that GMOs or their products, including medicines or food/feed consisting or containing GMOs, can only be authorized in the European Union if they have been evaluated in an environmental risk assessment. As a consequence of these regulations, products that have not been granted a marketing authorization by the Community and the Member State involved cannot be placed on the market.

### *1.2.2 Regulations and agencies involved in the USA*

The U.S. Food and Drug administration (FDA) is the USA agency involved in the regulation of amongst others human and veterinary drugs, biological products and food, by assessing the safety, efficacy and security of these products [6]. Various centres within the FDA may be involved in the regulation of genetically modified products. The Center for Biologics Evaluation and Research (CBER) regulates all biological products intended for human use, including genetically modified vaccines and gene therapy products. The Center for Veterinary Medicine (CVM) regulates drugs and food (additives) that are to be used in animals. Moreover, the CVM regulates genetically modified animals under the new animal drug provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA). Any new animal drug may not be commercially sold unless it has been the subject of an approved new animal drug application (NADA). According to this act, a recombinant DNA construct intended to affect the structure or function of an animal meets the definition of a new animal drug, regardless of whether the resulting 'genetically engineered' (GE) animals are intended for food, or to produce pharmaceuticals or any other substances [7].

Several other agencies may be involved in the regulation and licensing of GM products. The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) is an agency involved in the protection of agricultural health and the regulation of genetically modified organisms [8]. Within APHIS, the Center for Veterinary Biologics (CVB) is involved in the licensing of products intended for use in the treatment of animals, including genetically modified vaccines and viruses. The National Center for Import Export regulates the import and export of genetically modified animals, animal products, and biologicals. The Biotechnology Regulatory Services unit regulates the environmental release of genetically modified organisms considered to be

regulated articles. This includes for instance the environmental release of organisms that may have impact on plant health, such as insects, fungi, bacteria, and viruses [8].
The Environmental Protection Agency (EPA) is involved in the regulation and licensing of (most) pesticides, including insecticides, herbicides, fungicides, and various other substances used to control pests under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) [9].
The basic requirements and procedures for export of amongst others human drugs, animal drugs, biological products and food (additives) are laid down in the FDA Export Reform and Enhancement Act of 1996 [10]. If a product meets the requirements for sale and distribution in the United States, apparently there are no additional restrictions on its exportation. However, for (1) unapproved products or (2) products 'approved as distributed in the U.S., but to be exported with different or additional labelling requirements or conditions for use', a general requirement is that the product does not conflict with the laws of the importing country. This may be demonstrated either by a letter of an authorized foreign body stating that the product has marketing approval or does not conflict with that country's laws, or a notarized certification by a responsible company official in the United States stating that the product does not conflict with the laws of the importing country. For these two categories, different labelling requirements apply. EPA regulates both the import and export of pesticides. The export of pesticides from the USA has been laid down in the FIFRA act. According to this act, all registered pesticides which are exported to other countries must bear the product label approved by EPA [11].
The APHIS website indicates that the USA has minimal requirements for animals to be exported to other countries. Rules for entry of animals from the United States are established by the receiving country [12].

### *1.2.3 Cartagena protocol*

The Cartagena Protocol on Biosafety to the Convention on Biological Diversity is an international treaty that came into force in 2003 and that governs the movements of living GMOs from one country to another [13]. In the protocol these living GMOs are referred to as living modified organisms (LMOs). The main objective of the Protocol is to ensure that Parties have an adequate legal framework concerning biosafety of the use of LMOs. The Protocol contributes to ensuring an adequate level of protection in the field of the safe transfer, handling, and use of LMOs resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health. The protocol includes besides the requirements for handling, transport, packaging and identification (labelling) of LMOs, amongst others information regarding risk assessment and risk management of LMOs, environmental monitoring and reporting of LMOs, and requirements regarding public awareness and participation. Countries that are parties to the protocol include the countries in the European Union, Japan and China. Several countries in which GMOs have been placed on the market (i.e. the USA, Canada and Australia) are not a party to the protocol (see also Table I). However, according to the protocol, trans-boundary movements of living modified organisms between Parties and non-Parties should be consistent with the objective of this Protocol. The Biosafety Clearing-House (BCH) is an information exchange mechanism established by the Cartagena Protocol on Biosafety to assist Parties to implement its provisions and to facilitate sharing of information on, and experience with, living modified organisms [13].

## 1.3 Definition of a GMO for this report

The definition of a Genetically Modified Organism (GMO) for this report is the definition as has been laid down in the EU Directives 2001/18/EC on the deliberate release into the environment of genetically modified organisms and 2009/41/EC on the contained use of genetically modified micro-organisms. GMOs are defined as organisms, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination [3, 14].

# 2 Material and methods

Throughout the writing of this report the following databases and internet sites were consulted.

General information on the status of Biotechnology worldwide:
- The annual Global Agricultural Information Network (GAIN) country reports on biotechnology (e.g. designated Biotechnology – GE (Genetically Engineered) Plants and Animals) were a valuable source for generating the general overview of current developments and marketing status of GM animals for most countries involved in modern biotechnology [15].
- The BioDeC database, an initiative of the Food and Agriculture Organization of the United Nations (FAO), gives an overview of the state of Biotechnology in developing countries [16].
- Living Modified Organism (LMO) Registry of the Biosafety Clearing House [13].

For specific regions the following databases and sites were consulted:
European Union (EU): Deliberate Releases and placing on the EU market of genetically modified organisms - GMO Register [17], European Medicines Agency: European Public Assessment Reports for Human and Veterinary Medicines [18], European Food Safety Authority (EFSA) Journal [19]
EU register of genetically modified food and feed [20],
USA: Genetically Engineered Animals (FDA) [7], Complete List of Vaccines Licensed for Immunization and Distribution in the US (FDA), Veterinary Biological Products. Licensees and Permittees (USDA) [21], Biopesticide Active Ingredient Fact Sheets (EPA) [22], Federal register [23].
China: Chinese Biosafety Clearing House [24],
Japan: Biosafety Clearing House [25],
Korea: Biosafety Clearing House [26],
Canada: Veterinary Biologics Licensed in Canada (Canadian Food Inspection Agency) [27], Health Canada [28],
Australia: List of applications and licenses for Dealings involving Intentional Release of GMOs into the environment available from the Office of the Gene Technology Regulator (OGTR) [29].

To get more information on specific products from for instance patents databases and companies, we did specific searches on the internet applying the name of the product, and 'recombinant' or 'genetically modified' in combination with the modified species. Specific information was also gathered in PubMed by tracking down the papers in which the GMOs were originally described, usually by cross reference from the above sources.

A number of recent reports and literature reviews covering either novel developments in the generation of GM organisms, or risk (assessment) of GM organisms were a valuable source in generating the inventory. The following are examples of especially useful reviews/reports that cover many of the developments for specific categories of GMOs [1, 30-36].

In order to get an indication of the most recent developments involving the (potential) commercial application or illegal import of GMOs, software from Howards Home [37] was applied to search over 7000 reliable internet sources

with free content using a specific search profile that included the following search terms: ‘gene* therapy’, ‘gene* transfer’, ‘geneti* modif*’, ‘geneti* manipulat*’, ‘gmo’, ‘viral vector*’, ‘vector design’, ‘vector development*’, or ‘transgen* and animal*’. From these search results, relevant news items were subsequently selected. News items were found in essentially the same categories as found by consultation of the abovementioned databases and internet sites (GM ornamental fish, enhancement of GM animals for food/feed, gene therapy, GM vaccines, insect born-disease control, GM (micro-)organisms for production of substances. Relevant additional information found by this means was incorporated throughout the report. Especially with regard to the future environmental applications of GM micro-organisms, some interesting new developments were found that are mentioned in the report (see section 3.2.6).

# 3 Results

## 3.1 General overview of GM products that may lead to illegal introduction

### *3.1.1 GM products with (possible future) market authorization*

Illegal introduction of GMOs can be mostly expected of GMOs that are commercially sold in other countries, especially if they are available to the general public, simply because of their availability in numbers and the relative ease by which they can be obtained.
Such products may be commercially available because they have received a marketing authorization, but it is also possible that their commercial sale is not (yet) regulated, or that it has been decided to not regulate the product at all. In addition, GM products may be imported from countries where the product is also illegal, but where their sale is tolerated. The GM zebrafish is a good example of a GMO to which these different situations apply (see also section 3.2.1.1. for specific examples).

Using the methods and sources described in section 2, an inventory of GM animals and micro-organisms on the market (whether legal, illegal, or not regulated) and of the most relevant organisms that are near the market was generated. This inventory is presented in section 3.2 and in a number of tables that contain parameters useful for generating the priority list for the inspection. As indicated before, the aim was to identify information relevant for the priority list for inspection (e.g. modification technique, possible hazards of the GMO and information concerning availability and potential sources of illegal introduction).

Table I provides a general overview of developments in the commercialization of GM animals for several countries. The countries included are based on the Worldview Scorecard 2010, a list of innovation-capacity scores for 39 countries, published by Scientific American [38]. In the table, the countries of the European Union have been grouped together because marketing authorisations are, once given, valid in the entire European Union. The information in the table is partly based on the information provided in the annual USDA country reports on Agricultural Biotechnology that also cover the development of GE animals. In the writing of this report, additional information from other sources was added. Obviously, since no USDA report is available for the USA itself, developments in the USA were covered from other sources, most notably the website of the FDA [7]. In the course of this project, a number of other relevant countries in which development of GE animals is taking place were added to the table.

Table II includes the specifications of GM animal (products) commercially available and of GM animals that may be near a marketing status because, for instance, a market application has been submitted. Being part of the inventory, the table includes the essential parameters that are useful in generating the priority list for the inspection.

*Table I. Status of commercial GM animal development and availability in various countries*

| Country | Cartagena protocol ratified | GM animal development | Development phase | Type of developments | GM animal: | | |
|---|---|---|---|---|---|---|---|
| | | | | | Available | Market approval | Market application |
| Australia | No | Yes | Early research stage | farm animals | | No | GloFish (retracted) |
| Brazil | Yes | Yes | Early research stage | farm animals, GM mosquito (Field trial) | | No | |
| Canada | No | Yes | Market application | fish (food), biomedicines/substances (farm animals) | | No | Enviropig |
| China | Yes | Yes | Field trials | fish (food), biomedicines/substances (farm animals) | * | No | 'Biotech animals' |
| European Union | Yes | Yes | Research | biomedicines, disease resistance (farm animals) | * | No | |
| Hong Kong | No | No | | | (TK-1/2/3)[2] | No | |
| India | Yes | Yes | Early research stage | | * | No | |
| Indonesia | Yes | Yes | Early research stage | | * | No | |
| Japan | Yes | Yes | Research | biomedicines/substances, bio-organs | | No | |
| Korea (South) | Yes | Yes | Research | biomedicines/substances, bio-organs | | No | |
| Malaysia[1] | Yes | Yes | Field trial | GM mosquito | TK-1/2/3, * | Unclear | |
| Mexico | Yes | No | | | | No | |
| New Zealand | Yes | Yes | Field trial application | biomedicines/substances (farm animals) | | No | |
| Philippines | Yes | No | | | | No | |
| Russia[1] | No | Yes | Research | substances (farm animals) | * | No | |
| Singapore[1] | No | Yes | Research | ornamental fish | TK-1/2/3?, * | Unclear | TK-1/2/3 |
| South Africa | Yes | No | | | | No | |
| Switzerland | Yes | Not reported | | | | No | |
| Taiwan | No | Yes | Market application | ornamental fish, biomedicines (farm animals) | TK-1/2/3 | Yes | Ornamental fish (medium size) |
| Thailand | Yes | No | | | * | No | |
| USA[1] | No | Yes | Market application | fish (food), biomedicines/substances (farm animals) | GloFish (not regulated) | GTC goat | Enviropig, NeonMice AquAdvantage Salmon |

Main source for most countries: The annual Global Agricultural Information Network (GAIN) country reports on biotechnology available for most countries [15].
[1]Mainly other sources were used. For additional sources see text. [2]Regulated since 2011. *Examples of countries where GM danios are/were (illegally) available.

*Table II. Examples of GM mammals and fish (intended) for commercial use*

| **Trade name** | **Species** | **Applied technique for GM** | **Transgenic insert, regulatory sequences** | **Country of generation** | **Company** | **Commercially available in** | **(Proposed) conditions of use** | **Status market application** | **ERA available from** | **Niche[1] in NL** | **Risk** under condition of use | **Detection method** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NeonMice | Mouse | unknown | GFP/RFP, unknown regulatory sequences | USA | NeonPets | | Retail | USA (pending) | | Yes | t.b.d. | visual and PCR |
| GloFish | Zebrafish | DNA microinjection | GFP/YFP/ RFP, mylz2 promoter | Singapore | Yorktown | USA | Retail | Australia (retracted) | COGEM | No | Negligible | visual and PCR |
| TK-1 | Medaka | DNA microinjection | GFP/YFP/ RFP, actin promoter | Taiwan | Taikong | Taiwan, Malaysia | Retail | Approved | COGEM | No | Negligible | visual and PCR |
| TK-2 | Zebrafish | DNA microinjection | GFP/YFP/ RFP, actin promoter | Taiwan | Taikong | Taiwan, Malaysia | Retail | Approved | COGEM | No | Negligible | visual and PCR |
| TK-3 | Zebrafish | DNA microinjection | GFP and RFP, ubiquitous+muscle or skin specific promoter | Taiwan | Taikong | Taiwan, Malaysia | Retail | Approved | COGEM | No | Negligible | visual and PCR |
| N.A. | Angelfish, Cichlid | unknown (electroporation) | GFP, muscle-specific promoter | Taiwan | Jy Lin | | Retail | Taiwan (pending) | | No | t.b.d. | visual and PCR |
| Athryn[2] | Goat | DNA microinjection | rhAT | USA | GTC | USA, Europe (product) | Contained | Approved | FDA | Yes | Negligible | validated PCR |
| Rucin/ Ruconest[2] | Rabbit | DNA microinjection | conestat alfa | Netherlands | Pharming | Europe (product) | Contained use | USA (refused) | EMA | Yes | Negligible | |
| Enviropig | Pig | DNA microinjection | phytase (*E. coli*) | Canada | University of Guelph | | Contained | Canada, USA (pending) | | Yes | t.b.d. | |
| N.A. | Cow | DNA SCNT | rHLZ | China | | | | | | Yes | t.b.d. | |
| AquAd-vantage | Salmon | DNA microinjection | GH (chinook salmon), AFP promoter | Canada | Aqua-bounty | | Contained | USA (pending) | Applicant | Yes | t.b.d. | validated PCR |
| N.A. | Common Carp | DNA microinjection | GH (grass carp), B-actin promoter | China | | | | | | Yes | t.b.d. | |
| N.A. | Tilapia | DNA microinjection | GH (tilapia) | Cuba | | | | | | No | t.b.d. | |

Overview of approved products and market applications with their known specifications. For sources and more detailed information see text report and list of abbreviations.
[1]Environment in the Netherlands in which the species can survive. [2]Product is a substance.

For some categories of GM animals, e.g. GM insects, no marketing application has been done thus far. In Table V examples of GM insects that are commercially developed and that have been applied in deliberate release trials are listed.

Tables VI-VIII provide an overview of marketing authorizations of living GM micro-organisms worldwide. Table VI includes the specifications of approved GM veterinary vaccines, Table VII includes the specifications of gene therapy products and vaccines for use in humans, and Table VIII includes approved GM pesticides and products for crop enhancement.

The GMOs listed in the tables will be discussed in more detail in section 3.2

### *3.1.2 Other scenarios for illegal introduction: GMOs without marketing authorization*

Although less obvious, there is always a possibility that GMOs are introduced that do not have a (future) marketing license abroad. For instance, such an introduction may result from a scenario where the GMO is deliberately released into the environment abroad in a field trial, after which the GMO is accidentally introduced in a neighbouring country by trans-boundary movement of the GMO itself, or of a GMO containing host (with GM insects being an obvious example). Other scenarios are for instance the escape or theft of GMOs from a contained use facility. When applicable, such scenarios will be touched upon in this report, and in some cases examples will be given, but it is of course less predictable which specific kind of GMOs this will concern, since the amount of specific GMOs handled in contained use and deliberate release trials is vast, compared to the limited amount of GMOs available on the market.

A special scenario that is becoming reality is the illegal introduction of GMOs in the environment by their illegal generation within the country itself. Recombinant DNA plasmids are not considered GMOs in the Netherlands, and thus no (contained use) license is necessary to order, keep, or store them. However, a GMO license is obligatory as soon as the recombinant DNA plasmids are applied to modify cells or (micro-)organisms. In the Netherlands there have been several cases of schools using fluorescent bacteria (generated using kits that contain plasmid DNA and the *Escherichia coli* K12 strain) in their educational programme, without being aware of the GMO legislation. Another (potential) example of the illegal application of recombinant plasmids is their possible use in gene doping. A novel trend that definitely deserves more attention is ‘Do-it-yourself Biology’ in which individuals apply biotechnology at their own homes. These subjects will also be specifically discussed in the next sections.

## 3.2 Details of GM products on or near the market

### *3.2.1 Companion animals*

#### 3.2.1.1 GM Ornamental Fish

***Introduction***

A variety of fluorescent fish have been made commercially available on the market since 2003 in the USA and Taiwan. In 2009, the RIVM reported on the presence of illegal genetically modified ornamental fish in the Netherlands by order of the ILT Inspectorate in the report 'Genetically modified ornamental fish in the Netherlands. A glowing problem?' [1]. This investigation was initiated after genetically modified zebrafish (*Danio rerio*) were found to be illegally marketed via the internet by two Dutch ornamental importers/traders in 2008. The main conclusions from this report were that professional importers and retailers and private aquarium owners were at that time generally unaware of GMO regulations in the EU. According to this report, the scale of GM ornamental fish trade in the Netherlands and other countries is small, but is expected to stay, given the ongoing demand for these fish. The scale and trading of genetically modified (ornamental) fish were expected to increase in the future, given the improved biotechnology tools to generate these genetically modified fish [1].

In the next sections, we focus on the different companies involved in the production of genetically modified ornamental fish, using both information from the RIVM report and relevant (novel) information from other sources. Apart from the already identified companies Yorktown Technologies and Taikong Corporation, there is new player on the market: the Jy lin trading company, involved in the development of medium sized ornamental fish.

***Yorktown Technologies***

Yorktown Technologies (Austin, Texas, USA) is selling genetically modified zebrafish (*Danio rerio*) by the trade name GloFish. GloFish expressing green ('Electric green'), red ('Starfire red') or orange ('Sunburst orange') fluorescent proteins [39] were originally developed at the University of Singapore by microinjection of plasmid DNA encoding fluorescent proteins driven by the zebrafish muscle-specific mylz2 promoter [1, 40-42]. Recently, variants expressing blue and purple fluorescent proteins ('Cosmic Blue' and 'Galactic Purple') were added as new products. According to the company's website, each new GloFish inherits its unique colour directly from its parents, maintains the colour throughout its life, and passes the colour on to its offspring [39].
In the USA, after two years of extensive consultation with various agencies, a definitive risk assessment was generated by the FDA that decided not to regulate these fish. In a statement, the FDA has indicated that 'Because tropical aquarium fish are not used for food purposes, they pose no threat to the food supply. There is no evidence that these genetically engineered zebra danio fish pose any more threat to the environment than their unmodified counterparts which have long been widely sold in the United States' [43]. Following the introduction of illegally imported fluorescent zebrafish in the Netherlands in 2006, The Dutch Committee on genetic modification (COGEM) has also stated that there are no environmental hazards associated with these fish [44].
GloFish are exclusively sold in the United States (except California). Although Yorktown Technologies did submit an application in 2006 to import and supply GM zebrafish to the Australian wholesale and retail ornamental aquarium fish

trade, this application for commercial release was withdrawn and there are currently no plans to submit an application in either Australia, Canada or Europe [29, 39]. Interestingly, the GloFish have not yet been marketed in Singapore due to licensing problems [45] and are not for sale anywhere in Asia. In Australia, the Gene Technology Technical Advisory Committee (GTTAC) has advised that the fluorescent proteins in the GloFish are not likely to result in toxicity/allergenicity to humans and other organisms, and noted that data from previous releases (e.g. in the USA) would be useful in the preparation of the Risk assessment and risk management plan [46].
A patent issued by Yorktown Technologies for the development of fluorescent fish includes the application of alternative promoters (inducible, skin-specific, skeletal muscle-specific, etc.) for expression of different fluorescent genes and application in other fish (medaka, goldfish, carp, koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, and pangasius) [47].

***Taikong corporation***
Taikong corporation (Taiwan) has developed several transgenic lines of zebrafish (known by the trademark TK-2) and ricefish (Medaka) (known by the trademark TK-1) that are available on the market in Taiwan [1, 48]. Moreover, double fluorescent TK-3 Danios known by the name TK-3 Candycane are now for sale in Taiwan. These Danios emit red fluorescence from the front end of the body and green fluorescence from the rear end of the body [48]. For an overview of the different lines available see Table III.

*Table III. Overview of fish lines sold by Taikong*

| Species | Trade name | Insert | Comment |
|---|---|---|---|
| Fluorescent rice fish | TK-1 Green | GFP | |
| Fluorescent rice fish | TK-1 Green Diamond | GFP | |
| Fluorescent rice fish | TK-1 Red | RFP | |
| Fluorescent rice fish | TK-1 Red Diamond | RFP | |
| Fluorescent rice fish | TK-1 Golden | GFP+RFP | |
| Fluorescent rice fish | TK-1 Golden Diamond | GFP+RFP | |
| Fluorescent zebrafish | TK-2 Red | RFP | |
| Fluorescent zebrafish | TK-2 Yellow | GFP | Derivative? |
| Fluorescent zebrafish | TK-2 Green | GFP | |
| Fluorescent zebrafish | TK-2 Purple | RFP | Derivative? |
| Fluorescent zebrafish | TK-2 Red Leopard | RFP | |
| Fluorescent zebrafish | TK-2 Purple Leopard | RFP | Derivative? |
| Fluorescent zebrafish | TK-2 Platinum Red Leopard | RFP | |
| Fluorescent zebrafish | TK-2 Platinum Green Leopard | RFP | GFP? |
| Fluorescent zebrafish | TK-3 Candycane | GFP+RFP | |

The single-fluorescent zebrafish and medaka are produced, using micro-injection of linearized plasmid containing (from upstream to downstream inverted terminal repeats (ITR-R) of adeno-associated virus (AAV), actin gene promoter, fluorescent gene (either GFP, RFP, YFP, OFP, BFP, or CFP), SV40 poly A, and inverted terminal repeats (ITR-L) of AAV. The inclusion of AAV ITRs apparently leads to increased and more stable expression in the F0 and subsequent generations [49].

Taikong has also issued a patent for the development of fluorescent zebrafish and other ornamental fish in which the method for generation of fish that express two (or more) fluorescent genes at the same time (e.g. the already marketed TK-3 Danio Candycane) is described. This method applies micro-injection in fertilized eggs of recombinant plasmids containing one fluorescent gene (e.g. GFP) under control of a ubiquitous promoter, and a second fluorescent gene (e.g. RFP) under control of a muscle-specific or skin-specific promoter, wherein the ubiquitous promoter and the skin-specific or muscle-specific promoter have a reverse direction. According to the patent, transgenic fish from this invention will be selected from the group consisting of mekada, zebrafish, discus, goldfish, killifish, cichlid, guppy, arowana, koi, show betta and other (ornamental) fish. The patent also includes the use of heavy metal (cadmium, cobalt, chromium) inducible, or hormone (estrogen, androgen) inducible promoters to monitor environmental pollution and water quality. Apart from microinjection of plasmid DNA, also 'infection with recombinant vectors' is mentioned as a potential technique in this patent [50]. According to patents in the Taiwanese patent database [51], Taikong is applying breeding of fluorescent TK-1 and TK-2 fish with other non-modified *Oryzias* en *Brachydanio* species (i.e., *Oryzias curvinotus* and *Brachydanio frankei*) to generate 'novel fluorescent fish' [52]. Moreover, Taikong has issued a patent for 'Novel fluorescent cichlid and method for producing thereof' [53].
According to a newspaper from Singapore, TK-1 ricefish were for sale in 2003 in Hong Kong, Singapore, Japan and Malaysia [54]. Sales in Japan were apparently stopped by the authorities [55]. In 2003, Adec trading company in Singapore illegally imported fluorescent TK-1 fish from Taiwan and was fined by the authorities [54]. A market approval application for Taikongs fluorescent fish was done in Singapore in 2004 and was still under review in 2007, according to messages on the forum of the Taikong corporation website [48, 56, 57]. More information on the sales of these fish was gathered from the companies' website, in particular the forum in which information requests concerning the fluorescent fish were answered by representatives of the company. According to the information on the forum the fish are or have been for sale from agents in Malaysia and, of note, Greece [48]. On the forum, requests for the fluorescent fish were done from several countries including the USA, Canada, UK, Netherlands, Norway, Spain, Peru, Mexico, Italy, France, and India. In most cases, it was answered that there were patent problems (USA), problems with GMO legislation (Europe), or that the fish were not yet allowed for import in the particular country. Notably, a Dutch person requesting information on the sale of the fish in 2006 was given the address of a retailer in Belgium. On requests for the possibility of marketing of the fish from Mexico and USA, the company answered that the persons requesting would be contacted back. Furthermore, on the forum it was stated that the breeding of the fish is not allowed due to intellectual property and is, according to the company, not possible [48].

***Jy Lin trading company***
Another Taiwanese company, Jy Lin trading company, has recently developed the first medium sized fluorescent GM fish: green-fluorescent Angelfish (*Pterophyllum scalare*) and Convict cichlid (*Cichlasoma nigrofasciatus*). These GM fish were generated by a technique designated 'reproductive organ electroporation' in which fluorescent genes are delivered into the reproductive organs, after which male and female are mated to give rise to fluorescent offspring. The exact nature of the constructs (plasmid DNA or viral vector) is unknown, but the constructs include a whole-body muscle-specific promoter coupled to the GFP gene and an 'antimicrobial peptide gene'. These fish are not

yet for sale and are currently awaiting market approval by the Taiwanese Fisheries Agency, that is trying to confirm that these fish are not able to reproduce and sustain in the wild. If these fish pass field tests, they are expected to be on the market soon. According to the Jy Lin trading company website, many foreign biological museums actively invited the company to exhibit these fish. The company is now researching other colours and the possibilities for adapting the technology for even bigger fish [58, 59]. Unfortunately, no additional information about the development of these fish could be recovered by searching the Taiwanese patent database [51].

***Other species***

Other genetically modified Medaka (*Oryzias latipes*) and White Skirt Tetra (*Gymnocorymbus ternetzi)* expressing fluorescent proteins under muscle-specific fish promoters have been generated for reported 'ornamental purposes' in the past, using the DNA micro-injection technique that has also been applied in the GloFish [41, 42, 60, 61]. However there is no indication that these fish are currently being made available on the market.

***Reports of illegal introduction of genetically modified fish***

Table IV provides an overview of reports available on the internet of illegally imported GM fish, including (when available) the imported number of fish, the (suspected) country of origin and the method applied for detection. These reports were mainly found directly or indirectly applying the search terms ('Danio' or 'GM fish' and 'illegal import'). In addition to these reports, there is a limited amount of other relevant information concerning the countries of origin. According to information by an expert of the OFI (Ornamental Fish international) and by Dutch retailers, fluorescent zebrafish are being bred in India, Indonesia, Malaysia, Taiwan, China, Korea and Russia, and mainly imported in the Netherlands from Indonesia [1]. According to the UK, GM inspectorate countries where fluorescent zebrafish have been reported to be for sale include besides Taiwan and Malaysia, Cuba and Hong Kong [64]. In 2011, a Genetically Modified Organisms (Control of Release) Ordinance has been issued in Hong Kong, meaning that genetically modified fish are now regulated and require prior approval before they can be introduced into the environment. The objective of the Ordinance is to implement the Cartagena Protocol on Biosafety to the Convention on Biological Diversity in Hong Kong. Regulation will take effect in March 2011 [71]. Consequently the fish are currently not legally available in Hong Kong.

***Sales on the internet***

A limited search of trading sites was performed. Initially no offers of GM fluorescent fish could be traced on major Dutch trading sites. In August 2011, suspected pink danios were offered by a hobby breeder on a major Dutch trading site [62]. On an international trading site, suspect GM fish (e.g. designated 'pink (fluorescent) color danio', 'Pink Zebra Danio', 'Red danio', or 'Yellow Zebra Danio' are regularly offered from agents located in Thailand and Indonesia [72]. 'Red Zeebra' danios are also part of the catalogue of an ornamental fish exporter from Sri Lanka [73].

*Table IV. Overview of reports of illegally imported (suspected) GM fish*

| Year | Country | Details of 'illegal' import |
|---|---|---|
| 2011 | Netherlands | Sale of suspected GM zebrafish by retailer. Sale of suspected pink Danios by a hobby breeder via major Dutch trading site (Marktplaats) in August 2011. Confirmed by PCR. [62, 63]. |
| 2011 | United Kingdom | Sale of suspected 'glofish' at a local aquarium. Non GM glowlight tetra (*Hemigrammus erythrozonus*) and glowlight danio (*Danio choprae*) were for sale, but there was no evidence of GM fish [64]. In July, RFP expressing fish from China were intercepted at the border. |
| 2010 | United Kingdom | Interception of suspect GM fish (bright pink in colour) by an importer in August 2010 in a mixed population of fish. Tests confirmed that the fish were genetically modified and contained the Ds-Red type transgenic construct. The fish had been imported from Sri Lanka and had been substituted for fish that were 'not available'. None of the fish were placed on the market in England. Attempts to contact the supplier in Sri Lanka were unsuccessful. The industry body OATA circulated this to colleagues in the Far East to raise awareness that they must not send these fish to the EU [64]. |
| 2010 | United Kingdom | Interception of suspect GM fish (in a mixed population) in September 2010 at Manchester Airport. Tests confirmed the fish were genetically modified and contained the Ds-Red type transgenic construct. These fish were from Thailand. Efforts to contact the supplier were unsuccessful [64]. |
| 2009 | Ireland | GM Danios; probably from Indonesia [65]. Naturally occurring Glowlight danios (*Danio choprae*) were also present. |
| 2008 | United Kingdom | Suspected GM Yellow red zebra danios [66] |
| 2008/09 | Netherlands | Hundreds of RFP expressing danios from Indonesia seized at two importers and at different retail points including major Dutch trading sites on the internet (Marktplaats / Speurders). Confirmed by PCR [1, 63] |
| 2007 | United Kingdom | Coral pink danios (RFP)/Red danios (RFP and GFP). Modification confirmed by PCR [64]. Red danios were supposedly bred at home by an aquarist after being obtained from a retailer in UK. The fish were supposedly imported from breeders in the Czech republic that had imported a stock from Asia [67]. |
| 2007 | New Zealand | 300 Red/pink danios suspected to be originally imported from Singapore [68] |
| 2007 | Germany | Danios at an exhibition in Kiel from Czech republic or Poland [1, 67] |
| 2006 | Netherlands<br>Czech republic<br>Austria<br>Germany | 1400 RFP expressing danios from Singapore at an ornamental fish importer; of the fish 400 had been sold at a retail shop. Modification confirmed by PCR. The invoice stated that the fish were Glowlight Danio's, a name commonly used for (unmodified) *Danio choprae*. Also concurrent reports of illegal introduction in the Czech republic, Austria, Germany [1, 63, 69] |
| 2004 | Canada | Thousands of GloFish were imported from the USA [70]. |
| 2003 | Singapore | >400 TK-1 ricefish (produced in Taiwan) fish imported from Malaysia [54]. |

***Detection methods***
Unlabeled GM fluorescent fish can be detected by visual inspection, but this requires the use of UV black light and/or confirmation of the genetic modification by PCR. However, given the large number of ornamental fish that arrive at Schiphol airport each day, it is in practice rather difficult to detect these fish. The RIVM has already pointed out in a previous report that control and inspection of unlabeled (ornamental) fish with other non-visually detectable modifications (e.g. changes in temperature and pH) will be even more complex [1].

***Conclusions***
There are three companies that are currently actively engaged in the commercial development of GM ornamental fish: Yorktown Technologies (USA), Taikong corporation (Taiwan) and Jy Lin trading company (Taiwan). The GloFish marketed by Yorktown technologies are exclusively sold in the USA. The fluorescent fish marketed by Taikong corporation have been sold by agents in Singapore, Taiwan, Malaysia and Hong Kong, and possibly (in the past) also by agents in Greece and Belgium. Taikongs TK-1/2/3 fish are legally sold in Taiwan, Malaysia and possibly Singapore. According to the RIVM report from 2009 concerning the illegal import of GM ornamental fish in the Netherlands, breeding of GM fluorescent fish is taking place in a number of countries, including India and Indonesia. Since the writing of that report, the illegal import of GM fluorescent fish from South-East Asia to the European Union has continued throughout 2010 and 2011. In 2010, illegal imports of GM fish expressing red fluorescent protein originating from Sri Lanka and Thailand were reported in the United Kingdom. A limited search confirmed that such fish are (still) regularly offered on the internet by retailers and exporters from these countries. In 2011 again, illegal fish expressing RFP were imported in the Netherlands and in the UK (in the latter case from China). In August 2011, red fluorescent zebrafish were also offered on a Dutch trading site.
The exact nature and origin of the fish that have been illegally imported in the Netherlands and other countries remains illusive. Possibly, these are lines of fish derived from the original GloFish or TK-2 fish by breeding without allowance of the manufacturer, which would also imply a violation of the patent, apart from the possible violation of GMO laws (depending on the country). Novel ornamental fish variants have recently appeared on the market, or are expected to be on the market soon in Taiwan (TK-3 Danios and fluorescent Angelfish/Convict Cichlids). Given the frequency of illegal imports of GM fish in the past and the (future) availability of new GM fish variants, illegal imports of GM fish are expected to continue in the future.

#### 3.2.1.2 Other companion animals

***Introduction***
In the recent scientific/technical report 'Defining Environmental Risk Assessment Criteria for Genetically Modified (GM) Mammals and Birds to be placed on the EU market' submitted to the EFSA, the improvement of companion animals has been identified as an important driver for the current development of GM animals. According to this study, if GM companion animals are developed, their release in the environment will be very likely [32].

***Hypoallergenic cats and dogs***
In the past, (claimed) attempts have been made to develop genetically modified companion animals. An enterprise involving the development of allergy-free cats

and dogs failed in 2001 [32]. In 2004, the Allerca company claimed to be developing genetically modified cats with a hypoallergenic trait based on the deficiency of the gene encoding the Fel d 1 allergen. Later, Allerca was reported to have abandoned genetic engineering [74]. Indeed, the Allerca company is presently selling cats and dogs with claimed hypoallergenic traits based on naturally occurring divergences in the Fel d 1 and Can d 1 allergens, respectively. From the description on the Allerca website, it is clear that these cats and dogs were generated by natural breeding and selection and thus do not fall under the GMO legislation [75]. This is also true for hypoallergenic cats and dogs that are currently on the market, sold by other companies. Another company, Felix Pets, is presently using biotechnology to produce, as they claim, the world's first allergen-free cats. According to their website, hypo-allergenic cats are being generated by first removing the Fel d 1 gene from a single cat cell, after which this cell will be implanted into a surrogate cat to grow into an allergen free kitten. This description most probably implies that somatic cell nuclear transfer (SCNT) is the technique that will be applied by this company [76].

***Fluorescent animals***

Numerous lines of transgenic mice from different suppliers are commercially available for scientific research purposes that normally take place under contained conditions. A San Francisco based company called NeonPets is, according to their website, waiting for the FDA approval of the commercial sale of their NeonMice$^{TM}$ to the general public. The exact method applied for the modification of these mice is unknown. The company wants to make different varieties of fluorescent mice (Emerald Green, Ruby Red, Sapphire Blue, Yellow Quartz), including hairless variants, commercially available. The NeonMice that will be commercially available are exclusively sterilized males. The company is also trying to establish international markets outside of the USA and is considering to generate other glowing animals in the future [77].

Korean researchers have developed techniques to develop genetically modified cats that ubiquitously express the red fluorescent protein (RFP). The technique used is nuclear transfer, using somatic cells that were first genetically modified by infection with an RFP expressing retrovirus. These cats are able to transmit this modification directly to their offspring. Alternatively, the modification can also be recloned, using somatic cells from the first generation cat [78-80]. Dogs expressing RFP have also been generated, using retroviral transduction of somatic cells followed by the somatic cell nuclear transfer [81]. Although developed for research purposes, it is not impossible that these cats, dogs and other animals (e.g. rabbits, rats, ferrets) that have been modified with fluorescent proteins may appeal to the market as companion animals in the future, similar to what has happened with the GM zebrafish [32], and apparently, as described above, GM fluorescent mice. This is also illustrated by the fact that the Czech Environmental Inspectorate (CEI) recently discovered that a small number of unauthorized GM rats expressing GFP in the eye were held as pets and bred by individuals. The modification was detected under UV black light and confirmed by PCR. These rats are thought to originate from a rat that somehow escaped from a lab in the Czech republic. The rats were difficult to breed and posed no risk to the environment according to Czech GMO experts [82, 83].

***Detection methods***
Similar to fluorescent fish, fluorescent GM companion animals may be detected by visual inspection under UV black light and confirmation of the genetic modification by a PCR specific for the common fluorescent genes.

***Conclusion***
So far, no other GM companion animals than the fluorescent GM fish have been approved on the market worldwide. Thus, there is currently no requirement to have specific attention regarding the potential unintended or illegal introduction of such animals. However, this may change in the near future, given the market application of the NeonMice in the USA and the exploration of other markets by the company involved. The illegal introduction of unauthorized GM rats expressing GFP in the Czech Republic demonstrates that the Inspection should stay aware of illegally held 'escapees' from research labs (see also section 3.1.2).

### *3.2.2 GM Animals for food/feed, substances and other purposes*

#### 3.2.2.1 GM fish for food/feed, substances and other purposes

***Introduction***
An extensive overview of genetic modification in fish is provided in the recent report 'Defining Environmental risk assessment criteria for genetically modified fishes to be placed on the EU market', that was published by order of the European Food Safety Authority [31]. In this report, more than 50 fish species were identified that have been genetically modified, with over 400 fish/trait combinations. The report highlights that the development of (potentially commercial) GM fish is most commonly aimed at enhancing growth and/or environmental tolerance in food species, such as salmon and carp, with transgenic insertions of genes encoding for growth hormone genes and antifreeze proteins being the most common traits. Other major reasons for the development of GM fish that were identified include increased disease resistance (especially in intensively cultured fish), increased dietary performance, development of GM fish as bioindicators, and the production of fish for ornamental purposes (see also section 3.2.1.1). Less common but interesting traits that were designed in the past with a potential commercial aim include the use of GM fish as bioreactors for instance for the production of medicines (similar to GM mammals that are being developed for this purpose, see section 3.2.2.2), the enhanced growth of fish solely for prize course fishing and the transgenesis of hybrid fish (e.g. hybrids of goldfish and common carp, that are able to reproduce). According to the report, the GM traits that are being developed include traits that may lead to significant advantages of the GM fish over their wild type counterparts (e.g. by increased environmental tolerance, growth, survival, and food utilization), which could potentially lead to adverse environmental effects (e.g. increased invasiveness) and consequences (e.g. changes in the fish population and ecological effects), which would pose no problem in case of rearing in a closed facility, but which should be addressed in case dispersal in the environment is possible. Moreover, in case reproduction of the GM fish is possible, dispersal of transgenes into the wild may also lead to environmental risks [31].
Worldwide, several countries are actively involved in the development of transgenic fish for commercial food and feed production. These countries include China, Cuba, India, Korea, the Philippines and Thailand [31, 84]. No GM fish

(products) have been approved on the market worldwide, with the exception of GM ornamental fish, and so far, in the European Union no market approval application for transgenic fish has been done at all. However, since it is expected that applications involving the marketing of transgenic fishes will be submitted in the near future, and in fact transgenic fish would be the first GM animals expected on the EU market, guidelines for the environmental risk assessment and food/feed safety of transgenic fish are being developed by the European Food Safety Authority (EFSA) [31, 84]. Several countries (in particular USA, China and Cuba) have been reported to be near commercialization of transgenic fish for human consumption. For the purpose of this report, we focus on the characteristics of the few GM fish lines that supposedly are or have been near commercialization.

***USA: AquAdvantage Salmon***

In the USA, the application for the placing on the market (commercial sale and human consumption) of the AquAdvantage salmon is currently being reviewed by the FDA and the final decision may be made very soon. The AquAdvantage salmon, which has been developed by the Canadian company Aquabounty in conjunction with the University of Guelph, grows twice as fast as wild type salmon due to production of growth hormone throughout the year (instead of seasonal production). As a result, these fish reach the mature size earlier than their wild type counterparts. If approved, AquAdvantage Salmon will be the first genetically engineered animal intended for human food [85, 86].

The AquAdvantage salmon founder line was developed by microinjection of a construct (opAFP-GHc2) encoding the chinook salmon growth hormone (GH) gene into fertilized eggs of the Atlantic salmon (*Salmo salar*).

The environmental assessment of the applicant that is currently under review by the FDA and that is publicly available describes the potential environmental risks of the AquAdvantage Salmon under the specific conditions of its use being: production of eggs in Canada; shipment of eggs, grow-out and processing of fish in Panama; and shipment of processed fish to the USA for retail sale, and does not consider risks under other production or grow-out conditions [87].

According to the application, the likelihood of escape, establishment, and spread of AquAdvantage Salmon is extremely small due to redundant containment measures that are being implemented at the sites of egg production, grow-out, and disposal. These redundant measures include, apart from the biological containment (production of all-female salmons with a technique which is 100% efficient and pressure shock induction of triploidy, which induces sterility with an efficiency of >99%), physical containment measures (growth in tanks and use of screens and filters), physico-chemical measures (use of chlorine to kill any potential escapees) and geographical and geophysical containment (inhospitability due to poor habitat at the production sites). The environmental assessment concludes that the production of AquAdvantage Salmon under the conditions described will not result in significant effects on the environment due to the unlikely survival of the transgenic salmon in case of an accidental escape (which is estimated to be lower than 1%), the inability of the transgenic salmon to reproduce with wild type salmon and the inability of the transgene to be transmitted to wild type salmon or other species [87].

Apart from the GM salmon, Aquabounty is also developing other faster growing GM fish, notably tilapia and trout [31].

***China: transgenic Carp***

In China transgenic fish are being developed with the aim to produce desirable alterations in growth rates or feed-conversion efficiency [88, 89]. According to the GAIN reports on developments in biotechnology in China, the Heilongjiang Fishery Institute and the Institute of Hydrobiology of the Chinese Academy of Sciences are both involved in ongoing research (including field trials and mammal feeding studies) on transgenic carp that have been modified with constructs encoding growth hormone genes [88].

The Institute of Hydrobiology has conducted a medium scale trial of fast-growing transgenic common carp (*Cyprinus carpio*) generated by micro-injection in fertilized eggs using an 'all-fish' genomic construct encoding the grass carp growth hormone gene (gcGH). In this trial, strict measures were adopted to prevent escape of the transgenic fish. Enhanced growth and more efficient feed utilization of the F1 transgenic fish compared to control fish was demonstrated in this trial. By crossing the (F1) transgenic common carp diploids with a fertile tetraploid Carp strain, sterile transgenic triploids were produced that also showed an enhanced growth rate [89, 90]. More recently, consumption, movements and feeding hierarchy of juvenile transgenic common carp were compared with non-transgenic controls under conditions of limited food supply. The results indicated that transgenic fish possess an elevated ability to compete for limited food resources, which could be advantageous after an escape into the wild, but which may be compensated by other factors (e.g. reduced swimming ability) [91, 92]. The Chinese government has provided funding for the development of studies to assess the environmental safety and food-safety of these fish [89, 90]. Despite these efforts, so far in China no transgenic fish have been commercially produced or approved for consumption [88].

***Cuba: transgenic tilapia***

In Cuba, transgenic tilapia with accelerated growth was established in the nineties by microinjection into one-cell embryos of a construct in which expression of tilapia growth hormone (tiGH) was driven by human cytomegalovirus (hCMV) enhancer-promoter. Nine-month-old transgenic F1 progeny were 82% larger than non-transgenic fish [93, 94]. The national authorities required that environmental and food safety assessments would be conducted before the tilapia could be introduced into Cuban aquaculture. In a study published already in 1999, it was shown that transgenic tilapia had a lower food consumption and dominance status compared to controls. The food conversion efficiency was higher in the GM tilapia compared to controls [31]. Tilapia growth hormone had no biological activity when administered to non-human primates. After consumption of transgenic tilapia by human healthy volunteers, no effects were detected. It was concluded that the fish were safe for consumption and that the introduction of the transgenic tilapia line under the aquaculture conditions in Cuba was environmentally safe [93].

Although there have been reports that this transgenic tilapia is already on the market and being consumed in Cuba, this has been denied recently. According to a Cuban scientist, Cuba does not want to be the first country to release transgenic fish and even has no intention of marketing these transgenic fish at all [95].

***Detection methods***

In the environmental assessment for the AquAdvantage salmon it is indicated that a multiplex PCR is available that will be applied as a means for assessing the identity and integrity of the transgene (GHc cDNA and correct 5' and 3' junctions at the integration site) during commercial production [87] and that

is also of interest with respect to possible detection methods. For other transgenic GM animals, similar detection methods may be available.

***Conclusions***

In the report 'Defining environmental risk assessment criteria for genetically modified fishes to be placed on the EU market', the most important routes by which escape of GM fish could occur are summarized and these include escape or deliberate release during transportation, loss from research or experimental facilities, deliberate indiscriminate introductions to improve fishery performance, escape from commercial aquaculture facilities, and vandalism.

The transport of GM fish is deemed a high risk event in the production chain and may be caused by accidents during transport, failure of the transport containment, or unsatisfactory sterilization of containment water tanks. The receiving environment, including the specific properties of the production chain and the aquaculture facility (open versus closed), is an important element in the environmental risk assessment. The scenario of escape of GM larvae or fish during the commercial production cycle should always be considered. Thus as the report states: 'A thorough knowledge of all steps in the production chains, from import to farming, to delivery-to-market, is therefore a fundamental prerequisite for an effective evaluation of the environmental risks posed by likelihood of GM fishes entering the open water systems' [31].

So far, the only known market application for the commercial production of GM fish for food and feed involves the AquAdvantage Salmon. In this specific application, several levels of containment are implemented in the environmental risk assessment leading to, according to the applicant, a low risk of escape and the unlikely survival of the transgenic salmon in case of an accidental escape [87]. Although this application is still under review by the FDA, it is clear that for the production of the AquAdvantage salmon far more drastic containment measures are being undertaken compared to commercial culture of normal salmon, which usually takes place in intensive open systems without effluent treatment and from which escape would be considered more likely [31].

It is apparent that, in general, production and marketing conditions of (near future) GM fish intended for food/feed will be very different from those of (current) GM ornamental fish. GM ornamental fish are likely to be introduced in the wild, since the living fish itself is available on the market, and unwanted pet fishes are known to be frequently released by their owners in nearby ponds and streams. Moreover, the apparent illegal production of these fish increases the likelihood of dispersal in the environment [31]. In the case of GM fish intended for food/feed, in most cases only the product will be available on the market, with the larvae and fish being cultured in contained facilities that are being considered in the approval process.

Nevertheless, possible future applications of GM fish may include marketing conditions that impose a higher risk of dispersal like introduction on the 'live food market', use of GM fish for the purpose of fish stock enhancement activities, and use of GM fish for the purpose of biocontrol (e.g. to reduce or eliminate an unwanted population) [31].

From the description of the species near commercialization in the previous section, it appears that countries currently involved in the commercialization of GM fish for food/feed are aware of the environmental safety issues and are actively stimulating the development of studies to assess the environmental impact.

Given the fact that there are currently no GM fish for food/feed on the market (worldwide) and the above considerations (production under contained conditions and no marketing of live products in the near future), there is at this

moment no requirement to have specific attention regarding the potential unintended or illegal introduction of commercially available GM fish varieties intended for food/feed in the Dutch environment.

#### 3.2.2.2 Transgenic livestock

***Introduction***

A review of genetic modification in livestock (and companion animals) progressing to possible commercial use is included in the recent report 'Defining Environmental Risk assessment Criteria for Genetically modified Mammals and Birds to be placed on the EU Market' that was commissioned by the European Food Safety Authority [32]. In this report, a limited number of species (15) were identified that have been genetically modified, with approximately 50 species/trait combinations. Species that have been modified (in some cases as a proof concept that modification is possible) include mouse, rat, rabbit, pig, cattle, goat, sheep, yak, water buffalo, quail, chicken, marmoset, ferret, dog and cat. Various transgenesis techniques have been applied to establish germline transmission of the transgene, or knock-out of a gene, including micro-injection or electroporation of (plasmid) DNA, use of yeast artificial chromosomes, use of retroviral and lentiviral constructs and application of transposon-mediated transgenesis. Worldwide, GM livestock is being developed for several commercial reasons including sustainability (e.g. Enviropig), growth enhancement (increased meat production), enhanced disease resistance, production of pharmaceuticals and other substances of interest in milk and eggs, and as (improved) donors for xenotransplantation. Other potentially interesting traits that are mentioned in the report and that may be (further) developed include increased environmental tolerance (e.g. animals adapted to climate change), animals as environmental bioindicators (e.g. for detection of pollutants), and alteration with the goal to improve products derived from the animals (e.g. sheep with altered wool composition).

Similar to the traits developed in GM fish (see section 3.2.2.1), the traits that are used to develop transgenic livestock include traits that may lead to adverse environmental effects, for instance by dispersal of transgenes or by enhanced survival of transgenic animals over their wild type counterparts. Again, this may pose no problem if the opportunities for interaction with the environment are limited (e.g. in case of contained use), but these issues should be addressed in case dispersal in the environment is possible [31, 32].

Worldwide many countries are involved in the development of GM livestock (see Table I and [32]). In the European Union many of the animals that are being developed are kept under contained use restrictions and are therefore unlikely to be released in the environment. An example is the GM rabbits from the Dutch company Pharming (see below).

So far, there are only very few examples of market applications involving (products of) GM livestock animals worldwide. The known applications in the USA include an environmental assessment of the GM animal itself. In the next section we will briefly address these GM animals, the developments with GM animals in China and a few other very recent developments that are intended for market development.

***USA: ATryn producing goats (GTC Biotherapeutics)***

ATryn is the first drug produced by a transgenic animal that has been authorized for use in the European Union and the USA [96-99]. Atryn, the product name of recombinant human anticoagulant antithrombin (rhAT), can be applied in patients with a blood clotting disorder called hereditary antithrombin deficiency.

ATryn is manufactured by the U.S. company GTC Biotherapeutics [100]. The drug is made from the milk of transgenic GTC 155-92 goats that contain five copies of the Bc6 construct that consists of the coding region of rhAT and goat sequences (β casein promoter and other regulatory sequences) that drive expression of rhAT in the mammary gland. The transgenic goat founder animal was originally generated by micro-injection of the construct into the nucleus of a fertilized goat embryo [97, 99]. The drug was authorized for use in EU by EMA in 2006 [99] and in the USA in 2009 [98] for use in patients with hereditary antithrombin deficiency.
The approval of the FDA includes an approval of the recombinant construct that has been applied in the GE goats. The FDA's Center for Veterinary Medicine reviewed the goats as a new animal drug application (NADA) and determined that the transgenic goats have no significant impact on the environment. The goats or its products are not intended for food/feed and procedures were put in place to prevent entry of GM goats in the food supply. The goats are housed on a farm with active on-site-security and secure locked fencing around the entire campus, with double fencing around animal paddocks. Standard operating procedures ensure that waste materials (milk) and animals are adequately disposed at the end of use [97, 98]. GTC is developing additional therapeutic proteins with potential applications in hematology, oncology, and autoimmune diseases using transgenic goats [100].

***European Union: Rhucin producing rabbits (Pharming)***
The Dutch company Pharming [101] is producing drug products designated Rhucin or Ruconest (the two products differ in formula) in transgenic rabbits. Like the above-mentioned GTC-goats, this is an example of transgenic animals being used as bioreactors in order to produce therapeutic drugs.
Rhucin/ruconest contains conestat alfa, a human C1 esterase inhibitor that may be applied to treat patients suffering from hereditary angioedema, a condition that is similar to an allergic response and that may lead to severe swelling and suffocation of soft tissues. The GM rabbits produce the substance in their milk and after milking the rabbits, the drug is extracted with the remnants of the milk being destroyed. The company has declared that a herd of approximately 1000 rabbits is used for commercial production of Rhucin [101, 102]. In the Netherlands, the rabbits are kept under contained use conditions covered by a license from the Dutch government. In 2010, Ruconest received marketing authorization in the EU following a positive recommendation by the EMA [103]. In 2011, the FDA refused to review the marketing application of Rhucin by Pharming and partner firm Santarus, due to incompleteness of the provided data [104]. Apart from Rhucin/Ruconest, Pharming is developing other drugs and food additives (e.g. the human breast milk protein lactoferrin) that are produced in milk, using transgenic technology, for instance also utilizing cows [101].

***USA/Canada: Enviropig (University of Guelph)***
The University of Guelph in Canada has developed the Enviropig™, a transgenic line of Yorkshire pigs that produces the enzyme phytase in the salivary glands. The GM pigs were generated by pronuclear injection of a transgenic construct containing the phytase gene from *Escherichia coli* driven by the murine parotid secretory protein promoter gene. In these pigs the phytase enzyme aids in the digestion of phosphorous containing foods, leading to a reduction in output of fecal phosphorous of up to 75%. The applications (including food and environmental safety data) for the commercial use of these animals for human food consumption were submitted to the FDA in the USA in 2007, and to Canadian regulatory authorities (Health Canada for the assessment of food

safety and Environment Canada for the assessment of environmental safety) in 2009. The application is not publicly available. In February 2010, Environment Canada granted approval to the University of Guelph for the reproduction of the GM pigs in controlled facilities, segregated from other animals under specified conditions. In addition, the University of Guelph appears to be interested in the commercialization of the Enviropig in China [105-107].

***China***

So far, no transgenic live stock has been commercially approved in China [24, 108, 109]. However, there are several examples of GM livestock that is being developed for commercial purposes.

Notably, a herd of cloned transgenic cattle expressing recombinant Human lysozyme (rHLZ) in their milk has recently been generated the China Agricultural University in conjunction with the Biotechnology Company GenProtein. HLZ is a non-specific immune and anti-inflammatory factor that may be useful as an additive or preservative in food and medicinal products. In addition in cows, rHLZ may inhibit the bacteria that cause mastitis. The GM cows received a lot of attention in the media because according to the involved scientists, the development fulfils the conception of humanized bovine milk. The transgenic cows were generated by a construct containing the HLZ coding region driven by the goat ß-casein promoter. The cows were generated by somatic cell nuclear transfer (SCNT), applying the nucleus of modified somatic cells in which the construct had previously been transfected by electroporation [110, 111].

Other notable examples are transgenic cows expressing human fucosylated sugar transferase, transgenic goats expressing the human lactoferrin gene and transgenic goats expressing human lysozyme, of which the last two examples have been approved in enlarged and restricted field trials respectively [108].

***Other developments***

Disease resistance is suspected to be an important (future) driver in the development of GM livestock and GM companion animals. Possible diseases that may be future targets include viral and bacterial diseases in cats, dogs and parrots. In the case of livestock experimental transgenic disease resistant models for e.g. diseases in cattle (mastitis), pig (influenza, pseudorabies) and chicken (avian leucosis) are being developed, with variable results [32, 112].

Recently in the UK, GM chickens were developed (by lentiviral transgenesis) in which onward transmission of highly pathogenic avian influenza virus was prevented by the expression of a short-hairpin RNA that blocks the influenza virus polymerase. Although the GM chickens did not survive the primary challenge with the virus, the result is regarded as an important step in the development of completely disease resistant animals that may have a future commercial use [113].

Several companies are involved in the development of GM animals, in particular pigs, as xenotransplant donors for humans suffering from organ failure. A variety of GM pig lines have been established with the aim to overcome rejection mechanisms in humans. Clinically effective xenotransplantation of organs from GM pigs, however, is believed to require the development of GM pigs that combine several traits [32, 114]. Nevertheless, in 2006 an innovative biological wound dressing was introduced on the market in China as a medical device under the trade name Tiefu by Chongqing Zongshenjunhui Biotech. The dressing contains pigskin cells that have been genetically modified by an adenoviral vector containing the immunosuppressive CTLA4Ig gene [115].

Russian scientists at the Institute of Gene Biology at the Russian Academy of Sciences are developing production of the human breast milk protein lactoferrin

in mice. This milk protein stimulates the infant's immune systems and may be used to improve the formula of baby milk. For commercial purposes the production has to be scaled up to larger animals (goats, rabbits or cows) [116]. A development that recently received a lot of attention in the media were the GM goats producing spider silk in their milk developed by scientists at the University of Wyoming [117].

***Detection methods***

The market application of the ATryn producing goats (GTC Biotherapeutics) includes a method for identifying the construct in the blood or edible products of the GM goats. The method is a validated PCR that detects the presence of genes for the production of human antithrombin and goat β-casein and that is available from CVM [97, 98].

***Conclusions***

In previous and current market applications involving the use of GM livestock in the EU and the USA, the animals are held in closed (contained) facilities, which make accidental release unlikely. For animals most likely to be commercially developed in the near future (e.g. pigs, cattle, goats and chickens), this will most probably also be the case [32]. In particular animals intended for the production of substances and for medical applications (e.g. use of GM animals as xenotransplantation donors) are likely to be held in closed facilities. This may not be only for reasons of environmental safety, but also for reasons concerning defence of commercial value and intellectual property of the GM animals and their products.

Nevertheless, import of livestock with commercial value remains a possibility. This is illustrated by the import of embryo descendants from cloned cattle originating from the USA by a farmer in the United Kingdom in 2008 [118, 119]. Products (milk and meat) from these descendants were subsequently sold on the EU market in 2009, leading to a debate about the safety and the grounds for regulating food from descendants of cloned animals [118, 120]. It should be emphasized that cloned animals are not considered GMOs in the EU and therefore are not regulated by the EU Directive 2001/18/EC.

Risk assessment criteria have been defined for GM livestock (and companion animals) to cover any potential future commercial use, especially uses involving deliberate release, applying apart from scenarios of existing traits, also scenarios of traits that have not yet been developed (e.g. altered herding behaviour in sheep, and cats with increased longevity). Clearly, similar to the risk assessment criteria for GM fish, all steps in the production chain including transportation, level of confinement and receiving environment will be covered in such assessments [32].

Given the fact that currently there is no live GM livestock on the market worldwide and the above considerations about the production of products of GM animals under contained conditions, there is at this moment no requirement to have specific attention regarding the potential unintended or illegal introduction of commercially available GM livestock in the Dutch environment.

### *3.2.3 GM insects and other arthropods*

***Introduction***

Insects are essential to global ecology, and many species (e.g. the honey bee) are valuable as pollinators of agricultural crops. However, specific insects are also involved in the transmission of viral diseases (like malaria and dengue) to

humans and animals, and in induction of crop damage. In recent years GM insects have been developed in order to battle such insect-borne diseases and agricultural pests. Several unique benefits of GM insects have been proposed [121]:

- targeting of a single insect pest species would leave beneficial insects unharmed;
- pest populations inaccessible to traditional control methods could be eliminated;
- reducing the need for (toxic) insecticides;
- protection of everyone in the release area irrespective of socio-economic status and less requirement for involvement of the entire community (when applied in a disease control programme).

The release of GM insects has been criticized by environmental groups. Concerns are, for example, that reducing the wild type population may have an impact on the food chain and that it may create a niche in the environment that may be filled by other dangerous insects [122].

Like the other GMOs presented in this report, the release of a GM insect in the European Union is controlled by the EU Directive 2001/18/EC. In 2005, the COGEM released a notification (CGM/050202-05) on 'the use of transgenic mosquitoes as weapon in the battle against malaria'. COGEM stated that the step by step assessment of the deliberate release of GMOs - as is current policy for all GMOs that have introduced in the Netherlands - is a suitable approach for a safe and scientific solid introduction of GM insects [123].

In the recent scientific/technical report, 'Defining Environment Risk Assessment Criteria for Genetically Modified Insects to be placed on the EU Market' [30], the developments in the field of GM-arthropods are described, including an overview of modified species and introduced genetic traits. With respect to the ERA of GM insects, it is concluded that this should consider various issues regarding the genetic modification, the characteristics of the respective species, the purpose of the release, and the receiving environment. Potential risks are linked to (effects of) gene transfer, effects on other organisms (target and non-target), effects on agricultural management practices, and measures to control insect-born diseases, effects on biogeochemical processes, and effects on human health. In this report it is recommended to follow a case-by-case approach for the ERA of GM-arthropods.
The report by order of the EFSA describes 27 GM insects, and three other GM arthropods that have been generated in the laboratory worldwide. This list was reduced to a list of seven GM species of possible relevance for application within continental Europe in the next ten years: mosquitoes (*Aedes aegypti* (Yellow fever mosquito), and *Aedes albopictus* (Asian tiger mosquito)), flies (*Bactrocera oleae* (Olive fruit fly), *Ceratitis capitata* (Mediterranean fruit fly), and *Stomoxys calcitrans* (Stable fly)), and moths (*Cydia pomonella* (Codling moth), and *Pectinophora gossypiella* (Cotton pink bollworm)). Of these, only the stable fly and codling moth are currently established pest species in the Netherlands [30].

The honey bee (*Apis mellifera*) is currently not considered to be of enough relevance to reach the top ten. However, when beekeepers were asked if they would use GM bees (e.g. GM bees that are resistant to the varroa mite), 47 out of 63 voted yes on a forum where the opposition to the use of GMOs was outspoken [124]. Although such GM bees are not developed, yet this shows that also disease resistance may be an important driver in the development of GM insects.

***Tools to prevent spreading of insects: SIT and RIDL***

Important tools to prevent GM insects from spreading are the use of sterile insects, or insects carrying a dominant lethal trait. Both techniques (Sterile Insects Technology, or SIT, and Release of Insects carrying a Dominant Lethal, or RIDL) will be described below. It is important to note that only the application of the RIDL technology results in insects that should be considered GMOs. However SIT can be combined with other genetic traits that are the result of genetic modification techniques. Genetic modification in insects is usually based on introduction of genetic traits using transposon-based systems [30, 122] (see also Table V).

SIT is a method of insect control that involves the rearing of insects, followed by sterilization (mostly by irradiation) and mass release. The released insects are most often males since usually female insects cause damage to crops (e.g. by laying eggs in the crop) or are involved in disease spreading (e.g. female mosquitoes that sting humans). In the environment, the sterile males compete with the wild type males for mating with the female insects. Because females that have mated with sterile males do not give offspring, this will lead to a reduction of the next generation's population and repeated release of insects may eventually cause the total eradication of a population [125, 126].
SIT has been applied around the world resulting in for instance the control or eradication of the Screw-worm fly (in North and Central America), Mediterranean fly and Mexican fruit fly (in the United States, Central and South America, South Africa, and Europe), the pink bollworm (in the United States), and codling moth (in Canada) [126]. Since 1981, SIT has been applied by a private firm (De Groene Vlieg) in The Netherlands to control the onion maggot *Delia antique* [127]. Drawbacks of SIT are the use of irradiation that might weaken the newly sterilized insects, making them less able to compete with wild males and the need for sorting the male insects from the females. One way to address these problems has been the introduction of genetic modification in insects [126, 128].

RIDL is a method based upon a transgenic system that causes the offspring of released insects to die, unless the larvae are fed with the compound tetracycline. In RIDL insects, tetracycline represses the expression of a transcriptional transactivator (tTA) that controls the expression of gene product that is toxic to the larvae. While in the presence of tetracycline, the insects live and reproduce normally; the absence of tetracycline leads to a lethal phenotype. Several types of RIDL have been developed, involving either the use of transgenic systems that induce lethality in both male and female offspring (Bisex RIDL), or systems that induce repressible lethality that is specific to females (fsRIDL) (see also Table V). The different systems apply non-sex-specific or sex-specific regulatory elements that control the expression of the repressible transcription factor tTA in absence of tetracycline. Also female specific RIDL variants that apply female specific toxins in combination with non-sex-specific regulatory elements have been developed [129, 130]. Another female specific RIDL variant is the use of a transgenic system that uses a repressible female specific flightless and sterile phenotype, resulting in late-acting lethality. This system allows the release of eggs instead of mosquitoes [131].
According to Oxitec, the main company involved in control of insects using genetic modification, the conditional lethal attributes protect against the hazard of accidental release. If RIDL insects were to escape from a rearing facility, they would be unable to reproduce because they would not have access to tetracycline, will not survive and their genes are not spread [130]. Analysis in

the laboratory has shown that the penetrance of the lethal phenotype of the transgene in offspring from matings between *Aedes aegypty* Bisex RIDL (strain OX513A) homozygous males and wild type females (in the absence of tetracycline) was 96.5%. Survivors were weak and short-lived and Oxitec suspects that survival of these would even be lower in the field [128]. However, in case of fsRIDL, males survive, and their male progeny as well. In this case, extinction of the transgenes after environmental release of female-specific RIDL males may take longer depending on the phenotype in males.
In the United Kingdom, the organization Genewatch has questioned the effectiveness of the lethal phenotype in the GM mosquitoes, indicating that the incomplete penetrance of the lethal phenotype should be better understood and that the low fitness phenotype should be more thoroughly investigated [132].

***Oxitec Ltd.***
The GM pink bollworm strain OX1138 (a SIT strain that has been modified with the RFP fluorescent marker) has been evaluated in three years of open release trials by the Center for Plant Health Science and Technology (CPHST) in Phoenix, Arizona. According to the Oxitec website, CPHST has submitted the results of these studies to the cotton industry and is consulting stakeholders [130].
In the USA, APHIS has performed an environmental assessment in 2005 on the field study with OX1138 that is available on the internet, resulting in a finding of no significant impact (FONSI) [133]. The USDA addressed the impact of the use of OX1138 also in the context of the existing SIT programme by conducting an environmental impact assessment. This resulted in a record of decision in which it was declared that the use of the GM bollworm was 'the environmentally preferred alternative'[130, 134].
The results of the open field trials with the OX1138 strain were recently published, showing that the GM strain performed similarly (or sometimes better) in the field, compared to the standard SIT strain (that has been applied in the open field for several years) with respect to dispersal, persistence and ability to mate. The number of transgenic and non-transgenic moths that could be recaptured rapidly declined to zero approximately eight days after the last release. [135].
Oxitec has also developed a number of GM insects incorporating the RIDL technology [130]. RIDL strains of pink bollworm, Mediterranean fruit fly (*Ceratitis capitata*), Mexican fruit fly (*Anastrepha ludens*), and olive fly (*Bactrocera oleae*) are in different stages of evaluation. The company has strains of *Aedes aegypti* and *A. albopictus* designed to control the mosquitoes involved in transmission of dengue and chikungunya. The *A. aegypty* Bisex RIDL strain OX513A is being tested in several countries in Asia and the Americas. The female-specific flightless strain OX3604C is in testing in Mexico. The same technology is also being evaluated in contained experiments with the Asian tiger mosquito (*A. albopictus*) strain OX3688 in the USA. The above-mentioned *Aedes aegypti,* OX513A, has been approved for import and contained testing in Brazil, Cayman Islands, France, India, Malaysia, Singapore, Thailand, USA and Vietnam. Open field trials have been, or are being held the on the Cayman islands, Malaysia and Brazil. Recently, the results of open field trials in the Cayman islands were published. It was demonstrated that GM male mosquitoes mated with wild type female counterparts, and the estimated field competitiveness (a measure based on the density of wild type and sterile insects and the relative mating success) suggested that the transgenic males were able to compete with wild type males for mating in the field. After the last release, the numbers of GM male mosquitoes that could be recaptured rapidly declined.

*Table V. Examples of GM insects (intended) for commercial use*

| **Trade name** | **Species** | **Applied vector/technique** | **Transgenic insert, reg. sequences**[2] | **Spread control** | **Country of generation** | **Company** | **(Proposed) marketing conditions** | **Status of development** | **ERA available from** | **Niche**[1] **in NL** | **Risk** under conditions of use | **Detection** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OX513A | Yellow fever mosquito | DNA micro-injection(piggy Bac based) | *Act5Cp*-dsRed2, tRE-*hsp70p*-tTA | Bisex RIDL | United Kingdom | Oxitec | government programmes | Open field trials (Grand Cayman, Malaysia, Brazil) | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX3604C | Yellow fever mosquito | as above | *Hr5IE1p*-dsRed2, *AeAct4p*-sex-spec. intron-tTA, tRE-*hsp70p*-VP16 | fs RIDL (female flightless) | United Kingdom | Oxitec | government programmes | indoor cage suppression trials. Mexican permit to import, evaluation in large scale outdoor cages | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX3688 | Asian tiger mosquito | as above | dsRed2, tRE-tTA?, sex-spec., details unknown | fs RIDL (female flightless) | United Kingdom | Oxitec | government programmes | USA permits for import and contained evaluation, testing phase | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX3647 | Mediterranean fruit fly | as above | *hr5IE1p*-dsRed2, tRE-*hsp70p*-sex-spec.-intron (Cctra)-tTA | fs RIDL | United Kingdom | Oxitec | government programmes /retail? | product optimization, contained use (IAEA/FAO, USDA/Moscamed in Guatemala) | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX3713A | Olive fruit fly | as above | dsRed2, tRE-tTA?, sex-spec., details unknown | fs RIDL | United Kingdom | Oxitec | government programmes /retail? | optimized by Oxitec, available for evaluation by third parties | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX3713Q | Mexican fruit fly | as above | dsRed2, tRE-tTA?, sex-spec., details unknown | fs RIDL | United Kingdom | Oxitec | government programmes /retail? | tested by Oxitec, available for evaluation by third parties | | no | t.b.d. | fluorescent, PCR, diet-need |
| OX1138 | Cotton pink bollworm | as above | *Opie2p*- dsRed2, | SIT | United Kingdom | Oxitec | government programmes /retail? | commercial pilot (USA) | APHIS, USDA | no | 'findings of no significant impact' | fluorescent, PCR |
| OX3402 | Cotton pink bollworm | as above | dsRed2, tRE-tTA?, details unknown | Bisex RIDL | United Kingdom | Oxitec | government programmes /retail? | R&D (product optimization) | | no | t.b.d. | fluorescent, PCR, diet-need |

Overview of modified species that spread insect-borne diseases or agricultural pests with their specifications. Sources: [30, 130]. For more detailed information see text report. [1]Environment in the Netherlands in which the species can survive. [2]Regulatory sequences (promoter=p) are in italic.

Approximately two weeks after the last release no fluorescent eggs could be recovered from trapped females. According to the authors, these data suggest the feasibility of suppressing populations of *A. aegypty* in the field by sustained release of the GM mosquitoes [128]. Results of a second larger release trial have been presented in which a reduction of 80% of the wild type population was observed 11 weeks after release. This reduction was sustained for approximately seven weeks until the end of the trial [122].

***Other developments***
Other GM targets are silkworms (*Bombyx mori*) made to produce pharmaceutical and industrial proteins, like those used to create a particularly strong spider silk that could be used to make bullet-proof vests, parachutes, and artificial ligaments [136]. Insect control with genetically engineered micro-organisms, in a so-called paratransgenic strategy will be discussed later in section 3.2.6.

***Detection methods***
Similar to other fluorescent animals mentioned previously, fluorescent GM insects can be detected by visual inspection under UV black light and confirmation of the genetic modification by a PCR specific for the common fluorescent genes. The RIDL insects generally are designed to die when the appropriate supplement is not present in their diet, though as mentioned above, sometimes the lethal trait is exclusively female specific.

***Conclusions***
With the exception of the GM pink bollworm OX1138, GM insects are currently still in field trial stages. Illegal or unforeseen import of GM insects is currently highly unlikely due to the up till now (relatively) limited scale of production and use, in combination with the fact that open field releases have been exclusively performed in areas that are geographically situated far away from the Netherlands. More important, however, is the fact that the main traits that have been introduced so far (using the SIT or RIDL techniques) result in the self-limiting release of GM insects, that according to the involved company Oxitec is highly effective and that so far seems to be confirmed by the data presented in published trials.
The environmental releases of GM insects (both for disease and plant pest control) have been carried out in conjunction with the governments involved [128, 130, 135]. Oxitec has stated that it complies with all relevant regulations worldwide and that the public is actively informed [130]. However, the open field trial in the Cayman islands has been criticized regarding these aspects and also experts have stated that it is questionable whether this trial was carried out according to international guidelines, including the Cartagena protocol [122]. For future releases, it may also be expected that GM mosquitoes will be closely regulated and guided by governments, given the nature of the insects, their ease to spread, and the fact that potentially a large (human) population is affected. With regard to GM insects intended for pest control, commercial retail without governmental involvement may however also be a possibility.
Given the above considerations, there is at this moment no requirement to have specific attention regarding the potential unintended or illegal introduction of GM insects in the Dutch environment. This may, however, change in the future, for instance in case of open release trials in nearby countries or release of (novel) strains that are not, or less, self-limiting. Population replacement strategies using self-propagating genetic systems have been proposed as a strategy in insect control, but such strategies are still far away from being suitable for use [121].

### *3.2.4 Recombinant live veterinary vaccines and veterinary therapeutics*

***Introduction***

Live recombinant vaccines targeted at viral and bacterium-induced veterinary diseases are commercially available in the USA, Canada, EU and various other countries. The potential illegal introduction of live recombinant veterinary vaccines that are available on the international market has been acknowledged by the Hong Kong government in a GMO control of release ordinance that was issued to implement the Cartagena Protocol on Biosafety in Hong Kong. In the ordinance, a table with some examples of live recombinant veterinary vaccines available on the international market is provided [137]. We expanded on (the information in) this table in order to identify (a) the specific live recombinant veterinary vaccines that are commercially available in and outside the EU, focusing on the USA and Canada, (b) the genetic composition of these vaccines (viral vector and insert, or bacterial host and genetic alteration), (c) whether an environmental risk assessment is available, and (d) availability of the vaccines. Several databases were searched for this purpose: the European Public Assessment Reports (EPAR) database on Veterinary Medicines that have received a market authorization from the EMA; a database from the Canadian Food Inspection agency that contains an overview of Veterinary Biologics Licensed in Canada; and a document from the USDA that provides an overview of licensed Veterinary Biological Products in the USA. In some cases, additional information about the products was gathered from websites of the companies involved in the commercial production of these vaccines. The overview of products containing live recombinant veterinary vaccines is listed in Table VI.

***Live recombinant veterinary vaccines available on the market in the EU***

A small number of recombinant veterinary vaccines have received market approval by the EMA in the EU [18]. These include Proteqflu, Purevax FelV and Purevax rabies, that all consist of live canarypox vectors containing viral antigens targeted against Equine influenza, Feline leukemia virus and Feline rabies respectively. Other examples are Vaxxitek HVT+IBD, that is based on the Herpes virus of Turkey (HVT) vaccine targeted at Marek's Disease virus in chickens, but that has been genetically altered to include viral antigens targeted against infectious bursal disease virus (IBD), and Equilis StrepE, an attenuated *Streptococcus equi* strain containing a deletion of an auxotrophic gene, that is applied as a vaccine against *Streptococcus equi* infection in horses. Vaccines from the same companies containing the same as the above-mentioned GMOs have been approved in the USA and Canada, but it is important to note that in many cases the formulations of the approved recombinant vaccines differ between the EU and North-America, and that these formulations are available under (slightly) different product names. For instance the 'Purevax rabies' *suspension* vaccine approved in the EU contains a similar GMO (a genetically modified Canarypox vector) as the Purevax 3 and 4 Rabies' *lyophilized* vaccines approved in North-America. Apart from this first difference in formulation, a second difference in formulation is that the 'Purevax rabies' vaccine is a monovalent vaccine, while the 'Purevax 3 and 4' vaccines are *polyvalent* vaccines, that contain apart from the Canarypox vector additional non-modified live vaccines against other feline diseases. The recombinant Equine influenza vaccines that are marketed in North-America (Recombitec: lyophilized) and the EU (Proteqflu: suspension) also differ in formulation. It is unclear whether the import of a vaccine from North-America that is similar in GMO composition, but different in formulation compared to the vaccine approved in the EU, should be considered as illegal introduction from the GMO perspective.

*Table VI. Genetically modified micro-organisms and plasmid DNA used as veterinary vaccines*

| Trade name(s) | Target Species | Target micro-organism or disease | Company | Vector | Insert/deletion | Approval | | | ERA (summary) available from | Sale via internet |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EU | USA | Canada | | |
| Proteqflu/Proteqflu-TE[1] | Horse | Equine influenza (a.o.) | Merial | Canarypox | HA (EIV) | Yes | No | No | EMA (EU) | |
| Recombitek Equine Influenza | Horse | Equine influenza | Merial | Canarypox | HA (EIV) | No | Yes | Yes | CFIA (CAN) | y |
| Recombitek Equine rWNV(EWT)[2] | Horse | West Nile virus (a.o.) | Merial | Canarypox | viral antigen (WNV) | Yes | Yes | Yes | CFIA (CAN), EMA (EU) | y |
| Recombitek C3/C4/C6/(CV) | Dog | Canine distemper virus a.o. | Merial | Canarypox | HA and F (CDV) | No | Yes | Yes | CFIA (CAN) | y |
| Prevenile[3] | Horse | West Nile virus | Intervet | Yellow fever 17D | prM, Env (WNV) | No | Yes | Yes | CFIA (CAN) | y |
| AviPro Megan Vac1 | Chicken | Salmonella | Lohmann Animal Health Internat. | *Salmonella typhimurium* | cya, crp gene deletion | No | Yes | Yes | CFIA (CAN) | |
| Poulvac ST | Chicken | Salmonella | Fort Dodge Animal Health | *Salmonella typhimurium* | aroA gene deletion | No | Yes | Yes | CFIA (CAN) | |
| Innovax-ILT | Chicken | Marek's Disease virus, Infectious Laryngotracheitis virus | Intervet | Herpesvirus of Turkeys | viral antigen (ILTV) | No | Yes | Yes | CFIA (CAN) | |
| Innovax-ND/ND-SB | Chicken | Marek's Disease virus, Newcastle disease virus | Intervet | Herpesvirus of Turkeys | viral antigen (NDV, MDV) | No | Yes | Yes | CFIA (CAN) | |
| Purevax Ferret Distemper | Ferret | Canine Distemper virus | Merial | Canarypox | HA and F (CDV) | No | Yes | Yes | CFIA (CAN) | y |
| Vectormune FP-LT+AE | Chicken | Fowlpox, Infectious Laryngotracheitis virus a.o. | CEVA Biomune company | Fowl pox vector | viral antigen (ILTV) | No | Yes | Yes | | |
| Vectormune HVT IBD (+ SB1) | Chicken | Bursal Disease-Marek's Disease | CEVA Biomune company | Herpesvirus of Turkeys | viral antigen (IBDV, MDV) | No | Yes | No | | |
| Vectormune HVT NDV (+SB1) | Chicken | Marek's Disease, Newcastle disease virus | CEVA Biomune company | Herpesvirus of Turkeys | viral antigen (NDV, MDV) | No | Yes | No | | |
| Vectormune FP-LT | Chicken | Fowlpox, Infectious Laryngotr. virus | CEVA Biomune company | Fowl pox vector | viral antigen (ILTV) | No | Yes | No | | |
| Vectormune FP-N | Chicken | Fowlpox, Newcastle Disease virus | CEVA Biomune company | Fowl pox vector | viral antigen (NDV) | No | Yes | No | | |
| Vectormune FP-MG (+AE) | Chicken | Fowlpox, Mycoplasma galliseptum (a.o.) | CEVA Biomune company | Fowl pox vector | antigen (M. gallisepticum) | No | Yes | No | | |

*Table VI (continued from previous page)*

| Trade name(s) | Target Species | Target micro-organism or disease | Company | Vector | Insert/deletion | Approval | | | ERA (summary) available from | Sale via internet |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | EU | USA | Canada | | |
| Canine melanoma vaccine, DNA | Dog | Melanoma | Merial | Plasmid DNA | tyrosinase (human) | No | Yes | No | COGEM (NL), FR (USA) | |
| Once PMH SQ/Vista Once SQ | Cattle | *M. Haemolytica/ P. Multocida* (a.o) | Intervet | *M. Haemolytica/ P. Multocida* | unknown gene deletion | No | Yes | Yes | FR (USA) | y |
| Poulvac E. coli | Chicken | *Escherichia coli* | Fort Dodge Animal Health | *E. coli* | aroA gene deletion | No | Yes | Yes | CFIA (CAN) | |
| APEX-IHN | Salmon | Infectious Haematopoietic Necrosis Virus | Aqua Health | Plasmid DNA | env (IHNV) | No | No | Yes | CFIA (CAN) | |
| Purevax FelV/Recombinant Leukemia | Cat | Feline leukemia virus | Merial | Canarypox | env, gag, part pol (FeLV) | Yes | Yes | Yes | CFIA (CAN) | |
| Purevax RCP(Ch) FeLV | Cat | Feline leukemia virus a.o. | Merial | Canarypox | env, gag, part pol (FeLV) | Yes | No | No | EMA (EU) | |
| Equilis StrepE | Horse | *Streptococcus equi* | Intervet | *Streptococcus equi* | auxotrophic deletion | Yes | No | No | EMA (EU) | |
| Vaxxitek HVT+IBD | Chicken | Bursal Disease-Marek's Disease | Merial | Herpesvirus of Turkeys | VP2 (IBDV) | Yes | Yes | Yes | CFIA (CAN) | |
| Nobi-Porvac Aujeszky | Pig | Aujeszky disease virus | Vemie Veterinär Chemie Gmbh | Aujeszky's Disease Virus | deletion g and tk genes | Yes | No | No | EMA (EU) | |
| Suvaxyn Aujeszky | Pig | Aujeszky disease virus | Fort Dodge Animal Health | Aujeszky's Disease Virus | deletion gE and tk genes | Yes | No | No | EMA (EU) | |
| Hiprabovis IBR Marker Live | Cattle | Bovine Herpes Virus | Laboratorios HIPRA S.A. | BHV type 1 | deletion gE and tk genes | Yes | No | No | EMA (EU) | |
| Raboral V-RG | Fox | Rabies virus | Rhône-Mérieux | Vaccinia virus (BCG) | glycoprotein G (rabies virus) | Yes | No | No | EMA (EU) | |
| Purevax Feline (3/4) Rabies | Cat | Feline Rabies (a.o.) | Merial | Canarypox | viral antigen (RV) | Yes[4] | Yes | Yes | CFIA (CAN) | y (F3/4) |

Examples of approved products in European Union (EU), USA and Canada (CAN). Modified and expanded from GMO control of release ordinance [137]. Main sources used [18, 21, 23, 27, 256]. For other sources see text. Abbrevations: a.o.=and other diseases; y=yes. [1] Similar to Recombitek Equine Influenza (different in formulation), [2] Similar to Proteq Westnile vaccine approved in 2011 in EU (different in formulation), [3]recalled due to increased adverse events, [4]Exclusively 'Feline rabies' formulation is approved in Europe; 'Feline 3/4 formulations are not approved.

In addition a number of vaccines are available in the EU that have not been registered in the USA or Canada (see Table VI). For each vaccine registered in the EU, a summary of the environmental risk assessment is available in the EPAR database.

***Live recombinant veterinary vaccines available on the market in the USA and Canada, but not in the EU***

In Canada and the USA, a number of other live recombinant veterinary vaccines has received approval that has not (yet) been approved in the EU [21, 27]. These vaccines are listed in Table VI. Environmental risk assessment documents for most of these approved products are available in the Canadian Food Inspection agency database [138]. For most products licensed in the USA, a public risk assessment can be found in the Federal Register of the United States government [23]. A number of the vaccines that are approved in the USA and Canada, but not in the EU, can be ordered via the internet in the USA from a number of suppliers (for details see next page). These include vaccines for use in horses (Recombitek Equine Influenza, Recombitek West Nile and Prevenile), cattle (Vista Once SQ, Once PMH SQ), dogs (Recombitek Canine Distemper), cats (Purevax Feline Rabies) and ferrets (Purevax Ferret Distemper).
The Recombitek and Purevax vaccines are recombinant canarypox vectors containing viral antigens. Vaccines with a similar canarypox vector backbone have already received market approval in the EU (see Table VI).
Prevenile is recombinant vaccine based on the Yellow fever 17D vaccine strain with an insertion of the prM and E genes of West Nile virus. Both Yellow fever and West-Nile virus are pathogenicity class 3 viruses. The Netherlands Commission on Genetic Modification has advised several times on the (contained) use of recombinant YF-17D with the prM/E insertion of West Nile virus. Initially, the strain was classified as a pathogenicity class 3 strain [139]. In a later advice (CGM070724-01, not available), the COGEM was of the opinion that the recombinant strain, like the parental YF-17D strain can be regarded as an attenuated strain that should be classified as a pathogenicity class 2 strain.
The Once PMH SQ/Vista Once SQ vaccines contain recombinant gene deleted strains of *Mannheimia haemolytica* and *Pasteurella multocida* [140] that are pathogens causing pneumonia in cattle. A recombinant *M. haemolytica* and *P. multocida* vaccine is currently being evaluated for deliberate release in the Netherlands [141]. It is unknown whether these strains are identical to the recombinant strains in the Once PMH SQ/Vista Once SQ vaccine.
For all these vaccines, a public risk assessment is available from the Canadian and American authorities. In all cases the outcome was that the vaccine would be of no significant impact to the environment when applied according to the conditions of use.

***Veterinary uses of plasmid DNA***

In the USA, the plasmid Canine Melanoma Vaccine, DNA has been registered for treatment of melanoma in dogs, being the first approved vaccine for therapeutic use in either animals or humans [23]. A deliberate release trial with this plasmid in dogs was recently approved in the Netherlands. Three other plasmid DNAs have been approved on the market outside the European Union for use in animals (see also Table VI), a vaccine against West-Nile virus for use in horses, a vaccine against infectious haematopoietic necrosis virus for use in salmon, and a plasmid encoding growth hormone releasing hormone to protect against fetal loss in swine [142]. In the Netherlands animals injected with plasmid DNA are considered GMOs. The environmental risks of the majority of the plasmid DNA vectors will be negligibly small. However, in the environmental risk assessment,

there should be special attention for presence of sequences that may increase the environmental risk (e.g. viral sequences and transposons) [143].
In the report 'Gentechnologie bij Landbouwhuisdieren (Gene Technology in Farm animals)', potential future uses of gene technology in farm animals are mentioned, including the application of plasmid DNA in animals for production enhancement or to increase the performance of animals used in sports (gene doping). According to this publication from 2009, the known actual uses of injection of plasmid DNA in humans and animals only involved medical purposes [33]. However, a market application of the use of a porcine Growth-Hormone Releasing Factor (pGRF) plasmid for growth-enhancement in pigs has been submitted in China and the Philippines (see also section 3.2.8) [24].

***Sales of vaccines on the internet***
As already indicated above, at least some of the veterinary vaccines registered in the USA/Canada (and not approved in the EU) can be ordered in the USA through the internet, for example via web shops [144-148]. The product information on the USA and Canada product labels states that the vaccine products should be sold to veterinarians only. We checked for the above web shops whether it is indicated on their sites that the products are sold under restrictions (e.g. to veterinarians only) and whether there are any restrictions for international shipping. Only one of these sites clearly indicates that vaccines are sold to veterinarians only [146]. Another site indicates that for prescription drugs a prescription is needed, but it is unclear whether a prescription is obligatory for veterinary vaccines [144]. These two sites only do shipments within the USA. On the other three sites, no clear restrictions for the sale of vaccines could be found and these sites indicate that they perform international shipping of their products [145, 147, 148].

***Potential import of vaccines via other countries***
For our overview of GM vaccines, we have focused on the USA and Canada, as these are apart from the EU, the main countries where GM vaccines have been developed and registered [137]. The availability of GM vaccines within these countries is also visible to the general public, via information on the internet. It is less visible that some GM veterinary vaccines, developed and produced by companies from North America and Europe, are also registered in many (developing) countries. Especially GM veterinary vaccines used in poultry and cattle have been registered in several countries in Middle- and South-America, Africa, the Middle East and Asia (for examples see the international websites of the companies involved)[149, 150]. These are generally not the vaccines that are available to the general public in the USA as they are most often sold in large quantities for mass vaccination. To our knowledge, there is currently no general overview of the worldwide registration status of GM veterinary vaccines and of how these vaccines are distributed and sold within the involved countries. That the unauthorized introduction of veterinary vaccines (including GM vaccines) is possible and should not be neglected is illustrated by the outbreak of the Bluetongue virus strain BTV6/Net2008 in the Netherlands in 2008, that has been attributed to the introduction of a (non-recombinant) South-African BTV-6 vaccine strain that is not registered for use in the EU [151, 152].

***Detection methods***
The possibility of border detection of GM vaccines will depend on the information provided on the (outer) packaging and on the labelling of these products. The identification of GM vaccines by their label may be difficult because the recombinant nature of these products is often not (explicitly) indicated.

However, Table VI could be a helpful aid in the identification of non-registered GM vaccines by their product name or label. What will make detection probably more problematic is the fact that (GM) vaccines registered in the USA and Canada are regarded as biological products that are not subjected to specific shipping regulations. We anticipate that, although to some extent border detection may be possible through for instance the (required) invoice attached to a package and/or the UN1845 label that is required for dry-ice shipping [153], in general border detection will be very difficult.
Once administered, the detection of an illegal vaccine will be very difficult or impossible. The BTV-6 vaccine strain causing an outbreak in the Netherlands was detected because cows were routinely tested by PCR for presence of a previous outbreak strain (BTV8/neth2006) for export purposes. The PCR was positive but the PCR signal differed from the BTV8/neth2006 signal. Infected animals had infectious virus in their blood, since experiments showed that animals remained PCR positive for more than 30 days after infection, and inoculation of animals with PCR positive blood resulted in one successful infection. Molecular sequencing of BTV6/Net2008 in a reference lab showed that this strain was highly similar to the vaccine strain [152, 154]. This example shows that detection of an illegally introduced vaccine strain in animals is possible but is highly dependent on chance (especially in cases without any clinical symptoms) and the possibility to isolate infectious virus from an animal. Moreover, detection may be very time-consuming, requiring for instance full genome sequencing. To our knowledge, many of the currently approved GM vaccines will give limited clinical signs and will be temporarily/locally present in vaccinated animals, making detection after administration, similar to gene therapy products and gene doping, very difficult.

***Conclusions***
From the above it appears that some GM veterinary vaccines that have not been registered in the EU are offered for sale on the internet in the USA without clear restrictions and that some suppliers appear to perform international shipping of their products, including the vaccines sold through their websites. This route could potentially lead to illegal introduction and use in the EU.

### *3.2.5 GM human therapeutics and vaccines*

***Introduction***
The number of gene therapy products containing living GMOs that have yet received a marketing license is very limited (Table VII) [34, 35]. Two adenoviral vectors, a replication defective vector expressing p53 (Gendicine) and a replication-competent vector that has been attenuated by deleting the E1B gene (Oncorine) have received marketing approval in China, both for use in cancer therapy. A third GMO that is on the market is a replication-defective retroviral vector (Rexin-G) expressing a modified cell-cycle control gene that has received accelerated approval under monitored release for all solid tumours considering to be resistant to standard chemotherapy in the Philippines [155-157]. No gene therapy products have been approved in the EU or the USA. Two marketing applications with GMO containing gene therapy products that were done in the late 2000s (Advexin in the USA and Cerepro in the EU) were rejected.

*Table VII. Genetically modified micro-organisms used as gene therapy or as vaccines in humans*

| Trade name(s) | Target (disease) | Company | Vector | Insert | Country of approval | Market application | ERA (summary) available from | Commercial availability |
|---|---|---|---|---|---|---|---|---|
| Oncorine | Cancer (head/neck) | Shanghai Sunway Biotech | Ad5, (E1B, part E3 deleted (RC) | | China | | | Medical tourism |
| Gendicine | Cancer (head/neck) | Shenzhen SiBiono GeneTech | Ad5, E1 deleted (RD) | human p53 | China | | | Medical tourism |
| Rexin-G | Cancer (solid tumours) | Epeius | Retrovirus (RD) | dominant-neg. human cyclin G1 | Philippines | Thailand (suspended) | | Medical tourism |
| Glybera | LDL deficiency | Amsterdam Molecular Therapeutics | AAV1 (RD) | human lipoprotein lipase S(447)X | | Europe | | |
| IMOJEV | Japanese encephalitis virus | Sanofi Pasteur Pty | Yellow fever 17D | prM, Env (JEV) | Australia | Thailand | OGTR (AUS) | |
| Flumist/Fluenz | Influenza | MedImmune | Live recombinant influenza | | USA, Canada, EU | | | Retail (internet) |
| Orochol[1] | Cholera | CSL | *Vibrio cholerae* (ctxA, hlyA deleted) | Mer (*Shigella flexmeri*) | Australia, Switzerland and other | | OGTR (AUS) | |

Examples of approved products and current market applications. Abbrevations: LDL=low density lipoprotein, RD=replication deficient, RC=replication competent.
[1]Orochol is no longer available (worldwide).

Both of these products consisted of a replication defective adenoviral vector expressing the p53 gene for use in cancer therapy [34, 35]. The amount of current marketing applications involving GMOs also appears to be very limited. Epeius, the company that has received a marketing license for Rexin-G in the Philippines, has done a market application for the same product in Thailand. Dutch biotech company AMT (Amsterdam Molecular Therapeutics) has submitted a marketing application to the EMA for use of an adeno-associated vector expressing the enzyme lipoproteine lipase in patients with low-density lipoprotein (LDL) deficiency [35, 158].

***Medical tourism***

A marketing license for a gene therapy product usually involves the use of this product for a specific indication. Such products are generally not available to the general public and can only be prescribed or applied by a medical doctor. The direct import of such products is therefore not very likely. However, in a report that was written by order of the COGEM, medical tourism has recently been described as a route by which the import of GMOs may occur and that may give rise to environmental risks [34]. There have been reports that hundreds of foreign cancer patients, including a small number of Dutch residents were treated in China with Gendicine between 2004 and 2007, but the exact numbers are unknown. There have been two other cases documented of Dutch patients receiving experimental therapy with a genetically modified viral vector in a foreign clinic. In one case, the patient received an adenoviral vector within a clinical trial in the USA in which viral shedding was monitored (and was found to be absent). In the other case, the patient received an adenoviral vector of unknown nature in a compassionate use programme for cancer patients that is provided in a clinic in Helsinki, Finland [34]. This so-called Advanced Therapy Access Program was started in 2007, and since then apparently 200 patients from 18 countries have received a tailored treatment based on individual needs. Different genetically modified replication-competent viral vectors with the ability to kill tumour cells (oncolytic viruses) are applied in the programme and the modifications include the use of different therapeutic inserts and alteration of the tropism of the virus. The company involved (Oncos Therapeutics Ltd.) claims to comply with all national and international regulations. In the report on medical tourism, it has been noted that in the absence of detailed information about the study it is impossible to determine whether the treatment could have resulted in viral shedding and risk to thirds after return of patients to the Netherlands. The Advanced Therapy Access Program is currently on hold and the company is planning formal clinical trials in the future [34, 159]. Philippine Medical Tourism, Inc. (PMTI) is another company that started offering gene therapy treatment to foreigners via the internet; in this case the GM retroviral vector Rexin-G. Apparently however, the registration of Rexin-G in the Philippines was recently suspended [160].

***Gene doping***

Gene doping is defined as the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance. There is no clear indication that gene doping using, for instance, recombinant plasmids is currently indeed being applied in practice. This may however change in the near future since the necessary tools for the application of gene doping are available [161, 162]. Although the act of injecting plasmid DNA in a human being without a GMO license is an illegal activity, human beings themselves cannot be considered GMOs under Dutch legislation. This also raises the question to what

extent the Dutch ILT Inspectorate will be responsible for the detection of individuals that have applied or received gene doping.

***Vaccines for human use***

A limited number of live GM vaccines for human use has been registered worldwide. One example is IMOJEV, a live vaccine against Japanese encephalitis virus (JEV) that has been recently registered in Australia and has been filed for approval in Thailand. A risk assessment is available from the OGTR in Australia [29]. JEV causes a disease that is endemic in South-East Asia, but very rare in the Western pacific. In Australia, the vaccine is intended for people travelling to areas where JEV is found and can only be prescribed by registered medical practitioners [163]. This vaccine, although potentially (but exclusively) interesting for people travelling to endemic areas, is therefore currently not an obvious candidate for illegal import.

Another example is Flumist, a live oral influenza vaccine that has been registered in the USA and Canada [164, 165] and recently also in the EU (for a more restricted age group) under the product name Fluenz [166]. This vaccine is currently being produced using reverse genetics techniques [167]. Because of the application of this technique, in the Netherlands, Flumist is regarded as a GM vaccine. Flumist is available in the USA via the internet, but, according to one seller, sales of vaccines are available only to those registered medical providers and facilities authorized and licensed to purchase them and international orders are not accepted [168]. Another seller indicates that legal prescriptions are required [169].

The recombinant live Cholera vaccine Orochol, attenuated by the removal of cholera toxin subunit A and inclusion of a mercury resistance marker was registered in various countries (including Australia, Austria, Switzerland and Canada). For this vaccine, a risk assessment is available from the OGTR in Australia [29, 170]. During 1994-2004 more than 500,000 vaccinations were sold with limited side effects. This vaccine is no longer marketed (worldwide) for unknown reasons.

***Detection methods***

The detection of administered gene therapy products, GM vaccines, and gene doping in individuals is very difficult. Many of these vectors may be measurable only shortly after administration (most likely by PCR) and exclusively at the specific site of application, requiring tissue sampling [33, 161].

***Conclusions***

As indicated above, only a very limited amount of human therapeutics and vaccines that contain live GMOs have been registered or are under review abroad (Table VII). The current GM vaccines registered for use in humans abroad are no obvious candidates for illegal import. Moreover, vaccines and gene therapy products are not directly available to the general public because of the restriction that they can only be prescribed and/or applied by a registered medical practitioner. The direct import of such products is therefore highly unlikely. Medical tourism is an alternative route of import of GMOs that is occurring on a small scale and of which the environmental risks are unknown. This route has already drawn the attention of the Dutch ILT inspectorate that is currently trying to gather more information on this subject. This development requires the attention of the responsible authorities, but as with gene doping, it is presently unknown to what extent the Dutch ILT inspectorate is responsible in this matter, as human beings cannot be considered GMOs.

### *3.2.6 Genetically modified micro-organisms: other uses*

Recombinant bacteria, yeast and fungi are commonly applied as tools in biotechnology, especially for the cloning/amplification of expression constructs (in particular *Escherichia coli* and *Saccharomyces cerevisiae*), and as bioreactors for the small-scale and large scale production of substances and medicines, under contained use conditions. Various products that are produced by genetically modified bacteria, yeast or fungi (by fermentation), have been submitted for use on the market in the EU. EFSA opinions concerning these products are published online in the EFSA journal. For some examples, see references [171-177]. The applications usually include a proof of absence of the living recombinant production organism and its genetic material. An example is the feed additive Phytase SP 1002, that is produced by a genetically modified yeast *Hansenula polymorpha* and that is intended for fattening of pigs and poultry. In this application, the absence of the production organism from each enzyme batch is demonstrated. Furthermore, DNA from the production organism is below the limit of detection in the product [173]. Also killed biomass of recombinant bacteria or yeast may have a commercial application. For instance the biomass that remains after the fermentation and inactivation of the L-Lysine HCl producing *Brevibacterium lactofermentum* strain SO317/pCABL, or the recombinant *Saccharomyces cerevisiae* strains pMT742 and pAK729 ('Novo Yeast Cream') have been authorized for use as a feed additive by the European communion. Renewal of the authorization of these products is currently ongoing [20].
Apart form non-living GM products that are applied in food/feed, there is a variety of possible uses that may involve deliberate release and marketing of products containing live recombinant micro-organisms. In fact, the first genetically engineered organism ever released into the environment involved the use of a recombinant bacteria, i.e. the Ice minus bacteria that was released in California in 1987 for crop-protection (see below). In previous sections, examples of the use of attenuated live recombinant bacteria and viruses as veterinary/human vaccines and as gene therapy vectors have already been given. In the following sections, we will provide some other examples of (proposed) environmental uses of recombinant micro-organisms and of products that are currently on the market or being developed.

***Crop protection: Ice- bacteria (Frostban)***
*Pseudomonas syringae* and *P. fluorescens* are bacterial species that inhabit the surface of plants and that are implicated in frost injury of sensitive crops through the action of their ice nucleating protein that stimulates ice-crystal formation on the surface of plants. Recombinant *Pseudomonas* Ice- strains were developed to prevent the colonization of leaves and blossoms by wild type *Pseudomonas* (Ice+) strains, thereby protecting frost-sensitive plants against frost. The Ice- strains were generated through homologous recombination with a recombinant construct containing the Ice gene with an internal deletion [178, 179]. In 1987, a test was conducted in California by Advanced Genetic Sciences (AGS) in which strawberry crops were sprayed with the Ice- bacteria to show whether they could provide effective frost protection in the field. The field test had been approved by the California Department of Food and Agriculture (CDFA), based on tests proving that the bacteria are unable to spread to other plants and are non-pathogenic to animals and plants [179]. During the field trial, extensive studies of the dispersal of the sprayed inoculum and monitoring of presence of the bacteria on plants, soil, insects and water in the test area were required by the EPA. The application of the Ice- bacteria was found to be

safe and a lower frost-damage to the treated plants (strawberry, and in a later trial potato) was observed [178]. Although there were plans to market the Ice-bacteria under the trade name Frostban, the product has never been commercialized. This has been attributed to discouragement of the further development of the product due to high costs and too strenuous regulation by the US government [180]. Moreover, much controversy has surrounded this product from the beginning. For instance, the initial field test had been delayed by prolonged legal battles, and during the field test, crops were destroyed by activists [179].

***Biopesticides***

The EPA regulates pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Before a pesticide can be commercialized, it must be registered by the EPA and assigned an approved product label. This registration requires scientific studies showing that it can be used without posing unreasonable risks to human health or the environment. Some pesticides are classified as 'restricted use pesticides' and may only be applied by or under the supervision of certified applicators. In the USA, a number of recombinant bacterial strains have been approved and classified as pesticides for general use (Table VIII) [181, 182].

The recombinant *Agrobacterium radiobacter* Strain K1026 was registered as a bio-pesticide in Australia in 1988 (being the first live GMO commercially available to the public) [183], and in the USA in 1999 [184]. It was previously produced by Bio-care technology limited in Australia, and is currently produced under the brand name NOGALL by the company Becker Underwood [185]. The strain protects roots and stems of ornamentals, fruit trees and nut crops from crown root disease by producing compounds toxic to other *Agrobacterium* spp. involved in this disease, notably *A. tumefaciens* and *A. rhizogenes*. Strain K1026 is a deletion mutant of the naturally occurring strain K84 that is widespread in nature and has been applied for decades as a bio-pesticide without any reported adverse effects. K1026 was derived from K84 by introducing (using homologous recombination) a deletion in the transfer region of the plasmid pAgK84 in order to prevent the conjugational transfer of the main factor involved in crown gall biocontrol (agrocin 84) to pathogenic *Agrobacterium* strains, thereby preventing immunity [184, 186, 187]. In Australia, K1026 was not considered a GMO and was exempted from GMO regulation [186]. According to an environmental assessment available from the EPA in the USA, the pesticide is not expected to have adverse effects on man or the environment [184]. The exclusive importer and distributor of NOGALL in the USA is New BioProducts. Companies within the USA can order directly from them [185], but they do not export NOGALL outside the USA. NOGALL, however, appears to be directly available from international trading sites, e.g. from a trading company from Turkey [188].

*Bacillus thuringiensis* (Bt) is widely being applied worldwide as a biopesticide. Bt is a soil bacterium that forms spores that contain insecticidal crystal proteins (ICPs). Naturally occurring Bt strains harbour different combinations of ICPs (encoded by extrachromosomal plasmids). Each ICP has a specific insecticidal activity that is directed against certain caterpillars, beetles or flies. The ICP genes can be transferred by conjugation to other Bt strains. This is a process that also naturally occurs and that applies the naturally occurring plasmids. The resulting trans-conjugant strains are therefore considered non-recombinant strains in the USA, facilitating the registration process [189], and in fact, these strains are not considered GMOs in the Netherlands either. Various products containing non-recombinant Bt strains have been registered for use as a biopesticide in several countries [190-192].

*Table VIII. Genetically modified micro-organisms used as in plant protection or in nitrogen fixation*

| Trade name(s) | Target | Company | Vector | Insert | Country of approval | ERA (summary) available from | Commercial availability |
|---|---|---|---|---|---|---|---|
| NOGALL | *Agrobacterium* spp. (crown rot) | Becker Underwood | *Agrobacterium radiobacter* K1026 | deletion transfer region of plasmid pAgK84 | Australia, USA | EPA (USA) | Retail (internet) |
| Crymax WDG | Caterpillar pests | Certis | *B .thuringiensis* kurstaki | modified cry1C gene (Bt) | USA, Mexico | EPA (USA) | Retail |
| Lepinox WDG | Caterpillar pests | Certis | *B. thuringiensis* kurstaki | chimeric cry1Ac/1F gene (Bt) | USA, Mexico | EPA (USA) | Retail |
| Raven | Caterpillar, beetle pests | Certis | *B. thuringiensis* kurstaki | cry3Bb gene (Bt) | USA (discontinued) | | |
| Dormal plus | Alfalfa seeds (nitrogen fixation) | Becker Underwood | *Rhizobium melitoli* | nifA gene, dctABD gene, spec/strep resistance | USA (discontinued) | EPA (USA) | |

Examples of approved products. For references see text report.

As an alternative, genetic modification techniques, applying an indigenous site-specific recombination system, have been used to optimize activity spectrum of Bt strains. Three of such recombinant Bt strains, that contain exclusively Bt sequences, have been developed and authorised for use as a bioinsecticide to control pests of vegetables in the USA. Apparently, the regulatory approval process in the USA for these recombinant strains did not significantly differ from the approval process of trans-conjugant strains [193]. Because of the applied technique, the strains would be considered GMOs by the Dutch competent authority.
The first recombinant strain that was approved by the EPA in 1995 was Raven (EG7673) that was developed by the company Ecogen. This recombinant Bt *kurstaki* strain harbours different ICP proteins that have insecticidal activity against respectively caterpillars and beetles (in particular the Colorado beetle). One of the latter ICP proteins (cry3Bb) was introduced using the expression plasmid pEG930.9, that contains internal resolution sites of the Bt transposon Tn5401, that allowed the removal of foreign plasmid DNA sequences (including antibiotic resistance gene sequences) [191-194]. The production of Raven has apparently been discontinued in 2007 [195].
Two other products consisting of recombinant Bt strains are currently marketed by the company Certis in the USA. Both products, Crymax WDG (Bt *kurstaki* strain EG7841) and Lepinox WDG (Bt *kurstaki* strain EG7826), are applied against *Lepidoptera* caterpillar pests. These strains contain modified cry genes with improved insecticidal activity, respectively a modified cry1C gene containing a mutation with improved toxicity for the Beet Armyworm, (*Spodoptera exigua*) and a chimeric cry1Ac/1F gene with improved toxicity for the Fall Armyworm (*Spodoptera frugiperda*) [193, 196]. Lepinox and Crymax are also on the market outside the USA, for example in Mexico [196]. In the USA Crymax and Lepinox can be simply ordered by mail-order from an organic farm [196, 197].
Although for the individual Bt products no environmental risk assessment is available from the EPA website, a Re-registration Eligibility Decision (RED) document is available that concludes that the use of currently registered products containing *Bacillus thuringiensis* (including the recombinant strains) in accordance with approved labelling will not pose unreasonable risks or adverse effects to humans or the environment [198].
Recombinant Bt strains are also being developed for mosquito control for instance in the control of *Culex* species involved in the spread dengue and West Nile virus [199, 200].
It is important to note that apart from the above living recombinant bacteria, also killed recombinant bacteria have been registered for use as bio-insecticides in the USA. An example is a killed *Pseudomonas fluorescens* strains bearing *Bacillus thuringiensis* delta endotoxins [201]. Killed recombinant strains or their products are not considered GMOs. Their application, however, would be governed by biocide legislation in the Netherlands.

***Nitrogen fixation***
Dormal PLUS is the brand name of a genetically modified *Rhizobium melitoli* (a.k.a. *Sinorhizobium meliloti*) strain RMBPC-2 marketed by the company Becker Underwood, and approved for limited commercial release in the USA by the EPA in 1997. The inoculation of alfalfa seeds before planting with this strain may improve crop yields under certain soil conditions [202, 203]. The strain was modified using the integration plasmid pMW300, and contains extra copies of the regulatory gene *nifA* that increases nitrogen fixation, of a dicarboxylic acid transport gene (*dctABD*) that improves energy utilization, and of antibiotic

resistance cassette that confers resistance to spectinomycin and streptomycin [204]. In an available risk assessment summary from the EPA, it is indicated that the modifications would have no impact on man or the environment. The scientific advisory committee that reviewed the assessment, however, also considered the need for more information on persistence, dissemination, competitiveness, and genetic stability of the strain, but it was also indicated that limited commercial use could take place, during which such questions could be addressed. Because of these and other uncertainties associated with the behaviour of this micro-organism in the environment, the commercial production of the strain was limited to 500,000 pounds per year [203]. Although information on the Becker Underwood website indicates that the modification may be used with other *Rhizobium* strains, Dormal PLUS is currently not listed under their product lists, suggesting that the strain is not marketed anymore. Accordingly, no offerings of Dormal PLUS were found on the internet.

***Bioremediation***

Bioremediation involves the application of micro-organisms for the detoxification or removal of environmental pollutants in for instance soil, water or sludge [205-207]. Recombinant bacteria are believed to have great potential for bioremediation applications and may be applied against a wide range of chemical contaminants. The genetic engineering of these micro-organisms may improve their capability to degrade or immobilize compounds compared to wild type bacteria. The proposed strategies include the manipulation of rate-limiting steps in metabolic pathways in bacteria in order to increase degradation, the inclusion of completely new metabolic pathways in bacteria to facilitate the breakdown of notoriously difficult degradable compounds, and the use of recombinant bacteria as bio-indicators to monitor degradation [208]. A variety of organisms have been modified in the laboratory setting for bioremediation purposes during the past decades, and several genetic traits and approaches have been introduced in these organisms [209-211].

Although many studies in the laboratory have been performed, only very few engineered micro-organisms have actually been released into the environment. The first and only field release for bioremediation purposes was performed by the University of Tennessee in collaboration with Oak Ridge National Laboratory in 1996-1999, and involved the release of the *Pseudomonas fluorescens* strain HK44 in a semi-contained soil environment (a lysimeter facility, basically consisting of a steel silo with cover). This recombinant strain, that contains a naphthalene catabolic plasmid and a bioluminescent reporter gene that are regulated by increased naphthalene catabolic gene expression, is able to degrade naphthalene and produces a bioluminescent signal for in situ monitoring of bioremediation. The HK44 bacteria survived for the duration of the field trial and bioluminescence was detected, but a precise evaluation of the bioremediation effectiveness of the strain could not be performed due to the inadequacy of statistical models that are able to discern the contribution of the HK44 strain from concurrent processes that also contribute to degradation [208, 212].

The consensus in reviews that have addressed the development of bioremediation using recombinant bacteria throughout the last 20-30 years is that the further development is hampered by a lack of field data addressing the effectiveness and risks associated with the environmental introduction of recombinant bacteria and a lack of motivation or direction for the generation of more field data, which has been attributed to for instance the risk-based regulatory approach towards recombinant bacteria in general, and the perceived

need for engineered organisms in bioremediation and cost competitiveness compared to other solutions [205, 208-210, 213, 214].
Moreover, although bioremediation in general has the potential to restore contaminated environments in an inexpensive and effective manner, the implementation has been limited by the fact that the mechanisms and factors controlling the growth and metabolism of micro-organisms in contaminated environments are still not well understood. The application of various (high-throughput) genomic and computational strategies may address this issue in the future and increase the understanding of bioremediation in complex environments [206, 207, 211, 215].
To address the safety issues associated with recombinant bacteria (e.g. pathogenicity of some of the proposed organisms, horizontal gene transfer and presence of antibiotic resistance genes), several strategies are being developed to improve the suitability of recombinant bacteria for environmental release. These strategies include the use of biologically contained organisms and the use of safer recombinant DNA vectors, e.g. suicide vectors and non-replicating vectors [210, 213, 215].
Based on this information, market applications involving bioremediation applying recombinant bacteria are not expected in the near future.

***Other developments***
Using HowardsHome, a number of interesting news items concerning the commercial application of genetically modified micro-organisms were gathered. Apart from genetically modified bacteria and insects (see this section and section 3.2.3), also genetically engineered micro-organisms are being developed for mosquito control (so-called paratransgenic strategy). Recombinant strains of the yeast *Metarhizium anisopliae* have been produced that combat malaria parasites in mosquitoes by expressing molecules that target *Plasmodium* sporozoites [216], and symbiotic or commensal microbes of the host insects to *Trypanosoma cruzi* and *Leishmania* species have been modified, rendering the insects refractory to infection, and thus decreasing the possibility of transmission of the parasites that cause Chagas disease and leishmaniasis, respectively [217].
Recombinant bacteria and yeast are also being developed for novel environmental-friendly purposes. For instance, genetically engineered yeasts are being developed that are able to convert carbon dioxide emissions into carbonates that could be used as building materials [218], and the GM *Escherichia coli* B strain was successfully applied in air filters to extract pesticides from polluted air [219, 220]. Engineered bacteria are being developed to produce biodiesels or bioplastics from for instance biomass on an industrial scale [221-225].
Genetically engineered M13 and TMV viruses equipped with peptide groups that have affinity for nanomaterials are being used to improve the assembly of nanomaterials that are being applied in batteries and solar cells, to improve their power, or power conversion efficiency, respectively [226-231].

***Conclusions***
Currently, the live recombinant micro-organisms that are (besides vaccines) commercially available are limited to a small number of bio-pesticides that contain genetically modified bacteria. In the countries where they are produced and have been approved (e.g. USA and Australia), these engineered Bt and *Agrobacterium radiobacter* strains, that contain no foreign DNA, are regulated in a similar manner as non-engineered strains. The environmental risks associated with these strains appear to be small according to available risk assessment

documents from the EPA that incorporate a considerate amount of previous experience with non-engineered strains. For the general public it will be difficult to identify these strains as GM strains. The (accidental) import of these products cannot be entirely excluded, given the availability on international trading sites and the non-restricted availability in the USA. Also the possibility of import of recombinant live micro-organisms with sowing-seeds or other consumer-products should be considered. However, the regular import of these products will be unlikely, given the non-GMO alternatives that are widely available on the market in the EU.

### *3.2.7 Do-it-yourself biology (DIY biology)*

***Introduction***

DIY biologists perform experiments outside regular laboratories. DIY biologists (also known as 'Curious Ones' [232], 'Garagistas' [233], 'Biohackers' [234], or 'Citizen Scientists' [235]) range from scientists performing their experiments at home to individuals without any hands-on experience [236, 237]. Experience is gained through for instance meetings, exchange of experimental protocols and visitation of laboratories. In the USA, courses are organized that involve the isolation and modification of DNA and cloning of DNA into bacteria, the cost being $300 [238]. In the USA, there are known examples of DIY biologists that have genetically engineered bacteria. The Federal Bureau of investigation (FBI) has investigated DIY biology and has found no signs of DIY biologists that intend any harm. The FBI has therefore currently adopted the approach that DIY biologists should monitor their own community for 'threatening behavior' [236, 237].

Several organizations are involved in the promotion of DIY biology. Diybio.org is an organization dedicated to making biology as they state on their website 'an accessible pursuit for citizen scientists, amateur biologists and biological engineers who value openness and safety'. A map on their website shows the worldwide location of groups and individuals involved in DIY biology. The organization is aware that DIY biology comes with responsibilities that include the development of a code of ethics and the need for DIY biologists to increase their knowledge and skills [239]. There are currently questions asked and concerns in the DIY biology community about basic biosafety, since existing biosafety guidelines are aimed at institutions and not at individuals performing experiments in their garage [236, 237].

Humanity+, a non-profit organization for the ethical use of technology to extend human capabilities, published an article favouring open source drug development in 2010 [240]. The author, Andrew Hessel, founded a DIY drug company, Pink Army that allows people interested in tackling cancer to connect and focus their passion, skills, and other resources. (Fear of) Cancer is a strong motivator and initiatives like the ones described above could lead to local DIY biology initiatives.

***DIY biology in Europe***

The development of DIY biology could give rise to the illegal use of GMOs. It should be noted that the import, sales, and purchase of certain components that could be applied in DIY biology (e.g. the vector pGLO and the *E. coli* strains applied in kits to generate fluorescent bacteria) are outside GMO regulations in the EU, since the components are not GMOs. The actual experiments and the outcome (fluorescent bacteria) however are subject to GMO regulation.

In a recent publication in Nature, it has been stated that the amount of serious practitioners of DIY biology is unknown, but that DIY biology communities are

coalescing in Cambridge, New York, San Franscisco, London, Paris and the Netherlands [236, 237]. So far, there have however been no requests for permits that involve DIY biology by individuals in the Netherlands. However, the Dutch GMO office has been contacted recently by individuals who are interested in DIY biology and asked for information about the legal requirements in case activities with GMOs are performed. Further, it should be noted that several high schools have requested (and received) a GMO license in the Netherlands in order to make it possible to apply kits for generating fluorescent bacteria in their curriculum.
In March 2011, the Irish (GMO) Inspectorate reported a query from an individual who proposed to synthesize a cloning vector (via synthetic biology), incorporate it into *Bacillus subtilis* and subsequently make it commercially available for domestic use. The desire of the individual was initially that this application would fall outside the scope of the existing legislation [241].
Upon a short survey that followed the Irish report, sent out by the policy officer of the EC Directorate General Health and Consumers to members of the CAs under Directive 2009/41, the Czech Republic Department of Environmental Risks replied to have no information on any DIYbio activity going on in the Czech republic, and to be against the described DIY activities because the modified *Bacillus subtilis* cannot be exempted from the Directive 2009/41/EC [242], and the Austrian Federal Ministry of Health replied to be aware of DIY biology and to regularly check the internet. In addition, they noted that the term 'synthetic biology' has been becoming very fashionable in recent years. Upon closer examination of selected papers, the use of the term synthetic biology appeared to be largely referring to the synthesis of DNA *in vitro*, which was cloned into plasmids and subsequently transferred into host cells like *Escherichia coli* or *Saccharomyces cerevisiae*. The conclusion is that most of the work today, which is called synthetic biology, is simply 'classic' molecular biology using synthetic nucleic acids which is covered and regulated by 2009/41 [243].

***Conclusions***
DIY biology can encompass a wide range of activities, ranging from activities that could be regarded as hobbyism (e.g. extracting DNA from fruits, or isolating cultures from yogurt or beer), to development of ideas for the treatment of disease, or genetic modification of micro-organisms in garage-scale laboratories. Knowledge and protocols are shared through social media on the internet. Although the vast majority of DIY biology practices will not be within the scope of GMO legislation (culturing wild type organisms, DNA isolation and sequencing), some techniques are subject to GMO regulation and will require a (contained use) permit. A general concern with DIY biology is the (possible lack of) awareness of basic rules of biosafety. Regular visitations to internet forums on DIY biology are advised to spot new topics.

### *3.2.8 Status of market approval of GM products in China*

Because the developments concerning biotechnology in China appear to move fast and are difficult to follow it was decided to dedicate a special section to the current status of market approval of GM products in China. There are several databases and information resources in English that provide useful information. According to the most recent Global Agricultural Information Network (GAIN) reports on biotechnology in China, so far no so-called (bio)safety certificates have been granted for GM animals, but some GM animals may be in their final review stages. In China, this safety certificate is a requirement for agricultural

GMOs before they can enter the procedures for commercial approval. Consequently, there are no products derived from GM animals that are marketed in China. Specific examples of developments with GM animals in China can be found in the sections on GM fish and GM livestock (section 3.2.2) and in the GAIN reports on Biotechnology in China [108, 244]. The state of Biotechnology in developing countries, including China, can also be followed on the BioDeC database that is an initiative of the Food and Agriculture Organization of the United Nations (FAO) [16].

The Commercial Production DataBase of the Biosafety Clearing House of China [24] provides additional information of the commercial production of living modified organisms (LMOs) in China. While most entries in the database involve the culture of GM crops, a small number of other GM organisms either have been approved (temporarily) for commercial production, or have received the safety certificate for production. The following entries are included in the database. In most cases, additional information from other sources was added.

*Commercial production:*

- Transgenic *Bacillus thuringiensis* strains UV173A and G033A strains expressing the Cry3Aa gene in Hubei and Xinjiang province by Plant Protection Institute of CAAS (approval valid from 2006-2011).
- 'Young pig K88K99' genetic engineered bacteria 987p by Shanghai Academy of agricultural Sciences (valid 2001-2004). This probably involves the production of a veterinary vaccine against diarrhoea consisting of *E. coli* expressing antigens that can be used in pigs to target enterotoxic *E. coli* strains [245].
- 'Diarrhoea genetic engineered vaccine' in Ningxia autonomous region by Ningxia University (valid 2001-2004). This involves an inactivated *E. coli* BL21 strain expressing *Clostridium perfringens* beta toxin and *Escherichia coli* heat stable enterotoxin (CPB ST) fusion protein that can be applied as a vaccine in cows and sheep [246, 247].
- Genetic engineered vaccine of '0 Foot and mouth disease virus resistance' in Zhejiang by Fudan University (valid 2000-2005). This probably involves the production of VP1 protein of FMDV by an engineered *E. coli* strain (C500) [248].
- 'Genetic engineered vaccine Jisheng-1' based on 'Vaccinia virus Tian Tan strain' in Sichuan province by Nanjing Agricultural University (valid 2000-2004); probably involves the production of a veterinary vaccine.
- Recombinant yeast *Pichia pastoris* with high representation of phytase in Jiangxi (valid 2000-2004). The strain has been developed by Hopeland Chem-Tech co. with the intention of transferring the strain and production technology to thirds. Phytases produced by this technology will be restricted for either production or sale to China [249].

*Safety certificates:*

- Production of pig pGRF injection in Sichuan and Guangdong province (valid 2004-2008). The porcine Growth-Hormone Releasing Factor (pGRF) plasmid (product name Liberpro) enhances growth in pigs. According to the manufacturer of Liberpro, GreenPak Biotech. Limited, intramusculair injection of pGRF has several advantages in swine production, including improved feed conversion efficiency, shortened production cycle, decreased waste production, improved meat quality and healthier pigs. Production Scale Field Testing Phase trials were successfully completed in 2003, and the safety permit from the Ministry of Agriculture's Committee on the Safety of Recombinant Organisms was also granted in 2003. The company expected successful registration of pGRF Plasmid DNA in China and in the Philippines in 2009, but there is no indication that this has indeed happened [250].
- Live Pseudo-Rabies virus E-A line depletion vaccine in Henan and Hubei province by Huazhong Agricultural University (valid 2003-2005). This involves the production of a recombinant pseudorabies virus to be used as a veterinary vaccine [251].

- Recombinant chicken pox virus vaccine of chicken infectious laryngotracheitis providing resistance in Heilonhjiang province by Harbin Veterinary Institute (valid 2003-2005); involves the production of a veterinary vaccine.
- 'Phytase gene recombinant silkworm virus' in Sichuan (valid 2004-2008). This probably involves the use of the silkworms as biological reactor to produce phytase by infection with an insect virus that is able to express the protein [252].

Although a presentation about the current situation of research and application of agricultural GMOs in China (available from International Life Sciences Institute Focal Point in China) suggests that the information available on the BCH website is not entirely up to date, the presentation confirms that the approval of GMOs in China is so far mainly limited to GM veterinary vaccines, GM micro-organisms producing pharmaceuticals or feed additives and Bt pesticides. A notable addition not mentioned in the database is the production of a recombinant *Alcaligenes faecalis* strain*. A. faecalis* is a nitrogen fixing bacteria that is associated with rice roots [109]. There is no indication that any of the products is intended for export, with the notable exception of the market application of the pGRF plasmid in the Philippines.

Finally, reports about the current status of gene therapy in China have been published in the Netherlands (commissioned by the COGEM). As indicated before in section 3.2.5, two GM viral vectors have so far reached a market approval in China and apparently also a wound dressing containing genetically modified pig cells has been approved on the market as a medical device (see section 3.2.2.2) [34, 115].

# 4 Discussion

## 4.1 Overall conclusions from the inventory

Using a variety of (internet) resources, an inventory was compiled that provides a broad overview of the current status of commercialization of GMOs of non-plant nature (animals and micro-organisms) worldwide, and that is useful for prioritizing the activities of the ILT inspectorate regarding the control of the (potential) illegal import and/or illegal use of GMOs.

With the notable exception of GM ornamental fish and a few GM animals approved for the production of substances (that are held under contained conditions), no GM animals have received a market approval in any country in the world. However, in the near future, GM fish and livestock may be approved for food/feed in North America and in China since there are examples of products that are supposedly near a final decision. The commercial availability to the general public of GMOs outside of the EU is currently limited to GM ornamental fish, GM veterinary vaccines, a GM human influenza vaccine and a small number of pesticides consisting of GM bacteria. In all four categories, there are examples of products that are currently available to the general public through web stores or retailers on the internet.

The illegal import of GM ornamental fish in the Netherlands and the EU has continued despite efforts of the competent authorities in the EU to create awareness with distributors, retailers and exporting countries. GM ornamental fish, in particular RFP expressing 'Pink Danios' have been offered to the general public on the internet every now and then, both via Dutch and international trading sites. Novel ornamental fish variants have recently appeared on the market, or are expected to be on the market soon in Taiwan (TK-3 Danios and fluorescent Angelfish/Convict Cichlids). Given the current frequency of illegal imports of GM ornamental fish in the past and the (future) availability of new GM ornamental fish variants, illegal imports of GM ornamental fish are expected to continue in the future.

A number of GM veterinary vaccines intended for use in companion animals or livestock that have not been registered in the EU (see Table VI) are offered for (international) sale from the USA without any apparent restrictions (e.g. the necessity for a prescription by a veterinarian). Thus, these GM vaccines have the potential to be imported by interested pet-owners, farmers and veterinarians directly or indirectly (e.g. by ordering through an acquaintance in the USA) through these websites.

Compared to veterinary vaccines, the illegal import and use of GM live vaccines registered for use in humans appears less likely. A very limited number of GM live vaccines have been registered for use worldwide. Of note is Flumist, a live recombinant influenza vaccine that is currently being generated, using plasmid based reverse genetics. Flumist is sold to the general public in the USA via the internet, but requires a prescription by a medical practitioner and is not offered for international sale. Recently, Flumist was admitted to the market and registered under a different product name (Fluenz) in the EU.

Bio-pesticides consisting of GM bacteria are commercially available to the general public abroad. Examples of products are Crymax WDG (*Bt kurstaki* strain EG7841), Lepinox WDG (*Bt kurstaki* strain EG7826) and NOGALL (*Agrobacterium radiobacter* K1026). Since these products are regulated as non-engineered strains in countries where the products have been approved, it will be difficult for the general public to identify these strains as GM strains. The unapproved (accidental) import of these products cannot be entirely excluded, given the availability on international trading sites and the non-restricted availability in the USA.

Furthermore, medical tourism involving the application of gene therapy products and do-it-yourself biology are two other main developments that may give rise to the illegal introduction of GMOs in the environment.

## 4.2 Priority listing

The enforcement of GMO regulations, including the monitoring of unapproved GMOs, is a task of the ILT Inspectorate. This report provides a broad overview of GMOs (other than plants) that are internationally available on the market at present, or that could become available in the near future and which are not (yet) approved in the EU. This inventory includes examples of GMOs belonging to biologically very different categories (e.g. viral vaccines, pesticides, livestock, ornamental fish) that also show considerable differences in the conditions under which they are being made available on the market.

In Table IX a number of general characteristics of the different categories of GMOs are listed that were used to generate a relative priority or 'awareness' score that the ILT inspectorate may apply for prioritizing their current and future activities. We prefer to speak of an awareness score because, with the exception of the GM zebrafish of which illegal import has been frequently reported, there is no formal proof that illegal import or uses of any of the other of the categories GM animals or GM products listed (including ones that are available to the general public through e.g. the internet) has indeed occurred in the Netherlands or in other EU countries. This may be in part due to the fact that detection of these products is more difficult compared to products that can be visually detected (e.g. fluorescent zebrafish). However, there are examples of illegal import of similar unregistered (non-GM) products and organisms that demonstrate that illegal import of for example unregistered vaccines or engineered animals is not just a theoretical possibility. The relative priority/awareness score indicates whether the unregistered GM products are currently available and whether the ILT inspectorate should prepare for how to deal with these products once they arrive. When there are particular circumstances that could increase the priority score in the near future, this is indicated. Below, we summarize for each category of GMOs the main characteristics that were applied in assigning the relative priority.

**Ornamental fish** are currently the GMO category most likely to be introduced. Although the environmental risks associated with the illegal introduction of current GM fish are considered to be negligible, this category is currently prioritized as **medium**, because the import of these fish is societally and politically sensitive. Priority may increase upon the market approval of new species of ornamental fish generated with different techniques compared to the species currently on the market and/or the development of GM fish that are able to survive in the Dutch environment.

*Table IX. Suggestions to prioritize current awareness for possible illegal import and/or use of GMOs*

| Category of GMO | Priority | 1. (illegal) availability | 2. current (or (future) introduction likely? | 3. market approval | 4. market application | 5. restrictions | 6. environmental risks | 7. society sensitive | 8. Detection | 9. other jurisdiction | 10. example GMO |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ornamental fish | medium | x | x | x | x | no (retail) | n | x | ++ | | Glofish |
| companion animals | low* | | (x) | | x | no (retail) | ? | x | ++ | | NeonMice |
| fish food/feed | low | | (u) | | x | contained | ? | x | + | | AquAdvantage Salmon |
| livestock food/feed | low | | (u) | | x | contained | ? | x | + | | Enviropig |
| livestock substances | low | | | x | x | contained | n | | + | | GTC 155-92 Goat |
| insects | low* | | (p) | | | RIDL, SIT | ? | x | +/++ | | OX513A |
| veterinary vaccines | medium* | x | p | x | x | vets only[1] | n/? | | b | | Prevenile |
| human vaccines & medicines | low | x | (u) | x | x | prescription needed | ? | | - | | Flumist |
| medical tourism | high | x | x | | | prescription needed | ? | | - | x | Rexin-G |
| gene doping | low | ? | (p) | | | | ? | x | - | x | ? |
| bacteria (crop protection) | low | x | p | x | | no (retail) | n | x | - | | NOGALL |
| diy biology | high | x | x | | | | ? | x | f | | ? |

The relative priority/awareness score is based on the following characteristics*:* (1) Availability whether legal or illegal to the general public in NL*;* (2) the likelihood of introduction and/or use in the NL*;* (3) whether there are current market approvals in the category of GMOs listed*;* (4) whether there are currently market applications under evaluation; (5) whether there are or will be restrictions to the availability of the live GMO to the market; (6) the outcome of available environmental risk assessments; (7) to what extent the illegal use or introduction would be society sensitive; (8) the possibilities for detection and control; and (9) possible involvement of other inspectorates (e.g. of health or sports) due to other jurisdiction. In the last column (10), an example GMO of each category is listed. Abbreviations: x=yes, empty space=no, p=possible, ?=unknown, n= negligible, *indicates that the priority may increase in the (near) future. [1]Should be sold to veterinarians only, but restriction may not be always applied in practice. Regarding the possibility of future introduction, brackets in column 2 indicate that introduction would be (x)=very likely, (p)=possible or (u) unlikely in case of future market approval/commercial use. With regard to possibilities for control and detection, '++' indicates that detection is possible via non-invasive techniques (e.g. visual inspection, black light); '+' indicates that detection is possible but requires invasive techniques (e.g. PCR on tissue biopsies or blood); '-' indicates that currently detection possibilities are limited; 'b' indicates that border detection may be possible (dry-ice plus label); 'f' indicates that detection may be possible by following fora on the internet. Note that the table is a time snapshot, and that future updates are needed, based on new developments and alerts.

For the categories **companion animals, fish food/feed, livestock food/feed,** the priority is currently **low** because there are currently no examples of market approval and (hence) there is no availability to the public. However, market approval may be nearby because there are currently applications under evaluation in the USA. In our view, with respect to livestock and fish for food/feed, possible approval in the USA will not impact on the (low) priority because of the contained conditions under which the animals will be kept in the production chain. The environmental assessments involving these products appear to cover all steps in the production chain including for instance transportation, level of confinement and receiving environment. The priority of companion animals may increase upon the possible market approval of the NeonMice, which is likely to be imported upon approval (given the experience with ornamental fish) and of which currently no ERA is available.
The priority of **GM livestock intended for the production for substances is low** given the contained conditions under which the animals are generally kept and are also expected to be kept in the future.

**GM insects** are currently still in field trial stages, with the exception of the GM pink bollworm OX1138 for control of a pest of cotton that is at the 'commercial pilot' stage in the USA. Neither this pest nor crop species are present in the Netherlands. Moreover up till now, only a relatively limited scale of production and (open field) use of other GM insects has been carried out, mostly in tight conjunction with governments of the involved countries that are geographically situated far away from the Netherlands. Furthermore, the design of GM insects has so far focused on the self-limiting release of GM insects, using the SIT or RIDL techniques that according to the involved company Oxitec are highly effective. Illegal or unforeseen import of GM insects (both for pest and public disease control) is therefore currently highly unlikely, and the priority is **low**. Priority may, however, increase in the future upon the commercial release of GM insects that are able to survive in the Dutch environment and that are relevant for use in the Netherlands.

Currently registered **GM pesticides** are few and based on GM bacterial strains that do not contain any foreign DNA, and that are descendents of parental strains with a history of safe use. The current priority is therefore **low**, although these pesticides may possibly be introduced with relative ease (e.g. via the internet). There is no indication that novel GM pesticides are currently undergoing market approval.

**Veterinary vaccines** are freely available to the general public in the USA and may be introduced in the NL with relative ease (e.g. by ordering via the internet). Although the risks associated with the illegal introduction of the currently registered vaccines may be very limited (based on the known properties of the vaccines or familiarity with similar vaccines), in the absence of a risk assessment according to EU guidelines that takes into account the specific conditions of release in the EU, formally the risks are unknown. Several of the GM vaccines available in the USA and Canada have not been evaluated in the EU, including vaccines that (under contained use conditions) should currently be handled at GMO risk level 2 in the Netherlands. Moreover, the product label indicates that these products should be sold to veterinarians only. Availability to the general public without restrictions may lead to inappropriate uses. The introduction of the Bluetongue virus strain BTV6/Net2008 in the Netherlands in 2008 illustrates that illegal introduction of vaccines can indeed occur and may have a high impact (in this case mostly an economical impact). Because of the

possibility of introduction and the (partly) unknown risks, these vaccines should currently be given a **medium** priority. Priority may increase upon approval of novel GM vaccines in the USA and other countries. Whether detection and control of these vaccines 'at the gate' is possible is currently unknown and requires further research.

A small number of **GM vaccines** have been registered for human use worldwide. Flumist is a live recombinant influenza vaccine that would be classified as a GMO in the Netherlands because of the plasmid/bases reverse genetics method applied to generate the current vaccine. Although in the USA, Flumist can be ordered through the internet, a prescription is required. Recently, this vaccine has also been registered for use in the EU under the product name Fluenz. The priority of control of GM vaccines for human use is currently **low** but might increase in the future since novel GM live vaccines (e.g. targeted against dengue fever) are under development.

A small number of gene therapy products for human use have received a market approval in China and the Philippines. There is no indication that these medicines are available to the general public and could be directly imported. Priority for illegal import of **GM medicines** is **low**.

However, **medical tourism** involving the use of these registered gene therapy products, but also of experimental products may lead to the illegal introduction of GMOs in the environment. A small number of Dutch patients have already undergone treatment in foreign clinics with GM products. In absence of an available ERA, the risks of the GMOs involved are currently unknown. The priority of inspection for medical tourism solely based on the potential environmental risks should therefore in principle be **high**. However, it is currently unknown to what extent the current GMO regulations could be applied to control medical tourism and to what extent the ILT inspectorate and other inspections can take measures to control these events.

There is currently no indication that **gene doping** in man or animals is being applied in practice. Therefore the priority for inspection is currently **low**. However, this situation may change in the coming years. The IOC is expecting gene doping in five to ten years [253]. Similar to medical tourism, the question is what will be the responsibilities and the possibilities of the ILT inspectorate in the control of gene doping in humans, beyond the initial administration.

**DIY biology** may involve the generation of GMOs. There are indications that DIY biology is already taking place in the Netherlands and other countries in the EU. So far no contained use permits have been issued in the Netherlands that would allow the generation of GMOs by DIY biologists. The motives for the application of DIYbio might be diverse and it is unknown if and what kind of GMOs could potentially be generated and what would be the risks of these GMOs. A general concern that has been issued in publications covering the subject is the possible lack of awareness of basic biosafety rules in the DIY biology community. The priority score of DIY biology is therefore **high**.

## 4.3 Considerations for ILT inspectorate

In 2009, a report on the potential introduction of unapproved GM crops in the Netherlands resulted in a priority list for the monitoring of unapproved GM crops [2]. Although, the subject matter covered in the present report appears complementary to this report, resulting in a priority list of GMOs of non-plant origin (animals and micro-organisms), the two reports together still do not cover the entire spectrum of GM products that are available on the market. Developments regarding for instance GM ornamental flowers and plants are not covered. Although only a small number of cut flowers are currently marketed in the EU and in Asia by one particular company (Florigene) [254], this may be subject matter for future research.

The current inventory of GMOs is a snapshot in time of the marketing status of GMOs of non-plant origin worldwide. Given the many developments involving the use of GMOs, it is somewhat surprising to see how few GMOs have actually reached a marketing status worldwide. However, the expectation is that in the coming years, new GMOs will reach a marketing status and therefore, regular updates of the inventory should be considered.
At this moment, we advise the ILT inspectorate to consider a general update of the inventory within five years. This timeframe is mostly based on the relatively small number of GM products that we are aware of that is currently under review to reach the marketing status, and on the fact that review of these marketing applications can take (many) years. Moreover, companies and institutions that have new GMOs in their product pipeline usually indicate an expected marketing status of their products within five to ten years.

In the meantime, the sites and databases listed in this report under materials and methods are an excellent and reliable source for the ILT inspectorate to follow the developments concerning the marketing status of GMOs outside the EU. We especially recommend the site of the FDA to follow the marketing approval status of the products currently under review in the USA [7] and the yearly published USDA/GAIN reports to follow developments in other (developing) countries, including China [15]. The Netherlands Commission on Genetic Modification (COGEM) has prepared an inventory of the ethical and societal aspects surrounding the subject of GM animals, also addressing possible environmental impacts of their development [255]. This topic report is another useful source that can be applied by the ILT inspectorate to set their (future) priorities.

The inventory and priority/awareness list proposed in this report may be applied by the ILT inspectorate as one of their sources to generate an up-to-date priority list for inspection. Additional (future) sources that may be applied include other GMO inspectorates in the EU (for regular information exchange on illegal import and use), COGEM (e.g. for topic report on GM animals), and other inspectorates within the Netherlands (e.g. for additional information on possibilities for control and detection). As indicated before, it is currently unknown to what extent the current GMO regulations are suited to control some of the categories of use of GMOs listed in this report, e.g. introduction of GMOs in the environment as a consequence of medical tourism and gene doping. Therefore the initiation of discussions on these topics with the involved ministries and inspections may result in useful information for all participators.

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# Abbreviations

| | |
|---|---|
| AAV | adeno-associated virus |
| AFP | antifreeze protein |
| APHIS | U.S. Animal and Plant Health Inspection Service |
| BCG | Bacillus Calmette-Guérin vaccine against tuberculosis |
| BCH | Biosafety Clearing-House |
| BHV | Bovine Herpes Virus |
| Bt | Bacillus thuringiensis |
| BTV | Bluetongue virus |
| CA | Competent Authority |
| CBER | U.S. Center for Biologics Evaluation and Research |
| CDFA | California Department of Food and Agriculture |
| CDV | Canine Distemper virus |
| CEI | Czech Environmental Inspectorate |
| CFIA | Canadian Food Inspection Agency |
| COGEM | Dutch Committee on genetic modification |
| CPHST | U.S. Center for Plant Health Science and Technology |
| CTA4Ig | T lymphocyte associated antigen 4 immunoglobulin |
| CVB | U.S. Center for Veterinary Biologics |
| CVM | U.S. Center for Veterinary Medicine |
| DNA | Deoxyribonucleic acid |
| DIYbio | Do-it-yourself biology |
| EA | Environmental Assessment |
| EC | European Community |
| EEA | European Economic Area |
| EFSA | European Food Safety Authority |
| EFTA | European Free Trade Association |
| EIV | Equine Influenza virus |
| EMA | European Medicines Agency |
| EPA | U.S. Environmental Protection Agency |
| EPAR | European Public Assessment Reports |
| ERA | Environmental Risk Assessment |
| EU | European Union |
| F0/F1 | Parental generation/First filial generation |
| FAO | Food and Agriculture Organization of the United Nations |
| FBI | U.S. Federal Bureau of Investigation |
| FDA | U.S. Food and Drug administration |
| FeLV | Feline leukemia virus |
| FFDCA | U.S. Federal Food, Drug, and Cosmetic Act |
| FIFRA | U.S. Federal Insecticide, Fungicide, and Rodenticide Act |
| FMDV | Foot-and-mouth disease virus |
| FONSI | finding of no significant impact |
| FP | fluorescent protein (G(reen)FP, R(ed)FP, Y(ellow)FP, O(range)FP, B(lue)FP, C(yan)FP, Emerald Green, Ruby Red, Sapphire Blue, Yellow Quartz, etc) |
| FR | U.S. Federal register |
| fsRIDL | female-specific RIDL |
| GAIN | Global Agricultural Information Network (USDA) |
| gcGH | grass carp growth hormone gene |
| GE | genetically engineered |
| ggo | genetisch gemodificeerd organisme |

| | |
|---|---|
| GH | growth hormone |
| GHc(2) | growth hormone of chinook salmon |
| GM | genetically modified |
| GMO | genetically modified organism |
| GTTAC | Australian Gene Technology Technical Advisory Committee |
| hCMV | human cytomegalovirus |
| HVT | Herpes virus of Turkey |
| IAEA | International Atomic Energy Agency |
| IBDV | Infectious bursal disease virus |
| ICP | insecticidal crystal protein |
| IenM | Ministerie van Infrastructuur en Milieu (Ministry of Infrastructure and the Environment) |
| IHNV | Infectious Haematopoietic Necrosis virus |
| ILT | Inspectie Leefomgeving en Transport (Human Environment and Transport Inspectorate) |
| ILTV | Infectious Laryngotracheitis virus |
| ITR | inverted terminal repeats (ITR-L and -R) of adeno-associated virus |
| JEV | Japanese encephalitis virus |
| LDL | low density lipoprotein |
| LMOs | living modified organisms |
| MDV | Marek's disease virus |
| mylz2 | zebrafish myosin, light polypeptide 2 gene |
| NADA | new animal drug application |
| NDV | Newcastle disease virus |
| OGTR | Australian Office of the Gene Technology Regulator |
| PCR | polymerase chain reaction |
| pGRF | Porcine Growth-Hormone Releasing Factor plasmid |
| RED | Re-registration Eligibility Decision |
| rhAT | recombinant human antithrombin |
| rHLZ | recombinant Human Lysozyme |
| RIDL | Release of Insects carrying a Dominant Lethal trait |
| RIVM | Dutch National Institute for Public Health and the Environment |
| RNA | Ribonucleic acid |
| RC | Replication competent |
| RD | Replication deficient |
| RV | Rabies virus |
| SCNT | somatic cell nuclear transfer |
| SIT | Sterile Insects Technology |
| SV40 | Simian virus 40 |
| tiGH | tilapia growth hormone |
| t.b.d. | to be determined |
| TK | Taikong |
| TMV | Tobacco mosaic virus |
| tTA | tetracycline transactivator |
| tRE | tetracycline responsive elements |
| USDA | United States Department of Agriculture |
| UV | ultraviolet |
| VROM | Former Dutch Ministry of Housing Spatial Planning and the Environment, merged with the Ministry of Transport, Public Works and Water Management into the new Ministry of Infrastructure and the Environment on 14 October 2010 |
| WNV | West Nile virus |

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