

**Waste Stream Monitoring of  
Genetically Modified  
Micro-organisms**

**Research Report No. 16**  
Genetically Modified Organisms  
Research Report

## **PREFACE**

This study is one of a series of research reports previously published by the Department of the Environment, Transport and the Regions (DETR), now published by the Department for Environment, Food and Rural Affairs (DEFRA). The work was carried out under the DETR's *Genetically Modified Organisms (GMO) Research Programme*, which aims to underpin Government policy on the environmentally safe use of genetically modified organisms.

The study is the result of two contracts let by DETR. The original work was conducted by the Centre for Applied Microbiology and Research (CAMR), Porton Down, Salisbury, SP4 0JG. Due to the revision of the European Directive covering work with genetically modified microorganisms (GMMs) in containment, the original study has been substantially revised and updated to its current form through a contract with WS Atkins Environment, Woodcote Grove, Epsom, KT18 5BW.

This desk study reviews the work conducted with GMMs in the UK as a 'Contained Use' activity, and assesses the risks of such work to the UK environment and human health. The European Directive regulating work with GMMs in containment was revised in 1998 and was implemented in GB on 15 November 2000. This review describes the principal changes to the legislation and what effect they may have on the potential risks to the environment.

Reports published from this GMO Research Programme are peer-reviewed prior to publication.

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## **EXECUTIVE SUMMARY**

This report constitutes a review of the work being conducted with genetically modified microorganisms (GMMs) as a 'Contained Use' activity in the UK, and an assessment of the potential risks posed by such work to the environment and human health. The report was produced by WS Atkins Environment as part of the research programme of the Chemicals and Biotechnology Division of the Department of the Environment, Transport and the Regions (DETR), and is an update of an earlier review produced for the Department of the Environment (DoE) by the Centre for Applied Microbiology and Research (CAMR).

Work with GMMs as a 'Contained Use' activity in the UK was regulated, until very recently, by legislation that implemented European Directive 90/219/EEC. This directive has now been revised and the new directive (98/81/EC) was implemented in the UK on the 15 November 2000. The principal changes to the regulations that may affect the potential risks of 'Contained Use' work to the environment and human health, are the revision to the definition of 'Contained Use' and a revised classification system for GMMs to determine what procedures are required to limit their contact with the environment.

Under Directive 90/219/EEC a 'Contained Use' activity was defined as any operation in which physical barriers, on their own or in combination with chemical or biological barriers, are used to limit the contact between the GMM and the environment. The absolute requirement for physical barriers is removed in the revised directive (and in the changes to UK legislation), allowing chemical treatment or biological containment mechanisms to be used as the sole containment measures.

Directive 90/219/EEC also differentiated between work conducted with GMMs for industrial or commercial purposes, such as the production of biological catalysts, and work carried out for research or development. The review of the work conducted in the UK for this report (conducted just before the new Directive was implemented) showed that of the 30 industrial activities involving GMMs, only 19 used volumes of microbial culture of >50 litres, indicating that industrial processes were not necessarily large scale. All activities (industrial and research) will be assessed by the same risk-based criteria under Directive 98/81/EC, irrespective of the purpose of the work. Regulations implementing Directive 98/81/EC should also enable assessment of the risks to the environment and

human health to be more straightforward, as the regulations will be based directly on the containment level employed to conduct the work, rather than the classification of the GMM as Group I or II.

Chapter 3 of this report focuses on the types of contained use activities conducted in the UK. It is based on records held by HSE and DETR just before the new regulations came into force and thus the containment and work categories are those described under Directive 90/219/EEC. However, this provides a snapshot of the work likely to be carried out under the new Directive. The review showed that a total of 471 premises had notified the Health and Safety Executive (HSE) of an intention to work with GMMs. However, because activities involving non-pathogenic GMMs in a notified premises are exempt from further notification (except for GMMs used in Type B activities which are notifiable), it is likely that the actual number of activities involving GMMs is much greater than 471. A single notified research centre or university may conduct dozens of activities involving GMMs. For those activities notified to HSE, 84 percent of all operations used a culture volume of <1 l, with the large majority of the work conducted at containment level 2 (low risk activities). Only one activity (using GM morbillivirus, a bovine pathogen) was performed at containment level 4 (the most contained operating conditions).

Containment procedures primarily involved a combination of physical, chemical and biological techniques with contaminated waste usually being treated by autoclave or disinfectant. Where release of viable GMMs into the environment was possible, further biological based containment methods, usually the inability of the GMM to survive or transfer its genetic modification, were employed to minimise the potential for adverse environmental effects to occur. Activities at only three premises involved the detectable release of viable GMMs into the environment, and in each case biological containment measures were employed to ensure the GMM would not survive in the environment.

The risks posed by contained use work with GMMs are dependent on a combination of factors, all of which have to be taken into account in evaluating the likelihood of adverse consequences occurring. In addition to the host microorganism and the traits conferred by the genetic modification, the characteristics of the donor microorganism, and any vector involved must also be considered. The environment into which the GMM may be released or escape is a further consideration, as in many cases any pathogenic characteristics of the GMM are restricted to specific environments or are dependent on the presence of certain host organisms, such as Tsetse flies (not resident in the UK) in the case of the parasite *Trypanosoma brucei brucei*.

# **1. INTRODUCTION**

- 1.1 The use of genetically modified microorganisms (GMMs) has many applications, ranging from the elucidation of how microorganisms cause disease, to the production of enzymes for industrial processes or pharmaceuticals. The purpose of this report is to review the work that is being conducted with GMMs in the UK as a contained use activity, and to assess the risks of that work to the environment, particularly the risks posed by any waste produced.
- 1.2 The use of GMMs in the UK was regulated until very recently by legislation that implemented the European Directive 90/219/EEC. This directive was revised in 1998<sup>1</sup> and was implemented in the UK on the 15 November 2000<sup>2</sup>. This report reviews the contained use work conducted with GMMs under Directive 90/219/EEC and evaluates what may be expected under the revised Directive 98/81/EC.

## **SCOPE OF THE REPORT**

- 1.3 The scope of this report is to assess work with GMMs that has been, or is being, conducted as a 'Contained Use' activity in the UK.
- 1.4 Under the previous UK regulations<sup>3</sup>, a 'Contained Use' activity was defined as any operation in which physical barriers, on their own or in combination with chemical or biological barriers, are used to limit the contact of the genetically modified organism (GMO) with the general population and the environment. Although this definition is amended in Directive 98/81/EC, contained use work involving GMOs (including microorganisms) is characterised by some form of barrier between the GMO and the environment, and as such is distinct from 'Deliberate Release' activities, where the GMO is actively released into the

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<sup>1</sup> European Directive 98/81/EC

<sup>2</sup> The Genetically Modified Organisms (Contained Use) Regulations 2000

<sup>3</sup> The Genetically Modified Organisms (Contained Use) Regulations 1992 which implement Contained Use Directive 90/219/EEC in GB.

environment for experimental or commercial purposes. 'Deliberate Release' activities are regulated by separate legislation<sup>4</sup> and are not covered by the scope of this report.

- 1.5 A GMO is defined as any organism (except humans and human embryos<sup>5</sup>) whose genetic material (deoxyribonucleic acid (DNA)) has been altered in a way that could not have occurred naturally by mating and/or natural recombination. The use of a particular technique to alter an organisms genetic material does not necessarily result in that organism becoming genetically modified. For example, whilst the techniques of *in vitro* fertilisation, conjugation, transduction, transformation, and polyploidy induction can be used to produce a GMO, the organism produced is only defined under the regulations as GM if the process has involved the use of recombinant DNA or existing GMOs as recipient or parental organisms.
- 1.6 Because of the requirement for some form of separation between the GMO and the environment, contained use work is conducted in enclosed facilities, such as laboratories and glasshouses. With the exception of a small number of investigations involving fish, mice and in some case larger animals, the majority of work with GMOs in containment involves GMMs. Microorganisms are defined as any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, and include animal or plant cell cultures, viruses and viroids, the uncharacterised agent responsible for transmissible spongiform encephalopathy (such as bovine spongiform encephalopathy (BSE) and Creutzfeldt Jacob disease (CJD)). Naked nucleic acid (DNA or ribonucleic acid (RNA)), plasmids, and liposome gene delivery systems are not defined as microorganisms. This definition is unchanged in Directive 98/81/EC except that viruses and viroids are specifically included under the definition of microorganism.
- 1.7 Waste from contained facilities may be produced in gaseous, liquid or solid form (according to the nature of the activity being conducted). All waste is treated to minimise, or in some cases prevent, the release of GMMs to the wider environment. Solid waste is usually either autoclaved or incinerated, and then disposed of to landfill. Liquid waste is either treated with chemical disinfectants or autoclaved, and then disposed of to foul sewer, and gaseous waste may be filtered if necessary before being released to the atmosphere. The choice of treatment process depends on the assessment of the likelihood of the presence of GMMs in

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<sup>4</sup> The Genetically Modified Organisms (Deliberate Release) Regulations 1992 (amended 1995 and 1997) which implement Deliberate Release Directive 90/220/EEC in the UK.

<sup>5</sup> Exclusion of work with humans and human embryos means that human gene therapy does not fall under the Contained Use Regulations, unless the therapeutic agent is a GMM. In such cases the gene therapy is covered by the Contained Use Regulations, but not the human patient is not.

that particular waste stream and the suitability of a particular treatment process to render the microorganisms present non-viable.

## **AIMS AND OBJECTIVES OF THE REPORT**

1.8 The aim of this report is to assess the risks to the environment from work with GMMs in containment i.e. as a contained use activity, and specifically the risks arising from the waste generated from such work. Due to the enclosed nature of the work, waste streams represent the primary route of environmental exposure for GMMs used in containment. The information necessary to fulfil the aim was obtained through two objectives:

- to review the work being conducted with GMMs in containment, and
- to review the methods in place to prevent or control the escape of GMMs into the environment.

## **2. COMPARISON OF PREVIOUS AND CURRENT UK REGULATIONS RELEVANT TO THE USE OF GMMs IN CONTAINMENT**

### **OVERVIEW OF THE REGULATIONS**

- 2.1 Until very recently, the regulations in the UK governing the use of GMMs in containment implemented the European Directive 90/219/EEC. As already described (Chapter 1), this Directive was revised in 1998, and was implemented in GB legislation on the 15 November 2000. As part of the review of the legislation governing work with GMMs in containment, this report will first consider the regulations implemented under Directive 90/219/EEC, and then review the changes in the UK under the revised directive (98/81/EC) and the implications the revisions may have on work with GMMs in containment and the potential risks to the environment.
- 2.2 In the UK, work with GMMs in containment is regulated by the Health and Safety Executive (HSE). In their assessment of the potential risks of such work to human health and the environment, HSE are supported by DETR (on risks to the environment), and the Ministry of Agriculture, Fisheries and Food (MAFF). Technical advice is provided primarily by the Advisory Committees on Genetic Modification (ACGM). The Advisory Committee on Dangerous Pathogens (ACDP), which is referred to later in this report, is a joint Health and Safety Commission (HSC)/Department of Health (DH) committee which provides advice on biological agents in general, some of which may be GM.

### **BACKGROUND TO THE LEGISLATION**

- 2.3 The first UK regulations to control the genetic modification of organisms were introduced in 1978<sup>6</sup>, and prohibited any activity involving genetic modification which had not been notified to HSE. The Genetic Manipulation Regulations 1989 superseded these and included the requirement for users to carry out risk assessments for human health and safety for all

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<sup>6</sup> The Health and Safety (Genetic Manipulation) Regulations 1978, SI 1978/752.

activities, and that genetic modification safety committees were set up within each organisation conducting work with GMOs to advise on risk assessments. The 1989 Regulations, however, only required human health and safety assessments. Mandatory risk assessments for environmental safety were introduced within Directive 90/219/EEC.

## **OVERVIEW OF THE REGULATIONS UNDER EUROPEAN DIRECTIVE 90/219/EEC**

- 2.4 European Directive 90/219/EEC was implemented in GB by the Genetically Modified Organisms (Contained Use) Regulations 1992, as amended by the Genetically Modified Organisms (Contained Use) (Amendment) Regulations 1996 and 1998. The UK regulations covered contained use work involving both the generation of GMMs and the use of GMMs supplied by others. The regulations described the information required by the regulators, and whether prior consent was required before any work could commence. A central component of the information required was an assessment of the potential risks of the work to human and environmental health.
- 2.5 The sections of the regulations that are most relevant to this report relate to the definition of a 'Contained Use' activity, and the classification of contained use work as a 'Type A' or 'Type B' operation, involving either 'Group I' or 'Group II' microorganisms. The classification of the GMM and the work affects both the safeguards required whilst conducting the work, and also the information that is submitted to the regulatory authorities (HSE and DETR).

### **Type A and Type B Operations**

- 2.6 All contained use activities with GMMs were classified as either a Type A or Type B operation. Type A operations were defined as 'small-scale' non-industrial or non-commercial activities, although they could include research and development processes for subsequent industrial or commercial exploitation. The classification of Type A operations as 'small-scale' refers to the control systems in place, and not a fixed volume of culture used. Work was defined as 'small-scale' if it was conducted under good microbiological practice and occupational safety, and that the microorganisms used may be rendered inactive by standard laboratory decontamination techniques such as autoclaving or disinfection. However, because large volumes of microbial culture are more difficult to inactivate by standard techniques, Type A operations commonly involved small volumes of culture and were typically laboratory based research and development activities.

2.7 Under the regulations, all activities not defined as Type A were designated as Type B operations. These encompassed activities performed under industrial conditions to produce an industrial or commercial product, and usually involved larger volumes of microbial culture. Although predominantly commercial operations, some large-scale non-industrial research activities were also classified as Type B.

### **Group I and Group II Microorganisms**

2.8 In addition to the classification of the operation, the GMM used was also designated as a Group I or Group II microorganism. GMMs were classified according to the following criteria, with Group I microorganisms fulfilling all three requirements:

- the recipient or parental microorganism is unlikely to cause disease to humans, plants or animals,
- the nature of the vector used to insert the genetic material into the host organism, and the insert itself do not endow the GMM with a phenotype likely to cause either disease to humans, plants or animals, or adverse effects in the environment,
- the GMM is unlikely to cause disease to humans, plants or animals, and is unlikely to cause adverse effects in the environment.

2.9 All GMMs not classified as Group I, were designated as Group II microorganisms. Such microorganisms are therefore inherently pathogenic (able to cause disease), or are modified in such a way as to become potentially pathogenic to humans, plants or animals.

2.10 Work with all microorganisms in the UK in containment (GM and non-GM) is conducted at one of four containment levels, depending on the pathogenicity of the microorganism. Advice on the pathogenicity of individual microbial taxa to human health and the recommended containment level is given by ACDP, with containment level 1 the least effectively contained and applicable to non-pathogenic microorganisms. Containment level 4 employs the most effective containment methods and is used for highly pathogenic microorganisms that can survive in the environment, and may be spread by aerosol. Guidance on the containment level required for non-GM microorganisms is given by ACDP (ACDP, 1995). For GMMs the containment level required may however be influenced by the modification involved. Where the modification may increase the pathogenicity of the microorganism, for example through insertion of a gene for increased virulence, then a higher

containment level is applied. Details on the requirements for each containment level are available from HSE<sup>7</sup>.

### **Treatment of Waste**

- 2.11 The requirements for the treatment of all waste generated during work with GMMs are defined according to the containment level at which the work is being conducted. All Group II GMMs are required to be inactivated by validated means prior to their disposal into waste streams. Further information on waste treatment processes is given in Chapter 4.

### **Submission of Information to the Regulatory Authorities**

- 2.12 Information on activities involving GMMs must be submitted to HSE before any work can take place. There are three separate stages of notification:

- notification of first use of premises (CU1) - all organisations intending to work with GMMs are required to notify HSE of the premises in which the work will be conducted. A CU1 notification had to classify the GMM(s) as Group I or II, and Type A or B, and include an assessment of the risks of the proposed work to human health and the environment. This was usually either a generic risk assessment of the work that is proposed to take place at those premises, or a specific risk assessment for the first proposed activity. Where a premises had notified HSE of an intention to use Group I/Type A GMMs, subsequent use of Group II GMMs (or Group I GMMs as a Type B activity) could only proceed after consent had been given by the Competent Authority for the use of the premises and, for Group II/Type B only, the activity. For Group I GMMs in Type A activities work could commence 90 days after submission of the notification. For Group II GMMs work could not proceed until consent for the premises to be used for Group II activities had been issued by HSE. Group II activities (and Group I/Type B activities) also had to be notified separately on a CU2 or CU3 form,
- notification of individual activities (CU2 or CU3) - this was required for all Group II activities and Group I/Type B activities, and required a classification and categorisation of the work as Group I or II and Type A or B, and an assessment of

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<sup>7</sup> Equipment and handling requirements described in The Genetically Modified Organisms (Contained Use) Regulations 1992 (for work under Directive 90/219/EEC), and Council Directive 98/81/EC (1998), ACGM Compendium of Guidance.

the risks to human health and environment. Under the regulations a connected programme of work covering more than one activity could be notified as a single notification, for example a programme of work based around the study of a particular gene in a specific bacterial host/vector combination. Group II/Type B activities could not commence until a consent had been issued by HSE, and

- notification of significant change in risk assessment (10(4) notification) - all significant changes to work with GMMs in containment had to be notified. This applies to both a connected programme of work in which the scope of work changes during the programme, and a single notified activity. Where the change is sufficient to alter the risks of the work to the environment or human health then a 10(4) notification detailing the proposed changes is required by HSE.

2.13 The classification of the GMM and the activity as Group I or II, and Type A or B respectively was important in determining what information had to be submitted to the regulatory authorities. As described, all premises conducting work with GMMs had first to submit a CU1 for evaluation before that work could proceed. However, subsequent work at those premises which was designated as a Type A operation using Group I GMMs was not notifiable, including work where the risk assessment may have been different from that submitted in the CU1 notification. The regulations required that an assessment of the risks be made and kept by the relevant organisation, but not submitted to the regulators. Subsequent Type B activities using Group I GMMs, and all operations involving Group II GMMs were notifiable and subject to the CU2 (or CU3 for Group II/Type B activities) and 10(4) notifications described above.

2.14 The implications of these regulations to this report are that although all work with GMMs at a new premises was initially notifiable, the total number of activities involving GMMs in the UK in containment is unknown. Therefore it is not possible to review all the work conducted in the UK with GMMs as details of non-industrial/commercial work with Group I GMMs are not available. This is due to their exemption from notification at a previously notified premises. However, such work poses a low risk to human health and the environment as Group I GMMs are non-pathogenic and there should be no adverse effects to the environment.

## **OVERVIEW OF THE REGULATIONS UNDER THE REVISED EUROPEAN DIRECTIVE 98/81/EC**

- 2.15 The principal revisions to the new directive relevant to this report are the change in the classification system used to define the activity and the GMM, and the definition of a contained use activity.

### **Classification of Work with GMMs**

- 2.16 As described in Chapter 2, work with GMMs as regulated by Directive 90/219/EEC was classified according to the pathogenicity of the GMM (Groups I and II), and the scale and nature of the work (Types A and B). In the revised directive (98/81/EC), the system of classifying the work according to the activity and the GMM has been replaced by a single four-level classification system based on the containment level required for work with the specific microorganism. This has removed the distinction between research based and commercial/industrial activities. The purpose of this revision has been to simplify the classification procedure, and to bring the notification system in line with the assessment of the actual risks involved. Under Directive 90/219/EEC, work with the same volume of the same GMM was subject to different notification requirements depending on whether the work was being carried out for commercial or research purposes. However, the potential risks are likely to be similar, and Directive 98/81/EC recognises this under the revised single four-level classification system.
- 2.17 The four new classes are defined as those activities for which Level 1, 2, 3, or 4 containment facilities are appropriate to protect human health and the environment. Class 1 activities are defined as posing zero or negligible risk; Class 2, low risk; Class 3, moderate risk; and Class 4, high risk. The revised directive also contains more detailed guidelines on the facilities and procedures that should be used to conduct work in laboratories, glasshouses, animal units, and large scale fermenter units, at each of the four levels. Directive 90/219/EEC only had requirements for 'large-scale' activities.
- 2.18 The containment level classification was already used widely for GMMs alongside the previous system to ensure a smooth transition to the new classification. The use of a clear and easily applicable system is important, as the incorrect classification of a GMM has implications for the amount of information submitted to HSE and the containment measures employed.

- 2.19 However, the revision in the directive that is likely to have the greatest effect on the potential environmental risks of contained use activities with GMMs is the change of the definition of a 'Contained Use' activity. Under the Directive 90/219/EEC, there was an absolute requirement for the GMM to be physically contained to limit its contact with humans and the environment. Biological and/or chemical methods could be employed to support the physical barrier, but not to act independently of it. Under the Directive 98/81/EC, the absolute requirement for physical containment has been removed, with a contained use activity being redefined as any operation involving GMMs for which specific containment measures are in place to limit the contact of the GMM with the general population and the environment.
- 2.20 In the UK regulations implementing Directive 98/81/EC, there is a requirement to demonstrate in the notification that some form of containment, either physical, biological, or chemical is in place (acting independently or in unison), and that in all cases the waste must be rendered inactive before its release to the wider environment<sup>8</sup>. However, if an organisation was able to demonstrate that the risks to the environment and human health of the GMM used (in its viable state) were negligible, then contaminated waste can be disposed of without inactivation. Approval from the Competent Authority is required for all work (including Class 1 microorganisms) before such action can take place.

### **Treatment of Waste**

- 2.21 As work with GMMs under the Directive 98/81/EC is classified according to the containment level system already employed under the Directive 90/219/EEC, then the requirements for the treatment of any generated waste is similar to those already performed. However, the requirement for validated inactivation of waste may result in a shift away from chemical or biological techniques to physical waste treatment methods such as autoclaving and incineration.

### **Submission of Information to the Regulatory Authorities**

- 2.22 As with the requirements under Directive 90/219/EEC, not all work with GMMs in containment is notifiable under the revised directive. Activities defined as Class 1 are only notifiable when the premises are used for the first time. As with Group I, Type A work, no further notification is required for subsequent Class 1 activities. Activities designated as Classes 2, 3 or 4 will always be notifiable in the same way as Group II GMMs were under

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<sup>8</sup> Inactivation of waste does not require all the individual GMMs present to be inactivated.

the Directive 90/219/EEC. However, organisations that notify the regulators regarding a Class 2 activity in a notified premises will be able to start the proposed work on acknowledgement of their notification by HSE, rather than having to wait whilst the notification is evaluated, except for the first such activity, where there is a waiting period of 45 days. All notifications for work designated as Class 3 and 4 still requires evaluation by the regulatory authorities and a consent before the work can proceed.

### **IMPLICATIONS OF THE CHANGES IN THE DIRECTIVE TO THE ENVIRONMENTAL RISKS OF CONTAINED USE WORK WITH GMMs**

- 2.23 The change in the definition of a ‘Contained Use’ activity is significant as it removes the requirement for a physical containment system. The amalgamation of Type A and Type B operations means that potentially large-scale commercial work with GMMs, which are now non-notifiable after the initial CU1 notification, are, in the absence of physical containment, and on receipt of derogation from the Competent Authority, able to discharge potentially large volumes of untreated waste into waste streams. However, such disposal depends on the risk assessment showing that the GMM(s) present in the waste are completely non-pathogenic and unable to survive in the environment. This requirement highlights the importance of the risk assessment in defining what management options (including containment and waste treatment) are applicable to the work. The use and effectiveness of biological containment methods are discussed later in this report (Chapter 4).

### **3. OVERVIEW OF THE WORK BEING CONDUCTED IN CONTAINMENT IN THE UK**

3.1 The overview of work being conducted with GMMs in containment in the UK was compiled for this report from information held by HSE and DETR. As this review was carried out just before the new regulations came into force, the containment and work is described in relation to Directive 90/219/EEC. As described in the previous chapter, contained use work with all GMMs was classified, under the previous legislation, according to the pathogenicity of the microorganism and the scale of the work. According to HSE<sup>9</sup>, 471 premises have notified an intention to conduct work with GMOs in containment. Of these 471 first use (CU1) notifications, 196 were for activities involving Group II (pathogenic) GMMs, and only 34 were for Type B (industrial/commercial) operations. Although the figure of 471 notified premises is for work with all GMOs, only a very small minority involves work with non-microbial organisms. The number of individual activities involving GMMs will however be higher than the figure of 471 for the following reasons:

- further Type A work with Group I GMMs could proceed at a notified premises without subsequent notification, and
- where consent had been given for first use of premises for work with Group II GMMs, then notification for Type A work with Group I GMMs at those premises was not required.

3.2 A complete overview of the number and range of Type A activities involving Group I GMMs in the UK is therefore not possible from the records available, and for the purposes of this report the overview of the work with GMMs in containment will concentrate on the activities involving Group II GMMs, and Type B operations using Group I and Group II microorganisms.

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<sup>9</sup> Response to Parliamentary Question 56329.(Hansard, 26 October, 1998, Column 13 (Part 4))

## **OVERVIEW OF THE WORK BEING CONDUCTED IN CONTAINMENT IN THE UK AS A TYPE B OPERATION**

- 3.3 From the information held by DETR, a total of 30 Type B operations involving GMMs were identified<sup>10</sup>, working with culture volumes of GMMs of between 0.2 l and 200,000 l. All of the Type B operations identified were for non-pathogenic (Group I) GMMs, primarily GM bacteria (21 notifications). The other GMMs used in the Type B operations were the yeast *Saccharomyces cerevisiae* (5 notifications), the fungus *Penicillium chrysogenum* (2 notifications), and three mammalian cell lines (two modified by replication deficient adenovirus, and the other modified by an integrating plasmid). A complete list of all the 30 Type B operations is given at Appendix A detailing the type of GMM and the volume of microbial culture used.
- 3.4 Because all industrial and commercial activities were required under the previous regulations to be classified as Type B, not all of those identified involve the use of large culture volumes of GMMs. Indeed a culture volume of <20 l which is used in a third of the Type B operations is no greater than some of the Type A operations identified. Only three of the Type B activities identified used a culture volume of >10,000 l.

## **OVERVIEW OF THE WORK BEING CONDUCTED IN CONTAINMENT IN THE UK AS A TYPE A OPERATION INVOLVING GROUP II GMMs**

- 3.5 From the information held by DETR, a total of 452 notifications to conduct a programme of work with Group II GMMs were identified. All the notifications were for work with culture volumes of between 1 ml and 30 l, with 84 percent using a culture volume of  $\leq 1$  l (Table 3.1). Based on the highest containment level used during each programme of work, 23 notifications were for work at containment level one, 340 notifications at containment level two and 88 notifications at containment level three (Table 3.2). Only one notification, for work with GM Morbillivirus, a viral pathogen responsible for diseases such as canine distemper, and rinderpest (a highly infectious disease of cattle), was designated as containment level four.

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<sup>10</sup> The discrepancy between this number and the figure of 34 given in answer to PQ 56329 is that the response given to the Parliamentary Question was referring to large-scale uses of GMMs, and not specifically Type B activities. Large scale activities are typically those involving more than 50-100 l of culture medium and may include activities classified as Type A. The description of an activity as large scale depends on factors such as the level of containment as well as the volume of culture medium involved.

3.6 Table 3.2 shows that most of the work conducted at containment level one involves the use of GM bacteria. The majority of these are plant pathogens, such as *Septoria tritici*, *Erwinia carotovora*, and *Agrobacterium tumefaciens*. Apart from one notification for work with up to 10 l of culture of *Streptomyces avermitilis*, all the work with Group II GMMs at containment level one involves no more than 1 l of culture. A complete list of the Group II GMMs notified for use at containment level one is given in Appendix B.

**Table 3.1 - Volume of microbial culture used for all Type A activities using Group II GMMs.**

Volume of Microbial Culture (litres)	Percentage of activities
0 - <0.5	31
0.5 - <1	10.5
1	42
1 - <5	5
5 - <10	5
10 - 30	6.5

3.7 For work conducted at containment level two, the majority of the notifications (203) are for GM viruses, primarily adenovirus (43), retrovirus (37), and the poxvirus Vaccinia virus (48). A complete list of the Group II GMMs notified for use at containment level two is given in Appendix B. Of the GM bacteria notified (118), the majority are *E. coli* (34) usually modified with a virulence gene from another pathogenic microorganism. Most of the *E. coli* used are disabled strains, for example strain K12. Such organisms are not capable of surviving in the environment, as they have some form of biological limitation that restricts their ability to survive, such as the inability to synthesise the required nutrients (Oxford University, 2000). The other GM bacteria used at containment level two are mainly human pathogens, either opportunistic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* which are no more virulent than the wild type strains, or more

pathogenic bacteria such as *Neisseria meningitidis* and *Clostridium botulinum* where the bacteria has been modified for reduced virulence compared to the wild type. The other GMMs used at containment level two include the yeast *Candida albicans* which is an opportunistic human pathogen, and the trypanosome and plasmodium parasites responsible for sleeping sickness and malaria respectively. Although these parasites are significant human and animal pathogens, both require an insect vector to cause any disease. The insect vectors for both malaria and sleeping sickness are not present in the UK environment. Opportunistic human pathogens such as *S. aureus* only cause disease in people whose immune system is compromised or damaged in some way.

**Table 3.2 - Number of notifications submitted for Type A operations with GMMs at each containment level.**

Type of Organism	Containment Level			
	1	2	3	4
Bacteria	14	118	38	0
Viruses	6	203	39	1
Fungi	2	3	0	0
Parasites	1	9	6	0
Yeasts	0	7	0	0
Prions	0	0	5	0
Totals	23	340	88	1

3.8 Work at containment level three (88 notifications) involves primarily pathogenic microorganisms with a similar or potentially greater virulence than the wild type strain. All but three of the notifications to work with GM mycobacteria are conducted at containment level three. These include all of the notification for work with *Mycobacterium tuberculosis* (9 notifications) (the causative agent of tuberculosis). All of the work with GM prion proteins (5 notifications) is also performed at containment level three. The majority of work with GM viruses at containment level three (39 notifications) involve Hepatitis Virus (types B

and C) (11 notifications) and Human Immunodeficiency Virus (HIV) (12 notifications), all of which are human pathogens.

- 3.9 Whilst the *E. coli* used at containment levels one and two are all disabled strains, the *E. coli* strains used at containment level three are predominantly pathogenic strains such as the enterohaemorrhagic strain O157. A complete list of the Group II GMMs notified for use at containment level three is given in Appendix D.

## **4. REVIEW OF THE METHODS USED TO LIMIT THE CONTACT OF GMMs WITH THE ENVIRONMENT**

- 4.1 Under the definition of a ‘Contained Use’ activity (Chapter 1), all work with GMMs in containment was carried out with some form of physical barrier to limit contact between the GMM and the environment. Under the previous regulations the physical barriers may act independently or in combination with chemical or biological barriers. However, the implementation of Directive 98/81/EC into UK legislation replaces the absolute requirement for physical containment with a requirement for some form of containment, either physical, chemical, or biological.
- 4.2 The aim of this section is to review the physical, chemical, and biological methods used to limit the contact of GMMs with the environment and the general population, and to assess the techniques by which such methods may be validated. This section also reviews the containment methods used for activities involving more than 100 l of culture.

### **PHYSICAL AND CHEMICAL CONTAINMENT METHODS**

- 4.3 A GMM is defined as being physically contained if its contact with the environment is limited or prevented by a physical barrier, or if the GMM is destroyed by heat, pressure or other physical process prior to its discharge into waste streams and its subsequent entry into the environment. Chemical containment methods are characterised as those methods that involve a chemical process to inactivate or destroy the GMM before it can reach the environment.
- 4.4 In all contained use activities, the GMM is grown or stored in a culture vessel such as a flask or fermenter. Exposure of the GMM to the environment may potentially result from the failure of the containment vessel, removal of the GMM from the culture vessel as part of the programme of work, or release of the GMM in waste streams. Physical and chemical containment methods are therefore designed to minimise live GMMs from entering the environment by these three routes.

4.5 Release of live GMMs following failure of the containment vessel, or during routine removal of the GMM from that vessel, represents accidental releases to the environment, and are the least likely pathways for the environmental exposure of GMMs in containment. Virtually all work with GMMs in containment results in the generation of waste, therefore the release or escape of GMMs in waste streams (atmospheric, liquid, or solid) represents the most likely route of environmental exposure of GMMs. The physical and chemical methods for the treatment of atmospheric, liquid, and solid waste streams are described below.

### **Atmospheric Waste Streams**

4.6 Atmospheric waste streams from a culture vessel (technically referred to as the off gas) can be treated by single step processes or, more usually by a combination of methods. Off gas treatment is designed to have maximum efficiency in terms of power utilisation, gas flow and mass transfer (Rollinson, 1988) whilst ensuring that any microorganisms are inactivated, and that the system can be sterilised between runs. Off gas treatment systems often include the removal of excessive moisture from the waste stream which has the effect of either rendering any microorganism non-viable through desiccation, or returning it to the culture vessel with the condensed water. Industrial processes used include:

- condensers - these are normally the first stage of the exhaust gas treatment, and are designed to remove water vapour from the gas stream. Condensers normally use a chilled water coil placed on the outflow of the fermenter to cool the air so causing condensation of water vapour and allowing it to return to the fermenter. The condenser may reduce the microbial load in the exhaust air by flushing the microorganisms back to the fermenter,
- heat treatment - this is used to reduce the relative humidity of the off gas, and will consequently also inactivate airborne microorganisms. Heat treatment may involve incineration of the off gas in some cases to reduce offensive odours. This will inactivate all microorganisms present,
- sterile filtration - high efficiency filters (<0.2  $\mu\text{m}$  pore size) are used widely for the filtration of the off gas from both fermenters and smaller scale culture flasks. Filters are constructed from either hydrophobic or hydrophilic materials and are highly effective in the removal of airborne microorganisms so that <1 microorganism for every  $10^8$  microorganisms penetrating the filter will pass through. Because of their resistance to microbial growth, and to blockage under conditions of high humidity,

hydrophobic filters are the more widely used. If hydrophilic filters are used then the relative humidity of the exhaust air needs to be substantially reduced to ensure they maintain their high performance, and as such are seldom used in off gas applications.

Aerosol emissions of microorganisms from point sources such as steam escape valves or pressure release valves in fermenters can be prevented by adding high-efficiency particulate air (HEPA) filters to those points. A HEPA filter has been defined traditionally as an extended-surface dry-type filter having a minimum particle removal efficiency of 99.97 percent for all particles of 0.3  $\mu\text{m}$  diameter, with higher efficiency for both larger and smaller particles. To qualify as a 'true' HEPA filter, the filter must allow no more than 3 particles out of 10,000 to penetrate the filtration unit. More recently, filters made in the same physical style using less efficient filter paper have been produced. Such filters are described as 'HEPA-type' filters, and their actual efficiency may be 55 percent or less at 0.3  $\mu\text{m}$  (American Lung Association, 2000).

- cyclones - these reduce the water content in air streams by inducing rotary motion into the air stream causing deposition of droplets onto the walls of the cyclone. Because deposition is most effective at higher air velocities, then the use of cyclones is usually restricted to large scale fermenters,
- siphons - these involve the off gas being vented through a fluid, and will substantially reduce the number of airborne microorganisms (Winkler and Parke, 1992). Such systems are used widely in the UK fermentation industry,
- biofilters - these are ecosystems of microorganisms growing on a substrate, for example straw, through which the off gas may be pumped. They are primarily intended to reduce odours in the off gas, but also have a secondary function in the removal of the process microorganisms, either through interception in the biofilm matrix, or by inactivation by heat generated in the biofilm.

4.7 The use of GMMs in the production of a specific compound often involves downstream processing of the culture to extract the desired product. Both centrifuges and homogenisers have been shown to generate microbial aerosols at concentrations of  $10^4$  to  $10^5$  colony forming units (cfu) per cubic metre. Such aerosols may however be contained by conducting the work within rooms or cabinets where all exhausts are filtered before release to the environment.

## Liquid Waste Streams

- 4.8 The process used to treat the liquid waste depends on whether the purpose of the contained use work was to produce whole cells, or an intracellular or extracellular product. Treatment of the microorganisms within the primary culture vessel is the most effective treatment process in terms of minimising the release of live cells, as this avoids any downstream processing. Where inactivation in the culture vessel is unsuitable, for example if it denatures the required product, then the GMM must be transferred to a secondary vessel for extraction of the desired product before inactivation.
- 4.9 The treatment methods used to render microbial cells in liquid culture non-viable are heat treatment and chemical treatment. Heat treatment is the most commonly used method of inactivating liquid effluent, and involves heating the culture with steam, often under pressure to a set temperature for a fixed period of time. The temperature and time parameters are ideally evaluated by previous experimentation to result in an acceptable reduction in the number of viable cells present in the culture. Heat treatment has the advantage over chemical treatment in that:
- it is easily controlled and reproduced, and will therefore result in a consistent 'kill ratio' of live to dead cells in the culture every time,
  - it may be easily monitored by the use of thermocouples within the culture,
  - it does not involve the release of toxic chemicals to the environment, and
  - it is effective for the inactivation of a wide range of microorganisms.
- 4.10 Chemical treatment involves the addition of a bactericidal compound capable of inactivating the GMM, to the culture. For most chemical treatments this is a bleach based compound, although formaldehyde and alkali are also often used. Because of the volume of chemical required (usually 5 to 10 percent of the volume of culture to be inactivated), the treatment of large volumes of culture is usually performed by heat treatment, with chemical inactivation employed for smaller scale laboratory cultures. The primary drawback of chemical treatment is the use of the chemicals themselves, most of which are toxic to the environment and human health. The effectiveness of certain chemicals may also be dependent on the microorganism, and environmental factors, such as temperature. For example, the parasite

*Cryptosporidium* is resistant to disinfection by chlorinated compounds, and formaldehyde is ineffective below a temperature of 4 °C.

- 4.11 All notifications reviewed in the compilation of this report used chemical or heat treatment processes for the inactivation of liquid waste streams. For larger scale operations, the use of both physical and chemical processes for the treatment of liquid waste is less straightforward, due to the greater volume of culture within a single culture vessel. Complete inactivation of large volumes of culture with heat or chemicals is highly intensive in terms of energy or chemicals, and is usually only feasible for products with very high added value (Harrewijn and Kossen, 1989)<sup>11</sup>. Where complete inactivation of waste streams is not achieved then the notifier was required to state the level of live GMMs expected in the waste. In cases where complete inactivation of the microbial culture is not possible by physical or chemical means, then some form of biological containment measure is necessary to limit exposure of the GMMs to the environment.

### **Solid Waste Streams**

- 4.12 Solids containing potentially viable GMMs are produced by many contained use activities. In some cases a large proportion of the GMMs in the solid waste may have been already inactivated by downstream processing. These solids are often then resuspended with the resulting suspension treated as liquid waste. Otherwise solid waste may be autoclaved or incinerated, often on-site.

### **Containment in Case of Failure of the Culture Vessel**

- 4.13 Failure of the culture vessel is usually a result of a build up of high pressure within the vessel. Although flasks are used at ambient pressure, fermenter systems may sometimes operate at higher (positive) pressures. The possibility of complete failure of these systems can be reduced by the use of bursting discs at vulnerable seals, which are designed to fail without affecting the integrity of the system. Siting the culture vessel within a secondary vessel or enclosed area (a bund) will contain any potential spill within the proximity of the vessel. Culture contained in this way may then be treated with chemicals, or drained into another vessel or 'kill tank' for inactivation by the methods already described. The exterior of the culture vessel may also be sprayed with chemical disinfectant to kill any microorganisms released through minor leaks.

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<sup>11</sup> This however is an economic consideration, and as such is outside the Contained Use regulations which are risk-based, rather than cost/benefit-based.

### **Containment of GMMs During Routine Work**

- 4.14 The containment measures employed during routine work with the GMM are dependent on the containment level at which that work is conducted, and are designed to restrict the GMM to the vicinity of the laboratory or, more often the area immediate to the culture vessel. The use of aseptic technique when handling samples of microbial culture will largely confine the GMM to the culture vessel used, and conducting the work in a Class II (or higher) safety cabinet will reduce any airborne transmission of the GMM. The safety cabinets filter any aerosol produced through  $<0.2 \mu\text{m}$  filters before release to the atmosphere. Where necessary the use of laboratories with an atmosphere maintained at a lower (negative) pressure than the surrounding environment will prevent loss of airborne material, as any drafts will only flow into and not out of the room/laboratory. Negative pressure facilities are compulsory for work at containment levels 3 and 4. Aerosols, caused by the removal of samples from fermenter vessels can be prevented or reduced by the installation of dedicated sampling ports in the vessels.

### **BIOLOGICAL CONTAINMENT METHODS**

- 4.15 Biological containment represents the third form of barrier that may be used to limit contact between the GMM and the environment. A GMM is defined as being biologically contained if the genetic modification within that microorganism is limited from being transmitted to the other organisms by a biological process. The biological containment procedures employed are often specific to the individual GMM.
- 4.16 A widely used form of biological containment, particularly where large volumes of GMMs are being used is cell death. The lysis of the cell's membrane after death results in the exposure of the genetic material to environmental nucleases, and although fragments of the cell's DNA may persist in the environment (Paul *et al.*, 1987), the chance of any genes remaining intact is low.
- 4.17 Most microorganisms used in containment are laboratory or commercial strains and these are usually maintained under conditions of optimum temperature and nutrient availability. Mammalian cell lines such as Chinese Hamster Ovary (CHO) and HeLa cells will only survive at 37 °C and will therefore die if released into the environment from a contained facility, from either the reduced temperature or osmotic shock. Many laboratory strains of GMMs are poorly adapted to the marked fluctuations in growth conditions and nutrient levels experienced in the environment, and consequently their ability to survive outside the

laboratory environment is usually impaired compared to wild type strains of the same microbial species. Even if the GMM can survive in temperatures experienced in the environment, a probable inability to compete with wild type strains for nutrients means they will not persist in the environment.

- 4.18 The characteristics conferred by the genetic modification may also result in attenuating the ability of the GMM to survive in the environment. GMMs that are modified (usually for selection processes) to be dependent on a specific nutrient or growth condition are biologically contained if that requirement is not available or prevalent in the environment.
- 4.19 Where a microorganism is modified through the insertion of genetic material on a plasmid vector, the gene or genes that are inserted on that plasmid may become biologically contained within the GMM if the plasmid is non-mobilisable or mobilisation defective (Dougan *et al.*, 1978), or if the recombinant DNA is integrated into the microbial chromosome (Munthal *et al.*, 1996). The use of non-mobilisable plasmids does however not ensure complete biological containment, as genes can be transferred by other, non-active processes.
- 4.20 For many plasmid vectors the mobilisation capability is encoded in loci such as *bom*, *mob* and *tra*. Depending on the presence or absence of these loci, plasmid vectors may be non-mobilisable or mobilisation defective. Non-mobilisable vectors are defective in one or more functions required for transfer to other hosts. For *E. coli* non-mobilisable plasmid vectors should be  $Bom^-/(Nic^-)$ ,  $Mob^-$  and  $Tra^-$ , and include pAT153, pUC series, pBluescript II, pEX series, pCAT series, pT3/T7, pEUK-C1, pSP18, pXT1, pSUBeta, pEMBL19 and pSELECT (Oxford University, 2000).
- 4.21 Mobilisation defective vectors are defective in one or more transfer functions and can only be mobilised by the addition of other elements which supply the missing functions. In *E. coli* mobilisation defective plasmid vectors are  $Bom^+/(Nic^+)$  but  $Tra^-$  and  $Mob^-$ , and can be efficiently mobilised if they are co-resident with certain other plasmids. Examples of *E. coli* mobilisation defective plasmid vectors are pBR322, pBR325, pACYC177, pBTac-2, pBTrp2, pKT280, pFB series, pKSV-10, pGA482, pGA580, pNOS and RP4 1 (Oxford University, 2000).
- 4.22 Insertion of the recombinant DNA into the microbial chromosome can also confer a degree of containment, although recombinant DNA integrated into the microbial chromosome or on

a plasmid with its own origin of replication will of course be propagated as part of the natural replication of the GMM.

- 4.23 Biological containment is conferred through the use of mobilisation defective or non-mobilisable vectors or a GMM that is replication deficient. The modification of a bacterium, yeast or fungus with a non-mobilisable vector ( $Bom^-/(Nic^-)$ ,  $Tra^-$  and  $Mob^-$ ) means that the genetic modification is effectively biologically contained within the GMM and will only remain in the environment for the period that the individual GMM can survive. The use of mobilisation defective vectors ( $Bom^+/(Nic^+)$ ,  $Tra^-$  and  $Mob^-$ ) can also provide some degree of biological containment, although such vectors can be mobilised efficiently if they are supplied with other plasmids.
- 4.24 Viruses can be genetically modified so that they lack the specific gene or genes required to undergo replication and are therefore unable to multiply. Such microorganisms are described as replication deficient. Therefore even if the GM virus is able to survive in the environment following an accidental release or disposal into a waste stream, any exposure will be limited to the number of GM viruses that were released. The majority of the GM adenoviruses identified during this report have been genetically modified by the removal of one or more of their genes that are expressed at the start of viral replication (so called early genes). Two of the products of the early genes (E1 and E4) facilitate extensive modulation of the host cell's transcriptional machinery causing the host to express the viral genes, and one (E2) assembles the virus DNA replication complex (Murphy *et al.*, 1983). Removal of any of the DNA encoding for these products renders the adenovirus replication deficient.
- 4.25 Some microorganisms have specific properties or characteristics that allow them to survive in the environment, or to be pathogenic. Early gene product E3 in adenoviruses is involved in subverting host defence mechanisms. The modification or alteration of those characteristics will therefore alter the survival or virulence of that virus and may confer some degree of biological containment.
- 4.26 If the modified genes are inserted in the genome within a gene important for survival or pathogenicity then that gene becomes unreadable. However, because that point in the GMM's genome is now occupied by the inserted gene(s) the GMM is unable to regain its lost trait and a more permanent form of containment is conferred. Recombination with the wild type virus could result in the disrupted gene being reformed. However, as this would result in the inserted gene being lost from the virus, all that is produced is a non-genetically modified wild type virus (not a wild type virus with the inserted gene).

- 4.27 Where the GMM requires a specific host or vector to enable it to survive in the environment and/or cause disease, the absence of the required organism may result in the GMM being biologically contained. The best examples of this are the trypanosome, leishmania, and plasmodium parasites responsible for sleeping sickness, kala-azar and malaria respectively, which are included in 16 notifications. However each of these parasitic organisms require specific insect hosts to complete their lifecycle and to cause disease. *Leishmania major* for example requires a species of sandfly (of the genus *Phlebotomus*) to complete its lifecycle and this insect is not resident north of the Mediterranean. The insect vectors required by *Plasmodium sp.* (mosquito) and *Trypanosoma sp.* (Tsetse fly) are also not present in the UK environment. Therefore in this country, work with all three parasites is effectively biologically contained at the life cycle stage where the insect host is required. None of the parasites are able to complete their lifecycle and cause disease, and will therefore not survive in the environment. Other GMMs which cannot survive outside their specific host, particularly obligate pathogens, may also be biologically contained within the containment facility, if the host organism is not present in the local environment. Examples include GMMs that are plant pathogens such as *Septoria nodorum*, where a suitable host plant (wheat in the case of *S. nodorum*) may be some distance away from where the work is being conducted. If the GMM is unable to survive for sufficient time in the environment to reach the required host then it will not persist in the environment. In these situations the specific environment surrounding the containment facility has a strong influence on the potential risks of environmental exposure of the GMM.
- 4.28 Although the biological containment methods have been described separately in this section, they are frequently used in combination, particularly the mobilisation defective vectors and replication deficient microorganisms, to confer a high degree of biological containment.

#### **VALIDATION OF CONTAINMENT METHODS**

- 4.29 Validation of the effectiveness of the containment methods used is dependent on an ability to monitor or detect the presence of the GMMs in the waste streams. Monitoring or detection is significantly easier if conducted before the GMMs are discharged into the wider environment ('end of pipe' monitoring). Monitoring for microorganisms once they enter the wider environment is difficult due to the heterogeneity of many environments (particularly soil and sediment), and also the presence of a wide diversity of microbial taxa in the environment, many of which have similar characteristics and will be detected by the same tests. However, the existence of relatively simple media and a microbial culture consisting of one or maybe two strains of microorganism in waste streams from contained use facilities

make monitoring considerably easier. Although there was no specific reference to monitoring in the regulations, all containment measures used to limit the release of GMMs into the environment required validation (both before and during the use of the process). There was also a requirement to test for the presence of GMMs outside the area of primary containment, although this measure was only required where the risk assessment indicates a likely breach of primary containment, and was not an absolute requirement in all cases.

### **Collection of Microorganisms from Waste Samples**

4.30 Where high levels of viable microorganisms are present in solid and liquid wastes, monitoring may be conducted by direct sampling from the waste stream, before discharge to the wider environment. However, for most waste streams from contained use facilities, the concentration of viable GMMs is likely to be low following physical treatment processes<sup>12</sup>. In these cases some form of collection/concentration stage is necessary prior to analysis, although if the levels of microorganisms are too high, then the sample may need to be diluted prior to analysis. For liquid and solid waste, the sample can be centrifuged to concentrate the sample. Prior collection steps are however most applicable to sampling from off gas waste streams:

- impaction - this involves collection of the microorganism onto a solid growth medium such as agar plates or strips. The agar strips are then incubated and the number of microbial colonies formed are counted. The advantages of this system are that it is easy to use, portable and requires minimal handling of samples. However because of the incubation time period, impaction samplers are most suitable for regular monitoring of processes in clean environments where results are not required rapidly and the GMM is the only microorganism present. In waste streams containing other microorganisms in addition to the GMM, impaction sampling is only suitable if the GMM can be distinguished by specific growth characteristics such as pigment formation, or if it expresses a specific selectable marker, for example resistance to an antibiotic. In this case a selective growth medium containing the antibiotic will allow the GMM to be distinguished from the other antibiotic-sensitive microorganisms that are present,

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<sup>12</sup> The removal of the requirement for physical containment under the Directive 98/81/EC may however result in increased levels of viable microbial cells in waste streams, if the correct derogation is received from the Competent Authority. The revised Directive however still requires contact between the GMMs and the wider environment to be limited in some way.

- impingement - this involves collection of the microorganism into liquid, usually by a cyclone sampler. Fluid samples may be analysed by standard culturing techniques for the GMM of interest,
- filtration - here the microorganisms in the off gas are collected onto a filter. This is then washed and the filtrate analysed by standard microbiological techniques. Because vegetative microorganisms are likely to be inactivated through desiccation on standard filters, gelatin filters should be used to sample these non-spore forming microorganisms, and
- combined collection/detection systems - these were designed originally for the continuous monitoring of air for potentially pathogenic microorganisms, and provide a rapid detection system for air flows of up to 1000 l per minute.

### **Detection of Microorganisms in Waste Samples**

4.31 The method used for the detection of GMMs in waste stream samples is dependent on the aim of the monitoring process and also on the characteristics of the GMM to be detected. Where the purpose of the monitoring is to determine whether any GMMs are present in the waste stream, then a qualitative (yes/no) method will suffice. An example of this is the dehydrogenase assay, which is a colorimetric based method and shows the presence of metabolically active microorganisms. However, where a quantitative value is required, a technique such as viable counting on selective media is necessary. Unlike the detection of microorganisms in environmental samples, because waste streams consist of much less complex matrices containing a known microbial composition, then the detection and/or monitoring of GMMs is much easier. A range of methods are available, including:

- viable counting on selective media - this involves the incubation of liquid waste samples on a suitable growth medium (usually agar plates) and then counting the microbial colonies that grow on the medium. By altering the contents of the growth medium and the incubation conditions (time and temperature), this method can be tailored to detect individual or a range of microbial taxa, and can be designed to detect bacteria, yeasts, fungi, and bacteriophages. The length of incubation period is dependent on the growth rate of the GMM and ranges from 12 to 24 h for fast growing microorganisms such as *E. coli* and pseudomonads, to >3 d for slow growers such as mycobacteria and *Helicobacter pylori*. At concentrations of <10 microorganisms per ml of waste, sample concentration is necessary prior to analysis.

Selective media assays are however relatively simple and cheap to perform and can provide both a quantitative or qualitative result (Atlas, 1984). In situations where the microorganism of interest grows on poorly soluble hydrophobic substrates such as long-chain hydrocarbons then most-probable-number methods are more suitable than growth on selective media (Haines *et al.*, 1996).

Viable counting of microorganisms on selective media can of course only be performed with microorganisms that can be cultured. Studies have shown that in the environment only 1 percent of the microorganisms present can be cultured on selective media (Torsvik *et al.*, 1994). Many microorganisms are present in the environment in what is termed a viable but non-culturable (VBNC) state. Such microorganisms are proposed to be dormant, and are thought to be capable of returning to a more active state, should the environmental conditions, usually food availability improve. Because many of the GMMs used in contained facilities are not grown under oligotrophic (nutrient-limited) conditions, then it is likely that should GMMs enter the environment in a viable state then they are likely to become VBNC due to a lack of nutrients or adverse temperature or pH. This is another factor to support the monitoring for GMMs at 'end of pipe' or in the waste stream, rather than waiting until the GMMs have been discharged to the wider environment.

- colorimetric markers - the majority of colorimetric based methods only provide a qualitative result and cannot identify the size of the microbial population present (Kämpfer *et al.*, 1991; Knight *et al.*, 1997). However where the colorimetric marker is added to a range of substrates, as in the case of the Biolog™ and Pheneplate™ test assays (Bochner, 1989; Katoulie, 1996), individual strains of microorganisms can be identified (Fulthorpe and Allen, 1994). Tests such as Biolog and the dehydrogenase assay use a tetrazolium compound as the colorimetric marker. In the presence of metabolically active microorganisms, tetrazolium is taken up by the microorganism in its colourless form, and then oxidised within the microbial cell to form a visible red product (Guckert, 1996). If the tetrazolium compound is supplied in the presence of an utilisable substrate, then the assay can detect the presence of one viable microorganism per sample. If the GMM is likely to be present in the waste with other microorganisms then a more selective system is required to distinguish the GMM. The most effective of these systems are those where the GMM is modified with a unique marker gene (not present in the environment into which the GMM may be released), such as antibiotic or heavy metal resistance, luminescence (*lux* gene), or fluorescence (green fluorescent

protein). The sensitivity of the *lux* system without culture enrichment is approximately  $10^4$  cfu per ml,

- direct counting - involves enumeration of the microorganisms directly from the waste sample using a microscope. Depending on the size of the microorganism to be counted and the presence of any other particulate matter in the sample, some form of stain may be required to distinguish the viable microbial cells. Throughput of samples may be improved by automating the counting process with an image analysis system. The use of fluorescent stains such as acridine orange (Kämpfer *et al.*, 1991) and BacLight (Molecular Probes, Europe) (Cartwright, 1998) that can distinguish between viable and non-viable cells are also beneficial. If the waste samples contain organic particulate matter other than microbial cells, then direct counting methods can overestimate cell numbers as the fluorescent stain may also bind to non-microbial particles,
- spectrophotometric methods – these work by measuring the amount of light absorbed when a beam is shone through a liquid, and can provide a quantitative result for microbial cells in a liquid if the system has been correctly calibrated. The greater the concentration of particulate matter in the liquid then the greater the amount of light absorbed. As with direct counting methods, this technique is less suited to waste samples containing particles other than the microorganism of interest, including fragments of microbial cells,
- antibody-based methods - these are based on a specific antibody-antigen reaction and are generally used to monitor the presence of specific phenotypes. Although potentially highly specific, such methods have a low sensitivity (typically  $10^4$  to  $10^7$  microorganisms per ml) and are seriously affected by non-specific binding to background material. However in situations where sample quality is high and a pre-monitoring concentration stage is employed, for example in atmospheric waste streams, antibody-based methods such as ELISA (enzyme linked immunosorbent assay) can be very effective, although if the antigen is expressed after cell death, then the method will detect both viable and non-viable cells. The use of immuno-capture procedures, such as immunofilters and immunomagnetic beads can separate the labelled GMM from the background microflora, and thereby significantly improve the sensitivity of antibody-based methods,

- phage-based methods - these may be used for the detection of bacteria only, but with a sensitivity of only  $10^3$  to  $10^4$  cells per ml may be of limited use in the practical monitoring of GM bacteria,
- flow cytometry - involves the direct enumeration of microbial cells in solution. Because of its ease of automation, flow cytometry is particularly applicable to industrial process monitoring. However, unless the microorganism of interest expresses a fluorescent marker (such as *lux*), then flow cytometry is only applicable for the quantification of total microorganisms present in a sample,
- fatty acid analysis - because the individual fatty acids present in microbial membranes are discontinuously distributed between different microbial taxa, then analysis of the relative amounts of microbial fatty acids in a sample can be used for the identification of microorganisms in that sample (Descheemaker and Swings, 1995). Because fatty acids are catabolised rapidly after cell death, any fatty acids detected must be part of intact cell membranes and therefore indicative of the viable microorganisms in the waste stream at the time of collection (Zelles *et al.*, 1992). Fatty acid analysis has been used to identify certain microbial taxa down to a sub-species level on the basis of the presence or absence of specific fatty acids (Dowling *et al.*, 1986). However, because these so called 'biomarker' fatty acids may be expressed by other microbial taxa (Buyer and Drinkwater, 1997), this reduces their value as unique taxonomic signatures (Zelles *et al.*, 1995), and identification of microorganisms to the sub-species level by fatty acid analysis should therefore be treated with care (Griffiths *et al.*, 1997). However the expression of 'biomarker' fatty acids by more than one microbial taxon is only applicable where the waste stream contains a mixture of taxa,
- molecular methods - microorganisms can be detected and identified on the basis of their genetic material (Griffiths *et al.*, 1997). The use of molecular probes is particularly applicable to the detection of GMMs as the probe may be designed to detect the recombinant DNA inserted into the GMM (Sayler and Layton, 1990; Olson, 1991). The use of probes to detect ribosomal RNA represents a more sensitive detection technique than DNA based probes, due to the higher quantities of RNA in microbial cells compared to DNA (Leisack *et al.*, 1990; Stackenbrandt *et al.*, 1991). As with fatty acid analysis, molecular probes can be used without prior culturing of any microorganisms in the sample (Amann *et al.*, 1995).

- 4.32 From the information provided in the notifications reviewed for this report, the monitoring of GMMs in waste streams is confined largely to viable counting on selective media. The advantage of using viable counting on selective media to monitor GMMs in waste streams is that the contents of the waste stream are likely to contain a relatively low diversity of microorganisms. In many cases only the organism of interest is likely to be present. This avoids the problems of contamination of selective isolation plates that are often encountered in monitoring microorganisms from environmental samples on selective isolation plates. Also because the identity and characteristics of the organism to be monitored are known, then the selective isolation medium and culture conditions may be designed to provide a very sensitive detection system.
- 4.33 The use of selective isolation plates enables a large number of samples to be monitored relatively quickly and at a low cost. The technique is suitable to provide the information required, i.e. the presence and quantity of viable microorganisms present and is therefore proposed as particularly suitable to the monitoring of microorganisms in waste streams prior to discharge into the wider environment.
- 4.34 For the purposes of the monitoring required, the use of selective isolation plates represents the most suitable system. As discussed, this technique allows a high throughput of samples at relatively low cost and can be tailored to provide a quantitative or qualitative approach. The use of image analysis systems has the potential to automate the analysis of isolation plates after they have been incubated, leading to a greater throughput of samples. The frequently cited disadvantages of selective isolation plates are that they are subject to contamination from other microorganisms and can only identify culturable strains. These limitations are however not particularly relevant to monitoring the types of microorganisms used in contained facilities. Most strains used are culturable, and in many cases there is a commercially available selective isolation medium for the particular strain of GMM being used. Also the limited number of strains present in a single waste stream, mean that background contamination of isolation plates is likely to be low.

#### **CONTAINMENT OF “LARGER” VOLUME OPERATIONS**

- 4.35 As described (Chapter 3), 19 operations for work with GMMs have been notified in the UK, which involve work with more than 100 l of culture. Table 4.1 summarises the containment measures employed to limit environmental exposure of the GMM in these large volume operations.

4.36 Table 4.1 shows that the majority of the Type B operations using >100 l of microbial culture use a combination of both physical/chemical and biological containment methods, with the GMM usually possessing an attenuated capability to survive in the environment, and the recombinant DNA present on a mobilisation defective vector. Only three premises where large volume Type B operations were notified involved the detectable release of viable GMMs in waste streams, although in each case biological containment methods were employed to reduce the ability of the GMM to survive in the environment and/or transfer its recombinant DNA to other organisms.

**Table 4.1 - Containment and waste treatment methods employed for Type B operations involving more than 100 litres of microbial culture.**

GMM	Culture volume (litres)	Summary of containment measures and waste treatment methods used.
<i>Esherichia coli</i>	100	Waste autoclaved and disinfected. Plate counts used to confirm presence of GMMs in waste. No GMMs expected to survive in the waste stream.
Cultures of <i>Esherichia coli</i> and <i>Saccharomyces cerevisiae</i>	100	Waste autoclaved and disinfected. Swab sampling and plate counts used to monitor levels of GMMs in waste. No GMMs expected to survive in the waste stream.
<i>Saccharomyces cerevisiae</i>	200	Waste treated by heat sterilisation in a jacketed vessel at 60 °C for 1 h. GMM is recombination deficient and modified with a mobilisation defective vector. Waste contains live GMMs at a concentration of 10 cfu per ml.
<i>Saccharomyces cerevisiae</i>	200	Waste treated by heat sterilisation in a jacketed vessel at 60 °C for 1 h. GMM is recombination deficient and modified with a mobilisation defective vector. Waste contains live GMMs at a concentration of 10 cfu per ml.
<i>Esherichia coli</i>	500	Microbial cells lysed after growth to extract product. Culture then treated with alkali to pH 10 to kill any un-lysed cells and waste emptied into a sump containing disinfectant (<5 percent) before disposal by specialist contractor. All air exhausts filtered, and equipment steam sterilised after use. GMM is recombination deficient and modified with a mobilisation defective vector. No live GMMs discharged from plant.
<i>Esherichia coli</i>	500	Microbial cells lysed after growth to extract product. Culture then treated with alkali to pH 10 to kill any un-lysed cells and waste emptied into a sump containing disinfectant (<5 percent) before disposal by specialist contractor. All air exhausts filtered, and equipment steam sterilised after use. GMM is recombination deficient and modified with a mobilisation defective vector. No live GMMs discharged from plant.
<i>Alcaligenes xyloxydans</i>	750	Waste autoclaved and disinfected. The GMM will not survive in the environment, and is modified by a mobilisation defective vector. No GMMs expected to survive in the waste stream.

<i>Esherichia coli</i>	2300	Waste disinfected. GMM is recombination deficient and has limited survivability in the environment. No GMMs expected to survive in the waste stream.
Mammalian cells	2300	Work conducted in a closed system, with all waste treated by disinfection. No GMMs expected to survive in the waste stream.
Mammalian cells	2300	Work conducted in a closed system, with all waste treated by disinfection. Monitoring of airborne particulate matter. No GMMs expected to survive in the waste stream.
<i>Streptomyces clavuligerius</i>	3000	Waste treated at 60 °C for 1 h. Treatment process has been validated to kill all cells of this GMM in cultures up to 3000 l. No GMMs expected to survive in the waste stream.
<i>Streptomyces clavuligerius</i>	4500	Waste autoclaved and disinfected. GMM modified by mobilisation deficient vector, and is non-pathogenic.
<i>Streptomyces clavuligerius</i>	4500	Waste disinfected. GMM modified by mobilisation deficient vector, and is non-pathogenic.
<i>Agrobacterium</i> sp.	4500	All waste either heat treated or disinfected to ensure all GMMs are killed before disposal. Work conducted in a controlled area under negative pressure, and any releases will be localised. No GMMs expected to survive in the waste stream.
<i>Esherichia coli</i>	5000	Culture steam sterilised, and the filtrate from downstream processing treated with biocide. Release of 400 g of live cells to the environment per batch of culture. Equivalent to 20 kg live cells per annum. GMM not expected to survive in the environment.
Mammalian cells (myeloma NSO cells)	8000	Culture filtered (0.2 µm), heat treated or disinfected to remove live cells before disposal. All equipment steam sterilised after use. GMMs will lyse outside an isotonic environment, and have reduced viability and growth rate outside pH 5 to 8.5 and 30 °C respectively. Myeloma NSO cells have no means of survival in the environment and the viral vector is mobilisation defective.
<i>Esherichia coli</i>	15000	GMM is freeze dried and contained within glass vials for the entire operation. The GMM is also biocide sensitive and the modification is non-mobilisable.
<i>Penicillium chrysogenum</i>	200000	Culture extracted from fermenter with solvent, which kills the GMM. Effluent treatment system and effluent treatment tanks monitored for GMM, which is only rarely detected. Modification is mobilisation deficient.
<i>Penicillium chrysogenum</i>	200000	Culture extracted from fermenter with solvent, which kills the GMM. Effluent treatment system and effluent treatment tanks monitored for GMM, which is only rarely detected. Modification is mobilisation deficient.

## 5. ENVIRONMENTAL RISK ASSESSMENT OF WORK WITH GMMs IN CONTAINMENT

### INTRODUCTION

5.1 The integral part of the evaluation procedure for work with GMMs is an assessment of the risks posed by the work to the environment and human health. Activities involving GMMs in containment are only given approval to proceed by the regulatory authorities on the basis that the risks posed to the environment and human health are low to effectively zero. Under both the previous and current regulations, each risk assessment is based on a scientific evaluation of the probability of adverse consequences or 'harm' resulting from the release or escape of the GMM from containment into the environment. It should be noted that a contained use activity might not necessarily pose a high risk to human health or the environment if viable GMMs are released into the environment during the work.

5.2 Under the Environmental Protection Act (1990)<sup>13</sup> 'harm' is defined as:

*“harm to the health of humans or other living organisms or other interference with the ecological systems of which they form part and, in the case of man, includes offence caused to any of his senses or harm to his property”*

5.3 Therefore, the release or escape of GMM from containment<sup>14</sup> may result in either of the factors listed below. The probability of any of these 'harmful' consequences or hazards being realised is defined as the risk (The Royal Society, 1992).

- the infection of humans and wildlife; and

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<sup>13</sup> Section 107(6).

<sup>14</sup> If GMO is assessed to have a high potential to escape or be released from containment, then the application to use that GMO may have to be submitted under the Deliberate Release regulations rather than the Contained Use regulations. Poor containment does however not necessarily make the activity a deliberate release. There must be a reasonable intention of containment.

- the disruption of environmental processes as a consequence of any infection or disease caused.

5.4 Although the hazards of work with GMMs in containment have been defined as the infection of humans and wildlife and the disruption of environmental processes, for the purposes of risk assessment these hazards need to be subdivided to consider all aspects of the work involved. This includes all components of the GMM and the environment into which the GMM may be released or escape. Primary considerations are:

- pathogenicity of the GMM to both humans and the environment. Although the characteristics of the host (GM) microorganism are usually the most relevant, the pathogenic characteristics of all donor organisms from which any recombinant DNA has been derived, the recombinant DNA itself, and any vector sequences or vector organisms should also be assessed,
- ability of the GMM to survive and/or replicate in the environment. Characteristics of the environment, for example temperature and availability of suitable vector and host organisms are particularly relevant,
- ability of the genetic modification to be transferred to other organisms,
- effectiveness of the containment methods used, and the likelihood of GMMs being released into the environment. This includes treatment of all waste generated from the contained use activity.

5.5 The aim of this section is to assess the risks posed by the use of GMMs in containment to the environment and human health. Because the risk assessment for every contained use activity is dependent on the specific characteristics of the work involved, for example the GMM involved, each risk assessment is evaluated by the regulators on a case by case basis<sup>15</sup>.

### **PATHOGENICITY OF THE GMM**

5.6 Under the previous regulations, GMMs were classified as Group I or Group II microorganisms (Chapter 2), with Group I GMMs designated as microorganisms unlikely to

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<sup>15</sup> This is however only applicable to those activities notified to HSE, or which are reviewed during site inspections. Many Type A activities involving Group 1 GMMs may never been scrutinised.

cause disease to humans, plants or animals, or otherwise cause environmental harm. Due to their inability to cause disease, the release of Group I GMMs into the environment is extremely unlikely to result in an infection of humans or wildlife. However, although the risk of infection is negligible, there may still be a risk of the disruption of ecological processes. On the release of Group I GMMs into the environment, the cells are likely to die and lyse due to their inability to survive. Therefore instead of viable cells, the environment will be exposed to a readily assimilable supply of organic nutrients, principally carbon and nitrogen. Depending on the volume of microbial culture involved, such a release may cause a bloom in numbers of fast-growing microorganisms, and consequently cause a shift in the composition of the microbial community in terms of the number of microbial taxa and microorganisms present. Such an impact is applicable to releases of both non-GM and GM microorganisms, and is not a risk associated with releases of GMMs *per se*. The release of non-viable GMMs into the wider environment from waste streams is outside the scope of the Contained Use regulations, although other legislation may apply. The Contained Use regulations only apply to viable GMMs.

- 5.7 Sequential releases of the GMM culture to the environment are likely to have a potentially greater impact than a single release, particularly if the microbial community is affected and is unable to recover before subsequent exposures (Schuster and Schröder, 1990). However, the level of impact is dependent on the number of microorganisms released, and it is expected that any disruption caused will be short-lived and not detectable above the natural shifts in composition and size of the indigenous microbial population. Domsch *et al.* (1983) reported that microbial communities required 30 days to recover from natural perturbations such as fluctuations in temperature. Therefore the impact of compounds (including non-viable cells) whose effects on the microbial community lasting <30 days are considered as having no significant impact on the microbial community. Compounds that have an impact lasting 30 to 60 days are defined as tolerable, and those lasting >60 days as having a critical impact.
- 5.8 Because of their potential pathogenicity, Group II GMMs pose a greater risk of harm to the environment and human health. With Group II GMMs a number of factors will affect the risk of harm and the ability to disrupt ecological processes:
- the pathogenicity of the host microorganism. Where the host microorganism is pathogenic then the potential for the GMM to cause disease to wildlife and/or humans on release from containment is high. However because the more pathogenic microorganisms are used at the higher containment levels, then the probability of

release from containment is low, and the risk of any harm is consequently that much lower,

- the pathogenicity of the donor microorganism(s). This is an important component of the risk assessment if the donor microorganism is pathogenic itself, and the genes that are removed are involved in the expression of those pathogenic traits. Direct examples of this include genes responsible for toxin production, or tumour-inducing genes (oncogenes). Expression of such genes in the GMM may confer an ability on the GMM to become pathogenic, or more pathogenic. Less direct examples include those genes that the donor microorganism needs to express its pathogenic characteristics, such as the genes necessary to recognise and/or adhere to specific cells on the host, which enable subsequent entry of the microorganism into that host. Transfer of such genes into a host microorganism whose inability to cause disease is due to a failure to bind to the required cell membranes, may cause that GMM to become pathogenic, and
- the inserted gene(s) and any associated vector sequences. This is an important consideration, both in terms of the characteristics expressed by the inserted gene, such as pathogenic traits (see previous point), and also the position on the host's genome where the gene is inserted. Integration of the recombinant gene within any of the host's genes will render the host's gene unreadable and the gene will not be expressed. Where the host's gene is involved in the expression of a pathogenic trait, then the modification may lead to a potential reduction in the pathogenesis of the host microorganism. A well used example of this is the modification of Vaccinia virus (an opportunistic pathogen of humans and wildlife) by insertion of the recombinant DNA within the thymidine kinase gene of the Vaccinia, which inactivates that gene and reduces the virulence of the GM virus (Buller *et al.*, 1985).

5.9 Many of the GMMs notified for use in containment are modified to express a gene conferring resistance to a specific antibiotic. Such genes are used to allow the GMMs to be selectively isolated from other microorganisms and are therefore referred to as selectable marker genes. Other examples of selectable markers include expression of green fluorescent protein, and  $\beta$ -galactosidase. Although the expression of resistance to a specific antibiotic or group of antibiotics is extremely unlikely to confer any pathogenic characteristics, it does have the potential to disrupt ecological processes, and may affect the medical treatment of disease in humans and animals, although the latter point depends on the antibiotic involved. In consideration of the hazards of antibiotic resistance marker genes it is

important to evaluate the antibiotics to which the gene may confer resistance, and also the environment into which the GMM may be released.

- 5.10 Genes conferring resistance to antibiotics are widespread in the environment, and where the likelihood of antibiotic resistance genes being present is high, i.e. in stable environments with a rich microbial biodiversity, the release of a GMM expressing an antibiotic resistance gene will have a low risk of disrupting ecological processes in that environment as microorganisms expressing resistance to the same antibiotic will already be present.
- 5.11 However, where the gene confers resistance to an antibiotic that is used in human or veterinary medicine to treat infection by other pathogenic microorganisms, then release of that gene has a potentially higher risk of causing an infection with reduced treatment options, by conferring resistance to the antibiotic to the pathogenic microorganism. Where the pathogenic microorganism itself is modified to be resistant to an antibiotic used in its own treatment, then the available treatment options for that organism are reduced (should the GMM escape from containment and infect a suitable host). In the information reviewed for this report no GMMs were identified that had been modified to be resistant to an antibiotic used as the primary means of treating infections of the same microorganism, in either medical or veterinary applications.
- 5.12 Modification of the host microorganism with certain gene(s) may have a pathogenic effect if the host range of that microorganism, i.e. the organisms that the GMM can infect, is increased. Such modification may increase the probability of infection if the host range is altered to include organisms present in the environment into which the GMM may escape or be released. For example, if the host microorganism is a baculovirus capable of infecting species of butterfly larvae not resident in the UK, then because the required host is not present in the environment, there is a low risk that release of that baculovirus in the UK will cause disease. However, if the baculovirus is modified with a gene from another baculovirus that confers the ability to infect a wider species range of butterfly larvae, including some resident in the UK, then the risk of infection becomes much higher, if release from containment were to occur.
- 5.13 Assessment of the risks related to the pathogenicity of the GMM has not changed under Directive 98/81/EC. The only changes are that Group I GMMs have largely been re-classified as Class 1 (although some Group I GMMs have been reclassified as Class 2), and Group II GMMs re-classified into Classes 2, 3, and 4, according to the containment level employed.

## **SURVIVAL AND REPLICATION OF GMMs IN THE ENVIRONMENT**

- 5.14 In general, laboratory strains of microorganisms (including GM strains) are less hardy than their wild type counterparts. This is due to a number of factors, such as the presence of one or more disabling genetic mutations (possibly occurring as a result of specific modifications), or the absence of competition for resources from other microorganisms (a consequence of laboratory strains being maintained predominantly as pure cultures). Laboratory strains therefore exist under less stringent evolutionary pressures, and if released into the environment are unlikely to be able to compete with the resident microflora and consequently do not persist in the environment. However, the ability to survive and persist depends on the microorganism, the modification, and also the environment into which a release may occur.
- 5.15 For a GMM to persist in the environment it must be able to replicate. A number of methods are available to prevent replication of the GMM, ranging from absence of a necessary vector, host organism, or nutrient, to the deletion or inactivation of specific genes. For example, of the GM viruses notified for use in containment in the UK, 45 notifications were for GM adenovirus, with the majority modified to be replication deficient by the deletion of the early gene products E1, E2, or E4 (Chapter 4).

### **Survival of GMMs in Air**

- 5.16 Consideration of the ability of GMMs to survive in air is important, as aerosol transmission can result in the GMM being spread well beyond the local vicinity of the culture vessel. The survival of aerosolised bacteria depends on a number of both environmental and non-environmental factors:
- types of microorganism - Gram-positive<sup>16</sup> bacteria persist in aerosols much better than Gram-negative organisms, due to the possession of a relatively thicker cell wall. The spores formed by some Gram-positive bacteria, such as *Bacillus subtilis* are also particularly resistant to the conditions of desiccation and low temperature encountered in air streams. The survival of *E. coli* (a Gram-negative bacterium) in air differs considerably according to the strain tested, presumably due to differences in outer membrane or cell wall structure,

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<sup>16</sup> The Gram Stain is a method used to identify bacteria on the basis of their cell wall structure. Gram-negative bacteria possess a cell wall sandwiched between an inner and outer cell membrane, and stain red or pink under the Gram Stain. Gram-positive bacteria do not possess an outer cell membrane and stain purple.

- metabolic activity of the GMM - for *E. coli*, cells in resting phase were found to be more aerostable than cells in exponential phase. Further studies with *E. coli* and *Legionella* sp. supported this earlier work and showed an inverse relationship between metabolic activity and an ability to survive in aerosols (Hambleton *et al.*, 1983),
- composition of the liquid suspension - many compounds present in microbial cultures affect the subsequent survival of aerosolised bacteria. Poly-hydroxy compounds have been shown to provide some degree of protection against aerosol stresses, probably due to interactions between the compound and the microbial membrane, and the maintenance of membrane fluidity (Heipieper *et al.*, 1992),
- relative humidity and temperature - changes in the cellular water content represent the primary stress to airborne bacteria (Cox, 1968). Desiccation of bacterial cells is caused by the rapid evaporation of water from the water droplets containing the microorganisms. The degree and rate of evaporation is dependent on the ambient relative humidity and temperature, with the highest evaporation rates, and therefore the greatest desiccation and inactivation of microbial cells, occurring under conditions of low humidity and high temperature,
- oxygen - susceptibility of bacterial cells to oxygen usually increases with the degree of desiccation, whether it is through aerosolisation, freeze-drying or drying on surfaces, with vegetative aerosolised cells particularly vulnerable to inactivation (Cox, 1966; Benbough, 1967). Aerosolised spores are however insensitive to oxygen and survive equally in air and pure nitrogen. Hassan and Fridovich (1979) reported that the failure of the bacterial cell to induce peroxidase and catalase was associated with enhanced susceptibility of the bacteria to the lethal effect of oxygen,
- atmospheric pollutants - the reaction products of olefines (breakdown products of petroleum) and atmospheric ozone have been shown to cause rapid inactivation of *E. coli* in the atmosphere (May *et al.*, 1969; Dark and Nash, 1970; Cox, 1987),
- sunlight - light in both the visible and ultraviolet regions of the spectrum is inhibitory or toxic to aerosolised bacteria (Webb and Brown, 1976; Chang *et al.*, 1985). However, the exposure levels required for inactivation of 99.9 percent of the cells present depends on the microorganism (Chang *et al.*, 1985). For vegetative bacteria, including *E. coli*, *Salmonella typhi*, *Shigella sonnei*, *Streptococcus*

*faecalis*, and *Staphylococcus aureus* comparable doses were required for cell inactivation. However, for poliovirus type 1 and simian rotavirus SA11, *Bacillus subtilis* spores, and the cysts of the protozoan *Acanthamoeba castellanii*, inactivation to 99.9 percent required about 3 to 4 times, 9 times, and 15 times respectively, the dose required for *E. coli*. Negligible difference in UV sensitivity was also observed between surface and subsurface strains of bacteria (Arrage *et al.*, 1993), with microaerophilic, Gram-negative, and nonpigmented strains being the most susceptible to inactivation by UV,

- particle size - this affects the survival time of the microorganism and also the length of time the microorganism will remain airborne. Smaller microorganisms or clusters of microorganisms are inactivated more rapidly, but remain aloft for longer than larger cells or aggregates.

- 5.17 Very little work has been conducted directly on the survival of GMMs in air, and the survival of non-GMMs is often used to assess the survival of the host organism prior to any consideration of the effect of the modification itself. Because the mechanisms involved in the cell death of freeze-dried and air-dried bacteria are thought to be similar, freeze-drying has been used to investigate the differences in the survival of potentially airborne GM bacteria and equivalent wild type strains. Although exposure to light and air were found to increase the death rates of freeze-dried cells of both GM and non-GM *E. coli* and *Pseudomonas syringae*, the GM strains exhibited a significantly higher death rate than their non-GM parental strains (Israeli *et al.*, 1993).
- 5.18 Because of their ability to form spores, fungi and yeasts are probably more resistant to desiccation (Cox, 1987) and the effects of oxygen, pollution, and sunlight, than bacteria (except spore-forming species) and are therefore more likely to survive in aerosols.
- 5.19 Transmission of viruses in the air has been shown to occur over distances of up to 100 km (Gloster *et al.*, 1982). The primary factor affecting the survival of aerosolised viruses is the relative humidity of the air. Generally, viruses which have no structural lipids in their viral coats, such as poliovirus, pseudoviruses, picornaviruses (Benbough, 1971; and Donaldson, 1972) survive relatively poorly below 60 percent relative humidity. However viruses with structural lipids in their viral coats, such as Semliki Forest virus, vesicular stomatitis virus, Venezuelan equine encephalomyelitis virus, influenza virus, and pigeon poxvirus (Benbough 1971) were found to remain more infective at low relative humidities rather than intermediate or higher relative humidities.

## Survival of Microorganisms in Soil and Water

- 5.20 In consideration of the survival of microorganisms in soil and water it should be noted that studies on the survival of GM bacteria in soil have already been published by DETR in Research Report 7 'Risk assessment and the release of genetically modified microorganisms into the environment' (1995). In terrestrial and aquatic ecosystems, unlike the atmosphere, there is a larger and more diverse natural community of microorganisms. A primary consideration in assessing the likelihood of the GMM surviving in soil and water is therefore the ability of the GMM to compete with the indigenous microflora for nutrients and habitat. Due to the wide diversity of microorganisms present in most terrestrial and aquatic habitats, failure to compete means that the GMM will not survive. The exceptions to this are where the modification confers specific characteristics not expressed in the environment, that will give the GMM a competitive advantage over other microorganisms, for example the ability to degrade a relatively novel food source such as lignin or cellulose, or resistance to a compound present in the environment such as heavy metal, pesticide, or an antibiotic. However if the general fitness of the GMM is impaired it is unlikely to survive (Chao and Feng, 1990).
- 5.21 The ingestion of GMMs by macroorganisms such as earthworms has been suggested as a possible mechanism by which GMMs could survive in the environment for longer periods of time, and avoid competition with the indigenous microflora. However, whilst the diversity and number of microorganisms in the gastro-intestinal tract of macroorganisms such as earthworms may be low compared to levels in soil, there will be a natural microflora present in the gastro-intestinal tract, and the same problems of survival and competition for nutrients apply.
- 5.22 GMMs most likely to compete with the indigenous microflora and therefore survive are those with the most similarities to wild type strains, for example *Agrobacterium tumefaciens* (a plant pathogen) genetically modified to express green fluorescent protein as a selectable marker, but otherwise no different to the non-GM wild type strain.
- 5.23 Changes in outer membrane structure or composition are likely to have an effect on the ability of the GMM to survive, and should be considered for all GMMs, particularly GM viruses whose outer protein coat confers resistance to UV radiation. Outer membrane structures are also important as they often enable the microorganism to adhere to surfaces. Studies with *Klebsiella pneumoniae* found that cells that adhered to glass microscope slides were up to 150 times more resistant to disinfection than unattached cells (LeChevallier

*et al.*, 1988a). Other mechanisms which increased resistance to disinfection included the ability of the microorganism to form a biofilm, bacterial encapsulation, and previous growth conditions (e.g., growth medium and growth temperature). The choice of disinfectant was also shown to influence the type of resistance mechanism observed. Disinfection by free chlorine was affected by surfaces, age of the biofilm, encapsulation, and nutrient effects. Disinfection by monochloramine however, was only affected by surfaces (LeChevallier *et al.*, 1988b).

- 5.24 Survival of GMMs that require non-ambient growth conditions is extremely unlikely. For example tissue cell cultures must be maintained at body temperature (37°C) to survive, and will therefore die if they are released into the environment, although osmotic shock is likely to have an equally damaging effect on tissue culture cells. GMMs that are dependent on a specific nutrient, often an amino acid that they are unable to synthesise themselves are unlikely to survive unless that nutrient is readily available in the environment.

#### **TRANSFER OF THE GENETIC MODIFICATION TO OTHER ORGANISMS**

- 5.25 Genetic material can be transferred between microorganisms by one of the following three methods (Neidhart *et al.*, 1990):

- conjugation - this is the direct transfer of genes from one bacterial cell to another along a specialised pilus. Conjugation is mediated by conjugative plasmids, and occasionally chromosomal genes or other plasmids may be transferred,
- transduction - where DNA is transferred between bacterial cells by a bacteriophage, and
- transformation - where the bacterium takes up soluble DNA from its environment. This may include recombinant DNA lost from GMMs. However, because it is susceptible to degradation by nucleases and other enzymes present in the environment, naked DNA is only likely to survive for relatively short periods in the environment in an utilisable state. Most naked DNA is also unlikely to survive the inactivation processes used to treat the waste stream, although enough may be expected to remain intact through the waste stream for subsequent transformation in the wider environment, albeit at low frequencies.

- 5.26 Evaluation of the potential for the genetic modification to be transferred to other organisms is an important consideration of the risk assessment for contained use work. The transfer of modified genes is only applicable to GMMs that have been modified by the insertion of additional genes or modification of an existing gene, although this represents the large majority of GMMs used in containment in the UK.
- 5.27 The factors that affect the level of risk are the methods of containment employed and, particularly the treatment of any waste generated (Chapter 4). However, even if the risk of gene transfer is high, the potential for adverse consequences to be incurred may still be low, and is dependent on the gene(s) and microorganisms involved. 'Harm' to the environment and human health will only occur if dissemination of the modified genes through the environment leads to a possible disruption of ecological processes, or if alteration of the pathogenic characteristics of indigenous microorganisms occurs thereby resulting in harm to humans and wildlife.
- 5.28 Because of the selective pressures on microorganisms in the environment, dissemination of the gene throughout the natural microflora is unlikely to occur unless the gene confers a selective advantage relevant to that environment. An example would be the ability to utilise complex hydrocarbons in a soil contaminated with oil. The acquisition of such a capability would confer improved survivability of the microorganism in an oil contaminated environment, especially if the trait was not widespread.
- 5.29 Antibiotics are produced by organisms especially microorganisms, as a protection mechanism either for themselves or their immediate ecological niche. The majority of antibiotics have been developed from, or are produced by microorganisms isolated from the environment. Likewise antibiotic resistance genes have evolved in the environment to counter the selective advantage conferred by antibiotic expression, and the majority of antibiotic resistance genes have also been isolated from microorganisms taken from the environment. Therefore in an environment where the indigenous microflora possess antibiotic resistance genes, for example ones conferring resistance to the antibiotic kanamycin, the transfer of a kanamycin resistance gene from the GMM into the natural microbial community is of low risk, as it will not confer any selective advantage new to that environment. Therefore in assessing the risks of transfer of the modified gene, the environment in which the transfer may occur, i.e. the one into which the GMM may escape or be released, must be considered.

## EFFECTIVENESS OF THE CONTAINMENT METHODS USED

### Physical and Chemical Containment

- 5.30 Failure in the primary culture vessel such as the fermenter may occur either as a catastrophic failure resulting in a major spill, or as a small-scale leak causing a much more minor release. A major spill of the contents of the culture vessel would, in the majority of industrial locations be rapidly and effectively contained by the surrounding bund or drainage into appropriate kill tanks. With smaller laboratory scale work, a major spill is likely to be contained within the room housing the culture vessel. Where small-scale leaks are expected, for example with work involving high pressure systems, the use of burst valves and regular monitoring should be effective in minimising the risks to the environment.
- 5.31 Autoclave processes are routinely validated to ensure inactivation of viable microbial cells. Validation of autoclave cycles to ensure destruction of GMM would be necessary where initial risk assessment indicated the possibility of transfer of the genetic material into other organisms. Prion proteins, the causative agents of BSE and CJD, are thought to be resistant to wet (autoclave) and dry heat, ionising and UV radiation, proteases, phenols, and alkalis.
- 5.32 Liquid waste from large scale activities is commonly transferred to kill tanks for the inactivation of the GMMs. However, where the contained use work is being conducted at a site alongside other microbiology based activities, such kill tanks may contain wastes from a number of processes. The treatment process employed should therefore be suitable for a whole range of microorganisms, particularly if the genetic modification is capable of being transferred between different microbial strains. *Cryptosporidium* for example is resistant to disinfection. Where such a system exists, the monitoring techniques employed to validate the inactivation process should be capable of distinguishing between the GMM and other microorganisms present.

### *Containment of Downstream Processing Techniques*

- 5.33 Centrifugation of microbial cultures may cause significant aerosolisation, particularly the continuous desludging centrifuges favoured by industry, due to their higher throughput. However because of the more dilute nature of the processed suspension, this type of centrifuge generates more aerosols than other systems. In this case containment of the entire centrifuge and its desludging operation is desirable, if production of aerosols of a particular

GMM (or biological component of a GMM) is viewed in the risk assessment as likely to cause harm to human health and/or the environment.

- 5.34 Filtration systems used to concentrate GMMs generate less aerosol than centrifugation. However, because the concentrate produced contains a high proportion of water, this part of the filtration process may present a possible aerosol hazard during subsequent handling operations.
- 5.35 Where cell lysis is required to harvest intracellular products, physical shear techniques are usually employed. Such systems invariably generate significant aerosols containing viable cells. Containment of such systems should be considered whenever aerosolisation is deemed to present a risk to the environment and process worker. Downstream processing subsequent to cell lysis could result in low numbers of GMMs being released if inadequate sterilisation treatment was employed.

### **Biological Containment**

- 5.36 As described in Chapter 4, biological containment is based on the inability of the GMM to survive or replicate in the environment, and minimise the transfer of the genetic modification to other organisms. The effectiveness of such biological containment methods is therefore dependent on the permanence of each containment method. For GM viruses, the predominant biological containment method used is the deletion of the single gene or genes responsible for replication, rendering the GM virus replication deficient. The effectiveness of this biological containment method for GM viruses depends on whether the GM virus can reacquire the deleted gene or genes. This can only occur following the infection of a single cell with both the GM virus and a replication competent virus of the same strain. The likelihood of such an event occurring is low as the infection of a cell by a virus or replication of a virus within a cell, when that cell is already infected by a virus of a similar strain, is blocked by the host's immune system (a process known as superinfection immunity). Although low, the risk may be reduced further by improving the effectiveness of the containment system:

- the space available within a viral capsid for its DNA or RNA severely restricts the amount of genetic material a virus may possess. With GM viruses, the deletion of the replication genes and subsequent insertion of the recombinant genes usually means that the GM virus no longer has the physical space to reacquire its replication genes (although this does vary from virus to virus),

- removal of the viral replication genes, and insertion of the recombinant DNA in the viral genome at the position occupied by those replication genes, effectively blocks the virus from reacquiring its ability to replicate,
- insertion of the recombinant DNA in the viral genome within the replication gene. This inactivates the gene, rendering the GM virus replication deficient, and avoids having to delete the genes from the genome. This method however depends on the size of the inserted DNA. An important consideration in assessing the efficacy of this method, is that should the virus require a complete replication gene(s) by recombination, usually with a wild-type virus, then the resulting virus will be the same as the wild type, as it will have lost the inserted DNA due the recombination process.

5.37 In situations where transmission, survival, or replication of the GMM is dependent on other organisms, i.e. vectors or obligate hosts, the GMM is effectively contained if the required host or vector is not available. Microorganisms with narrow host ranges are therefore more likely to be biologically contained, than those with a diverse range of potential hosts.

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## 7. APPENDICES

### APPENDIX A – ALL NOTIFICATIONS FOR TYPE B ACTIVITIES

Operation number	GMM	Volume of microbial culture used (litres)
1	<i>Escherichia coli</i>	0.2
2	<i>Escherichia coli</i>	0.3
3	<i>Aeromonas salmonicilla</i>	0.5
4	<i>Saccharomyces cerevisiae</i>	0.5
5	<i>Escherichia coli</i>	1
6	<i>Escherichia coli</i> and <i>Pseudomonas</i>	2
7	<i>Saccharomyces cerevisiae</i>	2
8	<i>Salmonella enteridis</i>	5
9	<i>Escherichia coli</i>	10
10	<i>Escherichia coli</i>	20
11	<i>Escherichia coli</i>	50
12	<i>Escherichia coli</i>	100
13	<i>Escherichia coli</i> and <i>Saccharomyces cerevisiae</i>	100
14	<i>Saccharomyces cerevisiae</i>	200
15	<i>Saccharomyces cerevisiae</i>	200
16	<i>Escherichia coli</i>	500
17	<i>Escherichia coli</i>	500
18	<i>Alicialigenes xyloxydans</i>	750
19	<i>Escherichia coli</i>	2,300
20	Mammalian cells	2,300
21	Mammalian cells	2,300
22	<i>Streptomyces clavuligerus</i>	3,000
23	<i>Streptomyces clavuligerus</i>	4,500
24	<i>Streptomyces clavuligerus</i>	4,500
25	<i>Agrobacterium</i>	4,500
26	<i>Escherichia coli</i>	5,000
27	Human cytomegalovirus	8,000
28	<i>Penicillium chrysogenum</i>	200,000
29	<i>Penicillium chrysogenum</i>	200,000
30	<i>Escherichia coli</i>	15,000*

\* denotes an operation involving the irradiation of consignments of freeze dried GM *Esherichia coli* in glass vials. The GM bacteria were contained within the vials for the duration of the work.

**APPENDIX B – GROUP II GMMs NOTIFIED FOR USE IN TYPE A ACTIVITIES IN THE UK AT CONTAINMENT LEVEL 1**

Type of microorganism	GMM	Scale of the work (litres)
Bacterium	<i>Agrobacterium rhizogenes</i>	0.050
Bacterium	<i>Agrobacterium rhizogenes</i>	0.050
Bacterium	<i>Agrobacterium tumefaciens</i>	0.050
Bacterium	<i>Cladosporium fulum</i>	1.000
Bacterium	<i>Esherichia coli</i> , <i>Salmonella</i> sp.	0.050
Bacterium	<i>Esherichia coli</i> , <i>Streptomyces hygroscopicus</i>	1.000
Bacterium	<i>Erwinia carotovora</i>	1.000
Bacterium	<i>Mycobacterium grisea</i>	1.000
Bacterium	<i>Pseudomonas syringae</i>	1.000
Bacterium	<i>Salmonella typhimurium</i>	0.020
Bacterium	<i>Septoria nodorum</i>	1.000
Bacterium	<i>Septoria tritici</i>	1.000
Bacterium	<i>Streptomyces avermitilis</i>	10.000
Bacterium	<i>Yersinia enterocolitica</i> , <i>Aeromonas caviae</i>	0.010
Fungus	<i>Fusarium moniliforme</i>	1.000
Fungus	<i>Fusarium moniliforme</i>	1.000
Parasite	<i>Plasmodium falciparum</i>	1.000
Virus	<i>Adenovirus</i>	0.500
Virus	<i>Alfalfa mosaic virus</i> , <i>Tobacco mosaic virus (TMV)</i> , <i>Yellow mosaic virus</i> , <i>Cowpea chlorotic mottle virus</i>	0.500
Virus	<i>Avian leukaemia virus</i>	0.100
Virus	<i>Potato virus X (PVX)</i> , <i>Tobacco mosaic virus</i> , <i>Cucumber mosaic virus</i> , <i>Potato mop top virus</i>	1.000
Virus	<i>Sindai virus</i>	1.000
Virus	<i>Tobravirus</i>	1.000

**APPENDIX C – GROUP II GMMs NOTIFIED FOR USE IN TYPE A  
ACTIVITIES IN THE UK AT CONTAINMENT LEVEL 2**

<b>Type of microorganism</b>	<b>GMM</b>	<b>Scale of the work (litres)</b>
Bacterium	<i>Aeromonas salmonicilia</i>	0.010
Bacterium	<i>Aeromonas salmonicilia</i>	0.500
Bacterium	<i>Aeromonas salmonicilia</i>	1.000
Bacterium	<i>Agrobacterium rhizogenes</i>	1.000
Bacterium	<i>Agrobacterium tumefaciens</i>	0.100
Bacterium	<i>Bacillus cereus</i>	2.000
Bacterium	<i>Bordetella avium</i> , <i>Bordetella bronchiseptica</i> , <i>Bordetella parapertussis</i> , <i>Bordetella pertussis</i> , <i>Esherichia coli</i> , <i>Proteus mirabilis</i> , <i>Salmonella enterica</i> , <i>Shigella flexneri</i> , <i>Streptococcus suis</i> .	0.500
Bacterium	<i>Burkholderia cepacia</i> (formerly <i>Pseudomonas cepacia</i> )	0.100
Bacterium	<i>Burkholderia cepacia</i>	0.100
Bacterium	<i>Campylobacter</i> sp.	0.500
Bacterium	<i>Campylobacter</i> sp.	0.500
Bacterium	<i>Campylobacter</i> sp.	1.000
Bacterium	<i>Campylobacter fetus</i>	0.100
Bacterium	<i>Campylobacter</i> sp., <i>Neisseria meningitidis</i>	0.011
Bacterium	<i>Cichorium intybus</i>	1.000
Bacterium	species of <i>Citrobacter</i> , <i>Enterobacter</i> , <i>Klebsiella</i> , <i>Serratia</i> , <i>Pseudomonas</i> , <i>Aeromonas</i> , <i>E.coli</i> , <i>Acinetobacter</i> , <i>Proteus</i> , <i>Neisseria</i>	1.000
Bacterium	<i>Citrobacter freundli</i>	0.250
Bacterium	<i>Clostridium botulinium</i>	5.000
Bacterium	<i>Clostridium difficile</i>	0.050
Bacterium	<i>Clostridium septicum</i>	0.100
Bacterium	<i>Esherichia coli</i>	0.010
Bacterium	<i>Esherichia coli</i>	0.100
Bacterium	<i>Esherichia coli</i>	0.100
Bacterium	<i>Esherichia coli</i>	0.200
Bacterium	<i>Esherichia coli</i>	0.250
Bacterium	<i>Esherichia coli</i>	0.400
Bacterium	<i>Esherichia coli</i>	0.500
Bacterium	<i>Esherichia coli</i>	0.500
Bacterium	<i>Esherichia coli</i>	0.500
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000

Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	2.000
Bacterium	<i>Esherichia coli</i>	3.000
Bacterium	<i>Esherichia coli</i>	4.000
Bacterium	<i>Esherichia coli</i>	5.000
Bacterium	<i>Esherichia coli</i>	5.000
Bacterium	<i>Esherichia coli</i>	5.000
Bacterium	<i>Esherichia coli</i>	10.000
Bacterium	<i>Esherichia coli</i>	10.000
Bacterium	<i>Esherichia coli</i>	30.000
Bacterium	<i>Esherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa</i>	1.000
Bacterium	<i>enterotoxigenic E.coli</i>	0.250
Bacterium	<i>Erwinia amylovora</i>	0.200
Bacterium	<i>Esherichia coli</i>	10.000
Bacterium	<i>Esherichia coli, Pseudomonas syringae</i>	1.000
Bacterium	<i>Haemophilus influenzae</i>	0.010
Bacterium	<i>Haemophilus influenzae, Neisseria gonorrhoea</i>	0.010
Bacterium	<i>Helicobacter pylori</i>	0.050
Bacterium	<i>Helicobacter pylori</i>	0.200
Bacterium	<i>Helicobacter sp., Campylobacter sp., Salmonella enterica</i>	1.000
Bacterium	species of <i>Lactobacillus, Lactococcus</i>	1.000
Bacterium	<i>Listeria monocytogenes</i>	0.010
Bacterium	<i>Listeria monocytogenes</i>	0.500
Bacterium	<i>Listeria monocytogenes</i>	2.000
Bacterium	<i>Listeria monocytogenes</i>	10.000
Bacterium	<i>Mycobacterium sp.</i>	0.020
Bacterium	<i>Mycobacterium bovis</i>	0.050
Bacterium	<i>Mycobacterium microli</i>	0.500
Bacterium	<i>Neisseria gonorrhoeae</i>	10.000
Bacterium	<i>Neisseria gonorrhoeae</i>	0.200
Bacterium	<i>Neisseria meningitidis</i>	0.010
Bacterium	<i>Neisseria meningitidis</i>	1.000

Bacterium	<i>Neisseria meningitidis, Haemophilus influenzae</i>	0.100
Bacterium	<i>Neisseria meningitidis, Haemophilus influenzae</i>	10.000
Bacterium	<i>Porphyromonas gingivalis, Streptococcus pneumoniae, Streptococcus intermedius</i>	1.000
Bacterium	<i>Pseudomonas aeruginosa</i>	1.000
Bacterium	<i>Pseudomonas aeruginosa</i>	4.000
Bacterium	<i>Staphylococcus aureus</i>	1.000
Bacterium	<i>Salmonella</i> sp.	0.500
Bacterium	<i>Salmonella</i> sp.	1.000
Bacterium	<i>Salmonella</i> sp.	1.000
Bacterium	<i>Salmonella</i> sp.	0.250
Bacterium	<i>Salmonella enterica</i>	1.000
Bacterium	<i>Salmonella enteritidis</i>	0.010
Bacterium	<i>Salmonella enteritidis</i>	1.000
Bacterium	<i>Salmonella typhi, Salmonella enteritidis</i>	2.000
Bacterium	<i>Salmonella typhimurium</i>	0.500
Bacterium	<i>Salmonella typhimurium</i>	1.000
Bacterium	<i>Salmonella typhimurium, Plasmodium falciparum</i> (parasite), <i>Clostridium tetani</i>	0.200
Bacterium	<i>Septoria nodorum</i>	1.000
Bacterium	<i>Septoria nodorum</i>	1.000
Bacterium	<i>Septoria nodorum</i>	5.000
Bacterium	<i>Septoria nodorum, Septoria tritici</i>	0.100
Bacterium	<i>Septoria nodorum, Septoria tritici</i>	1.000
Bacterium	<i>Serratia marcescens</i>	1.000
Bacterium	<i>Shigella flexneri</i>	1.000
Bacterium	<i>Shigella flexneri</i>	1.000
Bacterium	<i>Staphylococcus aureus</i>	1.000
Bacterium	<i>Staphylococcus aureus</i>	5.000
Bacterium	<i>Staphylococcus aureus</i>	5.000
Bacterium	<i>Staphylococcus aureus, Staphylococcus epidermidis</i>	1.000
Bacterium	<i>Staphylococcus aureus, Staphylococcus epidermidis</i>	1.000
Bacterium	<i>Streptococcus cholerae-suis, Streptococcus dublin</i>	0.400
Bacterium	<i>Streptococcus pneumoniae</i>	0.020
Bacterium	<i>Streptococcus pneumoniae</i>	0.100
Bacterium	<i>Streptococcus pneumoniae</i>	0.100
Bacterium	<i>Streptococcus pneumoniae</i>	1.000
Bacterium	<i>Streptococcus pneumoniae, Neisseria meningitidis</i>	0.050
Bacterium	<i>Streptococcus pyogenes</i>	0.050
Bacterium	<i>Streptococcus pyogenes, Staphylococcus aureus</i>	0.100
Bacterium	<i>Streptococcus suis</i>	0.100
Bacterium	<i>Streptococcus uberis, Streptococcus dysgalactiae</i>	1.000
Bacterium	<i>Yersinia</i> sp.	1.000
Bacterium	<i>Yersinia pseudotuberculosis</i>	1.000



Virus	<i>Adenovirus</i>	1.000
Virus	<i>Adenovirus</i>	1.500
Virus	<i>Adenovirus</i>	2.000
Virus	<i>Adenovirus</i>	2.000
Virus	<i>Adenovirus</i>	2.000
Virus	<i>Adenovirus</i>	2.000
Virus	<i>Adenovirus</i>	2.000
Virus	<i>Adenovirus</i>	5.000
Virus	<i>Adenovirus</i>	5.000
Virus	<i>Adenovirus</i>	5.000
Virus	<i>Adenovirus</i>	10.000
Virus	<i>Adenovirus</i>	10.000
Virus	<i>Adenovirus, Canary pox virus, Vaccinia virus</i>	1.000
Virus	<i>Avian leukaemia virus</i>	1.000
Virus	<i>baculovirus</i>	0.010
Virus	<i>baculovirus</i>	2.000
Virus	<i>Bovine herpes virus</i>	1.000
Virus	<i>Bovine herpes virus</i>	1.000
Virus	<i>Bovine viral diarrhoea virus</i>	1.000
Virus	<i>Canary pox virus, Vaccinia virus, Adenovirus</i>	1.000
Virus	<i>encephalomyelitis virus</i>	0.400
Virus	<i>Eppstein Barr virus</i>	0.500
Virus	<i>Eppstein Barr virus</i>	2.000
Virus	<i>Eppstein Barr virus</i>	10.000
Virus	<i>Eppstein Barr virus, Papillomavirus, Simian virus 40, Human adenovirus</i>	5.000
Virus	<i>Feline herpes virus</i>	1.000
Virus	<i>Hepatitis B virus</i>	0.100
Virus	<i>Hepatitis C virus</i>	10.000
Virus	<i>Herpes simplex virus (HSV)</i>	0.001
Virus	<i>Herpes simplex virus</i>	0.010
Virus	<i>Herpes simplex virus</i>	0.010
Virus	<i>Herpes simplex virus</i>	0.010
Virus	<i>Herpes simplex virus</i>	0.100
Virus	<i>Herpes simplex virus</i>	1.000
Virus	<i>Herpes simplex virus 1</i>	1.000
Virus	<i>Herpes simplex virus 1</i>	1.000
Virus	<i>Herpes simplex virus 1 + 11</i>	1.000
Virus	<i>Turkey herpes virus</i>	1.750
Virus	<i>Herpes simplex virus 1</i>	0.250
Virus	<i>Human cytomegalovirus</i>	0.100
Virus	<i>Human cytomegalovirus</i>	1.000
Virus	<i>Human papillomavirus</i>	9.000
Virus	<i>Human rhinovirus</i>	0.500



Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	5.000
Virus	<i>Retrovirus</i>	5.000
Virus	<i>Retrovirus</i>	5.000
Virus	<i>Retrovirus</i>	10.000
Virus	<i>Retrovirus</i>	10.000
Virus	<i>Retrovirus</i>	20.000
Virus	<i>Avian leukaemia virus</i>	1.000
Virus	<i>Moloney murine leukaemia virus (MMLV)</i>	0.075
Virus	<i>Moloney murine leukaemia virus</i>	1.000
Virus	<i>Moloney murine leukaemia virus</i>	1.000
Virus	<i>Moloney murine leukaemia virus</i>	1.000
Virus	<i>Rubella virus</i>	1.000
Virus	<i>Semliki forest virus (SFV)</i>	1.000
Virus	<i>Semliki forest virus</i>	1.000
Virus	<i>Semliki forest virus</i>	0.100
Virus	<i>Semliki forest virus</i>	0.030
Virus	<i>Semliki forest virus</i>	0.030
Virus	<i>Semliki forest virus</i>	1.000
Virus	<i>Semliki forest virus</i>	1.000
Virus	<i>Semliki forest virus</i>	1.000
Virus	<i>Semliki forest virus</i>	5.000
Virus	<i>Sindbis virus expressing Hepatitis C virus</i>	0.100
Virus	<i>Sindbis virus</i>	0.100
Virus	<i>Sindbis virus</i>	0.100
Virus	<i>Vaccinia virus</i>	0.010
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	0.060
Virus	<i>Vaccinia virus</i>	0.075
Virus	<i>Vaccinia virus</i>	0.100
Virus	<i>Vaccinia virus</i>	0.200
Virus	<i>Vaccinia virus</i>	0.500
Virus	<i>Vaccinia virus</i>	0.500



Yeast	<i>Candida albicans</i>	1.000
Yeast	<i>Candida albicans</i>	2.000
Yeast	<i>Candida albicans, Candida glabrata</i>	1.000
Yeast	<i>Candida albicans</i> and <i>Esherichia coli</i> (bacterium)	0.050

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**APPENDIX D – GROUP II GMMs NOTIFIED FOR USE IN TYPE A  
ACTIVITIES IN THE UK AT CONTAINMENT LEVEL 3**

<b>Type of microorganism</b>	<b>GMM</b>	<b>Scale of the work (litres)</b>
Bacterium	<i>Aeromonas victoria</i>	0.100
Bacterium	<i>Bacillus anthracis</i>	0.050
Bacterium	<i>Bacillus pseudomallei</i>	1.000
Bacterium	<i>Brucellae melitensis, Brucellae suis</i>	0.010
Bacterium	<i>Citrobacter rodentium</i>	0.010
Bacterium	<i>Clostridium perfringens</i>	5.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	1.000
Bacterium	<i>Esherichia coli</i>	2.000
Bacterium	<i>Esherichia coli</i> (strain O157)	0.500
Bacterium	<i>Esherichia coli</i> (enterhaemorrhagic strain)	0.500
Bacterium	<i>Esherichia coli</i> (strain O157)	1.000
Bacterium	<i>Francisella tularensis</i>	0.200
Bacterium	<i>Helicobacter pylori</i>	0.010
Bacterium	<i>Mycobacterium</i> sp.	1.000
Bacterium	<i>Mycobacterium</i> sp.	1.000
Bacterium	<i>Mycobacterium</i> sp.	1.000
Bacterium	<i>Mycobacterium avium</i>	0.100
Bacterium	<i>Mycobacterium avium</i>	1.000
Bacterium	<i>Mycobacterium</i> sp. Knockout mutants	0.040
Bacterium	<i>Mycobacterium tuberculosis</i>	0.001
Bacterium	<i>Mycobacterium tuberculosis</i>	0.020
Bacterium	<i>Mycobacterium tuberculosis</i>	0.020
Bacterium	<i>Mycobacterium tuberculosis</i>	0.100
Bacterium	<i>Mycobacterium tuberculosis</i>	1.000
Bacterium	<i>Mycobacterium tuberculosis</i>	1.000
Bacterium	<i>Mycobacterium tuberculosis</i>	1.000
Bacterium	<i>Mycobacterium tuberculosis</i>	1.000
Bacterium	<i>Mycobacterium tuberculosis, Mycobacterium bovis</i>	1.000
Bacterium	<i>Pasteurella haemolytica</i>	1.000
Bacterium	<i>Pseudomonas pseudomallei</i>	0.200
Bacterium	<i>Salmonella typhi</i>	0.250
Bacterium	<i>Salmonella typhimurium</i>	0.250
Bacterium	<i>Salmonella typhimurium</i>	0.500
Bacterium	<i>Salmonella typhimurium</i>	10.000
Bacterium	<i>Yersinia pestis</i>	0.100
Bacterium	species of <i>Yersinia, Salmonella</i>	1.000
Parasite	<i>Leishmania major</i>	1.000
Parasite	<i>Plasmodium falciparum</i>	0.100

Parasite	<i>Trypanosoma brucei</i>	1.000
Parasite	<i>Trypanosoma cruzi</i> , <i>Leishmania</i> sp.	0.200
Parasite	<i>Trypanosoma cruzi</i> , <i>Leishmania</i> sp.	0.300
Parasite	<i>Trypanosoma</i> sp., <i>Leishmania</i> sp.	1.000
Prion	prion protein	1.000
Prion	prion protein	1.000
Prion	prion protein	1.000
Prion	prion protein	1.000
Prion	prion protein	1.000
Virus	<i>Adenovirus</i>	0.010
Virus	<i>Adenovirus</i>	0.010
Virus	<i>Cowpea mosaic virus</i>	1.000
Virus	<i>Hepatitis B virus</i>	2.000
Virus	<i>Hepatitis B virus</i>	0.100
Virus	<i>Hepatitis B virus</i>	0.250
Virus	<i>Hepatitis B virus</i>	1.000
Virus	<i>Hepatitis B virus</i>	1.000
Virus	<i>Hepatitis B virus</i>	1.000
Virus	<i>Hepatitis B virus</i>	2.000
Virus	<i>Hepatitis B virus</i>	2.000
Virus	<i>Hepatitis B virus</i>	10.000
Virus	<i>Hepatitis C virus</i>	0.100
Virus	<i>Hepatitis C virus</i>	10.000
Virus	<i>Herpes simplex virus</i>	0.100
Virus	<i>Human immunodeficiency virus (HIV)</i>	0.050
Virus	<i>Human immunodeficiency virus</i>	0.100
Virus	<i>Human immunodeficiency virus</i>	0.100
Virus	<i>Human immunodeficiency virus</i>	0.500
Virus	<i>Human immunodeficiency virus</i>	1.000
Virus	<i>Human immunodeficiency virus</i>	1.000
Virus	<i>Human immunodeficiency virus</i>	5.000
Virus	<i>Human immunodeficiency virus</i>	5.000
Virus	<i>Human immunodeficiency virus</i>	0.500
Virus	<i>Human immunodeficiency virus</i>	0.500
Virus	<i>Human immunodeficiency virus</i>	10.000
Virus	<i>Human immunodeficiency virus (attenuated strain)</i>	0.015
Virus	<i>Influenza virus</i>	1.000
Virus	<i>Jaagsiekte sheep retrovirus</i>	0.005
Virus	<i>Picornavirus</i>	0.250
Virus	<i>Rabies virus</i>	0.050
Virus	<i>Retrovirus</i>	0.500
Virus	<i>Retrovirus</i>	1.000
Virus	<i>Retrovirus</i>	10.000
Virus	<i>Simian immunodeficiency virus (SIV)</i>	1.000

Virus	<i>Simian immunodeficiency virus</i>	1.000
Virus	<i>Simian immunodeficiency virus, Human immunodeficiency virus</i>	1.000
Virus	<i>Vaccinia virus</i>	0.050
Virus	<i>Vaccinia virus</i>	1.000
Virus	<i>Varicella-zoster virus</i>	0.030

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