



**SLIPPING
THROUGH THE
REGULATORY NET:
'Naked' and
'free' nucleic acids**

by Mae-Wan Ho, Angela Ryan,
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*is published by
Third World Network
131 Jalan Macalister
10400 Penang, Malaysia.*

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Printed by Jutaprint
2 Solok Sungei Pinang 3, Sg. Pinang
11600 Penang, Malaysia.

ISBN: 983-9747-74-6

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Abstract

Biotechnological processes are creating an increasing variety of naked/free nucleic acids that are released unregulated into the environment. They range from oligonucleotides to artificial constructs thousands and millions of base-pairs in length, often containing heterogeneous combinations of genes from pathogenic bacteria, viruses and other genetic parasites belonging to every kingdom of living organisms. Most have never existed, or if they have, not in such large amounts. They are, by definition, xenobiotics – substances foreign to nature — with the potential to cause harm.

Nucleic acids are now known to persist in all environments, including the digestive tract. Transformation by the uptake of DNA is a significant route of horizontal gene transfer, and there is overwhelming evidence that horizontal gene transfer and recombination have been responsible for the recent resurgence of drug and antibiotic resistant infectious diseases.

Research findings in gene therapy and vaccine development show that naked/free nucleic acids constructs are readily taken up by the cells of all species including human beings. These nucleic acid constructs can become integrated into the cell's genome. Integration may result in harmful biological effects, including cancers.

Unfortunately, regulation has lagged far behind scientific evidence. The most serious is with regard to 'contained use'. Industrial contained users release large volumes of untreated transgenic microorganisms, which they judge to be 'safe', directly into the environment. Moreover, transgenic wastes consisting of killed microorganisms but *containing large amounts of transgenic nucleic acids are being recycled as food, feed and fertilizer, or disposed of in landfills*. These practices are precisely those that can enhance horizontal transfer and recombination of transgenic nucleic acids, *and should no longer be permitted*.

Chapter 1

'Naked' and 'free' nucleic acids

'Naked' nucleic acids are DNA/RNA produced in the laboratory for use in, or as the result of, genetic engineering.

'Free' nucleic acids refer to these laboratory-produced nucleic acids transfected into cells or organisms, whether incorporated as transgenic DNA or not, and subsequently released into the environment by secretion, excretion, waste disposal, death, industrial processing, or carried by liquid streams, or in airborne aerosol, dust and pollen.

Genetic engineering is a valuable research tool, which may have beneficial applications. But the continued research and development of the technology must go hand in hand with appropriate regulation in order that the benefits should not be outweighed by the harm it may cause.

The naked nucleic acids produced in the laboratory (see Box 1) range from oligonucleotides of less than 20 nucleotides to artificial chromosomes millions of base-pairs in length. The constructs typically contain antibiotic resistance marker genes plus a heterogeneous combination of genes from pathogenic bacteria, viruses and other genetic parasites belonging to every kingdom of living organisms on earth (1).

Most have never existed in nature, or if they have, not in such large quantities. They are, by definition, xenobiotics – substances for-

eign to nature — with the potential to cause harm.

Many novel constructs are incorporated into transgenic microorganisms, plant and animal cell cultures for commercial productions, and into crops, livestock, fish and other aquatic organisms for food, animal feed, and other purposes.

Box 1: Naked nucleic acids in genetic engineering biotechnology

DNA-based

Viral genomes, e.g., cauliflower mosaic virus, cytomegalovirus, vaccinia, baculovirus, adenovirus, SV40, many bacteriophages

cDNA of RNA viral genomes, e.g., retroviruses, SIV, HIV, Rous Sarcoma virus, mouse Moloney virus, Ebola virus

Plasmids, e.g., Ti of *Agrobacterium*, many plasmids from *E. coli* and yeast, often carrying antibiotic resistance genes

Transposons, e.g., many broad host-range transposons from *E. coli* with antibiotic resistance genes, some from *Drosophila*, such as mariner are found in all eukaryotic kingdoms

Artificial vectors made by recombining viral genomes, plasmids and transposons, carrying antibiotic resistance genes, and used for gene amplification, DNA sequencing, transfection, gene therapy, etc., many are shuttle-vectors designed for replication in more than one species, 'pan-tropic' vectors cross many species barriers

Naked DNA vaccines, both plasmid-based and viral vector-based

Artificial chromosomes, yeast (YAC), plasmid (PAC) and mammalian (MAC) (2)

Artificial constructs, transgene cassettes, often including antibiotic resistance gene cassettes, with promoters from viruses, or synthetic super-promoters and terminators

PCR amplified sequences

Oligodeoxynucleotides (antisense), hairpin-forming oligonucleotides used in gene therapy (3)

RNA-based

Antisense RNA used in gene therapy

Ribozymes (RNA enzymes that cut RNA) used in gene therapy (4)

Self-replicating RNA (linked to RNA-dependent RNA polymerase) used in gene therapy (5)

RNA vaccines

RNA viral vectors eg, alphaviruses, Venezuelan equine encephalitis virus

RNA-DNA hybrid

RNA-DNA hybrid sequence of 25 nucleotides used to introduce precise base-sequence changes in a target gene (6)

These constructs are therefore greatly amplified, and at the same time, introduced into foreign genomes where recombination with host genes and the genomes of the host's viral and bacterial pathogens may readily occur.

Transgenic wastes containing large amounts of free or potentially free transgenic DNA are being released into the environment, including those from microorganisms and cell cultures in 'contained use' (see Box 2).

With the possible exception of viral genomes, viral vectors and viroids (infectious naked RNA agents), neither naked nor free nucleic acids fall within the scope of the current EC Directive (7) on contained use and deliberate release.

They are also being excluded from the Cartagena Biosafety Protocol, an international agreement to regulate the international movement, handling and use of GMOs, negotiated in Montreal in January 2000, and signed by more than 60 nations to date.

Worse still, current European regulation allows users to release directly into the environment certain live transgenic microorgan-

Box 2: Free nucleic acids resulting from genetic engineering biotechnology

Transfected, unincorporated nucleic acids/constructs due to gene therapy, vaccination, and transgenesis, released into the environment by secretion, excretion, waste carcass disposal, cell death, etc.

Transgenic DNA released from live or dead cells in the following:

- Transgenic wastes from genetically engineered microorganisms in contained use
- Transgenic wastes from cell cultures in contained use
- Transgenic wastes from genetically engineered crops
- Transgenic wastes from genetically engineered fish and other aquatic organisms
- Transgenic wastes from genetically engineered farm animals
- Unprocessed transgenic food and animal feed
- Processed transgenic food for human use and animal feed
- Processed transgenic textiles such as cotton
- Transgenic dust from food processing
- Transgenic pollen

isms considered nonpathogenic or otherwise safe in liquid waste, although there is no agreement across European countries as to which bacteria are pathogens (8) (see Appendix).

Meanwhile, *all killed microorganisms and cells containing transgenic DNA are disposed of as solid waste, and are either recycled as food, feed and fertilizer, or disposed of in landfill and incineration, according to a recent paper in the 'safe biotechnology' series produced by the European Federation of Biotechnology (9).*

The paper from industry considered the DNA content of biotechnological waste, and reaffirmed the adequacy of existing practice, except "in cases where recombinant DNA is specifically constructed

to transform higher cells, such as gene vaccines or genetic-pill applications”, where “it will be necessary to inactivate waste by validated procedures rendering DNA nonfunctional by either reducing DNA fragment size below functional entities or altering the chemical composition and structure of the DNA.”

At present, the ‘validated procedure’ for treating viral genomes and viral vectors appears to involve no more than autoclaving, but whether this sufficiently degrades nucleic acids is not known.

Historically, the lack of regulation of naked/free nucleic acids is due largely to the assumption, *now proven to be erroneous*, that they would be rapidly broken down in the environment and in the digestive tract of animals (see the next chapter). Another assumption is that as DNA is present in all organisms, it is not a hazardous chemical, and hence there is no need to regulate it as such (10).

Chapter 2

DNA persists in all environments

Naked/free DNA is now known to persist in all natural environments. High concentrations of DNA are found in the soil, in marine and freshwater sediments and in the air-water interface, where it retains the ability to transform microorganisms (11, 12).

DNA also persists in the digestive tract (13), where sizable fragments may be taken up and incorporated by resident microbes and cells of the mammalian host.

A genetically engineered plasmid was found to have a 6% to 25% survival after 60 minutes of exposure to human saliva (14). The partially degraded DNA was capable of transforming *Streptococcus gordonii*, one of the bacteria that normally live in the human mouth and pharynx.

It has long been assumed that DNA cannot be taken up through intact skin, surface wounds, or the intestinal tract, or that it would be rapidly destroyed if taken up. Those assumptions have been overtaken by empirical findings. The ability of naked DNA to penetrate intact skin has been known at least since 1990.

Cancer researchers found that within weeks of applying the cloned DNA of a human oncogene to the skin on the back of mice, tumours developed in endothelial cells lining the blood vessels and lymph nodes (15).

Viral DNA fed to mice reached white blood cells, spleen and liver cells via the intestinal wall, to become incorporated into the mouse cell genome (16). When fed to pregnant mice, the viral DNA ended up in cells of the fetuses and the newborn, indicating that it had gone through the placenta (17).

Recent research in gene therapy shows how readily naked nucleic acids can enter practically every type of human cells and cells of model mammals.

Naked nucleic acids can be successfully delivered, either alone or in complex with liposomes and other carriers, in aerosols via the respiratory tract (18), by topical application to the eye (19), to the inner ear (20), to hair follicles by rubbing on the skin (21), by direct injection into muscle (22), through the skin (23), and by mouth, where the nucleic acid is taken up by cells lining the gut (24).

Naked DNA can even be taken up by sperms of marine organisms and mammals, and transgenic animals created (25). Researchers have also found unintended integration as, for example, of a plasmid-based naked DNA malarial vaccine injected into mouse muscle (26).

Chapter 3

The potential hazards of naked nucleic acids

The potential hazards of naked nucleic acids are summarised in Box 3.

Box 3: Hazards of naked nucleic acids

- Acute toxic shock from viral vectors
- Immunological reaction from viral vectors
- Autoimmune reactions from double-stranded DNA and RNA
- Non-target interference with gene function from antisense DNA, RNA and ribozymes
- Generation of virulent recombinant viruses
- Insertion mutagenesis
- Insertion oncogenesis
- Genetic contamination of germ cells

Naked viral genomes often have a wider host range than the intact virus. Human T-cell leukaemia viral genomes formed complete viruses (27), and naked genomes from the human polyomavirus BK (BKV) gave a full-blown infection when injected into rabbits, despite the fact that neither intact virus is infectious for rabbits (28). This is particularly relevant to the virus-based gene therapy vectors and naked DNA vaccines under development (29).

It is already known that even small modifications to viral genomes, such as insertions, deletions and single nucleotide substitutions, can have unexpected effects on virulence and host range (30). Re-

searchers in Canberra, Australia, accidentally created a deadly transgenic virus that killed everyone of its victims simply by inserting the gene for interleukin-4 into the relatively harmless mousepox virus (31).

Gene-therapy vectors and naked DNA vaccines have caused acute toxic shock reactions (32) and severe immune reactions (33). Between 1998 and 1999, scientists in US drug companies failed to notify the regulatory authorities of six deaths and more than 650 adverse events resulting from clinical trials of gene therapy, the precise causes of which are yet to be determined (34).

Naked DNA can also trigger autoimmune reactions. Any fragment of double-stranded DNA or RNA (down to 25 base-pairs) introduced into cells can induce those reactions, which are linked to rheumatoid arthritis, insulin-dependent diabetes and Graves disease of the thyroid (35). Double-stranded RNA mainly appears during viral infection, and is recognized as a trigger for activating genes that produce interferons (36).

Many 'spontaneous' mutations result from insertions of transposons and other invasive elements. Insertion mutagenesis is associated with a range of cancers of the lung (37), breast (38), colon (39) and liver (40). Finally, unintended modification of germ-cells can result from gene therapy and vaccinations (25).

Naked RNA genomes are also infectious. Viroids (41) and viroid-like satellite RNAs (42) from plants and the hepatitis δ virus (43) in humans are the smallest known agents of infectious disease. They are single-stranded RNA molecules that lack both protein coat and detectable mRNA activity.

Antisense RNA, like antisense DNA, will be expected to have biological effects either in blocking the function of homologous genes or genes with homologous domains.

RNA may also be reverse-transcribed into complementary DNA (cDNA) by reverse transcriptase, which is present in all higher organisms (44) and in some bacteria (45); the cDNA may then become incorporated into the cell's genome.

The direct uptake and incorporation of genetic material from unrelated species is referred to as *horizontal gene transfer*, as distinct from the usual *vertical* gene transfer from parent to offspring in reproduction.

Chapter 4

The horizontal transfer of transgenic DNA

Many geneticists accept that naked nucleic acids are transferred horizontally, especially to microorganisms, but dispute the transfer of transgenic DNA, which they regard to be no different from host-cell DNA. But there is already evidence of horizontal transfer of plant transgenic DNA; and there is no reason to believe that the same may not apply to transgenic DNA in animals – fish, laboratory mice and rats, livestock and insects – as all transgenic constructs are similar.

Secondary horizontal transfer of transgenic DNA to soil bacteria and fungi has been demonstrated in the laboratory. In the case of fungi, the transfer was obtained simply by co-cultivation (46). Successful transfers of a kanamycin resistance marker gene to the soil bacterium *Acinetobacter* were obtained using DNA extracted from homogenized plant leaf from a wide range of transgenic plants: potato, tobacco, sugar beet, oil-seed rape and tomato (47). About 2,500 copies of the kanamycin resistance genes (from the same number of plant cells) are sufficient to successfully transform one bacterium, despite the six million-fold excess of plant DNA present.

There are claims that horizontal gene transfer has close to zero probability of occurring in nature. But this is based more on assumptions than experimental investigations under realistic conditions. Gebbard and Smalla (48) have found horizontal transfer of kanamycin resistance genes from transgenic DNA to *Acinetobacter*, and

positive results were obtained using just 100µl of plant-leaf homogenate.

Horizontal transfer of transgenic DNA has actually been found in the field. Transgenic DNA was detected in the soil at least two years after the transgenic sugarbeet crop was harvested. Specific PCR probes show that different parts of the transgenic DNA may have been taken up by soil bacteria, although the bacterial strains themselves could not be isolated (49); which is not surprising as less than 1% of soil bacteria can be isolated.

There are also reasons to suspect that transgenic DNA may be more likely to transfer horizontally than the organism's own genes (see Box 4). Natural genetic material, say, in non-GM food, is generally broken down to provide energy and building-blocks for growth and repair. And in the rare event that the foreign genetic material gets into a cell's genome, other mechanisms can still put the foreign genes out of action or eliminate it. These are all part of the biological barrier that keeps species distinct, so gene exchange across species is held in check.

Almost by definition, genetic engineering involves designing artificial constructs to invade genomes and to overcome natural species barriers. Because of their highly mixed origins, transgenic constructs tend to be unstable as well as invasive, and may therefore be more likely to spread by horizontal gene transfer.

Box 4: Reasons to suspect that transgenic DNA may be more likely to spread horizontally than non-transgenic DNA

1. Artificial constructs and vectors are designed to be invasive to foreign genomes and overcome species barriers.
2. Artificial gene constructs tend to be structurally unstable (50, 51), and hence more prone to recombine and transfer horizontally.
3. The mechanisms enabling foreign genes to insert into the genome also enable them to jump out again, to reinsert at another site, or to another genome.
4. The integration sites of most commonly used artificial vectors for transferring genes are 'recombination hotspots', and so have an increased propensity to transfer horizontally.
5. Viral promoters, such as that from the cauliflower mosaic virus, widely used to make transgenes over-express, contain recombination hotspots (52), and will therefore further enhance horizontal gene transfer.
6. The metabolic stress on the host organism due to the continuous over-expression of transgenes may also contribute to the instability of the insert (53).
7. The foreign gene constructs, and the vectors into which they are spliced, are typically mosaics of DNA sequences from numerous species and their genetic parasites; that means they will have sequence homologies with the genetic material of many species and their genetic parasites, thus facilitating wide-ranging horizontal gene transfer and recombination.

Chapter 5

The hazards of horizontal gene transfer

Among the scientific advice given by the UK Ministry of Agriculture, Fisheries and Food (MAFF) to the US Food and Drug Administration (FDA) (54) at the end of 1998 are the following warnings:

1. Transgenic DNA can spread to farm workers and food processors via dust and pollen.
2. Antibiotic resistance marker genes may spread to bacteria in the mouth, as the mouth contains bacteria that readily take up and incorporate foreign DNA. Similar transformable bacteria are present in the respiratory tracts.
3. Antibiotic resistance marker genes may spread to bacteria in the environment, which then serves as a reservoir for antibiotic resistance genes.
4. DNA is not readily degraded during food processing nor in the silage (55), hence transgenic DNA in animal feed can spread to bacteria in animals.
5. Foreign DNA can be delivered into mammalian cells by bacteria that can enter the cells (56).
6. The ampicillin resistance gene in the transgenic maize undergoing 'farm-scale' field-trials in the UK and elsewhere is very mutable, and may compromise treatment for meningitis and other bacterial infections, should the gene be transferred horizontally to the bacteria.

The potential hazards of horizontal gene transfer are unlike those of any other process (see Box 5).

Box 5: Potential hazards from horizontal gene transfer of naked/free nucleic acids

- Generation of new viruses that cause disease
- Generation of new bacteria that cause diseases
- Spreading drug and antibiotic resistance genes among the viral and bacterial pathogens, making infections untreatable
- Random insertion into genomes of cells resulting in harmful effects including cancer
- Reactivation of dormant viruses, present in most, if not all, cells and genomes, which may cause diseases
- Multiplication of ecological impacts due to all the above

There is already overwhelming evidence that horizontal gene transfer has been responsible for creating new viral and bacterial pathogens and for spreading drug and antibiotic resistance among the pathogens (1).

Thus, it can be expected that a technology that enhances horizontal gene transfer, both by design and otherwise, will also contribute to creating new pathogens and to spreading drug and antibiotic resistance. We have already referred to the evidence for insertion mutagenesis earlier. The potential for reactivating dormant viruses cannot be dismissed.

We have drawn attention recently to the cauliflower mosaic virus (CaMV) 35 S promoter (57-60) widely used to make transgenes over-express constitutively, and is in practically all transgenic crops already released commercially or undergoing field trials.

This promoter not only has a recombination hotspot (see Chapter 4), but is completely promiscuous in that it functions efficiently in all plants, green algae, yeast and *E. coli*, as well as animal and human cells. It has a modular structure, with parts common to, and

interchangeable with promoters of other plant and animal viruses.

Dormant and relict viral sequences have been discovered in the human and other animal genomes at least 20 years ago (61). Proviral sequences and related retrotransposons have also been discovered recently in plant genomes (62).

Proviral sequences in animal genomes can be reactivated, most strikingly, in the phenomenon of xenotropism in which they become infectious for cells for other species (63), constituting a major safety issue for xenotransplantation. Plant proviral sequences can also be activated, in some cases simply during plant tissue culture (64).

Recombination between promoters is not a theoretical possibility any more than the reactivation of dormant viruses. Synthetic super-promoters for gene therapy have been created in the laboratory by random recombination of modules isolated from natural promoters (65).

In gene therapy, a major safety concern is, indeed, the generation of 'replication competent viruses' (RCV) due to recombination of viral vectors with proviral and other sequences in the genomes of cell-lines used to package the viral vectors (66).

Chapter 6

Conclusion

The naked/free nucleic acids created by genetic engineering biotechnology are potentially the most hazardous xenobiotics to pollute our environment.

Unlike chemical pollutants that dilute out and degrade over time, nucleic acids can be taken up by all cells to multiply, mutate and recombine indefinitely.

There is an urgent need to take appropriate measures to prevent the release of any of these naked/free nucleic acids into the environment.

In particular, the practices of recycling solid transgenic wastes as food, feed, fertilizer and landfills by the biotech industry should no longer be permitted until and unless they are demonstrated to be safe beyond reasonable doubt as the result of dedicated empirical investigations.

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Appendix

How the EC Directive on Contained Use Allows Dangerous GM Wastes to be Recycled as Food Feed and Fertilizer

The safety of GM crops and foods has been grabbing headlines over the past two years. However, a potentially much more serious source of hazard remains unregulated. We first pointed to fundamental flaws in the regulation on contained use in a comprehensive review published in a scientific journal in 1998 [1]. This paper was submitted to the World Health Organization, European Commission, the Biosafety Conferences at the UN, as well as to the UK Health and Safety Executive, with additional comments from Mae-Wan Ho and others.

More recently, we raised the matter again in an update calling attention to the increasing variety and volume of 'naked' and 'free' nucleic acids produced in the laboratory and biotech factories under contained use, that are in fact not contained at all, but discharged in one form or another into the environment, as sanctioned by the current EC Directive on Contained Use (Council Directive 90/219/EEC), last amended in 1998. Our paper was circulated at the Montreal meeting on Biosafety in January. But there has been no real change since to the Directive on Contained Use. This Directive is fundamentally inadequate for the following reasons:

- The scope covers only genetically modified microorganisms; transgenic animals, fish and plants are not included. It also excludes nearly all classes of naked or free nucleic acids, except for viroids (infectious naked RNAs that cause diseases in both plants and animals).
- Notification only and not explicit approval is needed for use of Group 1 GM microorganisms, GMMs, considered nonpathogenic or otherwise safe; however, there is no agreement among EU nations on which microorganisms are pathogens or not; and it is effectively left up to industry to decide.

- For Group 1 GMMs, only 'principles of good microbiological practice' applies, that is, there is no containment.
- 'Tolerated release' of Group 1 GMMs are allowed to take place, without treatment, directly into the environment.
- No treatment of GM DNA or RNA is required to break them down fully before release.
- There is no requirement to monitor for escape of GMMs or GM constructs, horizontal gene transfer, or impacts on health and biodiversity.

We have presented evidence alerting to the dangers of horizontal gene transfer, among which are the creation of new viral and bacterial pathogens and the spread of antibiotic and drug resistance among the pathogens.

Despite our efforts, successive versions of the Directive have been relaxed and shaped by the European Federation of Biotechnology. This industry-dominated group have produced a series of 'safe biotechnology' papers, one of which [9] specifically addresses DNA content of biotechnological wastes.

The paper admits that DNA persists in soil and aqueous environments, that it is transferred to bacteria and cells of animals, and that it may become integrated into their genomes.

But they defend current practice by claiming

- 1) horizontal transfer of GM DNA occurs, if at all, at very low frequencies, especially in nature,
- 2) the persistence of foreign DNA depends on selective pressure, especially in the case of antibiotic resistance marker genes, and
- 3) DNA taken up is unlikely to be integrated into the cell's genome unless designed to do so.

The first claim is unwarranted. Evidence of horizontal gene transfer from transgenic plants to soil bacteria has been obtained in the laboratory as well as in the field, although the researchers themselves are downplaying the findings, in violation of the precautionary principle [see ref. 67].

The second assumption has been shown to be false. There is now substantial evidence that antibiotic resistance can and does persist in the absence of the antibiotic [68], mainly because biological functions are, as a rule, all tangled up with one another, and cannot be neatly separated and selected one at a time.

The third point is false as well, for it has been demonstrated in gene-‘therapy’ experiments (see main text) that naked DNA constructs, not intended for integration, have nevertheless become integrated into the genome. Integration occurs not only in somatic cells, but also in germ-cells.

The most dangerous aspect of current practice, defended by industry, is that solid wastes, heat-treated, or autoclaved, containing large amounts of intact or incompletely degraded GM constructs and transgenic DNA are being recycled or disposed of as food, feed, fertilizer, land reclamation and landfill.

Only in cases where GM constructs are specifically made to transform higher organisms, such as gene vaccines and genetic-pill applications (for gene therapy) is there a recognition that there may be a need to ‘inactivate waste by validated procedures rendering DNA nonfunctional by either reducing DNA fragment size below functional entities or altering the chemical composition and structure of the DNA.’ However, no such validated procedures exist.

Our regulatory authorities at all levels persist in ignoring scientific advice and scientific evidence. It is an example of the anti-precautionary approach [69]. They, together with the biotech industry, should be held legally responsible for any harm resulting from the uncontrollable horizontal transfer and recombination of GM material.

Biotechnological processes are creating an increasing variety of naked/free nucleic acids that are released unregulated into (and which persist in) the environment. They are by definition, xenobiotics – substances foreign to nature. Research findings in gene therapy and vaccine development show that naked/free nucleic acid constructs are readily taken up by the cells of all species including human beings. These nucleic acid constructs can become integrated into the cell's genome and such integration may result in harmful biological effects, including cancers.

Of current and serious concern is with regard to 'contained use'. Industrial contained users release large volumes of untreated transgenic microorganisms, which they judge to be 'safe', directly into the environment. Moreover, transgenic wastes consisting of killed microorganisms but containing large amounts of transgenic nucleic acids, are being recycled as food, feed and fertilizer, or disposed of in landfills. These practices are precisely those that can enhance horizontal transfer and recombination of transgenic nucleic acids, and should no longer be permitted.

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BIOTECHNOLOGY & BIOSAFETY SERIES

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