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Ecotoxicological Hazard Assessment of Genotoxic Substances

E.W.M. Roex, T.P. Traas, W. Slooff

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Abstract

Mutations can be divided into somatic and germ-line mutations. This overview presents current knowledge on the ecological relevance of mutagenic substances. Since current screening methods in genotoxicology are focused on the protection of humans, somatic mutations are the most crucial. In the field, these mutations only pose a hazard for natural populations of species with a low reproductive output. These species are supposed to be protected by the current screening methods. Germ-line mutations seem to be more relevant for natural populations, but screening on these mutations does not take place in the regular testing of substances. The frequency of germ-line mutations is, however, probably much lower. Germ-line mutations can be divided into non-lethal and lethal mutations. Lethal mutations do not pose a risk since they disappear in the next generation. Non-lethal mutations only pose a risk when genes that influence the fitness of the phenotype negatively are affected. However, the current screening methods for somatic mutations are supposed to act as a safeguard for inherited effects. We can conclude therefore that the methods used in the regular hazard assessment of substances are likely to provide a base for protecting natural populations against mutagenic substances.

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Samenvatting

Een van de conclusies van de werkgroep CMR (Carcinogene, Mutagene en Reprotoxische Stoffen) binnen het nationale SOMS programma was dat de ecologische relevantie van genotoxische stoffen onduidelijk was en dat er meer aandacht aan mutageniteit binnen de ecotoxicologie moest worden besteed. Dit rapport geeft een beeld van de huidige kennis over de ecologische relevantie van genotoxische stoffen. Hierbij is alleen gekeken naar directe effecten, oftewel mutaties.

De huidige sreeningsmethoden binnen de genotoxicologie zijn gericht op bescherming van de mens. Hierbij spelen somatische mutaties een cruciale rol, omdat zij kanker tot gevolg kunnen hebben. Alhoewel deze mutaties een letale afloop voor het individu in kwestie kunnen hebben, vormen zij geen gevaar voor het voortbestaan van populaties. Alleen populaties met een lage reproductiesnelheid, zoals hogere zoogdieren, kunnen hierdoor bedreigd worden. Deze soorten worden beschermd door de eerder genoemde toxiciteitstesten binnen de humane genotoxicologie.

- Voor natuurlijke populaties en uiteindelijk ecosystemen vormen niet somatische, maar kiemcelmutaties de grootste bedreiging. Binnen de humane genotoxicologie treedt echter geen screening op kiemcelmutaties op. Het risico hangt af van de mutatiefrequentie en het effect van de kiemcelmutatie. De verwachte frequentie van kiemcelmutaties is veel lager dan die van somatische mutaties, omdat zowel het aantal cellen en de blootstelling bij kiemcellen lager zijn, daar kiemcellen beter beschermd zijn en moeilijker te bereiken.
- Effecten van kiemcelmutaties kunnen verdeeld worden in letale en niet-letale mutaties. Letale mutaties vormen geen risico voor de populatie, daar zij bij de volgende generatie alweer verdwenen zijn. Het risico van niet-letale mutaties hangt af van de functie van het gen dat wordt gemuteerd. Alleen mutaties die de fitness van het fenotype negatief beïnvloeden, kunnen een probleem zijn voor de stabiliteit van natuurlijke populaties. Daarom vormt alleen een gedeelte van de kiemcelmutaties een risico voor ecosystemen. Deze overerfbare risico’s worden verondersteld gedekt te worden door de huidige screeningstesten voor somatische mutaties binnen de humane genotoxicologie.

Concluderend kan gezegd worden dat de huidige screeningsmethoden in de reguliere risicobeoordeling een basis bieden voor de bescherming van natuurlijke populaties tegen mutagene stoffen.

Summary

One of the working groups in the national SOMS programme found the ecological relevance of genotoxic substances unclear and recommended paying attention to mutagenicity in ecotoxicology. This report explores the present knowledge on the ecological relevance of genotoxic substances, confining itself to the direct effects (mutations).

Current screening methods in genotoxicology are focused on the protection of man. Within this framework somatic mutations, with cancer and mortality as possible consequences, are the most crucial. Although potentially lethal for individuals, somatic mutations pose a negligible risk for natural populations. Only populations of species with a low reproductive output, like higher mammals are threatened. Therefore these species are supposed to be protected by the application of the screening methods used in human genotoxicology.

- For natural populations and consequently ecosystems, not somatic, but germ-line mutations seem to be the most relevant. However, screening on germ-line mutations does not take place in the regular testing procedures of substances. The hazard involved depends on the mutation frequency and the effects of germ-line mutations. The frequency of germ-line mutations is expected to be much lower than that of somatic mutations: both the number of cells and the risks of being exposed for germ cells are lower, as germ cells seem to be more protected and are difficult to reach.
- Effects of germ-line mutations can be divided between lethal and non-lethal mutations. Lethal mutations do not pose a risk for natural population, as the mutation will disappear in the next generation. With respect to non-lethal mutations, the risk depends on the function of the gene affected. Only mutations, which effect the fitness of the phenotype negatively, may cause problems for the stability of natural populations. Therefore only a part of the germ-line mutations will pose a hazard for ecosystems.

Somatic mutagenicity testing methods practically act as a safeguard against heritable effects as well, regardless of the life history of the species.

It is concluded that the current mutagenicity screening tests in regular hazard assessment of chemicals likely provide a base for protecting natural populations against potentially mutagenic substances.

1 Introduction

Crucial to all parts of the substances policy is the knowledge on the danger of substances. Hence, the EU regulation on existing substances aims to map and organise the knowledge that exists with respect to the risks and safety hazards associated with substances. However, the amount of knowledge available on the tens of thousands of substances that are commercially available today is by no means comprehensive [46]. Stagnation of the policy is to be expected with regard to existing substances reviewing the effects of continuation of the current practice of intensive risk assessment, and the resultant decision-making process on measures to be taken. Therefore, both at the international level [16] and national level [48] initiatives have been proposed or taken to accelerate the regulatory process. Fundamental to achieving the goal of sustainable development is the Precautionary Principle [17]. In this context it has been proposed, amongst others, to prioritise substances or to take (policy) measures based on the intrinsic (hazardous) properties of the substances. Important intrinsic properties that have been addressed are persistency, toxicity, bioaccumulative potential on the one hand (PTB-substances), and carcinogenicity, mutagenicity, reprotoxicity on the other hand (CMR-substances). Based on these criteria the thousands of substances may be classified into various concern classes, with different responsibilities of the stakeholders involved and measures to be taken.

One of the national working groups in the national SOMS programme found the ecological relevance of genotoxic substances unclear and recommended to pay attention to mutagenicity in ecotoxicology and suggested that mutagenicity should be incorporated as a predictor for carcinogenicity in the ecotoxicological assessment of CMR-substances. Against this background this report explores the present knowledge on the ecological relevance of genotoxic substances.

First the different types of genotoxic effects will be discussed, followed by the test methods currently in use. Next the ecological relevance of genotoxic effects will be discussed and their possible implications for risk assessment. To conclude, recommendations will be presented how to apply this information on classifying substances in the concern classes.

2 Types of genotoxic effects

The important difference between the group of genotoxic compounds and other classes of toxicants is the ability of genotoxicants to change the genetic structure of an organism and hence of populations. In principle, environmental contaminants can effect the genetic make up of populations in three distinct ways: via mutations, genetic drift, and genetic adaptation [10]. A difference has to be made between *direct* and *indirect* effects of toxic substances on the genetic code of populations, as the impact of both types of effect on populations can be substantially different. These types of effects are explained below.

2.1 Direct genotoxic effects: mutations

Mutagenesis is the major route of chemicals to execute their genotoxic effects. Mutation is defined as any sudden, heritable change in a genotype of an organism not explainable by recombination of pre-existing genetic variability [26]. Numerous studies in the literature have demonstrated a strong correlation between elevated levels of potentially mutagenic or carcinogenic substances and tumour incidences in organisms (see §4.). Screening for mutagenic properties of compounds is thus an essential first step in genotoxicological risk assessment [32].

Mutation is the direct way of genetic change, because the substance directly interferes with DNA molecules that contain all the genetic information of an organism. Hundreds of chemicals are known to have slight to very strong mutagenic effects, which can be divided in two major classes [26]:

(1) Compounds that are mutagenic to both replicating and non-replicating DNA: examples are alkylating agents and nitrous acid.

(2) Compounds that are only mutagenic to replicating DNA: acridine dyes and base analogs are examples of this class.

Mutation may occur in any cell and at any state in the cell cycle. Essential differences however, exist between the possible consequences of *somatic* and *germ-line* mutations.

2.1.1 Somatic mutations

It is widely accepted that mutations in somatic cells are a major cause for tumour formation, eventually leading to cancer. For a large number of compounds tested, a greater than 90 percent correlation was found between mutagenicity, as observed in the Ames test, and carcinogenity [26]. A substantial amount of eventually carcinogenic substances first have to be metabolised into mutagenic metabolites in eukaryotic cells. Addition of an enzymatic liver fraction (S9) while performing an Ames test may enhance the mutagenic properties of the sample significantly [47]. The substance most known for this bioactivation is the poly aromatic hydrocarbon benzo[*a*]pyrene (B[*a*]P).

2.1.2 Germ-line mutations

Germ-line mutation, spontaneous or induced, is one of the substrates of evolution. These mutations may cause deviations in the genetic material in the offspring, resulting in different effects [22]:

1. Gamete loss during cell death
2. embryo mortality as a consequence of lethal mutations
3. abnormal development
4. Heritable mutations, which may cause changes in genetic diversity
5. Heritable mutations changing gene expression, and consequently changing fitness

The first three categories result in direct effects on the progeny of the parent generation. The categories 4 and 5 may change the genetic material of future generations, and are the most important in studying effects of mutagenic substances on populations.

2.2 Indirect genotoxic effects

Next to germ-line mutations, two other processes can have heritable effects on natural populations, without necessary interference with DNA molecules. Genetic drift and genetic adaptation are indirect pathways of substances to influence the genetic code and possibly the fitness of a population.

2.2.1 Genetic drift

A decrease in genetic diversity may be caused by severe fluctuations in population sizes by other contaminants, which do not have a genotoxic potential [29]. This process of genetic

drift increases the changes of extinction for populations, as this genetic erosion may limit the ability of populations to adapt to a changing environment [11]. It may also lead to increased inbreeding and associated reductions in fertility and offspring viability as the exposed population becomes increasingly homozygous, allowing the expression of detrimental recessive genes [21].

2.2.2 Genetic adaptation

Genetic adaptation is a response to an environmental change, during which certain genotypes have advantages in terms of life-history costs compared to other genotypes. The higher fitness of one genotype over another will eventually change the frequency of genotypes [28].

3 Genotoxicity test methods

During the last decades a wide variety of tests has been developed to measure the possible effects of these chemicals. Most of these tests are based on principles developed in the field of human genotoxicology and measure a biochemical or molecular response.

3.1 *In vitro* tests

A variety of *in vitro* tests are used to measure the possible genotoxic effects of compounds on organisms. Beneath, some of the tests most used are shortly described [21]:

- Detection of the formation of DNA adducts, as a consequence of activated metabolites, for example from PAHs, binding covalently to DNA. The method of detection is ^{32}P postlabelling.
- Detection of the enhancement of DNA strand breaks by contaminants or as an indirect effect of the excision of adducts induced by contaminants. The method of detection is the alkaline unwinding assay and agarose gel electrophoresis.
- Detection of anaphasic chromosomal aberrations, resulting from clastogenesis (chromosome breakage), aneugenesis (uneven distribution of chromosomes over daughter cells after cell division), spindle malfunction or chromosome stickiness. These aberrations appear as attached/lagging fragments, bridges, and multipolar spindles.
- Induction of Sister Chromatid Exchanges (SCE). Method of detection is an enhancement of radiolabelled bases between the two chromatids of one chromosome. SCE's have not found to be lethal, and a relationship with other biological phenomena has also not been found [39].
- Micronuclei tests: the detection of whole or partial chromosomes that are not incorporated into daughter cells during replication. The detection is the easiest in erythrocytes.
- DNA fingerprinting. Random Amplified Polymorphic DNA (RAPD) is a technique that is used to study DNA alterations in the offspring of exposed organisms. This technique offers the possibilities of detecting germ-line mutations. Examples of the application can be found in [34], [41], in which mutations were induced in medaka fish, *Oryzias latipes* by irradiation.

3.2 *In vivo* tests

Beside the *in vitro* tests the earlier mentioned Ames test, which is an *in vivo* test, is widely used. Recent studies have shown the application of transgenic zebrafish in detecting mutations cause by aquatic pollution [1], [13].

Most of these tests, including the Ames test, are good biomarkers of exposure, the results of the test correlate well with the exposure concentration. The outcome of these tests only provides information about the potential genotoxic hazards of the compound. Substances with this potential are suspected of being carcinogenic, but the final proof can only be determined from experiments with organisms, which is the next step in the risk assessment of genotoxic compounds.

3.3 Genotype frequencies

Effects of toxicants on genotype frequencies can be studied in different ways. Recent advances in the field of molecular biology have enormously enhanced the possibilities to study the genetics of populations, and consequently also the possibilities to measure effects of toxicants on the genetic structure of a population [12], [3]:

- The first method is a step-wise approach: First sensitive individuals are detected by using biomarkers. Than the genotypes of the tolerant and the sensitive individuals at critical loci are determined. The next step is to determine whether some of these loci confer a greater fitness. The last step is to determine the frequencies of these loci in exposed and unexposed populations. This approach involves high costs, but is a direct way of determining genotoxic effects. The crucial question in this approach is how to measure the possible differences in fitness between populations.
- Measuring the genetic variation in exposed and unexposed populations by comparing series of allozymes. These are different forms of the same protein that can be separated with gel electrophoresis. Allozymes are specified by different alleles and therefore directly reflect the underlying genetic variation. This approach is cheaper, but is not so specific as the first approach, as the relationship between the changing frequencies of allozymes and the effects on fitness parameters is unknown.

4 Ecological relevance of genotoxic effects

Due to their direct or indirect interference with the genetic code of organisms not only exposed populations are at risk, but future generations may be influenced as well. This can have potentially major effects on the integrity and stability of natural populations and consequently on ecosystems.

One of the crucial questions in the field of environmental genotoxicology is how the potential hazards and risks of this group of substances should be evaluated further. To answer this question, a distinction has to be made between the different pathways along which a chemical is able to effect the genetic structure of an organism and the consequent effects this may have for the population in the field.

4.1 Somatic mutations

Little is known about the possible consequences mutagenic effects may have on higher levels of organisation like the stability of populations in the field or on total ecosystems. In many monitoring programs the potential mutagenic activity of environmental samples is measured by using rather simple bioassays like the Ames test. In the Netherlands for example, it has been shown that the mutagenic properties of samples from the rivers Meuse and Rhine have declined since the early nineties [47].

Numerous studies in the literature have demonstrated correlations between elevated levels of potentially mutagenic or carcinogenic substances and tumour incidences in organisms. Bauman and Harshbarger for example reported a long-term trend between liver cancer prevalence in brown bullheads inhabiting a river and PAH concentrations in the sediment of the same river [9]. The same correlation was found for English sole, *Parophyrus vetulus* [37].

Somatic mutations endanger the survival probability of the individual when they develop into malign tumours. In the field of human genotoxicology the elevated number of newly formed neoplasia in an *in vivo* test is the key parameter in extrapolating the results from tests with laboratory animals to elevated cancer risks for humans [5], [6]. Only in species with a relatively low reproductive output, like large mammals, these mutations may effect the stability and size of a population in the environment [38]. Especially when the organism is in

its juvenile or reproductive period, the existence of future generations will be endangered. However, no evidence has been found yet for correlations between high incidences of tumours and steady declines in population sizes in the field [21], [42].

More than 95% of the species in the animal kingdom consists of invertebrates, which have a relatively high reproductive output [15]. When individuals of these species are affected by an induced or spontaneous somatic mutation, this will not severely influence the population size.

In conclusion it can be stated that somatic mutations, induced by chemical agents, do not cause a substantial hazard, as their consequent effects on populations in the field are negligible for most species of concern.

4.2 Germ line mutations

Some types of germ-line mutations do not pose a major risk for populations. These mutations are so severe that the newly formed genotypes are not viable in most cases and consequently these mutations will disappear again after one generation. However, depending on the frequency and severity of the mutation and the life history of the organism under exposure (e.g. high or low reproductive output) these mutations may influence the size and stability of the current population. An example of such a germ-line mutation caused by exposure to chemical agents, is the observed increased number of congenital abnormalities in the population from a Hungarian village. This effect was possibly caused by the consumption of fish treated with high doses of the organophosphate trichlorfon [19].

Heritable mutations are the most important in terms of effects on populations, as they may change the genetic diversity and/or the fitness of populations structurally. The most popular example of selection is the case of industrial melanism in the moth *Biston betularia* during the industrial revolution in the UK in the late 1800s [15]. Other evidence of genetic adaptation as a consequence of exposure to a toxicant was found in the springtail *Folsomia candida* exposed to B[a]P for several generations. Initially, the population decreased in the F_0 due to B[a]P exposure. In the F_5 however, fitness had increased again as a result of a strong selection pressure for tolerant individuals [35]. Also the increasing number of cases of insecticide resistance is caused by genetic adaptation.

Depending on the stability of the mutation the genetic diversity in future generations may increase or decrease. Initially, the genetic diversity of the population will increase as a result

of the new genotype(s) caused by the germ-line mutations. For example, Yauk and Quinn found an elevated mutation rate in herring gulls, *Larus argentatus*, nesting in an industrialised urban site, compared to populations from rural sites. The industrialised site contained high levels of potentially mutagenic substances, like PAHs and heavy metals [51].
Most heritable mutations are correlated with a reduced fitness e.g. a lower survival probability of the offspring of the mutant, as shown in the next example. A few years after the nuclear accident in Chernobyl an increased frequency of partial albinism in barn swallows, *Hirundo rustica*, was discovered. This phenotype was found to be associated with a mutation which was at least partly of germ-line origin. Another effect of this mutation was a loss of fitness, as the number of nests with eggs or nestlings decreased in the affected region during the period after the accident [25].

4.2.1 Germ line mutation calculations

To explore the potential implications of chemically induced heritable mutations, use can be made of population genetics [27]. Selection operates on the phenotype and not on the genotype. The induced phenotypic change may pass unnoticed or induce visible aberrations. Thus, the function of the affected gene, not the presence of a mutated gene per se, determines whether the fitness of the individual is reduced or not. How strong the selection pressure is that operates against a certain phenotype depends on the fitness reduction caused by the affected gene. If the fitness reduction is severe enough, an extinction of the population may be the eventual effect. If the reduction in fitness is only moderate, the frequency of the mutant genotype will mostly decrease again (when the mutation-inducing event has stopped) as a consequence of natural selection and will eventually disappear, thus reducing the genetic diversity again. The pace at which this mutant disappears depends on the severity of the mutation on fitness parameters and again on the life history of the organism.

The effect of mutations in a single impacted generation is demonstrated in the following calculations. Cronin and Bickham [18] considered germ-line mutations at a single diploid locus due to an oil spill, in sexually reproducing organisms with an assumed population size (N) of 1000 individuals. As a worst case scenario, initial mutations occur in either 10 or 50% of the population. These estimates are based on field frequencies of liver carcinomas,

probably due to mutations at hotspot loci in oncogenes, induced by chronic exposure to a mixture of PAHs, PCBs, dioxins and heavy metals in the Hudson River (USA) as cited in [18].

We first consider a case when 50% of the population is affected and each mutation is heterozygous (i.e. the allele frequency is 50%/2N = 25%). When all genotypes that carry the mutated allele A_m are lethal, i.e. the mutation is dominant lethal (AA_m or A_mA_m), considerable mortality may result (Figure 1), but the mutant alleles disappear in one generation. Recessive lethal mutations (A_mA_m) result in very little mortality, but the decrease in mutant allele frequency is slow (Figure 1). Selection of the recessive genotype is not very effective in removing recessive mutations that are at a low frequency in a population. Many mutations appear to be partially recessive, i.e. the heterozygote (AA_m) survives but is less fit than the wild type (AA). The decrease of the mutant allele due to selection is in between the former genotypes (Figure 1). This example shows that the more deleterious a mutation is, the sooner it will be excised as a consequence of natural selection [18].

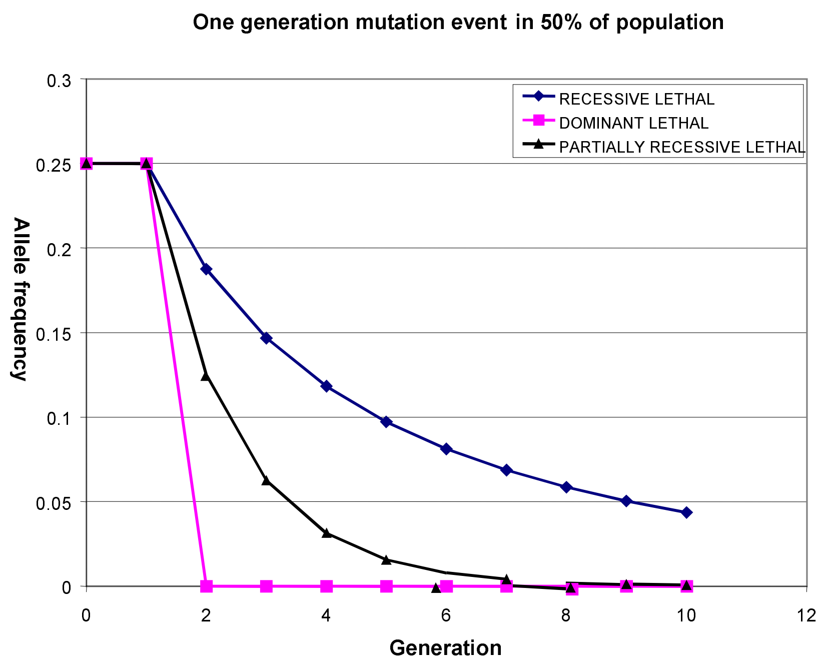

Figure 1: Change in a mutant allele frequency over 10 generations for recessive, partially recessive or dominant mutations, with initial frequency of 0.25 (50% of the population affected)

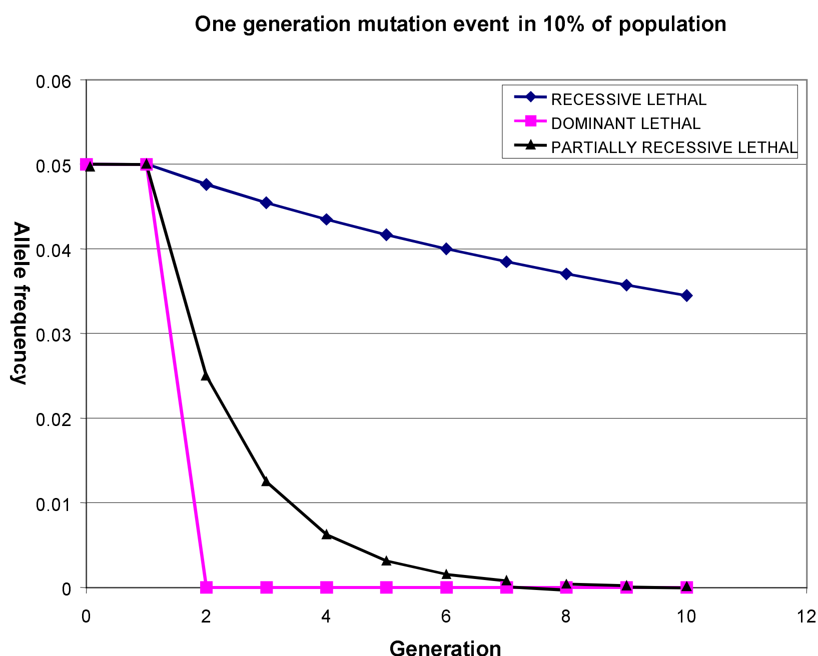

Figure 2: As Figure 1, but for an initial mutant allele frequency of 0.05 (10% of the population affected).

Most known lethal mutations in established natural populations are nearly totally recessive, suggesting that natural selection indeed removes those mutations that are dominant or partly recessive from the population [18]. Natural selection is ineffective in removing recessive mutations that are already at a low frequency in the population. Figure 2 shows that the selection of the recessive mutation proceeds much slower for the second case when the initial mutant allele frequency is chosen to represent a 10% affected population.

The previous example indicates that mutant allele frequencies soon decrease after a single generation event. In case of chronic pollutant stress, multi-generation exposure can occur e.g. for contaminated soil or sediments that act as a sink for pollutants with a high affinity for organic or mineral soil particles. This means that mutations are constantly occurring with a certain speed, depending on the type of chemical and exposure of the organism. How fast mutant allele frequencies increase in such a case, is shown in the next example calculations (Figures 3 and 4) according to [36]. Three different mutation rates are used (0.25, 0.05 and 0.0005) to represent a range from extremely worst case (0.25) to a case where one individual in a population with N=1000 is affected in each generation (0.0005).

The calculations in Figure 3 and 4 start at an allele frequency of 1.10^{-4}, chosen to represent a natural mutation rate. Spontaneous mutations for eukaryotes are observed to be in the range of 10^{-7} to 10^{-9} mutations per nucleotide pair per generation [27] [36]. Considering an average gene to be 1000 base pairs, the spontaneous mutation rate per gene is thus approximately 10^{-4} to 10^{-6} per gene per generation. When a worst case mutation rate of 0.25 is used (as in the previous example), allele frequencies are almost in equilibrium after 5 generations (Figure 3).

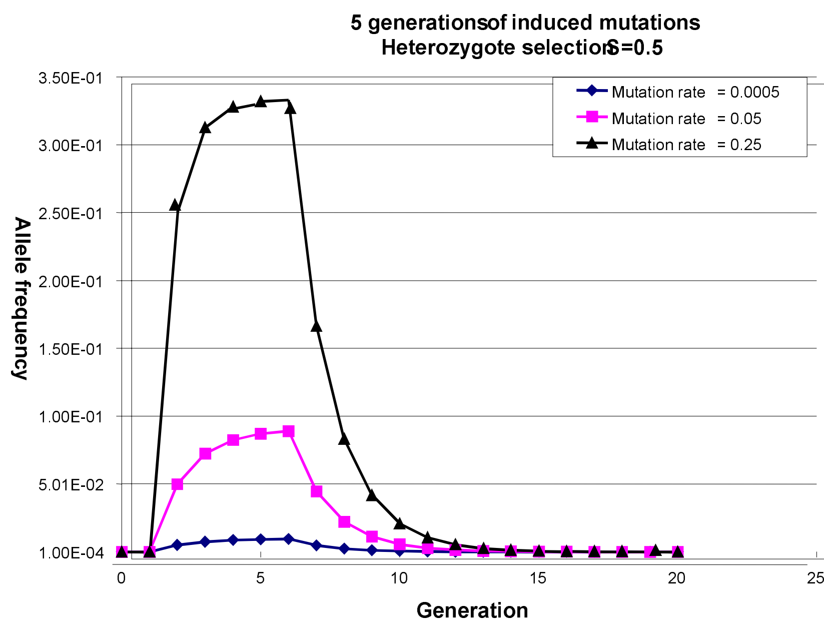

Figure 3: Change in mutant allele frequency for a partially recessive mutation (selected with s=0.5, cf. Lovell 1995 after 5 generations of chronic exposure to mutagens and the subsequent decrease afterwards

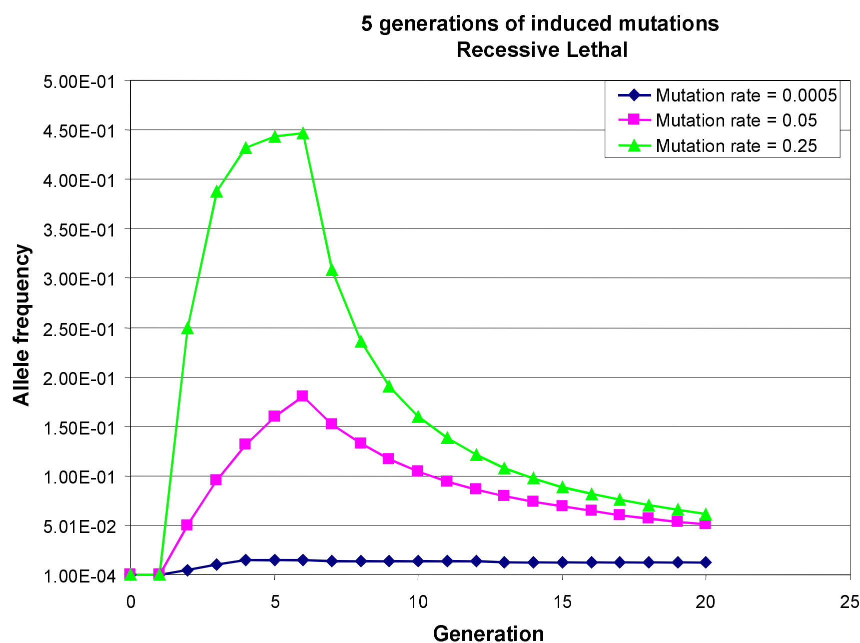

Figure 4: *Change in mutant allele frequency for a recessive mutation after 5 generations of chronic exposure to mutagens and the subsequent decrease afterwards*

This is also the case for lower mutation rates. For fast reproducing organisms such as microorganisms or insects, 5 generations can be produced in a relatively short time span, indicating that germ-line mutations are indeed of strong ecological relevance. The decrease of the mutant allele is however equally fast for the partially recessive mutants, as already shown in Figures 1 and 2.

In case the induced mutations are totally recessive (Figure 4), the allele frequency increases to a higher level than for partially recessive mutations (Figure 3) since only the homozygotes are removed from the population by natural selection. This means that the allele frequency of recessive mutations can increase almost unknowingly in a population. The occurrence of the genetic defect is not very likely, since it is a recessive trait (occurring at a frequency of A_m^2), but the spread in the population can go on for a long time. This could lead to an almost unnoticed accumulation of genetic defects in natural populations [36].

For a risk assessment of genotoxicants based on population genetics, it is important to know the mutation rate of the substance and the effect on fitness that a mutant allele might have. Both parameters are not routinely measured in genotoxicity tests. Without them, effects on population genetics of genotoxicants cannot be predicted.

4.2.2 Frequency of germ-line mutations

Little is known about the mutation rate and frequency of germ-line mutations caused by mutagenic chemicals, and in most studies difficulties arise in the detection of these specific mutations. In the field of human genotoxicology it is generally assumed that negative results in genotoxicity assays in somatic cells *in vivo* rule out possible effects in germ cells [7]. This assumption is based on the fact that the contrary has never been shown, and that a unique germ cell mutagen has not been identified until now [20]. This is not surprising as tests on germ cells are only required if positive *in vivo* somatic results are present. However these tests use very or extremely large numbers of animals and will not be normally required for industrial substances [7].
Heritable mutations are probably less frequent than somatic mutations [18] and Sommer postulates that most germ-line mutations are the result of endogenous rather than of exogenous processes [43].
These findings indicate that the deleterious effects of mutagenic substances are more severe for the population direct under toxic stress than for future generations.

4.2.3 How to detect possible germ-line effects?

To detect possibly heritable effects of mutagenic substances it is necessary to perform multi-generation toxicity studies, in which only the parent generation is exposed and effects on the progeny are determined. This approach enables the detection of pollutant-induced genetic changes passing from one generation to the next, as was also stated by Depledge [21] to be a major condition for heritable effects of toxicants. Some examples of multi-generation studies with the purpose to detect heritable mutagenic effect are described below.
In the laboratory showed daphnids, *Daphnia magna*, exposed to the mutagenic compound ENU (N-nitroso-1-ethyl-urea), a reduced number of offspring in the parental generation. However, the next four generations, raised from individuals exposed to the highest concentrations showed no effects on life-history parameters [44]. In a pilot report these life-history parameters were supposed to be the most promising parameters to detect mutagenic effects, because they are genetically controlled, they are important for the continued existence of the population and they are measurable in the laboratory [45].

Fathead minnows, *Pimephales promelas*, exposed to B[*a*]P for 4 months, showed no effects in the parental generation while the hatching frequency of eggs and the number of eggs laid by F1 females and the survival of the F2 larvae were significantly reduced. These findings indicate a heritable effect of the toxicant, accompanied with a reduced fitness although no genotoxic mechanism was found [49].

In an experiment with populations of *Drosophila melanogaster* exposed to the mutagenic substances DEB and ENU fitness reductions in the progeny occurred, indicating heritable mutations [33].

The studies mentioned above are examples of specific organisms exposed to selected substances. The effects on fitness caused by one specific compound however may vary from one species to the other, as a consequence of the differences in life history between organisms. Also the effects on fitness of different substances on one specific organism may differ, because of the different modes of action of the compounds. These processes hamper extrapolation from one species or substance to the other.

In conclusion it can be stated that germ-line mutations are ecologically relevant, but are hard to predict from screening on somatic mutations alone. Multi-generation experiments to quantify effects of germ-line mutations are feasible but expensive.

5 Risk assessment

In the risk assessment of chemicals, a first screening for mutagenicity takes place in a battery of three different *in vitro* genotoxicity tests. A positive result in one of these tests is qualitative, in the sense that the genotoxic potentials of a compound are determined. The next step, following after a positive result in an *in vitro* test is the quantitative phase, in which an *in vivo* carcinogenity test is carried out. Here is the relevance of the *in vitro* test assessed with respect to the *in vivo* situation [32], [7]. The results of these *in vivo* tests are extrapolated to carcinogenic risks for humans, by calculating a lifetime exposure level corresponding to a unit risk of 10^{-6}. This is accomplished by linear extrapolation from the lowest effective dose in the animal experiment to zero. Linear corrections are applied for the fractions of the total lifetime of the exposure duration and the observation period. It is assumed that no differences in pharmacokinetics, mechanisms of tumour induction, and sensitivities exist between test animals and humans [5].
In ecological risk assessment no procedures exist for potential mutagenic and/or carcinogenic substances, since the subject of protection is different. Ecological risk assessment concerns a wider range of species instead of a single one, and has to deal with the protection of populations instead of individuals [38], [50]. The type of tests used to assess the risk on humans can therefore not be used to quantify effects on the entire ecosystem as such.
An important difference between human and environmental genotoxicology is the method of risk assessment: DNA damage shows no threshold for response; in theory one single molecule can be enough to cause cancer. Regulations to protect against cancer are therefore mostly based on continuous probabilities of cancer rates. Ecological risk assessment however is based on dichotomous approaches, with the No Effect level as a central parameter [2].
The test animals that are used in carcinogenicity studies are mostly mice, rats or hamsters. For the extrapolation to the human situation these animals seem to represent the most suitable model available. However, for extrapolation to the ecosystem carcinogenity tests with more representative species should be more suitable. A simular test battery like the one that is used in regular ecological risk assessment procedures (e.g. fish, daphnid, and algae) would be more appropriate. The earlier mentioned work on transgenic zebrafish [1], [13] could be very promising, as it is possible to perform chronic toxicity tests with this species, enabling the

linking between different levels of biological organisation (mutation versus fitness parameters).

Some experiments in recent literature have tried to bridge the gap between results found in *in vitro* tests and effects on fitness parameters of more ecologically relevant species. These studies are summarised in Table 1. In most studies the applied genotoxicity test is the most sensitive, indicating that these tests can act as an early warning system. In these experiments no mechanistic link can be established between *in vitro* test results and effects on higher levels of biological organisation, like effects on growth, mortality or reproduction. These *in vivo* fitness parameters are supposed to be the most important in the extrapolation to effects on natural populations [14]. The effects that were found at higher levels of biological organisation could therefore also be caused by other modes of action, regardless of any genotoxic mechanisms.

At this moment ecological implications of genotoxic substances, positive in screening cannot be quantified with the available ecogenotoxicological data. One may speculate however on the relevance of risk levels used in human risk assessment. In the human risk assessment an arbitrary added risk of 10^{-6} is used as endpoint. The suitability of this factor in ecological risk assessment is questionable, since the subject of protection is different, e.g. individual versus population.

Germ-line mutations are expected to be less likely than this risk limit. Given the previous considerations on somatic effects of genotoxic compounds, this risk limit is quite low. However, interpretation of this risk limit strongly depends on the validity of the possible extrapolation factors that are incorporated. The results presented do not provide any evidence underpinning such factors.

6 Final considerations

The crucial problem with most of the tests, which are used to detect genotoxic effects, is extrapolation of these test results and the potentially heritable effects of a compound. Tests in regular genotoxicity testing are in principle not developed to detect effects on the progeny of exposed individuals, as most of these tests originate from the field of human genotoxicology. Performing *in vivo* tests with ecologically more relevant species as in regular toxicity testing is more appropriate. Modern molecular genetic engineering allows for the testing of genetically modified species, with reporter genes for genotoxic substances Multi-generation studies are feasible, but expensive, tools to generate data on population effects.

Heritable effects of genotoxic compounds seem to be the most threatening for populations and consequently ecosystems. The procedures developed in human genotoxicity testing are aimed at protecting individuals (and thus the population) against somatic mutations. Due to the higher incidence of somatic mutations compared to germ-line mutations, this practically acts as a safeguard against heritable effects as well.

We assume that the screening tests used in human genotoxicity are sufficiently sensitive to identify somatic genotoxic risks of substances, and thus are protective of heritable effects on natural populations as well.

From this reasoning it follows that natural populations and ecosystems are probably protected by the screening methods that are currently used in human genotoxicity testing.

Table 1. An overview of genotoxicity tests in which parameters on different levels of biological organisation are measured.

Species	Substance	Genotoxicity test	Fitness parameter	Most sensitive parameter	Reference
Nematoda					
Caenorhabditis elegans	ethyl methane sulfonate	Mutation frequencies	Number of viable offspring	Mutation frequencies	[4]
Annelida, polychaeta					
Pomatoceros lamarckii	carbendazim	Anaphase aberrations	Larval development	Anaphase aberrations	[24]
Pomatoceros lamarckii	di-butylphtalate	Anaphase aberrations	Larval development	Anaphase aberrations	[24]
Platynereis dumerilii	sewage effluent disinfected with sodium hypochlorite	Chromososme abberations	Development	Development	[31]
Arthropoda, crustacea					
Daphnia magna	B[a]P	Changes in DNA profiles, assayed by the RAPD technique	the intrinsic rate of natural increase, r_m	Changes in DNA profiles	[8]
Echinodermata					
Strongylocentrotus purpuratus	phenol	Anaphase aberrations	Development, fertilization rates	Anaphase aberrations	[2]
Strongylocentrotus purpuratus	benzidine	Anaphase aberrations	Development, fertilization rates	Anaphase aberrations	[2]
Strongylocentrotus purpuratus	pentachlorophenol	Anaphase aberrations	Development, fertilization rates	fertilization rates	[2]
Strongylocentrotus purpuratus	B[a]P	Cytologic and cytogenetic abnormalities	Fertilisation rate	Fertilisation rate	[30]
Chordata, vertebrata					
Xenopus laevis	B[a]P	erythrocytic micronuclei, DNA adducts	increased time to meta-morphosis, reduced wet weight	DNA adducts	[40]
Danio rerio	4-nitroquinoline-1-oxide	DNA repair, DNA strand breaks and deletions	F_0 reproduction, survival F_1	DNA repair	[23].

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Appendix 1 Mailing list

1. Dr. D. Jung (DGM-SAS)
2. plv. DG Milieubeheer
3. Hoofd afdeling stoffen, DGM-SAS
4. Dr. ir. C.A.M. van Gestel (VU/Amsterdam)
5. Dr. G.J. de Maagd (RIZA/Lelystad)
6. Drs. M. Koene, Stichting Natuur en Milieu
7. Depot Nederlandse Publikaties en Nederlandse Bibliografie
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9. Sectordirecteur Stoffen en Risico's
10. Hoofd Centrum voor Stoffen en Risicobeoordeling
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14. Dr. J. van Benthem (RIVM/LEO)
15. Dr. W. Slooff
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