Aquat. Sci. 66 (2004) 1–07
1015-1621/04/030001-07
DOI 10.1007/s00027-004-0721-x
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Aquatic Sciences

Overview Article

Characterization of environmental risk of genetically engineered (GE) organisms and their potential to control exotic invasive species

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Received: 13 October 2003; revised manuscript accepted: 5 April 2004

Abstract. Genetically engineered (GE) organisms could result in ecological harm in many ways in natural environments. Ecological harm can be assessed based on standard principles of risk assessment. Risk is the probability of harm as a result of a hazard, which in this case is a GE organism; its harm may not be known or knowable *a priori*, however, due to the large number of biotic interactions in nature in which it could be involved. We contend that for a GE organism to be a risk, it must be able to spread in nature. Thus, we do not have to determine all, or any, possible harms; we only need to be certain the organism will not spread if it escapes. Predicting the potential of a GE organism to spread is possible because the ultimate fate of a transgene will be determined by natural selection. Thus, environmental risk assessment can be accomplished by measuring six net fitness components that are common to all organisms, transgenic or wild type: juvenile and adult viability, fecundity, fertility, age at sexual maturity, and mating success. These components can be measured in secure settings. Previously, we focused on the environmental risk posed by GE organisms created to enhance agricultural productivity. Here we review the potential of using GE biotechnology for biological control of an existing undesirable exotic species. GE biological control might be employed to induce a 'Trojan gene effect' (Muir and Howard, 1999; 2002a,b) to eliminate such species, by introducing genes which cause male-biased sex ratios, inducible fatality, or selfish gene effects.

Key words. Environmental Risk Assessment; Genetically Engineered; GMO; Biocontrol; Aquatic; Trojan Gene.

Introduction

Genetically engineered (GE) organisms provide the potential for greatly enhanced agricultural production but may also pose environmental risks. Methods have been developed to predetermine such risks in secure settings. These methods can also be used to some degree to determine the potential invasiveness of exotic organisms. Alternatively, these methods might be used in biological control to contain or eliminate existing exotic species. Here we provide a brief review of environmental risk assessment methods for GE organisms, discuss their utility for exotic species, and finally examine GE methods for biological control of exotic species.

Environmental concerns created by GE organisms

Invasive exotic species often cause havoc in aquatic environments (Ehlers, 2003; Lukens, 2003; Schmitz, 2003) sometimes by eliminating other species (Drake and Mooney, 1986; Lodge, 1993; Bright, 1996). Genetically Engineered (GE) organisms, particularly fin fish, are

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Published on Web: ■

now commonly produced for a number of species (see Hackett and Alvarez, 2000; Muir and Hostetler, 2001, for review). In many respects, GE organisms present environmental concerns similar to those of invasive exotics because GE organisms can have novel or enhanced abilities relative to their wild-type conspecifics. As a result, GE organisms might interact differently with other native species including their wild-type counterparts. As a consequence, GE organisms might threaten the survival of wild-type conspecifics as well as other species in a community (Devlin and Donaldson, 1992; Kapuscinski and Hallerman, 1991; Muir and Howard, 1999; 2002a; and Tiedje et al., 1989).

The National Research Council (NRC, 2002) recently examined scientific concerns related to GE organisms and concluded that aquatic GE organisms (along with GE insects) created the greatest concern for environmental risk because they can escape captivity relatively easily in egg and larval stages, disperse rapidly through interconnected waterways, enter populations of native conspecifics and spread through inter-mating, and finally adapt quickly to natural settings and become feral.

GE animals are primarily created to enhance agriculture production by increasing resource utilization efficiency, extending the range of production (adaptability), or enhancing disease resistance (NRC, 2002). As such there are many potential economic benefits for production of such organisms. However, it is imperative that such advancements not come at a large environmental cost.

Overview of environmental risk assessment of GE organisms

Muir (2001a, b; 2002a, b) and Muir and Howard (2001; 2002a,b) have developed a method of environmental risk assessment based on population genetics theory. Classic risk assessment is based on degree of exposure to a hazard and risks resulting from such exposure. Living organisms do not clearly fit the paradigm of classic risk assessment because living organisms can expand on their own, and natural selection can modify the hazard. Thus, a modified form of risk assessment was needed for GE organisms as discussed by Muir (2001a, b; 2002a, b) and Muir and Howard (2001; 2002a, b) and accepted by the NRC (2002).

There are three important terms that need to be defined in risk assessment: harm, hazard, and risk. Harm is any defined adverse outcome as a result of exposure to a hazard. A hazard is the substance or action that brings about the harm. Risk is the probability of the harm from the hazard. In the context of GE organisms and the environment, the hazard is the GE organism itself because it is the agent that might cause a negative impact on community stability. The harm can be simple or complex, transient to permanent in time frame, and local to global in scope. The NRC (2002) defined ecological harm as ”gene pool, species, or community perturbation resulting in negative impacts to community stability”. These include displacement or reduction in the number or relative abundance of species that co-exist in a community. This definition is all-encompassing and broad, but can be further refined once a particular GE organism is identified and the environment into which it might escape or be released is known.

Potential harm

Some examples of potential harm that may result from GE fish are:

1) Transgenes might change predator-prey relationships. This might occur if fish transgenic for growth hormone are orders of magnitude larger than their native conspecifics. In mud loach, GE individuals grow 35 times faster than normal (Nam et al., 2001). At 2 months of age, some transgenic mud loaches were much larger even than any of their 12-year-old wild-type conspecifics. Most fish are gape limited; thus, larger adult fish consume larger prey. As a result, GE fish may prey on or size classes of their normal prey or even different species of prey. In addition, drastically larger GE fish might be too large for their normal predators to consume.

2) Transgenes might expand environmental adaptability. The geographic range of any species of fish is ultimately limited by abiotic factors such as water salinity and temperature. However, transgenes have the potential to remove these restrictions. For example, one of the first GE fish created was a salmon with an antifreeze protein gene that would allow the GE salmon to be grown further north (Fletcher et al., 1999). The transfer of genes that would allow saltwater fish to inhabit freshwater (or vice versa) would be another example of an increased range expansion in a GE fish. Transgenes with these effects would allow GE fish to invade new habitats, and thereby create a potential for them to become an invasive exotic species.

3) Transgenes might remove limiting biotic factors. The density of fish populations are typically limited by one or more biotic factors such as nutrients (minerals, vitamins, carbon source, protein sources, amino acids), predators, parasites, and diseases. If limiting biotic factors are eliminated or reduced in GE fish, even partially, then their population size can increase and intensify competition with other species, negatively impacting their population size and stability.

A common limiting nutrient in the environment is phosphorous. Hostetler and Muir (2004) transferred a phytase gene into Japanese medaka that allowed fish to liberate elemental phosphorous from phytic acid. Sur-

vival of these GE fish on diets low in phosphorous but high in phytic acid was as much as 50% greater than wild-type. Similarly, Golovan (2001a, b) transferred a phytase gene into mice and pigs, producing enhanced growth. If GE mice or pigs with a phytase gene escaped into nature, they could become even greater pests in some parts of the world than they already are now (Vtorov, 1993; Hone, 2002; King et al., 1996; Krebs et al., 1995).

Disease is another important biotic source of mortality that could influence population growth. An antibiotic gene, cecropin, was recently inserted into Japanese medaka (Sarmasik et al., 2002) and channel catfish (Dunham et al., 2002). Annual survivorship of GE channel catfish was more than twice that of wild-type controls (40.7% compared to 14.8%).

4) Transgenes might have opposite effects on different fitness components. Muir and Howard (1999, 2001) found that antagonistic pleiotropic effects of transgenes on different net fitness components can result in unexpected harm, such as the local extinction of GE-invaded conspecific populations (eliminating both native and GE individuals). We referred to transgenes which are predicted to cause such local extinctions as Trojan genes (Muir and Howard, 1999). A Trojan gene is defined as any gene which drives a population extinct as it spreads because of destructive self-reinforcing cycles of natural selection. For example, if a transgene enhances mating success of GE males while reducing the viability of their young, the mating advantage of the GE males quickly spreads the transgene into an invaded population but the low viability of its offspring (both male and female) results in a population decline. Thus, the downward spiraling of population continues until both wild-type and transgenic genotypes become locally extinct (Muir and Howard, 1999). These predictions were later independently verified by Hedrick (2001). An additional harm resulting from the local extinction of a GE-invaded population is a possible cascading, negative effect on the rest of the biological community.

The interaction of mating success and juvenile viability is not the only antagonistic pleiotropic effect which can produce a Trojan gene effect. Muir and Howard (2001) have also shown that a Trojan gene effect can result, if transgenes increase adult viability but reduce male fertility. This Trojan gene outcome is further enhanced if GE males also have a mating advantage. The effect of such a transgene parallels that obtained when sterile males were used in a biological control program to eradicate screwworms (Kempthorne and Pollak, 1970; Hedrick, 2000; Muir and Howard, 2002b), except that in the case of sterile males, males must be released continually to achieve control; a transgene that increases the viability component of fitness will spread on its own, while the reduced fertility brings about extinction, albeit over a longer time period.

Evaluating risk

Risk is the probability that harm will result from exposure to a hazard. Exposure is the key to environmental risk assessment because any effect of escaped GE organisms might be transitory if it cannot become established in nature. Thus, the NRC (2002) concluded that, provided the natural population is not already endangered, for a GE organism to prove a hazard, exposure must be more than just the release or escape of the organism into a community, the GE organism must spread into the community.

Stated in terms of mathematical probability, there are two mutually exclusive requirements for an environmental risk due to a GE organism: the first is a conditional probability of harm given that the GE organism will spread into the natural population, and the second is an unconditional probability that the GE organism will spread.

P(Harm from GE organism) = P(Harm given GE Organism Spreads) * P(GE Organism Spreads)

The conditional probability of harm given the gene has spread into the population is difficult to assess a priori because of the potentially large number of biotic interactions which could involve an invading GE organism. Some types of harm might be anticipated, as detailed in the previous section, but quantifying this probability would be exceedingly difficult.

Fortunately, the second part of the equation is more tractable. The probability a GE organism will spread can be further broken into two parts: a species-dependent component and a transgene-dependent component. The species-dependent component can be further broken into three parts: the natural ability of the organism to escape, disperse, and reproduce. Each of these can be estimated using prior information. For most fish species, each of these probabilities is rather high.

The transgene-dependent component is the ability of the transgene to persist or spread through natural selection. This part can be evaluated for any diploid sexually reproducing species, plant or animal, using population genetics theory (Muir and Howard, 2001; 2002b). The approach is based on estimating six critical life history characteristics, termed net fitness components: juvenile and adult viability, age at sexual maturity, female fecundity, male fertility, and mating success (Muir and Howard, 2001; 2002a, b). Our model is based on the assumption that natural selection acting through these components will determine the ultimate fate of the transgene. Fortunately, these components can be estimated in secure settings. Once the components are measured, the Muir and Howard (2001) model can be used to incorporate all components into one prediction equation. Computer programs utilizing the model have been provided by Muir (2001a,b; 2002a,b). The program provides three out-

comes: 1) loss of the transgene (no risk), 2) spread of the transgene (invasion risk), and 3) elimination of invaded natural populations (extinction risk involving a Trojan gene).

Because all the probabilities on the right-hand side of the overall risk equation are multiplicative, if one term is small or close to zero, the overall probability will also be close to zero, i.e. the maximum value of the left-hand side of the equation, the probability of harm, is the minimum value of the right-hand side of the equation. In view of the fact that the conditional probability of harm given spread is difficult or impossible to determine, the conservative assumption is that the conditional probability is one, i.e., there will be some harm if the GE organism spreads. This conclusion is based on results observed with introduction of exotic species, intended or accidental, most of which eventually caused environmental disruptions (Mooney and Drake, 1986; Bright, 1996).

Such an approach is attractive to regulators because it 1) provides a set of traits that can be measured for any diploid sexually reproducing organism, 2) uses quantitative estimates, and 3) does not require knowledge of the specific mechanisms that might cause the fitness components to differ between GE and wild-type individuals. Although study of such mechanisms might be of academic interest, they would likely require years and considerable expense to do. However, for predictive purposes related to risk, the phenotypic effects of a transgene on fitness components are the only factors natural selection acts upon and is thus the only information regulators need. This last point is critical because modern tools of molecular genetics allow us to determine gene action at a very fine scale. However, knowledge of the fine scale does not increase our predictive ability of a general outcome; here, a holistic phenotypic approach is needed.

Limitations of the net fitness approach

No method of risk assessment is perfect. Some limitations of the net fitness approach include the following:

1) Natural selection will always maximize viability fitness provided gene action is additive (Fisher, 1958). Thus, viability disadvantages observed initially will most likely be reduced through time.

2) Genotype by environment and epistatic interactions, if sufficiently large, can change the values of different fitness components and thus alter predictions. G × E interactions result when the phenotype produced by the same genotype differs significantly under different environmental conditions; as could be the case for the phenotype of a GM line in a laboratory versus natural setting or in two differing natural environments. Epistatic effects on the phenotype result from gene interactions. In the case of a GM line, the initial phenotypic characteristics observed are in the context of a particular genetic background, that of the original stock from which the GM line was created. The phenotypic effect of the transgene could vary across different genetic backgrounds, and natural populations invaded by GM organisms might well differ from the stock used to produce a GMO in genetic background. One solution to these problems would be to measure each component in each of the macro-environmental conditions where the organism is likely to occur and across multiple genetic backgrounds. Another approach is to examine the effect of a range of fitness component values on model predictions to determine robustness of predictions and identify which components are the most critical, and then concentrate efforts on measuring those components in a range of environments and genetic backgrounds (Muir and Howard, 2002b).

3) Estimation of fitness components in secure environments cannot replicate all conditions found in nature. Usually conditions found in confined situations will be more hospitable and less complex than those found in nature. As such, predictions of risk will be conservative, i.e. a high probability of risk of spread may be found when in fact the actual probability of spread may be much less. Alternatively, it is improbable that an organism will have low fitness components in confined situations and high in natural environments. Thus, our approach follows the principles of precaution (Muir and Howard, 2001).

One approach to this problem is to use surrogates that mimic a GE organism, such as fish implanted with growth hormones. The implanted fish could be released into natural environments and their survival rate and other fitness components could be measured (Johnsson et al., 1999; 2000). Unfortunately, it will not be possible to mimic all GE organisms, but the approach could be used to validate the method.

4) In its current state, model predictions do not incorporate uncertainty; that is, there is uncertainty in estimates of parameter values as well as stochasticity in response for many reasons such as changes associated with population density. Bayesian methods are currently being developed to address these concerns.

Use of GE organisms for biocontrol

As discussed under potential harms, one of the possible adverse outcomes of the spread of GE organisms in nature is local population extinction of its species from a Trojan gene effect. However, such an outcome might be desirable as a means of biological control of exotic invasive species (Muir and Howard, 1999). Up to now, three general approaches of maintenance control have been employed for invasive species when eradication programs have failed: chemical, mechanical, and biological control (Mack et al., 2000).

Chemical control is the most common method used on exotic agricultural pests and usually involves administering poisons non-discriminately in the habitat (e.g., an entire lake, pond, or stream). As a result, non-target species may be adversely affected or even eliminated along with the target organism, as was the case when using DDT for pest control (NRC, 1986). The high cost and potential for evolution of pesticide resistance in target pests often limit the utility of chemical control. Only in extreme cases, or in the early stage of infestation, should such draconian measures be considered. Mechanical control involves capture and removal. This approach is labor intensive, expensive, and often not effective except in the initial stages of invasion (Mack et al., 2000).

Biological control using natural control agents is considered by many as the most desirable management approach as it works within nature to achieve population stability. Although successful biocontrol projects are well known (e. g., control of prickly pear cactus in Australia by introducing a moth from Argentina (Pemberton and Cordo, 2001), the introduction of one exotic to control another has inherent risks of its own (Howarth, 1991). An introduced biocontrol agent may adversely affect non-target, native species as much or more than target species (Pemberton and Cordo, 2001), or, as a result of attaining a high population density, control agents might have a transient negative effect on less preferred non-target native species after they have eliminated the target species but before their own population size decreases (Lynch et al., 2002). An effective biocontrol method that should have little or no impact on non-target species involves release of sterile males (Knipling, 1955). Males are sterilized with irradiation resulting in the reduction or complete loss of progeny produced by their mates. To be effective, however, sterile males must be released repeatedly in sufficiently large numbers to swamp the reproduction of fertile males. The method was used to control screwworm flies in the Southwestern US (Whitten and Foster, 1975). However, other factors contributed to the success in eliminating screwworms besides the release of millions of sterile males (Baumhover, 2002): Female screwworms mate only once, thus females mating with sterile males cannot produce young by mating again with fertile males. Constant inspection and treatment of animal wounds provided a way to control larval screwworm abundance, thus increasing the ratio of sterile to wild males. In addition, a harsh winter assisted the program by eliminating northerly populations and reducing the size of surviving populations. Thus, success of the sterile insect technique may not readily extend to other species, and creating sterile males using genetic engineering rather than irradiation provides no additional advantage.

Three general categories of direct GE modification of exotic species have been considered: 1) production of male-biased sex ratios (Grewe, 1996), 2) introduction of inducible fatality genes (Grewe, 1996), and 3) induction of non-disposable genetic loads using Trojan genes (Muir and Howard, 1999; 2002b) or site-specific, selfish genes (Burt, 2002).

Male-biased sex ratios. Some steps have been taken to control a pest species by producing a male-biased sex ratio using GE techniques, particularly in Australia. Unfortunately, published information is limited to popular press reports, technical reports, and meeting proceedings. The CSIRO is currently developing a methodology to control carp using a GE gene referred to as 'daughterless' that blocks the female sex-determination gene, aromatase; hence, females with the gene are phenotypically male. These fish would then be released to mate with carp in the wild. As a result, carp populations should become male biased, and gradually decline to extinction over many generations.

The effectiveness of this approach or the mode of gene action of the daughterless gene (i. e., if the gene will be transmitted in a Mendelian fashion and whether it will be dominant or recessive) is not known. If the daughterless gene is a knockout gene, it will act as a recessive and will not be effective; if dominant, the method will be effective. However, population extinction will require several large releases because of selection against the daughterless gene. That is, natural selection should favor females without the transgene as the only fish producing female offspring are those who do not carry the gene, and only females can reproduce the species.

Inducible fatality genes (IFG) are neutral genes that become lethal when activated by some special agent, such as an antibiotic. The goal here is to drive an IFG into a population by successive releases, purge the population by releasing the activating agent, and then repeat the procedure until the population goes extinct. There are three pitfalls of this approach: neutral genes can only be driven into populations by extensive migration, in this case, by repeated extensive introductions of hatchery-reared exotic fish. Introduction of large numbers of exotic fish will, in the short term, exacerbate the problem. Secondly, there will be intense selection for fish resistant to the fatality gene. These resistant fish will become the founders of a future wave of exotic fish that will spread as the susceptible exotic fish die. Finally, when the fatal gene is activated there will be large die offs. The rotting carcasses, if not removed manually, will not only be unsightly and produce an objectionable odor, they may adversely affect other species through disease and oxygen depletion during decomposition.

Induction of non-disposable genetic loads. Trojan genes fall into this category because their pleiotropic effects involve increasing one fitness component while decreasing

another. As discussed above, there are two known ways to induce such an antagonistic pleiotropy (Muir and Howard, 1999; 2002): create a transgene that either increases the mating success of its carrier while decreasing either the viability of its offspring or decreases male fertility while increasing the viability of its offspring. The latter option differs from sterile male technique of biological control **(**Whitten and Foster, 1975**)** in which released sterile males have a viability of 1.0 and a fertility of 0. GE males have some fertility and the viability of their offspring must exceed that of wild type. As a consequence, repeated introductions of GE males are not required (Muir and Howard, 2002b). An example of such a transgene would be the cecropin gene (Dunham et al., 2002). The gene increases juvenile and adult survival but may also act as a spermicide reducing male fertility.

Both Trojan gene effect methods present problems in terms of biological control. With the first method, natural selection may eventually reduce the viability disadvantage of GE individuals while their mating success will be reinforced. Thus, rather than causing population extinction, the transgene may spread and invade, possibly creating a greater problem. The second method creates similar concerns. There will be strong selection for increased fertility in males while the positive impacts of the transgene on viability will be reinforced. Again, rather than causing population extinction, the transgene will spread and invade but with greater viability than before, creating a greater problem.

Among the alternative GE technologies presented to date for biological control, we suggest that selfish genes have the greatest promise (Burt, 2003). This method provides a means of gene conversion, in which homing endonuclease genes (HEG) copy themselves into a defined target DNA sequence, and thus spread on their own as a form of gametic selection or 'super-Mendelian' inheritance (a form of meiotic drive). Once infected, HEGs convert heterozygotes to homozygotes for the HEG. Hence, if such genes can be engineered to target new host sequences, then they can be used to control populations because they have a powerful drive mechanism and are effective even if released in relatively small numbers. Burt (2003) claims that "a genetic load sufficient to eradicate a population can be imposed in fewer than 20 generations, if the target is an essential host gene, the knockout is recessive, and the selfish gene has an appropriate promoter." A potential concern of this technology includes hybridization between the target species and closely related non-target species that contain a homologous sequence recognized by the endonuclease gene, resulting in the eradication of desirable species. Thus, as noted by Burt (2003), the potential of a target species for horizontal gene transfer and degree of sequence homology of the recognition site in the target gene among related species must be considered before using this technology in biological control.

In sum, the effective use of GE technology in biological control requires that the gene construct developed can spread in natural populations of only the target species and that subsequent evolution to counter the action of the gene construct in the target species is unlikely. GE approaches to produce a male-biased sex ratio in a target species or to induce fatality do not appear promising, as both methods require continuous reintroductions to be successful because the engineered genes will not spread on their own. However, the inability of the control gene to spread on its own limits the transgene and reduces environmental concern for such introductions. A non-disposable genetic load produced by a Trojan gene may spread but could be countered by subsequent evolution in the target organism to minimize its viability disadvantage. As a consequence, the target species may not go extinct, but rather be transformed by the transgene possibly producing an even greater menace (i.e., an invasion risk outcome; Muir and Howard, 2002b). The potentially rapid spread of a HEG selfish gene by a meiotic drive-type process and its ability to eliminate a target organism by destroying an essential gene function (Burt, 2003) is a powerful approach. However, more theoretical and empirical attention is needed to ensure the safety of this technology both in environments where the target species is considered to be a pest and in regions where it occurs naturally and is of no economic or ecological detriment.

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