Defusing the environment

Elaine Boyd & Neil Bruce

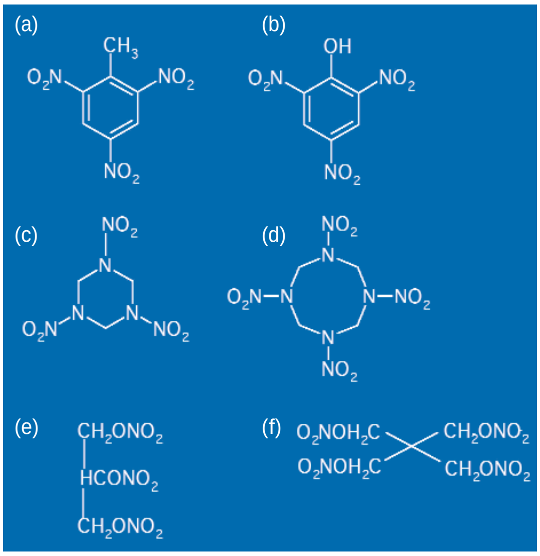
Environmental contamination by explosives presents a serious problem. Bacteria and plants may provide the solution, as Elaine Boyd and Neil Bruce describe.

Major international concern is growing over the wide-scale contamination of soil and ground waters with high explosives. For example, in the US an estimated 0·82 million cubic metres of soil at former ordinance sites and military proving grounds are contaminated with the explosive 2,4,6-trinitrotoluene (TNT). The manufacture, use and disposal of high explosives over the last hundred years have resulted in serious widespread contamination of the environment. The presence of these compounds not only presents the risk of detonation and a serious hazard to human health, but many explosives are also highly toxic and recalcitrant, persisting indefinitely in the environment. In addition to TNT, compounds of concern include nitroaromatic 2,4,6-trinitrotoluene (Picric acid), the nitroamines [hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; Royal Demolition Explosive) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; High Melting Explosive) are also widely used explosives as are the nitrate esters]; pentaerythritol tetranitrate (PETN) and glycerol trinitrate (nitroglyercin) (see Fig. 1).

Current remediation technologies

Effective treatment of the solid and liquid wastes emanating from production and decommissioning explosive processes is of paramount importance. Present and historical methods for the disposal of munitions include open burning, open detonation (Fig. 2), burial and incineration. These methods, however, are becoming increasingly unpopular due to environmental concerns. For example, current incineration of TNT-contaminated soil results in the production of thousands of tonnes of unusable ash. This raises concerns regarding further disposal and also presents a threat to the quality of the atmosphere through the release of fine particulate matter. Furthermore, the open detonation disposal option can result in the release of further contaminants, including dioxins and polychlorinated biphenyls. Open detonation can also result in major habitat destruction, ecosystem disturbance and extensive structural damage to any property in the vicinity. Alternative technologies must be developed to minimize air, water and soil pollution and to comply with present and anticipated environmental legislation.

BELOW:
Fig. 1. Structures of important explosives. (a) 2,4,6-trinitrotoluene (TNT); (b) 2,4,6-trinitrophenol (picric acid); (c) Royal Demolition Explosive (RDX); (d) High Melting Explosive (HMX), (e) glycerol trinitrate (nitroglycerin); (f) pentaerythritol tetranitrate (PETN).



Addressing the problem

At the Institute of Biotechnology, University of Cambridge, we have been focussing on both the potential of bacteria to bioremediate explosive-contaminated land and upon phytoremediation, the use of plants for explosives remediation. Such approaches can provide environmentally friendly and aesthetically pleasing alternatives to the harsher and more expensive methods of physical and chemical degradation approaches.

Bacteria – bioremediation potential

It was once thought that micro-organisms were infallible! It was generally considered that they were capable of oxidizing any organic molecule in the environment, providing conditions were favourable. This theory, however, began to be disproved when it was found that anthropogenic compounds (those synthesized by man) were in-fact persistent in the environment. These compounds were often foreign to biological systems and their structures were not easily recognized immediately by the microbial biomass. As a result, they accumulated in the environment and, through processes of bio-concentration and bio-magnification, began migrating to higher levels in the food chain.

Bacteria possess the natural ability to adapt and evolve degradative pathways to break down anthropogenic compounds, but to achieve this they need to be exposed to the contaminant for a considerable time. Our group was granted access to undisturbed soil samples contaminated with high explosives for over 60 years. This has provided us with the unique opportunity to isolate soil micro-organisms that have evolved the capacity to degrade or transform problem high explosives.

RDX degraders. Novel species have been isolated that can utilize RDX as a sole nitrogen source. Dispersion plates show 'zones of clearance' where RDX has been mineralized by *Rhodococcus rhodochrous* 11Y, a Gram-positive soil bacterium (Fig. 3). Detailed characterization of the primary metabolites has shown that RDX is broken down to simple carbon and nitrogen substrates such as formate, formaldehyde, nitrite and ammonia. All these compounds can then be further metabolized by the general soil microbial biomass.

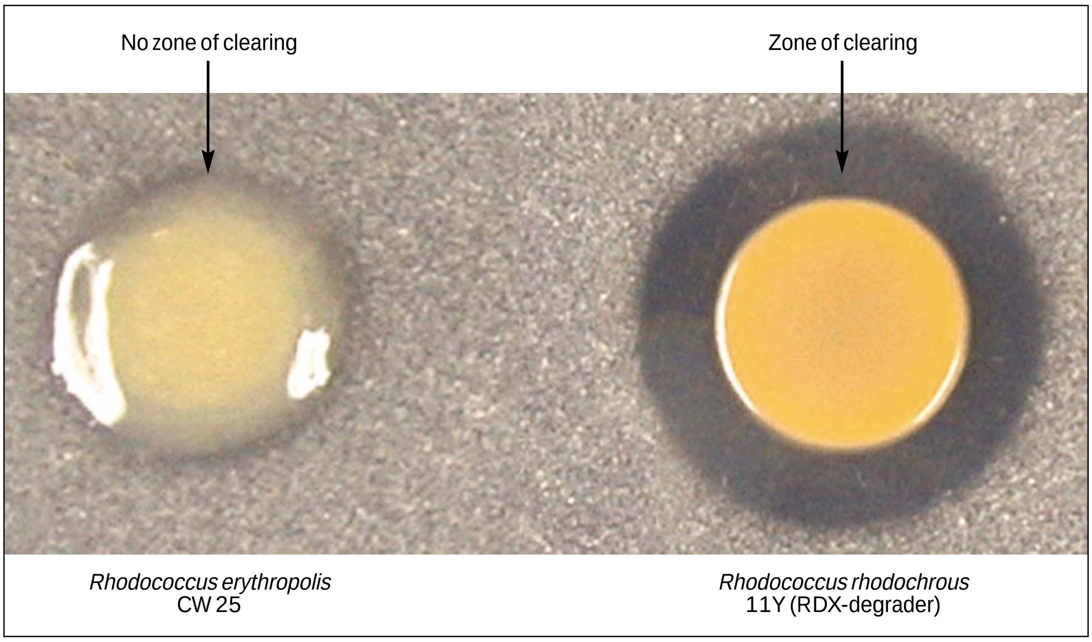
PETN and nitroglycerin. *Enterobacter cloacae* PB2 was isolated from explosives-contaminated soil in the laboratory on the basis of its ability to grow with nitrate ester explosives, such as PETN and nitroglycerin, as the sole nitrogen source. Characterization of the enzyme

responsible for the ability to transform PETN and nitroglycerin showed that the pathway was mediated by an NADPH-dependent reductase, designated PETN reductase, which reductively liberated nitrite from these compounds. The structural gene encoding PETN reductase was subsequently cloned and over expressed in *Escherichia coli* and the same reduction of PETN and nitroglycerin was observed. PETN reductase has now been characterized in considerable detail and the crystallographic structure of the enzyme was published in 2001. The ability of the innate bacterium and the recombinant species expressing PETN reductase was identified as having considerable potential for assisting in the bioremediation of explosives contaminated soils and groundwaters.

TNT. TNT has been found to be extremely resistant to breakdown by soil micro-organisms. This in part is due to the physio-chemical characteristics of the molecule that differ significantly from those of other explosives. The π electrons of the aromatic ring system are withdrawn by the nitro groups, making the nucleus electron-deficient and resistant to electrophilic attack. This has a profound effect on the mechanisms by which TNT can be transformed in the environment. The electron-deficient nature of the molecule makes it resistant to attack by oxygenases, the primary mode of attack by bacteria on organic pollutants. The electronic nature of TNT also means that it is readily bound in the soil to humic materials and its mobility and bioavailability (availability to the soil degrading community) within soil systems is thus drastically reduced. Reduction in bioavailability can result in the biomass never becoming exposed long enough in the environment; as a result catabolic pathways for the degradation of TNT may never actually evolve.

Nevertheless, some progress has been made to identify primary modes of attack on TNT. It is well established that TNT can be reductively transformed into Meisenheimer complexes, azoxydimers and diamino-nitrotoluenes, but these compounds have been shown to be resistant and persistent in the environment, being unsurprisingly termed dead-end metabolites. No aerobic bacteria had been isolated that could transform TNT beyond these products; we were surprised to find that PETN reductase could reduce TNT to a hydride–Meisenheimer complex, which was further reduced to dihydride–Meisenheimer complexes, which were yet further reduced to unknown products. Both purified and recombinant *E. coli* expressing PETN reductase were able to liberate nitrogen as nitrite from TNT, showing that, despite its recalcitrant nature, TNT could be degraded under reductive conditions.

LEFT: **Fig. 2.** Open detonation.
COURTESY DR DAVID J. WOOD, QINETIQ

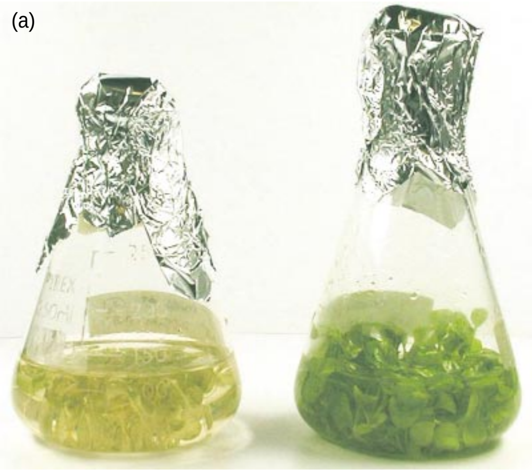


BELOW: **Fig. 3.** Zones of clearing.
COURTESY DR AMRIK BASRAN, UNIVERSITY OF CAMBRIDGE

Plants – phytoremediation potential

Recent attention in the group has focussed on phytoremediation as an approach to cleaning up high-explosive-contaminated environments. Plants possess the ability to extract compounds from the surrounding environment and their root systems are generally extensive, subsequently promoting increased microbial numbers and activity in the rhizosphere. It has been shown, for example, that this unique interaction between plants and bacteria can produce an increase in microbial biomass of an order of magnitude comparable with that of microbial populations in bulk soils. Plants sustain large microbial populations in the rhizosphere by secreting carbohydrates and amino acids through root epidermal cells and by the sloughing of root epidermal cells. The actual composition of the microbial community is a function of root type, plant species, soil

RIGHT:
Fig. 4. (a) Nitroreductase (NR) seedlings grow well compared to wild-type seedlings in TNT-spiked liquid medium. (b) Stunted seedling root growth of wild-type compared to the NR transgenic line when grown on TNT-spiked agarose.
COURTESY NERISSA HANNINK, UNIVERSITY OF CAMBRIDGE



Further reading

French, C.E., Nicklin, S. & Bruce, N.C. (1998). Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. *Appl Environ Microbiol* 64, 2864–2868.

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Hannink, N., Rosser, S.J., French, C.E., Basran, A., Murray, J.A.H., Nicklin, S. & Bruce, N.C. (2001). Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase. *Nat Biotechnol* 19, 1168–1172.

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type and exposure history. This interaction between plants and microbial communities in the rhizosphere is complex and has evolved to the mutual benefit of both organisms.

Despite this unique synergistic interaction, previous research has shown that innate biodegradative abilities of plants are less effective than those of adapted or recombinant bacteria alone. However, it was considered that since plants can cover a vast surface area and require low maintenance, then their incorporation into a remediation facility should be encouraged. This raised the interesting question therefore, of whether the biodegradative abilities of bacteria could be combined with the high biomass and stability of plants to yield an optimal system for *in situ* bioremediation of explosives. Seeds from transgenic tobacco plants expressing either PETN reductase or an aromatic reductase were able to germinate and grow in the presence of normally toxic levels of TNT. These concentrations inhibited germination and growth of wild-type (non-recombinant) seeds (Fig. 4a). Root growth of the resultant seedlings was severely stunted in the wild-type, although growth of the transgenic plant lines was prolific (Fig. 4b). Importantly, hydroponic studies showed that these transgenic plant lines could remove and sequester TNT from the medium. This suggests that transgenic plants expressing microbial degradative genes could be used for the bioremediation of explosive contaminated land. By harnessing the unique co-operative interaction between plant and bacterium the field of explosives remediation has been driven forward. Further investigations are currently underway to determine the ability of transgenic plants to remove TNT from soil aged under laboratory conditions.

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