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Executive Summary

The work in D3.4 seeks to produce engineered microbial strains for the degradation of problematic pollutants persisting in soil and ground water. The work focuses on engineering the bacterium *Pseudomonas putida* KT2440 as it is an industrially relevant microbial chassis with an extensive molecular toolkit available for its genetic manipulation. Key pollutants to target have been selected based on analysis of sites identified in WP1, combined with a search of the relevant literature to identify known metabolic pathways for degradation. Initial target compounds feature the pesticides lindane and atrazine, and additionally, polycyclic aromatic hydrocarbons (PAHs) are being investigated. Efforts will focus on the production of genetic constructs harbouring degradation pathways of target compounds. Engineered strains will be tested *in vitro* to validate recombinant degradation systems, before undergoing *in situ* microcosm assays in collaboration with UBU to evaluate the degradation abilities of the engineered microbial strains in a soil matrix. Data generated in the characterisation of engineered strains will be used in Task 2.2 to refine and improve Genome-Scale Metabolic Models (GEMs) initially constructed based on information available in public databases.

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Table of Abbreviations

Abbreviation	Definition
°C	Degrees Celsius
µg	Microgram
µL	Microlitre
ABC	ATP-binding cassette
bp	Base pairs
CDS	Coding sequence
CNB-CSIC	Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas
g	Gram
GEMs	Genome-Scale Metabolic Models
h	hour
IDT	Integrated DNA Technologies
kan	Kanamycin
LB	Lysogeny broth
MCS	Multiple cloning site
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
msfGFP	Mono-function superfolder green fluorescent protein
NCBI	National Centre for Biotechnology Information
OD	Optical density
oriT	Origin of transfer
PAH	Polycyclic aromatic hydrocarbons
PIA	Pseudomonas isolation agar
RBS	Ribosome binding site
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
WHO	World Health Organisation

1 Introduction

1.1 Overall strategy

The initial target pollutant is the γ-hexachlorocyclohexane isoform of lindane which has been used historically in agriculture as a broad-spectrum insecticide to target invertebrate pests. The World Health Organisation (WHO) classify lindane as 'moderately hazardous' due to its persistence in the soil following use and its neurotoxic effects[1]. In humans, lindane has been observed to affect the nervous system and impact liver and kidney function, as well as exhibiting carcinogenic effects. Although its use is now banned in most countries, its persistence in soils and groundwater mean that it still poses significant risk, particularly in areas of former production sites, and in certain locations where spillage and dumping has historically occurred. Within the BIOSYSMO project, soil and water samples obtained from Site 13 carry heavy lindane contamination, resulting from a lindane factory previously located on that site.

Current methods for the treatment and management of lindane-polluted soil and water involve excavation or pumping of contaminated sites and subsequent containment or disposal in dedicated landfill sites, however, this does not destroy or remove the contamination from the soil or water matrix itself. Combustion of contaminated waste is an alternative treatment method; however, it offers poor cost effectivity, and the combustion process also has the potential to release harmful toxins such as dioxins and furans. An alternative to these methods is to take a bioremediation approach which is the goal of BIOSYSMO. Biological treatment methods offer the greatest promise as a means to degrade lindane to lower toxicity degradation products, and also have the potential to accumulate compounds in biomass which can then be removed from affected sites.

There are a limited number of microbial species which exhibit lindane degradation abilities, many of which belong to the *Sphingomonas* and *Sphingobium* genera. In *Sphingobium japonicum* UT26, 15 genes have been identified dispersed within the genome which together permit *S. japonicum* to utilise lindane as a sole source of carbon and energy[2]. This includes 11 genes involved in the degradation of lindane through to acetyl-CoA (**Fig. 1**), and an additional four genes (*linKLMN*) comprising an ABC transporter system.

One of the goals of T3.4 is to engineer the model organism *Pseudomonas putida* KT2440 for the biodegradation of the pollutant lindane. There is a well-established toolkit for the genetic engineering of *Pseudomonas* spp. and moreover, *P. putida* is a popular chassis for industrial biotechnology with a high tolerance to physicochemical stresses[3].

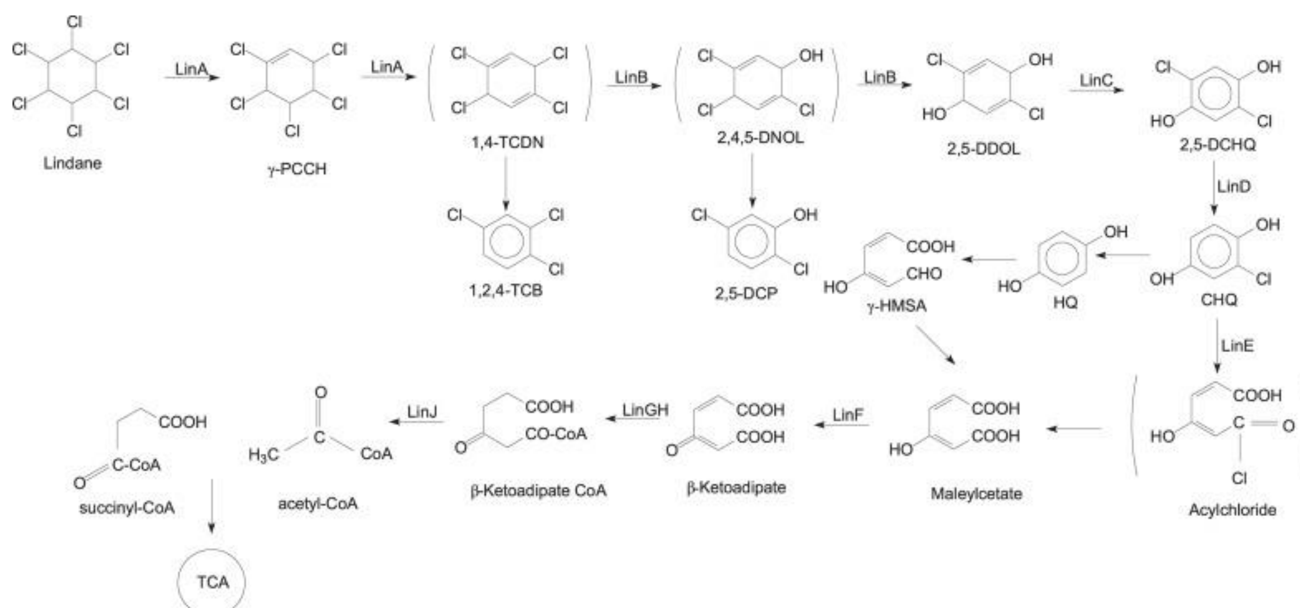

Figure 1. Lindane degradation pathway from *S. japonicum* UT26 [4].

2 Materials and Methods

2.1 Synthetic construct design

2.1.1 *In silico* design strategy

The nucleotide sequences for the genes comprising the lindane degradation pathway and ABC transporter system in *S. japonicum* UT26 were obtained from the National Centre for Biotechnology Information (NCBI). The Integrated DNA Technologies (IDT) Codon Optimization Tool (https://eu.idtdna.com/pages/tools/codon-optimization-tool) was used to generate coding sequences (CDSs) optimised for expression in *P. putida*. The 15 bp region upstream from each CDS was taken from the *S. japonicum* genome to include the native ribosome binding site (RBS) upstream of each gene. All genes and RBSs were arranged successively orientated in the forward direction. The constitutive pEM7 promoter from *P. putida* was included upstream of the RBS region preceding LinA. The complete lindane transcription unit, comprising a total of 15 genes with respective RBS regions, was flanked with XbaI sites for insertion into the multiple cloning site (MCS) of the pSEVA221 plasmid which also carries a kanamycin resistance gene (necessary for the selection of positive transformants), and RK2 origin of replication[5] for replication in a broad range of Gram-negative hosts. This resulted in the 16.94 kb plasmid pSEVA221-lindane (**Fig. 2**).

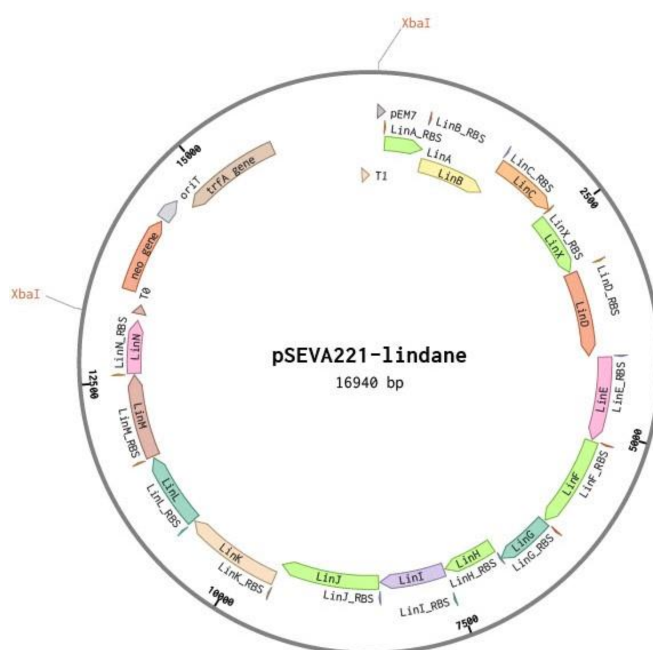

Figure 2. pSEVA221-lindane plasmid map.

2.1.2 Construct synthesis and assembly

The lindane transcription unit was synthesised by GenScript (genscript.com) using the GenBrick synthesis service with subcloning into the pSEVA221 vector. The plasmid vector was prepared using the QIAGEN Plasmid Midi Kit following the Quick-Start Protocol; the DNA quality and yield analysed by BioDrop spectrophotometer (biochrom.co.uk); and the plasmid supplied directly to GenScript for subcloning.

2.2 Microbial engineering

2.2.1 Transformation of *Escherichia coli* chemically competent cells

The synthetic DNA construct supplied by GenScript (pSEVA221-lindane) was delivered to One Shot TOP10 Chemically Competent *E. coli* cells (ThermoFisher Scientific) using the Chemical Transformation Procedure. Positive transformants were selected for by plating transformation mixture onto LB agar (LB Broth with agar (Miller) 40 g/L) supplemented with 50 µg/mL kanamycin and incubating overnight at 37°C. Long-term stocks of the strain were prepared by mixing equal quantities of overnight liquid culture (inoculated with a single colony) and 30 % glycerol in a cryovial for storage at -80°C.

2.2.2 Transformation of *Pseudomonas putida* by tri-parental mating

The pSEVA221-lindane plasmid was delivered to *P. putida* KT2440 *via* tri-parental mating which is preferred for the transfer of large genetic constructs[6]. Briefly, overnight 5 mL cultures of the donor, recipient, and helper (carrying the pRK600 plasmid with the *oriT* required for mobilisation of the target plasmid[7,8]) strains were grown in LB broth (LB Broth (Miller) 25 g/L) with the appropriate antibiotic selection and growth conditions (**Table 1**). Following incubation, 300 µL of each strain were combined in a 1.5 mL microcentrifuge tube and the cells pelleted for 1 min *via* centrifugation. The cells were washed twice in 10 mM $MgSO_4$, and the resultant cell pellet resuspended in 20 µL 10 mM $MgSO_4$ and spotted onto an LB agar plate void of antibiotics. The plate was air dried then incubated overnight at 30°C to allow conjugation to occur. The following day, the cells were scraped from the plate using an

inoculation loop and resuspended in 1 mL 10 mM $MgSO_4$; the suspension was then streaked onto selective medium (M9 agar + 10 mM citrate + 50 µg/mL kanamycin) and incubated at 30°C until transconjugant colonies appeared (1-2 days). M9 agar was prepared by combining 80 mL 5X M9 salts (M9 Minimal salts 56.4 g/L) with 320 mL ddH_2O autoclaved with 6 g agar giving a final concentration of 1.5% agar; additionally, the 400 mL 1X M9 agar was supplemented with 800 µL 1M $MgSO_4$, 40 µL 1M $CaCl_2$, 40 µL 10,000X vitamin solution (nicotinic acid 0.01 g/mL, thiamine 0.005 g/mL, pABA (aminobenzoic acid) 0.001 g/mL, biotin 0.1 mg/mL), 400 µL 1,000X trace elements ($ZnSO_4.7H_2O$ 0.1 g/L, $MnCl_2.4H_2O$ 0.03 g/L, H_3BO_3 0.3 g/L, $CoCl_2.6H_2O$ 0.2 g/L, $CuCl_2.2H_2O$ 0.01 g/L, $NiCl_2.6H_2O$ 0.02 g/L, $Na_2MoO_4.2H_2O$ 0.03 g/L).

Table 1. Strains and growth conditions for tri-parental mating.

Strain	Role	Antibiotic	Temperature
*E. coli*_pSEVA221-lindane	Donor	kanamycin 50 µg/mL	37°C
P. putida KT2440	Recipient	none	30°C
E. coli HB101	Helper	chloramphenicol 34 µg/mL	37°C

2.2.3 Microbial engineering using the pMATING plasmid series

In order to propagate beneficial traits within a microbial community, plasmids conferring the ability to transfer between individuals without the need for a helper strain can be implemented. To initially test the trait-propagation capability of pMATINGα-msfGFP, conjugations were performed using the *E. coli* strain carrying pMATINGα-msfGFP and *P. putida* KT2440. A collection of *E. coli* strains carrying plasmids from the pMATING series were obtained from Victor de Lorenzo's Molecular Environmental Microbiology Laboratory (CNB-CSIC, Madrid)[9]. Briefly, overnight cultures grown in 5 mL LB broth at 37°C and 30°C respectively were adjusted to an OD_{600} = 0.1. 1 mL of each diluted culture was spun to pellet the cells, and the pellet resuspended in 1 mL 10 mM $MgSO_4$. 100 µL aliquots of each strain were combined, 100 µL10 mM $MgSO_4$ added, the cells pelleted again, and the supernatant removed. The resulting pellet was resuspended in 20 µL 10 mM $MgSO_4$ and plated as a single drop onto an LB agar plate void of antibiotics, then incubated overnight at 30°C. Following incubation, cells were collected using an inoculation loop and resuspended in 1 mL 10 mM $MgSO_4$. 10-, 100-, 1,000-, and 10,000-fold dilutions were made and 50 µL of each was spread onto *Pseudomonas* isolation agar (PIA) (*Pseudomonas* isolation agar 45 g/L, glycerol 20 mL/L) supplemented with 50 µg/mL kanamycin then incubated overnight at 30°C.

2.3 Growth assays

2.3.1 Lindane toxicity assay

A range of media were prepared by supplementing 1 x M9 minimal medium (prepared as described in 2.2.2 but without agar) with different concentrations of lindane ranging from 0-5 mM; 0.4% glucose was also included to support growth of untransformed *P. putida* KT2440 lacking the pSEVA221-lindane plasmid. The conditions containing 0 mM lindane represent standard growth conditions used as a control. Growth assays were prepared as 1 mL cultures in a 24-well microtitre plate inoculated with 2 µL of an overnight *P. putida* KT2440 culture grown in LB broth at 30°C with shaking at 180 rpm. Growth in the 24-well plate was monitored at 15 min intervals for 24 h using a CLARIOstar® microplate reader to record absorbance at OD_{600} with incubation at 30°C and shaking at 200 rpm.

2.3.2 Lindane degradation assay

A range of media were prepared by supplementing 1 x M9 minimal medium (prepared as described in 2.2.2 but without agar) with different concentrations of lindane ranging from 0-5 mM to create a minimal growth medium with lindane as the sole carbon source; 50 µg/mL kanamycin was also included to select for *P. putida* KT2440_pSEVA-lindane. Conditions containing 0 mM lindane served as a negative control, in which no growth was expected due to the lack of an accessible carbon source. Growth assays were prepared as 1 mL cultures in a 24-well microtitre plate inoculated with 2 µL of an overnight *P. putida* KT2440_pSEVA-lindane culture grown in LB+kan at 30°C with shaking at 180 rpm. Growth in the 24-well plate was monitored at 15 min intervals for 24 h using a CLARIOstar® microplate reader to record absorbance at OD_{600} with incubation at 30°C and shaking at 200 rpm.

3 Results

3.1 Engineering microbial strains for biodegradation of lindane

3.1.1 Synthesis of pSEVA221-lindane

pSEVA221-lindane was successfully synthesised by GenScript and delivered to *E. coli* for preservation and subsequent delivery to *P. putida.* Colonies containing the plasmid were obtained through growth on selective media (LB agar+kan). Overnight cultures from single colonies were grown in LB broth+kan, combined in equal volumes with 30% glycerol, and stored at -80°C in cryovials for preservation.

3.1.2 Engineering microbial strains for lindane degradation

pSEVA221-lindane was successfully delivered to *P. putida* KT2440 *via* tri-parental conjugation, and transconjugants were obtained through growth on selective medium (M9 agar+citrate+kan) to counter select the donor and helper strains. Overnight cultures from single colonies were grown in LB broth+kan, combined in equal volumes with 30% glycerol, and stored at -80°C in cryovials for preservation.

3.1.3 Lindane toxicity and degradation assays

Preliminary assessment of the lindane degradation system was initially performed *via* growth assays to test for both lindane toxicity and the ability to use lindane as a sole carbon source. Untransformed *P. putida* KT2440 was grown in M9+glucose supplemented with a range of lindane concentrations. Engineered *P. putida* KT2440_pSEVA-lindane was grown in M9 minimal medium supplemented with a range of lindane concentrations as the sole carbon source (**Fig. 3**). Lindane exhibited poor solubility in M9 minimal medium, and as a result, undissolved solids in the medium contributed to a degree of noise in the plate reader measurements. Despite this, in the toxicity assay, the growth of *P. putida* KT2440 in M9+glucose without lindane was comparable to growth where lindane was also included. This suggests that the inclusion of lindane in growth assays at concentrations of 5 mM and below does not have a negative impact in growth.

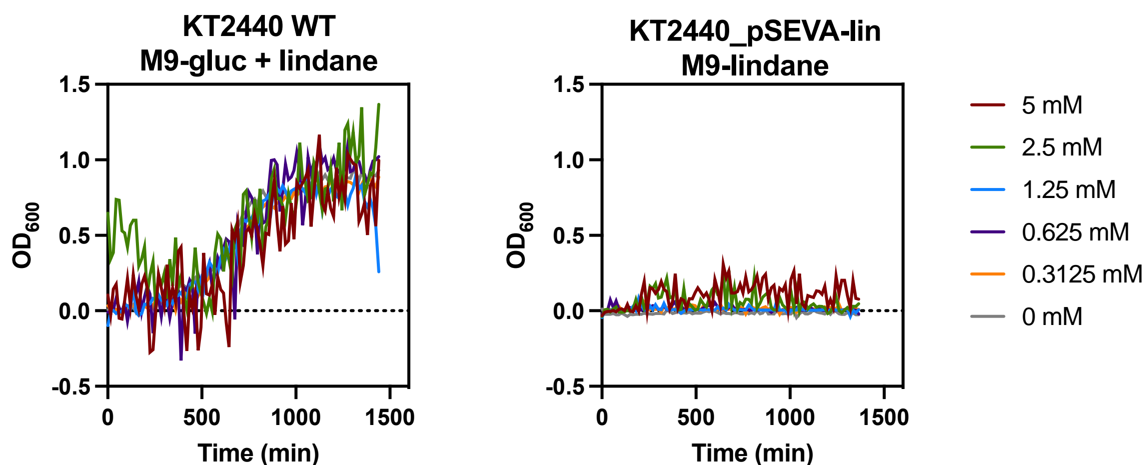

Figure 3. Growth assays to assess lindane toxicity in *P. putida* KT2440 (left), and the ability of engineered *P. putida* KT2440_pSEVA221-lindane to use lindane as a sole carbon source (right).

3.2 Engineering microbial strains for trait propagation

3.2.1 Validation of self-transferring plasmids

The pMATING series of plasmids have been constructed to contain the promiscuous conjugation machinery found in the RP4 plasmid. Microbial strains carrying variants of the pMATING plasmid series are able to transfer these plasmids to other individuals without the need for a helper strain. The long-term application of the pMATING plasmids is to engineer plasmid variants to carry degradation pathways for pollutants of interest, such that desirable traits are able to propagate within microbial communities.

Delivery of pMATINGα-msfGFP to P. putida KT2440

To confirm the activity of the RP4 machinery, the pMATINGα-msfGFP plasmid was used in initial tests to confirm that the plasmid is able to move between microbial strains without the need for a helper strain. The conjugation assay (2.2.3) was performed using *P. putida* KT2440 both with and without the addition of the *E. coli* strain carrying the pMATINGα-msfGFP plasmid. Dilution series of the conjugation mix were plated side-by-side onto M9+citrate and M9+citrate supplemented with kanamycin to select for the engineered individuals (M9-citrate provides an accessible carbon source to *P. putida* but not *E. coli*, so therefore counter selects for the recipient strain). Fluorescent colonies expressing the msfGFP protein were visible only where pMATINGα-msfGFP had been included in the transformation (**Fig. 4**). Comparisons of the plated transformation provide a qualitative indication of the efficiency of the conjugation.

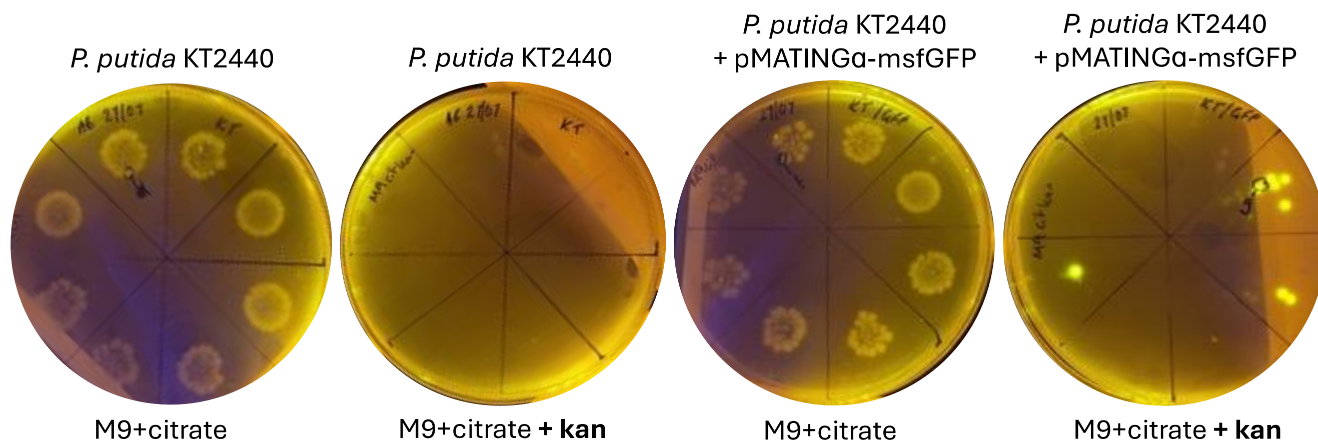

Figure 4. Dilution series of *P. putida* conjugations with and without *E. coli*_pMATINGα-msfGFP plated onto M9+citrate with and without kanamycin supplementation. Colonies expressing msfGFP are visible where the conjugation mixture was plated onto selective medium supplemented with kanamycin (right).

4 Conclusions

The pSEVA221-lindane plasmid has been successfully constructed and delivered to *P. putida* KT2440. The inclusion of lindane in culture medium to a maximum concentration of 5 mM does not have a negative impact on growth of the wild-type strain, however, solubility of lindane in water is poor and there is scope to improve this through the preparation of a stock solution in a more suitable solvent. The engineered *P. putida*_ pSEVA221-lindane did not show evidence of growth using lindane as a sole carbon source in the initial tests. This may be the result of poor lindane solubility reducing its availability as a carbon source; or the strain may require an alternative initial carbon source (e.g., glucose) to permit sufficient cell growth and enzyme production to access lindane as a substrate.

The RP4 propagation machinery present in the pMATING plasmid series has been tested using an *E. coli* strain carrying the pMATINGα-msfGFP plasmid which results in msfGFP expression and kanamycin resistance in successfully engineered individuals. *P. putida* KT2440 was successfully engineered using conjugation without the need for a helper strain. The efficiency of the conjugation was not particularly high, as can be observed by comparing the relative abundance of msfGFP fluorescent colonies in the plated transformations on plates with and without kanamycin.

5 Next Steps

5.1 Engineering microbial strains for biodegradation of lindane

The next steps in generating microbial strains for the biodegradation of lindane will be to continue with the validation and characterisation of *P. putida*_ pSEVA221-lindane. This will first involve modifications to the preparation of growth media containing lindane to improve the solubility and accessibility of the substrate. If growth on lindane as a sole carbon source still proves challenging, media will be supplemented with a low concentration of glucose to help with initial growth and production of the enzymes for lindane degradation. RT-PCR can also be used to confirm expression of all pathway genes in the engineered strain. This information and other growth parameters will then be added to the constraint-based, genome-scale metabolic model of *P. putida* in Task 2.2, to perform *in silico* simulations of the lindane degradation pathway.

Following verification of the lindane degradation pathway, the relevant transcription unit will be cloned from the pSEVA221 backbone into plasmids from the pMATING series using Gibson Assembly. This will result in a plasmid for trait propagation with the ability to move between individuals in a microbial community, without the need for a helper strain. Optimisation of the biparental conjugation method will be performed to improve the transformation efficiency. This will involve varying parameters such as the relative ratios of mating strains, and incubation times. Engineered strains will be shared with UBU to be used in *in situ* laboratory experiments in 200 g scale microcosms from lindane contaminated soil isolated from Site 13.

5.2 Engineering microbial strains for biodegradation of other pollutants

Other problematic pollutants identified in the consortium will also be addressed, and degradation pathways for specific compounds which have been previously reported in the literature will be constructed following the same format as the lindane degradation pathway, to create other microbial strains specialised to target different pollutants of interest. These strains will also be shared with UBU for *in situ* tests in microcosms.

One such additional target compound is the herbicide atrazine which has been identified as a pollutant present at Site 1 (WP1). Atrazine can be degraded enzymatically *via* a six-step pathway (*atzABCDEF*) yielding cyanuric acid which can subsequently provide an accessible nitrogen source for many bacteria (**Fig. 5**)[10]. Other potential target pollutants include the PAHs which are particularly abundant in Site 10. Potential enzymatic degradation pathways will be investigated for fluoranthene and benzo[a]pyrene.

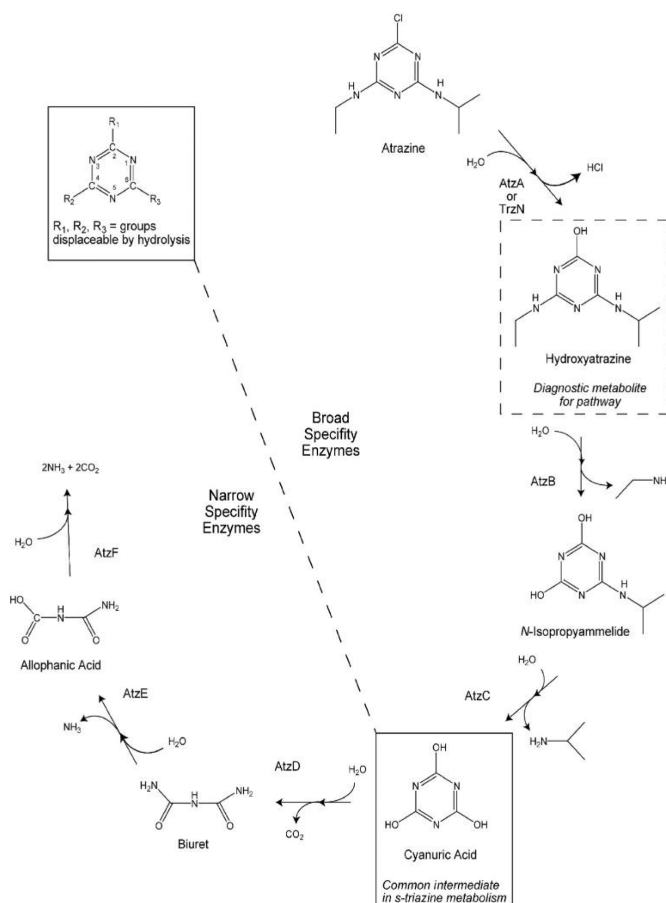

Figure 5. Microbial metabolism of atrazine[10].

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