Strategies for Metabolic Engineering of Environmental Microorganisms: Application to Degradation of Organophosphate Contaminants

Metabolic Engineering Working Group

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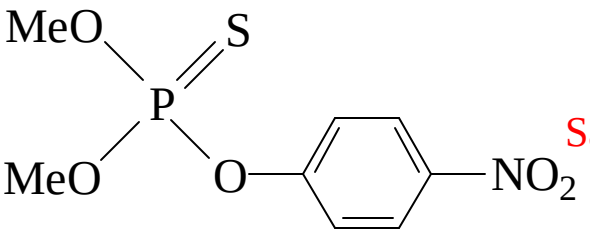
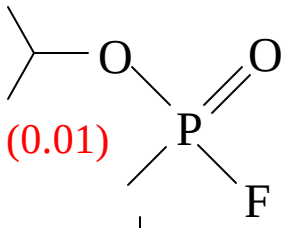
Research Goals

Goal -

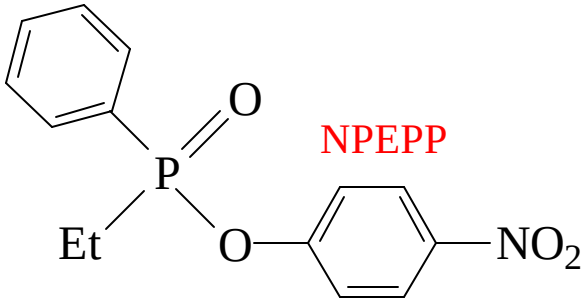
to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants.



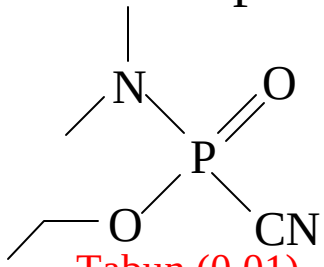
Methyl Parathion (14)



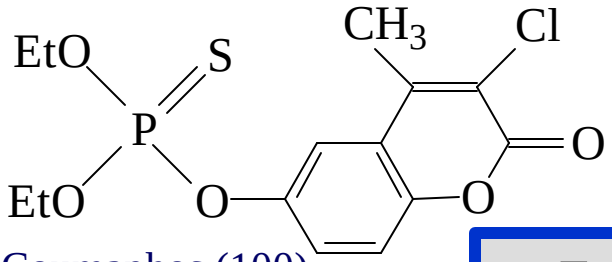
Sarin (0.01)



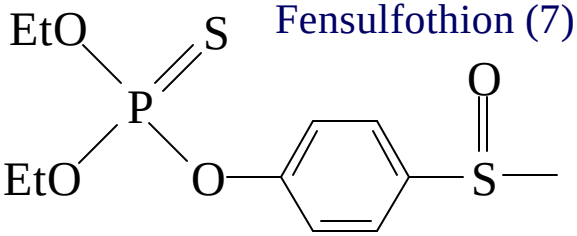
NPEPP



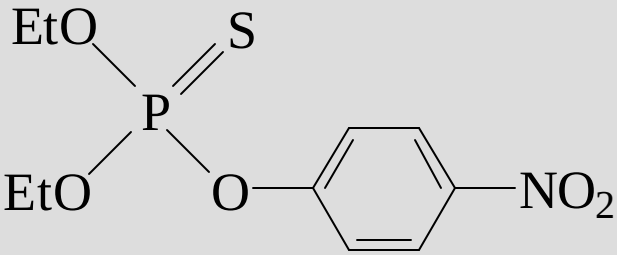
Tabun (0.01)



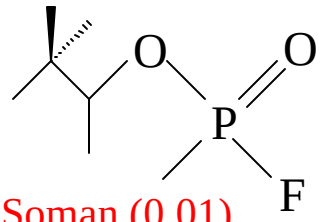
Coumaphos (100)



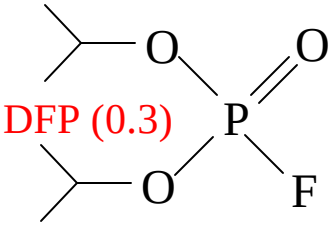
Fensulfothion (7)

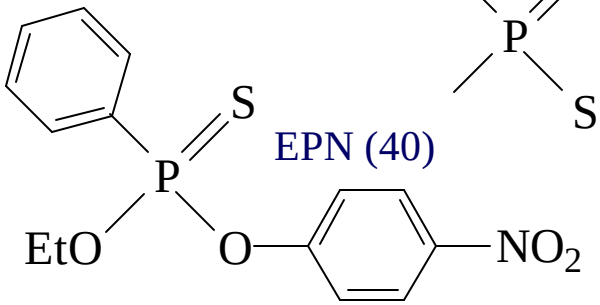


Parathion (LD_{50} 13 mg/kg)

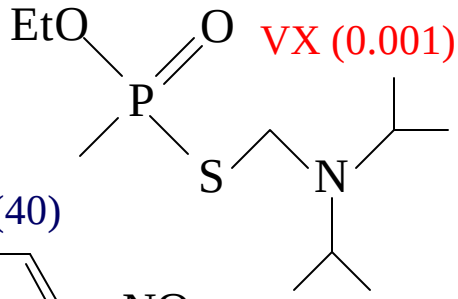


Soman (0.01)

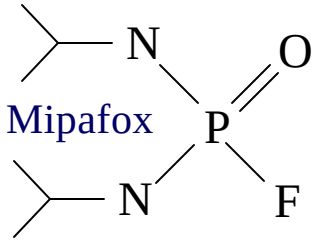


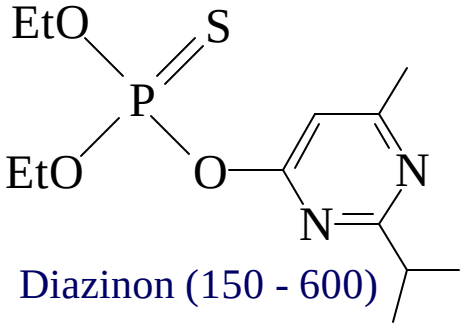


EPN (40)



VX (0.001)





Diazinon (150 - 600)

Justification

Pesticides

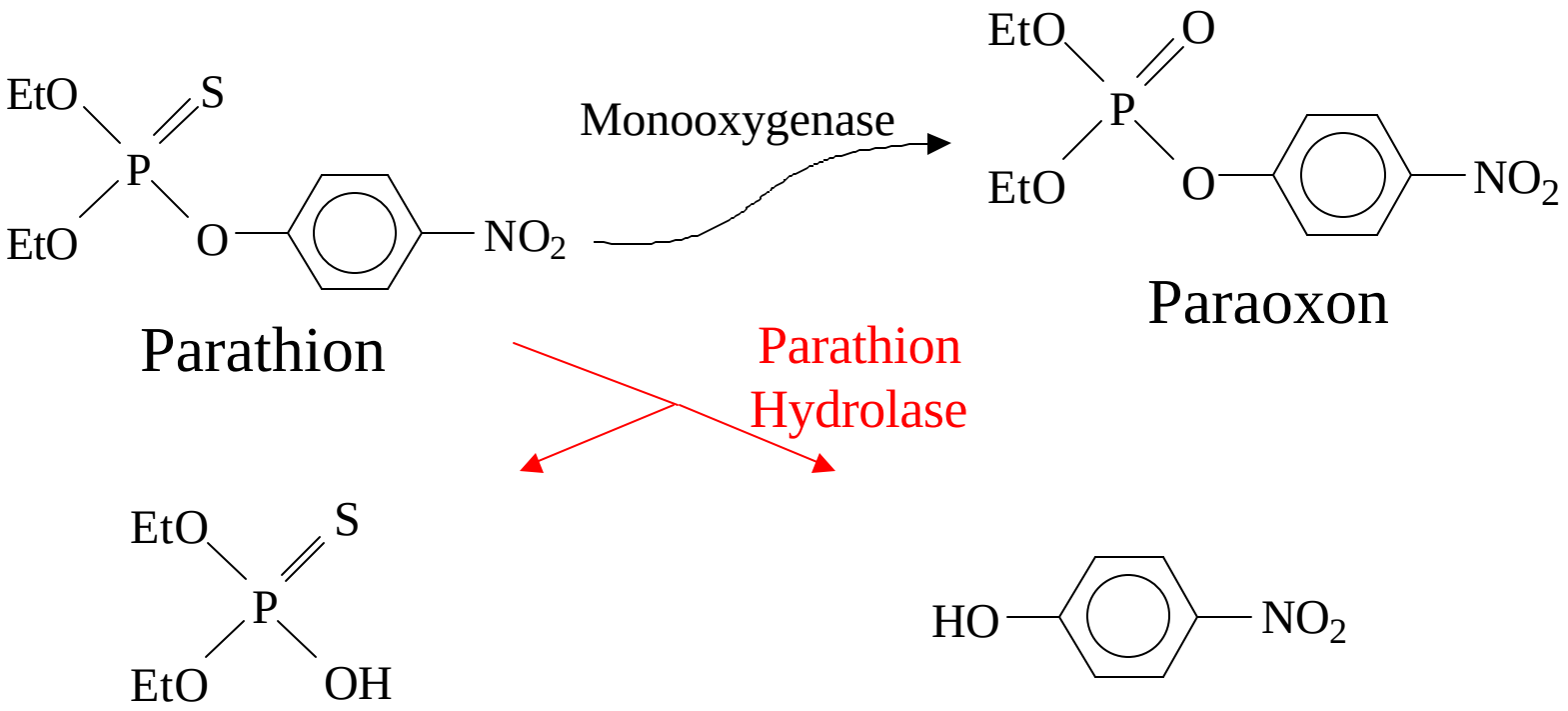
- ~ 60,000 tons of organophosphate pesticides are produced annually in the US
- U.S. Geological Survey reported 54.4% of groundwater sites sampled were contaminated with pesticides (1998)

Chemical Warfare Agents

- Chemical Weapons Convention calls for destruction of all chemical warfare stockpiles (1993)
- 30,000 metric tons of chemical agents to be destroyed in US

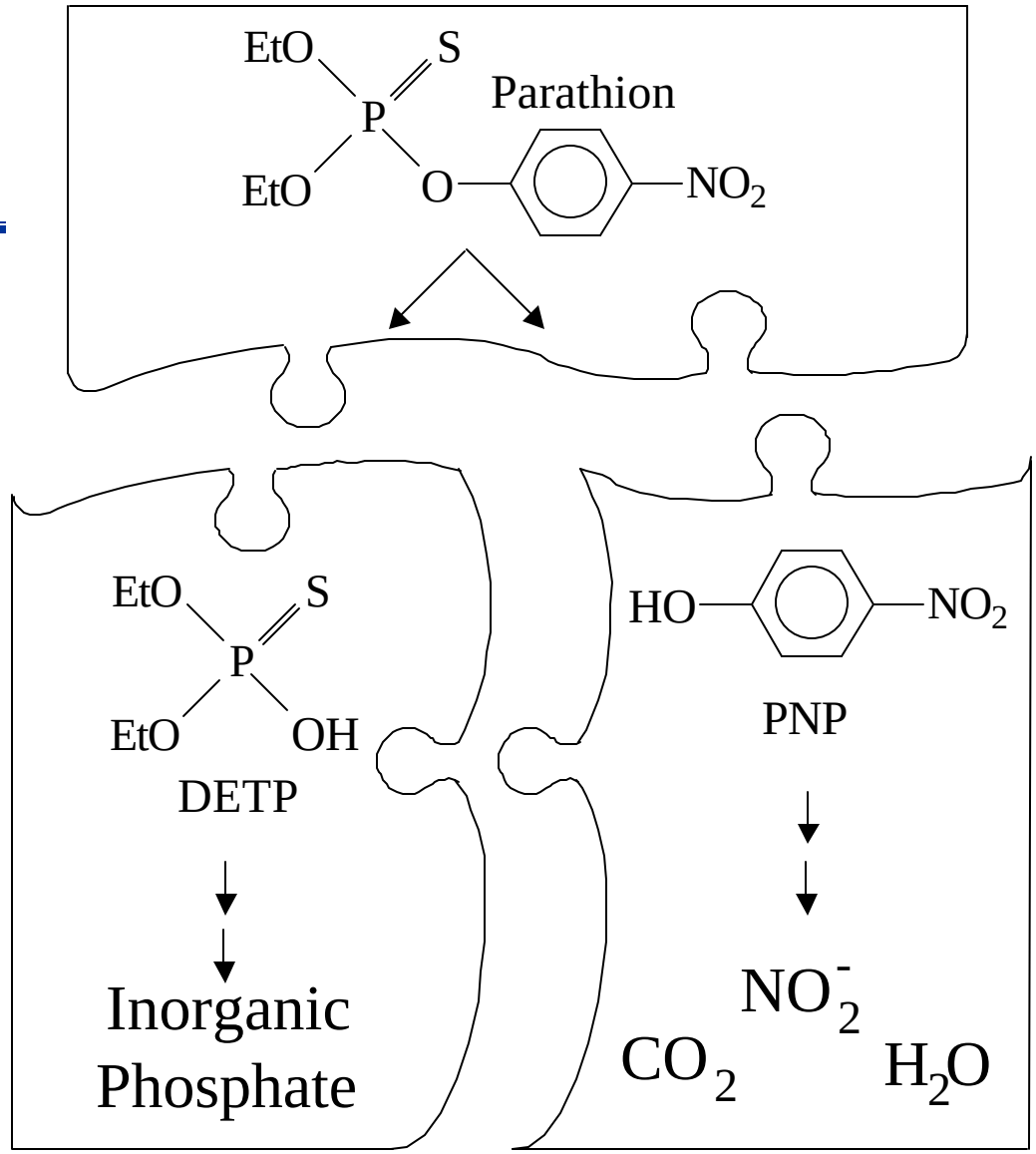
Parathion Degradation Background

- One of the most highly toxic compounds certified by EPA
- 4-7 million pounds are produced annually in the U.S.

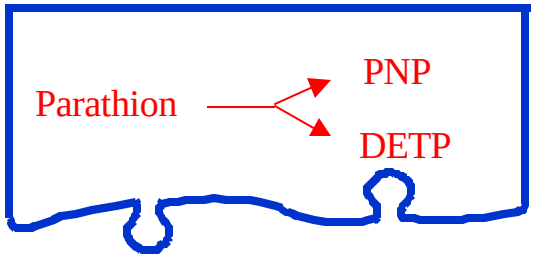


Parathion Degradation

A 3 piece puzzle:



Parathion Hydrolysis

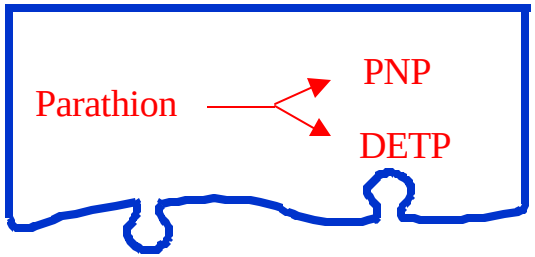


- Past work on parathion degradation has focused on initial hydrolysis
- Gene coding for parathion hydrolase (*opd*) has been cloned & sequenced from both *Pseudomonas* and *Flavobacterium*
- Two forms of *opd*:

Native – contains coding region for N-terminal leader sequence

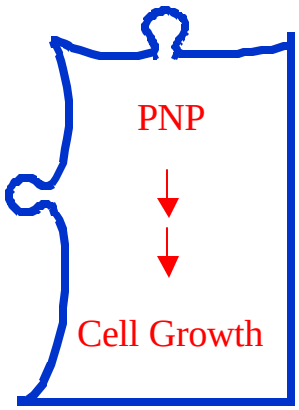
"Modified" – coding region for leader sequenced removed

Parathion Hydrolysis



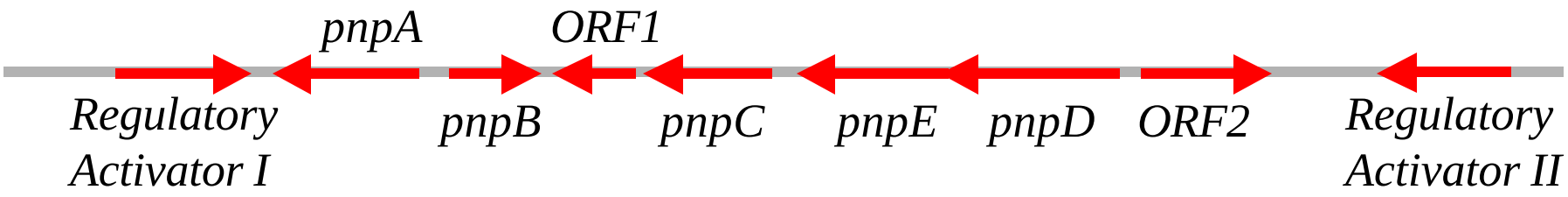
Plasmid:	pAWW01	pAWW02	pAWW04
Promoter:	P_{taclac}	P_{taclac}	P_{tac}
opd gene type:	"modified"	native	native

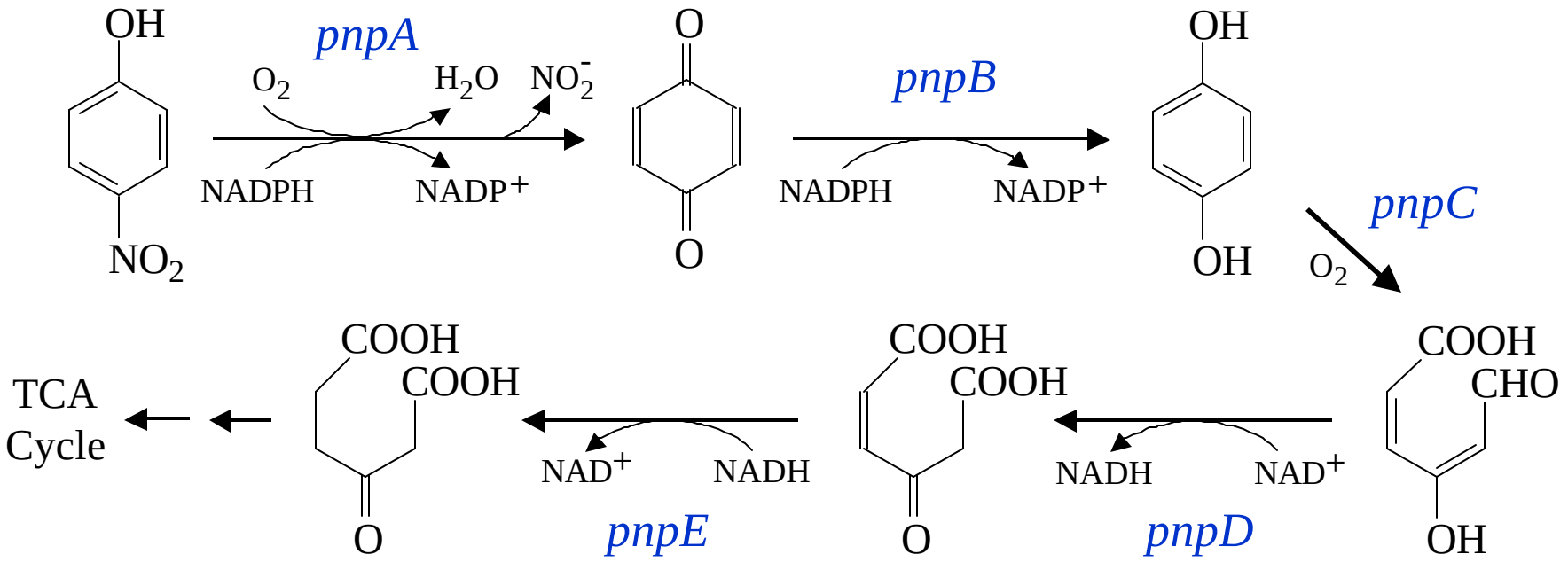
	Spec. Activity (μM/hour-OD)	Spec. Activity (μM/hour-OD)	Spec. Activity (μM/hour-OD)
E. coli DH5α:			
No induction	36.8	3.8	6.3
Full induction	88.5	10.2	13.9
P. putida KT2442:			
No induction	1.7	--	6.9
Full induction	1.8	--	7.3

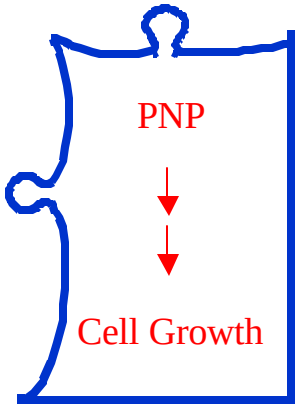


PNP Degradation

Bang & Zylstra isolated 18kb sequence from a Pseudomonad containing PNP degrading genes

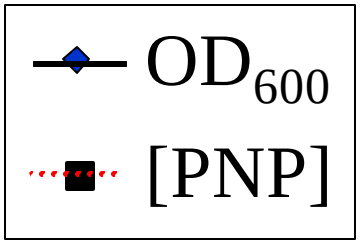


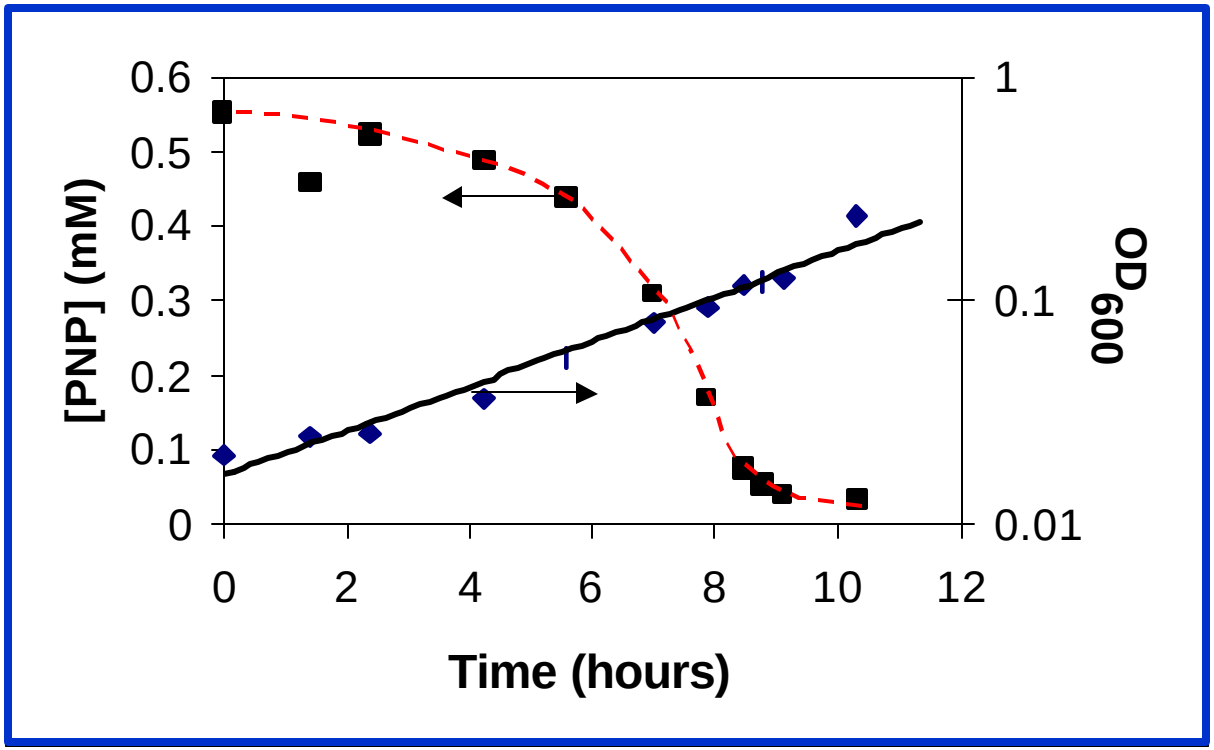




PNP Degradation

PNP degradation by *P. putida* KT2440 with pPNP

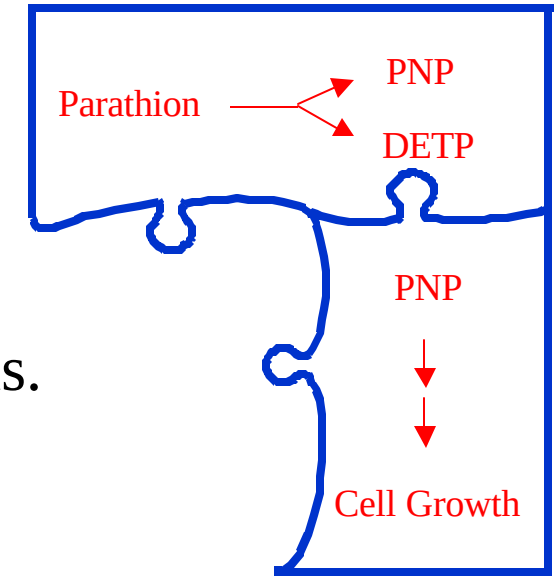




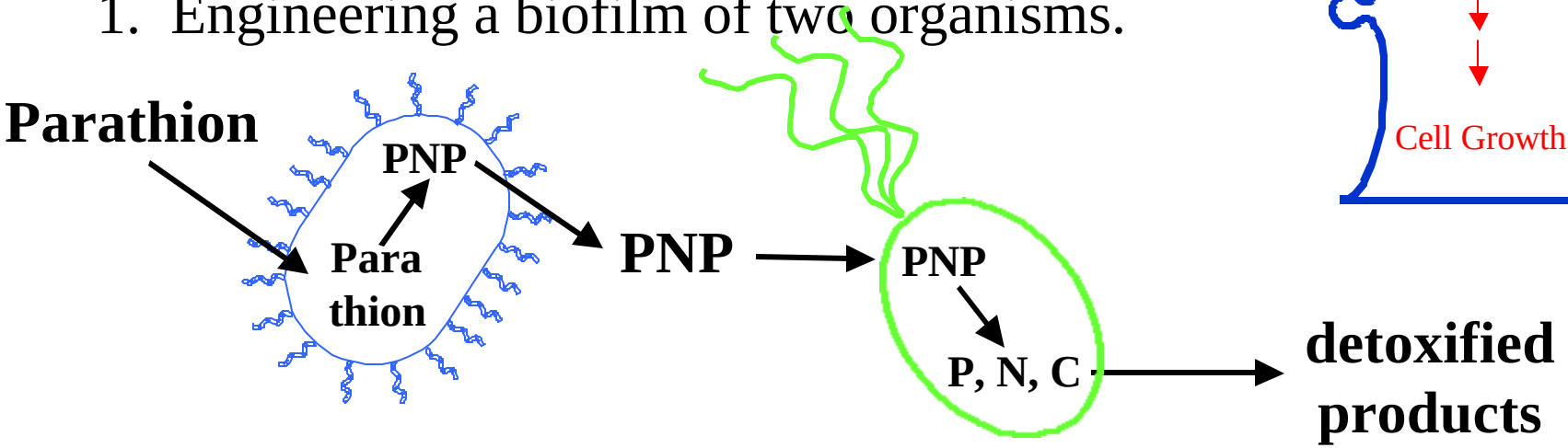
Specific Degradation Rate: 66 µmole/min-gDCW

Specific Growth Rate: 0.23 hour $^{-1}$

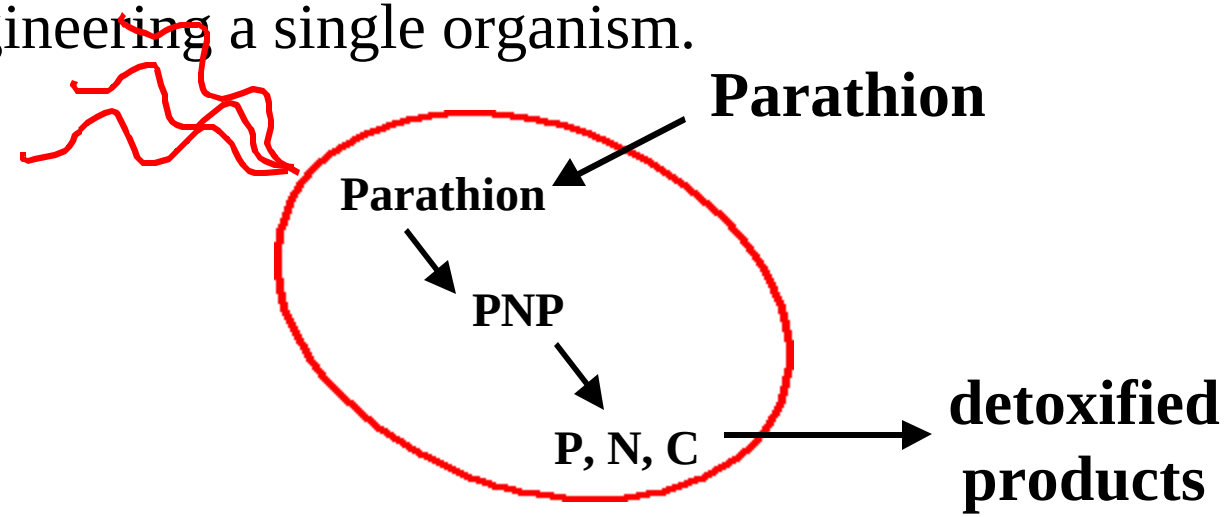
Two ME strategies



1. Engineering a biofilm of two organisms.

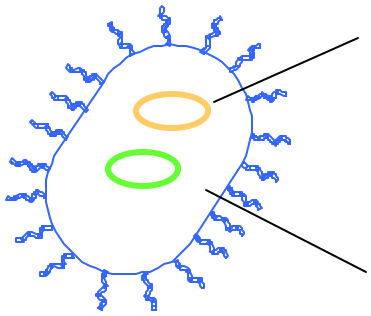


2. Engineering a single organism.



An engineered biofilm

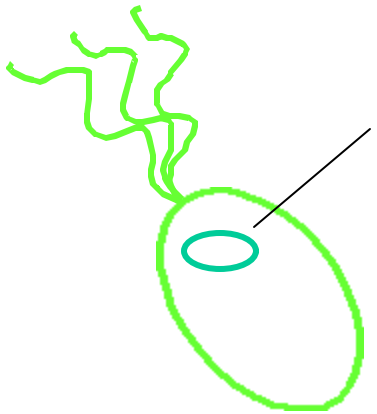
***E.coli* SD2**
hydrolyzes parathion



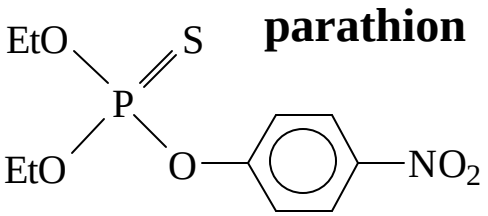
pWM513:
- parathion hydrolysis (*opd* genes)
- ampicillin resistance

pMAG1:
- *gfp* gene
- tetracycline resistance

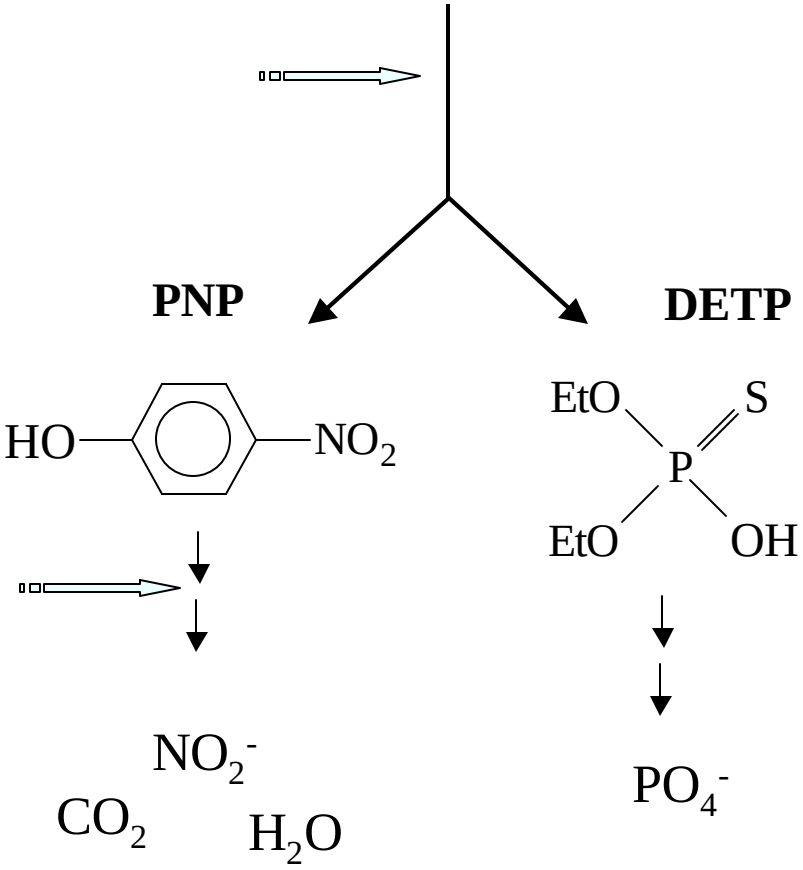
***Pseudomonas* KT2440**
mineralizes *p*-nitrophenol (PNP)



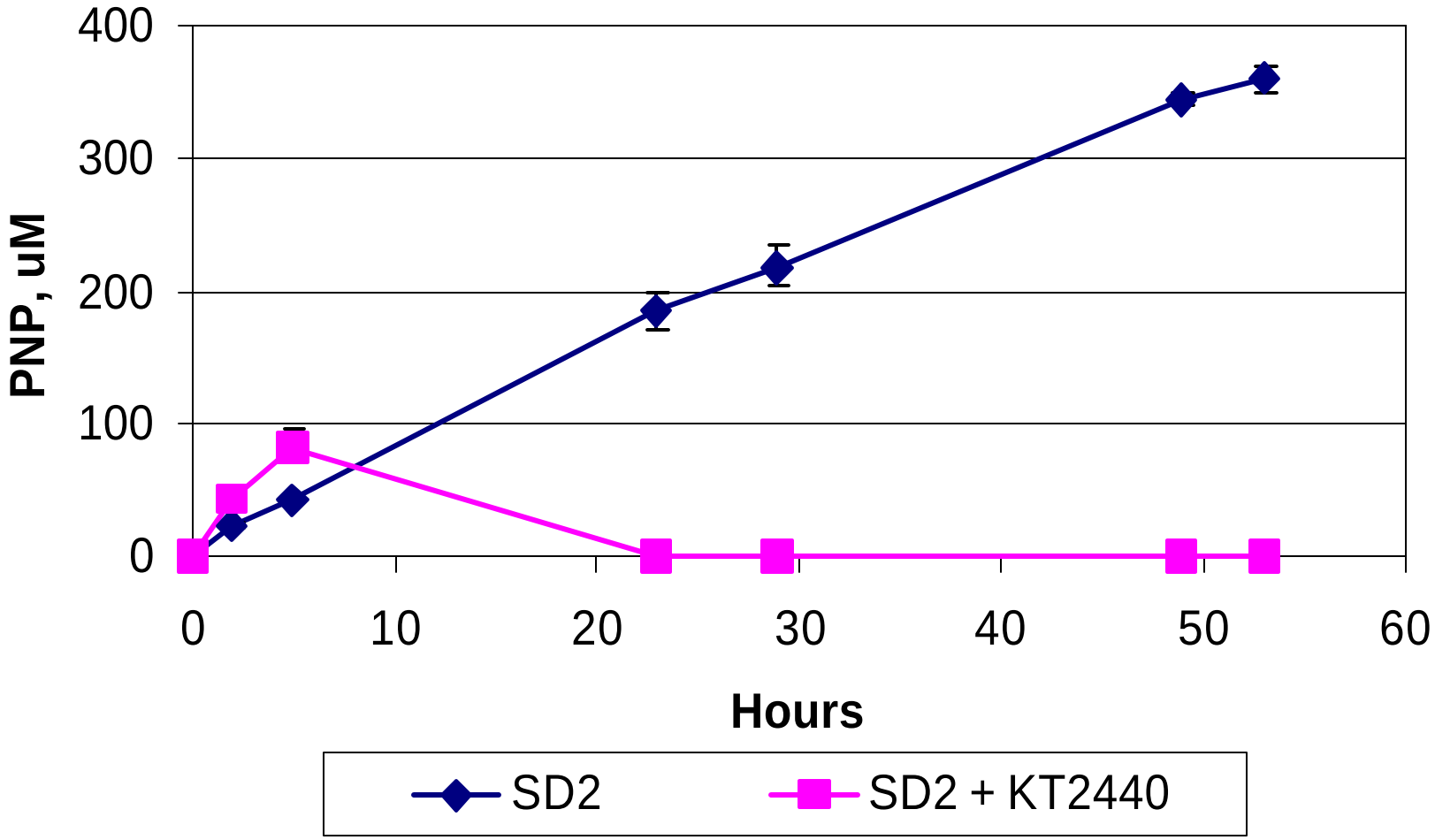
pPNP:
- PNP degradation
- tetracycline resistance
- natural ampicillin resistance



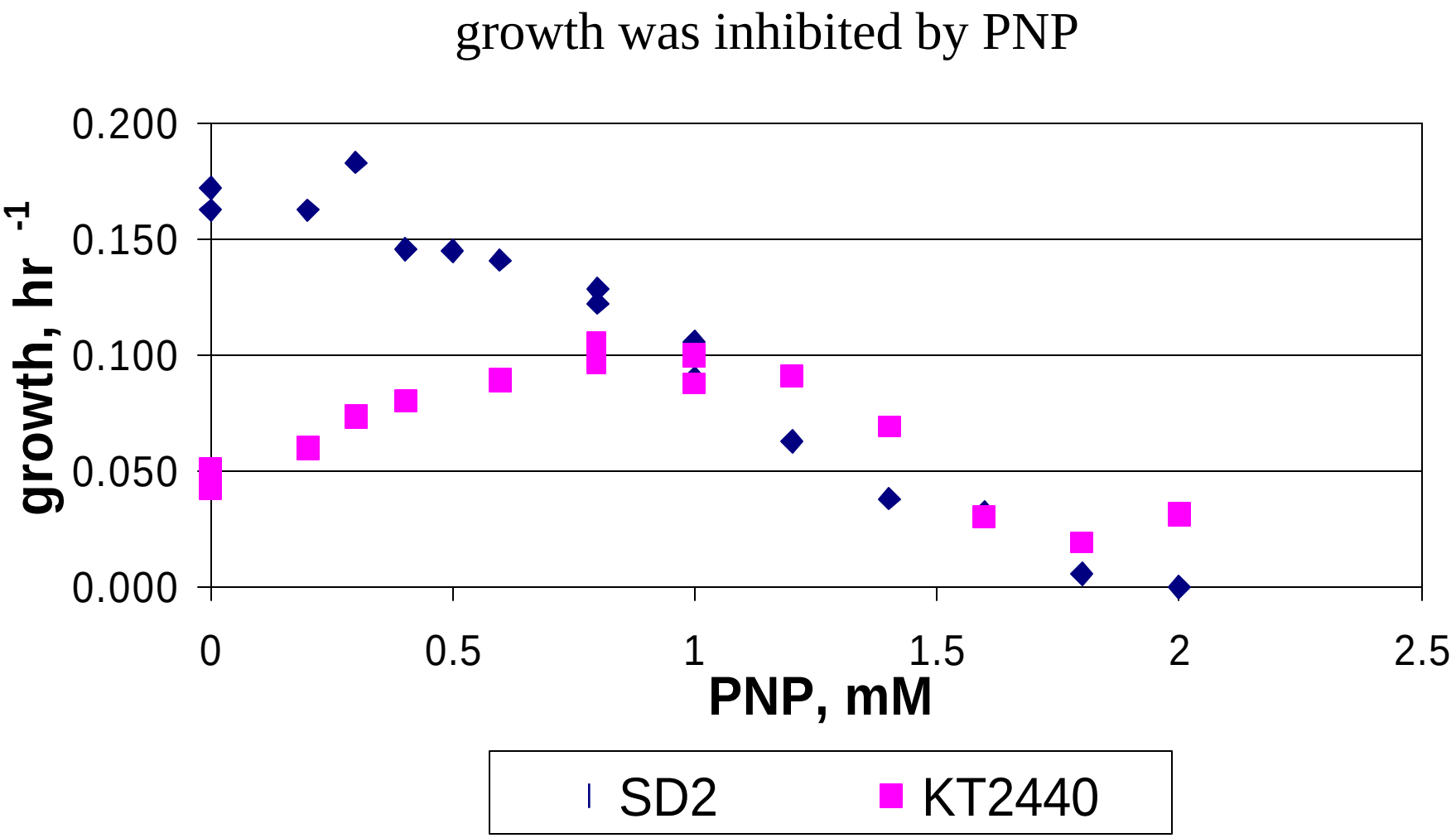
parathion



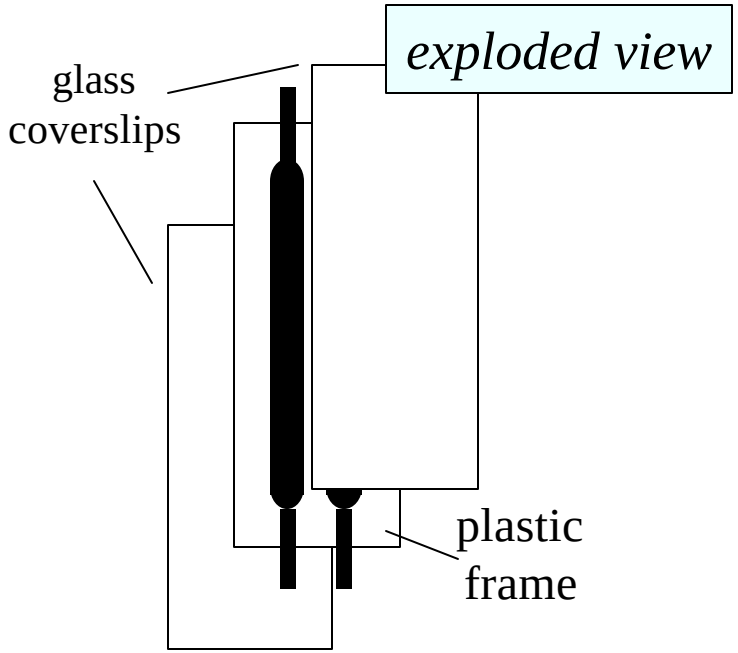
Biodegradation of parathion in suspended culture



Effect of PNP on cell growth

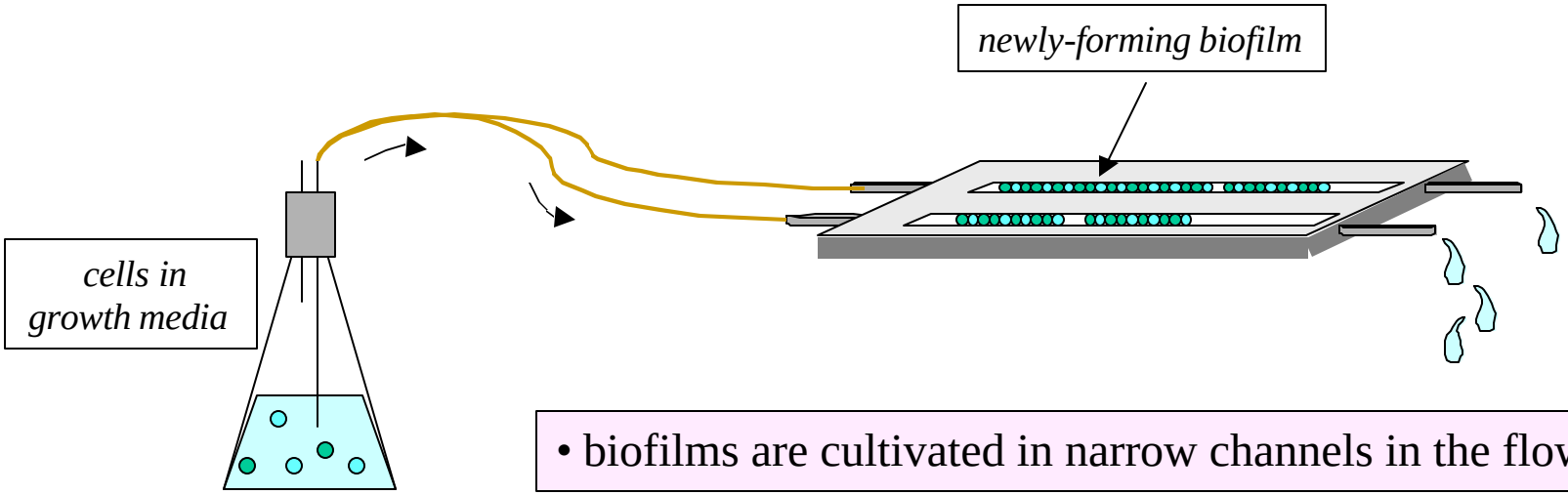


Flow cell for culturing biofilms



measurements

chamber size:
46 mm × 4 mm × 2 mm
chamber volume: 0.37 mL
flow: 0.86 mL min^{-1}
velocity: 41 mm min^{-1}



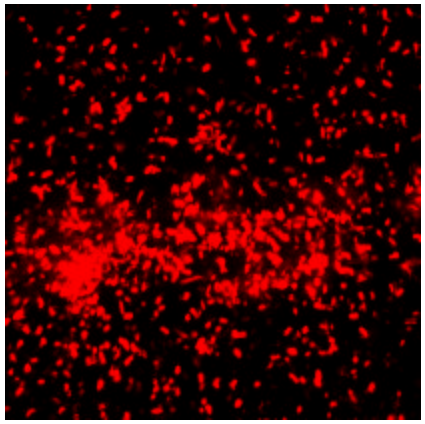
• biofilms are cultivated in narrow channels in the flow cell

Dual labeling with GFP and Syto 59

CSLM red channel

CSLM green channel

merged red and green channels



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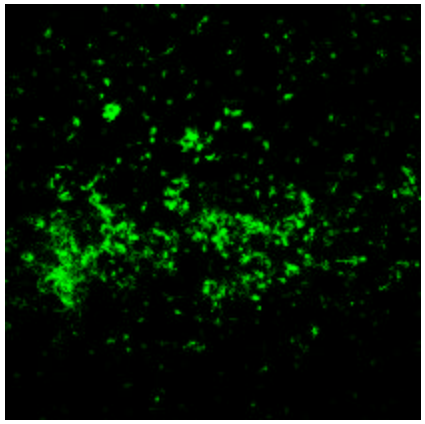
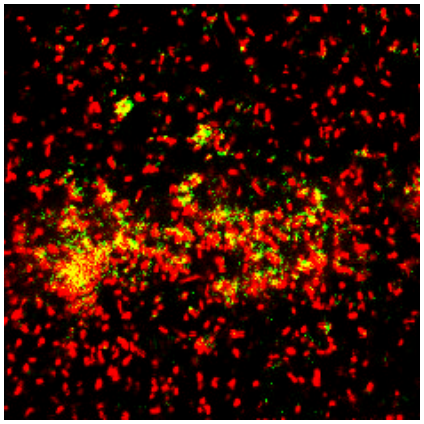
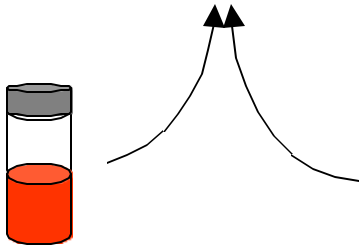




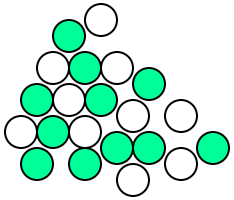
total population

subset of cells containing GFP

two identifiable populations



SYTO 59 red dye



mixed population of cells

= cell with GFP

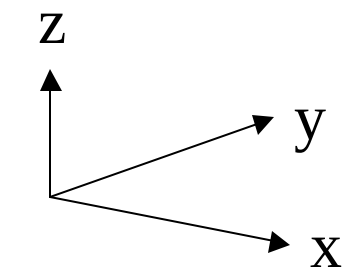
= cell

Development of a coculture biofilm for parathion biodegradation

red: *P. putida* KT2440

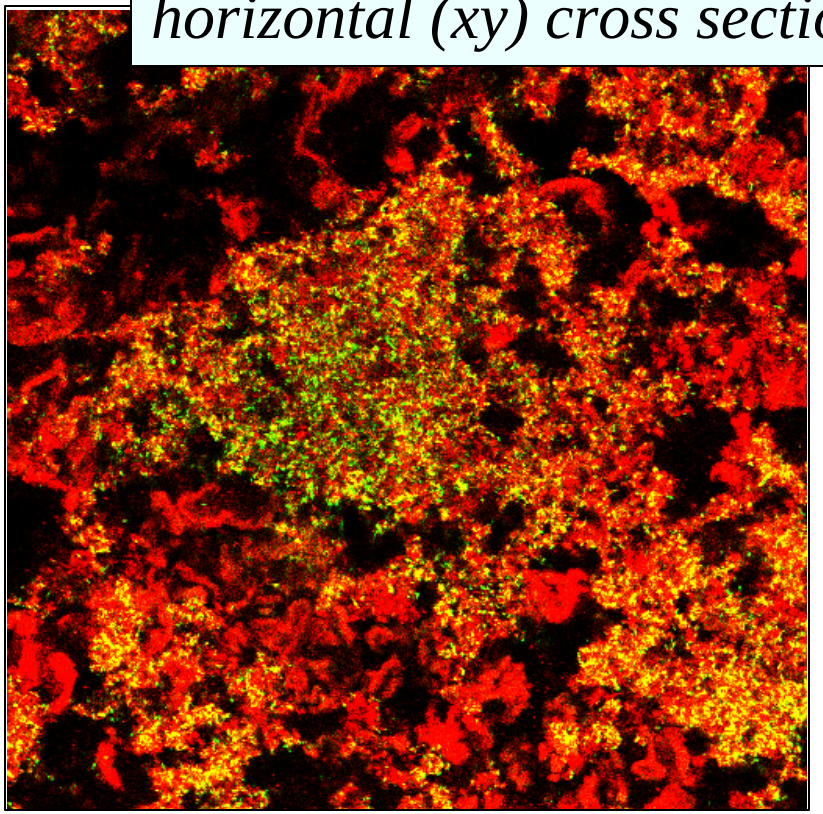
yellow/green: *E. coli* SD2

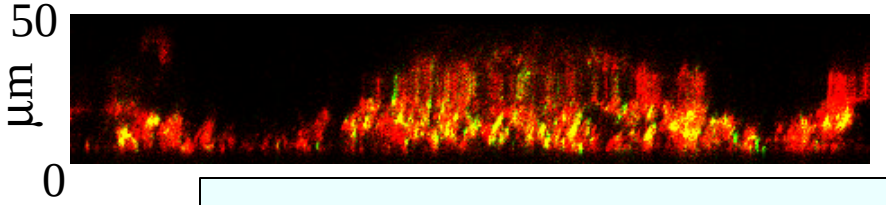
black: voids within the biofilm



orientation

horizontal (xy) cross section

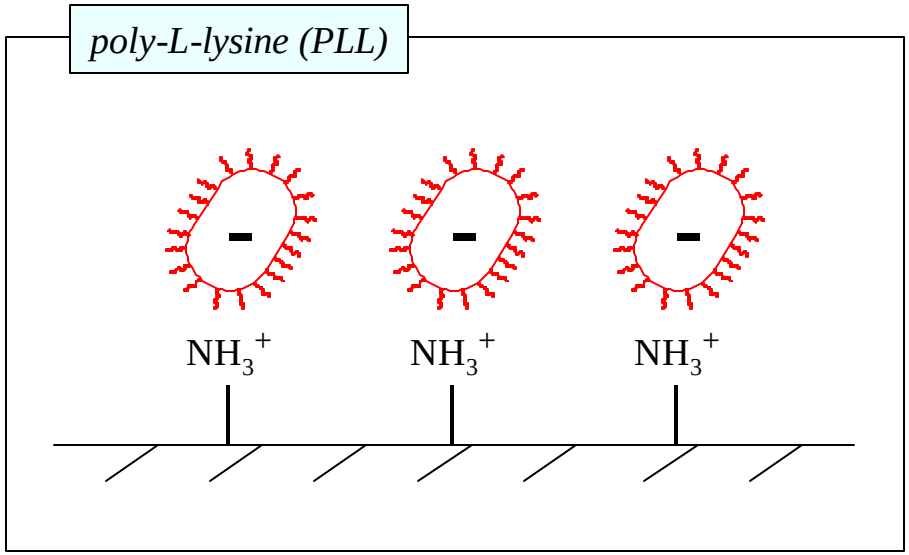




vertical (xz) cross section

Biofilm engineering

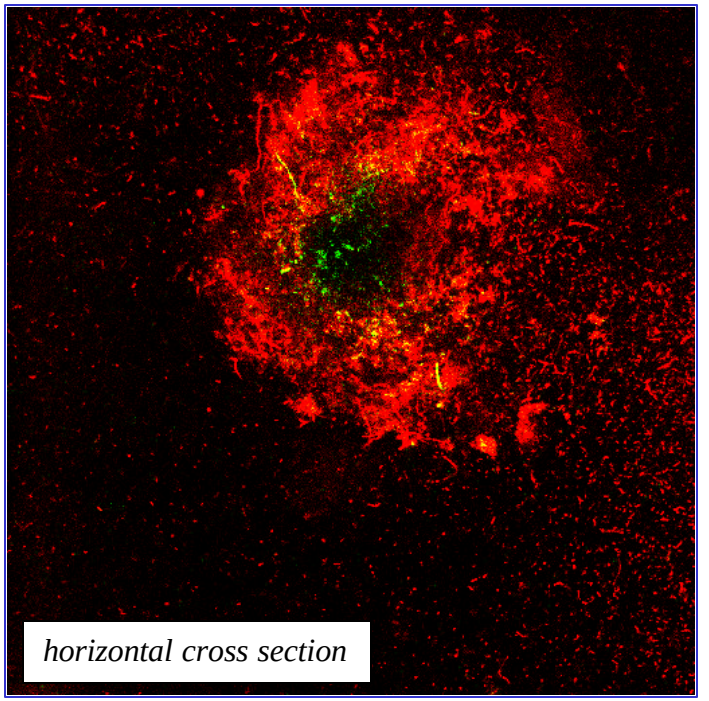
the activity of a biofilm potentially can be optimized by controlling the distribution of microorganisms

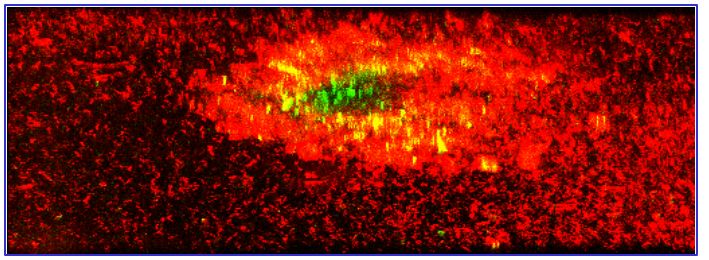


yellow/green: *E. coli* SD2 attached to glass sphere with PLL

red: *P. putida* KT2440

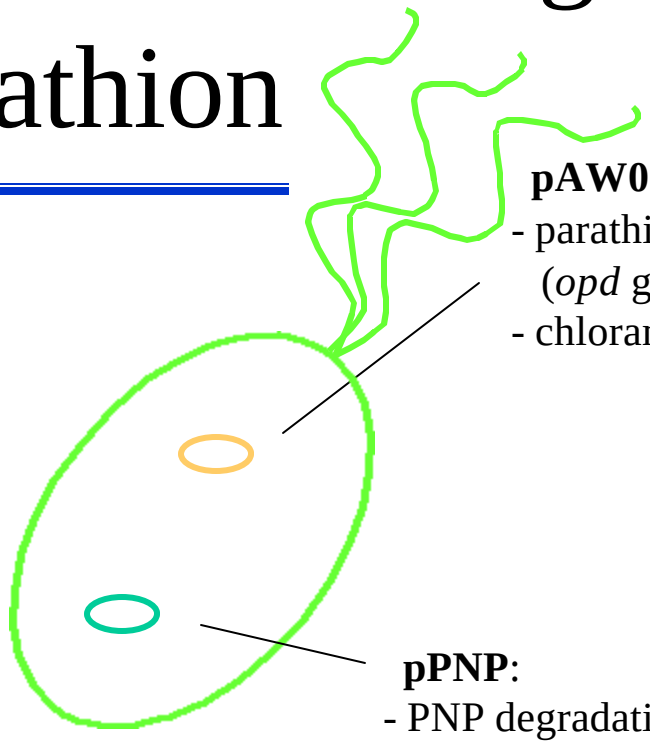
- strains were sequentially applied





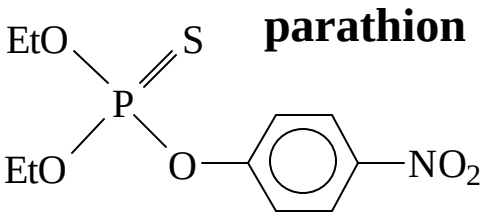
70 degree tilted projection

Engineering a single organism to degrade parathion



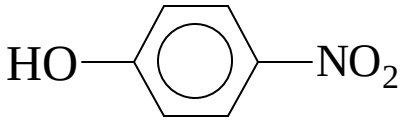
pAW02:
- parathion hydrolysis (*opd* genes)
- chloramphenicol resistance

pPNP:
- PNP degradation
- tetracycline resistance

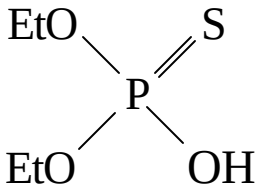


parathion

PNP



DETP



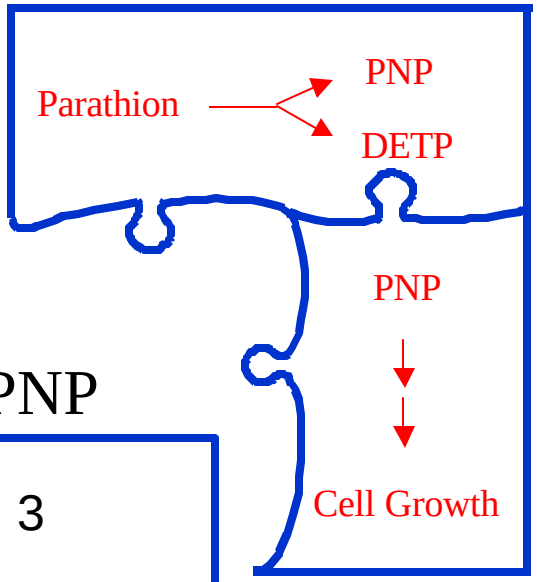
NO_2^-

CO_2 H_2O

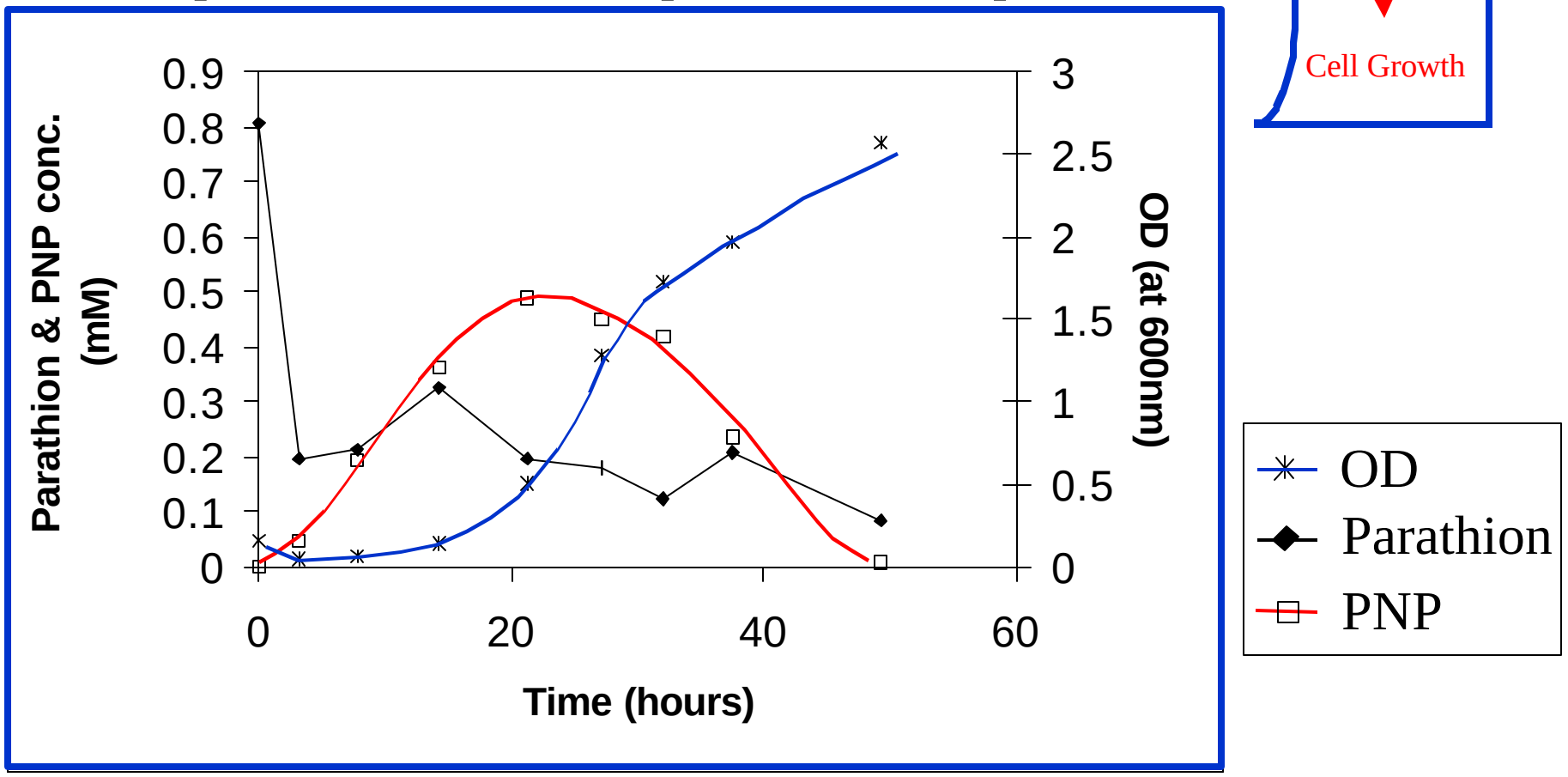
PO_4^-

***Pseudomonas* KT2440**
mineralizes *p*-nitrophenol (PNP)

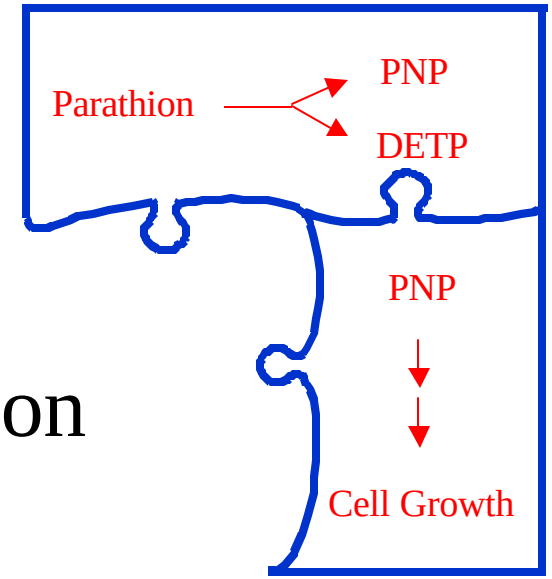
Parathion Utilization as a Carbon Source



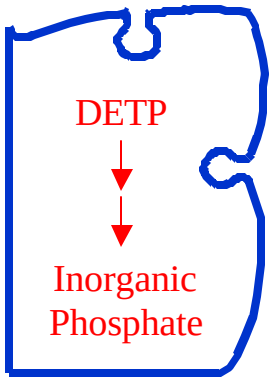
Parathion Degradation by
P. putida KT2442 with pAWW04 and pPNP



Parathion Utilization as a Carbon Source

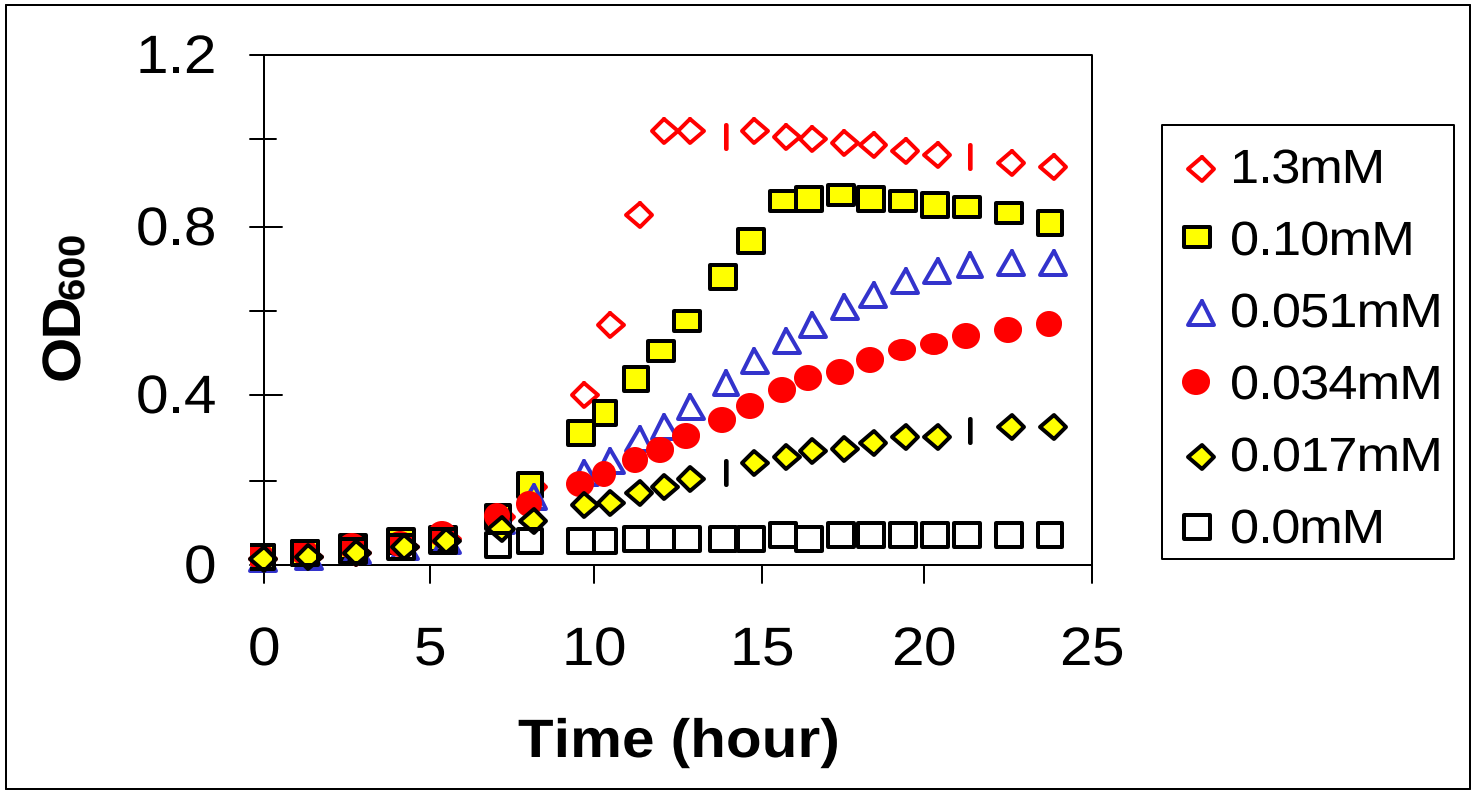


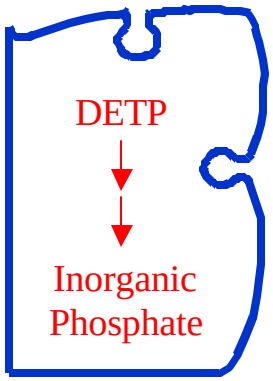
- Parathion is utilized as a carbon and energy source
- Parathion forms DNAPL, but is still bioavailable
- Measurement of aqueous phase parathion concentration is not a good indicator as to whether parathion degradation is occurring



DETP Degradation

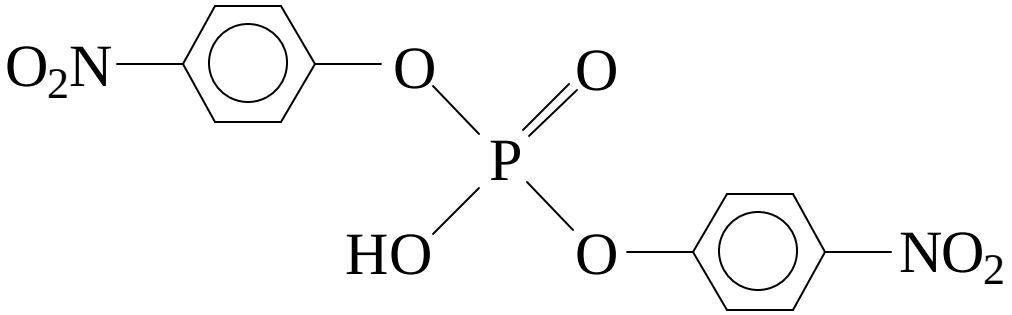
- *Comamonas acidovorans* is capable of utilizing DETP as a P-source:



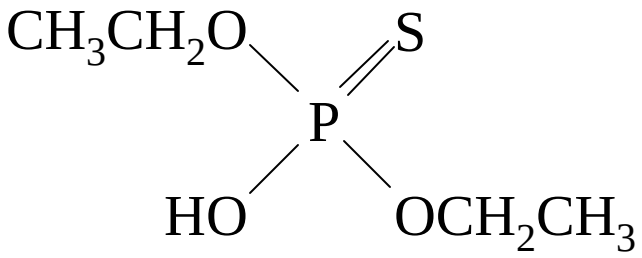


DETP Degradation

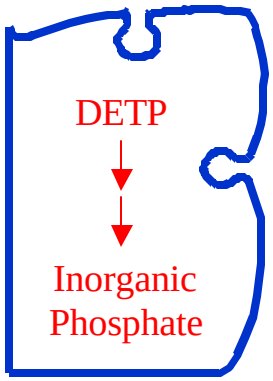
DETP degradation rates were estimated using a DETP analog, bis-(*p*-nitrophenol) phosphate (BNP).



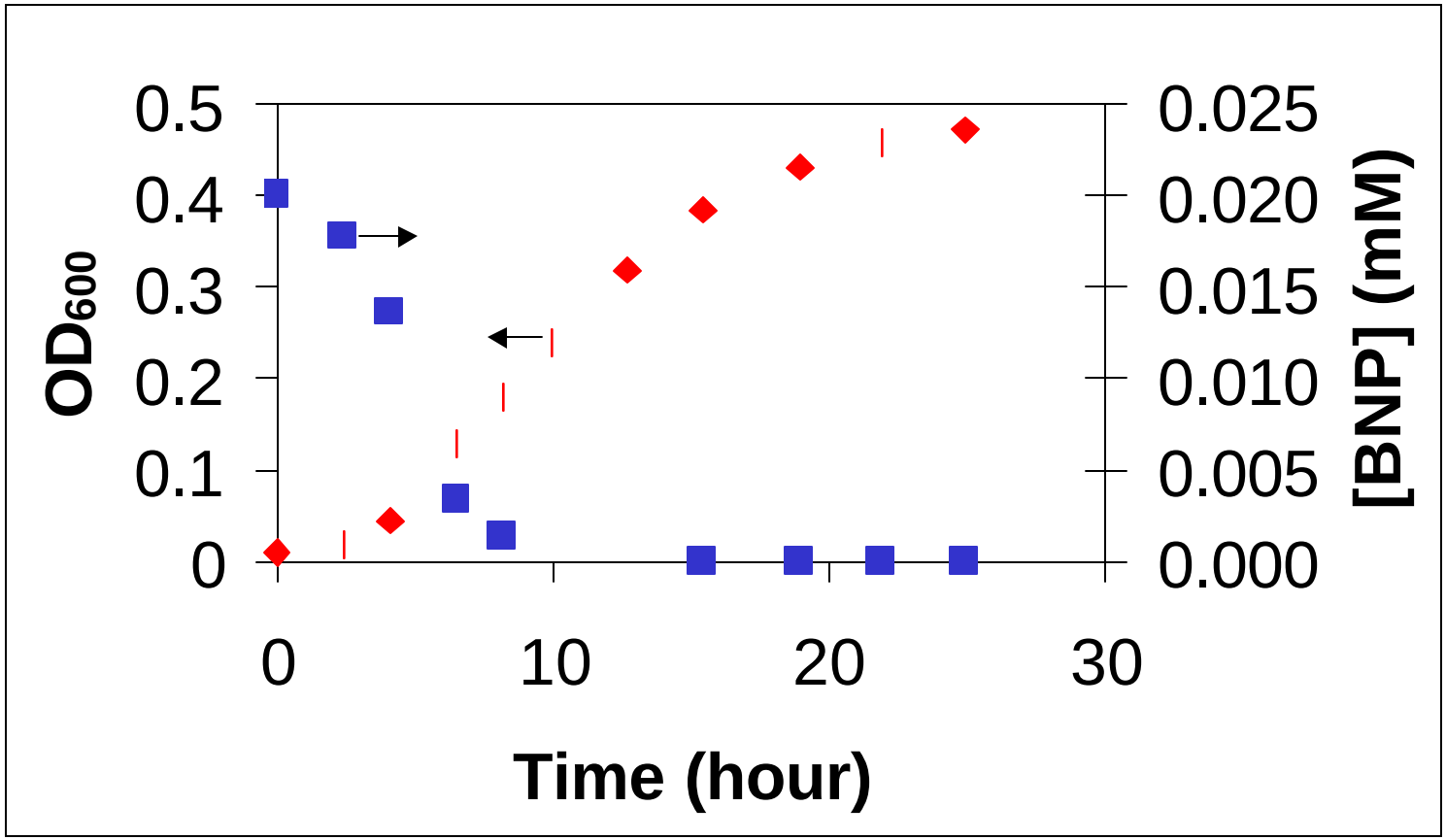
BNP



DETP



C. acidovorans growth and BNP disappearance



Purification and characterization of phosphodiesterase

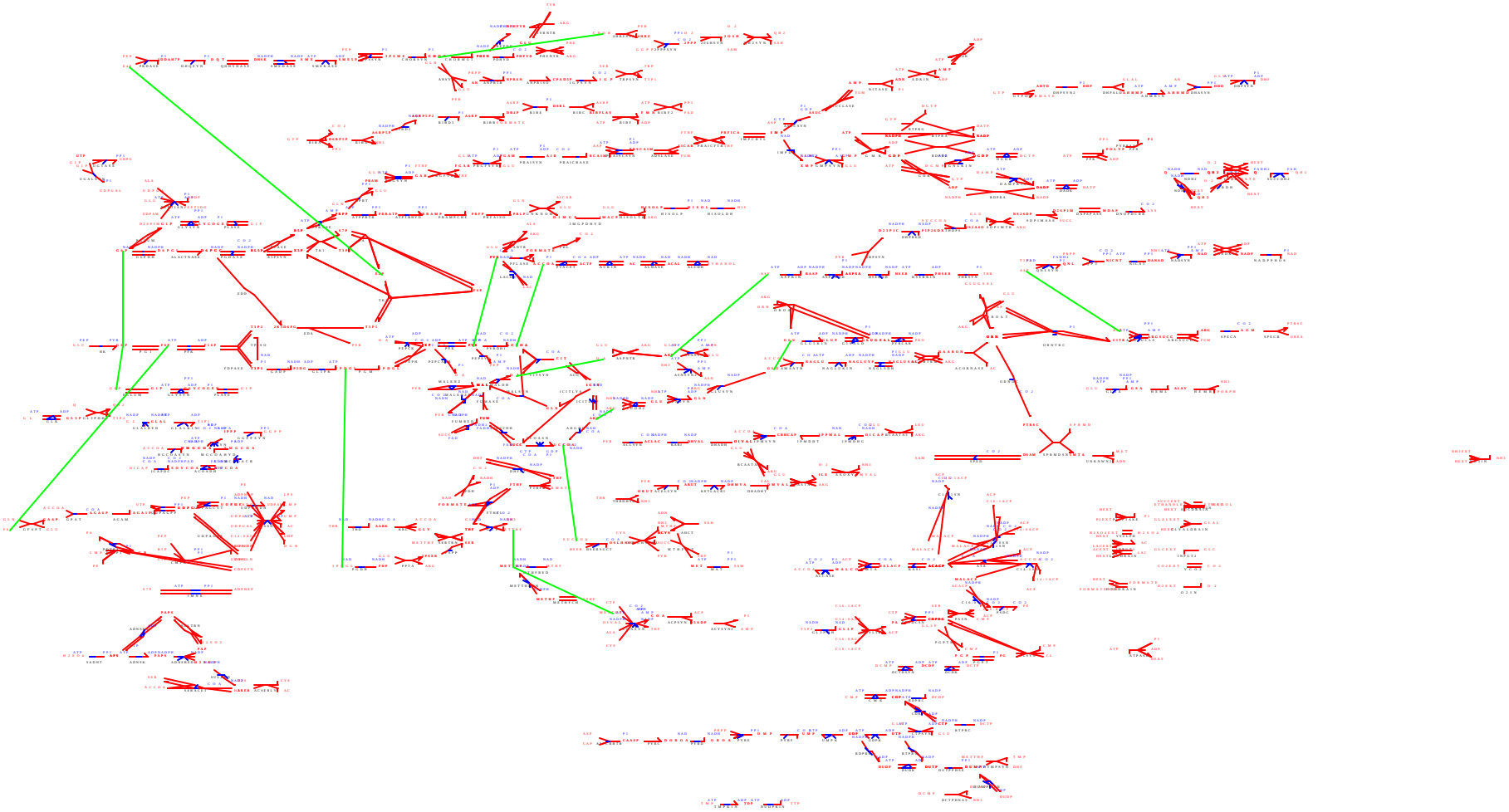
- The phosphodiesterase was purified to homogeneity
- N-terminal sequenced
- Degenerate primers synthesized
- Cloning of gene in progress

Flux-based pathway model

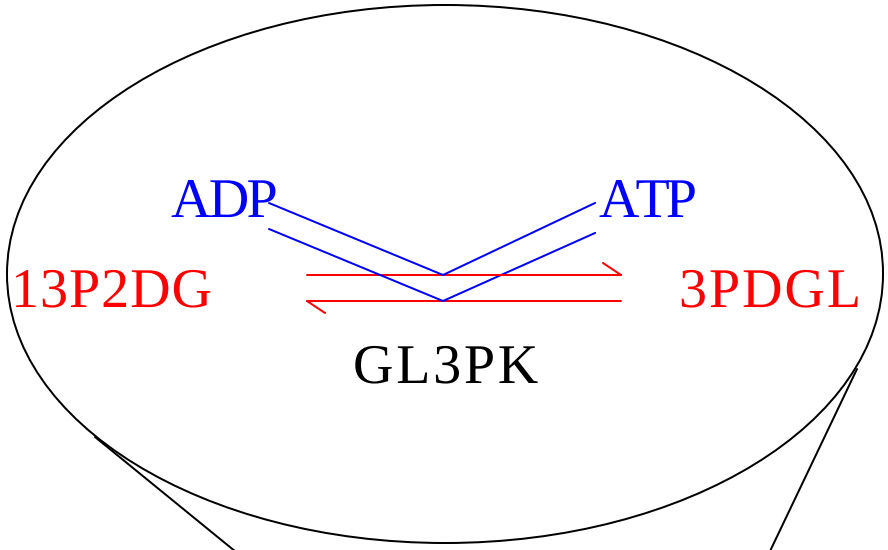
A model for the post-genomics era

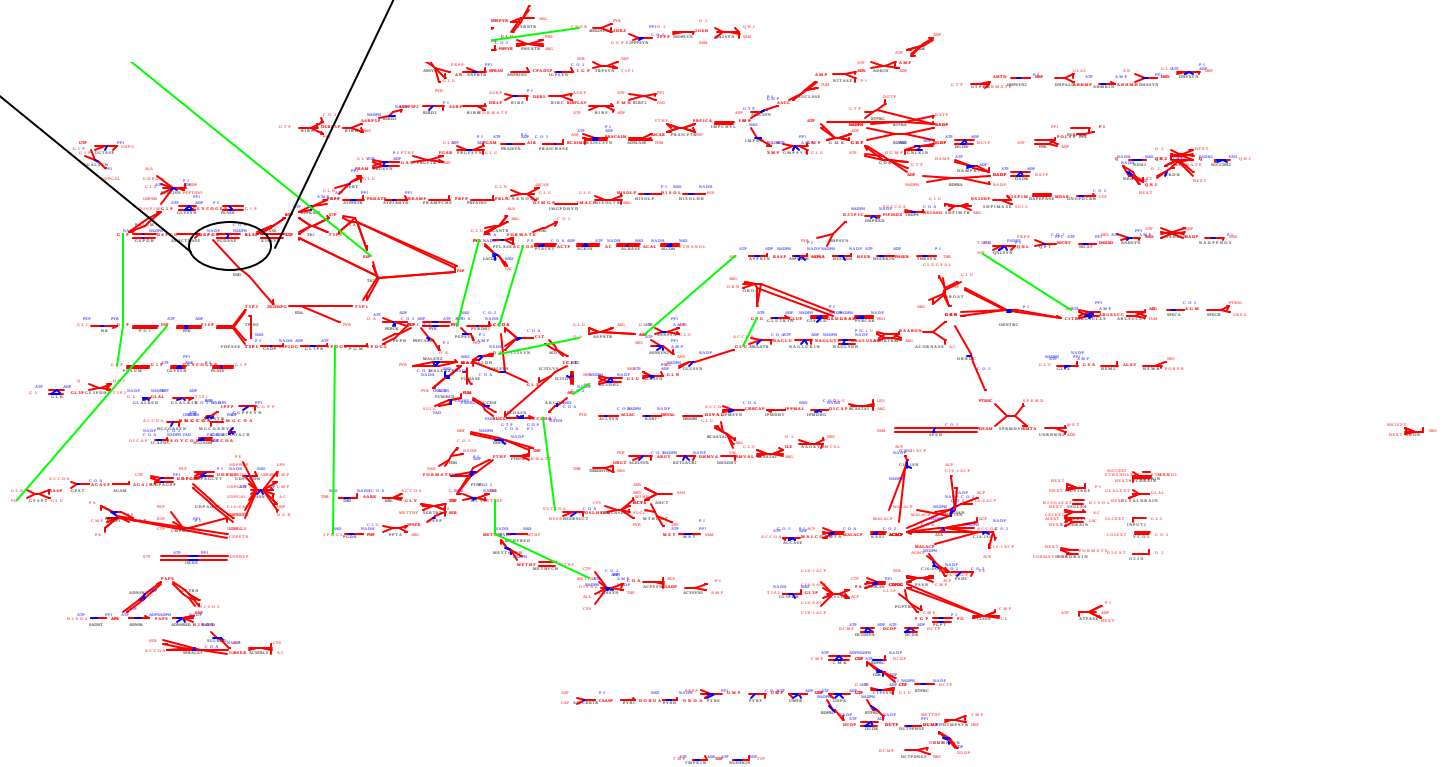
- Developed from the genome sequence of *E. coli* and other Gram-negative organisms
- Matlab based
- Capabilities:
  - Calculate and visualize metabolic fluxes
  - Visualize DNA microarray data

All pathways



All Pathways





Future Work

- Construction of one strain for parathion degradation
  - Introduce genes for DETP degradation into the engineered *P. putida*
  - Optimize degradation by gene/pathway DNA shuffling
- Introduce genes into an organism that expresses C-P lyase
- Flux predictions with DNA macroarray analysis

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