

# Influence of Treated Wastewater Irrigation on Antibiotic Resistance in the Soil Microbiome

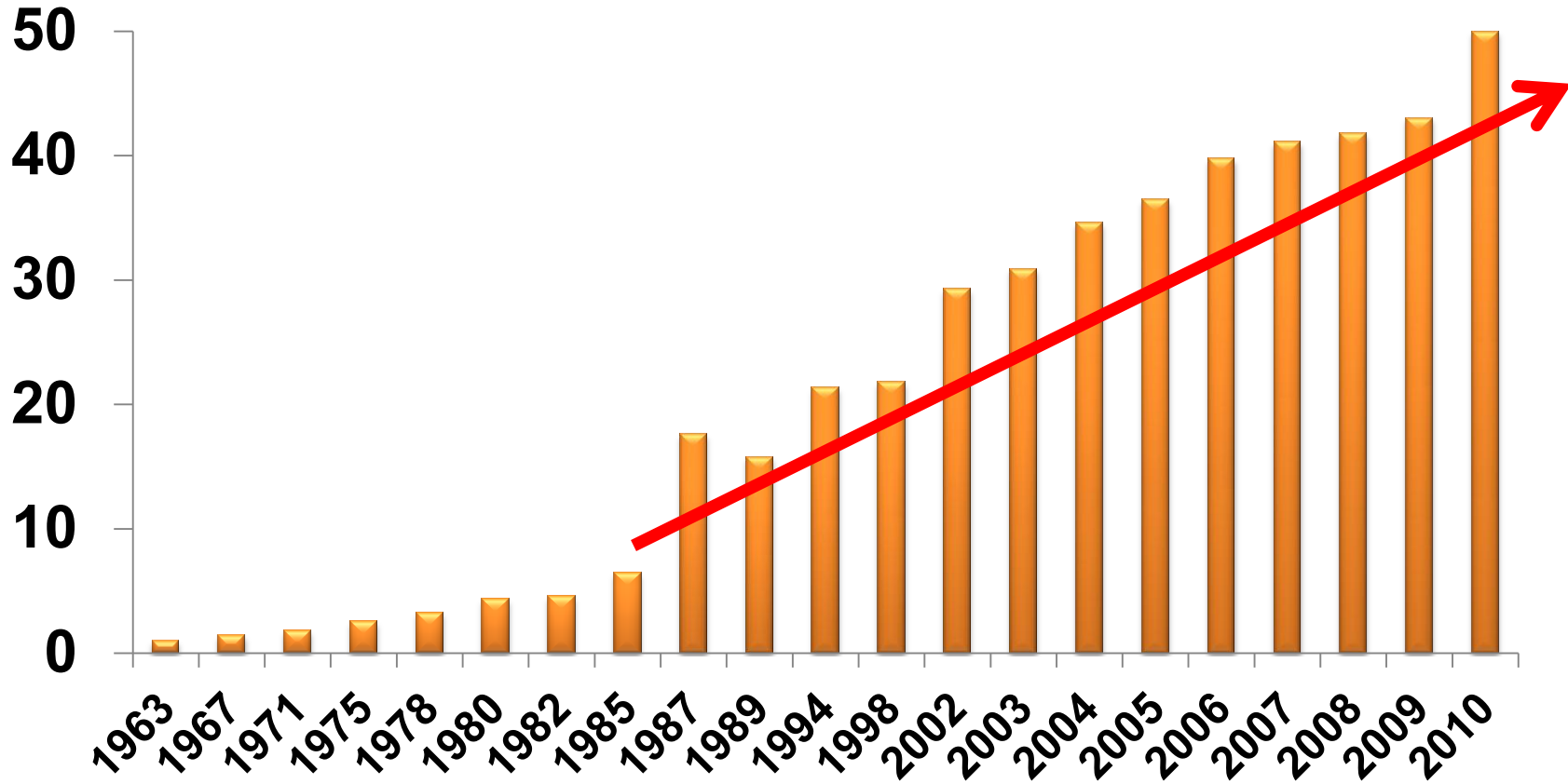
Yael Negreanu<sup>1,2</sup>, Joao Gatica-Arias<sup>1,2</sup>, Edouard Jurkevitch<sup>2</sup>, Zohar Pasternak<sup>2</sup>, Eddie Cytryn<sup>1</sup>

<sup>1</sup> Institute of Soil, Water and Environmental Sciences  
ARO, Volcani Agriculture Research Center, Israel

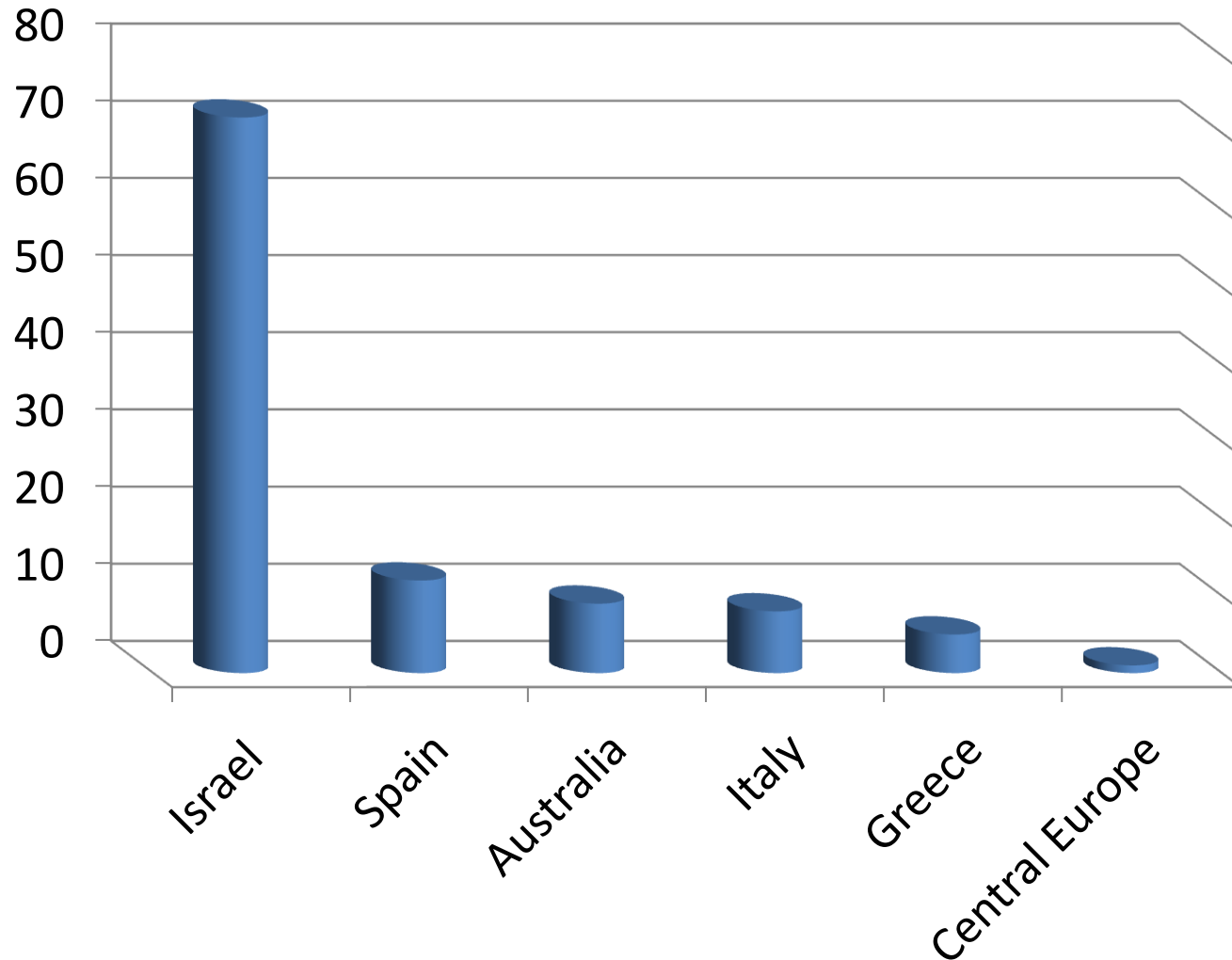
<sup>2</sup> The Department of Plant Pathology and Microbiology  
Robert H. Smith Faculty of Agriculture, Food and Environment  
The Hebrew University of Jerusalem



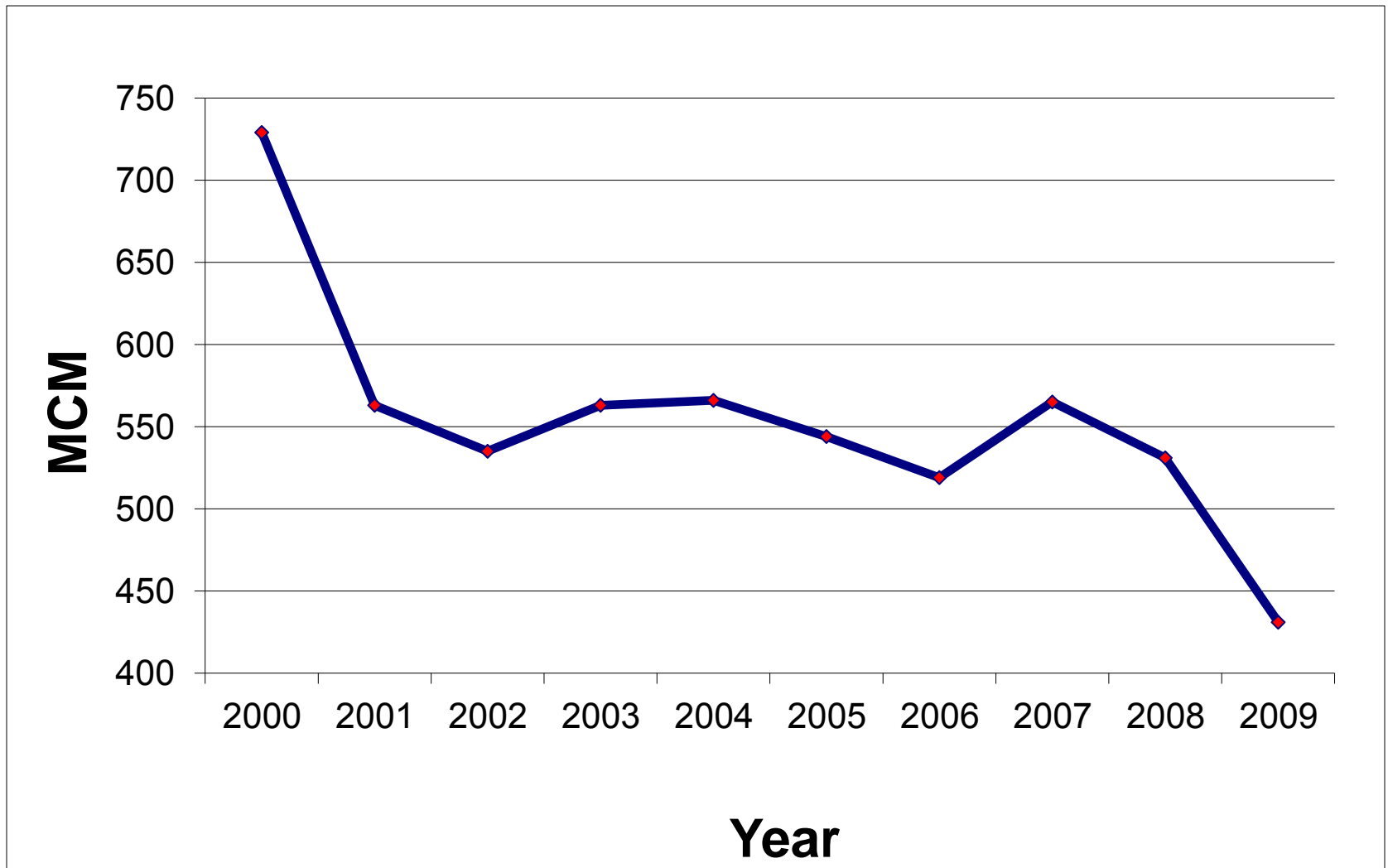
## Irrigation with Reclaimed Wastewater in Israel(% of total)



# Wastewater Reuse for Agriculture in Israel Relative to Other Countries



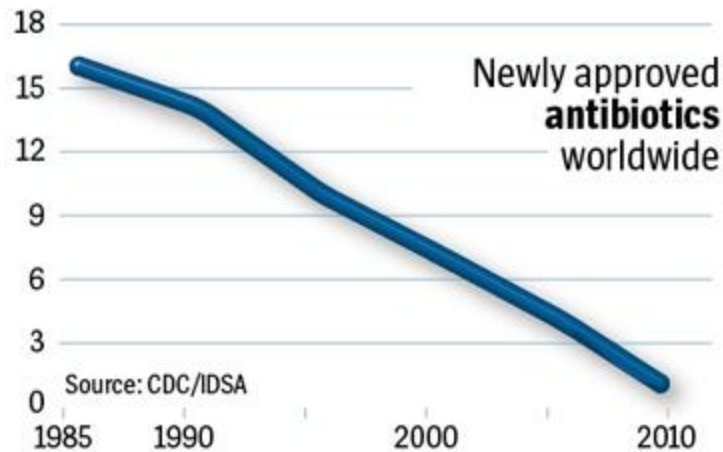
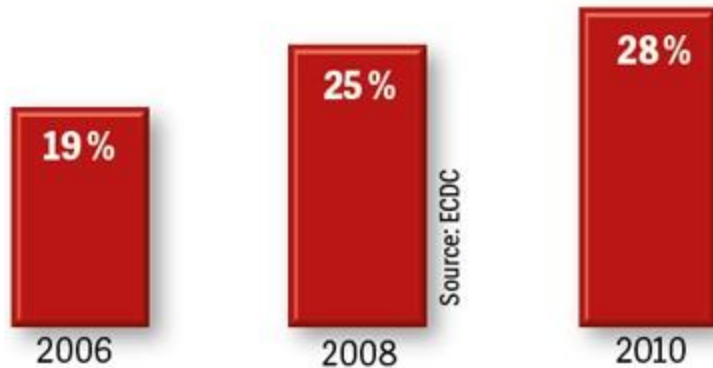
# Fresh-Water Allocation to the Agricultural sector 2000-2009



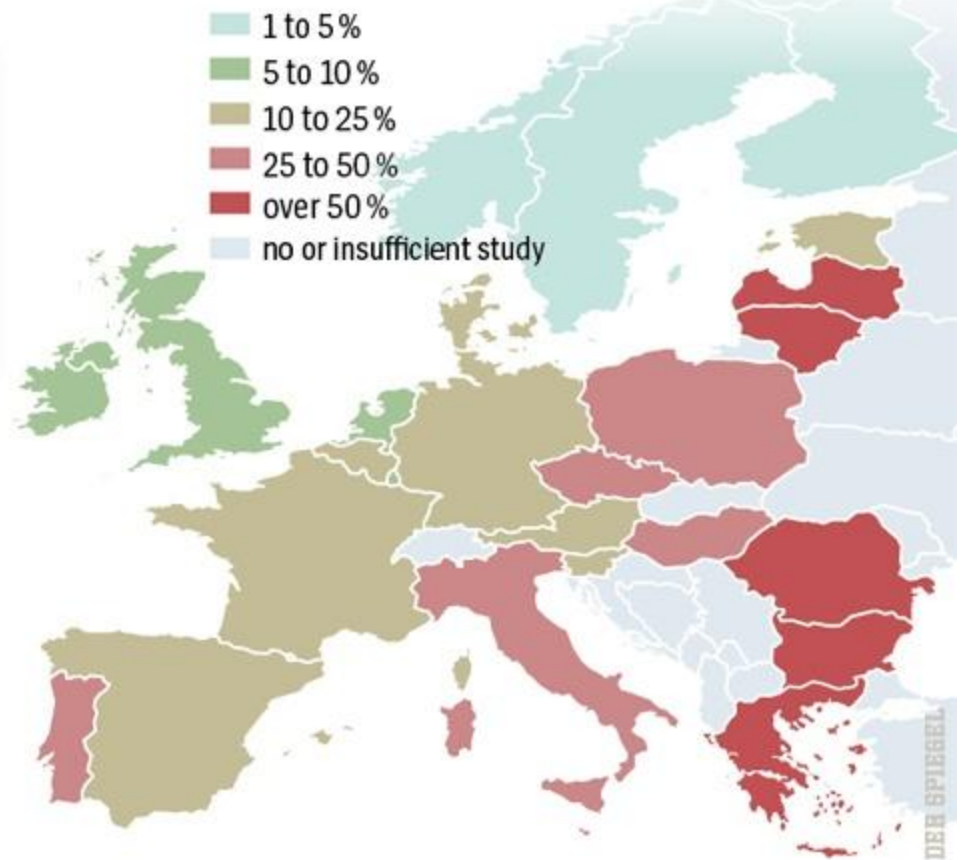
# The Rise of Antibiotic Resistance

## Victory of the Germs

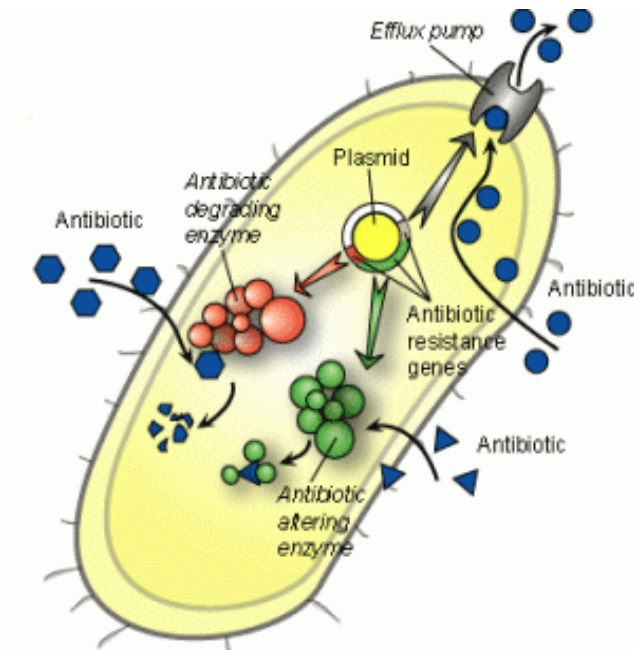
Share of **ESBL-forming bacteria** in Europe resistant to modern antibiotics



Proven resistance by country, 2010



# Dissemination of Antibiotic Resistance



<http://www.cheapmedicinechest.com/wp-content/uploads/2010/02/antibiotic-resistance-300x300.gif>

It was on a short-cut through the hospital kitchens that Albert was first approached by a member of the Antibiotic Resistance.

# Environmental Antibiotic Resistance Reservoirs

MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2010, p. 417-433  
 1092-2172/10/\$12.00 doi:10.1128/MMBR.00016-10  
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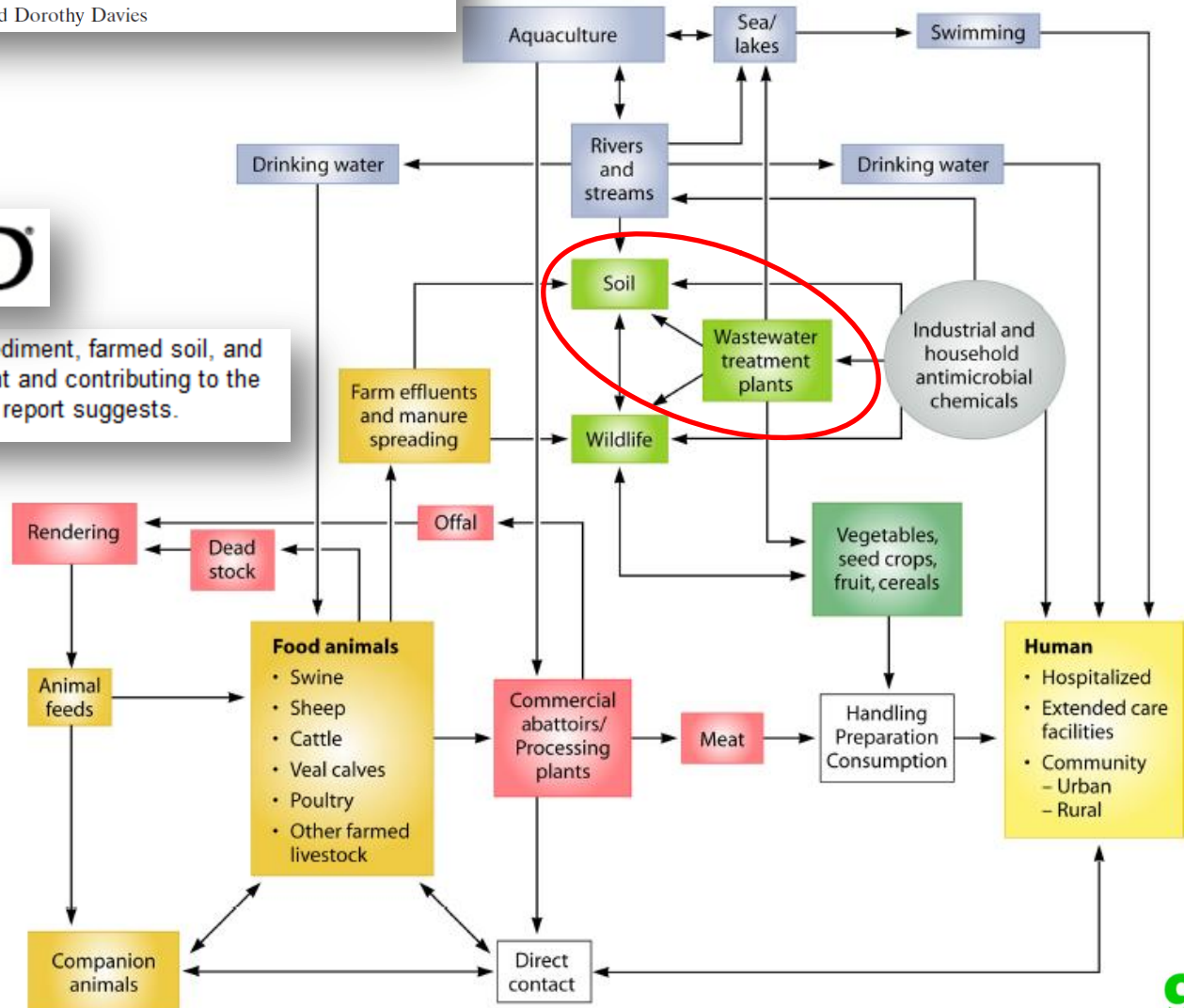
Vol. 74, No. 3

## Origins and Evolution of Antibiotic Resistance

Julian Davies\* and Dorothy Davies

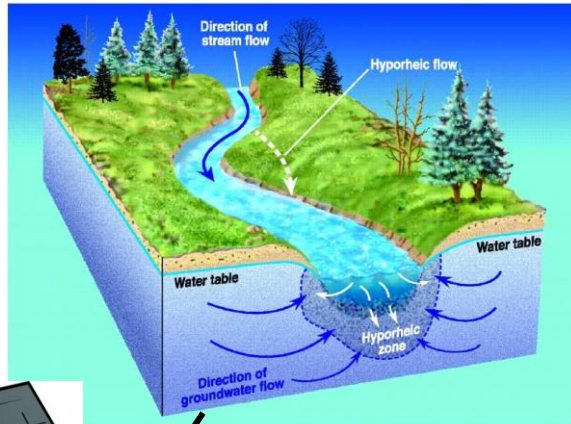


May 8, 2012 -- Antibiotics found in river sediment, farmed soil, and other sources are polluting the environment and contributing to the rising rates of antibiotic resistance, a new report suggests.





# Fate of Antibiotics and Antibiotic Resistant Bacteria in the Environment



Irrigation

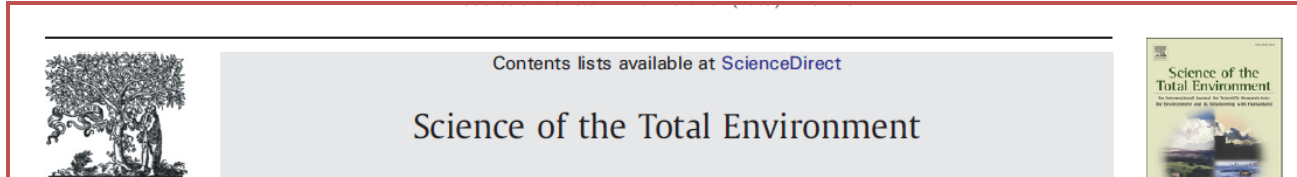
Runoff

Irrigation





# Antibiotics and antibiotic resistant bacteria and genes in wastewater effluents??



s in the

frontiers in  
**MICROBIOLOGY**

**ORIGINAL RESEARCH ARTICLE**  
published: 22 March 2012  
doi: 10.3389/fmicb.2012.00106



## Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland

*Nadine Czekalski<sup>1\*</sup>, Tom Berthold<sup>2</sup>, Serena Caucci<sup>2</sup>, Andrea Egli<sup>1</sup> and Helmut Bürgmann<sup>1</sup>*

<sup>1</sup> Department of Surface Waters – Research and Management, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland

<sup>2</sup> Department of Environmental Microbiology, UFZ, Helmholtz Centre for Environmental Research, Leipzig, Germany

Mariya Munir, Kelvin Wong, Irene Xagorarakis\*

[J Environ Qual.](#) 2010 Nov-Dec;39(6):2211-5.

**Broad-host-range plasmids in treated wastewater effluent and receiving streams.**

Akiyama *et al.*

Dep. of Crop, Soil, and Environmental Sciences, 115 Plant Science Bldg., Univ. of Arkansas, Fayetteville, AR 72701, USA.

# Impact of treated wastewater-irrigation on soil AR



Freshwater

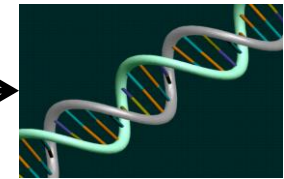


Treated wastewater



← Plate counts on dilute LB medium +/- selected antibiotics. Levels of AR (%) in FW and TWW-irrigated soils

↓ DNA Extraction



↓ Characterization and quantification of selected AR genes

# Impact of treated-wastewater irrigation on soil AR: study sites and conditions

Site	Soil type	TWW quality	FW quality	Irrigation period	Crop	Plot type	Duration of experiment
Akko	Vertisol (60% clay)	Secondary TWW from Shomrat-Agamit treatment facility	Fresh water	Irrigation season	Avocado	Orchard	12 years
Rishon LeZion	Dune quartz sand	wastewater effluent from the Shafdan mechanical-biological treatment plant	Injected TWW	All the time	Oroblanco Citrus	200 liters Lysimeters	12 years
Gilat	Loam (20% clay)	Secondary TWW from Sde-Timan treatment facility	Fresh water	Irrigation season	Cotton and wheat	Field	15 years
Kedma	Vertisol (52% clay)	Secondary-TWW from the city of Jerusalem	Well water from the local coastal aquifer	Irrigation season	Olive tree	Orchard	6 years



# Plate Assays

Antibiotic species	Antibiotic class	Mode of action	Chemical formula
Tetracycline	Tetracycline	Protein synthesis inhibitor	<chem>CN1C=CC2=C(C(=O)O)C(=O)C3=C(O)C(=O)C=C4C3=C(O)C(=O)C41</chem>
Ciprofloxacin	Fluroquinolone	Prevent unwinding & duplicating bacterial DNA	<chem>C1CCNCC1C2=CC(=C(C=C2)F)C3=C(C(=O)O)N(C3)C4CC4</chem>
Erythromycin	Macrolides	Protein synthesis inhibitor	<chem>C[C@H]1O[C@H]2[C@@H](O)[C@H](O)[C@@H](O)[C@H]2O[C@H]1C[C@H]3O[C@H]4[C@@H](O)[C@H](O)[C@@H](O)[C@H]4O[C@H]3</chem>

## Experimental sites

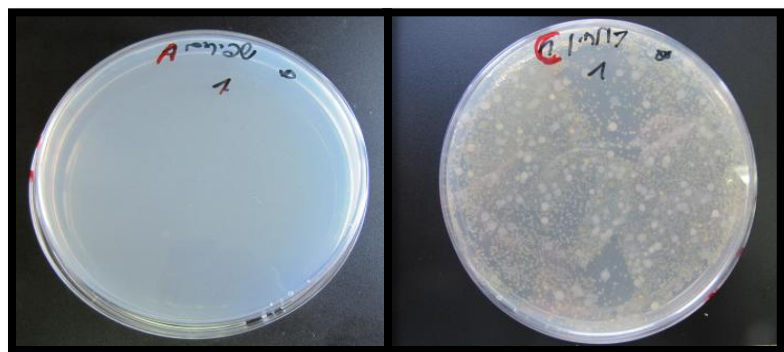
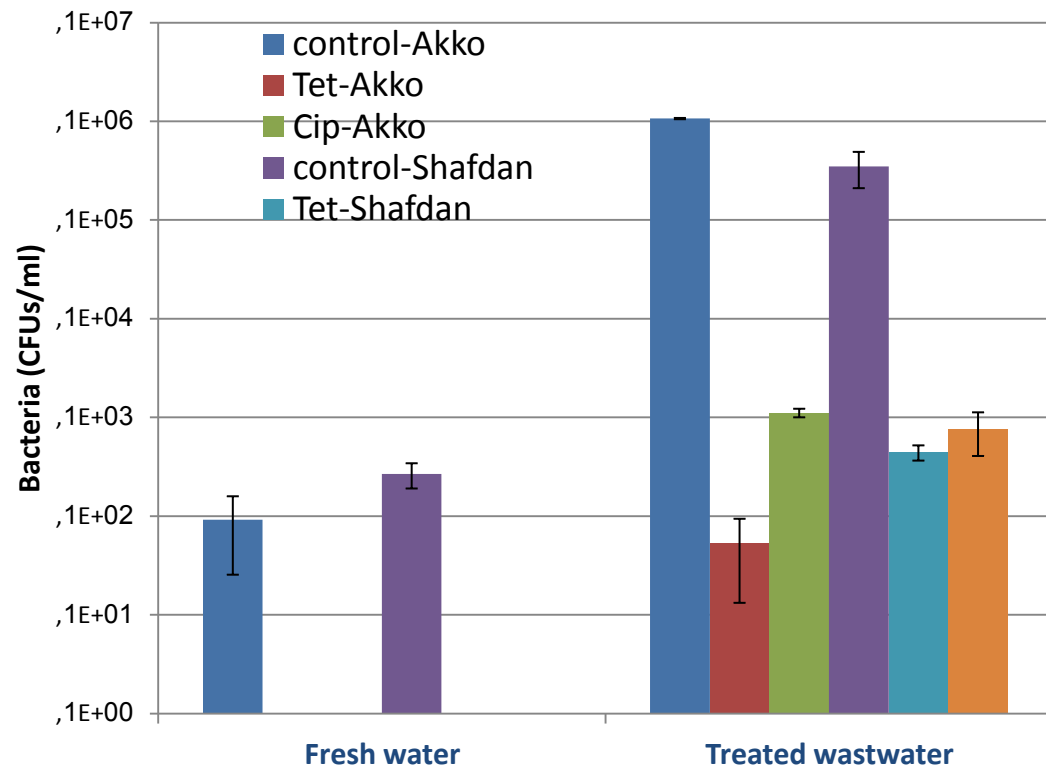


**Avocado orchard, Akko**



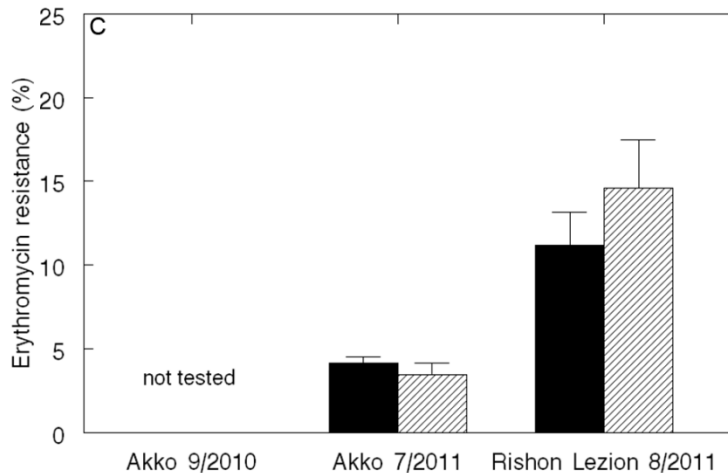
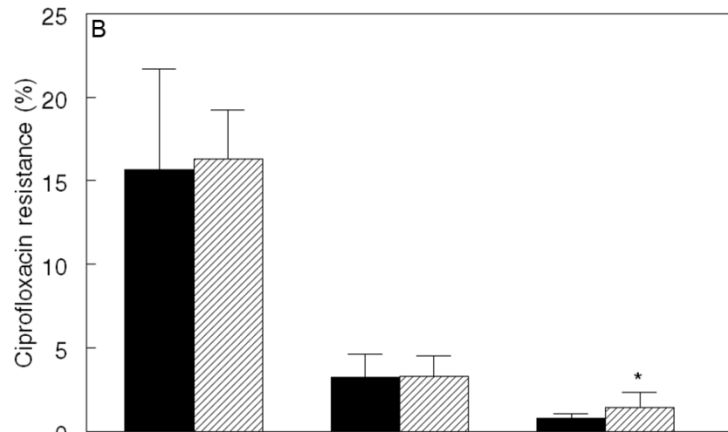
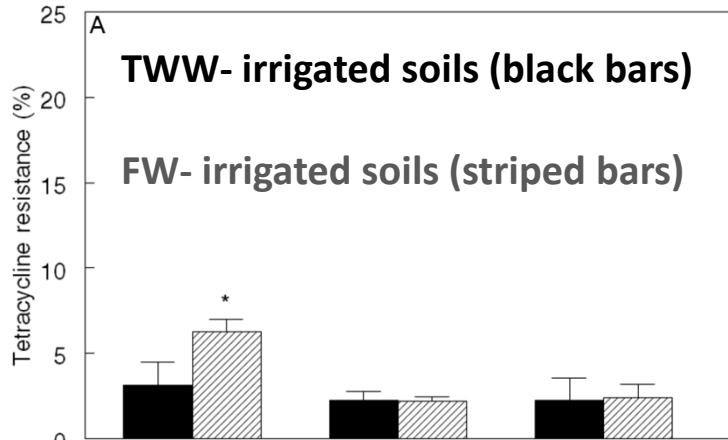
**Citrus Lysometers, Shafdan**

# Antibiotic resistance in freshwater and TWW samples





# Quantification of antibiotic resistance in “culturable” bacteria



**(A) Tetracycline resistant bacteria**

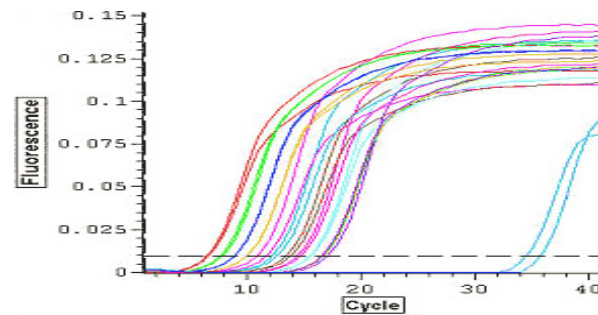
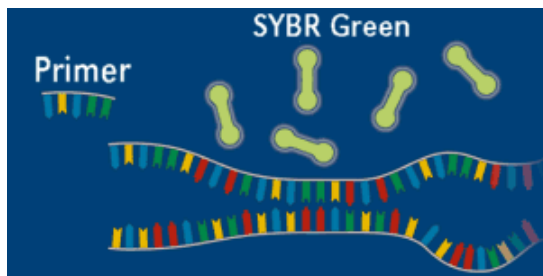
**(B) Ciprofloxacin resistant bacteria**

**(C) Erythromycin resistant bacteria**

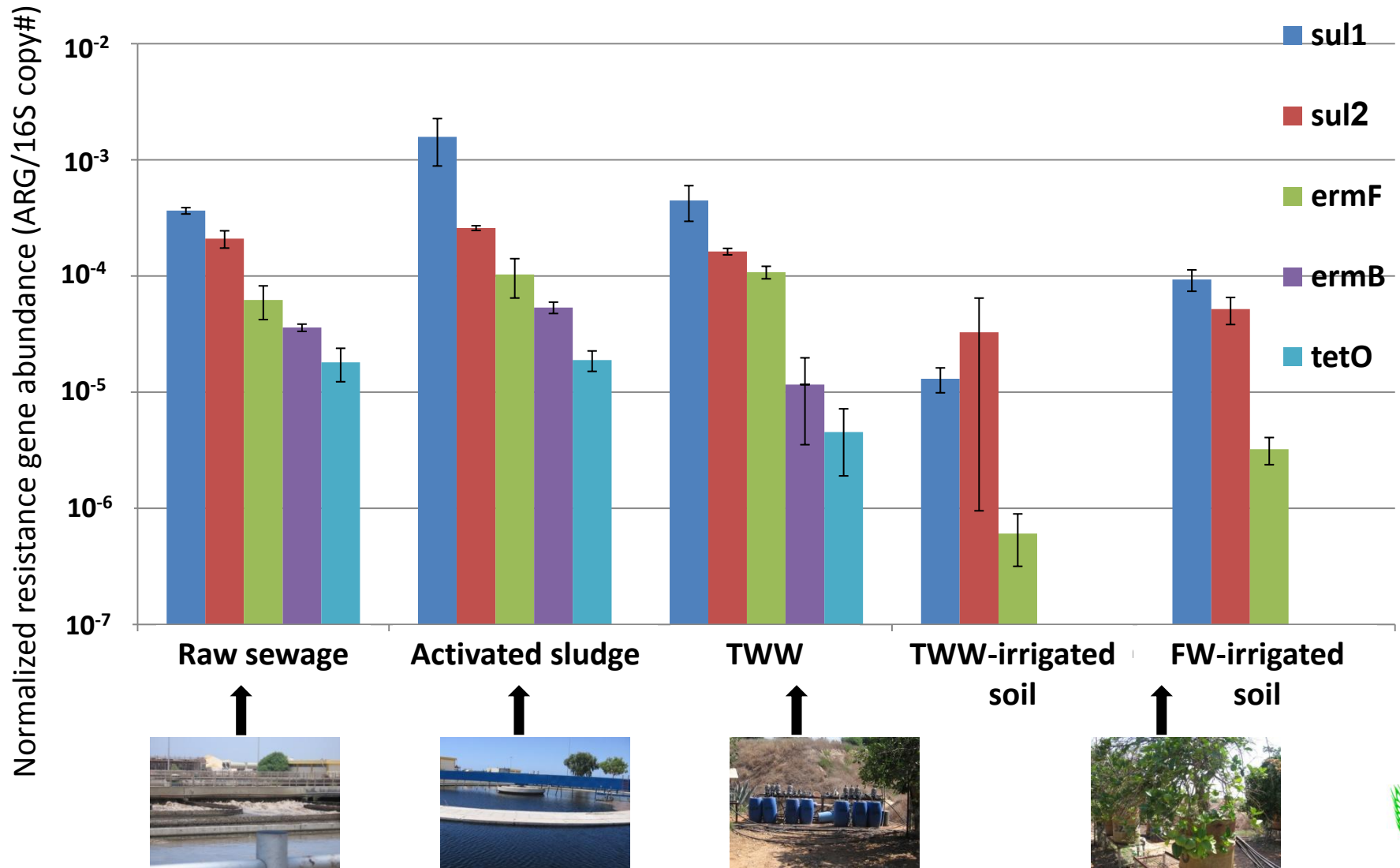
# “Culture independent” quantification of clinically-relevant AR genes using quantitative real-time PCR (qPCR)

Resistance	Targeted genes	Mode of protection
Tetracycline	<i>tet(O)</i>	Ribosome protection
Fluoroquinolone (Ciprofloxacin)	<i>qnr(A)</i>	Plasmid associated DNA gyrase protection (DNA synthesis)
Macrolide (Erythromycin)	<i>erm(B)</i>	Ribosome protection
	<i>erm(F)</i>	
Sulfonamide	<i>sul(1)</i>	Interferes with inhibition of folic acid synthesis
	<i>sul(2)</i>	

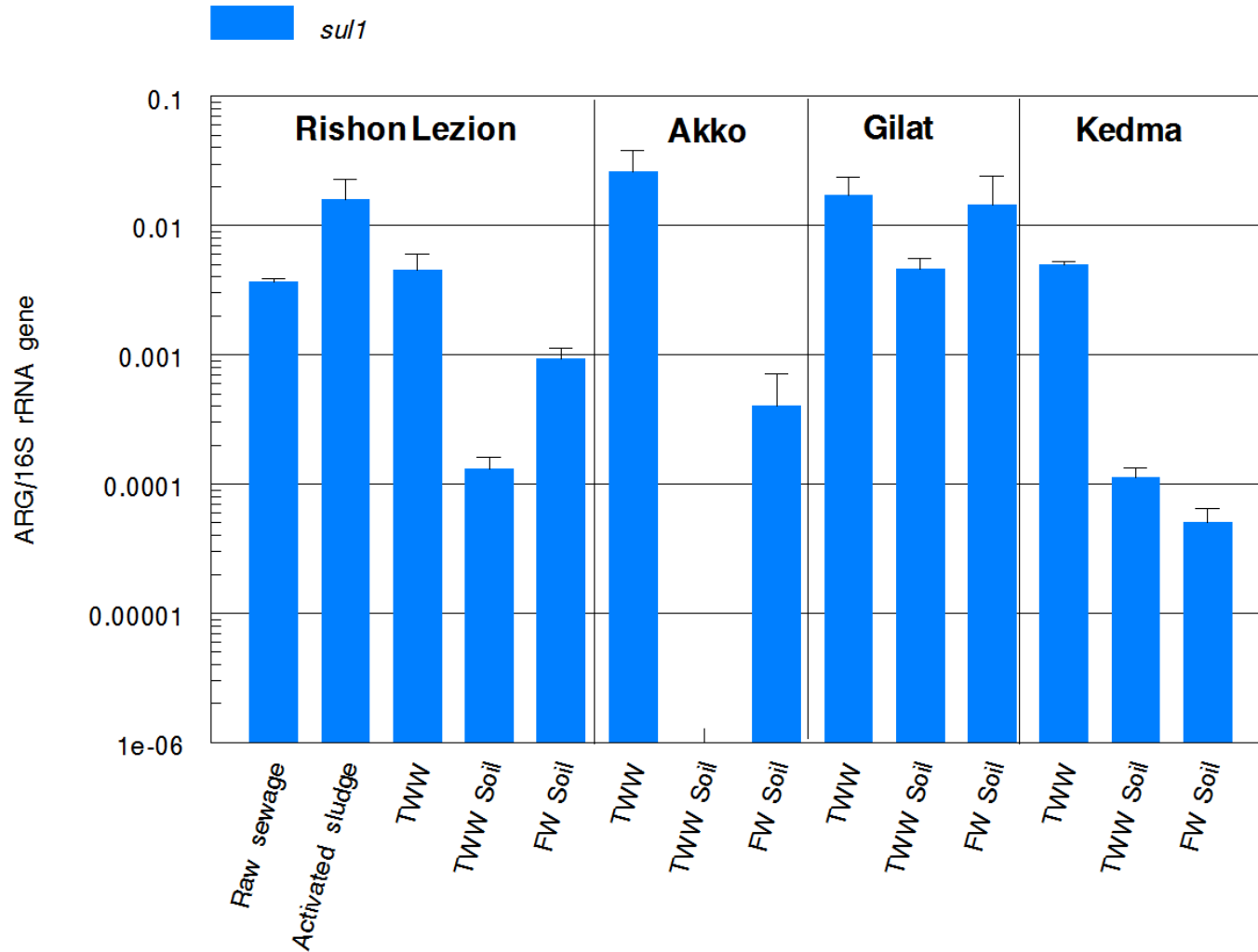
The relative abundance of the above genes were normalized by dividing the above AR gene amplicons by 16S rRNA gene amplicons



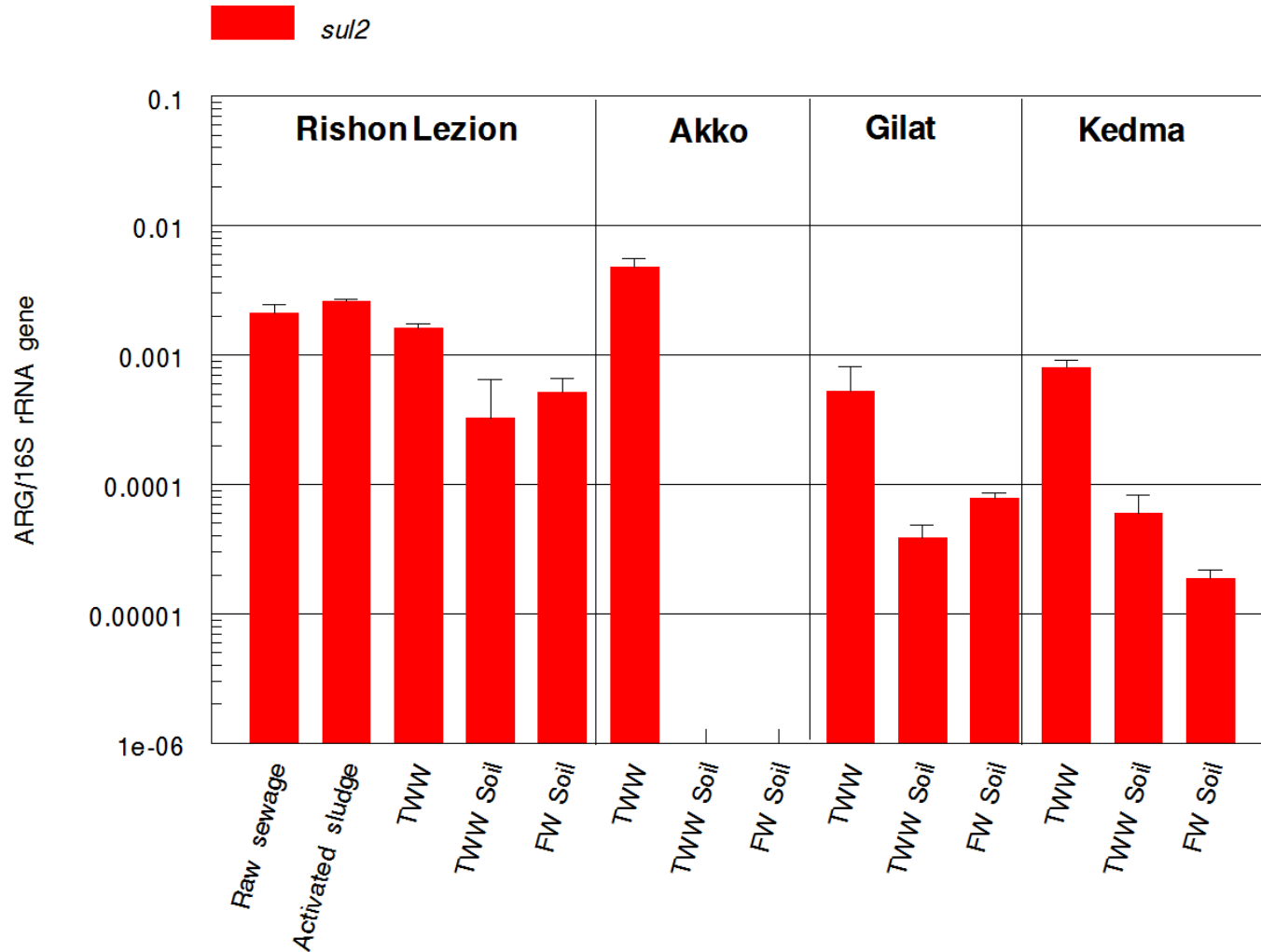
# Source tracking of antibiotic resistance genes (from sewage to soil) in Shafdan using qPCR



# qPCR targeting of AR genes in the four study sites

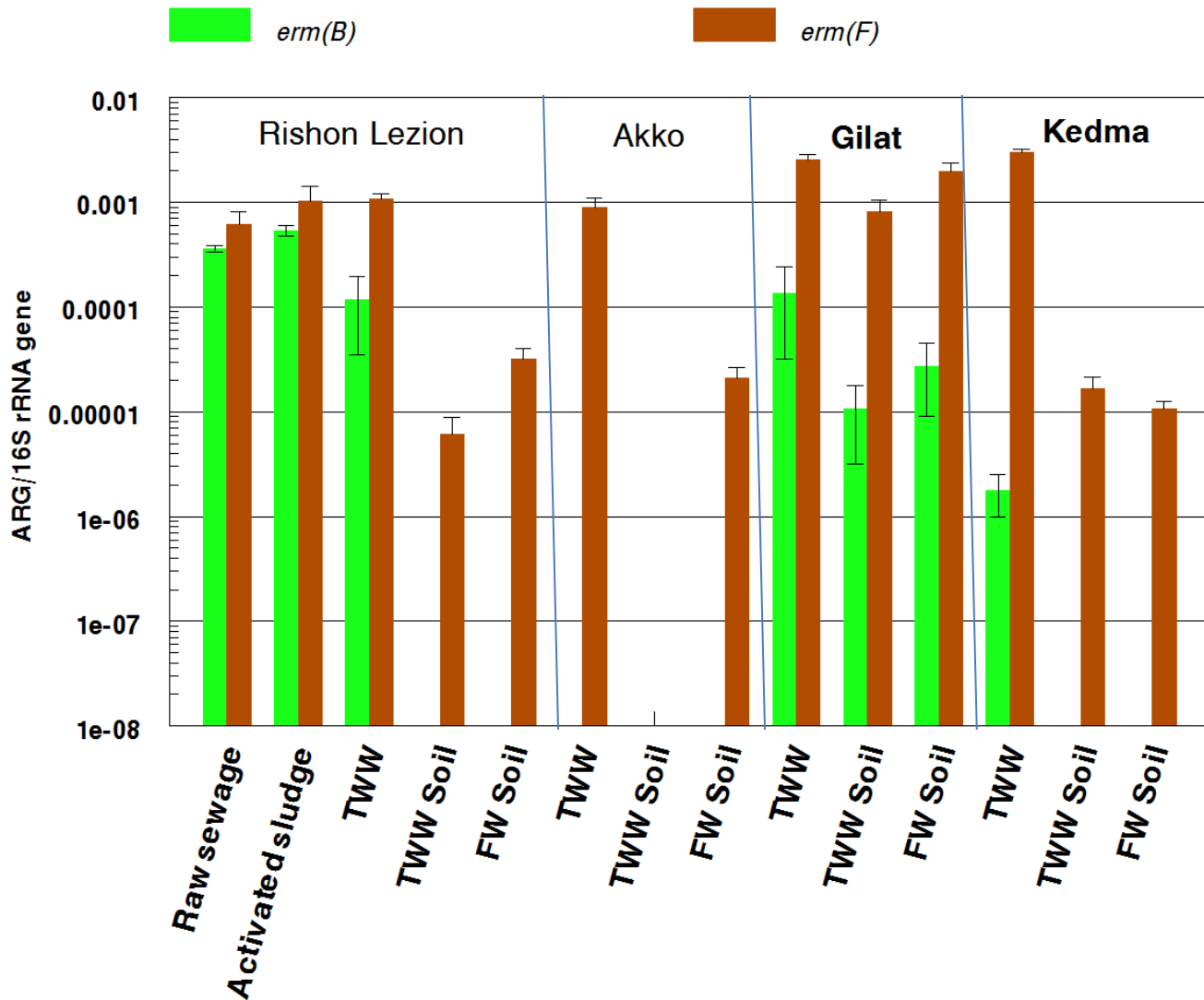


# qPCR targeting of AR genes in the four study sites

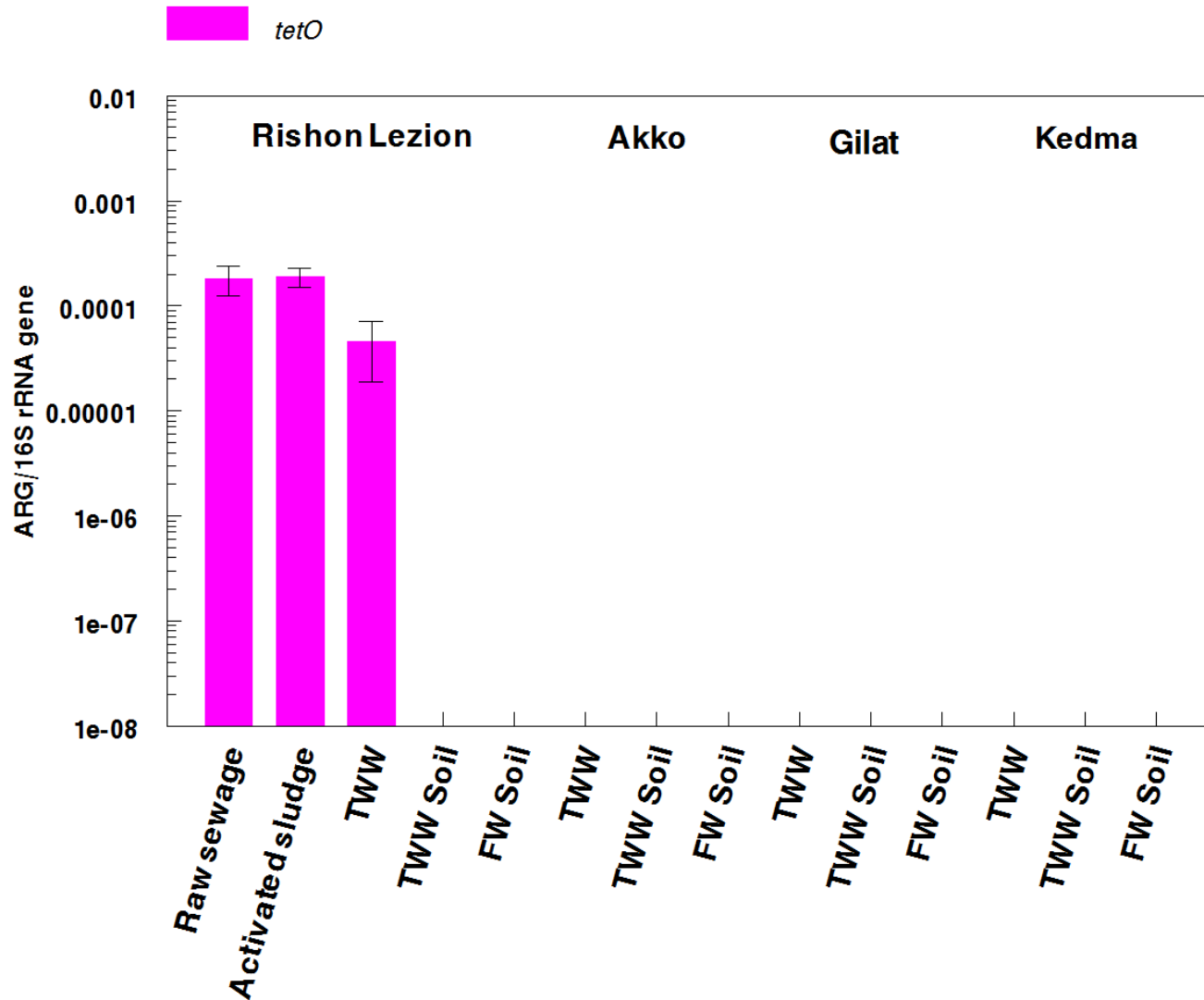




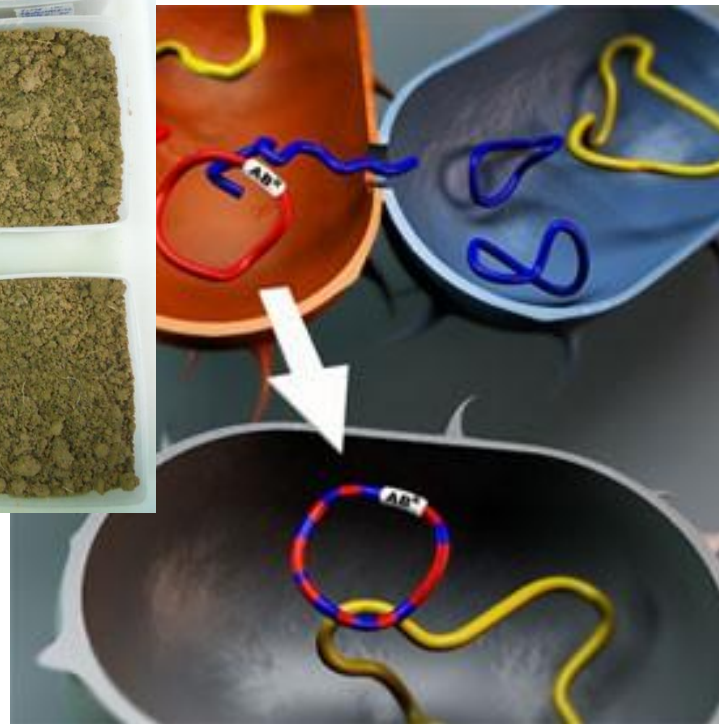
# qPCR targeting of AR genes in the four study sites



# qPCR targeting of AR genes in the four study sites

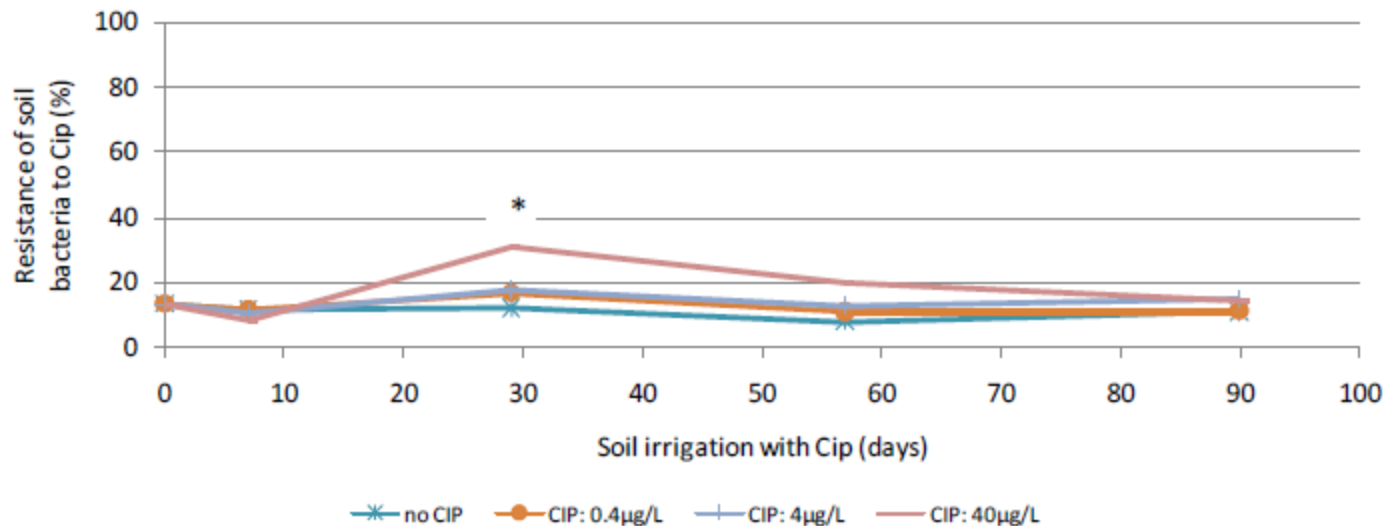


# How do Anthropogenic factors impact soil AR



# How do Anthropogenic factors impact soil AR- *Selective pressure*

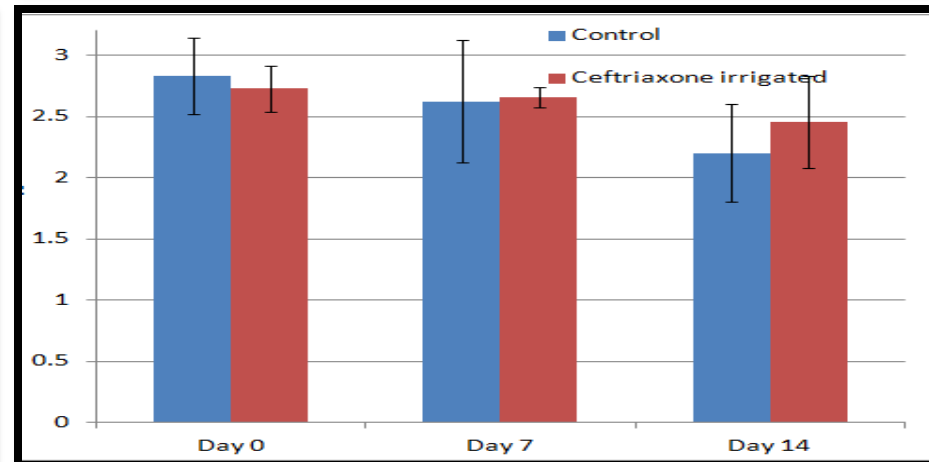
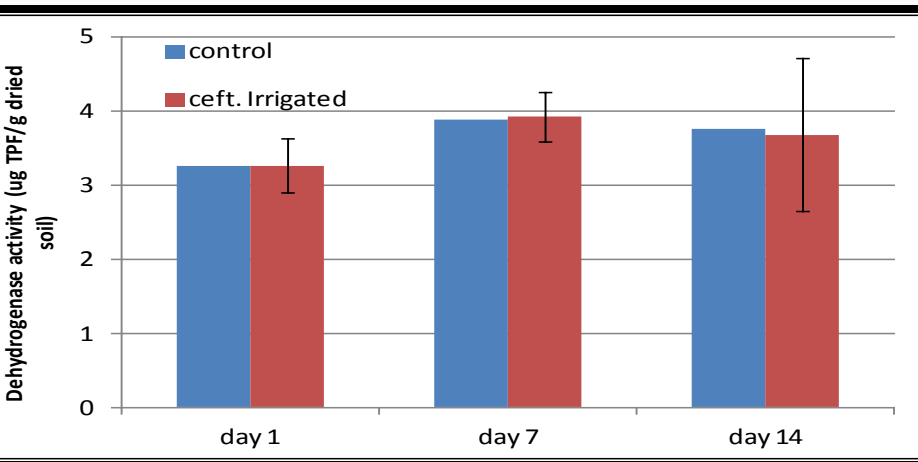
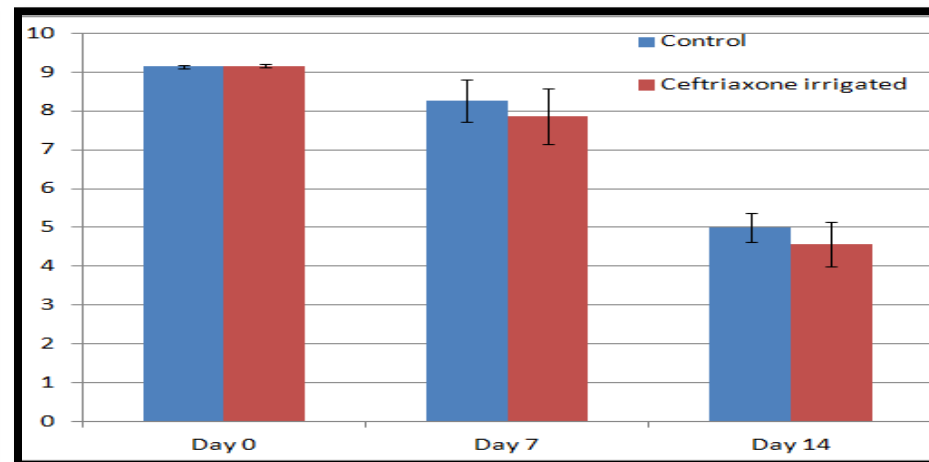
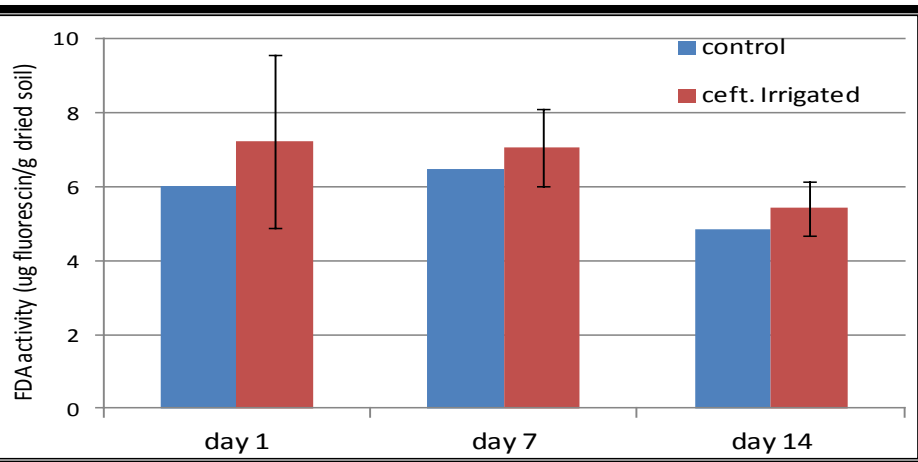
## Microcosm experiments



# How do Anthropogenic factors impact soil AR- *Selective pressure*

Gilat, Israel

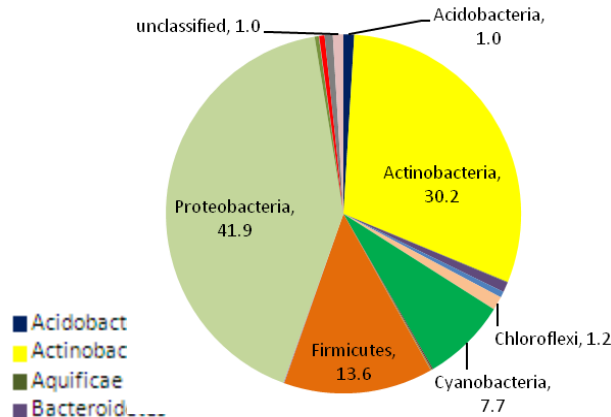
Hawaii, USA



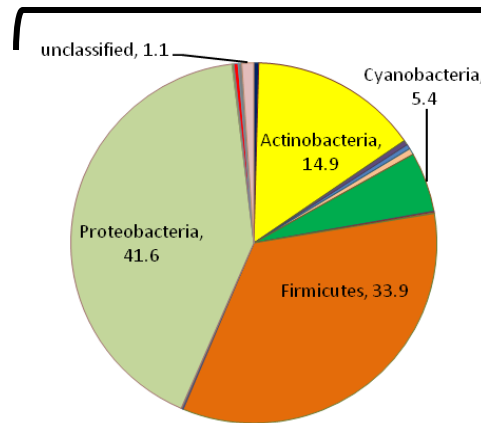


# Metagenomic analysis of bacterial community (phylum level) composition of pre- and post-incubated soil microcosms

## Original Soil

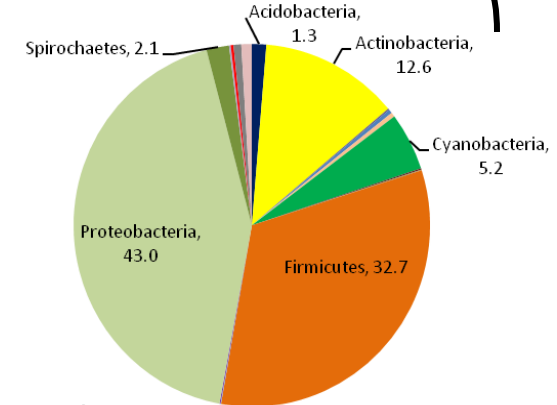


## 1 week

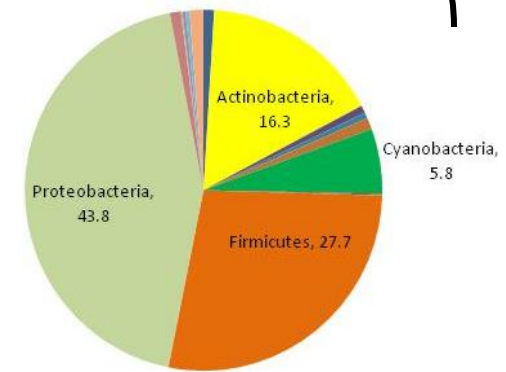
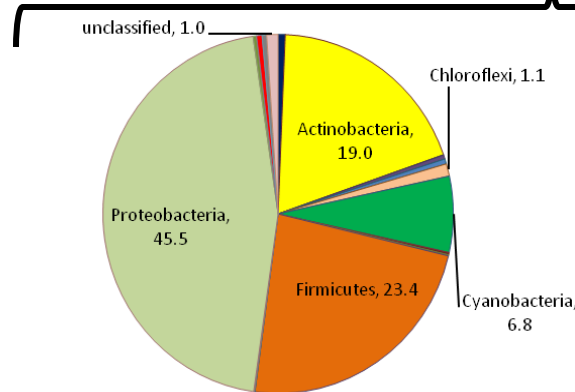


NO AB

## 2 weeks



## Ceftriaxone (2ug/ml)



- ❖ Each profile consists of average values from triplicate biological replicates (~3000 OTUs each).
- ❖ Data generated using the MG-RAST metagenomic analysis server

# Two dimensional distribution of bacterial community structure correlated to time and antibiotic exposure

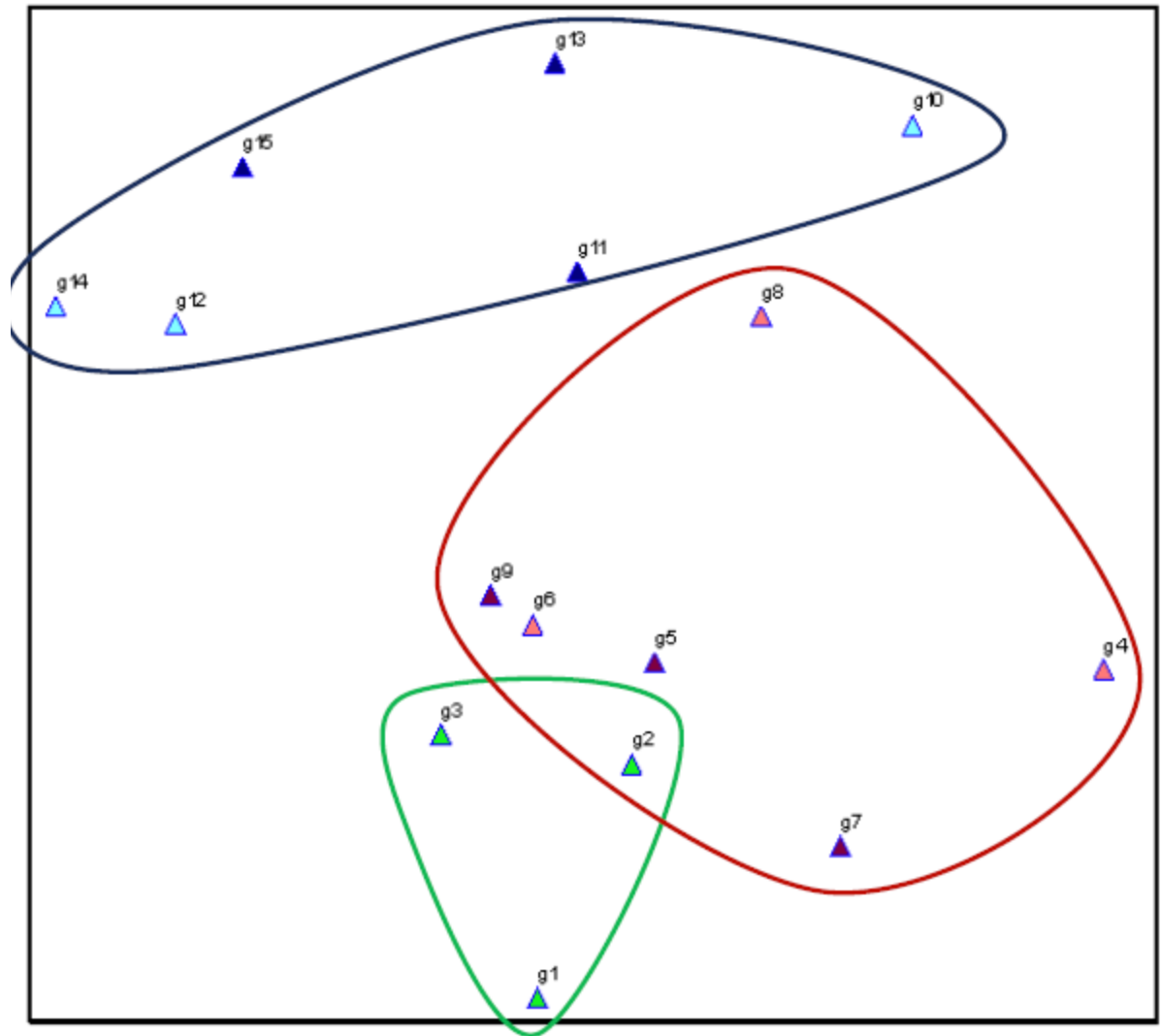
Green area- original soil

Red area- 7 days post-incubation

Blue area- 14 days post-incubation

Light colored triangles- non-amended (control);

Dark colored triangles- ceftriaxone amended.



❖ N-MDS analysis using PC-Ord

❖ Each triangle represents ~ 3000 OTUs

## Presence of ESBL genes in ceftriaxone-resistant isolates obtained from microcosms and slurry experiments

Soil site	Experiment	Total isolates	Extended spectrum beta-lactamase gene type					
			blaTEM	blaCTX-M	blaSHV	blaOXA	blaVIM	blaNDM
Gilat	microcosms	23	✓ (26%)	✓ (0%)	✓ (0%)	- (0%)	- (0%)	- (0%)
Gilat	slurry	30	✓ (87%)	✓ (0%)	- (0%)	- (0%)	- (0%)	- (0%)
Carmel	slurry	26	✓ (20%)	- (0%)	- (0%)	- (0%)	- (0%)	- (0%)
Ramat Menashe	slurry	25	✓ (84%)	- (0%)	- (0%)	- (0%)	- (0%)	- (0%)

✓ gene detected using direct molecular targeting  
(%) percent of resistant isolates harboring designated gene

## Conclusions

- 1. Treated wastewater-associated antibiotic resistant bacteria and genes do not appear to enhance antibiotic resistance in the irrigated soils.**

Plate-count assays estimated that TWW introduces  $\sim 10^5$ - $10^7$  AR bacteria per square meter of soil/day. → **Nonetheless, in most of the soils analyzed, AR bacteria and ARG levels in TWW-irrigated soils were on the whole identical (or sometimes even lower) than in FW-irrigated soils.**

## Conclusions

- 2. Antibiotics (at concentrations associated with STP effluents or even several orders of magnitude higher) do not appear to enhance antibiotic resistance in the irrigated soils.**
- 3. The soil microbiomes are characterized by significant levels of native antibiotic resistance**



# Native soil bacteria harbor antibiotic resistance genes that are prevalent in the clinical environment

## Sampling the Antibiotic Resistome

Vanessa M. D'Costa,<sup>1</sup> Katherine M. McGrann,<sup>1</sup> Donald W. Hughes,<sup>2</sup> Gerard D. Wright<sup>1\*</sup>

20 JANUARY 2006 VOL 311 SCIENCE

**nature** International weekly journal of science

NATURE | LETTER

Nature 477, 457–461 (22 September 2011)

## Antibiotic resistance is ancient

Vanessa M. D'Costa, Christine E. King, Lindsay Kalan, Mariya Morar, Wilson W. L. Sung, Carsten Schwarz, Duane Froese, Grant Zazula, Fabrice Calmels, Regis Debruyne, G. Brian Golding, Hendrik N. Poinar & Gerard D. Wright

**“Antibiotic resistance is a natural phenomenon that predates the modern selective pressure of clinical antibiotic use”.**

**“Targeted metagenomic analyses of ancient DNA from 30,000-year-old permafrost sediments revealed a highly diverse collection of genes encoding resistance to antibiotics that are highly similar to modern variants”.**

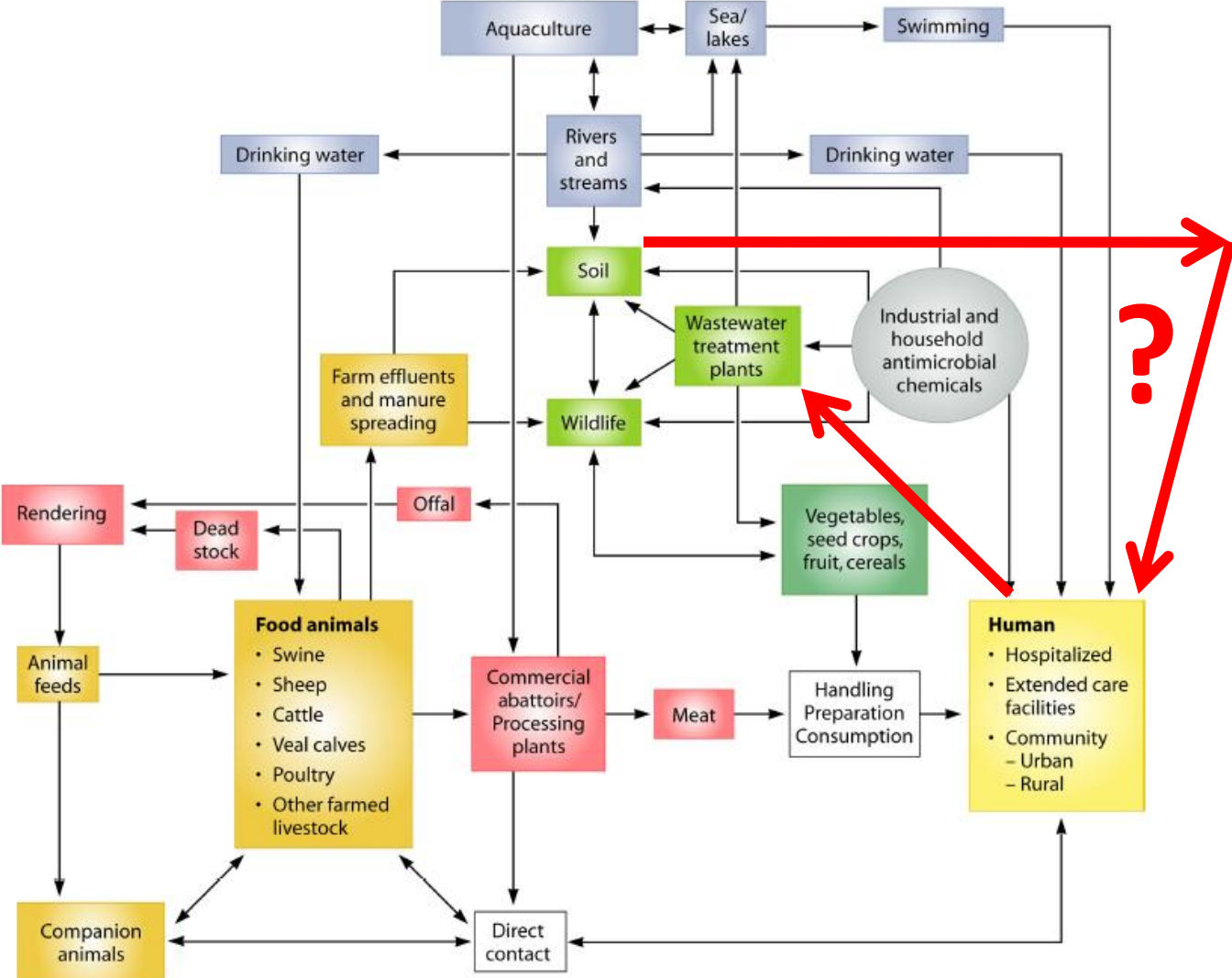
# The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens

Kevin J. Forsberg,<sup>1\*</sup> Alejandro Reyes,<sup>1\*</sup> Bin Wang,<sup>1,2</sup> Elizabeth M. Selleck,<sup>3</sup>  
Morten O. A. Sommer,<sup>4,5†</sup> Gautam Dantas<sup>1,2†</sup>

Soil microbiota represent one of the ancient evolutionary origins of antibiotic resistance and have been proposed as a reservoir of resistance genes available for exchange with clinical pathogens. Using a high-throughput functional metagenomic approach in conjunction with a pipeline for the de novo assembly of short-read sequence data from functional selections (termed PARFuMS), we provide evidence for recent exchange of antibiotic resistance genes between environmental bacteria and clinical pathogens. We describe multidrug-resistant soil bacteria containing resistance cassettes against five classes of antibiotics ( $\beta$ -lactams, aminoglycosides, amphenicols, sulfonamides, and tetracyclines) that have perfect nucleotide identity to genes from diverse human pathogens. This identity encompasses noncoding regions as well as multiple mobilization sequences, offering not only evidence of lateral exchange but also a mechanism by which antibiotic resistance disseminates.

*Science* 31 August 2012:  
Vol. 337 no. 6098 pp. 1107-1111

# Environmental Antibiotic Resistance Reservoirs



## Future “DARE INSPIRED” Questions that need to be addressed

1. What is the level of mobility of the anthropogenic-associated and native soil antibiotic resistance genes?-

*We need to look at antibiotic resistance genes on mobile genetic elements.*

1. What is the potential of these genes to be transferred from the soil reservoirs to commensal and pathogenic bacteria that are associated with humans

*We need to develop risk assessment models to predict horizontal transfer of antibiotic resistance genes.*

## Funding Agencies



Israeli Ministry of Agriculture



US-Israel Binational Science Foundation



Environmental Health Fund



The DARE COST action

# **Microbiological Quality Standards for Treated Wastewater-Irrigation**

**Guidelines for the microbiological quality of treated wastewater used in agriculture: recommendations for revising WHO guidelines**

Ursula J. Blumenthal et al. Bulletin of the World Health Organization, 2000, 78 (9)

## **New Standards For Treated Wastewater Reuse in Israel**

Yossi Inbar, Israel Ministry of Environmental Protection

Wastewater Reuse—Risk Assessment, Decision-Making and Environmental Security

NATO Security through Science Series, 2007

**BUT WHAT ABOUT ANTIBIOTIC RESISTANCE????**