

Methods development to detect antibiotic activity in water samples

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Who is who?

- **Grontmij|AquaSense**
 - Consultancy and ecotoxicology laboratories
- **Waterdienst**
 - Directorate-General for Public Works and Water Management (Rijkswaterstaat)
- **RIVM**
 - National Institute for Public Health and the Environment
- **RIKILT**
 - Institute of Food Safety
- **WUR**
 - Wageningen University and Research

Presentation

- Introduction to study on emerging substances
- Method development
- Application (examples)
- Method optimization and outlook

Background...

- NORMAN has identified a list of the currently most frequently discussed emerging substances and emerging pollutants today. Examples of this list are surfactants, pharmaceuticals and personal care products, methyl tert-butyl ether (MTBE) and other related petrol additives and their degradation products, polar pesticides and their degradation products and various proven or suspected endocrine disrupting compounds (EDCs). Another example is nanoparticles, which behave aerodynamically like gas molecules and have a large surface area per unit mass.

Background...

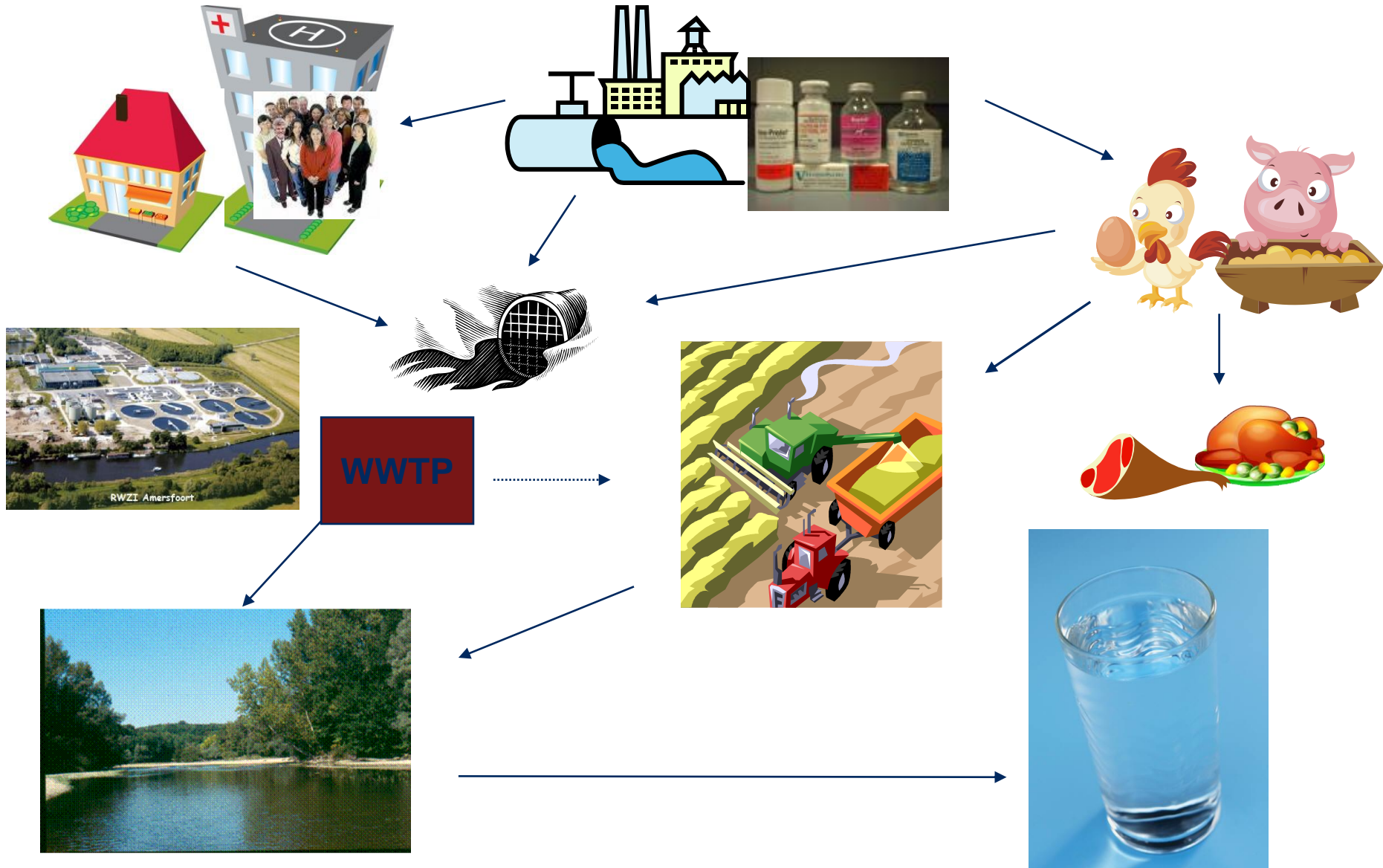
- NORMAN has identified a list of the currently most frequently discussed emerging substances and emerging pollutants today. Examples of this list are surfactants, **pharmaceuticals** and personal care products, methyl tert-butyl ether (MTBE) and other related petrol additives and their degradation products, polar pesticides and their degradation products, and various proven or suspected endocrine disrupting compounds (EDCs). An example is nanoparticles, which behave chemically like gas molecules and have a large surface area per unit mass.

'New substances', ask for 'new methods'

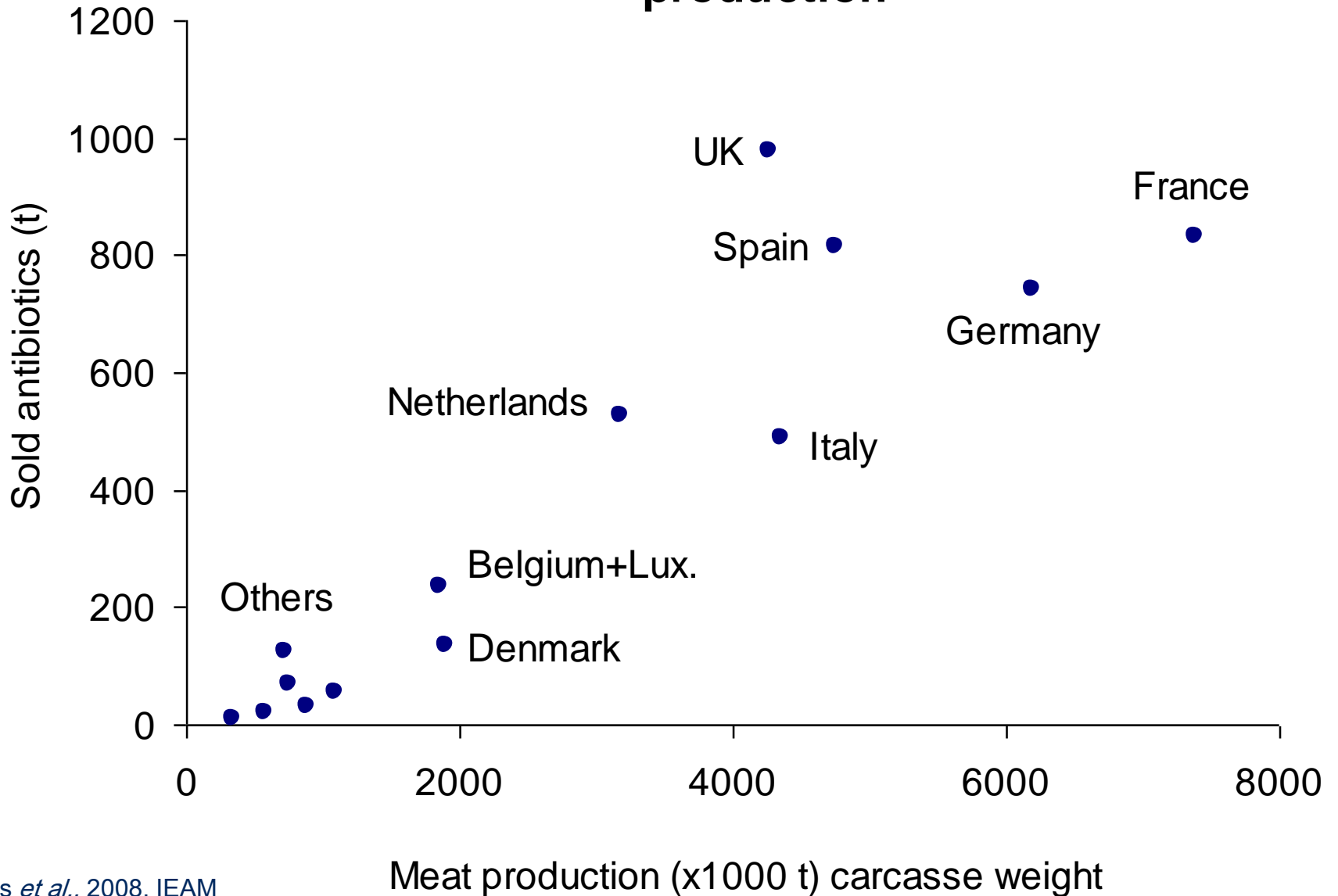
Pharmaceuticals – antibiotics

- Originally – natural substances against bacteria, fungi, protozoa:
 - Bactericidal – kill microbes
 - Bacteriostatic – inhibit growth
- 1928 – 1st antibiotic → penicillin
- Worldwide use: '*antibiotic era*'
 - Treatment in human and veterinary medicine
 - Threat: antibiotic resistance – prudent use
 - In veterinary medicine for prevention and as growth promoters (2004 stop in EU)

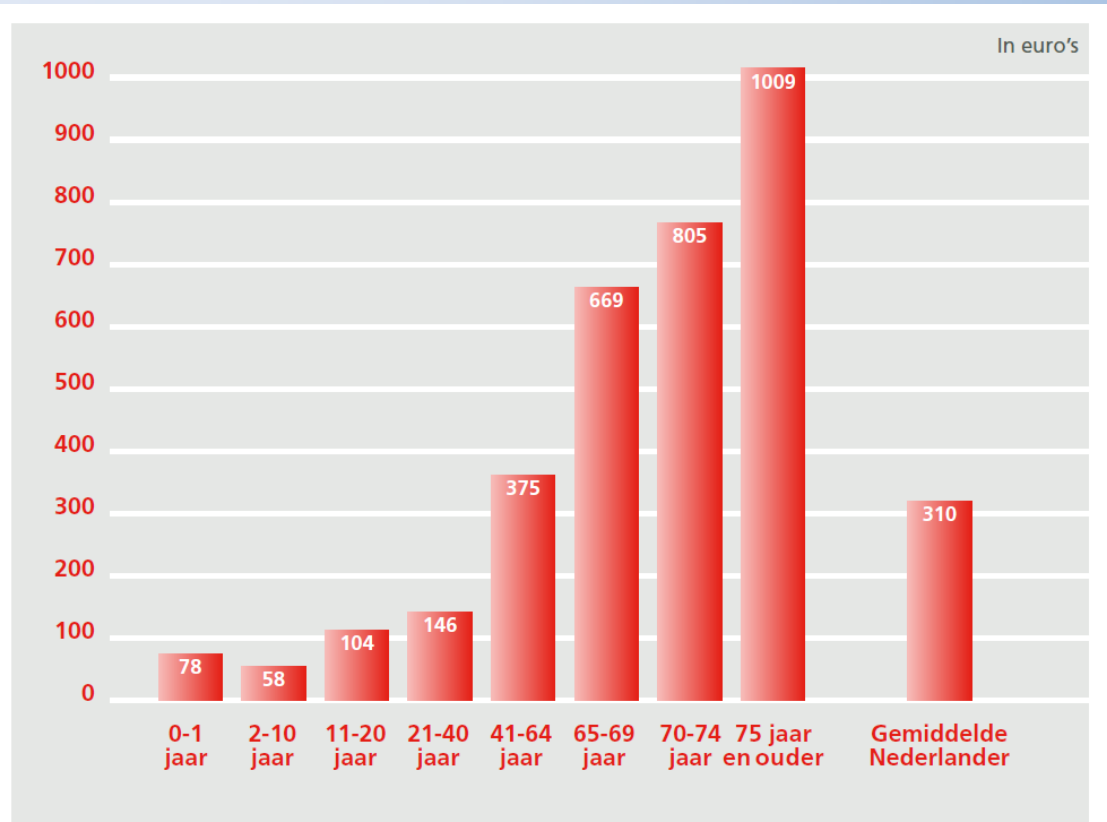
Antibiotics – fate and distribution



Relation between antibiotics and meat production



Use in households (NL)



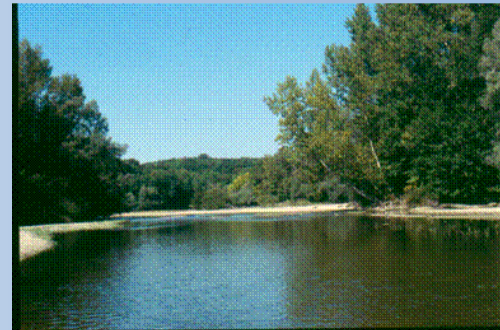
Bron: Stichting Farmaceutische Kengetallen

- Older population uses more medicines
- Expected life 2005-2025
 - >65 yr: +60%

Apples and oranges?

Antibiotics groups

- Aminoglycosides (A)
- Macrolides (M)
- Beta – lactams (B)
- Quinolones (Q)
- Sulfonamides (S)
- Tetracyclines (T)



Bioassay measurement

- Chemical analysis:
 - accurate identification? metabolites?
- Bioassays
 - Potential identification of activity
 - Cf. Steroids (Estrogens, Androgens, etc).
 - Cf. Dioxines
 - Including active metabolites

Methods

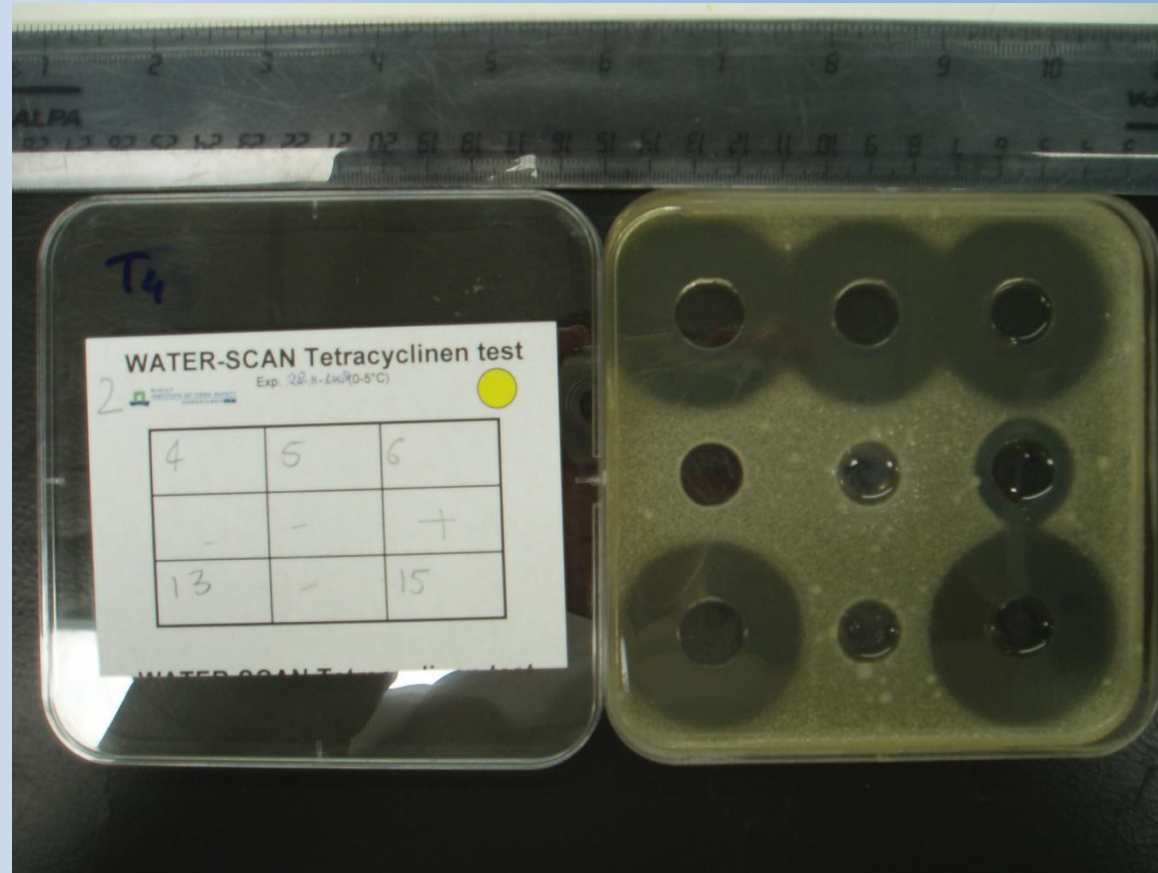


- Developed at RIKILT (Food Safety)
- The Nouws antibiotic test (NAT)
 - slaughter animals (residue analysis)
- 5 test plates – group-specific identification
 - T-plate; Q-plate; M&B-plate; A-plate and S-plate
- **PRINCIPLE**
 - **(Specific) BACTERIAL GROWTH INHIBITION IN PRESENCE OF ANTIBIOTICS**

Water – Scan Test



- Antibiotic present → growth inhibition
- 2 – 16 mm → linear correlation between inhibition zone and concentration



The tests

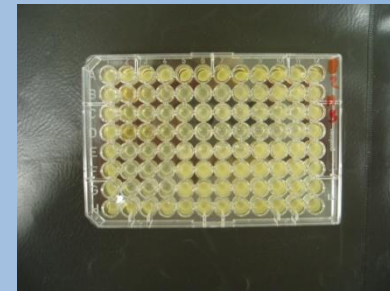


- Different agar compositions were used with different bacteria strains, antibiotic supplements to prepare them
- Applying samples on plates → incubate at 30 °C (T, Q, M/B)
37 °C (S,A)

1 concentration



Dilution series



- Measurements after 24 h incubation
 - Water – Scan Test → Inhibition zones were measured
 - 96 well plate test → Optical density (O.D.) (also at T “0”) was measured

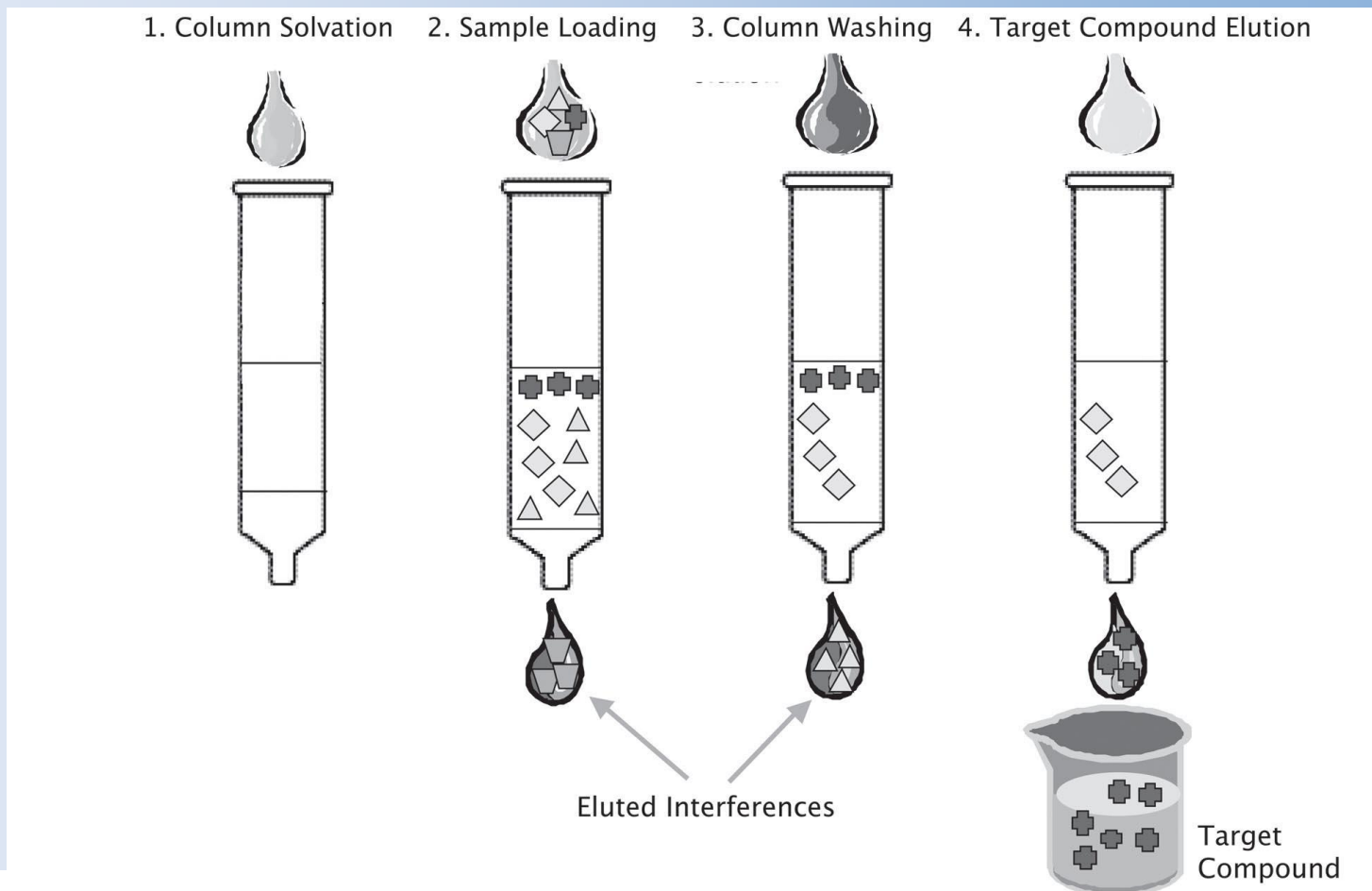
Method development



Questions?

- Extraction method needed ...
 - SPE, special resins (XAD)?
- Detection limit
- Sensitivity?
- Specificity? Cross-reactivity?

SPE – extraction

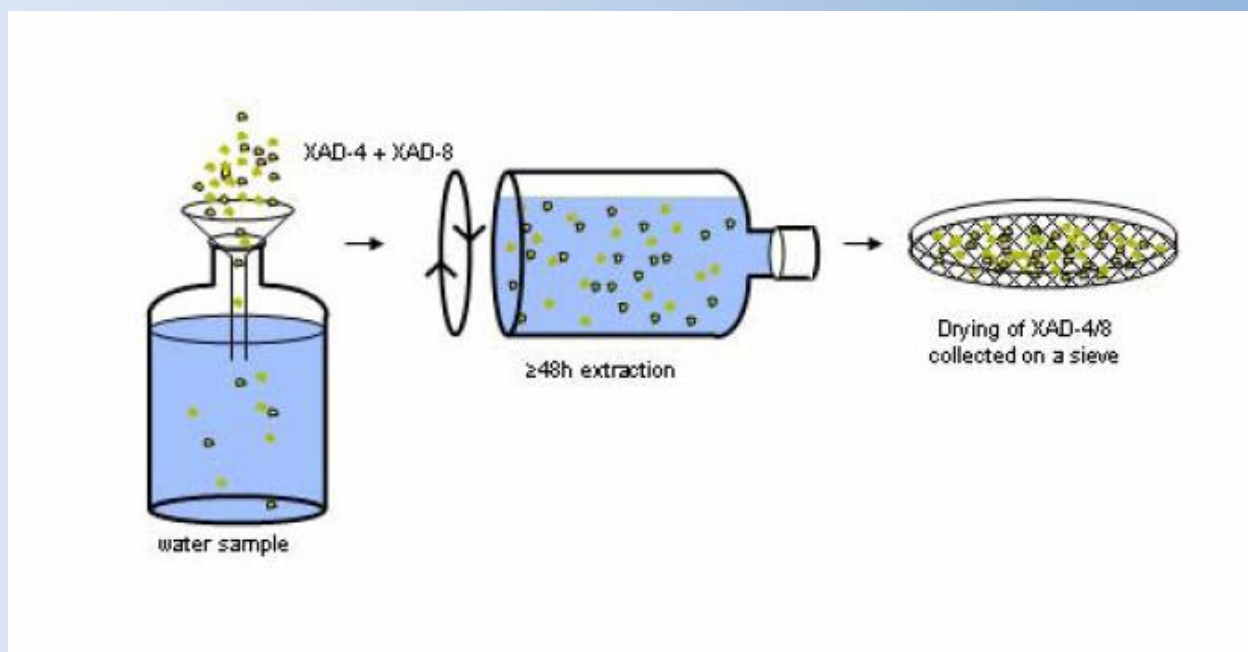


<http://www.biotage.com/graphics/9223.jpg>

XAD 4/8 – extraction



- XAD 4/8 – two synthetic resins used to extract substances

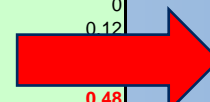


Spike (mix with log Kow range)



| Group | Antibiotic | CAS no | Molecular formula | log Kow | |
|------------------------|--------------------------------|-------------------|-------------------------|--------------|-----------------------|
| | | | | Experimental | Ecosar estim [kowwin] |
| Aminoglycosides | Neomycin B | 144-04-2 | C23H46N6O13 | -1.01 | -9.41 |
| Aminoglycosides | Streptomycin | 57-92-1 | C21 H39 N7 O12 | | -9.07 |
| Aminoglycosides | Apramycin | 37321-09-8 | C21 H41 N5 O11 | | -8.12 |
| Aminoglycosides | Kanamycin sulfate | | C18H36N4O11 | -6.7 | -6.7 |
| Tetracyclines | Oxytetracycline [SPE] | 79-57-2 | C22H24N2O9 | -0.9 | -2.87 |
| Cephalosporines | Cefazolin | 25953-19-9 | C14 H13 N8 O4 S3 | -0.58 | -2.19 |
| Aminoglycosides | Gentamicin | 1403-66-3 | C21 H43N5 O7 | | -1.88 |
| Cephalosporines | Cefalonium | 5575-21-3 | C20H18N4O5S2 | | -1.66 |
| Tetracyclines | Doxycycline=vibramycin | 564-25-0 | C22H24N2O8 | -0.02 | -1.36 |
| Tetracyclines | Tetracycline [SPE] | 60-54-8 | C22H24N2O8 | -1.3 | -1.33 |
| Cephalosporines | Cefacetile | 10206-21-0 | C13 H13 N3 O6 S1 | -0.45 | -1.12 |
| Quinolones | Marbofloxacin | 115550-35-1 | - | | -1.11 |
| Penicillines | Nafcillin | 985-16-0 | C21 H21 N2 O5 S1 Na1 | | -1.07 |
| Aminoglycosides | Spectinomycin | 1695-77-8 | C14 H24 N2 O7 | | -0.82 |
| Cephalosporines | Cefapirin | 21593-23-7 | C17H17N3O6S2 | -1.15 | -0.8 |
| Tetracyclines | Chlortetracycline [SPE] | 57-62-5 | C22H23ClN2O8 | -0.62 | -0.68 |
| Cephalosporines | Cefoperazone | 62893-19-0 | C25 H27 N9 O8 S2 | -0.74 | -0.42 |
| Sulfonamides | Sulfadiazine | 68-35-9 | C10 H10 N4 O2 S1 | -9 | -0.34 |
| Sulfonamides | Sulfadoxine | 2447-57-6 | C12 H14 N4 O4 S1 | 0.7 | -0.24 |
| Quinolones | Ciprofloxacin [SPE] | 85721-33-1 | C17H18FN3O3 | 0.28 | 0 |
| Quinolones | Sarafloxacin | 98105-99-8 | C20H17F2N3O3 | | 0.12 |
| Macrolides | Lincomycin | 154-21-2 | C18 H34 N2 O6 S1 | 0.56 | |
| Cephalosporines | Cefalexin | 15686-71-2 | C16H17N3O4S | 0.65 | |
| Sulfonamides | Sulfamethoxazole [SPE] | 723-46-6 | C10 H11 N3 O3 S1 | 0.89 | 0.48 |
| Quinolones | Enrofloxacin | 93106-60-6 | C19H22FN3O3 | | 0.7 |
| Sulfonamides | Trimethoprim | 738-70-5 | C14H18N4O3 | 0.91 | 0.73 |
| Sulfonamides | Sulfamethazine [SPE] | 57-68-1 | C12 H14 N4 O2 S1 | 0.89 | 0.76 |
| Sulfonamides | Dapsone | 80-08-0 | C12 H12 N2 O2 S1 | 0.97 | 0.77 |
| Tetracyclines | Chloramphenicol | 56-75-7 | C11H12Cl2N2O5 | 1.14 | 0.92 |
| Penicillines | Amoxicillin | 26787-90-3 | C16H19N3O5S | 0.87 | 0.97 |
| Macrolides | Tylosin | 1401-69-0 | C46 H77 N1 O17 | 1.63 | 1.05 |
| Sulfonamides | Sulfadimethoxine [SPE] | 122-11-2 | C12 H14 N4 O4 S1 | 1.63 | 1.17 |
| Quinolones | Danofloxacin | 112398-08-0 | C19H20FN3O3 | | 1.19 |
| Quinolones | Difloxacin | 98106-17-3 | C21H19F2N3O3 | 0.89 | 1.28 |
| Penicillines | Ampicillin | 69-53-4 | C16 H19 N3 O4 S1 | 1.35 | 1.45 |
| Quinolones | Oxolinic acid | 14698-29-4 | C13H11NO5 | | 1.7 |
| Macrolides | Oleandomycin | 3922-90-5 | C35 H61 N1 O12 | 1.69 | 1.83 |
| Penicillines | Penicillin G | 61-33-6 | C16 H18 N2 O4 S1 | 1.83 | 1.85 |
| Macrolides | Erythromycine | 114-07-8 | C37 H67 N1 O13 | 3.06 | 2.48 |
| Penicillines | Oxacillin | 66-79-5 | C19 H19 N3 O5 S1 | 2.38 | 2.57 |
| Quinolones | Flumequine | 42835-25-6 | C14H12FNO3 | 1.6 | 2.7 |
| Sulfonamides | Baquiloprim | 102280-35-3 | C17 H20 N6 | | 2.84 |
| Penicillines | Cloxacillin | 61-72-3 | C19 H18 Cl1 N3 O5 S1 | 2.48 | 3.22 |
| Penicillines | Dicloxacillin | 3116-76-5 | C19 H17 Cl2 N3 O5 S1 | 2.91 | 3.86 |
| Macrolides | Tilmicosin | 108050-54-0 | C46 H80 N2 O13 | 3.8 | 4.13 |
| Macrolides | Valnemulin | 101312-92-9 | C31H52N2O5S | | 4.16 |

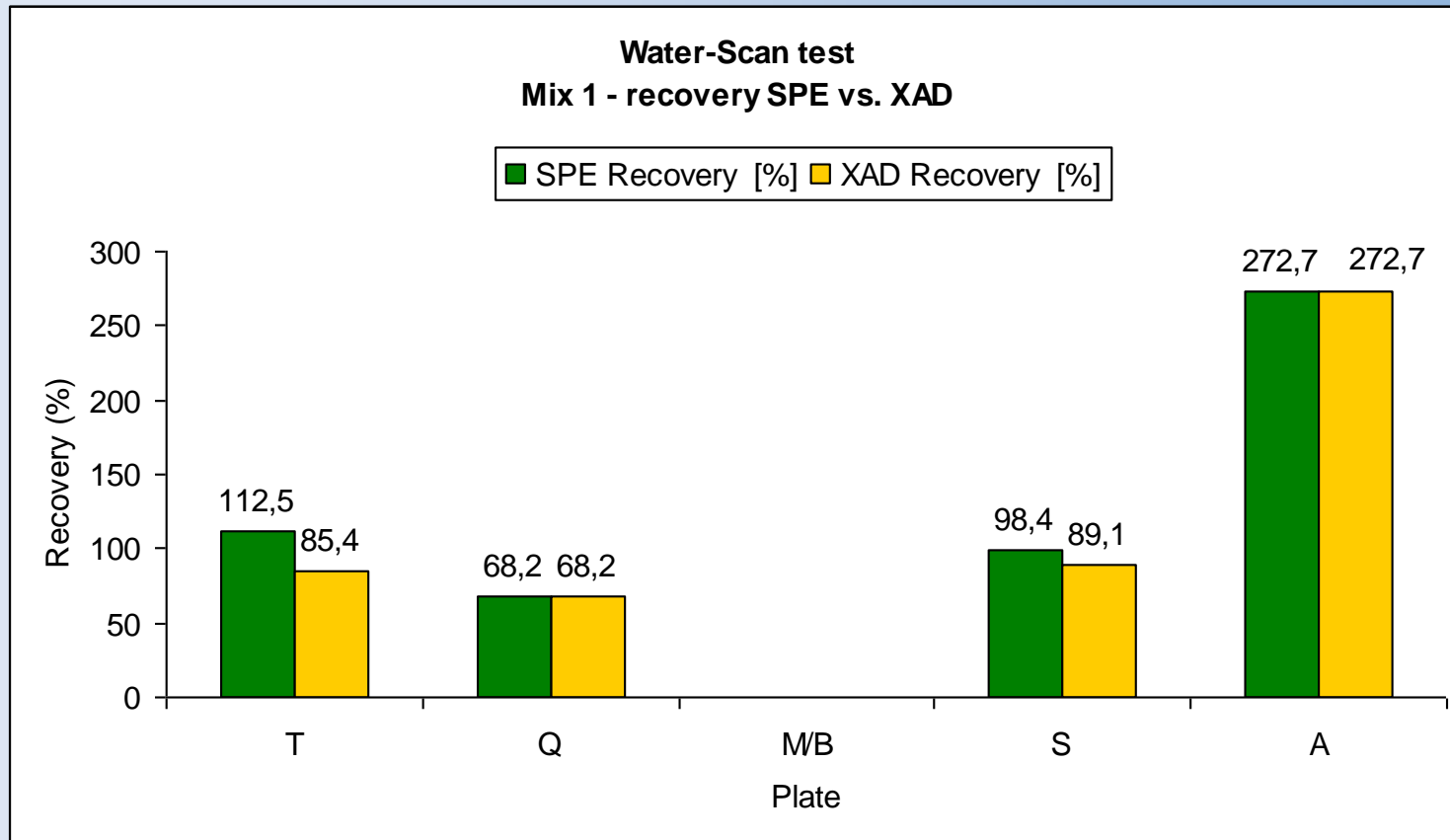
1. Neomycin
2. Oxytetracycline-OTC
3. Chlortetracycline-CTC
4. Sulfadiazine
5. Sulfamethoxazole-SMX
6. Oxolinic acid
7. Erythromycine
8. Flumequine



Log Kow clusters:



Recovery patterns (spiked water)



Conclusions



- Some cross-reactivity noted
 - At plate “A”: aminoglycosides
- Specific?
 - Limited effects (<< cyto toxicity) of others
 - RIVM data
- Overview of ‘indicative’ detection limits for >35 compounds (1-1000 ug/L)
 - Waterdienst data

Application, some examples



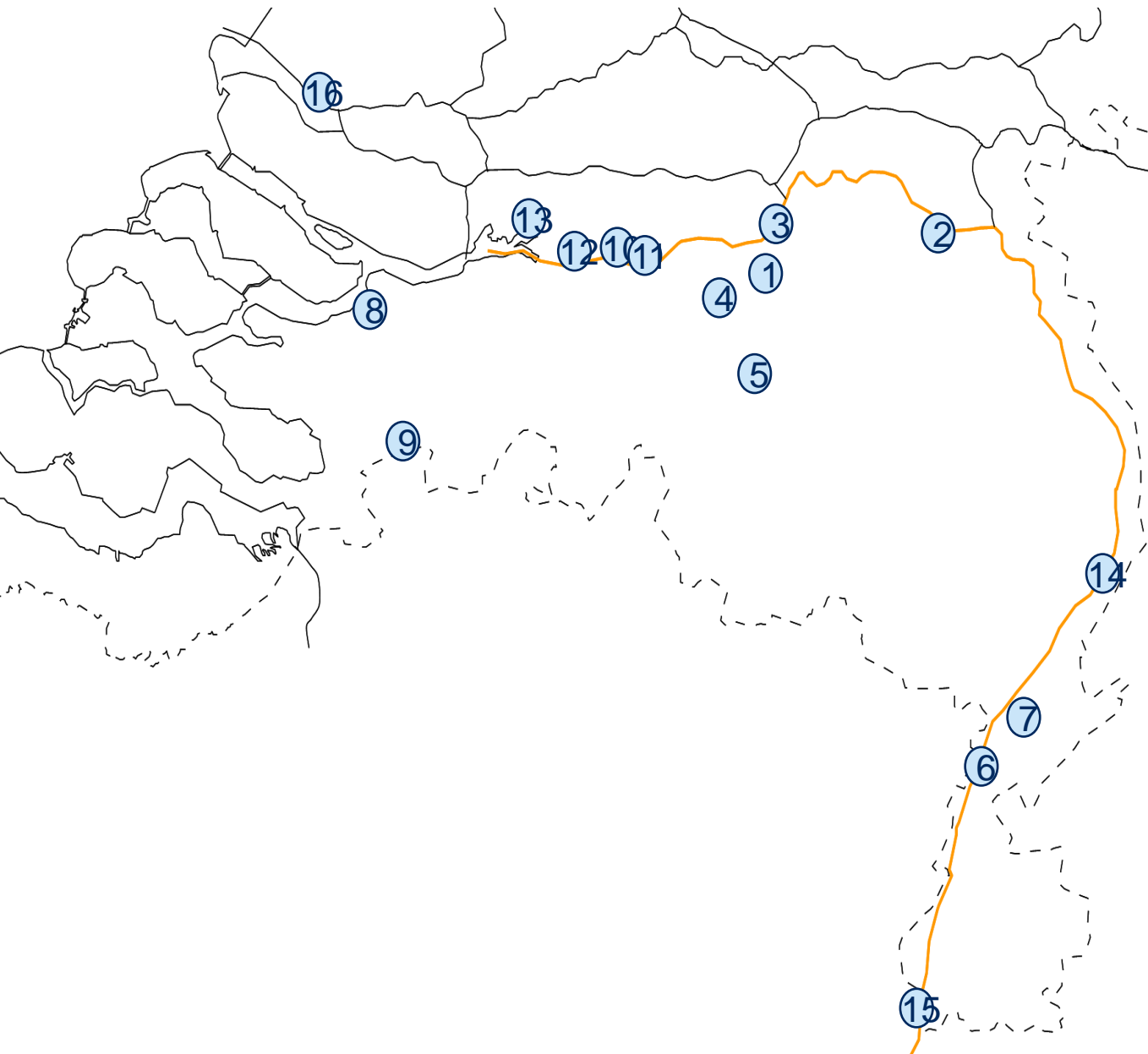
- Monitoring
- Screening locations
- Review of treatment techniques
- New treatments, do they remove?
- Prioritization of locations
- Identification of hot-spots

End-of-pipe?

- Antibiotics in river water
- Monitoring/screening
- Hot-spots?



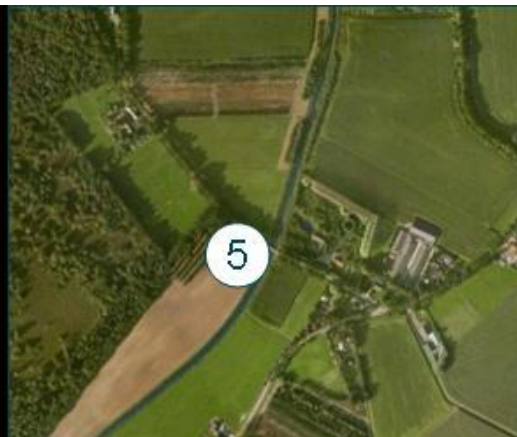
Screening the Dutch Meuse river basin (Waterdienst)



Screening the Dutch Meuse river basin (Waterdienst)



| | T | Q | S | M | A |
|----|---|---|---|---|---|
| 1 | ● | ● | ● | ● | ● |
| 2 | ● | ● | ● | ● | ● |
| 3 | ● | ● | ● | ● | ● |
| 4 | ● | ● | ● | ● | ● |
| 5 | ● | ● | ● | ● | ● |
| 6 | ● | ● | ● | ● | ● |
| 7 | ● | ● | ● | ● | ● |
| 8 | ● | ● | ● | ● | ● |
| 9 | ● | ● | ● | ● | ● |
| 10 | ● | ● | ● | ● | ● |
| 11 | ● | ● | ● | ● | ● |
| 12 | ● | ● | ● | ● | ● |
| 13 | ● | ● | ● | ● | ● |
| 14 | ● | ● | ● | ● | ● |
| 15 | ● | ● | ● | ● | ● |
| 16 | ● | ● | ● | ● | ● |

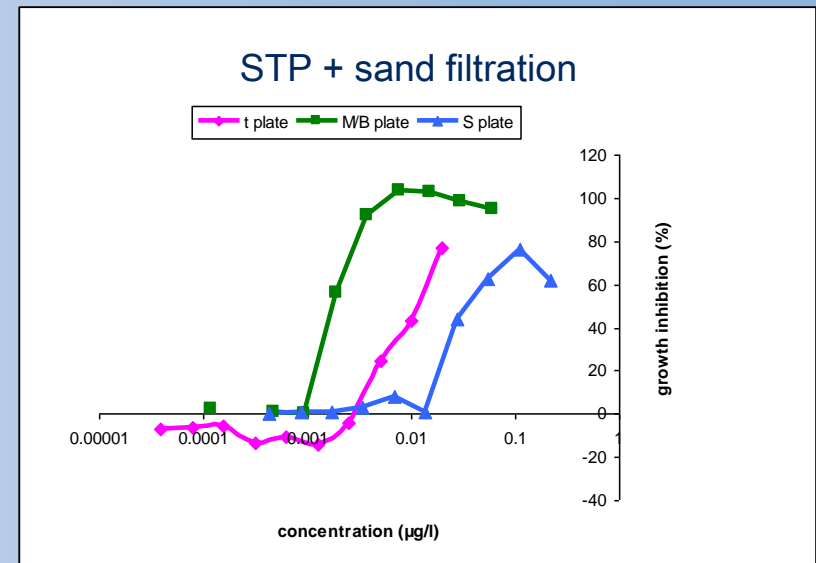
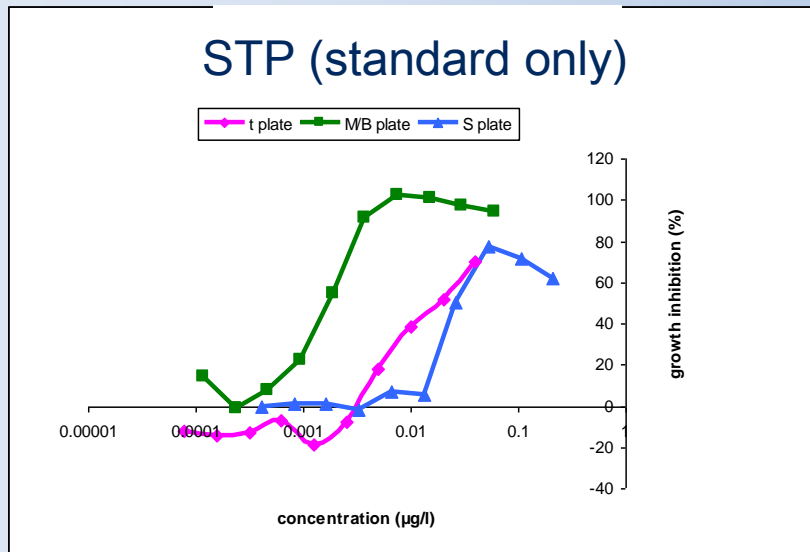


Zandleij near WWTP Ditch near a pig farm

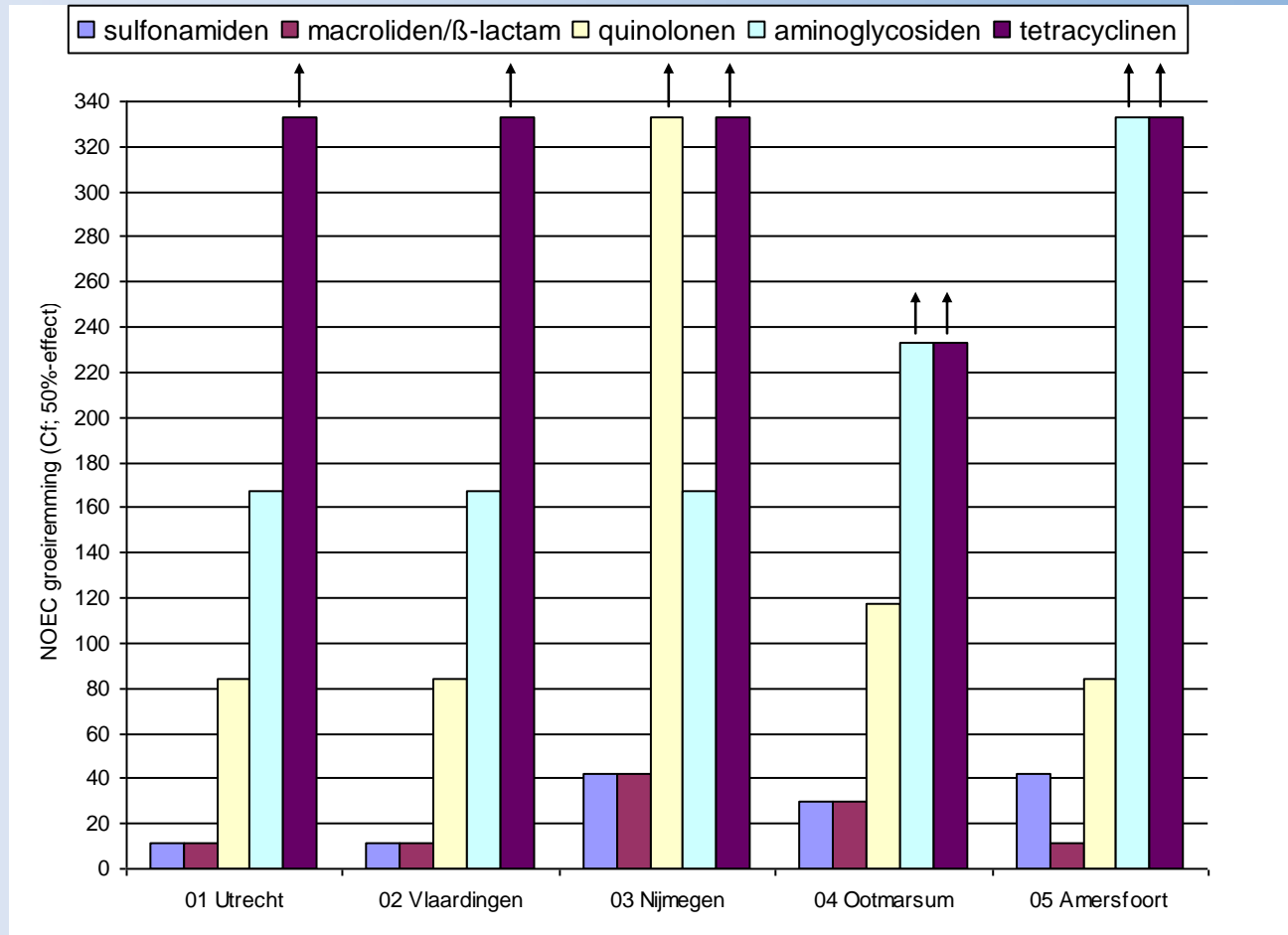
Example: use of antibiotic bioassay



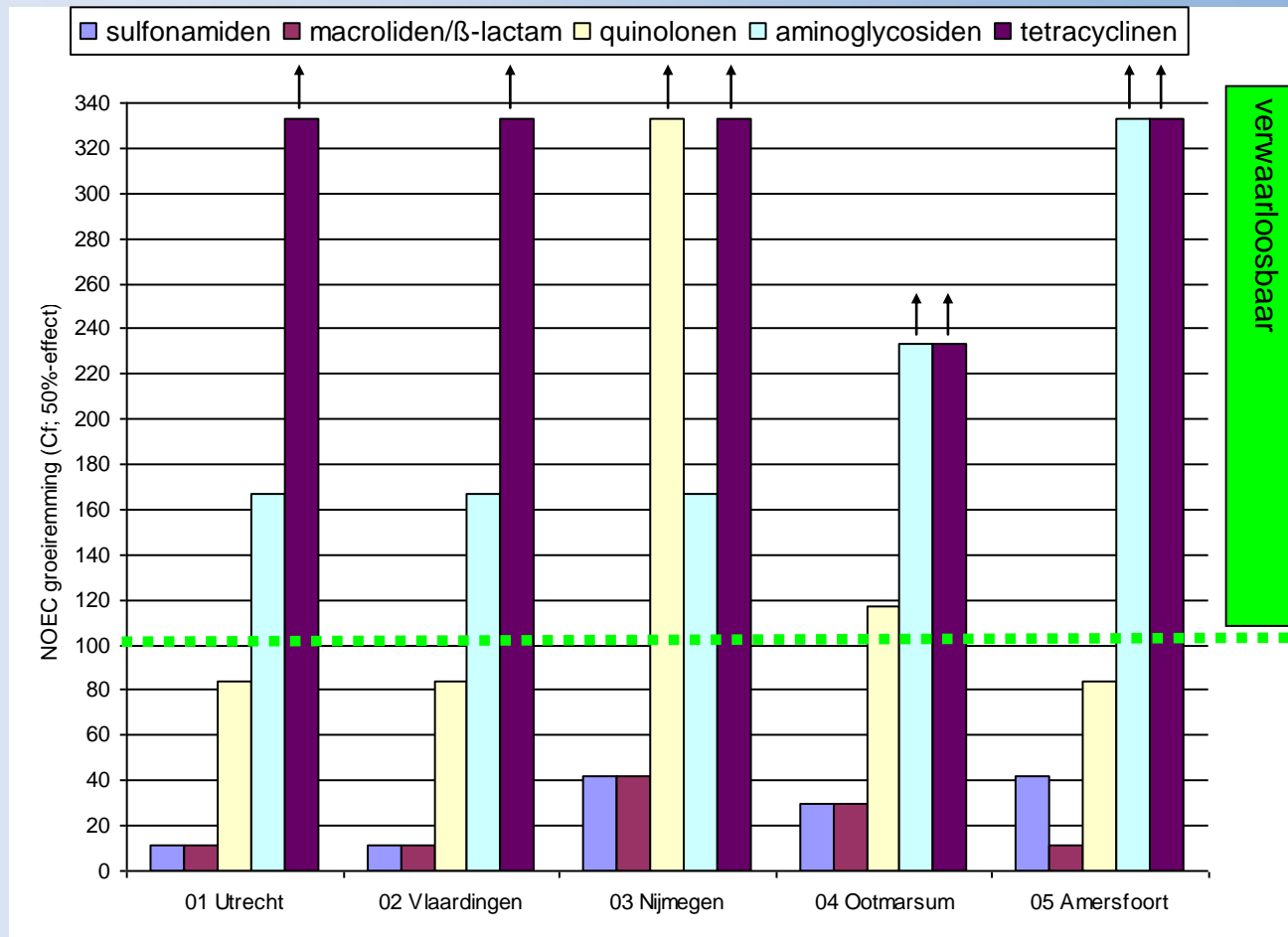
- STP standard sewage treatment plant
- STP + sand filter
 - Response patterns similar in 96-wells test



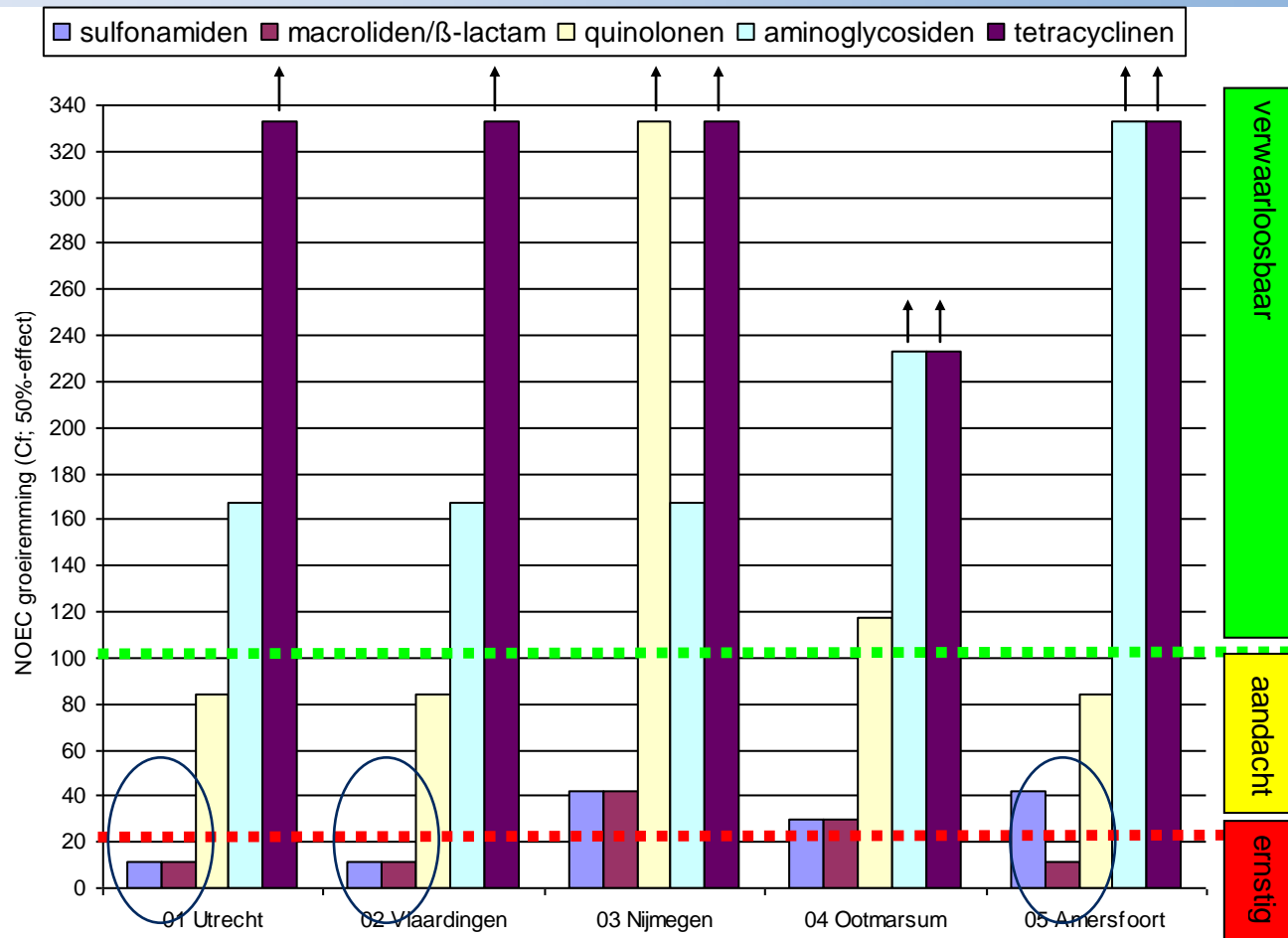
Antibiotics screening: locations



Antibiotics screening: assessment



Antibiotics screening: assessment



Conclusion

- Method optimization
 - (with interested parties)
- Available for routine screening
 - in combination with other bioassays
 - in combination with chemical analysis
 - (for interested parties)
- Outlook:
 - Possible application in research on the emission of antibiotics from households, hospitals and care-institutions ...

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Antibiotics screening in the Dutch Meuse river basin.

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Introduction

The need for screening of antibiotic agents surface waters is motivated by their wide use in both human and veterinary medicine and the possible spread of resistance caused by their constant environmental input. A low cost screening method (Water-SCAN) for detecting up to 48 antibiotic agents in surface water effluents was used to screen samples taken from the Dutch part of the Meuse and its river basin in the summer of 2007. The method consists of a 5-plate bacterial growth inhibition assay for different classes of antibiotics: Tetracyclines (T), Quinolones (Q), Sulphonamides (S), Macrolides (M) and Aminoglycosides (A). 50% and 100% methanol containing 125 ml sample equivalents were found sufficient for detecting antibiotics in 10 out of 16 locations. Typical LODs are 10 ng/l or less for 4 out of 48 agents. Besides the river basin survey, the results of a two-year screening effort (2005-2007) at the Epsen monitoring station are presented. Microbes proved to be the prevailing antibiotic class in this location. Effect directed analyses using HPLC fractionation into 50 fractions, followed by LC-MS/MS showed that Levonorgestrel was the causative agent.

Methods

Extraction: A sample of 1 liter of surface water is extracted through Solid Phase Extraction with ENVI-Clean H (Supelco). After desorption with methanol the extract is made up in 1 ml 50% methanol/water, concentration factor 1000.

Original format: Aliquots of 200 µl (or 375 µl on 96-plates) are transferred on to the inoculation plates. After inoculation overnight the growth inhibition zones are registered through digital photography.

Fractionation: An extract is fractionated with HPLC on C18 using a Methanol/Water gradient, injection volume 100 µl. Up to 50 fractions of 300 µl are collected in a inoculation plate, evaporated and reconstituted with inoculation buffer. Microbial format: Aliquots of 70 µl are transferred onto the inoculation plates. After inoculation overnight the difference in Optical Density is measured at 600 nm using a plate reader.

Table: Overview of detection of antibiotics in the Meuse (2007)

| Location | Antibiotic Class | Detection | |
|---------------------|-------------------|---------------|-------|
| Meuse basin | Tetracyclines (T) | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | Quinolones (Q) | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | Sulphonamides (S) | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | Macrolides (M) | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| | | 100% methanol | 10/16 |
| | | 50% methanol | 10/16 |
| Aminoglycosides (A) | 100% methanol | 10/16 | |
| | 50% methanol | 10/16 | |
| | 100% methanol | 10/16 | |
| | 50% methanol | 10/16 | |

Use and applicability of bioassays to measure effects of antibiotics in sewage water

Ever-Jan van den Brandhof, Esther van der Grinten, Remko Siers
Geert Bloembergen, Arno Stam, Serge Hofsteede, Maria Sijm
Rijkswaterstaat, Centre for Water Management, Grootes, The Netherlands

INTRODUCTION

- In the Netherlands the monitoring of the surface water quality involves the measurement of non-specific toxic effects by use of bioassays (B).
- This paper compares methods for specific antibiotic risk assessment (P) in the field.
 - Standard methods
 - Non-adapted multi-bacteria screening test
 - Cyanobacteria photosynthesis efficiency test

METHODS

Validation:

- Effects of laboratory-prepared solutions of six different antibiotics were measured with the standard Microtox test and with two specific tests: a 96-wells application of the multi-bacteria screening test and in cyanobacteria PAM test.

Field tests:

- Samples came from a hospital sewage, and five different STP (sewage treatment plant) effluents.
- Organic pollutants were concentrated by subsequent adsorption onto XAD resins (48 h), acetone elution and transfer into the water phase by distillation.

Test system

RESULTS

CONCLUSIONS

- The PAM test of the cyanobacteria green alga *P. subcapitata* appeared unexpectedly more sensitive to tetracyclines and the macrolide tylosine than the cyanobacteria *M. aeruginosa*, and also for tylosine in the multi-bacteria test.
- Specificity for joint-antibiotic inhibition needs more attention.
- The acute short term exposure microtox-test is unsuitable for measuring antibiotic effects.
- The multi-bacteria score without pre-screening results in detecting effects of antibiotics in the field.

WHAT'S NEXT?

- Optimization and automation of the multi-bacteria score by a second application.
- Determination of the specificity of the multi-bacteria score for other non-antibiotic components (opinion-modulators, disinfectants, cleaning agents, pesticides etc.).
- Further studies on the recovery of antibiotics with the concentration procedure and on additional extraction with resins like XAD-2.
- A fractionated sample approach Pto selected field samples can be tested to see which antibiotic fractions are responsible for the effects in the chosen antibiotic test methods.

References:

Acknowledgements:

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Grontmij | AquaSense

planning connecting
respecting
the future

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