QUANTIFICATION OF FIVE DIFFERENT CLASSES OF VETERINARY ANTIBIOTICS BEFORE AND AFTER TREATMENT OF SWINE MANURE USING A UHPLC-MS/MS METHOD

Van den Meersche Tina

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Institute for Agricultural and Fisheries Research

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Introduction

PhD project

Development and validation of a multi-compound analysis for the quantification of veterinary antibiotics in swine manure and soil using UHPLC-MS/MS

Optimization of DNA-extraction and qPCR to quantify antibiotic resistance genes in swine manure and soil samples

Screening for antibiotic residues, antibiotic resistance genes and pathogens (*Salmonella*, *Campylobacter* and *E. coli*)

Raw manure

Soil before and after spreading of manure

Manure during biological manure processing

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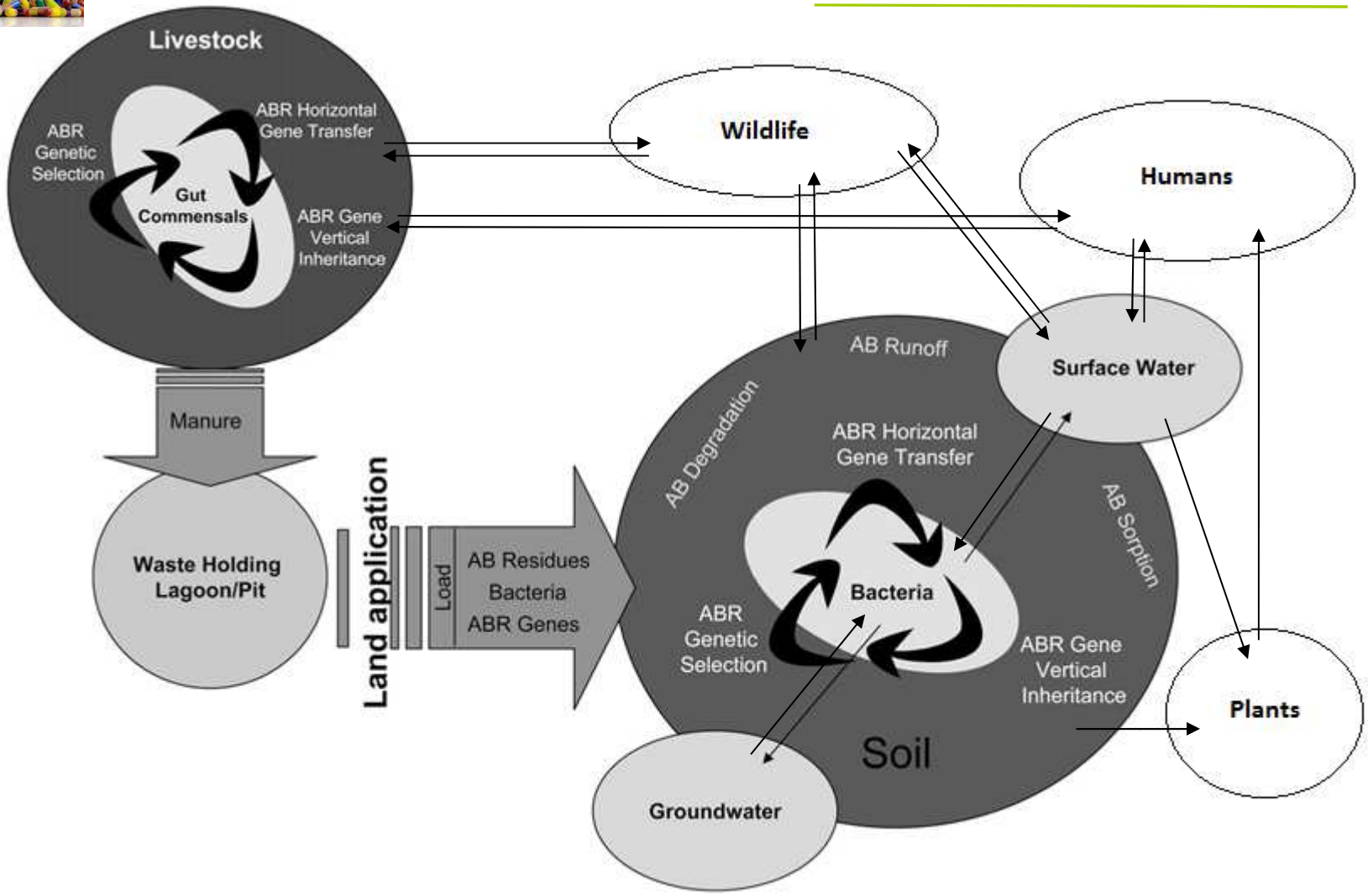
Introduction

PhD project



(Adapted from Chee-Sanford *et al.* (2009), Journal of Environmental Quality)

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Introduction

Use of manure in Belgium

Grasslands

Maize

Cereals

Vegetables

Introduction

Guidelines

Nitrates

Phosphates

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Introduction

Manure treatment

Manure treated in 2014: 39.3 kilotons N

- 120 plants
- Biological treatment
- 44% due to treatment and export of pig manure
- Unique situation due to the limited amount of arable land compared to the number of pigs

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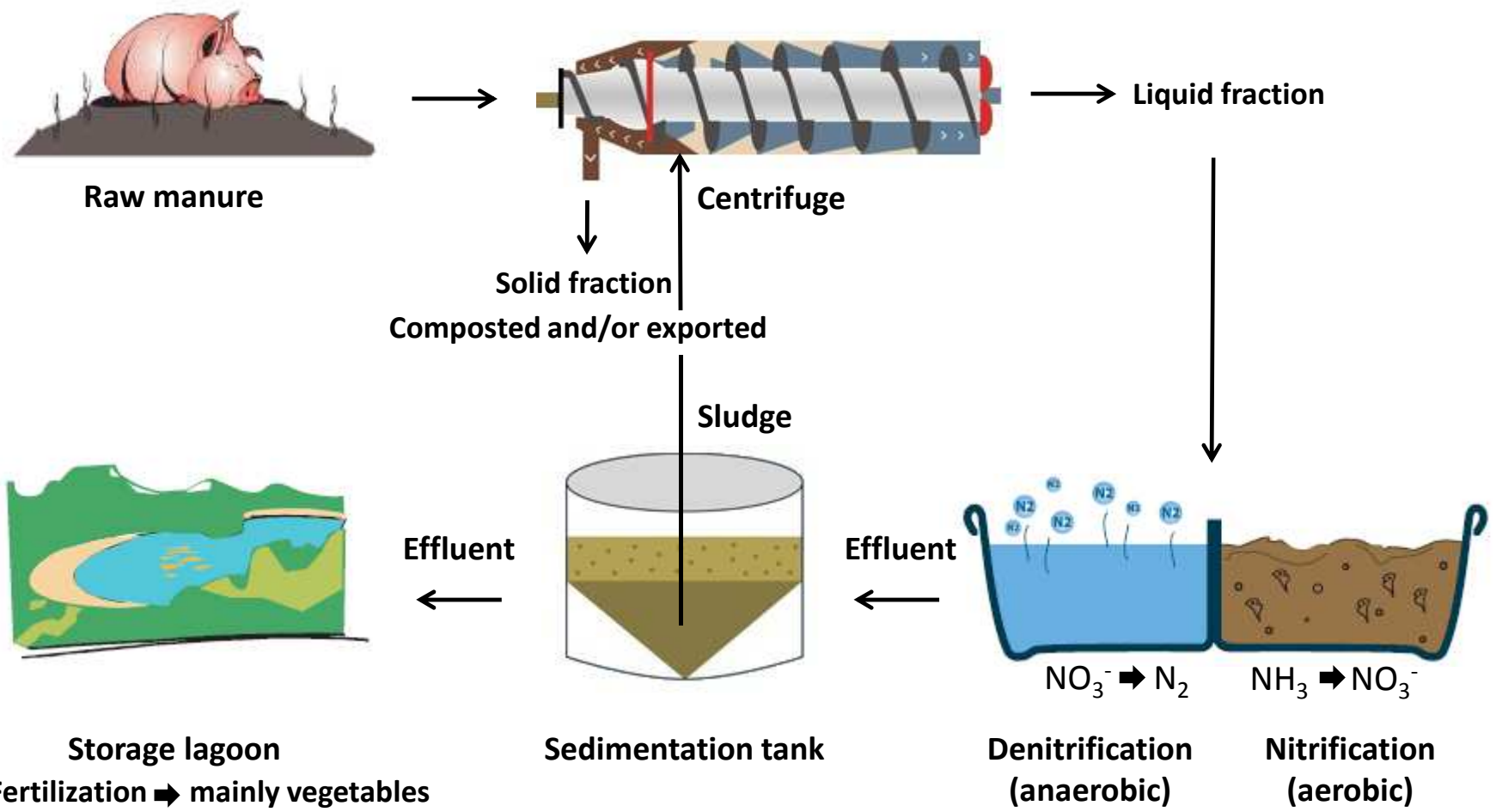
Introduction

Biological manure treatment



~ 35 days

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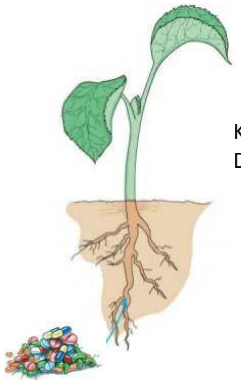
Introduction

But…



Kumar *et al.* (2005), Journal of Environmental Quality
Dolliver *et al.* (2007), Journal of Environmental Quality

Antibiotic residues

Pharmaceuticals

AB resistance genes

Introduction

Antibiotic use

Antibiotics used in pig production in Belgium

(Callens *et al.* (2012), Preventive Veterinary Medicine)

1. Penicillins (27,6%)
2. Polymyxins (27%)
3. Macrolides (17,7%)
4. Sulphonamides + trimethoprim (11,5%)
5. Tetracyclines (10%)

Approximately 75% excreted unchanged

(Chee-Sanford *et al.* (2009), Journal of Environmental Quality)

Aims

1. To develop and validate a method to quantify antibiotic residues from five different classes in swine manure in one run

2. To apply the method to swine manure samples

3. To assess whether biological manure treatment influences the antibiotic residues present in the manure

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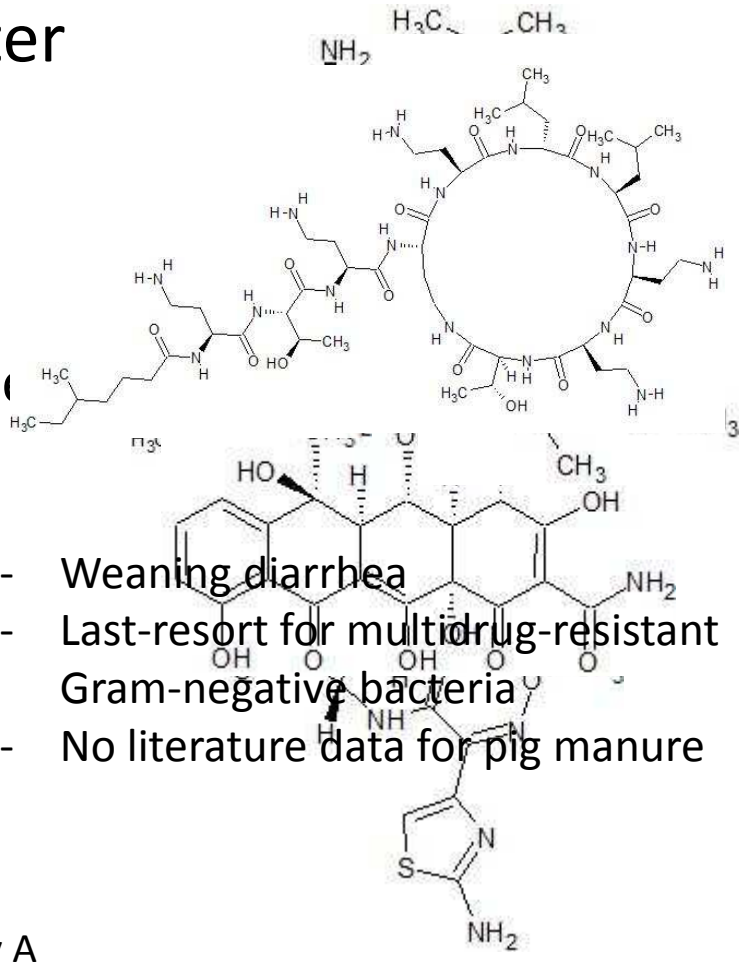
Results

Selection of antibiotics

– Administered via food or water
  - Amoxicillin
  - Colistin
  - Sulfadiazine and trimethoprim
  - Doxycycline and oxytetracycline
  - Tylosin

– Administered via injection
  - Ceftiofur



- Weaning diarrhea
- Last-resort for multidrug-resistant Gram-negative bacteria
- No literature data for pig manure

Van den Meersche *et al.* (2016), Journal of Chromatography A

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Results

UHPLC-MS/MS

Mass spectrometric parameters

(Xevo TQ-S, Waters)

- 2 product ions per precursor ion

Chromatographic separation

(Acquity UPLC H-class system, Waters – Kinetex C_{18}, Phenomenex)

- Mobile phases
  - H_2O/MeCN (95/5) + 0.5% formic acid + 0.1% ammonium formate (A)
  - MeCN + 0.1% formic acid (B)
- Gradient elution (15 min)

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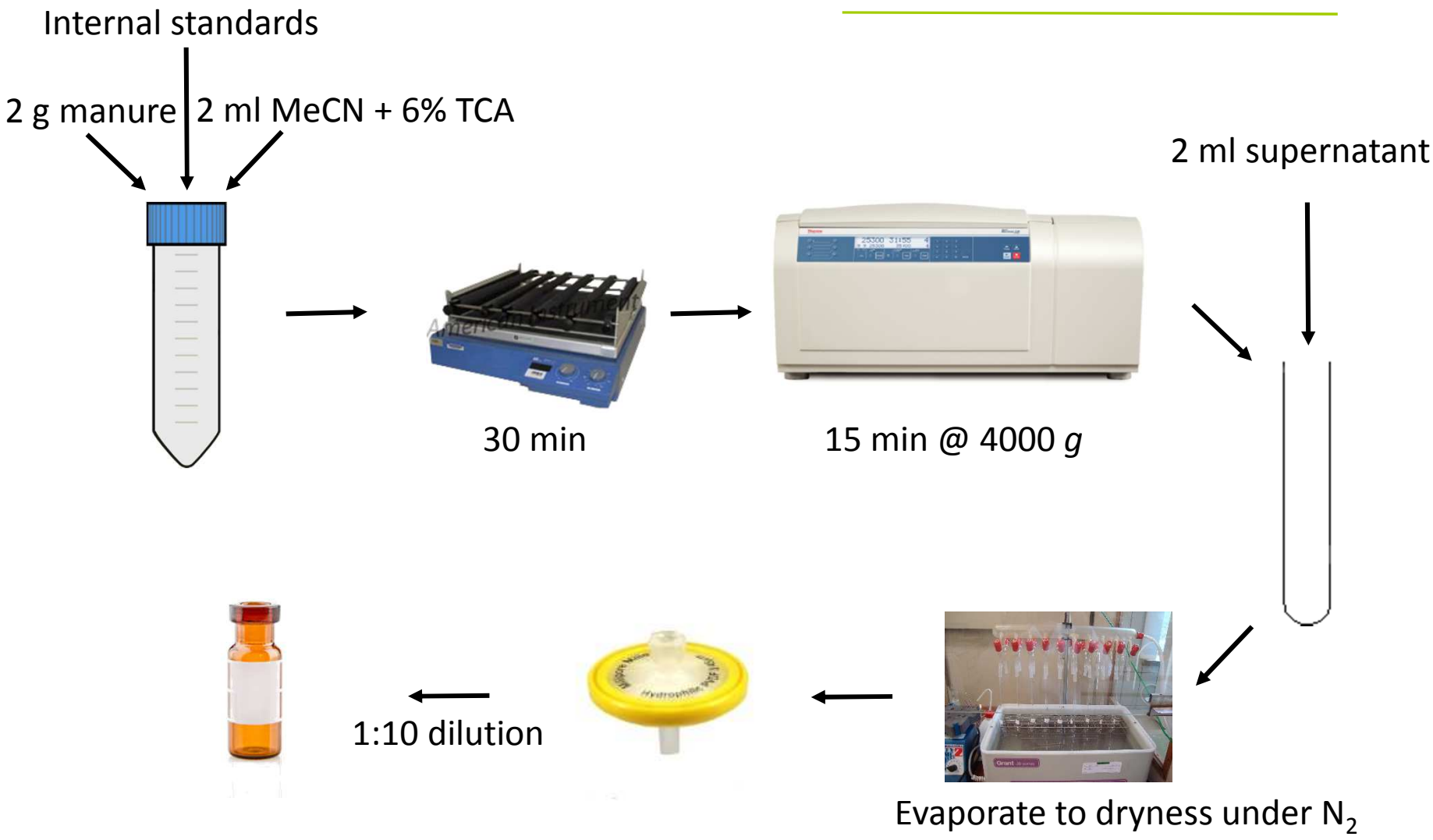
Van den Meersche *et al.* (2016), Journal of Chromatography A

Results

Extraction



Van den Meersche *et al.* (2016), Journal of Chromatography A

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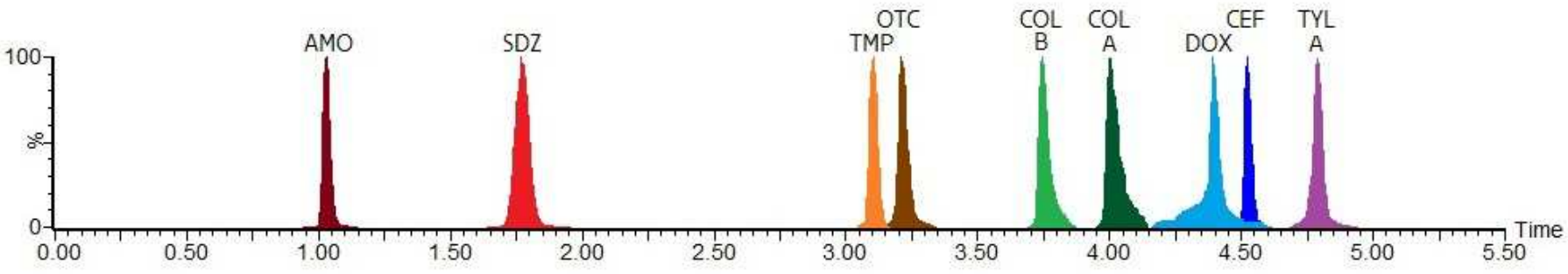
Results

Extracted spiked manure sample



Chromatogram of a blank swine manure sample spiked at 2000 µg/kg with the different antibiotics studied: amoxicillin (AMO), sulfadiazine (SDZ), trimethoprim (TMP), oxytetracycline (OTC), colistin B (COL B), colistin A (COL A), doxycycline (DOX), ceftiofur (CEF) and tylosin A (TYL A).

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Results

Validation levels: 100 – 500 – 1000 µg/kg

- Specificity: ✓
- Recovery: ✓
- Repeatability: ✓
- Reproducibility: ✓
- Linearity in the matrix: ✓
- Limit of detection: ✓
- Limit of quantification: ✓

Aims

1. To develop and validate a method to quantify antibiotic residues from five different classes in swine manure in one run

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3. To assess whether biological manure treatment influences the antibiotic residues present in the manure

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Results

Naturally contaminated samples

Overview of antibiotic residue concentrations (µg/kg) recovered from swine manure samples at different farms or from different manure pits on one farm

Compound	Farm 1a	Farm 1b	Farm 2a	Farm 2b	Farm 3a	Farm 3b	Farm 4	Farm 5	Farm 6	Farm 7
Ceftiofur					x	x				
Colistin A	**1 320**	**48 600**						**1 982**	**1 140**	
Colistin B	**627**	**40 800**						**1 435**	**957**	
Doxycycline	1 336		**11 960**	**22 760**	1 221			**1 400**	**19 525**	**2 715**
Oxytetracycline								18		**2 029**
Sulfadiazine			**763**	**2 980**		23	21		**217**	77
Trimethoprim			**<LOQ**	**6**		<LOQ			**4**	
Tylosin A				NQ			x		NQ	

Detected and used during the three months preceeding the sampling

Detected and not used during the three months preceeding the sampling

x = Used but not detected

Van den Meersche *et al.* (2016), Journal of Chromatography A

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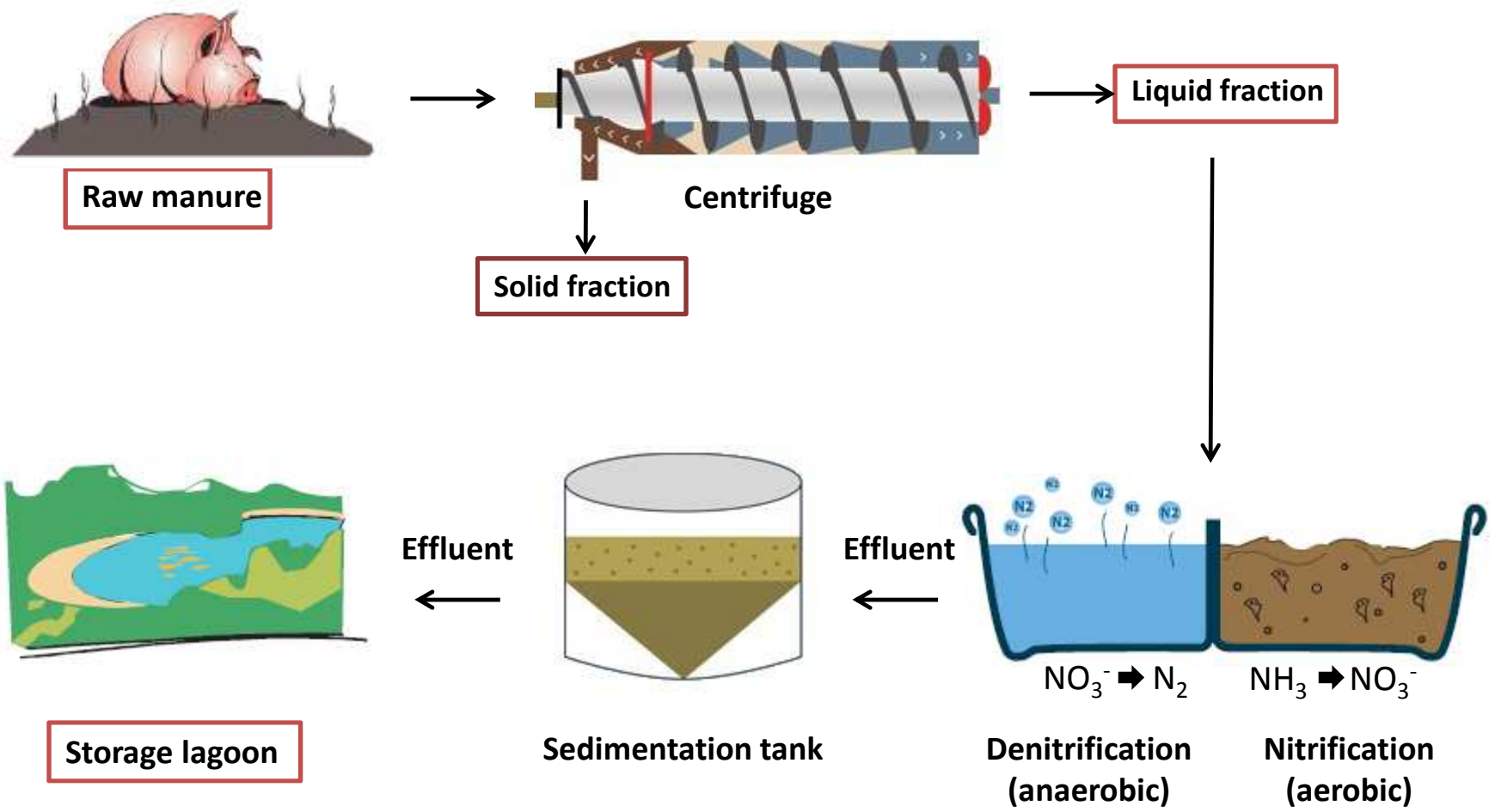
Results

Biological manure treatment



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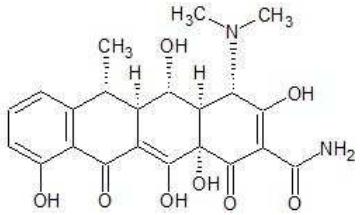
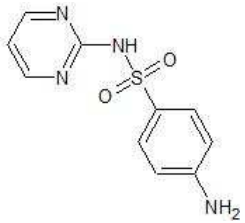
Results

Biological manure treatment

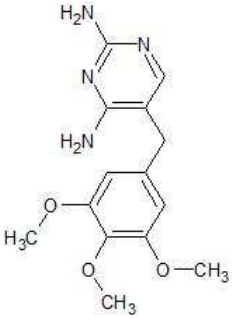
Raw manure



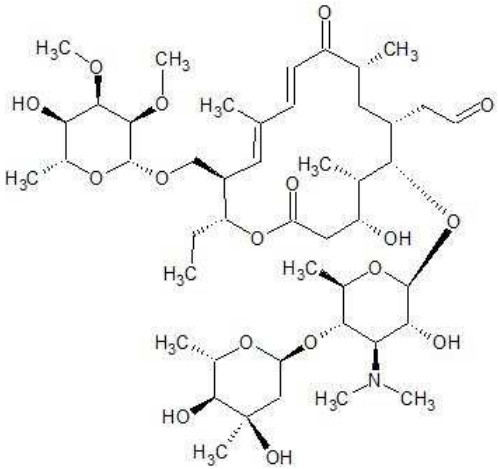
DOX



SDZ



TMP



TYL

Overview of antibiotic residue concentrations (µg/kg)

	Colistin A	Colistin B	Trimethoprim	Sulfadiazine	Doxycycline	Oxytetracycline	Tylosin A	Ceftiofur
Sample 1	0	0	0	5	231	0	0	0
Sample 2	0	0	0	9	196	0	0	0
Sample 3	0	0	0	9	7745	0	NQ	0

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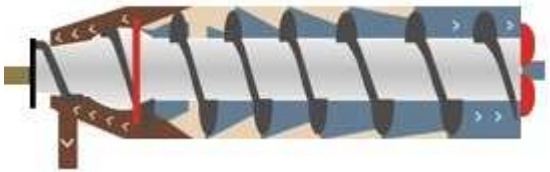
Results

Biological manure treatment



→ **Liquid fraction**

Overview of antibiotic residue concentrations (µg/kg)

	Colistin A	Colistin B	Trimethoprim	Sulfadiazine	Doxycycline	Oxytetracycline	Tylosin A	Ceftiofur
Sample 1	0	0	0	8	139	0	0	0
Sample 2	0	0	0	8	299	0	0	0
Sample 3	0	0	0	10	2541	0	NQ	0

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Results

Biological manure treatment

Solid fraction

Standard addition
Single-point calibration

Overview of antibiotic residue concentrations (µg/kg)

	Colistin A	Colistin B	Trimethoprim	Sulfadiazine	Doxycycline	Oxytetracycline	Tylosin A	Ceftiofur
Sample 1	0	0	5	663	1058	0	0	0
Sample 2	0	0	0	55	1658	0	0	0
Sample 3	0	0	0	79	14497	0	0	0

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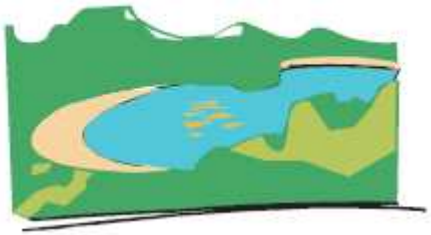
Results

Biological manure treatment



Storage lagoon

Standard addition
Single-point calibration

Overview of antibiotic residue concentrations (µg/kg)

	Colistin A	Colistin B	Trimethoprim	Sulfadiazine	Doxycycline	Oxytetracycline	Tylosin A	Ceftiofur
Sample 1	0	0	0	2	37	0	0	0
Sample 2	0	0	0	2	17	0	0	0
Sample 3	0	0	0	2	26	0	0	0

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Conclusions

1. A link can be found between the antibiotics used and detected in swine manure pits
2. High concentrations of antibiotic residues may be present in swine manure pits
3. Only the antibiotics which were used could be detected during biological manure treatment
4. Reduction of antibiotics after biological manure treatment

   ➡ To be confirmed by further sampling
5. Preliminary results indicate that biological manure treatment can be a tool to reduce the amount of antibiotic residues present in the manure

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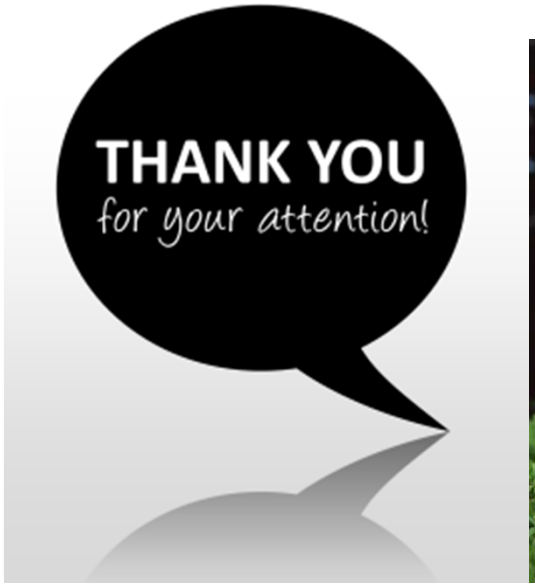

Tina.vandenmeersche@ilvo.vlaanderen.be

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Nitrification/denitrification

Nitrification	Denitrification
▪ NH3 to NO2 to NO3 ▪ Requires nitrifying bacteria ▪ Requires oxygen (aeration) ▪ Releases Hydrogen ions ▪ Consumes alkalinity, lowers ph ▪ Requires relatively clean environment (low soluble BOD)	▪ NO3 to N2 ▪ Requires denitrifying bacteria (very common) ▪ Requires low dissolved oxygen (less than 0.5 ppm) ▪ Increases alkalinity, increases pH ▪ Soluble organic food is required

MS parameters for the different antibiotics

Compound	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (eV)
Amoxicillin[1]	366.01	12	113.94	28
			133.96	26
			207.93	12
Ceftiofur[2]	523.87	26	125.05	58
			240.90	16
Colistin A[3]	585.56	30	101.03	27
			202.09	22
			241.17	22
Colistin B[3]	578.59	30	101.03	27
			227.27	22
Doxycycline[4]	444.99	22	98.01	42
			153.98	28
			320.98	30
Oxytetracycline[4]	460.99	20	200.98	42
			282.95	38
			425.97	18
Sulfadiazine[5]	250.89	22	92.03	26
			107.96	22
			155.93	14
Trimethoprim[6]	290.98	36	122.99	22
			230.01	22
			260.96	24
Tylosin A[7]	916.58	47	174.05	37
			772.08	27
Oxacillin	402.01	14	159.97	10
Ceftiofur-d_3	527.06	2	244.09	16
Polymyxin B1	602.54	27	101.03	25
4-epidemeclocycline	465.00	24	429.98	22
Sulfadimethoxine-$^{13}C_6$	316.96	30	155.94	22
Trimethoprim-d_9	300.04	36	234.01	24
Roxithromycin	837.37	30	158.01	32

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Validation parameters

RSD_r: repeatability, RSD_R: Intralaboratory reproducibility, LOD: limit of detection, LOQ: limit of quantification, U: expanded measurement uncertainty, ME: matrix effect

Compound	Validation level (µg/kg)	Recovery (%) n=24	RSD_r (%) n=24	RSD_R (%) n=24	LOD (µg/kg)	LOQ (µg/kg)	Mean linearity	U (%)	ME (%)
Ceftiofur	100	100	4.8	5.3				14.9	105
	500	99	2.7	3.6	2.3	7.6	0.998	9.8	106
	1 000	100	1.7	2.8				7.6	118
Colistin A	100	100	9.2	9.2				25.7	286
	500	102	4.9	4.9	20.2	67.3	0.996	14.0	158
	1 000	106	4.8	4.8				18.6	198
Colistin B	100	100	6.0	6.0				16.7	172
	500	101	4.6	5.0	15.0	50.0	0.996	14.3	172
	1 000	104	3.5	5.4				17.0	207
Doxycycline	100	98	5.6	6.5				18.1	173
	500	98	3.7	3.7	5.4	17.9	0.997	10.9	198
	1 000	100	5.0	5.3				15.0	173
Oxytetracycline	100	98	5.7	9.3				25.3	100
	500	94	2.1	6.0	3.4	11.3	0.995	19.0	107
	1 000	98	3.5	7.2				21.7	96
Sulfadiazine	100	107	4.4	6.1				22.0	121
	500	104	2.5	6.0	1.1	3.5	0.999	18.0	124
	1 000	106	1.8	6.5				21.4	151
Trimethoprim	100	98	3.6	6.3				17.0	91
	500	100	2.5	8.4	1.3	4.3	0.999	21.9	92
	1 000	103	1.9	6.8				19.0	126
Tylosin A	100	105	25.2	36.0				101.0	97
	500	103	15.6	24.8	5.2	17.3	0.969	68.0	103
	1 000	118	15.5	24.5				80.0	139

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