Food safety and the use of processed animal proteins in Atlantic salmon production

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Introduction salmon farming

Norwegian aquaculture of salmonids 2015

Salmonids in Norway: 1,386,575 mt

- Using in total **1,663,890 mt feed** in Norway
  - Fish meal and oil are traditionally the main feed ingredient to carnivorous fish such as salmon

- Use of wild pelagic fish species, such as blue whiting, to produce fish meal and oil which are included in high energy salmon diets

- Net importer of fish meal and fish oil
Sustainability in salmon farming
Recent trends - using more alternative ingredients towards balance in the “Fish in–fish out” concept (FIFO)

Wild fish used per kg salmon produced

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<tbody>
<tr>
<td>Salmon</td>
<td>7.5</td>
<td>5.4</td>
<td>4.9</td>
<td>4.0</td>
<td>3.0</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>All species</td>
<td>1.0</td>
<td>0.9</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

(Tacon & Metian 2008)

- In EU projects, RAFOA, AQUAMAX, ARRAINA
  - “Production of one **1kg of Atlantic salmon** on this diet required **800g of wild fish resources** (Fish in - Fish out<1)". (Lilland et al. 2013. Aquacult. Nutr. 289-300)
Introduction to salmon farming

Aquaculture grows while fish oil & meal availability stays the same.
Replacement of fish oil and meal with plant ingredients

Fig. 1. Nutrient sources in Norwegian salmon farming from 1990 to 2013. Each ingredient type is shown as its percentage of the total diet.

Rapeseed
Soya protein
EU re-authorization of the use of non-ruminant processed animal proteins in animal feeds, initially for aquafeeds

Represents large and flexible resources for fish feed which does not require new production of ingredients

Marine Harvest rapport "Sustainable seafood" (2010)
Earlier Norwegian research council ABP project and publications; PAPs are suited feed ingredients with nutrition values for Atlantic salmon.

Diet 1: control: fish meal and oil
Diet 2: Poulty meal and pork blood meal
Diet 3: poultry oil
Diet 4: Poulty meal and pork blood meal+poultry oil

<table>
<thead>
<tr>
<th></th>
<th>FI, %/d</th>
<th>SGR, %/d</th>
<th>FCR (feed:gain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal oil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal protein and oil</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Digestibility (%) of some nutrients

Diet 1: control: fish meal and oil
Diet 2: Poulty meal and pork blood meal
Diet 3: poultry oil
Diet 4: Poulty meal and pork blood meal+poultry oil

Diagram showing digestion of nitrogen, total AA, and crude fat across different diets.
Norwegian Research Council project; “Food safety and the use of terrestrial animal by-products in Atlantic salmon production” 2013-2017

• New feed ingredients give new challenges to feed safety and legislative compliance

• The project aims to assess feed and food safety of farmed salmon reared on PAP-feed.
Project outline

WP 1. Species (non ruminant)/tissue specific identification PAPs, see poster Josef Rasinger

WP 2. Screening therapeutically substances

WP 3. Carry-over chemical undesirables Feed-to-fillet
1. Species and tissue specific identification

- Positive feed samples even though non PAPs are used; need for an identification technique that can help with identifying the source of contamination
1. The use of proteomics and feed and food authenticity identification
1. Species (non ruminant)/tissue specific identification PAPs

- Primary amino acid sequences are considerably more resistant to food processing than DNA

- Tissue specific; provides identification of PAP type (milk, meat, bone, bloodmeal) and hence type of PAP material when ruminant material is detected.

- Quantifiable

- Complementary method to qPCR
<table>
<thead>
<tr>
<th>Product</th>
<th>Samples</th>
<th>Positive Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork Blood Meal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pork Meal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pork Greaves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poultry Blood Meal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poultry Meal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feather Meal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine Carcase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovine Carcase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovine Muscle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
WP 1. Species (non ruminant)/tissue specific identification PAPs

- Due to processing of PAP samples normal gel based methods can not be used; smears in stead of blots.
WP 1. Species (non ruminant)/tissue specific identification PAPs

- Use of digestion protocols on analyse directly on peptides.
Data analysis

Use of known database on chicken, bovine, ovine, pork to specific identify peptides

--- Classifier model (full training set) ---

Sequence:

- VAGTWYSLAMAASDISLLDAQSAPLR  →  BOVIN
- VYVEELKPTPEGDIEILLQK  →  BOVIN
- LLGNIVVVLAR  →  PIG
- LLVYPWTQR  →  PIG
- LASADIETYLEK  →  CHICK
- LGEHNIDVLEGNEQFINAAK  →  PIG
- VVAGVANALAHN  →  BOVIN
- TYPHFDSLHGSQVK  →  BOVIN
- MFLSFPTIK  →  BOVIN
- SYELPGQVITIGNER  →  CHICK
- EFTPVLQADFQK  →  BOVIN
- LRVDPVNFK  →  PIG
- VAPEEHPTILLTEAPLNPK  →  CHICK
Find a common peptide that differs in peptide sequence among species.

However, limited peptide data basis for some species causes limited identification of peptides that are different among species and tissues.
Solution, instead of specific identification of a peptide, use whole peptide profile and compare (pairwise spectrum comparison) to identify species and tissue.


Use of whole peptide profiles gives differentiation into both species and tissue

![Diagram](image)

**Fig. 3.** Species and tissues specific PAP differentiation. Direct comparison of spectra obtained by tandem mass spectrometry using a previously described method for phylogenetic analysis [33]. A and B depict representative technical parallels of PAP extracts obtained in laboratory A and B, respectively. Irrespective of the extraction method, PAP cluster according to the species and tissue origins of PAP.

Species and Tissue Specific!

Data mining
Future:
- Create public database for uploading of full spectrum peptides profile of PAP samples and possibility to identify bot species and tissue.
- Use databases to identify specific peptides, make artificial peptide marker (aptamer) that can be quantified by normal mass spectrometry.
WP 2. Screening and identification for chemical residues in PAPs. Jaime Nacher-Mestre, University Jaume II, IUPA

19 PAP samples
- pork blood meal, meal, greaves
- poultry blood meal, meal, feather meal

<table>
<thead>
<tr>
<th>GC- (APCI)QTOF MS</th>
<th>UHPLC-QTOF MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>~200 contaminants</td>
<td>~1300 contaminants</td>
</tr>
<tr>
<td>Pesticides</td>
<td>Pharmaceuticals, pesticides, dyes, ...</td>
</tr>
</tbody>
</table>

Quality Controls were injected for every matrix

Qualitative validation for “model compounds”

Confirmation & Quantification

GC or LC-(QqQ)MS/MS
- MRL for antibiotics under EU regulation
- All antibiotic use has to be registered
- Surveillance under EU directive
Screening and identification for chemical residues in pork PAPs.
Screening=present or not - quantification estimates
\[d=\text{detected not quantified}\]

<table>
<thead>
<tr>
<th>function</th>
<th>Pharmaceuticals &amp; Dyes</th>
<th>max-min</th>
<th>nr+/samples</th>
<th>max-min</th>
<th>nr+/samples</th>
<th>max-min</th>
<th>nr+/samples</th>
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<tr>
<td>antimicrobial</td>
<td>Enrofloxacin</td>
<td>3-1</td>
<td>3/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>antimicrobial</td>
<td>Cyprofloxacin</td>
<td>3-1</td>
<td>3/3</td>
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<td>-</td>
<td>-</td>
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<td>antimicrobial</td>
<td>Norfloxacin</td>
<td>5-d</td>
<td>3/3</td>
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<td>-</td>
<td>-</td>
<td></td>
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<tr>
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<td>Flumequine</td>
<td>3-1</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>antimicrobial</td>
<td>Chlortetracycline</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>antimicrobial</td>
<td>Trimethoprim</td>
<td>1-1</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>antimicrobial</td>
<td>Tylsosin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>antimicrobial</td>
<td>Tiamulin</td>
<td>1-1</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>anti-inflammation</td>
<td>Oxyphenylbutazone</td>
<td>d</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>coccidiostats</td>
<td>Monensin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>insecticide</td>
<td>Emamectin</td>
<td>d</td>
<td>1/3</td>
<td>-</td>
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<tr>
<td>anti-fungal Dye</td>
<td>Crystal violet</td>
<td>2-1</td>
<td>3/3</td>
<td>d</td>
<td>1/3</td>
<td>0.3-d</td>
<td>2/2</td>
</tr>
<tr>
<td>anti-fungal Dye</td>
<td>Leucocystal violet</td>
<td>6-d</td>
<td>3/3</td>
<td>d</td>
<td>1/3</td>
<td>d</td>
<td>2/2</td>
</tr>
<tr>
<td>anti-fungal Dye</td>
<td>Leucomalachite green</td>
<td>d</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>anti-fungal Dye</td>
<td>Malachite green</td>
<td>d</td>
<td>1/3</td>
<td>d</td>
<td>1/3</td>
<td>d</td>
<td>1/2</td>
</tr>
</tbody>
</table>
Screening and identification for chemical residues in poultry PAPs.

Screening = present or not - quantification estimates

d = detected not quantified

Higher levels of the metabolites leuco-crystal violet (LCV) and leuco-malachite green (LMG) than their mother compounds

We concentrate on LCV and LMG as they are not permitted to be used and are relative fat soluble with a potential to bioaccumulate

<table>
<thead>
<tr>
<th>Function</th>
<th>Pharmaceuticals &amp; Dyes (max-min)</th>
<th>Poultry blood meal nr+/samples (max-min)</th>
<th>Poultry meal nr+/samples (max-min)</th>
<th>Feather meal nr+/samples (max-min)</th>
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<tbody>
<tr>
<td>antimicrobial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>82-4</td>
<td>4/4</td>
<td>385-9</td>
<td>3/4</td>
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<tr>
<td>Cyproflaxacin</td>
<td>d</td>
<td>4/4</td>
<td>51</td>
<td>1/4</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>2-d</td>
<td>4/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flumequine</td>
<td>3-d</td>
<td>3/4</td>
<td>13-5</td>
<td>2/4</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>d</td>
<td>1/4</td>
<td>d</td>
<td>1/4</td>
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<tr>
<td>Trimethoprim</td>
<td>11-0.1</td>
<td>4/4</td>
<td>28-9</td>
<td>2/4</td>
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<tr>
<td>Tyllosin</td>
<td>28</td>
<td>1/4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tiamulin</td>
<td>-</td>
<td>-</td>
<td>0.3</td>
<td>1/4</td>
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<td>-</td>
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<tr>
<td>Monensin</td>
<td>-</td>
<td>-</td>
<td>d</td>
<td>1/4</td>
</tr>
<tr>
<td>Emamectin</td>
<td>-</td>
<td>-</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>0,5-d</td>
<td>4/4</td>
<td>d</td>
<td>2/4</td>
</tr>
<tr>
<td>Leucocrystal violet</td>
<td>3-d</td>
<td>4/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leucomalachite green</td>
<td>d</td>
<td>1/4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malachite green</td>
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<td>2/4</td>
<td>-</td>
<td>-</td>
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<th>2003</th>
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<td>4</td>
<td>8</td>
<td>4</td>
<td>0.01</td>
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<td>Florfenicol</td>
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<td>96</td>
<td>130</td>
<td>179</td>
<td>148</td>
<td>198</td>
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<tr>
<td>Suladiazine + trimethoprim</td>
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<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enrofloxacin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.1</td>
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<td>&lt;0.1</td>
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<tr>
<td>Flumequine</td>
<td>16</td>
<td>7</td>
<td>3</td>
<td>42</td>
<td>10</td>
<td>3</td>
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<tr>
<td>Oxolonic acid</td>
<td>434</td>
<td>405</td>
<td>877</td>
<td>578</td>
<td>911</td>
<td>1076</td>
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<tr>
<td>Procaine penicillin + dihydrostreptomycin&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.4</td>
<td>0</td>
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<td></td>
<td>599</td>
<td>529</td>
<td>1014</td>
<td>806</td>
<td>1073</td>
<td>1278</td>
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</tbody>
</table>
WP 3. Transfer of undesirables from feed-to-fillet
Which background level in feed gives concern for food safety after prolonged feeding?

Leuco-crystal violet
Leuco-malachite green

1) PBK compartmental model
2) Simple one compartmental model

Level in salmon fillet that are of concern for food safety
Feeding trial-kinetics

Leuco crystal violet/Malachite green

High dose
N=58
N=58
N=58

Low dose
N=58
N=58

40 days exposure
~580 g

~850 g

90 days depuration
~2110 g

N=58 N=58

~580 g

~850 g

~2110 g
Model approach 1
Simple one-compartmental bioaccumulation kinetic model after Sijm et al. 1992

\[ C_{\text{fish}}(t) = \frac{\alpha F t}{K + \gamma} C_{\text{feed}} (1 - e^{-(K+\gamma)t}) + C_{\text{filsh0}} e^{-(K+\gamma)t} \]

Concentration in fish \((C_{\text{fish}})\) at time point \((t)\) is a product of concentration in feed \((C_{\text{feed}})\) and relative parameters;

- Feed intake \((F)\) as % of BW/day
- Uptake \((\alpha)\) as % /day
- Growth \((\gamma)\) as % BW /day
- Elimination \((K)\) as loss/day
A general preliminary long-term transfer assessment based on the simple one-compartmental bioaccumulation kinetic model, compare model predicted with observed values.

- 40-120 days of experimental feeding period
- Feed concentration: 863 ng/g
- Feeding rate: 0.77% BW/day
- Growth rate: 0.92% BW/day
- Elimination and uptake rates assessed from uptake and depuration data
Preliminary general long term prediction of which leuco malachite green feed level will give fillet levels near RPA

- Reference point for action (RPA) to protect public health: 2 µg/kg
- EFSA Journal 2016;14(7):4530 “unlikely that exposure to food contaminated with MG/LMG at or below the RPA of 2 µg/kg represents a health concern”

Here one fixed growth and feeding rate for a production cycle

Fluctuation of growth and feeding rate depending on, life stage, season, and water temperature

Future models inclusion of fluctuating growth and feeding rate
Preliminary long term prediction of which leuco malachite green feed level will give fillet levels with food safety concern

- Reference point for action (RPA) to protect public health: 2 µg/kg (EFSA Journal 2016;14(7):4530)

6 µg/kg

200 g Post-smolt

Full sea water production cycle ~14 months with and average growth and feeding rate

<2 µg/kg

5 Kg Slaughter sized

Here one fixed growth and feeding rate for a production cycle

Fluctuation of growth and feeding rate depending on, life stage, season, and water temperature

Future models inclusion of fluctuating growth and feeding rate
Model approach-3 Physiological based kinetic (PBK) compartmental model

- $K_{ow}$ Leuco malachite green and leuco crystal violet = 5.7
- Elimination depends on body lipids (Bauer et al., 1988)
- Persistent compared to mother compound
- Some partitioning to the muscle part
A 3 compartmental model describes the feed-to-fillet transfer of leuco-malachite green and crystal violet.
Thank you for your attention