Intelligent Fish feeding through Integration of ENabling technologies and Circular principle

Grant Agreement (GA) No: 818036

D1.6: Valorisation of by-products and sludge

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Disclaimer:

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Glossary

Aquaculture: refers to fish farming in this document

Algae: refers to fish farming in this document

Concentrated sludge: sludge from RAS system, 15-20% dry matter, recovered using 60 µm filtration

Conc. sludge: concentrated sludge

Dirty water: wastewater from fish farming

MI: nutrients extracted from concentrated sludge by enzymatic or chemical hydrolysis (Medium Ingredient for microalgae and yeast)

Nanno: *Nannochloropsis gaditana*, renamed *Microchloropsis gaditana* in recent years

Raw sludge: non-concentrated sludge from RAS system, 0.5-4.3% dry matter

Partners:

- **AA**: Aller Aqua Research Gmbh
- **ABT:** Aquabiotech Limited
- **COVARTEC:** Covartec AS
- **GyE:** Gyori Elore Halaszati Termeloszovetkezet
- **LEITAT:** Acondicionamiento Tarrasense Associacion
- **NORCE:** Norwegian Research Centre AS
- **OXY:** Oxyguard International AS
- **SZIU/MATE:** Szent Istvan Egyetem
- **TTZ:** Verein Zur Forderung Des Technologietransfers an der Hochschule Bremerhaven Ev
- **VF**: Vitafort Elso Takarmanygyarto Es Forgalmazo Zartkoruen Mukodo Rt

Executive Summary

The iFishIENCi project, WP1 – *Task 1.5 Zero waste and valorisation of by-products and sludge* aims to design condition-based optimal valorisation processes for waste recirculation from aquaculture effluents for the recovery of nutrients (nitrogen, phosphorus, and carbon) within a circular economy. The valorisation processes were used for the production of the two iFishIENCi sustainable feed, namely microalgae and yeast (Task 1.3), and feed ingredients. Their sustainability and circularity were further evaluated as part of WP4.

The objective of task 1.5 is to identify and demonstrate potential valorisation routes for fish farming waste streams and by-products within a circular economy and zero waste strategy, taking into account all effluents generated within the whole value chain, from the production of ingredients (feeds) to fish farms themselves. The deliverable *D.1.6 Valorisation of by-products and sludge* details waste identification, collection plan, characterisation, selection for valorisation trials, methodologies, and results.

Identification, monitoring, and characterisation of different waste streams were investigated (LEITAT, NORCE) for the production of new feed (algae, yeast). MATE and ABT optimized waste collection and monitoring methods by testing the smart feeding technology (Product 3: SMART-RAS) and then demonstrating removal efficiency with swirl separators of 63% of Total Suspended Solids (TSS), with 79% of total N available for valorisation (WP2 and WP3). According to the characterisation of the various waste streams and taking the regulatory framework into account (assessed in WP4), two main sources of waste were selected to be investigated through different valorisation trials under a circular approach and towards zero-waste strategy: waste from the iFishIENCi test-sites from RAS system (ABT, AA) and Land-based flow-through system (GyE). Furthermore, the analysis of the different waste compositions provided the necessary information on carbon, nutrients, and mineral content to identify and select a number of key microorganisms (bacteria and fungi), used in industrial bioprocesses, and test their growth potential on RAS-waste as feedstock. Recommendation on priorities for further investigations are given.

As a result of the experiments for the reuse of dirty water as substrate for algae production conducted at pilot level as proof of principle (NORCE), it was proved that the outlet water from all fish production from RAS and land-based flow-through systems could be directly usable for the cultivation of photoautotrophic microalgae *(e.g., Nannochloropsis gaditana).* All batches of wastewater were found low in nutrient concentrations, phosphate being the main limiting nutrient for the microalgae. Therefore, process and/or reactor design to ensure high biomass productivity while treating large amounts of water is therefore necessary.
Additional experiments were conducted on the yeast *(Candida utilis)*. Candida could not grow on wastewater from RAS as feedstock, but significant growth was obtained on sludge, after appropriate pre-treatments (dilution, centrifugation to eliminate the insoluble material, and double autoclaving to kill all the microorganisms and spores).

A new methodology was developed to produce nutrients for microalgae and yeast feeding using concentrated sludge from RAS. The process involved pretreatments (ultrasounds), followed by enzymatic processes (endo and exoprotease) and chemical (KOH). The resulting total nutrient

recovery from waste was 36-46% nitrogen, 3-8% total phosphorus, and 13-60% total organic carbon, depending on the method. Nutrients extracted from concentrated RAS sludge, showed a good potential to be used as nutrients source for microalgae cultivation, in some specific cases, and as carbon and energy source for yeast production.

Besides the use of these nutrients for biomass production (algae, yeast) and as part of Waste2value, the medium ingredient obtained was characterized for potential use as fertilizing product in agriculture, showing promising potential.

The residue of this new process as well as the resulting residual fractions from the production of new feeds were also analysed for potential use as fertilizer, toward zero waste strategies. The residue showed promising characteristics, except for slightly above-permit cadmium levels. Further research is needed to assess pollutants under European and national fertilizer regulations.

1 Introduction

Aquaculture production has grown significantly, reaching 87.5 million metric tons in 2020. Despite the COVID-19 pandemic, the industry resumed rapidly, with a predicted growth of 201 mt in 2030 (FAO, SOFIA 2022). Common methods include cage aquaculture, land-based flow-through, ponds, and Recirculating Aquaculture Systems (RAS). However, these activities can negatively impact marine and freshwater environments, affecting biota levels and causing water eutrophication.

Nitrogen is mainly excreted through urine and faeces, with 7-30% of total nitrogen discharged from fish being particulate matter (Mommsen TP, 1992). About 7–30% of the total nitrogen discharged from fish occurs in the form of particulate matter (Cripps SJ, 2000) and if the waste is not collected, these nutrients are eventually discharged along with the uneaten pellets.

The type of cultivation system significantly impacts the environment. Closed-containment technologies and land-based systems like Flow-through and Recirculating Aquaculture Systems (RAS) can collect and concentrate waste, reducing habitat degradation. RAS uses nitrifying bacteria to convert ammonia into nitrate, and mechanical filtration removes solid wastes. This reduces water consumption and allows for the collection of nutrients-rich effluents and solids. As phosphorous is excreted with the faeces only, the removal of faeces through mechanical filters in RAS catches a rather small part of the excreted nitrogen but a larger proportion of the total phosphorous load (Bregnballe, 2015)

Research is exploring the potential of aquaculture waste products, such as solid waste and eutrophic water, to reduce water eutrophication and habitat degradation while creating extra income for fish farmers. Nutrients from aquaculture can be used in agricultural crops (Belmeskine, 2023) (Yep, 2019)). Soluble compounds extracted by bacteria, algae, and macrophytes can be used to build biomass and produce valuable compounds. Microalgae, with high growth and nutrient removal rates, are effective in aquaculture wastewater, producing value-added biomass that can be integrated into fish feed. Sludge from aquaculture wastewater can also be used as fertilizer.

In iFishIENCi, the iBOSS integrated smart feeding technology and added value to farming operations by preventing economic waste associated with overfeeding and food rejection while simultaneously reducing environmental impacts of feed waste. Further, by developing new feed innovations, iFishIENCi is helping to conserve natural resources (low fish meal content, valorising waste products for aquaculture, reducing water use) and make aquaculture more sustainable.

2 Waste identification, collection procedures, characterisation

2.1 Introduction

The waste collection, selection and valorisation trials were carried out under *Task 1.5.1 Identification of value chain valorisation*. Different fish farming-sites participating in the iFishIENCi project were assessed for potential waste collection and valorisation trials considering practical collection, characteristics, legislation, existing options for waste treatments and needs for further valorisation. The regulatory framework for waste management was also considered for waste collection and further detailed in deliverable D4.13 and D4.14. (TTZ). In parallel, in WP2 and WP3, waste data was manually added to iBOSS databases with the support of OXY and reported in D2.5. The AI demonstration at ABT in WP3, T3.4, proved the potential cost reduction of optimizing feeding through behavioral observation using smart feeding technology (Product 3: SMART-RAS).

2.2 Waste valorisation routes towards bioindustries

Sludge from fish-farming can be rich in carbon and nutrients, minerals and amino acids. It has been used as a fertiliser in some countries, following costly transport and dewatering. There has been growing interest in valorising the nutrient content of sludge by using it as a feedstock in fermentation processes. Both the carbon and nutrients can be used to replace or supplement sugar or cellulose based industrial fermentation processes using algae, yeast, bacteria, or fungi. Waste from fish-farming could thus be used for the production of protein or lipid-rich biomass to be used as feed ingredient to reduce production cost and environmental impact (as is being demonstrated through this project). It could also be used for the production of an array of chemicals such as lactic acid, acetate, ethanol, polymers for various applications, or precursors to jet fuel (Shi, 2018). Figure 1 presents different valorisation pathways of production waste-streams, under investigation in iFishIENCi (in blue) and links with other H2020 projects which have specific valorisation chains as main objectives, e.g., H2020 ASTRAL and H2020 IMPAQT for IMTA, and H2020-Gain for valorisation of fish protein hydrolysates, fish bones or fish skin.

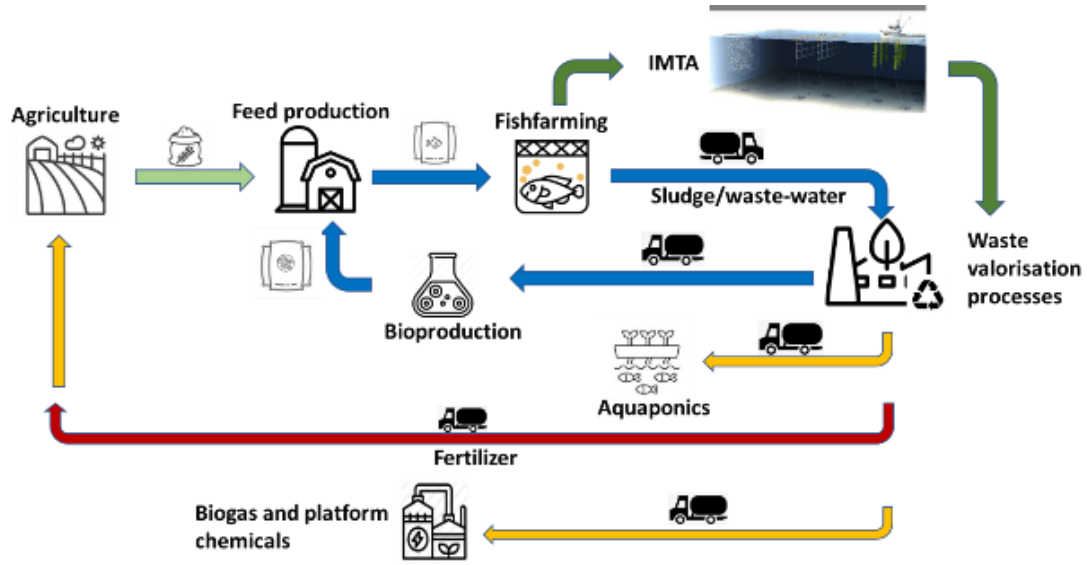


Figure 1 Value chains for valorisation of waste and by-products from the fish-farming sector. In Blue: valorisation addressed directly in iFishIENCi. In yellow, established (aquaponics) or innovative value chains to be further investigated. investigated. In dark green: Integrated multitrophic aquaculture.

The characterisation of the waste streams, the data from iBOSS collected from RAS systems by ABT in WP3 and the characterisation of medium ingredients obtained in iFishIENCi provided guidance and opportunity not only for algae and yeast growers but also to point out other potential uses such as fertilisers, platform chemicals, biogas – biofuel, IMTA and aquaponics. In WP5, an assessment of other potential different value chains was be pointed out based on physic-chemical characteristics to have a preliminary insight into wider scope of applications for further research in future projects.

2.3 Identification of waste-streams from fish farming-sites and from feeds production

Different fish farming-sites participating in the iFishIENCi project were assessed for potential waste collection and valorisation trials considering practical collection, characteristics, legislation, existing options for waste treatments and needs for further valorisation.

From this assessment, it was concluded that waste collection was not feasible in open systems but there would be possibilities to exploit waste from the following systems and sources:

From aquaculture :

- Waste from fish farming from Recirculation Aquaculture System (RAS): ABT (wastewater, raw sludge, concentrated sludge) and AA (dirty water, raw sludge).
- Wastewater from land-based flow-through system at GyE (dirty water)

From waste from the feeds production:

- Exhausted medium from yeast *Candida utilis*.
- Insoluble matter from the production of the antioxidant supplement (task 1.3) from microalgae *Nannochloropsis gaditana* supplied by NORCE.

2.4 Waste collection, characterisation and selection for valorisation trials

The Table 1 shows a summary of the different waste types selected at the iFishIENCi test-sites (partner fish-site, experiment, fish species, diet) from the different tasks in iFishIENCi. The results of characterisation are detailed in the following sub-sections.

Table 1 Waste collected by AA, ABT, GyE

AA – RAS system			
Experiments	**Fish**	**Diet**	**Type of waste**
RAS1_188 (Task 1.4)	Rainbow trout	Algae diet 30% and control diet	outlet water and sludge
RAS2_194 (Task 1.4)	Rainbow trout	Nanno extract and control diet	sludge
RAS5_243 (Task 1.4)	Rainbow trout	Candida diet 30% and control diet	outlet water and sludge
RAS6_275 (Task 3.4)	Rainbow trout	Control diet (with FM 15% and Astaxanthin 40 mg/kg) Candida diet (5% substituted for FM; 40 mg/kg Astaxanthin) Nanno diet 1 (5% substituted for FM; 40 mg/kg Astaxanthin)	outlet water and sludge

		Nanno diet 2 (5% substituted for FM; 20 mg/kg Astaxanthin)	
Experiments	**Fish**	**Diet**	**Type of waste**
RAS3_ABT3 (Task 1.3)	Rainbow trout	Conventional diet	outlet water and conc.sludge
IFN01_LC (Task 1.4)	Barramundi (salt water)	Nanno extract and control diet	outlet water
IFN02_RT (Task 3.4)	Rainbow trout	Conventional diet	outlet water and conc.sludge
IFN03_AC (Task 3.4)	African catfish	Candida diet 1 10% Candida diet 2 20%	outlet water and conc.sludge
Experiments	**Fish**	**Diet**	**Type of waste**
(Task 1.4)	African Catfish	Conventional diet	outlet water

Sludge from AA: sludge from faeces collector, 0.5-4.3% dry matter
Conc.sludge from ABT: concentrated sludge using 60 micron filter, 15-20% dry matter
MI: Medium Ingredient

2.4.1 Waste from Recirculation Aquaculture System (RAS) - ABT

The removal efficiency of Total Suspended Solids (TSS) in RAS combining mechanical filtration of swirl separator + drum filter has been described to be 88%, and 71-77% with microscreen with mesh pore sizes of 25-100µm (Couturier M, 2009). To simulate RAS sludge collection (RAS3_ABT3, IFN02_RT, IFN03_AC), faeces and uneaten feed were collected in swirl separators connected to cultivation tanks. The accumulated solids were "loose" and contained over 95% water. The mixture was periodically evacuated and collected in a flask. The solids settled, and the supernatant is removed manually. Concentrated sludge samples were produced through coarse and vacuum filtration. For fish trial IFN01_LC, with absence of swirl separator, sludge samples were collected from general waste tanks, mixed with effluents from both treatments, and filtered through a drum filter.

The Table 2 shows the physicochemical and microbiological characterisation and nutritive/pollutant elements (ICP-MS) of raw sludge, concentrated sludge and filtered water on fresh weight. Moisture, dry matter and ashes were analysed by gravimetry. TKN was analysed by kjeldahl method and NH_4^+-N, NO_2^--N and NO_3^--N by ion chromatography. For samples that were examined in duplicate (n=2), a deviation is provided. In addition, the Concentrate sludge, up to 10-21% dry matter, was rich in carbon 622888 mg/kg on fresh weight and total nitrogen, ranging from 5794-9753 mg/kg, with values up to 14 mg/kg of nitrates, which is the nitrogen form absorbed by algae. Sludge also contained phosphorus, ranging 2800-3600 in phosphate form, as well as other nutrients such as sodium, calcium. Pollutant elements were not detected or under 100 mg/kg (i.e. Zn). Salmonella and E.coli were not detected (under 100 CFU/g). In waste water, total nitrogen was lower ranging 128-148 mg/L fresh weight, but with higher values up to 195 mg/kg of nitrates. Nutrients were lower in comparison to sludge, phosphate was also lower, ranging 5-33 mg/Kg . Pollutant elements were not detected in water.

Table 3 and Table 4 show analysis of total amino acids (HPLC-DAD) and POPs (Bromide, Dioxine & Dioxine like PCB´s), respectively.

Table 2 Physicochemical and microbiological characterisation of raw sludge, concentrated sludge and water from Recirculation Aquaculture System (RAS) - ABT

Parameter	Waste from RAS3_ABT3 Rainbow trout and conventional feed			Waste from IFN01_LC Barramundi and feed with antioxidant Nanno extract				Waste from IFN02_RT Rainbow trout and conventional feed		Waste from IFN03_AC African catfish and new feed			
	Raw sludge	Conc. sludge	Water	Brackish water (BW)	Sea water (SW)					Candida diet 10%		Candida diet 20%	
				Raw sludge	Raw sludge	Conc. sludge	water	Conc. sludge	Water	Conc. Sludge	Water	Conc. sludge	Water
pH	7.3±0.1	6.6±0.0[(1)]	8.2	7.7±0.0	6.8±0.0	6.7 [(1)]±0.0	7.0	7.3±0.0[(1)]	7.1	5.0±0.0[(1)]	6.2[(1)]	6.7±0.0[(1)]	5.7[(1)]
Conductivity (mS/cm)	3.9±0.0	3.3±0.0[(1)]	n.a.	51.2±0.0	64.4±0.1	18.7[(1)]	n.a.	1.6±0.0[(1)]	n.a.	n.a.	n.a.	n.a	n.a
Salinity (ppt)	n.a.	n.a.	2.2	n.a.	n.a.	n.a.	36.7	n.a	1.9	n.a.	2.2	n.a.	2.3
Moisture (%)	99.5±0.2	79.3±2.5	n.a.	96.0±0.0	94.0±0.0	89.1±0.0	n.a.	84.5±0.1	n.a.	83.3±0.1	n.a.	84.5±0.3	n.a.
Dry matter (%)	0.5±0.2	20.7±2.5	n.a.	4.0±0.0	6.0±0.0	10.8±0.0	n.a.	15.5±0.1	n.a.	16.7±0.1	n.a.	15.5±0.3	n.a.
Ashes (%)	0.3±0.0	5.2±0.0	n.a.	3.2±0.2	3.7±0.0	4.2±0.0	n.a.	4.1±0.2	n.a.	n.a.	n.a.	n.a	n.a
TKN-N (mg/kg)	197±29	9755±112	n.a.	2070±42	1930±170	7130±0.0	n.a.	8360±650	n.a.	5794±199	n.a.	6286±226	n.a.
NH_4^+-N (mg/kg or L)	n.a.	159	1.3[(5)]	1.5	158	n.a.	1.1[(5)]	248	n.a.	520	n.a.	450	n.a.
NO_2^--N (mg/kg)	n.a.	4.3	n.a.	< 0.08	< 0.9	4.7	n.a.	7.6	n.a.	< 0.3	n.a.	< 0.3	n.a.
NO_3^--N (mg/kg or L)	n.a.	13	195[(6)]	52	0.3	7.0	59[(6)]	14	n.a.	< 3.5	117 [(6)]	6.5	136[(6)]
Total N (mg/Kg)[(2)]	n.a.	9773	n.a.	2122	1930	n.a	n.a.	8382	148[(8)]	5794	106 [(8)]	6293	128[(8)]
Organic N (mg/kg)[(3)]	n.a.	9597	n.a.	2069	1772	n.a	n.a.	8112	n.a.	5274	n.a.	5836	n.a.
Inorganic N (mg/kg or L)[(4)]	n.a.	176	196	51.8	0.4	n.a	60	270	n.a.	520	n.a.	456	n.a.
Phosphate (mg/kg or L)	n.a.	2800	7.0[(7)]	n.a.	n.a.	n.a	5.0[(7)]	3600	5.7	n.a.	25	n.a.	33
Total Organic Carbon (TOC) (mg/kg or L)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	68288	80	n.a.	n.a.	n.a.	n.a.
Chloride (mg/kg or L)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	1890	690	n.a.	n.a.	n.a.	n.a.
Sulphate (SO_4^{2-}) (mg/kg or L)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	970	175	n.a.	n.a.	n.a.	n.a.
Nutritive/pollutant ele ments (mg/kg)													
Na	709±1	983±68	561±1	12263±38	13294±576	12337±416	n.a.	791±38	n.a.	n.a.	n.a.	n.a.	n.a.
Mg	77±0	816±22	32±0	1428±2	1676±55	1810±60	n.a.	580±32	n.a.	n.a.	n.a.	n.a.	n.a.
P	184±4	10711±244	14±0	<10	373±34	1290±57	n.a.	8291±490	n.a.	n.a.	n.a.	n.a.	n.a.
S	<1000	1357±167	<1000	1132±20	1448±100	1665±81	n.a.	797±178	n.a.	n.a.	n.a.	n.a.	n.a.
K	41±1	246±10	260±0	450±1	697±24	929±28	n.a.	107±7	n.a.	n.a.	n.a.	n.a.	n.a.
Ca	336±6	17567±223	99±3	513±12	1303±64	2827±136	n.a.	13286±527	n.a.	n.a.	n.a.	n.a.	n.a.
Cr	<0.1	3±1	<0.1	<0.1	0.4±0.4	0.4±0.0	n.a.	0.7±0.1	n.a.	n.a.	n.a.	n.a.	n.a.
Mn	1.4±0.0	<0.1	<0.1	<0.1	1.1±0.1	4.4±0.2	n.a.	43±13	n.a.	n.a.	n.a.	n.a.	n.a.
Fe	12.3±0.1	809±75	<0.1	<1	23±2	92±6	n.a.	264±31	n.a.	n.a.	n.a.	n.a.	n.a.
Co	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	0.3±0.2	n.a.	n.a.	n.a.	n.a.	n.a.
Ni	<0.1	1±1	<0.1	<0.1	<0.1	0.4±0.0	n.a.	0.7±0.4	n.a.	n.a.	n.a.	n.a.	n.a.
Cu	0.2±0.0	5.8±0.0	<0.1	<0.1	1.2±0.1	3.7±0.0	n.a.	4.7±1.5	n.a.	n.a.	n.a.	n.a.	n.a.
Zn	2.7±0.4	130±2	<0.1	0.2±0.0	18±1	58±2	n.a.	99.1±4.2	n.a.	n.a.	n.a.	n.a.	n.a.

As	<0.1	<0.1	<0.1	<0.1	<0.1	0.4±0.0	n.a.	0.3±0.2	n.a.	n.a.	n.a.	n.a.	n.a.
Se	<0.1	<0.1	<0.1	<0.1	<0.1	0.4±0.0	n.a.	0.7±0.1	n.a.	n.a.	n.a.	n.a.	n.a.
Mo	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	n.a.	n.a.
Cd	<0.1	0.4	<0.1	<0.1	<0.1	<0.1	n.a.	0.4±0.04	n.a.	n.a.	n.a.	n.a.	n.a.
Pb	<0.1	<0.1	<0.1	<0.1	<0.1	0.3±0.0	n.a.	0.4±0.2	n.a.	n.a.	n.a.	n.a.	n.a.
Hg	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	<0.1	n.a.	n.a.	n.a.	n.a.	n.a.
Microbiology [9]													
Salmonella spp in 25 g	n.a.	absent	n.a.	absent	absent	absent	n.a.	absent	n.a.	n.a.	n.a.	n.a.	n.a.
E.coli (CFU/g)	n.a.	<100	n.a.	<10	<100	<100	n.a.	<100	n.a.	n.a.	n.a.	n.a.	n.a.
Total Aerobic count (CFU/g)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	1.0×10^8	n.a.	n.a.	n.a.	n.a.	n.a.
Total Fungi and Yeast count (CFU/g)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	4.2×10^3	n.a.	n.a.	n.a.	n.a.	n.a.
Enterobacteriaceae count (CFU/g)	n.a.	n.a.	n.a	n.a.	n.a.	n.a	n.a.	7.1×10^4	n.a.	n.a.	n.a.	n.a.	n.a.

n.a. not analysed

[1] pH and conductivity, sample preparation: the solid sample was mixed with distilled water (1:2.5), centrifuged at 10.000 rpm for 15 min. Next, the supernatant was analysed
[2] Total Nitrogen calculated as the sum of TKN + NO_2^--N + NO_3^--N
[3] Organic Nitrogen calculated as the difference between TKN and NH_4^+-N
[4] Inorganic Nitrogen calculated as the sum of NH_4^+-N + NO_2^--N + NO_3^--N
[5] Analysed with Photometer PF-12^{Plus} and NANOCOLOR ® Ammonium 3 test kit.
[6] Analysed with Photometer PF-12^{Plus} and VISOCOLOR ECO Nitrate test kit. Note: the nitrate test kit seems to continuously overestimate the total nitrate slightly, based on measurements of seawater with known addition of nitrate. The total N kit gives a better estimate based on the same standard seawater + nitrate solution.
[7] Analysed with Photometer PF-12^{Plus} and NANOCOLOR Ortho- and total Phosphate test kit.
[8] Analysed with Photometer PF-12^{Plus} and NANOCOLOR ® total Nitrogen TN_b kit
[9] Microbiology analysis:
Rapid detection method Salmonella. Rapid Salmonella. According to UNE EN ISO 16140
ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E.coli.
ISO 4833-2:2014 Colony count at 30ºC by surface plating techniques
ISO 21527:2008 Horizontal method for the enumeration of yeast and moulds
ISO 21528:2017 Horizontal method for the detection and enumeration of Enterobacteriaceae. Part 2: Colony-count

Concentrate sludge, up to 10-21% dry matter, was rich in carbon 622888 mg/kg on fresh weight and total nitrogen, ranging from 5794-9753 mg/kg, with values up to 14 mg/kg of nitrates, which is the nitrogen form absorbed by algae. Sludge also contained phosphorus, ranging 2800-3600 in phosphate form, as well as other nutrients such as sodium, calcium. Pollutant elements were not detected or under 100 mg/kg (i.e. Zn). Salmonella and E.coli were not detected (under 100 CFU/g). In waste water, total nitrogen was lower ranging 128-148 mg/L fresh weight, but with higher values up to 195 mg/kg of nitrates. Nutrients were lower in comparison to sludge, phosphate was also lower, ranging 5-33 mg/Kg . Pollutant elements were not detected in water.

Table 3 Analysis of total amino acids in concentrated sludge from recirculation Aquaculture System (RAS) - ABT fish trial IFN02_RT

Amino acid (mg/kg)	Result
ASP	2914
GLU	3733
ASN	n.d.
SER	1447
GLN	n.d.
HYS	900
GLY	2016
THR	1846
ARG	1156
ALA	2095

Amino acid (mg/kg)	Result
TYR	725
CYS-CYS	n.d.
VAL	1617
MET	277
TRP	n.d.
PHE	1660
ILE	1572
HYP	1471
LEU	2812
LYS	2224
PRO	1813

n.d. not detected

Total amino acids were 30270 mg/kg on concentrate sludge fresh weight , glutamic acid the most abundant.

Table 4 Analysis of POPs (Bromide, Dioxine & Dioxine like PCB´s) in concentrated sludge and water from recirculation Aquaculture System (RAS) - ABT (fish trial IFN02_RT)

Parameter	Concentrated sludge	Water
Bromide (Br)	na	2.4 mg/l
WHO-TEQ (PCDD/F + DL-PCBs) incl. LOQ	0.271 pg TEQ/g	0.242 ng TEQ/kg
Dioxine TEQ (WHO 2005) incl. LOQ	0.158 pg TEQ/g	0.160 ng TEQ/kg
2,3,7,8-TCDD	<0.05 pg/g	<0.05 ng/kg
1,2,3,7,8-PeCDD	<0.05 pg/g	<0.05 ng/kg
1,2,3,4,7,8-HxCDD	<0.05 pg/g	<0.05 ng/kg
1,2,3,6,7,8-HxCDD	<0.05 pg/g	<0.05 ng/kg
1,2,3,7,8,9-HxCDD	<0.05 pg/g	<0.05 ng/kg
1,2,3,4,6,7,8-HpCDD	<0.05 pg/g	<0.2 ng/kg
OCDD	<0.2 pg/g	<0.05 ng/kg
2,3,7,8-TCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,7,8-PeCDF	<0.05 pg/g	<0.05 ng/kg
2,3,4,7,8-PeCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,4,7,8-HxCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,6,7,8-HxCDF	<0.05 pg/g	<0.05 ng/kg
2,3,4,6,7,8-HxCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,7,8,9-HxCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,4,6,7,8-HpCDF	<0.05 pg/g	<0.05 ng/kg
1,2,3,4,7,8,9-HpCDF	<0.05 pg/g	<0.05 ng/kg
OCDF	<0.2 pg/g	<0.2 ng/kg
DL-PCB TEQ (WHO 2005) incl. LOQ	0.113 pg TEQ/g	0.083 ng TEQ/kg

PCB-77	<2 pg/g	<1 ng/kg
PCB-81	<2 pg/g	<1 ng/kg
PCB-126	<0.5 pg/g	<0.5 ng/kg
PCB-169	<2 pg/g	<1 ng/kg
PCB-105	<10 pg/g	<10 ng/kg
PCB-114	<10 pg/g	<10 ng/kg
PCB-118	<10 pg/g	<10 ng/kg
PCB-123	<10 pg/g	<10 ng/kg

Dioxin TEQ: macrocategory. TCDDD, PeCDDD and HxCDD are individual dioxins.
DL-PCB's TEQ: macrocategory. Specific PCB compounds: i.e PCB-105
(*) These values represent individual values. Incoming water changes every day

2.4.2 Waste from Recirculation Aquaculture System (RAS) - AA

Outlet water and sludge from faeces collector from Fish trials with Rainbow trout were collected at AA. The Table 2 shows the physicochemical and microbiological characterisation and nutritive/pollutant elements (ICP-MS) of raw sludge and water on fresh weight. Moisture, dry matter and ashes were analysed by gravimetry. TKN was analysed by kjeldahl method and NH_4^+-N, NO_2^--N and NO_3^--N by ion chromatography. For samples that were examined in duplicate (n=2), a deviation is provided.

Sludge from faeces collector from AA was a very different type of waste in comparison to the concentrated sludge from ABT (15-20% dry matter), because it wasn't concentrated, all samples ranging 0.5-4.3 % dry matter. Nutrients in sludge were similar to wastewater. Total nitrogen, ranged from 55-1372 mg/L, with values up to 62 mg/L of nitrates in water samples. Samples contained phosphorus, ranging 1.5-3 mg/L in phosphate form, as well as other nutrients such as sodium, calcium. Pollutant elements were not detected or they were under 5 mg/L (i.e. Zn). Salmonella and *E. coli* are were not detected (under 100 CFU/g).

Table 5 Physicochemical and microbiological characterisation of sludge samples from Recirculation Aquaculture System (RAS) - AA. Sludge samples (S), Water samples (W)

Parameter	Waste from RAS1_188 Digestibility trial with Nannochloropsis				Waste from RAS2_194 Trial with antioxidant supplement Nannochloropsis					Waste from RAS5_243 Digestibility trial with Candida				Waste from from RAS6_275 with optimised diets				
	RAS1_CD Control diet		RAS1_NA Nanno diet		RAS2_CD1 Control diet 15ºC	RAS2_CD2 Control diet 22ºC	RAS2_NA1 Nanno diet 1% 15ºC	RAS2_NA2 Nanno diet 2% 15ºC	RAS2_NA3 Nanno diet 3% 5+22ºC	RAS5_CD Control diet		RAS5_CA Candida diet		Control diet		Candida diet 10%	Nanno diet 10%	Nanno diet 20%
	S	W	S	W	S	W	S	W	S	S	W	S	W	S	W	S	S	S
pH	6.5 ±0.5	8.5	6.4 ±0.4	8.8	7.3 ±0.3	6.9 ±0.4	7.9 ±0.2	7.3 ±0.1	7.3 ±0.6	7.6 ±0.3	7.9	6.9 ±0.5	7.8	7.9 ±0.0	7.8	7.9 ±0.0	7.8 ±0.0	7.8 ±0.0
Conductivity (mS/cm)	5.8 ±0.7	n.a.	5.7 ±0.9	n.a.	5.3 ±0.0	0.9 ±0.0	4.5 ±0.0	4.5 ±0.1	3.9 ±0.2	6.4 ±0.0	n.a.	5.1 ±0.0	n.a.	5.7 ±0.0	n.a	5.6 ±0.0	5.7 ±0.0	5.6 ±0.0
Salinity (ppt)	n.a	2.7	n.a.	2.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.9	n.a.	2.9	n.a	3	n.a	n.a	n.a
Moisture (%)	95.7 ±0.1	n.a.	97.6 ±0.6	n.a.	97.1 ±0.8	97.6 ±0.1	99.3 ±0.0	96.9 ±0.2	97.9 ±0.0	99.5 ±0.00	n.a.	98.9 ±0.2	n.a.	99.5 ±0.0	n.a.	99.6 ±0.0	99.6 ±0.0	99.5 ±0.0
Dry matter (%)	4.3 ±0.1	n.a.	2.3 ±0.6	n.a.	2.9 ±0.8	2.4 ±0.1	0.7 ±0.0	3.0 ±0.2	2.1 ±0.0	0.5 ±0.0	n.a.	1.1 ±0.2	n.a.	0.5 ±0.0	n.a.	0.4 ±0.0	0.4 ±0.0	0.4 ±0.0
Ashes (%)	2.3 ±0.2	n.a.	0.7 ±0.5	n.a.	1.3 ±0.1	0.8 ±0.1	0.3 ±0.0	1.2 ±0.3	1.2 ±0.1	0.2 ±0.0	n.a.	0.2 ±0.0	n.a.	0.3 ±0.0	n.a.	0.3 ±0.0	0.3 ±0.0	0.32 ±0.0
TKN (mg/L)	1076 ±266	n.a.	1372 ±322	n.a.	606 ±10	349 ±73	594 ±1	925 ±0.	830 ±85	55 ±6	n.a.	156 ±40	n.a.	135 ±21	n.a.	95 ±7	110 ±0	105 ±7
NH_4^+-N (mg/L)	n.a.	0.43[4]	38.27	0.36[4]	n.a.	n.a.	n.a.	n.a.	12.7 [4]	n.a.	0.35[4]	2.9	0.47 [4]	n.a.	n.a	< 100	< 100	n.a.
NO_2^--N (mg/L)	n.a.	n.a.	14.5	n.a.	n.a.	n.a.	n.a.	n.a.	2.5	n.a.	n.a.	< 0.1	n.a.	n.a.	n.a	< 2.5	< 2.5	n.a.
NO_3^-- N (mg/L)	n.a.	54.7[5]	0.9	58.5 [5]	n.a.	n.a.	n.a.	n.a.	0.4 [5]	n.a.	50.1[5]	19	50.6[5]	n.a.	62[5]	< 23	< 23	n.a.
Total N (mg/L)[1]	n.a.	n.a.	1387	n.a.	n.a.	n.a.	n.a.	n.a.	833	n.a.	n.a	175	n.a.	n.a.	n.a	95	110	n.a.
Organic N (mg/L) [2]	n.a.	n.a.	1334	n.a.	n.a.	n.a.	n.a.	n.a.	817	n.a.	n.a.	153	n.a.	n.a.	n.a	n.a.	n.a.	n.a.
Inorganic N (mg/L)[3]	n.a.	55.1	54	58.9	n.a.	n.a.	n.a.	n.a.	16	n.a.	50.5	19	51.1	n.a.	n.a	n.a.	n.a.	n.a.
Phosphate (mg/L)[6]	n.a	2.4	n.a.	2.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	3.2	n.a.	3.0	n.a.	1.5[6]	n.a.	n.a.	n.a.
Nutritive/pollutant elements (mg/kg) (ICP-MS)																		
Na	962 ±256	n.a.	1046 ±29	n.a.	1077 ±63	56 ±16	1044 ±41	934 ±21	714 ±29	658 ±59	n.a.	1104 ±10	n.a.	n.a.	n.a.	1035±2	931±10	n.a.
Mg	28 ±2	n.a.	49 ±1	n.a.	12 ±1	8 ±0.3	8.2 ±0.5	8.5 ±0.3	11.9 ±0.1	2.9 0.3	n.a.	17.8 ±0.1	n.a.	n.a.	n.a.	12±2	11±2	n.a.
P	174 ±108	n.a.	88 ±3	n.a.	35 ±4	26 ±1	17 ±1	21 ±0	49 ±2	11 ±1	n.a.	203 ±6	n.a.	n.a.	n.a.	14±0	<10	n.a.

S	<1000	n.a.	<1000	n.a.	<1000	<1000	<1000	<1000	<1000	<1000	n.a.	<1000	n.a.	n.a.	n.a.	<100	<100	n.a.
K	10.92 ±0.01	n.a.	12 ±1	n.a.	14 ±1	8 ±0	12 ±0	11 ±0	13 ±0	5 ±0	n.a.	9 ±0	n.a.	n.a.	n.a.	11±3	7±1	n.a.
Ca	654 ±253	n.a.	290 ±15	n.a.	158 ±16	174 ±11	110 ±7	118 ±6	224 ±7	82 ±1	n.a.	432 ±32	n.a.	n.a	n.a	<100	<100	n.a
Cr	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	<0.1	<0.1	n.a.
Mn	<0.1	n.a.	0.3 ±0.0	n.a.	<0.1	<0.1	<0.1	<0.1	15 ±0	<0.1	n.a.	0.7 ±0.0	n.a.	n.a.	n.a.	0.2±0.0	0.1±0.0	n.a.
Fe	1 ±0.	n.a.	1 ±0	n.a.	8 ±1	8 ±0	5 ±1	5 ±0	<0.1	0.3 ±0.1	n.a.	8 ±0	n.a.	n.a.	n.a.	2.2±0.7	1.3±0.1	n.a.
Co	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a	n.a	<0.1	<0.1	n.a
Ni	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	<0.1	<0.1	n.a.
Cu	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	0.5 ±0.0	n.a.	n.a.	n.a.	0.2±0.0	0.1±0.0	n.a.
Zn	1 ±0.6	n.a.	0.2 ±0.1	n.a.	0.6 ±0.4	0.2 ±0.0	0.2 ±0.0	<0.1	0.4 ±0.0	<0.1	n.a.	4.5 ±0.3	n.a.	n.a.	n.a.	1.2±0.9	0.2±0.1	n.a.
As	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a	n.a	<0.1	<0.1	n.a
Se	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	<0.1	<0.1	n.a.
Mo	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	<0.1	<0.1	n.a.
Cd	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a.	n.a.	<0.1	<0.1	n.a.
Pb	<0.1	n.a.	<0.1	n.a.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	n.a.	<0.1	n.a.	n.a	n.a	<0.1	<0.1	n.a
Hg	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Salmonella spp in 25 g	n.a.	n.a.	abs.	n.a.	n.a.	n.a.	n.a.	n.a.	abs.	n.a.	n.a.	abs.	n.a.	n.a.	n.a.	abs.	n.a.	Abs.
E.coli(CFU/g)	n.a.	n.a.	<100	n.a.	n.a.	n.a.	n.a.	n.a.	<100	n.a.	n.a.	<100	n.a.	n.a.	n.a.	<10	n.a.	<10

n.a. not analysed ; abs. abasent

(1) Total Nitrogen calculated as the sum of TKN + NO_2^--N + NO_3^--N

(2) Organic Nitrogen calculated as the difference between TKN and NH_4^+-N

(3) Inorganic Nitrogen calculated as the sum of NH_4^+-N + NO_2^- -N + NO_3^--N

(4) Analysed with Photometer PF-12^{Plus} and NANOCOLOR ® Ammonium 3 test kit.

(5) Analysed with Photometer PF-12^{Plus} and VISOCOLOR ECO Nitrate test kit.

(6) Analysed with Photometer PF-12^{Plus} and NANOCOLOR Ortho- and total Phosphate test kit.7

(7) Microbiology: Rapid detection method Salmonella. Rapid Salmonella. UNE EN ISO 16140; ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E.coli

2.4.3 Waste from Land-based flow-through system – GyE

The outlet water from fish tanks from Fish trials with African catfish using commercial diet was collected during routine operation at the facility. The Table 6 shows the physicochemical and microbiological characterisation of water.

Table 6 Physicochemical characterisation wastewater from Land-based flow- through system - GyE

Parameter	Result
pH	7.5
Conductivity (µS/cm)	918
Total ion (mg/dm³)	578
NH_4-N (mg/L)	0.9
Ammonium ion (mg/L)	1.2
Free ammonia (mg/L)	0
NO_2^--N (mg/L)	0.3
Nitrite ion (mg/L)	0.1
NO_3^--N (mg/L)	2.8
Nitrate ion (mg L)	12
Total nitrogen (mg/L)	3.7
Orthophosphate ion (mg/L)	6.2
TOC - total organic carbon (mg/L)	1056
COD- chemical oxygen demand (mg/L)	315

Togal nitrogen in water, ranged from 3.7 mg/L, with values of 6.2 mg/L of nitrates. Samples contained phosphorus, ranging 6.2 mg/L in orthophosphate form and total organic carbon 1056 mg/L.

Sludge from faeces collector from AA was a very different type of waste in comparison to the concentrated sludge from ABT (15-20% dry matter), because it wasn't concentrated, all samples ranging 0.5-4.3 % dry matter. Nutrients in sludge were similar to wastewater. Togal nitrogen, ranged from 55-1372 mg/L, with values up to 62 mg/L of nitrates in water samples. Samples contained phosphorus, ranging 1.5-3 mg/L in phosphate form, as well as other nutrients such as sodium, calcium. Pollutant elements were not detected or they were under 5 mg/L (i.e. Zn). Salmonella and *E. coli* are were not detected (under 100 CFU/g).

2.5 Conclusions

Different fish farming-sites participating in the iFishIENCi project were assessed for potential waste collection and valorisation trials considering practical collection, characteristics, legislation, existing options for waste treatments and needs for further valorisation.

From this assessment, it was concluded that waste collection was not feasible in open systems but there would be possibilities to exploit waste from the following systems and sources since they could be exploited in the future*:*

- Waste from fish farming sites from Recirculation Aquaculture System (RAS) provided by ABT (wastewater, raw sludge 0.5-4% dry matter, concentrated sludge with 15-20% dry matter) and AA (dirty water, raw sludge 0.5-4.3% dry matter). Waste from land-based flow-through system provided GyE (dirty water).

- Waste collected from the production of iFishIENCi ingredients: the cell-free spent medium collected after harvesting the *Candida utilis* yeast biomass provided by NORCE and the insoluble matter collected after the production of the antioxidant supplement from *Nannochloropsis gaditana* provided by LEITAT.

Considering the legislation and results of the characterisation, it was concluded that the studied waste streams had potential for re-use and valorisation under a circular approach and towards zero waste. As recommendation, further investigation should be conducted to study the potential bioaccumulation of persistent organics pollutants (POPs) in yeast and microalgae as feed, and check permit limits (Directive 2002/32/EC).

The selection of various waste types and their re-use to be investigated for valorisation experiments for the production of new feeds is summarised in Table 7. This selection was based on the waste characterisation, the practical collection and needs for further valorisation. The valorisation trials for water reuse for microalgae production (NORCE), water and sludge reuse for yeast production (NORCE), and nutrient recovery from sludge (LEITAT) are detailed in the following chapters.

Table 7 Use of different waste for valorisation trials

AA – RAS system					
Experiments	**Fish**	**Diet**	**Type of waste**	**Used for**	**Production of biomass**
RAS1_188 (Task 1.4)	Rainbow trout	Algae diet 30% and control diet	outlet water and sludge	Algae	Photoautotrophic microalgae growth tests, small scale
			sludge	Recovery of nutrients (MI)	Microalgae tests on MI, small scale
RAS2_194 (Task 1.4)	Rainbow trout	Nanno extract and control diet	sludge	Recovery of nutrients (MI)	Microalgae tests on Mis, small scale
RAS5_243 (Task 1.4)	Rainbow trout	Candida diet 30% and control diet	outlet water	Algae	Photoautotrophic microalgae growth tests, small scale
			sludge	Recovery of nutrients (MI)	Microalgae tests on MI, small scale
RAS6_275 (Task 3.4)	Rainbow trout	-Control diet (with FM 15% and Astaxanthin 40 mg/kg) -Candida diet (5% substituted for FM; 40 mg/kg Astaxanthin) -Nanno diet 1 (5% substituted for FM; 40 mg/kg Astaxanthin) -Nanno diet 2 (5% substituted for FM; 20 mg/kg Astaxanthin)	outlet water sludge	Algae and Yeast Yeast Recovery of nutrients (MI)	Photoautotrophic microalgae, growth tests, small scale. Yeast growth tests, small scale Yeast growth tests, small scale Microalgae and yeast tests on MI
ABT – RAS system					
Experiments	**Fish**	**Diet**	**Type of waste**	**Use**	**Production of biomass**
RAS3_ABT3 (Task 1.3)	Rainbow trout	Conventional diet	outlet water	Algae	Photoautotrophic microalgae growth tests, small scale
			conc.sludge	Yeast	Yeast growth tests, small scale
			conc.sludge	Recovery of nutrients (MI)	Microalgae tests on MI

IFN01_LC (Task 1.4)	Barramundi (salt water)	Nanno extract and control diet	outlet water	Algae	Photoautotrophic microalgae growth tests, small scale **Proof of concept at pilot scale**
IFN02_RT (Task 3.4)	Rainbow trout	Conventional diet	outlet water	Algae	Photoautotrophic microalgae growth tests, small scale
			outlet water	Yeast	Yeast growth tests, small scale
			conc.sludge	Yeast	Yeast growth tests, small scale
			conc.sludge	Recovery of nutrients, **proof of concept at pilot scale**	Microalgae and yeast tests on MI, small scale
IFN03_AC (Task 3.4)	African catfish	-Candida diet 1 10% -Candida diet 2 20%	outlet water conc.sludge	characterisation characterisation	- -
GyE - Land-based flow-through System					
Experiments	**Fish**	**Diet**	**Type of waste**	**Use**	**Production of biomass**
(Task 1.4)	African Catfish	Conventional diet	outlet water	Algae	Photoautotrophic microalgae growth tests, small scale

3 Wastewater valorisation as microalgae production feedstock

3.1 Introduction

The reuse of dirty water was assessed under *Task 1.5.2 Proof-of-concept 1. Reuse of dirty water as algae production feedstock* by NORCE.

Interesting nutrients in the dirty water are the nitrogen, especially in the form of ammonium and nitrate, phosphate, and possibly some organic components and micronutrients. The concentrations as determined in Chapter 2 were generally lower than the concentrations used in commercial cultivation media for microalgae, but since the quantities of dirty water are very high, total amount of available ammonium and other nutrients are still very high and thus interesting for microalgae production. NORCE conducted a study on using dirty water for microalgae production, designing mediums, conducting preliminary testing, determining nutrient conversion efficiency, evaluating biomass quality, and ultimately achieving pilot scale proof-of-concept. Depending on whether the waste stream originates from freshwater or seawater, their applicability for freshwater (*Chlorella vulgaris NIVA-CHL 108*) or marine photoautotrophic microalgae (*Nannochloropsis gaditana* CCMP 526) was tested. *N. gaditana* was the strain of photoautotrophic microalgae that was used as feed ingredient for some of the fish trials that provided the waste for the circularity trials.

3.2 Experimental development

3.2.1 Medium design

Based on the results from the characterisation, various medium compositions were designed based on the nutrient requirements for either the seawater strain *N. gaditana* or the freshwater strain *C. vulgaris*. The dirty water was either used directly as it was received (after thawing, all dirty water was shipped frozen), or autoclaved (20 minutes at 121°C) or filtered to get rid of any possible contaminants that were already present. Furthermore, the addition of phosphate to obtain the ratio between nitrogen and phosphorous as found in our common cultivation media waste tested, as well as the addition of micronutrients.

3.2.2 Preliminary testing

In these trials, wells-plate experiments were used to test the growth of the microalgae on the designed media. Since some of the volumes provided were relatively small, wells plates cultivation was most suited to determine possible growth for a wide range of media compositions without the need for large quantities of medium. Main outcomes were to get a first indication of biomass growth, a rough estimation of limiting nutrients (N or P), and if there were any negative factors in the waste streams that would negatively influence/inhibit growth. All samples were tested at least in triplicate.

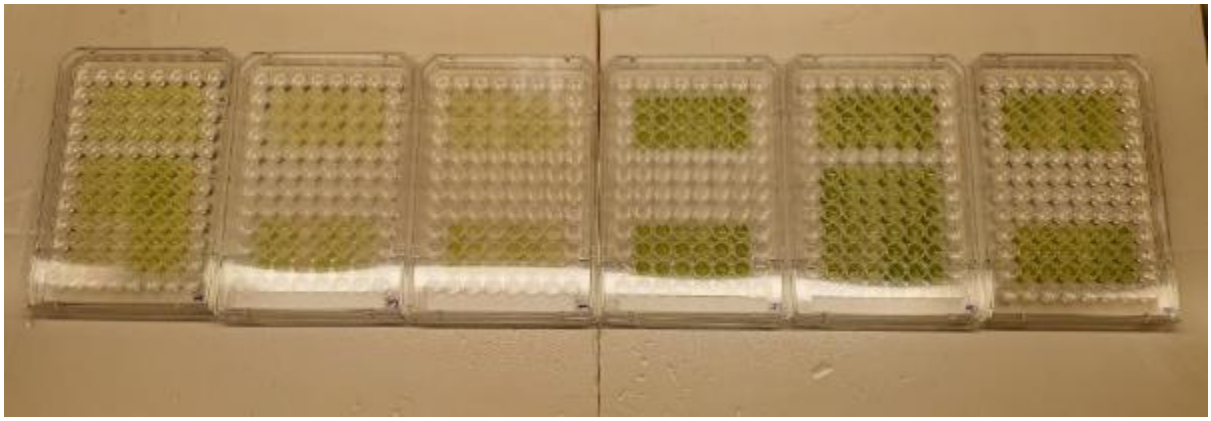


Figure 2 Preliminary testing of microalgae growth on dirty water in wells plates.

In Table 8 the results are shown from the wells plate experiments. G indicates that the microalgae grew on the tested medium, both with and without the addition of micronutrients (unless indicated differently). F indicates that no algae growth was registered, neither with nor without the addition of micronutrients (unless indicated differently). Different results were obtained for: [1] microalgae growth was only registered when micronutrients were added, not without; [2] microalgae growth was only registered without addition of micronutrients, not with; [3] no growth was measured in optical density, but visual inspection did indicate some microalgae growth, though uneven/in flocs. In none of the cases, there was an indication that phosphate was limiting, and addition of extra phosphate did not lead to different results than without the addition of phosphate [4] possibly some growth but seemed mainly flocs of other origin than microalgae. OD increased in the first days but crashed after day 4.

Overall, all the outlet waters were suitable as nutrient source regarding N and P for microalgae growth, both untreated and treated (autoclaving or filtration). In case of the direct use of raw sludge (non-concentrated sludge) coming from the RAS, one sample with microalgae culture crashed after the first day in the untreated sample (rainbow trout on control-diet, RAS1_188). However, good microalgae growth in the same sample that was autoclaved was observed, as well as in the other sample (rainbow trout on control diet).

With regards to the treated sludge, different approaches were tested to solubilize more nutrients, as described in Chapter 5, in the form of medium ingredients (MI). The results of growing microalgae on the medium ingredient from the treated sludge did not show consequent results. In most cases, there was no growth registered of *C. vulgaris* on the medium ingredient from the treated sludge (Table 8). In some cases, growth of the microalgae was observed, though it seems unlikely that this was related to the sludge origin. Most of the sludge samples were very turbid, which might have affected both the growth (blocking the light for the microalgae) as well as the measurement. Though through measuring the relative increase in absorption by chlorophyll (OD680), this should have been prevented.

Table 8 Microalgae growth (C. vulgaris and N. gaditana) on wastewater and sludge received from the RAS System at ABT and AA, as well as Medium ingredients (MI) from LEITAT

Aquaculture experiment					**Microalgae tested**	**Treatment of dirty water**		
Name	**Partner**	**Fish (water)**	**Diet**	**Waste collected**	**Species**	**None**	**Autoclaved**	**Filtered**
RAS1_188	AA	Rainbow trout (FW)	Algae	Outlet water	*C. vulgaris*	G	G	n.a.
				Sludge (direct)	*C. vulgaris*	G	G	n.a.
				MI from sludge – enzyme (Opt1)	*C. vulgaris*	F	n.a.	F
			Control	Outlet water	*C. vulgaris*	G	G	n.a.
				Sludge (direct)	*C. vulgaris*	F	G	n.a.
				MI from sludge – enzyme (Opt1)	*C. vulgaris*	F	n.a.	F
RAS2_194	AA	Rainbow trout (FW)	Control	MI from sludge – enzyme (Opt1)	*C. vulgaris*	F	n.a.	F
			Nanno-extract	MI from sludge – enzyme (Opt1)	*C. vulgaris*	F	n.a.	F
RAS5_243	AA	Rainbow trout (FW)	Candida	Outlet water	*C. vulgaris*	G	G	n.a.
				MI from sludge – enzyme (Opt1)	*C. vulgaris*	G	n.a.	G[3]
			Control	Outlet water	*C. vulgaris*	G	G	n.a.
				MI from sludge – enzyme (Opt1)	*C. vulgaris*	G	n.a.	G
RAS3_ABT3	ABT	Rainbow trout (FW)	Conventional	Outlet water	*C. vulgaris*	G	G	G
				MI from sludge – enzyme (Opt1)	*C. vulgaris*	G[1]	n.a.	F
				MI from conc. sludge – enzyme (Opt1)	*C. vulgaris*	F	n.a.	F
				MI from conc. sludge – enzyme (Opt2)	*C. vulgaris*	F	n.a.	F
				MI from conc. sludge – enzyme (Opt3)	*C. vulgaris*	F	n.a.	F
				Outlet water – blank enzyme (control)	*C. vulgaris*	G[2]	n.a.	G
				Outlet water – blank enzyme (control)	*C. vulgaris*	G	n.a.	G
RAS6_275	AA	Rainbow trout (FW)	Optimised Algae / Candida meal diets	MI from raw sludge – chem. (process 2)	*C. vulgaris*	G	n.a.	n.a.
IFN02_RT	ABT	Rainbow trout (FW)	Conventional	Nutrients recovered from conc. sludge (MI enzymatic process 1)	*C. vulgaris*	n.a.	n.a.	G
				Nutrients recovered from conc. sludge (MI chemical process 2, lab (filtered) + pilot (non-filtered)	*C. vulgaris*	F[4]	n.a.	G
IFNO1_LC	ABT	Barramundi (SW)	Nanno-extract	Outlet water (mix)	*N. gaditana*	G	n.a.	n.a.

Sludge from AA: sludge from faeces collector, 0.5-4.3% dry matter
Sludge from ABT: concentrated sludge (conc. Sludge), 15-20% dry matter
MI: Medium Ingredient containing nutrients recovered from sludge

3.2.3 Nutrient conversion efficiency and effect on biomass quality

Based on the outcomes of the preliminary testing and available quantities of dirty water, some freshwater waste streams (Table 9) were chosen for the cultivation of *C. vulgaris* in 300 mL bubble-column photobioreactors, to assess their potential for microalgal growth and yield of biomass. The waste streams were either used directly or diluted with freshwater to achieve similar starting concentrations of total nitrogen (Table 10). This was to ensure that the amount of nutrients would be limiting before the light would become limiting in the experiments, and thus the amount of biomass produced per available nutrients could be determined. Microalgal cultivation on the waste streams was compared to its cultivation in a control medium (BBM, a standard growth medium for *C. vulgaris* in the lab). All cultivation experiments were performed with 3 biological replicates.

For one waste stream (RAS3_ABT3) it was additionally tested whether supplementation with micronutrients or phosphate would lead to improved microalgal growth as those nutrients were expected to be limiting in the original waste streams. Therefore, in addition to the original waste stream (RAS3_O), two nutrient supplementations were tested: micronutrient addition (RAS3_µ), and phosphate and micronutrient addition (RAS_Pµ, same concentrations as in the control medium). Moreover, the quality of the microalgal biomass (e.g., lipid content) produced on the different RAS3_ABT3 treatments were studied.

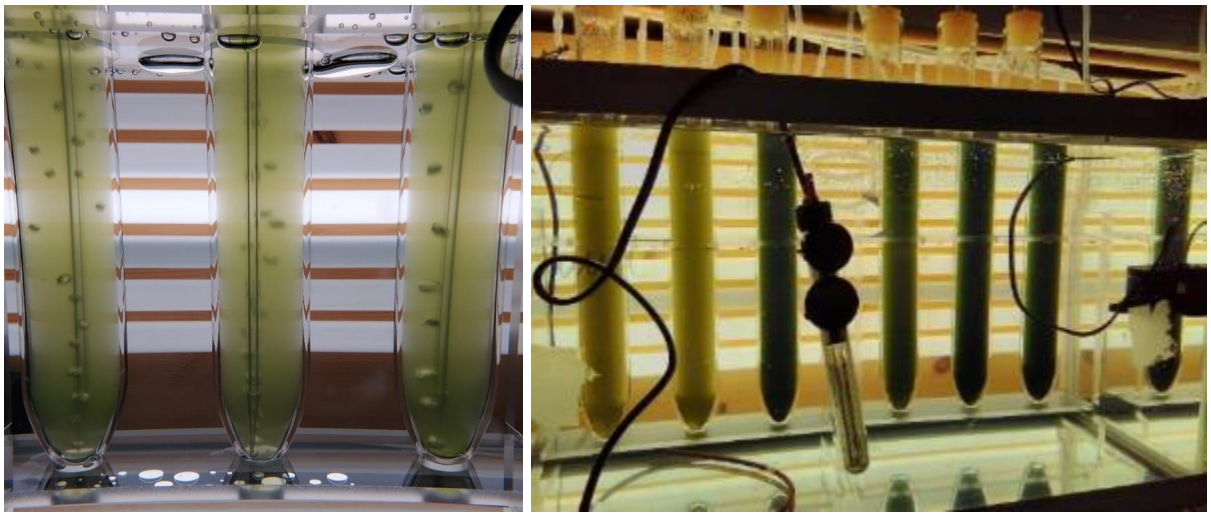


Figure 3 Bubble-column photobioreactors used for testing dirty water as nutrient source for microalgae growth (C. vulgaris).

Table 9 Tested waste streams for microalgae growth in screenatorium

Name	System	Fish (water)	Diet	Waste collected, medium
RAS1_188	RAS	Rainbow trout (FW)	Algae	Outlet water, with micronutrients
RAS5_243	RAS	Rainbow trout (FW)	Candida	Outlet water, with micronutrients
RAS3_ABT3	RAS	Rainbow trout (FW)	Conventional	Outlet water: original, with micronutrients, and with micronutrients and phosphate
IFN02_RT	RAS	Rainbow trout (FW)	Conventional	Outlet water, diluted, with micronutrients
GyE	Flow-through	African catfish (FW)	Conventional	Outlet water, with micronutrients

As can be seen in Figure 4, the algae grew well on all waste streams, and their performance was similar as on the control medium (BBM). As predicted, for all media, the growth started to flatten out towards

the end, due to nutrient limitation. This is confirmed by the decrease in quantum yield (QY), Figure 4. QY is an indicator of performance of the photosynthesis. Nutrient limitation generally leads to a decrease in QY. For RAS3_ABT3, the nutrient supplementation (RAS3_µ and RAS3_Pµ) resulted in a slightly higher biomass concentration compared to RAS3_O, though the difference was not statistically significant ($p>0.05$) based on the achieved dry weight (Table 11). The waste streams were obtained from different fish experiments, both from RAS and flow-through systems. The outlet water from RAS3_ABT3 was also tested with additional micronutrients (RAS3_µ) and additional phosphate and micronutrients (RAS3_Pµ). Values show the average and standard deviation of three biological replicates.

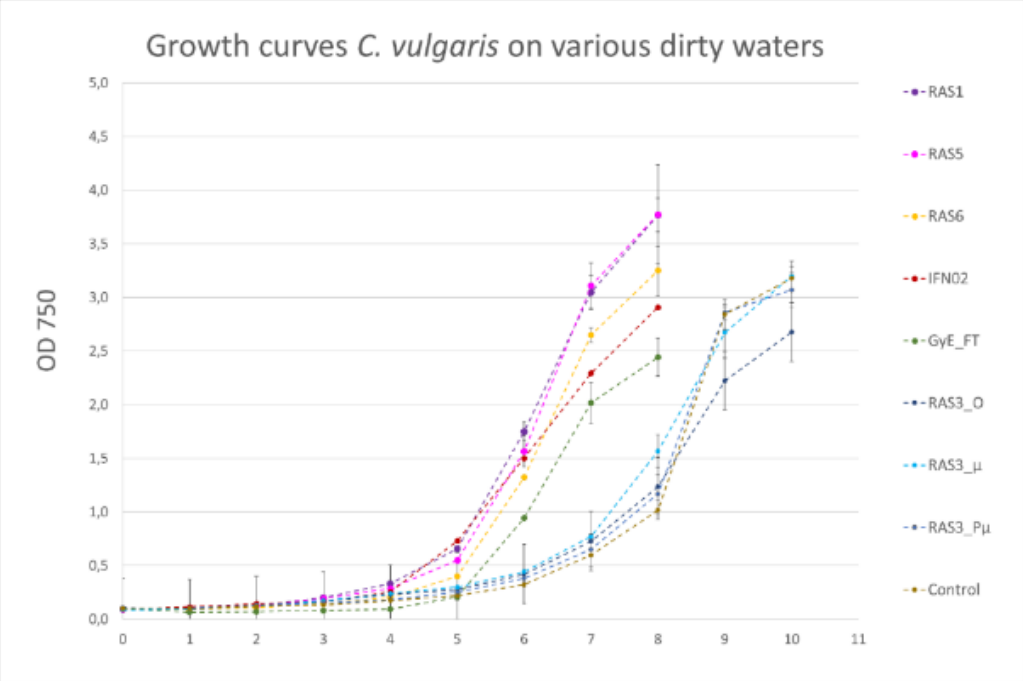


Figure 4 Growth curves of C. vulgaris on the various waste streams and a control medium (BBM) in lab-scale bubble column photobioreactors.

The waste streams were obtained from different fish experiments, both from RAS and flow-through systems. The outlet water from RAS3_ABT3 was also tested with additional micronutrients (RAS3_µ) and additional phosphate and micronutrients (RAS3_Pµ). Values show the average and standard deviation of three biological replicates.

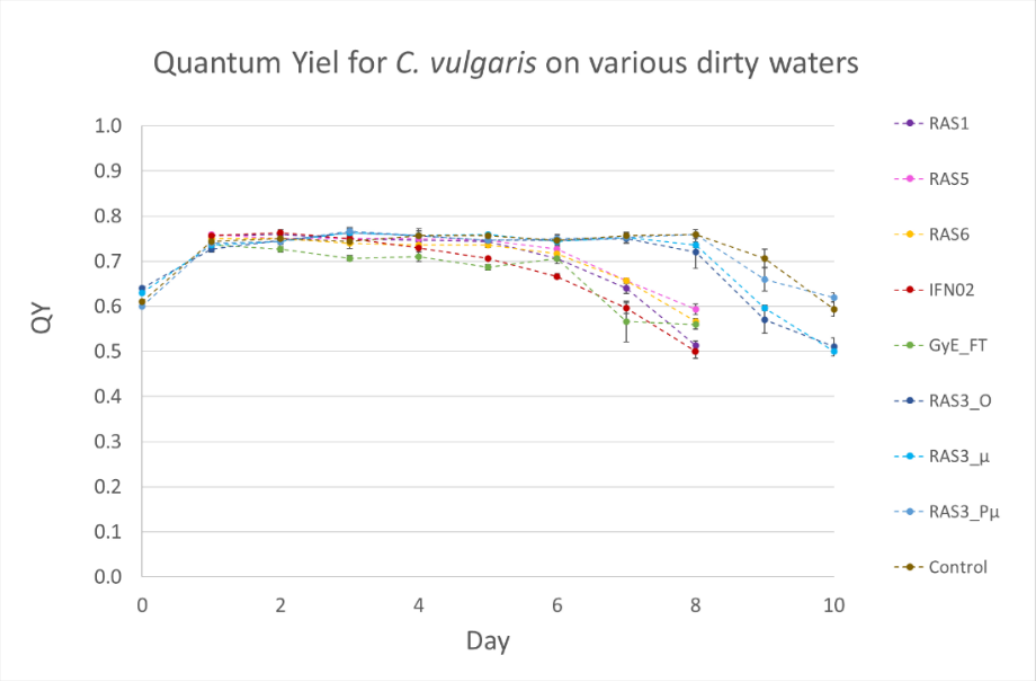


Figure 5 Quantum yield for C. vulgaris during growth on the various waste streams. and a control medium (BBM) in lab-scale bubble column photobioreactors.

In Table 10 the nitrogen and phosphate concentrations are shown that were available at the start of the cultivation experiments, as well as during and at the end of the experiments. As can be seen, especially phosphate was the first limiting nutrient, unless added at higher concentrations (RAS3_Pµ and in the control medium). In some cases, some P was still detected, possibly this was not in a bioavailable form for the microalgae to consume, or within the accuracy limits of the tests. The total N consisted mainly of nitrate and ammonium. Ammonium was available only at very low concentrations and was also completely consumed during the experiment. Very low concentrations of total nitrogen were still measured towards the end of the experiment (stationary phase), possibly because the phosphate already became limiting first. Or also here the nitrogen was not present in a bioavailable form for the microalgae to consume.

Table 10 Concentrations of main nutrients at the start and during the growth of C. vulgaris on the various waste streams.

Dilution rate from original waste stream		Nutrients (in start medium)		Nutrients (mid-growth phase)		Nutrients (stationary phase)	
		[mg/L]	[mg/L]	[mg/L]	[mg/L]	[mg/L]	[mg/L]
		tN	PO_4^{3-}-P	tN	PO_4^{3-}P	tN	PO_4^{3-}-P
RAS1_188	1	47.0	1.5	24.9	<0.2	8.1	<0.2
RAS5_243	1	41.0	2.0	22.1	<0.2	3.3	<0.2
RAS6_275	1	41.0	1.5	30.1	0.27	10.0	<0.2
IFN02_RT	2.5	61.0	1.6	42.4	<0.2	26.3	<0.2
GyE_FT	1	25.0	1.3	15.8	<0.2	3.7	<0.2
RAS3_ABT3_O	4.6	43.2	1.2	10.1	0.7	3.8	0.7
RAS3_ABT3_µ	4.6	46.0	0.5 [(1)]	8.4	0.6	6.0	0.4
RAS3_ABT3_Pµ	4.6	45.6	50.0	5.1	45	6.2	40.0
Control medium[(2)]	1	46.0	50.0	10.0	45	5.8	39.0

[(1)] Too low, should have been similar to value found for RAS3_O.

[(2)] Control medium is a medium with low nutrient concentrations for lab experiments.

In Figure 6, the content and profile of the various fatty acids in *C. vulgaris* is shown when grown on the different treatments of RAS3_ABT3 water (RAS3_O, RAS3_µ and RAS3_P) and control medium. These samples were taken on day 8 (see growth curves in Figure 4). When grown on RAS3_O and RAS3_µ, the total fatty acid content was higher than when grown on the media where extra phosphate was added (RAS_Pµ). Moreover, the amount of saturated and mono-unsaturated fatty acids increased in these samples, whereas the amount of poly-unsaturated fatty acids went down. This indicates that phosphate at this point was probably already becoming limiting, as nutrient limitation generally triggers lipid accumulation in *Chlorella vulgaris*, and a decrease in rate of saturation. However, *C. vulgaris* can accumulate more than three times the lipid content it has during growth under nutrient replete conditions, so the small increase seen here indicated that the stress was not that severe (Benvenuti G, 2015). Moreover, also based on the Quantum Yield, microalgae in the RAS3_ABT3 media without extra phosphate started to decrease in QY just before those with additional phosphate. Probably in those cases (control and RAS_Pµ), the nitrogen started to become the limiting nutrient.

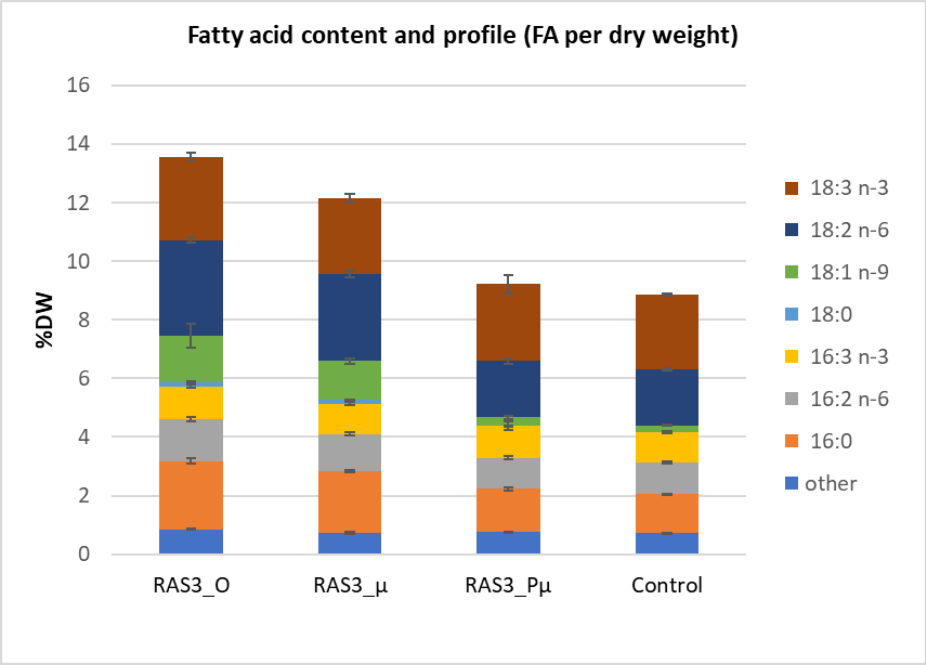


Figure 6 Fatty acid content and profile of C. vulgaris during growth on the RAS3_ABT3 water (RAS3_O), supplemented with additional micronutrients (RAS3_µ), additional micronutrients and phosphate (RAS3_Pµ), and when grown on a control medium. Values show the average and standard deviation of three biological replicates.

In Table 11 Biomass produced (per L medium, and possible per L original waste stream) and capture efficiency of nitrogen and phosphorous, as well as the amount of microalgal biomass that could potentially be produced per litre waste stream, if those would not have been diluted, is given. In none of the cases the waste streams would need to be diluted for commercial production of microalgae, as the medium used for commercial production generally has higher nutrient concentrations than the medium used for lab experiments. This is to ensure that nutrients are not limiting, and maximum biomass concentrations can be achieved.

Table 11 Biomass produced (per L medium, and possible per L original waste stream) and capture efficiency

	Biomass concentration		Capture Efficiency				Possible algae production per L waste stream	
	Mid-growth	Stationary	Mid-growth		Stationary		Mid-growth	Stationary
	[g/L]	[g/L]	%	%	%	%	[g/L]	[g/L]
	DW	DW	N	P	N	P	DW	DW
RAS1_188	0.16±0.01	0.64±0.03	47	100	83	100	0.16	0,64
RAS5_243	0.18±0.01	0.66±0.04	46	100	92	100	0.18	0,66
RAS6_275	0.13±0.01	0.55±0.01	27	82	76	100	0.13	0,55
IFN02_RT	0.23±0.02	0.53±0.03	30	100	57	100	0.58	1,33
GyE_FT	0.11±0.02	0.45±0.03	37	100	85	100	0.11	0,45
RAS3_ABT3_O	0.29±0.03	0.75±0.06	77	42	91	42	1.33	3,45
RAS3_ABT3_µ	0.40±0.05	0.84±0.03	82	-20*	87	-20*	1.84	3,86
RAS3_ABT3_Pµ	0.39±0.05	0.84±0.03	89	10	86	10	1.79	3,86
Control medium**	0.36±0.05	0.87±0.01	78	10	87	10	0.36	0,87

As can be seen in the table, both waste streams IFN02_RT and especially RAS3_ABT3 allowed for higher biomass concentrations to be achieved than the other wastewaters. This was because these streams were richer in nutrients than the other waste streams, most probably due to where and how they were collected from the RAS systems. However, the microalgae consumed in most cases almost all phosphorous already at the mid-growth phase. As indicated before, the biomass was already becoming stressed at this point due to nutrient limitation, which is indicated by the lowered QY and the very small increase in lipids, that already started during the mid-growth phase.

In case the objective is to use microalgae to consume as much nutrients as possible from the waste stream, this would be achievable by running a batch culture and aim for maximum biomass concentrations achieved. However, as can be seen in the growth curves, the growth rate of the microalgae will start decreasing towards the end, slowing down the productivity. The amount of algal biomass (*C. vulgaris*) that could be produced per litre of waste stream would in that case be predicted by the achievable biomass concentration measured here at stationary phase. This approach would have implications for the biomass compositions (higher in lipids, especially in saturated and mono-saturated fatty acids).

In case maximum biomass productivity for a production facility would be the main goal, it would be recommended to harvest already before the nutrients have run out, which would lead to lower maximum biomass densities and less algae grown per litre of waste stream. Though the medium would be faster replenished by new amounts of waste stream, providing more nutrients, thus allowing for more biomass being produced in the same time frame.

3.2.4 Proof of concept at pilot scale

The large quantity of wastewater obtained from ABT (IFN01_LC (Barramundi, seawater)), was tested for production of *N. gaditana* in our 25L GemTube® photobioreactors as a proof-of-concept of cultivation at pilot scale. The total biomass productivity, yield of microalgal biomass on wastewater, and the quality of the microalgal biomass were determined, and compared this to a control experiment on medium made with commercial fertilizers.

Figure 7 Photobioreactor used to test the dirty water from IFN_01_LC (Barramundi, seawater) for the production of N. gaditana. Explanation of points: 1. Main access point, 2. Tubular glass helix, 3. Aeration inlet and humidifier, 4. Deaeration, 5. pH-meter and thermometer, 6. Integrated LED-light panels.

As can be seen in Figure 8, *N. gaditana* grew equally well on the control medium (a rich medium developed for industrial production, with ~175 mg N/L) and the undiluted wastewater IFN01_LC, where only micronutrients were added. Only after 7 days the growth started to slow down on IFN01_LC, and the nitrate was fully consumed by the microalgae (100% capture efficiency). The quantum yield dropped only slightly at this point. Per litre wastewater it was possible to produce thus 2.4 gram microalgal biomass. S1 is the mid growth phase sampling point for both mediums. S2a and S2b is the stationary sampling point for IFN01_LC and NORCE, respectively.

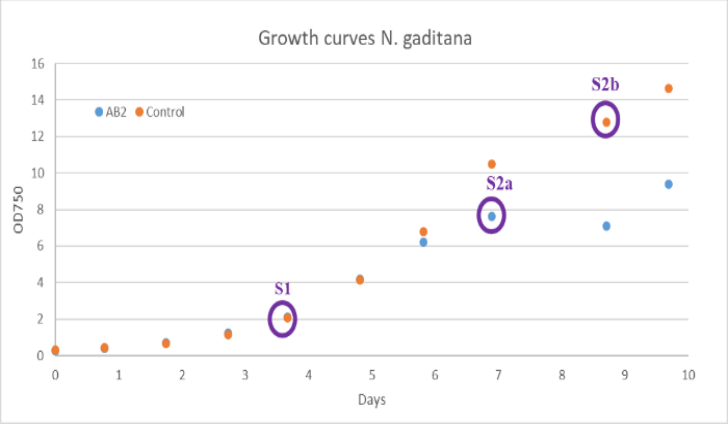


Figure 8 Left: Growth curves for N. gaditana on IFN01_LC and NORCE medium (based on OD750) during the photobioreactor experiment. Right: nitrogen concentration (NO3-N) and Quantum Yield for N. gaditana growth on IFN01_LC. Only one run was performed as there was not sufficient IFN01_LC wastewater available for multiple runs.

Table 12 Amount of microalgal biomass (C. vulgaris) produced per litre medium and per litre waste stream.

	Dry weight (mid growth)	Dry weight (stationary)	Biomass/L waste (mid growth)	Biomass/L waste (stationary)	Capture efficiency N (stationary)
	[g/L]	[g/L]	[g/L]	[g/L]	[%]
IFN01_LCIFNO	0.67	2.36	0.67	2.36	100
Control medium	0.60	4.81	-	-	-

3.3 Conclusions

The outlet water from all fish production could be directly used for the cultivation of microalgae. Treatment (sterilization) was not necessary, though this might lead to challenges at large-scale cultivation due to contamination with bacteria from the fish tanks. Addition of micronutrients seemed to lead to small improvements in achieved biomass concentrations. Phosphate was the first main limiting nutrient for the microalgae. Though the nutrient concentrations varied significantly between batches of fish-farming wastewater, all were lower in nutrient concentrations than growth medium for industrial production of microalgae. Process and/or reactor design to ensure high biomass productivity while treating large amounts of water is therefore necessary. Moreover, the capture efficiency can be up to 100% for phosphorous and up to 100% for nitrogen from the water. In some cases, not all P and/or N was captured, probably due to it being bound to organic particles. The final capture efficiency of a production facility will be determined based on the process design and the aim to be achieved: i) maximum biomass productivity in time, or ii) maximum capture efficiency and thus cleanest water produced.

The treated sludge could in some cases serve as nutrients for microalgae cultivation, though this needs further optimization, as the results were inconclusive. Moreover, it was only possible to determine growth or no growth for these samples.

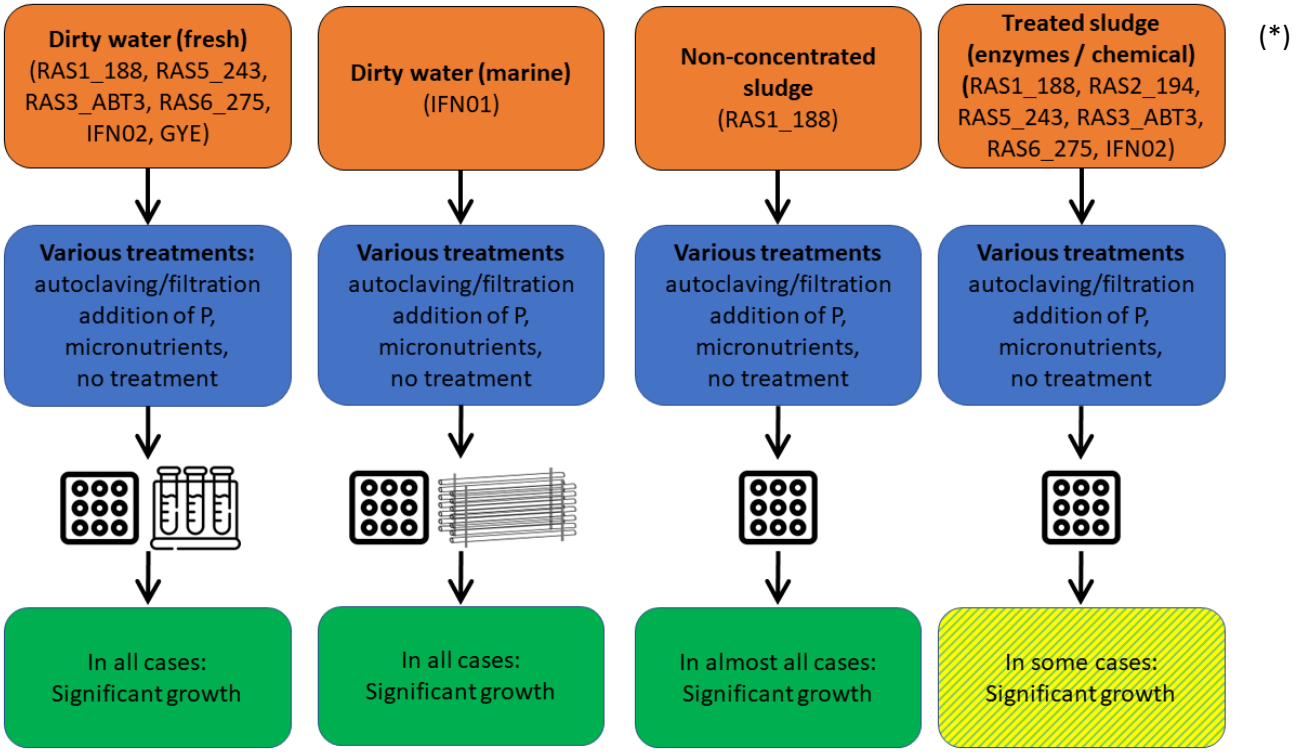


Figure 9 Overview of performance of microalgae growth (C. vulgaris or N. gaditana) on the various waste streams from fish-farming. (*) Nutrients recovered from sludge as Medium Ingredient (MI) by applying enzymatic and chemical treatments.

4 Wastewater and sludge valorisation as feedstock for the production of yeast and other industrial production organisms.

4.1 Introduction

The valorisation of wastewaters and sludge from the iFishIENCi fish farming sites was assessed under *Task 1.5.3 Proof-of-concept 3. Sludge valorisation into algae nutrients* by NORCE.

Sludge from fish-farming have been shown to contain carbohydrates, proteins, and amino acids as well as other nutrients which make it a potentially suitable feedstock for the growth of many different types of microorganisms. The use of sludge in industrial bioprocesses could lower production costs and environmental impact.

NORCE investigated microorganism growth on wastewater and sludge using a similar approach to microalgae growth by designing microbial growth media, performing preliminary growth tests on different media compositions, and conducting laboratory experiments to screen for suitable organisms while necessary growth supplements. The use of wastewater and sludge from feed trials for *Candida utilis* yeast growth was investigated.

4.2 Experimental setup for the production of yeast from wastes

4.2.1 Medium composition

All samples from fish farming and nutrients recovered from conc. sludge by LEITAT were received frozen in an isoprene box and stored at -20^{0}C until further processing. The list of samples received at NORCE is given in Table 13. The overview of the experimental set up for the growth tests is shown in Figure 10.

Table 13 Wastewater and sludge received from the ABT and AA. Medium ingredients from LEITAT

Name	Partner	Fish trial
RAS3_ABT3 concentrated sludge	ABT	Rainbow trout Conventional diet
IFN02_RT wastewater	ABT	Rainbow trout Conventional diet
IFN02_RT concentrated sludge	ABT	Rainbow trout Conventional diet
RAS 6_275 wastewater	AA	Rainbow trout Various new diets with Nanno and Candida
RAS6_275 sludge	AA	Rainbow trout Various new diets with Nanno and Candida
PILOT ING sludge IFN02 – Enzymatic (1)	LEITAT	Nutrients recovered from conc. sludge IFN02_RT (MI enzymatic process 1)
ING sludge RAS6_275 from Candida Enzymatic (1)	LEITAT	Nutrients recovered from non-conc. sludge RAS6_275 (MI enzymatic process 1)

Sludge from AA: sludge from faeces collector, 0.5-4.3% dry matter
Sludge from ABT: concentrated sludge (conc. Sludge), 15-20% dry matter
MI: Medium Ingredient containing nutrients recovered from sludge

4.2.2 Preliminary testing

The Figure 10 shows the yeast growth experiments.

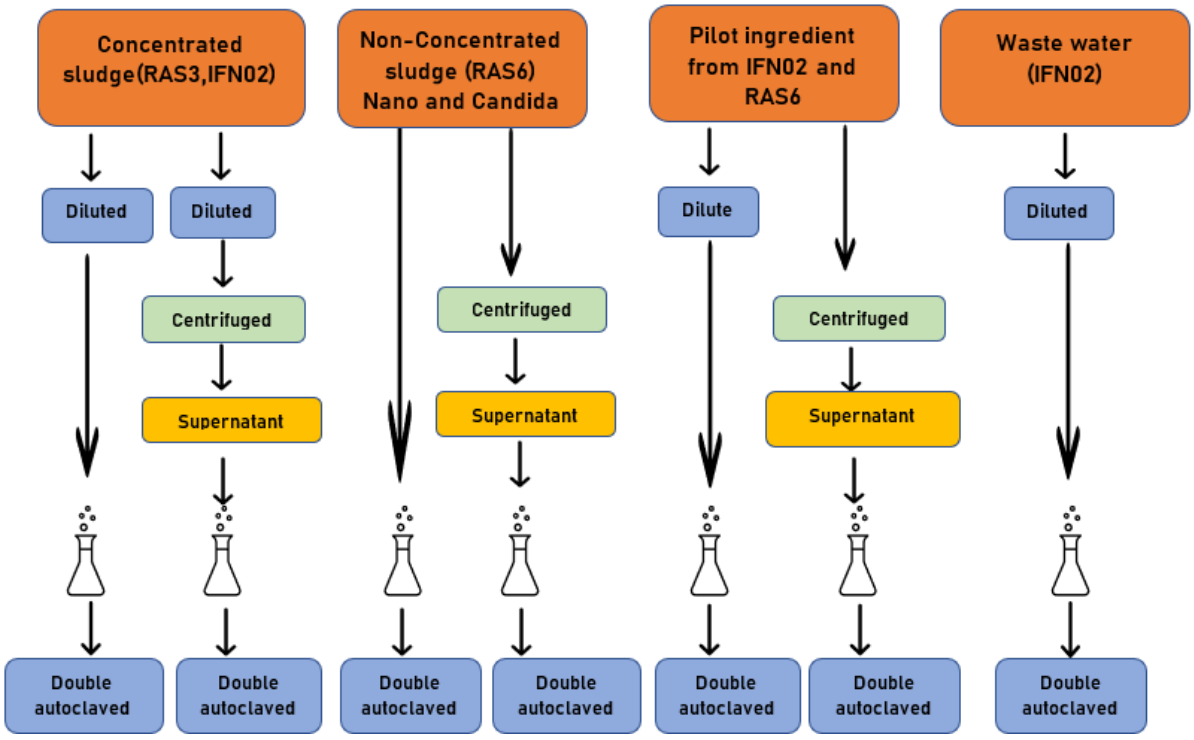


Figure 10 Yeast growth experiments

Concentrated sludge (RAS3_ABT3, IFN02_RT): The concentrated sludge was received in Ziplock bags. The samples were thawed and aliquoted to smaller sizes into smaller zip lock bags and stored in the -20^{0}C. The concentrated sludge had a very think consistency. To prepare a suitable media for the growth of yeast, *Candida utlilis*, a specific quantity of thawed sludge was diluted with distilled water in a Scott bottle. Different concentrations were made. The resulting diluted sludge was kept for mixing on a mechanical shaker at 120 rpm for 30 minutes at 4^{0}C as shown. This sludge was divided in to two portions and one portion was further referred as diluted sludge used as such to conduct the growth experiments. The other portion was centrifuged at 4500 rpm for 15minutes at 4^{0}C and the pellet was discarded, and the supernatant was distributed in to 250 ml shake flask (Erlenmeyer flask with baffles). 50ml of this processed sample was added to 3 of the 250ml shake flasks, two serving as the replicates and one as a control. The shake flasks were double autoclaved at 121^{0}C for 30minutes. Once the shake flasks were cooled to room temperature, 250µl of stock culture of yeast, *C.utilis* was added and kept for incubation in a shaking incubator at 200rpm and 28^{0}C. 2ml of the culture samples from each of the shake flask was taken aseptically every 5 hours in course of 48 hours. Optical density was determined using a spectrophotometer at 600nm wavelength.


RAS3 sludge & IFN02 sludge

Sludge diluted in shake flasks and inoculated with *C.utilis*

Figure 11 Yeast, Candida utilis growth experiments on the concentrated sludges RAS3_ABT3 and IFN02_RT

Non-concentrated sludge (RAS6_275): The non-concentrated sludge was received frozen in plastic bottles. The samples from Candida diet and Nannochloropsis diet1 were used for the shake flask experiments. The samples were thawed and dispensed into Scott bottles and kept for mixing at 120rpm for 30 minutes at 4^0C. This sludge samples were then divided in to two portions and one portion was used as such to conduct the growth experiments. The other portion was centrifuged 4500 rpm for 15minutes at 4^0C and the pellet was discarded, and the supernatant distributed in to shake flasks (Erlenmeyer flask with baffles). 50ml of this processed samples were dispensed to 3 of the 250ml shake flasks, two serving as replicates and one as a control. The shake flasks were double autoclaved at 121^0C for 30minutes. Once the shake flasks were cooled to room temperature, 250µl of stock culture of yeast, *C.utilis* was added and kept for incubation in shaking incubators at 200rpm and 28^0C. 2ml of the culture samples from each of the shake flask was taken aseptically every 5 hours in course of 48 hours. Optical density was determined using a spectrophotometer at 600nm wavelength.

RAS6 Sludge

Supernatant

Superanatant in shake flasks and inoculated with *C.utilis*

Figure 12 Yeast, Candida utilis growth experiments on the non-concentrated (RAS6_275) sludge.

Pilot medium Ingredient from conc. IFN02_RT and sludge RAS6_275 from LEITAT: The medium ingredient from IFN02_RT was received frozen in 5L plastic cans. The sample was thawed and distributed in to 1L plastic bottles and stored at -20^0C. The medium ingredient from RAS 6 was received frozen in smaller plastic bottles and were stored at -20^0C.


Pilot medium ingredient from RAS6 & Pilot medium ingredient from IFN02

Medium ingredients in shake flasks and inoculated with C.utilis

Figure 13 Yeast, Candida utilis growth experiments on the medium ingredient from LEITAT

Wastewater (IFN02_RT): The wastewater was received in plastic bottles and stored in -20^{0}C freezer. The samples were relatively clear without much insoluble matter. So, no further dilution or processing was needed. The samples were thawed and dispensed into Scott bottles and kept for mixing at 120rpm for 30 minutes at 4^{0}C. 50ml of this samples were added to 3 of the 250ml shake flasks, two serving as replicates and one as a control. The shake flasks were double autoclaved at 121^{0}C for 30minutes. Once the shake flasks were cooled to room temperature, 250µl of stock culture of yeast, *C.utilis* was added and kept for incubation in shaking incubators at 200rpm and 28^{0}C. 2ml of the culture samples from each of the shake flask was taken aseptically every 5 hours in course of 48 hours .Optical density was determined using a spectrophotometer at 600nm wavelength.


Waste water from IFN02

Waste water in shake flasks and inoculated with *C.utilis*

Figure 14 Yeast, Candida utilis growth experiments on the IFN02_RT wastewater.

C.utilis growth tests were performed on untreated RAS3_ABT3 sludge, IFN2_RT sludge and IFN02_RT sludge, as well as on waste which had been centrifuged to remove insoluble particles.

Results:

Experimental growth results for *C. utilis* on untreated RAS3_ABT3 sludge, IFN02_RT sludge or IFN02_RT wastewater as a source of carbon and nutrients are shown in Figure 15. The results from the growth of the microorganisms on wastes which had been centrifuged to remove insoluble particles through centrifugation are shown in Figure 16, Figure 17 and Figure 18. Figure 16 and Figure 17 show growth of *C. utilis* on different concentration of IFN02_RT sludge. Figure 18 shows growth of *C. utilis*

on RAS6_275 sludge. Experimental growth results from *C. utilis* grown on medium ingredients prepared by LEITAT are shown in Figure 19.

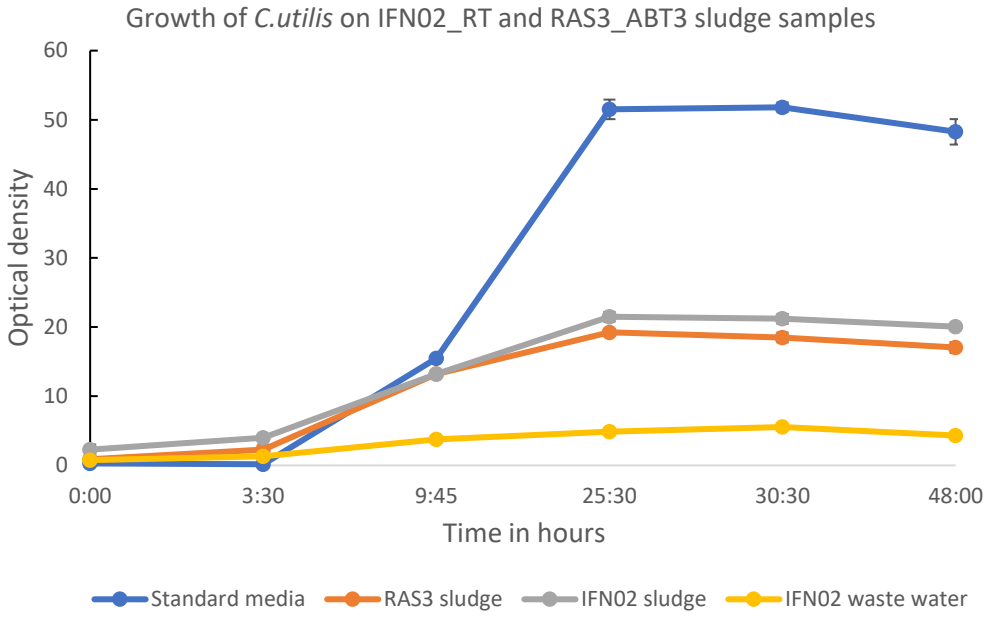


Figure 15 Growth of C. utilis on concentrate IFN02_RT and RAS3_ABT3 sludge without centrifugation step to remove the insoluble matter

There was no significant growth on any samples. So it was decided to centrifuge to remove the insoluble matter.

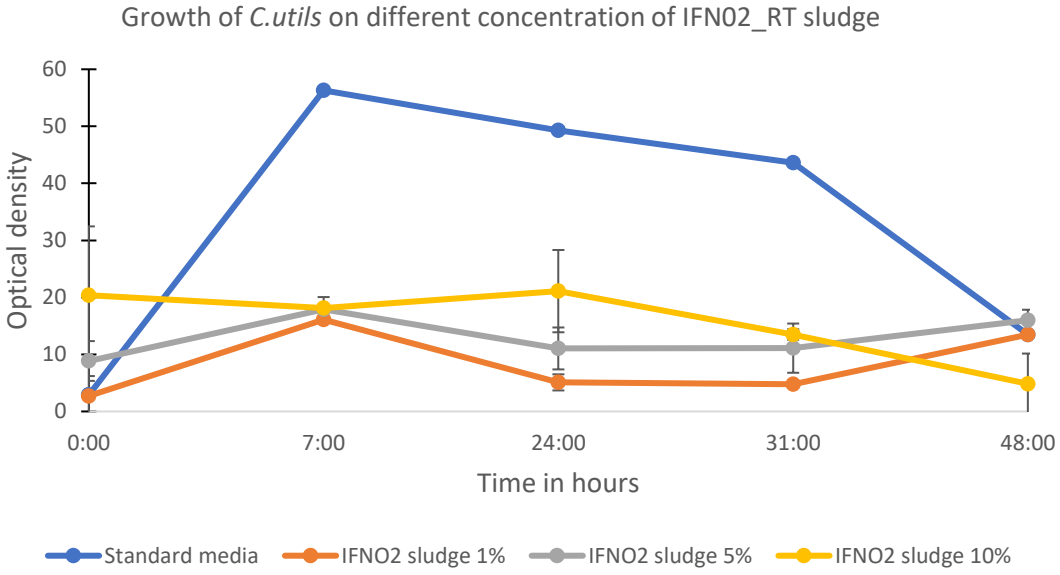


Figure 16 Growth of C. utilis on different concentration of sludge.

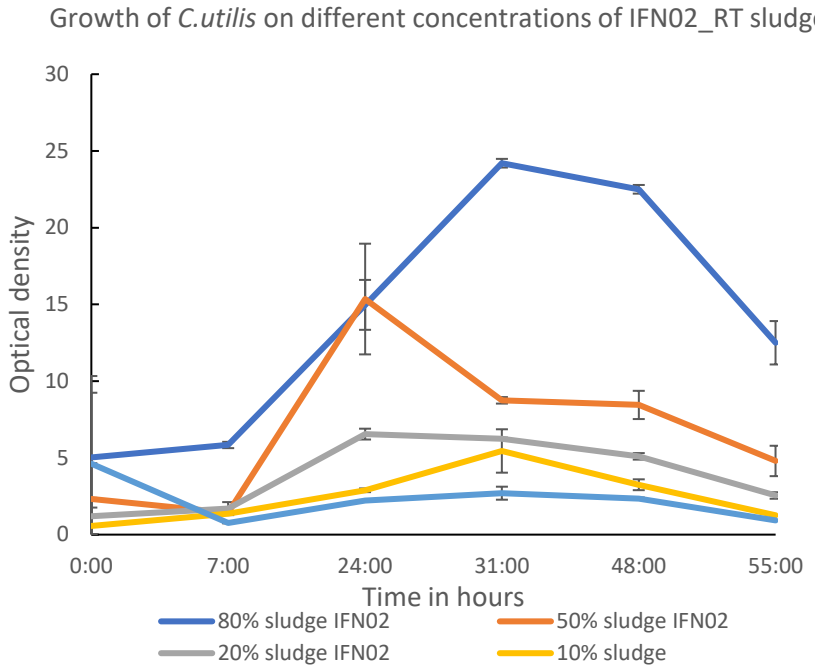


Figure 17 Growth of C.utilis on different concentration of IFN02_RT supernatant after removing the insoluble matter by centrifugation.

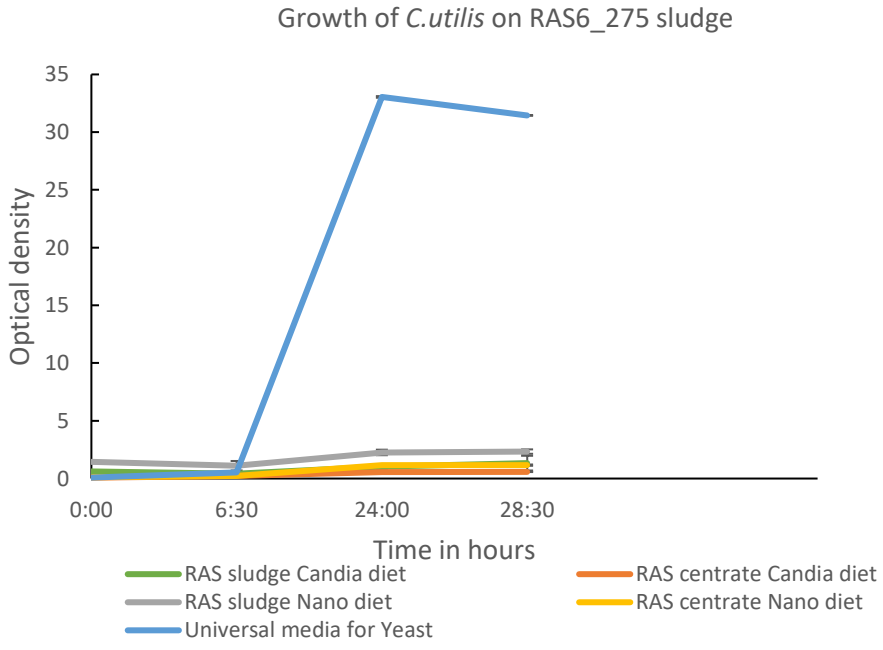


Figure 18 Growth of C. utilis on different sludge RAS6_275 (error was near zero).

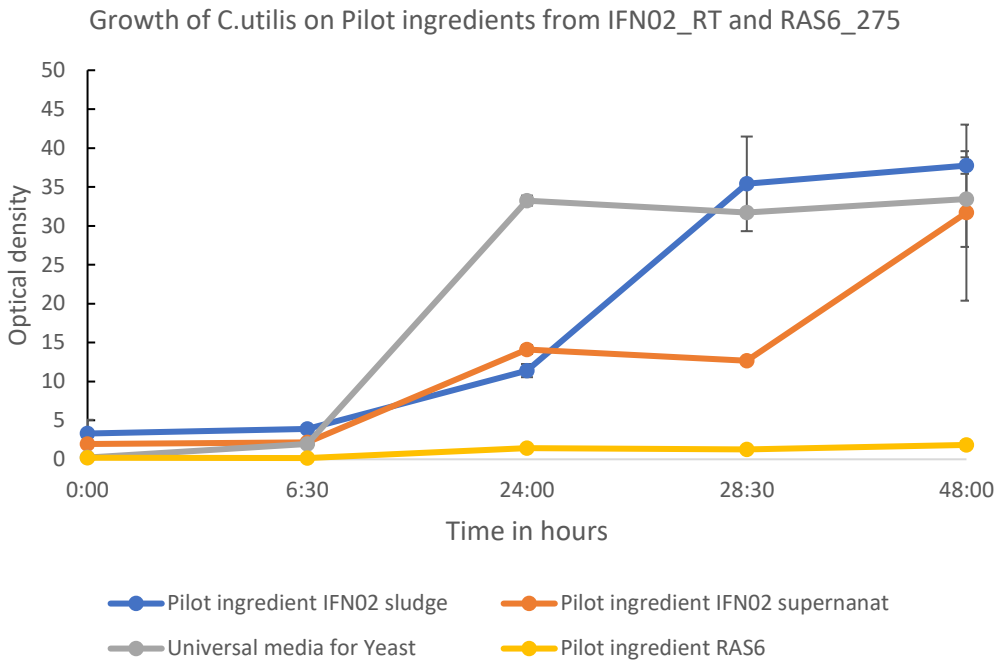


Figure 19 Growth of the medium ingredients from LEITAT.

The following observations were made:
- There was no significant growth on diluted sludge at any concentration.
- Significant growth of *C. utilis* was observed in IFN02_RT and RAS3_ABT3 supernatant from the 80% concentration but was still low in comparison to the standard media. Growth was poor in the lower concentrations of 50%, 20%,10% and 1%.
- No significant growth was observed on non-concentrated sludge from RAS6_275, either on nano or candida diet.
- No significant growth was observed on wastewater from IFN02_RT.
-There was significant growth on the Pilot ingredient from IFN02_RT but there was no growth on RAS3_ABT3.
With minimum growth observed and the scale of the experiment having remained at the laboratory scale, the full analysis of the media composition was not performed.

4.3 Experimental set up for the use of wastes as feedstock for production of other industrially relevant microorganisms

To evaluate the potential use of the different aquaculture sludges as a source of carbon, nutrient or energy source in industrial bioprocesses, promising sludges from iFishIENCi project feeding trials were selected, collected, and used as a feedstock for different type of microorganisms. Growth of the microorganisms was tested and recorded.

4.3.1 Selection of production organisms

Wastewater and sludge detailed in Table 13 were evaluated through for their potential use as feedstocks. The concentrated sludge from RAS3_ABT3, IFN02_RT and the pilot ingredient from IFN02_RT processed with enzymes were selected as the most promising potential sources of energy and nutrients based on their composition and ability to support growth of *C. utilis*. A literature search was conducted to identify a relevant list of different types of industrially relevant microorganisms with the potential to grow on several types of wastes including aquaculture sludge and fish waste. The selected microorganisms are listed in Table 14.

Table 14 Microorganisms selected for growth experiments using aquaculture sludge as a feedstock.

Industrial process	Product	Industry	Examples & Tested organisms
Biomass production	Feedstock Protein rich bacteria/yeast/fungi biomass	Fermentation industry	*Staphylococcus warneri* (fishmeal) *Pediococcus acidilactici* (fishmeal)
Lipid production	Lipids OM3	Feed / Food	*Enterococcus faecium* (lipid and proteins)
Production of enzymes	Oxireductases Proteases Lipases Invertases	Food Pharmaceuticals Chemicals and Detergents	*Yarrowia lipolytica* (fungus) (lipases) *Aspergillus ninger* (fungus) (amylases, pectinases, proteases) *Bacillus licheniformis* (proteases)
Production of useful microorganisms	Probiotics Bacteria/yeast/fungi	Food Pharmaceuticals Biorremediation	*Vibrio natriegens* (probiotics) *Bacillus licheniformis* *Bacillus pumilis* *Bacillus cereus*
Production of chemicals	Biopolymers Acetate Acetone Biofuels Ethanol Lactic acid Vitamins Amino acids Antibiotic	Feed / Food Chemicals Pharmaceuticals	*Pseudomonas oleovorans* (surfactants) *Pseudomonas oleovorans* (bioplastics - PHA) *Bacillus subtilis* (bioplastics - PHB) *Corynebacterium glutamicum* (amino acids)

4.3.2 Medium composition

IFN02_RT and RAS3_ABT3 sludge:

Sludge samples were collected from the -20^{0}C freezer where they had been stored after collection and weighed into a Scott bottle. Distilled water was added to a final concentration of 80% and left shaking on an orbital shaker for 1 hour at 4^{0}C.

The diluted sludge was centrifuged at 4500rpm for 15 minutes at 4^{o}c. The supernatant was collected. 5 ml of the supernatant was dispensed into 10ml test tubes and autoclaved at 121^{0}C for 30 minutes. The autoclavation was repeated to ensure inactivation of all potential spores present in the sample.

IFN02_RT sludge treated with enzymes:

Samples were collected from the -20^{0}C freezer and kept in the cooling room until completely thawed and 5 ml were dispensed into 10ml test tubes and autoclaved twice at 121^{0}C for 30 minutes.

4.3.3 Microorganisms´ preliminary growth tests

Preliminary growth tests were conducted to select the best microorganisms. 30µl of stock cultures from the -80ºC freezer were used to inoculate the sludge after which they were left to grow at room temperature for at least 48 hours. Growth was recorded by visual observation to select the best candidate for further growth testing. Growth was difficult to visualize due to cloudy nature of the sludge. The results are shown in Table 15. The microorganisms that showed some growth on the tubes or the were chosen to test further for the quantitative growth tests.

Table 15 Growth of the different microorganisms on the 3 types of sludge. (+: growth observed, -: no growth observed)

No	Micoorganism	Standard media	Growth on RAS3_ABT3	Growth on IFN02_RT	Growth on IFN02_ENZ
1	*E.gallinarum*	Trypticase soy yeast extract media	+	-	+
2	*S.warneri*	Trypticase soy yeast extract media	+	-	+
3	*B.licheniformis*	Nutrient agar	-	+	+
4	*S.agalacticiae*	Trypticase soy yeast extract media	+	-	+
5	*E.faceium*	Trypticase soy yeast extract media	+	-	+
6	*Y.lipolytica*	YPD media	+	+	+
7	*P.olevorans*	Nutrient agar	-	-	+
8	*C.glutamicum*	Trypicase soy agar	+	-	+
9	*P.acidilacticii*	MRS medium	-	+	+
10	*B.subtilis*	Nutrient agar	-	+	+
11	*B.pumilis*	Nutrient agar	-	-	+
12	*A.niger*	Potato dextrose Agar	+	+	+
13	*B.cerus*	Nutrient agar	-	-	+
14	*V.natrigens*	*Vibrio agar*	-	-	+

4.3.4 Quantitative growth tests

80% sludge was prepared using RAS3_ABT3, IFN02_RT and INF02_ENZ and double autoclaved as described in the preliminary test.

Inoculation:

Inoculated 30µl of stock cultures from the -80ºC freezer. Incubated at ambient temperature for at least 48hours. Observed for growth and serially dilute the contents of the tubes.

Serial dilutions:

After visual observation, serially diluted the cultures to get a countable number of Colony Forming Units (CFU) per plate.

Procedure:

Dispensed 900µl of sterile 0.9% sterile saline to sterile Eppendorf tubes. Dispensed 100 µl of the homogenized culture from the culture tube and vortex for 5 seconds. Took out 100 µl of the contents of this tube into another Eppendorf tube with 900 µl of saline. Repeated this four times to achieve 10^1, 10^2,10^3 and 10^4 dilutions. Placed 100 µl from each of the tubes to agar plates with standard media for each of the microorganism. Incubated for at least 48hrs at ambient temperature for each microorganism. Observed growth and count the colonies on the plates that show most countable CFU. After 48 hours incubation the colonies were counted, and results are shown in Table 16.

Table 16 Growth of the different microorganisms on the 3 most promising sludge samples.

Microorganism	Growth RAS3_ABT3 (microorganisms per ml)	Growth IFN03_AC (microorganisms per ml)	Growth IFN02_Enzy (microorganisms per ml)
E.gallinarum	$8.5x10^5$	NA	NA
S.warneri	$4.5x10^6$	NA	NA
B.licheniformis	NA	$1.8x10^8$	$5.4x10^7$
S.agalacticiae	750	NA	NA
E.faceium	$6.5x10^7$	NA	NA
Y.lipolytica	$8.9x10^9$	$1.5x10^7$	$4.1x10^5$
P.olevorans	NA	NA	NA
C.glutamicum	$2.6x10^5$	NA	NA
P.acidilacticii	1.5x108	NA	$5.6x10^7$
B.subtilis	NA	**250**	NA
B.pumilis	NA	poor	NA
A.niger	Matt	Matt	Matt
B.cerus	NA	NA	NA
V.natrigens	NA	NA	NA

4.4 Conclusions

The wastewater and the sludge from the different feed trials, including feed trials performed with novel feed ingredients were investigated as a source of carbon and/or nutrient for the growth of *Candida utilis* yeast. The medium ingredient obtained from Chapter 5 was also used in the valorisation trials. The total organic carbon content was the initial imperative parameter for the valorization experiments carried out with various sludge and wastewater streams.

The yeast could not grow in wastewater obtained from the drum filter after filtration with 60µm. There was significant growth in the media ingredient produced from concentrated sludge using the enzymatic treatment mentioned in the Chapter 5. But no significant growth in the media ingredient produced using the chemical treatment from more diluted waste stream.

These results suggested that the nutrient concentration in these sludges is likely not high enough to support significant growth of *C. utilis*. It is also likely that the compounds present in the sludges are not easily degraded by the candida to used successfully as a carbon and energy source to support growth. However, following hydrolysis of the sludge described in Chapter 5, the nutrients were made available to be used as a carbon and energy source by the microorganisms, making it a potentially viable feedstock for their production and it was concluded that

- The total organic carbon was the limiting factor in the valorization.
- The concentrated sludge required to be diluted to no more than 80%.
- It was crucial to centrifuge the diluted sludge to eliminate the insoluble material.
- The diluted sludge needs to be double autoclaved to kill all the microorganisms and spores.

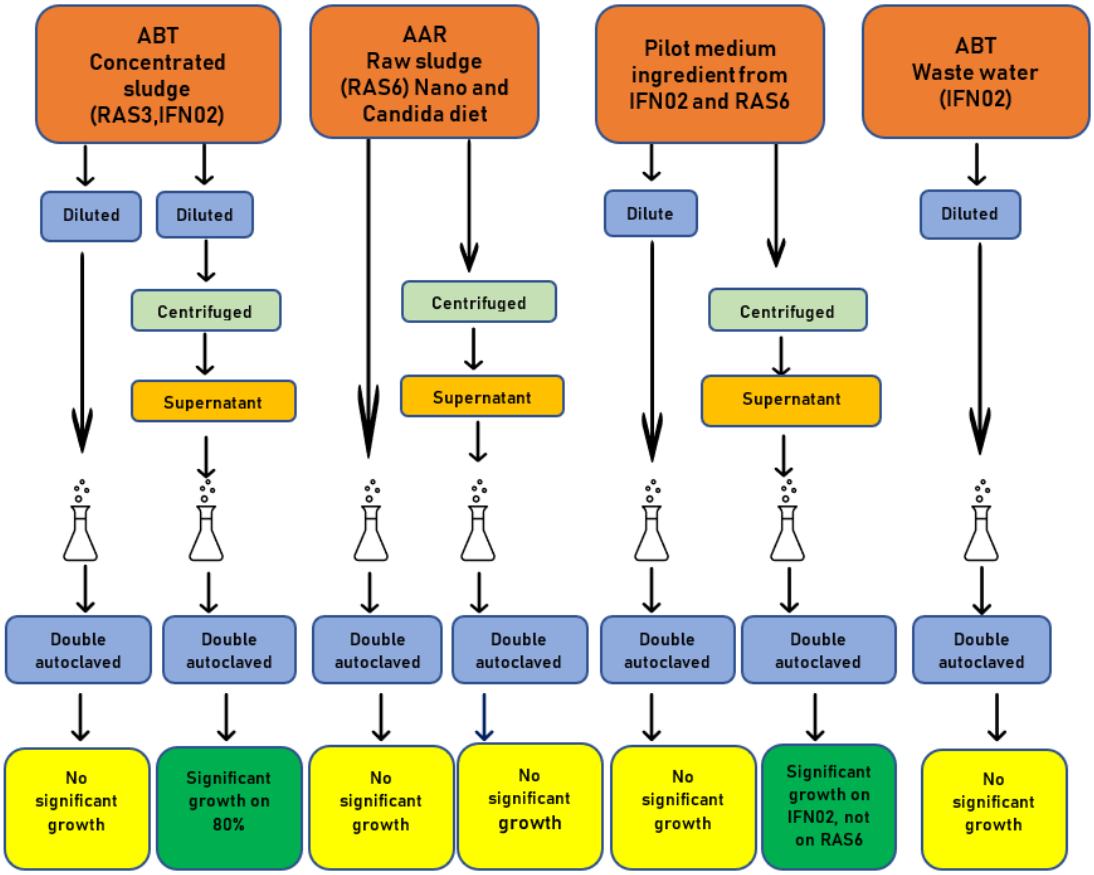


Figure 20 Overview of performance of yeast (Candida utilis) on the various waste streams from fish-farming.

Waste streams from RAS system have the potential to be re-used and valorised under a circular approach to produce new feeds and towards zero-waste. The ability for selected industrially relevant production organisms to use different sludges as a feedstock in bioproduction was further investigated. RAS3_ABT3 was found to support the growth *of E. glutamicum, S.warneri, E.faceum, Y.lipolytica* and *A. niger* and are the best candidates out of the 14 strains tested. IFN02supported the growth of *B.licheniformis, Y.lipolytica* and *A. niger* and are the best candidates out of the 14 strains tested. Samples of treated IFN02_RT (medium ingtedient) supported the growth of *B.licheniformis, Y.lipolytica, P.acidilactici* and *A. niger* and are the best candidates out of the 14 strains tested. *S.agalacticiae* had a weak growth on RAS3_ABT3 and *B. subtilis* on IFN03_03. They can be considered for further upscaling with some added nutrients.

Based on the composition of the different waste streams, industrially relevant microorganisms potentially able to use them as a source of carbon, nutrients and energy were identified and small-scale growth experiments were conducted. The results show that the sludge samples tested can support the growth on many organisms and could potentially be used as a feedstock in the industrial production of fishmeal, enzymes, or chemicals to develop new sustainable processes in different value chains.

5 Sludge bioconversion into nutrients for algae and yeast feedstock

5.1 Introduction

Nutrients in sludge are bound to solids but some treatments can enhance release nutrients into the water. Few studies have attempted to extracts nutrients from shrimp pond bottom sediments, mainly nitrogen (Yusoff, 2001). In this context, the sludge bioconversion into nutrients was investigated in iFishIENCi under the *Task 1.5.3 Proof-of-concept 3. Sludge valorisation into algae nutrients* by LEITAT. **The novelty of the approach is to valorise sludge into soluble nutrients (nitrogen, phosphorus, carbon) so that they can be taken up by microalgae and yeast.** The final achievement was the nutrients recovery, mainly, in the form of medium ingredient (culture medium, MI) to grow microalgae and yeast. LEITAT investigated nutrient recovery from sludge through screenings for pre-treatments, enzymatic and chemical hydrolysis, optimization of experimental designs, production of promising ingredients, pilot-scale validation, and ultimately physico-chemical characterisation of the medium ingredient for microalgae and yeast production.

5.2 Experimental development for sludge valorisation

A methodology was designed to recover nutrients from RAS system (15-20% dry matter, recovered using 60 µm filtration) (RAS3_ABT3), fish trial with rainbow trout and conventional feed) (Figure 21). The concentrated sludge was mixed with water. This was followed by the pre-treatment of the sludge and the enzymatic hydrolysis. Finally, the sample was centrifuged and filtered (43-48µm filter) to separate the undissolved particles from the liquid fraction. Most undissolved material would not be available for the microalgae; this would block the light for the microalgae and would impede the growth. Finally, the solid fraction was washed with water and then filtered to recover the remaining soluble nutrients that might have had left. Finally, the liquid fraction and the washing water were joined to recover the soluble nutrients (medium ingredient, MI).

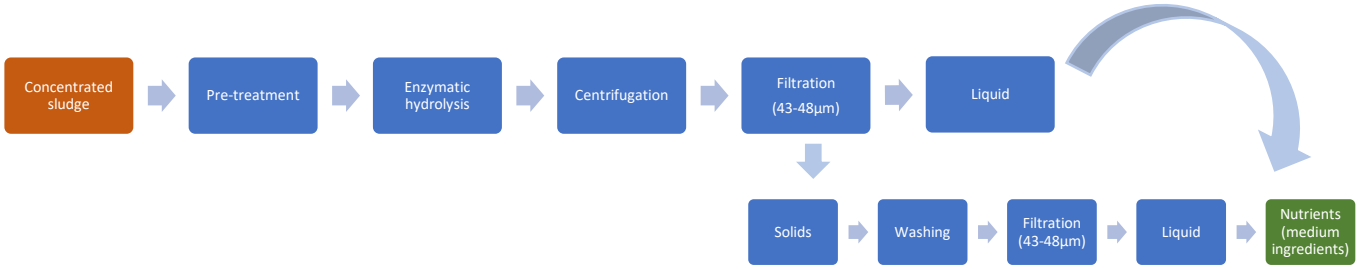


Figure 21 General diagram of sludge bioconversion process into nutrients (medium ingredients)

5.2.1 Optimisation (Response Surface Design)

iFishIENCi explored different screenings for pre-treatments (ultrasounds, thermal, chemical) to disintegrate and hydrolyse the concentrated sludge from RAS system (15-20% dry matter, recovered

using 60 µm filtration) and solubilise nitrogen. As a result of the screening, **ultrasounds were selected as the most promising pre-treatment to be further optimised in combination with enzymes.**

Next, the methodology was optimised to maximise the nutrient recovery from concentrated sludge using the response surface methodology (RSM) central composite by combining the ultrasounds and proteases (A or B). A four-factor design was used to study the interaction of factors simultaneously in a minimum number of trials. The factors included 2 categorical variables (Ultrasounds Assisted Extraction) as pre-treatment (UAE) (Yes/No) and type of enzyme (A/B)), and 2 numerical variables: dose of protease (0-2% dw) and time of enzymatic hydrolysis reaction (0-6h). As a response parameter, Total Kjeldahl Nitrogen (TKN) was analysed as a representative nutrient parameter. Furthermore, nitrogen is the main nutrient in the biorecovery cycle for the circularity assessment (WP 4). The design was analysed by the software Design Expert v.13.0.

Medium ingredients were analysed for nitrogen content as TKN and expressed in mg/L, fresh weight. The response was the Nitrogen recovery yield (%) expressed as Nitrogen (grams) in the medium ingredient expressed on the initial content of Nitrogen in the sludge (grams).

$$\text{Nitrogen recovery yield (TKN) (\%)} = \frac{\text{N (g) in medium ingredient}}{\text{Nitrogen (g) in sludge}} \times 100$$

Results

The TKN extraction yield (%) results were statistically analysed using the Design Expert v13 program. An approximate a linear model was given, statistically significant with a p-value 0.0001 and a confidence level α = 0.05 (

Figure 22). The ANOVA statistical analysis indicated that the variables of time, concentration enzyme and UAE pre-treatment had a significant effect on the TKN extraction yield response, with a p-value <0.05. In contrast, the type of protease (Protease A/Protease B) was found non significant.

ANOVA for Quadratic model

Response 1: TKN yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1000,65	12	83,39	7,72	< 0.0001	significant
A-Time	161,57	1	161,57	14,95	0,0005	
B-Concentration	247,57	1	247,57	22,91	< 0.0001	
C-Type enzyme	44,81	1	44,81	4,15	0,0503	
D-Pretreatment	417,06	1	417,06	38,60	< 0.0001	
AB	2,84	1	2,84	0,2624	0,6121	
AC	10,90	1	10,90	1,01	0,3229	
AD	0,1732	1	0,1732	0,0160	0,9001	
BC	23,14	1	23,14	2,14	0,1534	
BD	0,6067	1	0,6067	0,0561	0,8142	
CD	0,2836	1	0,2836	0,0262	0,8723	
A^2	78,49	1	78,49	7,26	0,0113	
B^2	89,30	1	89,30	8,26	0,0072	
Residual	334,95	31	10,80			
Lack of Fit	298,89	23	13,00	2,88	0,0624	not significant
Pure Error	36,06	8	4,51			
Cor Total	1335,60	43				

Figure 22 ANOVA results of nitrogen recovery yield (TKN) (%) from concentrated sludge recovered after 60µm filtration (20% dry matter) from RAS3_ABT3, fish trial with rainbow trout and conventional feed, and the interaction of variables Time (A), Concentration (B), Type enzyme (C), and Time Pretreatment (D)

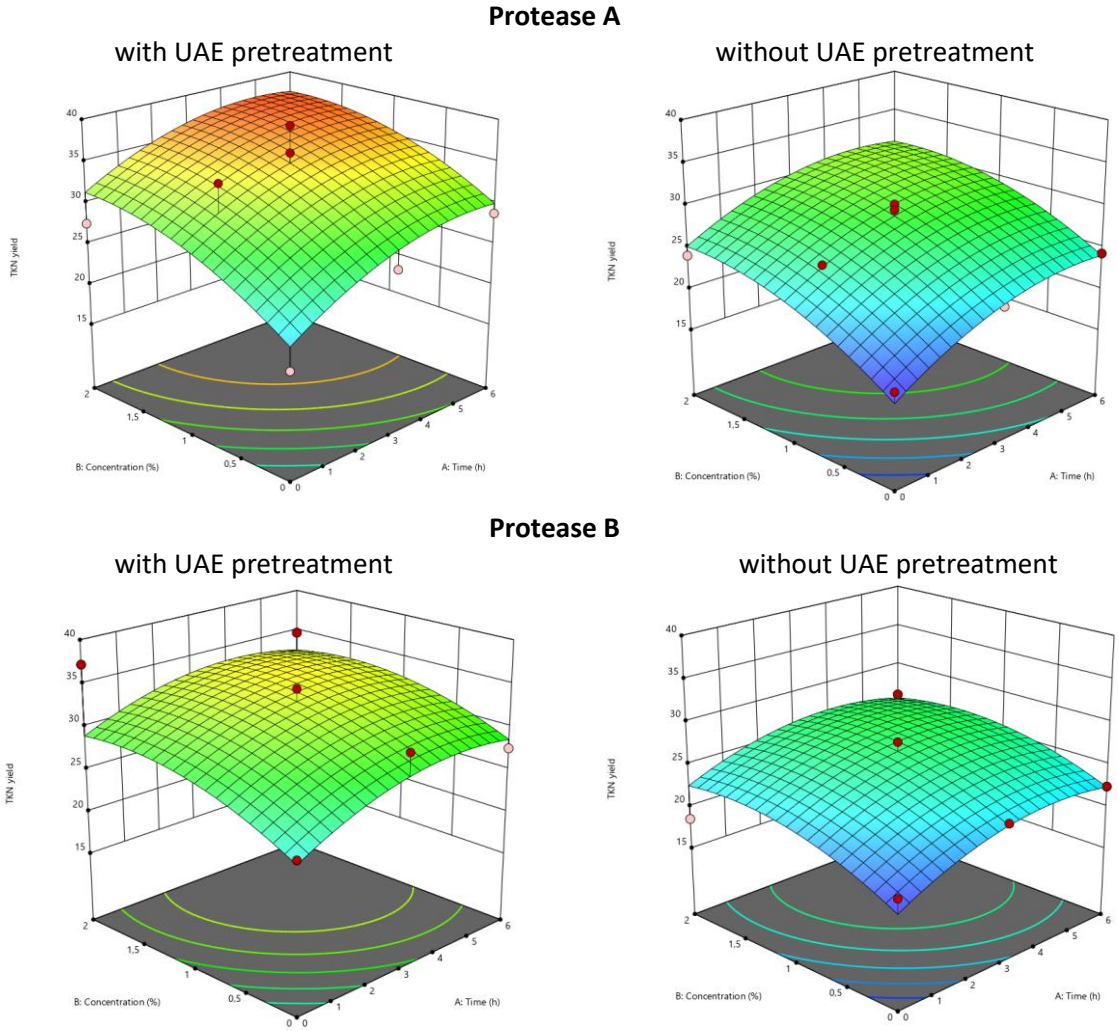


Figure 23 Analysis of Surface response of nitrogen recovery yield (TKN) (%) from concentrated sludge recovered after 60µm filtration (20% dry matter) from RAS3_ABT3, fish trial with rainbow trout and conventional feed, and the interaction of variables time, and the interaction of variables time, and enzyme dose. 3DSurface response models: Top 3D graphs with enzyme Pro A, bottom graphs with enzyme Pro B. Left graphs with UAE pre-treatment, right graphs without UAE pre-treatment.

The numerical optimisation through statistical modelling with the Design Expert v13 program was used to determine the optimal conditions under the criterion of "Maximum TKN extraction yield" and the optimal solutions were given. Based on the numerical optimisation, the optimal conditions to maximise the TKN extraction were found with the three following solutions:

-Opt1: 30 minutes UAE pre-treatment, 1.9% protease Pro A for 4.9h. Expected TKN yield 38%

-Opt2: 30 minutes UAE pre-treatment, 1.5% protease Pro B for 4.2h. Expected TKN yield 34%

-Opt3: without UAE pre-treatment, 1.8% protease Pro A for 4.8h. Expected TKN yield 31%

5.2.2 Proof of concept. Nutrient recovery yield

Preliminary ingredients were produced from concentrated and raw sludge from ABT and from raw sludge from AA (see Figure 24). Proof of concept (pilot validation) was done with conc.sludge (IFN02_RT) by applying ultrasounds followed by enzymatic hydrolysis (endo and exoprotease, Pro A and also Pro D, respectively) and also chemical hydrolysis (KOH). Results for algae and yeast growth are reported in Chapter 3 and 4.

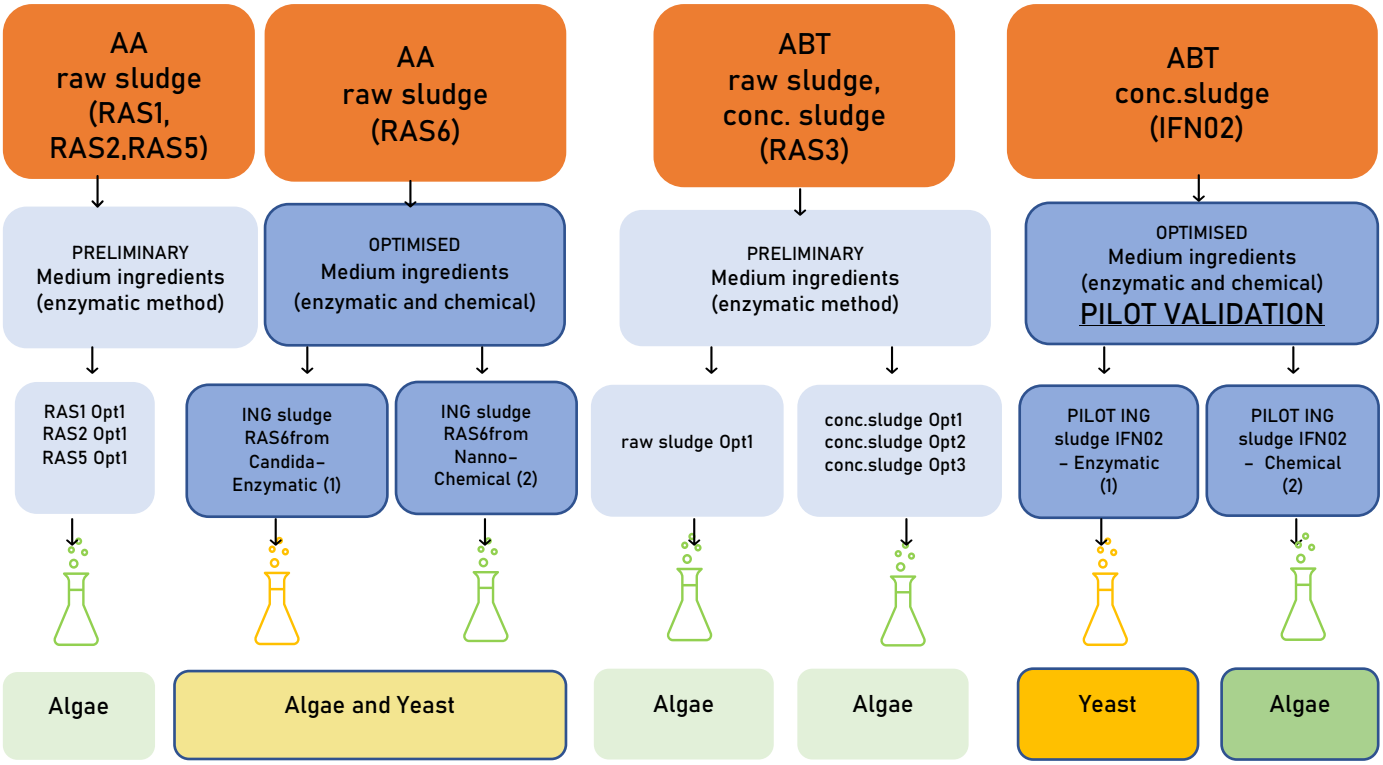


Figure 24 Summary of the medium ingredients (MI) obtained by LEITAT and delivered to NORCE for testing microalgae and yeast growth. In dark colour, the optimised medium ingredients. Conc.sludge from ABT: concentrated sludge using 60 micron filter, 15-20% dry matter. Sludge from AA: sludge from faeces collector, 0.5-4.3% dry matter

5.2.2.1 Medium ingredients from RAS System - ABT

The objective of this study was to validate at pilot scale the methodology previously developed and optimised at lab scale for the nutrients recovery (nitrogen, phosphorus, carbon) from concentrated RAS sludge (fish trial IFN02_RT) to growth microalgae and yeast.

Methodology

The concentrated sludge IFN02_RT, recovered after 60µm filtration (15% dry matter) from RAS (fish trial with rainbow trout and conventional feed), was weighed and diluted with distilled water (1:1) (final pH 5.32), and homogenised manually. Next, the samples were submitted to ultrasounds (Ultrasonic Processor HIELSCHER model UIP1000HD at 20Khz with 65% amplitude) for 30 min in batches (1kg sludge: 1 kg water). Next, two different hydrolytic processes were applied:

Process 1: enzymatic treatment with 1.9% Pro A (endoprotease) and 1.9% pro D (exoprotease) and the samples were placed in a thermal bath at 65ºC, under agitation at 70 rpm for 5 hours. Next, the enzymes were inactivated at 90ºC for 10 minutes.

Process 2: chemical treatment (KOH pH12) at 65ºC in thermal bath for 5h and placed in a thermal bath at 65ºC, under agitation at 70 rpm for 5 hours.

After the reaction, all samples were centrifuged 7500 rpm 10 min, and filtered at 43-48µm to obtain the liquid fraction. Next, the solid fraction was washed with water 1:1 to recover the remaining soluble nutrients that might have and then filtered to recover the washing water. The washing water and the liquid fraction were joined and referred it as medium ingredient (nutrients). The solids were also recovered as final residue to be analysed and assessed as potential fertilizer.

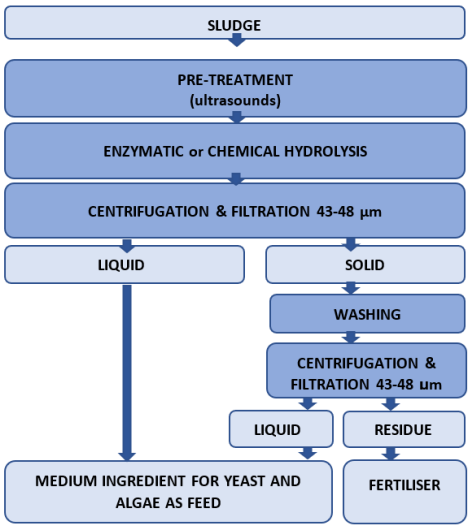


Figure 25 Optimised sludge bioconversion process into nutrients (medium ingredients) at pilot scale by applying ultrasound pre-treatment followed by enzymatic hydrolysis or chemical hydrolysis from concentrated sludge IFN02_RT, fish trial with rainbow trout and conventional feed.

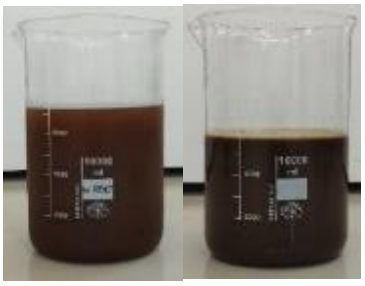

(1)



(2)


(3)


(4)


(5)

(6)

Figure 26 Medium ingredients obtained by applying ultrasound pre-treatment followed by enzymatic hydrolysis or chemical hydrolysis from concentrated sludge IFN02_RT, fish trial with rainbow trout and conventional feed. (1) Ultrasonic Processor HIELSCHER model UIP1000HD. (2): Hydrolysis reactor and tank. (3) Sludge after the enzymatic reaction and centrifugation. (4) Solid washing and filtration. Left: ING sludge IFN02_RT -Enzymatic (process 1); Right: ING sludge IFN02_RT-Chemical (process 2). (5) Left: ING sludge IFN02_RT_RT-Enzymatic (process 1); Right: ING sludge IFN02_RT - Chemical (process 2). (6) From left to right: PILOT ING sludge IFN02_RT-Enzymatic (process 1) and final residue, PILOT ING sludge IFN02_RT-Chemical (process 2) and final residue

The Table 17 shows the physicochemical characterisation of the medium ingredients obtained at pilot scale (proof of concept), including the characterisation of the final residue on fresh weight. Moisture, dry matter and ashes were analysed by gravimetry. TKN was analysed by kjeldahl method and NH_4^+-N, NO_2^--N and NO_3^--N by ion chromatography. Table 18 shows the free amino acids in the medium ingredients (HPL-DAD), Table 19 shows the nutritive/pollutant elements (ICP-MS) and Table 20 shows the microbiology results. For samples that were examined in duplicate (n=2), a deviation is provided.

Table 17 Physicochemical characterisation of the medium ingredients obtained from concentrated sludge IFN02_RT by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) and chemical hydrolysis (Process 2). Pilot validation.

Parameter	PILOT ING sludge IFN02_RT – Enzymatic (1)		PILOT ING sludge IFN02_RT – Chemical (2)	
	Medium ingredient	Residue	Medium ingredient	Residue
pH	5.4±0.0	5.8±0.0 (1)	9.9±0.0	9.9±0.1 (1)
Conductivity (mS/cm)	5.2±0.0	0.8±0.0 (1)	9.7±0.0	1.2±1.0 (1)
Moisture (%)	97.8±0.0	66.5±0.4	97.1±0.3	69.5±2.1
Dry matter (%)	2.2±0.0	33.5±0.4	2.9±0.3	30.5±2.1
Ashes (%)	0.3±0.0	10.0±0.3	0.9±0.1	12.3±2.0
TKN-N (mg/kg)	1300±14	11780±495	1510±28	6705±177
Free amino acids (mg/kg)	3721	n.a.	2401	n.a.
NH_4^+-N (mg/kg)	602	n.a.	665	n.a.
NO_2^--N (mg/kg)	LD <2.5	n.a.	LD <2.5	n.a.
NO_3^--N (mg/kg)	LD <22	n.a.	34	n.a.
Total N (mg/kg) (2)	1300	n.a.	1544	n.a.
Organic N (mg/kg) (3)	698	n.a.	845	n.a.
Inorganic N (mg/kg) (4)	602	n.a.	699	n.a.
Organic C (TOC) (mg/kg)	3760	n.a.	9880	n.a.
Phosphate (PO_4^{3-}) (mg/kg)	409	n.a.	65600	n.a.
Sulphate (SO_4^{2-}) (%)	LD <0.05	n.a.	LD <0.05	n.a.
Chlorides (Cl^-) (%)	LD <0.05	n.a.	LD <0.05	n.a.
Fats (%)	<0.1	n.a.	0.10	n.a.

(1) pH and conductivity, sample preparation: the solid sample was mixed with distilled water (1:2.5), centrifuged at 10.000 rpm for 15 min. Next, the supernatant was analysed
(2) Total Nitrogen calculated as the sum of TKN + NO_2^--N + NO_3^--N
(3) Organic Nitrogen calculated as the difference between TKN and NH_4^+-N
(4) Inorganic Nitrogen calculated as the sum of NH_4^+-N + NO_2^--N + NO_3^-N

Medium ingredients, were rich in carbon specially the one produce by chemical hydrolysis with values of 9880 mg/kg on fresh weight. Total nitrogen, ranged 1300-1510 mg/kg, with values up to 34 mg/kg of nitrates for the chemical process. Phosphate was 409-65600 mg/kg phosphate, the highest value from the chemical process. Remaining nutrients were also found in the residue.

Table 18 Free amino acids in the medium ingredients obtained from concentrated sludge IFN02_RT by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) and chemical hydrolysis (Process 2). Pilot validation.

Amino acid (mg/kg)	PILOT ING sludge IFN02_RT – Enzymatic (1)	PILOT ING sludge IFN02_RT – Chemical (2)
ASP	188	165
GLU	783	418
ASN	n.d.	n.d.
SER	224	151
GLN	n.d.	n.d.
HYS	57	21
GLY	207	125
THR	256	167
ARG	63	17
ALA	374	257
TYR	86	16
CYS-CYS	10	2
VAL	332	226
MET	97	25
TRP	n.d.	n.d.
PHE	226	169
ILE	249	183
HYP	n.d.	n.d.
LEU	395	291
LYS	60	46
PRO	112	122
Total	3721	2401

n.d. not detected

Total amino acids ranged 3721-2401 mg/kg on fresh weight, glutamic acid the most abundant.

Table 19 Nutritive/pollutant elements in the medium ingredients obtained from concentrated sludge IFN02_RT by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) or chemical hydrolysis (Process 2). Pilot validation.

Element (mg/kg)	PILOT ING sludge IFN02_RT Enzymatic (1)		PILOT ING sludge IFN02_RT Chemical (2)	
	Medium ingredient	Residue	Medium ingredient	Residue
Na	257±7	555±6	219±9	702±12
Mg	97±3	716±34	48±1	1002±27
P	118±9	18846±797	282±5	18426±682
S	LD<1000	1855±29	LD< 1000	1503±371
K	431±10	401±12	4350±12	4700±30
Ca	817±48	39517±1022	563±28	42536±1167
Cr	LD<0.1	2.5±0.1	LD 0.1	2.4±0.6
Mn	0. 4±0.0	91±3	1.5±0.0	85±4
Fe	2.5±0.7	679±42	32.0±0.3	472±7
Co	LD<0.1	0.3±0.0	LD<0.1	0.3±0.0
Ni	LD<0.1	2.1±0.1	LD<0.1	1.5±0.3
Cu	LD<0.1	10±1	0.9±0.0	3.7±0.0
Zn	0.8±0.3	226±14	8.6±0.0	163±0.0

As	LD<0.1	0.5±0.1	LD<0.1	0.3±0.0
Se	LD<0.1	1.5±0.1	LD<0.1	0.9±0.1
Mo	LD<0.1	0.60±0.04	LD<0.1	0.2±0.0
Cd	LD<0.1	1.1±0.0	LD<0.1	0.8±0.0
Pb	LD<0.1	0.7±0.1	LD<0.1	0.5±0.0

Medium ingredients contained nutrients such as sodium, calcium. Pollutant elements were not detected or they were under 9 mg/kg (i.e. Zn). Remaining nutrients and pollutants (cadmium) were found in the residue.

Table 20 Microbiology in the medium ingredients obtained from concentrated sludge IFN02_RT by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) or chemical hydrolysis (Process 2). Pilot validation.

Microorganism	**PILOT ING sludge IFN02_RT – Enzymatic (1)**		**PILOT ING sludge IFN02_RT– Chemical (2)**	
	Medium	**Residue**	**Medium**	**Residue**
Salmonella spp in 25 g	absent	absent	absent	absent
E.coli (CFU/ g)	<10	<10	<10	<10
Enterobacteriaceae count (CFU/g)	<10	<10	<10	<10
Total Aerobic count (CFU/g)	6.7×10^2	2.4×10^4	1.2×10^2	5.2×10^4
Total Fungi and yeast count (CFU/g)	<1	160	1	50

n.a. not analysed
Rapid detection method Salmonella. Rapid Salmonella. According to UNE EN ISO 16140
ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E.coli
ISO 21528:2017 Horizontal method for the detection and enumeration of Enterobacteriaceae. Part 2: Colony-count
ISO 4833-2:2014 Colony count at 30ºC by surface plating techniques
ISO 21527:2008 Horizontal method for the enumeration of yeast an moulds

Salmonella and E.coli were not detected (under 10 CFU/g).

The nutrient recovery yield was calculated as the nutrient content in the total volume recovered of the medium ingredient and expressed on the nutrient content in total sludge added in the process and expressed as percentage (see Figure 27).

Nutrient recovery yield (%) = $\frac{\text{Nutrient (g) in medium ingredient}}{\text{Nutrient (g) in sludge}} \times 100$

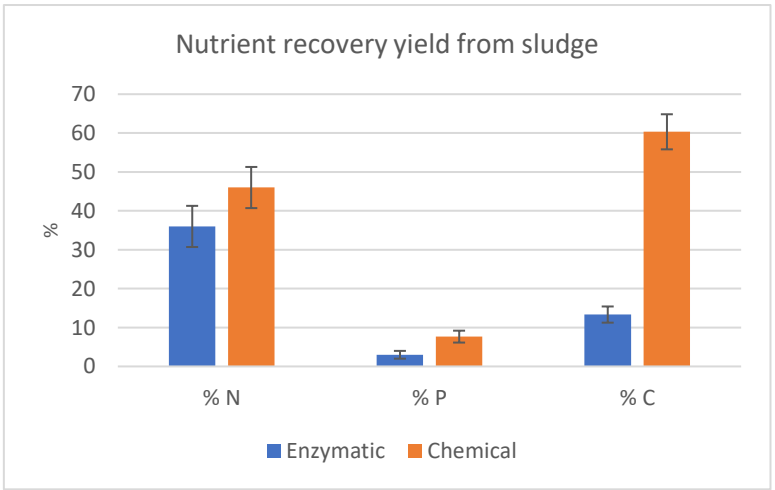


Figure 27 Nutrient recovery yield obtained from concentrated sludge IFN02_RT by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) and chemical hydrolysis (Process 2) at pilot scale (pilot validation). Results expressed as percentage. Bar graph illustrating the mean and standard deviation (error bars) with n=3.

The processes proved the following nutrients recovery yield:

- **Process 1.** Ultrasounds 30 min at 20Khz with 65% amplitude followed by enzymatic treatment with 1.9% Pro A (endoprotease) and 1.9% pro D (exoprotease) at 65ºC for 5h, under agitation, achieving a recovery yield of **36% total nitrogen, 3% total phosphorus and 13% total organic carbon.**
- **Process 2.** Ultrasounds 30 min at 20Khz with 65% amplitude followed by chemical treatment with KOH 4M at pH 12 at 65ºC for 5h, under agitation, achieving a recovery yield of **46% total nitrogen, 8% total phosphorus and 60% total organic carbon.**

The p-value corresponding to the F-statistic of one-way ANOVA for the recovery of nitrogen was 0.0816, higher than 0.05, suggesting that the treatments were not significantly different for that level of significance. The p-value corresponding to the F-statistic of one-way ANOVA for the recovery of phosphorus was 0.0114, lower than 0.05 suggesting that treatments were significantly different. The p-value corresponding to the F-statistic of one-way ANOVA for the recovery of carbon was <0.01, lower than 0.05 suggesting that treatments were significantly different. So, both processes recovered similar amount of nitrogen but the chemical treatment recovered more phosphorus and carbon.

The medium ingredients were shipped to NORCE to test yeast growth and microalgae. These results are described in Chapters 3 and 4.

5.2.2.2 Medium ingredients from RAS system - AA

Firstly, it is worth mentioning that sludge from faeces collector from AA (0.4-5% dry matter) was a very different type of waste in comparison to the concentrated sludge from ABT (15-20% dry matter), because it wasn't concentrated. The objective of this study was to replicate at lab scale the methodology previously developed on concentrated sludge from ABT for the nutrients recovery (nitrogen, phosphorus, carbon) in raw RAS sludge (fish trial RAS6_275). Although the majority of the nutrients were already soluble and available for microalgae and yeast, it was worthwhile to determine whether the raw sludge could be processed to solubilize nutrients that were bound in particles.

Methodology

The optimised methodology for concentrated sludge described in section 5.2.2 was adapted to raw sludge. In this case, no water was added since the sludge already contained enough water to solubilise enzymes. Next, the samples were submitted to ultrasounds (Ultrasonic Processor Sonics model VCX 750 at 20 Khz with 65% amplitude for 30 minutes in batches (200mL). Next, two different hydrolytic processes were applied:

For Process 1: the sample Candida diet pH was adjusted to pH 7.06 with sulfuric acid, next Enzymatic treatment with 1.9% Pro A (endoprotease) and 1.9% pro D (exoprotease) (pH 7.06) and finally, the samples were placed in a thermal bath at 65ºC, under agitation at 70 rpm for 5 hours. Next, the enzymes were inactivated at 90ºC for 10 minutes.

For Process 2: the sample Nanno diet 1 was adjusted to pH 12 with 4M KOH and placed in a thermal bath at 65ºC, under agitation at 70 rpm for 5 hours.

After the reaction, all samples were filtered at 43-48µm to obtain the liquid fraction. Next, the solid fraction was not washed to avoid further dilution of the medium ingredient but recovered as final residue.

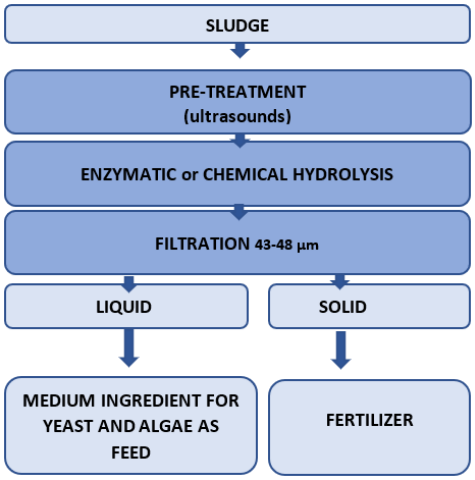
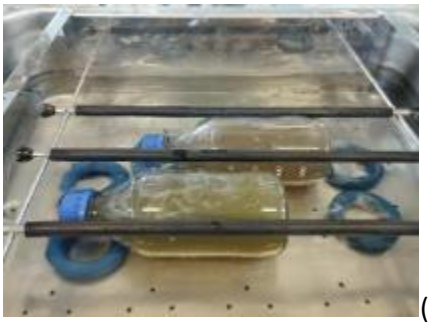


Figure 28 Sludge bioconversion process into nutrients (medium ingredients) at lab scale by applying ultrasound pre-treatment followed by enzymatic hydrolysis or chemical hydrolysis from raw sludge RAS6_275, fish trial with rainbow trout and optimised new feeds


(1)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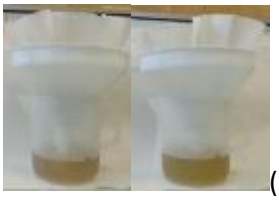
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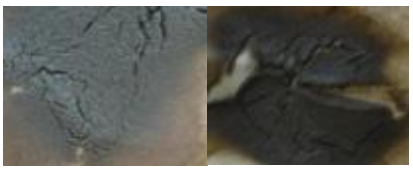
(5)

Figure 29 Medium ingredients obtained by applying ultrasound pre-treatment followed by enzymatic hydrolysis from raw sludge RAS6_275, fish trial with rainbow trout and optimised new diets. (1) Ultrasonic Processor Sonics model VCX 750. (2): Hydrolysis in water bath. (3) Sludge filtration. (4) Medium ingredients: Left- sludge C1 diet enzy (process 1 with Candida) and Right: Sludge N1 diet chem (Process 2 with Nanno diet 1). (4) Medium ingredients: Sludge filtration: Left-ING C1 diet enzy, Right-ING N1 diet chem. (5) Left-final residue Process 1 and Right: final residue Process 2.

Table 21 shows the results of the physicochemical of the medium ingredients obtained at **lab scale** on fresh weight. Moisture, dry matter and ashes were analysed by gravimetry. TKN were analysed by kjeldahl method and NH_4^+-N, NO_2^--N and NO_3^--N by ion chromatograph. Table 22 shows the nutritive/pollutant elements (ICP-MS) and Table 23 shows the microbiology results. For samples that were examined in duplicate (n=2), a deviation is provided.

Table 21 Physicochemical characterisation of the medium ingredients obtained from raw sludge RAS6_275 by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) and chemical hydrolysis (Process 2) at lab scale.

Parameter	ING sludge RAS6_275 from Candida-Enzymatic (1)	ING sludge RAS6_275 from Nanno 1-Chemical (2)
pH	8.2±0.0	10.1±0.0
Conductivity (mS/cm)	4.0±0.0	4.8±0.0
Moisture (%)	99.6±0.0	99.6±0.0
Dry matter (%)	0.4±0.0	0.4±0.0
Ashes (%) (gravimetry)	0.3±0.0	0.3±0.0
TKN-N (mg/kg)	72.2±0.4	73.5±7.1
NH_4^+-N (mg/kg)	< 100	< 100
NO_2^--N (mg/kg)	85	109
NO_3^--N (mg/kg)	< 23	< 23
Total N (mg/kg) [(1)]	157	183
Organic C (TOC) (mg/kg)	< 1000	< 1000
Sulphate (SO_4^{2-}) (%)	< 0.05	< 0.05
Fats (%)	0.2	0.2

n.a. not analysed

(1) Total Nitrogen calculated as the sum of TKN + NO_2^--N + NO_3^--N

Medium ingredients were low in carbon, values under 1000 mg/kg on fresh weight. Total nitrogen, ranged 72-73 mg/kg, with values under 23 mg/kg of nitrates.

Table 22 Nutritive/pollutant elements in the medium ingredients obtained from raw sludge RAS6_275 by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 1) and chemical hydrolysis (Process 2) at lab scale.

Element (mg/kg)	ING sludge RAS6_275 from Candida-Enzymatic (1)	ING sludge RAS6_275 from Candida-Chemical (1)
Na	1092±2	1083±20
Mg	10±0	2±0
P	<10	<10
S	<100	<100
K	12±0	347±5
Ca	<100	<100
Cr	<0.1	<0.1
Mn	<0.1	<0.1
Fe	<1	<1
Co	<0.1	<0.1
Ni	<0.1	<0.1
Cu	0.1±0.0	0.1±0.0
Zn	<0.1	<0.1
As	<0.1	<0.1
Se	<0.1	<0.1
Mo	<0.1	<0.1
Cd	<0.1	<0.1
Pb	<0.1	<0.1
Hg	<0.1	<0.1

Note: Potassium was higher in chemical treatment due to the addition of KOH

Medium ingredients contained nutrients such as sodium. Pollutant elements were not detected (under 0.1 mg/kg).

Table 23 Microbiology in the medium ingredients obtained from raw sludge RAS6_275

Microorganism	ING sludge RAS6_275 from Candida–Enzymatic (1)	ING sludge RAS6_275 from Candida–Chemical (1)
Salmonella spp in 25 g	absent	absent
E.coli (CFU/mL)	<10	<10

n.a. not analysed
Rapid detection method Salmonella. Rapid Salmonella. According to UNE EN ISO 16140
ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E.coli

Salmonella and E.coli were not detected (under 10 CFU/g).

The 2 medium ingredients were shipped to NORCE to test yeast growth (Process 1) and microalgae (Process 2) to test growth. These results are described in Chapters 3 and 4.

5.3 Conclusions

Presently, only sludge treatment options, such as landfill, incineration, biogas production, ensilage, and composting, are allowed for sludge applications (Regulation (EC) No 1069/2009). Sludge from Aquaculture Recirculation Systems (RAS) may be treated in regional waste treatment facilities or biogas plants, but generally the amount of sludge is not enough for RAS farms to have their own methane bio-digester. Sludge may be also used for agricultural purposes, but quality and nutrient content have to be appropriate as fertilizers. Besides, differences in rules and in quality and environmental standards hamper the circulation of fertilizers based on recycled nutrients in the EU.

The novelty of the approach in iFishIENCi was to valorise the sludge into soluble nutrients (nitrogen, phosphorus, carbon) in the form of medium ingredient (MI) so that they could be taken up easily by microalgae, yeast for feed applications.

The recovery of nutrients was demonstrated in iFishIENCi under the Task 1.5.3 Proof-of-concept "*Sludge valorisation into algae nutrients*". This subtask started with testing different pre-treatments (ultrasounds, thermal, and chemical with NaOH) and hydrolytic processes with enzymes (cellulases, proteases) on concentrated sludge from RAS system (15-20% dry matter, recovered using 60 µm filtration from fish trial with rainbow trout fed with conventional feed). As a result of the screening, ultrasounds pre-treatment was selected and applied to disintegrate the sludge. Next, two different processes, enzymatic and chemical hydrolysis, were successfully optimised, demonstrated and validated at pilot scale for the recovery of nutrients. The recovery of phosphorus and carbon varied significantly amongst the treatments. When compared to the enzymatic method, the chemical treatment yielded a greater recovery. The recovery of nitrogen did not differ statistically from the enzymatic method. The optimal conditions were determined as follows:

- **Process 1.** Ultrasounds 30 min at 20Khz with 65% amplitude followed by enzymatic treatment with 1.9% Pro A (endoprotease) and 1.9% pro D (exoprotease) at 65ºC for 5h, under agitation, achieving a recovery yield of **36% total nitrogen, 3% total phosphorus and 13% total organic carbon**
- **Process 2.** Ultrasounds 30 min at 20Khz with 65% amplitude followed by chemical treatment with KOH 4M at pH 12 at 65ºC for 5h, under agitation, achieving a recovery yield of **46% total nitrogen, 8% total phosphorus and 60% total organic carbon**

In addition, the medium ingredients were fully characterised to demonstrate potential application as **fertilising** products in the agriculture, as part of Waste2value. The medium ingredients fulfilled the physicochemical and microbiological characteristics for fertilising products (Regulation (EU) 2019/1009), for Product Functional Categories PFC4 (Growing medium) and PFC6 (Plant bio-stimulant). The final residue was also fully characterised as part **of zero waste strategy**. It showed good characteristics as potential fertilizer with the exception of cadmium, which was found slightly above permit limits.

In parallel, the enzymatic and chemical treatment were replicated on raw sludge from RAS system, containing mostly water (0.5-4.3% dry matter). Although most nutrients were already soluble and accessible for microalgae and yeast, the treatments were applied to assess the potential application of these treatments in non-concentrated sludge.

In conclusion, the treatment of the concentrated sludge from the RAS system which combines ultrasounds followed by enzymatic or chemical hydrolysis proved to be a promising technology to extract nutrients, mainly nitrogen and carbon.

Further investigation should be conducted to study the potential bioaccumulation of persistent organics pollutants (POPs) in yeast and microalgae as feed, and check permit limits in Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in **animal feed**.

6 Valorisation of waste derived from the production of ingredients

As indicated in Chapter 2.3., waste collected from the production of new ingredients were two types: spent medium from *Candida utilis* biomass and insoluble matter from the antioxidant supplement from *Nannochloropsis gaditana*. The expected outcome was to reuse these waste streams.

6.1 Exhausted media from Candida as substrate for algae

6.1.1 Introduction

Following the production of the fish feed ingredient through fermentation using *Candida utilis* yeast, the cell-free spent medium that was collected after harvesting and dewatering the Candida biomass and it was tested as nutrient source for photoautotrophic and/or mixotrophic microalgae production. Left-over ammonium and phosphate and produced dissolved organic components such as volatile fatty acids can be used by the microalgae as nutrients (Zhang Z. G., 2021)

6.1.2 Experimental development

The total nitrogen measured of the spent medium vas very high (465 mg N/L) compared to the other waste streams, however no ammonium or nitrate was measured, indicating that the available nitrogen might not have been available for the photoautotrophic microalgae. Moreover, there was no phosphate present. Moreover, the medium was very turbid, leading to challenging conditions for the microalgae regarding light penetration (Figure 30). Micronutrients were added to the medium to test in bubble columns for its suitability for *C. vulgaris* production.

In the bubble column experiments a minor increase in biomass in the first day was noticed (Figure 31), but after that growth ceased and a maximum biomass concentration of 0.17 g/L was achieved. Moreover, the quantum yield decreased significantly after day 6, indicating that the microalgae were not thriving on this medium.

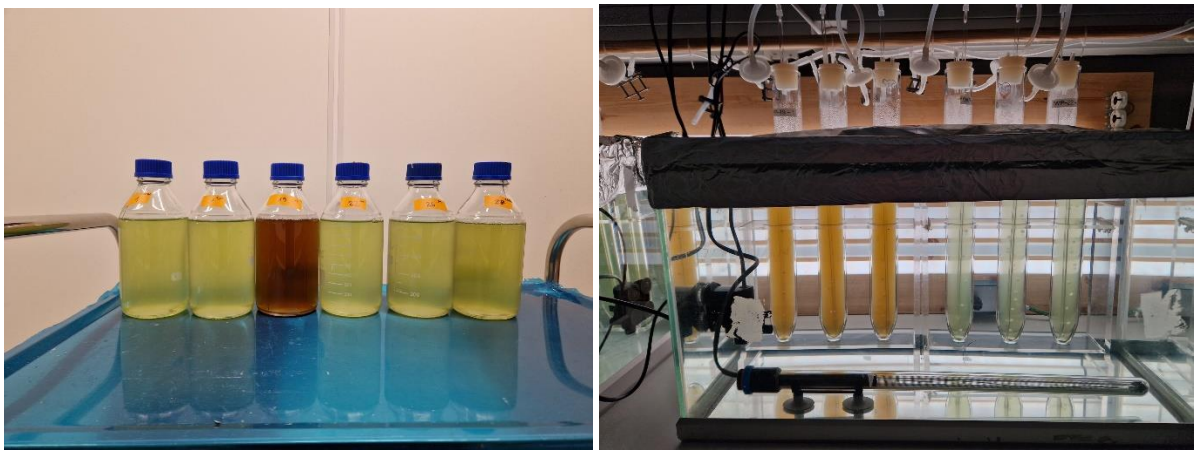

Figure 30 Left: Inoculum (C. vulgaris) on various waste streams (third bottle from left is on spent medium tested here, the other bottles contain wastewater from fish-farming (described in Chapter 3). Right: after inoculating in the bubble columns. The left three tubes are C. vulgaris on spent medium. The right three tubes are C. vulgaris on wastewater from fish-farming.

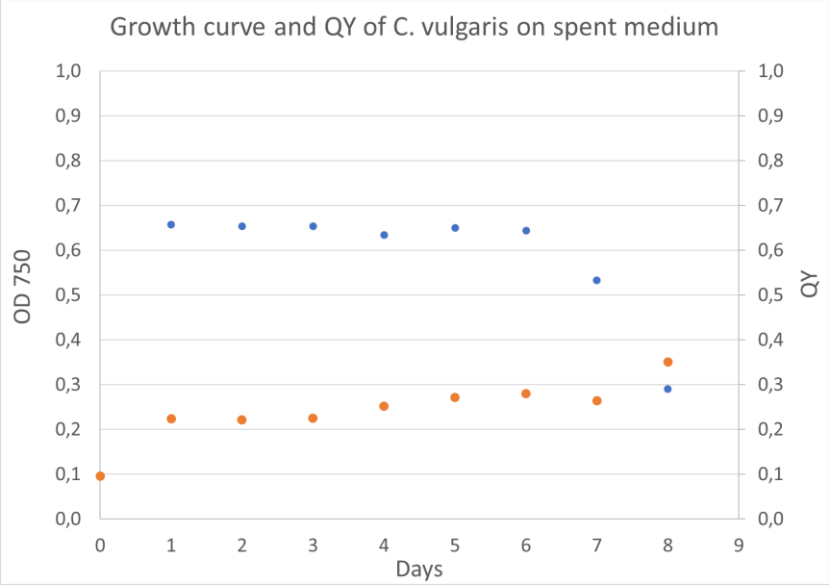

Figure 31 Growth curve and quantum yield of C. vulgaris grown on spent medium.

6.1.3 Conclusions

Spent medium from Candida fermentation does not appear to be a suitable medium for the production of the photoautotrophic microalga *C. vulgaris*.

6.2 Insoluble matter from *Nannochloropsis* – as nutrients for algae and yeast

6.2.1 Introduction

In this task, the insoluble matter retrieved from the production of the antioxidant supplement in task 1.3, was characterised and valorised into nutrients (medium ingredients) to be used for photoautotrophic microalgae and yeast growth tests at small scale. An experimental design to produce medium ingredients from the insoluble matter from *Nannochloropsis gaditana* by applying enzymatic processes was developed. Finally, a proof of concept for the nutrient recovery was developed.

6.2.2 Characterisation

The Table 24 shows the results of the physicochemical characterisation of the insoluble matter from *N. gaditana* on fresh weight. Moisture, dry matter and ashes were analysed by gravimetry. TKN was analysed by kjeldahl method.

Table 25 shows the nutritive/pollutant elements (ICP-MS) and Table 26 shows the microbiology results. For samples that were examined in duplicate (n=2), a deviation is provided.

Table 24 Physicochemical characterisation of the insoluble matter from N. gaditana collected in Task 1.3.

Parameter	Insoluble matter from Nannochloropsis
pH [(1)]	5.8
Conductivity (mS/cm) [(1)]	2.7±0.0
Moisture (%)(gravimetry)	77.5±0.0

Dry matter (%) (gravimetry)	22.5±0.0
Ashes (%) (gravimetry)	0.8±0.1
TKN-N (%) (kjeldahl method)	1.4±0.0

(1) pH and conductivity, sample preparation: the solid sample was mixed with distilled water (1:2.5), centrifuged at 10.000 rpm for 15 min. Next, the supernatant was analysed.

The insoluble matter from *N. gaditana* contained remaining nitrogen (1.4% on fresh weight).

Table 25 Nutritive/pollutant elements in insoluble matter from N. gaditana.

Nutritive/pollutant elements (mg/kg)	Insoluble matter from Nannochloropsis
Na	1250±50
Mg	490±17
P	1076±37
S	1709±89
K	1058±40
Ca	632±12
Cr	10±0.2
Mn	49±2
Fe	239±8
Co	0.3±0.0
Ni	1.2±0.0
Cu	3.9±0.1
Zn	24.3±0.4
As	<0.1
Se	<0.1
Mo	0.2±0.0
Cd	<0.1
Pb	<0.1

The insoluble matter from *N. gaditana* contained remaining nutrients, mainly sodium (1250 mg/kg), also phosphorus (1076 mg/kg).

Table 26 Microbiology in insoluble matter from N.gaditana

Microorganism	Insoluble matter Nannochloropsis
Salmonella spp in 25 g	absent
E.coli (CFU/g)	<100

n.a. not analysed
Rapid detection method Salmonella. Rapid Salmonella. According to UNE EN ISO 16140
ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E.coli

Salmonella and E.coli were not detected (under 10 CFU/g).

6.2.3 Proof of concept. Nutrient recovery yield

The objective of this study was to apply the optimised method for the nutrients' recovery from the insoluble matter from *Nannochloropsis gaditana*. Approach: Disintegrate the sludge and solubilise nutrients (nitrogen, phosphorus, carbon) as medium ingredient to growth microalgae and yeast. In addition, the chemical treatment developed for the sludge, was also applied.

Finally, the following medium ingredients were produced under the optimal condition by applying

- **Process 3** (enzymatic treatment). Enzymatic hydrolysis with protease D (exoprotease) to hydrolyse peptide bonds from proteins and enhance the release of free amino acids.
- **Process 4** (chemical treatment). Chemical hydrolysis KOH pH12

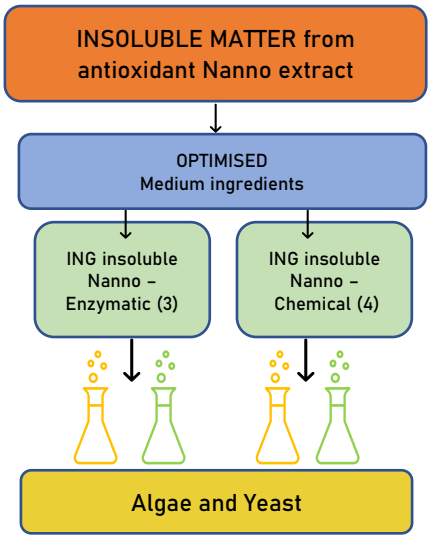

Figure 32 Summary of the medium ingredients obtained from Insoluble matter from the production of ingredients (antioxidant extract from Nannochloropsis) produced by LEITAT for testing the microalgae and yeast growth

The insoluble matter was weighed and diluted with distilled water (1:1) (final pH 5.32), and homogenised manually. Next, for **Process 3**: protease (Pro D) was added, and the sample was placed in a thermal bath at 50ºC, under agitation at 70 rpm for 5 hours. Next, the enzyme was inactivated at 90ºC for 10 minutes. For **Process 4**: samples was adjusted to pH 12 with 4M KOH and placed in a thermal bath at 50ºC, under agitation at 70 rpm for 5 hours. After the reaction, all samples were centrifuged 7500 rpm and filtered at 43-48µm to obtain the liquid fraction (medium ingredient). The solids were also recovered as final residue, since they could find application as fertilizer.

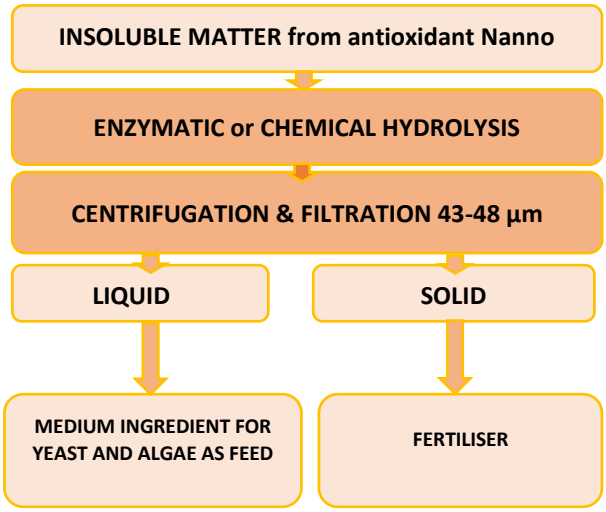

Figure 33 Optimised sludge bioconversion process into nutrients (medium ingredients) at lab scale by applying enzymatic hydrolysis or chemical hydrolysis from the insoluble matter from Nannochloropsis gaditana.

Figure 34 Medium ingredients obtained from insoluble matter Nannochloropsis. Left: ING insoluble Nanno – Enzymatic (process 3); Right: ING insoluble Nanno – Chemical (process 4)

The Table 27 shows the results of the physicochemical characterisation of the medium ingredients obtained lab scale. Moisture, dry matter and ashes were analysed by gravimetry. TKN was analysed by kjeldahl method and NH_4^+-N, NO_2^--N and NO_3^--N analysed by ion chromatography. Table 27Table 28 shows the nutritive/pollutant elements (ICP-MS) and Table 29 shows the microbiology results. For samples that were examined in duplicate (n=2), a deviation is provided.

Table 27 Physicochemical characterisation of the medium ingredients obtained from insoluble matter of N.gaditana, retrieved from the production of the antioxidant supplement collected in task 1.3. by enzymatic hydrolysis (Process 3) and chemical hydrolysis (Process 4) at lab scale.

Parameter	ING insoluble Nanno - Enzymatic (3)	ING insoluble Nanno - Chemical (4)
pH	5.9±0.0	10.3±0.0
Conductivity (mS/cm)	2	10
Moisture (%)	98.4±0.1	92.7±0.0
Dry matter (%)	1.6±0.1	7.3±0.0
Ashes (%) (gravimetry)	0.2±0.0	1.1±0.1
TKN-N (mg/kg)	2265±106	6125±21
NH_4^+-N (mg/kg)	30.4	1.3
NO_2^--N (mg/kg)	<0.08	<0.08
NO_3 -N (mg/kg)	0.5	3.2
Total N (mg/kg)[(1)]	2266	6128
Organic N (mg/kg)[(2)]	2235	6124
Inorganic N (mg/kg)[(3)]	30.9	4.5

[(1)] Total Nitrogen calculated as the sum of TKN + NO_2^--N + NO_3^--N
[(2)] Organic Nitrogen calculated as the difference between TKN and NH_4^+-N
[(3)] Inorganic Nitrogen calculated as the sum of NH_4^+-N + NO_2^--N + NO_3^--N

Medium ingredients, were rich in carbon specially the one produce by chemical hydrolysis with values of 6128 mg/kg on fresh weight. Total nitrogen, ranged 2265-6125 mg/kg, with values up to 3.2 mg/kg of nitrates for the chemical process.

Table 28 Nutritive/pollutant elements in the medium ingredients obtained from insoluble matter of N.gaditana by applying enzymatic hydrolysis (Process 3) or chemical hydrolysis (Process 4) at lab scale scale.

Element (mg/kg)	ING insoluble Nanno - Enzymatic (3)	ING insoluble Nanno - Chemical (4)
Na	525±21	535±15
Mg	146±4	111±3
P	257±20	369±10
S	406±65	816±40
K	538±12	7128±170
Ca	120±9	116±8
Cr	0.3±0.0	0.2±0.0
Mn	3.4±0.1	9.7±0.3
Fe	8.7±1.9	60.9±1.2
Co	<0.1	<0.1
Ni	<0.1	<0.1
Cu	<0.1	2.1±0.0
Zn	1.1±0.0	8.6±0.0
As	<0.1	<0.1
Se	<0.1	<0.1
Mo	<0.1	0.2±0.0
Cd	<0.1	<0.1
Pb	<0.1	<0.1

Medium ingredients contained nutrients such as sodium. Pollutant elements were not detected or they were under 9 mg/kg (i.e. Zn).

Table 29 Microbiology in the medium ingredients obtained from insoluble matter of N.gaditana by applying enzymatic hydrolysis (Process 3) or chemical hydrolysis (Process 4) at lab scale.

Microorganism	ING insoluble Nanno- Enzymatic (3)	ING insoluble Nanno–Chemical (4)
Salmonella spp in 25 g	absent	absent
E.coli (CFU/mL)	<10	<10

n.a. not analysed
Rapid detection method Salmonella. Rapid Salmonella. According to UNE EN ISO 16140
ISO 7251:2005 Horizontal method for the detection and enumeration of presumptive E. coli

Salmonella and E.coli were not detected (under 10 CFU/g).

The nutrient recovery yield was calculated as the nutrient content in the total volume recovered of the medium ingredient and expressed on the nutrient content in total sludge added in the process and expressed as percentage (see Figure 35).

Nutrient recovery yield (%) = Nutrient (g) in medium ingredient x 100
Nitrogen (g) in insoluble matter (g) in Nannoch.

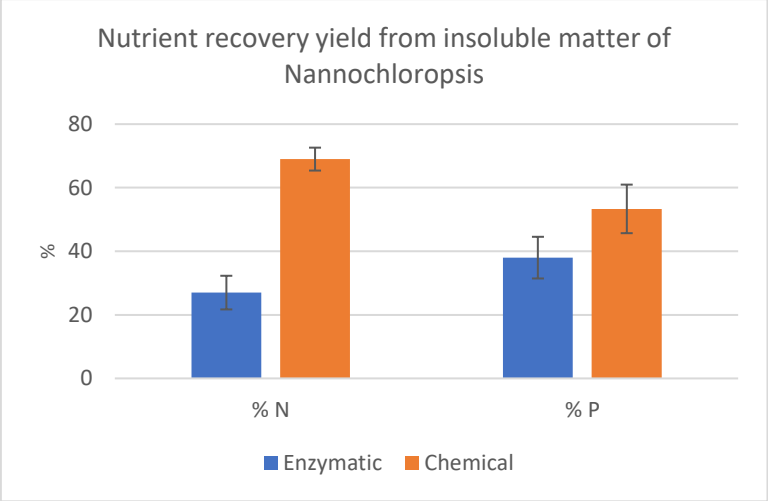

Figure 35 Nutrient recovery yield obtained from insoluble matter of Nannochloropsis by applying ultrasound pre-treatment followed by enzymatic hydrolysis (Process 3) and chemical hydrolysis (Process 4) at lab scale. Results expressed as percentage. Bar graph illustrating the mean and standard deviation (error bars) with n=3.

The processes proved the following nutrients recovery yield:

- **Process 3.** Enzymatic process with 2% pro D (exoprotease) at 50ºC for 5h, under agitation, achieving a yield of **27% total nitrogen and 38% total phosphorus**
- **Process 4.** Chemical treatment with KOH 4M at pH 12 at 50ºC for 5h, under agitation, achieving a recovery yield of **69% total nitrogen and 53% total phosphorus.**

The p-value corresponding to the F-statistic of one-way ANOVA for the recovery of nitrogen was 0.0010, lower than 0.05, suggesting that treatments were significantly different. The p-value

corresponding to the F-statistic of one-way ANOVA for the recovery of phosphorus was 0.0347, lower than 0.05, suggesting that treatments were significantly different.

The medium ingredients were shipped to NORCE to test yeast growth and microalgae. These results are described in Chapters 3 and 4.

6.2.4 Conclusions

One type of waste collected from the production of new ingredients was the insoluble matter from the production of the antioxidant supplement from microalgae *Nannochloropsis gaditana*. This waste stream was collected in task 1.3 as paste format (77% moisture, 1.4% TKN-N, 0.1% phosphorus).

This waste stream was characterised physicochemical and microbiologically and it showed potential for agriculture applications based on requirements and permits limits of Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003.

The novelty of the approach in iFishIENCi was to valorise the insoluble matter from *Nannochloropsis* into soluble nutrients so that they could be taken up easily by microalgae and yeast for feed production. The valorisation of waste derived from the production of ingredients into nutrients was demonstrated in iFishIENCi under the Task 1.5.3 Proof-of-concept "*Sludge valorisation into algae nutrients*". To do so, two different processes, enzymatic and chemical hydrolysis, were successfully optimised, demonstrated and validated at lab scale for the recovery of nutrients. The recovery of phosphorus and nitrogen varied significantly amongst the treatments. When compared to the enzymatic method, the chemical treatment yielded a greater recovery. The optimal conditions were determined as follows:

- **Process 3.** Enzymatic process with 2% pro D (exoprotease) at 50ºC for 5h, under agitation, achieving a yield of **27% total nitrogen and 38% total phosphorus**
- **Process 4.** Chemical treatment with KOH 4M at pH 12 at 50ºC for 5h, under agitation, achieving a recovery yield of **69% total nitrogen and 53% total phosphorus.**

Medium ingredients were characterized physicochemically and microbiologically, showing good results for yeast production but not suitable for microalgae growth due to their turbid nature.

Based on the physicochemical and microbiologically characteristics of both medium ingredients (liquid from), it can be concluded that they might also find application in agriculture. They could find potential application as fertilizer based on requirements of Product Functional Category PFC4: Growing medium and PFC6: Plant bio-stimulant of the mentioned fertilizer products regulation.

In conclusion, treatment of the insoluble matter from *Nannochloropsis* collected after the production of the antioxidant supplement is a promising technology to extract nutrients. These nutrients could be used as culture medium or in agriculture, but further optimisation should be carried out.

7 The iFishIENCi Waste2Value product

Waste2Value is one of the innovative products developed in iFishIENCi (together with Fish-Talk-to-Me, iBOSS, SMART-RAS) to contribute to improve sustainable aquaculture systems. The concept of Waste2Value is developing as a set of recommendations/guidelines to valorise different aquaculture waste streams (wastewater, sludge, waste from the production of ingredients).

The goal is to contribute to the generation of more circular value-chains within the aquaculture economy and beyond. The product will provide specific guidelines and recommendations on how to valorise aquaculture waste streams, regulatory framework/legislation, technical aspects (characterisation), as well as sustainability assessment (specific recommendations/conclusions from LCA-environmental assessment, and LCC-economic performance, and some insights into infrastructure, when possible). The product will also provide data of waste streams characteristics from RAS system (such as dissolved nitrogen, ammonia, nitrates), which were demonstrated in WP3 as integration into iBOSS at ABT (Product 3: SMART-RAS). This data could potentially be linked to water quality modelling and future development of FishMet.

The characterisation of the waste streams, the data from iBOSS collected at RAS system by ABT and the characterisation of medium ingredients obtained in iFishIENCi will provide guidance and opportunity not only for algae and yeast growers but also for further developments and other potential uses such as fertilisers, platform chemicals, biogas – biofuel, IMTA and aquaponics, enabling the partial or total valorisation of wastewater and sludge. Besides the valorisation routes addressed in iFishIENCi to produce new feeds, the analysis of the different waste compositions provided the necessary information on carbon, nutrients and mineral content to select a list of potential organisms used in industrial bioprocesses, which may be able to use the RAS-waste as source of carbon and energy to support growth. In WP5, an assessment of the technical potential of the above-mentioned value chains was conducted based on these data, in order to have a preliminary insight into wider scope of applications for further research in future projects. Discussions were held in WP5 to define the exploitation strategy of this know-how as a consulting service to provide information on the reuse of waste streams and to increase the impact of the iFishIENCi.

Task 1.5 will become the main source of guidelines to be further exploited as a “Key Exploitable Result” by WP5. The results from the proofs of concept for waste valorisation as new feeds (algae, yeast), as well as the data from the waste characterisation and bibliography research, will be the basis to point out alternative waste valorisation routes and prepare the **Guidelines** for further consideration for exploitation (WP5) and future research. The main contributors to develop these guidelines on Waste2Value will be NORCE, LEITAT, ABT, COVARTEC and VITAFORT. This know-how will be part of the iFishIENCi **Waste2Value product**.

The target groups of Waste2Value are producers of fish, algae feed and feed ingredient, bioprocessing companies, aquaponics, policymakers developing the circular economy policy and regulatory framework, stakeholders who wish to get involved in aquaculture waste reuse value chains, and obviously the companies involved in iFishIENCi and the scientific community.

8 Conclusions

In the iFishIENCi project, WP1 – *Task 1.5 Zero waste and valorisation of by-products and sludge,* which aimed at designing condition-based optimal valorisation processes for waste recirculation from aquaculture effluents and recovery of nutrients (nitrogen, phosphorus, carbon) within a circular economy and their use for the culture of two sustainable fish- feed ingredients addressed in iFishIENCi, namely microalgae and yeast, main conclusions were:

-Waste streams from RAS system have the potential to be re-used and valorised under a circular approach and towards zero-waste, according to the characterisation and the regulatory framework for waste-management.

-The analysis of the different waste compositions provided the necessary information on carbon, nutrient and mineral content to select a list of potential organisms used in industrial bioprocesses, which may be able to use the RAS-waste as source of carbon and energy to support growth.

- According to the results obtained in the proof-of-concept for the reuse of dirty water as algae production feedstock, it was concluded that the outlet water from all fish production from RAS and land-based flow-through systems could be directly used for the cultivation of **photoautotrophic microalgae *(e.g., Nannochloropsis gaditana).*** Sterilization was not necessary. Addition of micronutrients seemed to lead to limited improvements in achieved biomass concentrations. Phosphate was the main limiting nutrient for the microalgae. Though the nutrient concentrations varied significantly between batches of wastewater from fish-farming, all were lower in nutrient concentrations than growth medium for industrial production of microalgae. The capture efficiency could be up to 100% for both phosphorous and nitrogen from the water. In some cases, not all P and/or N was captured, probably due to chemical binding to organic particles. Regarding the results from the **yeast *(Candida utilis)*,** it could not grow in wastewater from RAS system. As for the sludge, it required to be diluted, centrifuged to eliminate the insoluble material, and double autoclaved to kill all the microorganisms and spores. The total organic carbon content was the limiting nutrients for yeast. The nutrient concentration was likely not high enough to support significant growth of yeast. Probably, the compounds present in the sludges were not easily degraded by the candida to be used successfully as a carbon and energy source to support growth. Further investigation should be conducted to study the potential bioaccumulation of persistent organics pollutants (POPs) in yeast and microalgae as feed, and check permit limits in Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in **animal feed**.

- According to the results obtained in the proof-of-concept for the sludge valorisation into algae and yeast nutrients, it was concluded that the **concentrated sludge** from RAS system could be treated to produce ingredient medium by applying ultrasounds followed by enzymatic (endo and exoprotease) or chemical hydrolysis (KOH), achieving a **recovery yield of 36-46% total nitrogen, 3-8% total phosphorus and 13-60% total organic carbon**. Medium ingredients were too turbid but they could in some cases serve as nutrients for microalgae cultivation. As for the yeast, the nutrients previously extracted from concentrated RAS sludge, they were found available as a carbon and energy source for yeast production, making it a potentially viable feedstock for their production.

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