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MSc. Aquaculture and Fishery

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Universidade do Algarve, 12 de Maio de 2021

Signiture

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I am grateful to have received the thorough and dedicated training at Landing Aquaculture B.V. in understanding the fundamentals of RAS engineering and designing. The months spent in the Netherlands were enlightening in developing exciting new research and development directions. I am also thankful to have received the opportunity to devise an interesting and certainly challenging thesis project, where I expanded the newly developed knowledge of RAS biological filtration with essential microbial ecology, untargeted metabolomics, and related statistical analyses. I feel my "luggage" of scientific experience has become substantially heavier. My special thanks go to Carlos for his creative possibilism, to Rob for his careful and methodological leadership and to Lola, for the patient and thorough guidance. Finally, I am grateful to every member of my family, to my partner Karina, and to my friends for having supported me through the ups and downs of this special time. With no doubt, the isolation induced by the Sars-Cov-2 pandemic and the hardship of this time would have been much worse without their vicinity.

Abstract

Land-based recirculating aquaculture systems (RAS) can boost food security and environmental sustainability worldwide. In RAS, the removal of nitrogenous waste is obtained via prokaryotic biodegradation. However, the spatiotemporal metabolic dynamics of microbial communities in RAS are poorly understood. Understanding these trends can generate operational improvements. The necessity for fast and cost-effectiveness analysis suggests the employment of top-down molecular techniques. This pilot study evaluated the applicability of untargeted metabolomics to describe metabolic trends in RAS bioreactors. We compared two reactor designs, a two-stage moving bed bioreactor (MBBR) and an anaerobic batch fed sludge bioreactor (BFDR), as well as different locations within each of these (i.e., inlets vs outlets). As expected, a differentiation in metabolomic fingerprints determined by different chemo-mechanical properties was highlighted between the two bioreactors. However, contrarily to our initial hypotheses, no differentiation was found within the MBBR. Possible explanation can be identified in a vessel-wide distribution of a single microbial community, as well as in the influence of ammonia-limiting conditions and short hydraulic retention time. Unexpectedly, however, a significant differentiation was found within the BFDR, where two distinct metabolic fingerprints were recorded, inferring on the presence of a community in suspension to be further investigated. We demonstrated how metabolomics analysis can reveal RAS bioreactor metabolic dynamics and at the same time expose the unsuccessful design of specific biomechanical properties.

Keywords

RAS, bioreactors, MBBR, BFDR, untargeted metabolomics, microbial community, metabolic fingerprinting

Resumo

O emprego de sistemas de aquacultura tecnologicamente avançados em terra pode aumentar a segurança alimentar e a sustentabilidade ambiental em todo o mundo. Os Sistemas de Recirculação em Aquacultura (SRA) produzem um maior rendimento por unidade de área com um menor impacto ambiental. No SRA, os parâmetros bióticos e abióticos são controlados, o consumo de água e a ocupação de terreno são reduzidos e os resíduos valorizados. No entanto, a avançada abordagem multidisciplinar e o elevado investimento permitiram a implementação de SRA em grande escala apenas em países ricos. A reutilização da água apresenta desafios de engenharia dadas as elevadas taxas de excreção de nitrogénio e carbono na alimentação. A remoção de resíduos nitrogenados em sistemas SRA é obtida por meio da biodegradação procariótica em bioreatores. Projetos de bioreatores aplicam pressão ambiental para conduzir a seleção natural de bactérias e arqueias desejadas, enquanto maximiza a área de superfície para biofilme de bactérias e seleciona o fluxo de água adequado, a turbulência, a proporção de carbono para nitrogénio e a aeração. Os microrganismos nitrificantes são responsáveis pela oxidação do nitrogénio amoniacal tóxico (NAT) em condições aeróbicas. Em condições anaeróbicas, os quimioheterotróficos anaeróbicos gram-negativos desnitrificantes, quebram as moléculas orgânicas, reduzindo os nitratos a gás nitrogénio (N2). No entanto, a dinâmica metabólica em bioreatores é muito mais complexa e diversa e a interação, a diversidade e a dinâmica espaço-temporal das comunidades microbianas em SRA ainda são pouco compreendidas. Regulamentações ambientais recentes encorajaram a atualização de SRA convencionais com bioreatores de desnitrificação. Além de melhorar os padrões de bem-estar dos animais de cultura, a recirculação da água é aumentada em até 99% e o custo de produção é decresce em 10% por kg de pescado, por meio da redução de resíduos, remineralização de nutrientes vegetais em sistemas aquapónicos e produção de biogás. No entanto, o emprego de reatores anaeróbicos também não tem sido extenso devido à falta de pesquisas fundamentais em ecologia microbiana. A sensibilidade microbiana às mudanças ambientais requer uma gestão pontual. Embora as ferramentas moleculares de DNA tenham descrito as estruturas das comunidades, elas não são confiáveis como uma ferramenta de diagnóstico para revelar prontamente as alterações espaço-temporais em resposta ao stresse. As interações químicas entre linhagens microbianas são desconhecidas e a descoberta e caracterização de metabólitos melhoraria a capacidade operacional do processo de nitrificação-desnitrificação. A necessidade de rentabilidade e ferramentas analíticas ágeis sugere o emprego de técnicas moleculares. Recentemente, análises metabolómicas foram propostas para melhorar a gestão de águas

residuais. As análises metabolómicas descrevem metabólitos, pequenas moléculas que constituem os biomarcadores mais precisos da natureza e a metabolómica não direcionada determina e compara os padrões dos sistemas em estados estacionários e perturbados. Os dados de alto rendimento requerem uma redução da dimensionalidade com estratégias de filtração específicas, enquanto as análises estatísticas identificam algumas variáveis principais resumindo completamente a complexidade geral. Até onde sabemos, a metabolómica nunca foi empregada para descrever SRA e a integração com a transcriptómica e metagenómica poderia ajudar a construir uma imagem holística da vida microbiana em bioreatores SRA. Este estudo piloto teve como objetivo avaliar a aplicabilidade da metabolómica não direcionada para descrever as tendências metabólicas em bioreatores SRA. Em 2019, a empresa Landing Aquaculture B.V. e a Universidade de Wageningen montaram um SRA semicomercial de 50 m3. O SRA foi equipado com um bioreator de leito móvel aeróbico de duas células de 10m3 (MBBR) e um reator anaeróbico de desnitrificação alimentado em lote de 1,2 m3 (BFDR). Os objetivos específicos deste estudo foram comparar os dois projetos de reator e diferentes locais dentro de cada um deles (ou seja, entradas vs saídas). Os reatores foram testados quanto à sua produção de diferentes identidades metabólicas: o reator aeróbico foi testado para sua capacidade de produzir identidades metabólicas específicas da célula, enquanto o reator anaeróbico era esperado desenvolver um único metaboloma. A cromatografia líquida - espectrometria de massa (LCMS) foi empregada com a plataforma analítica Quadrupole ExactivePlus Orbitrap Fourier Transformation e o fluxo de trabalho do software MetAlign-MSClust para a formação de identidades. Uma estratégia de filtração foi desenvolvida ad hoc e quatro multivariadas não supervisionadas projeções foram aplicadas para destacar a relação entre e dentro dos reatores. A diferenciação estatística global foi testada com PERMANOVA e testes de pares post hoc compararam os locais das amostras. No total, foram detetados 243.356 sinais no modo de ionização positiva e 89.870 sinais no modo de ionização negativa. Os compostos não foram anotados ou quantificados por métodos padrão, mas 23 compostos foram identificados com base na massa precisa dos iões moleculares presumidos. O MBBR resultou em uma identidade metabólica homogénea, enquanto o BFDR foi diferenciado em dois grupos distintos de metabolitos não sobrepostos. O teste de PERMANOVA confirmou a diferenciação global em todo o conjunto de dados (P <0,001). Todas as comparações de pares de grupos foram significativas, exceto para a comparação entre a entrada e a saída do MBBR (P = 0,4230). As análises metabolómicas foram bem-sucedidas na representação das diferentes identidades metabólicas entre e dentro de dois designs exclusivos de bioreatores SRA. Os resultados afirmam a sensibilidade do LCMS não direcionado, que rejeitou a hipótese inicial

determinada por projetos biomecânicos seletivos. O MBBR de 2 células se comportou como um recipiente totalmente misturado. A possível explicação pode ser identificada em uma distribuição em todo o vaso de uma única comunidade microbiana. Simultaneamente, o BFDR foi caracterizado por dois metabolomas altamente distintos, inferindo sobre a presença de uma comunidade em suspensão a ser investigada. Neste trabalho foi demonstrado como a análise metabolómica pode expor a dinâmica metabólica do bioreator e potencialmente se tornar uma ferramenta de sucesso para observar as diferenças quando as alterações do sistema são aplicadas. Ao mesmo tempo, a metabolómica pode revelar, como mostrado aqui, a falta de sucesso na designação de propriedades biomecânicas específicas. A metodologia proposta oferece a oportunidade de executar análises metabolómicas rápidas e económicas para descrever as tendências metabólicas dos bioreatores.

Palavras-chave

SRA, bioreatores, MBBR, BFDR, metabolómica não direcionada, comunidade microbiana, identidade metabólica.

List of Abbreviations

AHLs – acyl-homoserine lactones
AOA – ammonia oxidising archaea
AOB – ammonia oxidising bacteria
C/N – Carbon to Nitrogen ratio
DNA – deoxyribonucleic acid
DNRA – dissimilatory nitrate reduction to ammonia
DO – dissolved oxygen
DOM – dissolved organic matter
EPS – extracellular polymeric substances
FBBR – fixed bed bioreactor
FSB – fluidised sand biofilter
GCMS – gas chromatography-mass spectroscopy
GeoA – geosmin-synthesis gene
Geosmin – trans-1,10-dimethyl-trans-9-decalol
HCA – Hierarchical clustering analysis
HRT – hydraulic retention time
LCMS – liquid chromatography-mass spectroscopy
LDA – Linear Discriminant Analysis
MBBR – moving bed bioreactor
MIB – 2-methylisoborneol
NMDS – Nonmetric multidimensional scaling
NMR – Nuclear magnetic resonance
NOB –nitrite oxidising bacteria
NOx – nitrogen oxides
ORP – oxygen reduction potential ()
PCA – principal component analysis
PerMANOVA – permutational multivariate analysis of variance
POM – particulate organic matter
PTMs – posttranslational modifications
Q ExactiveP Orbitrap FTMS – Quadrupole ExactivePlus Orbitrap Fourier Transformation
RAS – Recirculating Aquaculture Systems
rt – Retention Time
RBC – rotating biological contactors
SWIS – Subsurface Wastewater Infiltration System
TAN – Total Ammonia Nitrogen
UASB – anaerobic sludge blanket reactor
UPLC – ultra-high performance liquid chromatography
WUR – Wageningen University & Research

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State of the Art

Chapters 1: RAS in modern aquaculture

The natural environment can no longer withstand the current magnitude and diversity of direct wildlife extraction imposed by fishing - the last remaining large-scale "hunter-gather" human activity (Ferri, 2010; Christensen et al., 2014; Timmons et al., 2018; Bindoff et al., 2019; Bradshaw et al., 2021). The forthcoming human population and consumption growth is associated with fading fish stocks in all projections, meanwhile the global aquaculture industry currently supplies approximately 50% of the total seafood demand (Tal et al., 2006; World Bank, 2014 Christensen et al., 2014; Eu 2017; Bindoff et al. 2019; FAO, 2020; Bradshaw et al., 2021). Consequently, the shrinking output from fisheries appoints to the aquaculture sector the future leading role as planetary, reliable, and necessarily sustainable seafood source (Tal et al., 2006; Timmons et al., 2018; Gentry et al., 2017).

Yet, many aquaculture-related socio-ecological adverse impacts have been exposed worldwide, and to the highest degree in low-income nations (Allsopp et al., 2008; FAO 2020). Commonly reported impacts include habitat loss, chemical disease-control pollution, exploitation of fish stocks for feed production, depletion and salinisation of potable water and agricultural land, nutrient pollution, farmed breeds escapes and "genetic pollution", diseases and parasites outbreaks, and introduction of non-native species (Tal et al., 2006; Tal et al., 2009; Allsopp et al., 2008; Gentry et al. 2017; Bindoff et al. 2019; FAO 2020; Ruiz et al., 2020). As a result, the necessity of environmentally responsible solutions to reach food security worldwide appeals for the international distribution of technologically advanced land-based aquaculture systems (Tal et al., 2006; Tal et al., 2009; Allsopp et al., 2008; Ferri, 2010; World Bank, 2014; Bostock et al., 2016; EU 2017; Bindoff et al. 2019; Xiao et al., 2019; FAO, 2020; Ruiz et al., 2020).

Recirculating Aquaculture Systems (RAS) are the result of over 40 years of research and development in the private, as well as public sector (Timmons et al., 2018). The advance of RAS technology has been favoured by the rising environmental regulations in countries with limited access to water (Goddek, 2019; Martins et al., 2010). At the same time, RAS increase the economic viability of aquaculture businesses by greatly improving production efficiency. RAS, in fact, allow the control of biotic and abiotic environmental parameters, the stabilisation of production capacities, guarantee high-quality standards, and infinitely expand the opportunities to produce seafood anywhere on land (Tal et al., 2009; Martins et al., 2010;

Bregnballe, 2015; Timmons et al., 2018; Goddek, 2019, Ruiz et al., 2020). RAS also reduce the consumption of land, water and heat energy, while upcycling waste products (Martins et al., 2010; Timmons et al., 2018; Goddek, 2019; Ruiz et al., 2020). As seafood production systems, RAS are potentially devoid of environmental pollutants, independent from site, salinity, and species restrictions, and are fundamentally biosecure (Tal et al., 2009). Today, RAS provide the highest yield per unit area with the smallest footprint requirements (Tal et al., 2009; Martins et al., 2010; Timmons et al., 2018; Goddek, 2019).

However, RAS are cursed with great complexity. The design of a RAS begins with biological planning of the species to produce, customised on the availability of space, desired yield, and investment. Once the food intake demand and conversion ratios are defined for all life stages in production with a mass balance, the water flow can be calculated for the size and capacities of culture units (Bregnballe, 2015). Subsequently, the compartments dedicated to restoring water quality parameters are laid out. The expired water requires specific treatment units for degassing CO_2 and dissolving oxygen, removing particulate and dissolved organic matter (POM and DOM), toxic inorganic compounds and for disinfection (Bregnballe, 2015; Goddek, 2019). The series of connected compartments are intensively monitored and ensure the re-establishment of high standard environmental parameters and welfare condition for cultured species (Bregnballe et al., 2015; Goddek, 2019). The multidisciplinary approach, with advanced competences in fluid mechanics, aquatic biology, biotechnology, environmental, electrochemical and process engineering, as well as the intense capital expenditure and high energy consumption, have so far permitted the development and implementation of large-scale RAS operations in wealthy nations only (Badiola et al., 2012; Bregnballe, 2015; Bostock et al., 2016; Xiao et al., 2019).

Chapter 2: RAS Biological filtration

Water re-use poses several engineering challenges to maintain welfare standards for species in culture. It is estimated that the assimilation performance of feed nitrogen by farmed fish reaches as little as 30%, while the remaining content is released in water (Yogev et al., 2017). Hence, the removal of metabolic wastes becomes a primary concern for cultured species welfare when high degree of dilution, typical of flow through systems, is replaced by land-based intensive recirculating systems (Goddek, 2019; Preena et al., 2017; Lekang, 2019; Ruiz et al., 2020).

Nitrogen is present in RAS water in several forms: bound in dissolved organic molecules (as in urea NH_2CONH_2), in inorganic ionic compounds and in gaseous compounds (Lekang 2019). Common inorganic forms are Ammonia gas (NH_3), Ammonium Ion (NH_4^+), Nitrite ion (NO_2^-), Nitrate ion (NO_3^-) and Nitrogen gas (N_2). The toxicity of each of these forms vary substantially (Lekang, 2019). Total Ammonia Nitrogen (TAN), referring to the sum of ammonia gas and ammonium ions-nitrogen only, is of major concern due to acute toxicity correlated to high mortality rates, while nitrates received recent attention due to chronic toxicity, correlated to oxygen transport impediment at gill level and growth depletion (Xiao et al., 2019; Goddek, 2019; Ruiz et al., 2020). Ammonia is the most toxic form of nitrogen compound in RAS water, with an approximate upper tolerance value of 0.01-0.025 mg/L and with an average LC_{50} of 0.068 mg/L (ECHA, n.d.; Lekang 2019). The other forms' tolerance is approximately 1 mg/L of NH_4^+, 0.08 mg/L of NO_2^- and 180 mg/L of NO_3^- (Bregnballe 2015; Timmons et al., 2018; Lekang 2019).

The removal of nitrogenous compounds in RAS systems is obtained via prokaryotic biodegradation occurring in specific filtration compartments called biofilters (van Rijn et al., 2006; Timmons et a., 2018; Azevedo et al., 2018; Del'Duca et al., 2019; Goddek, 2019; Lekang, 2019; Xiao et al., 2019; Ruiz et al., 2020). The working principle of biofilters lies in their ability to apply the correct environmental pressure and drive the natural selection of desired microorganic communities. Selective chemo-physical conditions, in fact, supports the natural occurrence of a large diversity of nitrifying and denitrifying bacteria and archaea (Goddek, 2019; Lekang, 2019; Xiao et al., 2019; Ruiz et al., 2020). Nitrifying microorganisms are responsible for the oxidation of TAN in aerobic conditions, while denitrifying microorganisms convert nitrates in anaerobic filters (Goddek, 2019; Lekang, 2019; Xiao et al., 2019; Ruiz et al., 2020). The diversity of these communities depends on variables such as fish species, geographic location, temperature, pH, fish feed, carbon to nitrogen ratio, organic load, filter design and mechanical properties (Goddek, 2019; Lekang, 2019).

In well aerated reactors, with low availability of organic carbon and high availability of TAN, aerobic autotrophs, such as ammonia oxidising bacteria (AOBs) and archaea (AOA), oxidise NH_3 and NH_4^+ through ammonia monooxygenase and hydroxylamine dehydrogenase enzymes to construct cell biomass and expand in number, synthesising ethyl cyanoacetate $C_5H_7NO_2$ (Del'Duca et al., 2019; Lekang, 2019; Ruiz et al., 2020). The product is NO_2, then further oxidised to NO_3 by nitrite oxidising bacteria (NOBs) through the enzyme nitrite-oxidoreductase (Lekang, 2019; Ruiz et al., 2020). Further ammonia protonation occurs as consequence of the decrease in NH_4^+ content, given a fraction of the NH_3 gas in the water reacts

with H^+ available, turning into NH_4^+ due to the equilibrium of the two species in solution (Goddek, 2019; Lekang, 2019). Precautionary C/N, chemical oxygen demand (COD) and total suspended solids (TSS) thresholds, as well as a suitable hydraulic retention time (HRT) need establishment to well control bioreactors (Christianson 2016; Goddek 2019). Such control concedes to maintain diverse and resilient communities, promoting a functional level of nitrification and avoiding pathogenic lineages (Rurangwa and Verdegem, 2015).

In anaerobic reactors, abundant facultative and obligatory anaerobic heterotrophs, also supported by some chemoautotroph counterparts, are responsible for dissimilatory nitrogen oxides reduction (van Rijn et al., 2006; Timmons et al., 2018; Lekang, 2019; Goddek 2019; Letelier-Gordo et al., 2020). These consortia grow and thrive by digesting organic compounds using nitrogen oxides in anoxic conditions. A common endogenous source of organic carbon is supplied by the sludge collected via mechanical filtration, to which permeate water from aerobic bioreactors is added to source nitrites and nitrates (van Rijn et al., 2006; Timmons et al., 2018; Lekang, 2019; Goddek 2019; Leterlier-Gordo et al., 2020). However, the use of external carbon sources, such as acetate, glucose, ethanol, and methanol has been largely employed to study and improve the denitrification performance (van Rijn et al., 2006; Timmons et al., 2018; Lekang, 2019; Goddek 2019; Leterlier-Gordo et al., 2020). In a single step reaction, denitrifying bacteria and archaea use the organic carbon molecules from uneaten food and faeces as source of electrons, reducing the nitrates mostly, but also nitrites, to nitrogen gas (N_2) (Tal et al., 2006; Lekang, 2019; Ruiz et al., 2020). In the absence of dissolved oxygen, the nitrogen oxides function as electron acceptors, receiving electrons removed from organic and inorganic carbon compounds (Timmons et al., 2018). The final product of denitrification is elemental nitrogen gas (N_2), although the accumulation of intermediate products, such as nitrous oxide (N_2O) and nitric oxide (NO) occurs under specific conditions, i.e., where low dissolved oxygen concentrations become available (van Rijn et al., 2006). The most crucial determining factor allowing denitrification is an adequate low C/N ratio, recommended in a range between 3.0 and 6.0 of g COD/g NO_3 (van Rjin et al., 2006; Timmons et al., 2018; Lekang, 2019), although factors such as low redox potential, carbon source, optimal pH range (7-8.5) and temperature (23-45 ºC) improve the denitrification performance (Timmons et al., 2018; Schmautz et al., 2020). The presence of minimum -100 oxygen reduction potential (ORP) is also essential to avoid fermentation, where the absence of oxygen compounds drives the synthesis of toxic sulphides (Lekang, 2019). The avoidance of high sulphide concentrations

is important to disfavour organisms operating dissimilatory nitrate reduction to ammonia (DNRA), counteracting the operate of aerobic nitrification (van Rjin et al., 2006).

Nevertheless, the metabolic dynamics in biological reactors are far more complex and diverse, as a wide range of bacteria have been reported to biodegrade TAN and nitrogen oxides in alternative pathways. For instance, species from the genus *Nitrospira*, have been described performing complete ammonia oxidation (comammox) (See in details Ruiz et al., 2020), while several studies described direct anaerobic ammonium oxidation (anammox) to nitrogen gas by several microbial consortia (Tal et al., 2006; Goddek, 2019; Eck et al., 2019; Ruiz et al., 2020). Although nitrate removal is traditionally performed by heterotrophic genera through anaerobic denitrification, special mention should be dedicated to recent findings on aerobic denitrification (Lv et al., 2017). Recently isolated species from the genus *Pseudomonas*, i.e., *P. putida, P. stutzeri* and *P. mendocina*, have been reported reducing N_2O and NOx compounds to N_2 in wastewater treatments and natural streams (Lv et al., 2017). These highly specialised obligatory and facultatively aerobic genera can reduce nitrites and nitrates in the presence of dissolved oxygen (Lv et al., 2017). These species thus infer on important bioaugmentation strategies potentially applicable to RAS to avoid the necessity of separated bioreactors.

The design of aerobic biological filters requires the maximisation of surface area for bacteria biofilm to form as well as the selection of a suitable substrate, water flow, turbulence, and oxygen supply (Lekang, 2019). Bacteria are commonly cultured in submerged substrates, such as in fixed bed and moving bed bioreactors (FBBR & MBBR), fluidised sand biofilter (FSB), where the sandy substrate is suspended by upward water current, but also in emerging substrates, such as trickling filters and rotating biological contactors (RBC) (Xiao et al., 2019; Ruiz et al., 2020). Conversely, denitrification reactors may be designed to hold viscous activated or granulated solid sludge blankets, which can grow in suspension, as in plug-flow or completely mixed reactors. However, biofilms in substrates may be used, e.g., in woodchip, packed bed and MBBR reactors (Christianson et al., 2016; Timmons et al., 2018). Reactors may be operated continuously, in distinct batches and in intermediate fed-batch mode (Fernandes and Cabral, 2016). Fed-batch designs have demonstrated particular tolerance towards flow and turbulence variations, improving construction and operation costs in wastewater treatment. Their applicability to RAS has recently been discussed (*see for details* Letelier-Gordo et al., 2020). In these reactors, the sludge is commonly removed periodically, although some designs do not necessitate a sludge exchange step (Timmons et al., 2018). Inversely to aerobic reactors, the maintenance of an anoxic, organic C and NOx-rich environment is a more fragile equilibrium to be maintained (Lekang, 2019).

Chapter 3: RAS bioreactors microbial communities

Microbial communities are essential to RAS. Consequently, the development of RAS depends on the understanding of all chemo-physical and biological processes determining the microbiota hosted (Goddek 2019). While the evolution of engineering know-how granted a high degree control of abiotic factors, the interaction, diversity, and spatiotemporal dynamics of microbial communities in RAS are still poorly understood and far from being controlled (Goddek 2019). On one hand, the microbial species diversity in RAS responds to environmental selection set by the design. On the other hand, the diversity also depends on specific microniches, thus each RAS compartment is characterised by unstable and unevenly distributed communities (Rurangwa and Verdegem, 2015). Both sessile and free-floating consortia are present in all compartments and piping systems and are constituted by desired bacteria lineages, but also by fungi and microalgae, partially contributing to nitrogen compounds alterations (Rurangwa and Verdegem, 2015).

Designated micro-organisms are commonly inoculated in RAS for 4 to 8 weeks with bioreactors start-up procedures, which aim to select beneficial consortia and achieve desired filtration performance (Rurangwa and Verdegem, 2015; Del'Duca et al., 2019; Ruiz et al., 2020). Inoculation can be carried out passively, transferring fish to the RAS, or actively with highly concentrated inactive "bacteria pasta". (Lekang 2019; Ruiz et al., 2020; Navada et al., 2020). In this case, inoculation is catalysed with acyl-homoserine lactones (AHLs) signalling molecules produced by gram-negative bacteria and extracellular polymeric substances (EPS) produced by heterotrophic bacteria (Lekang 2019; Ruiz et al., 2020; Navada et al., 2020). However, pathogenic, and opportunistic lineages may be unintendedly introduced via alternative pathways, particularly through make up water, air, feeds, stocked fish, and equipment, but also exposure to other animal carriers (i.e., human visitors and staff, insects, domestic and feral animals) (Rurangwa and Verdegem, 2015; Goddek 2019; Ruiz et al., 2020).

In aerobic reactors, the abundant clades of ammonia oxidising bacteria and archaea (AOB & AOA) are mainly constituted by β- and γ-proteobacteria such as *Nitrosomanas* and *Nitrosoccus* and nitrite oxidising bacteria (NOB) such as *Nitrospira*, *Nitrotonga* and *Nitrobacter* genera, but comammox taxa (*Nitrospira sp*.) are also present (French et al., 2012; Rurangwa and Verdegem, 2015; Eck et al., 2019; Ruiz et al., 2020). Although K-selected strategists have been reported to improve fish survival (Vadstein et al., 2018; Goddek 2019), both K-selected and r-selected microorganisms are present in aerobic bioreactors, supporting community changes due to TAN, nitrite, and oxygen shifts (Rurangwa and Verdegem, 2015).

The microbial sensitivity to environmental changes is, in fact, notoriously significant and requires careful management. If the concentration of suspended and dissolved organic matter increases, heterotrophic competitors swiftly develop in outer biofilm layers, compromising nitrification efficiency and producing metabolic by-products, potentially dangerous for autotrophic competitors (Chen et al., 2006; Rurangwa and Verdegem, 2015; Goddek 2019). Small changes in chemical parameters may cause the formation of moderate outer biofilm layers of fast-growing beneficial nitrate-reducing heterotrophs, constituting a physical protective barrier for autotrophic communities established below (Preena et al., 2017). On the contrary, a drastic increase in organic load can promote pathogenic lineages to arise, as in the case of *Vibrio* (Rurangwa and Verdegem, 2015).

Anaerobic denitrification reactors are colonised by gram-negative chemoheterotrophic Proteobacteria such as *Pseudomonas*, *Paracoccus* and *Comamonas* lineages, which play the essential mineralisation conversion of organic waste (Chen et al., 2006; Rurangwa and Verdegem, 2015; Eck et al., 2019). Besides abundant heterotrophic lineages, also chemoautotrophic genera (*Thiomicrospira, Thiothrix, Rhodobacter, etc.*), alternative anammox (*Planctomycetes*) and sulphate reducing groups have been reported harbouring in anaerobic sludge digesters (Rurangwa and Verdegem, 2015; Eck et al., 2019), whilst archaeal communities have so far received little attention in anaerobic reactors (Schmautz et al., 2021). In anoxic stratified substrates, bacteria are significantly distributed according to redox potential: as the dissimilatory pathways are dominant, the competition is based on the presence of electron acceptors, carbon uptake kinetics and efficiency (Robinson et al., 2016). Up to 21% of nitrogen loss efficiency was recently documented in anaerobic denitrification mesophilic digestion, i.e., operating at inner temperatures ranging between 25 and 45°C (Schmautz et al., 2020). This result supports the ongoing effort to develop stable control of anaerobic digesters and achieve wide integration in commercial size RAS.

Chapter 4: Emerging Use of Denitrification Reactors

Traditionally, the solids concentrated by RAS aerobic bioreactors have been either discharged directly into sewer systems or into decentralised water stabilisation ponds (Mirzoyan et al., 2010). In the last two decades, however, more stringent environmental regulations on greenhouse and ozone-depleting gases (CH_4, NO and N_2O), as well as the revealed chronic toxicity caused by nitrates accumulation, encouraged the update of conventional RAS with denitrification bioreactors (Lee et al., 2002; Tal et al., 2006; Hamlin et

al., 2008; Mirzoyan et al., 2010; Goddek 2019). Mirzoyan et al., (2010) published a detailed review on the history of RAS anaerobic sludge reactors development, focusing on the multiple benefits as the novel RAS constituent.

Besides improving the welfare standards of cultured animals, the addition of denitrification bioreactors has significantly increased the water reuse potential (Davidson et al., 2019). This factor suited the argument behind the development of RAS since its origin, i.e., to obtain efficient water recycling and reduce the discharge of wastewater (Xiao et al., 2019). The integration of anaerobic denitrification bioreactors, in fact, can increase the water recirculation capacity up to 99% by decreasing the necessity for make-up water to dilute the NO_3 concentration (Xiao et al., 2019). Supplementary motivation is given by the decrease of 10% in production cost per kg of fish, outweighing the operational and installation costs (Martins et al. 2010). Anaerobic microbial activity, in fact, releases heat and remineralises water, thus cutting expenses for alkalinity regulators and temperature control (van Rijn et al., 2006). More so, the cost of sewer discharge can be significantly reduced, despite initially introducing a new limiting factor: disposing concentrated solid waste (Goddek 2019)

Yet, the nutrients rich solid waste can be an opportunity in aquaponic systems, i.e., where RAS water supplies hydroponic horticulture production (Goddek 2019) Remineralization of aquaculture sludge is obtained via denitrification over long retention time and resulted particularly beneficial in hydroponics systems, given the solubilised macronutrients, with special reference to phosphorus and nitrogenous compounds, can be readily upcycled to plant biomass, adding new sustainable results to denitrification designs (Suhr et al., 2015; Davidson et al 2019; Goddek 2019, Eck et al., 2019; Panama et al., 2020). Both coupled and decoupled aquaponics designs, i.e., where the hydroponics system is fully integrated in the RAS loop or at the loop end, have resulted successful in improving plant welfare, growth rates, and biological protection of roots from pathogens (Eck et al., 2019; Goddek 2019; Panana et al., 2021). Besides promoting growth and plant welfare, the in-situ step of remineralisation has reduced waste products amounts and cut expenses for disposal and purchase of complementary minerals.

The possibility to further improve the economic feasibility of anaerobic reactors is also given by the synthesis of biogas, especially methane, produced by anaerobic activity (Mirzoyan et al., 2010; Goddek et al., 2019). Delaide et al., (2019) demonstrated how an up-flow anaerobic sludge blanket reactor (UASB) could reduce more than 90% of solid waste and convert over 50% of carbon introduced to methane (Goddek et al., 2019). Mirzoyan et al., (2010)

summarised the results of many similar studies and inferred that 2 to 5% of RAS energy requirement could be satisfied by the methane produced in-situ.

Important motivation is currently given by the potential biological control of 'earthy' and 'musty' off-flavouring metabolites released by opportunistic microorganisms in RAS. These compounds, primarily trans-1,10-dimethyl-trans-9-decalol (geosmin) and 2-methylisoborneol (MIB) in RAS water, are produced by cyanobacteria, myxobacteria and fungi and are associated to the accumulation of phosphates and organic solids (Rurangwa and Verdegem, 2015; Goddek 2019). These compounds are absorbed by the farmed fish and deposited in the tissue and fat, reducing palatability and market demand. Genetic tools developed so far, such as geosmin-synthesis gene (geoA) qPCR detection and quantification, promote successful early recognition but do not provide a robust avoidance strategy based on system conditions control by RAS operators (Rurangwa and Verdegem, 2015). Consequently, depuration from off-flavouring compound is commonly attempted at the expenses of water recycle, with fish temporarily residing in flow-through purging tanks before harvest (Guttman and van Rijn, 2009; Schram et al., 2017). Alternative ozonation and chemical removal protocols have not resulted successful on large scale and caused toxicity and bioreactors crashes. Preferably, a substantial reduction of off-flavouring compounds could be achieved by developing an effective system control promoting beneficial microbial competitors (Rurangwa and Verdegem, 2015). The biodegradation of geosmin and MIB was reposted in in vitro studies in denitrification sludge reactions primarily due to the presence of genus *Pseudomonas*. A complete removal of these compounds was described after 9 days incubation, although other lineages such as *Rhodococcus*, *Variovorax* and *Comomonas* were found greatly reducing these compounds in both aerobic and anaerobic conditions (Guttman and van Rijn, 2008; Rurangwa and Verdegem, 2015). Consequently, the stabilisation of bioreactors communities could inhibit the proliferation of opportunistic lineages and directly degrade off-flavouring compounds.

Although at present a large consensus on the effectiveness of denitrification reactors is published in the literature, the employment of these treatment technologies, either within or at the end of the recirculation loop, has not been extensive (Davidson et al., 2019; Goddek 2019). This consensus derives primarily from municipal wastewater treatment plants and some proof-of-concept designs, while limited information is published on RAS anaerobic digesters (Goddek 2019; Davison et al., 2019). While the list of potential environmental and economic benefits is extensive, the full-scale implementation of denitrification bioreactors has been counteracted by several challenges. On one side, wariness has characterised the private exploration of novel biological reactors, being these the most common source of system failure

in RAS (Xiao et al., 2019). On the other side, the high potential for toxic sulphide and ammonia production and the costs related to assuring external carbon sources motivated the lack of risks taken in employing an anaerobic denitrification step at commercial scale (Tal et al., 2006; Hamlin et al., 2008).

After all, the most essential shortcoming remains in the lack of fundamental research in microbial ecology, allowing to characterise and control microbial communities in RAS (Robinson et al., 2016; Azevedo et al., 2018; Goddek 2019). Heterotrophic lineages have received little attention compared to the autotrophic counterparts, thus large part of information on community composition, whether pathogenic or beneficial, is essentially missing (Riuz et al., 2020). While DNA molecular tools have revealed communities' structural alterations in response to changes in water chemo-physical parameters at RAS level, the chemical interaction between different microorganisms' lineages, whether allelopathic or benign, are unknown (Riuz et al., 2020). On one side, metagenomics and meta-transcriptomics analyses, high-throughput sequencing techniques, are bound to shine light on RAS maturation pathways by characterising the gene expression at different maturation stages (Riuz et al., 2020). On the other side though, the discovery and characterisation of metabolites, e.g., novel signalling molecules which promote substratum adherence and biofilm formation, would allow a direct control on bioreactor maturation speed, and improved the operational capacity of nitrification-denitrification (Riuz et al., 2020).

Besides improving bioreactors operational efficiency, the discovery and market development of microbial natural products could bring multiple other benefits, such as the characterisation of growth inhibiting factors, likely to impair development at embryonal and larval stages for some fish species (Martins et al., 2009a) and the rise of pathogenic competitors. Some diverse genera commonly harbouring in RAS, such as *Pseudomonas* and *Vibrio*, have already been described as source of natural products (Blunt et al., 2018), thus bioprospecting for bacterial metabolites can add a key motivation to characterise the compounds diversity of these systems. The necessity for cost effectiveness and agile analytical tools to explore the chemical dynamics of bioreactors suggests the employment of top-down molecular techniques to characterise key metabolites diversity and their possible employment (Vinayavekhin and Saghatelian, 2010). A potentially successful strategy can be identified in the application of targeted and untargeted metabolomics analyses.

Chapter 5: Untargeted Metabolomics.

Metabolomics are a diverse range of novel analytical tools allowing to describe the metabolome of a biological systems, i.e., the complete set of metabolites found within a sample. Metabolites are small-molecule compounds and constitute the direct product of enzymatic and protein activity. These compounds are the phenotypic outcome of all cellular metabolic processes and provide either primary or secondary metabolic functions (Vinayavekhin and Saghatelian, 2010; Worley and Powers, 2013). Primary metabolites are essential cell products, such as amino acids, organic acids, fatty acids, sugars, etc., and provide basic growth, reproduction, and maintenance functions, whilst other metabolites, as alkaloids and phenylpropanoids, support intercellular communications, defence, and other secondary functions (Commisso et al., 2013). Metabolites currently represent the most accurate biomarkers in nature (Worley and Powers, 2013) and have been employed in most fields of biology, ecology, ecotoxicology, and medicine (Pop et al., 2014). Alteration of these compounds, in fact, can only occur through changes in the expression level of source genes, differently from the changes in activity levels of proteins, promoted by several alternative factors (Worley and Powers, 2013), such as posttranslational modifications (PTMs, Kuile and Westerhoff, 2001; Patti, 2011; Friso and Van Wijk, 2015). Worley and Powers (2013) described metabolomics as "the quantitative measurement of the multiparametric metabolic response of living systems to pathophysiological stimuli and genetic modification". Thus, metabolomics analyses are essential nowadays to understand the impact of biotic and abiotic stressors on natural system of any level of complexity (Martin et al., 2015). Nevertheless, the highly reliable employability of metabolites as descriptive biomarkers is counteracted by immense diversity and abundance of these, cursing their understanding with great complexity (Worley and Powers, 2013).

Metabolomics analysis can have specific biomarker targets, in which case the presence/absence and concentration of target molecules define the system response to pre-determined conditions (Vinayavekhin and Saghatelian, 2010). This is the case of metabolic *profiling* and *footprinting*, a longer and more challenging procedure where compounds expected to be involved in a specific metabolic pathway are quantified (Krishnan et al., 2005; Vivanco et al., 2011). Conversely, untargeted metabolomics measures the entirety of ionised molecules present in a sample, providing clear ranges of mass values (Vinayavekhin and Saghatelian, 2010). This is the case for metabolic *fingerprinting*, which seeks to determine and compare the global spectral patterns of metabolomes at steady and perturbated states with

unbiased multivariate analyses (Krishnan et al., 2005; Vivanco et al., 2011, Worley and Powers, 2013). The substantial difference between these techniques is the aim: on one side targeted metabolomics answers to *which* health condition the metabolome corresponds to, provided a finite number of pre-defined possibilities, while untargeted analyses aim at defining *how* systems change at global metabolites level, without hypothesis a priori (Patti, 2011; Commisso et al., 2013). Thus, untargeted metabolomics allows to discover novel metabolites, describe new systems chemical dynamics/behaviours, and define potential reliable biomarkers for subsequent targeted approach (Vinayavekhin and Saghatelian, 2010).

Nuclear magnetic resonance (NMR), gas chromatography-mass spectroscopy (GCMS) and liquid chromatography-mass spectrometry are the most popular metabolomics assays (Commisso et al., 2013). NMR is a rapid and automated analysis capable of giving some structural information and it is based on how energy is absorbed and re-emitted by atom nuclei when variations of magnetic field are applied (Alonso et al., 2015). Conversely, mass/charge ratio (m/z), retention time and relative intensity per compound are the main spectral data collected by MS techniques. With these, the analytes are ionised to generate a molecule-specific spectrometric peak patterns, or fingerprints, which are recorded on a chromatogram over the retention time (Alonso et al., 2015). A chemical separation step is required before mass spectroscopy: volatile compounds are targeted with GCMS while thermally instable non-volatile compounds are measured with LCMS, considered the most sensitive analysis (Commisso et al., 2013). These approaches have however resulted highly consistent in representing metabolic profiles, regardless of the vast heterology of the methods (Martin et al., 2015). De facto, the performance of instruments, types of deconvolutions, configurations, and efficacy with or without prior standardisations, have revealed the strong inter-instrument's reliability of NMR, GCMS and LCMS and all their variants (Martin et al., 2015).

Metabolomics outputs enormous amounts of data which need to be analysed at high confidence levels. The recent advancement of automated spectral processing tools, such as XCMS and MetAlign, has granted the opportunity for rapid processing of high-throughput data (Commisson et al., 2013). These software allow the subtraction of background noise from initial batch chromatograms and the recognition, qualification, and alignment of signals to construct data matrices (Commisson et al., 2013). Yet, to extract meaningful patterns, the large variability of metabolomics data requires important reduction of dimensionality prior to and during statistical analyses. The validity of metabolomics analyses, in fact, can be highly affected by missing or below detection threshold values and outliers, especially when multivariate analyses are planned (Scholz and Selbig, 2007). Further biases are commonly

introduced by technical errors such as machine drift across samples and unpredictable variations of metabolites concentrations (Li et al., 2016). Hence, to avoid biased results, raw data require filtering strategies, and biological background information can often guide data-treatments techniques. Delineating context-based biological assumption can be propaedeutic to comprehend potential biotic and abiotic factors influencing the source of metabolites (van den Berg et al., 2006). Important procedures, namely filtering, missing values imputation and normalisation, ascertain the obtainment of cleaner datasets where relevant biological information are distinguished from measurement noise (van den Berg et al., 2006). Common baseline corrections consist in the removal of low and high frequency outputs when exceeding sensible retention time and peak intensity thresholds (Alonso et al., 2015). This is, for instance the case of the commonly employed "80% rule" or the class-adjusted version of this (Yang et al., 2015). A large variety of other strategies is published and the efficacy of one algorithm over another depends on the source of the metabolites, biotic and abiotic parameters (Grace and Hudson, 2016). Important is also the use of randomised value data imputation techniques to replace signals detected below thresholds and avoid biased results downstream the analyses (*see for details* van den Berg et al., 2006, Scholz and Selbig, 2007; Yang et al, 2015; Li et al., 2016; Shah et al., 2019). Nevertheless, the expertise of natural products scientists may result the most reliable resource available when novel biological systems with absent literature information are analysed.

Statistical analyses of untargeted metabolomics datasets aim to identify few key variables thoroughly summarising the overall metabolome complexity (Worley and Powers, 2013). This is the case of multivariate projections, which define biologically relevant spectral features by spatially summarising the dataset over few significant axis or clusters. Some of the most common statistical methods employed are principal component analysis (PCA), partial least square (PLS), independent component analyses (ICA) and hierarchical clustering analyses (HCA, Worley and Powers, 2013; Manier et al., 2019). These projection methods are usually followed by multivariate tests, where high collinearity and accumulation of false positive error need to be carefully considered (Worley and Powers, 2013).

Recently, the use of metabolomics analyses to improve wastewater management has been discussed. Yang et al., (2019) argued that DNA molecular markers are unreliable as a diagnostic tool to punctually describe the spatiotemporal structure of microbial communities in wastewater filtration technologies. While DNA sequencing analyses are successful in depicting subsurface wastewater infiltration systems (SWIS) at steady state, their applicability is inadequate to characterise the initial deviation of microbial community health, which

determines delayed community structure changes, and only at last causes the deterioration of effluent water quality. Consequently, a rapid detection method is necessary to ensure correct operation and exposing the spatiotemporal response relationships between microbial structures and water quality under the influence of multifactorial perturbations (Yang et al., 2019). The study supported the employment of untargeted metabolomics analysis such as UPLC-MS as a rapid and accurate alternative to analyse the presence of endogenous substances to then become reliable biomarkers for targeted assays (Yang et al., 2019).

Likewise, to our knowledge neither untargeted or targeted metabolomics assays have ever been applied to describe RAS bioreactors' microbial community's health and dynamics. The development of integrated analytical protocols, where untargeted and targeted metabolomics are combined with transcriptomics and RNA sequencing, can help constructing a holistic picture of microbial life, of changes in growth conditions due to disturbance and uncover unexpected metabolic pathways (Baran et al., 2013; Cho et al., 2015). To open the door for the employment of these techniques in RAS aquaculture, the first knowledge gaps to fill regard whether metabolomics analyses can successfully represent expected metabolic dynamics resulting from pre-selected chemical and physical properties. If successful, besides filling important biochemical knowledge gaps, these assays may become the source of cost-effective diagnostic tools to improve the operational control of RAS systems.

Chapter 6: Vida Project

During 2019 and 2020, as part of the ERANET project Geofood (www.geofood.eu) and the H2020 VIDA project (vidaproject.eu), Landing Aquaculture B.V. and Wageningen University ran a 12-month semi-commercial 50 m^3 RAS connected to a lettuce hydroponic sub-unit. The RAS was fitted with a 10 m^3 aerobic moving bed bioreactor (MBBR) and a 1.2 m^3 anaerobic batch-fed denitrification reactor (BFDR) to carry out nitrification and sludge denitrification-mineralization processes, respectively.

The MBBR was operated with a strong aeration to maintain bio-media in suspension. The vessel was split in two chambers connected via a slotted grid, where water moved horizontally due to unidirectional flow. The designed aimed at enhancing nitrification efficiency: the first cell aimed to harbour a larger abundance of heterotrophs, digesting the suspended solids escaped from the mechanical filter upstream, while the second chamber, aimed to better support autotrophic nitrifier development.

The experiment also assessed the denitrification performance of a novel anaerobic digester design developed by Landing Aquaculture. The BFDR was operated with a central airlift riser, operating for 15 minutes every hour, developed to apply an up-flow stream of large air bubbles. This design meant to maintain the sludge well mixed so to avoid the formation of a scum layer, which can cause severe fouling and impedes the correct functioning of common up-flow anaerobic sludge blanket reactors (UASB). Dissolved oxygen concentration was always kept below 0.2 mg/l to allow rapid resuming of denitrification reactor interrupted by the airlift mixing. The bottom sludge was expected to develop a diverse community of facultative and obligatory heterotrophic and autotrophic denitrifiers (Rurangwa and Verdegem, 2015; Letelier-Gordo et al., 2020)

By the end of the experiments, both biological reactor units were sampled for metabolomic fingerprinting. The sampling design aimed at evaluating the metabolic differences within and between the units and describe the metabolomes behaviour and dynamics. Liquid chromatography–mass spectrometry (LCMS) was employed to investigate the metabolite fingerprints present. Untargeted metabolomics analyses were performed by the plant metabolomics group at the business unit for Bioscience and Plant Sciences of Wageningen University & Research. The protocols employed were developed to obtain the accurate mass LCMS employing the platform Q Exactive Orbitrap FTMS. All compound signals detected were considered and raw data files were processed using a dedicated workflow for high-throughput untargeted data analyses.

Objectives

The proposed work for this master's thesis is to conduct a pilot prospective analysis of the acquired metabolomics database and identify significant metabolome dynamics.

This assessment will explore:

- the metabolomic differences within each biological reactor (inlet vs outlet and benthic sludge vs supernatant water), and
- the differences between the two biological reactors, to highlight their impact upon the metabolome of the RAS system.

The pilot work should conclude with an evaluation of the applicability of metabolomics analysis to successfully describe expected and unexpected metabolome trends across biofiltration units and promote the use of this technique to give insight on important knowledge gaps in the biochemistry of aquaculture circular production systems, opening the door for potential novel bioprospecting discoveries and operational improvements.

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Metabolic fingerprinting of Recirculating Aquaculture Systems: a pilot application of untargeted metabolomics to describe biological rectors.

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Abstract

Land-based recirculating aquaculture systems (RAS) can boost food security and environmental sustainability worldwide. In RAS, the removal of nitrogenous waste is obtained via prokaryotic biodegradation. However, the spatiotemporal metabolic dynamics of microbial communities in RAS are poorly understood. Understanding these trends can generate operational improvements. The necessity for fast and cost-effectiveness analysis suggests the employment of top-down molecular techniques. This pilot study evaluated the applicability of untargeted metabolomics to describe metabolic trends in RAS bioreactors. We compared two reactor designs, a two-stage moving bed bioreactor (MBBR) and an anaerobic batch fed sludge bioreactor (BFDR), as well as different locations within each of these (i.e., inlets vs outlets). As expected, a differentiation in metabolomic fingerprints determined by different chemo-mechanical properties was highlighted between the two bioreactors. However, contrarily to our initial hypotheses, no differentiation was found within the MBBR. Possible explanation can be identified in a vessel-wide distribution of a single microbial community, as well as in the influence of ammonia-limiting conditions and short hydraulic retention time. Unexpectedly, however, a significant differentiation was found within the BFDR, where two distinct metabolic fingerprints were recorded, inferring on the presence of a community in suspension to be further investigated. We demonstrated how metabolomics analysis can reveal RAS bioreactor metabolic dynamics and at the same time expose the unsuccessful design of specific biomechanical properties.

Keywords

RAS, bioreactors, MBBR, BFDR, untargeted metabolomics, microbial community, metabolic fingerprinting

1. Introduction

The natural environment can no longer withstand the current magnitude and diversity of direct wildlife extraction imposed by fishing (Ferri, 2010; Christensen et al., 2014; Timmons et al., 2018; Bindoff et al., 2019; Bradshaw et al., 2021). Declining wild fish stocks are coupled with unsustainable human population growth in all projections. Hence, the stagnant output from fisheries requires a substantial expansion of the aquaculture production to meet the forthcoming market demand (Tal et al., 2006; World Bank, 2014 Christensen et al., 2014; Eu 2017; Bindoff et al. 2019; FAO, 2020; Bradshaw et al., 2021). Additionally, the necessity for sustainable products appeals for the development of technologically advanced land-based systems (Tal et al., 2009; Allsopp et al., 2008; Ferri, 2010; World Bank, 2014; Bostock et al., 2016; EU 2017; Xiao et al., 2019; Ruiz et al., 2020). Among these, Recirculating Aquaculture Systems (RAS) have the potential to greatly expand provisions while substantially reducing socio-ecologic impacts associated with some traditional aquaculture systems (Timmons et al., 2018; Lekang 2019). Today, RAS provide the highest yield per unit area with the smallest environmental footprint and water requirements (Tal et al., 2009; Martins et al., 2010; Timmons et al., 2018; Goddek, 2019).

Nevertheless, water re-use in RAS poses several engineering challenges to maintain animal welfare and environmental compliance (Goddek, 2019; Preena et al., 2017; Lekang, 2019; Ruiz et al., 2020). Nitrogenous compounds are the main pollutants in RAS, as they can both affect fish welfare and promote eutrophication. (Letelier-Gordo et al., 2020; Robinson et al., 2021; UKRI De-risking RAS, 2021). The removal of nitrogenous compounds in RAS is achieved in bioreactors which promote prokaryotic organisms-mediated biodegradation (van Rijn et al., 2006; Timmons et a., 2018; Azevedo et al., 2018; Del'Duca et al., 2019). Bioreactors are designed to apply an environmental pressure that promotes microbial lineages performing the desired biodegradation activity. In RAS, chemoautotrophic lineages are employed for the oxidation of total ammonia nitrogen (TAN) to nitrites (NO_2) and then nitrates (NO_3) under aerobic conditions, while heterotrophic consortia are used to convert nitrate to nitrogen gas (N_2) under anoxic conditions (Goddek, 2019; Lekang, 2019; Xiao et al., 2019; Ruiz et al., 2020). Bioreactors are essential in RAS, which makes the understanding of their biochemical dynamics indispensable.

While bioreactor design provides a high degree of control over abiotic factors, the interaction, diversity, and spatiotemporal dynamics of microbial communities in RAS are still poorly understood (Goddek 2019). The most essential shortcoming remains the lack of

fundamental research in microbial ecology, allowing to characterise and control microbial communities' composition in RAS (Robinson et al., 2016; Azevedo et al., 2018; Goddek 2019). DNA molecular tools such as metagenomics and meta-transcriptomics have so far revealed RAS microbial communities' structure alterations in response to changes in water chemo-physical parameters (Riuz et al., 2020). However, the chemical interaction between different microorganisms' lineages, whether allelopathic or benign, remain unknown (Robinson et al., 2021). The necessity for cost effectiveness and agile analytical tools suggests the employment of top-down snapshot molecular techniques (Vinayavekhin and Saghatelian, 2010). A potentially successful strategy can be identified in untargeted and targeted metabolomics analysis. Metabolites, represent some of the most accurate biomarkers in nature (Worley and Powers, 2013) and metabolomics have been employed in most fields of biology, ecology, ecotoxicology, and medicine (Pop et al., 2014).

Recently, Yang et al., (2019) argued that while DNA sequencing analyses are successful in depicting wastewater systems at steady state, their applicability is inadequate to punctually characterise the initial deviation of microbial community's health, which determines delayed structural changes and only at last causes effluent water quality deterioration. Consequently, the employment of untargeted metabolomics analysis has been promoted as a rapid and accurate alternative to determine the presence of endogenous substances with the potential to become reliable biomarkers for targeted metabolomics (Yang et al., 2019). Similarly, to our knowledge, neither untargeted nor targeted metabolomics have ever been applied to describe RAS. These analytical tools could allow several improvements in the design and operational management for RAS builders i.e., by relating metabolic fingerprints to bioreactor functionality. Additionally, diverse bacteria genera commonly harbouring in RAS, such as *Pseudomonas* and *Vibrio*, have already been described as a source of natural products (Blunt et al., 2018), thus bioprospecting for bacterial metabolites can add a key motivation. The discovery and characterisation of commercially valuable microbial compounds, e. g., quorum sensing molecules promoting biofilm formation, could open the door to improve bioreactor performance (Solano et al., 2014, Ruiz et al., 2020). Microbial-mediated control of RAS off-flavouring compounds, such as trans-1,10-dimethyl-trans-9-decalol (geosmin) and 2-methylisoborneol (MIB) and isolation of plant bio-stimulant in aquaponic systems are only some of the possible beneficial outcomes (De Vos et al., 2007; Guttman and Van Rjin 2008; Goddek, 2019; Azaria et al., 2020).

This pilot study aimed to evaluate the applicability of untargeted metabolomics analysis to describe metabolome trends in two bioreactors connected to a RAS and operated with

different chemo-physical conditions. More specifically, our objective was to identify metabolic differences between a two-stage moving bed bioreactor (MBBR) and an aerobic batch fed sludge bioreactor (BFDR), as well as within each of these reactors (i.e., inlets vs outlets). Due to the bioreactors design and operation, the two reactors were hypothesised to produce different metabolic fingerprints, depicting the difference among microbial communities hosted.

The two-stage MBBR aerobic reactor was hypothesised to hold different metabolic fingerprints in each stage (Weiss et al, 2005; Ciesielski et al., 2010; Casas, 2015; Torresi et al., 2018). Due to reactor staging and unidirectional flow, this design was assumed to promote two distinct microbial communities: the first cell would harbour a larger abundance of heterotrophs, while the second cell would better promote autotrophic nitrifier development, once the organic matter content is reduced by in the previous chamber. Conversely, the anaerobic reactor lacked suspended biofilm carriers, hence it was expected to develop a single metabolome, produced by a diverse community of facultative and obligatory heterotrophic and autotrophic denitrifying microorganisms in the sludge (Rurangwa and Verdegem, 2015; Letelier-Gordo et al., 2020).

2. Materials and Methods

2.1 System Design and operation

During 2019 and 2020, as part of the ERANET project Geofood (www.geofood.eu) and the H2020 VIDA project (vidaproject.eu), Landing Aquaculture B.V. (Netherlands) and Wageningen University & Research (Netherlands) ran a 12-month semi-commercial RAS connected to a lettuce hydroponic sub-unit, located in Bleinswijk, the Netherlands. The RAS consisted of cylindrical and octagonal Cornell dual drain tanks with volumes from 0.5 to 10 m^3 and was fit with a 10 m^3 aerobic MBBR and a 1.2 m^3 anaerobic BFDR to carry out nitrification and denitrification-mineralization processes, respectively. The RAS was stocked with 3000 0.2 g red tilapia fingerlings (*Oreochromis niloticus*, Til-Aqua International, The Netherlands). The first batch of 1500 fingerlings entered the RAS on August 14th, 2019 and the second batch of 1500 fingerlings arrived on December 4th, 2020. During the experiments, the RAS was brought to its design feed load of 20 kg of feed/day. The MBBR was operational at first stocking, while the BFDR was started in November 2019. Both bioreactors were sampled for metabolomic fingerprinting from February to May 2020. A schematic diagram of the RAS bioreactors used in this study is shown in Fig. 1.

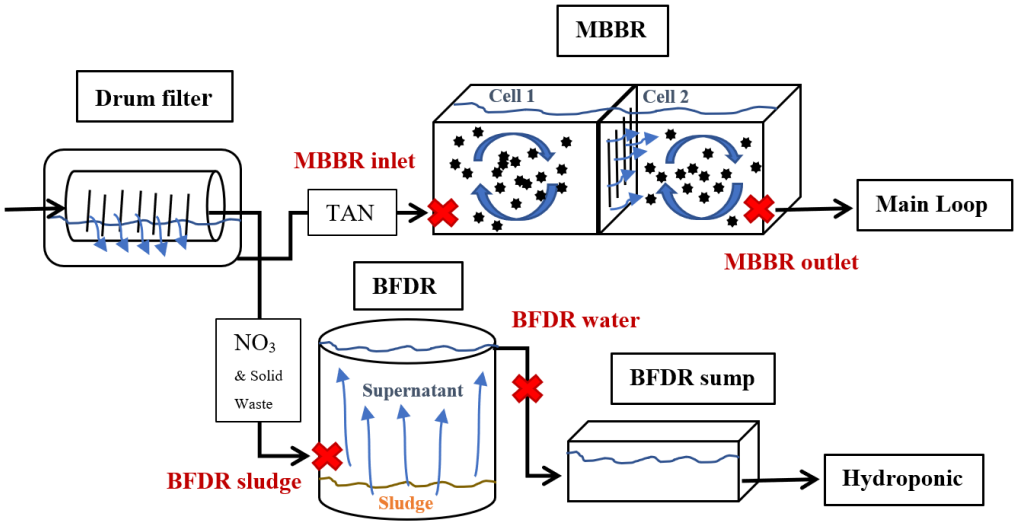

Figure 1. *Schematic diagram of the recirculating aquaculture system used in this study. The system included a two stages (cells) aerobic moving bed bioreactor (MBBR) and anaerobic bath fed denitrification reactor (BFDR) loops for complete removal of nitrogen by nitrifying and denitrifying processes, respectively. The solid black arrows indicate the general path of water flow connecting the compartments and the blue fine arrows the water flow within each unit. The sample locations are indicated by red crosses.*

The MBBR received process water from the drum filter, where particles greater than 60 µm were captured and moved to the BFDR. The MBBR was split in two cells (stages), each being fully mixed by strong aeration supporting vertical circular flow to maintain the carriers in suspension. Each cell was filled with 2.5 m^3 of RK Bio-elements media (RK Plast, Denmark) with a specific surface area (SSA) of 750 m^2/m^3. Filtered water flowed across each stage through a media sieve.

The BFDR was separated in three units: 1) a sump tank capturing drum filter sludge and pumping it into the main reactor, 2) a 2.1 m high and 1 m wide cylindrical reactor, with an inlet at 30 cm from the bottom and overflow weir outlet at the top, and 3) a separate pump sump capturing the reactor's outflow for disposal or further treatment. The BFDR received the sludge captured by the drum filter (> 1 % dry matter). The reactor was run in a sequence of fill and drain, mix, and react stages lasting 30, 15 and 120 minutes, respectively. Reactor filling from the sump tank caused the overflow of reactor bulk content to be discharged by the outlet. The fill and drain step changed 25 % of the reactor volume each time. An airlift riser at the centre of the reactor was used as mixing device, running for 15 minutes per hour. Thus, the reactor was always kept mostly anoxic, with dissolved oxygen increasing to 0.2 mg/l during mixing.

2.2 Sample collection and preparation

Four sample locations were determined, two per reactor (Fig. 1). In the MBBR, the two sample locations corresponded to the inlet and the outlet (Fig. 1) and 5 replicates per location-were collected, approximately 30 cm from the bottom. Both sampling locations were within the aerobic vessel. In the BFDR, 3 replicates were collected at the inlet, approximately 30 cm from the bottom and close to the deposited sludge. 5 replicates were collected past the BFDR outlet (Fig. 1), at the pipeline connecting the BFDR to the sump unit. Here, the cascading wastewater was sampled before it reached the sump vessel. The lower number of replicates collected at the BFDR inlet hinged on the difficult access and necessity for little disturbance.

The samples, each being 10 ml sample in 50 ml tube, were prepared as follows: 18 x 10 ml samples were placed in 50 ml tubes, then freeze-dried for transport and storage. 0.5 ml of a mixture of 75 % methanol + 0.1 % formic acid was added. The sludge samples had larger dry residue; thus 2 ml was added instead of 0.5 ml. Each sample underwent 15 min sonication, followed by 15 min centrifugation at 5000 g. The supernatant was transferred to 1.5 ml Eppendorf tubes and receive 15 min centrifugation at 20000 g. Lastly, 180 µl of protein-free, clear supernatant samples were transferred to high performance liquid chromatography (HPLC) vials.

2.3 Liquid Chromatography-Mass Spectrometry

Mass liquid chromatography – mass spectrometry (LCMS) was employed to investigate the metabolite profiles present in the bio-filtration units. The untargeted metabolomics analyses were performed by the plant metabolomics group at the business unit for Bioscience, Plant Sciences of Wageningen University & Research (WUR, The Netherlands). The following protocol was developed to obtain accurate mass LCMS, where all compound signals detected were considered and raw data files were processed using the in-house dedicated workflow for untargeted data processing.

The analytical platform Quadrupole ExactivePlus Orbitrap Fourier Transformation was employed for LC-MS/MS. The analytical workflow proceeded as following: 1) filtration: extracts were injected one-by-one into ultra-high performance liquid chromatography, where they were passed through a suitable LC column to separate the individual constituents before entering the photodiode array detector, 2) ionisation: extracts were ionized in both positive and negative modes at the source of the Quadrupole Exactive Orbitrap FTMS. The S-lens at the source filtered the ions from non-charged compounds and impurities, and 3) mass spectrometry: for untargeted analyses, the Q ExactivePlus Orbitrap FTMS mass analyser

detected simultaneously hundreds to thousands of metabolites within a wide m/z window at high mass resolution. The spectrometer has both the ability for scan-to scan polarity switching to cover as many metabolites as possible and simultaneously collecting high collision dissociation fragments for compound confirmation and partial characterization.

2.3 Untargeted Metabolomics Data Mining

The WUR standard, proven MetAlign-MSClust based workflow was applied for untargeted metabolomics analyses. MetAlign software (Lommen, 2009) was used for peak picking and alignment. MSClust (Fraley and Raftery, 1999) was used to group all signals originating from the same compound. The product of LCMS fingerprinting consisted of a database containing all compounds, comprehending both positive and negative ionization modes, and their relative intensity in each sample. The in-source mass spectra also included natural 13C isotopes, ionization adducts, ion-source mass fragments, and masses from co-eluting compounds. Metabolite intensities referred to the total of ion counts (i.e., all clustered ions per compound), in which, for each mass, its original intensity value is multiplied by its cluster membership.

Compounds were not annotated or quantified by standard methods. This pilot study aimed to complete a first explorative analyses applying a pragmatic and cost-effective approach, hence detecting global fingerprints differences between sampling locations, rather than identifying any compound. Therefore, no specific MS/MS of compounds was performed as it required additional LCMS runs and data processing. Nevertheless, the putative molecular ion mass of few compounds was identified based on the relative intensities. The identifications were manually performed solely based on the accurate mass of the putative molecular ions, with deduced elemental formula within 5 ppm mass accuracy. The putative molecule identification was obtained for future bio-prospective analyses.

2.5 Data pre-processing

The detection threshold was set at 50000 mass peak ion count based on our experience. As far as the authors are concerned, this is the first report of metabolomics analyses applied to RAS microbial communities and no clear consensus on the “best” employable mass peak ion count threshold was found in the literature.

A first filter was applied on retention time (rt) and variables were retained only between 5 and 45 minutes. The salts in solutions are commonly measured below 5 minutes rt while the peaks present above 45 minutes can represent noise and accumulated residuals from previous LCMS runs. In addition, variables were removed if not detected in at least one location for a

minimum of 3 replicates above detection threshold. Hence, all compounds at least present in more than 60 % of samples of MBBR inlet, MBBR outlet and BFDR water, and in all replicates of BFDR sludge met selection criteria.

Subsequently, all values below detection threshold were randomized between 20000 and 30000 mass peak ion count, with a mean set approximately to 50 % of detection threshold. The strategy aimed at avoiding biased results downstream the analyses in a time and cost-effective manner, as well as at avoiding the poor performance of low constant value imputation and other methods reported (*see for details* van den Berg, 2006; Scholz and Selbig, 2007; Li et al., 2016; Shah et al 2019).

2.6 Data pre-treatment

Transformation and scaling steps were performed in R (version 3.6.3). The reduced dataset was normalised using base 2 Logarithmic transformation (Grace et al., 2016). Pareto scaling was performed on log transformed data with mean centring applied (van den Berg et al., 2006). The scaled metabolite intensities were obtained using the "vegan" package (Oksanen et al., 2020).

2.7 Statistical Analyses

All analyses were performed in R (version 3.6.3). Four projection methods were tested to reduce the dimensionality of dataset and spatially visualise the variance between and within reactors using all variables and all replicates. First, the unsupervised multivariate projections principal component analysis (PCA) was employed using packages "MASS" and "factoextra" (Venables and Ripley, 2002; Kassambara and Mundt, 2020). The percentage contribution to the first three PCA axes' variance was assessed for the 20 most influential compounds using package "FactoMinerR" (Le et al., 2008). Hierarchical clustering analysis (HCA) was employed from package "ape" (Paridis and Schliep, 2019), followed by multiscale bootstrap resampling to validate groups (1000 permutations, package "pvclust", Suzuki et al., 2019). Nonmetric multidimensional scaling was also performed using "vegan" package (Oksanen et al., 2020; stress factor; 0.05, optimal number of axis: 2). Subsequently, a supervised Linear Discriminant Analysis (package "MASS"; optimal number of axes: 2) was performed considering location membership as projection supervisor to highlight relationship between and within reactors.

Normality and multivariate dispersion of data were checked using the Henze-Zirkler's test (package "MVN"; Wu et al., 2020) and the Marti Anderson's PERMDISP2 procedure (package "vegan", Oksanen et al., 2020), respectively. Data did not meet the normality

assumption, but the multivariate dispersion was respected (*see results*). Therefore, overall statistical differentiation was investigated using a permutational multivariate analysis of variance (PerMANOVA; 999 permutations, package "vegan"). Post hoc pairwise tests (package "vegan") were run to assess the pairwise difference within and across reactors and resulting *P*-values were corrected using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1998). The level of significance used in all tests was $P < 0.05$.

3. Results

3.1 Metabolomics fingerprints

In total, 243356 signals in positive ionization mode and 89870 signals in negative ionization mode were detected and submitted to peak picking and alignment. A total of 3000 compounds were found based on grouping signals originating from the same compound. Of this, however, only 1854 compounds (1052 in positive mode and 802 in negative mode) met selection criteria for biological meaningfulness and were considered in the statistical analyses. Figure 2 shows an example of the LCMS location fingerprint: the chromatograms represent all five BFDR water samples in positive ionization mode.

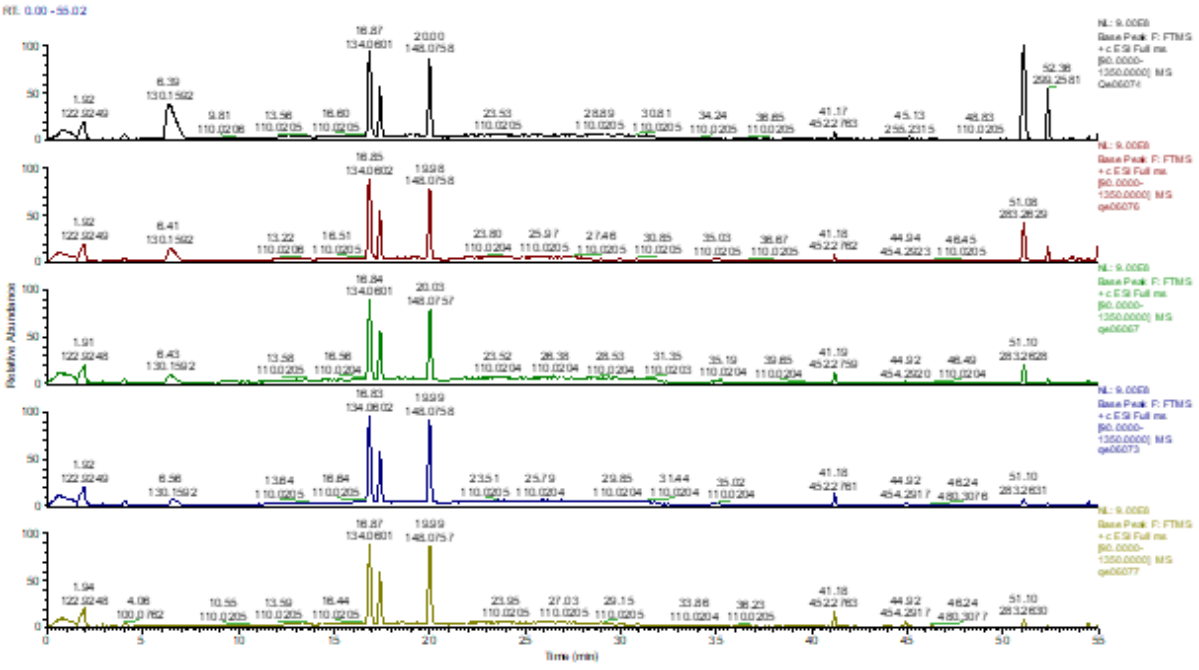

Figure 2. *LCMS profiles in positive ionization mode of replicate samples from BFDR water. The retention time (minutes) is read from left to right of each spectrum (top number on peaks) and the relative abundance of each compound on a vertical axis (bottom peak number).*

Overall, the putative and manually performed identifications pinpointed the presence of 23 compounds based on the accurate mass of the presumed molecular ions, where the deduced elemental formula are within 5 ppm mass accuracy. Between the compounds with potential market use, proline ($C_5H_9NO_2$) was predominantly found in the BFDR supernatant water.

3.2 Projections

All projections methods led to equivalent results, and we here only present results from PCA and HCA (see results for other projection methods in *annexes III and IV*).

3.2.1 Principal Component Analysis (PCA)

PCA highlighted the difference in the metabolome produced between the MBBR and BFDR (Fig. 3). The MBBR resulted in a homogeneous metabolic fingerprint, whilst the BFDR was differentiated in two distinct non-overlapping clusters of metabolites.

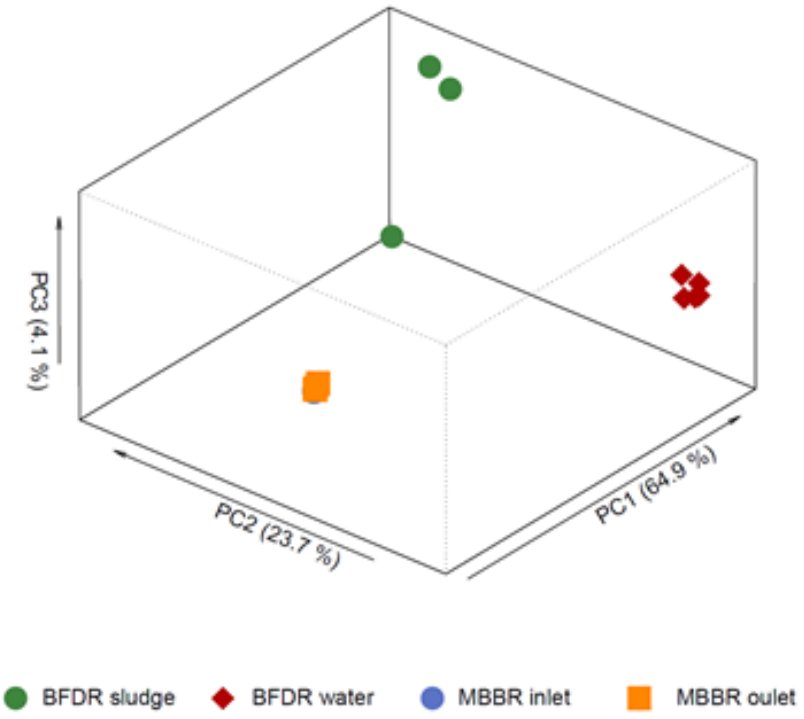

Figure 3. *PCA plot projecting the samples across the first three principal components, representing 92.8% of the total variance. The MBBR inlet and outlet locations are clustered along the three components and are represented with orange squares (inlet) and blue dots (outlet), respectively. The two BFDR compartments are represented by green round pointers for the sludge and red diamonds for supernatant water.*

The first three PCA axes represent 92.9 % of total variance (Fig. 3 and *Annex I*). The largest variance is described by PC1 (64.9 %), referring to the difference between the MBBR and BFDR. The second component describes the variability within the BFDR (23.7 %), distinguishing the sludge and the supernatant water. Lastly, PC3 does not highlight distinguishable differentiation in metabolomic trends, except for the BFDR sludge location. Here, one of the samples is visibly different from the two other BDFR sludge replicates (Fig. 3). However, the third component only describes a small percentage of overall variability (4.1 %). Due to the large number of variables, the contribution of each variable to the total variance is overall low, never exceeding 0.1 in PC1 and 0.18 % in PC2 (*Annex II*).

3.2.2 Hierarchical Clustering Analysis (HCA)

Hierarchical clustering projection (fig. 4) confirms the presence of three distinct metabolomes in the dataset, two in the BFDR (sludge vs supernatant water) and one in the MBBR. The MBBR corresponds to a single cluster indicating a single well mixed metabolome. On the contrary, a difference between the metabolic patterns sampled in the BDFR sludge compared to BFDR water can be precisely observed.

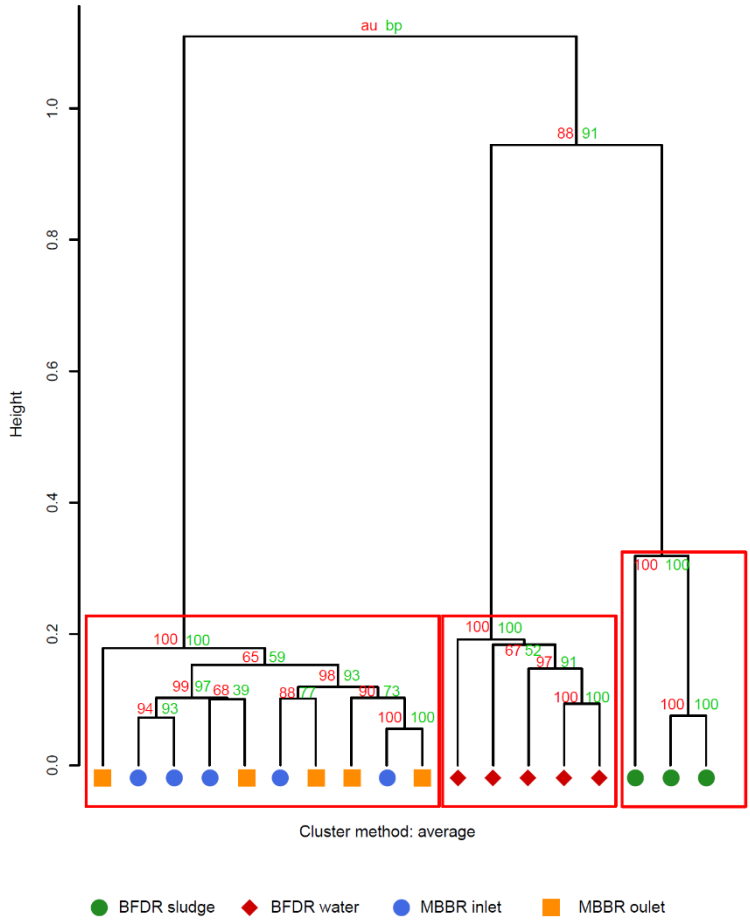

Figure 4. *Hierarchical cluster dendrogram. The green numbers represent the bootstrap probability (BP) (in %) of finding the resulting branching node after 1000 randomisations. The red number represents the percentage of confidence level of the approximately unbiased (AU) P-value test (in %). The red rectangles indicate major clusters with red-coloured AU p-values ≥ 99% (red values at branches)*

3.3 Within and between reactors differentiations

Data did not meet the normality assumption (HZ=72, df=3, $P<0.001$, Fig. 5a), while the homogeneity of variances was respected ($F=0.11$, df=3, $P=0.95$, Fig. 5b).

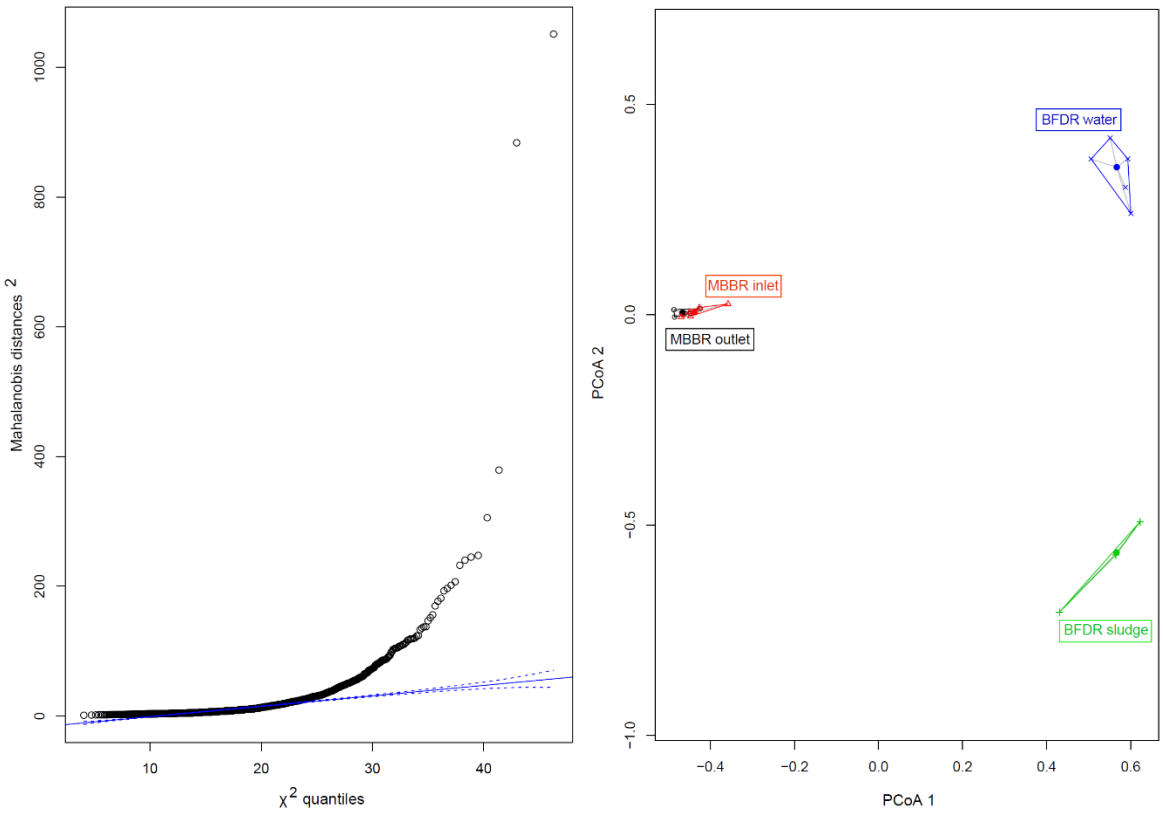

Figure 5. a) *Multinormal QQ plot;* ***b)*** *PCoA projection of the replicates' distribution.*

PerMANOVA confirms the presence of a highly significant global differentiation in the entire dataset (*P* =0.001, Table 1).

Table 1. *Permutational analysis of variance (PerMANOVA) results testing the global effect of location on variation in bioreactors based on Euclidean similarity matrices. Statistically significant p-value (P<0.05) is indicated in bold.*

Global variation between bioreactors locations						
Source of Variation	**Df**	**SS**	**MS**	**Pseudo-*F***	**R2**	***P* (*Perm*)**
Locations	3	56329	1877.30	32.92	0.88	**0.001**
Residuals	14	7986	570.40		0.12	
Total	17	64311			1.00	

All group-pair comparisons result statistically significant, except for the comparison between the MBBR inlet vs outlet (Table 2). These results confirmed the significant differences in metabolites content between the MBBR locations and the BFDR locations (Table 2). Within the BFDR, the sludge is significantly different from supernatant water (*P*=0.026; Table 2). However, no significant difference was found within the MBBR, where inlet and outlet resulted homogeneous (*P*=0.42; Table 2).

Table 2. *Post-hoc results testing the statistical differentiation between and within bioreactors. Statistically significant p-values (P<0,05) are indicated in bold*.

Pairwise variation between bioreactors locations				
Pair	**Pseudo-*F***	**R2**	***P***	**Adjusted *P***
MBBR Inlet vs BFDR sludge	31.86	0.84	**0.011**	**0.022**
MBBR Inlet vs BFDR water	60.98	0.88	**0.008**	**0.022**
MBBR Outlet vs BFDR sludge	31.07	0.84	**0.020**	**0.026**
MBBR Outlet vs BFDR water	59.15	0.88	**0.009**	**0.022**
Pairwise variation within bioreactors locations				
Pair	**Pseudo-*F***	**R2**	***P***	**Adjusted *P***
MBBR Inlet vs MBBR Outlet	0.98	0.11	0.42	0.42
BFDR sludge vs BFDR water	13.84	0.70	**0.022**	**0.026**

4. Discussion

In the present study, metabolomics analyses resulted successful in representing the different metabolic fingerprints between and within two unique RAS bioreactor designs. The results affirm the sensitivity of untargeted LCMS, which in the present study confirmed and rejected different initial hypothesis on metabolic dynamics determined by selective microbial growth in biological reactors. We employed several multivariate projections methods to highlight dissimilarities (HCA) and similarities (PCA), with supervised and non-supervised approaches (Alonso et al., 2015; Antonelli et al., 2017; Heinemann, 2019; Chanana et al., 2020). The negligible inter-analytical variation demonstrates the robustness of the results obtained. Statistically significant difference was found in the fingerprints between reactors, hence mirroring the clear effect of environmental conditions i.e., aerobic vs anaerobic and the reactors' hydraulic conditions. The bioreactors were designed with different environmental pressures to select unit-specific microbial communities and yielded three distinct metabolic fingerprints, denying the original design intents.

The MBBR was designed as a two-stage reactor and was hypothesised to produce two distinct metabolomes: the inlet being discrete, while the outlet being characterised by metabolites produced in loco and exogenous metabolites present upstream. Contrary to our expectations, the MBBR in this study did not create distinct metabolomes in each stage, the

reactor instead behaved as a fully mixed vessel. Reactor staging is a strategy to minimize the reactor size, as each stage can be designed to match the highest possible nitrification rates when these are limited by either available oxygen or available NH_3 (Weiss et al, 2005). In wastewater treatment practice, the first stage of an MBBR will encounter oxygen-limiting conditions and NH_3-limiting conditions in the subsequent stages (Weiss et al, 2005). In reactors treating low strength wastewater where dissolved oxygen is not limiting, most of the nitrification will already occur in the first stage (Casas et al., 2015). The reactors in these studies employed hydraulic retention times (HRTs) of several hours, which are often required for high degrees of pollutant removal (Aldris & Farhoud, 2020). In RAS, MBBRs are often operated in HRTs as low as 5 minutes, which may forbid the full oxidation of TAN to NO_3 in a single pass (Drennan et al., 2006). In this study, the reactor was not oxygen-limited during operation, with the first stage receiving low strength water from a microscreen drum filter with an oxygen concentration between 4 and 6 mg/l. Therefore, the MBBR was operated under NH_3-limiting conditions, with no further chemical substrates left for utilization. This, coupled with the low HRT (10 mins), may explain the absence of distinct metabolic fingerprints in each reactor cell. Another possible explanation can be identified in a vessel-wide distribution of a single microbial community, where the subdivision in cells and biomechanical properties did not create an abiotic gradient determining substantial metabolic - hence microbial assemblage - differences across cells.

The BFDR vessel mixed the reactor contents for ¼ of the operating time. Here, the unit was designed to allow sludge deposition for ¾ of the time at the bottom of the vessel. Hence, the operating principle suggested the possible presence of a homogenous microbial community and thus, a common metabolome. However, the intermittent mixing process did not homogenize the metabolites released by microbial community in the sludge. Interestingly, a significant differentiation was instead identified within the BFDR, which was characterised by two highly distinct metabolomes, inferring on the presence of two dissimilar communities of microbes in the sludge and supernatant water. The clear distinction in metabolic content suggests the presence of an unexpected consortium suspended in the supernatant water, breaking down much of the metabolic products released by the activated sludge below and producing its own. Free-swimming microbial growth in correlation with activated sludge reactors has not been discussed, while several studies have reported flocculating and fluidised bed systems associated with activated sludge in nitrifying and denitrifying reactors (Cowan et al., 1996; Singh and Kazmi, 2016; Wang et al., 2019). Cowan et al., (1996) concluded that the detachment and suspension of the activated sludge microorganism is dependent on microbe

relative abundance and taxonomic group. We suspect that the BFDR design, characterised by cyclical sludge deposition events intermittently disturbed by low dissolved oxygen shift, is responsible for the formation of two groups. Consequently, the significant difference of sludge-released metabolites from those in suspension in supernatant water exhorts to investigate the compounds source and characterise the filtration result of the communities in suspension. Worth mentioning is one of the three replicates drawn from the BFDR sludge. As seen on the PCA (Fig. 3), this sample largely contributed to the third principal component, yet the reason behind the higher variability is not identified and it might be due to the sampling location specific variability as to the sampling time, highlighting the need for more replicates to evaluate this location variability. Some significant variation might have been due to fluctuations in bacteria metabolic processes dependent on changing environmental stimuli, such as total suspended solids content and/or biochemical oxygen demand. Also, the specific nature of metabolites observed must be considered, given secondary metabolites such as signal molecules are far less abundant than primary ones such as ATP (van den Berg et al., 2006).

Although all replicates show similar patterns regarding between and within reactor differentiation, we cannot exclude that some variability could also be induced by technical biases, such as the decline of instrument sensitivity and separation ability, contamination of MS instruments (Do et al., 2018), sampling strategy and timing. Some potential technical biases might have been introduced during sample processing. The data collected, in fact, refers to one time point, hence the detection of metabolites over time in the LCMS machine remains unknown. Additionally, to avoid error measurements caused by cross-samples contamination of MS instruments, the analyses were run in blocks per sample location and were therefore only partially randomised. Furthermore, the homogeneous dilution factor across all locations may have constituted a significant factor shaping the results of the MBBR, where a weaker signal for most compounds was present compared to the BFDR locations, characterised by highly concentrated sludge samples. We, therefore, cannot exclude that meaningful compounds might have been present but not concentrated enough to be observed. To mitigate these biases, data pre-processing strategies are normally employed to filter out potentially meaningless signals and replace those below detection thresholds, usually outputted as zeros. In this study, a pre-processing method was devised *ad-hoc* due to the novel compound source, to the overall small number of samples collected and, especially, to the mismatch in sample numbers across locations, with the BFDR sludge being sampled two times less than other locations. No consensus currently exists on a standard data pre-processing for metabolomics studies and a large diversity of methods have been reported (Turck et al., 2020). On one hand, the most

successful strategies often employ quantitative information of correlated variables (Di Guida et al., 2016; Do et al., 2018). On the other hand, the expertise of natural products scientists may result in the most reliable resource available when novel biological systems with absent literature information are analysed, as in the present study. Schiffman et al. (2019) suggested that filtering methods should be data-adaptive and van den Berg et al., (2006) concluded that "the choice for a pre-treatment method depends on the biological question to be answered". The *ad-hoc* pre-processing method in this study was based both on our experience. Methods reported in the literature, such as "80% rule" and class-adjusted 80% rule (Yang et al, 2015), were not employable due to the mismatch in the number of replicates across locations. Therefore, the metabolites here characterising similarities and differences in bioreactors reflect the pre-treatment strategy choice. The method devised led to the deletion of over 48% of the original signal pool. Thus, the resulting metabolic pattern only depict the variation within thresholds selected, and there may have been meaningful compounds in low abundance not retained in the statistical analyses. Further metabolomics application to RAS bioreactors will improve the pre-selection strategies and draw more meaning out of RAS bioreactors metabolic samples. Nevertheless, the proposed methodology provides the opportunity to run fast and cost-effective metabolomics analyses to globally depict compound trends.

In this research trial, the analysis aimed at detecting fingerprint differences between sampling locations cost-effectively, rather than at identifying any specific compound. Therefore, compounds were not annotated or quantified by standard methods. Hence, no specific MS/MS of compounds was performed as it required additional LCMS runs and data processing. Consequently, the relative mass intensities obtained could not be compared across compounds, in view of their potential highly variable ionization efficiency, ultimately depending on their exact chemical structure. Furthermore, quantification of a compound could only be achieved using authentic standard, mostly not commercially available. Nevertheless, the putative molecular ion mass of few compounds was included based on the relative intensities. The putative identifications were manually performed solely based on the accurate mass of the putative molecular ions, with deduced elemental formula within 5 ppm mass accuracy. The putative molecule identification was obtained for a subsequent bio-prospective analyses, outside the scope of this master thesis. Between these, a compound predominantly present in the BFDR supernatant water was putatively annotated as proline, a proteinogenic amino acid used in plant bio-stimulant products, which enhances stress tolerance in plants when supplied in low concentrations (Hayat et al., 2012). This observation and the RAS effluent metabolite database created during the present study offer a starting point for future research

on denitrification bioreactor metabolites content and applicability in aquaponics and other commercial sectors. Desirable further data processing, selection and annotation of compounds is needed to confirm the presence of commercially viable compounds.

This pilot study represents the first step towards employing a new top-down, cost-effective, and untargeted snapshot analysis to describe metabolic dynamics of RAS bioreactors. To open the door for the employment of these and subsequent targeted assays in RAS aquaculture, the first knowledge gaps to fill regarded whether metabolomics analyses could successfully represent expected metabolic dynamics resulting from pre-selected chemical/physical properties. We demonstrated how metabolomics analysis can expose the bioreactor metabolic dynamics and potentially become a successful tool to observe differences when system changes are applied. But at the same time, metabolomics can reveal, as shown here, the lack of success in designing specific biomechanical properties. The metabolic fingerprints obtained, in fact, exposed the unsuccessful subdivision of MBBR vessel in two cells, which aimed to select different microbial assemblages and enhance overall TAN filtration. Hence, our results inferred in real time on the chemo-physical processes shaping the microbial community structures hosted. These findings disclose new methods to improve monitoring of microbial community compositions in RAS (Rurangwa and Verdegem, 2013). We believe that metabolomics analysis could be added to the toolbox of bioassays currently employed to describe the complex interacting mechanisms and cellular metabolic pathways in response to perturbations in RAS (Kim et al., 2007). The development of integrated analytical protocols, where untargeted and targeted metabolomics are combined with transcriptomics and RNA sequencing will help constructing a holistic picture of microbial life, revealing changes in growth conditions due to disturbance and uncovering unexpected metabolic pathways (Baran et al., 2013; Cho et al., 2015). This integration, if successful, besides filling important biochemical knowledge gaps, may become the source of new cost-effective diagnostic tools to improve the operational control of RAS systems and open the door to the discovery of commercially viable microbial natural products. Potential future applications can be identified in the description of H_2S shifts (Letelier-Gordo et al., 2020), improvement of biofilm maturation pathways (Riuz et al., 2020), bioprospecting for potentially useful denitrification by-products and commercially viable microbial products for drug discovery (Blunt et al., 2018). Additionally, we believe metabolomics bioassay can improve the management, detection and characterisation of bacteria producing off-flavour compounds and their potential bioremediation (Rurangwa and Verdegem, 2015; Azaria et al., 2020). Improving bioreactor

design and control can already be prospected given the successful application demonstrated in this study and in wastewater treatment plants (Yang et al., 2019).

5. Conclusion

This pilot study represents the first step towards employing untargeted metabolomics analyses as a new top-down, fast, and cost-effective technique to describe RAS bioreactor functionality. We demonstrated how this tool can expose the bioreactors' metabolic dynamics resulting from chemo-physical characteristics and supporting specific microbial assemblages. The two bioreactors resulted significantly different in their metabolic fingerprints, mirroring the unique design features. Simultaneously, here we demonstrate how metabolomics can expose the lack of success in designing specific biomechanical properties within a bioreactor. The MBBR resulted homogeneous in the metabolic fingerprint, while the BFDR produced to two distinct metabolic signatures, hence denying the initial hypotheses. These findings disclose on a new method to improve monitoring of microbial communities in RAS. We believe that metabolomics analysis could be added to the toolbox of bioassays currently employed to describe the complex interacting mechanisms and cellular metabolic pathways in response to perturbations in RAS. This integration may become the source of new cost-effective diagnostic tools to improve the operational control of RAS systems and open the door to the discovery of commercially viable microbial natural products.

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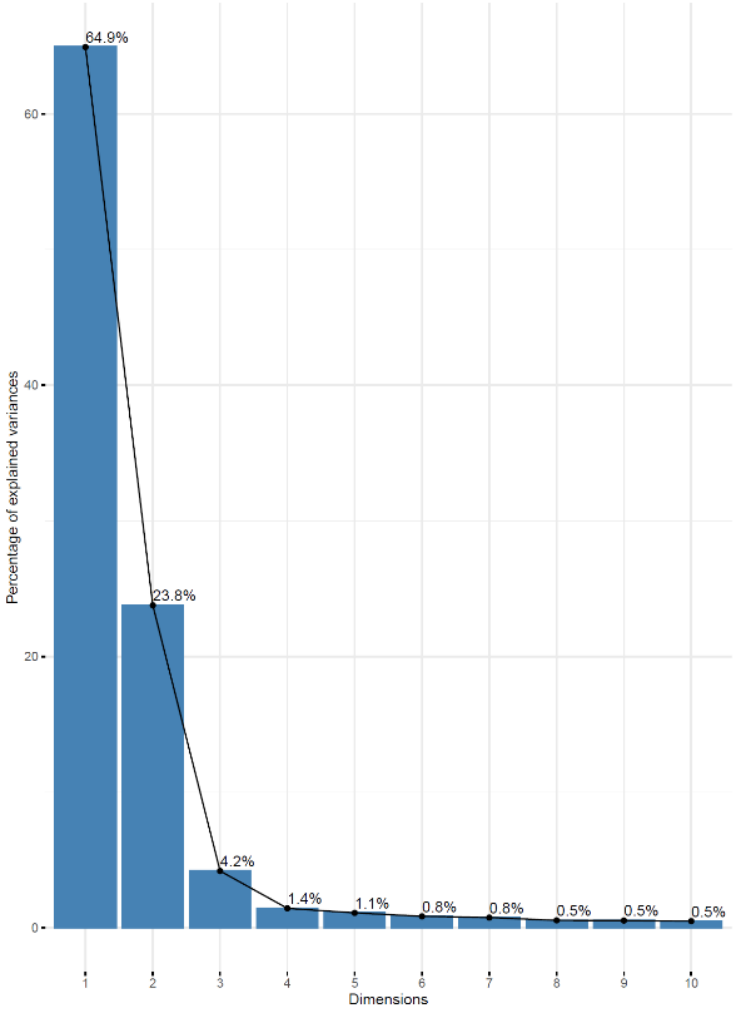
7. Annexes

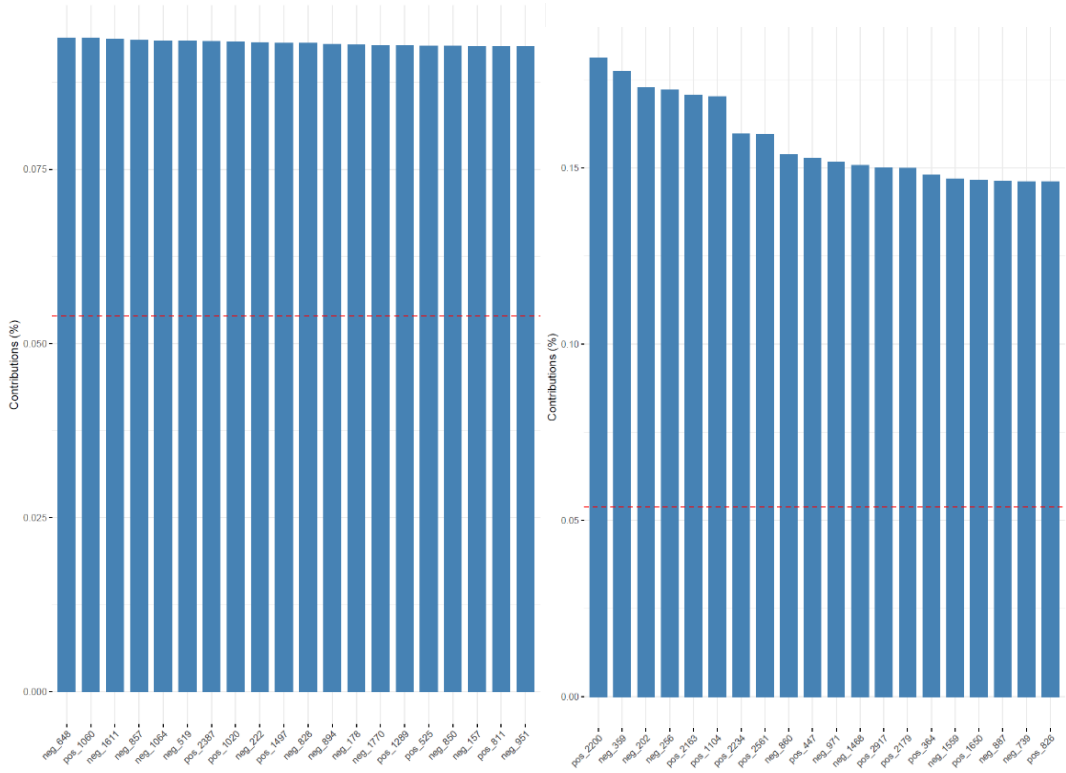
Annex I

Principal Component Analysis scree plot



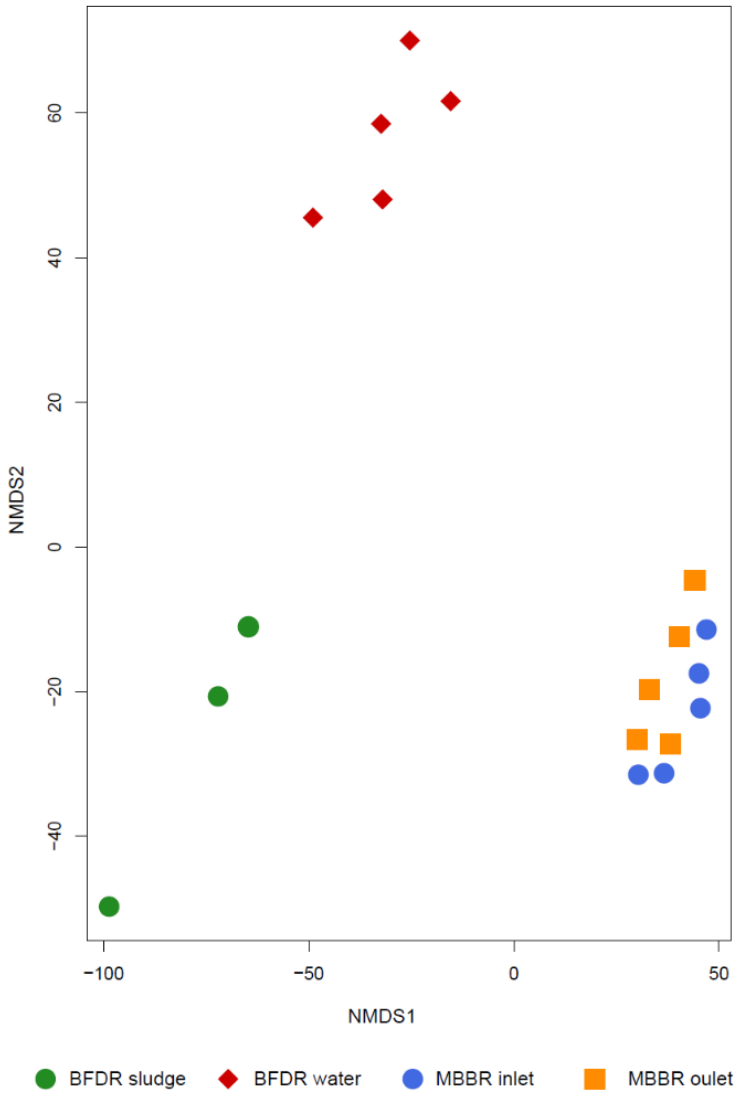
Annex II

Percentage contribution to variance for the 20 most influential compounds in principal components 1 (left) and principal component 2 (right).



Annex III

Non-metric multidimensional scaling score plot. The spatial distribution of variables spread result in clear differentiation between MBBR inlet (blue dots) and outlet (orange squares) cluster, BFDR sludge (green dots) and BDFR supernatant water (red diamonds).



Annex IV

Linear Discriminant Analysis score plot. The variance within and between location is reduced to two dimensions describing 99.8% of the total. The discrimination results with one cluster in the MBBR with inlet (blue dots) and outlet clustered (orange squares) and two distinct clusters in the BFDR, the sludge (green dots) and supernatant water samples (red diamonds).