AdriAquaNet

Enhancing Innovation and Sustainability in Adriatic Aquaculture

Deliverable WP4 task 4.2.1

Technical-scientific Report on efficacy of naturally extracted pyrethrins on the fish and parasites

PP3 IOF - Split, 30.06.2022

Project number:	**10045161**
Project Acronym:	**AdriAquaNet**
Project Title:	**Enhancing Innovation and Sustainability in Adriatic Aquaculture**
Start of the project:	**01/01/2019**
Duration:	**42 months**
WP/activity:	**WP4, Activity 4.2.1**
Deliverable name:	**Technical-scientific report of the fish microbiome, probiotic candidates and the effect of feeding trials on the fish growth and immunity**
WP leader:	**UNIUD**
Author (s):	**J. Hribar in collaboration with LP, PP1, PP2, PP4, PP8 and PP10**
Delivery date:	**30/06/2022**
Status:	**Final**

The technical scientific report WP 4.2.1

This technical scientific report is listed in Application Form as deliverable, focused on a specific WP and task results, outputs and on the impact of this activity on the area of cooperation generated by the project activities.

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This report has been prepared by the working group that is involved in the WP and coordinated by the WP leader or task leader. We suggest to be concise in part that you are dealing with- from 5 to 10 pages. Of course you can extend if you add images, photos, graphics…

It is divided in 2 parts:

- **PART 1**

  The first part provides details and assessment of the WP objectives related to the primary subject of the report and the implementation and results, compared to the information already provided in the different progress reports, in order to give back a cumulative illustration of what the project delivered in relation to this task;

- **PART 2**

  The second part provides the final results and a collection of data from the WP and project in relation to the General objectives at the Programme level, related to the final project report.

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PART 1

A. REPORT HIGHLIGHTS

Please provide a cumulative short overview of the project WP task 's achievements

Considering global climate changes aquaculture faces numerous challenges in meeting the market's demand for high-quality products. One of the crucial challenges of today's aquaculture is disease outbreaks, including parasitic diseases caused by both endo- and ectoparasites. Although several chemotherapeutics are available for treating parasitic infections in fish, there is a constant rise in resistance to these substances. Another aspect of their use is the environmental impact they have, leading to their ban in some regions. Among the most notable ectoparasites in Mediterranean and Adriatic aquaculture are the isopod Ceratothoa oestroides and gill monogenean Sparicotyle chrysophrii. Both parasites can have a detrimental effect on cultured fish, sometimes causing high economic losses.

For this reason, one of the objectives of the AdriAquaNet project was to test the antiparasitic activity of naturally occurring pyrethrins against these two significant fish parasites, in vitro or following a natural disease outbreak on a farm. Pyrethrins are secondary metabolites of Dalmatian chrysanthemum (Tanacetum cinerariifolium), a plant used in companion planting to repel pest insects from crops and other important plants. Six pyrethrins are known, but only two have insecticidal properties, pyrethrin I and pyrethrin II.

Institute of Oceanography and Fisheries (IOF) – Project Partner 3 (PP3) collected Dalmatian chrysanthemum for the extraction of natural pyrethrins to determine their lethal doses and safe concentration range on infected/uninfected fish in experimental tanks. Flower heads of Dalmatian chrysanthemum were hand harvested in their natural habitat near Split, Croatia. The flowers were then air dried under cool conditions. PP3 subcontractor (University of Osijek) extracted the pyrethrins using several state-of-the-art methods. The concentration of extracted pyrethrins was then quantified to determine the best extraction method. Additionally, we tested several solvents (ethanol, methanol, acetone) to obtain the highest concentration possible. Of all the extraction methods tested, only one was able to extract all six pyrethrins naturally occurring in Dalmatian chrysanthemum.

Following extraction and quantification of pyrethrins, we encountered several issues. Natural pyrethrins are known to be photo- and thermosensitive. Despite proper storage conditions, the concentration of pyrethrins in the extract progressively declined over time. Furthermore, due to the oceanographic conditions as well as improved zootechnical conditions on farms, parasite numbers were very low to enable us to properly conduct any downstream experiments using a range of concentrations and enough replicates. That was further impeded by the COVID-19 pandemics, preventing access to the farms.

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B. WP PROJECT OUTPUTS AND RESULTS

Specific 2: Promote fish health and provide "healthy and safe" fish to consumers

Summary and the presentation of the activities and the results:

Natural pyrethrins were extracted from Dalmatian chrysanthemum (*Tanacetum cineraiifolium*) to determine the safe concentration range to test on parasites infected/uninfected fish in experimental tanks (PP3) and aquaculture cages (PP8).

Hand picking of Dalmatian chrysanthemum flower heads on the island of Brač

Flower heads of Dalmatian chrysanthemum were hand-harvested at optimal maturity in mid-June 2019 at their natural habitat on Brač island. The flowers were then air dried in a dark and cool place and sent to the University of Osijek, a PP3 subcontractor who performed pyrethrin extractions. Dry flowers were pulverised and subjected to pyrethrin extraction. Seven different state-of-the-art methods were used for the extraction of six Dalmatian chrysanthemum pyrethrins (pyrethrine I, cinerine I, jasmoline I, pyrethrine II, cinerine II and jasmoline II). The methods used were Microwave assisted-extraction (MAE), Ultrasound-assisted extraction (UAE), High-voltage electric discharge (HVED), Deep eutectic solvents extraction (DES), Subcritical water extraction (SWE), Extraction with supercritical carbon dioxide (SFE) and High-performance liquid chromatography (HPLC).

Among the tested techniques, supercritical CO_2 extraction (SFE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) were suitable for the extraction of pyrethrins from the Dalmatian chrysanthemum. More specifically, supercritical CO_2 (SFE) at 300 bar and 40°C is appropriate for the extraction of all the pyrethrins tested (Table 1.)

Temp. (°C)	Preassure (bar)	Time (min)	Cinerin II ng/mg	Pyrethrin II ng/mg	Jasmolin II ng/mg	Cinerin I ng/mg	Pyretrin I ng/mg	Jasmolin I ng/mg	TOTAL ng/mg
40	300	60	10,30	48,22	5,33	9,95	46,72	3,85	124,37

Table 1. Process parameters and pyrethrin quantities obtained with supercritical CO2 extraction (SFE)

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We considered the UAE and MAE differently because different parameters and different solvents were used. In addition to the extraction method, the solvent used also played a role. For example, water was not suitable as a solvent, while aqueous solutions of ethanol and methanol, as well as acetone were suitable for extraction. With 50 and 80% ethanol and acetone, an increase in total pyrethrins with increasing temperature was observed in the case of MAE, while this trend is not evident with 50% methanol (Table 2.)

Solvent	Cinerin II ng/mg	Pyretrin II ng/mg	Jasmolin II ng/mg	Cinerin I ng/mg	Pyretrin I ng/mg	Jasmolin I ng/mg	TOTAL ng/mg
water	-	-	-	-	-	-	-
50% ethanol	-	16,2	-	-	-	-	16,2
80% ethanol	-	16,6	0,975	-	-	-	17,575
50% methanol	0,0	17,7	1,08	1,64	0,0		20,42
acetone	-	-	-	1,86	0,0	0,0	1,86
water	-	-	-	-	-	-	-
50% ethanol	-	-	-	-	-	-	-
80% ethanol	-	17,0	0,996	-	-	-	17,996
50% methanol	-	-	-	-	-	-	-
acetone	-	10,36	0,572	-	-	-	10,932
water	-	-	-	-	-	-	-
50% ethanol	-	16,7	0,983	-	-	-	17,638
80% ethanol	0,0	18,9	1,22	2,05	0,0	0,0	22,17
50%methanol	-	-	-	-	-	-	-
acetone	-	15,6	-	-	-	-	15,6
water	-	-	-	-	-	-	-
50% ethanol	-	16,9	0,991	-	-	-	19,687
80% ethanol	-	17,3	1,01	-	-	-	21,31
50% methanol	0,0	18,3	1,15	1,72	0,0	-	21,17
acetone	-	17,0	1,0	-	-	-	18,0
water	-	-	-	-	-	-	-
50% ethanol	0,0	19,1	-	1,95	0,0	0,0404	21,09
80% etanol	0,0	18,9	1,15	2,08	0,0	0,0	22,13
50% metanol	0,0	18,1	1,16	1,76	0,0	0,0	21,02
aceton	0,0	14,7	3,86	2,15	0,0	0,0	20,71
aceton	0,0	18,9	1,15	1,89	0,0	0,0	21,94
aceton	0,0	19,1	1,16	1,90	0,0	0,0	22,16

Table 2. Solvent and pyrethrin quantities obtained by Microwave Assisted Extraction (MAE)

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In the case of UAE, the same was observed with 80% methanol (Table 3.). Comparing the two mentioned techniques, UAE is still a more suitable technique considering that pyrethrin I was also extracted, i. e. it accounts for a larger proportion of the total pyrethrins extracted. The largest proportion of pyrethrins was extracted with 80% methanol followed by 80% ethanol at a temperature of 70°C and an extraction time of 30 min in an ultrasonic bath.

water	-	-		-	-	-	-
50 % Ethanol	-	8,247		-	-	-	8,24
50 % Methanol	-	18,668	0,907	0,7981	80,723	-	101,0
Acetone	-	18,053	0,1527	2,5987	72,656	0,0319	93,49
80% Ethanol	-	-		0,9612	76,331	-	77,29
water	-	-		-	-	-	-
80% Ethanol	-	27,672	1,5197	2,4098	73,051	-	104,6
80 % Methanol	0,012	20,888	1,4863	2,6631	72,491	-	110,0

Table 3. Solvent and pyrethrin quantities obtained by ultrasound-assisted extraction (UAE)

Considering all the extraction techniques the Extraction with supercritical CO_2 (SFE) is the only tested technique able to extract all listed pyrethrins and the largest amount of total pyrethrins, shown as the sum of six listed pyrethrins.

Reasons of discrepancies between planned and realized outputs (if any)

Despite successful extraction of all six pyrethrins from the Dalmatian chrysanthemum, we could not determine lethal doses for the parasites and the range of safe concentrations for the infected fish. The main reason for this is the low prevalence and intensity of infection at several farms during the project. Therefore, we could not obtain enough parasites to cover a range of concentrations with sufficient replicates to have reliable and reproducible results. COVID-19 restrictions further impeded this by preventing us from frequent visits to farms to inspect the fish and eventually collect the parasites.

Impact of outputs underachievement on project results

Due to the inability to determine the lethal concentration for the parasite and safe concentration ranges for the fish, we cannot conclude whether natural pyrethrins are effective and safe to use as antiparasitic agents. From the available literature, they will probably be highly effective against *Ceratothoa oestroides*,

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as is the case with other crustaceans. However, the effectiveness against *Sparicotyle chrysophrii* remains to be tested.
Additional results (was the project able to reach additional outputs /results besides those foreseen in AF? Not applicable.

C. DURABILITY AND TRANSFERABILITY OF THE PROJECT AND ITS RESULTS

Please describe shortly:

How will the outputs and results be maintained and developed further after project end? Within the project, we tested several extraction methods and selected three of those, resulting in the highest yield of the extracted natural pyrethrins. Nevertheless, we couldn't assess their efficacy on the target parasite species due to the low prevalence and intensity of infections. Furthermore, we also evidenced the relatively low chemical stability of natural pyrethrins. Considering that, different settings of tested methods could be evaluated, to check whether it is possible to achieve a higher yield of extracted pyrethrins. Furthermore, experimental infection in a controlled environment (experimental tanks) should also be performed to achieve a higher intensity of infection. That would enable a proper experimental design resulting in reliable and reproducible data.
How has the availability of project results and outputs for general public and other stakeholders been ensured during the project life and eventually after the project end? During the project, several online (during the COVID-19 pandemic) and on-site training and workshops were organised for the stakeholders and public interested in the topic. During these events the results of the specific task were presented. Furthermore, a scientific article is currently being prepared for publishing in a peer-reviewed journal after the end of the project, where the results of the task will be presented, discussed and new research perspectives proposed.

D. CAPITALISATION OF RESULTS

Please provide information about capitalisation:

Was the project able to capitalise or influence future calls or other projects? Please specify main results or output to be considered for future capitalisation action. **Are there any obstacles of legal or administrative nature that the project has encountered and which hampered cooperation? Is there any room to solve these obstacles?** We tested several extraction methods and selected three of those, resulting in the highest yield of the extracted natural pyrethrins, overcoming the first obstacle in obtaining enough of the extract. These results will be useful for future calls and other projects aiming at assessing the antiparasitic potential and safe concentrations range for fish of natural products, particularly pyrethrins. As mentioned above, future capitalisation actions should consider establishing experimental infections for testing the antiparasitic activity of natural pyrethrins, as natural disease outbreaks are highly unpredictable in terms of occurrence and intensity. We encountered no obstacles during the project implementation.

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E. PARTNERSHIP COOPERATION

Please provide an assessment of the participation and involvement of the partners in the project, answering the following questions:

Which Partners were active in your WP and the activities related to the report? Were all the Partners involved also active? Besides PP8 (Friškina Ltd.), at whose farm we conducted a part of the experimental work of the WP, LP (University of Udine), PP1 (Croatian Veterinary Institute), PP2 (University of Trieste) and PP4 (Istituto Zooprofilattico Sperimentale delle Venezie) were also involved in WP activities. The cooperation between the partners has been achieved in full sense. All the partners contributed to the WP within their area of expertise. Additionally, three companies (Biocentre, Naturalleva and Biotechvana) and one public University (University of Osijek) were also involved as subcontractors of PP3. In the activities conducted to achieve this deliverable only PP3 and subcontractor the University of Osijek were involved.
Were they all able to attract other local/regional actors and involve them in the project activities? No, as the PP3 subcontracted three companies and one public University to cover several of the activities of the WP.
What was the added value given by the cooperation? The subcontractor, the University of Osijek, has extensive experience in the extraction of natural products with desirable properties. Through cooperation, we were able to test a plethora of methods and select those resulting in the highest yield of natural pyrethrins.
Which were the main problems encountered? The main problem encountered was the low prevalence and intensity of natural infections with target parasites species, due to which we couldn't test the antiparasitic activity of natural pyrethrins.
Was the project able to create links with other projects? **Not applicable.**
Will the PPs cooperate in the future even without funding (if yes explain the main aims of this cooperation)? Yes, but mainly in trainings organisation and results dissemination.

F. TARGET GROUPS INVOLVEMENT

Please list the main target groups that benefited from your WP project's achievements as inserted in the relevant Report Section in SIU that you will find on the left (the numbers are our project numbers). In few word provide further details on how they were able to make use of the outputs/ results of the project.

TARGET GROUPS	***Description***
SMEs (50)	*1 farm directly involved in the feeding trial.*
Universities, technology transfer institutions, research institutions (10)	*LP, PP1, PP2, PP3, PP4, University of Osijek*
NGOs, associations, innovation agencies, business incubators, cluster management bodies and networks (5)	
Centers of R excellence (5)	

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Local, regional and national public authorities (10)	
General public (1000)	*Dissemination of the results on PP3 online channels (website, social media), on the AdriAquaNet project website and as a published scientific article in a high impact journal.*

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PART 2

A. CONTRIBUTION TO EUSAIR

Please provide a description of the project contribution to the EUSAIR in terms of synergy with the Strategy's pillars and alignment of implemented project's activities with the Action Plans and labelled projects.

The AdriAquaNet project contributes to the EUSAIR strategy within Pillar 1 "Blue growth", Topic 2 "Fisheries and aquaculture", aiming at enhancing the Adriatic area aquaculture by developing a sector that is both economically and environmentally sustainable. The activities within task 4.2.1. are necessary for environmental and economic sustainability. Introducing novel natural products with antiparasitic activity could be an alternative to the use of conventional chemotherapeutics, thus lowering the environmental impact. The project results and outputs can easily be transferred to other territories of the EUSAIR region.

B. CONTRIBUTION TO HORIZONTAL PRINCIPLES

Please provide a description of the project contribution to the horizontal principles of equality between men and women, non-discrimination and sustainable development.

The AAN project aims were developing and promoting healthy and sustainable aquaculture products from the Adriatic region. The project brought together different interested parties: scientists, farmers, consumers, and veterinarians. To achieve this staff participating in the project were engaged based on their characteristics, complying with equal opportunities and without discrimination based on gender, race, nationality, ethnic origin, religion, disability, age or sexual orientation.
A social component of the project should also be accentuated, as increased sustainability and reduced production costs will help ensure continuous production and higher profitability for the farmers, providing at the same time permanent employment opportunities.

C. COMMUNICATION ACTIVITIES

Please refer to the Final Communication Report template and provide a summary on the main achievements trying also to identify which were the most successful communication tools in reaching general public/decision makers/other target groups.

Activities within task 4.2 performed to produce this deliverable, in particular, fieldwork at the Friškina Ltd. farm and dissemination of the results have been documented with photos and videos by PP3 staff and PP2 subcontractor.
Task 4.2 main activities and results have been presented to a broad audience of scientists and specialists dealing with fish health at the EAFP 2021 conference as an oral presentation. The project and part of the results of the present task have also been presented as part of a conference workshop "How outputs from EU projects can upgrade health management in Mediterranean aquaculture". Furthermore, the activities and main results have been communicated with different stakeholders (farmers, veterinarians, local authorities) during one online (February 24th, 2022) and one on-site training held in Zadar (June 2nd, 2022), with both pieces of training reaching a high number of attendees. Finally, a summary of the main activities and most important results has also been presented at two final conferences in Zadar (June 3rd, 2022) and Udine (June 20th, 2022),

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D. NATURA 2000

Please describe, if it is the case, measures foreseen and implemented by the project:

a) In case the project involved Natura 2000 sites, describe what measure the project envisaged and implemented to avoid any negative impact: Not applicable.
b) In case the project had a positive effect on Natura 2000 sites, please describe which measure the project has foreseen and implemented in order to reach a direct or indirect positive impact: Not applicable.

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E. TYPES OF ACTIONS ADDRESSED (as defined in the Cooperation Programme)

These are our primary objective's types of actions, that we addressed by the Project:

Specific Objectives	***Types of action***	***the most relevant one within the SO addressed by your project***
1.1 Enhance the framework conditions for innovation in the relevant sectors of the blue economy within the cooperation area	*Joint projects and actions aimed at creating platforms, networks and at supporting exchange of good practices in order to enhance the knowledge transfer and capitalization of achieved results in the field of blue economy*	***X***
	Actions aimed at cluster cooperation, joint pilot initiatives in order to boost the creation of marketable innovative processes and products, in the field of blue economy	***X***

F. TYPES OF OUTPUTS PRODUCED

Specify the types of outputs generated by your activity that are reported here and provide a brief description

Output typology	**Description**
Trainings	Two training sessions have been performed (online and on-site) where the extraction and properties of natural pyrethrins have been presented. Additionally, their potential use as antiparasitic agents has been discussed.
Monitoring systems	
SMEs clusters	
New networks	
Platforms	
Adaptation plan	
Building renovation	
Others (please specify)	

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G. TYPOLOGY OF IMPACTS

Please indicate what type of impact(s) your project has had. You can choose more than one answer. For each tangible impact selected, please provide a concrete example from your project, where possible supported by quantitative information.

TANGIBLE IMPACTS

Tangible impacts	**Example/ quantitative information**
Improved access to services	/
Cost savings	/
Time savings	/
Reduced energy consumption	/
Reduced environmental impact	Reduced environmental impact through the use of natural products for the treatment of parasitic diseases over synthetic ones.
(Man-made, natural) risk reduction	/
Business development	/
Job creation	/
Improved competitiveness	/
Other tangible impacts (specify)	/

INTANGIBLE IMPACTS

Intangible impacts	**Example/quantitative information**
Building institutional capacity	/
Raising awareness	/

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Changing attitudes and behavior	/
Influencing policies	/
Improving social cohesion	/
Leveraging synergies	/
Other intangible impacts (Specify)	Final consumers are increasingly aware of the environmental impact of aquaculture and becoming more prone to consuming fish that has been cultured without the use of synthetic chemotherapeutics and with reduced environmental impact.

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