Quality enhancement of alternative fish feeds using high-power ultrasound processing

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Abstract

Background: The rising costs of current fish feeds has led to an increase in the demand for alternative sources of nutrients to supplement the feed with, in the hope of lowering the overall price. Fish nutrition involves an intricate balance of macro and micronutrients which need to be met to ensure the highest quality fish meat. Plant nutrients extracted from sustainable sources, namely *Alaria esculenta*, *Chlorella vulgaris* and *Lemna minor*; have been evaluated for their nutritional value, in an attempt to make the aquaculture industry more environmentally friendly. Furthermore, the implementation of green technology (mainly ultrasound) to extract these nutrients from the alternative biomasses was studied extensively and compared to more traditional techniques.

Method: Various experiments were performed to establish the best parameters for nutrient extraction. Characterisation of raw material was performed initially. This included colorimetry, microscopy, water activity and moisture content assessments. The impact of different ultrasound set-ups was evaluated to determine which ultrasonic treatment is the most efficient out of bath sonicators and probe sonicators set at different amplitudes. The impact of ultrasound on biomass treatments was assessed to determine which ultrasonic treatment is the most efficient out of enzyme-assisted, conventional or alkali protein extraction methods.

Results and discussion: The raw material characterisation gave insight on parameters that affect the quality of the end-product. The UP400St probe sonicator was the most efficient ultrasonic device. Ultrasound extraction using the probe sonicator resulted in a higher extraction yield overall. Ultrasound as a pre-treatment for enzyme-assisted extraction gave the best protein extraction yields.

Conclusion: This study delved into relatively untouched territory in nutrient feeding ingredients in aquaculture research. Ultrasonic probe devices were found to be more efficient in extraction of compound than bath sonicators. Furthermore, ultrasound technology fared better than conventional or alkali extraction methods. *Lemna minor* was found to have the greatest extraction yield of nutrients, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

Keywords: Ultrasound; Aquaculture; Fish feeds; Green technology; Bioactive compound extraction.

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40T1	UP400St set at 40% amp. time 1 min
40T20	UP400St set at 40% amp. time 20 min
40T5	UP400St set at 40% amp. time 5 min
80T1	UP400St set at 80% amp. time 1 min
80T2	UP400St set at 80% amp. time 2 min
80T5	UP400St set at 80% amp. time 5 min
BSA	Bovine serum albumin
BT1	Bath time 1 min
BT20	Bath time 20 min
BT5	Bath time 5 min
CBS	Capture-based species farming
CCS	Closed-cycle species farming
CFP	Common Fisheries Policy
CT1	Control time 1 min
CT2	Control time 2 min
CT20	Control time 20 min
CT5	Control time 5 min
dH_2O	Distilled water
DNS	3,5-Dinitrosalicylic acid
EDX	Energy-dispersive X-ray spectroscopy
FAO	Food and Agriculture Organization
FOX	Ferrous oxidation xylenol
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HPLC	High Performance Liquid Chromatography
KOH	Potassium hydroxide
MRD	Maximum recovery diluent
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NSO	National Statistics Office
OPA	O-phthaldialdehyde
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PES	Polyethersulfone
PoU	Prevalence of undernourishment
SD	Standard deviation
SDGs	Sustainable Development Goals
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
STD	Standard error of the mean
SEM	Scanning electron microscopy
TFAA	Total free amino acid
TRS	Total reducing sugar
TSA	Tryptone soya agar
UCD	University College Dublin
UN	United Nations
UoM	University of Malta
US	Ultrasound
YPGA	Yeast extract peptone glucose agar

Chapter 1 Literature review

1.1 Introduction

Malnutrition and hunger remain two major global concerns. Many efforts have been made to resolve these issues; however, it is evident that more needs to be done to safeguard both current and future generations. Statistics from recent reports on chronic hunger show that it is on the rise worldwide, with 815 million chronically undernourished people in 2016 (FAO, 2021). According to the Food and Agriculture Organization (FAO) of the United Nations (UN), in 2020 there was an increase by 1.5% in the prevalence of undernourishment (PoU) when compared to the previous year (Fig. 1.1).

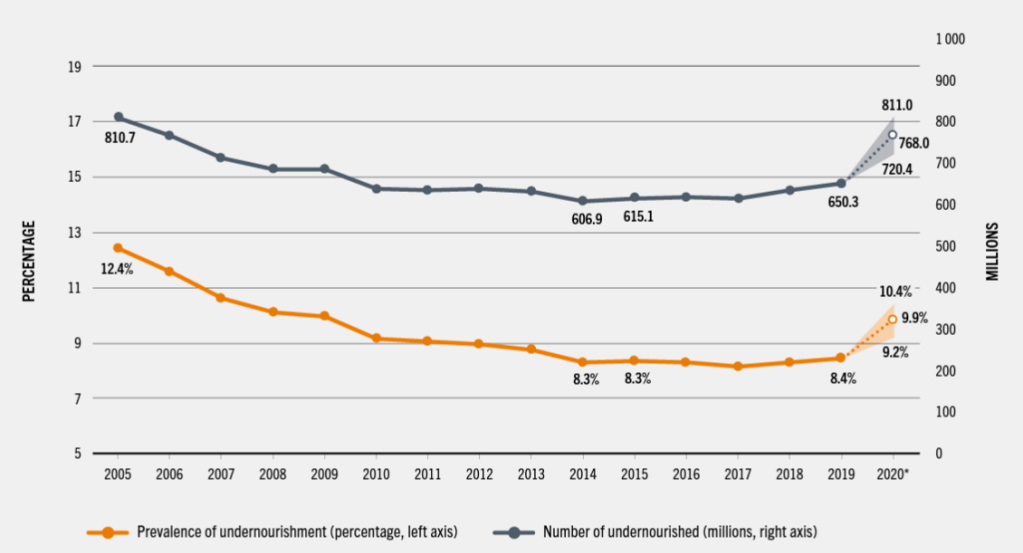

Fig. 1.1 Line graphs depicting the PoU (orange) and the number of undernourished persons (grey) globally throughout the years. "2020*": the values for 2020 were estimated by calculating the lower and upper bounds, the shaded region, and then averaging these two values, shown by dotted lines (source: FAO, 2021).

This is rather worrying considering that the PoU had remained unchanged in 2014 and 2019 (FAO, 2021). This increase is attributed to the COVID-19 pandemic. The pandemic will inevitably have long-lasting effects on global food security. The FAO estimates that 660 million individuals will be struggling with hunger in the year 2030, an increase of 30 million people from their estimate of the situation had the pandemic not occurred (FAO, 2021). The aim is that by 2030, hunger and malnutrition will be

reduced through the use of more sustainable agricultural techniques and food systems. This would guarantee a stable food supply and means for suitable nutrition and good health (FAO, 2021).

Furthermore, it is calculated that by 2050, developing countries in South Asia and sub-Saharan Africa will see a rise in population by 2.4 billion, which will inevitably put further strain on our food resources (Cottrell et al., 2019; Lipper et al., 2014). Sustainable food production is extremely challenging to achieve, due to the restrictions of agricultural land caused by various factors, including desertification, salinization and urbanization (Béné et al., 2020; Godfray et al., 2010). The depletion of natural resources due to overuse thereof and climate change – which causes extreme variations in rainfall and harsh temperature fluctuations – are causing agriculture and aquatic biodiversity as well as crop production systems to dwindle (Cottrell et al., 2019). In order to create sustainable food production systems that will keep up with the increase in human demand, innovative systems that require less energy consumption and produce less stress on water and land, while keeping in mind the possible alterations in dietary and consumption trends, must be designed (Eitzinger et al., 2018; Ramirez-Villegas et al., 2012).

It has long been established that the aquaculture and fisheries sector is crucial for a food secure future. Data by the FAO shows that out of all the seafood consumed globally, 78.7% is produced from the 66% of monitored fisheries that were found to be at biologically sustainable levels (FAO, 2020). Hence it is critical that aquaculture becomes the main source of fish globally, including in the less developed countries (Belton et al., 2018; Supartini et al., 2018).

It is estimated that global fish production reached around 179 million tonnes in 2018, out of which 156 million tonnes are available for human consumption (FAO, 2020). Fish supply close to 20% of the average per capita intake of animal protein to about 3.3 billion individuals (FAO, 2018). Research in aquaculture is linked to three of the UN's sustainable development goals (SDGs): Goal 2 "End hunger, achieve food security and improved nutrition and promote sustainable agriculture", Goal 12 "Ensure sustainable consumption and production patterns" and Goal 14 "Conserve and sustainably use the oceans, seas and marine resources for sustainable development".

Goal 2 is indirectly linked with research in aquaculture, as with more available fish feed, more fish shall be available for human consumption. This will result in a decrease in hunger and food insecurity. Goal 12 is directly linked as this research applies a novel way of producing alternative fish feeds from sustainable sources using green technology. Goal 14 is also connected to this study. The amount of fish stocks which are at biologically sustainable levels is the principal indicator in measuring the progress towards achieving SDG 14. Regrettably, the levels of biologically sustainable fish stocks had reduced to 65.8% by 2017 compared to 90% back in 1974 (FAO, 2020). This puts further stress on aquaculture.

Aquaculture has been of great aid in ensuring food security globally since it inflated fish accessibility to regions of the world with limited or no access to cultured fish species. Furthermore, it usually provides food at a lower price hence leads to better nutrition in low-income groups. Aquaculture has been the principal source of fish suitable for human consumption internationally since 2016. It is estimated that dependence on aquaculture will increase rapidly due to the depletion in levels of the captured fish production. Fish consumption is predicted to increase by 18% between 2018 and 2030 (FAO, 2018, 2020). This further emphasizes the importance of the use of sustainable alternative fish feed on fish farms, to supplement the use of soymeal and fishmeal and support the demand of protein in aquaculture.

The aim of this study is to delve into sustainable and alternative fish feeds that can be used for aquaculture operations. With the increasing demand for fish and seafood, aquaculture production facilities had to intensify their outputs. Currently, aquaculture feeds are mainly based on fish and soybean meal, which pose sustainability issues (Kim et al., 2019; Zhang et al., 2018). The extensive use of soybean meal has made the EU reliant on third-country imports, whilst fishmeal poses higher and increasing fish feed costs. This also causes further strain on the agriculture and fishing sector to keep up with the increasing demand. The use of alternative biomass, such as macroalgae, microalgae and duckweed, and the development of innovative processing technologies to integrate them in current feeds are required to tackle these issues (Henchion et al., 2017; Venugopal, 2021).

1.2 Need for alternative protein sources in fish feed

There is an increasing need for alternative protein sources to serve as an additive to current fish feeds. This is mainly due to the accelerated rate at which the global population is increasing, coupled with the inadequate allocation of arable land and water, which is also escalating problems (FAO, 2021). Macroalgae (seaweed e.g. *Alaria esculenta*) (Albrektsen et al., 2022), microalgae (unicellular algae e.g. *Chlorella vulgaris*) (Reis et al., 2022) and simple plants like duckweed (the smallest flowering plants e.g. *Lemna minor*) (Fiordelmondo et al., 2022) are all great sustainable alternative sources of protein. They are considered sustainable since they do not compete with the traditional fish feeds for resources and space. These biomasses are sources of alternative proteins and are all high in nutritional value, which makes them a viable option as additives to current fish feeds.

Natural resources are being depleted due to the increase in their demand all over the world. This is causing further stresses on the allocation and distribution thereof. As the world's population is increasing rapidly, more of these natural resources need to be redirected for food production. Fish aquaculture production facilities have been intensified and further developed to meet the demand of animal protein. Interestingly, the FAO estimates that 16.6% of the global demand for all animal protein intake is being fulfilled by fish and seafood. It is also believed that this percentage will increase steadily in the near future (Garlock et al., 2022; Kobayashi et al., 2015). The role of aquaculture, together with its opportunity for growth, is highlighted in Fig. 1.2.

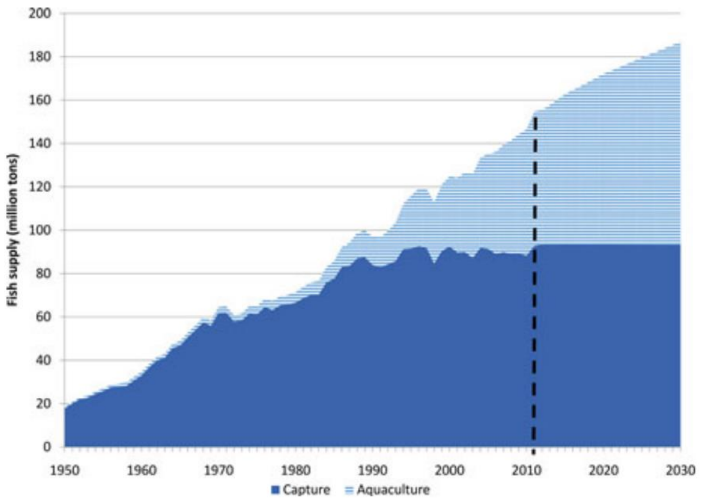

Fig. 1.2 Graph showing the global fish supply between 1950-2030, as described in the FishStat and IMPACT model projections. The vertical dashed line marks projection after 2011 (source: Kobayashi et al., 2015).

Contrary to common belief, the main limitation of the aquaculture industry is not related to simply land and water restrictions, but rather to the high cost of fish feed (Farmery et al., 2022; Gentry et al., 2017). Reducing fish feed costs through alternative protein sources would result in the aquaculture facilities achieving lower operational costs (Gasco et al., 2018).

The limitations of current fish feeds are also related to their negative environmental impact. Soybean meal and fish meal, which are the two most common types of feeds used, are both lacking in the aspect of sustainability. The current plant protein ingredients (e.g. rapeseed meal, corn gluten meal and soybean meal) which are being used as feeds in aquaculture are still crucial staples however the industry has little potential to increase production since it is already causing ample stress on arable land, water and even phosphorous resources (Hua et al., 2019; Malcorps et al., 2019).

Although fishmeals, which are made from animal byproducts (e.g. fish/meat and bone meal, poultry meal), are a great way to decrease food waste since the trimmings which are usually discarded by the food industry can be used to create feed, they pose another issue. It is extremely hard to tell the nutrient composition of this feed since it depends on the byproduct. Different parts of the animal's body will have different nutrient contents. This results in nutrient variability in the fishmeal (Hua et al., 2019).

Apart from this, setting up and maintaining an industrial-scale fishmeal production site is extremely costly. A constant supply of raw materials is required in very large quantities. Furthermore, the raw material is usually collected from remote fish processing plants that tend not to supply large enough quantities per day (Hua et al., 2019).

1.3 Aquaculture in Malta

Aquaculture, also known as fish farming, is the natural/controlled cultivation of fish, shellfish or seaweed in marine/freshwater environments. Over the last three decades, aquaculture became the fastest growing food sector nationally (Ministry for Sustainable Development, 2014). Due to this fact, it is a crucial contributor to national economic development, global food supply and food security (Farmery et al., 2022; Kobayashi et al., 2015; Ministry for Sustainable Development, 2014).

The aquaculture industry in Malta, as reported by the National Statistics Office (NSO) at the end of 2020, accounts for a total output of €178.7 million. This value marks a 1.4% increase over the corresponding value of €176.3 million that was registered in 2019. This means that its output grew by €2.4 million in 2020, following a €62.9 million drop in 2019 (NSO, 2021). The output and intermediate consumption of the industry between 2017 and 2020 can be followed in Fig. 1.3.

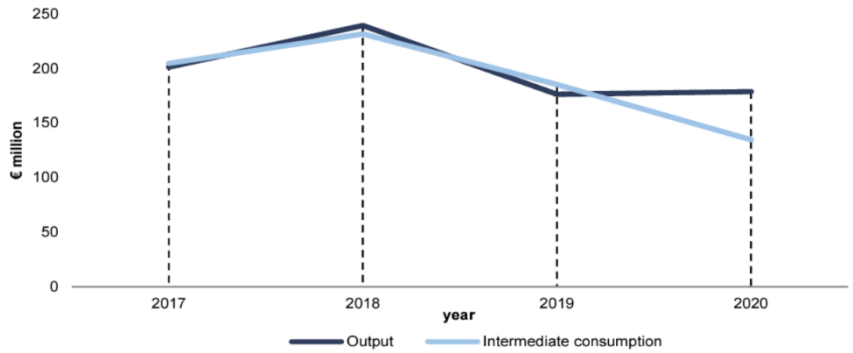

Fig. 1.3 Output and intermediate consumption of the aquaculture industry in Malta. The gap in between lines is the value added (gross). Statistics in this graph should be interpreted in the context of the COVID-19 situation (source: NSO, 2021).

This industry is based on two sectors which are capture-based species (CBS) farming and closed-cycle species (CCS) farming. CBS farming is used for the cultivation of Atlantic bluefin tuna (*Thunnus thynnus*, "tonn" in Maltese). In Malta, six tuna farms operate from specifically assigned Aquaculture Zones, five of which are situated 6 km off the south eastern coast, while the other one is towards the north of Malta (Sterling Aquaculture, 2012) (Fig. 1.4).

Fig. 1.4 One of the tuna ranches that is operating in the southeastern Aquacultural Zone, limits of Marsaxlokk Bay. Photo taken during a site visit to the fish farm.

CBS farming in Malta occurs in large cages bought from England, Italy and Spain. These were anchored 6 km from the coast, 90 m deep in the sea. Their diameters range from 35-60 m and their depth is around 30 m (Sterling Aquaculture, 2012). CBS farming involves the capturing of wild adult tuna (usually weighing around 100kg) in May/June, mainly from the southern Mediterranean region, followed by the transfer of this fish into the pens in Malta. Hereafter, they are fattened for a period of 6 months or more (Sterling Aquaculture, 2012). This sector comprised 91% of the total output generated in 2020 (NSO, 2021).

The slaughtered fish are exported fresh or frozen to Asian markets, mainly Japan. Bluefin tuna is highly sought after for raw fish dishes such as sushi and sashimi. The fresh product is exported on ice and transported by air, while the frozen product is slaughtered and cut up before being frozen at -80°C and subsequently transported by sea (Sterling Aquaculture, 2012). Tuna is partly fed locally-caught fresh mackerel, but mostly imported frozen mackerel, sardine and herring (Fig. 1.5). This is posing an environmental threat as the frozen fish decomposes fairly quickly in the warm waters during the summer months. A slimy effluent results, which floats along the coast. This oily substance is not only an eyesore, but also soils fishing nets and makes them hard to pull due to the slipperiness. To combat this problem, the frozen baitfish must be thawed on shore before being thrown into the tuna pens at sea, which is quite time-consuming (Borg, 2018).

Fig. 1.5 An image of the sardines that were fed to the captured tuna. Photo taken during a site visit to the fish farms in the South Aquacultural Zone.

Unfortunately, fish that has been caught in the wild, tends to refuse to eat fish feed in pellet form. This makes the tuna farming process rather unsustainable. Recently, aquaculture research groups around the world have been favouring an extruded sausage-shaped fish feed that has proven to be palatable to captured wild fish. Malta is aiming to create a similar product in terms of texture, shape and mouthfeel. This is planned to comprise alternative protein sources to make the tuna farming industry more sustainable in the coming years.

CCS farming, on the other hand, consists of gilt-head sea bream (*Sparus aurata*, "awrat" in Maltese), European sea bass (*Dicentrarchus labrax*, "spnott" in Maltese), meagre (*Argyrosomus regius*, "gurbell rar" in Maltese) and amberjack (*Seriola dumerili*, "aċċola" in Maltese) that are raised from eggs produced in hatcheries and fed on manufactured dry feed. The fingerlings are imported from approved hatcheries in Italy, France and Spain and then grown in Malta. There are two CCS farms, one that operates close to shore at Mistra Bay, St Paul's Bay, Mellieħa on the north coast; and another found close to Xrobb l-Għaġin at the southern end of the Maltese Islands (Sterling Aquaculture, 2012). All closed-cycle fish farmed in Malta are reared in floating sea cages. Nursery cages usually measure 5 m x 5 m x 5 m with a depth of 10 m, circular nursery cages measure 12 m in diameter with a depth of 10 m, whereas offshore cages are 20 m in diameter x 10 m deep (Sterling Aquaculture, 2012).

This sector comprised 8% of the total output generated in 2020 (NSO, 2021). The total sales of farmed fish increased dramatically by 43.4% (six million kilograms) when compared to 2019. This was mostly due to the 4.6 million kilogram increase in the volume of sales of farmed tuna (NSO, 2021). The bulk of sea bream and sea bass produced in Malta is exported by truck fresh, on ice, to Italy. They are sold as whole portion-sized fish, with an average weight of 300-400 g. Producers have shown some interest in the production of meagre over recent years. Growth rates are impressive, with fish reaching an average weight of 1.2 kg within 12 months. The main constraint to the expansion in the production of this species is poor market demand. Therefore, production volumes are expected to remain low unless there is some market development that increases demand.

In CCS farms, 2 g fingerlings are stocked in cages and fed on dry pellets imported from Europe. The pellets are a mix of fish and soybean meal. The pellet size is increased

according to the fish's life stage from 1 mm to 1.5 mm to 2 mm and finally to 5 mm. The fish are fed 3 to 4 times daily (Fig. 1.6). They are grown to a market size of 350-450 g. The average farm cycle is approximately 15 months.

Fig. 1.6 One of the CCS cages that is operating in the South Aquacultural Zone, limits of Marsaxlokk Bay. Photo taken during a site visit to the fish farms. An employee can be seen feeding fishmeal pellets. The multiple ripples seen on the water surface are caused by the fish surfacing to eat.

1.4 Fish nutrition

The most important task of any animal production system is to supply the farmed animal with a balanced and nutritious diet. This ensures a high-quality end product. Interestingly, feed accounts for about 50% of the overall cost of a fish farming plant. Hence, there is a huge demand for affordable high-quality fish feeds. Species-specific diet formulations are the way forward, so as to help the fish reach the desired weight in the shortest timeframe possible (Aragão et al., 2022; Daniel, 2018).

With protein being the costliest nutrient in fish feed, it is common practice for fish farms to determine the exact protein requirement at different life stages for specific species that are being cultivated. Fish cannot synthesize the following 10 amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Hence, they are referred to as essential amino acids. It is worth mentioning that feeds made from plant protein sources, e.g. soybean meal, tend to be deficient in

methionine. Furthermore, feeds that are made from protein which was extracted from yeast or bacteria tend to be deficient in methionine as well as lysine. Care must be taken to supplement fish with the essential amino acids in a feed formulation, so as to ensure optimal health and growth of the animal (Gatlin & Yamamoto, 2022; Hua et al., 2019).

As a general rule, herbivorous and omnivorous fish have lower protein requirements than carnivorous fish. Furthermore, fish grown in fish farms having low-density culture systems (e.g. ponds) have a lower protein requirement than ones grown in high-density systems (e.g. recirculation aquaculture). Smaller fish need more protein than fish which grow large in size. Similarly, fingerlings in their early life stages require more protein than adult fish. Interestingly, external factors such as environment, water temperature, water quality and feeding rates, all cause variations in the protein requirements. An in-depth evaluation of the fish farm needs to be carried put before deciding on the best protein percentages to be fed to any specific fish (Gatlin & Yamamoto, 2022; Hua et al., 2019).

Fats tend to be added as alternatives to proteins for supply of energy. Apart from this, they act as vessels to carry fat-soluble vitamins. Fats need to be added sparingly however, as they decrease the shelf-life of feeds, and pose a risk of liver disease and general ill-health in fish. Interestingly, algal oils are naturally high in omega-3s (unsaturated fatty acids) which are healthy for human health (Byreddy et al., 2019; Norambuena et al., 2015). These omega-3 fatty acids are consumed by the fish, and deposited into fish muscle. This makes the end-product more nutritious to humans (Kwasek et al., 2020).

Carbohydrates are the cheapest source of energy for fish. They are not really required as proteins and fats can provide energy to fish; however, the addition of carbohydrate lowers the feed costs. Apart from this, starches used during the extrusion process of formulating feed has been found to increase the bioavailability of nutrients (Ansari et al., 2021). Vitamins are organic water/fat-soluble compounds which are crucial to fish health. Water-soluble vitamins include all the B vitamins and vitamin C (ascorbic acid). Vitamin C is extremely important due to its immune system-boosting properties as well as being a great antioxidant. The fat-soluble vitamins are A, D E and K, out of which vitamin E (tocopherols) is the most prioritized due to its antioxidant nature. Also worth including is that due to their antioxidant effects, both vitamin C and E hinder lipid

oxidation and thus prevent feed spoilage and nutrient degradation (Ansari et al., 2021; Halver, 2003).

The last components required for good fish nutrition are minerals. These are inorganic compounds which can either be supplied in the feed or taken up from the surrounding water through the fish's skin and gills. Macrominerals like calcium, phosphorous and sodium are essential for the regulation of osmotic balance and bone growth. Microminerals like zinc, iron and selenium are only required in trace amounts and help in hormone and enzyme systems (Lall & Kaushik, 2021).

1.5 Current fish feed and their limitations

The high costs associated with baitfish to maintain CBS operations and that of other fish feeds used in CSS operations is a major challenge to make fish farming cost-effective. The main source of protein in conventional CSS feeds is derived from fishmeal and the increase in its demands has brought higher feed costs. The FAO reports that during the 2000-2008 period, a 62% increase in global aquaculture production was achieved with global fishmeal demand declining by 12%. This was due to various research efforts to reduce the inclusion of the fishmeal component in the major farmed species (Kobayashi et al., 2015; Zhang et al., 2018).

Fishmeal is being replaced by plant-based protein sources such as soybean meal, which gives rise to both nutritional and sustainable issues (Fig. 1.7). Soybean meal poses nutritional issues due to the presence of a variety of antinutritional factors (Francis et al., 2001; Zhang et al., 2018). This kind of meal contains antivitamins, lectins, phytic acid, phytoestrogens, protease inhibitors and saponins, all of which were reported to cause a decline in fish growth (Zhang et al., 2018).

Fig. 1.7 The fish feed used at the Mount Lucas Wind Farm, County Offaly, Ireland. This is a mixture of fish and soybean-meal extruded in pellet form in different sizes, depending on species and the life stage of the fish. Photo taken during a visit to the wind farm.

Using soymeal and fishmeal in aquaculture is a tried and tested process that has been optimized greatly over the years. However, there will not be enough feed production to sustain the ever-growing demand of fish from human consumption. Soymeal and fishmeal must now be supplemented by some other sources of protein to meet this high demand. From a sustainability perspective, the extensive use of soybean meal as a substituent protein source has led the EU to have a 70-80% production deficit due to its reliance on third-country imports (Kim et al., 2019; Martin, 2014). If efforts were made to account for this deficit, it would require the EU to allocate 20-30% of its arable land to eliminate this reliance on imports. This would cause further strain on the agriculture sector to keep up with the increasing demand when arable land is already a limited resource. Therefore, alternatives to plant-based protein sources are needed to establish new sustainable sources that keep up with the growth of these sectors and relieve current pressure on natural resources (Kim et al., 2019).

Animal production systems require adequate nutrition in order to result in a high-quality end-product that is both healthy for human consumption and relatively cheap. With feed constituting about 50% of the variable production cost, nutrition is of the essence in fish farming. Recently there has been substantial improvement in fish nutrition. Scientists have created profit-oriented diets that are also balanced in nutrients, that assist in optimal health and growth in fish. There have also been advancements in the formulation of species-specific feeds, which aid the aquaculture industry greatly (Craig et al., 2017; Yarnold et al., 2019).

Formulated feeds can be supplemental or complete. As suggested by the name, complete feeds provide all the required nutrients (macronutrients: carbohydrates, protein, fats; micronutrients: vitamins and minerals) needed for the best growth and overall health of the fish. Due to their ease of use, the majority of fish farmers utilize complete feeds. Table 1.1 depicts the usual quantities of each nutrient.

Table 1.1 The typical components found in a complete feed (adapted from Craig et al., 2017).

Components	**Percentage ranges (%)**
Protein	18-50
Lipid	10-25
Carbohydrate	15-20
Ash	<8.5
Phosphorus	<1.5
Water	<10
Vitamins and minerals	Trace amounts

Supplemental feeds are used to fortify the naturally occurring food in outdoor raceways or ponds. These supplemental feeds do not comprise a full array of vitamins and minerals, instead they tend to have extra protein, lipid of carbohydrate to strengthen the nutritional value of the ordinary diet. With protein being the costliest ingredient in the fish feeds, it is wise to establish the exact protein requirements for specific fish species and the respective life stage during which they are cultured (Yarnold et al., 2019).

1.6 Alternative fish feeds

The use and integration of alternative fish feed with current aquaculture fed practices poses numerous challenges including variable protein content, feed production feasibility and consumer trends. The variable protein content of these alternative feeds requires feed formulations to be made of multiple alternative ingredients. It is unlikely that a singular protein source would be established to meet the requirement for all feed operation or a completely substituted fishmeal. Yet these multi-formulated feeds would provide more diverse nutritional profiles and offer manufacturers more flexibility when ingredient prices fluctuate (Hua et al., 2019).

Ingredient price is a significant factor in establishing feed production feasibility but other factors such as the availability of processing technologies and their scalability also influence this factor. Several candidate alternative protein sources have been identified, including macroalgae, microalgae and duckweed. These alternative biomasses also contain other important metabolites like polyunsaturated fatty acids, polysaccharides, vitamins and micronutrients such as zinc (Zn), and iron (Fe) (Aragão et al., 2022; Daniel, 2018; Norambuena et al., 2015).

Macroalgae (seaweed) have been increasing in popularity as a potential alternative source of protein to supplement fish feeds. A great benefit of using seaweeds is that their harvest does not require the use of arable land. They are easy to access along the coastal regions and also simple to farm. Furthermore, macroalgae have been used for many years as sources of iodine and have the potential to supply trace elements in animal feeds (Wan et al., 2019). Macroalgae have recently been studied more intently, and it was found that they enhance stress resistance, physiology, fish growth, fillet muscle quality and fish immune system (Annamalai et al., 2021). Interestingly, the water surrounding fish farms is the ideal environment to cultivate macroalgae. This water is concentrated in fish waste (effluent) and nutrients which has been found to proliferate the growth of seaweed (Dhingra & Kandiannan, 2021).

Microalgae (phytoplankton) have similar protein content levels when compared to other traditional meat and soybean-based sources. This is also coupled with a greater protein yield per unit area used, in contrast to terrestrial crops (4–15 tons/ha/year against 2.5-7.5 tons/ha/year, respectively) (Bleakley & Hayes, 2017; Byreddy et al., 2019). Microalgae production is more sustainable as it does not require extensive land and water use, it can be harvested all-year-round with high biomass yields, and its cultivation can utilize wastewater.

Aquatic floating plants such as duckweed have similar advantages as microalgae, with the addition of being more easily harvested, requiring no agitation system and having a low sensitivity to pests and diseases (Iqbal, 1999; Slembrouck et al., 2018). Interestingly, duckweed grows readily as a byproduct of freshwater fish farms (Fig. 1.8).

Fig. 1.8 A pond at the Mount Lucas Wind Farm, County Offaly, Ireland. The bright, light green parts floating on top of the water's surface are duckweed. Photo taken during a visit to the wind farm.

Duckweed was initially a problem to the freshwater fish ponds at Mount Lucas Wind Farm, in Co. Offaly, Ireland. These ponds were man-made just a few years ago, and started to be used for the cultivation of trout. Duckweed started growing rapidly and was acting as a filter feeder by soaking up nutrients from the fish's waste. However, it grew so much that the trout could not eat all of it and this resulted in depleted oxygen levels. To combat this, several troughs were dug up to link all the ponds together and paddle pumps were set up in each pond (Fig. 1.9). This created a slight water current which directed the extra duckweed which grow in the ponds to migrate to the last pond where it can be collected, dried and sold as raw material for animal feed. The pumps also serve as aerators.

Fig. 1.9 A pond with a paddle pump at the Mount Lucas Wind Farm. Photo taken during a visit to the wind farm.

Although there is an increasing interest in alternative protein sources from macroalgae, microalgae and duckweed, more research needs to be done. Findings are limited as of yet and there is a gap in the literature. Furthermore, fish trials are needed to ensure that these alternative fish feeds do have the predicted effect on different fish species. Gilthead sea bream is a good fish species to start with as they are not picky with food and are omnivores (Pulido-Rodriguez et al., 2021).

1.7 Novel approaches using ultrasound- and enzyme-assisted extraction

As previously stated, macroalgae, microalgae and duckweed are all rich in proteins, fats, carbohydrates, vitamins and minerals. A dehydration step is usually carried out before the biomasses are sold, as dry biomass is easier to handle, transport and store. However, the drying process can lead to denaturation and loss of functionality of proteins (Soto-Sierra et al., 2018). Having said this, protein extraction from dried biomass was found to be easier than extracting from the fresh biomass. Macroalgae have viscose intercellular matrices held together with a rigid polysaccharide cell wall. This cell wall inhibits the release of intracellular components, hence pretreatment such as chopping is required to facilitate cell disruption (Bleakley & Hayes, 2017; Cunha & Pintado, 2022). Non-food grade enzymes and chemicals must be avoided at all costs, since the end-product (fish feed) will be given to fish meant for human consumption.

There are barely any studies on the extraction of proteins from macroalgae and duckweed to date. Protein extraction from microalgae has been studied more extensively and findings from these studies can serve as a starting point for macroalgae and duckweed protein extraction. Protein extraction serves to make nutrients more digestible and bioaccessible as the process breaks down the tough polysaccharide cell walls (Bleakley & Hayes, 2017; Cunha & Pintado, 2022).

With consumers becoming more environmentally conscious, the aquaculture industry is experiencing a shift from so-called “brown technology” to “green technology”. Green technology performs the same function as its traditional counterpart but with significantly less environmental impact (Yu et al., 2016; Q. Zhang et al., 2020). Fig. 1.10 describes various green technologies which can be categorised as bio-preservation,

electromagnetic wave heating, electric and magnetic fields, and non-thermal technologies (Inguanez, 2020; Ngadi et al., 2012).

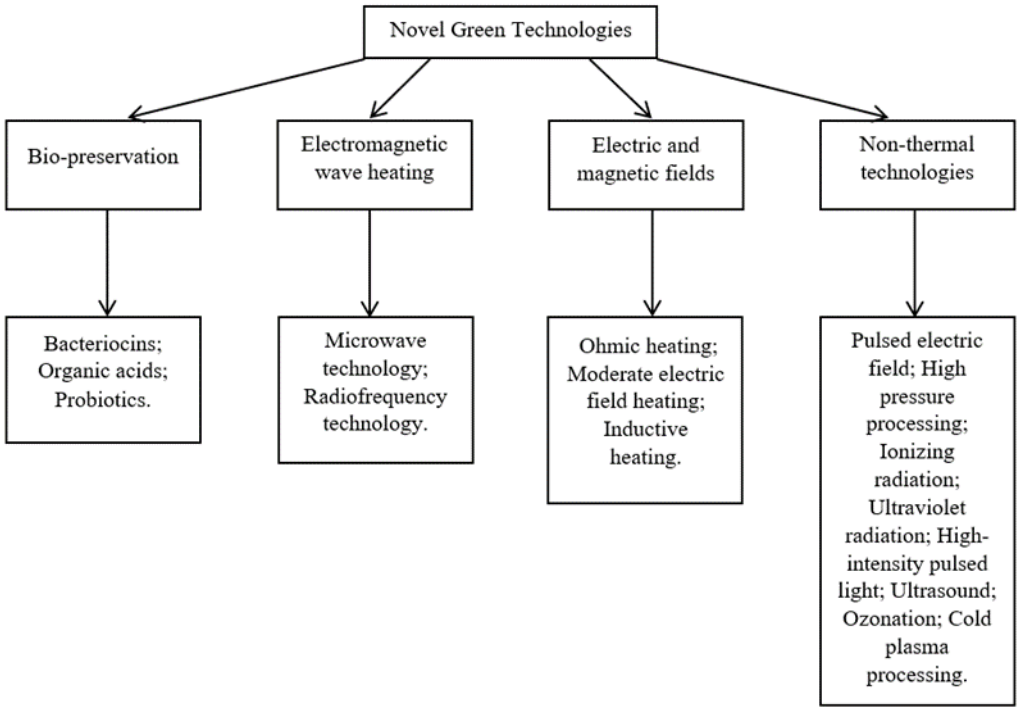

Fig. 1.10 Representative developing technologies for microbial control in food processing (source: Inguanez, 2020; adapted from Ngadi, Latheef, & Kassama, 2012).

Ultrasound technology works by creating bubble cavitation. Microbubbles grow and collapse repeatedly, creating shock waves that are powerful enough to create microtears in cell structures, causing the release of intracellular matter (Fig. 1.11). Sonication has a high energy expenditure and causes pressure and temperature to increase in regions of cavitation. Care must be taken to control these parameters so as to ensure a good quality protein.

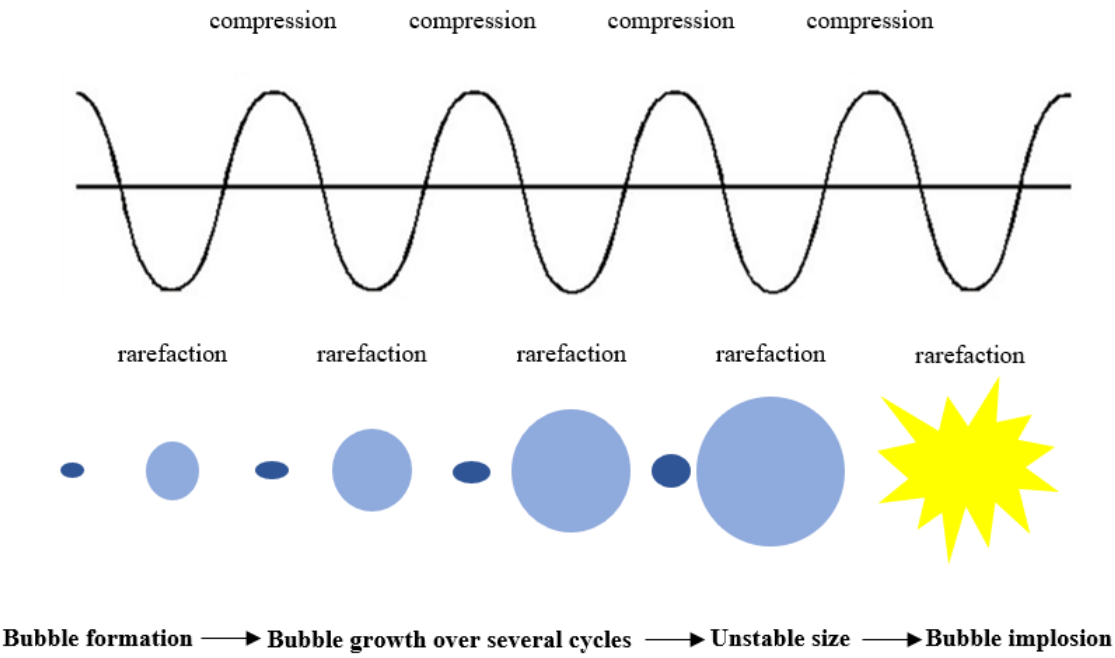

Fig. 1.11 Ultrasonic cavitation. Depiction of how sonication applies sound energy to agitate particles in a sample, eventually causing the cells to burst and release their contents (source: Inguanez,2020; adapted from Hielscher Ultrasound Technology website).

High-power ultrasound has been extensively used in the food industry due to its relatively cheap apparatus, simple operation, and efficiency in increasing yields (Bhargava et al., 2021; Pojić et al., 2018). Using ultrasound speeds up the extraction rate and promotes the extraction yield in solid-liquid extraction. It can also decrease the time of operation and the temperature, which is particularly favoured for the extraction of thermolabile compounds such as proteins (Zhu et al., 2018). Likened to other techniques such as high-pressure application, the apparatus for ultrasound is much more cost-effective and easy to operate, while it also consumes less energy (Bhargava et al., 2021; Chemat et al., 2017).

There is a gap in the literature regarding the combination of green technologies. Enzyme-assisted extraction alone is a very mild process that does not achieve cell lysis, however, coupled with other green technology it is predicted to make extraction more efficient overall (Fig. 1.12). A crucial step is to understand the composition of the cell walls for the specific biomass being used as enzymes are highly specific (Nadar et al., 2018).

Fig. 1.12 Extraction of various biomolecules by using enzyme-assisted extraction (adapted from Nadar et al., 2018).

Algal cell walls have a very complex composition. Hence, enzymes should be chosen based on the target species and its matrix. One must be careful when adding enzymes as they can impact the structure and functionality of proteins, which must be retained for food implementation. However, there are also benefits to using enzymes as they can disrupt the cell wall and act as hydrolyzing agents to create protein hydrolysates. The cost of creating enzyme cocktails to target such complex matrices is quite high. However, the energy requirements are very low and the operating conditions are mild (Amorim et al., 2020). The search strategy of the literature review is presented in the Appendix.

1.8 Overall problem statement, aims and objectives

With wild fish stocks depleting, there is an increased need for sustainable aquaculture. Current fish feeds are mainly created from fish trimmings and soya beans, both of which

are unsustainable sources. Furthermore, the use of green technology such as ultrasound is still relatively undocumented as a means to extract bioactive compounds. Fish must be provided with nutritionally balanced feed as a prerequisite to cost-effective production. There is an evident need for alternative protein sources to act as additives in fish feeds. It is also clear that studies are needed to optimise the production of alternative protein rich sources that have enhanced stability following their treatment with green technologies such as ultrasound.

Overall, this research aimed to tackle global food security issues. The study fulfils both national and EU objectives by improving fish production. Brood and fish stocks shall be improved due to the addition of high nutritional valued biomass (such as macroalgae, microalgae and duckweed) into fish feed.

More specifically, this research aims to create alternative feed formulations by using biomasses of *Alaria esculenta, Chlorella vulgaris* and *Lemna minor* which are nutritious and easy to cultivate. Hence, these biomasses are considered sustainable sources of protein. The main objectives of this study were:

1. To optimise an ultrasound treatment for achieving extraction of nutritional compounds
2. To identify and retain nutritional compounds post-treatment for treated fish feed components
3. To mitigate undesirable compounds in fish feed components by the selected ultrasound treatment

Chapter 2 Research methodology

This project aims to address the concerns discussed in Chapter 1 by investigating ultrasound processing technologies to further enhance the quality and feasibility of these new sustainable sources to keep up with the growth of the aquaculture sector. Enzyme-assisted extraction, another form of green technology, was also studied and included in this research. Various biochemical, microbiological and physical assessments were conducted to further support the feasibility and ensure the quality of the processed alternative feeds. The resultant research acquired shall provide critical knowledge for the local aquaculture sector to further substantiate the integration of these alternative feeds in their operations. This would have the potential to increase the sustainability and competitiveness of the local maritime aquaculture sector.

2.1 Characterisation of raw material

2.1.1 Colorimetry

The colorimetry tests were performed in the bioactive isolation lab at Teagasc, Dublin, Ireland. Colour measurement was used as an indicator to determine whether a food is appetizing or not. The test is usually performed before and after a treatment, to determine the colour change caused by the process. Absorbance readings were only taken prior to treatment since the end product will be used as fish feed and is not meant for human consumption.

The colorimeter (CR-410 Chroma Meter, Konica Minolta) was calibrated by reading the absorbance of a white tile (Fig. 2.1). Around 100 g of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1), respectively, were placed into separate, identical plastic bags and sealed. Each biomass was shaken vigorously to ensure a homogenous sample. Absorbance readings of four random points were taken for each sample (Fig. 2.2).

Fig. 2.1 Calibrating the colorimeter against a white tile.

Fig. 2.2 Taking an absorbance reading of *Chlorella vulgaris*.

The data was averaged and the standard deviation was calculated in Excel. GraphPad Prism version 8 was used to perform statistical tests. The statistical analysis was carried out systematically. First, the data were checked for outliers using the ROUT method, and if any were found, these values were excluded from the dataset. Next, the data were tested for normality. If they were found to be normally distributed, a parametric test was performed. If, however, they did not pass the normality test, the data were transformed to attempt to make them normally distributed. If the transformation succeeded, the parametric test was performed. If on the other hand, no transformation managed to normalise the dataset, the nonparametric equivalent was performed. Denotation of significant differences (p <0.01) in each result table followed these rules:

- Superscripts were assigned in alphabetical order.
- The largest mean or means bore the superscript that comes the earliest in the alphabet.
- Same superscripts were placed next to means which did not significantly differ one another.

- Different superscripts were placed next to means which were significantly different.
- Where *mean x* and *mean y* were significantly different from each other, but *mean z* did not significantly differ from either *mean x* nor *mean y*, *mean z* bore both superscripts that were placed next to *mean x* and *mean y* (e.g. *mean* x^{a}, *mean* y^{b} and *mean* z^{ab}).

In this set of results, one-way ANOVA was used as the parametric test and Kruskal-Wallis was used as the nonparametric test, in order to compare any statistical differences between the biomasses.

2.1.2 Microscopy

A small amount of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1) was placed on three separate microscope slides. The biomasses were viewed under a magnification of x40, x100 and x400 using a microscope (CME binocular microscope, Leica, Germany) (Fig. 2.3).

Fig. 2.3 Leica CME binocular microscope.

Images of the grid of a haemocytometer were taken under a magnification of x40, x100 and x400 (Fig. 2.4).

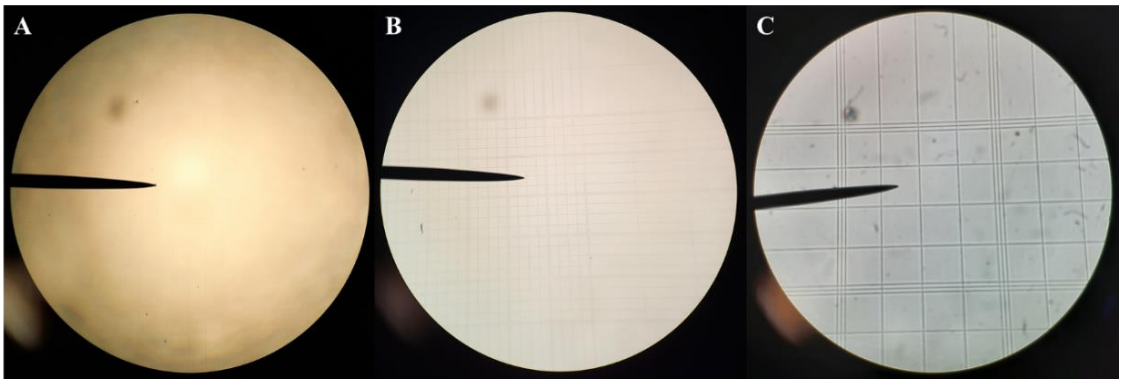

Fig. 2.4 Light microscope images of a haemocytometer as seen under different magnifications. (A) Haemocytometer as seen under low-power Mag. x40. (B) Haemocytometer as seen under medium-power Mag. x100. (C) Haemocytometer as seen under high-power Mag. x400.

These three images were then processed using ImageJ. A yellow line was drawn on the side of a 200 µm square for each image (Mag. x40, x100, x400, respectively). Each yellow line was then analysed to determine its length in pixels (Fig. 2.5). This length was used to create an accurate scale bar for each magnification, and was added to the microscopy images of the biomasses.

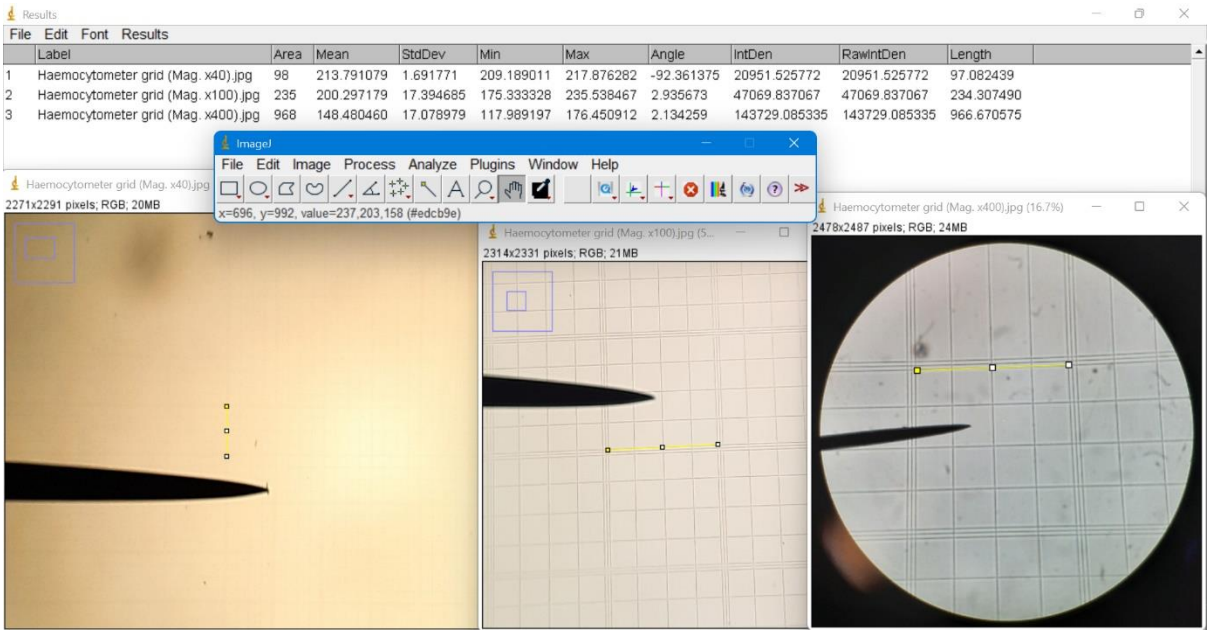

Fig. 2.5 Screenshot of ImageJ software in use. The table at the top shows the length of each yellow line (200 µm length of haemocytometer's square) in pixels. Below the table there is the ImageJ toolbar. At the bottom there are the images of the haemocytometer at different magnifications (Mag. x40, x100, x400 from left to right).

Each biomass image was opened in Microsoft Paint and analysed to determine the most ideal scale. After the desired image was opened in Microsoft Paint, the line tool was selected and a line was drawn across the length of a biomass particle. The line's length was noted in pixels and this value was compared to the length of the yellow line under the same magnification from Fig. 2.5 above. The most appropriate scale was chosen by creating the following formula in Excel: "**Scale (µm) = [biomass length (pixels) * 200 (µm)] / length of yellow line (pixels)**". The 200 µm is the actual length of the yellow line. The rectangle tool was then selected and a white line with the correct pixel length (to ensure an accurate scale) was created. The text tool was selected and the scale was written above the white line (Fig. 2.6). The images were then saved and merged to compare the different biomasses to each other.

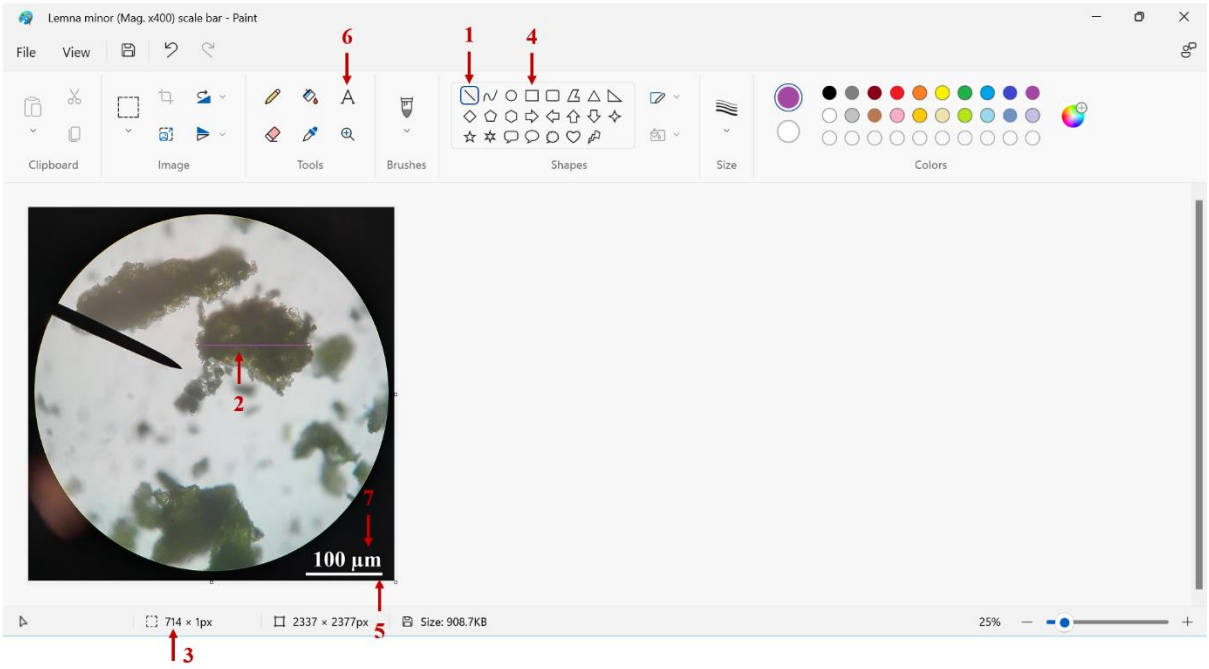

Fig. 2.6 Screenshot of Microsoft Paint software in use. An image of *Lemna minor* as seen under high-power (Mag. x400) was opened. (1) Line tool was selected. (2) Line was drawn across the length of the *Lemna minor* particle. (3) Line's length was noted in pixels and an appropriate scale was chosen. (4) Rectangle tool was selected. (5) White rectangle with the correct pixel length was created. (6) Text tool was selected. (7) Scale was written above the white line.

2.1.3 Water activity

Each biomass was placed in 5 separate sample cups. It was ensured that the bottom of all sample cups was completely covered with biomass, but the volume did not exceed more than half the cup. To ensure this, 3.5 g of *Alaria esculenta* (type 1 and 2); 3.5 g of *Chlorella vulgaris*; and 1.5 g of *Lemna minor* (type 1 and 2) were weighed out for each replicate. The machine was set to perform the following test: $\mathbf{a_w = P/P_0}$, where P is the vapour pressure in the sample and P_0 is the vapour pressure of pure water. Five replicates for each biomass were analysed using the dew point and water activity meter (4TE, Aqua Lab, Munich, Germany) (Fig. 2.7).

Fig. 2.7 The water activity meter taking a reading from a biomass sample, with two biomasses prepared in a sample cup on the bottom right corner.

The data was analysed as described in section 2.1.1. In this set of results, one-way ANOVA was used as the parametric test and Kruskal-Wallis was used as the nonparametric test, in order to compare any statistical differences between the biomasses.

2.1.4 Moisture content

This experiment was carried out on of *Alaria esculenta* (type 1 and 2); *Chlorella vulgaris*; and *Lemna minor* (type 1 and 2). The readings were taken using the moisture analyzer (PMB 53, Adam, United Kingdom) (Fig. 2.8). Samples had to be placed on the pan in an even homogenous layer to ensure uniform heating and allow the moisture to be on the surface. All the biomasses were in powder form so no further sample preparation was required. The analyzer was left to reach a stable internal temperature for about 30 min until the stable sign ~ was shown on the bottom left of the display screen (Fig. 2.9). The [Test] key was pressed and the machine was set to perform the

following moisture test: moisture → **% moisture = 100 x ((initial mass - dry mass)/initial mass)**. The single option was pressed for the heat setting and 140°C was inputted, meaning that there was only one single temperature setting to heat to. The time interval at which results were to be computed was set at 10 seconds. The rapid heat option was selected, which heats the sample to 145% of the set temperature value (in this case 203°C) for 3 min to initiate the process quickly. The timer was set for 15 min to stop automatically and then the manual option was selected to manually start the process by pressing the start button.

Fig. 2.8 The moisture analyzer, PMB 53, Adam, United Kingdom.

Fig. 2.9 The stable sign ~ is seen once the machine reaches a stable internal temperature.

After the test procedure was set up, an empty aluminium pan was weighed and tared (Fig. 2.10). The pan was removed and filled with 8 g of sample (Fig. 2.11). The filled pan was then placed on the analyzer, the lid was lowered and the start button was pressed (Fig. 2.12). Once the test was complete, the end result (% moisture) was seen on the display screen (Fig. 2.13). The [Dspl] key was pressed to show the initial mass and final mass. Three replicates for each biomass were performed.

Fig. 2.10 The aluminium pan was weighed and tared.

Fig. 2.11 The aluminium pan was filled with 8 g of sample.

Fig. 2.12 The lid was lowered and the once the process started, light was emitted from the red-hot filament.

Fig. 2.13 The end result (% moisture) was seen on the display screen.

The data was analysed as described in section 2.1.1. In this set of results, one-way ANOVA was used as the parametric test and Kruskal-Wallis was used as the nonparametric test, in order to compare any statistical differences between the biomasses.

2.2 Impact of different ultrasound set-ups on biomass treatments

2.2.1 Ultrasound energetics

2.2.1.1 Bath and UP200St probe sonicators at 150 W

The bath sonicator (Elma S 60 Elmasonic, Germany) was filled with 2300 mL of distilled water (dH_2O), enough volume to ensure that the sample is completely immersed and surrounded by water. A beaker was filled with 350 mL dH_2O and placed in the bath. The bath had a fixed power of 150 W. A digital thermometer was used to monitor the internal temperature of the beaker (Fig. 2.14). After three trials, the time it takes for the sample to reach an internal temperature of around 50°C was determined.

Fig. 2.14 The set-up of the bath sonicator.

A 14 mm diameter (Ø) sonotrode was fitted to the probe sonicator (Ultrasonic Processor-Model UP200St, Hielscher Ultrasonic, Germany). The probe was inserted 4.5 cm deep in a beaker containing 350 mL dH_2O. The probe was set at a power of 150 W (continuous/no pulses, amplitude at 20%). The digital thermometer was used to monitor the internal temperature of the beaker (Fig. 2.15). After three trials, the time it takes for the sample to reach an internal temperature of around 50°C was determined.

Fig. 2.15 The set-up of the UP200St probe sonicator.

The data was analysed using Excel and GraphPad Prism version 8. The energy output was determined using the following equations:

Absolute ultrasonic power $$\boldsymbol{P = mC_p \left(\frac{dT}{dt}\right)_{t=0}}$$

Ultrasound intensity $$\boldsymbol{UI = \frac{4P}{\pi D^2}}$$

Acoustic energy density $$\boldsymbol{AED = \frac{P}{V}}$$

Table 2.1 gives a guide to how each equation was calculated.

Table 2.1 Guide to determine the energy output of ultrasonic treatments.

Symbol	Meaning
Equation 1	
P	Absolute ultrasonic power
m	Mass *Bath sonicator: weight of water in beaker and basin* *Probe sonicator: weight of water in beaker*
C_p	Specific heat capacity at time = 0 *Calculated from the CRC Handbook of Chemistry by referring to the average temperature at the start of experiments*
(dT/dt)	Initial rate of change of temperature during sonication *Calculated from the equation of the line from each data set*
Equation 2	
UI	Ultrasonic intensity
D	Diameter of emitter/probe *Bath sonicator: surface area of inner sides of basin* *Probe sonicator: diameter of probe*
P	Absolute ultrasonic power
Equation 3	
AED	Acoustic energy density
P	Absolute ultrasonic power
V	Volume of medium *Bath sonicator: volume of water in beaker and basin* *Probe sonicator: volume of water in beaker*

2.2.1.2 UP200St probe sonicator at different amplitude settings

This test served to determine the time it takes to heat 500 mL dH_2O to 70°C using different parameters. The UP200St machine does not allow changing both power and amplitude settings at one go. For this reason, the experiment focused on changing the amplitude to 75%, 50% and 25%, respectively; the power varied accordingly and the ultrasonic pulse was set as continuous. Tests were initially performed using a 14 mm Ø sonotrode and a 40 mm Ø sonotrode (Fig. 2.16), immersed 4.5 cm deep into the sample. The 40 mm Ø sonotrode was not used further as it was creating cracks in the glass beakers (a circular cut just beneath the probe), causing water to flow out. The digital thermometer was used to monitor the internal temperature of the beaker. Tests on each

of the three amplitude settings were repeated thrice. The time it took to reach 70°C, for each of the three amplitude settings, was divided by 5. From this value, 5 different time points to reach 70°C (or the threshold temperature) were specified. The temperature was recorded for each of the 5 time points until the internal temperature of the beaker reached 70°C.

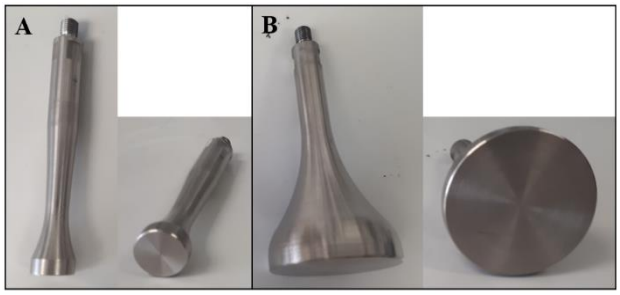

Fig. 2.16 Different sonotrode Ø. (A) 14 mm Ø; (B) 40 mm Ø.

The data was analysed using Excel and GraphPad Prism version 8. The energy output was determined using the equations described in Table 2.1 (refer to section 2.2.1.1).

2.2.1.3 UP400St probe sonicator at different amplitude settings

A beaker was filled with 80 mL dH_2O. The UP400St machine (Ultrasonic Processor-Model UP400St, Hielscher Ultrasonic, Germany) was set to automatically stop the treatment when the internal temperature of a sample reached 70°C. The amplitude was set at 60%, 55%, 50%, 45%, 40%, 25%, and 20%, respectively; the power varied accordingly and the ultrasonic pulse was set as continuous. The tests were performed using a 14 mm Ø sonotrode at a depth of 4.5 cm in the sample (Fig. 2.17). The fixed-in digital thermometer was used to monitor the internal temperature of the samples.

Fig. 2.17 The set-up of the UP400St probe sonicator.

Tests on each of the 7 amplitude settings were repeated three times. The CSV file generated by the machine was analysed using Excel and GraphPad Prism version 8. The energy output was determined using the equations described in Table 2.1 (refer to section 2.2.1.1).

2.2.2 Ultrasound extraction

Three alternative proteinaceous fish feeds were chosen to be analysed: *Alaria esculenta* (type 1) (macroalgae, brown seaweed); *Chlorella vulgaris* (microalgae); and *Lemna minor* (type 1) (smallest flowering plant). The three biomasses were purchased from different suppliers and pre-processed in different ways. *Alaria esculenta* (type 1) was supplied by Connemara, Organic Seaweed Company, Ireland. It was delivered pre-dried and roughly ground but a finer powder was desired, thus it was ground further using a coffee grinder (Silvercrest SKEE 150 A1) (Fig. 2.18, Fig 2.19 A). *Chlorella vulgaris* was purchased from Mega Foods, Greece. It came pre-dried and as a loose fine powder (Fig. 2.19 B). *Lemna minor* (type 1) was purchased from a farmer in Greece in pre-dried form and it was ground to a fine powder using the Silvercrest coffee grinder (Fig. 2.18, Fig 2.19 C).

Fig. 2.18 Silvercrest coffee grinder was used to grind *Alaria esculenta* and *Lemna minor* into fine powders. Plastic tub on the right contains unground *Lemna minor*.

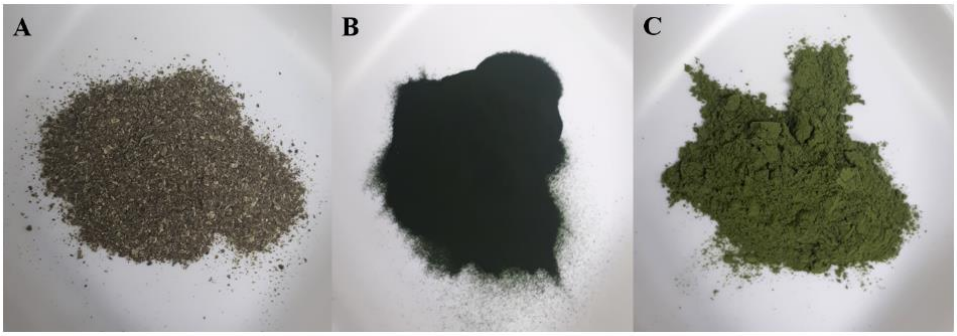

Fig. 2.19 The three biomasses. (A) *Alaria esculenta* (type 1), (B) *Chlorella vulgaris*, (C) *Lemna minor* (type 1).

2.2.2.1 Bath vs probe ultrasound-assisted extraction

Approximately 1 g of biomass (*Alaria esculenta* (type 1), *Chlorella vulgaris* and *Lemna minor* (type 1)) was weighed out using an analytical balance (TE64, Sartorius, Germany) (Fig. 2.20). The biomass was put in a 50 mL centrifuge tube (Fig. 2.21). About 40 mL of dH_2O were measured and poured into the tube (Fig. 2.22). The mixture was stirred well with a glass rod (Fig. 2.23).

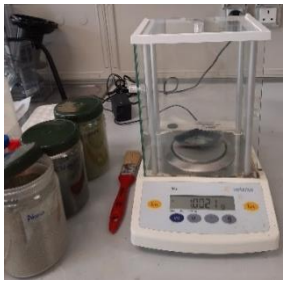

Fig. 2.20 The biomasses being weighed to 1 g on the analytical scale. On the left are samples of *Alaria esculenta* (type 1), *Chlorella vulgaris* and *Lemna minor* (type 1) from front to back.

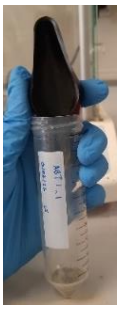

Fig. 2.21 The biomasses being placed in a 50 mL centrifuge tube.

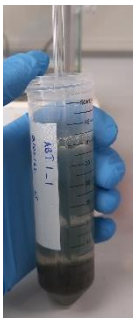

Fig. 2.22 The biomass being topped up with 40 mL of dH_2O.

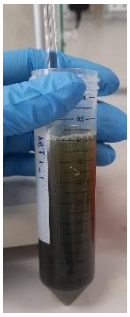

Fig. 2.23 The mixture being stirred well with a glass rod.

Each of the three biomasses was subjected to 13 different conditions at various time intervals, using the bath sonicator, the UP400St probe sonicator at 40% amplitude, the UP400St probe sonicator at 80% amplitude and no ultrasound as controls. Three replicates per biomass per time point were set for 1 min, 5 min and 20 min in the bath sonicator, respectively (Fig. 2.24). Another three replicates per biomass per time point, were set at 40% amplitude (upper temperature limit set at 55°C) and treated with the UP400St device using a 3 mm Ø sonotrode, immersed 4.5 cm deep, for the following durations: 1, 5, 20 min (Fig. 2.25, 2.26). Another three replicates per biomass per time point, were set at 80% amplitude (upper temperature limit set at 55°C) and treated with the UP400St device using a 3 mm Ø sonotrode, immersed 4.5 cm deep, for the following durations: 1, 2, 5 min (Fig. 2.25, 2.26). Lastly, three replicates per biomass per time point were set as controls and left untreated for 1, 2, 5 and 20 min, respectively.

Fig. 2.24 The set-up for the treatment with the bath sonicator. The 50 mL centrifuge tubes were held upright and in place with the aid of a rack placed inside the tank. On the bottom left there is the ear protection used during all ultrasound treatments as a precaution.

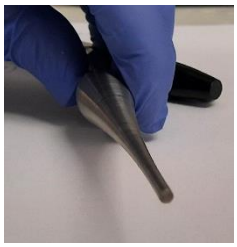

Fig. 2.25 The 3 mm Ø sonotrode.

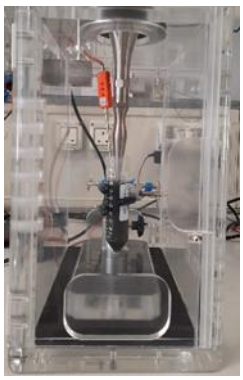

Fig. 2.26 The set-up for the treatment with the UP400St probe sonicator. The 50 mL centrifuge tube was held in place using a clamp. The temperature probe was inserted in the sample to detect the internal temperature and make sure that the process stops if the temperature exceeds 55°C.

All samples were centrifuged for 30 min at 3,820 RCF (g-force) and set at 4°C (PrO-Analytical centrifuge, Centurion Scientific limited, United Kingdom) (Fig. 2.27 A, B). The supernatants (top layer) were separated from the pellets (bottom layer) (Fig. 2.28). The supernatants were poured through a fluted filter paper (Fig. 2.29) and decanted into 15 mL centrifuge tubes for storage at -20°C until further analyses to assess chlorophyll spectra (Fig. 2.30). The filtered supernatants were also analysed using four assays to assess the protein content, the peroxide content, the total free amino acid (TFAA) content and total reducing sugar (TRS) content. A small amount of each pellet was

transferred to Eppendorf tubes for storage at -20°C (Fig. 2.31 A, B), to be used for scanning electron microscopy (SEM) imaging later on.

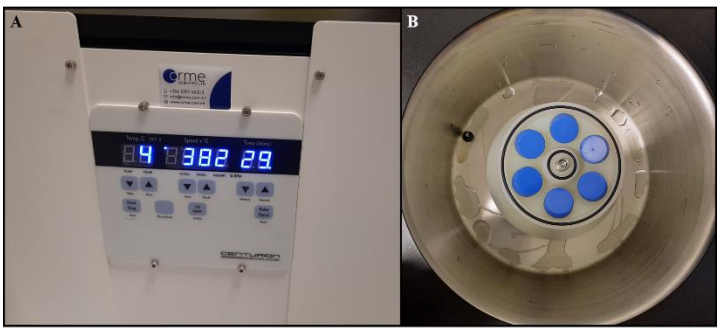

Fig. 2.27 The centrifuge. (A) The centrifuge settings. (B) Six centrifuge tubes were fit into the rotor.

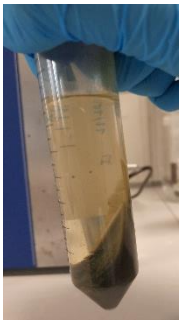

Fig. 2.28 The pellet settled at the bottom while the supernatant rests on top.

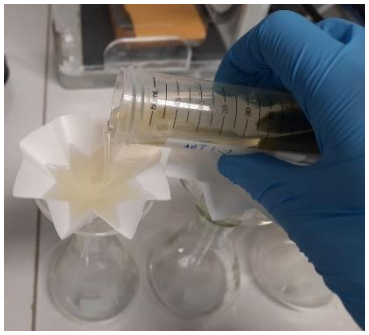

Fig. 2.29 The supernatant being filtered through a fluted filter paper.

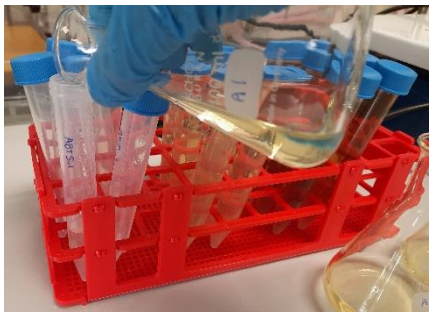

Fig. 2.30 The supernatants being decanted in 15 mL centrifuge tubes for storage at -20°C until further analyses.

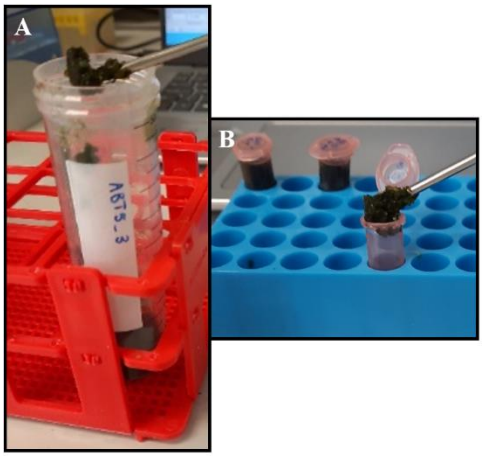

Fig. 2.31 The pellets being transferred from the 50 mL centrifuge tubes (A) to 2 mL Eppendorf tubes (B) for storage at -20°C until SEM analyses.

To summarize, approximately 39 g of each biomass (*Alaria esculenta* type 1, *Chlorella vulgaris*, *Lemna minor* type 1) was used for these experiments. These 39 g were split into 1 g portions, each of which was added to 40 mL dH_2O. Then, 12 of these sample dilutions were left untreated to act as controls (three left for 1 min, three left for 2 min, three left for 5 min, three left for 20 min). Next, 9 of these sample dilutions were treated using the bath sonicator (three left for 1 min, three left for 5 min, three left for 20 min). Another 9 sample dilutions were treated using the UP400St probe sonicator set at 40% amplitude (three left for 1 min, three left for 5 min, three left for 20 min). Finally, the last 9 sample dilutions were treated using the UP400St probe sonicator set at 80% amplitude (three left for 1 min, three left for 2 min, three left for 5 min). Hence, 13 treatments (three replicates each) per biomass were performed.

2.2.2.2 Assays

All the filtered supernatants were analysed using four different assays. Bradford's assay was used to assess the protein content of the samples. Ferrous oxidation xylenol (FOX) assay was used to check the hydrogen peroxide (H_2O_2) content in the samples. The TFAA content was found by using o-phthaldialdehyde (OPA) reagent. The TRS content was analysed using 3,5-dinitrosalicylic acid (DNS) reagent. All the four assay protocols were adapted to have the total volume fit in a well of a 96-well plate. Each plate was placed in the UV-spectrophotometer (Sunrise, Tecan, Austria) (Fig. 2.32) and the machine was set to read the absorbance of each well at 600 nm, 560 nm, 540 nm and 340 nm according to Table 2.2 below.

Fig. 2.32 The UV-spectrophotometer.

Table 2.2 Standard curves were made for each assay and the respective absorbances were read at the appropriate wavelength as follows:

Assay	Standards	Absorbance
Bradford's	BSA standard solutions of 0, 100, 300, 600, 900, 1200, 1500 µg/mL were prepared for the standard curve. A 5 mg/mL BSA stock solution was used.	600 nm
FOX	H_2O_2 standard solutions of 0, 1, 3, 5, 7, 9, 11 µg/mL were prepared for the standard curve. A 100 µg/mL H_2O_2 stock solution was used.	560 nm
TFAA	Leucine standard solutions of 0, 10, 20, 30, 40, 50, 60, 70 µg/mL were prepared for the standard curve. A 1 mg/mL (1000 µg/mL) leucine stock solution was used.	340 nm
TRS	Glucose standard solutions of 0, 300, 600, 900, 1200, 1500, 1800 µg/mL were prepared for the standard curve. A 2 mg/mL (2000 µg/mL) glucose stock solution was used.	540 nm

The Reader-Server RdrOle program was used to define the appropriate filter slides since the filter for the TFAA assay was on a different holder to the rest (Fig. 2.33 A, B). This was set-up by pressing 'Instrument', 'Connect', 'Set-up', 'Define filter slides', inputting the appropriate wavelengths in the write position, 'OK', 'disconnect' and closing the program.

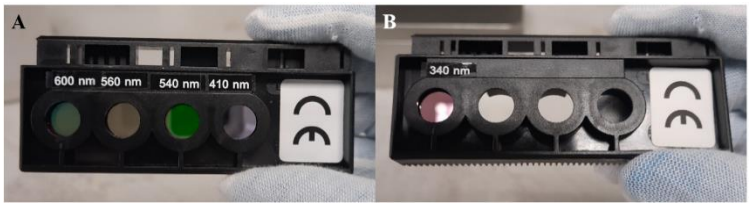

Fig. 2.33 The wavelength filters. (A) 600, 560 and 540 nm filters were used for Bradford's, FOX and TRS assays, respectively. (B) 340 nm filter was used for TFAA assay.

After this was completed, the appropriate volume of standards/samples was placed in the wells of a 96-well plate, followed by the reagent (Fig. 2.34 A, B). These were then left to acclimatize for a number of min (depending on the assay), and afterwards placed in the UV-spectrophotometer. The X Fluoro4 software was opened and the following were pressed: ‘Add-Ins’, ‘X Fluoro’, ‘connect’, ‘OK’, ‘X Fluoro’, ‘edit measurement parameters’, ‘read absorbance at – 600/560/ 540/340 nm’, ‘shaking’, ‘shaking before measurement – 30 seconds’, ‘inside – normal’, ‘settle time – 30 seconds’. Each assay resulted in a change in colour/opaqueness, which indicated the amount of the desired component as in Fig. 2.35 A, B. Bradford’s assay caused the Bradford’s reagent to change from brown to varying intensities of blue in the presence of proteins. FOX assay caused the reagent to change from orange to varying intensities of fuchsia. TFAA assay caused the OPA reagent to change in opaqueness, however the turbidity increase was so slight that it was hard to detect with the naked eye. TRS assay caused the DNS reagent to change from bright yellow to varying intensities of orange.

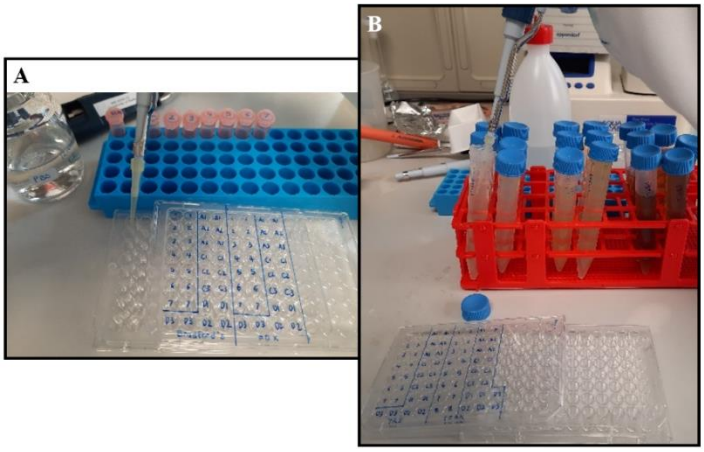

Fig. 2.34 The appropriate amount of (A) standard and (B) sample was placed into the well of a 96-well plate, followed by the reagent.

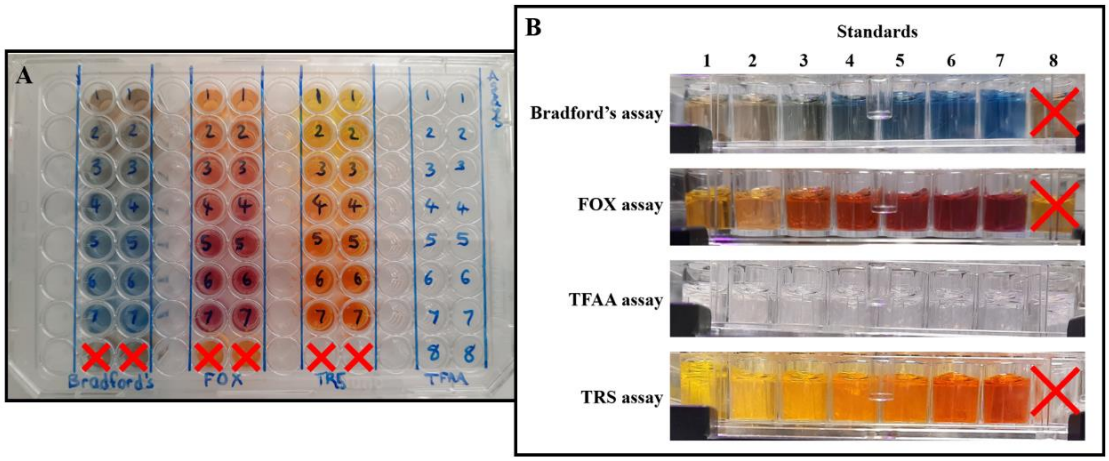

Fig. 2.35 Each assay results in a change in colour/turbidity. (A) A 96-well plate showing the standards of all four assays, Bradford's, FOX, TRS, TFAA assays going from left to right, top-view. Standard 1 to 7/8 going down, increasing in their concentration. (B) Side-view of all 4 assays.

The results generated from X Fluoro4 software were then analysed on Excel. GraphPad Prism version 8 was used to create bar graphs for all of the samples. The data was analysed as described in section 2.1.1. For almost all of the results, one-way ANOVA was used as the parametric test and Kruskal-Wallis was used as the nonparametric test, in order to compare any statistical differences between the biomasses. For the 2-min treatments of UP400St set at 80% and the control, the T-test (or the nonparametric equivalent, Mann-Whitney test) was used as there were only 2 groups to compare.

2.2.2.2.1 Bradford's

The following protocol was adapted from Marion Bradford's paper (Bradford, 1976).

Preparation of Bradford's reagent

This procedure was carried out in a fume hood. In a 1 L glass beaker, 50 mg of Coomassie Blue G250 (Carl Roth, Germany) were dissolved in 50 mL of methanol (Carlo Erba, France) and mixed thoroughly with a glass rod. Next, 100 mL of 85% w/v phosphoric acid (Sigma-Aldrich, Germany) were added to the Coomassie Blue solution. The mixture was then stirred with a glass rod to ensure dissolution. Finally, 500 mL of dH_2O were added and mixed thoroughly. The resulting solution was taken out of the fume hood and filtered through a 0.22 µm vacuum filter unit (Merck Millipore, Sweden) fitted to a 1 L Duran bottle, to remove any precipitate (Fig. 2.36). The mixture was

topped up with 350 mL of dH_2O. The Duran bottle was wrapped in aluminium foil to protect Bradford's reagent from light and stored at 4°C. Any leftover/expired reagent was disposed of in the sink.

Fig. 2.36 The Bradford's reagent after being filtered through a 0.22 µm vacuum filter unit.

For the stock culture a bovine serum albumin (BSA) solution was prepared. This procedure was carried out in a class 2 cabinet to ensure the sterility of the final product, which decreases protein degradation. Phosphate buffered saline solution (PBS) was prepared by dissolving one PBS tablet (Oxoid, England) in 100 mL dH_2O. To achieve a stock solution of 5 mg/mL BSA, 200 mg of crystalline BSA (Carl Roth, Germany) were weighed out in a weighing boat. The BSA was then transferred to a small beaker and dissolved in 40 mL of PBS with the help of a magnetic stirrer. The BSA stock solution was filtered in a clean 50 mL centrifuge tube through a 0.22 µm syringe filter (Alwasci, China) to remove any particulate matter. This was finally aliquoted into 1.5 mL Eppendorf tubes and stored at -20°C.

A standard curve was created and used to determine the concentration of protein in the biomass supernatants. All standards were prepared fresh on the day of running the assay. A volume of PBS was first added to an Eppendorf tube, followed by a volume of BSA stock solution as per Table 2.3.

Table 2.3 Composition of BSA dilutions.

Standard number	Concentration of dilution (µg/mL)	Volume of PBS (µL)	Volume of BSA stock solution (µL)
1	0	300	0
2	100	294	6
3	300	282	18
4	600	264	36
5	900	246	54
6	1200	228	72
7	1500	210	90

After preparing the dilutions, 5 µL of standard/sample were placed in a well of a 96-well plate (Nunclon Delta Surface, Thermo Scientific, Denmark). This was followed by the addition of 250 µL of Bradford's reagent (Fig. 2.37). The procedure was performed in duplicates for each standard and sample (Fig. 2.38). The plate was placed in the UV-spectrophotometer and left to incubate for 5 min at room temperature. The absorbance was then read at 600 nm. When a sample's absorbance resulted in a value which was greater than 1, the test was repeated using a dilution of the sample.

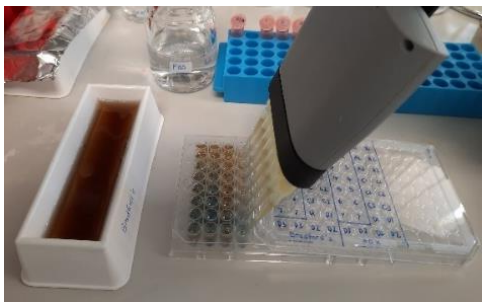

Fig. 2.37 Bradford's reagent was added to the standards/samples by using a multichannel micropipette.

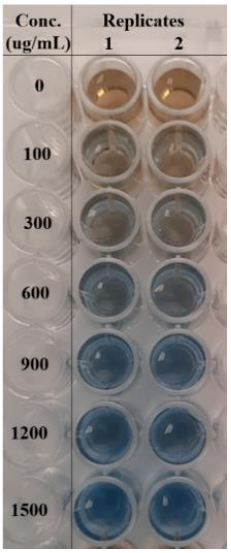

Fig. 2.38 Annotated diagram to explain how the standards were placed inside the wells. The increase in protein concentration can be seen through the colour change from brown to varying intensities of blue.

2.2.2.2.2 FOX

The following method was adapted from a paper on the detection of lipid hydroperoxides in plant tissue (DeLong et al., 2002).

Preparation of FOX reagents A & B

This procedure was carried out in the fume hood. In a 250 mL volumetric flask, about 150 mL of dH_2O were added, followed by 33.421 mL of 96% sulfuric acid (H_2SO_4) (Carlo Erba, France). The dilution was topped up with dH_2O until it reached the 250 mL mark to achieve a 2.5 M H_2SO_4 solution. This was stored in the fume hood to be used later on. Any leftover/expired reagent was disposed of in the sink after neutralising (pH 7) with a 1 M solution of sodium chloride (NaCl).

Reagent A, 25 mM ammonium ferrous (II) sulfate in 2.5 M H_2SO_4, was then prepared. In a 100 mL amber volumetric flask, around 50 mL of 2.5 M H_2SO_4 added, followed by 0.9804 g of ammonium ferrous (II) sulfate hexahydrate (Carl Roth, Germany). This was then topped up with 2.5 M H_2SO_4 until the 100 mL mark and stored at 4°C.

Finally, reagent B, 100 mM sorbitol and 125 µM xylenol orange, was made. In a 250 mL amber volumetric flask, around 100 mL of dH_2O was added, followed by 4.5545 g of D-sorbitol (Carl Roth, Germany) and 0.0224 g of xylenol orange (Carlo Erba, France). The flask was topped up with dH2O until the mark and stored at 4°C.

The working reagent was prepared fresh on the day of running the assay by adding 1 mL of reagent A to 100 mL of reagent B (Fig. 2.39 A, B, C). Any leftover reagent was disposed of in containers used specifically for acidified waste.

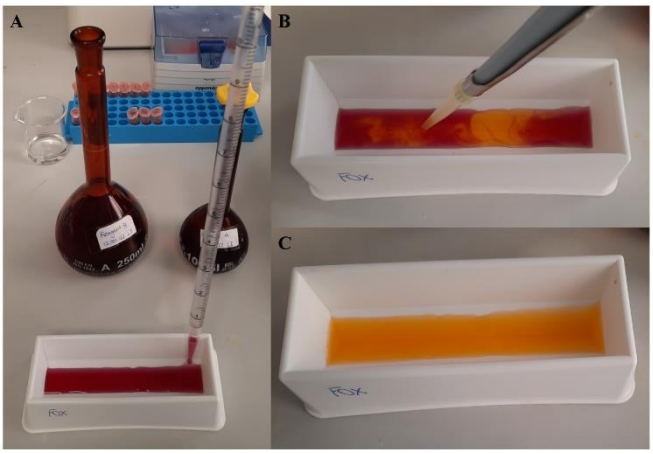

Fig. 2.39 The preparation of the working reagent for FOX assay, by mixing 1 volume of reagent A with 100 volumes of reagent B. (A) 100 volumes of reagent B (purple) were added to the white container, followed by (B) 1 volume of reagent A (colourless). (C) The resulting working reagent turns orange.

A stock solution of 100 µg/mL H_2O_2 was prepared fresh on the day of running the assay. In a 100 mL volumetric flask, about 50 mL of dH_2O were added, followed by 30 µL of 30% wt. H_2O_2 (Scharlab, Spain). This was topped up with dH_2O until the mark.

A standard curve was created and used to determine the concentration of peroxide in the biomass supernatants. All standards were prepared fresh on the day of running the assay. About 50 mL of dH_2O was added to a 100 mL volumetric flask, followed by a volume of H_2O_2 stock solution as per Table 2.4. The flask was then topped up with dH_2O until the mark.

Table 2.4 Composition of H_2O_2 dilutions.

Standard number	**Concentration of dilution (µg/mL)**	**Volume of dH_2O (µL)**	**Volume of H_2O_2 stock solution (µL)**
1	0	100	0
2	1	99	1
3	3	97	3
4	5	95	5
5	7	93	7
6	9	91	9
7	11	89	11

After preparing the dilutions, 6 µL of standard/sample were placed in a well of a 96-well plate (Nunclon Delta Surface, Thermo Scientific, Denmark). This was followed by 240 µL of FOX working reagent (Fig. 2.40). The procedure was performed in duplicates for each standard and sample (Fig. 2.41). The plate was placed in the UV-spectrophotometer and left to incubate for 20 min at room temperature. The absorbance was then read at 560 nm. When a sample's absorbance resulted in a value which was greater than 1, the test was repeated using a dilution of the sample.

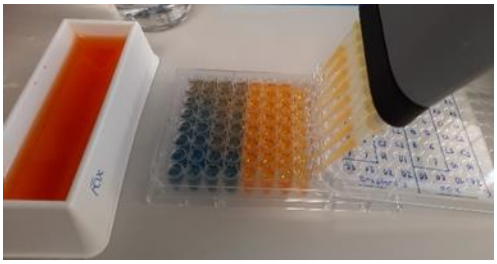

Fig. 2.40 FOX working reagent was added to the standards/samples by using a multichannel micropipette.

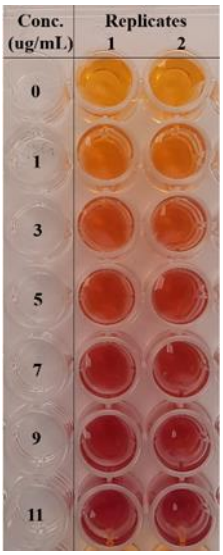

Fig. 2.41 Annotated diagram to explain how the standards were placed inside the wells. The increase in peroxide concentration can be seen through the colour change from orange to varying intensities of fuchsia.

2.2.2.2.3 TFAA

The following protocol was adapted from a method developed to detect the total free amino acids in soil solutions (Jones et al., 2002).

Preparation of OPA reagent

A 100 mM sodium tetraborate solution was prepared by adding 38.137 g of sodium tetraborate (VWR Chemicals BDH, Belgium) to 1 L of dH_2O. A 10% sodium dodecyl sulfate (SDS) (w/w) (Carl Roth, Germany) solution was made in the fume hood by weighing 10 g of SDS and adding them to 90 mL of dH_2O. The making of OPA solution was carried out in the fume hood and prepared fresh on the day of running the assay. This was achieved by adding 0.04 g of OPA (Alfa Aesar, China) to 1 mL methanol (Carlo Erba, France). The OPA reagent was carried out in the fume hood and prepared fresh on the day of running the assay. In a 50 mL volumetric flask, 25 mL of 100 mM sodium tetraborate, 5 mL of 10% SDS (w/w), 1 mL of OPA solution and 100 µL of β-mercaptoethanol (Acros Organics, Germany) were added and mixed well. The flask was topped up with dH_2O until it reached the 50 mL mark. Any leftover reagent was disposed of in containers used specifically for OPA reagent.

A stock solution of 1000 µg/mL (1 mg/mL) L-leucine (Biochem Chemopharma, France) was prepared by adding 1 mg leucine to 1 mL dH_2O. A standard curve was created and

used to determine the concentration of total free amino acids in the biomass supernatants. All standards were prepared fresh on the day of running the assay. A volume of leucine stock solution was first added to a well of a 96-well plate, followed by a volume of OPA reagent as per Table 2.5.

Table 2.5 Composition of leucine dilutions.

Standard number	Concentration of dilution (µg/mL)	Volume of leucine stock solution (µL)	Volume of OPA reagent (µL)
1	0	0	200
2	10	2	198
3	20	4	196
4	30	6	194
5	40	8	192
6	50	10	190
7	60	12	188
8	70	14	186

After preparing the standards inside the wells, 10 µL of sample were placed in a well of a 96-well plate (Nunclon Delta Surface, Thermo Scientific, Denmark). This was followed by 190 µL of OPA reagent (Fig. 2.42). The procedure was performed in duplicates for each standard and sample (Fig. 2.43). The plate was placed in the UV-spectrophotometer and left to incubate for 3 min at room temperature. The absorbance was then read at 349 nm. When a sample's absorbance resulted in a value which was greater than 1, the test was repeated using a dilution of the sample. It was ensured that any bubbles from the OPA reagent were popped using a sterile needle before reading the absorbance, to minimize errors in the results (Fig. 2.44).

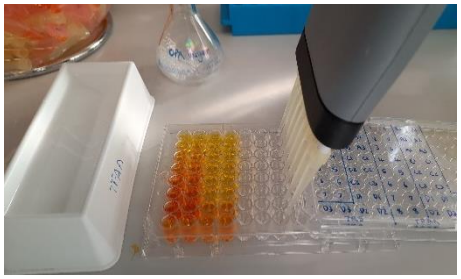

Fig. 2.42 OPA reagent was added to the samples by using a multichannel micropipette.

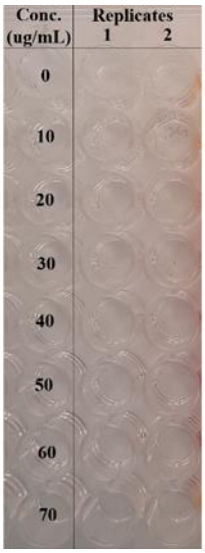

Fig. 2.43 Annotated diagram to explain how the standards were placed inside the wells. The increase in total free amino acid concentration can be detected through the increasing turbidity which is hard to see with the naked eye.

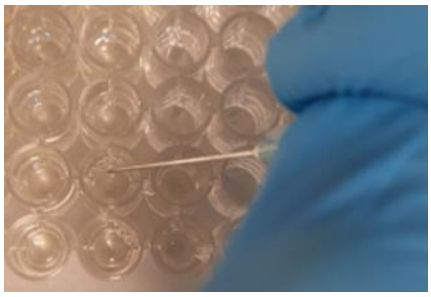

Fig. 2.44 It was ensured that any bubbles from the OPA reagent were popped using a sterile needle before reading the absorbance, to minimize errors in the results.

2.2.2.2.4 TRS

The following procedure was adapted from an improved DNS protocol (Wood et al., 2012).

Preparation of DNS reagent

In a 100 mL volumetric flask, 1.6 g of sodium hydroxide (Sigma-Aldrich, Czech Republic) were dissolved in 40 mL of dH_2O. Subsequently, 30 g of potassium sodium tartrate tetrahydrate (Carlo Erba, France) and another 40 mL of dH_2O were added. Finally, 1 g of 3,5-dinitrosalicylic acid (Acros Organics, India) was added slowly and the flask was topped up with dH_2O until the mark. The DNS reagent was filtered through a polyethersulfone (PES) syringe filter (Kinesis Scientific Experts, USA) before use.

Any leftover/expired reagent was disposed of in containers used specifically for alkaline soluble water waste.

To achieve a stock solution of 2 mg/mL glucose, 8 mg of glucose (Eurostar Scientific Ltd, UK) was added to 4 mL of dH_2O. A standard curve was created and used to determine the concentration of total reducing sugars in the biomass supernatants. All standards were prepared fresh on the day of running the assay. A volume of dH_2O was first added to an Eppendorf tube, followed by a volume of glucose stock solution as per Table 2.6.

Table 2.6 Composition of glucose dilutions.

Standard number	**Concentration of dilution (µg/mL)**	**Volume of dH_2O (µL)**	**Volume of glucose stock solution (µL)**
1	0	1000	0
2	300	850	150
3	600	700	300
4	900	550	450
5	1200	400	600
6	1500	250	750
7	1800	100	900

After preparing the dilutions, 50 µL of standard/sample were placed in a polymerase chain reaction (PCR) tube. This was followed by 50 µL of DNS reagent (Fig. 2.45). The PCR tubes were then placed in a thermal cycler (T100, BioRad, Malta) and boiled at 100°C for 5 min (Fig. 2.46). After the contents of the tubes cooled down to room temperature, 80 µL of each tube was transferred to an Eppendorf tube with 400 µL of dH_2O. From this, 250 µL were transferred into wells of a 96-well microtiter plate (Fig. 2.47). The procedure was performed in duplicate for each standard and sample (Fig. 2.48). The plate was placed in the UV-spectrophotometer and the absorbance was then read at 540 nm. When a sample's absorbance resulted in a value which was greater than 1, the test was repeated using a dilution of the sample.

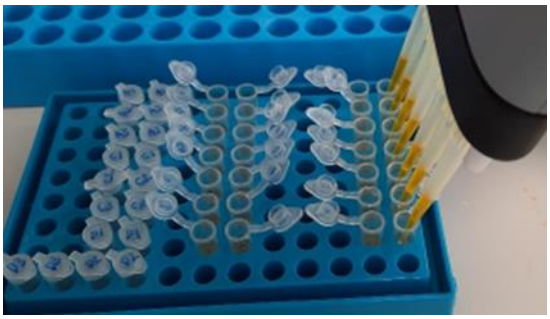

Fig. 2.45 DNS reagent was added to the standards/samples by using a multichannel micropipette.

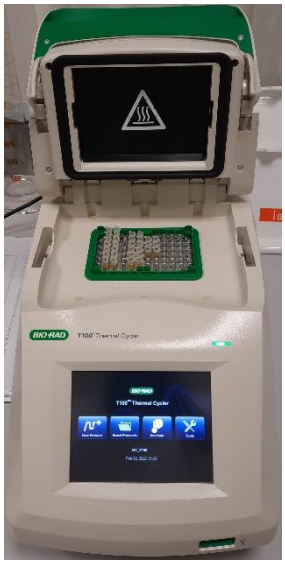

Fig. 2.46 Standards and samples inside the thermal cycler (T100, BioRad, Malta).

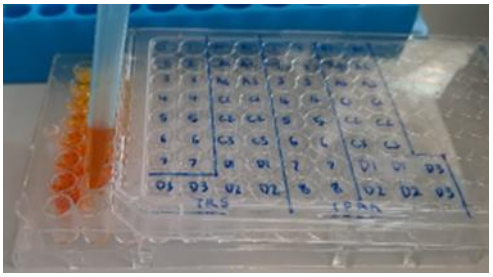

Fig. 2.47 Standards/samples being placed in a 96-well plate.

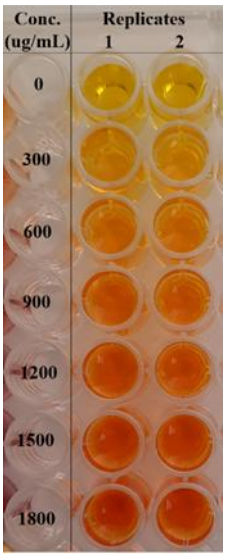

Fig. 2.48 Annotated diagram to explain how the standards were placed inside the wells. The increase in total reducing sugar concentration can be seen through the colour change from bright yellow to varying intensities of orange.

2.2.2.3 Chlorophyll spectra

The supernatants had very distinct colour shades and intensities (Fig 2.49 A, B, C), hinting that chlorophyll was being released from the biomass cells during all treatments. A spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan) (Fig. 2.50) was used to measure the absorbance of all supernatants at wavelengths between 750 to 550 nm. A blank (dH_2O in a glass cuvette) was first conducted, followed by the readings for all supernatants. The programme used to take these readings was UV probe (Version 2.42). The procedure was as follows: the UV-spectrophotometer and the computer to which it is attached were switched ON. Gloves were worn to prevent leaving fingerprints on the glass cuvette. UV probe 2.42 software was opened and the method tab was pressed. Measurements were set to be taken from 750 to 550 nm. Around 3 mL of dH_2O were poured into the glass cuvette and the baseline button was pressed, followed by the Autozero button to set the blank. The Start was pressed to run the blank. The run was saved as a spectrum file (shows a line graph on the software) and as a data print file (shows all the absorbance values at the different wavelengths when opened through Excel). After this was completed, the same procedure was carried out using 3 mL for each supernatant sample (Fig. 2.51 A, B).

Fig. 2.49 The different shades of the supernatants of each biomass [(A) *Alaria esculenta* (type 1), (B) *Chlorella vulgaris*, (C) *Lemna minor* (type 1)] that were treated with the bath sonicator for 20 min, probe sonicator at 40% amplitude for 20 min, and probe sonicator at 80% amplitude for 5 min (from left to right).

Fig. 2.50 The spectrophotometer which was used to run the chlorophyll spectra.

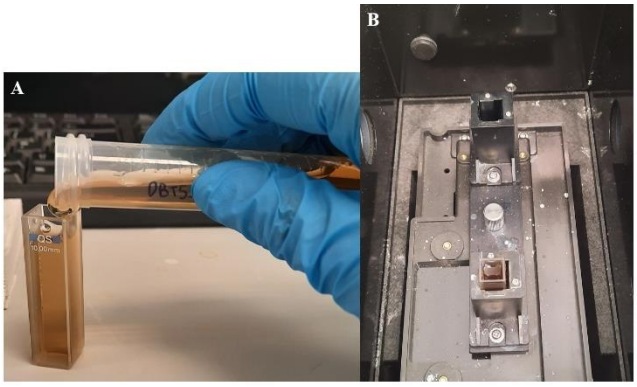

Fig. 2.51 A supernatant being set for a chlorophyll spectrum run. (A) Around 3 mL of sample was poured in the glass cuvette. (B) The glass cuvette was placed in the holder inside the spectrophotometer.

The results generated from UV probe 2.42 were then analysed on Excel. GraphPad Prism version 8 was used to create line spectra for all of the samples. The amount of chlorophyll could not be quantified as a chlorophyll standard was not available. The absorbance values at a wavelength of 670 nm were analysed as this peak marks the region for chlorophyll *a*, which is present in all three biomasses. The results were

inputted in GraphPad Prism version 8. The statistical analysis was carried out systematically, as described in section 2.1.2.2.

2.2.2.4 SEM

Five PBS tablets (Oxoid, England) were dissolved in 500 mL dH_2O to achieve a 100 mM solution and autoclaved at 121°C for 15 min. To this, 50 mL of 25% glutaraldehyde (Alfa Aesar, Germany) were added to achieve a 2.5% glutaraldehyde solution. This was stored at 4°C until later use. The osmium tetraoxide (Carl Roth, Germany) was in a 500 mg ampule, hence the ampule was broken into 12.5 mL dH_2O to achieve 1% osmium tetraoxide. It was made sure that the lid was closed properly. The flask was wrapped in aluminium foil and left to dissolve for 3 days at 4°C.

Fixation

Throughout the fixation process, the centrifuge (PrO-Analytical centrifuge, Centurion Scientific limited, United Kingdom) was set for 10 min at 1,120 RCF (g-force) and 4°C (Fig. 2.52). The frozen biomass pellets were defrosted and a small amount of each was transferred into new Eppendorf tubes (0.035 g for *Alaria esculenta* (type 1) samples, 0.03 g for *Chlorella vulgaris* samples, 0.02 g for *Lemna minor* (type 1) samples). Each pellet was resuspended in 500 µL PBS and vortexed. The tubes were centrifuged and the PBS supernatant was removed. The pellets were topped up with 200 µL of the 100 mM phosphate buffered 2.5% glutaraldehyde fixative. The tubes were vortexed and placed at 4°C for 30 min. The tubes were then centrifuged and the supernatant was discarded. The pellets were re-suspended again in 500 µL glutaraldehyde fixative, vortexed, and left for 2 hours at 4°C. The samples were vortexed and placed back at 4°C for a further 2 hours. The supernatants were discarded and the pellets were topped up with 500 µL of PBS to remove excess of the primary fixative. The tubes were vortexed and centrifuged. This was repeated once more by removing the supernatants, adding another 500 µL of PBS to each tube and centrifuging again. The supernatants were removed and the pellets were then post fixed in 500 µL of 1% osmium tetroxide fixative for 1.5 hours at 40°C in the dark, with the tubes being vortexed every 30 min. After secondary fixation the biomasses were re-pelleted by centrifugation. The pellets were topped up with 500 µL of PBS to remove excess of the secondary fixative. The tubes

were vortexed and centrifuged. The pellets were topped up with another 500 µL of PBS and stored at 4°C for later use.

Fig. 2.52 The centrifuge set for 10 min at 1,120 RCF (g-force) and 4°C.

Alcohol dehydration

The biomasses which were stored in 500 µL of PBS at 4°C were first vortexed and then centrifuged for 10 min at 1,120 RCF and 4°C. The supernatant was discarded while the pellet was dehydrated with an ethanol series, prepared as described in Table 2.7. Each biomass pellet was placed in 1 mL of 30%, 50%, 70%, 80%, 90%, and 100% ethanol absolute anhydrous (Carlo Erba, France) for 15 min each, respectively. After the 15 min passed, the supernatants were discarded after centrifuging for 10 min at 140 RCF and 4°C. Each sample was stored in 100% ethanol at 4°C.

Table 2.7 Ethanol series for sample dehydration before SEM.

Concentration of ethanol (%)	**Volume of dH_2O (mL)**	**Volume of ethanol (mL)**
30	10.5	4.5
50	7.5	7.5
70	4.5	10.5
80	3	12
90	1.5	13.5
100	0	15

Gold coating

This was performed at Heritage Malta. SEM pin stubs (Agar scientific, UK) were sprayed with some isopropanol and wiped dry to ensure that no dust settled on them. A small square of conductive carbon double-sided tape (NEM, Tokyo, Japan) was cut and

placed on each stub. The samples stored in 100% ethanol were vortexed and a few drops of each mixture were dropped on the carbon tape of several stubs. Every sample was allowed to air dry in a slightly open SEM storage box (AGAR scientific, UK) placed in the fume hood. The automatic sputter coater (Agar scientific, UK) (Fig. 2.53) was switched ON. The stubs holding the dehydrated samples were placed in the holders and a vacuum of 0.1 mbar was set. The machine was set to manual and the time was set to 120 seconds. Argon gas was flushed for 5 seconds and then 'Leak' was pressed to allow the pressure to return to 0.1 mbar. The 'Start' button was pressed to start coating the samples with gold. This process released a violet beam of light (Fig. 2.54). The stubs were completely covered in gold (Fig. 2.55 A, B), making them ready for SEM imaging.

Fig. 2.53 The automatic sputter coater with two stubs holding samples fitted in the holders.

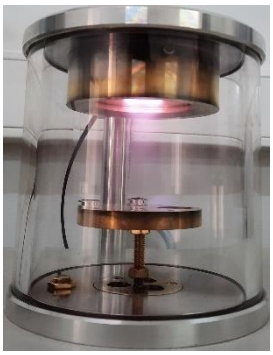

Fig. 2.54 A violet beam of light being emitted during the gold excitation process.

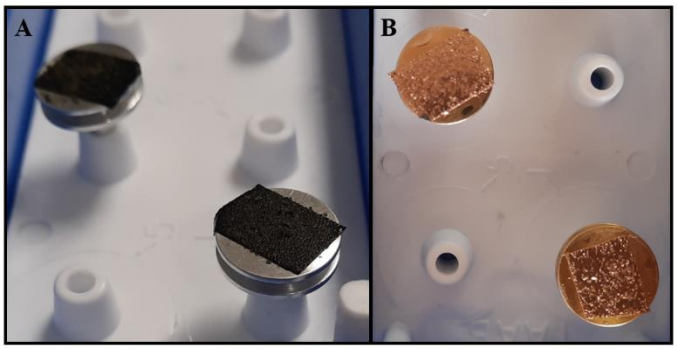

Fig. 2.55 Two samples on stubs before (A) and after (B) gold coating.

SEM imaging

This was performed at the Department of Metallurgy and Materials, Faculty of Engineering, UoM. The 6 samples were placed in the holder of the field emission scanning electron microscope (ZEISS GeminiSEM 360, Germany) as in Fig. 2.56. The 6 samples were the control treatment for 20 min and the probe sonicator at 80% amplitude for 5 min of all 3 biomasses. Each sample was imaged at 1, 5, 10, 20 and 50 K magnifications.

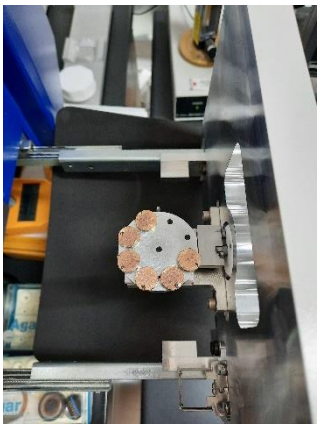

Fig. 2.56 All the 6 samples placed in the holder of the SEM machine, ready to be imaged.

The resulting images were compared qualitatively with each other, to determine if there were any changes in the structure of the biomasses after the different treatments. It was decided to only compare the 20-min controls with the 5-min treated samples using UP400St at 80%. In this way the differences in the structure were clearer, as the most hydrated samples were being compared to the samples treated with the harshest conditions.

2.3 Impact of assisted ultrasound on biomass treatments

2.3.1 Probe ultrasound vs enzyme-assisted extraction

These tests were performed in the bioactive isolation lab at Teagasc, Dublin, Ireland. For the following experiments, two biomasses were used; fresh *Alaria esculenta* (type 2) which was provided by Dúlra Seaweed, harvested from County Mayo, Ireland, in March 2020 (Fig. 2.57 A), and fresh *Lemna minor* (type 2) which was harvested at the Mount Lucas Wind Farm, County Offaly, Ireland (Fig 2.57 B). The two fresh biomass samples were washed thoroughly with tap water to remove any salt and surface impurities. The samples were then wiped with tissue to remove surface water and were ground until they were approximately 1-2 cm in length. They were then stored at -20°C prior to further processing.

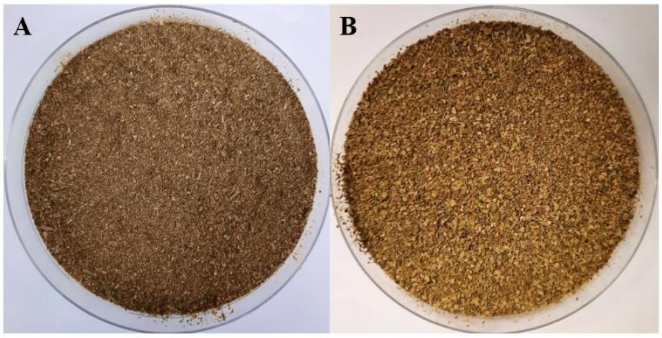

Fig. 2.57 The two biomasses. (A) *Alaria esculenta* (type 2), (B) *Lemna minor* (type 2).

Various protein extraction methods were explored: ultrasound-assisted extraction and enzyme-assisted extraction (both of which are considered novel techniques), followed by conventional extraction and alkali extraction.

2.3.1.1 Protein extraction using ultrasound as a pre-treatment for enzyme-assisted extraction

Around 10 g of both dried biomasses (*Alaria esculenta* (type 2) and *Lemna minor* (type 2)) were weighed (WVR, USA) in a beaker. The biomasses were rehydrated with 250 mL dH_2O for 10 min, with constant stirring. Both biomasses were pre-treated as follows:

a. Sonicated with a 40 mm Ø sonotrode set at 50% amplitude for 10 min (UIP1000hdT, 1000W, 20kHz, Hielscher Ultrasonic, Germany).

b. Sonicated with a 40 mm Ø sonotrode set at 100% amplitude for 10 min (UIP1000hdT, 1000W, 20kHz, Hielscher Ultrasonic, Germany).
c. Not sonicated, left for 10 min (**enzymes only, buffer only and water only controls**).

Both biomasses were then prepared for the treatment as follows:

a. pH adjusted to 4.5 with citric acid (isoelectric precipitation) with 0.1 M citric acid (Sigma-Aldrich, Germany) solution.
b. pH not adjusted (**water only control**).

The sample mixtures were incubated in a water bath (Optima TC120 Heated Circulating Bath, Grant, UK) until an internal temperature of 50°C was reached. Both biomasses were then treated by adding:

a. 1 mL of cellulase (Sigma-Aldrich, Denmark).
b. 1 mL of dH_2O (**ultrasound (US) probe only, buffer only and water only controls**).

The mixtures were poured into culture media bottles and incubated in two ways:

a. 6 hours at 50°C and 150 rpm (MaxQ 8000 Incubated/Refrigerated Stackable Shakers, Thermo Scientific, Denmark).
b. 18 hours at 50°C and 150 rpm (MaxQ 8000 Incubated/Refrigerated Stackable Shakers, Thermo Scientific, Denmark).

The flasks were then heated in the water bath to 90°C for 10 min (enzyme inactivation). The mixtures were left to cool to room temperature in centrifuge tubes, and then centrifuged (Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Scientific, Denmark) at 8,000 RCF (x g) for 10 min at 14°C. The supernatants were poured through a muslin cloth and into new containers, and their weights were recorded. The pellets were transferred to separate containers, and their weights were recorded. Both the supernatants and pellets were freeze-dried (Lyovapor L-300, Büchi, Switzerland) and the new weights were recorded. The freeze-dried samples were milled into a fine powder using a ball miller (Mixer Mill MM 400, Retsch, Germany) and stored for further analysis. A summary of all the treatments is given in Table 2.8 and Fig. 2.58 below.

Table 2.8 Applied treatments for protein extraction using ultrasound as a pre-treatment for enzyme-assisted extraction.

Summary (the same for both *Alaria esculenta* and *Lemna minor*)	
Treatments	**Conditions**
Ultrasound probe pre-treatment + enzymes	50% amplitude, 10 min + 6-hour incubation
Ultrasound probe pre-treatment + enzymes	100% amplitude, 10 min + 6-hour incubation
Ultrasound probe pre-treatment + enzymes	50% amplitude, 10 min + 18-hour incubation
Ultrasound probe pre-treatment + enzymes	100% amplitude 10 min + 18-hour incubation
Ultrasound probe only	50% amplitude, 10 min + 6-hour incubation
Ultrasound probe only	100% amplitude, 10 min + 6-hour incubation
Ultrasound probe only	50% amplitude, 10 min + 18-hour incubation
Ultrasound probe only	100% amplitude 10 min + 18-hour incubation
Enzymes only	No US, 10 min + 6-hour incubation
Enzymes only	No US, 10 min + 18-hour incubation
Buffer only	No US, 10 min + 6-hour incubation
Buffer only	No US, 10 min + 18-hour incubation
Water only	No US, 10 min + 6-hour incubation
Water only	No US, 10 min + 18-hour incubation

Fig. 2.58 Summary of protein extraction using ultrasound as a pre-treatment for enzyme-assisted extraction.

2.3.1.2 Protein extraction using the conventional method used in the food industry

Around 10 g of both dried biomasses (*Alaria esculenta* (type 2) and *Lemna minor* (type 2)) were weighed (WVR, USA) in a beaker. The biomasses were rehydrated with 250 mL dH_2O for 10 min, with constant stirring. Both biomasses were poured into culture media bottles and left to incubate for 24 hours at 50°C and 150 rpm (MaxQ 8000 Incubated/Refrigerated Stackable Shakers, Thermo Scientific, Denmark). The mixtures were left to cool to room temperature in centrifuge tubes, and then centrifuged (Sorvall

LYNX 6000 Superspeed Centrifuge, Thermo Scientific, Denmark) at 8,000 RCF (x g) for 10 min at 14°C. The supernatants were poured through a muslin cloth and into new containers, and their weights were recorded. The pellets were transferred to separate containers, and their weights were recorded. Both the supernatants and pellets were freeze-dried (Lyovapor L-300, Büchi, Switzerland) and the new weights were recorded. The freeze-dried samples were milled into a fine powder using a ball miller (Mixer Mill MM 400, Retsch, Germany) and stored for further analysis. A summary of all the treatments is given in Table 2.9 and Fig. 2.59 below.

Table 2.9 Summary of protein extraction using the conventional method used in the food industry.

Summary (the same for both *Alaria esculenta* and *Lemna minor*)	
Treatments	**Conditions**
Water only	24-hour incubation

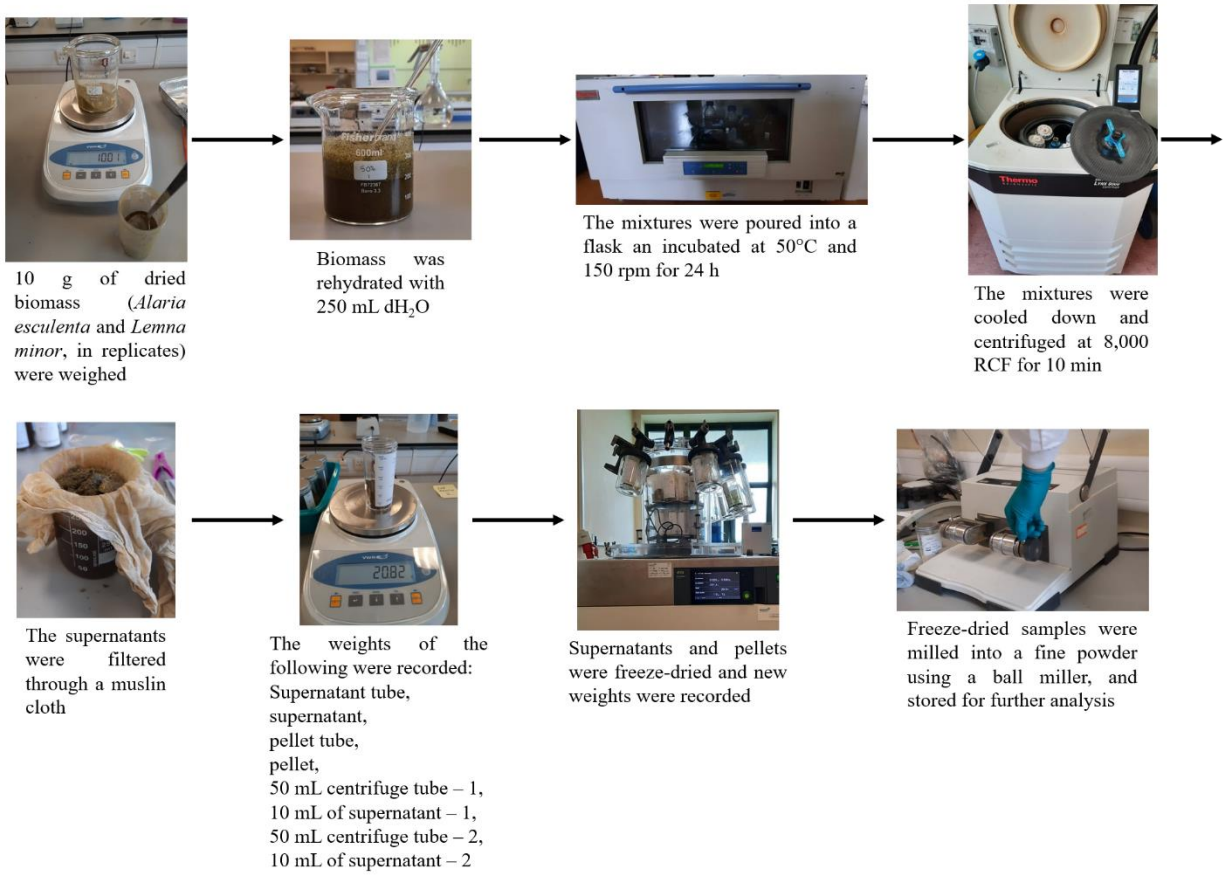

Fig. 2.59 Summary of protein extraction using the conventional method used in the food industry.

2.3.1.3 Protein extraction using alkali extraction as a pre-treatment for ultrasound-assisted extraction

Around 10 g of both dried biomasses (*Alaria esculenta* (type 2) and *Lemna minor* (type 2)) were weighed (WVR, USA) in a beaker. The biomasses were rehydrated with 250 mL dH_2O for 10 min, with constant stirring.

Both biomasses were then prepared for the treatment by adjusting the pH to 11 (isoelectric precipitation) with 0.2 M sodium hydroxide (NaOH) (Sigma-Aldrich, Czech Republic) solution. Both biomasses were treated as follows:

a. Sonicated with a 40 mm Ø sonotrode set at 100% amplitude for 30 min (UIP1000hdT, 1000W, 20kHz, Hielscher Ultrasonic, Germany).
b. Not sonicated, left to incubate for 6 hours at 50°C and 150 rpm (MaxQ 8000 Incubated/Refrigerated Stackable Shakers, Thermo Scientific, Denmark) (**control**).

The mixtures were left to cool to room temperature in centrifuge tubes, and then centrifuged (Sorvall LYNX 6000 Superspeed Centrifuge, Thermo Scientific, Denmark) at 8,000 RCF (x g) for 10 min at 14°C. The supernatants were poured through a muslin cloth and into new containers, and their weights were recorded. The pellets were transferred to separate containers, and their weights were recorded. Both the supernatants and pellets were freeze-dried (Lyovapor L-300, Büchi, Switzerland) and the new weights were recorded. The freeze-dried samples were milled into a fine powder using a ball miller (Mixer Mill MM 400, Retsch, Germany) and stored for further analysis. A summary of all the treatments is given in Table 2.10 and Fig. 2.60 below.

Table 2.10 Summary of protein extraction using alkali extraction as a pre-treatment for ultrasound-assisted extraction.

Summary (the same for both *Alaria esculenta* and *Lemna minor*)	
Treatments	**Conditions**
Alkali + ultrasound probe pre-treatment	100% amplitude 30 min
Alkali only	6-hour incubation

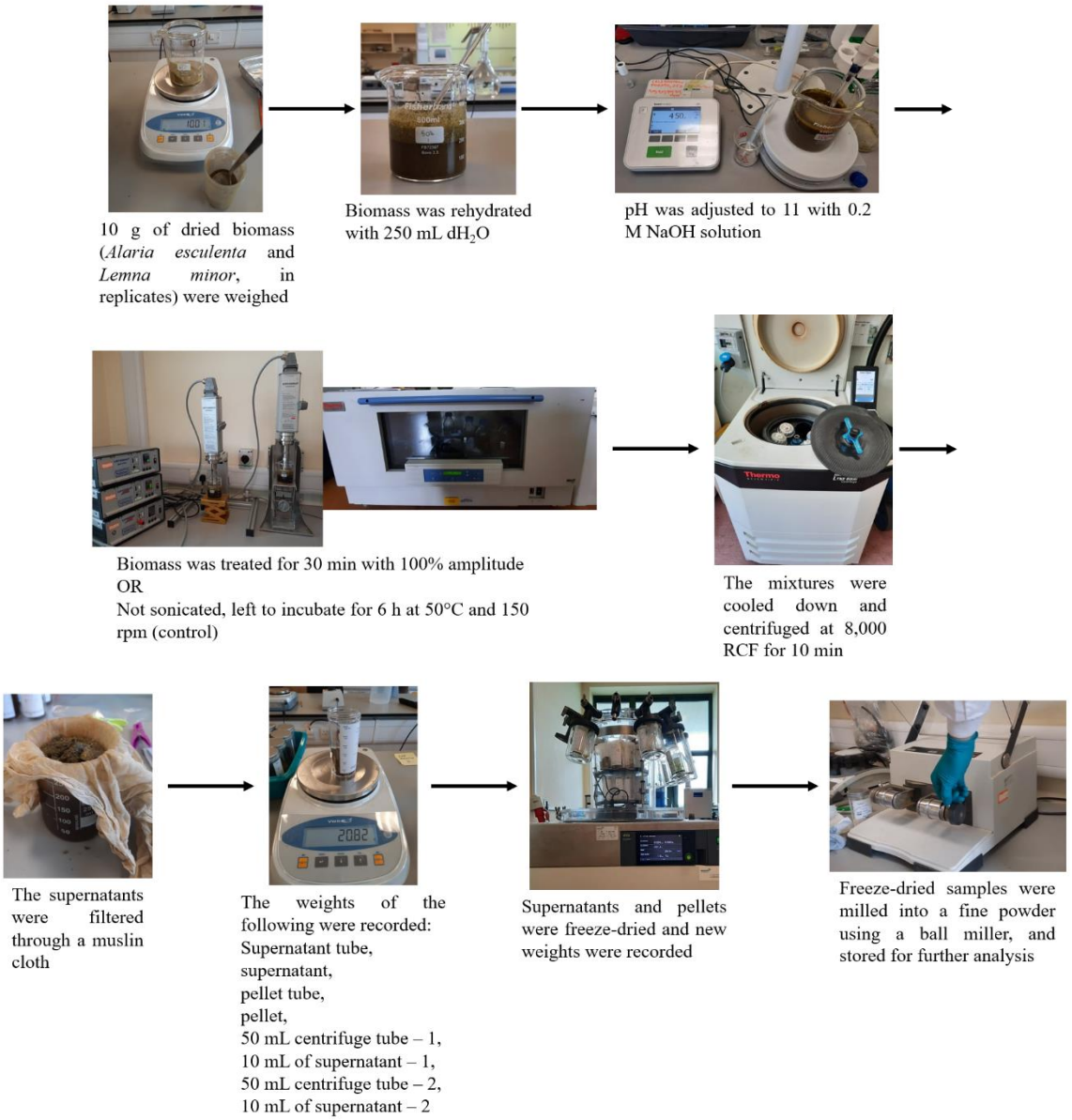

Fig. 2.60 Summary of protein extraction using alkali extraction as a pre-treatment for ultrasound-assisted extraction.

2.3.2 Nitrogen content

This test was performed with the aid of Ms Karen Hussey. The nitrogen content (%) was assessed to determine which treatment resulted in the highest protein yield. This was determined using LECO FP628 protein analyser (LECO Corp., MI, USA) (Fig. 2.61). The supernatants were first rehydrated in 20 mL of dH_2O and then added to tin capsules. About 500 µL of rehydrated freeze-dried supernatants were loaded into a metal capsule.

Fig. 2.61 The LECO FP628 protein analyser.

On the computer screen across the top tab, ‘Sample’ was chosen followed by ‘Login’. The sample login box appeared and the following information was inputted in the dialog box: description (sample code), mass (weight of sample in grams) and factor (nitrogen factor). After selecting ‘OK’ the sample codes appeared on screen in grey colour. The samples were placed in the carousel on Leco 628, starting from the space to the right of the arrow and going clockwise. After making sure that the location number on the screen allocated to the sample matches that of the carousel number, the ‘Analyse’ button was pressed. This caused the carousel to turn and drop the sample into the instrument. The sample on screen changed font colour from grey to blue. Once complete, the sample code appeared in a black font on screen. Each sample was analysed in two replicates. After Analysis was finished, the drop-down arrow beside ‘Gas Standby’ was selected and ‘Select Gas off’ was pressed.

The protein concentration determined using the LECO FP628 protein analyser is based on the Dumas method and according to AOAC method 992.15, 1990. The nitrogen to protein conversion factor used for *Alaria esculenta* was 5, while for *Lemna minor* it was 6.25. The results generated were then analysed on Excel. GraphPad Prism version 8 was used to create bar graphs for all of the samples.

2.3.3 SEM

The first step was to rehydrate the samples. In an Eppendorf tube, 1 g of freeze-dried pellet was added. To each tube, 1.5 mL of dH_2O were added to rehydrate the samples overnight (12-24 hours). The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded.

The second step was pre-fixation. About 1 mL of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was added to each sample. The samples were incubated for 6 hours at 4°C. The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded. Approximately 1.5 mL of 0.1 M phosphate buffer (pH 7.4) were added to each sample. The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded. The addition of 1.5 M 0.1 M phosphate buffer, followed by centrifugation, was repeated twice more.

The third step was post-fixation. About 1 mL of 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) was added to each sample. The samples were incubated for 1.5 hours at 4°C. The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded. Approximately 1.5 mL of 0.1 M phosphate buffer (pH 7.4) were added to each sample. The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded. The addition of 1.5 M 0.1 M phosphate buffer, followed by centrifugation, was repeated twice more. Around 1.5 mL of dH_2O were then added to the samples. The samples were left to incubate for 15 min. The tubes were then vortexed for 1 to 2 min each, followed by centrifugation for 10 min at 10,000 RCF (x g) at 4°C. The supernatant was discarded.

The fourth step was dehydration using an ethanol series of 30, 50, 70, 80, 90 and 100% in dH_2O, for 15 min each. The final step was critical drying using nitrogen gas. This was performed by sealing each Eppendorf tube in parafilm wax and inserting a needle with a connected nitrogen gas supply. Gold coating and imaging were done by Dr Ian Reid, in the Nano Imaging and Material Analysis Centre (NIMAC), School of Chemical and Bioprocess Engineering, UCD. Each sample was imaged at 1, 5, 10, 20 and 150 K magnifications.

The resulting images were compared qualitatively with each other, to determine if there were any changes in the structure of the biomasses after the different treatments. It was decided to only compare the following treatments of each biomass were imaged as they are representative of the remaining treatments: water only (6 h), buffer only (6 h), enzyme only (6 h), US 100% only (6 h) and US 100% + enzymes (6 h).

To summarize, approximately 340 g of each biomass (*Alaria esculenta* type 2 and *Lemna minor* type 2) was used for these experiments. These 340 g were split into 10 g portions, each of which was added to 250 mL dH_2O. In total, 17 treatments (two replicates each) per biomass were performed. The first 14 treatments were done using ultrasound as a pre-treatment for enzyme-assisted extraction; the 15^{th} treatment was done as the conventional method used in the food industry; the last 2 treatments were done using alkali extraction as a pre-treatment for ultrasound-assisted extraction.

After the treatments, samples were cooled and centrifuged. Both the supernatants and the pellets were freeze-dried for further testing to quantify the nitrogen content, and hence the protein content and to take SEM images were developed to analyze the surface structure of each biomass before and after treatments.

Chapter 3 Results

3.1 Characterisation of raw material

3.1.1 Colorimetry

Colorimetry was used to determine the concentration of coloured compounds in three different biomasses, *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1). Absorbance readings of four random points were taken for each sample and Table 3.1 was created. The meaning behind the symbols is summarized in Table 3.2.

Table 3.1 Statistical analysis to compare the absorbance values from *Alaria esculenta* (type 1), *Chlorella vulgaris* and *Lemna minor* (type 1). Data are presented as mean (STD), +/- SD. Four separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Biomass	L*	a*	b*
Alaria esculenta (type 1)	61.91 ± 0.31^{a}	$(-2.90) \pm 0.05^{b}$	7.36 ± 0.23^{b}
Chlorella vulgaris	44.01 ± 0.43^{c}	$(-3.10) \pm 0.29^{b}$	3.21 ± 0.50^{c}
Lemna minor (type 1)	56.85 ± 0.06^{b}	$(-7.18) \pm 0.05^{a}$	14.64 ± 0.13^{a}

Table 3.2 Summary of the meaning behind the colour coordinates.

Symbol	**Meaning**		
L*	Lightness	0 = black	100 = white
a*	Green-red	Negative is green	Positive is red
b*	Blue-yellow	Negative is blue	Positive is yellow

From the statistical analysis, one can conclude that there is a significant difference between the lightness of all the biomasses. There is a significant difference between the green tone in *Lemna minor* compared to that found in *Alaria esculenta* and the one found in *Chlorella vulgaris*. Also, there is a significant difference in the yellow tones found in all the biomasses.

3.1.2 Microscopy

A small amount of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1) was placed on three separate microscope slides. The biomasses were viewed under a magnification of x40, x100 and x400 using a microscope (Fig. 3.1).

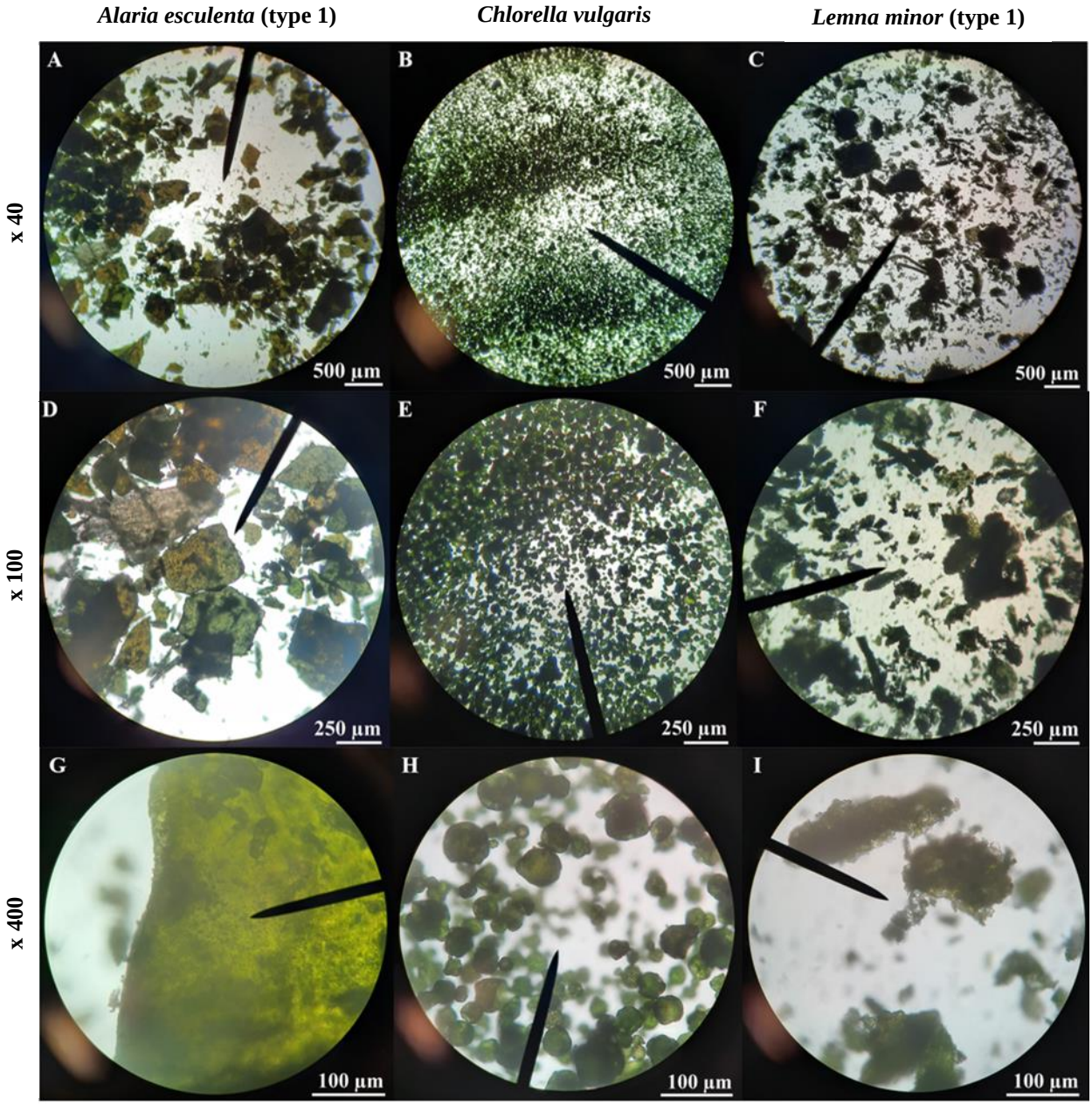

Fig. 3.1 Merged images of *Alaria esculenta* (type 1), *Chlorella vulgaris* and *Lemna minor* (type 1) [left to right], as seen under the microscope using a magnification of x 40, x 100 and x 400 [top to bottom]. (A) *Alaria esculenta* (type 1) as seen at mag. x 40. (B) *Chlorella vulgaris* as seen at mag. x 40. (C) *Lemna minor* (type 1) as seen at mag. x 40. (D) *Alaria esculenta* (type 1) as seen at mag. x 100. (E) *Chlorella vulgaris* as seen at mag. x 100. (F) *Lemna minor* (type 1) as seen at mag. x 100. (G) *Alaria esculenta* (type 1) as seen at mag. x 400. (H) *Chlorella vulgaris* as seen at mag. x 400. (I) *Lemna minor* (type 1) as seen at mag. x 400. Biomass particles can be scaled to their actual size by using the respective scale bars, found at the bottom right of each image.

It is quite clear that *Alaria esculenta* (type 1) has the largest particle sizes overall, after the drying and grinding steps. Furthermore, the surface looks quite rigid. *Chlorella vulgaris* has the smallest particle sizes, with small spherical shapes that also look quite rigid in structure. *Lemna minor* (type 1) has varying mid-sized particles that have a very porous structure. This hints that the drying and grinding process made the *Lemna minor* have a more permeable surface.

3.1.3 Water activity

Five replicates for each biomass were analysed using the dew point and water activity meter. Around 3.5 g of *Alaria esculenta* (type 1 and 2); 3.5 g of *Chlorella vulgaris*; and 1.5 g of *Lemna minor* (type 1 and 2) were used for each replicate. Table 3.3 presented an analysis to determine any significant differences between the raw biomasses.

Table 3.3 Statistical analysis to compare the water activity values from *Alaria esculenta* (type 1), *Chlorella vulgaris*, *Lemna minor* (type 1), *Alaria esculenta* (type 2) and *Lemna minor* (type 2). Data are presented as mean (STD); +/- SD. Five separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Biomass	**Water activity**
Alaria esculenta (type 1)	0.50 ± 0.00^{e}
Chlorella vulgaris	0.58 ± 0.00^{b}
Lemna minor (type 1)	0.50 ± 0.00^{d}
Alaria esculenta (type 2)	0.51 ± 0.00^{c}
Lemna minor (type 2)	0.67 ± 0.00^{a}

From the statistical analysis, one can conclude that there is a significant difference (p <0.001) between the water activity values of all the samples. It was interesting to see how the same biomass (*Alaria esculenta* types 1 and 2; *Lemna minor* types 1 and 2), harvested from a different place and processed differently, resulted in different water activity values.

3.1.4 Moisture content

This experiment was carried out on 8 g of *Alaria esculenta* (type 1 and 2); *Chlorella vulgaris*; and *Lemna minor* (type 1 and 2). The readings were taken using the moisture analyzer and each biomass had three replicates. Table 3.4 reports the analysis to determine any significant differences between the raw biomasses.

Table 3.4 Statistical analysis to compare the water activity values from *Alaria esculenta* (type 1), *Chlorella vulgaris*, *Lemna minor* (type 1), *Alaria esculenta* (type 2) and *Lemna minor* (type 2). Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Biomass	Moisture content (%)
Alaria esculenta (type 1)	12.08 ± 0.18^{e}
Chlorella vulgaris	9.58 ± 0.06^{b}
Lemna minor (type 1)	10.44 ± 0.11^{d}
Alaria esculenta (type 2)	13.22 ± 0.14^{c}
Lemna minor (type 2)	17.54 ± 0.21^{a}

From the statistical analysis, one can conclude that there is a significant difference (p <0.001) between the moisture content values of all the samples. This was expected as it is similar to what was found for the water activity parameter. Once again, it was interesting to see how the same biomass (*Alaria esculenta* types 1 and 2; *Lemna minor* types 1 and 2), harvested from a different place and processed differently, resulted in different water activity values.

3.2 Impact of different ultrasound set-ups on biomass treatments

3.2.1 Ultrasound energetics

3.2.1.1 Bath and UP200St probe sonicators at 150 W

This initial experiment was performed to determine changes in the internal temperature of a liquid sample over time, during its treatment with a bath and a probe sonicator, respectively (Fig. 3.2). Graph A depicts how the water sample in the bath sonicator reached an internal temperature of 52.13°C after the 70-min mark. Graph B shows that an identical water sample reached an internal temperature of 54.47°C after treatment

using the probe sonicator for just 7 min. However, it was noted that bath temperature readings had less variability than those measured using the probe sonicator. From these graphs it can be concluded that in order to decrease the chances of protein denaturation, treatments using a bath sonicator should be less than 1 hour, while treatments using the probe sonicator should be less than 7 min.

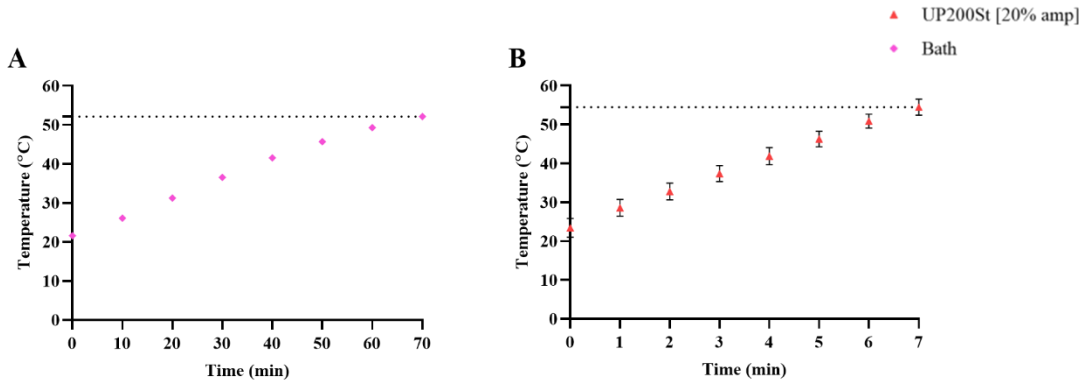

Fig. 3.2 Temperature changes with respect to time during ultrasound treatments with the **bath sonicator** (graph A) and the **probe sonicator (UP200St)** (graph B). Data are presented as mean (standard error of the mean – STD). The dots represent mean +/- standard deviation (SD). Three separate trials were performed for the data in both graphs. (In graph A the error bars are too small to be seen as the different trials gave consistent values.)

It is worth mentioning that when the temperature values in graph A and B of Fig. 3.2 were analysed, it was found that their energy output was increasing with time at approximately the same rate (Fig. 3.3). Hence, it can be concluded that the same power in both sonicators (150 W) was truly achieved when using the 20% amplitude setting on the probe sonicator.

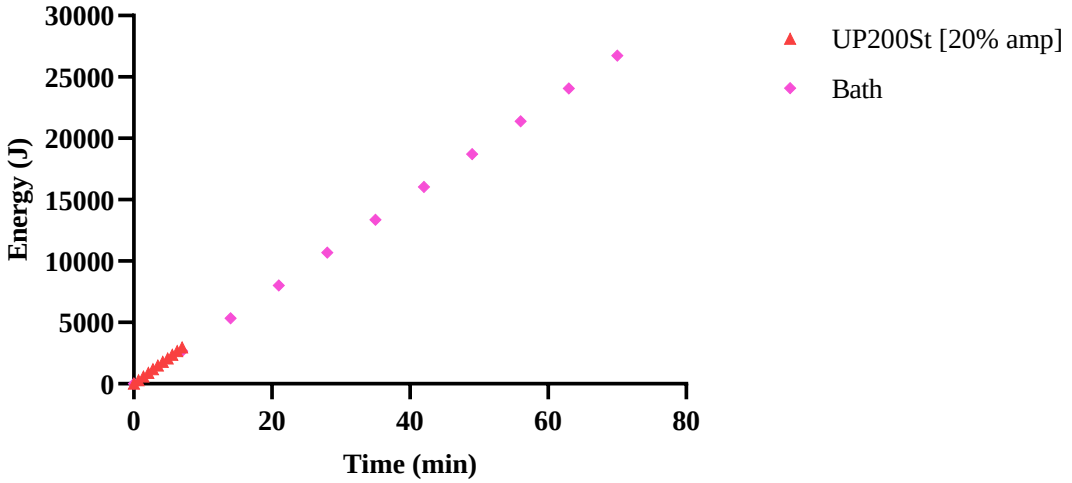

Fig. 3.3 Energy changes with respect to time during ultrasound treatments with the **bath** and the **probe (UP200St)** sonicators.

3.2.1.2 UP200St probe sonicator at different amplitude settings

This experiment was performed to determine changes in the internal temperature of a liquid sample over time, during its treatment with a probe sonicator set at different amplitudes (Fig. 3.4). The graph depicts how the water sample reached an internal temperature of 70°C after treatment using the probe sonicator at different amplitudes. As expected, the rate was fastest for the 75% amplitude setting followed by 50% and 25%, respectively. Interestingly, when set at the 25% amplitude setting, the temperature of the water sample can be seen to reach a plateau at around 150 min. This might be a useful feature for long US treatments in ensuring that the protein denaturation does not occur.

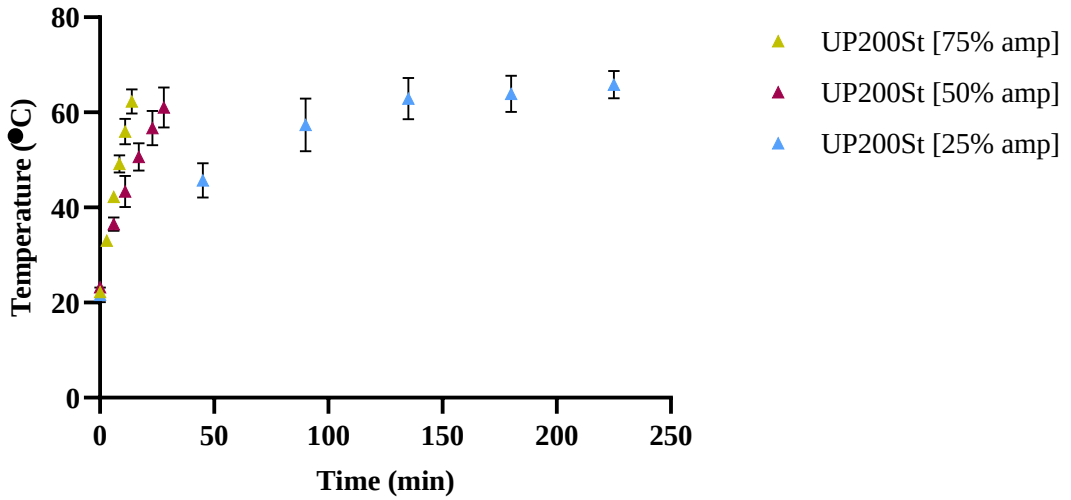

Fig. 3.4 Temperature changes with respect to time during ultrasound treatments with the UP200St set at **75%**, **50%** and **25%** amplitude settings. Data are presented as mean (STD). The dots represent mean +/- SD. Three separate trials were performed for the data in both graphs.

The data of Fig. 3.4 were then analysed to determine the energy output of each treatment with time (Fig. 3.5). As expected, the 75% amplitude setting had the greatest power, followed by 50% and 25%, respectively.

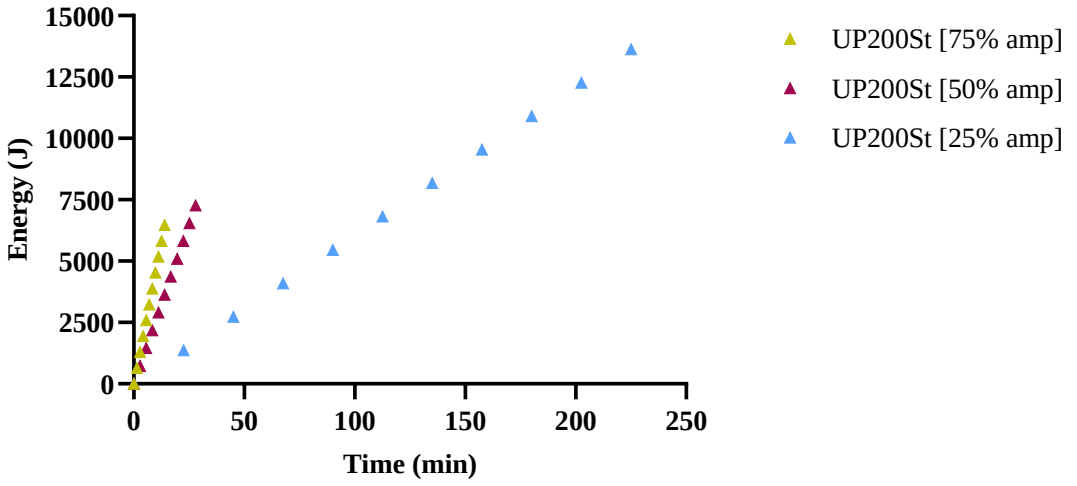

Fig. 3.5 Energy changes with respect to time during ultrasound treatments with the UP200St set at **75%**, **50%** and **25%** amplitude settings.

3.2.1.3 UP400St probe sonicator at different amplitude settings

This experiment was performed to determine changes in the internal temperature of a liquid sample over time, during its treatment with a probe sonicator of a different model, set at different amplitudes (Fig. 3.6). The graph depicts how the water sample reached an internal temperature of 70°C after treatment using the probe sonicator at different amplitudes. As expected, the rate was fastest for the 60% amplitude setting followed by 55%, 50%, 45%, 40%, 25% and 20%, respectively.

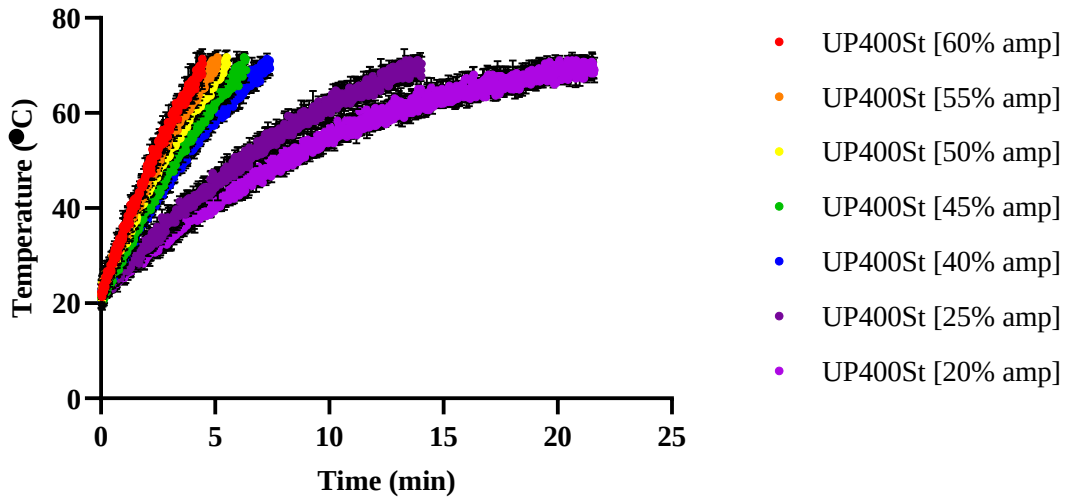

Fig. 3.6 Temperature changes with respect to time during ultrasound treatments with the UP400St set at **60%**, **55%**, **50%**, **45%**, **40%**, **25%** and **20%** amplitude settings. Data are presented as mean (STD). The dots represent mean +/- SD. Three separate trials were performed for the data in both graphs.

The graph in Fig. 3.6 was then analysed to determine the energy output of each treatment with time (Fig. 3.7). As expected, the 60% amplitude setting had the greatest power, followed by 55%, 50%, 45%, 40%, 25% and 20%, respectively.

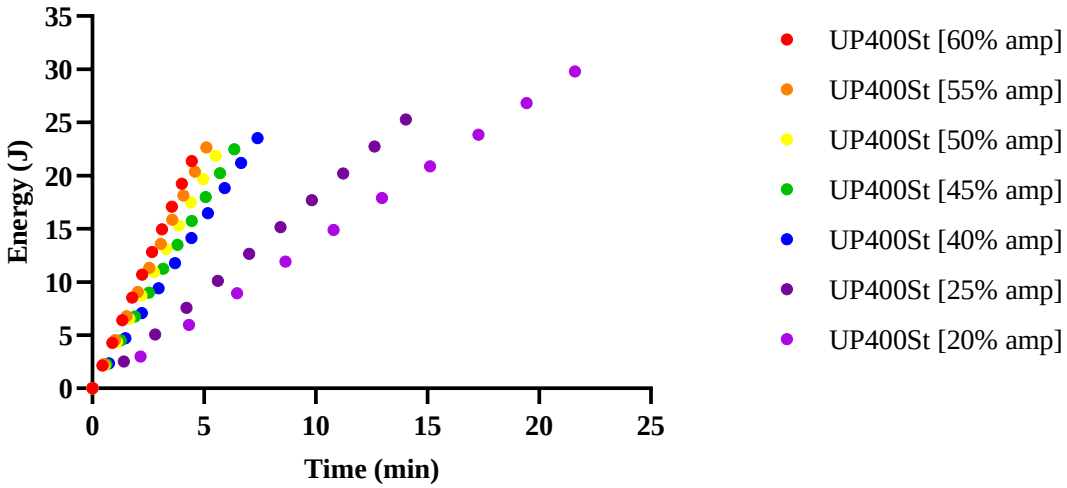

Fig. 3.7 Energy changes with respect to time during ultrasound treatments with the UP400St set at **60%**, **55%**, **50%**, **45%**, **40%**, **25%** and **20%** amplitude settings.

It can be concluded that the bath sonicator requires the most power to run, followed by the UP200St probe sonicator and the UP400St, respectively. Interestingly, the two different probe sonicators had differences in energy output and power for the same amplitude settings. This leads to the inference that sonication parameters should be translated to different models using power (W), ultrasonic intensity (W m^{-2}) or acoustic energy density (W m^{-3}) rather than amplitudes.

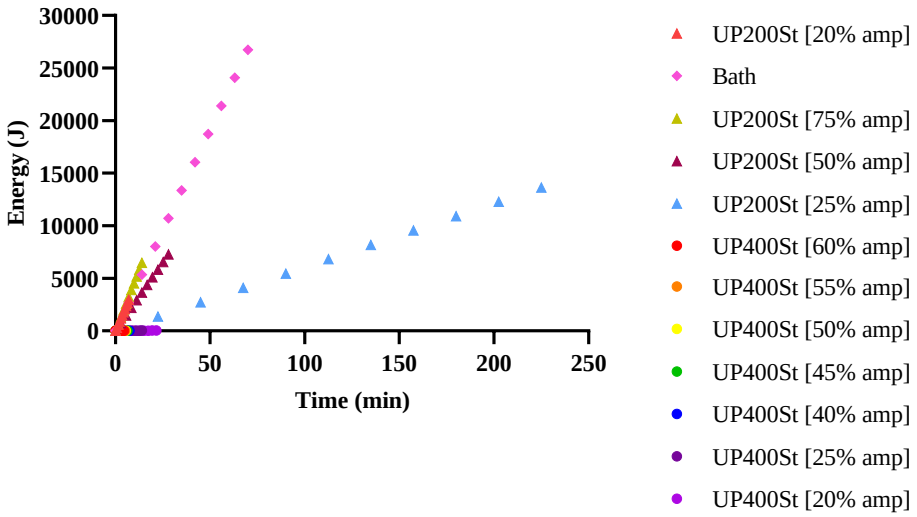

Fig. 3.8 Energy changes with respect to time during all the different ultrasound treatments.

Table 3.5 summarizes the power, ultrasonic intensity and acoustic energy density of each treatment that was used.

Table 3.5 Summary of the power, ultrasonic intensity and acoustic energy of each US treatment.

Ultrasonic device data	**Power (W)**	**Ultrasonic Intensity (W m^{-2})**	**Acoustic Energy Density (W m^{-3})**
Bath	6.367	46.851	2,402.642
UP200St [75%]	7.704	702.821	15,408.000
UP200St [50%]	4.328	394.835	8,656.000
UP200St [25%]	1.010	92.145	2,020.000
UP400St [60%]	0.080	7.248	1,000.000
UP400St [55%]	0.074	6.704	925.000
UP400St [50%]	0.066	5.980	825.000
UP400St [45%]	0.059	5.345	737.500
UP400St [40%]	0.053	4.802	662.500
UP400St [25%]	0.030	2.718	375.000
UP400St [20%]	0.023	2.084	287.500

The trend for the UP400St data is as expected. The 60% amplitude setting resulted in the highest power, ultrasonic intensity and acoustic energy density. This was followed by 55%, 50%, 45%, 40%, 25% and 20%, respectively. However, the bath and UP200St sonicators gave varying values, seemingly without a trend. This leads to the conclusion that UP400St is more time efficient than UP200St and the bath sonicators, but might lead to protein denaturation quicker.

3.2.2 Ultrasound extraction

3.2.2.1 Bath- vs probe-assisted extraction

After the treatments, samples were tested further to quantify the protein, peroxide, total free amino acid and total reducing sugar concentrations (refer to section 2.2.2.2). Moreover, the supernatants were analysed to determine each of their chlorophyll spectra and their surface structure. The objective for this particular experiment was to determine if ultrasound facilitates the extraction process for three different biomasses. Moreover, it served to establish whether different sonicators and different settings aided or hindered this extraction. The legends were abbreviated as described in Table 3.6.

Table 3.6 Description of the abbreviations used to make the legends in the graphs of section 3.1.2.

Abbreviated treatment label	Full treatment label
CT1	Control time 1 min
CT2	Control time 2 min
CT5	Control time 5 min
CT20	Control time 20 min
BT1	Bath time 1 min
BT5	Bath time 5 min
BT20	Bath time 20 min
40T1	UP400St set at 40% amp. time 1 min
40T5	UP400St set at 40% amp. time 5 min
40T20	UP400St set at 40% amp. time 20 min
80T1	UP400St set at 80% amp. time 1 min
80T2	UP400St set at 80% amp. time 2 min
80T5	UP400St set at 80% amp. time 5 min

3.2.2.2 Assays

3.2.2.2.1 Bradford's

Protein quantification was performed using Bradford's assay. The following graphs demonstrate the protein concentrations present in the supernatants of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1), respectively; after each of the 13 treatments. *Lemna minor* was found to have the greatest protein content, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

***Alaria esculenta* (type 1)**

The graph shows that for a fixed time, UP400St at 80% resulted in the most protein extraction, followed by UP400St at 40%, the bath sonicator and the control, respectively (Fig. 3.9). For most cases, each treatment gave a higher protein value with increasing the duration of treatment.

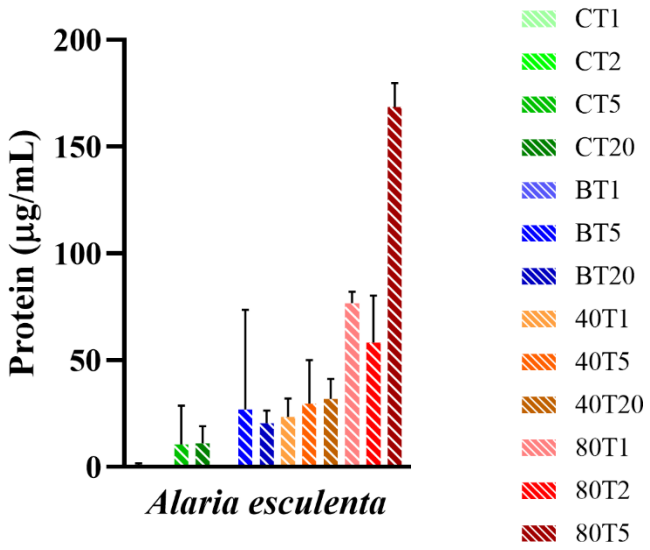

Fig. 3.9 Protein concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

An analysis was done to compare how different treatments performed when set at the same duration (Table 3.7). It was concluded that there was no significant difference

between any of the treatments for a given duration. However, it was interesting to see that for the control treatments, approximately the same protein content was extracted after 20 min (11.15 µg/mL) when compared to 5 min (10.54 µg/mL). This shows that after 5 min, the extraction of protein seemingly plateaus.

Table 3.7 Statistical analysis to compare how different treatments performed when set at the same duration. Protein concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.67 ± 1.16[a]	0.00 ± 0.00[a]	23.38 ± 8.76[a]	76.77 ± 5.29[a]
2	0.00 ± 0.00[a]	n/a	n/a	58.25 ± 21.96[a]
5	10.54 ± 18.26[a]	26.93 ± 46.65[a]	29.72 ± 20.28[a]	168.55 ± 11.19[a]
20	11.15 ± 8.01[a]	20.53 ± 5.95[a]	31.84 ± 9.36[a]	n/a

Analysis between different times was performed to determine how the same treatment performed with different durations (Table 3.8). One can conclude that there is a significantly higher amount of protein present in the supernatants after treatment with UP400St at 80% for 5 min, when compared to 1-min (p <0.001) and 2-min (p <0.001) durations of this same treatment. The 5-min treatment resulted in a 2.20-fold higher protein concentration than the 1-min treatment and a 2.89-fold higher protein concentration than the 2-min treatment.

Table 3.8 Statistical analysis to compare how the same treatment performed with different durations. Protein concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.67 ± 1.16^{a}	0.00 ± 0.00^{a}	23.38 ± 8.76^{a}	76.77 ± 5.29^{b}
2	0.00 ± 0.00^{a}	n/a	n/a	58.25 ± 21.96^{b}
5	10.54 ± 18.26^{a}	26.93 ± 46.65^{a}	29.72 ± 20.28^{a}	168.55 ± 11.19^{a}
20	11.15 ± 8.01^{a}	20.53 ± 5.95^{a}	31.84 ± 9.36^{a}	n/a

Chlorella vulgaris

Figure 3.10 shows that for a fixed time, UP400St at 80% resulted in the most protein being extracted, followed by UP400St at 40%, the bath sonicator and the control, respectively (Fig. 3.10). Generally, each treatment gave a higher protein value with increasing the duration.

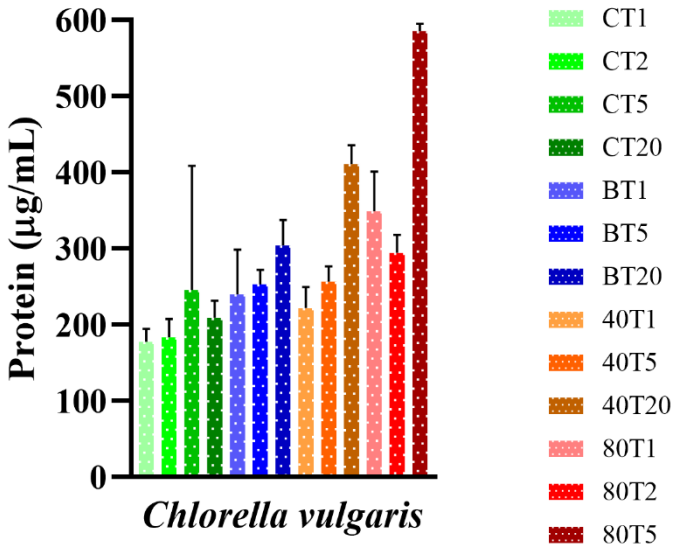

Fig. 3.10 Protein concentration in µg/mL from *Chlorella vulgaris* after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.9). For the 1-min treatments, it was concluded that there was a significant difference in protein extraction after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 2.96-fold higher protein concentration than the control. The same was observed for the 2-min treatments. There was a significant difference in protein extracted after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 1.60-fold higher protein concentration than the control.

With the 5-min treatments, significant differences resulted between the protein extracted after using the UP400St set at 80% when compared to the control (p <0.01), bath sonicator (p <0.01) and UP400St set at 40% (p <0.01). The 80% amplitude setting resulted in a 2.38-fold higher protein concentration than the control, a 2.31-fold higher protein concentration than the bath, and a 2.28-fold higher protein concentration than the UP400St set at 40%.

Lastly, the 20-min treatments were found to have significant differences between the UP400St set at 40% and the control (p <0.001) and bath treatments (p <0.01). The 40% amplitude setting resulted in a 1.97-fold higher protein concentration than the control, a 1.35-fold higher protein concentration than the bath.

Table 3.9 Statistical analysis to compare how different treatments performed when set at the same duration. Protein concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	177.79 ± 16.72^{b}	240.47 ± 58.42^{ab}	222.20 ± 27.72^{ab}	349.15 ± 52.29^{a}
2	183.96 ± 23.84^{b}	n/a	n/a	294.69 ± 23.63^{a}
5	245.97 ± 162.89^{b}	253.19 ± 19.24^{b}	256.94 ± 20.02^{b}	585.76 ± 9.59^{a}
20	209.26 ± 22.52^{b}	304.26 ± 33.38^{b}	411.27 ± 24.70^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.10). One can conclude that there is a significantly higher amount of protein present in the supernatants after treatment with UP400St at 40% for 20 min, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 1.85-fold higher protein concentration than the 1-min treatment and a 1.60-fold higher protein concentration than the 5-min treatment.

Similarly, there is a significantly higher amount of protein present in the supernatants after treatment with UP400St at 80% for 5 min, when compared to 1-min (p <0.001) and 2-min (p <0.001) durations of the same treatment. The 5-min treatment resulted in a 1.68-fold higher protein concentration than the 1-min treatment and a 1.99-fold higher protein concentration than the 2-min treatment.

Table 3.10 Statistical analysis to compare how the same treatment performed with different durations. Protein concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	177.79 ± 16.72[a]	240.47 ± 58.42[a]	222.20 ± 27.72[b]	349.15 ± 52.29[b]
2	183.96 ± 23.84[a]	n/a	n/a	294.69 ± 23.63[b]
5	245.97 ± 162.89[a]	253.19 ± 19.24[a]	256.94 ± 20.02[b]	585.76 ± 9.59[a]
20	209.26 ± 22.52[a]	304.26 ± 33.38[a]	411.27 ± 24.70[a]	n/a

Lemna minor (type 1)

Figure 3.11 shows that for a fixed time, UP400St at 80% resulted in the most protein being extracted, followed by UP400St at 40%, the bath sonicator and the control, respectively. Overall, each treatment gave a higher protein value with increasing durations.

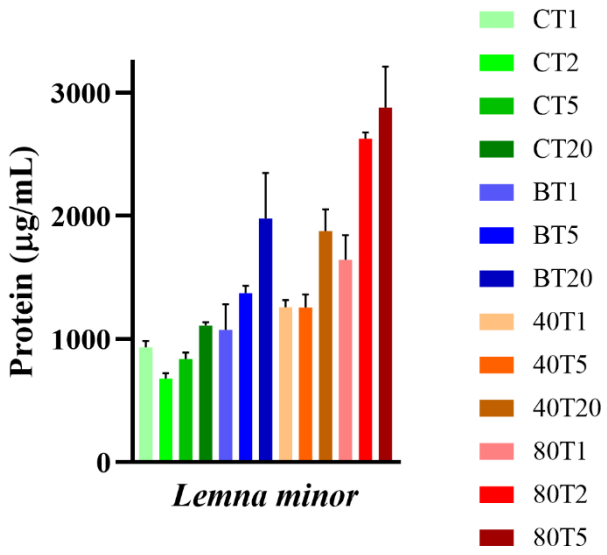

Fig. 3.11 Protein concentration in µg/mL from *Lemna minor* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.11). For the 1-min treatments, it was concluded that there was a significant difference in protein concentration after using the UP400St set at 80% when compared to the control (p <0.01) and bath sonicator (p <0.01). The 80% amplitude setting resulted in a 1.76-fold higher protein concentration than the control and a 1.53-fold higher protein concentration than the bath.

For the 2-min treatments, there was a significant difference in protein extracted after using the UP400St set at 80% when compared to the control (p <0.001). The 80% amplitude setting resulted in a 3.87-fold higher protein concentration than the control.

With the 5-min treatments, significant differences resulted between the protein extracted after using the UP400St set at 80% when compared to the control (p <0.001), bath sonicator (p <0.001) and UP400St set at 40% (p <0.001). The 80% amplitude setting resulted in a 3.43-fold higher protein concentration than the control, a 2.10-fold higher protein concentration than the bath, and a 2.29-fold higher protein concentration than the UP400St set at 40%.

Table 3.11 Statistical analysis to compare how different treatments performed when set at the same duration. Protein concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	933.18 ± 51.05^{b}	1074.09 ± 209.24^{b}	1258.20 ± 57.55^{ab}	1641.29 ± 202.85^{a}
2	678.69 ± 43.39^{b}	n/a	n/a	2627.03 ± 51.17^{a}
5	839.13 ± 50.72^{b}	1372.84 ± 58.16^{b}	1255.75 ± 105.23^{b}	2876.82 ± 334.05^{a}
20	1110.15 ± 24.87^{a}	1977.50 ± 369.94^{a}	1874.35 ± 177.46^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.12). One can conclude that there is a significantly higher amount of protein present in the supernatants after the 20-min control, when compared to 1-min (p <0.01), 2-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 1.19-fold higher protein concentration than the 1-min treatment, a 1.64-fold higher protein concentration than the 2-min treatment and a 1.32-fold higher protein concentration than the 5-min treatment. Likewise, there was a significant difference between the controls left for 5 min and those left for 2 (p <0.01), resulting in a 1.24-fold higher protein concentration for the 5-min treatment. Strangely, there was a significant dip (p <0.001) in protein extraction between the controls left for 1 min and those left for 2, resulting in a 0.73-fold decrease in protein concentration for the 2-min treatment.

Similarly, there is a significantly higher amount of protein present in the supernatants after treatment with UP400St at 40% for 20 min, when compared to 1-min (p <0.01) and 5-min (p <0.01) durations of the same treatment. The 20-min treatment resulted in a 1.49-fold higher protein concentration than the 1-min treatment and a 1.49-fold higher protein concentration than the 5-min treatment.

Furthermore, when using the UP400St at 80% there were significant differences between the protein values of 1-min and 2-min treatments (p <0.01), with the 2-min treatment resulting in a 1.60-fold higher protein concentration. The same was seen

between the 1-min and 5-min treatments (p <0.01), with the 5-min treatment resulting in a 1.75-fold higher protein concentration.

Table 3.12 Statistical analysis to compare how the same treatment performed with different durations. Protein concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	933.18 ± 51.05^{b}	1074.09 ± 209.24^{a}	1258.20 ± 57.55^{b}	1641.29 ± 202.85^{b}
2	678.69 ± 43.39^{c}	n/a	n/a	2627.03 ± 51.17^{a}
5	839.13 ± 50.72^{b}	1372.84 ± 58.16^{a}	1255.75 ± 105.23^{b}	2876.82 ± 334.05^{a}
20	1110.15 ± 24.87^{a}	1977.50 ± 369.94^{a}	1874.35 ± 177.46^{a}	n/a

All the biomasses followed the same trends. All graphs show that for a fixed time, UP400St at 80% resulted in the most protein being extracted, followed by UP400St at 40%, the bath sonicator and the control, respectively. Furthermore, each treatment gave a higher protein value with increasing durations. It is worth noting that *Lemna minor* extracted the highest quantity of protein followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

3.2.2.2.2 FOX

Peroxide quantification was done using FOX assay. The following graphs demonstrate the peroxide concentrations present in the supernatants of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1), respectively; after each of the 13 treatments. This serves as an indicator of the antioxidant content in the biomasses. *Lemna minor* was found to have the greatest peroxide being generated after treatments, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

***Alaria esculenta* (type 1)**

The graph shows that after treatments with UP400St at 40%, the bath sonicator and the control, more peroxide is being generated with increasing durations, however, after treatment with UP400St at 80% the peroxide concentration is seen to decrease with longer durations of treatment (Fig. 3.12).

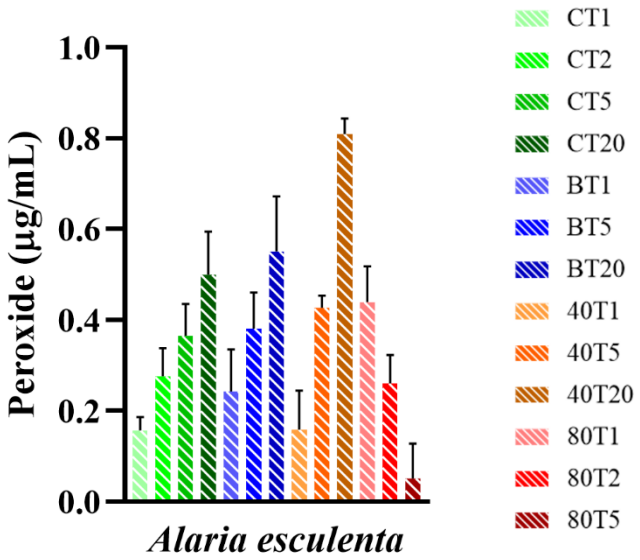

Fig. 3.12 Peroxide concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.13). For the 1-min treatments, there was a significant difference in peroxide generation after using the UP400St set at 80% when compared to the control (p <0.01) and the UP400St at 40% (p <0.01). The 80% amplitude setting resulted in a 2.75-fold higher peroxide concentration than both the control and the UP400St at 40%.

For the 5-min treatments, there was a significant difference in peroxide generation after using the UP400St set at 80% when compared to the control (p <0.01), the bath (p <0.01) and the UP400St at 40% (p <0.001). The 80% amplitude setting resulted in a 0.14-fold decrease in peroxide concentration than the control, a 0.13-fold higher peroxide concentration than the bath, and a 0.12-fold higher peroxide concentration than the UP400St set at 40%.

Table 3.13 Statistical analysis to compare how different treatments performed when set at the same duration. Peroxide concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.16 ± 0.03^{b}	0.24 ± 0.09^{ab}	0.16 ± 0.09^{b}	0.44 ± 0.08^{a}
2	0.28 ± 0.06^{a}	n/a	n/a	0.26 ± 0.06^{a}
5	0.37 ± 0.07^{a}	0.38 ± 0.08^{a}	0.43 ± 0.03^{a}	0.05 ± 0.08^{b}
20	0.50 ± 0.09^{a}	0.55 ± 0.12^{a}	0.81 ± 0.03^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.14). One can conclude that there is a significantly higher amount of peroxide generated in the supernatants after the control for 20 min, when compared to the 1-min (p <0.01) duration of this same treatment. The 20-min treatment resulted in a 3.13-fold higher peroxide concentration than the 1-min.

There is a significantly higher amount of peroxide present in the supernatants after treatment with UP400St at 40% for 20 min, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 5.06-fold higher peroxide concentration than the 1-min treatment and a 1.88-fold higher peroxide concentration than the 5-min treatment. Similarly, the UP400St at 40% for 5 min resulted in a peroxide concentration which was significantly different from that after 1 min (p <0.01). The 5-min treatment had a 2.69-fold increase in peroxide concentration when compared to the 1-min treatment.

Also, there was a significant difference in the peroxide generated after treatment with the UP400St at 80% set for 5 min compared to 1 min. The 5-min treatment had a 0.11-fold decrease in peroxide concentration compared to the 1-min treatment.

Table 3.14 Statistical analysis to compare how the same treatment performed with different durations. Peroxide concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.16 ± 0.03^{b}	0.24 ± 0.09^{a}	0.16 ± 0.09^{c}	0.44 ± 0.08^{a}
2	0.28 ± 0.06^{ab}	n/a	n/a	0.26 ± 0.06^{ab}
5	0.37 ± 0.07^{ab}	0.38 ± 0.08^{a}	0.43 ± 0.03^{b}	0.05 ± 0.08^{b}
20	0.50 ± 0.09^{a}	0.55 ± 0.12^{a}	0.81 ± 0.03^{a}	n/a

Chlorella vulgaris

The graph shows no real trend in peroxide generation, however, the UP400St treatment is seen to decrease the amount of TRS in the supernatant (Fig. 3.13).

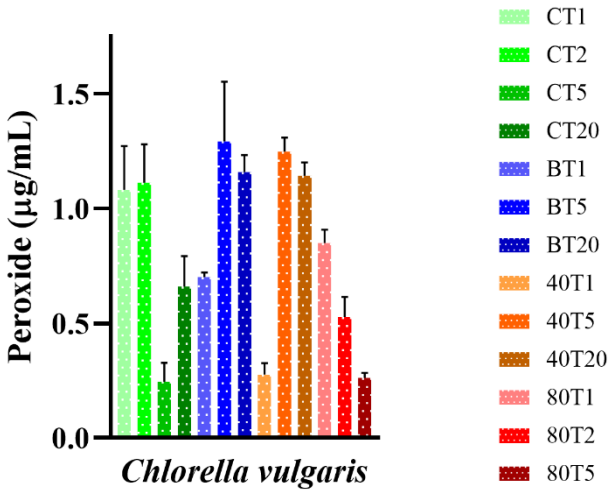

Fig. 3.13 Peroxide generated in µg/mL from *Chlorella vulgaris* after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.15). For the 1-min treatments, it was concluded that there was a significant difference in peroxide generation after using the UP400St set at 80% when compared to the UP400St set at 40% (p <0.001). The 80% amplitude setting

resulted in a 3.04-fold higher peroxide concentration than the UP400St set at 40%. Furthermore, the UP400St set at 40% treatment generated a significantly lower amount of peroxide than the control (p <0.001) and bath (p <0.01) treatments. The 40% amplitude setting resulted in a 0.26-fold decrease in peroxide concentration compared with the control and a 0.4-fold decrease in peroxide content compared with the bath. There was also a 0.65-fold significant decrease in peroxide in the bath treatment compared to the control (p <0.01).

For the 2-min treatments, there was a significant difference in peroxide generated after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 0.48-fold lower peroxide concentration than the control.

With the 5-min treatments, significant differences resulted between the peroxide content after using the UP400St set at 80% when compared to the bath sonicator (p <0.001) and UP400St set at 40% (p <0.001). The 80% amplitude setting resulted in a 0.20-fold lower peroxide concentration than the bath and a 0.21-fold lower peroxide concentration than the UP400St set at 40%. There was also a significant difference in the values of the control treatment when compared to the bath (p <0.001) and UP400St set at 40% (p <0.001) treatments. The bath resulted in a 5.16-fold increase in peroxide concentration compared to the control, while the UP400St set at 40% resulted in a 5-fold increase in peroxide concentration compared to the control.

Lastly, the control 20-min treatments were found to have significant differences with the bath (p <0.01) and the UP400St set at 40% (p <0.01) treatments set at the same time. The bath resulted in a 1.76-fold higher peroxide concentration than the control, while the UP400St set at 40% resulted in a 1.73-fold higher peroxide concentration than the control.

Table 3.15 Statistical analysis to compare how different treatments performed when set at the same duration. Peroxide concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	1.08 ± 0.19^{a}	0.70 ± 0.02^{b}	0.28 ± 0.05^{c}	0.85 ± 0.06^{ab}
2	1.11 ± 0.17^{a}	n/a	n/a	0.53 ± 0.09^{b}
5	0.25 ± 0.08^{b}	1.29 ± 0.26^{a}	1.25 ± 0.06^{c}	0.26 ± 0.02^{b}
20	0.66 ± 0.13^{b}	1.16 ± 0.07^{a}	1.14 ± 0.06^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.16). One can conclude that there is a significantly lower amount of peroxide present in the supernatants after the control for 5 min, when compared to 1-min (p <0.001) and 2-min (p <0.001) durations of the same treatment. The 5-min treatment resulted in a 0.25-fold lower peroxide concentration than the 1-min treatment and a 0.23-fold lower peroxide concentration than the 2-min treatment.

On the contrary, there is a significantly higher amount of peroxide present in the supernatants after treatment with the bath for 5 min, when compared to 1-min (p <0.01) durations of the same treatment. The 5-min treatment resulted in a 1.84-fold higher peroxide concentration than the 1-min treatment.

When treating with UP400St at 40%, there was a significant increase in the peroxide generated during 5-min (p <0.001) and 20-min (p <0.001) treatments, as compared to 1-min treatments. The 5-min treatment resulted in a 4.46-fold higher peroxide concentration than the 1-min treatment, while the 20-min treatment resulted in a 4.07-fold higher peroxide concentration than the 1-min treatment.

For the UP400St at 80% treatments, there was a significant decrease in the peroxide generated during 2-min (p <0.01) and 5-min (p <0.001) treatments, as compared to 1-min treatments. The 2-min treatment resulted in a 0.62-fold lower peroxide concentration than the 1-min treatment, while the 5-min treatment resulted in a 0.31-fold lower peroxide concentration than the 1-min treatment. Furthermore, the there was a significant decrease in the peroxide generated during the 5-min (p <0.01) treatments,

as compared to 2-min treatments. The 5-min treatment resulted in a 0.49-fold lower peroxide concentration than the 1-min treatment.

Table 3.16 Statistical analysis to compare how the same treatment performed with different durations. Peroxide concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly ($p < 0.01$).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	1.08 ± 0.19^{a}	0.70 ± 0.02^{b}	0.28 ± 0.05^{b}	0.85 ± 0.06^{a}
2	1.11 ± 0.17^{a}	n/a	n/a	0.53 ± 0.09^{b}
5	0.25 ± 0.08^{b}	1.29 ± 0.26^{a}	1.25 ± 0.06^{a}	0.26 ± 0.02^{c}
20	0.66 ± 0.13^{ab}	1.16 ± 0.07^{ab}	1.14 ± 0.06^{a}	n/a

Lemna minor (type 1)

The same trend that was seen in *Alaria esculenta* was found in *Lemna minor*. The graph shows that after treatments with UP400St at 40%, the bath sonicator and the control, more peroxide was generated with increasing durations, however, after treatment with UP400St at 80% the peroxide concentration was seen to decrease with longer durations of treatment (Fig. 3.14).

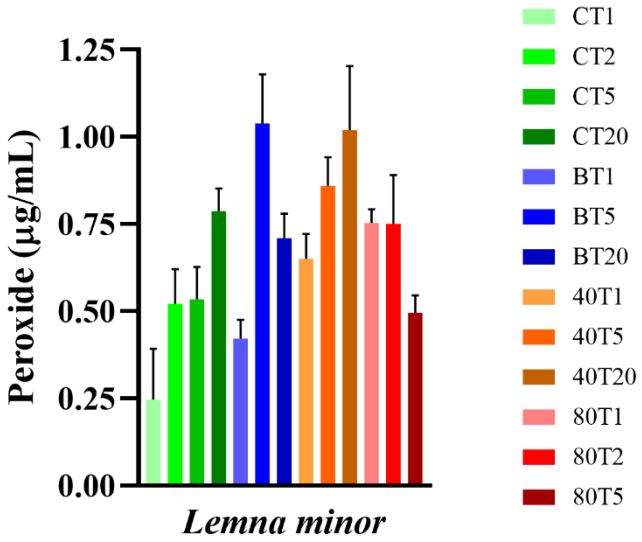

Fig. 3.14 Peroxide concentration in µg/mL from *Lemna minor* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.17). For the 1-min treatments, it was concluded that there was a significant difference in peroxide concentration after using the UP400St set at 40% when compared to the control (p <0.01). The 40% amplitude setting resulted in a 2.6-fold higher peroxide concentration than the control. Similarly, there was a significant difference in peroxide concentration after using the UP400St set at 80% when compared to the control (p <0.001) and bath (p <0.01) treatments. The 80% amplitude setting resulted in a 3-fold higher peroxide concentration than the control and a 1.79-fold higher peroxide concentration than the bath.

With the 5-min treatments, significant differences resulted between the peroxide generated after using the UP400St set at 80% when compared to the bath (p <0.001) and UP400St set at 40% (p <0.01). The 80% amplitude setting resulted in a 0.47-fold lower peroxide concentration than the bath and a 0.57-fold lower peroxide concentration than the UP400St set at 40%. Furthermore, the bath treatment had a significantly higher (p <0.01) peroxide concentration than the control. The bath sonicated sample resulted in a 1.96-fold higher peroxide concentration than the control.

Table 3.17 Statistical analysis to compare how different treatments performed when set at the same duration. Peroxide concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.25 ± 0.15^{b}	0.42 ± 0.05^{bc}	0.65 ± 0.07^{ac}	0.75 ± 0.04^{a}
2	0.52 ± 0.10^{a}	n/a	n/a	0.75 ± 0.14^{a}
5	0.53 ± 0.09^{b}	1.04 ± 0.14^{a}	0.86 ± 0.08^{ab}	0.49 ± 0.05^{bc}
20	0.79 ± 0.06^{a}	0.71 ± 0.07^{a}	1.02 ± 0.18^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.18). One can conclude that there is a significantly higher amount of peroxide present in the supernatants after the 20-min control, when compared to 1-min (p <0.01) duration of the same treatment. The 20-min treatment resulted in a 3.16-fold higher peroxide concentration than the 1-min treatment.

There was a significant difference between the bath treatments left for 5 min and those left for 1 (p <0.001), resulting in a 2.48-fold higher peroxide concentration for the 5-min treatment.

Table 3.18 Statistical analysis to compare how the same treatment performed with different durations. Peroxide concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.25 ± 0.15^{b}	0.42 ± 0.05^{b}	0.65 ± 0.07^{a}	0.75 ± 0.04^{a}
2	0.52 ± 0.10^{ab}	n/a	n/a	0.75 ± 0.14^{a}
5	0.53 ± 0.09^{ab}	1.04 ± 0.14^{a}	0.86 ± 0.08^{a}	0.49 ± 0.05^{a}
20	0.79 ± 0.06^{a}	0.71 ± 0.07^{ab}	1.02 ± 0.18^{a}	n/a

Alaria esculenta and *Lemna minor* followed the same trend. Supernatants had more peroxide after treatment with UP400St at 40%, the bath sonicator and the control, with increasing durations, however, after treatment with UP400St at 80% the peroxide concentration was seen to decrease with longer durations of treatment. *Chlorella vulgaris* did not follow the trend completely but it also showed a decrease in peroxide after UP400St set at 80%. It is worth noting that *Lemna minor* extracted the highest quantity of peroxide followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

3.2.2.2.3 TFAA

TFAA quantification was done using OPA reagent. The following graphs demonstrate the TFAA concentrations present in the supernatants of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1), respectively; after each of the 13 treatments. *Lemna minor* was found to have the greatest amount of TFAA released after treatments, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

***Alaria esculenta* (type 1)**

The graph shows that overall, after treatments with UP400St at 40% and UP400St at 80%, more TFAA was released. However, after treatment with the bath, the TFAA content seems to decrease with longer durations of treatment (Fig. 3.15).

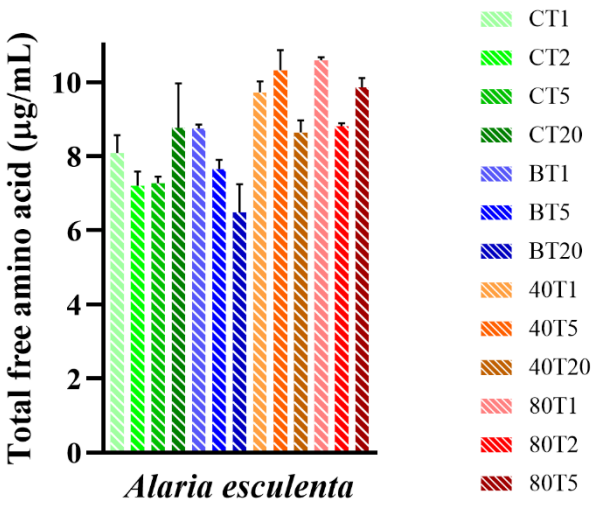

Fig. 3.15 TFAA concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.19). For the 1-min treatments, there was a significant difference in TFAA release after using the UP400St set at 80% when compared to the control (p <0.001) and the bath (p <0.001). The 80% amplitude setting resulted in a 1.31-fold higher TFAA concentration than the control and a 1.21-fold higher TFAA concentration than the bath. Furthermore, there was a significant difference in TFAA release after using the UP400St set at 40% when compared to the control (p <0.001), which resulted in a 1.20-fold higher TFAA concentration.

For the 2-min treatments, there was a significant difference in TFAA release after using the UP400St set at 80% when compared to the control (p <0.01), which resulted in a 1.22-fold higher TFAA concentration.

For the 5-min treatments, there was a significant difference in TFAA release after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 1.42-fold decrease in TFAA concentration than the control, and a 1.35-fold higher TFAA concentration than the bath. There was also a significant difference in TFAA release after using the UP400St set at

80% when compared to the control (p <0.001) and the bath (p <0.001). The 80% amplitude setting resulted in a 1.36-fold decrease in TFAA concentration than the control, and a 1.29-fold higher TFAA concentration than the bath.

Table 3.19 Statistical analysis to compare how different treatments performed when set at the same duration. TFAA concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	8.08 ± 0.49^{b}	8.74 ± 0.11^{bc}	9.72 ± 0.29^{ac}	10.58 ± 0.08^{a}
2	7.20 ± 0.39^{b}	n/a	n/a	8.80 ± 0.08^{a}
5	7.27 ± 0.18^{b}	7.65 ± 0.25^{b}	10.32 ± 0.54^{a}	9.86 ± 0.25^{a}
20	8.75 ± 1.21^{a}	6.48 ± 0.75^{a}	8.64 ± 0.33^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.20). One can conclude that there is a significantly lower amount of TFAA released in the supernatants after the bath treatment for 20 min, when compared to the 1-min (p <0.01) duration of this same treatment. The 20-min treatment resulted in a 0.74-fold lower TFAA concentration than the 1-min.

There is a significantly lower amount of TFAA present in the supernatants after treatment with UP400St at 40% for 20 min, when compared to the 5-min (p <0.01) duration of the same treatment. The 20-min treatment resulted in a 0.84-fold lower TFAA concentration than the 5-min treatment.

There was also a significant difference in the TFAA released after treatment with the UP400St at 80% set for 5 min compared to the 1-min (p <0.01) and 2-min (p <0.001) durations of the same treatment. Strangely, the 5-min treatment had a 0.93-fold decrease in TFAA concentration compared to the 1-min treatment but a 1.12-fold increase in TFAA concentration compared to the 2-min treatment. Furthermore, there was a significant difference in the TFAA released after treatment with the UP400St at 80% set for 2 min compared to the 1-min (p <0.001) duration of the same treatment. The 2-min treatment resulted in a 0.83-fold lower TFAA concentration than the 1-min treatment.

Table 3.20 Statistical analysis to compare how the same treatment performed with different durations. TFAA concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	8.08 ± 0.49^{a}	8.74 ± 0.11^{a}	9.72 ± 0.29^{ab}	10.58 ± 0.08^{a}
2	7.20 ± 0.39^{a}	n/a	n/a	8.80 ± 0.08^{c}
5	7.27 ± 0.18^{a}	7.65 ± 0.25^{ab}	10.32 ± 0.54^{a}	9.86 ± 0.25^{b}
20	8.75 ± 1.21^{a}	6.48 ± 0.75^{b}	8.64 ± 0.33^{b}	n/a

Chlorella vulgaris

The graph shows that there is no particular trend and that there is only a slight increase in TFAA concentration with the treatments (Fig. 3.16).

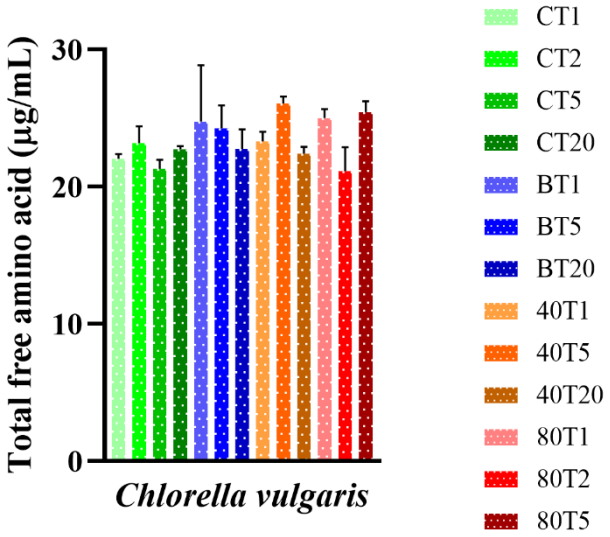

Fig. 3.16 TFAA released in µg/mL from *Chlorella vulgaris* after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.21). With the 5-min treatments, significant differences resulted between the TFAA content after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 1.19-fold increase in TFAA concentration than the control. There was also a significant difference in the values of the UP400St set at 40% when compared to the control (p <0.01). The UP400St at 40% resulted in a 1.22-fold increase in TFAA concentration compared to the control.

Table 3.21 Statistical analysis to compare how different treatments performed when set at the same duration. TFAA concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	22.04 ± 0.34^{a}	24.75 ± 4.12^{a}	23.30 ± 0.70^{a}	25.02 ± 0.64^{a}
2	23.19 ± 1.22^{a}	n/a	n/a	21.13 ± 1.76^{a}
5	21.32 ± 0.65^{b}	24.26 ± 1.68^{ab}	26.07 ± 0.53^{a}	25.44 ± 0.79^{a}
20	22.73 ± 0.23^{a}	22.77 ± 1.41^{a}	22.42 ± 0.50^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.22). One can conclude that there is a significantly higher amount of TFAA present in the supernatants after the UP400St set at 40% for 5 min, when compared to 1-min (p <0.01) and 20-min (p <0.001) durations of the same treatment. The 5-min treatment resulted in a 1.12-fold increase in TFAA concentration than the 1-min treatment and a 1.16-fold greater TFAA concentration than the 20-min treatment.

For the UP400St at 80% treatments, there was a significant increase in the TFAA released during 5-min (p <0.01) as compared to 2-min treatments. The 5-min treatment resulted in a 1.20-fold higher TFAA concentration than the 2-min treatment.

Table 3.22 Statistical analysis to compare how the same treatment performed with different durations. TFAA concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly ($p < 0.01$).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	22.04 ± 0.34^{a}	24.75 ± 4.12^{a}	23.30 ± 0.70^{b}	25.02 ± 0.64^{ab}
2	23.19 ± 1.22^{a}	n/a	n/a	21.13 ± 1.76^{b}
5	21.32 ± 0.65^{a}	24.26 ± 1.68^{a}	26.07 ± 0.53^{a}	25.44 ± 0.79^{a}
20	22.73 ± 0.23^{a}	22.77 ± 1.41^{a}	22.42 ± 0.50^{b}	n/a

Lemna minor (type 1)

No trend was present for the TFAA release, and minimal extraction took place after using the different treatments as compared to the controls (Fig. 3.17).

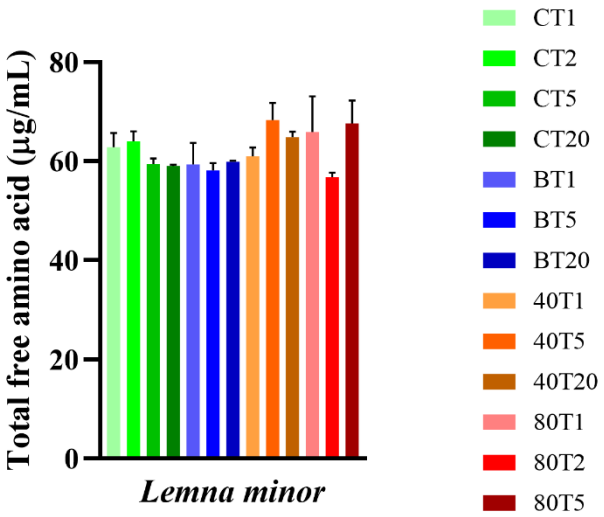

Fig. 3.17 TFAA concentration in µg/mL from *Lemna minor* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.23). For the 2-min treatments, there was a significant difference in TFAA concentration after using the UP400St set at 80% when compared

to the control (p <0.01). The 80% amplitude setting resulted in a 0.89-fold lower TFAA concentration than the control.

With the 20-min treatments, significant differences resulted between the TFAA released after using the UP400St set at 40% when compared to the control (p <0.001) and bath (p <0.001). The 40% amplitude setting resulted in a 1.10-fold higher TFAA concentration than the control and a 1.08-fold higher TFAA concentration than the bath.

Table 3.23 Statistical analysis to compare how different treatments performed when set at the same duration. TFAA concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	62.86 ± 2.86^{a}	59.39 ± 4.29^{a}	60.94 ± 1.84^{a}	65.93 ± 7.20^{a}
2	64.01 ± 2.05^{a}	n/a	n/a	56.77 ± 0.94^{b}
5	59.41 ± 1.16^{a}	58.19 ± 1.45^{a}	68.32 ± 3.42^{a}	67.63 ± 4.61^{a}
20	59.00 ± 0.29^{b}	59.89 ± 0.22^{b}	64.81 ± 1.18^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.24). No significant differences were found in the amount of TFAA after using different time points.

Table 3.24 Statistical analysis to compare how the same treatment performed with different durations. TFAA concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	62.86 ± 2.86^{a}	59.39 ± 4.29^{a}	60.94 ± 1.84^{a}	65.93 ± 7.20^{a}
2	64.01 ± 2.05^{a}	n/a	n/a	56.77 ± 0.94^{a}
5	59.41 ± 1.16^{a}	58.19 ± 1.45^{a}	68.32 ± 3.42^{a}	67.63 ± 4.61^{a}
20	59.00 ± 0.29^{a}	59.89 ± 0.22^{a}	64.81 ± 1.18^{a}	n/a

There was no general trend for TFAA release overall. However, it is worth noting that *Lemna minor* extracted the highest quantity of TFAA followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

3.2.2.2.4 TRS

TRS quantification was done using DNS reagent. The following graphs demonstrate the TRS concentrations present in the supernatants of *Alaria esculenta* (type 1), *Chlorella vulgaris*, and *Lemna minor* (type 1), respectively; after each of the 13 treatments. *Lemna minor* was found to have the greatest TRS release after treatments, followed by *Alaria esculenta* and *Chlorella vulgaris*, respectively.

***Alaria esculenta* (type 1)**

The general trend is that after treatments with UP400St at 40% and UP400St at 80%, more TRS is being released with increasing durations, however, after treatment with the bath the TRS concentration is seen to decrease with longer durations of treatment (Fig. 3.18).

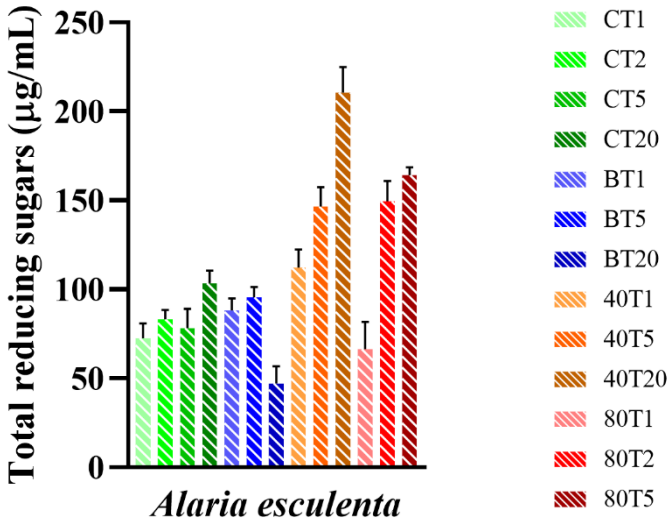

Fig. 3.18 TRS concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.25). For the 1-min treatments, there was a significant difference in TRS release after using the UP400St set at 40% when compared to the control (p <0.01) and the UP400St at 80% (p <0.01). The 40% amplitude setting resulted in a 1.54-fold higher TRS concentration than the control, but a 0.59-fold lower TRS concentration than the UP400St at 80%.

For the 2-min treatments, there was a significant difference in TRS release after using the UP400St set at 80% when compared to the control (p <0.001). The 80% amplitude setting resulted in a 1.80-fold higher TRS concentration than the control.

For the 5-min treatments, there was a significant difference in TRS content after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 1.87-fold increase in TRS concentration than the control and a 1.53-fold higher TRS concentration than the bath. There was also a significant difference in TRS content after using the UP400St set at 80% when compared to the control (p <0.001) and the bath (p <0.001). The 80% amplitude setting resulted in a 1.87-fold decrease in TRS concentration than the control and a 1.72-fold higher TRS concentration than the bath.

Lastly, for the 20-min treatments, there was a significant difference in TRS content after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 2.04-fold decrease in TRS concentration than the control and a 4.46-fold higher TRS concentration than the bath. There was a significant difference in TRS content after using the control when compared to the bath (p <0.01), which resulted in a 0.46-fold decrease in TRS concentration when using the bath.

Table 3.25 Statistical analysis to compare how different treatments performed when set at the same duration. TRS concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	72.65 ± 8.30^{b}	88.32 ± 6.63^{ab}	112.22 ± 10.20^{a}	66.41 ± 15.37^{b}
2	83.31 ± 5.18^{b}	n/a	n/a	149.56 ± 11.38^{a}
5	78.19 ± 10.97^{b}	95.51 ± 5.88^{b}	146.47 ± 10.99^{a}	164.31 ± 4.43^{a}
20	103.37 ± 7.09^{b}	47.20 ± 9.50^{c}	210.57 ± 14.27^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.26). One can conclude that there is a significantly higher amount of TRS released in the supernatants after the control for 20 min, when compared to the 1-min (p <0.01) duration of this same treatment. The 20-min treatment resulted in a 1.42-fold higher TRS concentration than the 1-min.

There is a significantly lower amount of TRS present in the supernatants after treatment with bath for 20 min, when compared to 1-min (p <0.01) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 0.53-fold lower TRS concentration than the 1-min treatment and a 0.49-fold lower TRS concentration than the 5-min treatment.

There is a significantly higher amount of TRS present in the supernatants after treatment with UP400St at 40% for 20 min, when compared to 1-min (p <0.001) and 5-min (p <0.01) durations of the same treatment. The 20-min treatment resulted in a 1.88-fold higher TRS concentration than the 1-min treatment and a 1.44-fold higher TRS concentration than the 5-min treatment.

Table 3.26 Statistical analysis to compare how the same treatment performed with different durations. TRS concentration in µg/mL from *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	72.65 ± 8.30^{b}	88.32 ± 6.63^{a}	112.22 ± 10.20^{b}	66.41 ± 15.37^{a}
2	83.31 ± 5.18^{ab}	n/a	n/a	149.56 ± 11.38^{a}
5	78.19 ± 10.97^{ab}	95.51 ± 5.88^{a}	146.47 ± 10.99^{b}	164.31 ± 4.43^{a}
20	103.37 ± 7.09^{a}	47.20 ± 9.50^{b}	210.57 ± 14.27^{a}	n/a

Chlorella vulgaris

The general trend is that, compared to the controls, after treatments with UP400St at 40% and UP400St at 80%, more TRS is being released with increasing durations, however, after treatment with the bath the TRS concentration is seen to decrease with longer durations of treatment (Fig. 3.19).

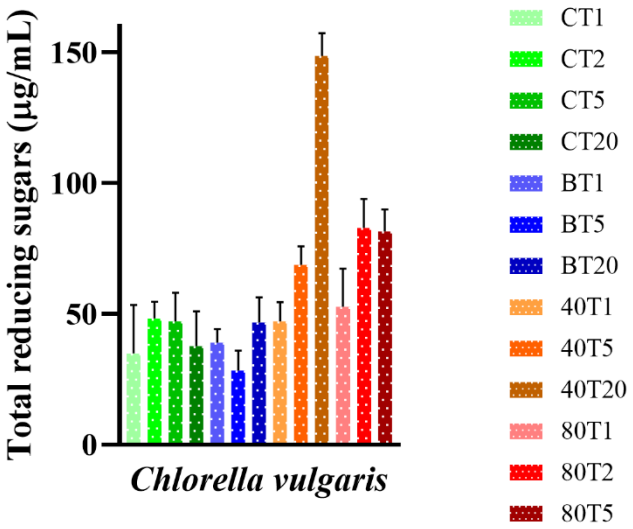

Fig. 3.19 TRS released in µg/mL from *Chlorella vulgaris* after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.27). For the 2-min treatments, there was a significant difference in TRS released after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 1.72-fold higher TRS concentration than the control.

With the 5-min treatments, significant differences resulted between the TRS content after using the UP400St set at 80% when compared to the control (p <0.01) and bath sonicator (p <0.001). The 80% amplitude setting resulted in a 1.73-fold higher TRS concentration than the control and a 2.88-fold higher TRS concentration than the bath. There was also a significant difference in the values of the bath treatment when compared to the UP400St set at 40% (p <0.01) treatments. The UP400St set at 40% resulted in a 2.43-fold increase in TRS concentration compared to the bath.

Lastly, the UP400St set at 40% 20-min treatments were found to have significant differences with the control (p <0.001) and the bath (p <0.001) treatments set at the same time. The control resulted in a 3.94-fold higher TRS concentration than the control, while the bath resulted in a 3.18-fold higher TRS concentration than the bath.

Table 3.27 Statistical analysis to compare how different treatments performed when set at the same duration. TRS concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	34.82 ± 18.63^{a}	38.96 ± 5.25^{a}	47.13 ± 7.28^{a}	52.73 ± 14.58^{a}
2	48.15 ± 6.48^{b}	n/a	n/a	82.92 ± 10.97^{a}
5	47.09 ± 10.98^{bc}	28.31 ± 7.68^{b}	68.71 ± 7.03^{ac}	81.58 ± 8.36^{a}
20	37.70 ± 13.31^{b}	46.78 ± 9.53^{b}	148.59 ± 8.73^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.28). One can conclude that there is a significantly higher amount of TRS present in the supernatants after the UP400St for 20 min, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment.

The 20-min treatment resulted in a 3.15-fold higher TRS concentration than the 1-min treatment and a 2.16-fold higher TRS concentration than the 5-min treatment.

Table 3.28 Statistical analysis to compare how the same treatment performed with different durations. TRS concentration in µg/mL from *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly ($p < 0.01$).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	34.82 ± 18.63^{a}	38.96 ± 5.25^{a}	47.13 ± 7.28^{b}	52.73 ± 14.58^{a}
2	48.15 ± 6.48^{a}	n/a	n/a	82.92 ± 10.97^{a}
5	47.09 ± 10.98^{a}	28.31 ± 7.68^{a}	68.71 ± 7.03^{b}	81.58 ± 8.36^{a}
20	37.70 ± 13.31^{a}	46.78 ± 9.53^{a}	148.59 ± 8.73^{a}	n/a

Lemna minor (type 1)

No trend was present for the TRS release, and minimal extraction took place after using the different treatments, however, it is worth nothing that the bath treatments results in a decrease in TRS concentration with longer durations (Fig. 3.20).

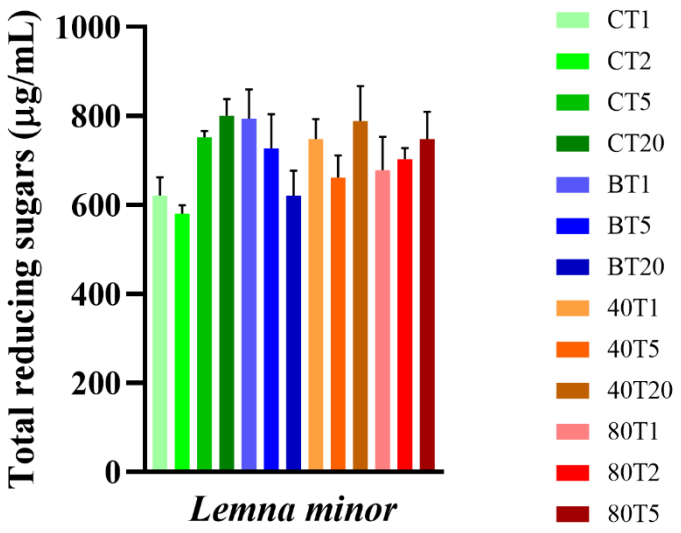

Fig. 3.20 TRS concentration in µg/mL from *Lemna minor* (type 1) after treatment with bath and probe sonicators at different settings and durations. Data are presented as mean (STD). The error bars represent mean +/- SD. Three separate trials were performed for all the data.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.29). For the 2-min treatments, it was concluded that there was a significant difference in TRS concentration after using the UP400St set at 80% when compared to the control (p <0.01). The 80% amplitude setting resulted in a 1.21-fold higher TRS concentration than the control.

Table 3.29 Statistical analysis to compare how different treatments performed when set at the same duration. TRS concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	621.31 ± 40.85^{a}	793.57 ± 66.25^{a}	747.52 ± 45.76^{a}	677.85 ± 75.35^{a}
2	580.56 ± 19.27^{b}	n/a	n/a	702.80 ± 25.18^{a}
5	751.72 ± 14.38^{a}	726.77 ± 77.13^{a}	660.95 ± 50.46^{a}	748.24 ± 61.30^{a}
20	800.76 ± 37.37^{a}	621.16 ± 55.73^{a}	788.97 ± 78.46^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.30). One can conclude that there is a significantly higher amount of TRS present in the supernatants after the 20-min control, when compared to 1-min (p <0.001) and 2-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 1.29-fold higher TRS concentration than the 1-min treatment and a 1.38-fold higher TRS concentration than the 2-min treatment. Similarly, there was a significantly higher amount of TRS present in the supernatants after the 5-min control, when compared to 1-min (p <0.01) and 2-min (p <0.001) durations of the same treatment. The 5-min treatment resulted in a 1.21-fold higher TRS concentration than the 1-min treatment and a 1.29-fold higher TRS concentration than the 2-min treatment.

Table 3.30 Statistical analysis to compare how the same treatment performed with different durations. TRS concentration in µg/mL from *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	621.31 ± 40.85^{b}	793.57 ± 66.25^{a}	747.52 ± 45.76^{a}	677.85 ± 75.35^{a}
2	580.56 ± 19.27^{b}	n/a	n/a	702.80 ± 25.18^{a}
5	751.72 ± 14.38^{a}	726.77 ± 77.13^{a}	660.95 ± 50.46^{a}	748.24 ± 61.30^{a}
20	800.76 ± 37.37^{a}	621.16 ± 55.73^{a}	788.97 ± 78.46^{a}	n/a

Alaria esculenta and *Chlorella vulgaris* followed the same trend. Supernatants had slightly more TRS release after treatment with UP400St at 40% and 80%, however, after treatment with the bath sonicator the TRS concentration was seen to decrease compared to the control samples. *Lemna minor* showed no particular trend but also experienced a decrease in TRS release while using the bath sonicator, when compared to the controls. It is worth noting that *Lemna minor* extracted the highest quantity of TRS followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

3.2.2.3 Chlorophyll spectra

A spectrophotometer was used to measure the absorbance of all supernatants at wavelengths between 750 to 550 nm. The chlorophyll spectra served as estimates of the

degree chlorophyll release after applying the different treatments. The applied experiment clearly shows how the different treatments had different effects on the three biomasses. The longest duration of each treatment was used to create the following graphs, to depict the trend in all biomasses more vividly.

Alaria esculenta

Supernatants of *Alaria esculenta* (type 1) treated with UP400St set at 40% and 80% showed a very minute peak at the 670 nm region (Fig. 3.21). However, no peak was seen after the control and bath treatments. Thus, chlorophyll was only extracted when the probe sonicator was used.

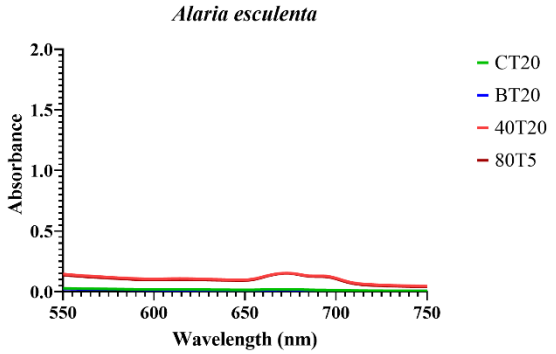

Fig. 3.21 Absorbance spectra from *Alaria esculenta* (type 1) after treatment with bath and probe sonicators at different settings and durations.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.31). For the 1-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.01) and the bath (p <0.01). The 40% amplitude setting resulted in a 4.69-fold higher chlorophyll content than the control and a 2.83-fold higher chlorophyll content than the bath. Similarly, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001) and the bath (p <0.001). The 80% amplitude setting resulted in a 10.67-fold higher chlorophyll content than the control and a 6.43-fold higher chlorophyll content than the bath. There was also a significant increase (p <0.001) in chlorophyll content

after using the UP400St at 80% as compared to when it was set at 40%, resulting in a 2.27-fold increase.

For the 2-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001), resulting in a 10.13-fold higher chlorophyll content than the control.

For the 5-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 7.50-fold higher chlorophyll content than the control and a 4.06-fold higher chlorophyll content than the bath. Similarly, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001) and the bath (p <0.001). The 80% amplitude setting resulted in a 10.80-fold higher chlorophyll content than the control and a 5.85-fold higher chlorophyll content than the bath. There was also a significant increase (p <0.001) in chlorophyll content after using the UP400St at 80% as compared to when it was set at 40%, resulting in a 1.44-fold increase. Furthermore, there was a significant increase (p <0.01) in chlorophyll content after using the bath as compared to the control, resulting in a 1.85-fold increase.

For the 20-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 7.24-fold higher chlorophyll content than the control and a 9.54-fold higher chlorophyll content than the bath.

Table 3.31 Statistical analysis to compare how different treatments performed when set at the same duration. Chlorophyll absorbance at 760 nm from supernatants of *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.01283 ± 0.00^{c}	0.02130 ± 0.00^{c}	0.06020 ± 0.00^{b}	0.13693 ± 0.02^{a}
2	0.01443 ± 0.00^{b}	n/a	n/a	0.14620 ± 0.02^{a}
5	0.01390 ± 0.00^{d}	0.02567 ± 0.00^{c}	0.10423 ± 0.00^{b}	0.15010 ± 0.00^{a}
20	0.02017 ± 0.00^{b}	0.01530 ± 0.00^{b}	0.14603 ± 0.01^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.32). One can conclude that there is a significantly higher amount of chlorophyll release in the supernatants after the 20-min control, when compared to 1-min (p <0.001), 2-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 1.57-fold higher chlorophyll content than the 1-min treatment, a 1.40-fold higher chlorophyll content than the 2-min treatment and a 1.45-fold higher chlorophyll content than the 5-min treatment.

Interestingly, there is a significantly lower amount of chlorophyll release in the supernatants after the 20-min bath sonicator treatment, when compared to 1-min (p <0.01) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 0.72-fold decrease in chlorophyll content than the 1-min treatment and a 0.60-fold decrease in chlorophyll content than the 5-min treatment.

There is a significantly higher amount of chlorophyll release in the supernatants after the 20-min treatments using UP400St set at 40%, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 2.43-fold higher chlorophyll content than the 1-min treatment and a 1.40-fold higher chlorophyll content than the 5-min treatment.

Table 3.32 Statistical analysis to compare how the same treatment performed with different durations. Chlorophyll absorbance at 760 nm from supernatants of *Alaria esculenta* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.01283 ± 0.00^{b}	0.02130 ± 0.00^{b}	0.06020 ± 0.00^{c}	0.13693 ± 0.02^{a}
2	0.01443 ± 0.00^{b}	n/a	n/a	0.14620 ± 0.02^{a}
5	0.01390 ± 0.00^{b}	0.02567 ± 0.00^{b}	0.10423 ± 0.00^{b}	0.15010 ± 0.00^{a}
20	0.02017 ± 0.00^{a}	0.01530 ± 0.00^{a}	0.14603 ± 0.01^{a}	n/a

Chlorella vulgaris

Supernatants of *Chlorella vulgaris* treated with UP400St set at 40% and 80% showed a peak at around 670 nm (Fig. 3.22). The bath treatment also resulted in a small peak at the same absorbance region, while the control treatment seemed to release no chlorophyll.

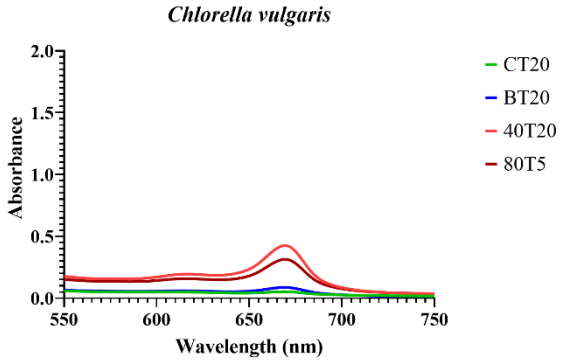

Fig. 3.22 Absorbance spectra from *Chlorella vulgaris* after treatment with bath and probe sonicators at different settings and durations.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.33). For the 2-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when

compared to the control (p <0.001), resulting in a 3.82-fold higher chlorophyll content than the control.

For the 5-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001), the bath (p <0.001) and the UP400St set at 40% (p <0.001). The 80% amplitude setting resulted in a 5.26-fold higher chlorophyll content than the control, a 3.07-fold higher chlorophyll content than the bath and a 2.36-fold higher chlorophyll content that the UP400St set at 40%.

For the 20-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 7.43-fold higher chlorophyll content than the control and a 4.49-fold higher chlorophyll content than the bath.

Table 3.33 Statistical analysis to compare how different treatments performed when set at the same duration. Chlorophyll absorbance at 760 nm from supernatants of *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.04803 ± 0.00^{a}	0.14120 ± 0.10^{a}	0.07770 ± 0.00^{a}	0.14717 ± 0.00^{a}
2	0.04140 ± 0.01^{b}	n/a	n/a	0.15797 ± 0.01^{a}
5	0.05887 ± 0.01^{b}	0.10093 ± 0.05^{b}	0.13107 ± 0.00^{b}	0.30977 ± 0.02^{a}
20	0.05647 ± 0.01^{b}	0.09350 ± 0.03^{b}	0.41963 ± 0.00^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.34). There is a significantly higher amount of chlorophyll release in the supernatants after the 20-min treatments using UP400St set at 40%, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 5.40-fold higher chlorophyll content than the 1-min treatment and a 3.20-fold higher chlorophyll content than the 5-min treatment. Additionally, there is a significantly higher amount of chlorophyll after the 5-min

treatment (p <0.001) compared to the 1-min treatment, resulting in a 1.69-fold increase of chlorophyll content.

There is a significantly higher amount of chlorophyll release in the supernatants after the 5-min treatments using UP400St set at 80%, when compared to 1-min (p <0.001) and 2-min (p <0.001) durations of the same treatment. The 5-min treatment resulted in a 2.10-fold higher chlorophyll content than the 1-min treatment and a 1.96-fold higher chlorophyll content than the 2-min treatment.

Table 3.34 Statistical analysis to compare how the same treatment performed with different durations. Chlorophyll absorbance at 760 nm from supernatants of *Chlorella vulgaris* after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.04803 ± 0.00^{a}	0.14120 ± 0.10^{a}	0.07770 ± 0.00^{c}	0.14717 ± 0.00^{b}
2	0.04140 ± 0.01^{a}	n/a	n/a	0.15797 ± 0.01^{b}
5	0.05887 ± 0.01^{a}	0.10093 ± 0.05^{a}	0.13107 ± 0.00^{b}	0.30977 ± 0.02^{a}
20	0.05647 ± 0.01^{a}	0.09350 ± 0.03^{a}	0.41963 ± 0.00^{a}	n/a

Lemna minor

Supernatants of *Lemna minor* (type 1) treated with UP400St set at 40% and 80% showed very distinct peaks at the 670 nm region (Fig. 3.23). The bath treatment and control also resulted in small peaks at the same absorbance.

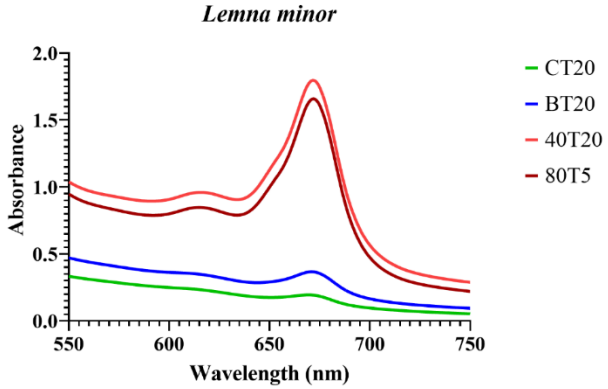

Fig. 3.23 Absorbance spectra from *Lemna minor* (type 1) after treatment with bath and probe sonicators at different settings and durations.

Statistical analysis was done to compare how different treatments performed when set at the same duration (Table 3.35). For the 1-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.001) and bath (p <0.001). The 40% amplitude setting resulted in a 2.15-fold higher chlorophyll content than the control and a 1.45-fold higher chlorophyll content than the bath. There was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001), the bath (p <0.001) and the UP400St set at 40% (p <0.001). The 80% amplitude setting resulted in a 4.48-fold higher chlorophyll content than the control, a 3.03-fold higher chlorophyll content than the bath and a 2.08-fold higher chlorophyll content that the UP400St set at 40%. Furthermore, there was a significant difference in the chlorophyll released after using the bath when compared to the control (p <0.01), resulting in a 1.48-fold higher chlorophyll content than the control.

For the 2-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001), resulting in a 5.35-fold higher chlorophyll content than the control.

For the 5-min treatments, there was a significant difference after using the UP400St set at 40% when compared to the control (p <0.001), resulting in a 3.52-fold higher chlorophyll content. There was a significant difference in the chlorophyll released after using the UP400St set at 80% when compared to the control (p <0.001), the bath (p <0.001) and the UP400St set at 40% (p <0.001). The 80% amplitude setting resulted in a 9.67-fold higher chlorophyll content than the control, a 5.74-fold higher chlorophyll content than the bath and a 2.47-fold higher chlorophyll content that the UP400St set at 40%.

For the 20-min treatments, there was a significant difference in the chlorophyll released after using the UP400St set at 40% when compared to the control (p <0.001) and the bath (p <0.001). The 40% amplitude setting resulted in a 9.06-fold higher chlorophyll content than the control and a 4.82-fold higher chlorophyll content than the bath.

Table 3.35 Statistical analysis to compare how different treatments performed when set at the same duration. Chlorophyll absorbance at 760 nm from supernatants of *Lemna minor* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a row, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.17273 ± 0.00^{d}	0.25593 ± 0.00^{c}	0.37157 ± 0.04^{b}	0.77443 ± 0.01^{a}
2	0.20183 ± 0.02^{b}	n/a	n/a	1.08027 ± 0.06^{a}
5	0.17533 ± 0.00^{c}	0.29510 ± 0.03^{b}	0.61773 ± 0.07^{b}	1.69497 ± 0.23^{a}
20	0.19597 ± 0.00^{b}	0.36807 ± 0.10^{b}	1.77530 ± 0.20^{a}	n/a

Statistical analysis was performed to determine how the same treatment performed with different durations (Table 3.36). There is a significantly higher amount of chlorophyll release in the supernatants after the 20-min treatments using UP400St set at 40%, when compared to 1-min (p <0.001) and 5-min (p <0.001) durations of the same treatment. The 20-min treatment resulted in a 4.78-fold higher chlorophyll content than the 1-min treatment and a 2.87-fold higher chlorophyll content than the 5-min treatment.

There is a significantly higher amount of chlorophyll release in the supernatants after the 5-min treatments using UP400St set at 80%, when compared to 1-min (p <0.001) and 2-min (p <0.01) durations of the same treatment. The 5-min treatment resulted in a 2.19-fold higher chlorophyll content than the 1-min treatment and a 1.57-fold higher chlorophyll content than the 2-min treatment.

Table 3.36 Statistical analysis to compare how the same treatment performed with different durations. Chlorophyll absorbance at 760 nm from supernatants of *Chlorella vulgaris* (type 1) after treatment with control, bath and probe sonicators at different settings and durations. Data are presented as mean (STD), +/- SD. Three separate trials were performed for all the data. Within a column, means without the same superscript letter differ significantly (p <0.01).

Time of treatment (min)	Control	Bath	Probe 40% amp.	Probe 80% amp.
1	0.17273 ± 0.00^{a}	0.25593 ± 0.00^{a}	0.37157 ± 0.04^{b}	0.77443 ± 0.01^{b}
2	0.20183 ± 0.02^{a}	n/a	n/a	1.08027 ± 0.06^{b}
5	0.17533 ± 0.00^{a}	0.29510 ± 0.03^{a}	0.61773 ± 0.07^{b}	1.69497 ± 0.23^{a}
20	0.19597 ± 0.00^{a}	0.36807 ± 0.10^{a}	1.77530 ± 0.20^{a}	n/a

It is evident that more chlorophyll was released with longer durations of treatment. The UP400St set at 80% gave the highest chlorophyll content, followed by the UP400St set at 40% and the bath sonicator. However, both *Alaria esculenta* and *Chlorella vulgaris* experienced a dip in chlorophyll concentration when using the bath as compared to the controls. The bath sonicator seems to inhibit chlorophyll extraction in these two biomasses.

It is worth noting that *Lemna minor* resulted in the most chlorophyll extraction, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively. This is in line with the results in section 3.2.2.2. Hence, it can be concluded that *Lemna minor* has the highest concentration of these substances and is possibly the most porous in structure, thus releases the compounds more readily. On the other hand, *Alaria esculenta* must have a very tough and rigid structure that is not easily broken-down.

3.2.2.4 SEM

Samples of the control treatment for 20 min and the probe sonicator at 80% amplitude for 5 min of all 3 biomasses were fixed and gold coated. Each sample was imaged at 1, 5, 10, 20 and 50 K magnifications.

Fig. 3.24, 3.25 and 3.26 below show quite clearly that the UP400St probe sonicator set at an amplitude of 80% (top rows) causes surface roughness on the biomass when compared to the control samples (bottom rows). It is also worth mentioning that small perforations in the structure can be seen after treatment with the probe, indicating that mechanical break-down occurred.

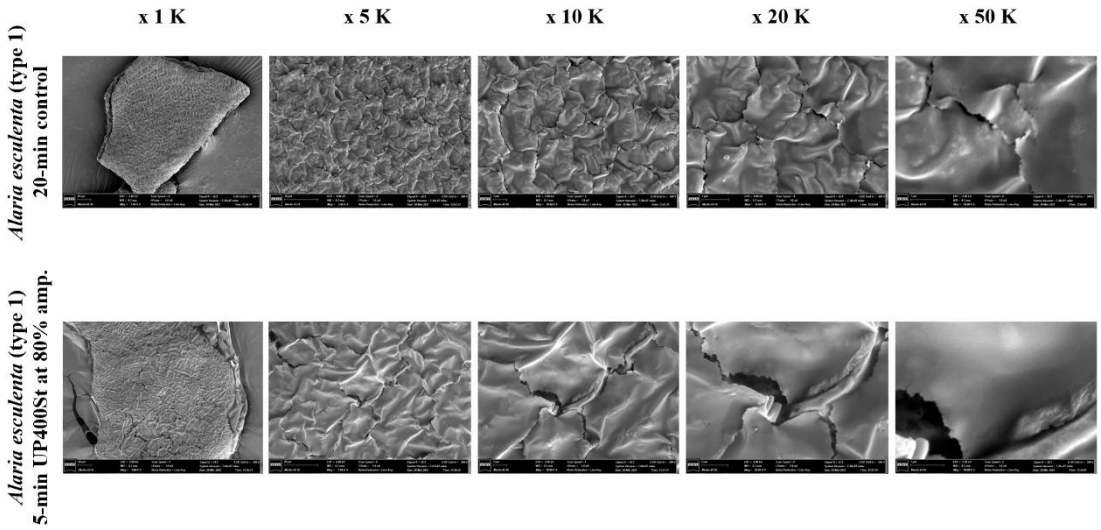

Fig. 3.24 SEM images for *Alaria esculenta* (type 1), taken at magnification of 1, 5, 10, 20 and 50 K, respectively.

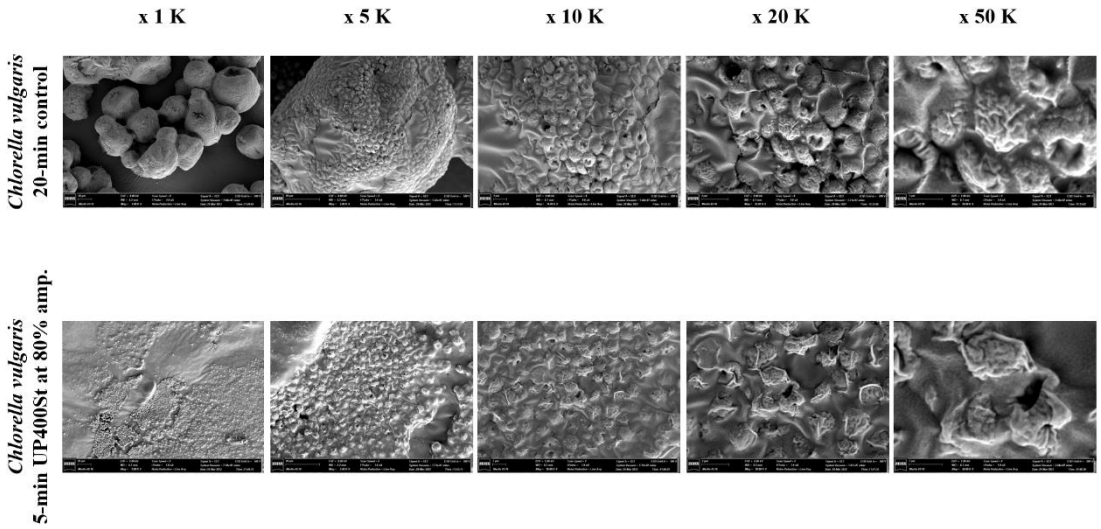

Fig. 3.25 SEM images for *Chlorella vulgaris*, taken at magnification of 1, 5, 10, 20 and 50 K, respectively.

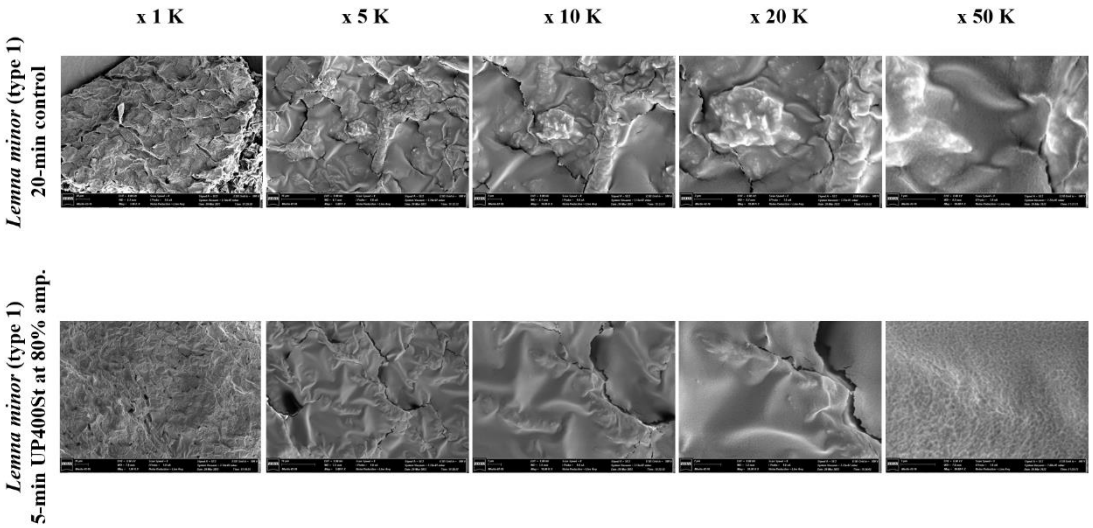

Fig. 3.26 SEM images for *Lemna minor* (type 1), taken at magnification of 1, 5, 10, 20 and 50 K, respectively.

3.3 Impact of assisted ultrasound on biomass treatments

The objective for this particular experiment was to determine if ultrasound facilitates the extraction process for two different biomasses. Moreover, it served to establish whether using ultrasound with enzymes or ultrasound with alkali give better results than the conventional method for protein extraction that is currently being used in the food industry. The treatments were summarized in Table 3.37.

Table 3.37 Summary of all the treatments used to assess the impacts of assisted ultrasound. Applied treatments for protein extraction using ultrasound as a pre-treatment for enzyme-assisted extraction (1-14); using the conventional method used in the food industry (15); and using alkali extraction as a pre-treatment for ultrasound-assisted extraction (16-17).

Summary (the same for both *Alaria esculenta* and *Lemna minor*)		
No.	**Treatments**	**Conditions**
1	Ultrasound probe pre-treatment + enzymes	50% amplitude, 10 min + 6-hour incubation
2	Ultrasound probe pre-treatment + enzymes	100% amplitude, 10 min + 6-hour incubation
3	Ultrasound probe pre-treatment + enzymes	50% amplitude, 10 min + 18-hour incubation
4	Ultrasound probe pre-treatment + enzymes	100% amplitude 10 min + 18-hour incubation
5	Ultrasound probe only	50% amplitude, 10 min + 6-hour incubation
6	Ultrasound probe only	100% amplitude, 10 min + 6-hour incubation
7	Ultrasound probe only	50% amplitude, 10 min + 18-hour incubation

8	Ultrasound probe only	100% amplitude 10 min + 18-hour incubation
9	Enzymes only	No US, 10 min + 6-hour incubation
10	Enzymes only	No US, 10 min + 18-hour incubation
11	Buffer only	No US, 10 min + 6-hour incubation
12	Buffer only	No US, 10 min + 18-hour incubation
13	Water only	No US, 10 min + 6-hour incubation
14	Water only	No US, 10 min + 18-hour incubation
15	Water only	24-hour incubation
16	Alkali + ultrasound probe pre-treatment	100% amplitude 30 min
17	Alkali only	6-hour incubation

3.3.1 Nitrogen content

Protein quantification was done by using the LECO FP628 protein analyser, which is based on the Dumas method. The nitrogen to protein conversion factor used for *Alaria esculenta* was 5, while for *Lemna minor* it was 6.25. The following sections demonstrate the protein extracted results for the supernatants and pellets of *Alaria esculenta* (type 2), and *Lemna minor* (type 2), respectively; after each of the different treatments. *Lemna minor* was found to have the greatest protein content in the supernatants, followed by *Alaria esculenta*.

3.3.1.1 Protein extraction using ultrasound as a pre-treatment for enzyme-assisted extraction

Enzyme extraction

The graph in Fig. 3.27 depicts how treatment with enzymes only gave approximately the same percentage yield of protein as the water only and buffer only controls for *Alaria esculenta* samples. It is also worth noting that increasing the duration does not really help in increasing the protein yield.

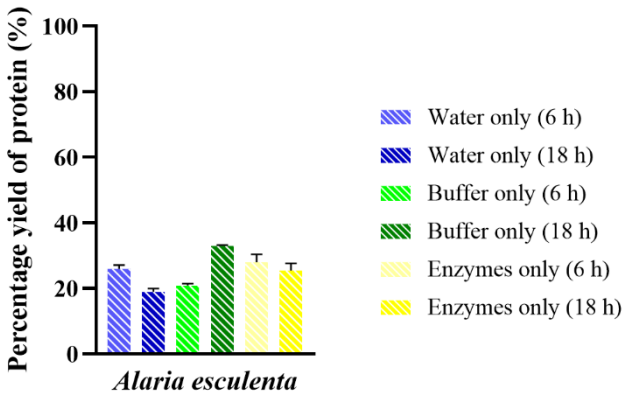

Fig. 3.27 Percentage yield of protein (%) from *Alaria esculenta* (type 2) after treatment with enzymes at different durations, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

The graph in Fig. 3.28 shows how treatment with enzymes only fared worse than the water only and buffer only controls for *Lemna minor* samples. However, in this case increasing the duration led to an increase in the protein yield for all the three treatments. It is also interesting to note that *Lemna minor* extracted around double the amount of protein when compared with *Alaria esculenta*.

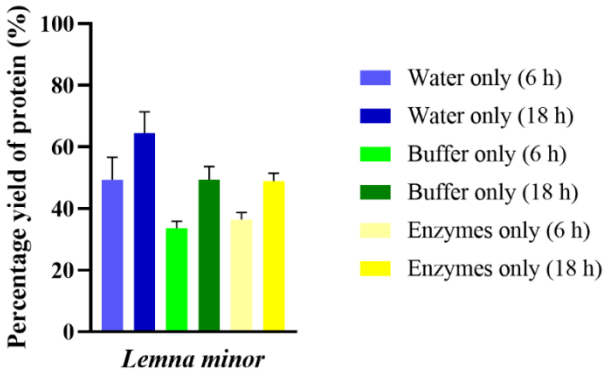

Fig. 3.28 Percentage yield of protein (%) from *Lemna minor* (type 2) after treatment with enzymes at different durations, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

US extraction

The graph in Fig. 3.29 depicts how treatment with different US settings gave a slightly higher percentage yield of protein than the water only and buffer only controls for *Alaria esculenta* samples. Furthermore, the US treatment at 100% amplitude released more protein when left for 18 hours as compared to the same treatment left for 6 hours.

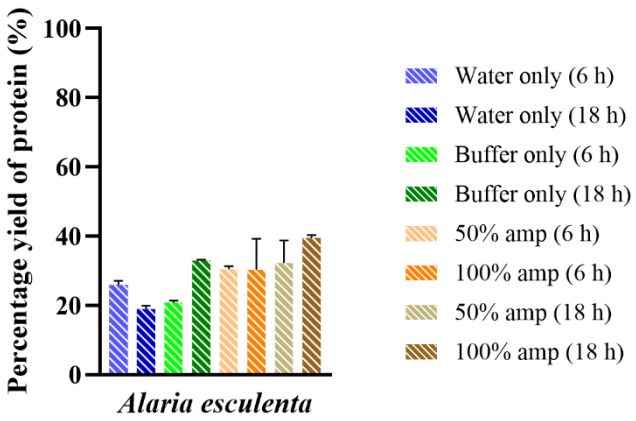

Fig. 3.29 Percentage yield of protein (%) from *Alaria esculenta* (type 2) after treatment with US at different amplitudes and durations, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

The graph in Fig. 3.30 shows that for *Lemna minor*, treatment with different US settings gave a slightly higher percentage yield of protein than the buffer only controls. However, the water control seemed to release more protein than the ultrasonic treatments. It is also worth noting that increasing the duration does not really help in increasing the protein yield. Interestingly, the extraction yield is about three times greater for *Lemna minor* than the *Alaria esculenta* samples.

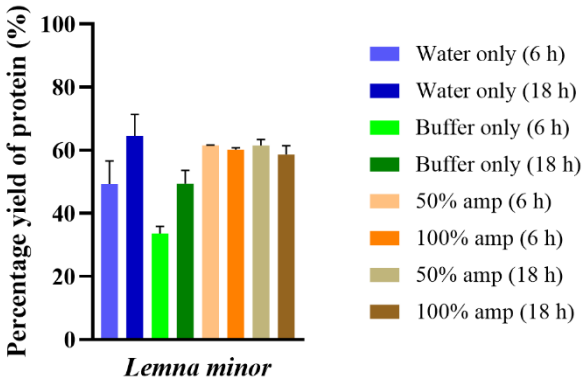

Fig. 3.30 Percentage yield of protein (%) from *Lemna minor* (type 2) after treatment with US at different amplitudes and durations, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

US pre-treatment for enzyme extraction

The graph in Fig. 3.31 depicts how treatment with different US settings coupled with enzymes gave a higher percentage yield of protein than the water only and buffer only controls for *Alaria esculenta* samples. Furthermore, the US treatments at 50% and 100% amplitude released much more protein when left for 18 hours as compared to the same treatments left for 6 hours.

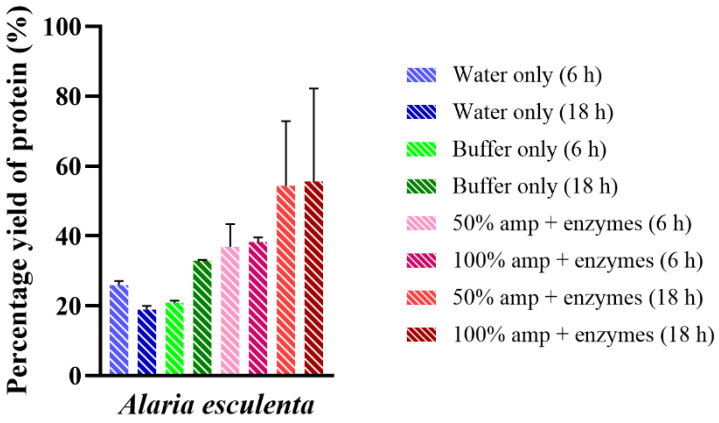

Fig. 3.31 Percentage yield of protein (%) from *Alaria esculenta* (type 2) after treatment with US at different amplitudes and durations coupled with enzymes, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

The graph in Fig. 3.32 shows that for *Lemna minor*, treatment with different US settings and enzymes gave a lower percentage yield of protein than the water only controls. Strangely, the water only control, left for 18 hours, seemed to release a lot more protein than the ultrasonic treatments. It is also worth noting that increasing the duration does not really help in increasing the protein yield. Interestingly, the extraction yield is about three times greater for *Lemna minor* than the *Alaria esculenta* samples with the water only treatment, but similar with the buffer and ultrasonic treatments with enzymes of 6-hour incubations. One the other hand, *Lemna minor* released far less protein than *Alaria esculenta* for the ultrasonic treatments with enzymes of 18-hour incubations.

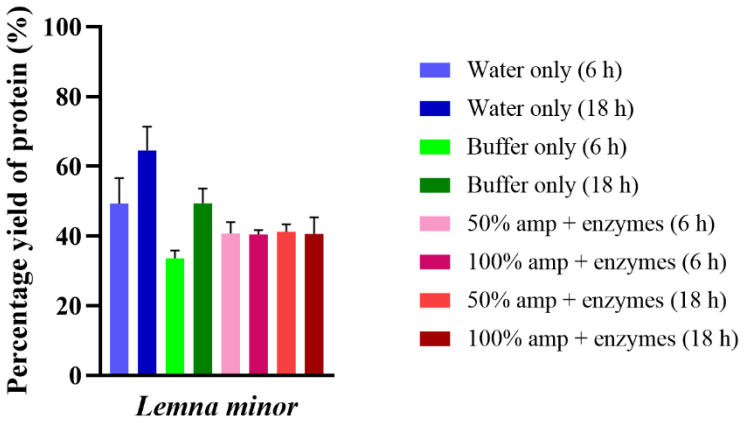

Fig. 3.32 Percentage yield of protein (%) from *Lemna minor* (type 2) after treatment with US at different amplitudes and durations coupled with enzymes, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

3.3.1.2 Protein extraction using the conventional method used in the food industry

Conventional extraction

The graph in Fig. 3.33 depicts how the conventional extraction method gave the same percentage yield of protein as the water only and buffer only controls for *Alaria esculenta* samples, regardless of the duration. This percentage is far less than the extraction yield achieved from US only and US coupled with enzymes treatments.

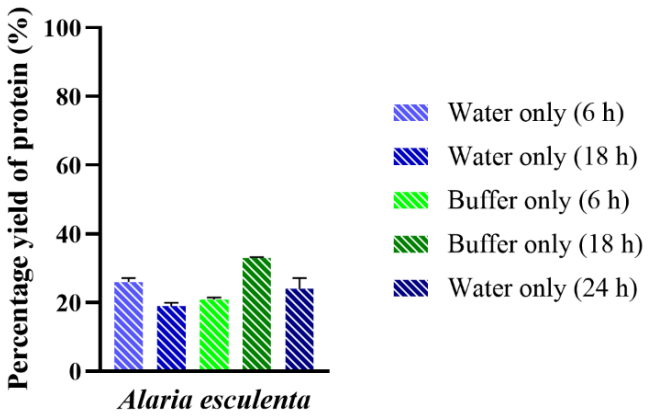

Fig. 3.33 Percentage yield of protein (%) from *Alaria esculenta* (type 2) after treatment with the conventional method, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

Similarly, the graph in Fig. 3.34 depicts how the conventional extraction method did not give a higher percentage yield of protein, when compared to the water only and buffer only controls for *Lemna minor* samples. This percentage is slightly less than the extraction yield achieved from US only and US coupled with enzymes treatments.

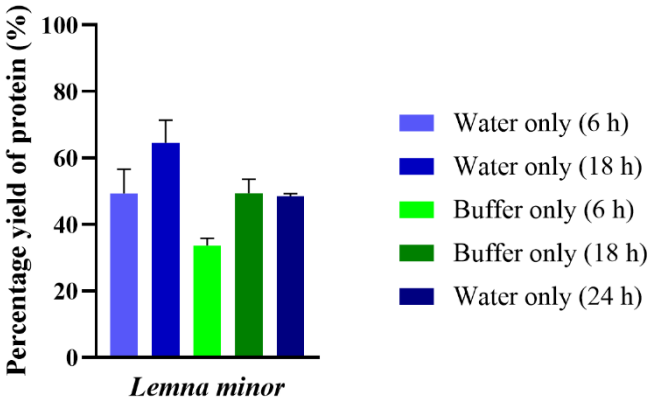

Fig. 3.34 Percentage yield of protein (%) from *Lemna minor* (type 2) after treatment with the conventional method, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

3.3.1.3 Protein extraction using alkali extraction as a pre-treatment for ultrasound-assisted extraction

Alkali extraction

The graph in Fig. 3.35 depicts how the alkali extraction method gave the same percentage yield of protein as the water only and buffer only controls for *Alaria esculenta* samples, regardless of the duration. This percentage is far less than the extraction yield achieved from US only and US coupled with enzymes treatments.

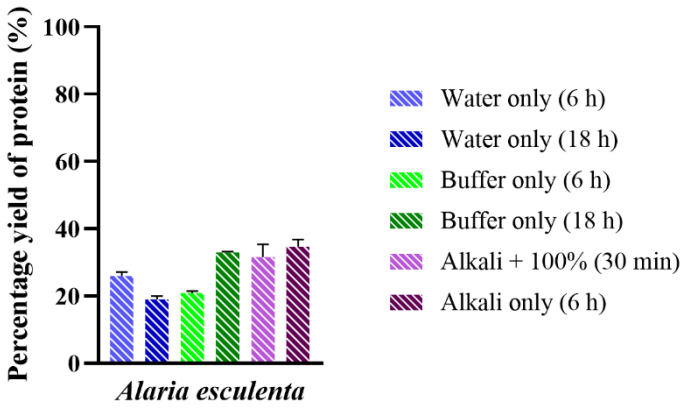

Fig. 3.35 Percentage yield of protein (%) from *Alaria esculenta* (type 2) after treatment with the alkali extraction method, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

Similarly, the graph in Fig. 3.36 depicts how the conventional extraction method did not give a higher percentage yield of protein, when compared to the water only and buffer only controls for *Lemna minor* samples. This percentage is slightly less than the extraction yield achieved from US only and US coupled with enzymes treatments.

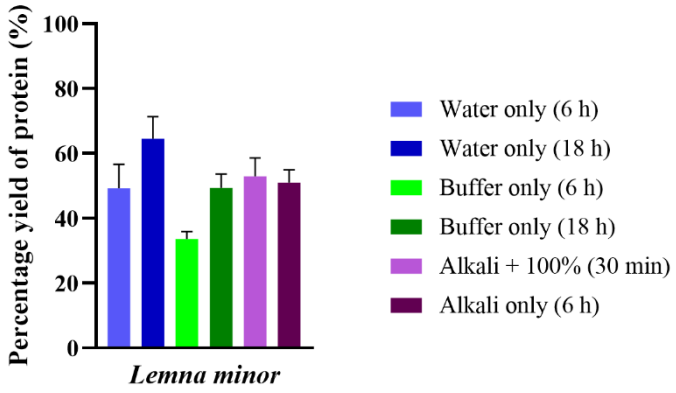

Fig. 3.36 Percentage yield of protein (%) from *Lemna minor* (type 2) after treatment with the alkali extraction method, as compared to water and buffer controls. Data are presented as mean (STD). The error bars represent mean +/- SD. Two separate trials were performed for all the data.

Overall, the enzyme treatments alone had no impact on the extraction. *Alaria esculenta* had no noticeable increase in percentage yield overall and longer durations had no favourable effect. *Lemna minor* experienced a decrease in percentage yield, however, it was noted that longer durations gave more favourable outcomes.

The ultrasound treatments alone did result in increased protein extraction. *Alaria esculenta* gave a higher yield than the controls and with longer durations of treatment, more extraction occurred. *Lemna minor* also gave a higher yield than the controls, however, longer durations had minimal benefits. For the combined ultrasound and enzyme treatments, *Alaria esculenta* had a noticeable increase in extraction yield which improved when using longer durations, while *Lemna minor* actually experienced a decrease in yield, and no improved effects with longer durations. Interestingly, the conventional method did not result in an increase in extraction for both biomasses. Hence, short durations should be considered as the degree of extraction will be the same. For the alkali extraction method, both biomasses had minimal changes to their extraction yield when compared to the controls. Increasing the duration will not increase the yield.

3.3.2 SEM

The following treatments of each biomass were imaged as they are representative of the remaining treatments: water only (6 h), buffer only (6 h), enzyme only (6 h), US 100% only (6 h) and US 100% + enzymes (6 h).

Fig. 3.37 and 3.38 show quite clearly that there is little change in the surface roughness of both biomasses when treated with the water, buffer and enzyme controls. However, noticeable perforations formed on the surface of the biomasses after treatment with US at 100% only and US at 100% with enzymes. This leads to the conclusion that the break-down of the biomasses occurred due to the ultrasonic treatments.

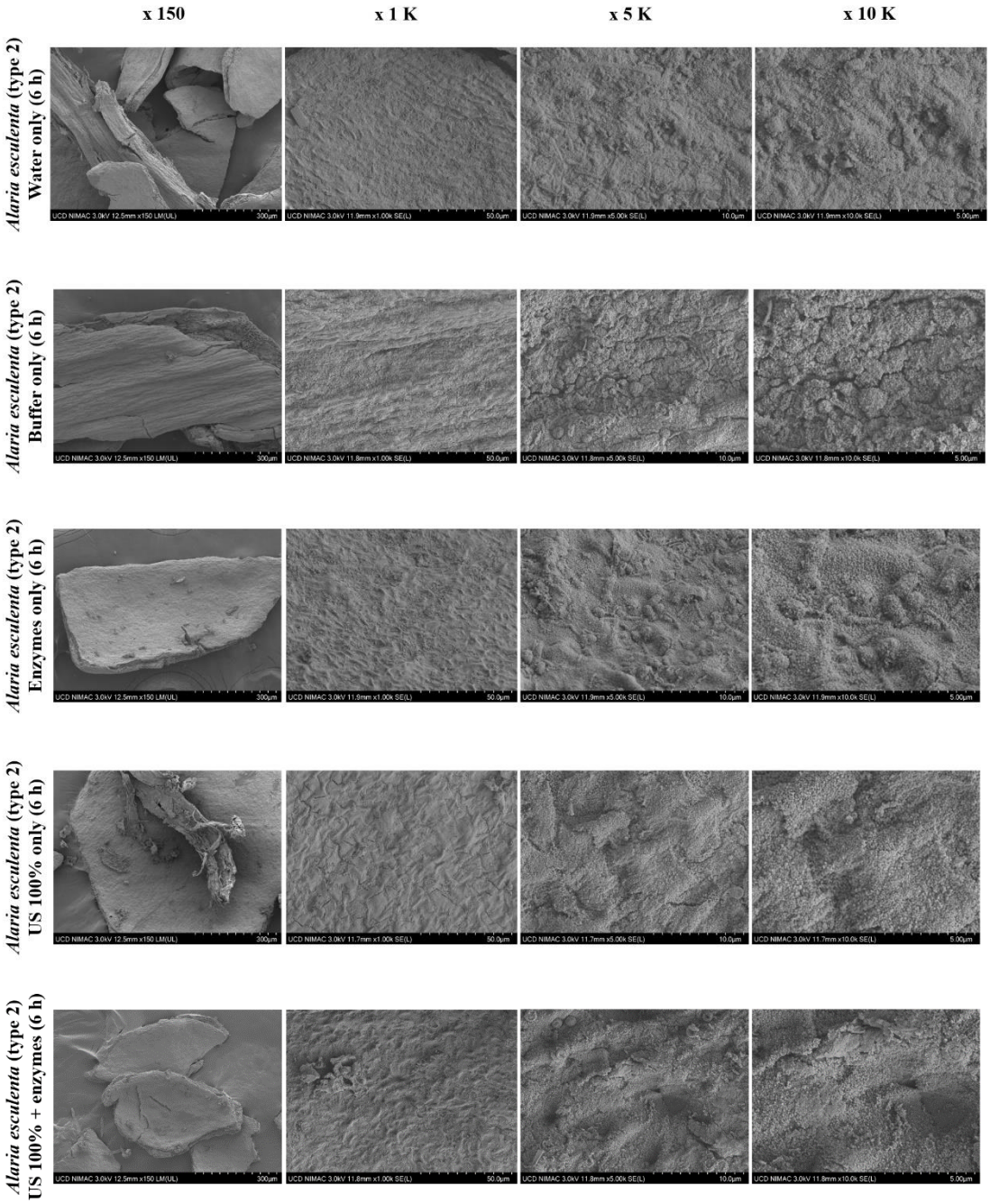

Fig. 3.37 SEM images for *Alaria esculenta* (type 2), taken at magnification of 150, 1 K, 5K and 10 K, respectively.

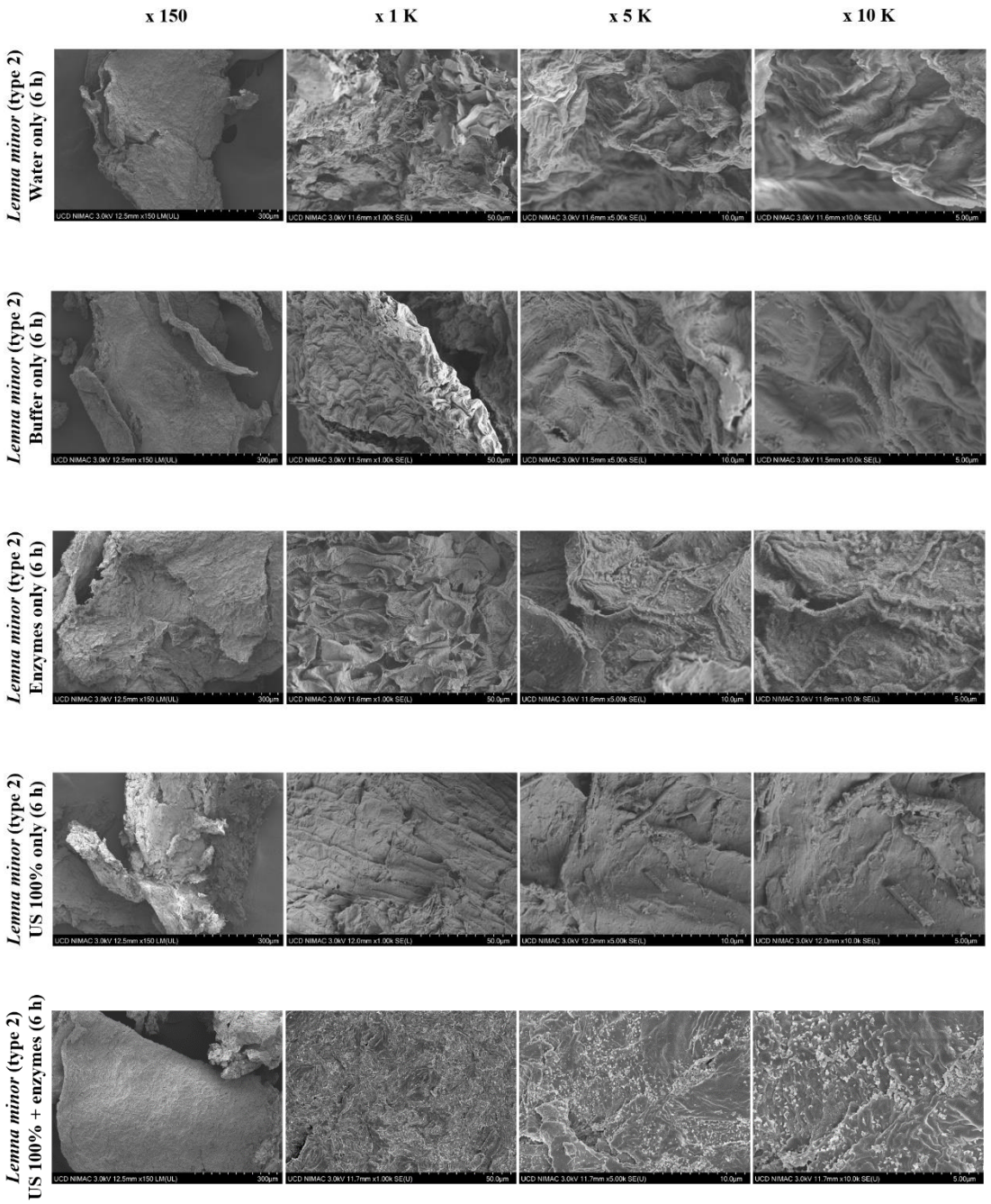

Fig. 3.38 SEM images for *Lemna minor* (type 2), taken at magnification of 150, 1 K, 5K and 10 K, respectively.

Chapter 4 Discussion

4.1 Characterisation of raw material

Characterisation of the raw biomasses was the initial set of experiments performed. Colorimetry measurements were taken to quantify the difference in the hues between each biomass. *Lemna minor* was found to be greener than *Alaria esculenta* and *Chlorella vulgaris*. If the biomasses were meant for human consumption sensory perception would be a priority to ensure marketability of the end-product (Hosseinizand et al., 2017).

Microscope images were taken to determine any obvious differences in the particles of each biomass. The particle size of *Alaria esculenta* was found to be the largest with the most sharp/rigid edges. This could be linked to the fact that *Alaria esculenta* showed the least extraction of compounds out of all biomasses, as there was less surface area for the sonication treatments to work on. On the other hand, *Lemna minor* particles had a fuzzy edge and looked frail, which might be the reason behind the high extraction rates of compounds from this biomass. Even though this was a qualitative test that could not give robust findings, it was valuable as there are no similar images documented in the literature.

Water activity and moisture content were quantified for all the dried biomasses. The results from these two tests can be used to make inferences on the stability and shelf-life of the dried biomass. Usually, an a_w value of 0.9 or lower indicates that the foodstuff is stable. All the biomasses had an a_w value lower than 0.7 so they can be considered as non-perishable (Dhaliwal et al., 2021). Similarly, a moisture content value of 10% or less is considered as safe (Hosseinizand et al., 2017). Only *Chlorella vulgaris* had a moisture content value of less than 10%, so care should be taken to dry the other biomasses better to ensure that they do not degrade in quality.

4.2 Impact of different ultrasound set-ups on biomass treatments

4.2.1 Ultrasound energetics

The next set of experiments dealt with assessing the impact of different ultrasound set-ups on biomass treatments. An extensive set of tests were performed to determine ultrasound energetics. It was determined that probe sonicators heat a sample quicker and

have more energy output than bath sonicators. Furthermore, probe sonicators set at higher amplitudes heat a sample quicker than lower amplitude settings. A very interesting finding was that different models of probe sonicators resulted in different energy outputs when set at the same amplitudes. This confirms the need to stipulate the acoustic energy density rather than the amplitude setting being used, to create more repeatable methodologies (Tiwari & Mason, 2012).

4.2.2 Ultrasound extraction

These tests were followed by a set of ultrasound extraction treatments comparing bath and probe sonicators. The supernatants were analysed using various colorimetric measures. For all the different compounds quantified for the degree of extraction, the *Lemna minor* samples extracted the highest yields, following *Chlorella vulgaris* and *Alaria esculenta*, respectively. The probe treatments always resulted in an increased extraction compared to the control. The bath treatments rarely caused an increase in extraction compared to the controls. Overall, the longer the duration of treatment the more extraction takes place. This concurs with what was reported in the literature, where it was stated that probe sonicators are more efficient than bath sonicators when it comes to extraction (Carreira-Casais et al., 2021). *Lemna minor* had a lot more amino acids being extracted than the other two biomass, but this was probably because it contains the highest quantities of this compound (Appenroth et al., 2018). The peroxide content was also quantified as the literature states that the sonication of water creates radicals and H_2O_2 (Duco et al., 2016). However, the results show that sonication had little effect on peroxide generation. This might be due to the peroxide being short-lived, hence the test was not able to detect changes quick enough, but it can be concluded that the peroxide which might be generated will not affect the biomass quality.

When it came to chlorophyll spectra, it was found that *Lemna minor* had the most chlorophyll extraction, followed by *Chlorella vulgaris* and *Alaria esculenta*. There is not enough data in the literature to confirm this, however, the trend of duckweed being the easiest to extract was seen throughout the study. Furthermore, it is evident that more chlorophyll was released with longer durations of ultrasound treatment. SEM images gave clear indications that the sonication treatments caused mechanical destruction of

all three biomasses. This is in line with what was reported in the literature for all three biomasses (Hildebrand et al., 2020b; Ummat et al., 2020; Yilmaz et al., 2022).

4.3 Impact of assisted ultrasound on biomass treatments

When the impact of assisted ultrasound on biomass treatments was assessed, the enzyme treatments alone did not result in an increase in protein yield for either of the biomasses. This was not expected as the same enzyme, cellulase, was found to extract proteins efficiently from *Macrocystis pyrifera* and *Chondracanthus chamissoi*, two types of seaweed (Vásquez et al., 2019). The possible reason for this is that cellulase is not as well-suited for the matrix of the three biomasses. The ultrasound treatments alone did result in increased protein extraction. This matches with what was reported in the literature for the same biomasses (Kopczyk, 2020; Krupka et al., 2021). For the combined ultrasound and enzyme treatments, a very noticeable increase in extraction yield resulted. This is very intriguing as such findings have not been documented for these particular biomasses as yet. As expected, longer durations of treatment gave a higher protein yield.

Interestingly, the conventional method did not result in an increase in extraction for either biomass. For the alkali extraction method, neither biomass had changes to their extraction yield when compared to the controls. Increasing the duration did not increase the yield. Several drawbacks for both of these methods have been published recently (Garcia-Vaquero et al., 2020). This study mentioned how time-consuming these two methods are, how wasteful and expensive they can be due to the large amounts of toxic organic solvents required, and also how hazardous the exposure to these chemicals can be to workers and consumers alike. Hence the adoption of ultrasound extraction, especially when coupled with enzymes should be considered by the industry.

SEM images of the biomasses before and after ultrasound-assisted enzyme extraction gave clear indications that the sonication treatments caused mechanical destruction of all three biomasses. These images can serve as a useful reference as so far, no similar results were published in the literature.

4.4 Comparing findings with recent literature

Ultrasound technology improves both the yield and the rate of extraction of compounds in solid-liquid extraction from cells (Vernès et al., 2019). Ultrasound is a form of green technology that has become of increasing interest in the food industry, due to promising findings related to increased extraction yields with minimal requirements of reagents. Bath sonication is reported to provide a weak sonication with non-uniform distribution whilst probe sonication is said to provide stronger sonication that is much more focused and uniform in distribution (Mellado et al., 2019).

During a study performed by Vernès et al. (2019), it was found that for spirulina (a microalgae), ultrasonic treatments caused the fragmentation of the filaments. It was concluded, that this increase in surface area increased the protein to solvent contact, which in turn amplified the extraction rate and yield (Vernès et al., 2019). A similar inference can be applied to the findings of the current research. The SEM images do suggest fragmentation of all the biomasses, and thus an increase in surface area.

Furthermore, a study carried out by Görgüç et al., (2019) found that combining ultrasound with the enzyme alcalase exhibited the highest protein recovery in sesame brans. Alcalase was found to be more effective than viscozyme, highlighting the importance of finding a highly specialized enzyme for each respective biomass (Görgüç et al., 2019). Cellulase was likely better suited for *Lemna minor* than it was for *Alaria esculenta*. Carrying out further tests using different types of enzymes would be ideal so as to determine which is the best suited for each biomass. Ultrasound treatments might also lead to improved digestibility of the biomass. It was found that plant-based proteins treated with ultrasound were more bioavailable and readily absorbed in the intestines (Rahman & Lamsal, 2021a).

In a study carried out by Sapatinha et al. (2022), it was found that the enzyme alcalase (a type of peptidase) had a significant enhancing effect on the extraction yield of compounds from macroalgae. Alcalase was found to be better suited than cellulase of viscozyme for assisted extraction. It is also worth pointing out that brown seaweeds (10.5–33.2%) were found to extract much fewer compounds than red seaweeds (24.6–63.3%) (Sapatinha et al., 2022).

Similarly, a study on *Chlorella vulgaris* performed by Hildebrand et al. (2020) found that using the enzyme lysozyme coupled with ultrasound, resulted in protein recoveries

ranging from 33 to 42%, while using proteases results in an enhanced recovery of proteins (58–82%). Furthermore, SEM imaging depicted significant changes in the morphology of the cell surface (Hildebrand et al., 2020a).

No research has been reported on the use of ultrasound and enzyme extraction of compounds from *Lemna minor*. However, with duckweed being the smallest flowering plant, it is safe to assume that it behaves in a similar way to other plant matrices. Cellulase was the enzyme of choice in this study as there is little documentation on its effect on extraction, however ample findings on the presence of cellulose in plant cells and hence in all the three studied biomasses of this dissertation (Broxterman & Schols, 2018; Shafizadeh & McGinnis, 1971).

A fast and economical extraction method, with minimal hypothermic effects, is possible with ultrasound technology. As a result, the functional qualities of the extracted nutrients are improved, and their deterioration is decreased (Celotti et al., 2021; Rahman et al., 2021; Rahman & Lamsal, 2021b). This technique can be utilized to produce high yields of thermo-labile proteins. Protein aggregation is stimulated because disulfide bonds between cysteine residues are formed as a result of the hydroxyl free radicals produced by ultrasound-assisted extraction (Kumar et al., 2021). Long-term acoustic agitation brought on by cavitation during sonication causes the soluble protein fractions to become denatured, ultimately lowering the protein recovery. Therefore, it is essential to optimize ultrasound-assisted extraction parameters for various plant matrices in order to maximize yields while retaining the functional qualities of protein as well as other nutrients. Furthermore, ultrasound technology coupled with enzyme-assisted extraction can increase nutrient yields even more (Kumar et al., 2021).

To summarize, there were three main findings from this research. The first is that ultrasonic probe devices were found to be more efficient in the extraction of compounds than bath sonicators. This implies that the industry would be better off opting to invest in a probe sonicator rather than a bath sonicator device. Secondly, this study confirms that ultrasound technology fared better than conventional or alkali extraction methods. This indicates that the industry would benefit greatly in terms of increased extraction yields if it were to take the leap and change from current extraction methods to the more novel sonication method proposed. Lastly, one can conclude that *Lemna minor* (duckweed) was found to have the greatest extraction yield of nutrients, followed by

Chlorella vulgaris and *Alaria esculenta*, respectively. Thus, the aquaculture industry would be better off starting fish trials with duckweed, since this biomass is the cheapest to harvest and the easiest to extract compounds of interest from.

Chapter 5 Conclusions and recommendations

The Common Fisheries Policy (CFP) states that fish stocks are finite, albeit renewable. Due to this, EU countries are currently taking the required actions to guarantee that the European fishing industry is sustainable. This will diminish threats concerning fish population size and productivity in the long run. In line with the current CFP, this research will revamp brood and fish stocks by including sustainable high nutritional value feed ingredients from alternative protein sources. This shall be accomplished by refining existing practices as required by the CFP, which will include strategies for the addition of selected biomass with high nutritional value into fish feed. Also, this research coincides with Directive 2008/56/EC which suggests a sustainable ecosystem-based approach to the management of human activities in fisheries while permitting sustainable use of nutrients for fish feeding.

Overall, the following three outcomes resulted from this research:

1) Ultrasonic probe devices were found to be more efficient in the extraction of compounds than bath sonicators.
2) Ultrasound technology fared better than conventional or alkali extraction methods.
3) *Lemna minor* was found to have the greatest extraction yield of nutrients, followed by *Chlorella vulgaris* and *Alaria esculenta*, respectively.

The study shows that ultrasound technology does improve the extraction yield of nutrients from the three biomasses. Very little data on this topic was available before this study was carried out. The findings are promising as the use of ultrasound was found to surpass the extraction yields of conventional extraction methods. Current fish feeds are cheaper than any of the three proposed biomasses for now. This means that further efforts will be needed to reduce costs and make them more relevant for food security and hence food availability of our future generations. However, with the cultivation of such biomasses being relatively low to maintain, this endeavour will surely be worth it in the long run.

5.1 Strengths

Built on what has been executed and accomplished, this study has the following strengths:

- Ultrasound technology is a novel (green) technology for extracting nutrients. The study compares many different ultrasonic devices and settings.
- The study compares three different biomasses (*Alaria esculenta*, *Chlorella vulgaris* and *Lemna minor*) using the same extraction methods, and an approach that has not been extensively researched previously.
- Various methods to determine the protein content were utilized as Bradford's reagent adheres more willingly to arginine and lysine than it does to other amino acids, which might lead to misleading results. Hence, amino acids were quantified using the TFAA assay.

5.2 Limitations

The following shortcomings were encountered in this research project:

- The biomasses which were used do not represent the seasonal variation. Seaweed harvested in August has more bioactive compounds like carotenoids, polyphenols, phytosterols and fatty acids, while that harvested in December has more proteins (Afonso et al., 2021).
- The assays which were used in this study all rely on likening the absorbance of the sample to that of a standard. If the compound being analysed does not correspondingly respond to the dye as the standard, the concentration measured will likely be imprecise (Palladino et al., 2019).
- More compounds must be analysed to get a clearer idea of the nutrient content in the biomasses.
- Further resources will be required to create a prototype of the fish feed additive from these biomasses.

5.3 Suggestions for further research

The following is a list of tests that pairs well with the research presented in this thesis:

- Use high performance liquid chromatography (HPLC) to determine the nitrite, nitrate and vitamin C content in the supernatants after the different treatments.
- Use sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the protein distribution.
- Use gas chromatography to analyse the free fatty acids present in the biomass before and after sonication.
- Review techniques for inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES) to assess trace amounts of the heavy metal content in the biomasses.
- Explore other forms of green technology, such as microwave or pulse electric field, to extract protein and compare which method is better.
- Assess the antimicrobial properties of the biomasses through an absorbance method and determine the minimum inhibitory concentration (MIC) at different biomass concentrations
- Explore the cytotoxic effects of the three biomasses.
- Create a prototype of the fish feed additive from these biomasses and carry out fish trials to establish the effects on fish health as compared to current fish feeds.
- Introduce more experiments using different types of macro- and microalgae.
- Introduce new experiments with enzyme-assisted extraction using various enzymes to find the best-suited enzyme for each biomass.

Appendix

Search strategy

The following tables depict the search strategy followed for each of the above-mentioned sub-chapters. These keywords were searched for in Google Scholar between August 2020 and August 2022.

Need for alternative protein sources in fish feed

Terms connected by OR	AND	Terms connected by OR	AND	Terms connected by OR
alternative OR replacement OR substitute		protein OR polypeptide OR amino acids OR macromolecule OR essential nutrient		fish feed OR food for fish OR fodder for fish OR fish fodder OR soymeal OR fishmeal

Aquaculture in Malta

Terms connected by OR	AND	Terms connected by OR
aquaculture OR fish farming		Malta OR Maltese Islands OR Mediterranean

Fish nutrition

Terms connected by OR	AND	Terms connected by OR
fish OR carnivorous fish OR herbivorous fish OR omnivorous fish		nutrition OR diet OR food OR meal OR nutriment OR nourishment

Current fish feed and their limitations

Terms connected by OR	AND	Terms connected by OR
fish feed OR food for fish OR fodder for fish OR fish fodder OR soymeal OR fishmeal		limitation OR drawback OR restriction OR hinderance

Alternative fish feeds

Terms connected by OR	AND	Terms connected by OR
alternative OR replacement OR substitute		fish feed OR food for fish OR fodder for fish OR fish fodder OR soymeal OR fishmeal

Novel approaches using ultrasound- and enzyme-assisted extraction

Terms connected by OR	AND	Terms connected by OR	AND	Terms connected by OR
novel OR new OR latest OR advanced		ultrasound OR sonication OR sonography OR ultrasonic OR ultrasound bath OR ultrasound probe		enzyme OR enzyme extraction OR enzyme-assisted extraction OR EAE

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