A Mega (Omega) Need for the Integration of Bioengineered *Porphyra yezoensis* into a Modern Aquaculture System

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I. Project Summary:

The mighty seaweed is a culturally and nutritionally valuable crop consumed across the globe. Despite its relatively fast growth rate, the amount proposed to be grown for the purposes of extending this research is undermined by a lack of interest in daily consumption of seaweed products. With a shelf life of 2-5 years when flash frozen and excellent nutrient removal properties, there is desire to increase the value of this commodity[6].

This research seeks to transfect *Porphyra yezoensis* with the isFAD6 gene from *Isochrysis* using a homology directed repair (HDR) plasmid and CRISPR/Cas9 technology. Upregulating the gene which codes for the ω-3 fatty acid desaturase protein will increase expression of ω-3 and 6 fatty acids. If transfection rates are successful, these fatty acids can be extracted from farmed *P. yezoensis* and sold for human consumption or supplemented into fish feed, providing an alternative and lower mercury feed in finfish aquaculture systems. The remaining carbohydrates can be converted into bioplastics, taking slight pressure off of the petroleum industry.

Macroalgae need nitrogen, phosphorus, carbon dioxide, light energy, and various trace nutrients to grow efficiently. Seawater is the ideal medium, and in fact it can yield a surplus of these nutrients. However, excessive nutrients can damage vital ecosystems through eutrophication, an excessive overgrowth of algae and phytoplankton. The finfish aquaculture industry faces this pressing issue. Technologies with economical and environmental sustainability are necessary to reduce the environmental impact of effluents coming from those systems. Plants such as *Porphyra* can drastically lower nutrient levels in waste streams through photoautotrophic assimilation and simultaneously increase profit of industrialized mariculture[6].

In this system, *Porphyra* functions to treat eutrophication, enhance the growth of aquaculture systems necessary to meet rising global food demands, provides nutraceuticals and food, and recovers phosphorus from waste streams[5]. This process flow seeks to integrate sustainability into its system[3].

II. Introduction:

Excessive enrichment of water with nutrients, or eutrophication, is a concern across U.S. coasts as dependency on marine finfish aquaculture increases with rising food demands. Liquid waste or sewage discharge are effluents rich in inorganic nitrogen and phosphorus may be the culprit in these excessive nutrient loads[1]. Specifically, animal waste, fertilizer run off, and bacterial breakdown of leftover aquaculture feed are some of the sources of these nutrients. In addition, an estimated 3 million tons of phosphorus are discharged every year by wastewater treatment plants into our waterways[4]. The influx of these nutrients in rivers, lakes, groundwater, and coastal waters can cause harmful blooms of phytoplankton and algae leading to hypoxic conditions[2,3]. If left untreated, eutrophication can destroy vital ecosystems.

Furthermore, our modern agriculture system is so highly dependent on inorganic phosphorus sourced from phosphate rock that global reserves are expected to be depleted in 50-100 years. With increasing demand and production costs, it is of global interest to recover phosphorus for food security[5]. In a sustainable aquaculture production system, the nitrogen and phosphorus in the effluent would ideally be recycled into the production of valuable commodities.

Macroalgae, also known as seaweed, have demonstrated the ability to grow in eutrophic waters. A variety of species in the genus *Porphyra,* which is a coldwater algae that can grow in

the intertidal zone of temperate waters worldwide and commonly used for consumption in Asia under the name "Nori", can function as bioremediation agents, successfully removing "70-100% of Nitrogen within 3-4 days at nitrogen concentrations up to 150 μM" and 35-91% of the inorganic phosphorus in experimental conditions (Figure 1)[6]. Often ammonium toxicity is a concern in algal cultivation. However, research suggests that supplementing with ammonium does not reduce growth rate and in fact, results in higher tissue N contents than another common bioavailable nitrogen form, nitrate[6]. It can be concluded that *Porphyra* is reliable for nutrient removal of eutrophic waste effluents.

This red seaweed has remained culturally important and nutritionally valuable for human consumption. The tissue is harvested in great numbers, dried before processing, and served in various forms as a food product. Coastal indigenous people from British Columbia and Alaska have "expressed concerns about potential commercialization of *Porphyra* and impacts from pollution and global climate change.[7]". However, the research proposed in this document adds an ethical benefit by cultivating transfected *Porphyra* downstream of aquaculture systems to: (a) treat eutrophication, (b) optimize growth of species with enhanced omega (ω) 3 production, (c) provide an alternative to fossil fuels in plastic production (bioplastics) and (d) create a cost-effective means for managing solid (uneaten food, feces) and effluent wastes (dissolved metabolic wastes: CO_2, NH_4, PO_4).

Various nutraceuticals can be extracted from *Porphyra* sp. This dried purple laver contains a substantial amount of Vitamin B_{12}, approximately 32.3 μg/100 g dry weight[9], and research suggests it is bioavailable[10]. Yields of up to 3600 μg of Vitamin A, 10.7 mg of iron, and 1.19 g of *n*-3 polyunsaturated fatty acids per 100g can also be obtained[9]. Individuals with diets

deficient in these compounds, such as vegans and those in developing countries, could take supplements to counter malnutrition.

The most common plant ingredients in fish feed are corn, wheat, and soy. Protein and fatty acid content make macroalgae a superior ingredient to corn and wheat, but not soy[8]. Fish fed a diet primarily composed of cereal lack ω-3 poly-unsaturated fatty acids (PUFAs). This fatty acid can reduce cardiovascular disease by decreasing inflammation. In cold-water marine species, ω-3 PUFAs are crucial for physiological functions such as temperature regulation and membrane viscosity[8]. In addition, seaweed has a high mineral content, particularly iodine and selenium, which can be obtained from seawater. Iodine and selenium are essential for the synthesis of thyroid hormones, which are important metabolism regulators in both humans and fish[8].

However, the consumption of heavy metals taken up and stored by algae may pose potential problems to human health. Consuming arsenic may lead to peripheral vascular disease and other cancers, the uptake of cadmium could lead to renal tubular dysfunction, and impaired mental development may be associated with mercury consumption[8]. For this reason, extracting proteins, minerals and/or fatty acids, as single compounds for use as ingredients in food and feed production is the optimal strategy when utilizing the valuable products of these macroalgae species.

Transfection is the insertion of foreign or altered genes into target cells.While transfection rates are historically poor with *Porphyra* (ability for acceptance of foreign/altered genes), we propose a new approach where *Porphyra yezoensis* will be transfected with the isFAD6 gene from *Isochrysis* using an HDR plasmid and CRISPR/cas9 technology. Thus far, the

unicellular red alga *Porphyridium* sp. has seen successful genetic transfection through "integration of the gene encoding AHAS(W492S) into the chloroplast genome by homologous recombination, resulting in sulfometuron methyl (SMM) resistance at a high frequency in SMM-resistant colonies[18]." Other unicellular alga have undergone successful transfection but the preliminary experiments conducted for multicellular alga remains weak in establishing a viable procedure. An efficient gene transfer and expression system must be resolved. With limited knowledge behind this field, it is critical to receive funding for future experimentation.

III. Rationale & Significance:

Attempting to transfect *Porphyra* will increase understanding of how a protein in seaweed can be manipulated to express more ω-3 and 6 fatty acids. If successful, this proposed research will determine whether transfection of the entire homology directed repair is applicable to *Porphyra*. The experiments conducted will validate whether particle bombardment or utilization of a gene gun is a useful method in transfecting *Porphyra*. The transfected seaweed can then be integrated into a pilot-scale aquaculture system where it will function to remove nutrients from the water prior to harvest. Valuable commodities can be extracted from *Porphyra* such as ω-3 supplements, fish feed, be made into bioplastics (carbohydrate utilization), or flash-frozen for 2-5 years for human consumption.

IV. Research Objectives & Hypothesis:

- The primary objective of this proposal is to genetically modify a protein in *Porphyra* to yield a higher lipid content, specifically ω-3 and ω-6.
- Omega-3/6 obtained from *Porphyra* can be used to produce valuable products such as nutraceuticals.
- If the predictions are not met, this project will still be useful in deterring the levels of eutrophication in marine finfish aquaculture, and protein and carbohydrates can still be extracted for creating bioplastics. Another transfection attempt will provide future researchers a new angle to consider regarding seaweed biotechnology.
- Phosphorus can be recovered into seaweed, a food stock, which will decrease reliance on depleting phosphate rock reserves.
- Effluents rich in inorganic nitrogen and phosphorus from animal waste, fertilizer run off, a bacterial breakdown of leftover aquaculture feed will be recycled and used to make valuable products rather than flowing into rivers, lakes, groundwater, and coastal waters.
- The decrease of nutrients flowing into these bodies of water can decrease the harmful blooms of phytoplankton and algae, thus diminishing the hypoxic conditions created, which are destructive to vital marine ecosystems.

Hypothesis: *Porphyra* can be used in the bioremediation of eutrophic waters. Genetically engineering the species to yield a higher ω 3/6 fatty acid content which can be isolated and sold as a nutraceutical post-harvest will increase the desire for continuous use of this crop.

V. Methods:

General Procedure

In order to achieve our goal, crucial aspects of the experiment must be assessed: Isolation of the isFAD6 gene from *Isochrysis,* integration of the isFAD6 gene into the HDR plasmid, transfection of the CRISPR/Cas9 and HDR plasmids into *Porphyra yezoensis*. Artificial selection of species that express the desired phenotype, must take place and then these sporophytic cells must be propagated. The main obstacle is the engineering of a foreign gene of *Isochrysis* and performing stable transfection into *Porphyra*. However, it has been shown that this can be achieved by integrating silent mutations into the gene of interest to convert it to a high guanine-cytosine (GC) content gene in the DNA, as well as by pairing it with an endogenous promoter[11]. The gene of interest, isFAD6, which codes for the ω-3 fatty acid desaturase protein, has an endogenous promoter, CaMV 35S[11,14]. In theory, this would upregulate lipid content[13].

Determination and Modification of Gene

The protein that needs to be upregulated for increased polyunsaturated fatty acid production is ω-3 fatty acid desaturase as well as elongase[13]. However, either or is sufficient but one may do. The cDNA of ω-3 fatty acid desaturase gene has been determined and deemed isFAD6 in Marine Microalgae *Isochrysis spp*[14]. In order for transfection to occur correctly the G-C content must be determined. The gene sequence mentioned later in the procedure will be observed and it will be determined if GC content is higher than 66.6%. If it is not silent mutations will be inserted into the genome not affecting the amino acid sequence.

Isolation of isFAD6 Gene

In order to isolate the DNA sequence of ω-3 fatty acid desaturase gene (isFAD6) from *Isochrysis spp.* a clone will need to be made from said *Isochrysis.* In order to do this *Isochrysis* will be grown up to a relatively high density, where it will then be ground up to a powder in liquid nitrogen. The total mRNA will be extracted via CTAB methods using a mini plasmid kit[15]. The purified mRNA will then be reversed transcribed. This will be done using reverse transcriptase and RACE CDS primers.This will be conducted with isFAD6 SP1 and SP2[14]. Products will be ran on an electrophoresis gel where the gel will be cut and dissolved using a gel purification kit. The gene will be ligated into a pMD-18 cloning vector. The sample will be sent of for sequencing and the sequence will be amplified with TAQ polymerase as well as the primers RV-M and M13-47 via a polymerase chain reaction[16].

Amplification of Plasmid

Once PCR is complete verification of the correct plasmid will be confirmed via gel electrophoresis. This will be done with the 5' and 3' RACE CDS techniques and then amplified in order to obtain a usable segment for gel electrophoresis. The target RNA segment should be ~1500 bp. There should also be two DNA fragments ~1200 bp. This is with the segments involved 5`-UTR and the 3`-terminal UTR. This DNA segment should encode the n ω-3 fatty acid desaturase protein[14].

Integration of isFAD6 into CRISPR/HDR plasmid

The isFAD6 DNA segment will be sent to OriGene in order for it to be integrated into an HDR Plasmid. The product that will be sent back will be a CRISPR/Cas9 Plasmid as well as the

integrated HDR Plasmid. The HDR Plasmid also holds a gene segment for determination of transfection (These are Puromycin resistance and Red fluorescent Protein (RFP). The CRISPR/Cas9 will be co-transfected with the HDR plasmid; in other words, the CRISPR/Cas9 will cause a break in a specific portion of the genome upstream from an endogenous promoter of *Porphyra yezoensis* a director such as CaMV 35S. The HDR plasmid will then act to repair the site inserting the isFAD6 gene as well as puromycin resistance and RFP genes.

Transfection of HDR Plasmid

One of the most difficult matters of transfecting the target genus, *Porphyra* or Red seaweed is translocation across the cell wall of the species. In order to do this many transfection methods have been attempted yet only a few have shown significant positive results. The method that has had the most success in transfection of *Porphyra* is particle bombardment or the use of a gene gun. For full expression into *Porphyra* it is necessary to have the isolated gene with high GC content, this is because anything less of ~65.0% GC content will not express well. Expression will also not occur without an endogenous promoter such as CaMV 35S[11]. Ideally the transfection method will be outsourced to a school or company with access to a gene gun. If that is not this step is still necessary. Gold or tungsten particles will be coated with both of the plasmids[11]. High pressure helium will be applied via the gene gun. Transfection is seen regardless of cell wall or its composition among *porphyra* and other algal species. The expected outcome is the transfection of the entire HDR plasmid, this includes RFP (red fluorescent protein), puromycin resistance gene, and the isFAD6 gene and the promoter caMV 35s.

Determination of Plasmid Expression

To determine if the plasmid is being expressed observation of the RFP protein will be observed either by fluorescent microscopy or fluorescence spectrometry. The expressing cells cannot be selected for via puromycin resistance since *Porphyra yezoensis* is not susceptible to puromycin. Although even the RFP protein may not express yet isFAD6 still might be expressed. In order to determine this lipid quantification must be observed via staining with Nile Red or Bodipy, polar lipid stains, after the proliferation of seaweed cells a few days-weeks after growth. This would give output of relative lipid production in the cells.

Proliferation of Porphyra into Aquaculture

Porphyra will be proliferated into an aquaculture. This will allow for growth of the seaweed in a controlled and monitored environment, To do this two sets of two tanks will be set up. One tank will house finfish with an input for food and "new" re-circulated water. PO_4, CO_2, and NH_4 will be excreted by the fish and filtered through a mechanical filter to remove solid waste before entering the second tank that houses the *Porphyra*. Along with the suspected transfected *Porphya,* the aquacultures will be seeded with the initial strain of untransfected *Porphyra* and noted to act as a control. The CO_2, PO_4, and NH_4 will feed into the *Porphyra* as it utilizes energy from overhead LED lights *;* the *Porphyra* will release O_2 which will be recycled to the finfish tank. There will also be an outlet in the *Porphyra* tank for wastewater. The temperature, light level, filtration, and aeration will be maintained and monitored. Ideally seawater will be utilized instead of a specialized media. Fish waste will provide the essential nutrients for *Porphyra* growth as stated above (Figure 2, 3). Nitrate, ammonia, biological oxygen demand, dissolved oxygen, and phosphorus will be quantified daily at the start and sampling

adjusted as needed as the experiment continues. Analysis will be conducted in the environmental engineering lab at Cal Poly.

Fluorescence Assay of Lipid Content

As mentioned above, a lipid quantification assay will be performed on the seaweed after two weeks of growth. This will be done by staining with Nile Red Fluorescent tag; this stain specifically stains polar lipid bodies[17]. Ideally this output will show a larger amount in the transfected *Porphyra* when compared to the control set of *Porphyra*. The sporophytic cells will then be selected for and propagated for future cultures, although it should be noted that even though sporophytes do express, expression in sporophytes is much lower than expression in gametophytes[12]. The overarching goal of this project is to replace many strains of *Porphyra* currently used for commercial agriculture purposes.

VI. Research Timeline:

Expected time	Task
Two Weeks	Determination and Modification of Gene
One Week	Isolation of isFAD6 Gene
1-2 Days	Amplification of Plasmid
Two Weeks (outsourced)	Integration of isFAD6 into CRISPR/HDR Plasmid
1-Day to Week (Dependent on ability to outsource)	Transfection of HDR Plasmid
1-2 Days	Determination of Plasmid Expression
3 Weeks	Proliferation of *Porphyra* into Aquaculture

(aquaculture built slowly throughout experiment)	
1-2 Days	Fluorescence Assay of Lipid Content

Overall time: Ten weeks (ideal), Eleven-fifteen weeks (less ideal)

VII. Budget:

Supplier	Item Description	Equipment	Number of Items	Cost per Item	Total Cost
Qiagen	Plasmid Giga Kit		1	$508.00	$508.00
Carolina	Electrophoresis Gel		5	$87.00	$435.00
Qiagen	Gel Extraction Kit		1	$113.00	$113.00
BioAssay Systems	EnzyChrom Free Fatty Acid Assay Kit		1	$329.00	$329.00
Functional Biosciences	Plasmid Sequencing		30 Rxns	$3.50	$105.00 (outsourced)
Bio-Rad	Helios Gene Gun System		1	$17,000	$17,000 (ideally outsourced)
OriGene	CRISPR/Cas9-HDR Plasmid		1	$450.00	$450.00
Cal Poly		PCR machine	1	$0.00	$0.00
Cal Poly		Fluorescent/Light Microscope	1	$0.00	$0.00
Cal Poly		Air pump	1	$0.00	$0.00
Cal Poly		Water circulator	1	$0.00	$0.00

Home Depot		LED lights	1	$97.00	$97.00
Home Depot		Rubbermaid 54 gallon bin	2	$25.97	$51.94
MicroBio Engineering	To serve as fish culturing system	Deep tanks	2	$0.00	$0.00
Cal Poly	Finfish		12+	$0.00	$0.00
Hach	Nitrate TNTplus vial test kit	Low-medium range	20	$41.85	$837.00
Hach	Ammonia test kit	High range	8	$89.85	$719.60
Hach	Phosphate as Phosphorus	Medium-high range	5	$177.00	$885.00
Cal Poly	Spectrophotometer		1	$0.00	$0.00
Cal Poly	Nitrate, Ammonia, Phosphate Standards		1	$0.00	$0.00
Cal Poly	Biological Oxygen Demand			$0.00	$0.00
Cal Poly	Dissolved Oxygen (D.O.)	D.O. probe	1	$0.00	$0.00
Carolina	Living *Isochrysis*		1 vial	$7.50	$7.50

Total estimated cost: $21,539.

FIGURES:

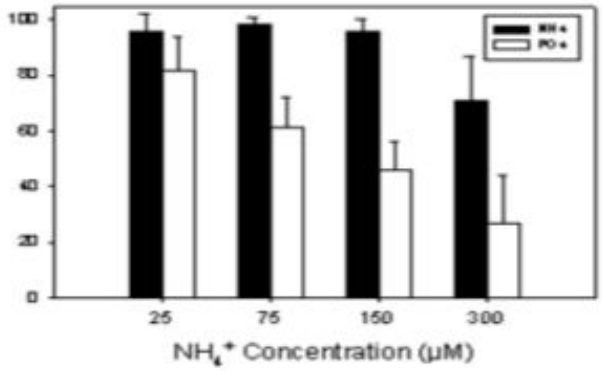

Figure 1. Percent of inorganic nutrient removed from culture medium over a 3 day period.

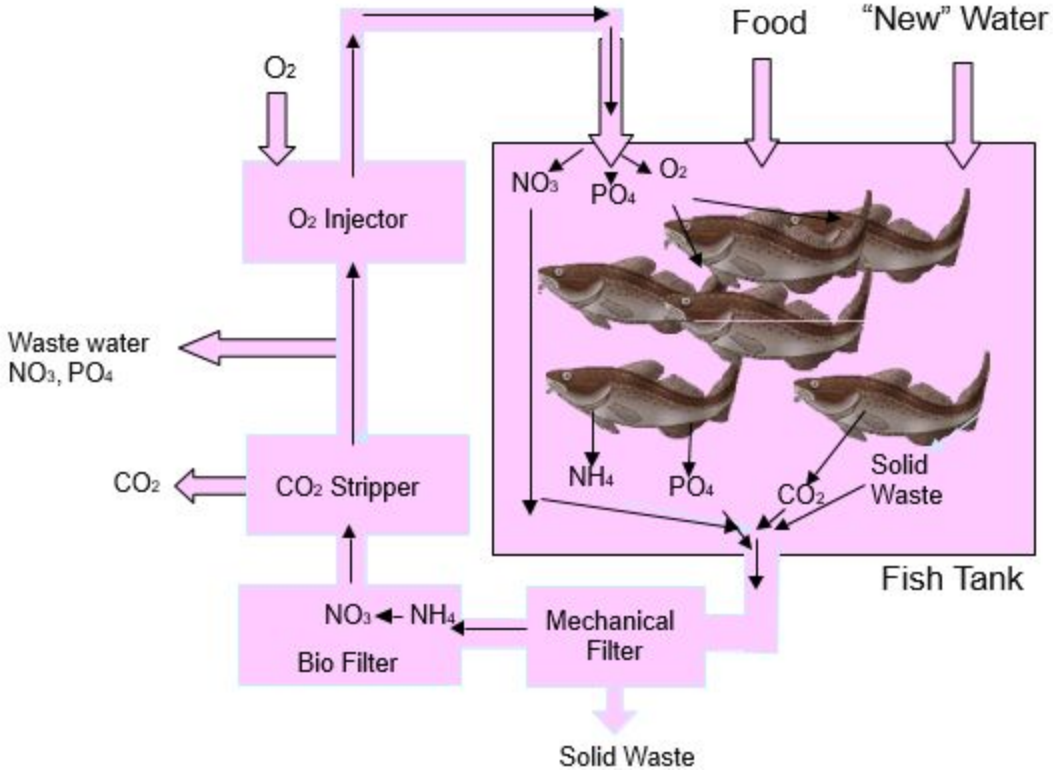

Figure 2. Conventional finfish recirculating aquaculture system (courtesy of Charles Yarish at University of Connecticut).

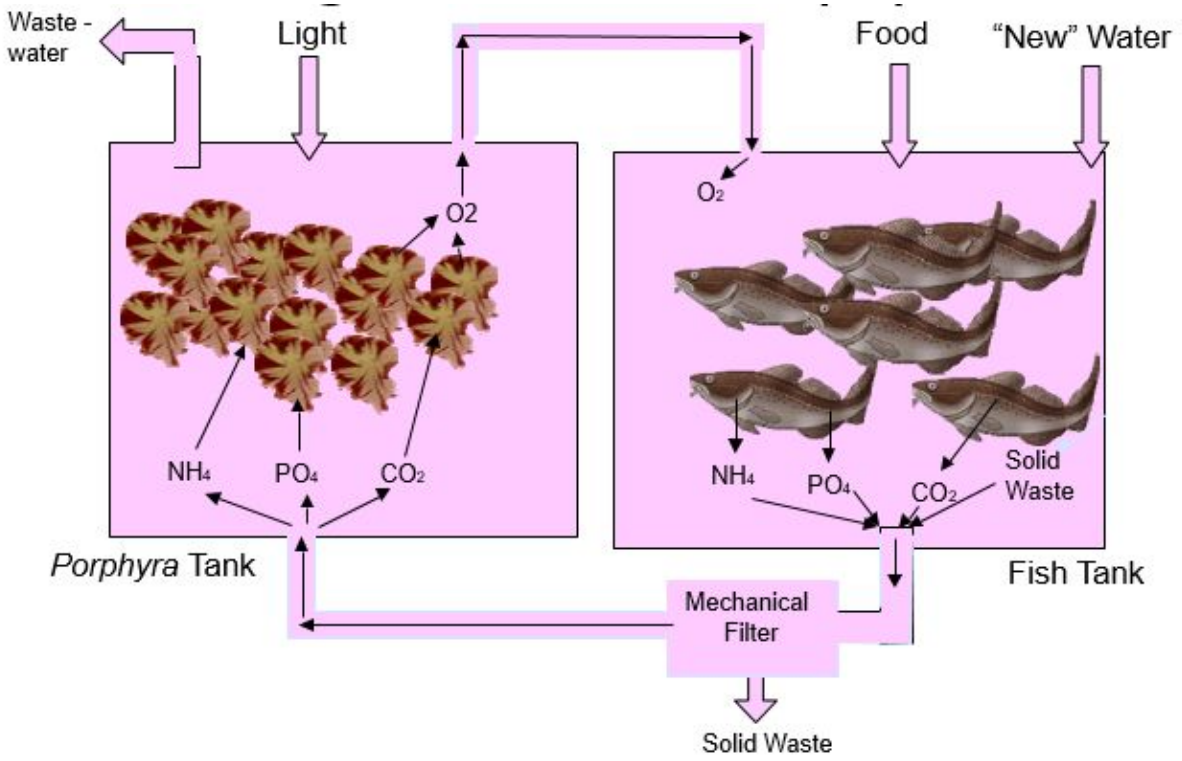

Figure 3. Alternative design: recirculating aquaculture system integrating finfish and *Porphyra* (courtesy of Charles Yarish at University of Connecticut).

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