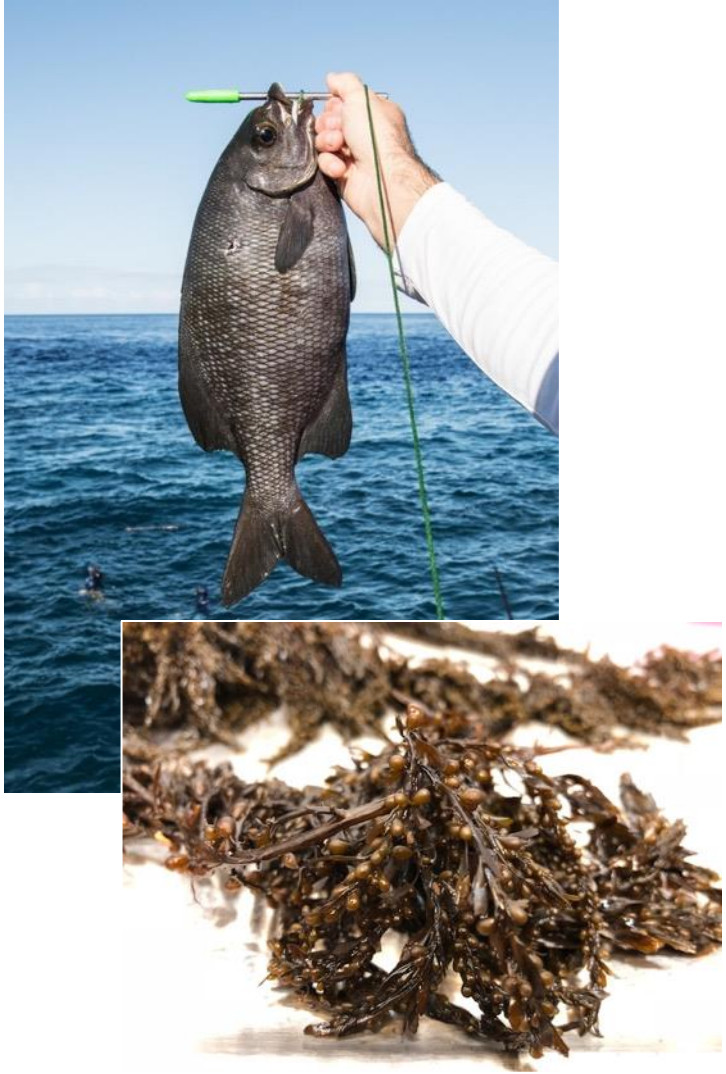


Kyphosid Ruminant Microbial Biodigestion of Seaweed (KRuMBS)

Harnessing the Biological Model of Herbivorous Fish Gut Microbiome to Improve Seaweed Bioconversion

Lieve M. Laurens, Simona Augyte, Neil Sims

ARPA-E Virtual Workshop

November 16-18, 2020

Can rudderfish (Family Kyphosidae – an herbivorous, active-swimming reef-fish) provide a biological model for macroalgae biodigestion as a route to renewable bioenergy?

OCEAN ERA

NREL
NATIONAL RENEWABLE ENERGY LABORATORY

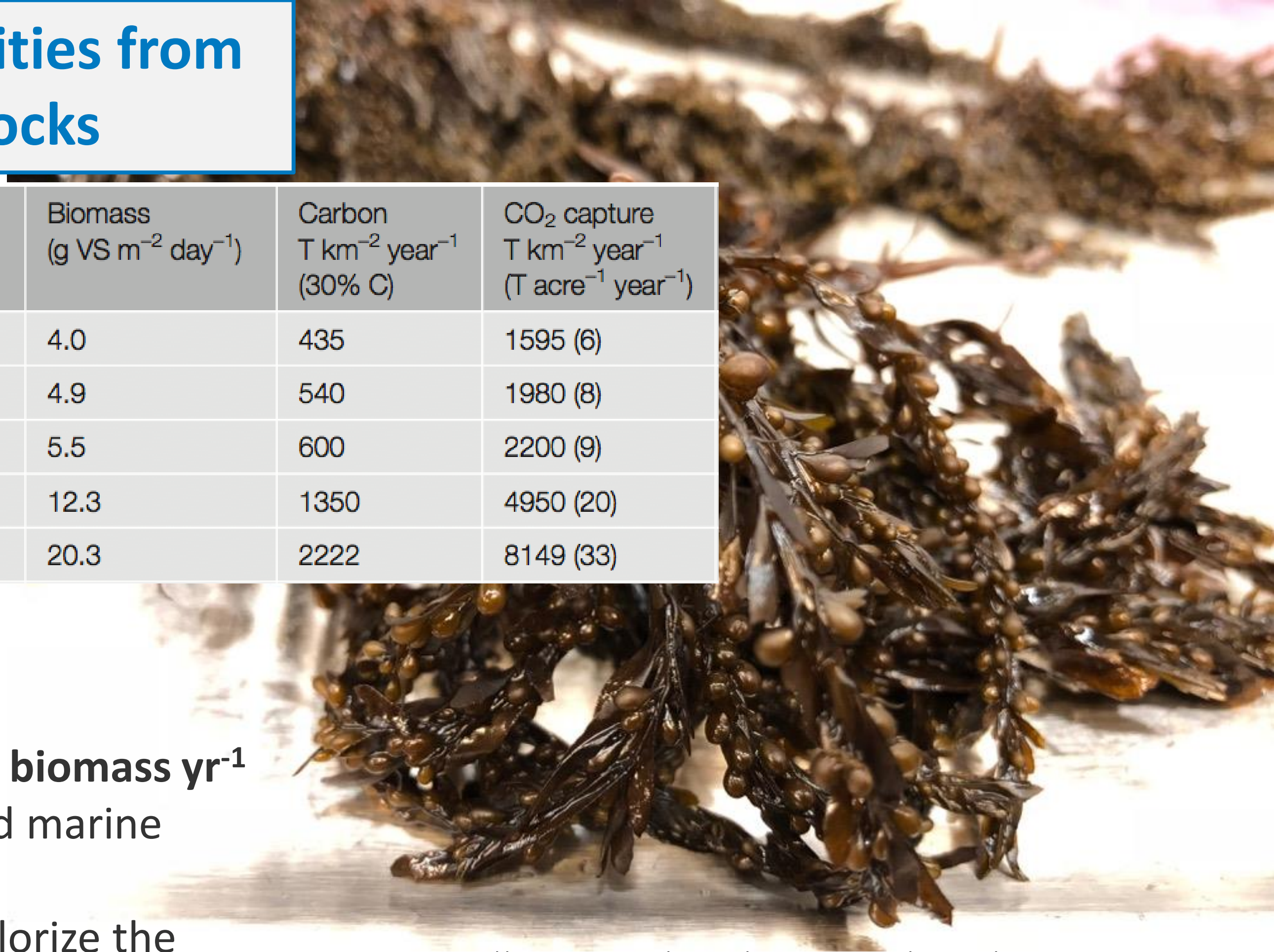
BERKELEY LAB
Lawrence Berkeley
National Laboratory

Neil Anthony Sims - PI, Simona Augyte (Ocean Era)
Lieve Laurens, Rob Nelson (NREL)
Craig Nelson, Wesley Sparagon (University of Hawaii)
Linda Wegley-Kelly (San Diego State University)
Eric E. Allen, Jake Minich, Pieter Dorrestein, Emily Gentry (UC San Diego)
Blake A. Simmons, Steve W. Singer, Lydia Rachbauer (LBNL)

arpa·e
CHANGING WHAT'S POSSIBLE

Bioenergy opportunities from Marine Feedstocks

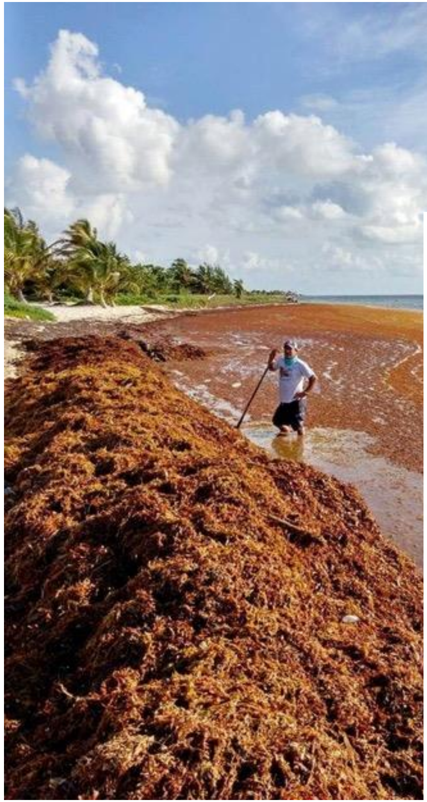
Species	Biomass T VS km^{-2} $year^{-1}$ (T VS $acre^{-1}$ $year^{-1}$)[a]	Biomass (g VS m^{-2} day^{-1})	Carbon T km^{-2} $year^{-1}$ (30% C)	CO_2 capture T km^{-2} $year^{-1}$ (T $acre^{-1}$ $year^{-1}$)
ND	1450 (6)	4.0	435	1595 (6)
Macrocystis pyrifera	1800 (7)	4.9	540	1980 (8)
M. pyrifera	2000 (8)	5.5	600	2200 (9)
Ulva lactuca	4500 (18)	12.3	1350	4950 (20)
Laminaria longicruris	7407 (30)	20.3	2222	8149 (33)

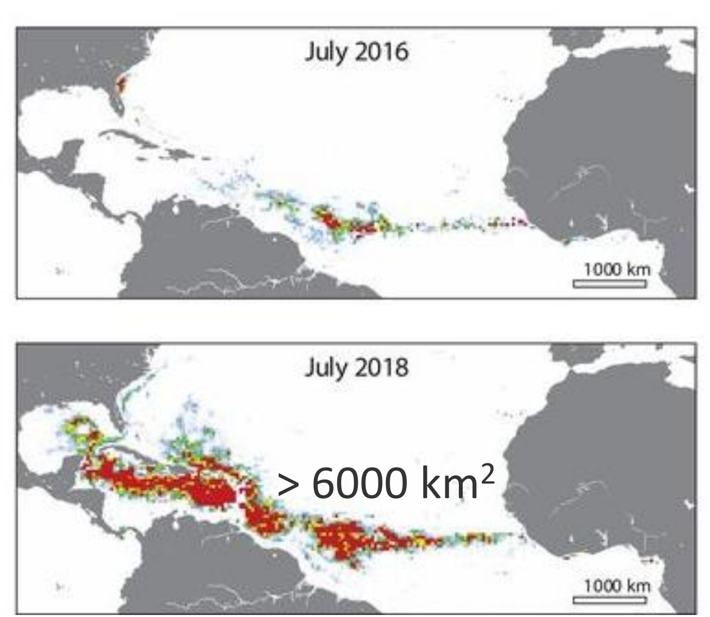


- Potential for up to **300 MT biomass yr^{-1} production** with developed marine agronomy in EEZ
- Important to retain and valorize the captured **carbon** and **nitrogen**

https://www.cell.com/trends/biotechnology/fulltext/S0167-7799(20)30090-1

No shortage of marine biomass



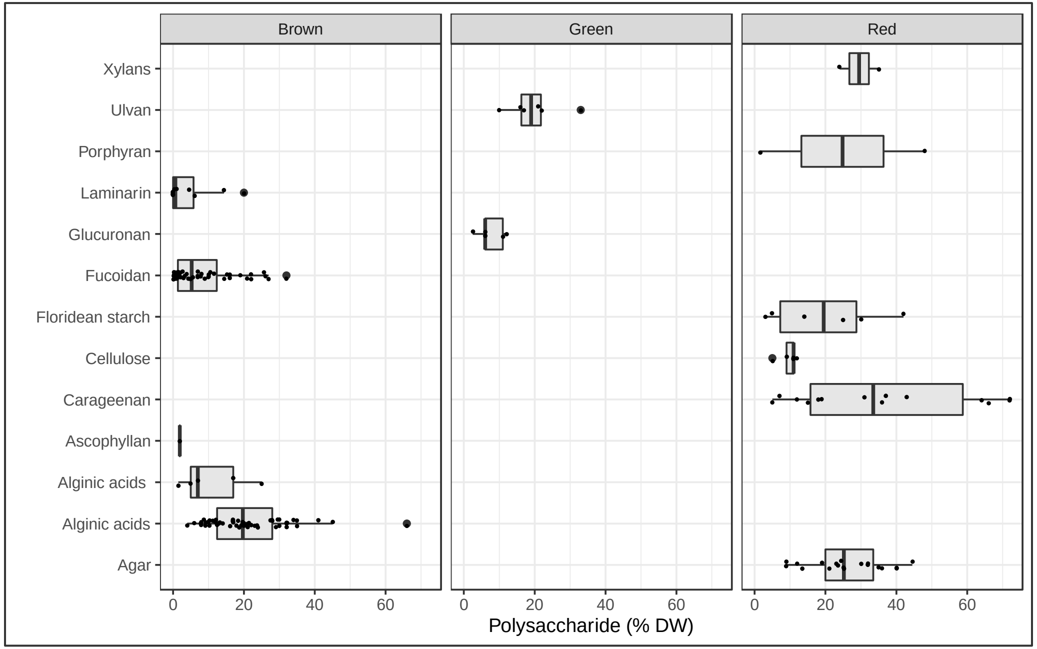


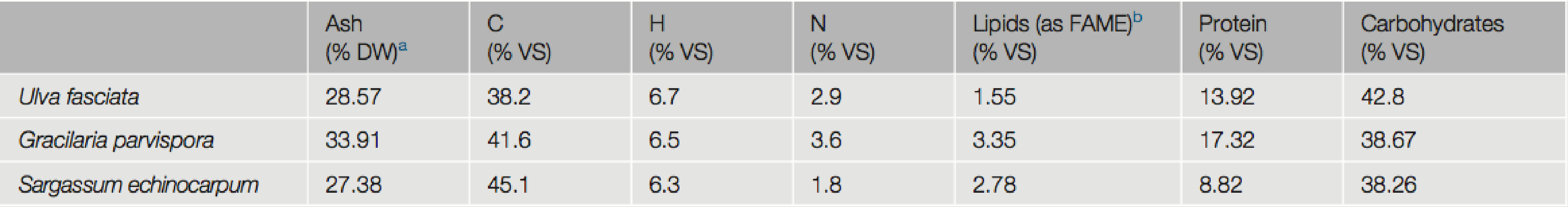
> 6000 km^2

Wang, et al., Science, 2019: 365, 6448, 83-87

Bioenergy challenges from marine feedstocks

1. Water content >80%
2. Saline environment >35 ppt
3. High ash content > 30% (DW)
4. Seasonal supply
5. Complex composition profile

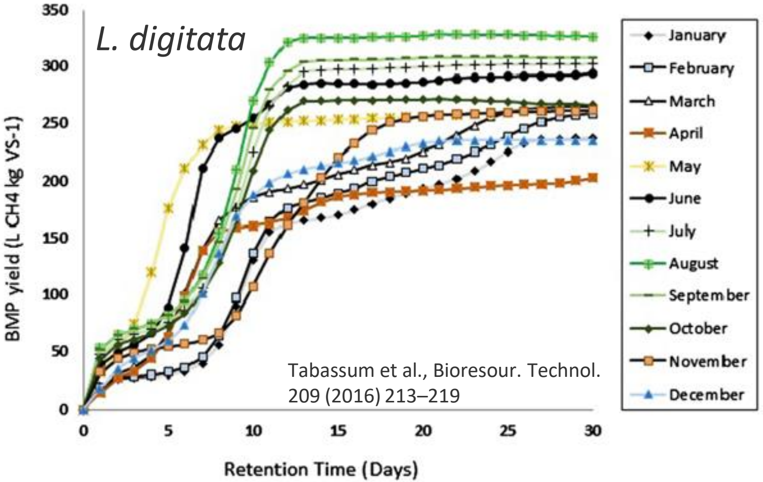




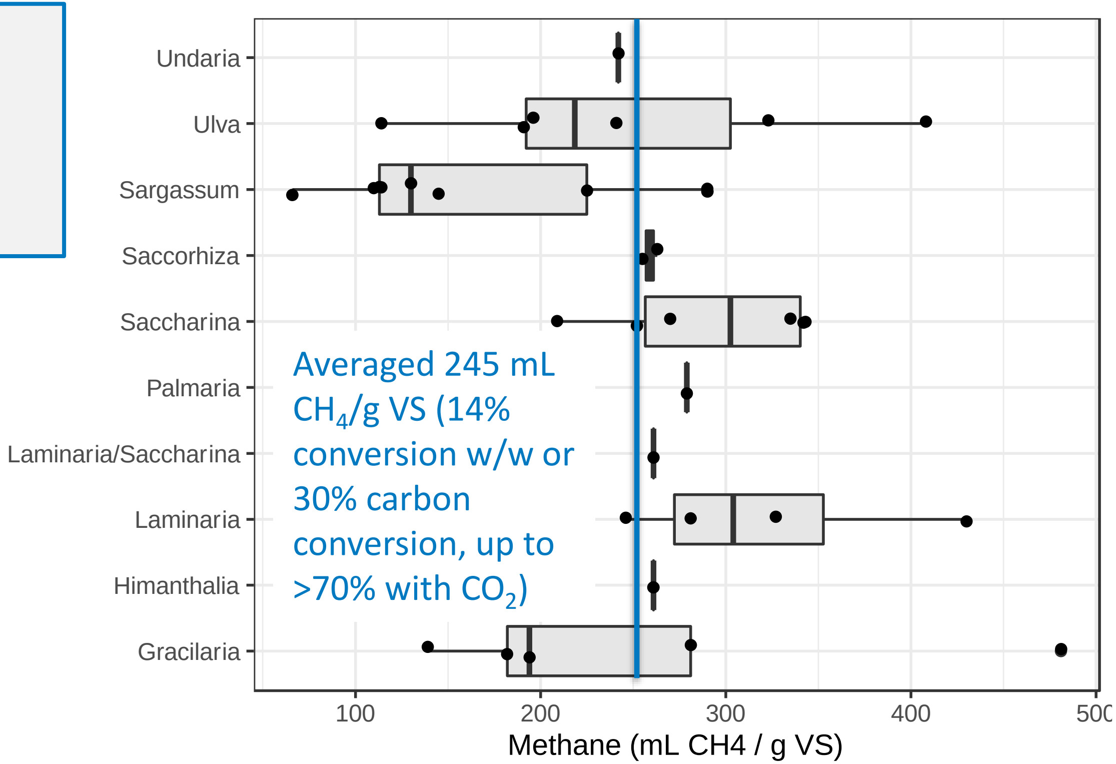
	Ash (% DW)[a]	C (% VS)	H (% VS)	N (% VS)	Lipids (as FAME)[b] (% VS)	Protein (% VS)	Carbohydrates (% VS)
Ulva fasciata	28.57	38.2	6.7	2.9	1.55	13.92	42.8
Gracilaria parvispora	33.91	41.6	6.5	3.6	3.35	17.32	38.67
Sargassum echinocarpum	27.38	45.1	6.3	1.8	2.78	8.82	38.26

- **Critical to tailor microbial deconstruction to seaweed chemical compositional profile and saline environment to maximize conversion efficiency**

Anaerobic digestion to methane production as scalable energy solution



Tabassum et al., Bioresour. Technol. 209 (2016) 213–219



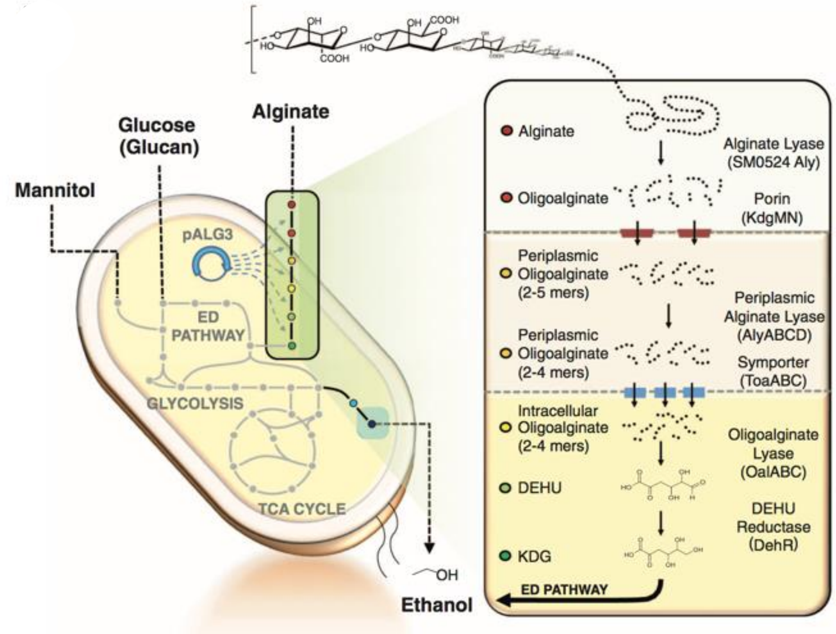
Averaged 245 mL CH_4/g VS (14% conversion w/w or 30% carbon conversion, up to >70% with CO_2)

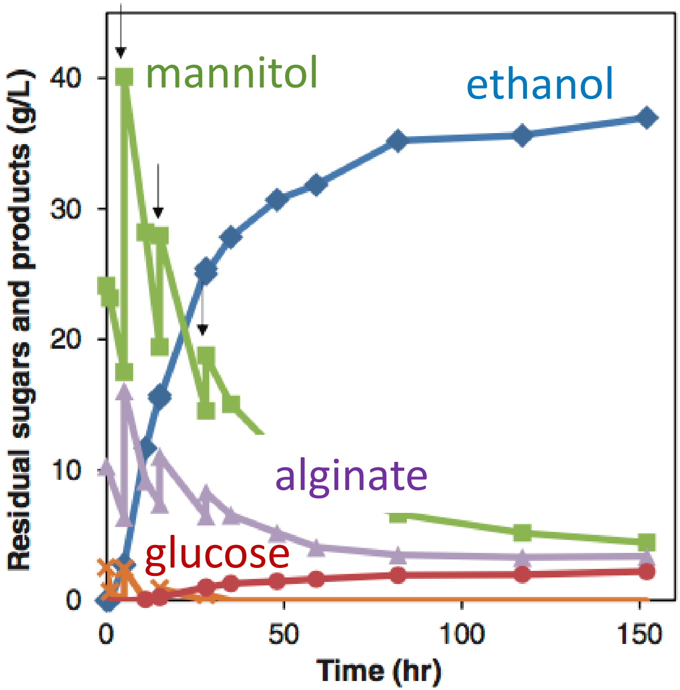
mmol C/g VS/hr	Total wt% VS (conversion yield to product)	
0.032	18.5	Tabassum 2016 (CH_4)
0.072	19.3	Chynoweth 1993 (CH_4)
0.050	15.5	Hessami 2019 (CH_4)
0.028	26.6	Oliveira 2014 (CH_4)

- With a projected **300 MT seaweed** produced (10x current global production) could generate **4 Quad BTU**

Alternative, more rapid, microbial fermentation to higher value products

mmol C/g VS/hr	Total wt% VS (conversion yield to product)	
0.091	5.4	Hwang 2012 (Lactic acid)
0.102	19.2	Lin 2020 (Lactic acid)
0.281	**28.1**	Wargacki 2012 (Ethanol)

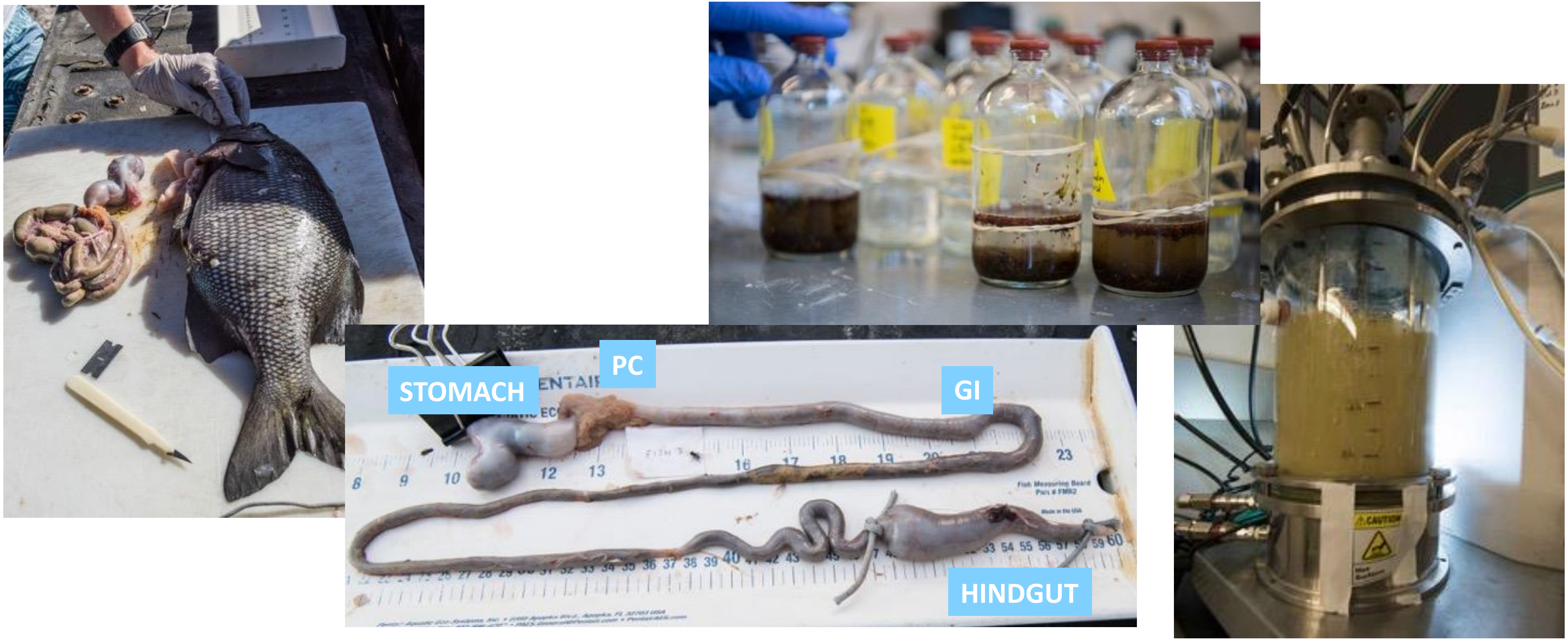


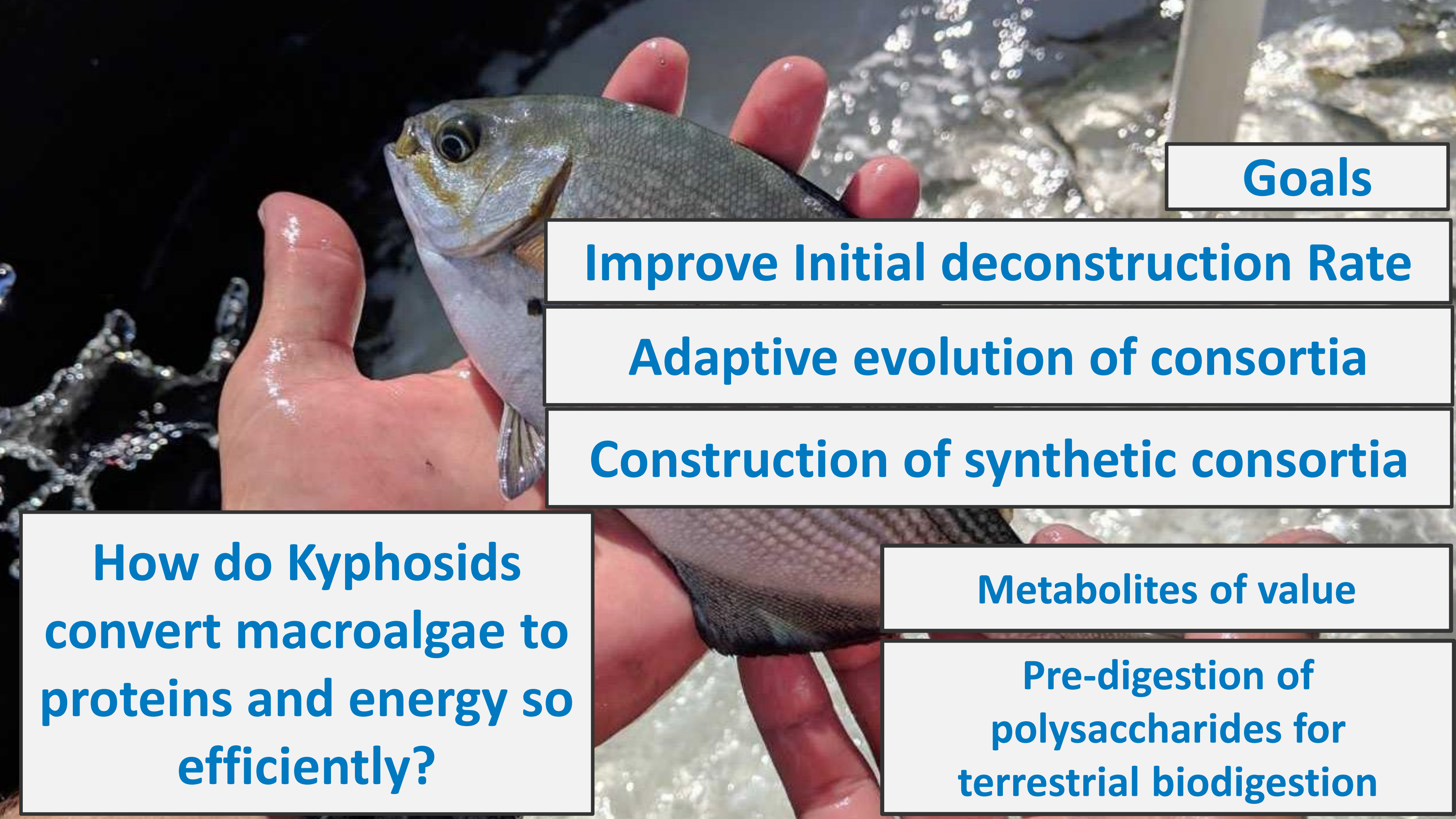


Wargacki, et al., Science, 2012 335(6066):308-13

Can we **leverage and adapt the marine microbiome** to optimize the conversion efficiency and valorization of seaweeds?

The <u>KRuMBS goal</u>: Reducing the Complexity of a Reef Fish Gut Microbiome to a Reproducible, Scalable Biodigestion Process, with Metabolites of Commercial Value





Goals

Improve Initial deconstruction Rate

Adaptive evolution of consortia

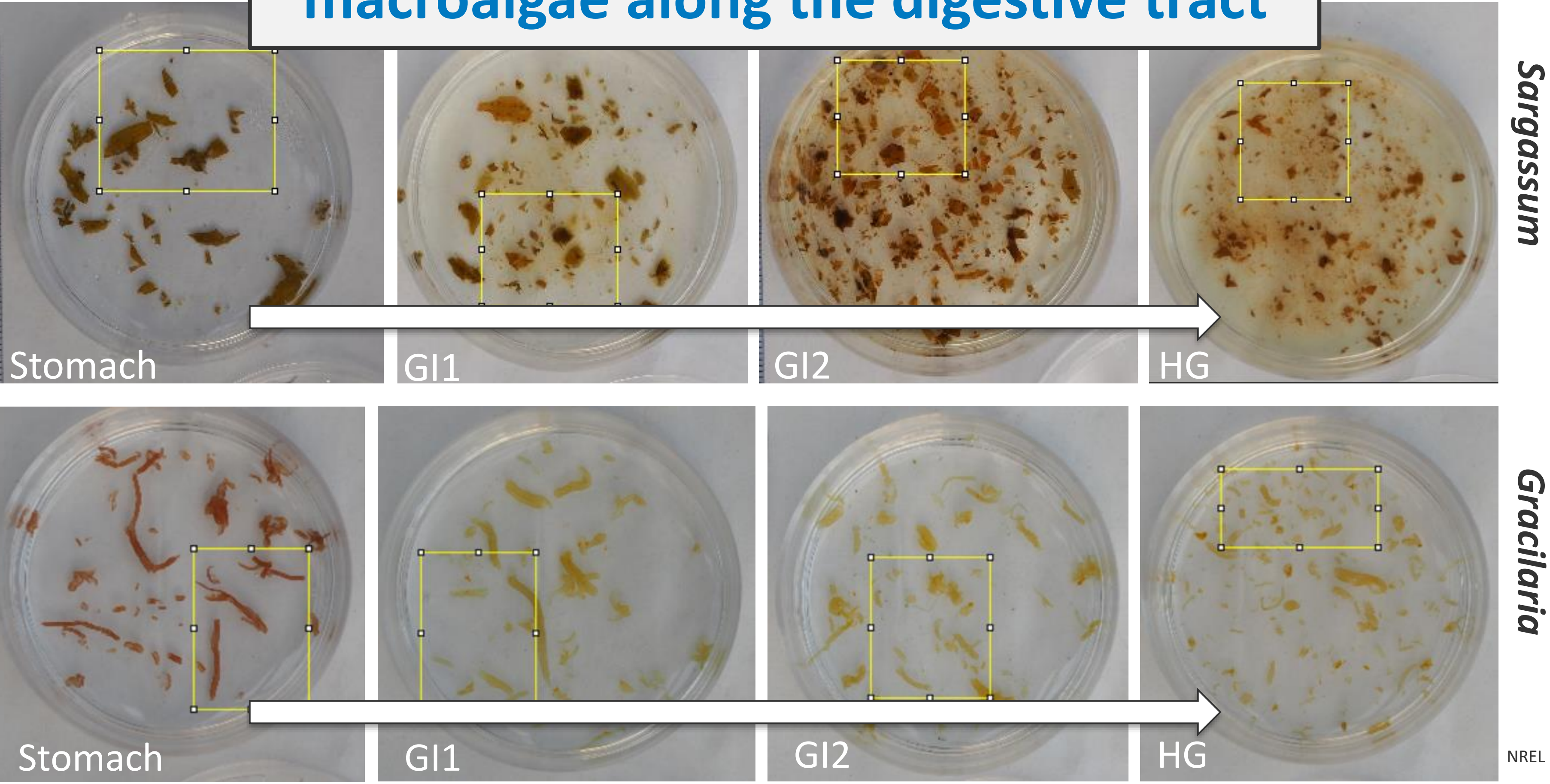
Construction of synthetic consortia

How do Kyphosids convert macroalgae to proteins and energy so efficiently?

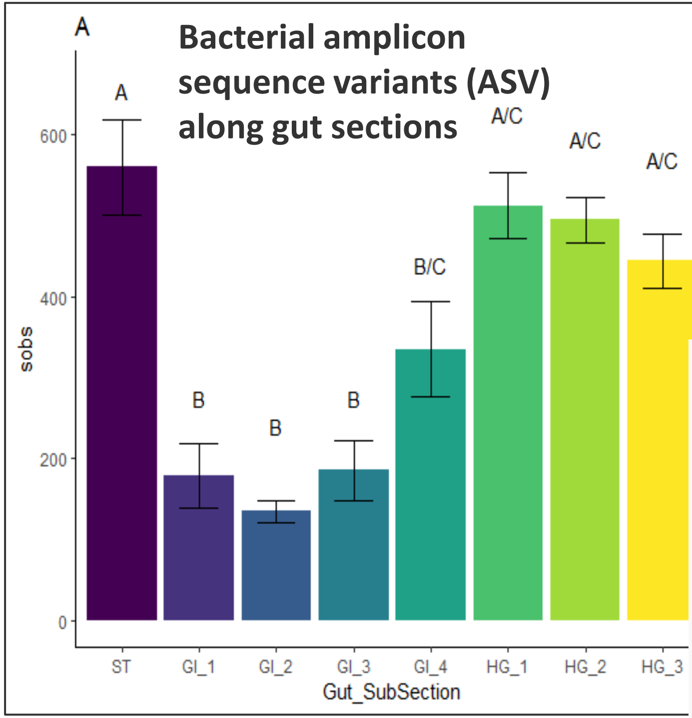
Metabolites of value

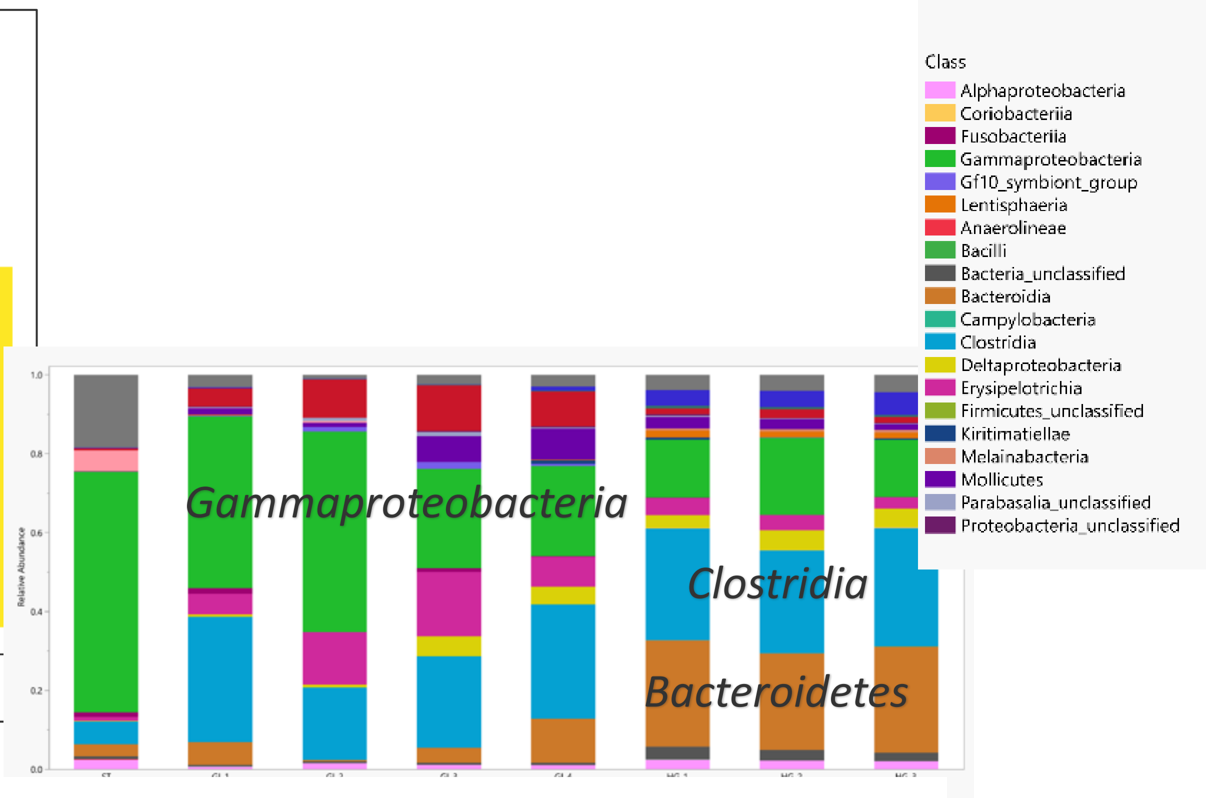
Pre-digestion of polysaccharides for terrestrial biodigestion

Kyphosids are efficient at converting macroalgae along the digestive tract



… and have highly specialized microbial communities associated with the different gut sections


Bacterial amplicon sequence variants (ASV) along gut sections


Gammaproteobacteria

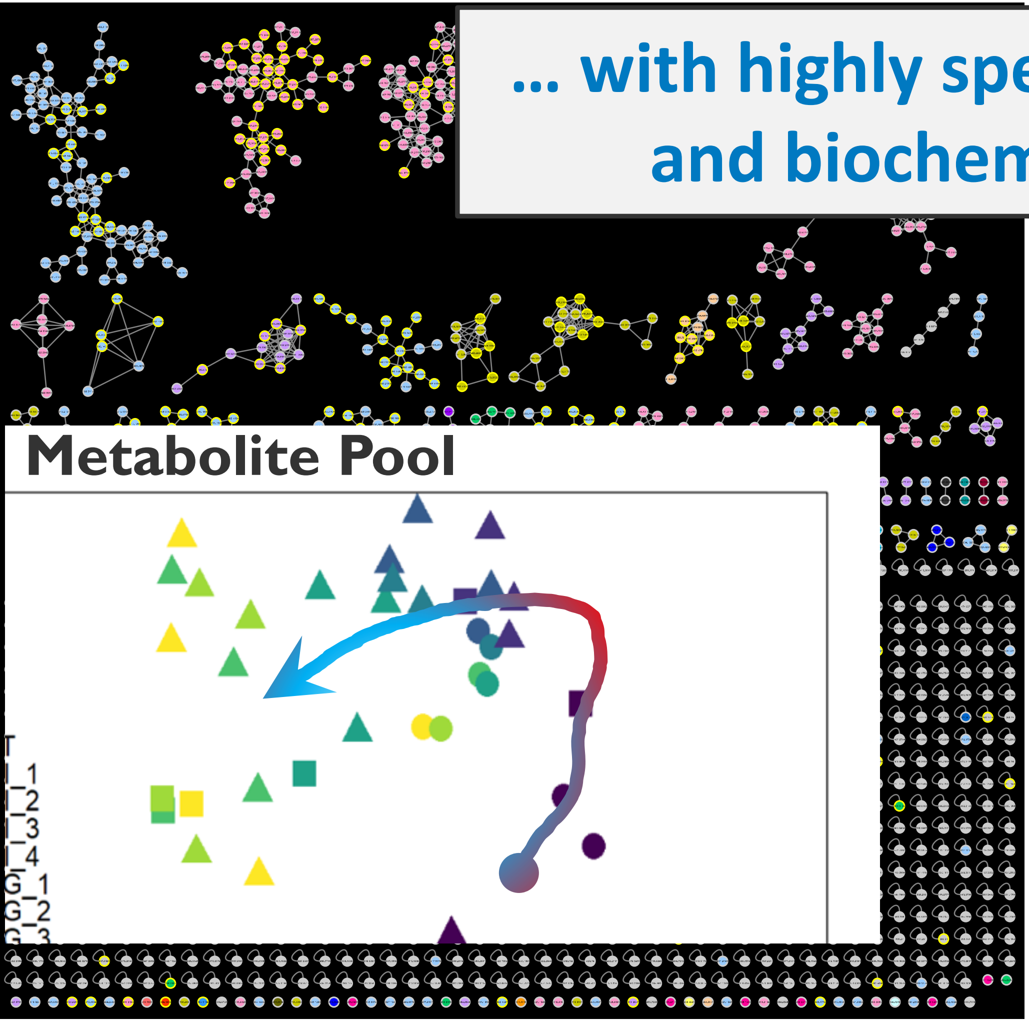
Clostridia

Bacteroidetes

STOMACH FOREGUT HINDGUT

Sparagon, Nelson, *unpublished*

… with highly specialized metabolic and biochemical functions



Gentry, Dorrestein, *unpublished*

Carboxylic acids and derivatives

Prenol lipids

Glycerophospholipids

Fatty acyls

Steroids and derivatives

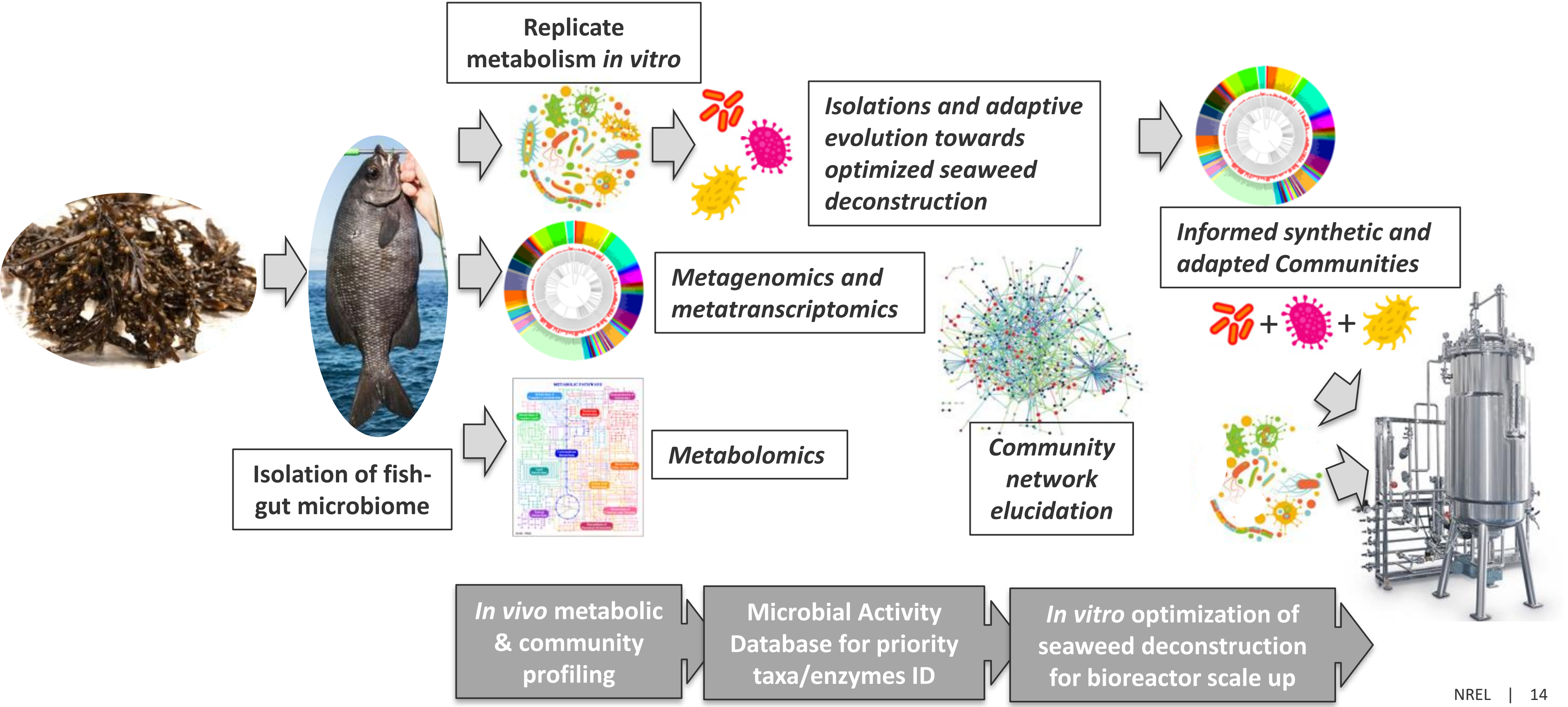
Benzene and substituted derivatives

Sphingolipids

Organooxygen compounds

1847 LC-MS/MS features detected
240 GNPS library hits
13% Annotation rate

Evolution of natural community metabolism toward highly valuable consortia



Isolation of fish-gut microbiome

Replicate metabolism *in vitro*

Metagenomics and metatranscriptomics

Metabolomics

Isolations and adaptive evolution towards optimized seaweed deconstruction

Community network elucidation

Informed synthetic and adapted Communities

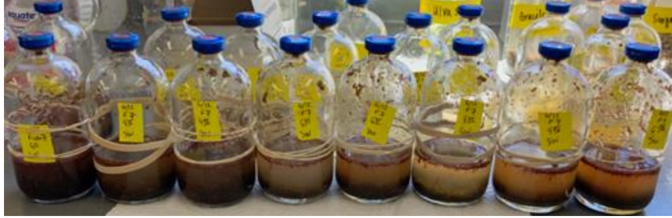
In vivo metabolic & community profiling

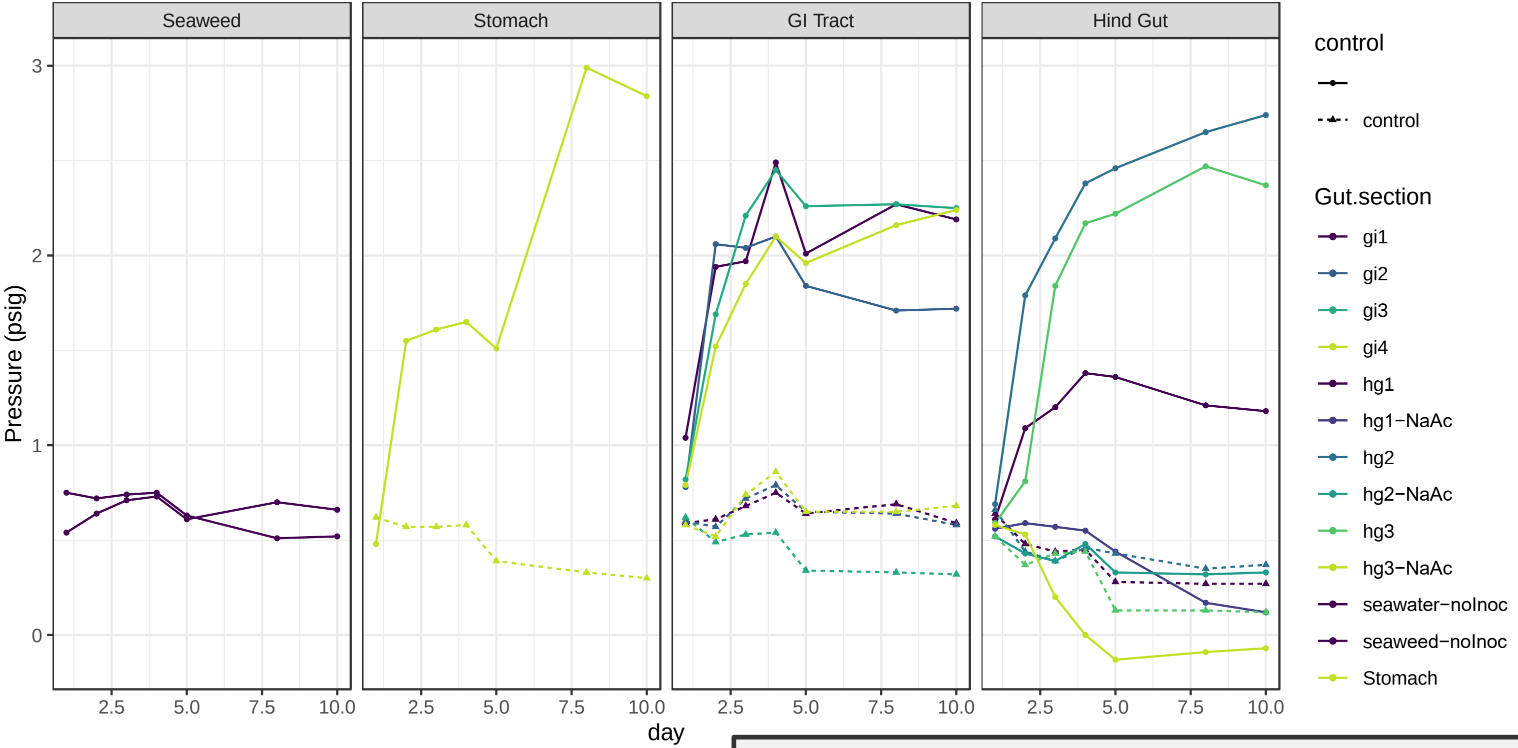
Microbial Activity Database for priority taxa/enzymes ID

In vitro optimization of seaweed deconstruction for bioreactor scale up

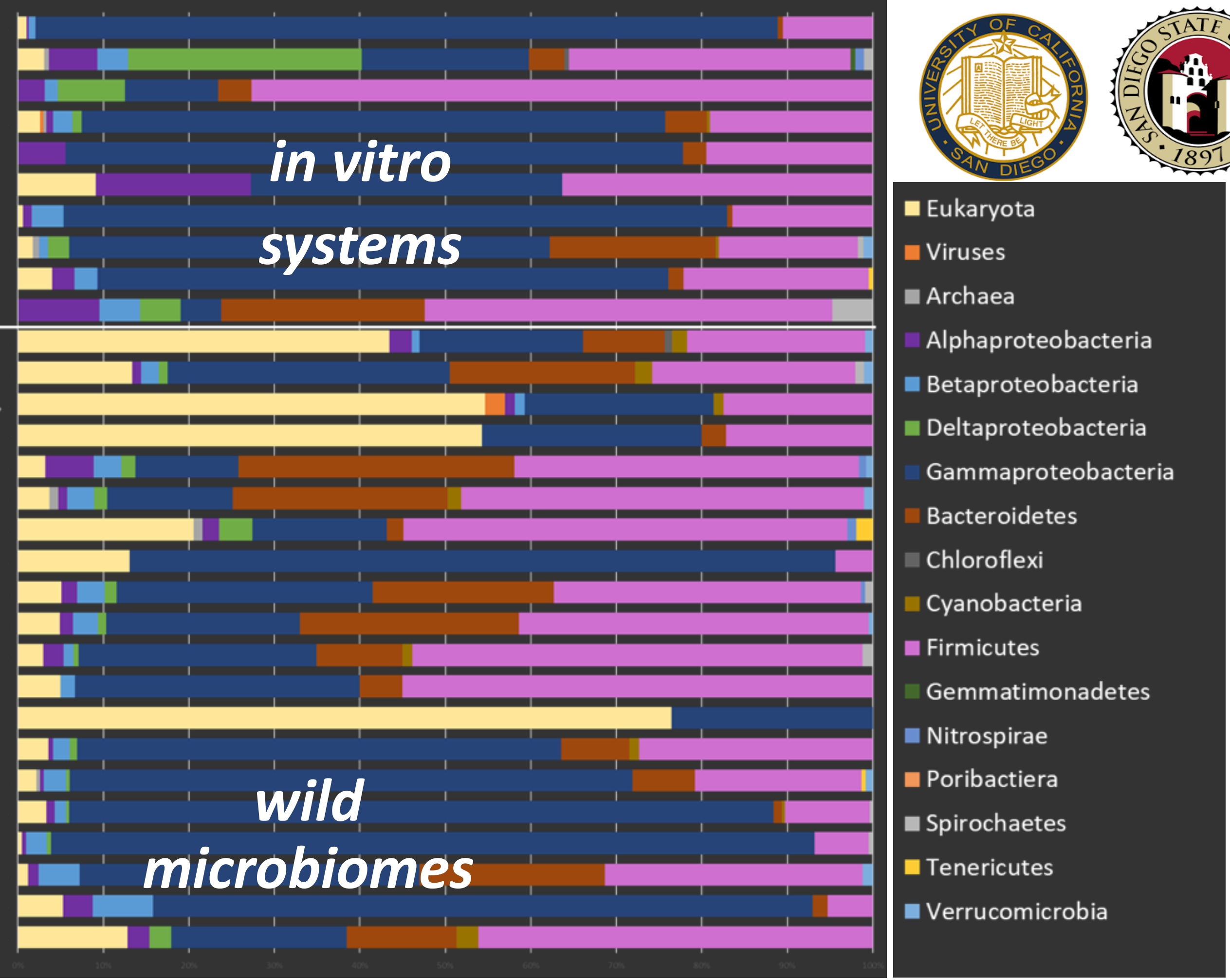
… can we **replicate the microbial conversion** in fully controlled *in vitro* systems?

Rapid and Gut-section-specific microbial activity *in vitro*





… that is seaweed specific



UNIVERSITY OF CALIFORNIA SAN DIEGO

SAN DIEGO STATE UNIVERSITY 1897

In vitro digestions repeatably mimic fish gut microbiome

Assembled genomes for dominant populations will deliver candidate taxa and enzymes involved in complex polysaccharide hydrolysis

Wegley Kelly, Allen, Minich, Sparagon, Nelson, *unpublished*

How do we achieve **commercial impact**?

Commercialization opportunities extend to processing sustainably farmed seaweed

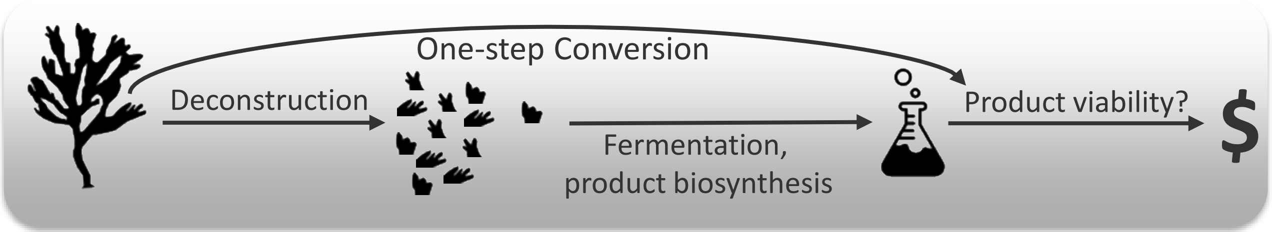
1. Microbial deconstruction of seaweed to more digestible/fermentable substrates with isolated, adapted or synthetic communities
2. Two-stage fermentation (e.g. microbial pretreatment with sequential fermentation), e.g. halophilic lactic acid fermentation
3. Direct production of high-value metabolites from seaweed based on unique microbial synthetic consortium
4. Direct isolation and pretreatment of high-value seaweed polymers

Beyond bioenergy:

- Human food/ingredients
- Feed for aquaculture
- Feed for livestock
- Skin care
- Fertilizer

Conclusions

- *In vitro* propagation of microbiome achieves biomass carbon deconstruction >60% demonstrated, primarily as VFA on par with AD methane production
- Microbiome continuously evolving over experiments, selectivity for substrate improving
- Metagenomic identification of seaweed polymer degradation activities in microbiome
- Commercialization opportunities exist in bioconversion to bioenergy and higher value seaweed-derived ingredients



Questions?

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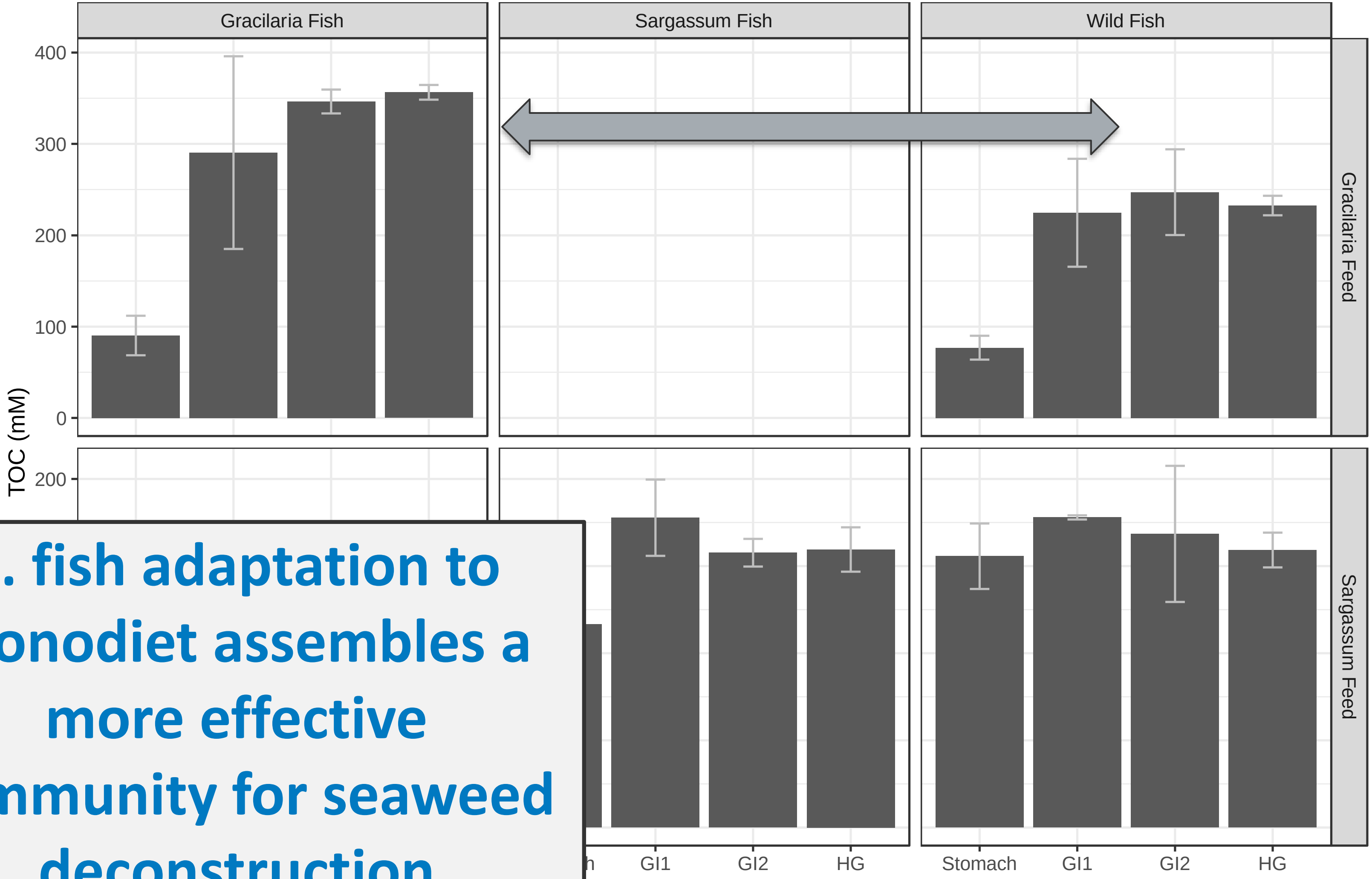
Simona@ocean-era.com

U.S. DEPARTMENT OF ENERGY

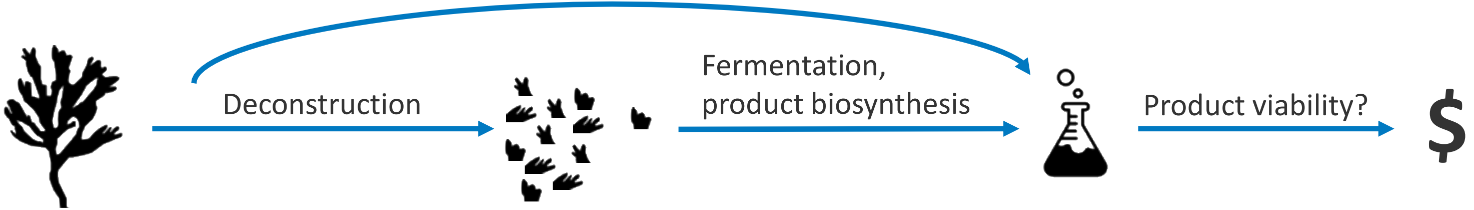
arpa·e
CHANGING WHAT'S POSSIBLE

Additional Slides



… fish adaptation to monodiet assembles a more effective community for seaweed deconstruction

One-step Conversion



Deconstruction

Fermentation,
product biosynthesis

Product viability?

	Deconstruction	Product Biosynthesis	Products
Baseline/reference	Rotting seaweed Known pretreatment/hydrolysis?	Known fermentation organism/inocula: *S. Cerevisiae* | *E. coli* Clostridia Terrestrial AD inoculum	[full fermentation product suite +] Ethanol, butanol, isopentanol [literature] Butyric acid, propionic, valeric, … [literature] Methane (terrestrial inoculum) [literature]
Fish microbiome contribution	Novel enzymatic activities, unique co-culture physiology	Novel isolated organisms, and synthetic microbial consortia, capable of one-step conversion (seaweed-to-product)	Novel products from metabolomics, enhancement of product yield (e.g. methane yield/rate)
Microbiome (metagenome)	Encoded hydrolytic activities, enriched with *in vitro* fermentation	Biosynthetic pathways to novel products	Targets for heterologous overexpression
Metabolome (product ID and quantification)	Polymer deconstruction pathway reconstruction (for known polysaccharides)	Novel products microbially produced *in vivo* that can be replicated in *in vitro* fermentations	Metabolic flux optimization, relative to theoretical yield (e.g. relieve inhibition of methanogenesis)
Metric of success	• Solubilization (g C hr^{-1} g^{-1} seaweed) • Completeness of polymer deconstruction • Particle size reduction • Polymer size distribution (SEC)	• Rate, titer, yield • C conversion efficiency (mol % or wt % basis) to known products • Mass spec ID and quantification of products	• Techno-economic analysis (TEA) • target concentration and purification route (g/L) • Market value/size (x **Ocean Era commercialization interest**)

Isolation of microbial community members indicates selective polysaccharide deconstruction activity

NREL
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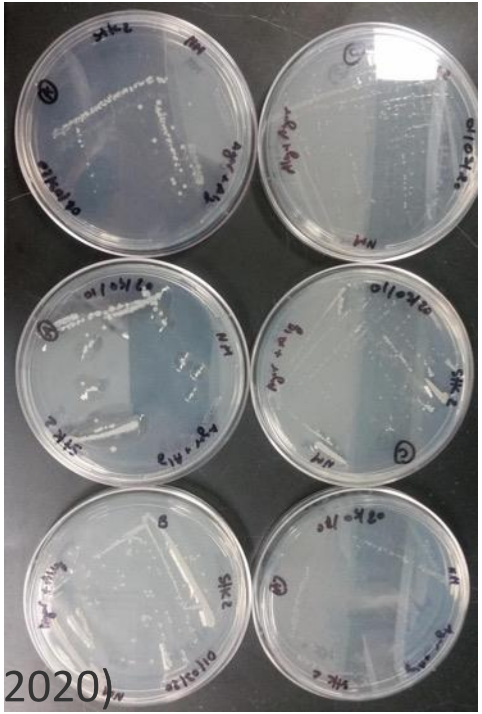
BERKELEY LAB
Lawrence Berkeley National Laboratory

- *Pseudoalteromonas carrageenovora* KCTC 22325
- *Pseudoalteromonas atlantica* ECSMB14104
- *Pseudoalteromonas* sp. BSw20018
- *Cobetia pacifica* strain GPM2
- *Cobetia* sp. strain Aga-AMLN-15-1
- *Cupriavidus necator* strain GX_5
- *Paraglaciecola aestuariivivens* strain JDTF-33
- Uncultured proteobacterium clone SPLON073

Aerobic isolation



➢ Aerobic and anaerobic isolation ongoing from fish gut inocula (June 2020)

Hydrolytic Enzymes	CAZY	Bacterial species
κ-carrageenase (ZgCgkA, ZgCgiA1, ZgCgiA2, ZgCgiA3), β-agarase (ZgAgaA, ZgAgaB, ZgAgaC, ZgAgaD), β-porphyranase (ZgPorA, ZgPorB), α-1,3-(3,6-anhydro)-L-galactosidases (ZgAhgA), Laminarinases (Algal laminarin-specific β-glucanase ZgLamA), Alginate lyases(ZgAlyA4, Endo-guluronate lyase ZgAlyA1, Exo-alginate lyase ZgAlyA5, Alginate lyase ZgAlyA7),	GH16, GH82, GH117, PL6, PL7	*Zobellia galactanivorans* Dsij
ι-carrageenase (CgiA, CgiB)	GH82	*Cellulophaga* sp
β-agarase (AagA	GH16, GH82	*Pseudoalteromonas gracilis* B9
β-agarase	GH16,	*Pseudoalteromonas* sp. AG4
β-agarase AgaG1	GH16,	*Alteromonas* sp. GNUM-1
β-agarase II (AgrA)	GH86	*Pseudoalteromonas atlantica* T6c
Alginate lyases(Poly-mannuronate lyase AlxM)	PL7	*Photobacterium* sp. ATCC 43367
Alginate lyase Aly1	PL7	*Streptomyces* sp. ALG-5
Alginate lyase AlyVI, Poly-MG alginate lyase	PL7	*Vibrio* sp. QY101, *Pseudoalteromonas elyakovii* IAM14594
Alginate lyase Aly-SJ02	PL18	*Pseudoalteromonas* sp. SM0524

Marine Microbiome – Activity characterization

Strain isolation and physiology characterization of carbohydrate utilization and metabolite synthesis

Seaweed microbiome

Genus	family (seaweed)	Oxygen Tolerance
Acinetohacter	Moraxellaceae	Aerobe
Aeromonas	Aeromonadaceae	Facultative Anaerobe
Algoriphagus	Cyclobacteriaceae	Anaerobe
Bacillus	Bacillaceae	Aerobe & Facultative anaerobes
Bacillus	Bacillaceae	Aerobe or Facultative anaerobes
Cellulophaga	Flavobacteriaceae	Aerobe
Corynebacterium	Corynebacteriaceae	Aerobe or Facultative anaerobes
Flammeovirga	Flammeovirgaceae	Aerobe
Flammeovirga	Flammeovirgaceae	Aerobe
Flavobacterium	Flavobacteriaceae	Aerobe or Facultative anaerobes
Glaciecola	Alteromonadaceae	Aerobe
Lewinella	Saprospiraceae	Aerobe
Mesorhizobium	Phyllobacteriaceae	Aerobe
Microbacterium	Microbacteriaceae	Aerobe
Paenibacillus	Paenibacillaceae	Facultative anaerobe
Phaeobacter	Rhobacteraceae	Facultative Anaerobe
Photobacterium, Vibrio	Vibrionaceae	Facultative anaerobes
Pseudoalteromonas	Pseudoalteromonadaceae	Aerobe
Roseobacter	Rhobacteraceae	Aerobe
Staphylococcus	Staphylococcaceae	Facultative anaerobes
Zobellia	Flavobacteriaceae	Aerobe

Martin, Appl Microbiol Biotechnol (2014) 98:2917–2935

Hydrolytic Enzymes	CAZY	Bacterial species
κ-carrageenase (ZgCgkA, ZgCgiA1, ZgCgiA2, ZgCgiA3), β-agarase (ZgAgaA, ZgAgaB, ZgAgaC, ZgAgaD), β-porphyranase (ZgPorA, ZgPorB), α-1,3-(3,6-anhydro)-L-galactosidases (ZgAhgA), Laminarinases (Algal laminarin-specific β-glucanase ZgLamA), Alginate lyases(ZgAlyA4, Endo-guluronate lyase ZgAlyA1, Exo-alginate lyase ZgAlyA5, Alginate lyase ZgAlyA7),	GH16, GH82, GH117, PL6, PL7	*Zobellia galactanivorans* Dsij
ι-carrageenase (CgiA, CgiB)	GH82	*Cellulophaga* sp
β-agarase (AagA	GH16, GH82	*Pseudoalteromonas gracilis* B9
β-agarase	GH16,	*Pseudoalteromonas* sp. AG4
β-agarase AgaG1	GH16,	*Alteromonas* sp. GNUM-1
β-agarase II (AgrA)	GH86	*Pseudoalteromonas atlantica* T6c
Alginate lyases(Poly-mannuronate lyase AlxM)	PL7	*Photobacterium* sp. ATCC 43367
Alginate lyase Aly1	PL7	*Streptomyces* sp. ALG-5
Alginate lyase AlyVI, Poly-MG alginate lyase	PL7	*Vibrio* sp. QY101, *Pseudoalteromonas elyakovii* IAM14594
Alginate lyase Aly-SJ02	PL18	*Pseudoalteromonas* sp. SM0524

Seaweed Composition
Kona/San Diego Seaweeds

Challenges in effective hydrolysis of complex polymers and detection/quantification of novel monomers – complicates deconstruction characterization

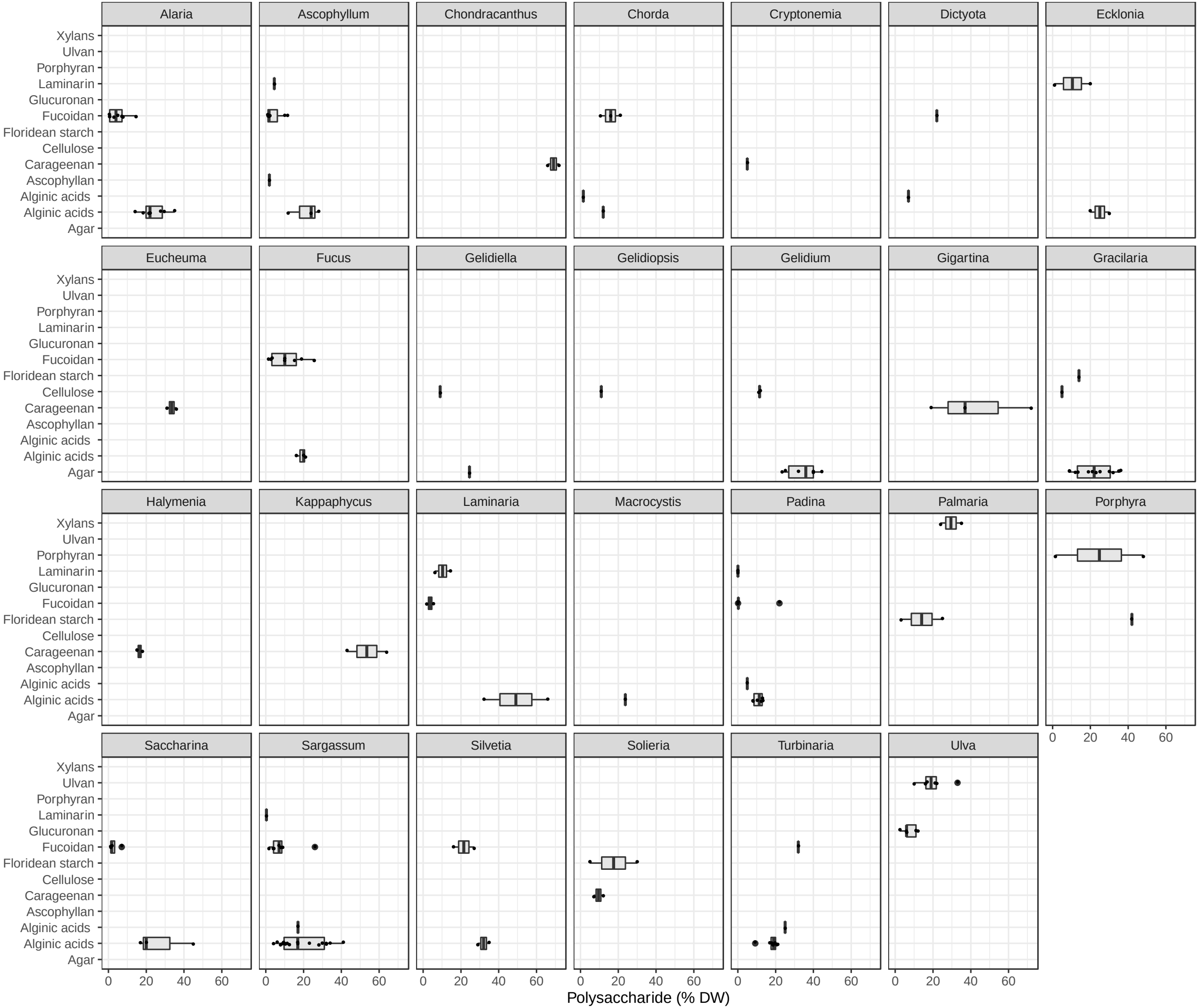
%DW	Ash	FAME	Protein	Carbs	Uronics	Mannitol	Sum
Unknown Red (San Diego, Oct 2019)	28.4	0.9	14.9	33.1	ND	ND	77.3
Sargassum Spp. (San Diego)	30.9 ± 0.4	2.3 ± 0.4	12.9 ± 2.2	9.6 ± 0.6	10.8 ± 0.6	2.9 ± 0.8	69.4 ± 1.4
Macrocystis pyriferia - Bladder	38 ± 0.4	0.8 ± 0.3	3.7 ± 1.7	8.2 ± 0.9	13.5 ± 0.2	20 ± 4.6	**84.2 ± 3.7**
Macrocystis pyriferia - Stipe	31.6 ± 1.7	1 ± 0.3	4.4 ± 2.1	9.9 ± 2.8	17.2 ± 1.7	17.2 ± 2.8	**81.4 ± 3.3**
Macrocystis pyriferia - Blade	32.1 ± 4.2	0.9 ± 0.1	5.6 ± 1.9	10.3 ± 0.2	14.2 ± 0.6	16.1 ± 5.9	**79.2 ± 0.4**
Sargassum and Red Blend	29.1	1.7	14.3	19.8	5.7	1.2	71.9
Seaweed Mix (June 2019)	43.6	1.4	7.3	7.5	1.7	4.5	65.8
Sargasssum (Oct 2018)	27.38	2.02	7.21	12.84	3.7	11.29	64.4
Sargassum (May 2019)	35.1	1.3	3.1	8.4	3.6	11.2	62.7
Sargassum (June 2019)	42.1	1.2	2.9	6.1	4.1	9	65.3
Ulva (Oct 2018)	28.57	1.11	10.67	23.15	<loq	7.42	70.9
Ulva (May 2019)	36.6	1.3	6.4	10.5	<loq	4.6	59.4
Ulva (June 2019)	49.2	0.9	4.4	4.9	<loq	2.9	62.3
Gracilaria (Oct 2018)	33.91	2.21	10.99	25.1	<loq	0.46	72.7
Gracilaria (May 2019)	53	1.6	11.7	6.3	<loq	0.3	72.9
Gracilaria (June 2019)	38.4	1.8	14.6	11.2	<loq	0.4	66.4

Milestone:

✓	Demonstrate analytical pipeline for algae biomass analysis. Characterize macroalgae biomass with respect to baseline protein, lipid, ash and polysaccharide composition for at least three of the primary species of macroalgae (Sargassum, Gracilaria, Ulva). Analyze other native or invasive algae available in lab scale amounts. [NREL, Kampachi, LBNL/JGI] – **Metric = achieve mass balance accounting of >80%**	April 30, 2019

All Seaweed Polysaccharides – wide diversity in published data

- 44 papers, 180 values summarized showing species-specific polysaccharide composition
- 27 genera, 58 species
- Not all reported values are trustworthy (analytical methodology varies significantly)
- Data indicative of which polysaccharides are present, not be used for absolute value referencing



Biomass Deconstruction
Kona #2 sampling

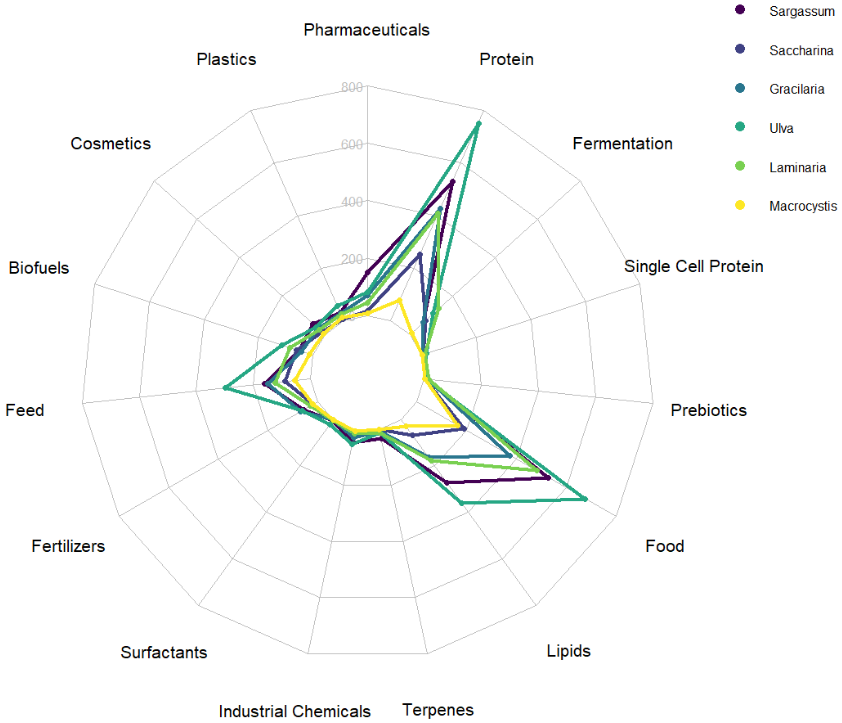
- Monodiet fish microbiome incubated with respective seaweed feed (50 mL scale)
    - ➢ 3 Fish x
    - ➢ 3 diets (Sargassum, Gracilaria, Wild) x
    - ➢ 4 sections x
    - ➢ 2 Feeds (Sargassum, Gracilaria)
- Question: **“Are mono-diet adapted fish microbiomes better at digesting their respective seaweed feed?”**
- Tracking solids deconstruction, total organic carbon and known carboxylates, and gas composition (testing accuracy and precision and interpretation of these metrics)

Phase II High-value Bioproduct Pursuits

- **Microbial breakdown** products of macroalgae
- **Metabolites microbially produced** that have utility as feedstuff, biopolymers, and other industrial products

Options:

1. Deconstruction of seaweed to more digestible/fermentable substrates
2. Two-stage fermentation (e.g. microbial pretreatment with sequential fermentation)
3. Direct production of high-value metabolites from seaweed based on unique microbial synthetic consortium



Literature research and categorization of >10,000 papers:

- Protein sourcing, including single cell protein
- Food/feed formulation
- Lipid isolation (>**1050 unique seaweed lipids**)

Marine Microbiome – Activity characterization

Seaweed microbiome

Genus	family (seaweed)	Oxygen Tolerance
Acinetohacter	Moraxellaceae	Aerobe
Aeromonas	Aeromonadaceae	Facultative Anaerobe
Algoriphagus	Cyclobacteriaceae	Anaerobe
Bacillus	Bacillaceae	Aerobe & Facultative anaerobes
Bacillus	Bacillaceae	Aerobe or Facultative anaerobes
Cellulophaga	Flavobacteriaceae	Aerobe
Corynebacterium	Corynebacteriaceae	Aerobe or Facultative anaerobes
Flammeovirga	Flammeovirgaceae	Aerobe
Flammeovirga	Flammeovirgaceae	Aerobe
Flavobacterium	Flavobacteriaceae	Aerobe or Facultative anaerobes
Glaciecola	Alteromonadaceae	Aerobe
Lewinella	Saprospiraceae	Aerobe
Mesorhizobium	Phyllobacteriaceae	Aerobe
Microbacterium	Microbacteriaceae	Aerobe
Paenibacillus	Paenibacillaceae	Facultative anaerobe
Phaeobacter	Rhobacteraceae	Facultative Anaerobe
Photobacterium, Vibrio	Vibrionaceae	Facultative anaerobes
Pseudoalteromonas	Pseudoalteromonadaceae	Aerobe
Roseobacter	Rhobacteraceae	Aerobe
Staphylococcus	Staphylococcaceae	Facultative anaerobes
Zobellia	Flavobacteriaceae	Aerobe

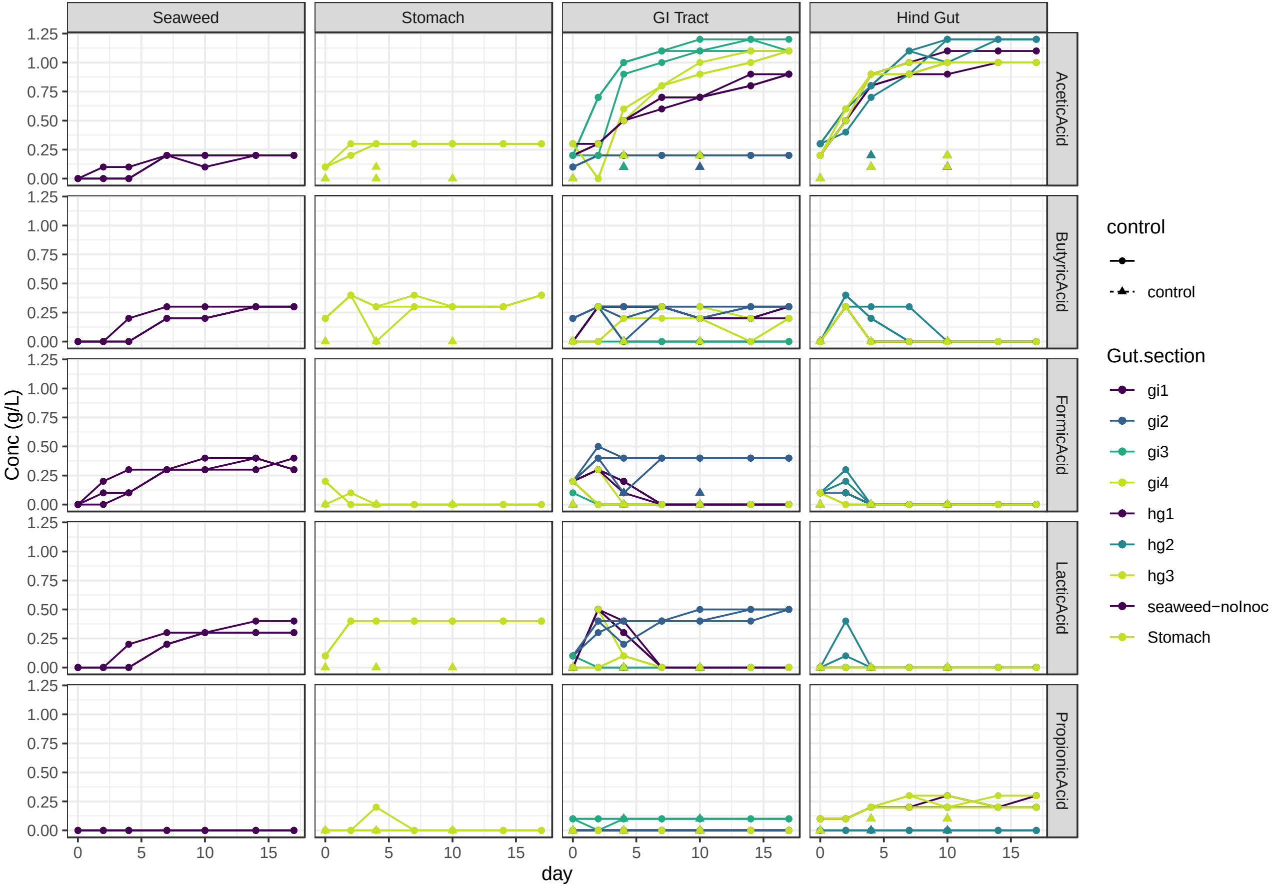
Martin, Appl Microbiol Biotechnol (2014) 98:2917–2935

Fish gut microbiome (literature)

Genus	family (fish-gut)	Oxygen Tolerance
Acholeplasma	Acholeplasma	facultative anaerobes,
Aeromonas	Aeromonadaceae	facultative anaerobe
Agathobaculum	Butyricicoccaceae	Anaerobe
Aliivibrio	Vibrionaceae	facultative anaerobes,
Bacillus	Bacillaceae	aerobe or facultative anaerobe
Bacteroides	Bacteroidaceae	obligate anaerobe
Clostridioides	*Clostridiaceae*	Strictly anaerobic
Clostridium	Clostridiaceae	Anaerobe
Desulfovibrio	Desulfovibrionales	Anaerobic
Faecalibacterium	Ruminococcaceae	Anaerobe
Micrococcus	Micrococcaceae	Aerobe
Mycoplasma	Mycoplasmataceae	aerobic or facultative anaerobic
Paeniclostridium	Clostridiaceae	Anaerobe
Papillibacter	Ruminococcaceae	Anaerobe
Paraclostridium	Peptostreptococcaceae	obligate anaerobe
Photobacterium	Vibrionaceae	aerobe or facultative anaerobe
Plesiomonas		aerobe or facultative anaerobe
Psychromonas	Psychromonadaceae	facultative anaerobes,
Spiroplasma	Spiroplasmataceae	Aerobe or Anaerobe
Streptococcus	Streptococcaceae	facultative anaerobes, or strictly anaerobe
Terrisporobacter	Peptostreptococcaceae	Anaerobe
Vibrio	Vibrionaceae	Facultative Anaerobe

Carboxylates are Substrate and Gut-specific

Sargassum feed (3rd passage)



Gas Composition – CO_2

Sargassum feed (3rd passage)