



Trophic Transfer of Macroalgal Fatty Acids in Two Urchin Species: Digestion, Egestion, and Tissue Building

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Sea urchins are ecosystem engineers of nearshore benthic communities because of their influence on the abundance and distribution of macroalgal species. Urchins are notoriously inefficient in assimilation of their macroalgal diets, so their fecal production can provide a nutritional subsidy to benthic consumers that cannot capture and handle large macroalgae. We studied the assimilation of macroalgal diets by urchins by analyzing the profiles of trophic biomarkers such as fatty acids (FAs). We tracked macroalgal diet assimilation in both *Strongylocentrotus droebachiensis* and *S. purpuratus*. Juvenile *S. droebachiensis* and adult *S. purpuratus* were maintained for 180 and 70 days, respectively, on one of three monoculture diets from three algal phyla: *Nereocystis luetkeana*, *Pyropia* sp., or *Ulva* sp. We then analyzed FA profiles of the macroalgal tissue fed to urchins as well as urchin gonad, gut, digesta, and egesta (feces) to directly evaluate trophic modification and compare nutritional quality of urchin food sources, urchin tissues, and fecal subsidies. In the *S. purpuratus* assay, there were significantly more total lipids in the digesta and egesta than in the algae consumed. The FA profiles of urchin tissues differed among urchin species, all diets, and tissue types. Despite these differences, we observed similar patterns in the relationships between the urchin and macroalgal tissues for both species. Egesta produced by urchins fed each of the three diets were depleted with respect to the concentration of important long chain polyunsaturated fatty acids (LCPUFAs), but did not differ significantly from the source alga consumed. Both urchin species were shown to synthesize and selectively retain both the precursor and resulting LCPUFAs involved in the synthesis of the LCPUFAs 20:4 ω 6 and 20:5 ω 3. *S. droebachiensis* and *S. purpuratus* exhibited consistent patterns in the respective depletion and retention of precursor FAs and resulting LCPUFAs of *Pyropia* and *Ulva* tissues, suggesting species level control of macroalgal digestion or differential tissue processing by gut microbiota. For both *S. droebachiensis* and *S. purpuratus*, macroalgal diet was a surprisingly strong driver of urchin tissue fatty acids; this indicates the potential of fatty acids for future quantitative trophic estimates of urchin assimilation of algal phyla in natural settings.

Keywords: fatty acids, biomarkers, trophic ecology, egesta, macroalgae, trophic modification, PUFA

INTRODUCTION

Lipids are dense sources of metabolic energy that provide building blocks of cellular membranes and are involved in intracellular signaling and localized hormone control (Christie and Han, 2010). Fatty acids (FAs) from multiple lipid classes are integral in membrane function, energy storage, and many physiological processes (Arts et al., 2009). Fatty acids that cannot be synthesized by animals *de novo* in biologically relevant amounts and must be obtained from dietary sources are generally referred to as “essential fatty acids” (EFAs; Arts et al., 2001). Marine primary producers, particularly macroalgae and phytoplankton, are rich sources of lipids including polyunsaturated fatty acids (PUFAs) that are important for growth and reproduction of marine organisms (Dalsgaard et al., 2003; Wacker and Von Elert, 2004). Specifically, long chain polyunsaturated fatty acids (LCPUFAs, more than 20 carbon atoms), many of which are considered to be EFAs for consumers, have been identified as a key dietary component supporting growth and have been used to evaluate food quality (Sargent et al., 1999; Bell and Sargent, 2003; Winder et al., 2017).

Many FAs have been used as dietary biomarkers or tracers to explore trophic relationships in aquatic, pelagic, and benthic consumers (reviewed by Dalsgaard et al., 2003; Müller-Navarra, 2008; Kelly and Scheibling, 2012). However, dietary FAs are often modified by the consumer, either by elongation, desaturation, or *de novo* synthesis of FAs from precursors present in dietary tissues (Cook et al., 2007; González-Durán et al., 2008; Brett et al., 2016). Our understanding of FA trophic modification by marine invertebrates is still developing, and sea urchins are an interesting case study for this question because they have been studied for the sake of aquaculture (Castell et al., 2004). Additionally, when a certain EFA is limited in food sources, many consumers, including sea urchins, can selectively store the EFAs for later use, and as a result the FAs observed in consumer tissues often do not directly reflect their food sources (Castell et al., 2004; González-Durán et al., 2008).

Organismal FA composition can also vary seasonally and spatially, complicating the use of FA as biomarkers (Hughes et al., 2005; Dethier et al., 2013). FA profile variation in producers and consumers may be influenced by local abiotic conditions such as nutrient availability, temperature, salinity, and light (Galloway and Winder, 2015). However, more variation in FA profiles is explained by phylogeny, reflecting relatedness, than by abiotic conditions in aquatic ecosystems (Galloway and Winder, 2015). Because multivariate FA profiles can be reliably associated with a variety of primary producers and consumers, the use of FAs as biomarkers is supported despite local seasonal and site-specific variation (Galloway et al., 2012, 2013; Kelly and Scheibling, 2012; Dethier et al., 2013).

Sea urchins are ecologically and economically important organisms that can exert a strong influence on community composition primarily through grazing pressure (Steneck et al., 2002; Pearse, 2006). Urchins are known to transform significant amounts of kelp-derived biomass into particulate organic matter (e.g., Koike et al., 1987) through their fecal production (Sauchyn and Scheibling, 2009; Sauchyn et al., 2011), which enhances

growth of associated microbes. Moreover, urchins are notoriously inefficient assimilators of their diet (Vadas, 1977), such that a large portion (e.g., 40–80%) of the macronutrients from *Strongylocentrotus droebachiensis* algal diets are left intact in the resulting feces (Mamelona and Pelletier, 2005). Urchin trophic modification of macroalgal tissue may represent a trophic hub between benthic and pelagic systems (Hughes et al., 2011). Urchins can produce tissues of high nutritional quality, as measured by content of key FAs, as a subsidy to larval pelagic and benthic consumers (direct predation on gonad tissue) while generating lower quality tissues for benthic detritivores (fecal material; Hughes et al., 2011).

Urchin gonad material (uni) is an important fishery (Andrew et al., 2003). Aquaculture studies have extensively investigated ratios of lipids in urchin diets that maximize the incorporation of essential FAs into gonad production and growth (Castell et al., 2004; Cook et al., 2007) for the sake of improved uni production. In pursuit of ideal FA ratios in diets, researchers have noted that urchins are capable of *de novo* synthesis of novel FAs, including non-methylene interrupted dienes (NMID; Takagi et al., 1980; Castell et al., 2004; González-Durán et al., 2008). This capability exists in multiple species of urchin including *S. droebachiensis* and is likely the result of $\Delta 5$ desaturase activity, which is also involved in the synthesis of arachidonic acid (ARA, 20:4 ω 6) from 20:3 ω 6 and of eicosapentaenoic acid (EPA, 20:5 ω 3) from 22 carbon chain monounsaturated fatty acids (MUFAs; Takagi et al., 1980).

To better understand dietary FA trophic transfer in urchins and the universality of the role that urchin digestion of macroalgae plays in either enhancing or depleting the nutritional quality of the material egested, we performed controlled laboratory feeding assays with two species of urchin, *S. droebachiensis* and *S. purpuratus*. *S. droebachiensis* experiments were conducted with newly settled juveniles, and *S. purpuratus* experiments used field-collected adults. We addressed four primary objectives: (1) Assess similarities and differences in the total lipid and FA content of tissues of urchins maintained on controlled algal diets; (2) Evaluate whether urchins alter the nutritional quality of their own tissue and of egested material by quantifying the concentrations of total lipid and long-chain polyunsaturated fatty acid (LCPUFA) of gonad, gut, alga consumed, digesta, and egesta tissues; (3) Evaluate if the urchins selectively retain LCPUFAs and their precursors; (4) test whether urchin tissue FAs can reliably be used to differentiate phylum level differences in their known algal diets.

MATERIALS AND METHODS

Strongylocentrotus droebachiensis Feeding Assays

In autumn 2016, 240 recently settled *S. droebachiensis* (1.95 \pm 0.19 mm, mean test diameter \pm SD) resulting from *in vitro* breeding at Friday Harbor Laboratories (FHL: 48°32.7'N, 123°0.8'W) were raised on monoculture macroalgal diets for 180 days (September 2016–March 2017). Multiple juvenile urchins ($N = 15$) were maintained in replicate ($N = 4$ per diet treatment)

clear, lidless, flow-through plastic bins (20 × 20 cm). Bins were kept in seawater tables at FHL on San Juan Island, WA using ambient seawater from Friday Harbor. Replicate bins were randomly assigned to one of three diet treatments, representing a species from Ochrophyta (*Nereocystis luetkeana*—*Nereocystis* throughout), Rhodophyta (*Pyropia* sp.—*Pyropia* throughout), or Chlorophyta (*Ulva* sp.—*Ulva* throughout). These algae are regularly available to urchins as attached benthic species or as drift in the intertidal and shallow subtidal, and their fatty acid (FA) signatures have clear phylogenetic resolution at the phylum level (Galloway et al., 2012). Algae were collected from the FHL property, either as drift (*Nereocystis*) or growing in the intertidal (*Ulva* and *Pyropia*). During the experimental period, seawater temperature declined (13–8°C) and there were some fluctuations in salinity (25–30 ppt) at the algal collection sites. Urchins were fed *ad libitum*; uneaten food was removed several times a week as needed and bins were cleaned regularly to prevent waste accumulation and diatom growth. Egesta were collected weekly and immediately stored at –20°C for later FA analyses. At the termination of the experimental period, urchins were euthanized and dissected to isolate urchin gonad, gut, and digesta (Hakim et al., 2015).

***Strongylocentrotus purpuratus* Feeding Assays**

Forty-two relatively small, but unknown age, adult *S. purpuratus* (5.5 ± 0.6 cm, mean test diameter ± SD), were collected by hand from tide pools at Cape Arago, Oregon (43°20′ 24″ N, 124°19′48″ W) in August 2016. Urchins were collected from two low intertidal pools with similar temperature (13.1 and 13.0°C) and salinity (30.2 and 30.6 ppt). Urchins were immediately transported to the Oregon Institute of Marine Biology (OIMB) and haphazardly assigned individually to 4.7 L flow-through containers filled with 1 μm-filtered seawater. Filtered seawater was successfully used to impede diatom growth in the experimental chambers, which could have confounded the controlled feeding experiment if the urchins grazed on this alternative resource. Each container had both an airstone and constant filtered seawater flow. Urchins were given 2 weeks to purge their guts to remove remnants of previous diets and to acclimatize to laboratory conditions prior to the start of experimental feeding trials. During this period, fecal material was removed daily to avoid fouling. At the end of the acclimatization period, each urchin was randomly assigned to a *Nereocystis*, *Pyropia*, or *Ulva* diet treatment. A representative subset of the urchins maintained for the acclimatization period were dissected at the end of the acclimatization period to determine the amount of gonad tissue present at the start of the experiment.

Urchins were fed 6.0 g (± 0.1 g) of their designated alga every other day. Fresh macroalgae were collected weekly from OIMB property, either from a nearshore *Nereocystis* bed or intertidally (*Pyropia* and *Ulva*). The macroalgal collection site is adjacent to the seawater intake for OIMB and experiences fluctuations in seawater temperature (12–14°C) and salinity (30–33 ppt). Macroalgae were maintained in ambient flow-through seawater tables in the lab prior to presentation to urchins. No

macroalgae were kept for longer than 1 week, as aging has been shown to affect kelp FA (Raymond et al., 2014). To prepare macroalgae, thalli were rinsed briefly in freshwater to dislodge mobile epibionts, e.g. amphipods, gastropods, or isopods, then inspected for sessile epibionts, which were removed if present. Cleaned macroalgae were spun dry and briefly blotted before one final inspection immediately prior to recording wet weights. Before new food was added to urchin containers, egesta (fecal material) and uneaten macroalgal thalli were removed. At three time points over the course of the 70 day experimental period, we collected and rinsed macroalgal thalli and egesta for later FA extraction and analysis. Adult *S. purpuratus* were euthanized and dissected as described for *S. droebachiensis*.

Fatty Acid Analysis

At the termination of the 180 and 70 day experimental periods, *S. droebachiensis* and *S. purpuratus* (respectively) were carefully dissected to isolate gonad, gut, and gut contents (digesta) from all other tissues. The same analysts performed all extractions on both species to maintain methodological consistency. Isolated tissues were briefly rinsed with freshwater and drained before placing samples in labeled plastic microcentrifuge tubes and frozen at –20°C. All tissues collected for total lipid and FA analysis were freeze dried for a minimum of 48 h (or until tissues were dry) within 3 weeks of collection. Following freeze-drying, each tissue type was carefully homogenized and extracted for total lipids as described by (Parrish, 1999). Briefly, homogenized tissue samples were digested for a minimum of 12 h in 2 ml chloroform and then sonicated, vortexed, and centrifuged three times in a 4:2:1 chloroform:methanol:0.9% NaCl solution. Following each of these cycles, the chloroform layers containing organics were removed and pooled. From this pooled solution, subsamples were removed for total lipid analysis by gravimetry (Kainz et al., 2017). The pooled chloroform solutions containing the organics were evaporated to dryness under N₂ flow and the organics were re-suspended in a toluene and 1% sulfuric-acid methanol solution for 16 h at 50°C to transesterify FA methyl esters (FAME). Following transesterification, FAME mixtures were allowed to cool before neutralizing the acid with 2% KHCO₃. The neutralized FAME solution was then diluted with 5 ml of hexane, and the mixture was briefly vortexed and centrifuged before carefully removing the FAME layer. The hexane addition, vortex, centrifugation and removal was repeated once more. Following the second addition of hexane, the pooled hexane solution was evaporated to dryness under N₂ flow and the FAME were re-dissolved in 1.5 ml hexane in 2 ml glass vials for gas chromatography.

FAME were analyzed using a gas chromatograph equipped with a mass spectrometer (GC-MS, Model QP2020, Shimadzu) with a DB-23 column (30 × 0.25 mm × 0.15 μm, Agilent, Santa Clara, CA, USA) using helium as a carrier gas. A split/splitless injector was used to inject 1 μl of sample for analysis. The following heating program, based on Taipale et al. (2013), was used: 50°C was maintained for 1 min, then the temperature was increased at a rate of 10°C min⁻¹ to 140°C, after which the warming rate was adjusted to 1.40°C min⁻¹ to 190°C and held for 3 min, followed by 5°C min⁻¹ to 220°C, and finally

heated at $13^{\circ}\text{C min}^{-1}$ to 240 and held for 2 min. The FA were identified from relative retention times and specific ions and then quantified using peaks of the major ions (Taipale et al., 2016). Concentrations of FAs were calculated based on calibration curves (Pearson correlation coefficient ≥ 0.99) of serial dilutions of known standard FAME mixtures (GLC standard mixture 566C, Nu-Chek Prep, Elysian, MN, USA). The FA concentrations were then standardized against the dry weight of the tissue extracted to obtain the concentrations in $\mu\text{g mg}^{-1}$ dry weight. FA proportions represent the percentage of the sum of the total weight of FAs identified.

Statistical Analysis

We used the Shapiro–Wilks test to evaluate the normality of the total lipid content of gonad, gut, alga, digesta, and egesta tissues from both feeding assays. We then separately analyzed the total lipid of the alga tissue fed to *S. droebachiensis* and to *S. purpuratus* to evaluate differences in monoculture diets on which urchins were maintained, using a 2-way (diet \times urchin species/location) analysis of variance (ANOVA, $\alpha \leq 0.05$) on untransformed data in R (R Core Team, 2015). Additionally, we used a 2-way ANOVA (diet \times tissue) to evaluate the total lipid content based on diet consumed and tissue analyzed.

To evaluate the multivariate FA profiles, we used multiple routines in Primer v6.1.13 (Clarke and Gorley, 2006) with the PERMANOVA+ v.1.0.3 add-on (Anderson et al., 2008). Differences in the untransformed FA profiles of *S. droebachiensis* and *S. purpuratus* tissues were analyzed using a 3-way factorial permutational multivariate analysis of variance (PERMANOVA, $\alpha \leq 0.05$), with 9999 permutations with Type III sums of squares, mixed factors, and Euclidian distance for all tissues. The results were not sensitive to arcsine square-root transformations of FA data; we therefore report the untransformed results. To visualize the relationships between urchin species, diets, and tissue types we used non-metric multidimensional scaling (nMDS) plots. To identify the FAs contributing to differences between species, diets, and tissues we calculated the similarity percentage (SIMPER). Vector overlays of the FA identified by SIMPER analyses were then mapped onto a subset of the nMDS graphics to visualize relationships between diets for the gonad and gut (collectively referred to throughout as urchin tissues) and diet alga, digesta, and egesta (collectively referred to throughout as macroalgal tissues).

To evaluate the nutritional quality differences in tissues analyzed, we used a 2-way ANOVA of the Σ LCPUFAs, defined here as the sum of arachidonic (ARA, 20:4 ω 6), eicosapentaenoic (EPA, 20:5 ω 3), and docosahexanoic (22:6 ω 3) acid (Arts et al., 2001; Hughes et al., 2011). We also used 2-way ANOVAs to evaluate the mean content of EPA and ARA and precursor FAs including α -linoleic acid (LIN, 18:2 ω 6) and combined α -linolenic acid (ALA, 18:3 ω 3) and stearidonic acid (SDA, 18:4 ω 3) in urchin and macroalgal tissues.

RESULTS

Based on the increase in test diameter (mm), juvenile *S. droebachiensis* grew an average of $873 \pm 195\%$ over 180 days,

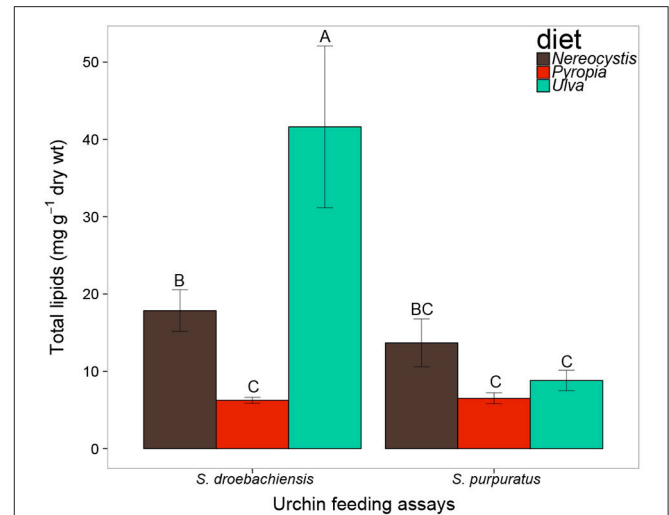
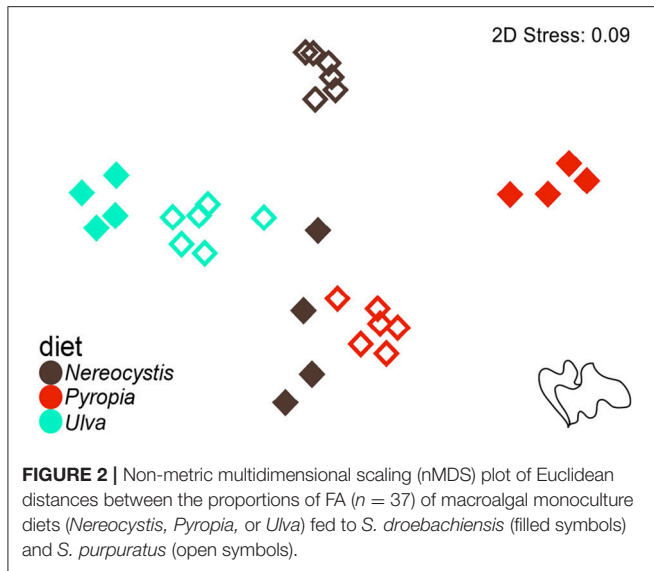


FIGURE 1 | Total lipid (mg g^{-1} dry tissue weight) of macroalgae fed to *S. droebachiensis* and *S. purpuratus*. Bars with the same letter are not significantly different (2-way ANOVA, Tukey HSD, $p < 0.05$). Concentrations represent mean \pm standard deviation, (SD).

to an average test diameter of 19 ± 4 mm (mean \pm standard deviation, SD) and an average wet weight of 2.77 ± 1.38 g. Growth of *S. droebachiensis* was extensive due to the fact that initial *S. droebachiensis* were newly settled larvae at the start of the experiment (initial test diameter 1.95 ± 0.19 mm). *S. purpuratus* also grew in their experiment, resulting in an average test size of 5.9 ± 0.6 cm, a total of $4.8 \pm 6.9\%$ change in diameter, and a $13.5 \pm 9.5\%$ increase in the mean wet weights across all diet treatments with a final mean gonad index of 0.12 ± 0.03 . The mean gonad index of *S. purpuratus* dissected at the beginning of the experiment was 0.02 ± 0.01 (gonad wet weight: whole body wet weight), resulting from the presence of gonad material in 8 of the 10 urchins dissected. A total of 37 FAs were identified in *S. droebachiensis* and *S. purpuratus* tissues and their associated diets, digesta, and egesta, with 32 FAs contributing $\geq 1\%$ to any of the tissues analyzed (Supplementary Tables 1–6). The total lipids extracted from the algae fed to urchins at both locations exhibited significant differences based on macroalgal species (2-way ANOVA, $F = 31.52$, $df = 2$, $p < 0.001$), location ($F = 63.57$, $df = 1$, $p < 0.001$), and the interaction between diet algae and location ($F = 40.32$, $df = 2$, $p < 0.001$). The most total lipid was available in *Ulva* tissues fed to *S. droebachiensis* at FHL and the least total lipid was available in *Pyropia* fed to *S. droebachiensis* as well as *Pyropia* and *Ulva* fed to *S. purpuratus* (Figure 1). The FA contents of algae consumed by *S. droebachiensis* and *S. purpuratus* were more similar for the *Ulva* fed to urchins in both experiments, with greater separation between the FA profiles of the *Nereocystis* and *Pyropia* consumed (Figure 2).

The total lipids in tissues of juvenile *S. droebachiensis* were not significantly different based on urchin diet (Table 1, 2-way ANOVA, $F = 1.02$, $df = 2$, $p = 0.37$) and there was no interaction between urchin diet and total lipids in tissues ($F = 2.06$, $df = 8$, $p = 0.55$) but there were lower levels of total lipid in the



macroalgal tissues compared to the urchin tissues ($F = 19.03$, $df = 4$, $p < 0.001$). In contrast, the tissues of adult *S. purpuratus* differed in total lipid based on urchin diet (Table 1, 2-way ANOVA, $F = 6.17$, $df = 2$, $p < 0.001$) and tissue type tested ($F = 236.77$, $df = 4$, $p < 0.001$) with no interactions between these factors ($F = 1.51$, $df = 8$, $p = 0.17$). For *S. purpuratus*, tissues from urchins fed *Pyropia* differed from those fed *Nereocystis* (Tukey HSD, $p < 0.001$), and the total lipids in the macroalgal tissue were lower than the digesta, gut, and gonad tissues (Tukey HSD, $p < 0.001$), but not different from the egesta (Tukey HSD, $p = 0.08$). There also were no differences between the total lipids of the egesta compared to the digesta (Tukey HSD, $p = 0.98$) or the gut compared to the gonad (Tukey HSD, $p = 0.77$).

The multivariate FA profiles of both urchin species were significantly different for all three factors tested (urchin species, macroalgal diet, urchin tissue type) and there were significant interactions between all factors (Table 2). SIMPER analysis (Table 3) identified FAs contributing to the differences between urchin species. There were high proportions of 16:0 and 20:5 ω 3 in both urchin species (Table 3). However, *S. droebachiensis* were characterized by 16:1 ω 7, 18:3 ω 3, and 16:4 ω 3 while the distinguishing FAs for *S. purpuratus* were 18:4 ω 3, 18:1 ω 7, and 14:0. We found 20:2 Δ 5,11 NMID in the urchin gut and gonad tissues of both urchin species but this FA was not detected in the algae consumed or the associated digesta and egesta (Supplementary Tables 1–6). Despite significantly different FA profiles among urchins, both exhibited similar distributional patterns with respect to the relative relationships between the urchin and macroalgal tissues (Figures 3, 4).

The gut and gonad from each species tended to group together by dietary treatment, and tissues from *S. droebachiensis* (Figure 3A) are grouped tighter than tissues from *S. purpuratus* (Figure 4A). Because there were significant differences between urchin species, we performed additional SIMPER analyses separately for *S. droebachiensis* and *S. purpuratus* to identify the

TABLE 1 | Total lipid (mean \pm SD) of tissues from *S. droebachiensis* and *S. purpuratus* feeding assays.

Diet	Tissue	Total lipids		
		(mg g ⁻¹ dry wt)		
		N	Mean	SD
<i>S. droebachiensis</i>				
<i>Nereocystis</i>	gonad ^A	3	319.9	48
	gut ^A	4	228.1	55.3
	alga ^B	4	17.8	2.7
	digesta ^B	4	36.3	6.4
	egesta ^B	4	23.6	6.5
<i>Pyropia</i>	gonad ^A	3	299.4	111.3
	gut ^A	4	283.6	93.1
	alga ^B	3	6.2	0.4
	digesta ^B	4	60.3	23.8
	egesta ^B	4	34.4	8.9
<i>Ulva</i>	gonad ^A	3	155.1	37.3
	gut ^A	4	317.6	232.6
	alga ^B	4	41.6	10.5
	digesta ^B	4	93.3	65
	egesta ^B	4	40.5	16.5
<i>S. purpuratus</i>				
<i>Nereocystis</i> ^a	gonad ^c	6	220.9	40.9
	gut ^c	6	264.4	52.5
	alga ^d	5	13.7	3.1
	digesta ^e	6	58.4	19.9
	egesta ^{de}	5	40.6	9.9
<i>Pyropia</i> ^b	gonad ^c	5	242	52.9
	gut ^c	5	251.6	42.6
	alga ^d	6	6.5	0.7
	digesta ^e	6	34	11.3
	egesta ^{de}	6	35.7	5.9
<i>Ulva</i> ^{ab}	gonad ^c	6	244.4	51.3
	gut ^c	5	214.5	34.7
	alga ^d	6	8.8	1.3
	digesta ^e	5	42.3	14.2
	egesta ^{de}	4	38.1	8

Letters indicate significant differences (Tukey HSD, $p \leq 0.05$) and for the *S. purpuratus* results, letters a,b indicated significant differences based on diet and letters c–e indicated differences based on tissue.

FAs characterizing diets and tissues (Figures 3, 4, Tables 4, 5; Supplementary Tables 7–11). Both 16:0 and 20:5 ω 3 were present in high proportions in all three diets for both urchin assays (Table 4). There was more overlap between the FA profiles of *S. droebachiensis* and *S. purpuratus* fed *Nereocystis* compared to those fed *Pyropia* or *Ulva* (Figures 3A, 4A). The urchin and macroalgal tissues associated with the *Ulva* diet were separated from the other diets by 16- and 18- ω -3 PUFAs (Figures 3, 4, Table 4).

Urchin tissues were dominated by many of the same FAs that were prominent contributors to the *Nereocystis*, *Pyropia*, and *Ulva* diets, particularly the presence of 20:5 ω 3 (Figures 3B, 4B,

TABLE 2 | Results of PERMANOVA analysis of proportions of identified FA contributing $\geq 1\%$ of identified FA for *S. droebachiensis* and *S. purpuratus* (data untransformed, Euclidean distance) in a 3-way design with factors of species, diet, and tissue.

Source	df	MS	Pseudo-F	P(perm)	Unique permutations
Species	1	1499.3	37.303	0.0001	9934
Diet	2	7525.6	165.48	0.0001	9930
Tissue	4	7662.8	1121.7	0.0001	9942
Species*Diet	2	1618	39.197	0.0001	9912
Species*Tissue	4	779.24	23.983	0.0001	9912
Diet*Tissue	8	1134.6	37.899	0.0001	9878
Species*Diet*Tissue	8	632.63	22.631	0.0001	9899
Residual	118	31.31			
Total	147				

Analyses used fixed effects, with Type III sums of squares 9999 permutations of data residuals to determine significance. Significant differences ($p < 0.05$) are indicated in bold.

TABLE 3 | SIMPER results for analysis of combined FA of tissues from *S. droebachiensis* ($N = 12$) and *S. purpuratus* ($N = 18$).

Species	FA	Mean FA %	Cont (%)
<i>S. droebachiensis</i>	16:0	26.10	28.01
	20:5 ω 3	14.40	27.48
	16:1 ω 7	7.13	10.04
	18:3 ω 3	4.05	5.95
	16:4 ω 3	2.51	3.68
<i>S. purpuratus</i>	16:0	24.60	39.07
	18:4 ω 3	4.78	10.19
	20:5 ω 3	10.50	9.64
	18:1 ω 7	5.12	5.76
	14:0	7.23	5.15

The top five FA contributing to the FA profiles based on species are shown along with the mean proportion relative to the total FA (Mean FA %) and their contribution to the similarity within species (Cont %).

Table 5), which was found in high proportions in all urchin and macroalgal tissues investigated (**Table 5**). The presence of 20:5 ω 3 and 20:1 ω 9 were important in distinguishing *Pyropia*-derived tissues from urchin tissues from other macroalgal treatments, particularly gonad, gut, as well as *Pyropia*-alga tissues (**Figures 3, 4**). The SIMPER analysis identified 18:3 ω 3 as a prominent component of *Nereocystis* tissue (**Table 5**), which separated *Nereocystis* from the other macroalgal tissues (**Figures 3C, 4C**).

Overall, the LCPUFAs in *S. droebachiensis* tissues exhibited similar trends to those observed in *S. purpuratus* tissues (**Figure 5**). The LCPUFAs in gut and gonad tissues were higher than in the macroalgal, digesta, or egesta tissues (**Figure 5**). There were significant differences in the concentration of LCPUFAs in the tissues from *S. droebachiensis* assays as a result of diet (2-way ANOVA, $F = 21.70$, $df = 2$, $p < 0.001$) and tissue ($F = 70.83$, $df = 4$, $p < 0.001$), and there was an interaction between these factors ($F = 11.38$, $df = 8$, $p < 0.001$). The LCPUFAs were higher (Tukey HSD, $p < 0.05$) in the gonad tissue of *S. droebachiensis* fed

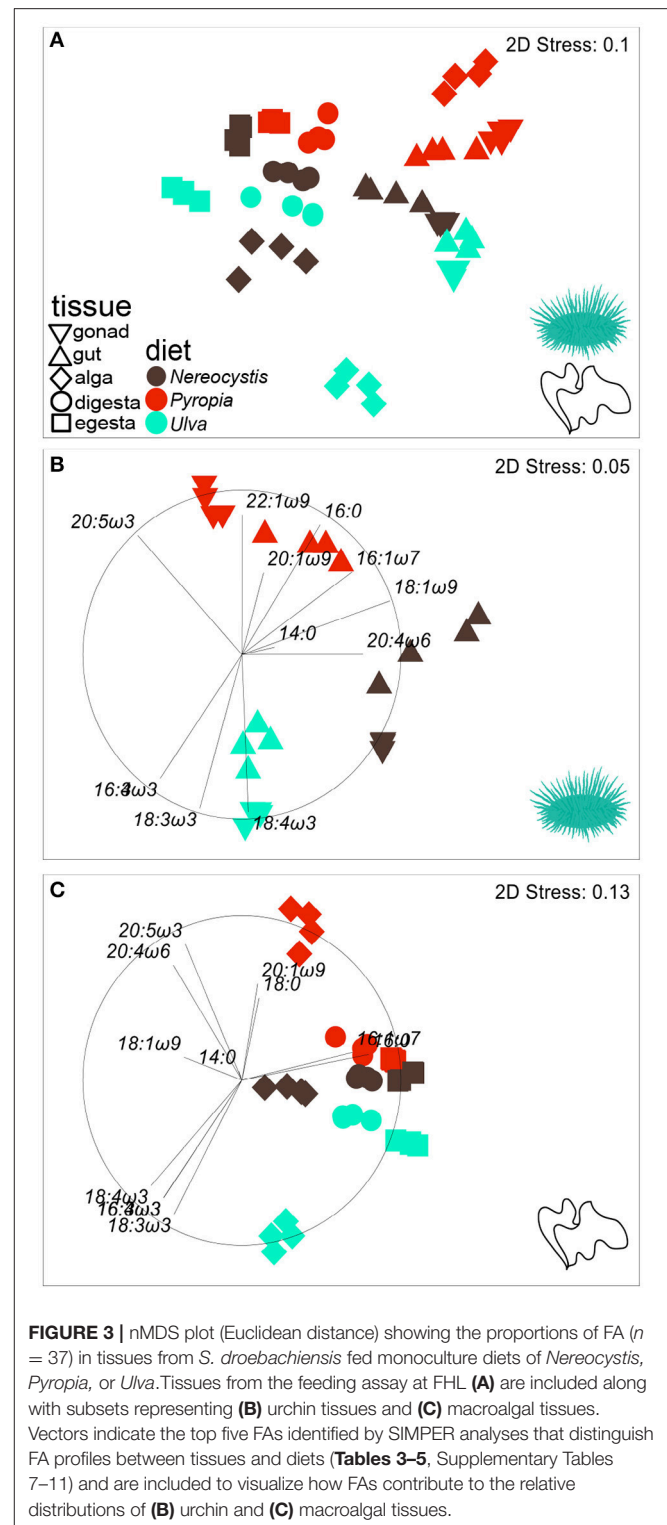


FIGURE 3 | nMDS plot (Euclidean distance) showing the proportions of FA ($n = 37$) in tissues from *S. droebachiensis* fed monoculture diets of *Nereocystis*, *Pyropia*, or *Ulva*. Tissues from the feeding assay at FHL (**A**) are included along with subsets representing (**B**) urchin tissues and (**C**) macroalgal tissues. Vectors indicate the top five FAs identified by SIMPER analyses that distinguish FA profiles between tissues and diets (**Tables 3–5**, Supplementary Tables 7–11) and are included to visualize how FAs contribute to the relative distributions of (**B**) urchin and (**C**) macroalgal tissues.

Nereocystis or *Pyropia* than all other tissues quantified (**Figure 5**). LCPUFA concentrations differed based on diet (2-way ANOVA, $F = 12.40$, $df = 2$, $p < 0.001$) and tissue ($F = 174.19$, $df = 4$, $p < 0.001$), and there was an interaction between factors

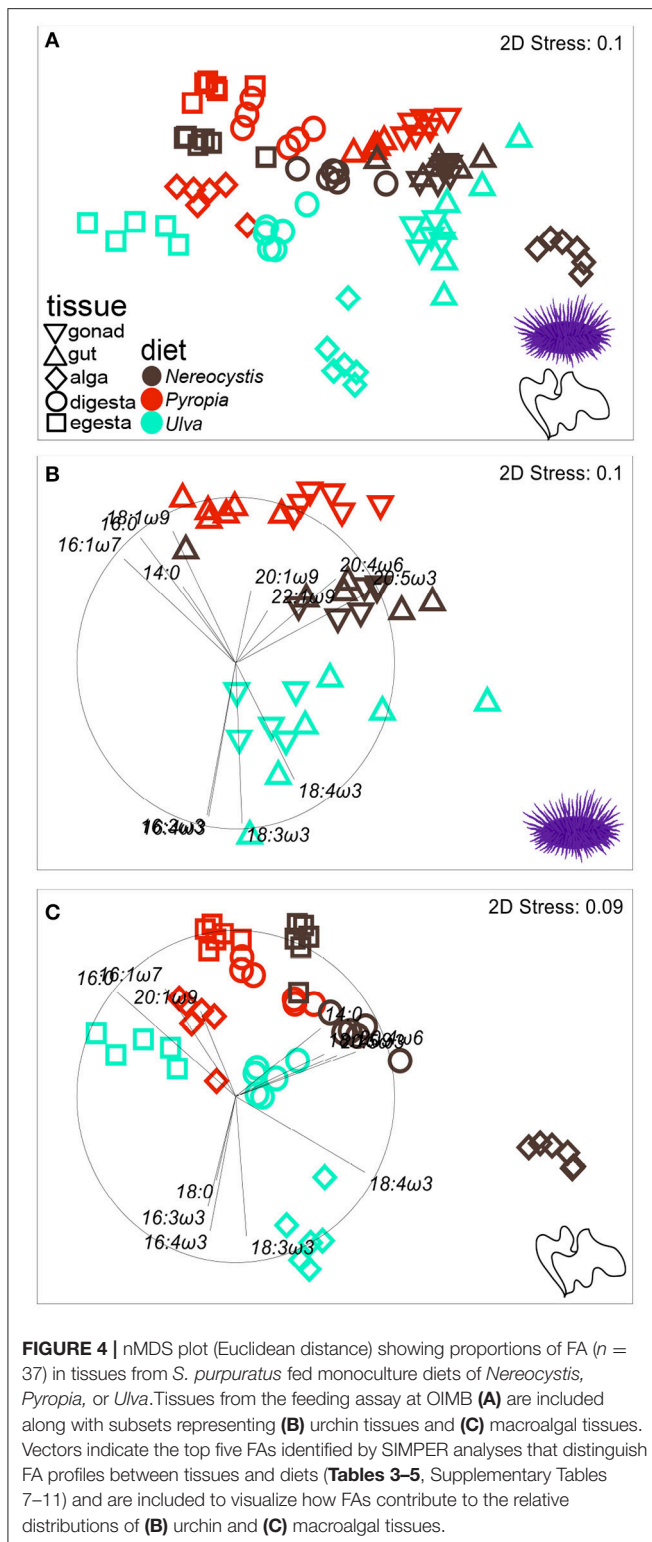


FIGURE 4 | nMDS plot (Euclidean distance) showing proportions of FA ($n = 37$) in tissues from *S. purpuratus* fed monoculture diets of *Nereocystis*, *Pyropia*, or *Ulva*. Tissues from the feeding assay at OIMB (**A**) are included along with subsets representing (**B**) urchin tissues and (**C**) macroalgal tissues. Vectors indicate the top five FAs identified by SIMPER analyses that distinguish FA profiles between tissues and diets (Tables 3–5, Supplementary Tables 7–11) and are included to visualize how FAs contribute to the relative distributions of (**B**) urchin and (**C**) macroalgal tissues.

($F = 5.62$, $df = 8$, $p < 0.001$). As with *S. droebachiensis*, the highest concentrations of LCPUFAs were found in the gonad tissues of *S. purpuratus* fed *Nereocystis* and *Pyropia* (Tukey HSD, $p < 0.05$), however these concentrations did not differ from the

gut tissue of *S. purpuratus* fed *Nereocystis* (Tukey HSD, $p \geq 0.05$, Figure 5B).

Similar to LCPUFA analysis, there were significant differences in LIN (18:2ω6), ARA (20:4ω6), ALA + SDA (18:3ω3 + 18:4ω3), and EPA (20:5ω3) based on urchin diet, tissue, and the interaction between both factors (Table 6). To visualize these relationships between the mean concentrations of precursor and resultant LCPUFAs, we plotted the concentrations of LIN against ARA (Figure 6) and concentrations of ALA + SDA against EPA (Figure 7). For all comparisons, the gonad and gut tissues of *S. droebachiensis* and *S. purpuratus* concentrations of LIN, ARA, ALA + SDA, and EPA were greater than the concentrations in the macroalgal tissues (Figures 6, 7). In *S. droebachiensis*, the macroalgal tissues exhibited intermediate FA concentrations of LIN and ARA relative to the digesta and egesta (lower relative concentrations) and the gut and gonad (higher relative concentrations) tissues associated with *Pyropia* and *Ulva* (Figure 6A). There is a similar pattern between the concentrations of the precursors ALA + SDA and resultant EPA in *S. droebachiensis* tissues (Figure 7A) with a slightly more pronounced increase in EPA in digesta along with a decrease in ALA + SDA (Figure 7A). In the *S. purpuratus* assays, *Pyropia* and *Ulva* macroalgal tissues had the lowest relative LIN and ARA concentrations, while *Nereocystis* macroalgal tissues had intermediate LIN and ARA concentrations compared to digesta and egesta tissues (Figures 6B, 7B). This same trend is also reflected in the comparison of ALA + SDA and EPA for *S. purpuratus* (Figure 7B). PUFAs (ARA and EPA) tended to be increased in digesta tissue and reduced in egesta compared to precursor FAs (Figures 6, 7).

DISCUSSION

We observed clear correspondences between the fatty acid composition of the diets urchins consumed and the new tissues they produced. One reason we pursued these experiments with two urchin species, and over relatively long durations, is that there have been very few controlled feeding trials with urchins fed different macroalgal diets, with mixed results reported in literature on urchin incorporation of dietary FA. Past feeding experiments with *S. droebachiensis* have shown, for example, that they have considerable ability to modify their dietary FA over long experiments (14 week duration; Kelly et al., 2008), or that they do not incorporate their dietary FA signal into their tissues in very short experiments (3 week duration; Wessels et al., 2012). A long (17 week) feeding experiment with adult red urchins (*Mesocentrotus franciscanus*) showed that urchin FAs were strongly differentiated in the gonad tissues of urchins fed two kelp species (Raymond et al., 2014). Our results show that when fed for a sufficiently long period (e.g., 25 weeks in *S. droebachiensis*, 10 weeks in *S. purpuratus*), both urchins' multivariate FA compositions are strongly affected by their diets; in fact, both species can be differentiated based on whether they were fed brown, red, and green algae (NMDS and PERMANOVA results). Interestingly, this was the case for both the gonad and gut tissues. To our knowledge, this is the first experiment that has

TABLE 4 | SIMPER results for analysis of combined FA of tissues from *S. droebachiensis* (N = 12) and *S. purpuratus* (N = 18).

Diet	FA	Mean FA %	Cont (%)
<i>S. droebachiensis</i>			
<i>Nereocystis</i>	16:0	26.30	26.37
	16:1 ω 7	10.20	19.30
	18:1 ω 9	7.06	14.88
	20:5 ω 3	7.80	9.97
	18:4 ω 3	4.23	8.57
<i>Pyropia</i>	16:1 ω 7	9.16	41.20
	20:5 ω 3	26.30	27.13
	16:0	27.60	13.11
	20:4 ω 6	4.32	11.08
	18:1 ω 9	2.17	2.27
<i>Ulva</i>	16:0	24.50	43.55
	18:4 ω 3	6.11	19.53
	20:5 ω 3	9.00	7.40
	16:4 ω 3	7.48	6.27
	16:3 ω 3	4.83	5.49
<i>S. purpuratus</i>			
<i>Nereocystis</i>	20:5 ω 3	12.00	20.41
	16:0	20.80	19.58
	20:4 ω 6	8.72	11.06
	14:0	10.50	9.22
	18:4 ω 3	8.86	8.80
<i>Pyropia</i>	16:0	29.90	31.44
	20:5 ω 3	12.80	16.69
	14:0	6.14	11.41
	20:4 ω 6	6.11	10.04
	20:1 ω 9	5.15	8.02
<i>Ulva</i>	20:5 ω 3	6.33	26.88
	16:3 ω 3	2.94	19.84
	18:4 ω 3	6.83	7.69
	16:0	23.10	7.53
	18:3 ω 3	7.89	7.13

The top five FA contributing to the FA profiles based on diets of both species are shown along with the mean proportion relative to the total FA (Mean FA %) and their contribution to the similarity within diets (Cont %).

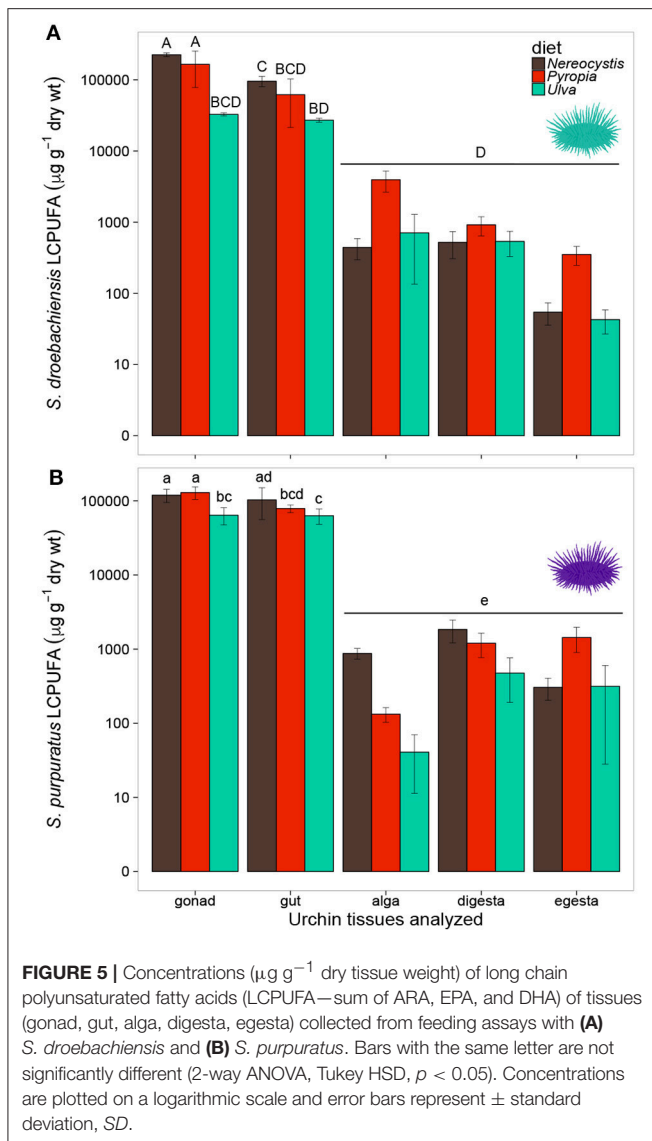
evaluated multiple urchin tissue types in multiple urchins after controlled feeding trials with different algae.

Some differences in urchin tissue FAs may have resulted from the total lipids and FAs available in local algae fed to urchins at the two experimental sites. It is likely that macroalgal tissue consumed by urchins at FHL (inland marine waters of Washington) and OIMB (southern coastal Oregon) experienced different abiotic conditions, resulting in some of the differences in lipid and FA content observed. FA biosynthesis is subject to environmental conditions that also influence photosynthesis, including but not limited to light, temperature, nutrient, and salinity (reviewed by Galloway and Winder, 2015). Here, we observed greater similarity between the FA profiles of *Ulva* tissues from the two areas, with less overlap between *Nereocystis* and

TABLE 5 | SIMPER results for analysis of combined FA of tissues from *S. droebachiensis* (N = 12) and *S. purpuratus* (N = 18).

Tissue	FA	Mean FA %	Cont (%)
<i>S. droebachiensis</i>			
gonad	20:5 ω 3	24.00	54.53
	16:0	11.40	11.81
	20:1 ω 9	4.94	6.54
	22:1 ω 9	3.20	3.70
	14:0	5.02	3.41
Gut	20:5 ω 3	21.00	32.64
	16:1 ω 7	6.35	25.50
	16:0	14.30	17.39
	20:4 ω 6	5.50	8.26
	18:4 ω 3	4.51	3.59
Alga	18:1 ω 9	7.77	17.85
	18:4 ω 3	4.28	17.56
	16:0	26.00	17.52
	20:5 ω 3	15.50	15.24
	20:4 ω 6	4.74	13.39
Digesta	16:1 ω 7	10.30	48.62
	16:0	36.40	33.21
	20:5 ω 3	8.55	6.94
	20:4 ω 6	2.98	2.15
	18:4 ω 3	1.93	1.17
Egesta	16:0	42.60	44.91
	16:1 ω 7	16.40	28.74
	14:0	5.37	3.94
	18:0	1.86	3.52
	20:5 ω 3	3.06	3.36
<i>S. purpuratus</i>			
gonad	20:5 ω 3	14.70	20.00
	20:1 ω 9	5.47	19.88
	14:0	1.25	13.78
	16:0	14.30	9.80
	20:4 ω 6	6.37	9.68
gut	20:5 ω 3	13.80	46.36
	16:0	14.60	9.76
	18:4 ω 3	3.80	7.24
	20:4 ω 6	5.98	6.40
	14:0	8.16	6.15
alga	16:3 ω 3	2.37	35.84
	16:0	22.20	19.73
	20:5 ω 3	10.10	10.93
	18:4 ω 3	11.20	6.30
	16:4 ω 3	4.71	5.28
digesta	16:0	30.60	28.82
	14:0	8.80	23.85
	20:5 ω 3	8.31	9.44
	20:4 ω 6	6.06	7.83
	18:3 ω 3	3.86	7.01
egesta	16:0	41.90	21.89
	20:4 ω 6	3.76	14.88
	20:5 ω 3	5.36	9.93
	18:1 ω 9	5.95	8.75
	16:1 ω 7	6.97	7.29

The top five FA contributing to the FA profiles based on tissues of both species are shown along with the mean proportion relative to the total FA (Mean FA %) and their contribution to the similarity within tissues (Cont %).



Pyropia fed to urchins in both feeding trials. Some variation between study sites may arise from our inability, without molecular identification, to guarantee that we were working with the same *Pyropia* and *Ulva* species at each study site. Despite the observed differences in lipid and FA content, there was sufficient similarity between monoculture diets of the same phylum from FHL and OIMB to support new tissue growth with significantly different FA profiles.

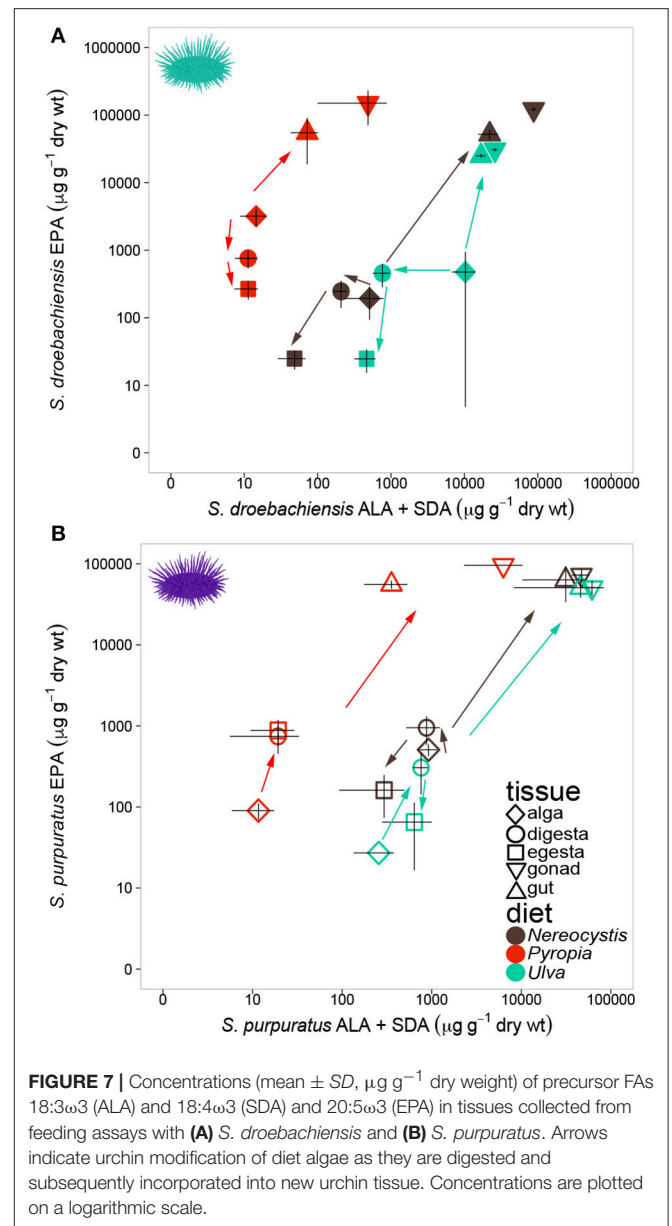
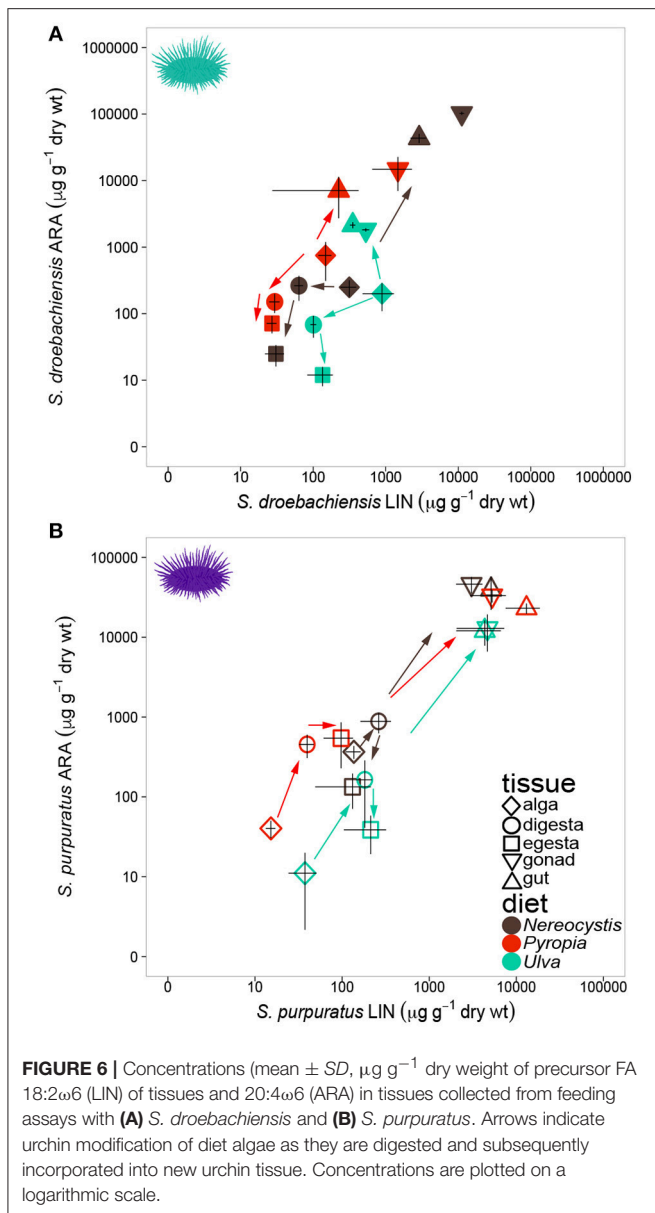
Remarkably, even though certain key LCPUFA content declined in egesta, the total lipid content tended to increase from macroalgal tissue to digesta to egesta, suggesting that the nutritional quality of macroalgal tissues was modified by urchin digestion. This result is consistent with other studies, using different metrics, that have shown a general increase in the nutritional value or energy content of urchin fecal material derived from macroalgae (Mamelona and Pelletier, 2005) or seagrass (Koike et al., 1987), as a consequence of microbial

TABLE 6 | Results from 2-way analysis of variance (ANOVA) for the concentration ($\mu\text{g g}^{-1}$ dry tissue weight) of precursor (LIN and ALA + SDA) of the corresponding most abundant (ARA and EPA, respectively) long chain polyunsaturated fatty acids (LCPUFA) identified in tissues from *S. droebachiensis* and *S. purpuratus* assays.

Source	df	Sum of Squares	F	p
<i>S. droebachiensis</i>				
LIN (18:2ω6)				
Diet	2	83950950	302.20	<0.001
Tissue	4	160738215	289.30	<0.001
Diet*Tissue	8	214498922	193.00	<0.001
ALA + SDA (18:3ω3 + 18:4ω3)				
Diet	2	4.84E+09	342.00	<0.001
Tissue	4	1.23E+10	434.30	<0.001
Diet*Tissue	8	1.26E+10	222.40	<0.001
ARA (20:4ω6)				
Diet	2	9.59E+09	438.80	<0.001
Tissue	4	1.46E+10	334.50	<0.001
Diet*Tissue	8	1.86E+10	212.30	<0.001
EPA (20:5ω3)				
Diet	2	1.02E+10	9.92	<0.001
Tissue	4	9.38E+10	45.49	<0.001
Diet*Tissue	8	2.32E+10	5.61	<0.001
<i>S. purpuratus</i>				
LIN (18:2ω6)				
Diet	2	72165813	6.86	<0.001
Tissue	4	809556110	38.45	<0.001
Diet*Tissue	8	223528851	5.31	<0.001
ALA + SDA (18:3ω3 + 18:4ω3)				
Diet	2	6.18E+09	21.85	<0.001
Tissue	4	2.14E+10	37.81	<0.001
Diet*Tissue	8	9.40E+09	8.31	<0.001
ARA (20:4ω6)				
Diet	2	2.27E+09	31.49	<0.001
Tissue	4	1.68E+10	116.52	<0.001
Diet*Tissue	8	3.20E+09	11.11	<0.001
EPA (20:5ω3)				
Diet	2	4.84E+09	7.34	<0.001
Tissue	4	1.23E+10	196.38	<0.001
Diet*Tissue	8	1.26E+10	4.94	<0.001

Significant differences ($p < 0.05$) are indicated in bold.

enrichment of the substrate. Increased lipid content in the egesta across all diets was a trend, albeit insignificant, in the juvenile *S. droebachiensis* experiment, and was significant with the adult *S. purpuratus*. Differences in the total lipid content of macroalgal tissues and the resulting digesta and egesta is likely to be influenced by the urchin gut microbiome, which is thought to play a role in carbohydrate, amino acid, and lipid metabolism (Hakim et al., 2016) and can be directly influenced by immediate culture environment (Hakim et al., 2015). It is therefore possible that some of the differences we observed in urchin digestion of *Nereocystis*, *Pyropia*, and *Ulva* between our experiments are driven not just by the difference in FA metabolism between urchin species, but also by variations in



the local microbiota at Friday Harbor, WA and Charleston, OR. This may in part explain the consistent patterns in the relative depletion of LIN and ARA the digesta and egesta of *S. droebachiensis* for *Pyropia* and *Ulva* compared to increased in concentration of LIN, ARA, ALA + SDA, and EPA in the *S. purpuratus* assay as tissues were digested (e.g., **Figures 6, 7**). Local microbiota availability and retention in urchin gut tissue may have contributed to the increased ARA and EPA concentrations in digesta and subsequent depletion in egesta of *Nereocystis* tissues. Microbial abundance on macroalgal tissue can influence the biochemistry of macroalgae consumed (Sosik and Simenstad, 2013) and could contribute to differences in urchin gut microbiota (Hakim et al., 2015, 2016). Conspecific urchin microbiome comparisons between these regions could be an interesting area of future investigation.

Urchin tissues from each diet treatment exhibited parallel differences to those that distinguish brown, red, and green algae from one another (Galloway et al., 2012; Kelly and Scheibling, 2012). The FA profiles of both urchin species displayed elevated levels of FAs previously associated with the same macroalgal phyla, even though tissues were not characterized by all of the same FAs (Kelly and Scheibling, 2012). As a result, urchins fed the same macroalgal diet grouped more closely together in the nMDS, with the tightest groupings among *S. droebachiensis* fed monoculture diets from which all tissues were generated. Convergence on similar profiles between the two assays occurred despite *S. purpuratus* having a unquantified amount of “legacy” FAs in soft tissues from previous *in situ* diets. The substantial growth we quantified in both species (e.g., in wet weight) suggests

that urchins rapidly generated new tissues from experimental diets. This higher abundance of food in the laboratory may have over-ridden some of the within-species or site variation we would have otherwise expected due to season or sex (Dethier et al., 2014; Zárate et al., 2016). Urchin “tissue turnover” time is still unknown, and thus the ideal experimental duration for tracking macroalgal diets into urchin tissues can be estimated using growth rate as a proxy, until future experiments quantify tissue turnover rates. However, it appears that urchin tissue turnover is slow enough that experiments of several weeks (e.g., Wessels et al., 2012) are insufficient to allow urchin tissues to incorporate the dietary FA biomarker signal. Urchins, like other animals, can elongate and modify (i.e., biosynthesize) their dietary precursor FA to achieve heightened concentrations of key PUFA (Floreto et al., 1996; Cook et al., 2000; Castell et al., 2004). For example, the urchin *Psammechinus miliaris* has the ability to synthesize small amounts of EPA (20:5 ω 3) from ALA (18:3 ω 3) among other PUFA modifications (Bell et al., 2001). It is presently unknown whether the patterns we observed here, resulting from very regimented and controlled diets offered in the lab, would transfer to wild urchins that are presumably integrating a range of macroalgal diets over the course of the year.

The higher concentrations of LCPUFAs observed in urchin tissues compared to macroalgal tissues highlight the urchins’ ability for *de novo* synthesis and selective retention of some FAs (Cook et al., 2000; Bell et al., 2001). This is particularly evident in the *S. droebachiensis* experiment, in which juvenile urchins generated nearly all of their tissues from a monoculture diet, as compared with the *S. purpuratus* experiment, which started with adult urchins with significant “legacy FA” from their previous diets in nature. We documented relatively high proportions of 20:2 Δ 5, 11 NMID in the gonad and gut tissue of *S. droebachiensis* and *S. purpuratus*, as high as 8.38 ± 0.83 and $9.68 \pm 0.94\%$ (gonad, gut of *S. purpuratus* fed *Pyropia*, Supplementary Table 5). González-Durán et al. (2008) documented similar proportions of 20:2 Δ 5, 11 NMID in urchin gonad (5.9%) and gut (8.61%) while Castell et al. (2004) documented much higher synthesis in tissues of *S. droebachiensis* fed formulated diets, and demonstrated that urchins can synthesize 20:2 Δ 5, 11 NMID from either 20:1 ω 7 or 20:1 ω 9. González-Durán et al. (2008) hypothesized that when there is an EFA deficiency, taxa such as echinoids may utilize NMIDs in place of some EFAs to maintain physiological functions.

S. droebachiensis and *S. purpuratus* also synthesized and retained LCPUFAs including ARA and EPA in gonad and gut tissues. These elevated LCPUFAs can become available to pelagic and benthic consumers upon gamete release (Hughes et al., 2011; Fuiman et al., 2015), subsidizing marine consumers. FA profiles of gonad tissue and larvae subsequently released from mature gonads share similar concentrations of these important LCPUFAs (Hughes et al., 2011). However, Hughes et al. (2011) only took FAs into account to evaluate nutritional quality. In the present study, we demonstrated that even though there were no significant differences in the FA profiles between algae consumed and egested, the urchins increased the nutritive quality of egesta in the form of increased total lipid content. This has significant implications for marine

food webs, because increased lipid content in a food source is likely to benefit consumers, and suggests a mechanism for why macrophyte-derived carbon (generally) and kelp-derived carbon (more specifically) may be a valuable trophic subsidy (Sauchyn and Scheibling, 2009; Sauchyn et al., 2011; Britton-Simmons et al., 2012; Lowe et al., 2015) to benthic organisms that might not otherwise utilize these sources directly.

There are multiple factors influencing FA profiles that could explain some of the differences we documented between *S. droebachiensis* and *S. purpuratus* assays. These include using two different urchin species (Dethier et al., 2013), under different local conditions (Hughes et al., 2005; Dethier et al., 2013), and with different prior *in situ* diets. Additionally, the feeding assays spanned different time periods (180 vs. 70 days) and used urchins at different life history stages (Hughes et al., 2006), which likely resulted in gonad tissues in different stages of gametogenesis (Zárate et al., 2016). The gonads present in the juvenile *S. droebachiensis* were unlikely to be mature and more likely represent their role in nutrient storage rather than reproduction. We did not quantify sex ratios, but in some cases females and males have different FAs (Hughes et al., 2006; Zárate et al., 2016). Despite these differences, particularly between urchin species and life stage, it is nonetheless clear that growing urchins, whether from increased tissue biomass, or in gonad development, incorporated multivariate FA profiles of the algae provided. The macroalgal diets selected here are known to have strong differences in their FA; future work could investigate whether it is possible to trace the FA of more closely related algae, e.g., different species of Laminariales (e.g., Raymond et al., 2014), into these urchin species.

Our results indicate that urchin digestion of different macroalgae following maintenance on the same monoculture diets generates similar changes in total crude lipid and FA content. The FA profiles of urchin tissues differ significantly from the FA profiles of the macroalgal diets. This is expected, as animal FA profiles will never completely align with those of macroalgal diets, even if they are strongly structured by them. Additionally, neither urchin species significantly altered the LCPUFA content of macroalgae consumed. However, we did find different patterns in *S. droebachiensis* and *S. purpuratus* processing of ARA and EPA precursors that may be related to differences in gut microbiota. We found that both urchin species selectively retained EFAs that were relatively depleted in diets and may have synthesized some of these EFAs from precursors along with 20:2 Δ 5, 11 NMID. The similar patterns in trophic modification we documented suggest that it is feasible to develop FA resource libraries for future use in Bayesian mixing models for further evaluating *in situ* urchin trophic linkages (Galloway et al., 2014). Common resource libraries representing urchin trophic modification of macroalgae make it possible to estimate the diets of a wider array of urchins. We have also demonstrated that it is possible to obtain a good estimation of urchin modification of macroalgal tissue over a relatively short time period if macroalgae are made available in quantities to support new soft tissue generation. This rapid convergence of FA profiles despite a large quantity of legacy tissue from previous

diets makes the development of resource libraries for benthic consumers more feasible.

AUTHOR CONTRIBUTIONS

JS, AG, MD, and JK: experimental conception, writing; JS and JK: sample collection, experimentation, total lipid/FA extraction; JS and AG: statistical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2018.00083/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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