Text S1. Supplemental Methods

The V4 region of the 16S rRNA gene was targeted for microbial community profiling using the primer pair 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′, Parada et al. 2016) and 806RB (5′-GGACTACNVGGGTWTCTAAT-3′, Apprill et al. 2015), using a fusion primer-based approach (Kozich et al. 2013). Triplicate 25µl PCR reactions were performed using the following recipe: 5.5µl nuclease free water, 12.5µl 2X FroggaBio Master Mix, 1µl Bovine Serum Albumin (BSA), 2µl each of forward and reverse fusion primers (515F/816R, 2.5µM) and 2µl of normalized DNA (2.5ng/µl). Fusion primers sequences contained not only the primer but also Illumina adapters and indexes as detailed by Comeau et al. 2017. Thermocycler conditions were as follows: 3 minutes at 94°C, 45 seconds at 94°C, 60 seconds at 50°C, 90 seconds at 72°C, repeating steps 2-4 29 times, followed by 10 min at 72°C, and a hold at 10°C. Successful amplification was verified by gel electrophoresis.

If a visible band was present, PCR triplicates were pooled. If no band was visible, a series of troubleshooting steps were carried out, including increasing DNA volume in the reaction, diluting samples 1:10 and/or using a Zymo Research OneStep PCR Inhibitor Removal Kit to remove potential PCR inhibitors. When at least a faint band was detected, two additional PCR reactions were run with the successful troubleshooting method. Successful triplicate PCR reactions were pooled, purified using SPRI paramagnetic beads (Beckman Coulter, Inc.) and quantified using a Quant-iT™ dsDNA Assay Kit. Samples were pooled in equimolar concentration (7.5ng of DNA per sample) and sequenced, along with the five extraction negatives and one PCR negative, using an Illumina MiSeq using V3 600-cycle kit.

Sequences were quality controlled and denoised using QIIME2 (Bolyen et al. 2019). Briefly, primers were trimmed using cutadapt (Martin 2011) and denoised using dada2 (Callahan et al. 2016; trim-left-r 5, trunc-len-f 230, trunc-len-r 210, max-ee-f 3, and max-ee-r 5). Rare amplicon sequence variants (ASVs), with a frequency of less than 0.1% of the mean sample depth after denoising, were filtered out of the ASV table and representative sequences were then classified using the QIIME2 naive Bayes Classifier (Bokulich et al. 2018) trained to the Silva database v 138 (Quast et al. 2013). Eukaryotic, mitochondrial, and chloroplast sequences were removed prior to further analysis.

Due to several abundant ASVs being not being classified beyond domain, all sequences were then classified with two additional classifiers: (1) SINA classifier, available on the Silva Database website (https://www.arb-silva.de/aligner/, Pruesse et al. 2012) and (2) QIIME2's implementation of consensus BLAST, using a percent ID of 0.7. We then selected the sequences classified only to Bacteria using the QIIME2 naive Bayes Classifier (n=31) and compared the new taxonomy assignments from SINA and BLAST. SINA provided many more assignments, but not always with a high percent ID. If these additional taxonomic assignments had a percent ID below 0.7, assignments were not updated (and in 50% of these cases were ID'd as being mitochondrial) and ASVs were subsequently removed (n = 14) from the dataset. Several ASVs with SINA taxonomic assignments with percent identities greater than 70% but less than 75% (n =5) still remained unclassified with BLAST (2/5), had conflicting BLAST assignments (2/5), or were classified as mitochondrial (1/5) and were also removed. For several ASVs with a SINA taxonomic assignment with a percent ID of 75% or greater (n = 12), a consensus taxonomic assignment was also available from BLAST but did not agree at the Phylum level in 25% of the cases and thus we could not confidently assign these ASVs beyond Bacteria. These ASVs were

therefore removed from the dataset (3/12). For the remaining 75% of these hard-to-classify ASVs (9/12) the SINA and BLAST classifications agreed to at least the Phylum level. An updated lowest-common ancestor (LCA) classification was used for these ASVs. Additionally, unclassified ASVs were removed from the dataset before further analysis.

We controlled for potential contamination in the dataset using the *decontam* package in R (Davis et al. 2018). This process identified 72 of 2993 ASV's as contaminants, and these ASVs were removed from the dataset. Further, some samples had low read counts (similar to the extraction and PCR negatives), and thus we pruned the dataset to remove samples with <9000 read counts, which resulted in removal of eight swabs, five extraction negatives and one PCR negative, leaving 46 samples containing 2921 ASVs, with a mean read count of 287,362 per sample (ranging from 18,425 to 550,473 reads). All subsequent analyses were performed on this decontaminated and pruned dataset. The 16S rRNA gene sequence data are available in the NCBI Sequence Read Archive under BioProject number PRJNA839850. Details of bioinformatic processing of the data and the code and associated input files for data visualization and statistical analysis are available at https://github.com/hakaigenomics/seastar-swab-extmethods.

All visualizations and statistical tests were conducted in R version 4.2.2 (R Core Team 2022). A significance value of 0.05 was used for all statistical tests. Richness was calculated using the *breakaway* package in R (Willis & Bunge 2015), while alpha diversity (Shannon Index) was calculated using the *DivNet* package (Willis & Martin 2022). Both packages were developed to make more robust estimates of species richness in microbiome datasets by accounting for species interactions (Willis & Bunge 2015). The effect of extraction kit on estimates of richness and diversity was determined by fitting repeated measures analysis of variance (ANOVA) models (anova_test in the *rstatix* package, Kassambara 2022) to account for the same specimens being used in each of the five extraction kits. Further, because two sea star species were included in our dataset and microbiomes are often host-specific, we tested for differences in richness and diversity among kits within leather star (*D. imbricata*) and ochre star (*P. ochraceous*) specimens separately. We also tested for differences in richness and diversity between the two sea star species using t-tests, temporarily excluding the effect of extraction kit. Within each subset of the data, we tested homogeneity of variances using the bartlett.test in the *stats* R package and used ggqqplot in the *ggpubr* package (Kassambara 2022) to visually examined if the data were normally distributed. If these assumptions were not met, we used the Skillings-Mack test in the *PMCMRplus* package (Pohlert 2022) as a non-parametric alternative to the repeated measures ANOVA with an unbalanced block design, and the Wilcoxon test as an alternative to the t-test in the *rstatix* package (Kassambara 2022).

A Principal Coordinates Analysis (PCoA), using the Bray-Curtis dissimilarity index based on ASV abundances normalized to the total reads per sample (i.e. proportions), was used to test for differences in community composition among extraction kits. Homogeneity of dispersions was also tested among the five kits using the betadisper function and Permutational multivariate analysis of variance (PERMANOVA, using the adonis2 function) was used to test for differences in beta diversity (community composition) among extraction kits and between sea star species – both analyses were conducted in the *vegan* R package (Oksanen et al. 2022). To further examine differences in prokaryotic community composition among the five extraction kits and between the two sea star species we created heat maps showing % read abundance at the Phylum level using the *ampvis2* package (Anderson et al. 2018).

Supplemental References:

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Table S1. Results from statistical analyses examining the effect of extraction kit and sea star species on microbial richness and diversity

A. Repeated measures ANOVA results

Dataset	Fixed Effect	Repeated Measure	Response	Degrees of Freedom (dfn, dfd)	F-value	p-value
All data	Extraction Kit	Specimen	Richness estimate	4, 16	1.13	0.38
P. ochraceus				4, 8	0.926	0.50
D. imbricata				4, 4	0.816	0.58
D. imbricata	Extraction Kit	Specimen	Shannon diversity	4, 4	0.703	0.63

B. Skillings-Mack test results

Dataset	Group	Block	Response	Degrees of Freedom	Skillings-Mack chi-squared	p-value
All data	Extraction Kit	Specimen	Shannon diversity	4	3.68	0.45
P. ochraceus				4	1.34	0.85

C. T-test and Wilcoxon test results

Dataset	Group	Response	Statistical Test Used	Degrees of Freedom	Test statistic	p-value
All data	Species	Richness estimate	T-test	24.8	-2.61	0.015
		Shannon diversity	Wilcoxon Test	NA	95	0.003

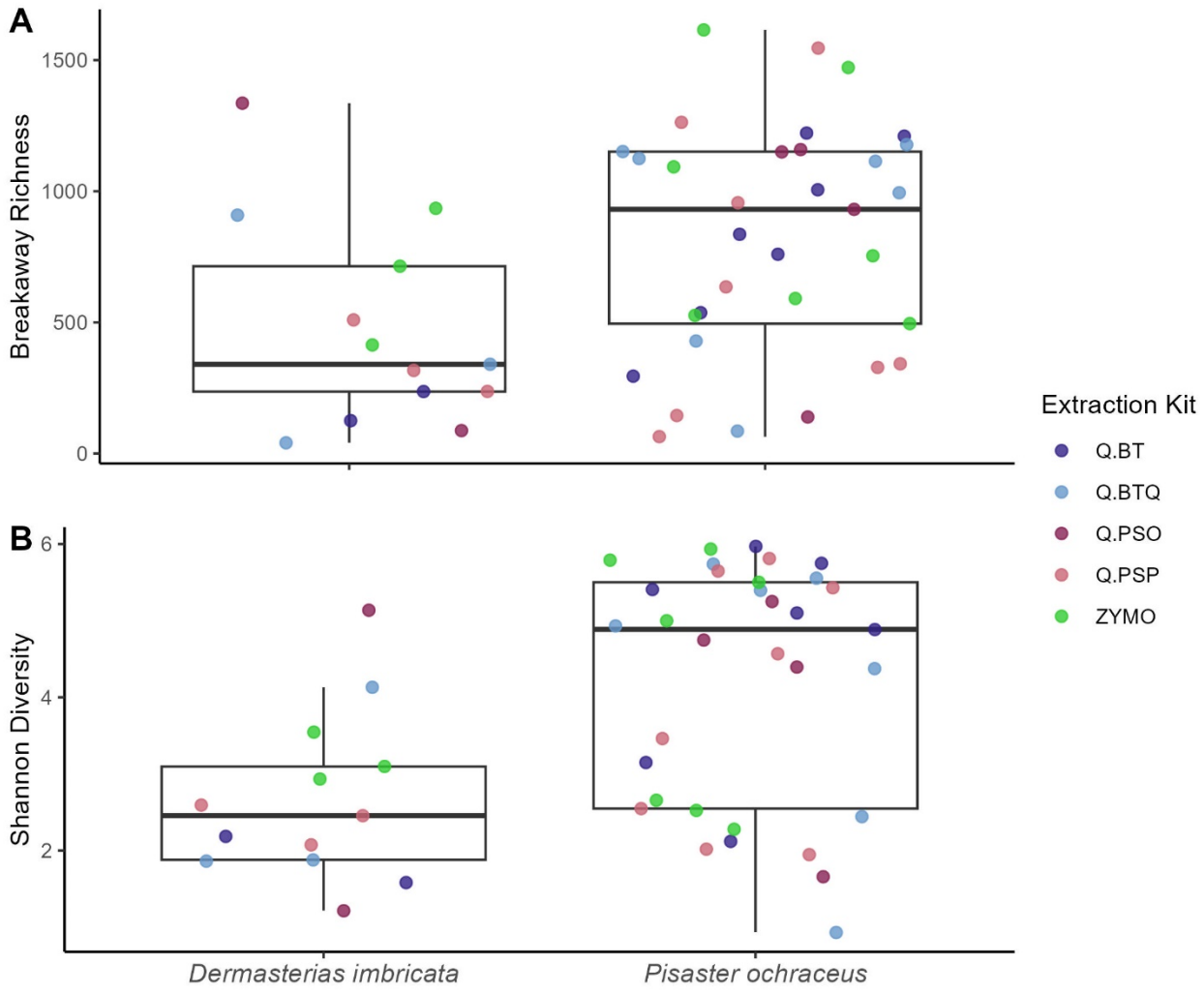

Figure S1: Estimates of (A) richness and (B) Shannon diversity for the two sea star species, with extraction kit indicated by color