**Working Title:**

**Effects of environmental contamination on diet metabarcoding data of invertebrate consumers in mesocosms and natural environments**

**Target Journal: *Molecular Ecology Resources (8000 words excluding references, unclear if this means including components after references)***

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**Abstract ( ≤ 250 words)**

**Introduction**

Biological communities and ecosystem function are shaped by the interactions between organisms (Brown et al. 2001, Hooper et al. 2005, Delmas et al. 2019, Schleuning et al. 2015). Among the many interaction types (e.g. mutualism, consumption, competition), consumptive interactions (including herbivory, predation, and parasitism) can shape the stability of biologically diverse communities (Ings et al. 2009, Delmas et al. 2019). For decades, methods for determining consumptive interactions through assessing diet contents have emerged and provided key insights into consumptive interactions across ecosystems and consumer groups (Hylsop 1980, Nielson et al. 2018). These have included many developments specific to environments or consumer groups and rely on visual observation of consumption events (live or via recordings) or recognition of diet items in diet contents, either unaided or through the use of microscopy (i.e. Baker et al. 2014, Duffy and Jackson 1986, for a review of methods best suited for different contexts and goals see Birkhofer et al. 2017).

This dependence on visual identification or observation is unfeasible or impossible for many consumer groups; specifically, for consumers that are too small for dissection and food identification, have feeding habits which render food items unidentifiable, have food items that contain few components which pass through digestion in recognizable form, or have cryptic habits that prevent them from being observed in large enough numbers for diet analyses, to name a few (Sheppard and Harwood 2005). Observation-based methods become especially limiting in community studies in which it is necessary to ascertain the diets of multiple consumers and for consumers who feed on many diet items (e.g. Polis 1991). In these cases, the most promising avenue for determining consumptive interactions has been the exploration and expansion of DNA-based diet analyses either through gut or fecal contents, initially through species-specific approaches, and now through high throughput sequencing of the DNA of all species in gut contents (Pompanon et al. 2012, Sheppard and Harwood 2005).

The use of high throughput sequencing methods for molecular gut content analysis allows for the identification of a suite of diet species at once and provides a comprehensive and efficient method for determining intraspecific, intra-population, and interspecific diets (Lucas et al. 2018, Soininen et al. 2015, Quemere et al. 2013). These methods have already illuminated interesting new interactions and ecological trends in a variety of environments (e.g. host-parasitoid: Wirta et al. 2014; plant-herbivore: Kartzinel; host-parasite: Schnell et al. 2012, predator-prey: Toju et al. 2018). As these methods have continued to advance, however, they need to be validated so that the ecological inference made from them is robust. Specifically, for organisms where small body size has limited other diet analysis methods, DNA diet analyses often necessitate the extraction of diet data from full organisms, and so the possibility of surface contamination altering the detection and species composition of presumed diet items is an important consideration.

Surface contamination could alter the results of diet metabarcoding via altering the detection and richness of presumed diet items, either through falsely inflating diet estimates (i.e. contaminants are potential diet items) or by depressing diet estimates (i.e. contaminants are not potential diet items). The effects of surface contamination could vary by environmental, ecological, or methodological aspects of a DNA metabarcoding study (e.g. collection method: Greenstone CITATIONS). Environmentally, in more closed ecosystems (i.e. aquatic pools, mesocosm experiments, soil environments), DNA can persist in the environment for months to years (Strickler et al. 2015, Barnes et al. 2014, Neilson et al. 2007, Carini et al. 2016). Combined with a high likelihood that organisms share contained substrates with each other in these environments, the likelihood of environmental contamination could be quite high. Environments where DNA contaminants may be lower risk are those with larger substrates with high cycling (i.e. marine environments; Collins et al. 2018) or environments where UV light and biological activity degrades DNA on surfaces (e.g. many terrestrial environments; effects in aquatic environments shown in Strickler et al. 2015). In any environment, broadly used DNA metabarcoding methods for determining the diet of multiple consumers which potentially feed on a wide variety of diet items are dominated by the DNA of the consumer (e.g. diet comprise 0.03 – 8.43 percent of all sequencing reads due to the inability to use blocking primers; Krehenwinkel et al. 2016, Piñol et al. 2014). In any of these studies, low abundance potential diet DNA could be even more hidden by high-biomass contaminant DNA (e.g. biomass and metabarcoding analysis in Elbrecht et al. 2017) and so recognizing and mitigating the risk of contamination is paramount.

While it has not been systematically used in diet metabarcoding studies, surface sterilization to reduce potential surface contamination has been utilized across disciplines in both single-interaction and high-throughput sequencing methodologies (e.g. single species: Greenstone et al. sterilization one, Linville et al. 2002; high-throughput: Zimmerman and Vitousek 2012, Burgdorf et al. 2014). While some fields (e.g. fungal endophytes) that use high throughput sequencing to describe community diversity use surface sterilization as standard practice, these methods can vary greatly (e.g. using washes of different disinfectants or sonication; Burgdorf et al. 2014) and often depend on the aspects of the tissues used (e.g. how sensitive they are to sterilization-induced degradation; Hallman et al. 1997). The field of fungal endophyte research has developed informed protocols based on decades of research into best practices and study-specific considerations (Brown et al. 2018). Conversely, the field of diet metabarcoding, particularly when determining diet from full individuals, has not developed a similarly systematic approach (e.g. ethanol washes in Doña et al. 2019, bleach washes in Anslan et al. 2016, no sterilization in Wirta et al. or Jacobsen et al. 2018). This lack of systemic surface sterilization in diet metabarcoding when using full individuals limits the ability to confidently assign DNA sequences to ingested diet items and to help discern study-specific considerations (e.g. likelihood of contamination or sensitivity of different consumer species to sterilization-induced degradation). Indeed, the same methods seem to lead to degradation in one study (Greenstone) while providing a robust dataset in another study (Linville et al 2002), suggesting that a broader analysis of the costs and benefits of surface sterilization as well as limitations and study-specific considerations needs to be undertaken by the field.

In this study, we look at the effects of surface contamination and surface sterilization on our understanding of consumer diets where the DNA of full organisms is used in analyses. We use high throughput sequencing results of the CO1 gene from the full body parts (opisthosomas) of invertebrate consumers (the spider, *Heteropoda venatoria*) from two environments – a “contained” mesocosm environment in which we offered consumers a potential diet item, and a natural field environment in which consumers could feed on available diet items. In each environment, we surface sterilized half of the consumers prior to DNA extraction using a series of washes in a 1:10 dilution of NaOCl and deionized water; we left the other half of consumers unsterilized. Specifically, we ask: Does surface sterilization alter the 1) detection of potential diet items or 2) the proportion of potential diet DNA, suggesting that contaminants either hide or inflate diet data, and does this effect matter more in contained environmental contexts (mesocosm versus field)? Then, because DNA metabarcoding is used to examine diet composition, we ask 3) Does surface sterilization alter the richness or composition of potential prey items in a field environment, suggesting that surface contamination could alter ecological interpretations of community-scale species interactions? Our results suggest minimal to no significant impacts of surface contamination on diet detection or diversity, though in the mesocosm environment, surface contamination marginally influenced potential diet item detection. Given equivocal results suggesting that some environments may be prone to surface contamination and the low cost (of time, data, or money) of many surface sterilization protocols, it may be judicious to surface sterilize full consumers prior to DNA extraction for diet metabarcoding.

**Materials and Methods**

*Field site and collections*

We conducted this work on Palmyra Atoll National Wildlife Refuge, Northern Line Islands (5º53’ N, 162º05’W). We targeted an abundant, generalist, active hunting spider species (*Heteropoda venatoria*) as the consumer for this project because this species occurs in high abundance on the atoll and is therefore easy to collect and observe; furthermore, Palmyra Atoll has a well-characterized species list, and like many atolls, is relatively species poor, allowing for relatively easy characterization of potential diet species (Handler et al. 2007, Food webs if published). We collected consumer individuals during two field seasons. In 2017, we collected consumer individuals which we kept in mesocosm environments in the lab (explained below). In 2015, we collected individuals in the field in natural habitats across the atoll. All individuals were collected individually in clean collection containers to avoid contamination (Greenstone et al. 2011).

*Mesocosm consumer set-up and feeding*

In the 2017 field season, we conducted mesocosm trials because we were interested in testing whether DNA metabarcoding would detect DNA from diet items a consumer was offered in a common “contained” environment (mesocosms) often used in studies of feeding interactions (e.g. Gao 2017, Rudolf et al. 2014, Srivistava et al. 2004). We created feeding mesocosms out of one-liter yogurt containers with holes for air transfer. We placed an individual *H. venatoria* in each of these mesocosms. After a 12-hour period alone in the mesocosms, all *H. venatoria* individuals were offered one individual of a common large grasshopper species (*Oxya japonica*), which is a common introduced species on the island (Handler et al. 2007). While many mesocosm experiments include natural “habitats” for consumers and diet to hide, we did not include anything in mesocosms to avoid contamination from DNA on these items. We left all mesocosms for 24 hours, after which we immediately froze *H. venatoria* individuals which had killed (though ingestion was not confirmed) an *O. japonica* individual at -20°C. All mesocosms were cleaned between each trial with a 10% bleach solution and kept closed to avoid contact with other organisms with the inside of the mesocosms.

*Field consumer collection*

In the 2015 field season, we collected consumers in field environments because we were interested in whether DNA metabarcoding would detect DNA from consumers occurring in natural environments. We froze all individuals at -80°C immediately following collection until surface sterilization and DNA extraction in 2019.

*Both mesocosm and field consumers: surface sterilization*

Because we planned to extract DNA from entire consumer individuals or from the opisthosoma of larger individuals (following methods from Krehenwinkel et al. 2016 and Macias-Hernandez et al. 2018), we wanted to determine whether contaminant DNA on the outside of *H. venatoria* consumer individuals altered potential diet DNA detection, and for consumers from a natural environment potential diet item richness and composition. We used a surface sterilization treatment to remove possible contaminants from some consumer individuals while leaving some individuals unsterilized. We used surface sterilization techniques common in other fields of molecular ecology (i.e. plant endophytes Schulz et al. 1993, Burgdorf et al. 2014) by submerging and stirring each sample in 10% NaOCl by volume for 2 minutes and then washing each sample by submerging and stirring in deionized water for 2 minutes. We surface sterilized mesocosm consumers (2017 field season) in the field lab on the atoll following freezing at -20°C and then stored each sample in individual vials of 80% ethanol because no long-term refrigeration was available on the atoll at the time (n = 10 surface sterilized; n = 14 not surface sterilized). Field-collected consumers (2015 field season) had been frozen at -80ºC since collection; these consumers were surface sterilized in a sterilized laminar flow hood in 2019 just before DNA extraction (n = 22 surface sterilized, n = 25 not surface sterilized; Table). Prior to DNA extraction, all samples from both 2015 and 2017 were allowed to dry for 1-3 hours in a sterilized laminar flow hood.

*DNA extraction and removal of consumer DNA with Ampure XP beads*

We extracted DNA from each *H. venatoria* consumer individual following a modified CTAB extraction protocol (Fulton et al. 1995). At least twenty-four hours following extraction, we quantified DNA using a Qubit (Invitrogen) fluorometer and the high sensitivity DNA quantification kit with 1mL of DNA per reaction. We used methods developed by Krehenwinkel et al. (2016) to remove a proportion of consumer DNA prior to PCR steps. To do this, we diluted each DNA sample to 20ng/ml (a total of 40mL per sample), bead cleaned each sample using Ampure XP beads (0.75x bead ratio) and keeping the supernatant from the bead cleaning step. Because Ampure XP beads preferentially bind to heavier molecules, during this step, more intact consumer DNA binds to the beads (Supp figure). Thus, by keeping the supernatant, we aimed to work with a sample that had a larger proportion of diet DNA after removing some consumer DNA that bound to beads (Krehenwinkel et al. 2016). With the supernatant, we repeated the CTAB protocol steps for precipitating DNA pellets with isopropanol and 5M potassium acetate and cleaning DNA pellets with ethanol washes. Waiting at least another twenty-four hours, we quantified DNA again using a Qubit fluorometer and high sensitivity kit with 1mL of DNA per reaction tube and diluted all samples to 10ng/mL prior to PCR steps. All DNA pellets were stored in and diluted with TE buffer.

*PCR amplification, library preparation, and sequencing*

The COI gene is well-represented in the GenBank sequencing database (Porter et al. 2018), so we chose to amplify this gene using general metazoan primers (Krehenwinkel et al. 2016, Yu et al. 2012, Leray et al. 2013). We performed all PCR amplification steps in a UV-sterilized biosafety cabinet. We used a standard desalted primer set tested by Krehenwinkel et al. (2016) for use in diet analyses of invertebrate predatory consumers (Table). Our primers included overhang adapters compatible with the Illumina indexing PCR (Illumina 2009).

We amplified the COI gene in our samples with an initial PCR with a 25mL reaction volume, including 9mL nuclease free water, 12.5mL GoTaq Green Master Mix (Promega Corp.), 1.25 mL of each of the primers, and 1 mL of DNA template (at 10ng/mL). When DNA concentrations were lower than 10ng/mL, we added more DNA to the sample to equal 10ng of total template and reducing the amount of water added. Each sample was run in duplicate until after the Illumina indexing PCR, and we ran a duplicated negative sample each PCR run. We ran each reaction with an initialization step at 95°C for 3 minutes, and then 35 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at 46°C for 30 seconds, an elongation step at 72°C for one minute. We ended each run with a final elongation step at 72°C for 5 minutes and then held samples at 4°C until placed in a 4°C refrigerator until attaching Illumina indices via an additional PCR step. To remove reaction dimer, we performed a bead cleaning step with Ampure XP beads at a 0.8x bead ratio prior to the Illumina index PCR. Samples were re-suspended from beads using a 10mM TRIS resuspension buffer.

We then attached Illumina index primers with an additional PCR step (Nextera XT Index Kit v2). Each total reaction volume was again 25mL, with 5mL of nuclease free water, 12.5mL GoTaq Green Master Mix, 1.25mL of each primer, and 5mL of PCR product. These were run in a standard PCR protocol for these primers: an initialization step at 95°C for 3 minutes, followed by 10 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an elongation step at 72°C for 30 seconds. We ended each run with a final elongation step at 72°C for 5 minutes and then held samples at 4°C until placed in a 4°C refrigerator.

We verified PCR amplification by visualizing 3-4 mL of each PCR product in a 1.5% agarose gel using GelRed (Biotium) at 100V and 170mA for 30-40 minutes. Gels were visualized with a Bio-Rad Gel Doc XR+ imager using Image Lab 5.0. We kept samples for which both duplicates successfully amplified during the PCR steps and only kept samples on PCR runs in which both negative control duplicates resulted in no product detection. For successful samples, we combined duplicates and bead cleaned with an Ampure XP bead ratio of 0.7x. We determined the average length of the gene region using an Agilent TapeStation with a D1000 ScreenTape System following the standard protocol from the quick start guide. We then quantified these final PCR products using a Qubit fluorometer and a high sensitivity kit with 1mL of sample per reaction tube and diluted each sample in 10mM TRIS to a final concentration of 5nM.

We multiplexed all samples along with one negative control and three positive controls (cloned fungal species of the ITS gene region; GenBank accession numbers: MG840195 and MG840196, Apigo and Oono 2018; Toju et al. 2012). We submitted multiplexed samples for sequencing at the University of California, Santa Barbara Biological Nanostructures Laboratory Genetics Core. Samples were run on an Illumina MiSeq platform (v2 chemistry, 500 cycles, paired end reads). Following sequencing, samples were demultiplexed using Illumina’s bcl2fastq conversion software (v2.20) at the Core facility.

*Sequence merging, filtering, and clustering with UNOISE3*

We merged, filtered, and denoised (clustered) our sequences around exact sequence variants (ASVs) using the UNOISE3 algorithm (unoise3 command in the open-source USEARCH 32-bit version 11.0.667; Edgar 2016). This ASV denoising approach incorporates sequence abundance, quality, and error rates to cluster reads in high throughput sequencing data into a smaller subset of real biological units (Supp Figure). We also repeated analyses with the DADA2 algorithm run through R (dada2 package version 1.1.14.0; Callahan et al. 2016) and with a data cleaning step run through BBSplit (Bushnell 2018) to remove consumer DNA prior to ASV assignment (since ASV assignment is abundance-sensitive); however, UNOISE3 produced more sequence reads and assigned more ASVs per sample, so we chose to continue analyses from this algorithm only (summary and comparisons in Supplement).

Prior to denoising, we used cutadapt (version 1.18, Martin 2011) to remove primers from each sequence. With trimmed sequences, we ran UNOISE3 in USEARCH with a maximum error rate of 1 in the filtering step. From the output, we created a list of unique ASVs and a matrix of ASV abundances across samples. We matched ASVs to taxonomies both in the GenBank and BOLD databases. For GenBank, we used BLAST (version 2.7.1) with the blastn command for taxonomic assignment of each ASV using the computing cluster at UC Santa Barbara, comparing against the GenBank nucleotide database with an evalue of 0.01 (downloaded on November 20, 2019). We visualized and exported taxonomic alignment using MEGAN Community Edition (version 6.18.0, Huson et al. 2016), selecting the subtree with all possible diet items for this species (Kingdom: Animalia, Clade: Bilateria). For BOLD, we used the BOLD IDEngine of the COI gene with Species Level Barcode Records (accessed February 5-16, 2020; 3,825,490 Sequences, 216,704 Species, and 95,537 Interim Species in database) to match each ASV list to taxonomies. We combined taxonomic assignments from both programs, keeping the highest matching taxonomic match between databases and discarding assignments which did not match across both (following protocol in Elbrecht et al 2017).

*Sampling completeness and error correcting*

Following ASV assignment, we assessed sampling completeness using interpolation and extrapolation methods in the iNEXT package in R (Hsieh and Chao 2016, 2.0.20). We kept samples with above 95% sampling completeness to ensure our metrics of diversity were accurate and comparable (Hsieh and Chao 2017). We then checked our data for error that arises during the sequencing process (from either sequence hopping or crosstalk; Weng et al. 2017, van der Valk et al. 2019). We based sequencing error on the number of sequences assigned to the negative control, since we verified that the concentration of the negative control was zero prior to sequencing and so any non-zero values in the negative control represent the rate at which sequences are mis-assigned on the sequencing platform. We used the distribution of read count values in the negative control to build an error distribution and correct our raw sequencing data prior to community analyses, if needed. To do this, we fit the ASV abundances for the negative control to a Poisson and negative binomial distribution and used BIC to fit the best of the two distributions to the data (Olds et al. 2016). We then used the distribution of the best-fitting model to predict whether values of different abundances were likely due to error or not (if significant p-value, i.e. 0.001 or lower, these values correspond to real biological diversity and not to sequencing error). Any ASV in a sample with a read value which was likely due to sequencing error were removed prior to community analyses. In addition to accounting for and correcting error from the sequencing process, we also used positive controls of fungal clones to assess the specificity of UNOISE in assigning reads to ASVs. Because these fungal positive controls are clones, they should be assigned to one or very few ASVs in the final dataset.

*Hypothesis 1: Does surface sterilization alter the detection of potential diet items?*

For consumers from both mesocosm and field environments, we wanted to know whether surface contamination altered the detection of potential diet items for each consumer (either by increasing detection because of “false” diet detection or by decreasing detection because of abundance of non-diet DNA). We analyzed consumers from the mesocosm environment separate from the field environment because we wanted to know whether the risk of surface contamination varies by environmental differences related to how likely consumers and potential diet items are to interact without consumption via physical contact or shared surfaces (mesocosm = “high risk”, field = “low risk”). For mesocosm consumers, we focused our detection analysis on the potential diet item we had offered the consumers in the mesocosm environment (*O. japonica*, which all consumers were observed to have killed, but not necessarily ingested). For field consumers, we examined all potential diet items (which could represent either real diet or surface contaminants). Because sequencing depth (total number of DNA sequences assigned) can vary considerably across samples in high throughput sequencing runs, we first rarefied our samples so that we were comparing samples with equal sampling effort (McKnight et al. 2018). We did this using the rrarefy() function in the vegan (version 2.5.6) package in R and we rarefied based on the sample with the lowest sequencing depth. We rarefied the mesocosm dataset separate from the field dataset since these samples had been preserved in different ways and for different times, which can have large effects on DNA extraction outcomes (Murphy et al. 2002). Following rarefying, we selected all ASVs which matched to the known potential diet item for the mesocosm consumers (species: *Oxya japonica*, genus: *Oxya*, family: Acrididae), and all potential diet items for the field collected consumers (Kingdom: Animalia; Clade: Bilateria, excluding consumer DNA). In addition, for field consumers, because BLAST and BOLD matched multiple ASVs to the same species taxonomy, we concatenated all ASVs based on shared taxonomic assignment (i.e. multiple ASVs matched to *diet species A* were combined into one *diet species A* taxonomy with cumulative read abundance). For both mesocosm and field consumers, we assessed per sample detection of potential diet (offered diet item for mesocosm consumers, all potential diet items for field collected consumers) using generalized linear models with potential diet item detection (presence-absence per sample) as the response variable, surface sterilization treatment as a fixed effect, and a binomial distribution.

*Hypothesis 2: Does surface sterilization alter the proportion of potential diet DNA?*

Because potential diet DNA can represent a rare subset of total sequence abundance in DNA metabarcoding studies (e.g. 0.03 – 8.43 percent of all sequencing reads in one study; Krehenwinkel et al. 2016), we also wanted to determine whether surface sterilization altered this proportion of reads assigned to possible diet. Again, because contaminants can represent artificial diet or can be non-diet items, surface contamination, and therefore, surface sterilization, could lead to either an increase or decrease in the proportion of reads representing potential diet. To test whether surface sterilization altered the proportion of DNA representing potential diet items, we assessed per sample potential diet DNA proportion for both sets of consumers (mesocosm and field) separately. For this analysis, we subset only consumer individuals for which we detected diet DNA, since non-zero values are informative in indicating whether surface sterilization altered our detection of potential diet DNA when we detect potential diet DNA. We assessed diet proportional abundance in consumers using generalized linear models with the number of potential diet DNA reads per sample (only *O. japonica*, the offered potential diet item, for mesocosm consumers, all potential diet DNA for field consumers) as the response variable, surface sterilization treatment as a fixed effect, total read abundance of the sample (constant across all) as an offset term, and a Poisson or negative binomial distribution (to correct for overdispersion when needed). We also verified that any variation in detection or read abundance in either environment was due to contaminant removal and not a systematic relic of the extraction, amplification, or sequencing process by also examining the total proportion of DNA sequence reads assigned to consumers, and for the mesocosm consumers, all potential diet DNA (including *O. japonica*) expected to be found in each sample within the timeframe of our mesocosm trials based on results from another study (Marcias-Hernandez et al. 2018). These results are summarized in the Supplement.

*Hypothesis 3: Does surface sterilization alter the richness and composition of potential diet items in field consumers?*

In addition to allowing detection of diet items, DNA metabarcoding also enables the analysis of diet communities, allowing us to ask questions about individual-, population-, and species-level diet richness and composition. If surface contaminants alter these metrics, ecological interpretation of these community-level data could be misleading, either by increasing the number of consumptive interactions we attribute to a consumer, or by hiding interactions that occur more rarely or further back in time (e.g. Marcias-Hernandez et al. 2018, CITE RARITY and SAMPLILNG EFFORT). We assessed whether surface contamination, and thus, surface sterilization, altered species richness and species composition of potential diet items in our field-collected consumers. For per sample potential diet richness, we assessed per sample potential diet richness using generalized linear models with the number of potential diet species per sample as the response variable, surface sterilization treatment as the fixed effect and a Poisson or negative binomial distribution (to correct for overdispersion when needed). We assessed differences in potential diet species composition between surface sterilized and unsterilized consumers using a presence-absence PERMANOVA model fit with a binomial mixed effects model with surface sterilization treatment as a fixed effect, a random intercept term for potential diet species, and a random slope term for surface sterilization treatment. Incorporating a random intercept term for potential diet species combined with a random slope term for surface sterilization treatment allows the effect of surface sterilization treatment to vary by potential diet species, such that some potential diet species may increase in presence with surface sterilization (i.e. hidden by contaminants), while others may decrease in presence (i.e. potential diet item is a contaminant; Zurr, or other random slopes citation here). We repeated the field consumer potential diet item PERMANOVA with abundance data and also conducted both presence-absence and abundance based PERMANOVA analyses on all potential diet items (including offered item) for mesocosm consumers; Supplement.

*Model selection*

For all generalized linear models, we performed model selection by comparing the full model (including the fixed effect of surface sterilization treatment) to a null model without this effect. All models were called in the glmmTMB package (version 1.0.0, Brooks et al. 2017) in R (version 3.6.1) We chose the best fitting model based on size corrected AIC values (MuMIn package version 1.43.15). For responses for which the best model included the surface sterilization treatment term, we examined the model summary to determine the standardized coefficients () and p-value of the significance between marginal means of the levels of the surface sterilization fixed effect. We assessed model fit using diagnostics in the DHARMa package (version 0.2.7), including tests for heteroskedasticity, and for count models (Poisson or negative binomial), zero inflation and overdispersion (Zurr, Bolker citations here). All raw data, data cleaning, and data analyses can be found in the data (CITE data here).

**Results**

*PCR success, sequence merging, filtering, and clustering with UNOISE3 and DADA2*

Amplification success across all samples was 78%, with 56 of 72 initial samples successfully amplified and sequenced (mesocosm: n = 8 surface sterilized, n = 11 unsterilized; field: n = 18 surface sterilized, n = 19 unsterilized). The Illumina MiSeq run yielded 33,332,804 unpaired reads and had a Q30 quality score of 78.03%. After quality filtering and denoising with UNOISE3, we had a set of 8,029,959 paired-end reads that corresponded to 176 ASVs. Eighty-two percent (145/176) of the ASVs received a taxonomic assignment. All BOLD and BLASTed taxonomic assignments matched and the BOLD IDEngine did not assign taxonomies to any ASVs not assigned by BLAST (the BOLD taxonomic assignments were to species level versus order, family, or genus level for those ASVs that matched in BOLD; 44 total).

*Sampling completeness and error checking*

Based on sampling completeness curves, all samples were sequenced enough to capture 99 – 100% of the species richness in samples (supplement). The negative control was assigned only one ASV with a read abundance of one and were best fit by a negative binomial distribution. Based on this distribution, all reads with a read abundance of one or more represent real biological diversity (p-value < 0.001). ASVs were matched with good specificity, with each positive control assigned to 3 ASVs (with one dominating read abundance by a factor of 105 and the other two being much less abundant).

*Detection and abundance of diet*

We detected known diet in 74% of mesocosm and diet of all species in 86% of field-collected consumers. For mesocosm consumers, one ASV matched to the known diet (*O. japonica*), and the best model for diet detection included the fixed effect of surface sterilization treatment with a marginally significant difference between marginal means (( = -2.3; p-value = 0.07). Based on this model, known diet detection decreased with surface sterilization treatment from a detection in 91% of all consumers when unsterilized to 50% of all consumers when surface sterilized. For field consumers, 28 concatenated ASVs corresponded to diet items. The best model for diet detection was the null model that did not include surface sterilization treatment as a fixed effect (Figure). (Supplement).

We detected an average per-sample potential diet read proportion (of total abundance) of 0.8% (± 0.7% SE) for known diet reads in the mesocosm consumers and 2.0% (± 1.0%) diet read proportion of all species per sample for field-collected consumers (Figure). For both the mesocosm and field-collected consumers, the null models which did not include surface sterilization treatment as a fixed effect, were the best models of prey DNA read proportion.

*Diet richness and composition in field spiders*

For field consumers, the best model for per sample diet richness was the null model which did not include surface sterilization treatment as a fixed effect. Diet richness per consumer was an average 1.97 (± 0.04) per individual sample, with a maximum of 6 diet species in one consumer diet. The best model for potential diet species composition also did not include surface sterilization treatment as a fixed effect. Potential diet species composition consisted of nineteen species from nine arthropod orders (Insecta: Dermaptera, Blattodea, Diptera, Orthoptera, Odonata, Hymenoptera; Arachnida: Araneae, Scorpiones; Chilopoda: Geophilomorpha) and one infraclass (Insecta: Neoptera; winged insects) (Figure; Supplement).

**Discussion:**

[Main takeaways]

[Surface sterilization broadly]

[Surface sterilization by environment]

[limitations/ways forward for diet DNA metabarcoding]

[what we learn broadly from diet studies and why new tools are important]

Surface contamination may alter DNA metabarcoding results more in contained environments than in field environments, a result highlighted here in a comparison of contained versus field-collected individuals of the predatory consumer *Heteropoda venatoria*. Diet detection decreased with surface sterilization for consumers in a contained mesocosm environment, suggesting that shared surfaces or handling in the mesocosm environment could artificially inflate estimates of diet consumption by consumers in these types of environments. Contrasted with field collected consumers, where surface sterilization did not alter diet detection, this outcome highlights that some environments may be more prone to surface contamination that could alter the ecological interpretations of diet metabarcoding studies. Neither diet species richness nor the composition of diet species in field-collected consumers changed with surface sterilization, suggesting that the diet DNA detected in these consumerss represents diet items which were consumed by this predator. Overall, our DNA metabarcoding protocol had a high rate of diet detection (74% with a fed diet item, 86% with natural-fed diet items), suggesting that this protocol has broad usage for other invertebrate consumers. While the results of diet read proportional abundance in mesocosms was highly skewed by one datapoint, for both environments, diet proportional reads did not change with surface sterilization, suggesting that contamination neither inflates or hides diet reads, even when they are relatively rare in sequencing datasets (0.2 – 2.0% of the current dataset, similar to other studies; Krehenwinkel et al. 2016). Because surface sterilization did not decrease diet DNA detection, abundance, or richness in field-collected consumers (suggesting that sterilization method did not degrade DNA, further validated by our supplementary results, SUPPLEMENT), surface sterilization may be an appropriate conservative approach prior to any diet DNA metabarcoding study. In any case, the diet data revealed in our dataset suggest a wide range of continued and future applications for diet DNA metabarcoding studies across a range of environments (e.g. distinguishing consumption from fear responses; interactions including plant-herbivore, predator-diet, host-parasitoid, plant-pollinator, parasite-host, parasite predation; Kartzinel, Gao et al., Wirta, Bell et al. 2019, Orlofske et al. 2012).

These environments are often used to examine food web effects and functional responses of diet communities to different consumer communities (e.g. built mesocosms: Gao 2017, Rudolf et al. 2014; ponds, lakes, and natural microcosms: Srivistava et al. 2004, De Meester et al. 2005)

Diet detection in DNA diet contents is the key first diagnostic for determining consumptive interactions. While in the field environment diet detection did not vary with surface sterilization (and thus, contamination), in the mesocosm environment, contamination inflated the predicted rate of consumption for our consumer species. This outcome highlights that the decision to surface sterilize prior to DNA metabarcoding may matter more in some experimental set ups than others. For example, if a study is designed to understand the functional response of diet individuals or populations to consumer presence (e.g. Rudolf et al. 2014, Os Schmitz PAPERS, Carol Blanchette), then the distinction between altering the diet population via consumptive or non-consumptive effects (including killing but not ingesting) is less important than the key response, which is that a diet population changed (through loss of individuals, changes in population vital rates, or behavior) because of consumer presence. Conversely, if the study is designed to determine biomass of energy transfer, for example, in understanding how environmental variables (e.g. oxygen) or biotic variables (e.g. predator or diet size diversity) influence food web dynamics and nutrient cycling (e.g. Degerman et al. 2018, Garcias-Comas et al. 2016), then being able to validate consumption is key.

In addition to environmental context, various aspects of the experimental design and the ecology of the organisms involved in mesocosm experiments may lead to more or less chances of surface contamination. From our results from the field environment, we can hypothesize that this type of environment poses a relatively low risk of surface contamination. Any environment is shaped by both abiotic and biotic factors, so determining which of the components of this environment contribute to relatively low contamination risk could help build predictive frameworks of when surface sterilization may be necessary. In aquatic and marine eDNA environments, some abiotic conditions are attributed to DNA persistence (e.g. pH and salinity; Collins et al. 2018), while others are not or show inconclusive results (e.g. sunlight; Machler et al. 2018, Pilliod et al. 2014). Others attribute biotically-mediated responses to the environment in DNA persistence (e.g. microbial growth due to ideal abiotic conditions: Nielson et al; Strickler et al. 2014). In mass-collected arthropods, surface contamination influences diet detection (Greenstone et al. 2011/12) suggesting that diet density (and potentially size), and therefore, likelihood of surface contact, is also an important environmental consideration. Our field-collected consumers handle similar-sized diet items in their natural diet (all Blattodea observed in wild diets are similar in size to *O. japonica*), suggesting that shared environmental surfaces may contribute more to surface contamination than just handling diet during consumptive interactions.

Detection is a more common metric of interactions or presence than DNA read abundance in diet metabarcoding and eDNA studies (Elbrecht et al. 2015, Deagle et al. 2018, Zinger et al. 2019) and the high variability in values we observed for known diet DNA read abundances in both groups of consumers (0 – 5,391 reads per predator for mesocosm; 0 – 4,082 for field) highlight why the field has chosen to conservatively approach interactions with detection-only data. Many factors could contribute to this variation in read abundances, including how much diet was ingested (i.e. sample biomass: Elbrecht et al. 2017). how recently diet was ingested (Marcias-Hernandez), random variability in the DNA extraction and PCR protocols (e.g. primer bias; Elbrecht 2015), and randomness in the sequencing platform (Wen et al. 2017). While the randomness introduced via DNA extraction, PCR, and sequencing is harder to control, if variability is due to aspects of the interaction (e.g. ingestion amount or time), future studies could elucidate how to correct for this so that read abundances can be used with confidence in understanding both the frequency and biomass of interactions in DNA metabarcoding studies.

Adding to the ability to merely detect diet, common for decades in species-specific molecular diet approaches, DNA metabarcoding also allows for analyses of diet richness and composition (e.g. Kartzinel et al.). We found that these metrics of diet communities were not affected by surface contamination in field-collected consumers. Again, this suggests that environments similar to our field environment (factors that include sunlight, temperature, and collection method, among others) may pose relatively low-risk for surface contamination in DNA diet metabarcoding studies. In other environments and collection methods (i.e. those similar to our mesocosms), however, surface contamination may be more of an issue, and alter ecological inference from DNA metabarcoding studies in profound ways. For example, based on results from our contained mesocosm environment, we over-estimated population-level diet consumption by 41 percent. Based on the average diet richness observed per consumer individual in the field environment (roughly 2 species), if other contained environments have this level of over-estimation, estimates of population-level diet richness for a similar consumer population could include up to fifteen spurious diet items. These diet items could include species already detected in the diet of the population or new species that could represent up to 40% (15/38 diet items in this example) of the total diet diversity detected. This could have profound effects on the predictions drawn from these systems related to food web structure (e.g. food web resolution and aggregation: Martinez 1991), intraspecific diet specialization (e.g. Quevedo et al. 2009), and other important emergent properties of combinations of multiple species interactions (e.g. species distributions, ecological functions, and species adaptation or evolution: Kissling and Schleuning 2015, Weber et al. 2017).

Our mesocosm environments represented the potential risk of contained environments to inflate diet richness estimates; conversely, in other environments where consumers share surfaces with non-diet items that are also amplified with selected PCR primers, diet richness could be under-estimated as non-diet amplification drowns out potentially rare diet items. In our study, we used a general metazoan primer set (Leray et al.; other, Krehenwinkel) because our predator is a known generalist that will consume invertebrates and vertebrates in this system. This means that this primer set could also detect a large suite of non-diet items. In our study system on Palmyra Atoll, which is species poor and in which *H. venatoria* represents a top consumer that can eat most other species, most DNA amplified by these primers is a possible diet item for this consumer. However, in an environment where a consumer had a generalist diet (thus necessitating general primers) and shared substrates with many other animals that were likely not diet, these items could contaminate sequencing results and drown out diet items (e.g. soil environments; pools, and lakes). This could both hide low-biomass diet items (e.g. Elbrecht et al. 2017) as well as items of food that had been eaten longer ago (Marcias-Hernandez et al.), thus both decreasing the diet richness in samples and also reducing the snapshot of time from which DNA diet data is drawn. This reduction in the time frame for which diet data could be detected is particularly important in consumer interactions where consumers go long periods between feeding events (i.e. when diet densities or predator-diet ratios are low; Abrams and Ginzberg 2000).

The outcomes of our study highlight that the environmental aspects of any DNA metabarcoding study may warrant surface sterilizing consumers prior to diet metabarcoding. We have highlighted one aspect of this environmental context: namely, that there are environments where consumers are more likely to come into contact with contaminant DNA (either diet or non-diet), and that this seems to be driven through the degree with which consumers and diet share substrate or environmental space. There are other factors that may contribute to this pattern, including abiotic conditions which favor the persistence or degradation of DNA in the environment (e.g. pH, salinity, sunlight; Strickler et al. 2019). We have also suggested that ecological factors of an environment or consumer could shape this rate of contamination, including diet density, consumer-diet population ratios, and consumer-diet body size ratios, which can increase contact probability and contact (handling) times, predisposing consumers to having diet DNA on body surfaces even if ingestion has not occurred (Scharf et al. 1998, Jeschke et al. 2002, Samu and Biro 1993). Finally, collection method may contribute to contamination risk, as highlighted with mass-collection methods in Greenstone et al. (2011/2012); and this contamination is doubly important in metabarcoding studies (as opposed to studies of one or few diet items, as these studies) where not only diet detection, but also diet diversity and composition could be greatly influenced by contaminant DNA. Our findings also highlight that surface sterilization does not seem to be degrading DNA (also see Supplementary analyses), and so despite any environmental, ecological, or methodological considerations, it appears that surface sterilization is a conservative approach in any context that will not lead to the loss of diet information and will solidify confidence in findings and interpretation.

DNA metabarcoding is providing the first glimpse at comprehensive diet for a suite of consumers important to the field of food web ecology and to the maintenance of biodiversity on the planet (Nielson et al. 2018). DNA metabarcoding could be used in combination with other diet methods to understand how consumptive processes that scale from between individuals to between functional groups may regulate ecosystems and their functions (Birhofer et al. 2017). Like any method for determining consumptive interactions in nature, DNA metabarcoding needs refinement (e.g. discussion in Zinger et al. 2019). As DNA methods become more standardized and cost-efficient (including extending to metagenomics, which can differentiate individuals of a species in a sample; Gomez-Rodriguez et al. 2017), online taxonomy databases become more complete (Kvist 2013), and new techniques arise (e.g. real-time field sequencing with MinION technology; Jain et al. 2015), DNA-based diet approaches may surpass many other similar-performing methods (e.g. Riccioni et al. 2018). Being able to determine consumptive interactions for many species and environments for the first time will only continue to build a bigger picture of the complex structure of nature, and how species interactions can scale up to create unique ecosystems and ecosystem functions (Pilosof et al. 2017, Ives et al. 2005, Rudolf and Lafferty 2011, Brophy et al. 2017, Orlofske et al. 2012, Dunne et al. PARASITES). In addition to building more realistic models of community interactions, a more complete picture of how consumptive interactions shape ecosystems could be key to predicting and curbing future biodiversity loss due to human change (Tylianakis et al. 2008, Harvey et al.2017).

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**References**

Will be putting in after some rounds of edits.

**Data Accessibility**

Data will be available on Dryad and I will be uploading my sequence data to GenBank.

**Author Contributions**

AM-tK, AA, and HY conceived the idea for this study. AM-tK collected field samples and conducted mesocosm study. AM-tK and AA designed laboratory analyses for this study. AM-tK performed all lab processing and data analyses for the study. AA and HY provided feedback on data analysis methods. AM-tK led the writing of the manuscript. All authors contributed to editing of the manuscript.

**Figures and Tables:**

Table: Sample sizes for successfully DNA extracted and PCR amplified samples of surface sterilized and unsterilized *H. venatoria* individuals in the mesocosm and field environments. Bold numbers indicate final sample sizes for analyses.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | *Surface sterilized* |  | *Unsterilized* |  |
| *Environment* | Extracted | Amplified | Extracted | Amplified |
| Mesocosm | 10 | **8** | 14 | **11** |
| Field | 22 | **18** | 25 | **19** |

Table: Primers with Illumina overhang adapters (in bold) used to amplify the COI region in this study.

|  |  |  |
| --- | --- | --- |
| Primer | Sequence (5’ – 3’) | Source |
| mICOIintF | **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGWACWGGWTGAACWGTWTAYCCYCC | Yu et al. 2012 |
| Fol-degen-rev | **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TANACYTCNGGRTGNCCRAARAAYCA | Leray et al. 2013 |

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Figure: a) Detection of fed diet (*Oxya japonica*) DNA in mesocosm consumers that were and were not surface sterilized. Significant decrease with surface sterilization. B) Detection of all diet DNA in field-collected consumers that were and were not surface sterilized. Non-significant difference.

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Figure: a) In mesocosm consumers for which the fed diet was detected, the proportional abundance of DNA from that diet item in individual spiders. The difference is significant, but driven by one very high data point, and when that is removed, the difference is no longer significant. b) In field-collected consumers for which diet DNA was detected, the proportional abundance of DNA from that diet item in individual consumers. The difference is non-significant.

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Figure: Diet richness (number of unique diet taxonomic assignments) for field collected consumers that were and were not surface sterilized. There is no significant difference.

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Figure: Presence (indicated by a colored box) and abundance (indicated by color depth) of diet items in field-collected consumers that were and were not surface sterilized. Neither presence- or abundance-based community metrics demonstrate a significant difference between these two groups of consumers. Because of the wide range of abundances per diet item, color depth is divided by quartiles of DNA sequence abundance.

**Supplementary Info and Figures:**

Data: Raw data files as well as code for each step of the cleaning and analysis process.

Supplement A: model outputs for GLMMs in main text

Supplement B: Full DNA extraction and PCR protocol

Supplement C: Comparison of UNOISE3 and DADA2 in diet DNA detection

Supplement D: Additional figures and statistical analyses, including consumer and other diet DNA abundance for mesocosm consumers, by-species presence and composition effect graphs from both mesocosm and field PERMANOVA analyses.

A picture containing dark, man, holding, standing

Description automatically generated

Supp Figure: Ampure XP bead cleaning of DNA to remove consumer DNA.



Supp Figure: Library prep, starting with attaching the COI primer pair with Illumina tag to diluted, bead-cleaned DNA. Then, this PCR product is bead cleaned at a 0.8x ratio and run through a subsequent PCR step to attach Illumina tag, index, and P5/P7 identifiers. This PCR product is then cleaned again at a 0.7x bead ratio, diluted to 5nM, and pooled for sequencing on an Illumina MiSeq.

A picture containing screenshot, food

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Supp Figure: Denoising algorithms like UNOISE3 and DADA2 take into account DNA sequence abundance and error rates to assign groups of similar sequences to one amplicon sequence variant (ASV).



Supp Figure: Sequencing depth determined via interpolation and extrapolation in the iNEXT package in R. All samples were sequenced to 99-100% sequencing depth, meaning we could compare communities derived from these samples without removing any from the analysis.

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Supp Figure: Consumer DNA read abundances from A) mesocosm consumers and B) field-collected consumers and potential diet DNA reads from C) mesocosm consumers and D) field consumers that were and were not surface sterilized. The surface sterilized/not surface sterilized treatment groups are not significantly different for any type of other DNA, suggesting that the reduction in diet detection for mesocosm-fed consumers is due to surface contamination as opposed to a relic of the DNA extraction, amplification, or sequencing process.



Supp Figure: The composition by-species of other diet in the mesocosm consumers, demonstrating that both presence- and abundance-based diet communities did not shift with surface sterilization treatment. (more positive means more present/abundant in unsterilized; more negative means more present/abundant in sterilized). Species are ranked by their overall presence in the population (A) or their overall abundance in the population (B) to demonstrate that there is no skew for relatively abundant or rare species.



Supp Figure: The composition by-species of diet in the field consumers, demonstrating that both presence- and abundance-based diet communities did not shift with surface sterilization treatment. More positive values mean more present/abundant in non-sterilized; more negative values mean more present/abundant in surface sterilized consumers). Species are ranked by their overall presence in the population (A) or their overall abundance in the population (B) to demonstrate that there is no skew for relatively abundant or rare species.