Assessing the persistence of environmental DNA and environmental RNA for zooplankton biodiversity monitoring by metabarcoding

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Abstract

Environmental DNA (eDNA) metabarcoding has proven successful at detecting low abundance species due to the stability and ubiquity of DNA in the environment. While this is ideal for efficient monitoring over large geographic ranges, eDNA persistence and transport within aquatic systems often introduces false positive detection of species which are spatially or temporally removed. Environmental RNA (eRNA) metabarcoding may allow better spatial and temporal resolution by detecting only present, local species due to the faster degradation rate of the RNA molecule; however, little is known about eRNA persistence in the environment. Here I test species detection in zooplankton communities using eDNA and eRNA metabarcoding of two barcode markers (COI and 18S) across seven time points spanning from one hour to one month after organism removal. The metabarcoding results were validated with morphologically identified voucher specimens and mock communities. Community composition as detected by eDNA and eRNA was similar, however, species assignment at the COI and 18S markers differed. Zooplankton were detectable with eDNA throughout the experiment with the COI marker and at all but one time point with the 18S marker, whereas detection with eRNA ceased at 24 hours after organism removal for both markers, with only rare detections at 48 hours with 18S, and four and seven days with COI. There was an unexpected increase in detection at the 28-day time point for all methods, possibly due to concentration of eDNA and eRNA adhered to the container walls. Through comparing the two metabarcoding techniques, I have demonstrated that eRNA metabarcoding stops detecting zooplankton species shortly after organism removal, while detection by eDNA metabarcoding persists for at least seven days. Environmental RNA metabarcoding could be applied in parallel with eDNA metabarcoding to distinguish current,

local diversity, which is particularly relevant for high-resolution sampling, such as for species at risk or invasive species monitoring.

Résumé

Récemment, les méthodes moléculaires utilisées pour la biosurveillance sont devenues populaires, notamment les méthodes de métacodage à barre de l'ADN environnemental (ADNe). Cette méthode est efficace pour détecter des organismes rares à cause de la stabilité et l'abondance de l'ADN dans l'environnement. Ces caractéristiques sont idéales pour la surveillance dans des grandes régions, par contre la persistance et le transport de l'ADNe dans les systèmes aquatiques peuvent introduire des résultats faux positifs en détectant des espèces qui ne sont pas présent localement. L'utilisation de métacodage à barre d'ARN environnemental (ARNe) a été proposée pour améliorer le problème de la résolution spatiale et temporale, menant seulement à la détection des espèces locales en raison de la dégradation rapide d'ARN. Ici, je teste la détection des espèces de zooplanctons avec le métacodage à barre d'ADNe et d'ARNe avec deux marqueurs moléculaires (COI et 18S) entre une heure et un mois après l'enlèvement des organismes. Les résultats moléculaires ont été vérifiés avec des spécimens identifiés morphologiquement et le séquençage des communautés artificielles. La composition des communautés détecté par l'ADNe et l'ARNe était similaire, mais l'identification des espèces par les marqueurs COI et 18S était diffèrent. Des zooplanctons étaient détectables pendant toute l'expérience avec le marqueur COI sur l'ADNe, et à tous les temps sauf un avec le marqueur 18S. D'un autre côté, rien n'était détectable par l'ARNe à 24 heures après l'enlèvement des organismes, avec seulement des rares détections à 48 heures avec 18S, et quatre et sept jours avec COI. Il y avait un augmentation inattendue de la détection des crustacés par toutes les méthodes après 28 jours, possiblement causée par la concentration de l'ADNe et l'ARNe adhéré

au mures des contenants. En comparant les deux techniques de métacodage à barre, j'ai démontré que l'ARNe arrête de détecter des espèces de zooplancton plus tôt après l'enlèvement des organismes que l'ADNe. Ceci est causé par les différentes caractéristiques de dégradation et de persistance de l'ADN et l'ARN dans l'environnement. Le métabarcodage d'ARNe peut être utilisé avec l'ADNe pour distinguer des organismes présents et locaux, ce qui est nécessaire pour la surveillance des espèces en péril ou envahissantes.

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Contribution of Authors

I am the main author of this thesis and the manuscripts herein.

I designed this experiment with the help of my supervisor, Dr. Melania Cristescu. I collected water samples from three artificial ponds. I filtered and extracted eDNA and eRNA. I performed all molecular laboratory work, including DNA and RNA extractions, DNA digestion, reverse transcription, PCR amplification and library preparation for high throughput sequencing. Quality control and high throughput sequencing was conducted by Genome Quebec, Montreal, QC. I analysed the high throughput sequencing data with the help of Dr. Sergio Hleap, with a bioinformatics pipeline modified from Dr. Frederic Chain and Gillian Martin.

I collected water from ponds in a large-scale collaborative experiment of the McGill University Department of Biology, hereafter LEAP 2017. The LEAP 2017 experiment was designed by Andy Gonzalez and Vincent Fugère. Morphological species identification was done by Jorge Negrin Dastis.

My supervisor, Dr. Melania Cristescu, contributed my thesis proposal, experimental design, experimental guidance and comments on earlier drafts of this thesis and the manuscript herein. My committee members, Dr. Gregory-Eaves and Dr. Whyte, provided feedback on the proposal and comments on earlier drafts of this thesis.

General Introduction

Environmental DNA (eDNA) metabarcoding is increasing in popularity as a non-invasive biomonitoring method capable of providing species level identification with reduced dependency on taxonomic expertise (Thomsen et al. 2012). This method is particularly relevant for monitoring ephemeral or low abundance species, such as species at risk or the early stages of species invasion (Jerde et al. 2011; Thomsen et al. 2012; Mahon et al. 2013). These rare species are often difficult to detect by traditional monitoring methods. The use of eDNA can increase the chances of detection, in large part because DNA is a stable molecule capable of persisting in an environmental medium for up to several weeks after being shed from an organism (Dejean et al. 2011; Thomsen et al. 2012; Barnes et al. 2014; Barnes and Turner 2016). During this time, eDNA may be transport in lotic systems, and eDNA from an entire catchment area can be funnelled to a downstream sampling site (Deiner et al. 2016; Pont et al. 2018). While this can be ideal for monitoring large geographic ranges, it may introduce confounding signals when monitoring for local, currently present species. While eDNA metabarcoding may increase the probability of detection over traditional methods, thereby reducing false negatives, it may also be increasing false positive detection of species that are not present in the sampled site.

The use of environmental RNA (eRNA) has been proposed as a complimentary barcode template molecule with a faster degradation time, and so, limited persistence in the environment (Laroche et al. 2017; Pochon et al. 2017, Cristescu and Hebert 2018). It is well known that RNA is less stable than DNA, due to its single-stranded molecular structure and the ubiquity of RNAses (Karl and Bailiff 1989; Deutscher 200). Furthermore, ribosomal RNA (rRNA) is more stable than messenger RNA (mRNA) due to its secondary structure and protein association (Fontaine and Guillot 2003; Deutscher 2006). These differences in molecular stability between

DNA, mRNA and rRNA could serve to refine the window of species detection from environmental samples. Used in parallel with eDNA metabarcoding, eRNA metabarcoding could allow us to distinguish local and currently present species from those which are spatially or temporally removed.

Literature Review

Molecular Biomonitoring

Environmental DNA metabarcoding is rapidly becoming an efficient, reliable and non-invasive way to assess biodiversity in freshwater ecosystems (Thomsen et al. 2012). DNA barcoding is a method for taxonomic identification by comparing the sequence of a standardized barcode marker, obtained from a single sample organism, against a reference database of sequences from taxonomically-identified voucher specimens (Hebert et al. 2003). Metabarcoding is taxonomic identification at a community level by using high throughput sequencing to simultaneously sequence barcode markers collected from many species (Taberlet et al. 2012b; Shokralla et al. 2015). Metabarcoding can be performed on either bulk DNA sampled directly from organisms (e.g., a plankton tow) or on environmental DNA (eDNA) shed into the air, soil or water (Ficetola et al. 2008). Therefore, the term eDNA metabarcoding refers to simultaneous species identification of a community, based on high throughput sequencing of DNA barcodes collected from environmental samples (Shokralla et al. 2012).

Molecular based methods increase biomonitoring efficiency over traditional morphology based methods by reducing the need for taxonomic expertise in species identification (Darling and Blum 2007). For example, zooplankton species are notoriously difficult to identify by traditional methods due to morphologically diverse life stages and cryptic species (Bucklin et al. 2016). Metabarcoding can provide simultaneous species level identification of organisms across

a community, thereby offering an advantage over targeted species monitoring by enabling the detection of unanticipated organisms (Klymus et al. 2017). While molecular methods allow for taxonomic identification of larval or juvenile life stages and partial specimens, which are often unidentifiable by morphological methods, they cannot currently distinguish the sex, age, viability, or exact number of organisms (Rees et al. 2014). Furthermore, the reduced need for taxonomic expertise in sampling is accompanied by a dependency on well-curated databases of sequences from voucher specimens. Species identification depends on the availability of a complete reference database with accurately identified voucher specimens (Clarke et al. 2017). Incomplete databases result in false negative detection, failing to identify species without vouchers, while an inaccurate database result in false positive species detection by matching a sequence to a misidentified voucher specimen (Bucklin et al. 2016).

Ideal barcode markers must have sufficient interspecific variation to distinguish species, yet low intraspecific variation to group organisms of a species together (Bohle and Gabaldón 2012). Markers used in metabarcoding must have conserved regions for primer binding, flanking divergent regions for taxonomic identification. There is a trade-off between taxonomic recovery and resolution in barcodes. Barcode markers that are more universal (i.e., more conserved) may not be sufficiently divergent to distinguish all species; whereas, markers that are more distinct between species may not be amplifiable by a common set of universal primers. Differences in taxonomic recovery and resolution between common zooplankton barcode markers are often reported in DNA metabarcoding literature (Corell and Rodríguez-Ezpeleta 2014; Bucklin et al. 2016; Clarke et al. 2017; Zhang et al. 2018). The 18S barcode marker is commonly used for zooplankton metabarcoding and can distinguish taxa to the family or genus level (Bucklin et al. 2016). The COI barcode marker is more variable and can distinguish closely related species,

however it may require group specific primers to amplify (Corell and Rodríguez-Ezpeleta 2014; Bucklin et al. 2016). Degenerate primers, which allow for some base mismatch when binding to the template, can also broaden taxonomic recovery but may be more prone to non-specific binding (Corell and Rodríguez-Ezpeleta 2014; Clarke et al. 2017). Ultimately, species detection relies on the amplification (i.e., primer binding) of a barcode marker that corresponds to a unique voucher taxon in the sequence database.

Environmental DNA

The use of environmental samples offers advantages over traditional sampling methods, particularly when monitoring for low abundance organisms. For example, locating species at risk, monitoring the early stages of a species invasion, or detecting surviving invaders after eradication efforts all require detection of species at low abundance (Jerde et al. 2011; Mahon et al. 2013; Dunker et al. 2016; Balasingham et al. 2018; Takahashi et al. 2018). Surveying these rare organisms is costly and time consuming by traditional methods, and eDNA based methods are an efficient alternative. Environmental DNA has been shown to outperform electrofishing, fyke net sampling and visual surveys for both species detection and cost effectiveness (Civade et al. 2016; Hinlo et al. 2017; Takahashi et al. 2018). When monitoring for invasive American Bullfrog, eDNA detected the species in five times as many ponds as visual surveys (Dejean et al. 2012). While monitoring invasion fronts, it required 93 person days of surveying to detect one carp in a tributary that tested positive by eDNA based methods (Jerde et al. 2011). The same study estimated that every 100 eDNA samples required 40 person hours, or 0.174 person days per sample.

Traditional monitoring methods rely on observing the organism, which requires increased sampling effort for low abundance species. Environmental DNA based methods are capable of

detecting the same organism from trace amounts of DNA shed into the waster (Jerde et al. 2011). Concentrations as low as 0.00045 ng/L (Pilliod et al. 2014), or as little as 25 eDNA copies (Thomsen et al. 2012) have been reported as detection limits. One study reported single copy detection from control treatments (Barnes et al. 2014). While this level of sensitivity highlights the susceptibility of eDNA samples to contamination and false positive detection, it is also the underlying reason for the success of eDNA based biomonitoring.

Environmental DNA metabarcoding has proven successful at capturing the biodiversity of an area, largely due to the relative stability of the DNA molecule and its ability to persist in the environment. Studies have shown that eDNA can persist for days, up to several weeks in freshwater and several months in sediment (Dejean et al. 2011; Thomsen et al. 2012; Barnes et al. 2014; Pilliod et al. 2014; Strickler et al. 2015; Turner et al. 2015; Barnes and Turner 2016). During this time, eDNA can be transported in lotic systems, resulting in an "conveyor belt" funnelling eDNA from an entire catchment area downstream (Deiner et al. 2016; Pont et al. 2018). Reported eDNA transport distances range from tens of meters to over 12 km, and vary with flow rate and streambed substrate (Deiner and Altermatt 2014; Jane et al. 2015; Jerde et al. 2016; Wilcox et al. 2016; Shogren et al. 2017). Signals from lake dwelling fish have been detected up to 60 km downstream of their source population in large rivers (Pont et al. 2018). Downstream sampling has been shown to increase detection probability and enable detection of both aquatic and terrestrial organisms (Deiner et al. 2016; Rice et al. 2018). This can facilitate sampling over large geographic ranges, further adding to sampling efficiency.

Though the persistence and transport of eDNA increases efficiency for large-scale biodiversity surveys, it introduce confounding signals when trying to assess current, local diversity. Distinguishing between local organisms and those residing upstream may not always

be possible with eDNA. The method may also fail to detect rapid species turnover or eradication, with eDNA persisting in the environment for weeks after a species becomes absent (Dunker et al. 2016; Kamoroff and Goldberg 2018). This indiscrimination between present organisms, and those that are spatially or temporally removed is problematic for small scale, high-resolution studies. Recent studies have proposed the use of environmental RNA (eRNA) as a complementary barcode marker with faster degradation rates, and therefore limited persistence in the environment (Laroche et al. 2017; Pochon et al. 2017). With a shorter window of detection, eRNA metabarcoding could be a useful tool for assessing locally present species.

Environmental RNA

RNA has been widely used in metabarcoding studies as a complementary barcode marker to assess microbial community activity (reviewed in Blazewicz et al. 2013). Despite limitations in directly correlating RNA to microbial community growth, RNA and normalized RNA:DNA ratios have been used to identify currently active species in microbial communities (Blazewicz et al. 2013). The parallel use of DNA and RNA allows for monitoring of presence and metabolic activity in benthic microbial and meiofaunal communities along gradients radiating away from aquafarms (Dowle et al. 2015; Pochon et al. 2015; Pawlowski et al. 2016) or oil platforms (Laroche et al. 2016). RNA has been used to distinguish between microbial species living below a chemocline and detritus sinking from above (Stoeck et al. 2007), and to detect microbial species composition after seasonal turnover (Charvet et al. 2018). It has even been suggested that RNA may be a stronger indicator of in-situ microbial diversity patterns by targeting living cells (Vargas et al. 2009; Egge et al. 2015; Massana et al. 2015; López-escardó et al. 2018).

Until recently, the use of RNA alongside DNA has been limited to microbial metabarcoding studies. Though collected as water or sediment, these are samples of microbial

nucleic acids from intact, living organisms as opposed to nucleic acids shed into the environmental medium (Taberlet et al. 2012; Cristescu and Hebert 2018). One recent study has paired environmental RNA (eRNA) with eDNA metabarcoding of macroorganisms to distinguish between living organisms and detritus in ship bilge water (Pochon et al. 2017). The study found a large overlap in Operational Taxonomic Units (OTUs) detected by eDNA and eRNA metabarcoding, with OTUs unique to eDNA attributed to legacy DNA from detritus and OTUs unique to eRNA attributed to increased cellular activity in rare organisms, or artefacts of reverse transcription and amplification. With the recent expansion in eDNA metabarcoding studies, it has been suggested that eRNA metabarcoding could be applied to refine species detection (Barnes and Turner 2016; Laroche et al. 2017). This method has the potential to apply spatial and temporal range limits to species detection, based on the understanding that, while DNA is stable, RNA is subject to rapid degradation and will not persist in the environment. However, to date few studies have applied eRNA metabarcoding to extra-organismal RNA and the fundamental groundwork for this method is still needed to establish the window for detection.

Degradation of eDNA and eRNA

It is well known that DNA is more stable than RNA, as RNA has a single-stranded molecular structure and a 2'-hydroxyl group that makes it prone to hydrolysis (Deutscher 2006). This effect is increased under alkaline conditions where the hydroxyl group becomes more volatile. Moreover, RNA is more difficult to collect from environmental samples than DNA, due to the ubiquity of RNA degrading enzymes, or RNAses (Stoeck et al. 2007). Thus, RNA samples must be flash frozen to -80°C or stored in a preservative immediately after filtration. Even then, differences in DNA and RNA degradation rates in stored filtered water samples have been observed. Karl and Bailiff (1989) noted 10% loss filtered DNA by 28 days, and a 25% loss in

RNA over the same time when samples were frozen without a preservative. Differences in molecular structure also result in variable stability among different types of RNA molecules; ribosomal RNA (rRNA) is more stable than messenger RNA (mRNA) due to the secondary structure and folding patterns of rRNA (Fontaine and Guillot 2003; Deutscher 2006). Molecule length, protein association, copy number and location within a cell or organelle will also contribute to the stability of nucleic acids (Deutscher 2006). As such, choosing short, high copy number molecular markers will increase the probability of detection from environmental samples. The stability of eDNA and eRNA will further depend on initial state; whether it is free floating, membrane bound in cells or organelles, or contained within relatively large pieces of detritus (Barnes et al. 2014; Turner et al. 2014). These differences in molecular stability translate to potential differences in environmental persistence, and therefore species detection by metabarcoding.

The decay pattern of eDNA under varying environmental conditions has been extensively studied and is agreed to follow an exponential decay model:

$$D(t) = D_0 e^{-rt}$$

where D(t) is the concentration of eDNA at time t, D_0 is the initial eDNA concentration and r is the decay rate (Thomsen et al. 2012; Barnes et al. 2014; Pilliod et al. 2014; Strickler et al. 2015; Lance et al. 2017). Decay rates (r) ranging from 0.105 hr^{-1} to 0.37 $days^{-1}$ for carp (Barnes et al. 2014; Lance et al. 2017), and 0.05 $days^{-1}$ to 0.35 $days^{-1}$ for bullfrog (Strickler et al. 2015) have been reported. The degradation rate has been shown to vary with temperature, pH and UV treatments, while the exponential shape of the curve is independent of these variables and of the initial concentration of eDNA. (Thomsen et al. 2012; Pilliod et al. 2014; Strickler et al. 2015; Eichmiller et al. 2016; Lance et al. 2017). Microbial activity is thought to be among the most

causal factors in eDNA decay. In fact, the temperature and pH that result in maximum degradation rates are associated with conditions favouring microbial growth rather than physical or chemical damage to DNA (Barnes et al. 2014; Strickler et al. 2015).

To date, little is known about how eRNA persists in the environment. To the best of my knowledge, this thesis is the first account of comparative degradation of eDNA and eRNA. Based on the recent success of eDNA metabarcoding in monitoring macrobiotic diversity, and the use of parallel DNA and RNA metabarcoding in microbial studies, there is great potential for developing a complementary eRNA metabarcoding method to refine macroorganism biomonitoring. However, the fundamental work is still needed to demonstrate that eRNA metabarcoding will refine species detection. This thesis is the next step in developing the novel method in molecular ecology.

Objectives

In order for eRNA metabarcoding to be an effective biomonitoring technique capable of distinguishing present organisms, we must demonstrate that 1) eRNA based methods provide comparable species detection to eDNA metabarcoding for present communities, and that 2) the eRNA based method fails to detect species shortly after they are removed from the system. If eRNA degrades so rapidly that eRNA metabarcoding cannot reliably detect the same diversity of present species as eDNA metabarcoding, then eRNA metabarcoding cannot become an effective biomonitoring tool. Alternatively, if eRNA persists as long as eDNA after organism removal, then the application of eRNA metabarcoding is redundant.

In this thesis, I compare zooplankton species detection by eDNA and eRNA metabarcoding from samples collected at McGill University's Large Experimental Array of Ponds (LEAP). The ponds contain well-documented communities that resemble natural assemblages. Zooplankton

samples were collected for morphological community identification, DNA sequencing of voucher specimens, and to assemble mock communities. I compare the degradation rates of eDNA and eRNA by comparing species detection across time points spanning from one hour to one month after organism removal. I used two common barcode markers: The COI marker located on mitochondrial DNA and transcribed as mRNA, and the 18S marker on nuclear DNA and transcribed as rRNA. I predict that eDNA will persist longer in the environment than eRNA, resulting in species detection over a longer time span by eDNA metabarcoding than eRNA metabarcoding. Furthermore, within eRNA samples, I expect species detection with the rRNA based marker will outlast that of the mRNA based marker over time, due to the increased molecular stability of rRNA.

Assessing the persistence of environmental DNA and environmental RNA for zooplankton biodiversity monitoring by metabarcoding

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Abstract

Molecular methods of species identification are increasingly used in biodiversity studies and conservation. Especially attractive are methods based on environmental DNA (eDNA) largely due to its ubiquity and stability, thereby increasing the chances of detecting ephemeral or low abundance species. While this can be ideal for efficient monitoring over large geographic ranges, eDNA persistence and transport within complex, interconnected aquatic systems may introduce confounding signals from species which are spatially or temporally removed, increasing false positive detection. The use of environmental RNA (eRNA) metabarcoding may allow finer spatial and temporal resolution by only detecting present, local species due to the faster degradation time of the RNA molecule. Here we test species detection in zooplankton communities by eDNA and eRNA metabarcoding with two barcode markers (COI and 18S) across seven time points spanning up to one month after organism removal. Zooplankton community composition as determined by eDNA and eRNA based methods were similar; however, species assignment by the COI and 18S barcode markers differed. Zooplankton were detected throughout the experiment with eDNA with the COI barcode marker, and at all but one time point with the 18S marker; whereas detection with eRNA ceased at 24 hours after organism removal for both markers, with only rare detections at 48 hours with 18S, and four and seven days with COI. An unexpected increase in zooplankton detection was observed at 28 days with all methods. The results indicate that eRNA metabarcoding is capable of capturing similar species diversity as the established eDNA based method, and that detection be eRNA metabarcoding stops shortly after organism removal, while detection by eDNA metabarcoding persists.

Key Words: Environmental DNA; Environmental RNA; Zooplankton; Metabarcoding

Introduction

In the face of the current extinction crisis, there is an urgent need for accurate and efficient monitoring of low abundance species, such as early invaders or species at risk, which have a disproportionately large impact on biodiversity and ecosystem health (Barnosky et al. 2011). There is a need to move beyond single species monitoring, towards community assessment in order to properly evaluate the health of our ecosystems (Yamanaka and Minamoto 2016; Balasingham et al. 2018). As a non-invasive sampling technique that allows for community-wide species level identification with reduced need for taxonomic expertise, eDNA metabarcoding is rapidly becoming an efficient and reliable way to assess biodiversity in freshwater ecosystems (Shokralla et al. 2012). This is particularly relevant for monitoring zooplankton species, as they are notoriously difficult to identify by traditional methods due to morphologically diverse life stages and cryptic species (Bucklin et al. 2016). An organism's probability of detection depends on the amount of DNA present in the environment; the release, degradation and transport of eDNA, collectively referred to as the "ecology" of eDNA (Barnes and Turner 2016). One of the reasons that eDNA metabarcoding has proven so successful at capturing diversity is the stability of the DNA molecule and its ability to persist in the environment. Studies have shown that DNA is stable enough to last weeks in fresh water and even endure transport up to 12 km in lotic systems (Dejean et al. 2011; Barnes et al. 2014; Deiner and Altermatt 2014; Barnes and Turner 2016). Though this is useful when assessing the biodiversity of a catchment area, it may introduce confounding signals when trying to assess current, local diversity. For example, we may not be able to detect rapid species turnover with eDNA metabarcoding, or distinguish between local and regional (upstream) organisms. As eDNA based surveys move beyond single organism identification and are applied to community assessment in both natural and

experimental settings, the indiscrimination between the present community and communities removed in space or time could be problematic (e.g., Dejean et al. 2012; Pont et al. 2018).

A potential solution to this problem is the use of an alternative barcode molecule, such as RNA, which has a history of use in microbial studies to assess community metabolic activity (Blazewicz et al. 2013). Until recently, the use of RNA alongside DNA has been limited to microbial samples (e.g., Pawlowski et al. 2014; Dowle et al. 2015; Pochon et al. 2015), and though collected in environmental samples (e.g., water or sediment), the nucleic acids are sampled directly from intact, living organisms (Taberlet et al. 2012). The use of eDNA has since expanded to include biomonitoring of diverse macroorganism taxa by sampling extra-organismal DNA (Rees et al. 2014), and it is only recently that this form of eRNA metabarcoding has been proposed (Barnes and Turner 2016; Laroche et al. 2017). One study used environmental RNA (eRNA) along side eDNA metabarcoding to distinguish between living macroorganisms and detritus in ship bilge water (Pochon et al. 2017). It has been hypothesized that eRNA metabarcoding could be applied to detecting current, local diversity, without the residual signals introduced by eDNA persistence and transport (Laroche et al. 2017). This could allow us to detect species turnover within a community, or apply spatial-temporal range limits on detected organisms. However, in order to apply eRNA metabarcoding for environmental monitoring, we must first understand how species detection compares to that of the established eDNA method.

In order to verify that eRNA metabarcoding could be developed as an efficient method for sampling present communities, we must 1) establish that eRNA based methods are efficient for species detection by providing comparable results to eDNA metabarcoding for current or recent species assemblages, and 2) understand the degradation time of eRNA to ensure the detection of only the most recent community. If eRNA degradation occurs so rapidly outside of the organism

that there is insufficient product to detect present species from environmental samples, then the method is not reliable for biomonitoring. Moreover, if eRNA persistence in the environment is no different than eDNA, then the use of an alternative RNA-based marker is redundant.

Differences in molecule stability, and therefore persistence in the environment, will affect metabarcoding species detection success. It is well established that RNA is less stable than DNA, in part due to the single-stranded structure of RNA and the presence of hydroxyl groups that make it prone to hydrolysis, especially under alkaline conditions. When collecting and storing nucleic acids from filtered water samples, RNA must be immediately frozen at -80 °C or stored in preservative (Stoeck et al. 2007). Additionally, within RNA, ribosomal RNA (rRNA) is more stable than messenger RNA (mRNA) due to a combination of molecular length, secondary structure and folding, protein association, copy number or expression and location within the cell or organelle (Fontaine and Guillot 2003; Deutscher 2006).

In addition to differences in molecular stability, the choice of barcode marker may also affect detectability. Short markers with high copy number are more likely to be detected in the environment. Here we use two common barcode markers for zooplankton identification: the cytochrome *c* oxidase I fragment, hereafter COI (mICOIintF and HCO2198; Leray et al. 2013) and the V4 region of the nuclear 18S marker, hereafter 18S (Uni18S and Uni18SR; Zhan et al. 2013). The COI marker is located on mitochondrial DNA and transcribed as mRNA. The COI marker used here is half the length of the original COI barcoding marker (Folmer et al. 1994) and was developed to detect degraded plankton DNA in fish gut content (Leray et al. 2013). The 18S marker is located on nuclear DNA and transcribed as rRNA. The primers were designed to detect low abundance species in zooplankton communities (Zhan et al. 2013).

We evaluate the degradation rates of eRNA and eDNA using a comparative metabarcoding approach based on two distinct markers (COI and 18S) to analyse zooplankton communities maintained in artificial ponds. We compare species detection across seven time points spanning from one hour to one month after organism removal. Due to the variation in molecular stability and persistence in the environment, we expect species detection by eDNA to persist longer than that of eRNA over time. Furthermore, within eRNA samples, species detection at the rRNA based 18S marker is expected to outlast that of the mRNA based COI marker.

Materials and Methods

Experimental Set Up and Sample Collection

This experiment occurred at McGill University's Large Experimental Array of Ponds (LEAP) at the Gault Field Station, Mont St. Hilaire, QC. The array comprises 100 artificial ponds filled with approximately 300 gallons of water – including microbial, phytoplankton and zooplankton communities – from Lac Hertel in May 2017. Nutrient spikes of 50 mL of each of 600 μg/L nitrogen and 40 μg/L phosphorus were added on 31 May 2017 to facilitate planktonic community establishment. Three types of samples were collected from the field station ponds (Figure 1): eDNA and eRNA samples were filtered in June and August to sample early-season community composition; bulk zooplankton samples were collected in July and September for morphological identification, voucher specimen sequencing and building mock communities; and in September, water was removed from three ponds while excluding zooplankton, and was subsampled for eDNA and eRNA at seven time points after organism removal.

Water samples were collected from field station ponds early in the season on 22 June and 24 August 2017, to account for past community composition and eDNA signals that may have persisted after community turnover. For each pond, two 400 mL replicates were filtered through

0.7 μm glass microfiber filters (Millipore). Each filter was cut in half; while one half was preserved in ATL buffer (Qiagen DNEasy) and used for eDNA extraction, the other half was preserved in RLT buffer (Qiagen RNEasy) with 1% β-mercaptoethanol and used for eRNA extraction. The preserved filters were stored at -80°C immediately after filtration.

Bulk zooplankton samples were collected on 27 July and 26 September 2017 for morphological identification and assembling mock communities. Three litres of water were collected from each pond and the contents were concentrated on a 64 μm sieve. The zooplankton were anesthetized in tonic water and the concentrated sample was stored in 50 mL of 95% ethanol. Two thirds of the sample were used for morphological identification of crustaceous zooplankton in the pond communities, and the remaining third of the sample was used to build mock communities from taxonomically identified voucher specimens.

On 11 September 2017, approximately seven litres of water were siphoned from each of three ponds. The water was siphoned through 50 μm Nytex to remove crustaceous zooplankton, hereafter crustaceans, and halt eDNA and eRNA input from these organisms. Subsamples were collected from the siphoned water at seven time points: Within an hour after siphoning, two hours, 24 hours, 48 hours, four days, seven days and 28 days after siphoning. For each pond at each time point, filtration and preservation of eDNA and eRNA occurred as above. A filtration blank of 400 mL MilliQ water was filtered at the end of sample collection for each time point.

Extraction and High Throughput Sequencing of eDNA and eRNA

Environmental DNA and eRNA were extracted using DNEasy and RNEasy kits (Qiagen) according to the manufactures instructions with the following modifications for extracting the nucleic acids from filters. Environmental DNA samples were thawed and 20 μL of protinase-K was added to the ATL buffer with the filter. The tubes were incubated at 56°C for 24 hours and

vortexed thoroughly three times throughout the incubation. The eRNA samples were thawed at room temperature and vortexed thoroughly as soon as the buffer liquefied. The tubes were spun at 14 000 rpm for three minutes and the liquid was pipetted out from around the filter. The extractions proceeded as per the manufacturer's instructions with two rounds of 30 µL elutions, for a total of 60 µL. Blanks were included in each round of extractions.

The digestion of DNA present in eRNA samples was conducted with the DNA-Eraser Genomic DNA Removal kit (iNtRON Biotechnology) according to the manufacturer's instructions. The reaction contained: 8 µL of eRNA, 2 µL Reaction Buffer and 2 µL of Reaction Stopper, with a 10 min incubation period. Complete DNA digestion was verified with failed PCR amplification of the post digestion eRNA product, as RNA will not amplify prior to revers transcription. Reverse transcription to cDNA was conducted with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The reaction contained: 2.0 µL RT buffer, 0.8 µL dNTP mix, 2.0 µL random primers, 1.0 µL MultiScribe Reverse Transcriptase, 4.2 µL nuclease free water and 10 µL eRNA. The thermocycler regime was: 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Blanks for DNA digestion and reverse transcription were included on each plate.

Samples containing eDNA and cDNA (from eRNA) were PCR amplified at the 18S and COI barcode markers with primers modified to include Illumina adaptors: Uni18S AGGGCAAKYCTGGTGCCAGC and Uni18SR GRCGGTATCTRATCGYCTT (Zhan et al. 2013); miCOIintF GGWACWGGWTGAACWGTWTAYCCYCC (Leray et al. 2013) and HCO2198 TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al. 1994). Environmental DNA and cDNA were not amplifiable with the same PCR reactions, and so separate optimized protocols were applied. The eDNA reactions for both COI and 18S amplification contained:

8.0 µL nuclease free water (Qiagen), 1.25 µL 10x Taq Buffer (GenScript), 0.5 µL 25 mM $MgCl_2$ (Thermo Scientific), 0.125 µL 10 mM dNTP Mix (GeneDire), 0.2 µL each of forward and reverse primers (10 mM), 0.05 µL BSA (Fisher Scientific), 0.25 µL Taq DNA Polymerase (5U/µL) and 2 µL DNA, for a total of 12.6 µL. The eRNA reactions for both COI and 18S contained: 6.25 µL MyFi master mix (BioLine), 3.25 µL nuclease free water, 0.5 µL of each of the forward and reverse primers (10mM) and 2 µL of cDNA, for a total of 12.5 µL. The thermocycler regime for 18S amplification on eDNA and cDNA consisted of an initial denaturing step at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 90 s, and an elongation step at 72°C for 10 min (Zhan et al. 2013). The thermocycler regime for COI amplification on both eDNA and cDNA consisted of touchdown PCR with 16 initial cycles of denaturation at 95°C for 10 s, annealing at 62°C for 30 s (-1°C per cycle) and extension at 72°C for 60 s, followed by 25 cycles with a 46°C annealing temperature (Leray et al. 2013). Blanks for PCR were included on each plate. Amplification was verified on a 1% agarose gel. Technical replicates were pooled and cleaned with a 0.875 ratio of Ampure beads (New England BioLabs). Filtration, extraction, DNA digestion and reverse transcription, and PCR blanks were pooled for sequencing within each marker/ molecular template combination (i.e., eDNA COI, eDNA 18S, eRNA COI and eRNA 18S). Environmental DNA and eRNA samples from past communities were also pooled within marker/ molecular template. Samples were indexed with Nextera Indexing Kit (Illumina) and sequenced in a single lane, Illumina MiSeq run at Genome Quebec. Libraries were submitted for three ponds sampled at seven time points for both eDNA and eRNA, each amplified at the COI and 18S barcode markers (84 libraries). Additionally, libraries of pooled-blanks and past community samples were included for each marker/

molecular template combination (four libraries each). Finally, mock community libraries were included for the COI and 18S markers, for a total of 94 libraries on the run.

To avoid cross-contamination, all equipment was thoroughly bleached and rinsed with sample water prior to filtration. Filtrations were conducted in a wet laboratory separate from the molecular laboratory. The molecular laboratory workspaces and equipment were cleaned with bleach and RNAse Away prior to use. Sterile filter pipette tips were used for all pre-amplification steps.

Voucher Specimens and Mock Communities

DNA was extracted from voucher specimens of nine morphologically identified species collected from the LEAP field station: *Eubosmina longispina, Cyclops scutifer, Diaphanosoma* sp.*, Sida crystallina, Chydorus sphaericus, Daphnia pulex, Simocephalus* sp.*, Ceriodaphnia* sp.*,* and *Daphnia ambigua.* These single individual extractions were amplified and sequenced at the 18S and COI barcode markers with the intention of building a local species database specific to the populations in this experiment. However, the DNA extractions from the ethanol preserved specimens were largely unsuccessful despite the use of two protocols, and the resulting Sanger sequences were unusable.

Three specimens of each species were extracted with a CTAB Chloroform:Isoamyl procedure and two were extracted with DNEasy Blood and Tissue Kits (Qiagen) as per the manufacturer's instructions. Amplification at the COI and 18S barcode markers was conducted as in the above-described MyFi protocol. Amplification failed for most replicates of most species at both barcode markers, regardless of extraction method or amplification protocol. The amplified barcode markers were used for both Sanger sequencing of individuals, to associate

morphological species identity to sequences, and pooled into mock communities order to identify PCR bias or high throughput sequencing errors.

Amplicons were prepared for Sanger sequencing with the following reaction: 5.25 µL Nuclease free water (Qiagen), 3 µL 5mM $MgCl_2$ (Thermo Scientific), 0.5 µL 10mM primer, 0.25 µL Big Dye (AppliedBiosystems) and 1.0 µL PCR product. Samples were submitted for Sanger sequencing at Genome Quebec. Amplicons from each species were pooled in equal volume within markers to form two mock community libraries, one for 18S and one for COI. The mock community libraries were prepared for high throughput sequencing as per the other samples, and were sequenced in the same Illumina MiSeq run by Genome Quebec.

Bioinformatic Analyses

Raw reads underwent initial quality assessment, removal of Illumina adaptors and short or low quality read, merging of paired ends reads, removal of primer sequences, final read quality assessment and dereliction. The COI and 18S barcode pipelines were customized with primer sequences and expected read length (COI: 313 bp; 18S: ~400 bp) specific to each marker. Raw read quality was assessed with FastQC (Andrews 2010). Trimmomatic (Bolger et al. 2014) was used to remove Illumina adaptors and low-quality reads less than 100 bp or containing regions of mean quality < 20 over 5 bp. Overlapping paired reads were merged using FLASh (https://ccb.jhu.edu/software/FLASH/) using a minimum overlap of 10 bp and maximum overlap of 280 bp. Merged reads were used in the proceeding analysis. Trailing primer and adaptor sequences were removed with Cutadapt (Martin 2011). FastQC was re-run on the processed reads. Singletons were included to maximize the probability of detection rare eDNA and eRNA sequences. Reads were dereplicated with SeqKit (Shen et al. 2016) and a BLASTn (Altschul et al. 1990) search was conducted on the NCBI Nucleotide Database to assign reads to species identity.

The top blast result with minimum 97% identity and 95% query cover was retained. The TaxonKit (http://bioinf.shenwei.me/taxonkit/) was used to obtain lineages matching the taxonomic identification numbers of the subject sequences in the NCBI database. Read counts were determined with SeqKit (Shen et al. 2016) after each step in the pipeline. All taxa were documented and organized according to phylum. Taxa of the subphylum Crustacea, and the number of reads corresponding to these taxa were documented and used for subsequent analysis.

Statistics

Sequencing read counts were compared between metabarcoding methods with two-way ANOVAs run in R (version 3.5.1). Both raw read counts and processed read counts were compared between molecular template (eDNA and eRNA), markers (18S and COI), ponds, and degradation time points.

Principal Components Analysis (PCA) was conducted on species presence/absence in each sample, and samples were grouped according to molecular template (eDNA or eRNA), barcode marker (COI or 18S), pond and degradation time point in order to visualize similarities and differences in zooplankton community composition. The PCA was conducted in R (version 3.5.1) with the devtools package (https://github.com/r-lib/devtools) and plotted with the ggbiplot package (Wickham 2016).

Exponential decay curves were fit to the decline in zooplankton detection over the first seven days of the experiment, excluding the anomalous results observed at the 28-day time point. This curve has previously been applied directly to the decline in eDNA concentration. In this study, concentrations of crustacean eDNA and eRNA could not be measured independently from the DNA and RNA of microorganisms and phytoplankton living in the carboys, and so the curve was fit to the decline in number of taxa detected by metabarcoding. The exponential decay

models were fit with scipy.optimize package (Jones et al 2011) and plotted with matplotlib.pyplot (Hunter et al 2007) in Python (version 3.6.3). The models were fit to the decline in detected zooplankton taxa observed in the first seven days of degradation, according to the equation:

$$D(t) = D_0 e^{-rt}$$

where D(t) is the number of taxa detected at time t, D_0 is the initial number of taxa detected and r is the decay rate. The decay rate (r) was compared between eDNA and eRNA metabarcoding at each of the COI and 18S barcode markers.

Results

Sequencing

The Illumina MiSeq run resulted in 17,551,664 reads from 92 eDNA and eRNA based libraries (Table S1). The 42 experimental eDNA based libraries, representing three ponds sampled at seven time points with two markers, contained 9,274,384 reads (5,263,014 reads form COI and 4,011,370 reads from 18S). The 42 experimental eRNA based libraries contained 7,210,787 reads (3,763,376 reads from COI and 3,447,411 reads from 18S). Within the initial raw reads, there was no significant difference in read count among ponds or between time points. However, there were significant effects of molecular template (i.e., eDNA or eRNA) ($F=10.65$, $p=0.0016$) and marker (i.e., COI or 18S) ($F= 18.46$, $p< 0.0001$) on initial read count. After processing with the bioinformatics pipeline, the only significant difference was between markers, with final 18S read counts exceeding COI read counts by 35% ($F=35.32$, $p<0.0001$).

Morphological Identification, Voucher Specimens and Mock Communities

Nine crustacean species were present in the ponds at the LEAP field station, based on morphological identification (Table 1, taxa in bold). Of these, seven were observed directly in at

least one of the three sampled ponds on either 27 July or 26 September 2017. Voucher specimens of these nine species were sequenced at the 18S and COI barcode markers with the intention of building a local species database specific to the populations in this experiment. Extraction and amplification of DNA at both markers resulted in low quality Sanger sequences. Of the nine voucher taxa, five failed to amplify identifiable sequences at both markers: *Sida crystallina, Daphnia pulex, Diaphanosoma* sp., *Ceriodaphnia* sp. and *Cyclops scutifer* (Table 2). *Sida crystallina* and *Ceriodaphnia* sp. were assigned identities of other voucher taxa, *Diaphanosoma* sp. and *Simocephalus serrulatus*, respectively. This likely resulted from contamination of specimens poorly preserved in ethanol. Three taxa were assigned identities matching morphological identification to at least the genus level. *Eubosmina longispina* was identified as *Bosmina longirostris*, *Chydorus sphaericus* was identified as itself and *C. brevilabris,* and *Simocephalus* sp. was identified as *Simocephalus serrulatus* and *Simocephalus vetulus*.

Amplified voucher specimen DNA was pooled to assemble mock communities for the COI and 18S markers. Five species were identified in the 18S mock community: *Chydorus brevilabris, Daphnia ambigua, Diaphanosoma* sp., *Mesocyclops leuckarti* and *Simocephalus serrulatus* (Table 2). The COI mock community resulted in species assignment of 40 crustacean taxa (Table 2). Of these, nine matched morphological identifications of voucher specimens to the genus level.

Community Composition

Species of Fungi, Metazoa, Viridiplantea, Amoebozoa, Alveolata, Rhizaria, Stramenoplies, Hacrobia and Bacteria were detected in samples collected throughout the experiment (Table S2). The COI barcode marker detected 155 and 93 taxa from eDNA and eRNA, respectively. The 18S barcode marker detected 498 and 44 taxa from eDNA and eRNA, respectively.

A total of 50 crustacean taxa were identified by metabarcoding, either in past community pond samples or during the degradation experiment (Table 1). Environmental DNA and eRNA metabarcoding at the COI marker detected 17 and 11 crustacean taxa respectively in the past community samples, and detected 19 and 10 taxa respectively during the degradation experiment. Metabarcoding at the 18S marker detected 16 and 15 taxa by eDNA and eRNA metabarcoding in the past community samples, and nine and seven taxa, respectively during the degradation experiment (Table 1).

Principal Components Analysis (PCA) was conducted on the presence/absence of crustacean taxa in each sample. The primary axis accounted for 21.9% of the variation, while the secondary axis accounted for 12.9% (Figure 2). Samples were grouped according to pond (Figure 2A), degradation time point (B), molecular template (C) and barcode marker (D). Past community samples separated from samples collected during the degradation experiment. Within the degradation experiment, samples collected from all three ponds overlapped (Figure 2A), and samples collected across all time points overlapped (Figure 2B), indicating similar community composition across these variables. Community composition as determined by eDNA and eRNA based metabarcoding was similar for both past community samples and samples from the degradation experiment (Figure 2C). There was little overlap between samples sequenced at the COI and 18S barcode markers, indicating that distinct community compositions were assigned by each marker (Figure 2D).

Degradation Experiment

Metabarcoding of eDNA samples detected crustaceans with the COI marker at all time points following organism removal, and at all but the 48-hour time point with 18S (Figure 3 and 5). Environmental RNA metabarcoding at both markers failed to detect crustaceans by 24 hours

of degradation time, with only few taxa detected from trace reads at 48 hours with 18S, and four and seven days with COI. At the 28-day time point, eDNA and eRNA metabarcoding with both markers experienced an increase in crustacean taxa detection. This could be the result of contamination; concentration of eDNA and eRNA adhered to the carboy walls as sample water was removed; or small cysts, eggs or nauplia passing through the Nytex and growing to detectable levels over the span of one month.

Between the first and second hour after organism removal, the mean number of crustacean taxa detected with the COI marker (± standard error) decreased from 4.67 (± 0.31) to 4.33 (± 0.41) by eDNA metabarcoding, and decreased from 2.00 (± 0.27) to 1.33 (± 0.62) by eRNA metabarcoding (Table 3 and Figure 3). Mean detection by eDNA metabarcoding varied between 4.67 (± 0.56) and 1.67 (± 0.15) taxa between the 24-hour and 28-day time points. Environmental RNA metabarcoding with the COI marker did not detect crustacean taxa at the 24-hour and 48-hour time points, and detected only one taxon at each of the four-day (*Macrothrix* sp., pond 3) and seven-day (*Chydorus brevilabris*, pond 2) time points. Detection by eRNA metabarcoding increased to 4.67 (± 0.41) taxa at the 28-day time point, exceeding detection by eDNA metabarcoding (2.66 ± 0.31 taxa) for the first time in the experiment.

Mean (± standard error) crustacean detection by eDNA metabarcoding with the 18S marker decreased from over 2.33 (± 0.56) taxa in the first two hours, to 0.67 (± 0.154) taxa at 24 hours, four days and seven days (Table 4 Figure 5). Mean crustacean detection by eRNA metabarcoding with 18S decreased from 2.00 (± 0.27) to 1.33 (± 0.41) taxa in the first two hours, and no crustaceans were detected by eRNA metabarcoding at 24 hours, four days and seven days after organism removal. Crustacean detection increased for both eDNA and eRNA at the 28-day time point, with mean numbers of crustacean taxa of 3.00 (± 0.27) and 1.33 (± 0.41), respectively.

The exponential decay curves fit to the number of taxa detected with the COI marker in the first seven days of degradation had decay rates (r ± variance) of $0.69e^{-3}$ ($\pm 5.34e^{-6}$) hr^{-1} and 0.405 (± 0.25) hr^{-1} for eDNA and eRNA metabarcoding, respectively (Figure 4). The curves fit to the number of taxa detected by eDNA and eRNA metabarcoding with 18S had decay rates (r) of 0.058 ($\pm 2.44e^{-3}$) hr^{-1} and 0.405 (± 0.25) hr^{-1}, respectively (Figure 6).

Blanks

Amplification was not detected by gel electrophoresis in the filtration, extraction, reverse transcription, and PCR blanks at any time point. The blanks were pooled within molecular template and marker, and sequenced. The pooled COI blanks resulted in 572 reads from eDNA and 78 reads from eRNA after bioinformatic processing (Table S1), with 57 eDNA reads and 14 eRNA assigned to crustacean taxa (Figure 3). *Chydorus brevilabris* (14 reads), *Leptodiaptomus minutus* (7 reads), *Cyclops* sp. (10 reads), Bosminidae sp. (23 reads) and *Macrothrix* sp. (3 reads) were detected in COI eDNA blanks; while *Chydorus brevilabris* (2 reads), *Simocephalus serrulatus* (2 reads), *Ceriodaphnia dubia* (1 read), *Diaphanosoma* sp. (3 reads) and *Daphnia ambigua* (6 reads) were detected in COI eRNA blanks (Table 1). The pooled 18S blanks resulted in 2317 reads from eDNA and 37266 from eRNA after processing (Table S1). Four eDNA reads and 69 eRNA reads were assigned to crustacean taxa (Figure 5). *Acanthodiaptomus pacificus* (eDNA: 2 reads; eRNA: 16 reads), Copepoda sp. (eDNA: 1 read; eRNA: 35 read) and *Microcyclops varicans* (eDNA: 1 read; eRNA: 15 read) were detected in both 18S eDNA and 18S eRNA blanks, and *Eurytemora affinis* (3 reads) was detected exclusively in 18S eRNA blanks.

Discussion

Here we have conducted a comparative metabarcoding experiment to evaluate the use of eRNA as a complementary barcode molecule to eDNA for high resolution monitoring. We have demonstrated that eRNA based metabarcoding is capable of detecting comparable species diversity to the established eDNA based methods. We further demonstrate that after removing organisms, detection by eRNA decreases more rapidly than detection be eDNA over the span of seven days. We discuss possible explanations for the increase in zooplankton detection by all methods at the 28-day time point.

Community Composition

The PCA conducted on crustacean taxa presence/absence in each sample indicated that the past community samples were distinct from those collected during the degradation experiment. Community composition as determined by eDNA and eRNA metabarcoding was similar, whereas some distinction in community assemblages was observed from sequencing the COI and 18S barcode markers. This is likely due to differences in species distinguished by each marker and the availability of voucher sequences in the NCBI database. Within the degradation experiment, the PCA indicated similar community composition among ponds and across time points.

We can assess the comparative performances of eDNA and eRNA metabarcoding for taxa detection by examining true positive detection (identification of taxa known to inhabit the ponds), false positive detection (identification of taxa known not to inhabit the ponds) and false negative detection (failure to identify taxa known to inhabit the ponds). In this experiment, we used morphological identification to validate zooplankton community composition as determined by metabarcoding methods. We had planned to use voucher specimens to associate sequences to

morphological identification, and mock communities to test for PCR or sequencing biases; however, the genomic DNA extractions failed for many of the zooplankton taxa.

True positive detection was achieved for seven out of nine of the morphologically identified taxa, which were identified by eDNA and eRNA metabarcoding to the genus or species level. In two cases, metabarcoding was able to further refine identification from genus to species level (*Ceriodaphnia dubia* and *Simocephalus serrulatus*). In two cases eDNA and eRNA metabarcoding detected taxa that were known to be present at the field station despite not being captured in the zooplankton samples from these ponds (*Daphnia ambigua* and *Diaphanosoma* sp.). Finally, two cases resulted in identification to the genus level. *Chydorus sphaericus* was accurately identified with the 18S marker, and identified as *C. brevilabris* with the COI marker, as was expected based on the BLAST results of the voucher specimen. *Eubosmina longispina* was identified as *E. coregoni* by eDNA metabarcoding at both markers; the voucher specimen indicated *Bosmina longirostris*. These mismatches could be the result of misidentification of our voucher specimens by our taxonomist, or misidentification of sequences within the database.

False negative detection by eDNA and eRNA metabarcoding occurred for two of the nine crustacean species. *Cyclops scutifer* and *Sida crystallina* were not identified by metabarcoding at either marker, despite being collected from the ponds in morphological samples. *Cyclops* sp. was identified once by eDNA metabarcoding, but only in a blank; the order Cyclopidae was identified by metabarcoding in past community samples and in the degradation experiment. The voucher specimens for *Cyclops scutifer* and *Sida crystallina* did not amplify at either barcode marker, so it is possible that these species were missed due to PCR bias.

More taxa were detected by all metabarcoding methods than were known to be present in the experiment; these are considered false positive detections. Most of these species were

identified by only one marker/template combination and were detected inconsistently in only one of the past community samples, blanks or the degradation experiment. These additional taxa are likely the result of PCR or sequencing error resulting in matches to a closely related species. For taxa detected uniquely by eRNA (Chthamalidae sp., *Daphnia tibetana*, *Simocephalus heilongjiangensis* and *Thermocyclops* sp.), additional error may have been introduced during the reverse transcription step, as reverse transcriptase is not a proofreading enzyme. Fourteen taxa were detected uniquely by eDNA during the degradation experiment, and thought to be attributed to eDNA persistence from past communities; however, only four of these taxa were detected in past community samples. Though false positive detections occurred in every marker/template combination, they were most prevalent in eDNA based COI samples. This could be the result of increased variability in the COI marker, making it more prone to mismatching with fewer errors.

Degradation of eDNA and eRNA

Crustacean taxa were detected by eDNA metabarcoding throughout the 28 day experiment, while detection with eRNA metabarcoding was not possible at 24 hours and 48 hours using the COI marker, or at 24 hours, four days and seven days using the 18S marker. The observed declines in crustacean taxa detection over the first seven days of degradation were fit with exponential decay models. The decay rates produced by the models were greater for eRNA based detection than eDNA based detection. The decay rates were the same for eRNA detection at both markers, whereas the decay rate for 18S eDNA based detection was greater than that of COI eDNA based detection.

The differences in taxa detection over time are attributed to differences in nucleic acid persistence in the water, resulting from differences in molecular stability. The differences in molecular stability result from the initial state of the nucleic acid (e.g., free-floating, membrane

bound in cells or organelles), the length, molecular structure (e.g., single/ double stranded, folding and secondary structure) and protein association (Deutscher 2006; Nielsen et al. 2007; Turner et al. 2014; Barnes and Turner 2016). Once released from the organism, degradation of nucleic acids occurs by enzymatic digestion, chemical or physical breakdown (e.g., radiation exposure or shearing), or adsorption to mineral or sediment (Nielsen et al. 2007). Persistence in the environment is further influenced by the amount of initial material, which will depend on the number or biomass of the organisms previously in the system, the duration of their occupancy, activity level or eDNA input rates (e.g., shedding, spawning) and the copy number of the marker for each individual (King et al. 2008, Wilcox et al. 2016).

Both nuclear (18S) and mitochondrial DNA (COI) persisted in the water with similar relative species detection throughout the degradation experiment. The molecular structure of both markers was similar; both are double stranded and similar length target fragments (COI: 313 bp; 18S: ~400 bp) (Leray et al. 2013; Zhan et al. 2013). The markers differ in location within the cell, with the COI marker packaged within mitochondria, possibly offering prolonged protection from cellular nucleases (Nielsen et al. 2007). The markers may also differ in initial abundance due to copy number variation (King et al. 2008). The copy number of ribosomal DNA varies widely across eukaryotes, and is correlated to genome size (Prokopowich et al. 2003). For zooplankton, 18S copy numbers range from 730 in *Mesocyclops edax* to over 33 000 in *Calanus glacialis* (Wyngaard et al. 1995). Goodall-Copestake (2018) determined nuclear ribosomal DNA (nrDNA) to mitochondrial DNA (mtDNA) ratios in two species of zooplankton by comparing qPCR quantification of 18S (nrDNA) and 16S (mtDNA). The nrDNA:mtDNA was 9:1 and 3:1 for *Salpa thompsoni* and *S. fusiformis* sampled, respectively. The amount of mtDNA was similar between species, and the variation in ratios resulted from differences in nrDNA amounts. The

amounts of nrDNA and mtDNA in a cell translate to amounts of initially available 18S and COI barcode template, respectively. This can inform marker choice, as more abundant markers are more likely to be detected as eDNA than single copy genes.

Detection by eRNA metabarcoding largely ceased by 24 hours after organism removal, with only few detection from trace reads later on. Differences in persistence could be attributed to variation molecular stability resulting from secondary and tertiary structure of the RNA molecules. Ribosomal RNA (18S) has increased stability over messenger RNA (COI) due to folding patterns and protein association (Deutscher 2006). Variation in transcription rates and RNA half-life will also influence how much initial material is available for detection by metabarcoding. The vast majority of RNA in a cell, up to 98%, is rRNA thereby making rRNA based markers more abundant and durable (Deutscher 2006).

It is worth noting that species detection from eRNA is dependant on reverse transcription, while eDNA does not undergo this process. The conversion of RNA to cDNA may not be 100% efficient and may vary between markers. Furthermore, as reverse transcriptase is not a proofreading enzyme, additional sequence errors may be introduced during this step.

28-Day Time Point

Despite experiencing prolonged periods of time where detection was not possible, crustaceans were detected with both the 18S and COI eRNA markers at the end of the experiment, 28 days after organism removal. This is possibly due to small eggs, resting cysts, or nauplia passing through the 50 µm Nytex when siphoning the water from the artificial ponds, and growing over the span of one month. The pore size was selected to collect water that resembled the natural setting, including microbial and phytoplankton communities, while excluding living zooplankton and allowing for timely collection from the heavily fouled ponds. Microscopic

examination of the siphoned water revealed rotifers and phytoplankton, as expected. No crustaceans were observed, however this does not preclude their presence.

Lance et al. (2017) observed similar spikes in carp eDNA in the early (day 2 – 3) and late (day 21 – 28) stages of their degradation experiment. They attributed the anomalies to eDNA adhering to the walls of the plastic containers and resisting degradation. The eDNA was thought to have later dissociated from the walls and resulted in spikes in later samples. This is another possible explanation for what we observed, as we also used polypropylene carboys to store water during our degradation experiment. Furthermore, eDNA and eRNA adhering to the plastic or to biofilms on the walls of the carboys would have become more concentrated as water was removed from the carboys with each sample.

In either case, the eDNA and eRNA detected likely originated from the sampled community rather than external contamination, as the PCA indicated similar community composition at the 28-day time point as in the rest of the experiment. Most of the taxa detected at the 28-day time point were previously detected in samples from their respective carboys throughout the experiment. This supports the explanation of growth in the carboys, or the concentration of residual eDNA and eRNA protected on the walls of the container. Four taxa were detected exclusively at the 28-day time point (COI: Chthamalidae sp., *Pleuroxus varidentatus* and *Daphnia laevis*; 18S: *Cobanocythere japonica*) and are more likely the result of misidentification due to amplification or sequencing error of degrading eDNA and eRNA. For example, though *Daphnia laevis* was not previously detected, *D. ambigua* was detected from the same carboy earlier in the experiment. The remaining three taxa detected for the first time in their respective ponds at 28 days were *Diaphanosoma sp.* (COI pond 2 and 18S pond 3), *Ceriodaphnia dubia* (COI pond 3) and *Chydorus brevilabris* (COI and 18S pond 3). These taxa

were all detected in the other carboys earlier in the experiment, and therefore could be the result of cross contamination. Though all blanks failed to amplify, *Ceriodaphnia dubia*, *Chydorus brevilabris* and *Diaphanosoma* sp. were identified in the pooled blanks. In order to be confined to all of the 28-day samples, cross contamination would have occurred at or before sample filtration, as all other laboratory processing was conducted simultaneously across all time points.

Detection of crustaceous zooplankton taxa by eRNA exceeded that of eDNA at the 28-day time point. This is the only time in the experiment where this occurs and could be the result of increased RNA levels and elevated transcription rates. Zooplankton have been shown to experience elevated RNA concentration and RNA:DNA ratios associated with growth or egg production (Saiz et al. 1998; Wagner et al. 1998).

Conclusions

Despite inconsistencies in taxonomic assignment between morphological and molecular methods, which are an ongoing challenge for metabarcoding studies, species identification between eDNA and eRNA metabarcoding was similar. This demonstrates that eRNA is capable of capturing the same species diversity as the established eDNA metabarcoding method and can be reliably applied to biomonitoring studies. Furthermore, despite the unanticipated resurgence in both eDNA and eRNA at the end of the experiment, we have demonstrated that eRNA metabarcoding experiences prolonged periods of time where species detection did not occur, while detection by eDNA metabarcoding continued from the same samples. This demonstrates that eRNA metabarcoding can be applied alongside eDNA metabarcoding to refine the window of species detection to within 24 hours of species presence.

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Tables

Table 1. Crustacean taxa identified at the LEAP field station by morphology and by eDNA and eRNA metabarcoding with the COI and 18S barcode markers. Taxa in bold were identified by morphology at the field station; taxa marked as present under "Morphology" were identified in any of the three artificial ponds sampled in this study. Morphological samples were collected on 27 July and 26 September 2017. Metabarcoding results are separated into blanks, past community and degradation experiment samples. "Blanks" represents pooled filtration, extraction, DNA digestion and reverse transcription, and PCR blanks; "Past" community represents samples collected from the field station ponds on 22 June and 24 August 2017; "Degradation" experiment represents samples collected at any of the seven time points of the degradation experiment, after excluding live zooplankton.

Class			COI						18S					
Order	Morphology		Blanks		Past		Degradation		Blanks		Past		Degradation	
Genus species			eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Bosmina longirostris	-		-	-	+	+	-	-	-	-	+	+	-	-
Bosminidae sp. (Family)	-		+	-	-	+	-	-	-	-	-	-	-	-
Ceriodaphnia dubia	-		-	+	+	+	-	+	-	-	+	+	-	-
***Ceriodaphnia* sp.**	+		-	-	-	-	+	-	-	-	-	-	-	-
Chydorus brevilabris	-		+	+	+	+	+	+	-	-	+	+	+	-
Chydorus sphaericus	+		-	-	-	-	-	-	-	-	+	+	+	+
Daphnia ambigua	-		-	+	+	+	+	+	-	-	-	+	+	+
Daphnia catawba	-		-	-	-	+	+	-	-	-	-	-	-	-
Daphnia laevis	-		-	-	-	-	+	-	-	-	-	-	-	-
Daphnia pulex	-		-	-	-	-	-	-	-	-	-	-	-	-
Daphnia tibetana	-		-	-	-	-	-	-	-	-	-	+	-	-
Eubosmina coregoni	-		-	-	-	-	+	-	-	-	+	-	-	-

Eubosmina longispina	+	-	-	-	-	-	-	-	-	-	-	-	-
Eurycercus longirostris	-	-	-	+	+	-	-	-	-	-	-	-	-
Macrothrix sp.	-	+	-	+	-	+	+	-	-	-	-	-	-
Pleuroxus validentatus	-	-	-	-	-	+	-	-	-	-	-	-	-
Simocephalus heilongjiangensis	-	-	-	-	-	-	-	-	-	-	+	-	-
Simocephalus serrulatus	-	-	+	+	+	+	+	-	-	+	+	-	-
***Simocephalus* sp.**	+	-	-	-	-	-	-	-	-	-	-	-	-
Ctenopoda													
***Diaphanosoma* sp.**	-	-	+	+	+	+	+	-	-	+	+	+	+
Holopedium gibberum	-	-	-	+	-	+	+	-	-	-	-	-	-
Sida crystallina	+	-	-	-	-	-	-	-	-	-	-	-	-
Hexanauplia (Formerly Maxillopoda sp.)	-	-	-	+	-	+	+	-	-	-	-	-	-
Copepoda sp. (Subclass)	-	-	-	-	-	-	-	+	+	+	+	+	+
Calanoida													
Acanthodiaptomus pacificus	-	-	-	-	-	-	-	+	+	-	-	-	-
Calanus finmarchicus	-	-	-	+	-	-	-	-	-	+	+	-	-
Calanus glacialis	-	-	-	+	-	-	-	-	-	-	-	-	-
Centropages abdominalis	-	-	-	-	-	-	-	-	-	+	-	-	-
Eurytemora affinis	-	-	-	-	-	-	-	-	+	-	-	-	-
Leptodiaptomus minutus	-	+	-	+	+	+	-	-	-	-	-	-	-
Pontellidae sp. (Family)	-	-	-	+	+	-	-	-	-	-	-	-	-
Pseudocalanus acuspes	-	-	-	-	-	-	-	-	-	+	-	-	-
Pseudocalanus newmani	-	-	-	-	-	-	-	-	-	+	-	-	-
Skistodiaptomus oregonensis	-	-	-	-	-	+	-	-	-	-	-	-	-
Temora turbinata	-	-	-	-	-	-	-	-	-	-	-	+	-
Cyclopoida sp.	-	-	-	+	-	-	-	-	-	-	-	-	-
Cyclopidae sp. (Family)	-	-	-	+	-	+	-	-	-	+	+	-	+
Cyclops scutifer	+	-	-	-	-	-	-	-	-	-	-	-	-
Cyclops sp.	-	+	-	-	-	-	-	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	-	+	-	-	-	-	-	-	-
Microcyclops varicans	-	-	-	-	-	-	-	+	+	+	-	+	+
Oithona similis	-	-	-	+	-	-	-	-	-	+	+	-	-

Onychocorycaeus catus	-	-	-	-	-	+	-	-	-	-	-	-	-
Thermocyclops sp.	-	-	-	-	-	-	-	-	-	-	+	-	-
Thecostraca (Subclass)													
Sessila													
Balanus balanus	-	-	-	-	-	-	-	-	-	+	+	-	-
Chthamalidae sp. (Family)	-	-	-	-	-	-	+	-	-	-	-	-	-
Ostracoda													
Podocopida sp.	-	-	-	-	-	+	-	-	-	-	-	-	-
Cobanocythere japonica	-	-	-	-	-	-	-	-	-	-	-	+	-
Cypridopsis uenoi	-	-	-	-	-	-	-	-	-	+	+	+	+
Cypridopsis vidua	-	-	-	+	+	+	+	-	-	-	-	-	-
Total	6	5	5	17	11	19	10	3	4	16	15	9	7

Table 2. Voucher specimen identity as determined by morphological identification, Sanger sequencing of individuals and High Throughput Sequencing (HTS) of mock communities. Molecular identification by DNA barcoding with the 18S and COI markers was assigned by BLASTn search against the NCBI Nucleotide Database. "NA" indicates that DNA amplification or Sanger sequencing was of insufficient quality to assign species identity from any replicate specimens. Species are sorted by class and order, and mock community results are aligned to the closes matching voucher specimen.

Class	Morphology	Sanger Sequencing		HTS Mock Communities	
Order		COI	18S	COI	18S
Branchiopoda					
Anomopoda	*Ceriodaphnia sp.*	*Simocephalus serrulatus*	*Simocephalus serrulatus*	*Ceriodaphnia dubia*	
	Chydorus sphaericus	*Chydorus brevilabris*	*Chydorus sphaericus*	*Chydorus brevilabris*	*Chydorus brevilabris*
				Chydoridae sp. (Family)	
				Acroperus harpae	
				Acroperus sp.	
				Aloninae sp.	
				Eurycercus longirostris	
				Ophryoxus gracilis	
				Pleuroxus varidentatus	
				Polyphemus pediculus	
	Daphnia ambigua	*Daphnia ambigua*	*Daphnia galeata*	*Daphnia ambigua*	*Daphnia ambigua*
	Daphnia pulex	NA	NA	*Daphnia longiremis*	
				Daphnia sp.	
				Scapholeberis mucronata	
	Eubosmina longispina	NA	*Bosmina longirostris*	*Eubosmina coregoni*	
	Simocephalus sp.	*Simocephalus serrulatus*	*Simocephalus vetulus*	*Simocephalus* sp.	*Simocephalus serrulatus*

Anostraca				*Simocephalus serrulatus* *Artemia franciscana*	
Ctenopoda	*Diaphanosoma* sp. *Sida crystallina*	NA *Diaphanosoma* sp.	NA *Diaphanosoma* sp.	*Diaphanosoma* sp. Ctenopoda sp. (Order)	*Diaphanosoma* sp.
Copepoda (Subclass)					
Calanoida				*Acartia californiensis* *Eudiaptomus gracilis* *Eurytemora carolleeae* *Leptodiaptomus minutus* Pontellidae sp. (Family) *Pseudocalanus minutus*	
Cyclopoida	*Cyclops scutifer*	NA	NA	Cyclopidae sp. (Family) *Acanthocyclops americanus* *Macrocyclops albidus* *Mesocyclops leuckarti* *Thermocyclops crassus*	*Mesocyclops leuckarti*
Thecostraca (Subclass)					
Sessila				*Balanus balanus* *Balanus crenatus* Chthamalidae sp. (Family)	
Malacostraca				*Carcinus maenas*	
Decapoda				*Palaemon suttkusi*	
Ostracoda				Podocopida sp. (Order) *Cypridopsis* sp. *Cypridopsis vidua*	

Table 3. Crustacean taxa detection with the COI barcode marker by eDNA and eRNA metabarcoding at seven time points following organism removal. Taxa are sorted by class and order and results are separated by artificial pond.

Pond 1														
Class	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
Order														
Genus species	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Ceriodaphnia dubia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ceriodaphnia sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chydorus brevilabris	+	-	-	-	-	-	+	-	+	-	-	-	-	+
Daphnia ambigua	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Daphnia catawba	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Daphnia laevis	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Eubosmina coregoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Macrothrix sp.	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Pleuroxus varidentatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simocephalus serrulatus	+	+	+	-	-	-	-	-	+	-	-	-	-	-
Ctenopoda														
Diaphanosoma sp.	+	-	-	-	-	-	-	-	+	-	-	-	-	+
Holopedium gibberum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maxillopoda sp. (Hexanauplia)	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Calanoida														
Leptodiaptomus minutus	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Skistodiaptomus oregonensis	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclopidae sp.	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Onychocorycaeus catus	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Sessila														
Chthamalidae sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ostracoda														
Podocopida sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cypridopsis vidua	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pond 1 Total	6	1	3	0	3	0	2	0	6	0	1	0	2	3

Pond 2														
Class	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
Order														
Genus species	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Ceriodaphnia dubia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ceriodaphnia sp.	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Chydorus brevilabris	+	+	+	+	+	-	-	-	+	-	-	+	-	+
Daphnia ambigua	-	-	-	-	-	-	-	-	-	-	+	-	-	+
Daphnia catawba	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Daphnia laevis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eubosmina coregoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Macrothrix sp.	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Pleuroxus varidentatus	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Simocephalus serrulatus	+	-	+	+	+	-	-	-	-	-	-	-	-	+
Ctenopoda														
Diaphanosoma sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Holopedium gibberum	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Maxillopoda sp. (Hexanauplia)	-	-	+	+	-	-	-	-	-	-	+	-	+	+
Calanoida														
Leptodiaptomus minutus	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Skistodiaptomus oregonensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclopidae sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Onychocorycaeus catus	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Sessila														
Chthamalidae sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ostracoda														
Podocopida sp.	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Cypridopsis vidua	+	+	+	-	+	-	-	-	-	-	-	-	-	-
Pond 2 Total	4	2	6	4	7	0	1	0	2	0	4	1	4	5

Pond 3														
Class	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
Order														
Genus species	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Ceriodaphnia dubia	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ceriodaphnia sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chydorus brevilabris	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Daphnia ambigua	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Daphnia catawba	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Daphnia laevis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eubosmina coregoni	-	-	+	-	-	-	+	-	-	-	-	-	-	-
Macrothrix sp.	+	-	+	-	+	-	-	-	+	+	+	-	+	-
Pleuroxus varidentatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simocephalus serrulatus	-	+	-	-	-	-	-	-	-	-	-	-	-	+
Ctenopoda														
Diaphanosoma sp.	+	-	-	-	-	-	-	-	+	-	-	-	-	+
Holopedium gibberum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maxillopoda sp. (Hexanauplia)	+	+	+	-	+	-	-	-	+	-	-	-	+	+
Calanoida														
Leptodiaptomus minutus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Skistodiaptomus oregonensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclopidae sp.	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Mesocyclops leuckarti	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Onychocorycaeus catus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sessila														
Chthamalidae sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ostracoda														
Podocopida sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cypridopsis vidua	+	-	+	-	+	-	+	-	+	-	-	-	-	-
Pond 3 total	4	3	4	0	4	0	2	0	4	1	2	0	2	6

Table 4. Crustacean taxa detection with the 18S barcode marker by eDNA and eRNA metabarcoding. Taxa are sorted by class and order and results are separated by artificial pond.

Pond 1														
Class	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
Order														
Genus species	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Chydorus brevilabris	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chydorus sphaericus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Daphnia ambigua	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eubosmina coregoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simocephalus serrulatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ctenopoda														
Diaphanosoma sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexanauplia														
Copepoda sp. (Subclass)	-	+	-	-	-	-	-	+	-	-	-	-	+	-
Calanoida														
Acanthodiaptomus pacificus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eurytemora affinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temora turbinata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclopoida														
Cyclopidae sp. (Family)	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microcyclops varicans	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Ostracoda														
Podocopida														
Cobanocythere japonica	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Cypridopsis uenoi	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pond 1 Total	0	1	0	0	0	0	0	2	1	0	0	0	3	0

Pond 2														
Class Order Genus species	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Chydorus brevilabris	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chydorus sphaericus	+	-	+	+	-	-	-	-	-	-	-	-	-	-
Daphnia ambigua	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Eubosmina coregoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simocephalus serrulatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ctenopoda														
Diaphanosoma sp.	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Hexanauplia														
Copepoda sp. (Subclass)	+	+	+	-	-	-	-	-	-	-	+	-	+	+
Calanoida														
Acanthodiaptomus pacificus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eurytemora affinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temora turbinata	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Cyclopoida														
Cyclopidae sp. (Family)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microcyclops varicans	+	-	+	+	-	-	-	-	-	-	-	-	+	+
Ostracoda														
Podocopida														
Cobanocythere japonica	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Cypridopsis uenoi	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Pond 2 Total	4	2	5	3	1	0	0	2	0	0	1	0	2	2

Pond 3														
Class Order Genus species	1 Hour		2 Hours		24 Hours		48 Hours		4 days		7 days		28 days	
	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA	eDNA	eRNA
Branchiopoda														
Anomopoda														
Chydorus brevilabris	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Chydorus sphaericus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Daphnia ambigua	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Eubosmina coregoni	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simocephalus serrulatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ctenopoda														
Diaphanosoma sp.	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Hexanauplia														
Copepoda sp. (Subclass)	+	+	+	-	-	-	-	-	+	-	+	-	+	+
Calanoida														
Acanthodiaptomus pacificus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eurytemora affinis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Temora turbinata	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cyclopoida														
Cyclopidae sp. (Family)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mesocyclops leuckarti	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Microcyclops varicans	+	+	+	+	-	-	-	-	-	-	-	-	+	+
Ostracoda														
Podocopida														
Cobanocythere japonica	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cypridopsis uenoi	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Pond 3 total	3	3	2	1	1	0	0	0	1	0	1	0	4	2

Figures

Figure 1. Experimental set up and sampling procedure. Zooplankton communities were established in three artificial ponds. Bulk zooplankton samples were collected on 27 July and 26 September 2017. Past community eDNA and eRNA samples were collected on 22 June and 24 August 2017. Water was siphoned from the ponds, excluding living zooplankton on 11 September 2017. Water was filtered at one hour, two hours, 24 hours, 48 hours, four days, seven days and 28 days following removal from the ponds. eDNA and eRNA were extracted. eRNA was reverse transcribed to cDNA. eDNA and cDNA were amplified at the 18S and COI markers. Technical replicates were pooled and submitted for high throughput sequencing.

Figure 2. Principal Components Analysis of crustacean taxa presence/absence in eDNA and eRNA samples. The data points are coloured according to (A) source pond, (B) degradation time point, (C) template molecule and (D) barcode marker.

Figure 3. Mean (± standard error) number of crustacean taxa detected by eDNA and eRNA metabarcoding with the COI marker in blanks (n = 1), past community samples (n = 3) and degradation samples across seven time points (n = 3). The mean (± standard error) number of crustacean reads prior to dereplication is indicated above each bar.

Figure 4. Number of crustacean taxa detected by eDNA and eRNA metabarcoding with the COI barcode marker across the first seven days of degradation. An exponential decay model was fit to the declines in taxa detection with the equation: $D(t) = D_0e^{-rt}$. Decay rate (r) is $0.69e^{-3}$ ($\pm$ $5.34e^{-6}$) hr^{-1} for eDNA based taxa detection and 0.405 ($\pm$ 0.25) hr^{-1} for eRNA based taxa detection.

Figure 5. Mean (± standard error) number of crustacean taxa detected by eDNA and eRNA metabarcoding with the 18S marker in blanks (n = 1), past community samples (n = 3) and degradation samples across seven time points (n = 3). The mean (± standard error) number of crustacean reads prior to dereplication is indicated above each bar.

Figure 6. Number of crustacean taxa detected by eDNA and eRNA metabarcoding with the 18S barcode marker across the first seven days of degradation. An exponential decay model was fit to the declines in taxa detection with the equation: $D(t) = D_0e^{-rt}$. Decay rate (r) is 0.058 (± $2.44e^{-3}$) hr^{-1} for eDNA based taxa detection and 0.405 (± 0.25) hr^{-1} for eRNA based taxa detection.

Figure 1

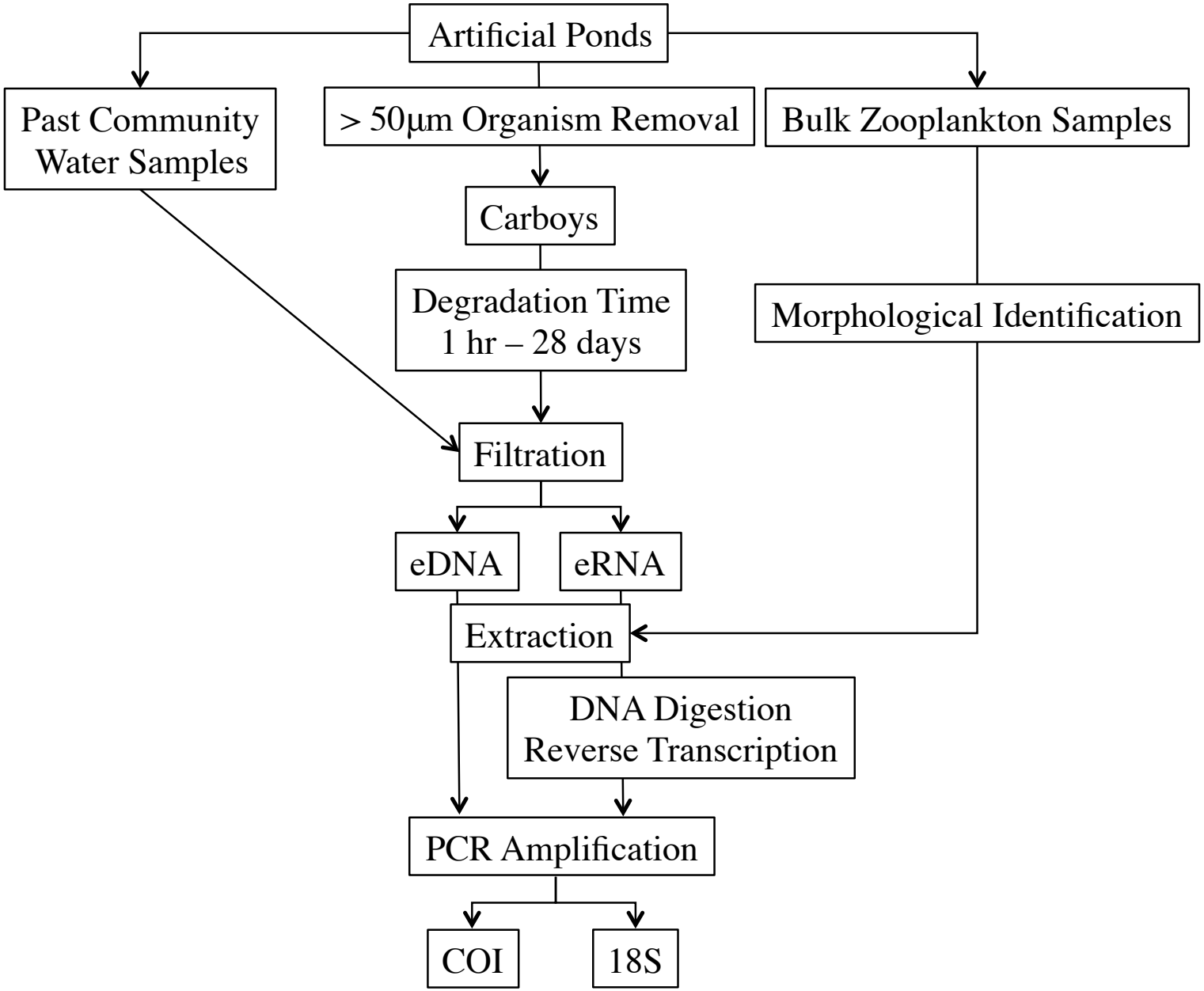

Artificial Ponds
Past Community Water Samples
> 50μm Organism Removal
Bulk Zooplankton Samples
Carboys
Degradation Time 1 hr – 28 days
Morphological Identification
Filtration
eDNA
eRNA
Extraction
DNA Digestion Reverse Transcription
PCR Amplification
COI
18S

Figure 2

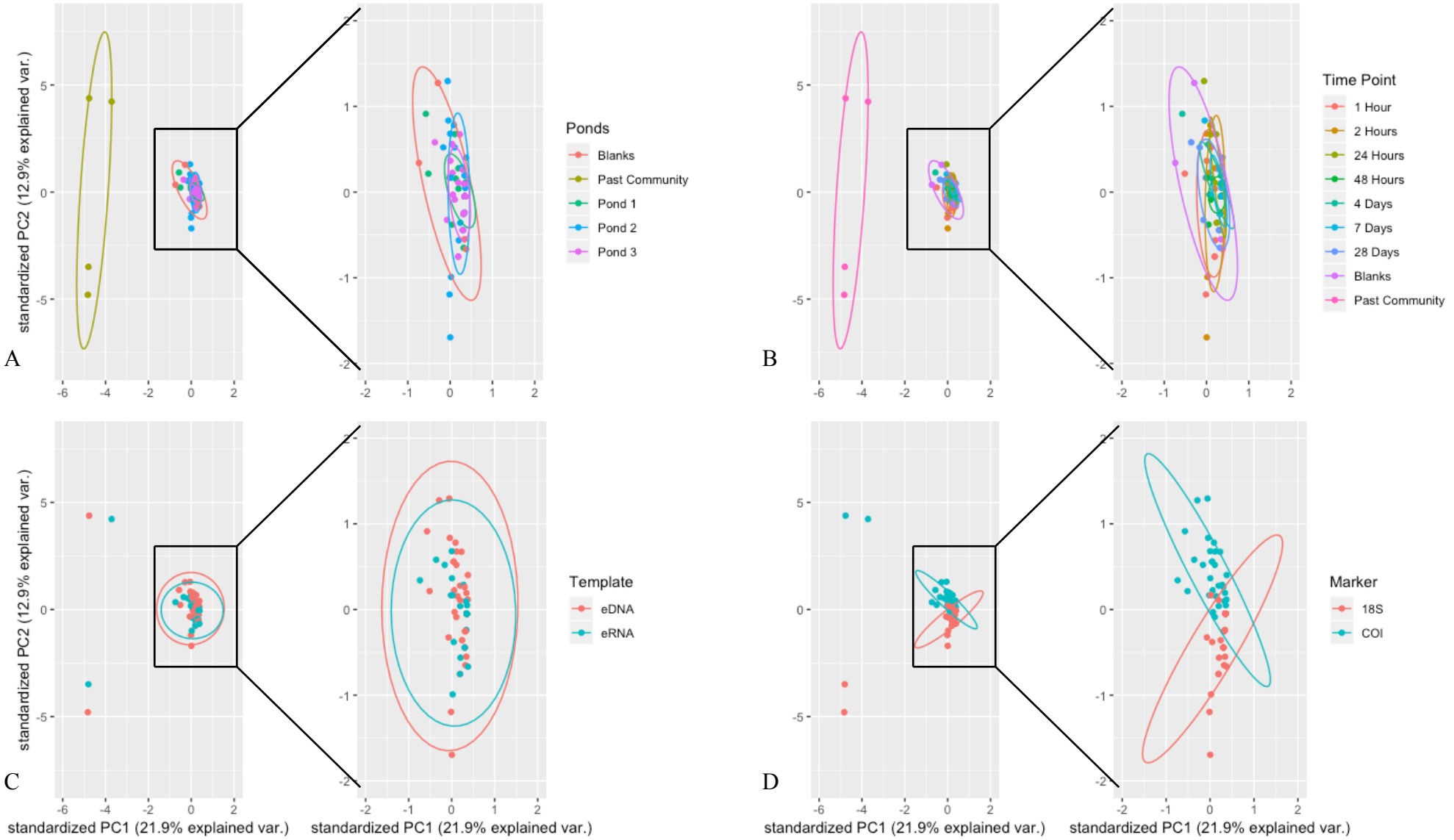

A
B
C
D
standardized PC2 (12.9% explained var.)
standardized PC1 (21.9% explained var.)
Ponds
Blanks
Past Community
Pond 1
Pond 2
Pond 3
Time Point
1 Hour
2 Hours
24 Hours
48 Hours
4 Days
7 Days
28 Days
Blanks
Past Community
Template
eDNA
eRNA
Marker
18S
COI

Figure 3

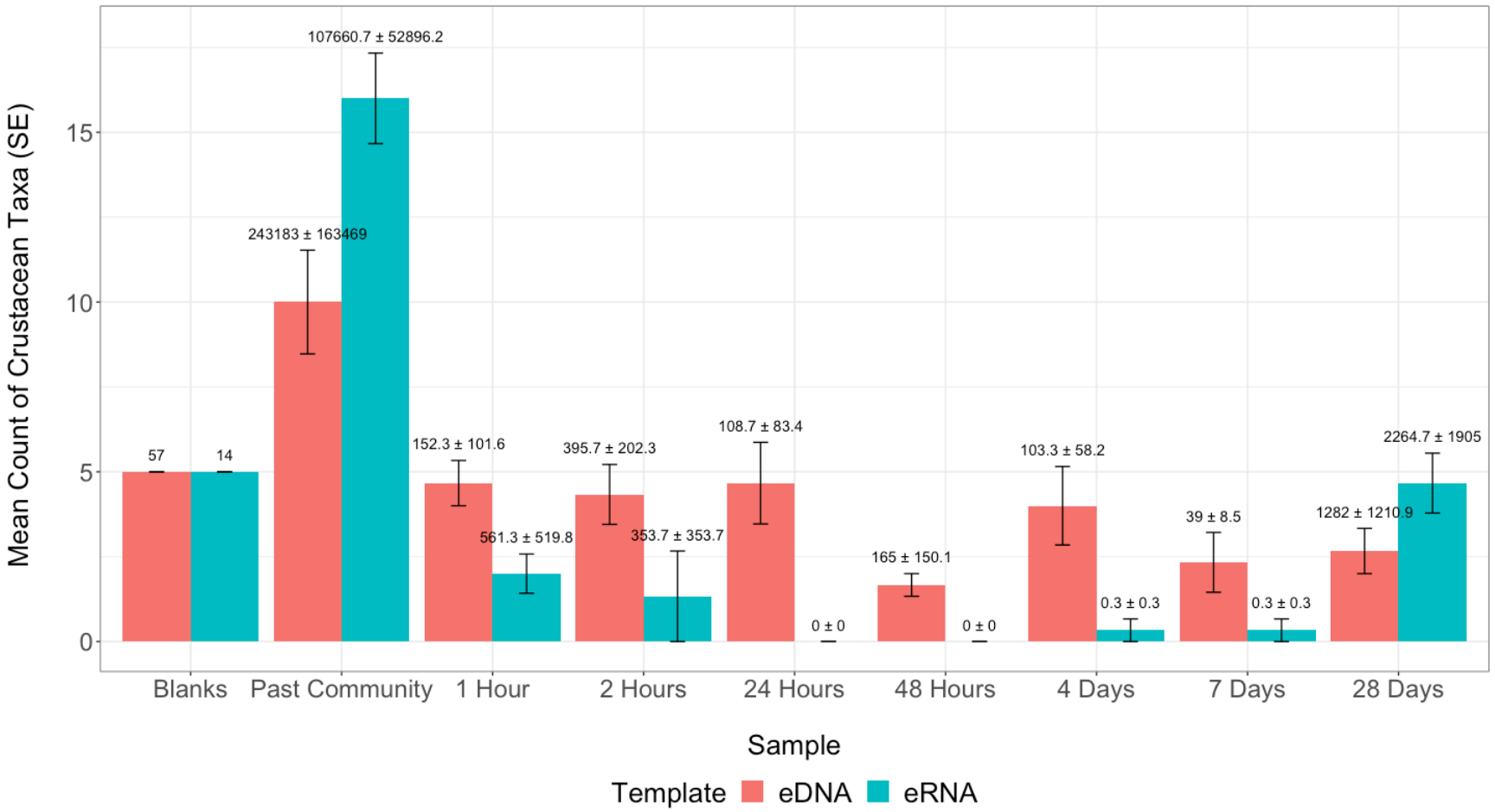

Mean Count of Crustacean Taxa (SE)
0
5
10
15
57
14
243183 ± 163469
107660.7 ± 52896.2
152.3 ± 101.6
561.3 ± 519.8
395.7 ± 202.3
353.7 ± 353.7
108.7 ± 83.4
0 ± 0
165 ± 150.1
0 ± 0
103.3 ± 58.2
0.3 ± 0.3
39 ± 8.5
0.3 ± 0.3
1282 ± 1210.9
2264.7 ± 1905
Blanks
Past Community
1 Hour
2 Hours
24 Hours
48 Hours
4 Days
7 Days
28 Days
Sample
Template
eDNA
eRNA

Figure 4

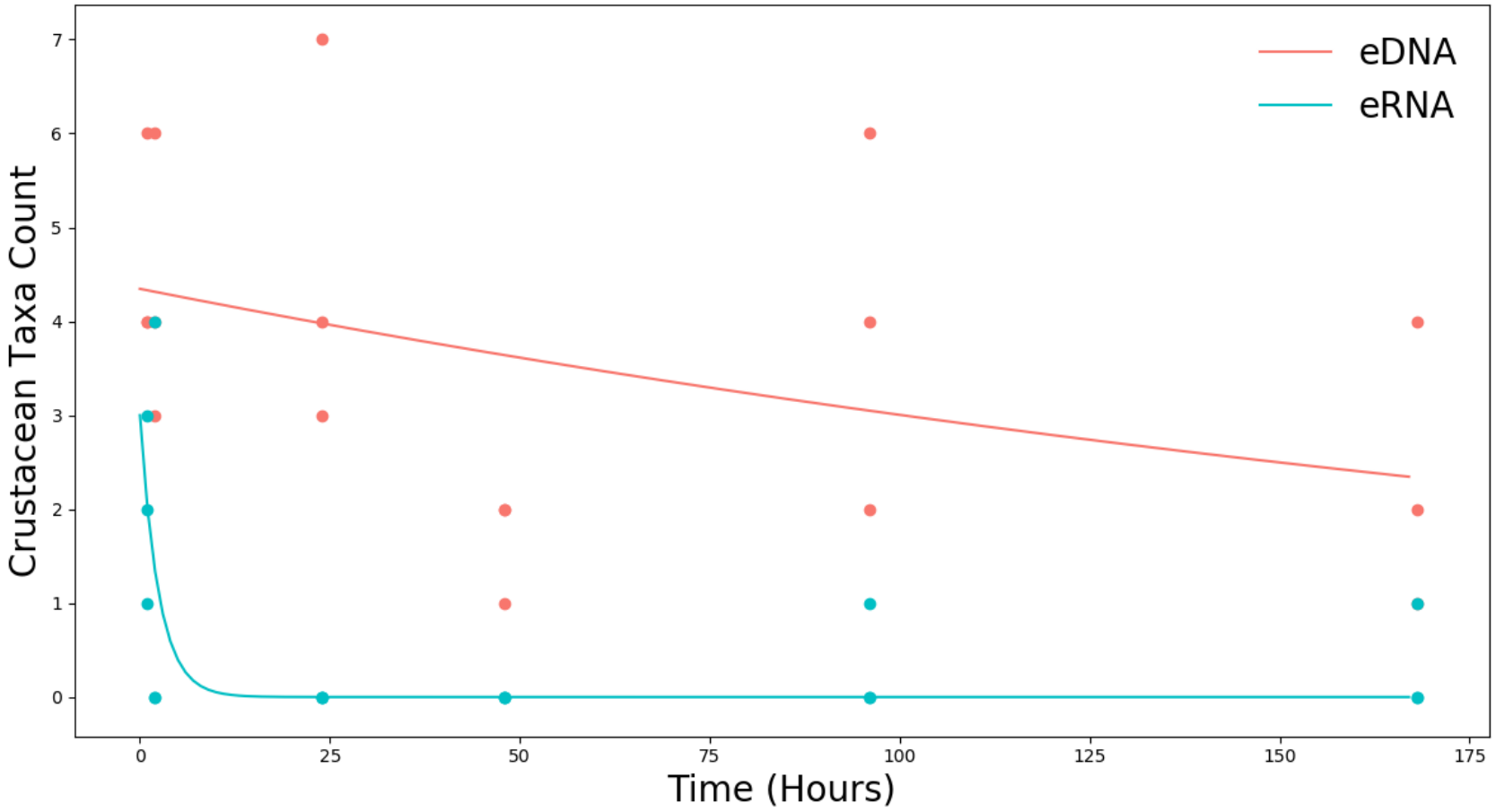


Figure 4

Figure 5

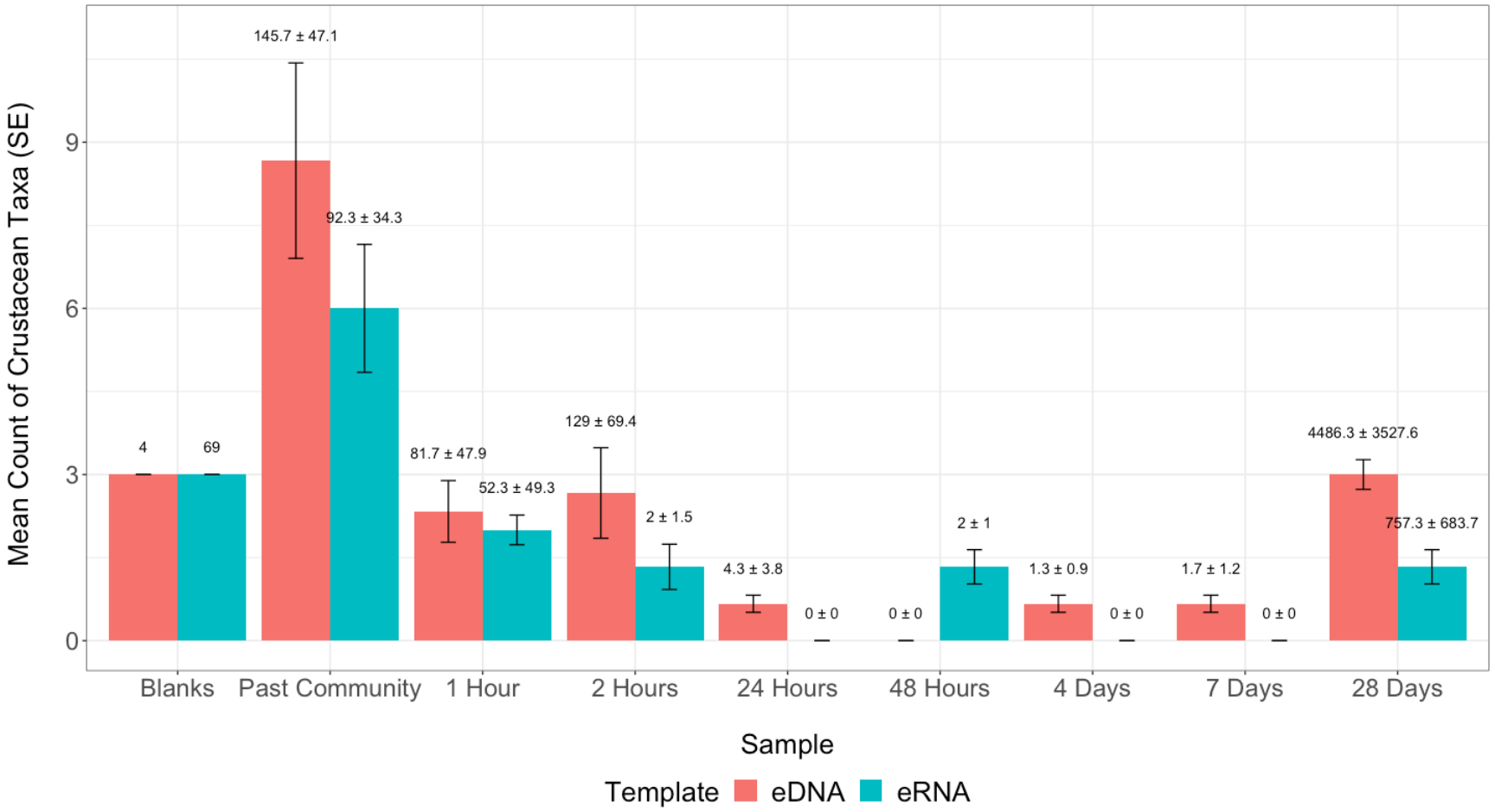
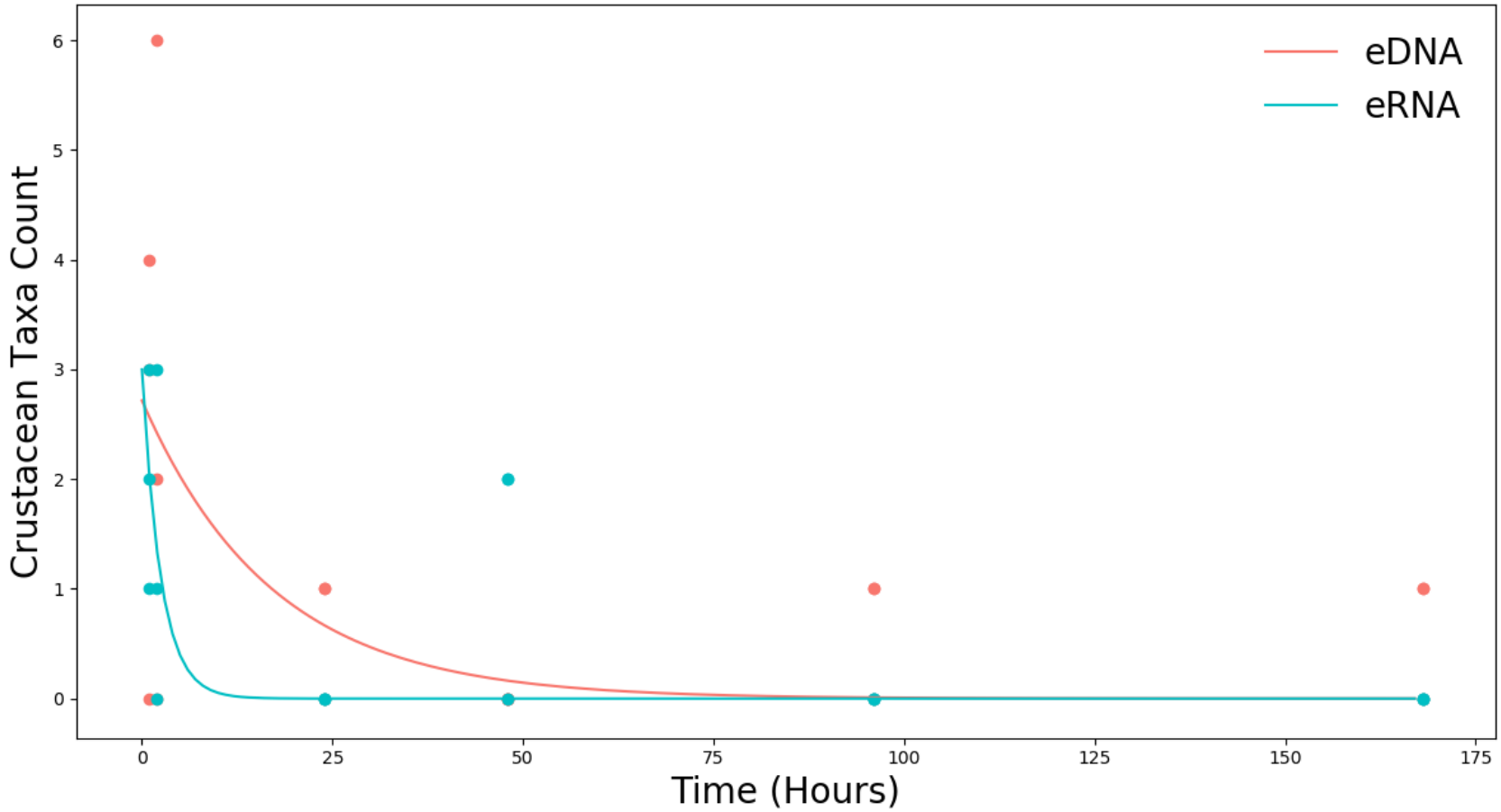

Mean Count of Crustacean Taxa (SE)
0
3
6
9
4
69
145.7 ± 47.1
92.3 ± 34.3
81.7 ± 47.9
52.3 ± 49.3
129 ± 69.4
2 ± 1.5
4.3 ± 3.8
0 ± 0
0 ± 0
2 ± 1
1.3 ± 0.9
0 ± 0
1.7 ± 1.2
0 ± 0
4486.3 ± 3527.6
757.3 ± 683.7
Blanks
Past Community
1 Hour
2 Hours
24 Hours
48 Hours
4 Days
7 Days
28 Days
Sample
Template
eDNA
eRNA

Figure 6


eDNA
eRNA
Crustacean Taxa Count
Time (Hours)
0
1
2
3
4
5
6
0
25
50
75
100
125
150
175

General Discussion

The work presented in this thesis represents the initial steps in validating eRNA metabarcoding as a novel method for molecular biomonitoring. I have demonstrated that eRNA from the COI and 18S barcode markers declined to undetectable levels by 24 hours after organism removal, with rare detection thereafter. Meanwhile, zooplankton detection by eDNA metabarcoding was possible throughout the experiment. This reflects the faster degradation rate of eRNA, resulting from the relative instability of RNA compared to DNA. With a range in molecular stabilities, DNA and RNA based markers can be applied in parallel to establish time and range limits on species detection. Such fine scale measurements are necessary for prioritising protection of preferred habitat for species at risk (Takahashi et al. 2018), for identifying patterns of co-occurrence (Balasingham et al. 2018), and for monitoring the advances of invasion fronts (Jerde et al. 2011). Environmental RNA also offers the potential to distinguishing between living and dead individuals of invasive species (Pochon et al. 2017). The use of eRNA could refine biomonitoring by detecting rapid species turnover, providing high-resolution activity monitoring, or confirming eradication efforts. In this discussion, I will explore how eDNA has recently been applied to such challenges, and the shortcomings it has encountered that could be overcome with the application of eRNA based biomonitoring.

Both the success and challenges of eDNA based monitoring stem from the stability of DNA and its ability to persist in the environment. Laboratory and field based studies have shown eDNA persistence ranging from days to weeks after organism removal (Dejean et al. 2011; Thomsen et al. 2012; Barnes et al. 2014; Pilliod et al. 2014; Strickler et al. 2015; Barnes and Turner 2016; Lance et al. 2017). While this persistence increases the probability

of detection, particularly for low abundance or transient species, it may lead to the detection of species that are no longer present. When monitoring for the American Bullfrog, Dejean et al. (2012) reported concerns that eDNA persistence caused an overestimation of ponds testing positive for the invasive species. As the frogs move from one pond to the next, their DNA can persist for weeks in ponds they no longer inhabit. The faster degradation rate of eRNA offers refined temporal resolution over the eDNA based method. With a shorter period of persistence in the environment, detection by eRNA would more confidently indicate which ponds are currently occupied by the invasive species and give a more accurate representation of the scale of the invasion.

The effects of eDNA persistence are further complicated in flowing systems, where eDNA can be transported away from its source location. Studies have reported detection of lake dwelling species kilometers downstream (Deiner and Altermatt 2014; Civade et al. 2016; Wilcox et al. 2016; Pont et al. 2018). While eDNA transport can facilitate large-range sampling by funnelling eDNA to downstream sites (Deiner et al. 2016; Rice et al. 2018), it introduces confounding signals for high resolution monitoring. For example, when monitoring an invasion front, knowing the precise range of a non-native species will inform management action like the installation of barriers, or localized eradication to prevent further spread. Jerde et al. (2011) praised eDNA based monitoring for detecting Asian Carp upstream of a barrier months before a fish was observed. This is remarkable and shows the great potential for molecular monitoring; however, such detection would not be possible in a system with reverse flow, where the species is progressing downstream. With limited environmental persistence, and therefore possible transport distance, eRNA based methods could apply range limits on species detection in flowing systems.

As eDNA based monitoring methods improve, there is a growing desire to apply molecular techniques at finer and finer spatial-temporal scales. For example, Takahashi et al. (2018) were interested in low abundance detection and monitoring changes in seasonal and diel activity of the Eastern Hellbender salamander. The study showed that eDNA was successful in monitoring seasonal changes by detecting increased activity around mating season; however, there was no detectable change in eDNA signal over diel cycles. The authors determined that the pattern was likely too subtle to detect against environmental noise. Pilliod et al. (2013) also expected variation between daytime and night-time samples due to increased activity in nocturnal salamanders, and similarly noted that such patterns were not detectable in eDNA samples. It is possible that eRNA based monitoring may have the resolution to detect these changes in activity. With the faster degradation rates of eRNA relative to eDNA, eRNA is less likely to persist through the remainder of the cycle. This could result in a sharper contrast between signals detected during active and inactive periods.

Though eDNA based monitoring has been shown to outperform traditional methods such as electrofishing, fyke netting and snorkelling surveys, in terms of both cost and detection of rare species (Jerde et al. 2011; Dejean et al. 2012; Hinlo et al. 2017; Takahashi et al. 2018), the current advantage of traditional sampling techniques is their ability to distinguish living and dead organisms (Rees et al. 2014). Recent studies have applied eDNA to monitor lakes after invasive species eradication efforts in hopes that the method would detect survivors posing a risk of recolonization. However, when euthanized fish were returned to the lakes for nutrient cycling, the eDNA from the carcases was detectable for over a month (Dunker et al. 2016; Kamoroff and Goldberg 2018). Living and dead sources of eDNA could not be differentiated by particle size distribution (Kamoroff and Goldberg 2018). There is a clear need to distinguish beteween living

organisms and detritus, and given the short-lived persistance of eRNA, detection by eRNA would provide better evidence for organisms surviving eradication efforts.

In addition to invasive species eradication, distingushing between living and dead organisms is necessary for invasion prevention. To date, the only co-application of extra-organismal eDNA and eRNA has been to sample ship bilge water in an effor to distingush living organisms from detritus (Pochon et al. 2017). The study compared detection from both eDNA and eRNA metabarcoding and noted considerable overlap, with approximately 60% of taxa identified by both methods. The approximately 20% of taxa identified uniquely by eDNA were mostly fungi, and were attributed to legacy DNA persisting in the water samples. The approximately 20% of taxa detected uniquely by eRNA were attributed to high expression rates in low abundance species. This study identified the need to distingush between living organisms that pose an invasion risk and detritus that remains after sucessful eradication. However, no validation was conducted in order to detrmine if the speces detected by each method were infact living or dead. The authors showed the great potentinal for the co-application of eDNA and eRNA by demonstating similar species coverage; however, further groundtruthing was needed to demonstrate that detection by eRNA metabarcoding is limited to currently living species. The results presented herein compare eDNA and eRNA metabarcoding in a system where living zooplankton are known to be absent. With the knowledge that eRNA does, infact, degrade more rapidly than eDNA and offeres a confined window of detection, we can be more confident in the results of future studies using comparative eDNA and eRNA methods.

Conclusions and Summary

This study is the first of its kind to my knowledge to compare eDNA and eRNA degradation and metabarcoding detection of macroorganisms. Environmental DNA has been applied to a wide range of taxa, and degradation rates have been studied to understand eDNA persistence and transport in aquatic systems. The work presented here is the natural next step in metabarcoding environmental samples. This thesis represents the first steps in validating the use of eRNA as an effective barcode marker, capable of detecting similar diversity as eDNA metabarcoding, but with a shorter window of detection. While crustaceans were detectable throughout the experiment with eDNA metabarcoding, species detection by eRNA metabarcoding declined by 24 hours after species removal, at both the COI and 18S barcode markers. Reducing the window of species detection to between one and four days greatly refines the spatial and temporal resolution of environmental sampling, and applies rage and time limits to the species detected. This resolution can be applied to monitoring critical habitat or activity of endangered species, precisely locating an invasion front, or confirming eradication of invasive species.

References

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Appendix

Supplementary Table 1. Read counts for eDNA and eRNA metabarcoding at the COI and 18S barcode markers. Read counts are indicated for initial read 1 and read 2 files, reads 1 and 2 after trimming adaptors (Trimmomatic), after merging paired end reads (Flash), after trimming trailing adaptors (CutAdapt) and after dereplication (SeqKit). The total number of crustacean reads, prior to dereplication, is indicated (Crustacean).

Sample		eDNA COI Initial	Trimmed	Merged	Cutadapt	Dereplicated	Crustacean
Blanks		122,160	1,012	924	924	572	57
Past Community 22 July		766,525	451,138	409,419	409,336	152,371	554,523
Past Community 22 July		606,054	349,565	305,054	304,978	116,151	173,924
Past Community 24 August		214,847	161,975	156,066	156,057	50,361	1,102
1 Hour	Pond 1	186,083	98,387	94,505	94,502	22,039	39
	Pond 2	238,488	84,674	81,057	81,054	20,281	355
	Pond 3	92,777	9,727	9,315	9,315	4,078	63
2 Hours	Pond 1	160,080	96,960	92,609	92,600	22,914	6
	Pond 2	290,866	164,491	157,550	157,539	37,441	496
	Pond 3	297,510	98,817	94,616	94,600	27,411	685
24 Hours	Pond 1	324,395	209,111	200,415	200,403	40,757	14
	Pond 2	163,915	79,268	68,717	68,706	24,351	37
	Pond 3	361,473	103,399	97,431	97,400	29,551	275
48 Hours	Pond 1	237,092	159,200	152,523	152,518	34,210	23
	Pond 2	344,511	132,922	126,995	126,988	30,501	7
	Pond 3	330,420	135,541	130,426	130,388	35,224	465
4 days	Pond 1	264,741	180,298	172,513	172,486	45,826	72
	Pond 2	352,573	169,432	160,002	159,966	40,176	22
	Pond 3	357,801	187,260	180,618	180,542	39,506	216
7 days	Pond 1	173,643	109,651	104,617	104,617	34,422	56
	Pond 2	195,765	130,000	122,555	122,529	29,841	29
	Pond 3	194,330	146,181	140,976	140,927	31,562	32
28 Days	Pond 1	245,126	165,949	159,112	159,097	36,879	17
	Pond 2	238,936	168,483	161,369	161,348	43,417	126
	Pond 3	212,489	167,679	161,353	161,350	37,702	3,703
Total of All Time Points		**5,263,014**	**2,797,430**	**2,669,274**	**2,668,875**	**668,089**	**73,6344**

Sample		eRNA COI Initial R1	Trimmed R1	Merged	Cutadapt	Dereplicated	Crustacean
Blanks		151,961	115	88	88	78	14
Past Community 22 July		498,715	343,562	310,469	310,425	97,117	159,308
Past Community 22 July		689,298	515,399	472,325	427,325	137,085	161,769
Past Community 24 August		200,332	166,405	158,641	158,641	37,705	1,857
1 Hour	Pond 1	192,361	160,456	154,012	153,998	43,633	2
	Pond 2	251,946	210,897	201,975	201,967	46,083	82
	Pond 3	199,830	172,823	166,726	166,711	43,977	1,600
2 Hours	Pond 1	189,988	158,952	152,297	152,282	41,154	0
	Pond 2	210,874	174,869	167,360	167,355	40,369	1,061
	Pond 3	175,095	148,924	142,728	142,717	40,126	0
24 Hours	Pond 1	147,439	124,351	119,023	119,014	35,329	0
	Pond 2	188,027	155,571	148,548	148,540	37,359	0
	Pond 3	273,716	223,239	214,108	214,086	57,576	0
48 Hours	Pond 1	19,305	7,136	5,627	5,626	3,750	0
	Pond 2	195,716	158,341	149,100	149,081	41,474	0
	Pond 3	154,828	133,036	127,339	127,324	35,116	0
4 days	Pond 1	137,198	112,114	106,858	106,854	36,165	0
	Pond 2	186,051	153,476	144,483	144,462	40,457	0
	Pond 3	213,148	186,011	179,334	179,294	42,692	1
7 days	Pond 1	144,994	121,807	116,208	116,201	36,634	0
	Pond 2	140,661	119,496	113,375	113,352	33,390	1
	Pond 3	208,246	161,202	154,767	154,728	37,085	0
28 Days	Pond 1	122,497	93,881	89,136	89,129	28,369	5
	Pond 2	188,485	156,338	149,686	149,661	42,494	738
	Pond 3	222,971	182,009	173,449	173,422	44,047	6,051
Total of All Time Points		**3,763,376**	**3,114,929**	**2,976,139**	**2,975,804**	**807,279**	**332,489**

Sample		eDNA 18S Initial R1	Trimmed R1	Merged	Cutadapt	Dereplicated	Crustacean
Blanks		68,692	4,951	3,289	3,287	2,317	4
Past Community 22 July		716,194	488,644	277,282	277,243	146,176	169
Past Community 22 July		758,887	514,732	293,727	293,688	159,853	213
Past Community 24 August		226,030	199,313	148,464	148,459	67,423	55
1 Hour	Pond 1	187,284	161,797	122,903	122,902	43,196	0
	Pond 2	209,772	180,432	131,883	131,875	48,850	166
	Pond 3	227,562	194,170	140,691	140,687	60,930	79
2 Hours	Pond 1	191,018	165,813	127,196	127,193	44,175	0
	Pond 2	192,471	168,104	125,780	125,773	46,900	238
	Pond 3	163,581	140,871	101,947	101,945	44,731	149
24 Hours	Pond 1	190,986	166,692	127,461	127,457	40,479	0
	Pond 2	189,672	162,555	119,780	119,774	45,440	12
	Pond 3	197,718	174,208	128,895	128,894	53,477	1
48 Hours	Pond 1	171,342	148,619	112,279	112,273	38,442	0
	Pond 2	167,956	143,913	105,497	105,489	41,736	0
	Pond 3	192,056	165,820	120,007	120,004	53,029	0
4 days	Pond 1	178,402	154,771	116,707	116,702	46,004	3
	Pond 2	197,028	171,777	131,071	131,061	45,597	0
	Pond 3	226,113	198,741	152,172	152,161	55,070	1
7 days	Pond 1	215,560	186,210	135,395	135,387	58,597	0
	Pond 2	229,584	198,977	147,766	147,758	56,368	4
	Pond 3	153,213	133,776	101,207	101,205	42,131	1
28 Days	Pond 1	216,868	184,063	124,023	124,015	56,114	4
	Pond 2	123,783	100,094	58,824	58,815	32,099	2,009
	Pond 3	189,401	164,696	123,377	123,366	54,873	11,446
Total of All Time Points		**4,011,370**	**3,466,099**	**2,554,861**	**2,554,736**	**1,008,238**	**14,554**

Sample		eRNA 18S Initial R1	Trimmed R1	Merged	Cutadapt	Dereplicated	Crustacean
Blanks		140,096	115,245	80,975	80,974	37,266	69
Past Community 22 July		575,366	426,976	249,705	249,683	125,502	107
Past Community 22 July		644,101	452,002	275,775	275,751	139,527	143
Past Community 24 August		135,465	115,827	77,918	77,914	43,776	27
1 Hour	Pond 1	155,162	135,780	106,473	106,472	41,636	1
	Pond 2	103,865	89,571	63,881	63,878	32,139	5
	Pond 3	238,644	208,597	157,132	157,128	64,053	151
2 Hours	Pond 1	173,785	153,196	121,683	121,682	45,078	0
	Pond 2	221,910	192,012	143,368	143,353	63,394	5
	Pond 3	183,783	161,588	121,555	121,554	52,191	1
24 Hours	Pond 1	171,638	151,516	118,065	118,064	46,561	0
	Pond 2	83,161	72,062	53,208	53,207	27,606	0
	Pond 3	195011	170,925	127,879	127,878	56,853	0
48 Hours	Pond 1	247,744	213,080	157,843	157,838	66,744	3
	Pond 2	200,246	178,341	137,655	137,648	59,562	3
	Pond 3	155,061	136,671	102,146	102,145	45,812	0
4 days	Pond 1	165,219	140,926	100,292	100,282	48,694	0
	Pond 2	138,506	114,477	68,739	68,736	39,961	0
	Pond 3	146,704	123,294	89,202	89,197	43,050	0
7 days	Pond 1	152,171	125,245	83,132	83,127	43,824	0
	Pond 2	156,429	127,227	83,025	83,019	46,671	0
	Pond 3	150,900	126,889	90,006	90,003	45,342	0
28 Days	Pond 1	144,714	120,849	78,940	78,939	39,100	0
	Pond 2	117,112	94,756	61,034	61,032	32,130	150
	Pond 3	145,646	117,763	76,812	76,807	40,888	2,122
Total of All Time Points		**3,447,411**	**2,954,765**	**2,142,070**	**2,141,989**	**981,289**	**2,787**

Supplementary Table 2. Species detection of all taxa by eDNA and eRNA metabarcoding with the COI and 18S barcode markers. Taxa are grouped according to Phylum or the highest classification assigned by TaxonKit.

		COI		18S	
	Taxonomic Identification	**eDNA**	**eRNA**	**eDNA**	**eRNA**
Fungi					
	Acephala applanata	-	-	-	+
	Acremonium sp.	-	-	+	-
	Adelosphaeria catenata	-	-	-	+
	Alternaria alternata	-	-	-	+
	Alternaria citri	-	-	+	+
	Alternaria papavericola	+	-	-	-
	Alternaria sp.	-	-	+	-
	Alternariaster centaureae-diffusae	-	-	+	-
	Apiotrichum dulcitum	-	-	-	+
	Apiotrichum porosum	+	-	+	-
	Aquamyces chlorogonii	-	-	-	+
	Ascochyta hordei	-	-	+	-
	Ascomycota sp.	-	-	-	+
	Aspergillus fumigatus	+	+	+	+
	Aspergillus niger	-	-	+	+
	Aspergillus restrictus	-	-	+	+
	Aspergillus ruber	+	-	-	-
	Aspergillus sp.	-	-	+	-
	Aspergillus terreus	-	-	-	+
	Aspergillus unguis	-	-	+	-
	Astragalicola vasilyevae	-	-	+	+
	Aureobasidium pullulans	-	-	+	-
	Auxarthron umbrinum	-	-	-	+
	Avachytrium platense	-	-	+	+
	Basidioradulum radula	-	-	+	-
	Bipolaris sorokiniana	-	-	+	-
	Blumeria graminis	-	-	-	+
	Boeremia exigua	-	-	-	+
	Cadophora fastigiata	-	+	-	+
	Cadophora interclivum	-	-	+	-

Celerioriella dura	-	-	+	-
Cercophora sp.	-	+	-	-
Cerrena unicolor	-	-	+	-
Chaetomium grande	+	-	-	-
Chaetomium homopilatum	-	-	-	+
Chaetomium nozdrenkoae	-	-	+	-
Chaetomium sp.	-	+	-	-
Chaetothyriales sp.	-	-	+	+
Chalara austriaca	-	-	+	-
Chrysomyxa arctostaphyli	-	-	+	-
Chytridiomycota sp.	-	-	-	+
Cladochytrium replicatum	-	-	+	+
Cladophialophora sp.	-	-	-	+
Cladosporium cf. herbarum	-	+	-	+
Cladosporium cladosporioides	-	-	-	+
Cladosporium endophytica	-	-	+	-
Cladosporium pseudocladosporioides	-	-	+	-
Cladosporium sp.	-	-	+	+
Clarireedia homoeocarpa	-	-	+	-
Clitocybe odora	-	-	+	-
Comoclathris rosarum	-	-	-	+
Coniochaeta rosae	-	-	+	-
Conocybe apala	-	-	+	-
Cordyceps farinosa	-	-	+	-
Cortinarius bolaris	-	-	+	-
Cortinarius cotoneus	-	-	+	-
Cryptosporella hypodermia	-	-	-	+
Cuphophyllus pratensis	-	-	+	-
Curvularia spicifera	-	-	+	+
Cutaneotrichosporon cutaneum	-	-	+	-
Cyberlindnera jadinii	-	-	+	-
Cyclothyriella rubronotata	-	-	-	+
Cystofilobasidium infirmominiatum	-	-	+	-
Diaporthe foeniculina	-	-	+	-
Diatrype palmicola	-	-	-	+
Diatrypella tectonae	-	-	+	+
Dissoconium aciculare	-	-	-	+
Donkia pulcherrima	-	-	+	-
Elsinoe brasiliensis	-	-	+	+
Endosporium aviarium	-	-	-	+
Entyloma ficariae	-	-	+	-
Epicoccum nigrum	-	+	+	-
Eurotiomycetes sp.	-	-	+	-

Eutypella sp.	-	-	-	+
Favolus subtropicus	-	-	+	-
Flammulina velutipes	-	+	-	-
Flavomyces fulophazii	-	-	+	+
Fusarium cf. solani	-	+	-	-
Fusarium culmorum	-	-	-	+
Fusarium fujikuroi	+	+	-	-
Fusarium graminearum	+	-	-	-
Fusarium oxysporum	+	+	+	+
Fusarium solani	-	-	+	+
Fusarium sp.	-	-	+	-
Fusarium tricinctum	-	-	+	-
Fusarium verticillioides	+	+	-	-
Gelasinospora sp.	+	+	+	-
Gibbera conferta	-	-	+	-
Gibellulopsis nigrescens	-	+	+	-
Gliomastix murorum	-	-	+	-
Golovinomyces orontii	-	-	-	+
Golovinomyces sp.	-	-	-	+
Gymnopus androsaceus	-	-	-	+
Gymnopus dryophilus	-	-	+	-
Gymnopus luxurians	-	-	+	-
Halorosellinia rhizophorae	-	-	+	-
Hansfordia pulvinata	-	-	+	+
Helotiaceae sp.	-	-	-	+
Helotiales sp.	-	+	-	-
Humicola grisea	-	-	+	+
Hyaloraphidium curvatum	-	-	+	+
Hymenoscyphus ohakune	-	-	-	+
Hyphoderma definitum	-	-	+	-
Hyphodontia alutaria	-	-	+	-
Hyphodontia rimosissima	-	-	+	-
Hypochnicium sp.	-	+	+	-
Hypoderma rubi	-	-	-	+
Hypogymnia vittata	+	-	-	-
Hypoxylon fragiforme	-	-	-	+
Irpex lacteus	-	-	+	-
Itersonilia perplexans	-	-	+	-
Keissleriella rosae	-	-	+	-
Lactarius cf. volemus	-	-	+	-
Lambertella subrenispora	-	-	-	+
Lasiosphaeriaceae sp.	-	-	-	+
Leotiomycetes sp.	-	-	+	-

Lepista sordida	-	-	+	-
Leucosporidium scottii	+	-	+	-
Lophium mytilinum	-	-	+	-
Malassezia restricta	-	-	-	+
Megacollybia platyphylla	-	-	+	-
Metarhizium granulomatis	-	-	+	-
Metarhizium marquandii	+	-	-	-
Microdochium chrysanthemoides	-	-	+	+
Microdochium nivale	-	-	+	-
Microsporidium sp.	-	-	-	+
Mortierella elongata	+	+	-	-
Mortierella sp.	-	+	-	-
Mucor moelleri	-	-	-	+
Murispora hawksworthii	-	-	+	-
Mycosphaerella hyperici	-	-	-	+
Myrothecium sp.	-	-	+	-
Nadsonia starkeyi-henricii	-	+	-	-
Neocatenulostroma microsporum	-	-	+	-
Neocucurbitaria cava	-	-	+	-
Neopaucispora rosaecae	-	-	-	+
Neurospora udagawae	-	-	+	-
Nigrograna mackinnonii	-	-	-	+
Nowakowskiella multispora	-	-	-	+
Occultifur sp.	-	-	+	-
Ophiognomonia clavigignenti-juglandacearum	-	-	+	-
Ophiostoma sp.	-	-	+	-
Pandora neoaphidis	-	-	+	+
Paraglomus sp.	-	-	+	-
Paraphaeosphaeria sporulosa	-	-	+	-
Paraphoma radicina	-	-	+	-
Parastagonospora forlicesenica	-	-	-	+
Parmotrema cetratum	-	-	+	-
Patinella hyalophaea	-	-	+	-
Paxillus vernalis	-	-	+	-
Penicillium brevicompactum	-	-	+	+
Penicillium chrysogenum	-	-	+	+
Penicillium citreonigrum	+	+	-	-
Penicillium glabrum	-	-	-	+
Penicillium javanicum	-	-	-	+
Penicillium murcianum	+	-	-	-
Penicillium sclerotiorum	+	+	-	-
Penicillium sp.	-	-	+	+
Penicillium westlingii	+	-	-	-

Phacidium sp.	-	-	-	+
Phaeococcomycetaceae sp.	-	-	-	+
Phialemoniopsis curvata	-	-	+	-
Pholiota sp.	-	+	-	-
Phoma herbarum	-	-	+	-
Phoma sp.	-	-	+	-
Phomatospora biseriata	-	-	-	+
Pichia sp.	-	-	+	-
Pithomyces chartarum	-	-	+	+
Plectosphaerella cucumerina	-	-	+	-
Plectosphaerella sp.	-	-	+	-
Pleurotus ostreatoroseus	-	-	+	-
Pluteus cervinus	+	-	-	-
Podosphaera longiseta	-	-	-	+
Polyporus hapalopus	-	-	+	-
Postia floriformis	-	+	-	-
Psathyrella candolleana	-	-	+	-
Pseudeurotium ovale	-	-	+	-
Pseudocamarosporium propinquum	-	-	+	+
Pseudocercospora fici	-	-	+	-
Pseudomicrostroma glucosiphilum	-	-	+	-
Pseudoophiobolus mathieui	-	-	+	+
Pseudotruncatella arezzoensis	-	-	-	+
Psilocybe caerulipes	-	-	+	-
Purpureocillium lilacinum	-	-	+	-
Ramichloridium apiculatum	-	-	+	-
Rhizoclosmatium globosum	-	-	+	-
Rhizoctonia solani	-	-	+	-
Rhizophydiales sp.	-	-	+	+
Rhizophydium sp.	-	-	+	+
Rhizopus microsporus	+	+	-	+
Rhodotorula mucilaginosa	-	-	+	-
Rhodotorula sp.	-	-	+	-
Roseodiscus formosus	-	-	-	+
Saccharomyces cerevisiae	-	-	+	-
Scedosporium boydii	-	-	+	-
Schizothyrium pomi	-	-	+	+
Scutellinia sp.	-	+	-	-
Setomelanomma holmii	-	-	-	+
Setophoma terrestris	-	-	+	+
Sistotrema brinkmannii	-	-	+	-
Sistotrema resinicystidium	-	-	+	-
Smittium culicis	-	-	+	+

	Solicoccozyma terricola	-	+	+	-
	Spongipellis pachyodon	-	-	+	-
	Sporidiobolus pararoseus	-	-	+	-
	Sporisorium sp.	-	-	+	-
	Stereum hirsutum	-	-	-	+
	Sulcispora sp.	-	-	+	-
	Synchytrium decipiens	-	-	+	-
	Thysanorea sp.	-	-	-	+
	Trametes sp.	-	-	+	-
	Trematosphaeria hydrela	-	-	+	+
	Trichocladium sp.	+	-	-	-
	Trichoderma hamatum	+	+	-	-
	Trichoderma harzianum	-	-	-	+
	Trichoderma spirale	-	+	-	-
	Tuckermannopsis chlorophylla	-	-	-	+
	Verruconis gallopava	-	-	+	-
	Vishniacozyma carnescens	-	-	+	-
	Westerdykella multispora	-	-	+	-
	Zoophthora radicans	-	-	+	+
	Total Fungi	**22**	**27**	**139**	**88**
Metazoa					
	Ablabesmyia americana	+	-	-	-
	Ablabesmyia sp.	+	-	-	-
	Acanthodiaptomus pacificus	-	-	+	+
	Acricotopus sp.	+	-	-	-
	Anoecia sp.	+	-	-	-
	Anogdus secretus	+	-	-	-
	Anopheles punctipennis	+	-	-	-
	Aphelenchoides bicaudatus	-	-	+	-
	Aphelinidae sp.	+	-	-	-
	Appendiseta robiniae	+	-	-	-
	Ascidia ceratodes	-	-	-	+
	Baetis cf. tricaudatus	+	-	-	-
	Balanus balanus	-	-	+	+
	Beauchampia crucigera	-	-	-	+
	Bitectipora retepora	-	-	+	+
	Bosmina longirostris	+	+	+	+
	Bosmina sp.	-	+	-	-
	Bosminidae sp.	+	+	-	-
	Brachionus calyciflorus	-	-	+	+
	Brachionus urceolaris	-	-	+	+
	Bradysia placida	+	-	-	-
	Calanus finmarchicus	+	-	+	+

Calanus glacialis	+	-	-	-
Callibaetis ferrugineus	+	-	-	-
Callibaetis fluctuans	+	-	-	-
Cecidomyiidae sp.	+	-	-	-
Centropages abdominalis	-	-	+	-
Cephalodella forficula	-	-	+	+
Cephalodella gibba	-	-	+	+
Ceriodaphnia dubia	+	+	+	+
Ceriodaphnia sp.	+	-	-	-
Chaetonotus sp.	-	-	+	+
Chaoboridae	+	+	-	-
Chironomidae sp.	+	+	+	-
Chironomus atrella	+	-	-	-
Chironomus sp.	+	-	-	-
Chthamalidae sp.	-	+	-	-
Chydorus brevilabris	+	+	+	+
Chydorus sphaericus	-	-	+	+
Cloeon dipterum	+	-	-	-
Cloeon sp.	-	-	+	+
Cobanocythere japonica	-	-	+	-
Copepoda sp.	-	-	+	+
Corixidae sp.	-	-	-	+
Corynoneura arctica	+	+	-	-
Corynoneura sp.	+	+	+	+
Coturnix coturnix	-	-	+	-
Cricotopus trifascia	+	-	-	-
Crisularia plumosa	-	-	-	+
Ctenophora sp.	-	-	-	+
Culex theileri	-	-	+	-
Cyclopidae sp.	+	+	+	+
Cyclops sp.	+	-	-	-
Cylindrostoma fingalianum	-	-	-	+
Cypridopsis uenoi	-	-	+	+
Cypridopsis vidua	+	+	-	-
Cyprinus carpio	-	-	-	+
Daphnia ambigua	+	+	+	+
Daphnia catawba	+	+	-	-
Daphnia laevis	+	-	-	-
Daphnia tibetana	-	-	-	+
Dasyhelea sp.	-	+	-	-
Diaphanosoma sp.	+	+	+	+
Dicrotendipes modestus	+	-	-	-
Dicrotendipes sp.	-	-	+	-

Dicrotendipes tritomus	+	+	+	+
Drepanaphis sp.	+	-	-	-
Eburia sp.	-	-	+	-
Endochironomus sp.	-	-	+	-
Entomobrya unostrigata	+	-	-	-
Entomobryomorpha sp.	-	-	+	-
Epiphanes senta	-	-	+	+
Erigone prominens	-	-	-	+
Eriosoma americanum	+	-	-	-
Eubosmina coregoni	+	-	-	-
Euchlanis alata	-	-	+	+
Euchlanis alata	-	-	+	+
Euchlanis dilatata	-	-	+	+
Eupodidae sp.	+	+	-	-
Eurycercus longirostris	+	+	-	-
Eurytemora affinis	-	-	-	+
Exogone heterosetosa	-	-	+	-
Felis silvestris	+	-	-	-
Forcipomyia sp.	+	-	-	-
Frankliniella occidentalis	-	-	-	+
Gattyana cirrhosa	-	-	+	-
Glyptotendipes sp.	-	-	+	-
Halichondria bowerbanki	-	-	+	+
Halichondria panicea	-	-	-	+
Hemiptera sp.	+	+	-	-
Hogna cf. frondicola	-	-	-	+
Holopedium gibberum	+	+	-	-
Homo sapiens	+	+	+	-
Kellicottia sp.	+	+	-	+
Keratella cochlearis	+	+	+	+
Keratella quadrata	-	-	+	+
Lecane inermis	-	-	+	+
Lecane ungulata	-	-	-	+
Lepadella patella	-	-	+	+
Leptodiaptomus minutus	+	+	-	-
Limnophyes sp.	+	-	-	-
Lindia tecusa	-	-	-	+
Lindia torulosa	-	-	+	+
Macrochaetus collinsi	-	-	+	+
Macropsis basalis	+	-	-	-
Macrosiphum euphorbiae	+	-	-	-
Macrothrix sp.	+	+	-	-
Maxillopoda sp.	+	+	-	-

Mesocyclops leuckarti	+	-	+	-
Microcyclops varicans	-	-	+	+
Micropsectra nigripila	+	-	-	-
Microtendipes pedellus	+	-	-	-
Monellia sp.	+	-	-	-
Monommata maculata	-	-	-	+
Monostyla sp.	-	-	+	+
Monostyla sp.	-	-	+	+
Myzocallis walshii	+	-	-	-
Neocondeellum brachytarsum	+	-	-	-
Neodermation sp.	-	+	-	-
Nereis pelagica	-	-	+	-
Notommata allantois	-	-	+	+
Notommata cordonella	-	-	+	+
Oecetis inconspicua	+	-	-	-
Oithona similis	+	+	+	+
Onychocorycaeus catus	+	-	-	-
Panopoda rufimargo	+	-	-	-
Paratanytarsus grimmii	+	-	-	-
Paratanytarsus laccophilus	+	-	-	-
Paratanytarsus sp.	+	-	+	-
Pectinaria granulata	+	-	-	+
Plationus patulus	-	-	+	+
Pleuroxus cf. varidentatus	+	-	-	-
Ploesoma hudsoni	-	-	+	+
Ploesoma hudsoni	-	-	+	+
Ploesoma truncatus	-	-	+	+
Plumatella casmiana	-	-	+	-
Podocopida sp.	+	-	-	-
Polyarthra dolichoptera	+	+	-	-
Polyarthra remata	+	+	+	+
Polypedilum sp.	-	+	-	-
Polypedilum sp.	+	+	-	-
Pontellidae sp.	+	-	-	-
Prionospio steenstrupi	-	-	+	-
Proales doliaris	+	-	+	+
Psectrocladius cf. limbatellus	+	+	-	-
Psectrocladius sp.	-	-	+	+
Pseudocalanus acuspes	-	-	+	-
Pseudocalanus newmani	-	-	+	-
Rotaria magnacalcarata	-	-	+	-
Sciaridae sp.	+	+	-	-
Sigara alternata	+	-	-	-

	Simocephalus cf. serrulatus	+	+	+	+
	Simocephalus heilongjiangensis	-	-	-	+
	Sinhomidia bicolor	-	-	+	+
	Skistodiaptomus oregonensis	+	-	-	-
	Smittia cf. stercoraria	+	-	-	-
	Stenostomum bryophilum	-	-	+	-
	Stenostomum sp.	-	-	+	-
	Sympetrum pedemontanum	-	-	+	-
	Synchaeta pectinata	+	+	+	+
	Synchaeta tremula	-	-	+	+
	Synchaeta tremuloida	-	-	+	+
	Tanytarsus mendax	+	-	-	+
	Tanytarsus wirthi	+	-	+	-
	Temora turbinata	-	-	+	-
	Tetraneura nigriabdominalis	+	-	-	-
	Tetraspora sp.	-	-	+	+
	Thelepus sp.	-	-	-	+
	Therioaphis riehmi	+	-	-	-
	Thermocyclops sp.	-	-	-	+
	Thrips tabaci	+	-	-	-
	Trichotria tetractis	-	-	+	+
	Uroleucon erigeronensis	+	-	-	-
	Ursus arctos	-	-	+	+
	Willowsia buskii	+	-	-	-
	Total Metazoa	**91**	**37**	**80**	**75**
Viridiplantae					
	Acer rubrum	-	-	+	-
	Acutodesmus bajacalifornicus	-	-	+	+
	Acutodesmus deserticola	-	-	+	+
	Ajania potaninii	+	+	+	+
	Ankistrodesmus fusiformis	+	-	+	+
	Asterarcys quadricellulare	-	-	+	+
	Asterococcus sp.	-	-	-	+
	Athyrium filix-femina	-	-	-	+
	Atractomorpha porcata	-	-	-	+
	Attalea speciosa	-	-	-	+
	Auxenochlorella pyrenoidosa	-	-	-	+
	Avena fatua	-	-	+	-
	Betula pendula	-	-	+	-
	Botryococcus braunii	-	-	+	+
	Brachiaria fragrans	-	-	-	+
	Brachypodium distachyon	-	-	+	-
	Bracteacoccus deserticola	-	-	+	+

Bracteacoccus glacialis	-	-	-	+
Bracteacoccus ruber	-	-	+	+
Bracteacoccus sp.	-	-	-	+
Brassica napus	-	-	+	-
Carolibrandtia ciliaticola	-	-	+	+
Carpinus betulus	-	-	+	-
Carteria crucifera	-	-	+	+
Castanea seguinii	-	-	+	-
Castanopsis tibetana	-	-	+	-
Chaetopeltis orbicularis	-	-	+	+
Chaetosphaeridium globosum	-	-	+	+
Characiopodium hindakii	-	-	+	+
Characiopodium sp.	-	-	+	+
Chasechloa madagascariensis	-	-	+	+
Chlamydomonadaceae sp.	-	-	+	+
Chlamydomonas applanata	-	-	+	+
Chlamydomonas fasciata	-	-	+	-
Chlamydomonas perpusilla	-	-	-	+
Chlamydomonas pulvinata	-	-	+	+
Chlamydomonas raudensis	-	-	+	+
Chlamydomonas reinhardtii	-	-	+	-
Chlamydomonas sordida	-	-	+	-
Chlamydomonas sp.	-	-	+	+
Chlamydomonas sphaeroides	-	-	+	-
Chlamydopodium starrii	-	-	-	+
Chlorella heliozoae	-	-	-	+
Chlorella singularis	-	-	-	+
Chlorella sorokiniana	-	-	+	+
Chlorella sp.	-	-	+	+
Chlorella volutis	-	-	-	+
Chlorella volutis	-	-	-	+
Chlorella vulgaris	-	-	-	+
Chlorococcum ellipsoideum	-	-	+	+
Chlorococcum oleofaciens	-	-	-	+
Chlorococcum sp.	-	-	+	+
Chlorococcum sphacosum	-	+	-	-
Chloromonas oogama	-	-	+	+
Chlorophyta sp.	-	-	+	+
Chlorosarcinopsis eremi	-	-	-	+
Chlorosarcinopsis sp.	-	+	-	-
Choricystis sp.	-	-	+	-
Chrysanthemum indicum	+	+	-	+
Closterium spinosporum	-	-	+	-

Coelastrella aeroterrestrica	-	-	+	+
Coelastrella saipanensis	-	-	+	+
Coelastrum astroideum	-	-	+	+
Coelastrum microporum	-	-	+	-
Coelastrum microporum	-	-	+	+
Coelastrum morum	-	-	+	+
Coelastrum sp.	-	-	+	-
Coelastrum sphaericum	-	-	+	+
Commiphora wightii	-	-	+	-
Cosmarium bioculatum	-	-	+	+
Cosmarium debaryi	-	-	+	-
Cosmarium depressum	-	-	+	-
Cosmarium humile	-	-	-	+
Cosmarium impressulum	-	-	+	+
Cosmarium laeve	-	-	-	+
Cosmarium meneghinii	-	-	+	+
Cosmarium phaseolus	-	-	+	-
Cosmarium punctulatum	-	-	+	-
Cosmarium regnellii	-	-	+	-
Cosmarium sp.	-	-	-	+
Cucumis sativus	-	-	+	-
Cucurbita pepo	-	-	+	-
Cystomonas sp.	-	-	+	-
Deasonia sp.	-	-	+	+
Desmochloris cf. halophila	-	-	-	+
Desmodesmus abundans	-	-	+	-
Desmodesmus bicellularis	-	-	-	+
Desmodesmus brasiliensis	-	-	+	-
Desmodesmus communis	-	-	-	+
Desmodesmus costato-granulatus	-	-	-	+
Desmodesmus intermedius	-	-	+	+
Desmodesmus maximus	-	-	-	+
Desmodesmus pannonicus	-	-	+	+
Desmodesmus sp.	-	-	-	+
Desmodesmus tropicus	-	-	-	+
Dictyosphaerium sp.	-	-	-	+
Dysoxylum spectabile	-	-	+	+
Ecballocystopsis dichotomus	-	-	+	+
Fagus grandifolia	-	-	+	-
Fasciculochloris boldii	-	-	-	+
Fernandinella sp.	-	-	+	+
Follicularia paradoxalis	-	-	+	+
Franceia amphitricha	-	-	+	+

Glycine max	-	-	+	-
Gonium pectorale	-	-	+	+
Graesiella emersonii	-	-	+	+
Haematococcus lacustris	-	-	+	+
Hafniomonas conica	-	-	-	+
Hamakko caudatus	-	-	-	+
Hazenia basiliensis	-	-	+	+
Heterochlorella luteoviridis	-	-	-	+
Hindakia tetrachotoma	-	-	-	+
Hormotila blennista	-	-	-	+
Hormotilopsis gelatinosa	-	-	-	+
Ipomoea leptophylla	-	-	+	-
Jacobaea vulgaris	+	-	+	-
Juglans nigra	-	-	+	+
Kalenjinia gelatinosa	-	-	-	+
Kirchneriella lunaris	-	-	+	+
Lactuca sativa	-	-	-	+
Laennecia sophiifolia	-	-	+	-
Lagerheimia ciliata	-	-	-	+
Lemna turionifera	-	-	-	+
Lobomonas monstruosa	-	-	-	+
Lolium multiflorum	-	-	-	+
Makinoella tosaensis	-	-	+	+
Medicago truncatula	-	-	+	-
Messastrum gracile	-	-	+	-
Micractinium pusillum	-	-	-	+
Micractinium sp.	-	-	-	+
Microglena monadina	-	-	+	-
Monomastix minuta	-	+	-	+
Monomastix sp.	-	-	+	+
Monoraphidium convolutum	-	-	+	+
Monoraphidium minutum	-	-	+	-
Monoraphidium saxatile	-	-	+	+
Monoraphidium sp.	-	-	+	+
Mychonastes sp.	-	-	+	+
Neglectella peisonis	-	-	+	+
Neglectella solitaria	-	-	+	-
Neochloris conjuncta	-	-	+	-
Neochloris sp.	-	-	-	+
Neochlorosarcina sp.	-	-	-	+
Oedogonium pusillum	-	-	+	+
Oocystaceae sp.	-	-	+	+
Oocystella heteromucosa	-	-	+	+

Oocystella nephrocytioides	-	-	-	+
Oocystella oogama	-	-	+	+
Oocystidium polymammilatum	-	-	+	+
Oocystis marina	-	-	+	+
Oocystis parva	-	-	+	+
Oocystis rhomboidea	-	-	+	+
Oocystis sp.	-	-	+	+
Oophila amblystomatis	-	-	+	+
Oophila sp.	-	-	+	-
Panicum hallii	-	-	+	+
Parastrephia quadrangularis	-	-	+	-
Paulschulzia pseudovolvox	+	+	+	+
Phyllostachys heteroclada	-	-	+	+
Pinus armandii	-	-	+	+
Pinus elliottii	-	-	+	+
Pinus luchuensis	-	-	-	+
Pinus morrisonicola	-	-	+	+
Pinus taeda	-	-	+	-
Planktosphaeria sp.	-	-	+	+
Planophila laetevirens	-	-	+	+
Plantago lanceolata	-	-	+	+
Platanus occidentalis	-	-	+	-
Polytoma oviforme	-	-	+	-
Populus trichocarpa	-	-	+	+
Protodesmus sp.	-	-	+	+
Pseudomuriella sp.	-	-	+	+
Pseudopediastrum alternans	-	-	+	+
Pseudoschroederia antillarum	-	-	-	+
Quercus suber	-	-	+	+
Radiococcus polycoccus	-	-	+	+
Radiococcus sp.	-	-	-	+
Rotundella rotunda	-	-	+	+
Rotundella sp.	-	-	+	+
Rubus allegheniensis	-	-	+	-
Sanionia uncinata	-	-	-	+
Sarcinofilum mucosum	-	-	+	+
Scenedesmaceae sp.	-	-	+	+
Scenedesmus sp.	-	-	+	+
Scenedesmus vacuolatus	-	-	+	+
Selenastraceae sp.	-	-	-	+
Selenastrum capricornutum	-	-	+	+
Silene antirrhina	-	-	+	-
Silene vulgaris	-	-	+	+

	Sphagnum strictum	-	-	-	+
	Spirotaenia condensata	-	-	-	+
	Staurastrum avicula	-	-	+	+
	Staurastrum paradoxum	-	-	+	+
	Staurastrum pinnatum	-	-	-	+
	Staurastrum quadricornutum	-	-	-	+
	Stauridium privum	-	-	+	+
	Stigeoclonium helveticum	-	-	-	+
	Stigeoclonium sp.	-	-	+	+
	Stigeoclonium tenue	-	-	-	+
	Taraxacum obtusifrons	-	-	+	-
	Taraxacum officinale	-	-	+	-
	Taraxacum sp.	-	-	+	-
	Tetracystis sarcinalis	-	-	+	+
	Tetracystis sp.	-	+	-	-
	Tetradesmus dimorphus	-	-	+	-
	Tetradesmus obliquus	-	-	+	+
	Tetraedron minimum	-	-	-	+
	Tetranephris brasiliensis	+	-	+	+
	Tetraspora cylindrica	-	-	+	+
	Tortitaenia alpina	-	-	-	+
	Treubaria triappendiculata	-	-	+	+
	Triticum aestivum	+	-	+	-
	Tsuga chinensis	-	-	+	-
	Tumidella tumida	-	-	-	+
	Tupiella akineta	-	-	+	+
	Tupiella speciosa	-	-	+	+
	Ulmus glaucescens	-	-	+	-
	Ulothrix zonata	-	-	-	+
	Urtica angustifolia	-	-	+	+
	Vitis vinifera	-	-	+	-
	Volvox aureus	-	-	+	-
	Willea vilhelmii	-	-	+	+
	Wolffia brasiliensis	-	-	-	+
	Zea mays	-	-	+	-
	Total Viridiplantae	**7**	**7**	**157**	**166**
Amoebozoa					
	Amoebozoa sp.	-	-	+	-
	Cochliopodium kieliense	+	+	-	-
	Cochliopodium larifeili	+	+	+	+
	Echinamoeba exundans	-	-	+	+
	Fuligo sp.	-	-	-	+
	Korotnevella jeppesenii	-	-	+	+

	Lesquereusia spiralis	-	-	+	+
	Platyamoeba sp.	-	-	-	+
	Protostelium nocturnum	-	-	-	+
	Ripella tribonemae	-	-	+	+
	Vannella persistens	-	-	+	+
	Vannella planctonica	-	-	+	+
	Vannella simplex	-	-	+	+
	Vannella sp.	-	-	+	+
	Vexillifera expectata	+	+	-	-
	Total Amoebozoa	**3**	**3**	**10**	**12**
Alveolata					
	Alexandrium affine	-	+	-	-
	Alexandrium minutum	-	+	-	-
	Alexandrium tamarense	+	-	-	-
	Apicoporus sp.	-	-	+	-
	Arcuospathidium cultriforme	-	-	+	+
	Asulcocephalium miricentonis	-	-	+	+
	Azadinium concinnum	-	-	+	-
	Azadinium dalianense	+	-	-	-
	Balantidion pellucidum	-	-	+	-
	Biecheleriopsis adriatica	-	-	+	+
	Bryophyllum sp.	-	-	+	-
	Colepidae sp.	-	-	+	+
	Colpoda sp.	-	-	-	+
	Cryptoperidiniopsis sp.	+	+	-	-
	Cyclidium glaucoma	-	-	-	+
	Cyrtohymena citrina	-	+	-	-
	Cyrtolophosis mucicola	+	-	+	+
	Dexiostoma sp.	-	-	-	+
	Enchelyodon sp.	-	-	+	+
	Ensiculifera imariensis	-	-	+	-
	Epicarchesium pectinatum	-	-	-	+
	Frontoniidae sp.	+	+	+	+
	Glaucoma sp.	-	-	+	+
	Gymnodinium sp.	-	-	-	+
	Gyrodiniellum shiwhaense	-	-	+	+
	Halteria grandinella	-	-	+	+
	Halteria sp.	-	-	+	-
	Haptoria sp.	+	+	+	+
	Hemiurosomoida longa	+	-	+	+
	Heterocapsa rotundata	-	-	+	+
	Lagynophrya acuminata	-	-	+	+
	Microdiaphanosoma arcuatum	-	-	+	+

Oligohymenophorea sp.	-	-	+	+
Ostreopsis cf. ovata	+	-	-	-
Ostreopsis cf. siamensis	+	-	-	-
Oxytricha sp.	-	-	-	+
Paraschneideria metamorphosa	-	-	+	-
Paruroleptus lepisma	-	-	+	+
Parvodinium inconspicuum	-	-	+	+
Parvodinium mixtum	+	-	+	+
Parvodinium trawinskii	-	-	+	+
Parvodinium umbonatum	-	-	+	+
Peridiniales sp.	-	-	-	+
Peridiniopsis polonicum	-	-	-	+
Peridinium sp.	+	-	-	-
Peridinium wisconsinense	-	-	+	+
Pfiesteria piscicida	-	+	-	+
Pfiesteriaceae sp.	+	-	-	-
Platyophrya bromelicola	-	-	+	+
Podolampas elegans	-	-	+	+
Podolampas spinifera	-	-	-	+
Protoceratium reticulatum	-	+	-	-
Pseudocyrtolophosis alpestris	-	-	+	+
Pseudokeronopsis pararubra	-	+	-	-
Scrippsiella aff. acuminata	-	-	+	+
Scrippsiella cf. erinaceus	-	-	+	+
Scrippsiella sp.	-	-	+	+
Scrippsiella trochoidea	+	-	+	+
Scyphidia sp.	-	-	-	+
Spathidium foissneri	-	-	+	+
Spathidium papilliferum	-	-	+	+
Spathidium polynucleatum	-	-	+	+
Spathidium sp.	-	-	+	-
Stichotricha aculeata	-	-	+	+
Stichotrichia sp.	+	+	+	+
Tetrahymena glochidiophila	-	-	+	+
Tetrahymena pigmentosa	-	-	+	+
Tetrahymena setosa	-	-	+	-
Tetrahymena sp.	-	-	+	+
Theleodinium calcisporum	-	-	-	+
Thoracosphaera heimii	+	-	-	-
Trachelophyllum brachypharynx	-	-	+	+
Tripos platycornis	-	-	+	-
Trochiliopsis australis	-	-	+	-
Uroleptus gallina	-	-	+	+

	Uroleptus pisces	-	-	+	+
	Uroleptus sp.	-	-	+	+
	Urosoma emarginata	-	-	+	+
	Urosoma salmastra	-	-	-	+
	Vorticella aequilata	-	-	+	+
	Vorticella campanula	-	+	+	+
	Vorticella gracilis	-	-	+	-
	Vorticella sp.	-	-	+	+
	Vorticellides aquadulcis	-	-	+	+
	Total Alveolata	**15**	**11**	**58**	**59**
Rhizaria					
	Bodomorpha minima	-	-	+	+
	Bodomorpha sp.	+	-	+	+
	Cercomonas sp.	-	-	-	+
	Cercozoa sp.	-	-	+	+
	Fisculla asini	+	-	+	+
	Fisculla siemensmai	-	-	+	+
	Glissomonad sp.	-	-	+	+
	Gymnophrys sp.	-	-	+	+
	Heteromita globosa	-	-	-	+
	Leptophrys vorax	-	-	+	+
	Neocercomonas sp.	-	-	+	+
	Paracercomonas sp.	-	-	+	+
	Pseudodifflugia cf. gracilis	+	+	+	+
	Rhogostoma cylindrica	-	-	+	-
	Spongomonas sp.	-	-	+	-
	Vernalophrys algivore	-	-	+	+
	Viridiraptor invadens	-	-	+	+
	Total Rhizaria	**3**	**1**	**15**	**15**
Stramenopiles					
	Achnanthidium minutissimum	-	-	+	-
	Achnanthidium pyrenaicum	-	-	-	+
	Aphanomyces cf. repetans	-	-	+	-
	Aphanomyces sp.	+	+	+	-
	Chrysochaete britannica	-	-	+	+
	Chrysamoeba tenera	-	-	+	-
	Chrysophyceae sp.	-	-	+	-
	Lagenidium sp.	-	-	+	-
	Leptolegnia caudata	+	-	-	-
	Nannochloropsis limnetica	+	+	-	-
	Nannochloropsis oculata	-	-	-	+
	Nitzschia frustulum	-	-	+	+
	Ochromonas cf. sphaerocystis	-	-	+	-

	Ochromonas perlata	-	-	+	+
	Ochromonas sp.	-	-	-	+
	Paraphysomonas mantoni	-	-	+	-
	Paraphysomonas solis	-	-	+	-
	Paraphysomonas sp.	+	+	+	+
	Poterioochromonas stipitata	-	-	+	+
	Poteriospumella sp.	-	-	+	-
	Pylaiella littoralis	-	-	+	-
	Pythium adhaerens	+	-	-	-
	Rhopalodia gibba	-	-	+	-
	Saprolegnia bulbosa	+	-	-	-
	Saprolegnia ferax	+	+	-	-
	Saprolegnia sp.	-	-	+	+
	Total Stramenopiles	**7**	**4**	**18**	**9**
Hacrobia (Cryptophyta, Centroheliozoa & Haptophyceae)					
	Centroheliozoa sp.	-	-	+	-
	Choanocystis symna	-	-	+	+
	Chrysochromulina parva	+	+	+	+
	Chrysochromulina sp.	+	+	+	+
	Cryptomonas curvata	-	-	+	+
	Cryptomonas lundii	-	-	+	-
	Cryptomonas platyuris	-	-	-	+
	Cryptomonas pyrenoidifera	+	+	+	+
	Cryptomonas reflexa	-	-	+	+
	Cryptomonas sp.	-	-	+	+
	Cryptomonas tetrapyrenoidosa	-	-	+	+
	Pterocystis sp.	-	-	-	+
	Raineriophrys sp.	+	-	+	+
	Raphidiophrys ambigua	+	-	+	+
	Sphaerastrum fockii	-	-	+	+
	Total Hacrobia	**5**	**3**	**13**	**13**
Bacteria					
	Escherichia coli	+	-	+	+
	Escherichia fergusonii	-	-	-	+
	Mucilaginibacter sp.	+	-	-	-
	Planctopirus hydrillae	-	-	+	-
	Planctopirus limnophila	-	-	+	-
	Ralstonia pickettii	-	-	+	-
	Rhodococcus erythropolis	-	-	+	-
	Total Bacteria	**2**	**0**	**5**	**2**
Choanoflagellida					
	Codosiga botrytis	-	-	-	+
	Salpingoeca punica	-	-	+	+

	Salpingoeca ventriosa	-	-	+	+
	Sphaeroeca leprechaunica	-	-	+	+
	Total Choanoflagellida	**0**	**0**	**3**	**4**
Euglenozoa					
	Peranema trichophorum	-	-	-	+
	Total Euglenozoa	0	0	0	1
Total		**155**	**93**	**498**	**444**