Title: Monitoring the land and sea: Enhancing efficiency through CRISPR-Cas driven depletion and enrichment of environmental DNA

Running Title: Biomonitoring ecosystems using CRISPR-Cas

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Abstract

Characterising biodiversity using environmental DNA (eDNA) represents a paradigm shift in our capacity for biomonitoring complex environments, both aquatic and terrestrial. However, eDNA biomonitoring is limited by biases towards certain species and the low taxonomic resolution of current metabarcoding approaches. Shotgun metagenomics of eDNA enables the collection of whole ecosystem data by sequencing all molecules present, allowing characterisation and identification. CRISPR-Cas based methods have the potential to improve the efficiency of eDNA metagenomic sequencing of low abundant target organisms and simplify data analysis by enrichment of target species or non-target DNA depletion prior to sequencing. Implementation of CRISPR-Cas in eDNA has been limited due to a lack of interest and support in the past. This perspective synthesizes current approaches of CRISPR-Cas to study underrepresented taxa and advocate for further application and optimization of depletion and enrichment methods of eDNA using CRISPR-Cas, holding promise for eDNA biomonitoring.

Introduction

Assessing the health of the vast marine and terrestrial biomes poses a significant challenge. Currently, most biomonitoring techniques rely on visual identification of species or measurements of physico-chemical quality attributes as a proxy for ecosystem health. While visual methods provide valuable estimates of population health and size, they often miss or underestimate cryptic taxa, juveniles, “damaged” specimens, or camouflaged animals, rely on specialised equipment (SCUBA, video, or camera traps) and require taxonomic expertise [1-4]. Likewise, physico-chemical properties offer valuable real-time insights into ecosystem health but are not capable of measuring organism presence or abundance [5,6]. With the ever pressing need for faster, more comprehensive, and consistent monitoring of marine environments driven by challenges such as habitat loss and degradation; and unsustainable anthropogenic activities such as overfishing and pollution [7-10], advancements in molecular-based monitoring techniques are needed to address these challenges more effectively and monitor environments across the blue-green interface.

Molecular-based monitoring techniques using environmental DNA (eDNA) from water [11-13], sediment [14], and air [15] for biodiversity detection have grown rapidly over the past decade offering increased reliability, accuracy, and species interaction detection [16-18]. Environmental DNA biomonitoring can surpass traditional methods in efficiency and species detection [19-22], with health indices developed for routine monitoring of bacterial assemblages from various sources [23] and freshwater health [24] and best practise guidelines applied for consistent eDNA sampling and analysis from water and sediment samples [14,25-27].

Metabarcoding is a widely adopted technique that uses taxonomically broad eDNA assays to target specific groups, such as arthropods [28], fish and elasmobranchs[29], or corals [30], or broader ‘universal’ targets such as eukaryotes [31,32] or vertebrates [33]. However, metabarcoding relies on PCR amplification of barcoding gene regions for species detection, thereby introducing PCR amplification bias through variable thermodynamic binding affinities of primers for different taxonomic groups, leading to incomplete or unrepresentative results, at worst, causing false-negative species detections, with no correlation to species relative abundance [19,21]. Primer set and bioinformatic pipeline choice can further influence the accuracy of metabarcoding results, causing variable results from the same data based on the complexity of an eDNA sample and the different criteria the data is tested against [12,20,21,25,34,35]. While metabarcoding holds promise for rapid ecosystem monitoring, reliance on PCR amplification presents challenges for widespread adoption as a monitoring method for management and the knowledge-transfer to end-users [19,36].

Metagenomics, or shotgun sequencing of native eDNA, sequences the genomic DNA of all species in a sample, which avoids the limitations of PCR amplification. It enables monitoring of species at the individual level through population genetics and Single Nucleotide Polymorphism (SNP) analysis [37-41]. However, incomplete reference databases for eukaryote genomes [42] and the 4.5-fold higher cost to run the assay compared to metabarcoding [43] limits the utility and scalability of shotgun sequencing. Efforts to expand reference databases [44] and decrease sequencing costs [45] will reduce these two limitations. Nevertheless, data dominated by uninformative repeats and highly abundant non-target species DNA is the most significant obstacle to shotgun sequencing in routine biomonitoring [42,46,47]. For example, [46] found that in marine eDNA samples, most reads were of bacterial origin (94.5%), followed by viruses (3.0%), with only 2.4% of reads originating from eukaryotes, of which only 0.00004% of reads were fish (class Actinopterygii, Chondrichthyes, and Cyclostomata). Hence, depletion of over-abundant organisms and sequences must be carried out. However, current methods of removing unwanted DNA before sequencing rely on the physical differences in the DNA (e.g. mitochondria and chloroplast organelles [48] or RNA sequence types [49]) which restricts the amount of data that can be removed.

Here, we explore two options for Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated proteins (Cas) technologies to circumvent current drawbacks of metagenomic sequencing: (1) the removal of non-target sequences to limit DNA exploration to target species, and (2) selectively enriching for taxa of interest in a precise and sequence-specific method.

The promise of CRISPR-Cas deployment in environmental DNA studies

CRISPR-Cas was discovered as part of an adaptive immune system in bacteria and archaea [50]. Due to its ability to precisely target DNA, CRISPR-Cas has since been adapted to selectively target both nucleic and non-nucleic acids for various applications such as cancer treatment [51], agriculture [52], or controlling pest populations [53]. The system uses a short guide RNA (sgRNA) probe in complex with a Cas protein to precisely cleave the complementary DNA to the sgRNA (~20 bp long) downstream of a protospacer-adjacent motif (PAM) site. Despite the early stage the field is in, the expanding repertoire of commercial Cas enzymes (e.g., dCas9, mutated Cas9 nickases, and recombinant Cas12a [IDT Technologies]), the discovery of new enzymes [54-56], and the significant private investment (i.e., JumpCode genomics, Mammoth biosciences, Sherlock biosciences) has resulted in diverse CRISPR-Cas based studies encouraging their use in eDNA.

Depleting over-abundant DNA

One of the first approaches to deplete non-target sequences using CRISPR-Cas is the Depletion of Abundant Sequences by Hybridization (DASH, Figure 1a) method developed by [57]. DASH works by cleaving target DNA in a sequencing library so that only non-targeted sequences with adapters at both ends remain to be sequenced. [58] demonstrated the effectiveness of using the DASH method by using 566,766 sgRNAs to deplete repetitive elements in a lentil genome to improve genotyping methods. DASH-depletion resulted in a 37.7%–41.2% reduction of repetitive DNA sequences, with an increase of up to 160% in target DNA reads sequenced. This led to the identification of ~4.5–to ~18-fold more variants in the DASH-depleted samples when compared to non-depleted samples. Other CRISPR-Cas based depletion methods have been used in microbiome analyses [59,60], single cell transcriptomics [61], immunotherapy for cancer treatment by down-regulating amino acid uptake by tumor cells [62], used as an antimicrobial agent by selectively depleting antibiotic resistant strains of bacteria [63], and recently in host depletion for metabarcoding analysis in faeces and blood[64]. The diverse range of demonstrated applications of DASH and other CRISPR-Cas technologies provide an encouraging foundation for its potential use in eDNA monitoring of diverse terrestrial and aquatic ecosystems.

For the use of DASH and other CRISPR-Cas depletion technologies to be useful, eDNA samples must first be shotgun sequenced to find the most abundant sequences to be targeted. Once this is done however, eDNA samples from marine environments are similar, comprising common bacteria, viruses and repeats with predictable patterns of most abundant species [65,66]. This means the same set of guides can be used in multiple areas reducing most of the cost to develop these depletion assays. Terrestrial environments are less well studied using eDNA [67] and are much more heterogenous than marine ecosystems. However, depletion of common contamination sources that often complicate air eDNA analysis can also improve and optimise terrestrial eDNA studies. CRISPR-Cas depletion can fill a gap in sequence specific depletion methods that have not been possible yet without taking advantage of structural differences in unwanted DNA (i.e., rRNA) or removing organelles to remove their unwanted DNA.

Enrichment of low-abundant DNA

Conversely to CRISPR depletion, CRISPR enrichment techniques offer a promising means of selectively targeting and enriching DNA sequences in an eDNA sample. By using Cas enzymes to treat the DNA and isolate it in various ways, as extensively detailed by [68], CRISPR-Cas-based enrichment becomes a powerful and highly adaptable tool to enrich almost any sequence of target taxa before sequencing, thereby enabling enhanced species and individual identification with ultra-conserved

elements [69] or SNP analysis [37-39]. Quan et al. (2019) [70] demonstrated the use of CRISPR-Cas enrichment through a method called "Finding Low Abundance Sequences by Hybridisation" (FLASH, Figure 1b), a simple way of targeting specific DNA sequences for enrichment. FLASH first dephosphorylates the ends in DNA libraries, preventing adapters from ligating onto these ends, and then uses CRISPR-Cas9 to cut target DNA to allow sequencing adapters to be ligated onto the cut ends. FLASH treatment of simulated clinical dried blood spot samples targeting drug resistant malaria sequences produced far higher on-target sequencing efficiency with 85.6% on-target sequence reads, compared to <0.02% on-target reads without FLASH enrichment [70]. Moreover, with costs of <$1 US per library, FLASH also provides a cost-effective option for detection of rare sequences [70]. Targeted single species enrichment using other CRISPR-Cas technologies has been demonstrated in several studies, such as in detecting SARS-CoV-2 [71], endangered delta smelt [72], harmful algal blooms [73], Atlantic salmon [74,75], and invasive insects [76,77]. Although multiplex species enrichment and detection with FLASH is possible and comparable to metabarcoding studies in freshwater bulk eDNA samples [78], and despite FLASH's capability to detect very rare and low abundance species, its application beyond human disease studies remains largely unexplored.

CRISPR-Cas enrichment offers distinct advantages compared to other enrichment techniques allowing for a high degree of multiplexing. For example [70] used 5,513 sgRNAs to target 127 genes, and [79] used more than 4,500 sgRNAs in one assay to detect 169 different species with no detectable reduction in reaction efficiency. Additionally, CRISPR-Cas enrichment and depletion offer solutions to several challenges encountered in current eDNA monitoring methods, including the potential for quantitative assessment by avoiding PCR limitations, streamlining lengthy laboratory stages, and by increasing specificity/accuracy, allowing for portable and isothermal assessment [80-82].

However, before CRISPR-Cas deployment in environmental DNA studies can begin, factors affecting interaction efficiency between the Cas enzyme, sgRNA, and target DNA must be addressed. Consequently, ongoing efforts include the development of deep learning models to account for these factors systematically [83-85]. In conjunction with more accurate sgRNA design capabilities, research into the generation of RNA guides is underway [86], allowing for assays that can be customised in a matter of hours [85] without compromising the outcome quality of the desired assay.

eDNA limitations and CRISPR opportunities

CRISPR-Cas based methods have shown promise in eDNA studies [72,74,75,78,87]. So far, we have explored the possible improvements that can be made to eDNA monitoring through selective depletion of highly abundant DNA using DASH and selective enrichment of low-abundant DNA using FLASH.

Besides FLASH/DASH, we explore three more promising avenues of CRISPR-based biomonitoring approaches.

Detecting eDNA interactions and dynamics

The accuracy of eDNA studies is limited by our knowledge of factors including eDNA decay [88], exogenous DNA interactions (e.g., biofilms from bacteria [89]), and endogenous DNA interactions (e.g., nucleosomes inhibiting protein binding and cleavage [90]). Additionally, the vast diversity in organism types in an eDNA sample (e.g., Bacteria, Archaea, Eukarya domains) means that DNA condition or DNA interactions within the eDNA collected can be hard to predict [89], which may impact the ability to isolate the DNA of interest. By using CRISPR-Cas-based techniques to sequence specific species to a higher read depth, we can shed light on these questions behind eDNA interactions.

CRISPR-based screens of extracellular DNA can shed light on the mechanisms of DNA release and decay. Using CRISPR screens similar to those used on cell free DNA in human studies [91] we can detect changes in expression levels of target DNA, which would not normally be seen in diverse eDNA samples, and as a result, inform targets for eDNA based monitoring if certain DNA fragments are more readily released than others, for example, to form biofilms.

The benefits of identifying and quantifying all organisms and their interactions in an environmental sample are becoming more apparent [8,92] and CRISPR-Cas methods can assist this process. Specifically, CRISPR-Cas-based depletion of uninformative sequences from non-target taxa can increase the depth of information gathered on low-abundant organisms. These rare or understudied organisms can shed further light onto inter-species interactions, for example, polymicrobial interactions creating biofilms are common in disease [93], they may also be common in harmful algal bloom biofilms. Targeting a single species will miss these exogenous DNA interactions. In this scenario, once interacting species are identified, enrichment of the specific species can then further show what endogenous DNA interactions are occurring by avoiding PCR (which removes epigenetic marks on the DNA) and, in turn, let us learn more about the abiotic/biotic factors influencing persistence, and toxin production in harmful algal blooms. Though other CRISPR-based enrichment methods, such as CAPTURE [94] must be used instead of FLASH, as FLASH does have a short PCR amplification step to attach barcode primers. Additionally, CRISPR-Cas-based biomonitoring can potentially be applied for accurate and sensitive early detection of blooms by sampling eDNA instead of relying on spectrophotometry methods [95].

eRNA-based biodiversity mapping

In addition to eDNA, environmental RNA (eRNA) is a growing field in environmental biomonitoring, offering distinct advantages, but also some disadvantages, over eDNA due to its faster degradation rate and the need for a cDNA conversion step for most sequencing technologies [96]. Its rapid degradation means that eRNA can signal the presence of live organisms at the time of sampling (i.e., if it is found present in a sample then it can be assumed that the organism was still alive at the time of sample collection). Comparatively, eDNA persists longer in the environment with eRNA potentially having 4-5 times faster half-life than eDNA depending on many environmental factors [96], potentially leading to false positives if an organism has left the area or organism DNA has been carried into the area by predators.

CRISPR-Cas methods such as DASH and FLASH can address the limitations of RNA analyses. While RNA depletion kits exist to optimise RNA analysis [49], these kits lack the sequence-specific targeting capability of CRISPR-Cas technologies, limiting the efficiency of RNA analysis. This presents an opportunity for DASH, which has already been successfully adapted to RNA sequencing by [97] and in MAD-DASH by [98], to remove adapter dimer and abundant miRNAs, respectively. In addition, FLASH represents a promising alternative for RNA enrichment, underscoring the potential for CRISPR-Cas to contribute to the advancement of eRNA techniques.

In-field detection challenges and solutions

For eDNA detection and monitoring methods to be most effective, there is a growing demand for in-field detection capabilities in all environments regardless of the resources available. Advancements such as SHERLOCK – Specific High Sensitivity Enzymatic Reporter UnLOCKing [99] or DETECTR – DNA Endonuclease-Targeted CRISPR Trans Reporter [87] which use Cas13a and Cas12a, respectively, for florescence readings, have paved the way for such developments. Many CRISPR technologies have been harnessed for targeted single species detection in a portable and compact way (e.g., SARS-CoV-2, Dengue, Zika), exemplified by lateral flow assays [100], fluorescence-based detection [79,87,99], and automated systems using robots for sampling and detection of COVID-19 variants in the environment [101]. These developments in the medical field for biosensing that are even moving towards wearable devices [102] can be applied to eDNA monitoring fairly seamlessly due to the programmability of sgRNAs.

For using depletion and enrichment in the field, both DASH and FLASH methods can already be used at sea if molecular laboratories are available on board. These methods both have relatively short lab protocols as the most time-consuming step is a two-hour incubation. In more remote field testing

(e.g., remote locations for biosecurity monitoring), the requirement of a thermocycler and sequencing machine, pose logistical challenges. Despite these challenges, ongoing advancements in in-field DNA extraction methods (e.g., PDQeX [103], ExCad [104,105], and HUDSON [106]), hand-held lab equipment such as PCR machines [107], and portable sequencers [108] offer potential solutions for more accessible in-field monitoring.

Environmental DNA promises to be an effective method acquiring comprehensive ecosystem information [11,16,17,25,109]. CRISPR-based approaches, such as DASH and FLASH combined with shotgun sequencing, can significantly improve the efficiency of eDNA data analysis by removing non-target DNA and streamlining enrichment, thereby enhancing the overall accuracy and quality of results and reduce computational burden. Integration of CRISPR-Cas technologies offers avenues for refining monitoring approaches, overcoming PCR bias, and enabling efficient high-throughput applications. Moreover, the adaptability and scalability of CRISPR-Cas systems provide a customizable toolset to meet diverse research needs and study limitations. Broader interest and support for more accurate monitoring methods may lead to wider adoption of CRISPR-Cas techniques, revolutionizing our capacity to monitor natural ecosystems on a global scale.

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