

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

3 **Running Title:** Biomonitoring ecosystems using CRISPR-Cas

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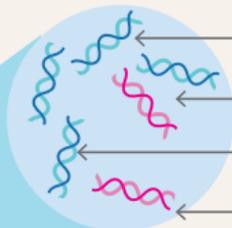
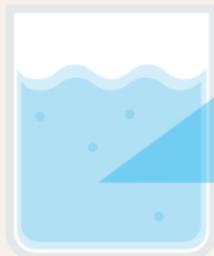
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46 Abstract

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

Marine water sample



Bacteria



Fish



Virus



Dolphin

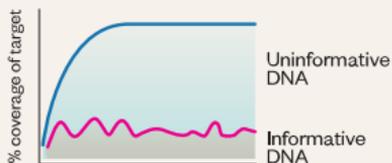
DNA



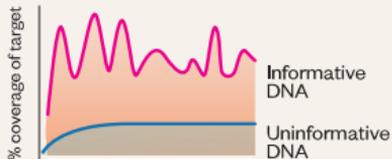
Cas

Depletion

Before Cas treatment

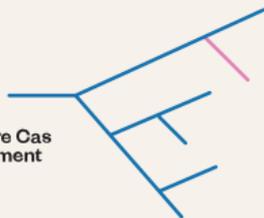


After Cas treatment



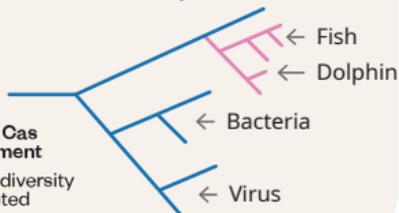
Enrichment

Before Cas treatment



After Cas treatment

More diversity detected



59 Introduction

60 Assessing the health of the vast marine and terrestrial biomes poses a significant challenge.
61 Currently, most biomonitoring techniques rely on visual identification of species or measurements of
62 physico-chemical quality attributes as a proxy for ecosystem health. While visual methods provide
63 valuable estimates of population health and size, they often miss or underestimate cryptic taxa,
64 juveniles, “damaged” specimens, or camouflaged animals, rely on specialised equipment (SCUBA,
65 video, or camera traps) and require taxonomic expertise¹⁻⁴. Likewise, physico-chemical properties
66 offer valuable real-time insights into ecosystem health but are not capable of measuring organism
67 presence or abundance^{5,6}. With the ever pressing need for faster, more comprehensive, and
68 consistent monitoring of marine environments driven by challenges such as habitat loss and
69 degradation; and unsustainable anthropogenic activities such as overfishing and pollution⁷⁻¹⁰,
70 advancements in molecular-based monitoring techniques are needed to address these challenges
71 more effectively and monitor environments across the blue-green interface.

72 Molecular-based monitoring techniques using environmental DNA (eDNA) from water¹¹⁻¹³, sediment
73¹⁴, and air¹⁵ for biodiversity detection have grown rapidly over the past decade offering increased
74 reliability, accuracy, and species interaction detection¹⁶⁻¹⁸. Environmental DNA biomonitoring can
75 surpass traditional methods in efficiency and species detection¹⁹⁻²², with health indices developed
76 for routine monitoring of bacterial assemblages from various sources²³ and freshwater health²⁴ and
77 best practise guidelines applied for consistent eDNA sampling and analysis from water and sediment
78 samples^{14,25-27}.

79 Metabarcoding is a widely adopted technique that uses taxonomically broad eDNA assays to target
80 specific groups, such as arthropods²⁸, fish and elasmobranchs²⁹, or corals³⁰, or broader ‘universal’
81 targets such as eukaryotes^{31,32} or vertebrates³³. However, metabarcoding relies on PCR amplification
82 of barcoding gene regions for species detection, thereby introducing PCR amplification bias through
83 variable thermodynamic binding affinities of primers for different taxonomic groups, leading to
84 incomplete or unrepresentative results, at worst, causing false-negative species detections, with no
85 correlation to species relative abundance^{19,21}. Primer set and bioinformatic pipeline choice can
86 further influence the accuracy of metabarcoding results, causing variable results from the same data
87 based on the complexity of an eDNA sample and the different criteria the data is tested against
88^{12,20,21,25,34,35}. While metabarcoding holds promise for rapid ecosystem monitoring, reliance on PCR
89 amplification presents challenges for widespread adoption as a monitoring method for management
90 and the knowledge-transfer to end-users^{19,36}.

91 Metagenomics, or shotgun sequencing of native eDNA, sequences the genomic DNA of all species in
92 a sample, which avoids the limitations of PCR amplification. It enables monitoring of species at the
93 individual level through population genetics and Single Nucleotide Polymorphism (SNP) analysis ³⁷⁻⁴¹.
94 However, incomplete reference databases for eukaryote genomes ⁴² and the 4.5-fold higher cost to
95 run the assay compared to metabarcoding ⁴³ limits the utility and scalability of shotgun sequencing.
96 Efforts to expand reference databases ⁴⁴ and decrease sequencing costs ⁴⁵ will reduce these two
97 limitations. Nevertheless, data dominated by uninformative repeats and highly abundant non-target
98 species DNA is the most significant obstacle to shotgun sequencing in routine biomonitoring ^{42,46,47}.
99 For example, ⁴⁶ found that in marine eDNA samples, most reads were of bacterial origin (94.5%),
100 followed by viruses (3.0%), with only 2.4% of reads originating from eukaryotes, of which only
101 0.00004% of reads were fish (class Actinopterygii, Chondrichthyes, and Cyclostomata). Hence,
102 depletion of over-abundant organisms and sequences must be carried out. However, current
103 methods of removing unwanted DNA before sequencing rely on the physical differences in the DNA
104 (e.g. mitochondria and chloroplast organelles ⁴⁸ or RNA sequence types ⁴⁹) which restricts the
105 amount of data that can be removed.

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

110 The promise of CRISPR-Cas deployment in environmental DNA 111 studies

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

122 Depleting over-abundant DNA

123 One of the first approaches to deplete non-target sequences using CRISPR-Cas is the Depletion of
124 Abundant Sequences by Hybridization (DASH, Figure 1a) method developed by ⁵⁷. DASH works by
125 cleaving target DNA in a sequencing library so that only non-targeted sequences with adapters at
126 both ends remain to be sequenced. ⁵⁸ demonstrated the effectiveness of using the DASH method by
127 using 566,766 sgRNAs to deplete repetitive elements in a lentil genome to improve genotyping
128 methods. DASH-depletion resulted in a 37.7%–41.2% reduction of repetitive DNA sequences, with an
129 increase of up to 160% in target DNA reads sequenced. This led to the identification of ~4.5–to ~18-
130 fold more variants in the DASH-depleted samples when compared to non-depleted samples. Other
131 CRISPR-Cas based depletion methods have been used in microbiome analyses ^{59,60}, single cell
132 transcriptomics ⁶¹, immunotherapy for cancer treatment by down-regulating amino acid uptake by
133 tumor cells ⁶², used as an antimicrobial agent by selectively depleting antibiotic resistant strains of
134 bacteria ⁶³, and recently in host depletion for metabarcoding analysis in faeces and blood⁶⁴. The
135 diverse range of demonstrated applications of DASH and other CRISPR-Cas technologies provide an
136 encouraging foundation for its potential use in eDNA monitoring of diverse terrestrial and aquatic
137 ecosystems.

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

149 Enrichment of low-abundant DNA

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

155 elements⁶⁹ or SNP analysis³⁷⁻³⁹. Quan et al. (2019)⁷⁰ demonstrated the use of CRISPR-Cas
156 enrichment through a method called “Finding Low Abundance Sequences by Hybridisation” (FLASH,
157 Figure 1b), a simple way of targeting specific DNA sequences for enrichment. FLASH first
158 dephosphorylates the ends in DNA libraries, preventing adapters from ligating onto these ends, and
159 then uses CRISPR-Cas9 to cut target DNA to allow sequencing adapters to be ligated onto the cut
160 ends. FLASH treatment of simulated clinical dried blood spot samples targeting drug resistant malaria
161 sequences produced far higher on-target sequencing efficiency with 85.6% on-target sequence
162 reads, compared to <0.02% on-target reads without FLASH enrichment⁷⁰. Moreover, with costs of
163 <\$1 US per library, FLASH also provides a cost-effective option for detection of rare sequences⁷⁰.
164 Targeted single species enrichment using other CRISPR-Cas technologies has been demonstrated in
165 several studies, such as in detecting SARS-CoV-2⁷¹, endangered delta smelt⁷², harmful algal blooms
166⁷³, Atlantic salmon^{74,75}, and invasive insects^{76,77}. Although multiplex species enrichment and
167 detection with FLASH is possible and comparable to metabarcoding studies in freshwater bulk eDNA
168 samples⁷⁸, and despite FLASH’s capability to detect very rare and low abundance species, its
169 application beyond human disease studies remains largely unexplored.

170 CRISPR-Cas enrichment offers distinct advantages compared to other enrichment techniques
171 allowing for a high degree of multiplexing. For example⁷⁰ used 5,513 sgRNAs to target 127 genes,
172 and⁷⁹ used more than 4,500 sgRNAs in one assay to detect 169 different species with no detectable
173 reduction in reaction efficiency. Additionally, CRISPR-Cas enrichment and depletion offer solutions to
174 several challenges encountered in current eDNA monitoring methods, including the potential for
175 quantitative assessment by avoiding PCR limitations, streamlining lengthy laboratory stages, and by
176 increasing specificity/accuracy, allowing for portable and isothermal assessment⁸⁰⁻⁸².

177 However, before CRISPR-Cas deployment in environmental DNA studies can begin, factors affecting
178 interaction efficiency between the Cas enzyme, sgRNA, and target DNA must be addressed.
179 Consequently, ongoing efforts include the development of deep learning models to account for these
180 factors systematically⁸³⁻⁸⁵. In conjunction with more accurate sgRNA design capabilities, research into
181 the generation of RNA guides is underway⁸⁶, allowing for assays that can be customised in a matter
182 of hours⁸⁵ without compromising the outcome quality of the desired assay.

183 eDNA limitations and CRISPR opportunities

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

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

189 Detecting eDNA interactions and dynamics

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

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

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

217 eRNA-based biodiversity mapping

218 In addition to eDNA, environmental RNA (eRNA) is a growing field in environmental biomonitoring,
219 offering distinct advantages, but also some disadvantages, over eDNA due to its faster degradation
220 rate and the need for a cDNA conversion step for most sequencing technologies⁹⁶. Its rapid
221 degradation means that eRNA can signal the presence of live organisms at the time of sampling (i.e.,
222 if it is found present in a sample then it can be assumed that the organism was still alive at the time
223 of sample collection). Comparatively, eDNA persists longer in the environment with eRNA potentially
224 having 4-5 times faster half-life than eDNA depending on many environmental factors⁹⁶, potentially
225 leading to false positives if an organism has left the area or organism DNA has been carried into the
226 area by predators.

227 CRISPR-Cas methods such as DASH and FLASH can address the limitations of RNA analyses. While
228 RNA depletion kits exist to optimise RNA analysis⁴⁹, these kits lack the sequence-specific targeting
229 capability of CRISPR-Cas technologies, limiting the efficiency of RNA analysis. This presents an
230 opportunity for DASH, which has already been successfully adapted to RNA sequencing by⁹⁷ and in
231 MAD-DASH by⁹⁸, to remove adapter dimer and abundant miRNAs, respectively. In addition, FLASH
232 represents a promising alternative for RNA enrichment, underscoring the potential for CRISPR-Cas to
233 contribute to the advancement of eRNA techniques.

234 In-field detection challenges and solutions

235 For eDNA detection and monitoring methods to be most effective, there is a growing demand for in-
236 field detection capabilities in all environments regardless of the resources available. Advancements
237 such as SHERLOCK – Specific High Sensitivity Enzymatic Reporter UnLOCKing⁹⁹ or DETECTR – DNA
238 Endonuclease-Targeted CRISPR Trans Reporter⁸⁷ which use Cas13a and Cas12a, respectively, for
239 fluorescence readings, have paved the way for such developments. Many CRISPR technologies have
240 been harnessed for targeted single species detection in a portable and compact way (e.g., SARS-CoV-
241 2, Dengue, Zika), exemplified by lateral flow assays¹⁰⁰, fluorescence-based detection^{79,87,99}, and
242 automated systems using robots for sampling and detection of COVID-19 variants in the environment
243¹⁰¹. These developments in the medical field for biosensing that are even moving towards wearable
244 devices¹⁰² can be applied to eDNA monitoring fairly seamlessly due to the programmability of
245 sgRNAs.

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

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

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

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