IWEG

International Workshop on Environmental Genomics

STANDARDIZATION AND EVALUATION OF EDNA APPROACHES: METHODOLOGICAL, SOCIETAL & LEGAL IMPLICATIONS

JULY 13 - 14, 2022

Introduction

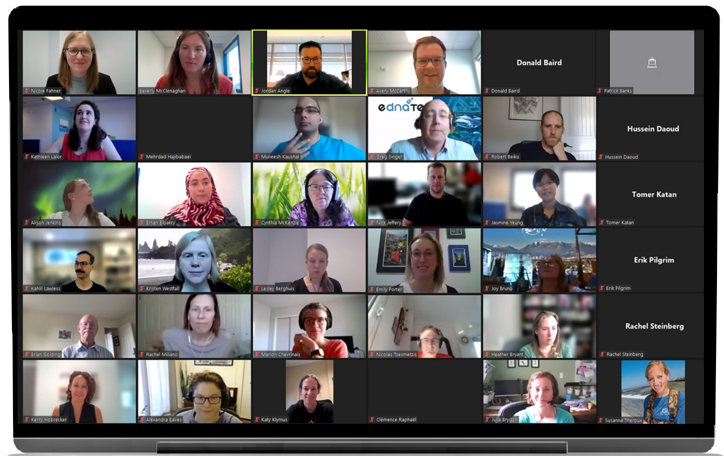
The 7th annual International Workshop on Environmental Genomics (IWEG) was held virtually and hosted in St. John's, Newfoundland and Labrador, Canada from July 13 - 14th, 2022.

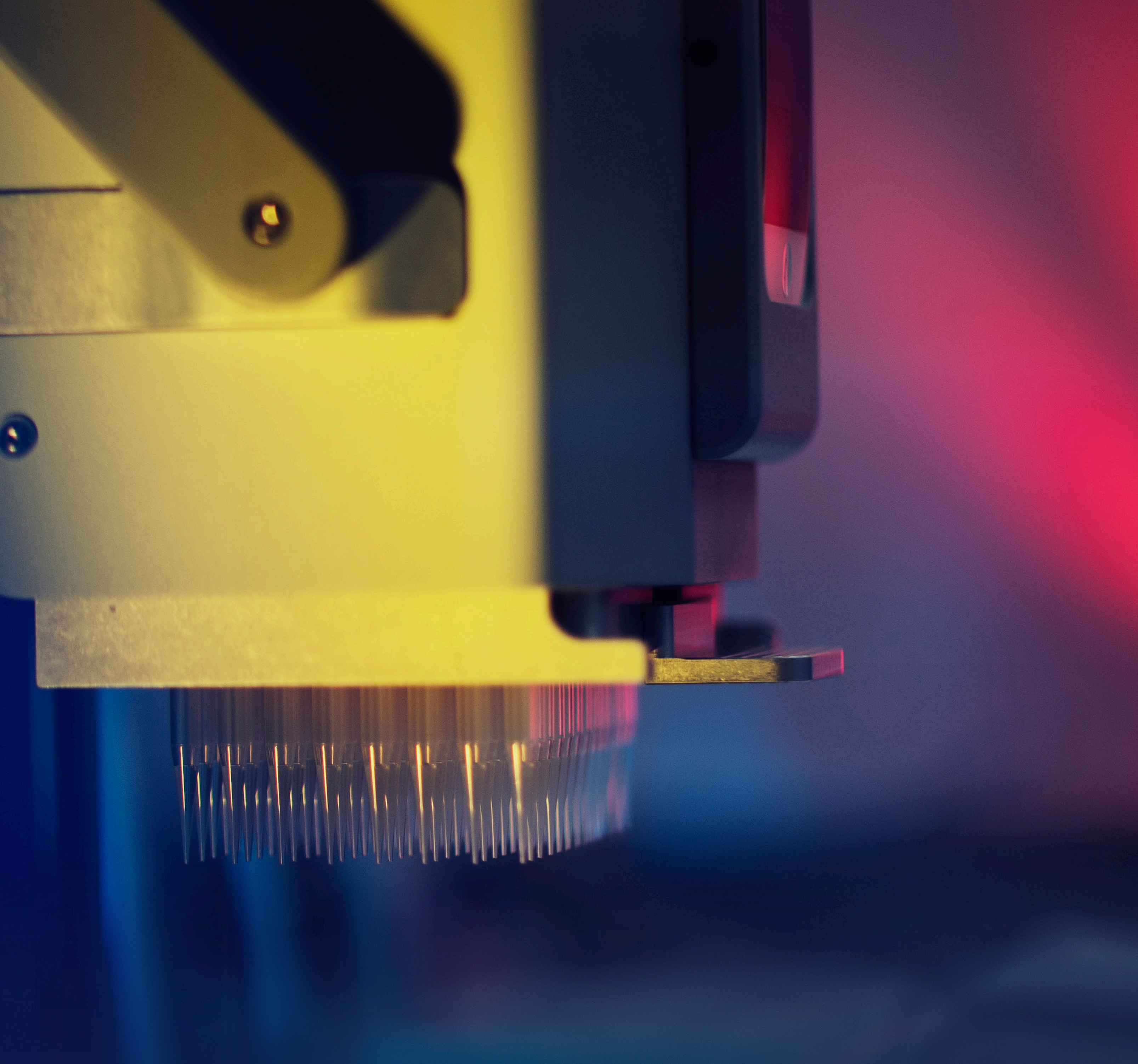
This was the third virtual IWEG due to ongoing travel restrictions and health concerns related to the global COVID-19 pandemic. This year's workshop focused on the theme "Standardization and Evaluation of eDNA Approaches: Methodological, Societal & Legal Implications". The workshop brought together participants from across the globe to discuss how we evaluate and validate environmental genomics methods to move standardization efforts forward and the wide-ranging implications of these efforts. Participants represented stakeholders across many sectors, including the oil and gas industry, environmental consulting, regulatory agencies, and academia.

This year's workshop included a keynote presentation from Dr. Donald Baird (Environment and Climate Change Canada), three presentation sessions with presenters from NORCE, Fisheries and Oceans Canada, Institute of Oceanology, Polish Academy of Sciences, ExxonMobil, Illumina, Genome Atlantic and the Centre for Environmental Genomics Applications. A discussion panel was held on the first day and an interactive session on the second day.

The presentations demonstrated eDNA applications in diverse ecosystems as well as how eDNA has become a critical tool for ecosystem management under rapidly changing environmental conditions. Our keynote demonstrated how eDNA can be used to prioritize areas for management action by detecting adverse change in rivers based on a consistent eDNA signal derived from a greater segment of the local biodiversity than observed using conventional methods. Regulatory agencies have many end-users interested in using eDNA including those involved with species at risk, aquaculture, fisheries management, and Marine Protected Areas (MPAs). Presenters highlighted the opportunity for eDNA metabarcoding as a standardized biomonitoring tool for MPAs, which are challenging to monitor routinely given the limitations on the use of common survey methods like ground-trawls, their large areas, and their dispersed locations. Other presentations focused on optimizing eDNA workflows for various environments, including evaluating filter types, comparing extraction methods and detection chemistries, and developing metabarcoding-based methods that could replace the use of macroinvertebrates as bioindicators for benthic monitoring.

In addition to advances in eDNA research and applications, several presentations focused on the challenges and opportunities for standardization in the environmental genomics field. The complexity in validating eDNA methodologies for standards development was a common theme. Lab and field-based experiments with controlled parameters are not analogous to real world environmental conditions and comparing eDNA to morphology, remote sensing, visual methods, and acoustics all have serious limitations related to sensitivity, specificity, and cost. Presenters discussed the importance of considering the drivers for standardization and why they matter, how other sectors achieve standardization, and how applying technology readiness evaluations to eDNA methods may facilitate the transition towards regulatory compliance. These ideas were further developed through the discussion panel and the interactive session.





Discussion Panel

The discussion panel brought together four experts from industry and regulatory agencies on a panel to lead the conversation and included input from all IWEG participants on a series of topics related to the theme of standardization.

The results of this discussion session are summarized below under three themes:

1. The need for standards in environmental genomics
2. Standardization and regulatory approval of eDNA for biomonitoring
3. Drawing on previous standardization journeys

The Need for Standards in Environmental Genomics

Environmental genomics is a rapidly advancing field that includes a broad range of current and potential users and applications. As these tools and techniques are increasingly applied outside of academia, the impact that environmental genomics will have on the broader environmental monitoring field will depend on how standards are established.

Developing standards for the field will build confidence among regulators in environmental genomics data for biomonitoring and help progress regulatory acceptance across industries and applications. Using best practices to ensure environmental genomics data meet quality standards will minimize the need for redundant or complementary experiments that come with added costs to prove the quality of environmental genomics data for each application. Standards facilitate the accreditation of service or diagnostic labs as providers of environmental genomics methods. These labs will be necessary to increase the global capacity to generate reliable, high-quality results and meet the demands of industry and large monitoring programs. Procedural requirements like routine external quality audits would ensure these service providers are adhering to guidelines and maintaining records to satisfy certification standards and support regulatory compliance. As the volume of environmental genomics data generated increases, standards will also be needed for data management practices and to achieve consistency and reliability in data reporting. This, in turn, will build trust between environmental genomics practitioners, decision makers, and end users.

While standards will help progress the field of environmental genomics, they must not act as a barrier to innovation. There is a risk that once standards are embedded within regulatory frameworks, it will be very challenging to update or repeal them because they have become part of legislation . Choosing where and how standards are implemented is critical.

Standardization and Regulatory Approval of eDNA for Biomonitoring

Standardization and regulatory approval go hand-in-hand: we need standardization for regulatory approval, and we need regulatory approval to push forward the development of standards. Which should come first and who will set these standards?

In the scientific community, peer review is used to assess the quality of the research before it is published. Similarly, peer review from an interdisciplinary standards committee can bring together experts from various sectors and coordinate efforts around a living standards document An approach like this would create standards that could evolve as eDNA methods get integrated into regulations.

Regulatory approval is a driving force behind the implementation of standardization of monitoring approaches. When biodiversity data from eDNA methodologies are not directly comparable to previous data sets obtained through conventional survey methods, regulators are hesitant to provide these approvals. However, eDNA methods can immediately service applications where other tools are unsuitable (e.g., Marine Protected Areas). Overall, there is a large knowledge gap to fill between science and policy. Scientists specialized in environmental genomics are often not familiar with policy, and likewise, regulators are not familiar with genomics science. This information exchange will be key to progression towards standardization and will be an important feature of any standards committee. While standards are important, they may not be the only approach to gaining regulatory acceptance. Current methods to study and monitor biodiversity are not all standardized, they simply have credibility and validity from use over long periods of time. eDNA methods can be used without standardization across service providers, when employed properly. For example, involving an expert in the field to provide external review of methodologies, similar to the use of expert witnesses in a court of law, provides integrity, which is what standards try to establish at a broader temporal and spatial scale.

Drawing on Previous Standardization Journeys

Developing standards and demonstrating reproducibility and repeatability in a technology that is constantly advancing is not a new concept. We can draw on the experiences from other fields to inform progress towards these goals for environmental genomics.

Some fields we can look towards include those with similar applications, such as non-molecular biomonitoring programs. There are many standardized coastal and stream monitoring programs that exist from local to national levels around the world (e.g., CABIN in Canada, Water Framework Directive Monitoring Program in Europe). These programs do not all follow identical protocols, but they generate similar data types and share similar goals. We can also look to fields that use similar tools and techniques for different applications, such as the medical and forensic fields. While these applications are subject to different regulatory and legal frameworks, they have developed standard methodologies using the same molecular techniques that are used for environmental genomics. While drawing on these fields, we must be aware that the standards for human health diagnostics and forensics are very strict due to a high threshold of accuracy and precision needed for these applications. The thresholds for accuracy and precision in biomonitoring decisions should be considered to avoid setting excessively strict or costly standards that cannot be sustained in this field long term.

Other examples are a little further afield, but they have parallels to the environmental genomics field. For example, electronics and computing technology is a field that has and continues to rapidly evolve. Standards were developed successfully despite the continuous development in the field. Similarly, geographical Information systems for mapping are an example where standards were developed within the industry allowing and enabling continued technological advancement. Multiple geospatial standards have been developed but this hasn't impeded technological progression. Finally, there are relevant fields that are currently undergoing standardization processes that may reveal useful insights, such as the maturing carbon offset market and emerging biodiversity credit market.

Interactive Session:
Current Status and Opportunities for Standardization

We conducted an interactive session with the goals of:

1. **Documenting consensus and variability between users at key steps in environmental genomics workflows**
2. **Determining why variability exists at certain stages**
3. **Using this knowledge to identify opportunities for implementing standards, benchmarks, or best practices**
4. **Identifying roadblocks to standardization and potential solutions**

In order to achieve the first goal of documenting consensus and variability in environmental genomics workflows, we conducted a survey that included questions about several decision points in eDNA/ metabarcoding workflows that fell within five broad categories: design, field, lab, bioinformatics, and analysis. The questions in this survey did not encapsulate all possible decision points but were chosen to represent the most common decision points encountered in eDNA workflows. During the interactive session, the results of this survey were presented (the full results can be found in Appendix #) and focused discussion groups concentrated on addressing the goals 2-4 within each of the five categories. Participants represented various sectors, used eDNA for a range of applications (e.g., whole community, targeted species, bioindicators) and included users studying all types of organisms from microbes to macrofauna.

The discussion revealed that variability in workflows most often exists because multiple options are available and there is no single, best option either because research is lacking or there is a lack of consensus within the research. Other major contributors to variable workflows are project-specific characteristics, including the application, study environment, logistics, and budget. Furthermore, there is some disagreement between users on whether standards are even needed for certain steps (e.g., should sampling equipment be standardized across different projects?). Identifying these sources of variability highlights some of the barriers that are currently slowing down the development of standards within the environmental genomics field. First, there is a need for flexibility at many stages throughout workflows to accommodate and fit the range of projects that exist, both in terms of biological scope and resources available. Second, more research is needed to fill knowledge gaps and guide users in making informed decisions for their projects. Finally, there is a lack of consensus among stakeholders on where standardization efforts should be focused.

In order to address the first barrier identified above (the need for flexibility), participants in the workshop identified steps that could be taken towards standardization that do not prevent the customization of workflows for specific projects. First, there is a need to standardize the training of personnel and maintenance of lab and field equipment. Standards frameworks, such as ISO, can provide reliable means to collect and maintain essential operational records. Second, reporting standards are an important step in standardization that apply throughout workflows. Transparency is essential for interpreting results, but also in facilitating the further development of standards. Reporting should include information on key decision points in the workflow and capture the variation in workflows between users. Reporting standards do not require users to change their workflows or adopt new methods. Flexibility within workflows will not be impeded by transparently communicating methods and results. The development of reporting standards will require the clarification of terminology to ensure language is being used consistently (see Box 1). Reporting standards will facilitate the presentation, interpretation, and usage of eDNA data for all stakeholders.

Standardizing Terminology – what is a replicate?

Standardization includes establishing precise descriptions and terminology to facilitate communication. Environmental genomics workflows include many levels of replication, both biological and technical, and replication can be added in different ways, even at the same step. Given the many ways replication can be used, this provides good example for where shared terminology will facilitate communication and comparability between projects.

Biological replicates are defined as biologically distinct samples representing an identical time point, location, or treatment. However, following this definition a biological replicate could refer to multiple samples from within a single Niskin bottle, samples from multiple Niskin bottles in the single rosette cast, or samples from separate Niskin bottles on rosette casts at a location. The same logic applies to sediment samples collected from a corer or grab sampler. How do we distinguish true biological replicates and pseudo-replicates?

Technical replication involves performing the same test on the same sample multiple times. This most often occurs at the level of DNA extraction, PCR, or both, leading to replicates of replicates. Replicates may get pooled together at subsequent stages. How can we clearly communicate levels of replication and pooling at various lab processing stages?

Box #1

In addition to the overarching need for reporting standards and records, participants identified several steps that are appropriate for the development of either best practices, decision trees, and/or benchmarks (see Box 2). Best practices can be employed where a lot of flexibility is required, and the decision can be informed by pilot studies or previous research. Best practices would provide guidelines for how to develop and use pilot studies or previous research to inform decisions. Decision trees can be used for decision points where multiple options are required but these are limited and associated with specific scenarios. The decision tree would present options based on project specific information (e.g., study environment or application). Finally, benchmarks act as a default option or minimum standard. Variation from a benchmark would be accepted, but would require justification based on project-specific needs or goals. Below, we've described two steps in the workflow where we identified an opportunity to employ one of these standardization approaches given the knowledge and information currently available. Not surprisingly, there was no step where a single universal standard could be applied (i.e., everyone was already using the same method) and not all steps that were discussed lent themselves well to one of these standardization approaches at this time.

What types of standard requirements can be implemented in environmental genomics workflows?

Universal standard: A single standard that must be applied across projects and applications for reliable results. No deviation from this standard would be acceptable.

Example: All molecular assays must be run with a negative control to detect DNA contamination of reagents.

Benchmark: A default option or minimum standard that can be used across projects for reliable results. Deviation from a benchmark is acceptable provided justification is included.

Example: The minimum recommended sequencing read depth per sample per DNA marker for community biodiversity analysis is 100,000 reads. If a project used a read depth of 50,000 per sample per DNA marker for a community analysis, a justification of this choice would need to be provided (e.g., required assay sensitivity achieved with this sequencing depth in a pilot study).

Decision tree: A limited series of options that are to be applied depending on project specific criteria. A decision tree guides users through these criteria to the option that is appropriate for their purpose.

Example: The recommended environmental material to collect depends on the environment of interest, taxonomic groups of interest, as well as application/ research question. In order to select a sample type, a decision tree would guide a user through questions (e.g., What type of environment are you studying: marine or freshwater or terrestrial? What type of organism(s) are you studying: benthic or pelagic or both? Water.)

Best Practices: System-level guidelines or a standardized process for decision-making, development, and/or parameter selection that draws from previously available information or experimentation (e.g., pilot study). This leaves room for flexibility depending on project-specific variation, while establishing a repeatable process. ISO 9001 quality management systems and good laboratory practices fall under this category.

Example: If a target species of interest is identified and no species-specific primers exist for this species, best practices would provide guidelines for the development and validation of a new primer set. The quality of a new primer set can be trusted if it was developed following established best practices and meets specified quality checks or benchmarks.

Box #2

Two steps were identified as opportunities for implementing benchmarks: the number of biological replicates collected and the denoising step of bioinformatics. At the level of biological replication, survey respondents indicated that they either always use the same standard or that replication is informed by previous research. The most common response was sampling in triplicate. Given a relatively broad consensus on a minimum of three replicates, this could be used as a benchmark for the default minimum number of replicates. If users wanted to deviate from this benchmark, they would need to provide justification. For example, participants raised the scenario of reducing replication to increase spatial coverage within a given project budget. This may allow users to achieve their goals with fewer biological replicates, however replicates can act as quality control flag, so this limitation would need to be addressed if choosing to use reduced replication. For the denoising step in bioinformatics, survey results showed that most participants use one standard workflow across projects for denoising, most frequently to create ASVs, sometimes followed by OTU clustering. However, the same software may not be used to conduct this step (e.g., DADA2 vs vsearch/usearch). Conducting the denoising step to create ASVs could be a benchmark that is conducted by default. Users could then add additional steps, such as OTU clustering, or work directly with ASVs.

The choice of sequencing depth was identified as an opportunity to use a combination of benchmarks and a decision tree. Respondents largely indicated that sequencing depth was optimized within projects or that it varied for non-scientific reasons (primarily budget). A decision tree would distinguish different applications and for each application a minimum threshold or benchmark could be identified. For example, for a metabarcoding survey of eukaryote biodiversity a higher sequencing depth may be recommended compared to a targeted sequencing project. The minimum thresholds would be decided based on empirical data and, if a project needed to use a lower sequencing depth due to budgetary constraints, this justification could be provided.

The DNA marker selection process was identified as an appropriate step for a decision tree. The most frequent survey response was that markers are chosen from a library of standard markers. While users are all pulling from their own custom libraries at the moment, this library could be standardized to include the most commonly used published primer sets. The library could be paired with a decision tree to guide the selection of markers based on target species and project goals. If no markers are available that meet the needs of a project, new markers can be developed.

The development and validation of new primer sets was raised as a step where best practices could be implemented. Requirements for validation could be set and new primer sets could only be added to the standard set once appropriate testing and validation have been conducted. Functionally, this is how the field works at the moment with the academic literature functioning as a library of pre-tested markers and the publication process acting as validation. However, the documentation and validation of primers sets varies between publications. With best practices in place, new, custom primer sets would not necessarily need to be published in the academic literature as long as the validation requirements can be demonstrated.

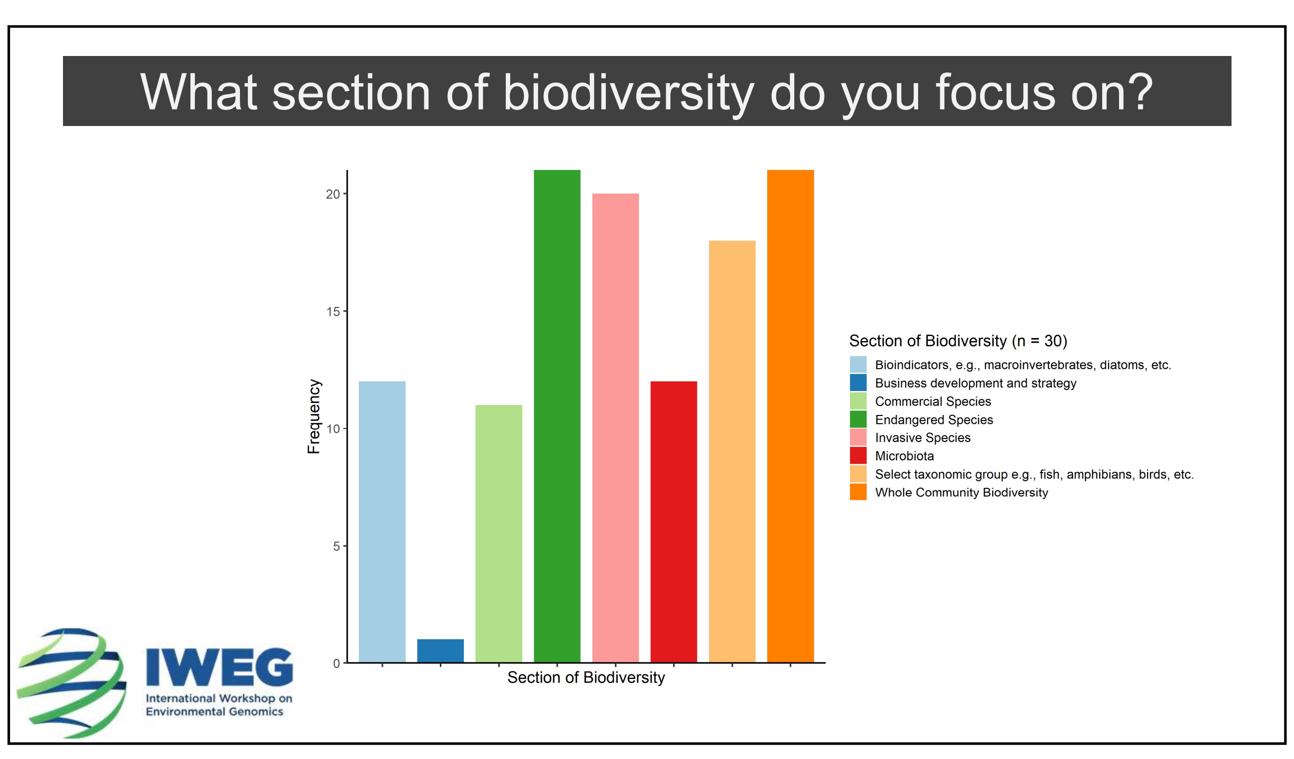
While we were able to identify several opportunities to accommodate flexibility in a standardized workflow, there remains variability and uncertainties in workflows that may present roadblocks to standardization at certain steps. For example, more research is needed to understand the effects of bioinformatics parameterization, even when using the same software. As another example, a round robin approach was recommended to compare filter pore sizes, materials, and preservation methods for different environments and applications. In order to conduct the research needed to fill these knowledge gaps, different research groups need to coordinate efforts and longer-term studies will be required. To achieve this, the environmental genomics community needs to engage in strategic, multi-year planning and secure funding to support this research. Only through coordinated efforts will we be able to address uncertainties remaining in environmental genomics workflows across applications and environments. Increased communication and dialogue among stakeholders within the environmental genomics community would improve consensus on where standardization effort should be focused.

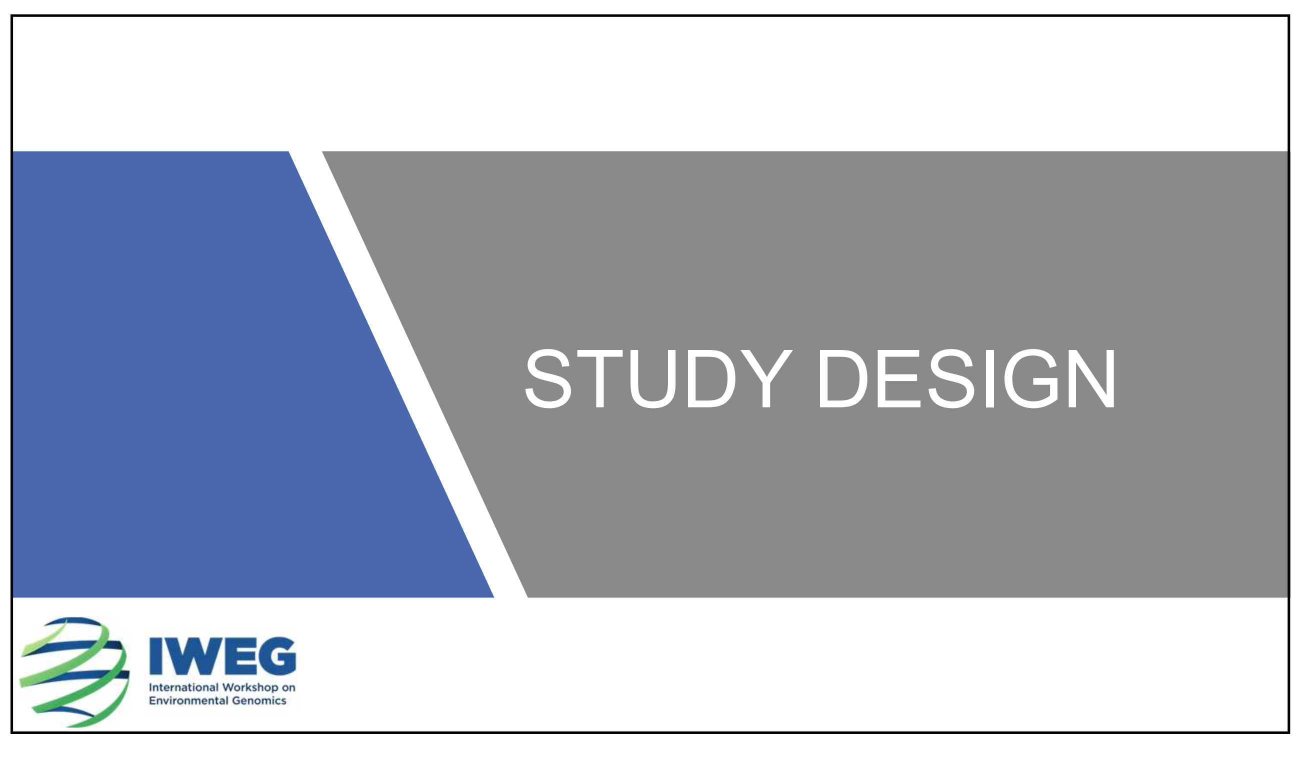
By pursuing the opportunities, tackling the challenges, and filling the knowledge gaps identified here, the environmental genomics community can take important steps towards standardization and make environmental genomics more transparent, reproducible, and reliable, which will further broaden the use and application of this technology.

The complete set of survey questions and responses can be found in the Appendix.

APPENDIX:

Interactive Session Survey Results




STUDY DESIGN
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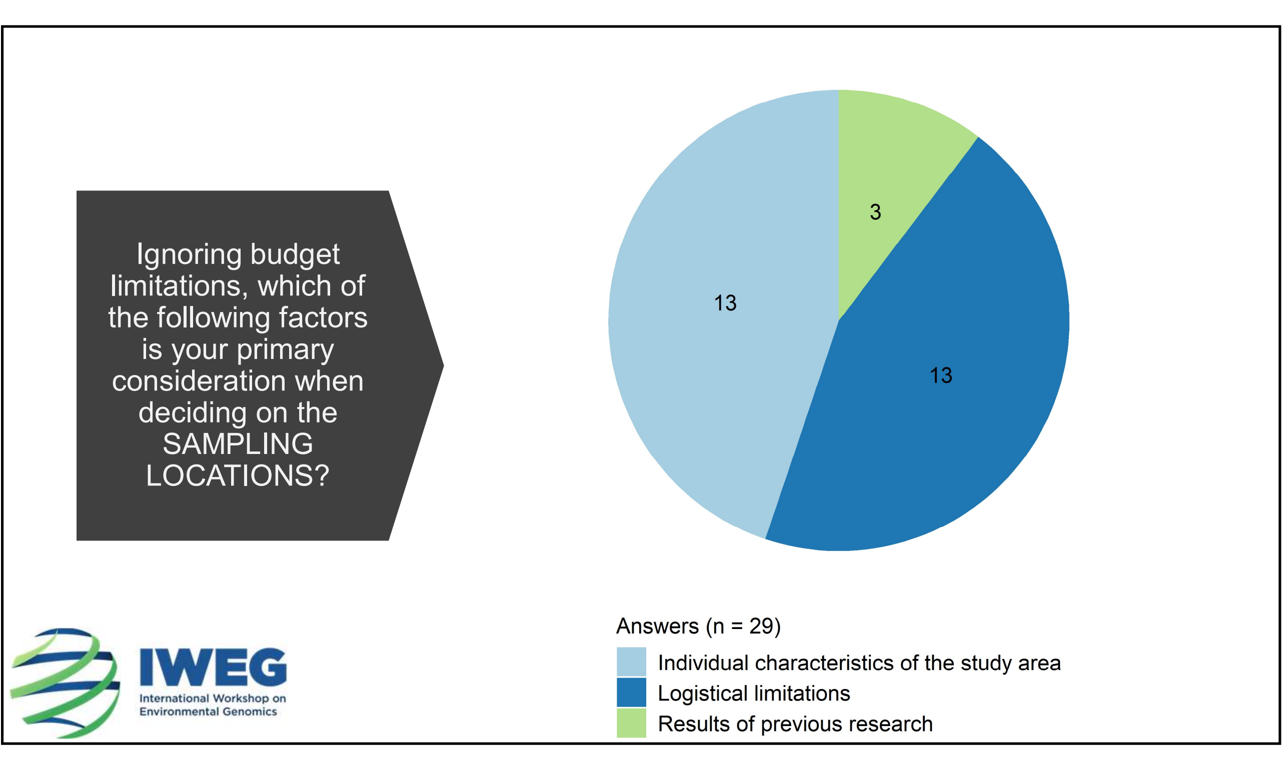
Ignoring budget limitations, which of the following factors is your primary consideration when deciding on the SAMPLING LOCATIONS?
3
13
13
Answers (n = 29)
Individual characteristics of the study area
Logistical limitations
Results of previous research
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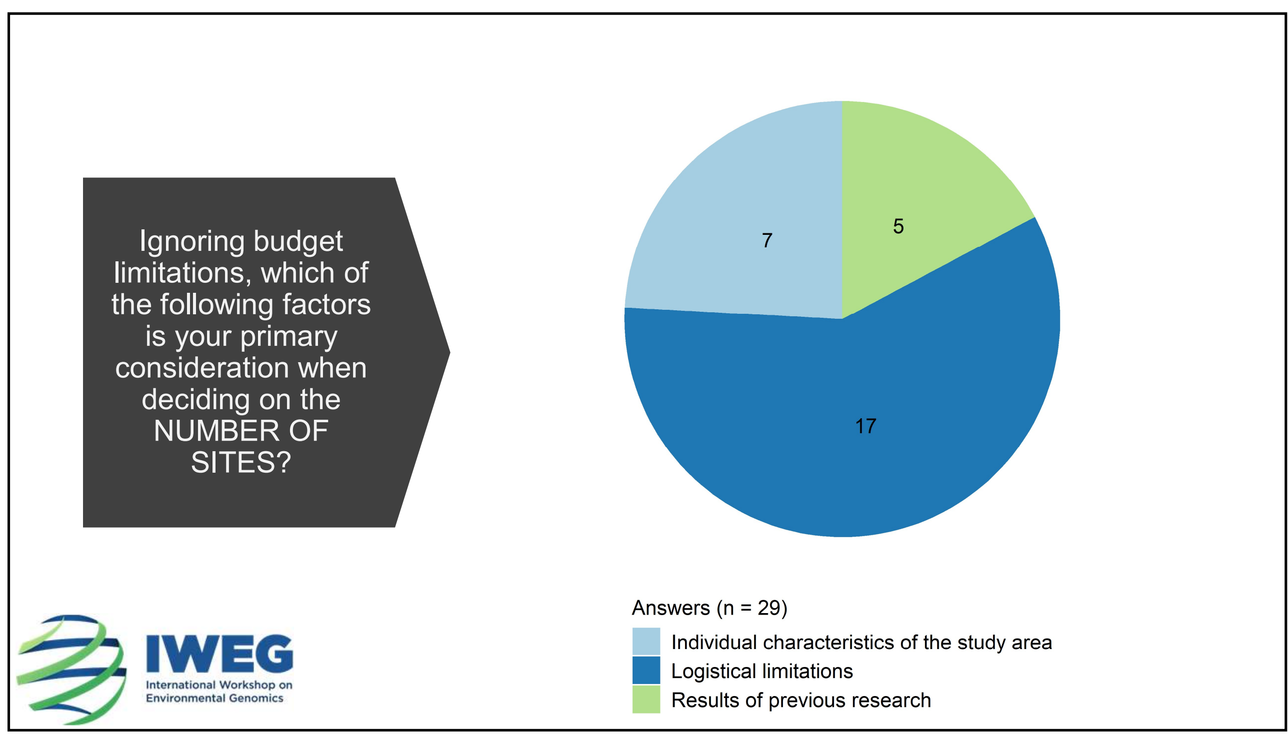
Ignoring budget limitations, which of the following factors is your primary consideration when deciding on the NUMBER OF SITES?
7
5
17
Answers (n = 29)
Individual characteristics of the study area
Logistical limitations
Results of previous research
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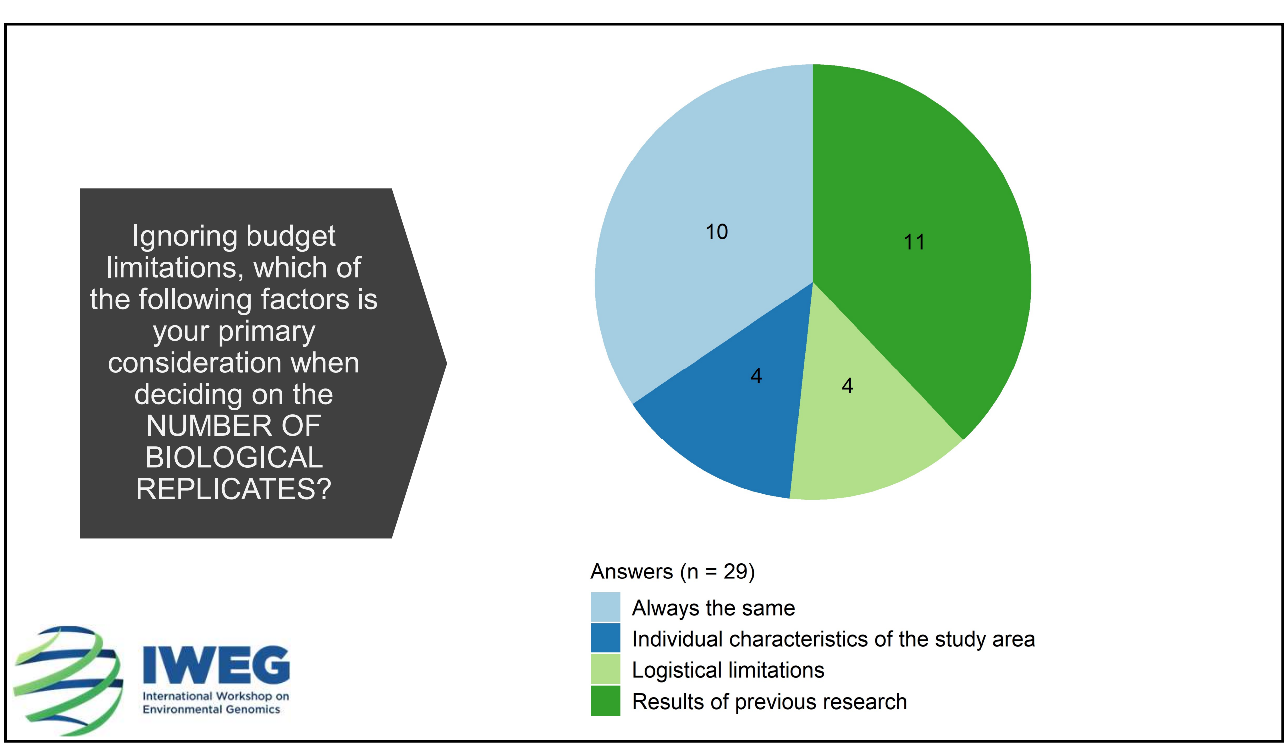
Ignoring budget limitations, which of the following factors is your primary consideration when deciding on the NUMBER OF BIOLOGICAL REPLICATES?
10
11
4
4
Answers (n = 29)
Always the same
Individual characteristics of the study area
Logistical limitations
Results of previous research
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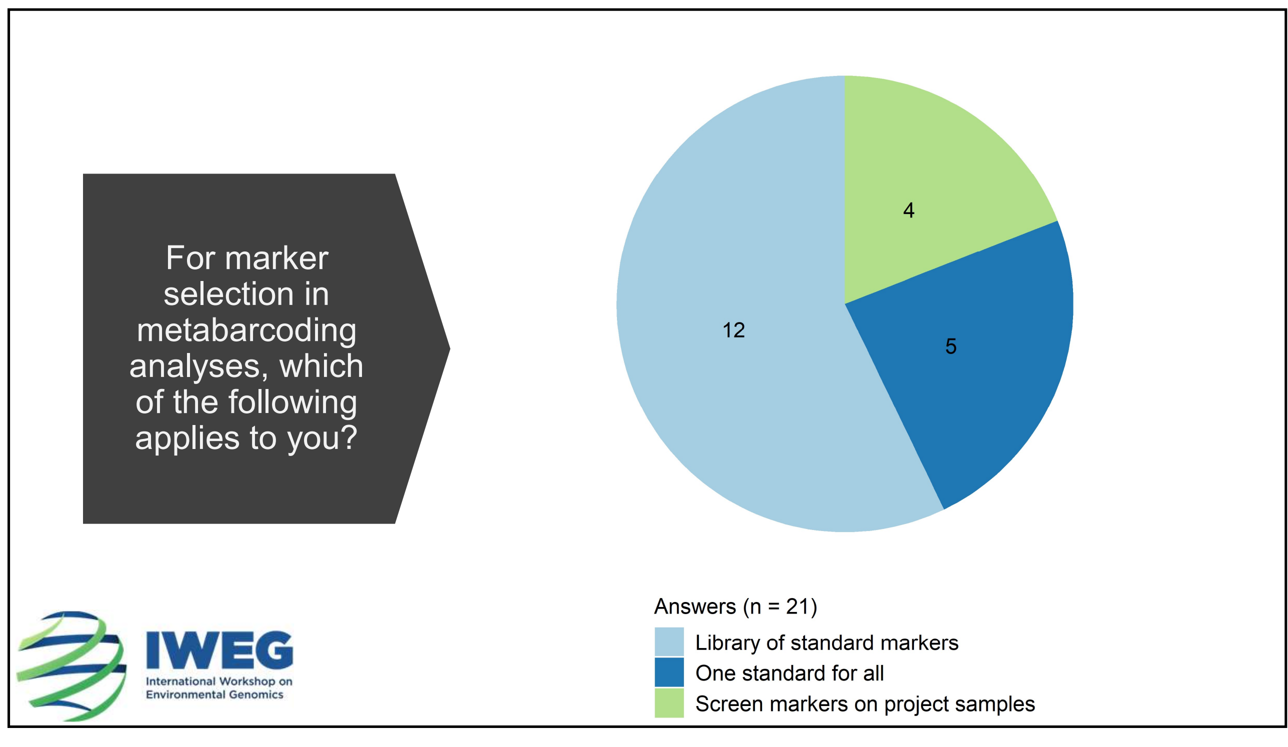
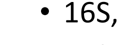


What is the standard marker (or set) that you use across projects/applications?

Fish:

- 16S, 12S



Invertebrates:

- COI

Animals:

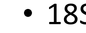
- COI, CytB, 16S

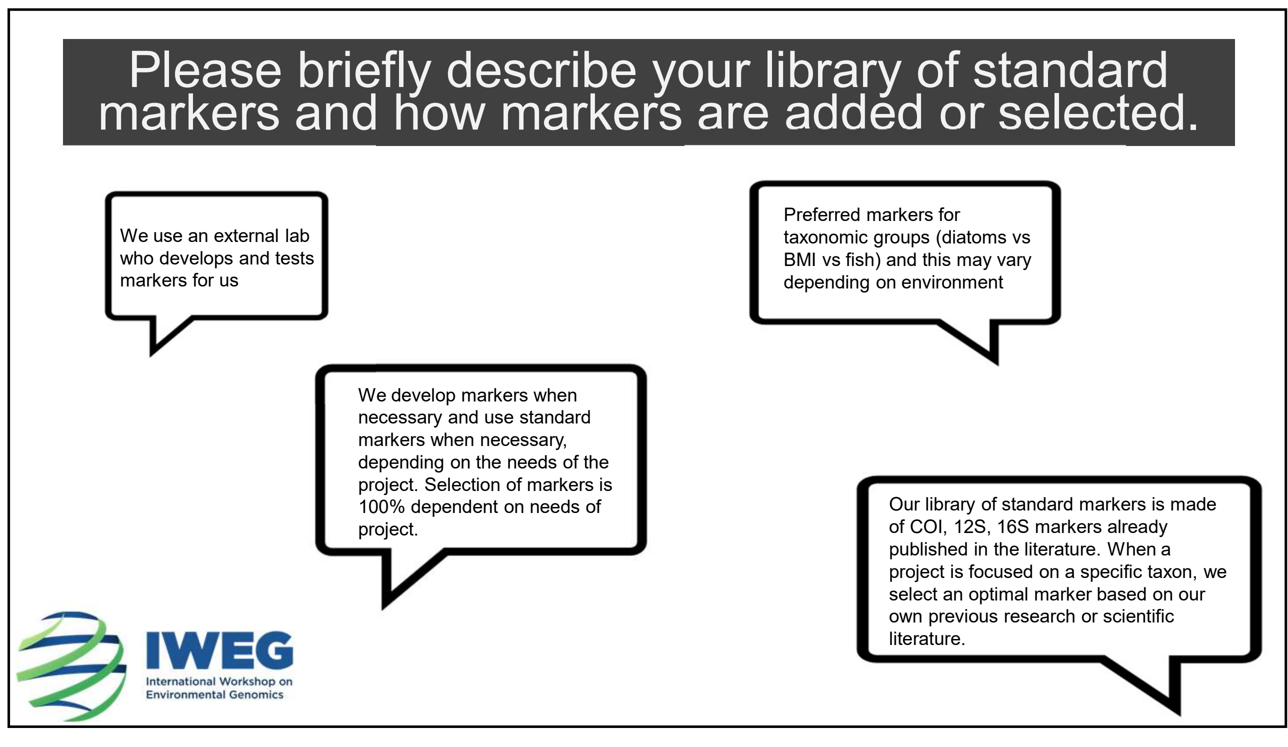
Plants:

- rbcL, trnH, psbD, rpoC1, ITS2

Eukaryotes:

- 18S




Please briefly describe your library of standard markers and how markers are added or selected.
We use an external lab who develops and tests markers for us
Preferred markers for taxonomic groups (diatoms vs BMI vs fish) and this may vary depending on environment
We develop markers when necessary and use standard markers when necessary, depending on the needs of the project. Selection of markers is 100% dependent on needs of project.
Our library of standard markers is made of COI, 12S, 16S markers already published in the literature. When a project is focused on a specific taxon, we select an optimal marker based on our own previous research or scientific literature.
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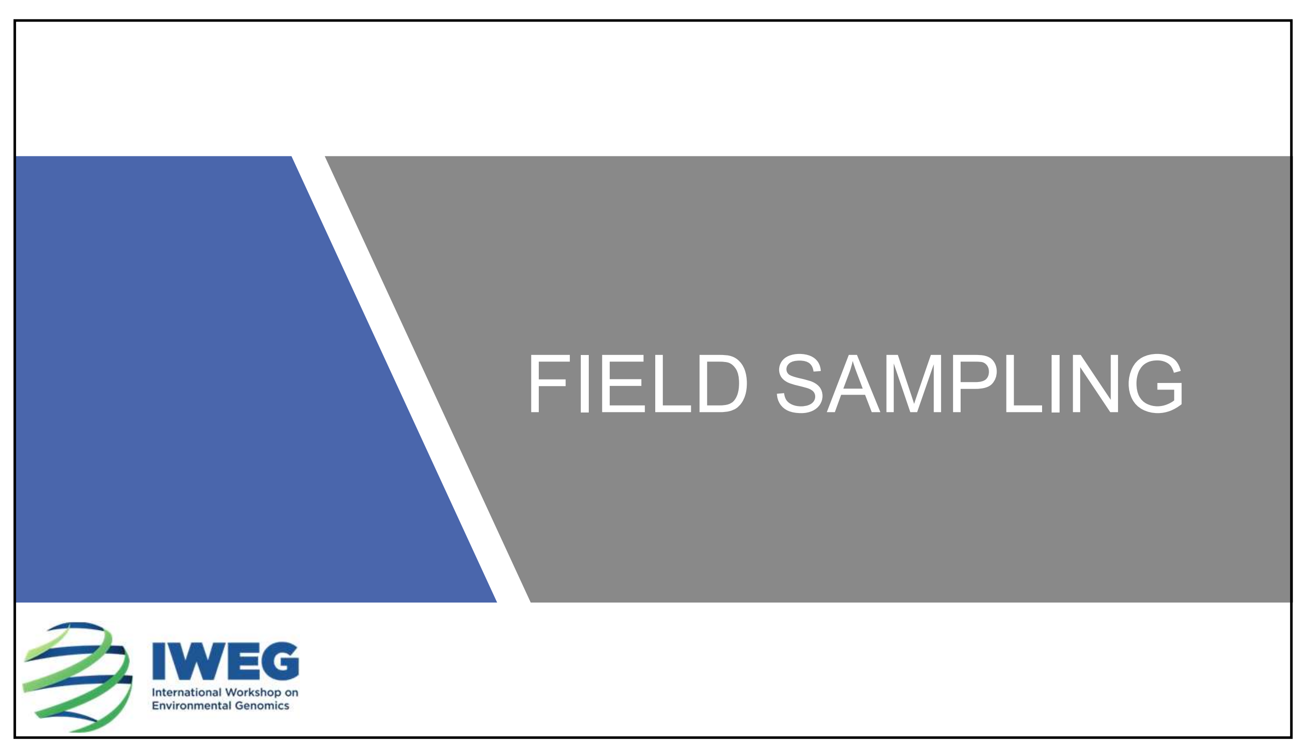
FIELD SAMPLING
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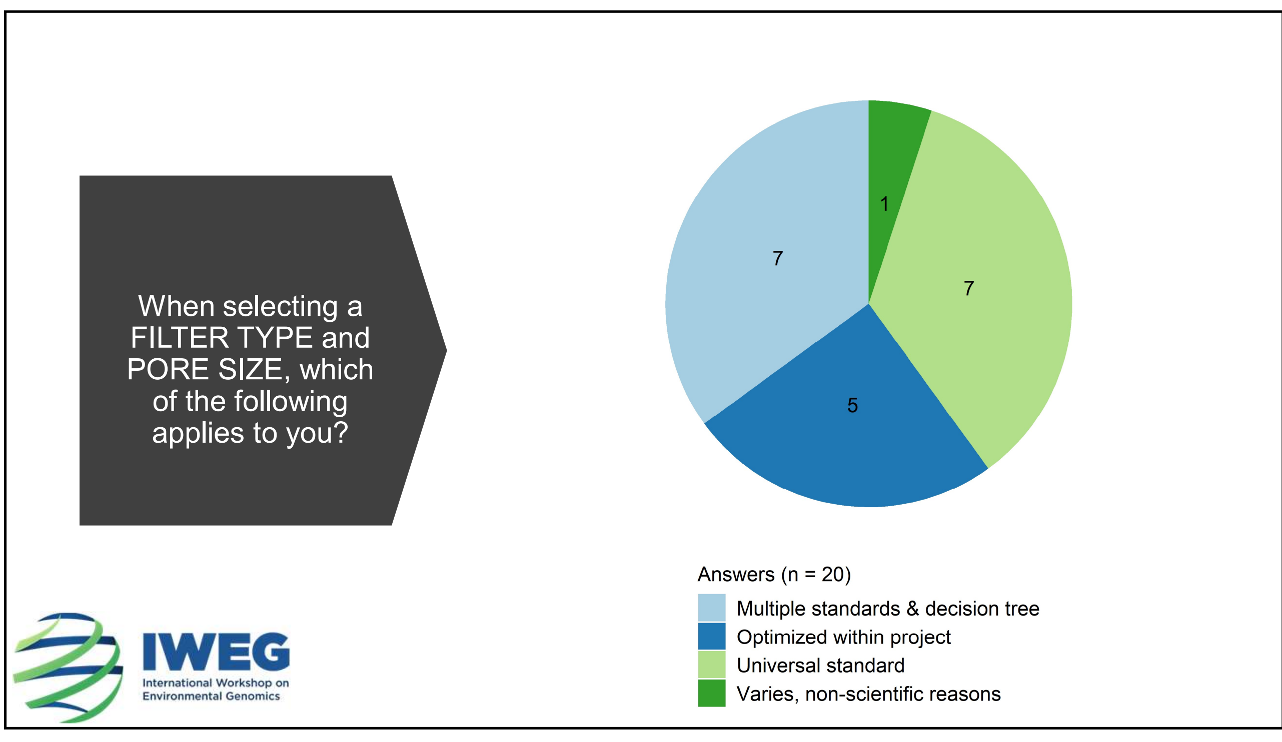


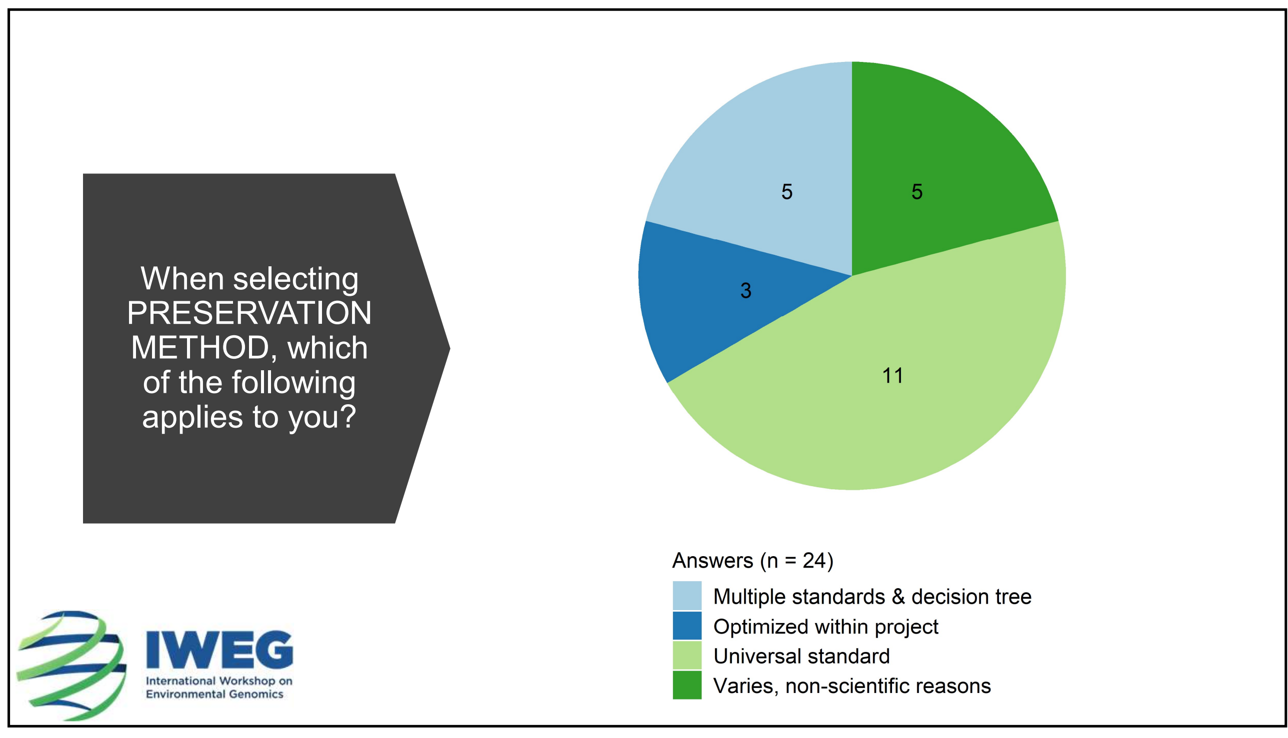
Please describe your standard FILTER TYPE/PORE SIZE.

Pore Sizes:

- 0.22um or 0.45um
- 0.45 to 1.2 um
- 0.7 um
- 1.5 um
- 400 um

Membrane Types:

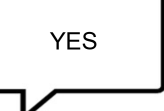
- Sterivex PVDF
- Glass Fiber Filter
- PES
- Cellulose



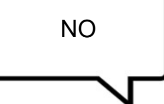
Please describe your standard PRESERVATION METHOD.

- Freezing
- Lab grade ethanol
- Preserved in silica beads
- Longmire buffer
- Isopropanol
- Self desiccating filter cups
- Depends on the type of substrate

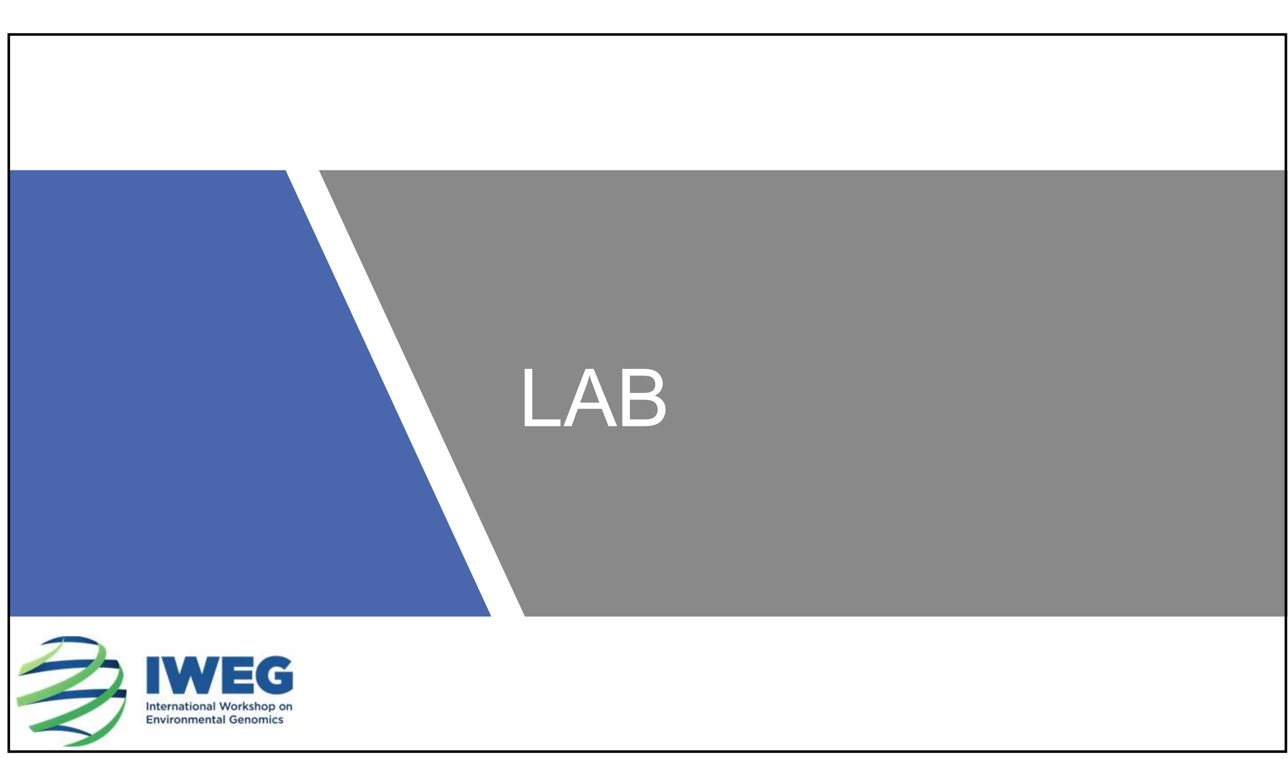
Does sampling EQUIPMENT need to be standardized and why?

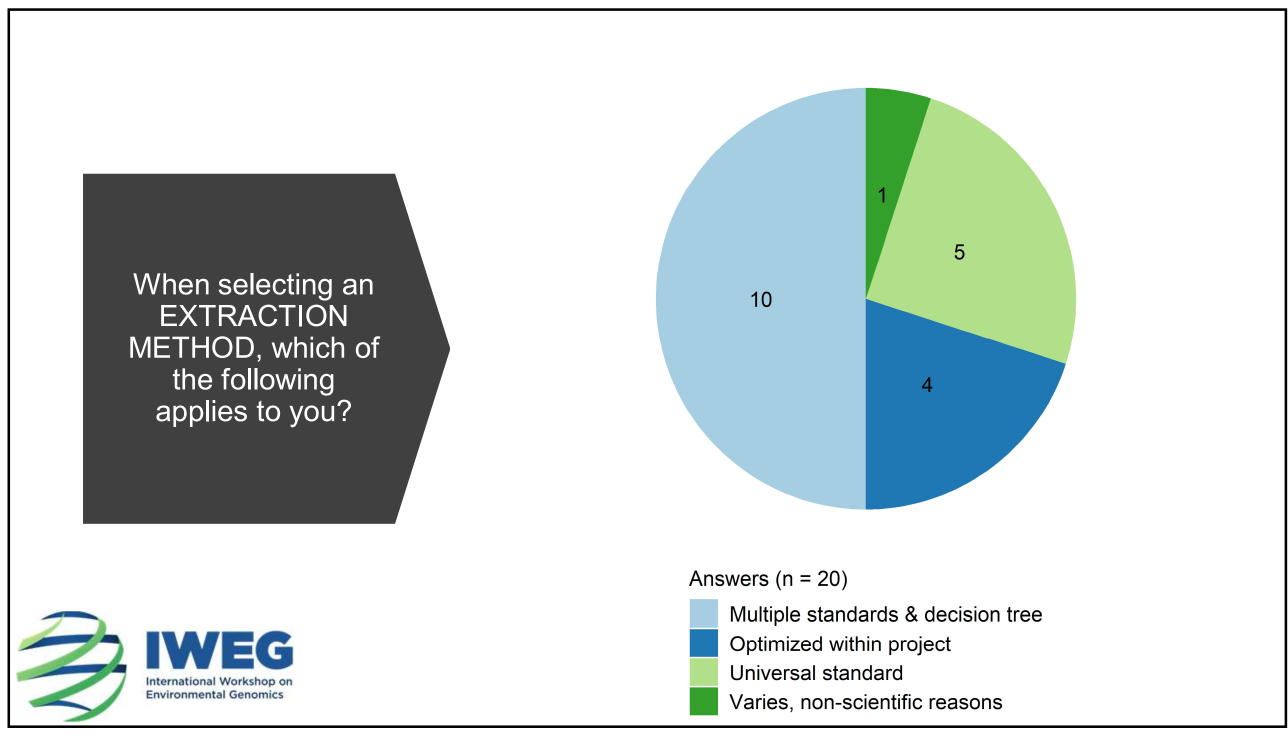


- Yes, for comparability among projects
- Yes, for a particular project or for long term monitoring because the sampling equipment can introduce variation in results (e.g., rare species drop out)
- Yes, to ensure consistent sampling volume, pathway, timeline, etc.



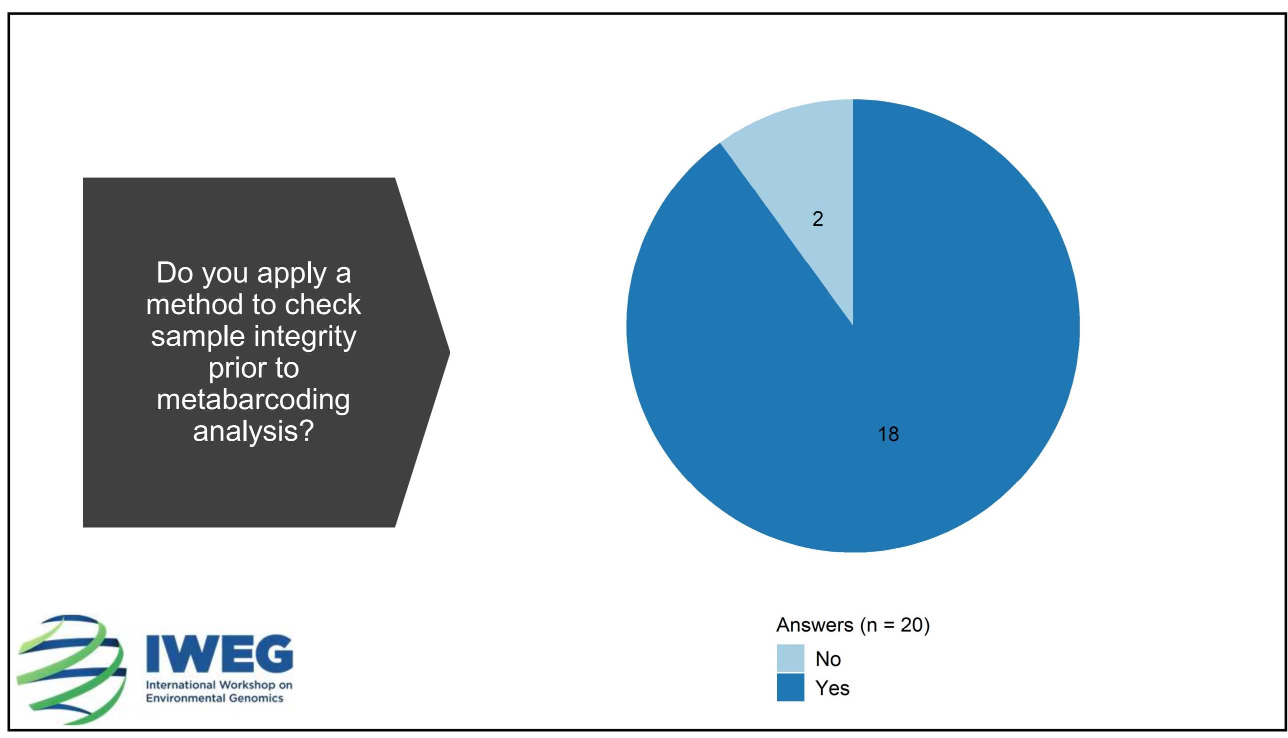
- No, diversity in sampling equipment is key to allow sampling gear to be selected to meet the needs of specific projects (depth, volume, deployment method)
- No, the amount of environmental material sampled, where it's sampled from, and proper decontamination are what need to be standardized. Should be aware of possible biases from equipment.
- No, there is less concern about equipment and more about sampling volume, filter, preservation, extraction.





Please describe your standard EXTRACTION METHOD.

- Separate Qiagen kits for tissue, water, and sediment/soil DNA. Different kit for RNA.
- Zymo (depending on preservation method)
- Autogen 12S DNA isolation robots and kits


Do you apply a method to check sample integrity prior to metabarcoding analysis?
2
18
Answers (n = 20)
No
Yes
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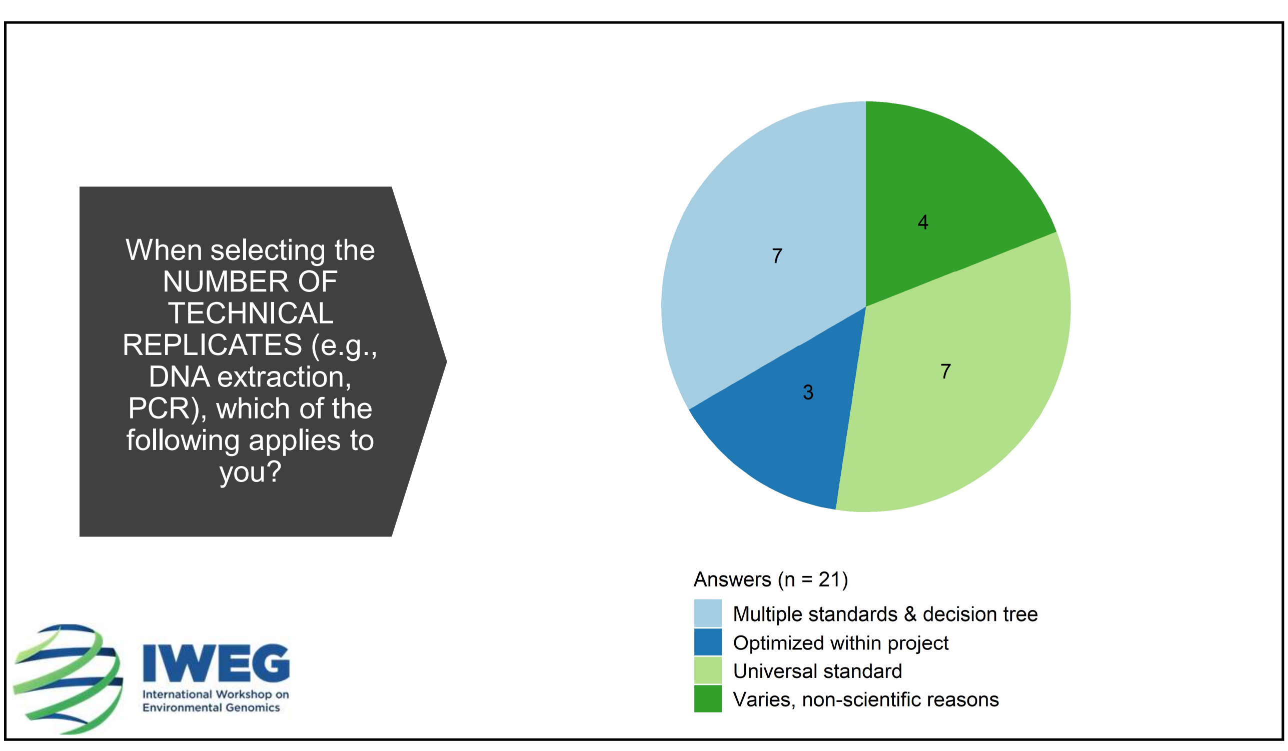
When selecting the NUMBER OF TECHNICAL REPLICATES (e.g., DNA extraction, PCR), which of the following applies to you?
7
4
3
7
Answers (n = 21)
Multiple standards & decision tree
Optimized within project
Universal standard
Varies, non-scientific reasons
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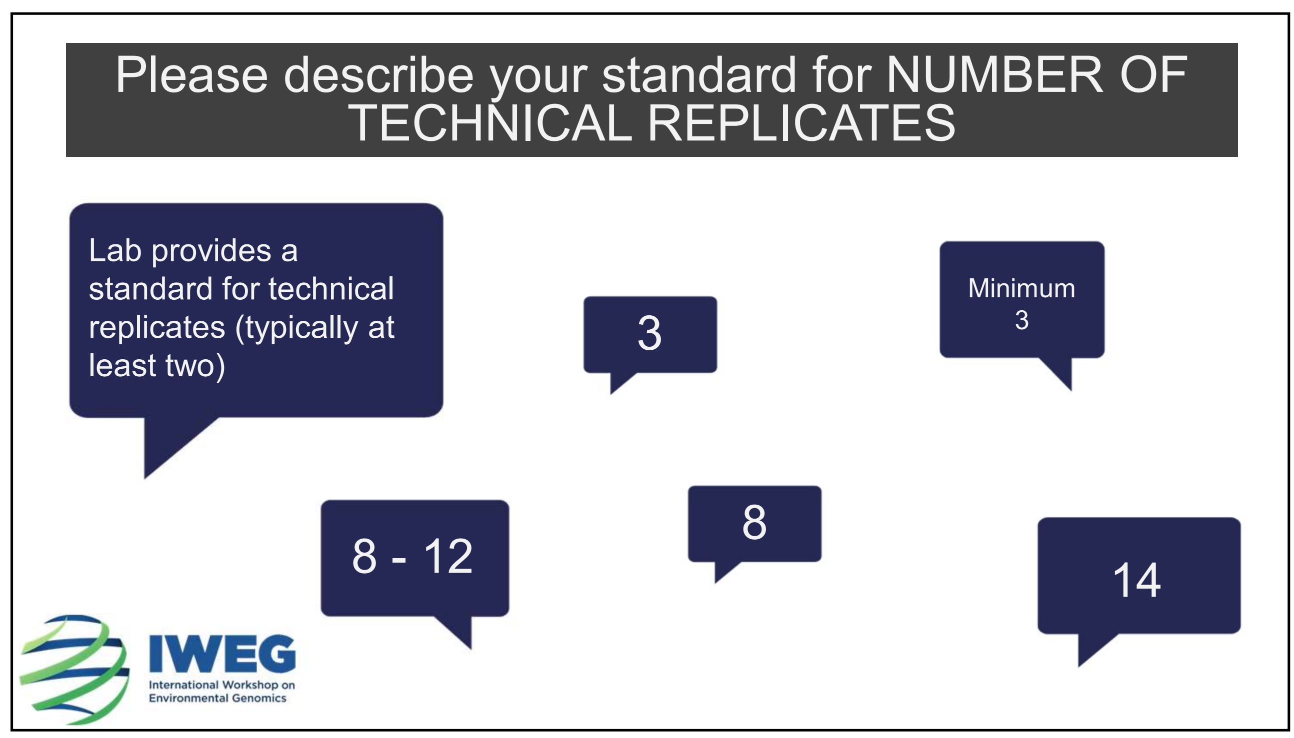
Please describe your standard for NUMBER OF TECHNICAL REPLICATES
Lab provides a standard for technical replicates (typically at least two)
3
Minimum 3
8 - 12
8
14
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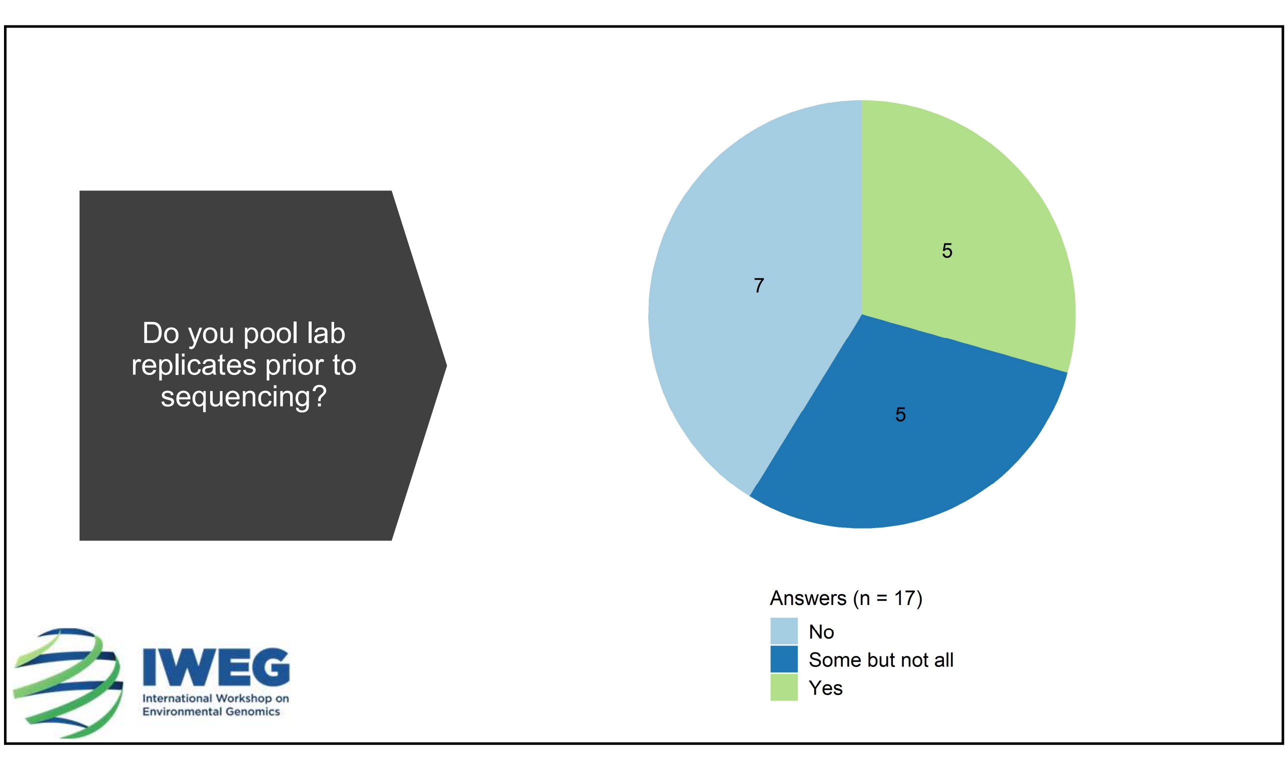
Do you pool lab replicates prior to sequencing?
7
5
5
Answers (n = 17)
No
Some but not all
Yes
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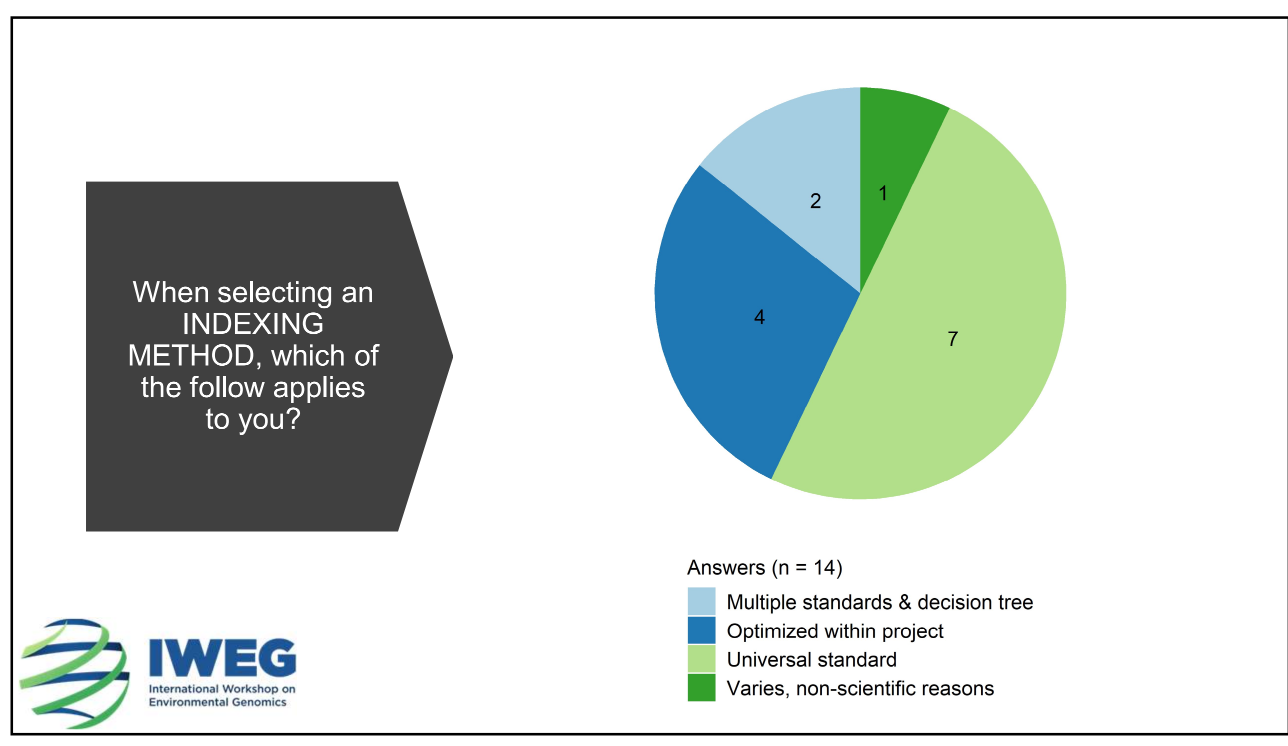
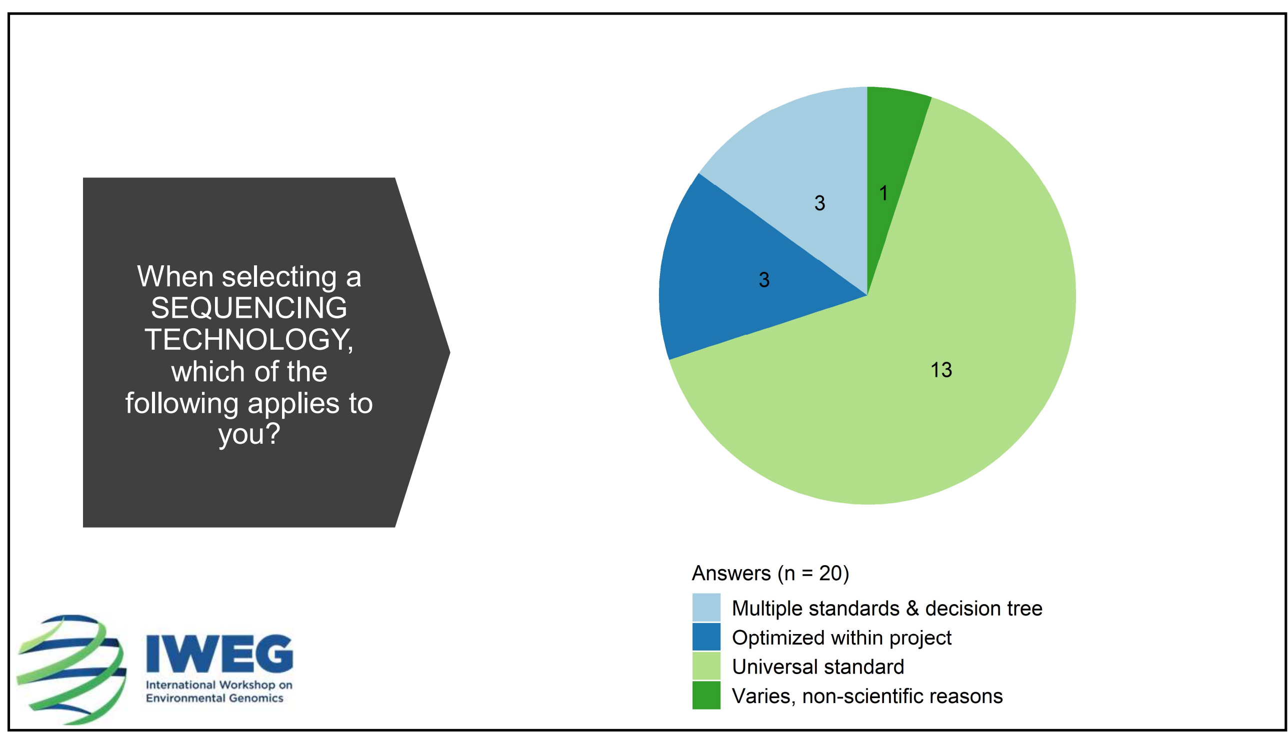


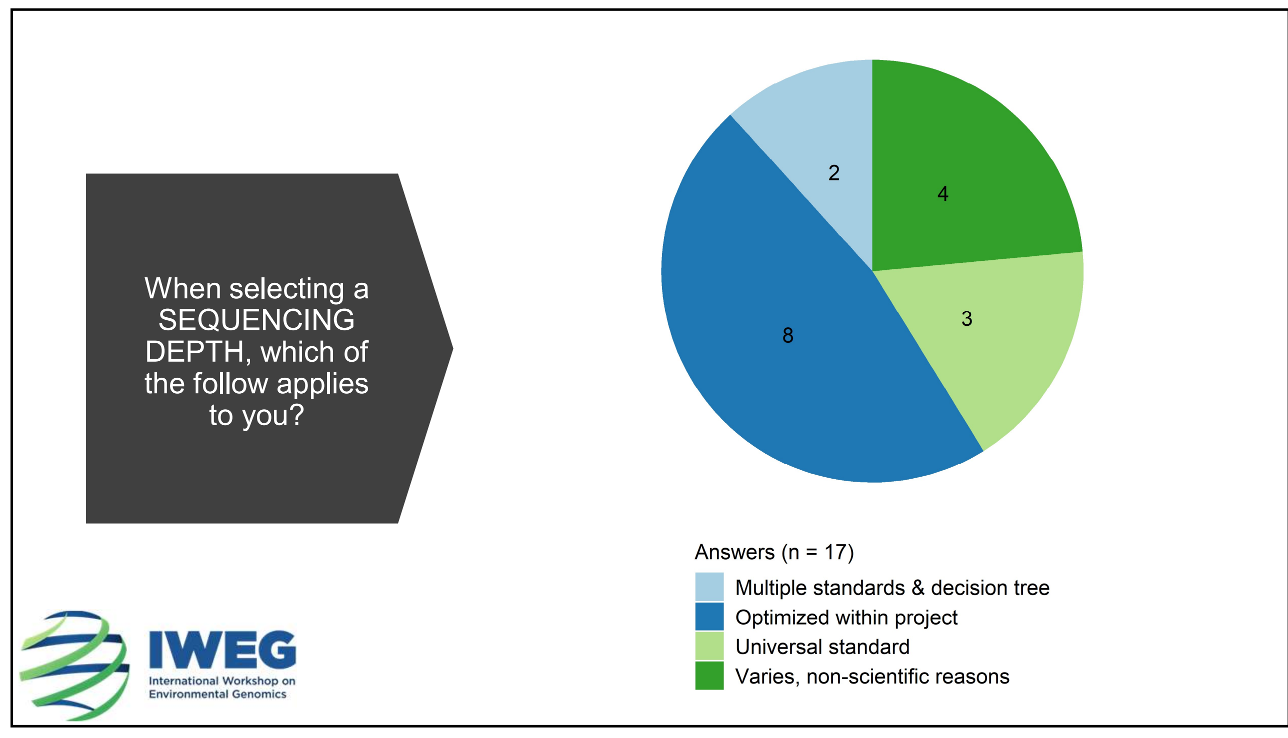
Please describe your standard INDEXING METHOD.

- Unique Dual indexing
- Nextera
- Tagged primers



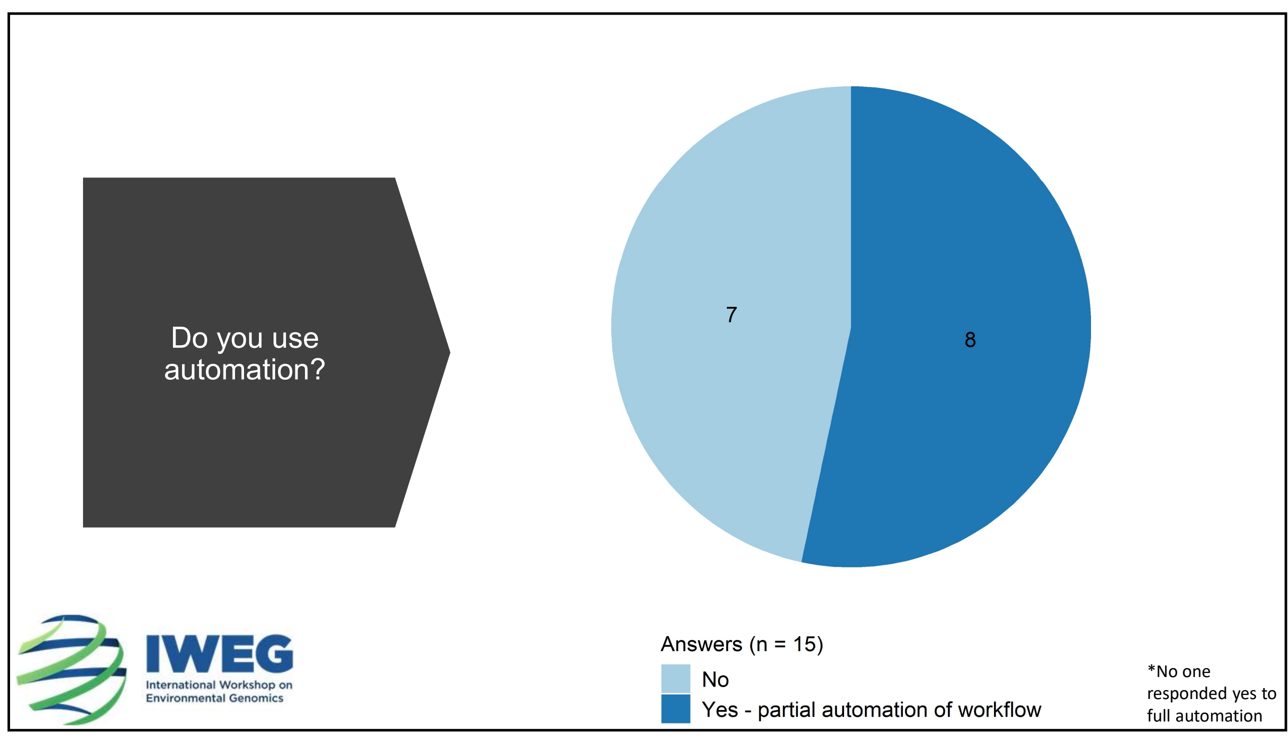
Please describe your standard for SEQUENCING TECHNOLOGY.

- MiSeq (9 mentions)
- NovaSeq (4 mentions)
- Oxford Nanopore Minion (1 mention)



Please describe your standard for SEQUENCING DEPTH.

- In general, 100-200k per sample for community study
- Maximum number of samples we include in a MiSeq library is 96.
- Varies by type of analysis (targeted, community, metagenomics, etc.) along with read length.



Please describe the automation of your workflow.

- For some samples we use an extraction robot
- Extractions are automated and starting to use automation for plating
- Partial automation for both DNA extraction and library prep (including 96 pipettor for indexing and automated liquid handler for PCR plate prep).

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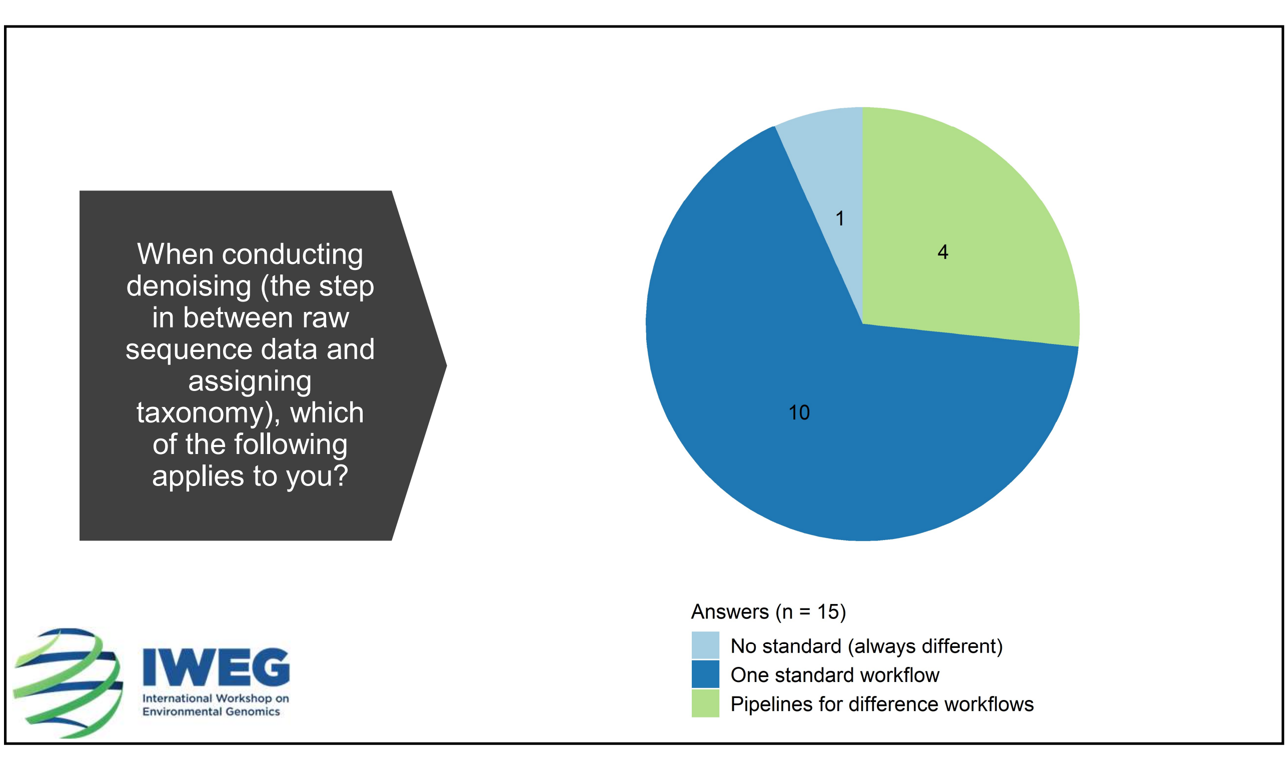

When conducting denoising (the step in between raw sequence data and assigning taxonomy), which of the following applies to you?
1
4
10
Answers (n = 15)
No standard (always different)
One standard workflow
Pipelines for difference workflows
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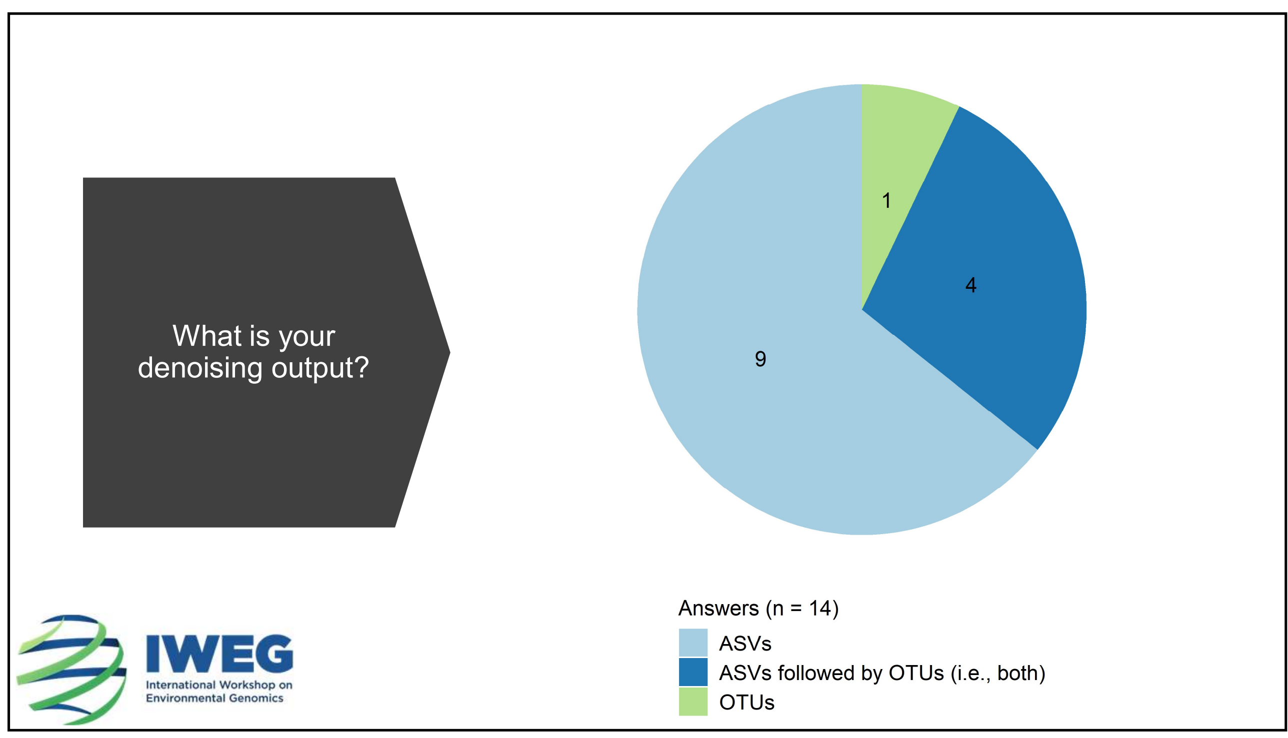
What is your denoising output?
1
4
9
Answers (n = 14)
ASVs
ASVs followed by OTUs (i.e., both)
OTUs
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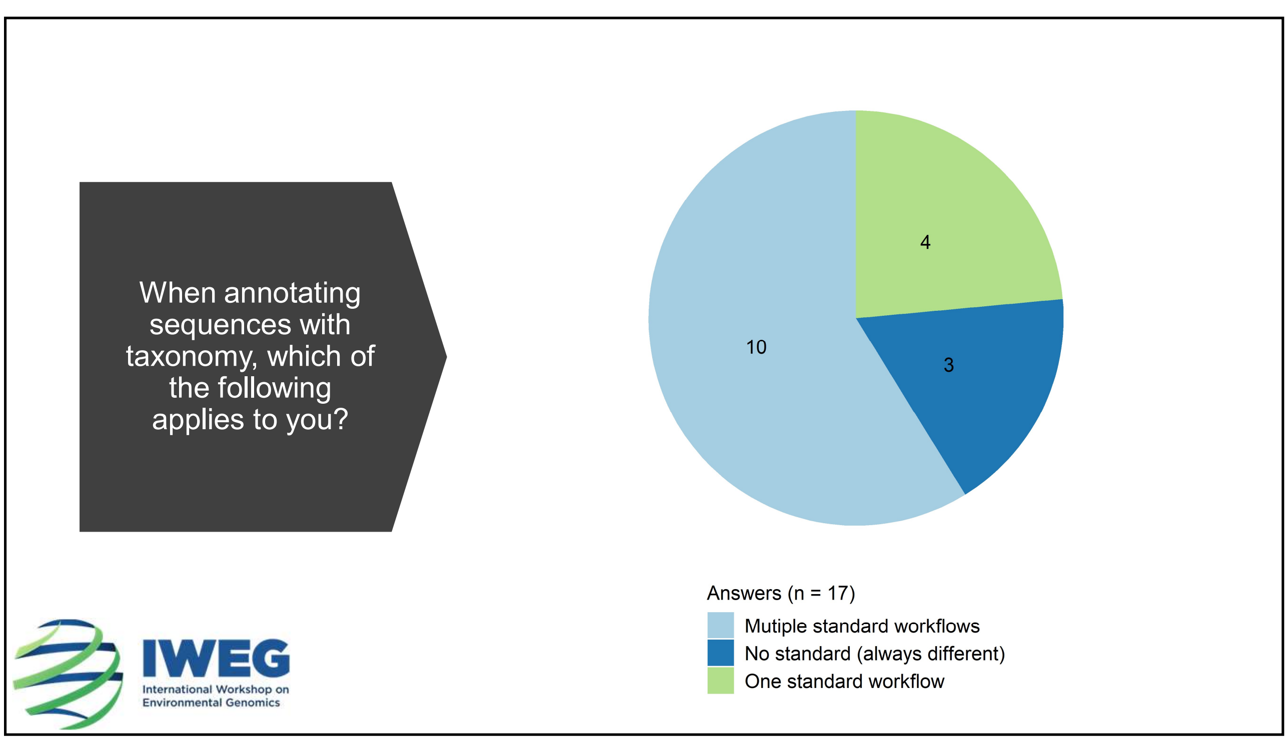
When annotating sequences with taxonomy, which of the following applies to you?
4
10
3
Answers (n = 17)
Mutiple standard workflows
No standard (always different)
One standard workflow
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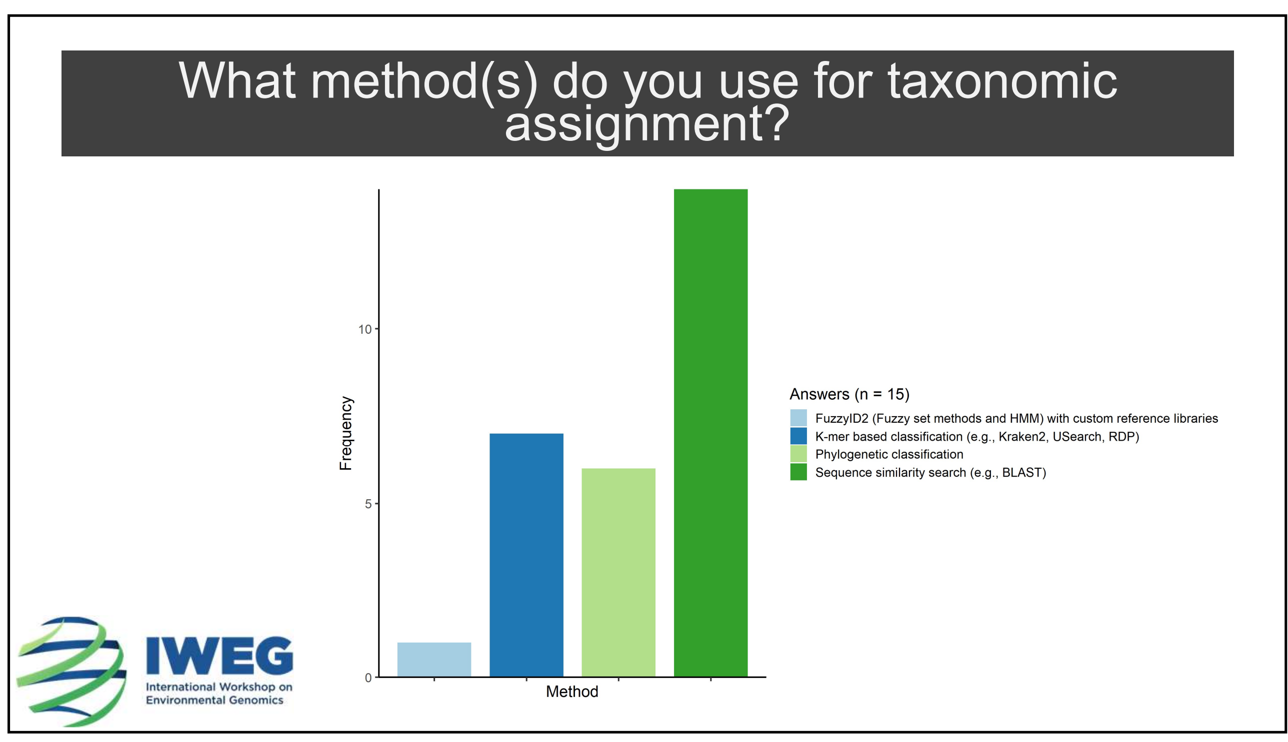
What method(s) do you use for taxonomic assignment?
Frequency
10
5
0
Method
Answers (n = 15)
FuzzyID2 (Fuzzy set methods and HMM) with custom reference libraries
K-mer based classification (e.g., Kraken2, USearch, RDP)
Phylogenetic classification
Sequence similarity search (e.g., BLAST)
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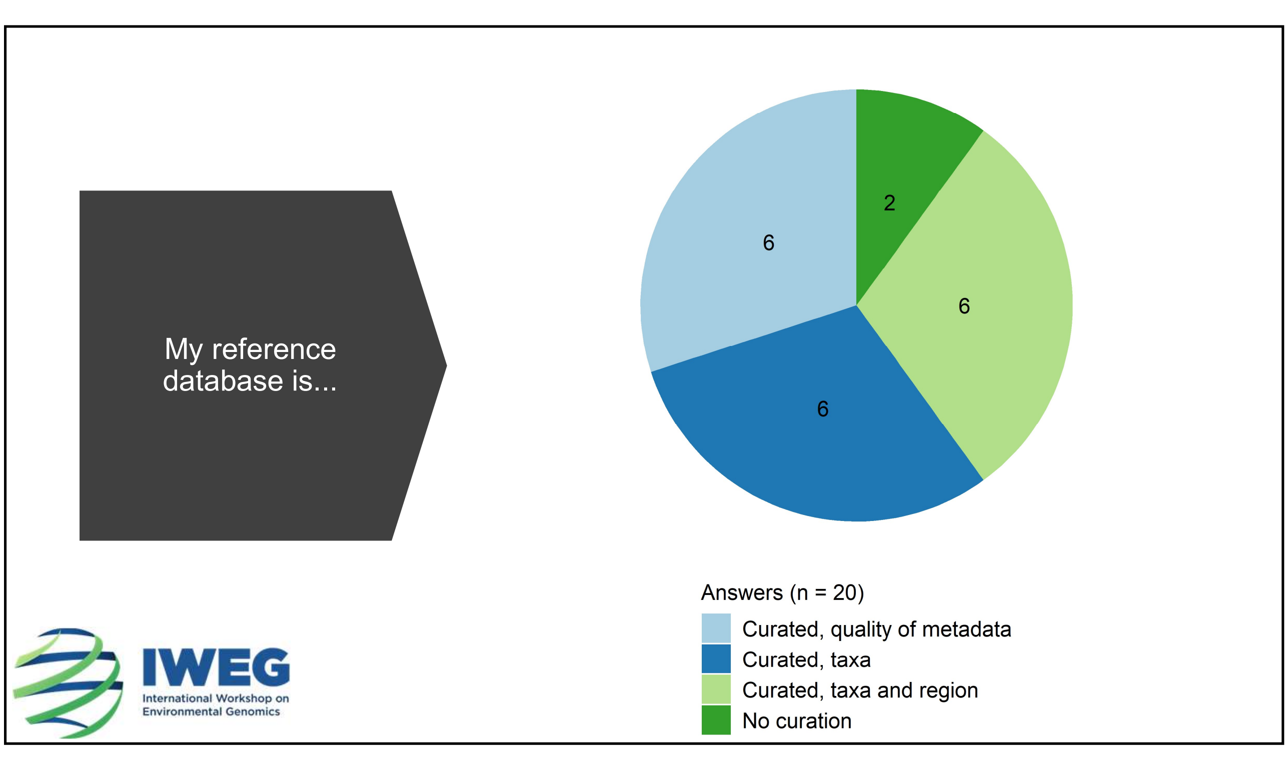
My reference database is...
2
6
6
6
Answers (n = 20)
Curated, quality of metadata
Curated, taxa
Curated, taxa and region
No curation
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ANALYSIS
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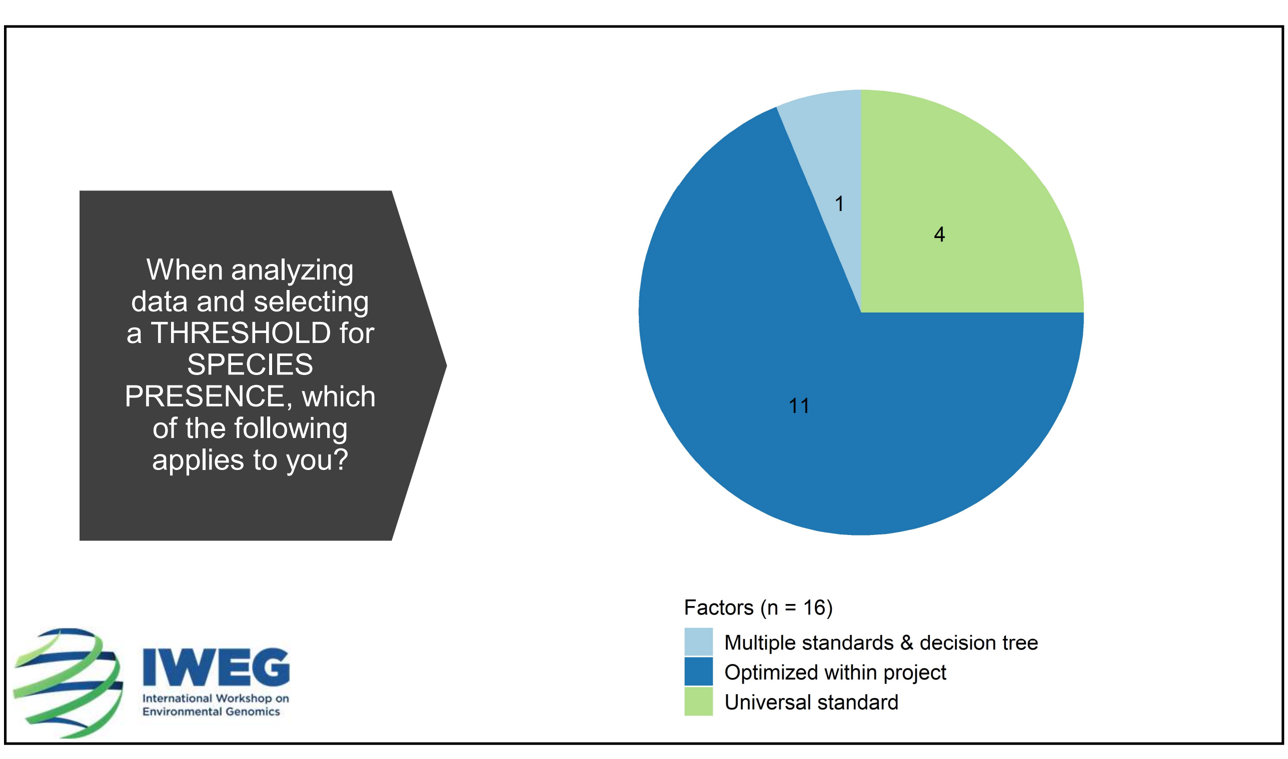
When analyzing data and selecting a THRESHOLD for SPECIES PRESENCE, which of the following applies to you?
1
4
11
Factors (n = 16)
Multiple standards & decision tree
Optimized within project
Universal standard
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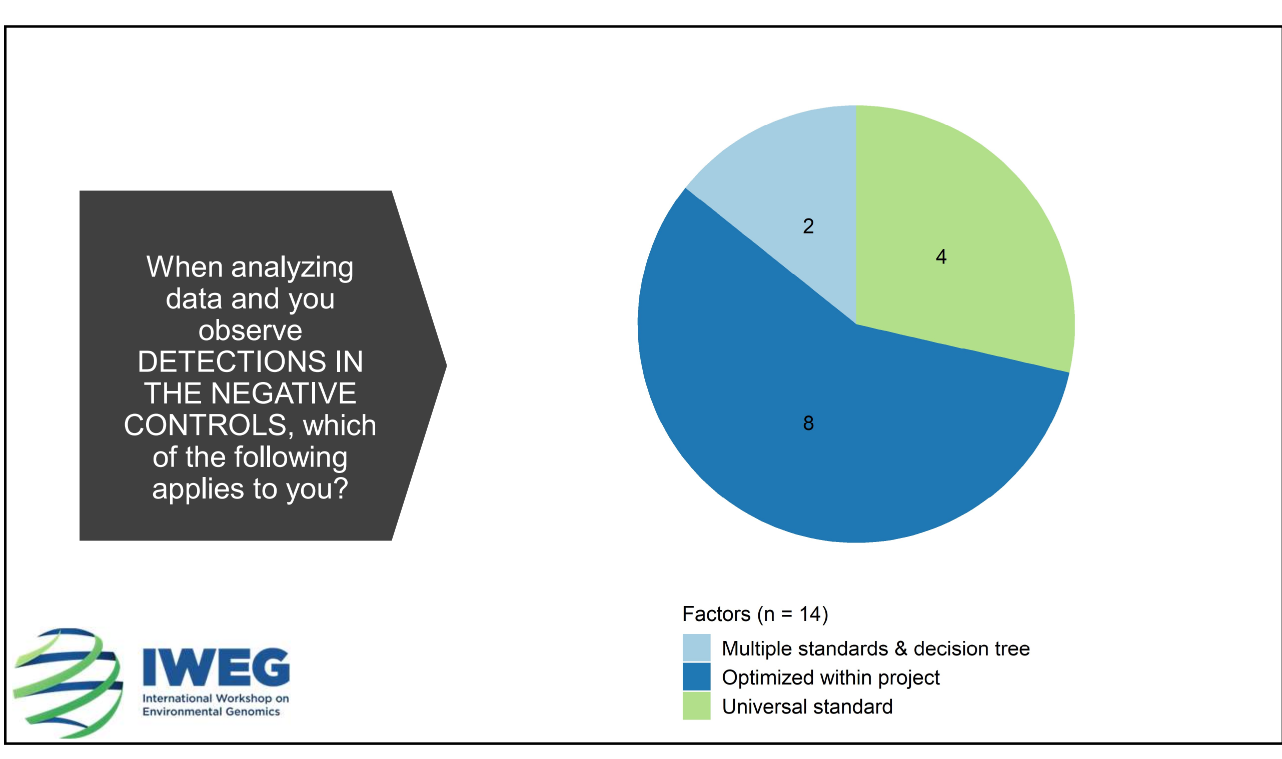
Please describe your standard THRESHOLD for SPECIES PRESENCE.

- 0.1% of reads
- We use a 1% read cut-off, but we aren't doing complex mixtures - for our sequence analysis.
- Score thresholds for similarity/alignment length to reference for each taxonomic level. Typically, no cut-off for number of reads.



Please describe your standard approach for handling DETECTIONS IN THE NEGATIVE CONTROLS.

- We run a positive control sample as well, and use the relative proportion of reads of the positive control found in our negative controls as a threshold for removal of read in all samples
- We subtract the number of reads for each species in the negative control from the number of reads for each sample affected by that control (i.e., field control per site, extraction control for extraction batch, etc)
- Remove ASVs found in blanks from associated samples. Or subtract reads of each ASV found in blank from detections in related samples.
- We interpret and report on levels of detections
- We have negative controls throughout the extraction, amplification, purification process and if DNA is detected in a negative control at any point, we go back to the last successful step and repeat the work. If we are unable to eliminate contamination in a negative control (sometimes it is too costly or time-consuming to repeat work), then we carry the negative control through to sequencing and evaluate the data. If the contamination can be explained, we will endeavour to account for it, so that we can use our sample data. If the negative controls confound interpretation of the sample data, we start over and repeat the full workflow. This is time-consuming and costly, but we cannot report data that may be subject to alternate interpretation.