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DNA extraction bias influences resistome profiles in seahorse skin microbiomes

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Abstract. *Ortega-Kindica RCMH, Balcazar JL, Tabugo SRM. 2025. DNA extraction bias shapes resistome profiles in seahorse skin microbiomes. Biodiversitas 26: 5049-5056.* Antimicrobial Resistance Genes (ARGs) are emerging environmental risks, particularly in coastal environments impacted by anthropogenic activities. Seahorses (*Hippocampus* spp.), as benthic and ecologically sensitive species, are in constant contact with biofilm-enriched sediments and can act as bioindicators of environmental ARGs. Their unique ecological niche and sensitivity to environmental changes make them ideal candidates for monitoring ARGs in marine ecosystems. In this study, the skin microbiome of *Hippocampus barbouri* was analyzed using shotgun metagenomics to assess the impact of two commercial DNA extraction kits, the HiMedia Water DNA Purification Kit (HMS) and the Qiagen DNeasy PowerSoil Pro Kit (QKS), on the detection of ARGs. DNA was extracted from the skin microbiome of 30 adult *H. barbouri* (Barbour's seahorse) collected from Cantiasay Island, Surigao del Norte, Philippines, and subsequently subjected to shotgun metagenomic sequencing. Across the samples, 17 ARG families were found with recovery varying between the extraction kits. The QKS kit yielded higher DNA concentrations and revealed a broader range of ARGs, including multidrug, macrolide, and β-lactam. In contrast, the HMS kit recovered fewer ARG categories, possibly due to reduced cell lysis efficiency or the presence of inhibitors. Statistical analyses (p<0.05) confirmed significant kit-dependent differences in resistome profiles. These findings highlight that DNA extraction protocols can influence ARG detection and emphasize the need for rigorous methodological standardization in metagenomic studies. Furthermore, the detection of diverse ARGs in the skin microbiota of *H. barbouri* supports its potential as a sentinel species for environmental resistome monitoring in marine ecosystems.

Keywords: ARGs, DNA extraction bias, Hippocampus barbouri, marine resistome, shotgun metagenomics

INTRODUCTION

The emergence of Antibiotic Resistance Genes (ARGs) in aquatic environments poses a critical challenge to both public health and environmental safety. Rivers, lakes, seas, and oceans serve as major reservoirs of ARGs, facilitating their dissemination across diverse microbial communities and potentially impacting human populations (Chen et al. 2019; Amarasiri et al. 2020). The presence of ARGs in these ecosystems is largely driven by anthropogenic activities, including agricultural runoff, wastewater discharge, and pharmaceutical pollution, which introduce both antibiotics and Antibiotic-Resistant Bacteria (ARBs) into aquatic systems (Givens et al. 2023). These dynamics highlight the urgent need for robust approaches to detect and monitor ARGs in aquatic environments.

In molecular research, detection begins with the DNA extraction process, where the choice of extraction kit significantly affects DNA yield, purity, and integrity, ultimately influencing downstream applications such as sequencing and bioinformatic analysis (Zielińska et al. 2017). Different extraction methods can produce distinct microbial community profiles, which may, in turn, alter the detection and quantification of ARGs (Flint et al. 2022). Thus, choosing an optimal DNA extraction method is crucial to ensure the reliability and reproducibility of research findings. Despite substantial evidence that protocol choice can generate divergent outcomes, DNA extraction procedures are often used interchangeably for microbiome studies (Evans et al. 2018). Recent advancements in microbiome research have promoted the use of shotgun metagenomics for the comprehensive characterization of microbiome communities across diverse hosts and habitats. This approach offers a broader and more detailed detection of ARGs compared to targeted methods such as high-throughput qPCR, thereby enhancing the accuracy of AMR surveillance (Habibi et al. 2023; Kumar et al. 2024). With the growing application of shotgun metagenomics, there is a substantial increase in demand for DNA extraction methods that deliver high-quality and optimal yields. However, extraction biases remain pervasive, making the recovery of high-quality DNA from microbial communities a critical step for accurately characterizing both microbiome profiles and associated ARGs. Moreover, marine organisms, particularly those with distinctive biological characteristics like seahorses (*Hippocampus*), offer a unique opportunity to investigate

microbial interactions and potential reservoirs of ARGs. Belonging to the Syngnathidae family, seahorses are recognized for their complex life cycles, symbiotic relationships with microbes, and unique host-microbiome dynamics (Beemelmanns et al. 2019; Ortega et al. 2021).

Studying ARGs in seahorse skin is critical for understanding the potential role of marine organisms as reservoirs and vectors of resistance traits in aquatic ecosystems. Seahorses (*Hippocampus* spp.) are considered habitat-linked sedentary organisms that exhibit restricted but ecologically significant mobility (Harasti 2016). Being benthic and slow-moving teleosts, they are constantly exposed to sediments and biofilms, which are environments known to harbor dense microbial communities and facilitate horizontal gene transfer (Zhao et al. 2023). Their skin microbiota, shaped by environmental conditions and host-specific factors, may serve as a niche for bacteria carrying Antimicrobial Resistance Genes (ARGs) (Lira et al. 2021). With increasing anthropogenic activities such as aquaculture, antibiotic runoff, and coastal pollution, marine organisms like seahorses may accumulate ARGs from their surroundings and contribute to their environmental dissemination (Reichertet al. 2021; Inda-Díaz et al. 2023). Furthermore, since seahorses are ecologically important and often threatened by habitat degradation and overexploitation, understanding their microbiome's resistance profiles could provide insights into their health, disease vulnerability, and ecosystem risks (Koning and Hoeksema 2021). Hence, examining ARGs in the skin microbiota of seahorses not only supports marine conservation and biosecurity efforts but also contributes to the global monitoring and mitigation of the spread of ARGs in natural environments.

Thus, this study evaluates the effectiveness of two DNA extraction kits in detecting and characterizing Antimicrobial Resistance Genes (ARGs) through shotgun metagenomics of the skin microbiome of *Hippocampus barbouri* (Jordan & Richardson, 1908) (Barbour's seahorse). This work is significant for both ecological and health-related research, as understanding ARG distribution in marine environments can uncover potential reservoirs of resistance that threaten marine life and human health. A major challenge, however, is the absence of standardized DNA extraction protocols for marine organisms, which can introduce methodological biases and distort resistome profiles. To address this, the study tests the hypothesis that different extraction kits yield distinct ARG detection patterns in *H. barbouri* skin microbiomes, thereby influencing interpretations of antimicrobial resistance in marine ecosystems.

MATERIALS AND METHODS

Sample collection, DNA extraction, and shotgun sequencing

This research was carried out in accordance with national laws and adhering to the national guidelines set by the Bureau of Fisheries and Aquatic Resources (BFAR) as part of the Gratuitous Permit (GP No. 0249-23). In addition, all procedures were reviewed and approved to ensure that there was compliance with ethical and sustainable methods in handling organisms. Opportunistic sampling was done where 30 adult *Hippocampus barbouri* were collected from Cantiasay Island, Surigao (9°51'34"N 125°36'04"E), weighing an average of 7.5 g. Three biological replicates were included in this study for each DNA extraction kit, each containing 5 adult seahorses (approved and within the allowable number of samples as per GP No. 0249-23). Fifteen seahorses for the DNeasy PowerSoil Pro Kit (Qiagen) (QKS) and 15 for the HiPurA Water DNA Purification Kit (HiMedia) (HMS). Preparation of samples for DNA extraction of bacterial communities was performed following the protocol described by Ortega et al. (2021).

DNA extractions were performed using the manufacturer's protocols for the following kits: DNeasyPowerSoil Pro Kits (Qiagen; Hilden, Germany; QKS) and HiPurA Water DNA Purification Kit (MB 577) (Mumbai, India; HMS). The DNeasy PowerSoil Pro kit provides flexible homogenization options, ranging from standard vortexing to high-energy bead beating, combined with a defined Inhibitor Removal Technology (IRT) step. This approach facilitates efficient disruption of diverse microbial cells and the removal of humic substances commonly encountered in soil and sediment matrices. In contrast, the HiPurA Water DNA Purification Kit employs a filter-based microbial capture step followed by vortex-assisted bead beating in lysis solution, representing a streamlined method optimized for aqueous samples. These methodological differences can substantially influence DNA yield, fragment size, microbial community representation, and the degree of inhibitor carryover, parameters that have been shown to affect downstream microbiome profiling in comparative DNA extraction studies significantly (Feng et al. 2023; Gand et al. 2023; Wright et al. 2023). DNA samples for both kits had a minimum concentration of 20ng/uL, a crucial factor in ensuring the success of downstream applications such as sequencing. During the final DNA elution step, 50 µL of elution buffer (10 mM Tris-Cl, pH 8.0) was used, which is important for maintaining the stability and integrity of the DNA. The quality of DNA samples was checked using agarose gel electrophoresis. Samples with the best yield from the extracted total DNA were stored at -80°C for sequencing. Approximately 1.053 µg on average of a DNA aliquot proceeds for shotgun metagenomic sequencing using a NovaSeq 6000 system sequencing platform and TruSeq PCR-free library (350bp). DNA was quantified through the QuantiFluor dsDNA system method using Victor Nivo Multimode Microplate Reader (a fluorescence-based quantification). To verify the DNA integrity (DIN), the DNA was evaluated through 2100 Bioanalyzer and 4200 TapeStation, which determines the degree of fragmentation of the genomic DNA of each sample by measuring the distribution of signals of various sizes and finally checking the size of the DNA using the Femto Pulse System to determine the actual size distribution of each gDNA sample.

Bioinformatics analysis

Bioinformatics analysis followed the protocol outlined by Ortega-Kindica et al. (2024) and Padasas-Adalla et al. (2024). Initially, raw reads were filtered to remove low-quality sequences using the FASTX-Toolkit

(https://github.com/agordon/fastx_toolkit) with a minimum quality score threshold of Q30, where shorter reads less than 50 bp were discarded and adapter sequences were trimmed to ensure efficient downstream analysis. High-quality reads were then aligned against the Comprehensive Antibiotic Resistance Database (CARD) version 3.2.4 (Alcock et al. 2023) and an in-house database (Gionchetta et al. 2022) using DIAMOND v2.1.7 (Buchfink et al. 2015) to identify ARGs. Only BLAST hits with ≥90% amino acid identity over ≥90% of the read length were classified as ARGs, ensuring stringent criteria to minimize false positives (Gionchetta et al. 2022). The abundance of ARGs was normalized to the total number of reads annotated as 16S rRNA genes using METAXA2 (Bengtsson-Palme et al. 2015). Taxonomic classification was performed using Kraken 2 v2.1.3, with a standard Kraken 2 database, which includes RefSeq complete genomes and sequences for archaea, bacteria, plasmids, and viruses (O'Leary et al. 2016; Lu et al. 2017; Wood et al. 2019). Significant differences between skin samples were assessed via one-way ANOVA or Student's t-test where appropriate, with significance set at $P<0.05$. Heatmaps of the most abundant ARGs to the total number of reads annotated as 16S rRNA genes using METAXA2 were generated using the "pheatmap" package in R (version 4.3.0; R Core Team, 2023; https://www.R-project.org). Data were filtered to reduce network complexity, considering only strong (Spearman's $\rho>0.7$) and highly significant ($p<0.01$) correlations. Network analysis was performed in R using the "vegan" and "Hmisc" packages and visualized in Gephi v0.10 with the Fruchterman-Reingold layout algorithm (Bastian et al. 2009).

Nucleotide sequence

Metagenome sequence data derived from bacterial communities from the skin of seahorses from Surigao del Norte were submitted to the NCBI Short Read Archive under Bioproject PRJNA1333584.

RESULTS AND DISCUSSION

To evaluate the efficiency of DNA extraction methods in detecting ARGs from the skin microbiome of *Hippocampus barbouri*, a statistical comparison was performed between the two commercial kits. Table 1 summarizes the quantitative analysis of ARG recovery. For ARG families, the two-tailed *p*-value was 0.0453, indicating a statistically significant difference between the HMS and QKS samples according to conventional thresholds. The mean difference between HMS and QKS is 6.68, with a 95% confidence interval of 0.23 to 13.13. For ARGs, the two-tailed *p*-value was 0.0025, reflecting a highly significant difference, with a mean difference of -2.44 and a 95% confidence interval of -3.44 to -1.44. The higher recovery of ARG families and genes with the QKS method suggests improved detection sensitivity compared to HMS. While these results confirm statistically significant differences in resistome profiles, the key challenge remains interpreting their ecological relevance. Future research should investigate functional linkages to better elucidate the broader ecological implications of such methodological differences.

Figures 1 and 2 illustrate the relative abundance of Antimicrobial Resistance Gene (ARG) families and dominant ARGs detected from the skin microbiome of *H. barbouri*. The DneasyPowerSoil Pro Kit (Qiagen) (QKS) consistently yielded higher ARG detection rates than the HiPurA Water DNA Purification Kit (HiMedia) (HMS), particularly across multidrug, macrolide, MLS (Macrolide-Lincosamide-Streptogramin), and fluoroquinolone resistance gene families. Multidrug resistance genes emerged as the most dominant class, with significantly greater relative abundance in QKS-derived samples. Specifically, Figure 1 shows that the QKS kit was consistently higher relative abundances across a wide range of ARG families, particularly in multidrug, macrolide, MLS (Macrolide-Lincosamide-Streptogramin), and fluoroquinolone resistance genes. These ARGs are essential indicators of environmental and clinical antibiotic resistance threats. They are commonly linked to mobile genetic elements, suggesting that the QKS kit may be more efficient in lysing cells and recovering DNA from both Gram-positive and Gram-negative bacteria, including those embedded in biofilms or carrying low-copy plasmids (Gerasimidis et al. 2016; Costea et al. 2017).

Furthermore, our results aligned with previous work on diverse human microbiota, where the QKS kits yield relatively pure DNA and good results in terms of microbiome profiles (Lim et al. 2020; Le Gall-David et al. 2023). In contrast, the HMS kit consistently underrepresents multiple ARG families, especially Fosmidomycin, Fosfomycin, Glycopeptide, Phenicol, and Rifampin resistance genes, all of which show minimal to undetectable levels. This result may be attributed to differences in the kits' chemical lysis strength, bead-beating efficiency, or capacity to retain DNA from low-abundance or hard-to-lyse taxa such as Actinobacteria and certain Proteobacteria, which frequently harbor these ARGs (Yuan et al. 2012; Knudsen et al. 2016). Such biases can significantly alter downstream interpretations of resistome composition and ecological risk, highlighting the importance of careful consideration of DNA extraction methods in microbiome studies.

Table 1. Quantitative analysis comparing the yields of ARG families and individual ARGs from *Hippocampus barbouri* skin using HiMedia (HMS) and Qiagen kits (QKS)

	ARG families		AR genes	
	HMS	**QKS**	**HMS**	**QKS**
Mean	-3.06853	-9.74377	-0.8269	1.613516
SD	3.983266	0.568399	0.604824	0.147759
SEM	2.299739	0.328165	0.349196	0.085308

Note: Values represent log2-transformed counts normalized to 16S rRNA gene abundance

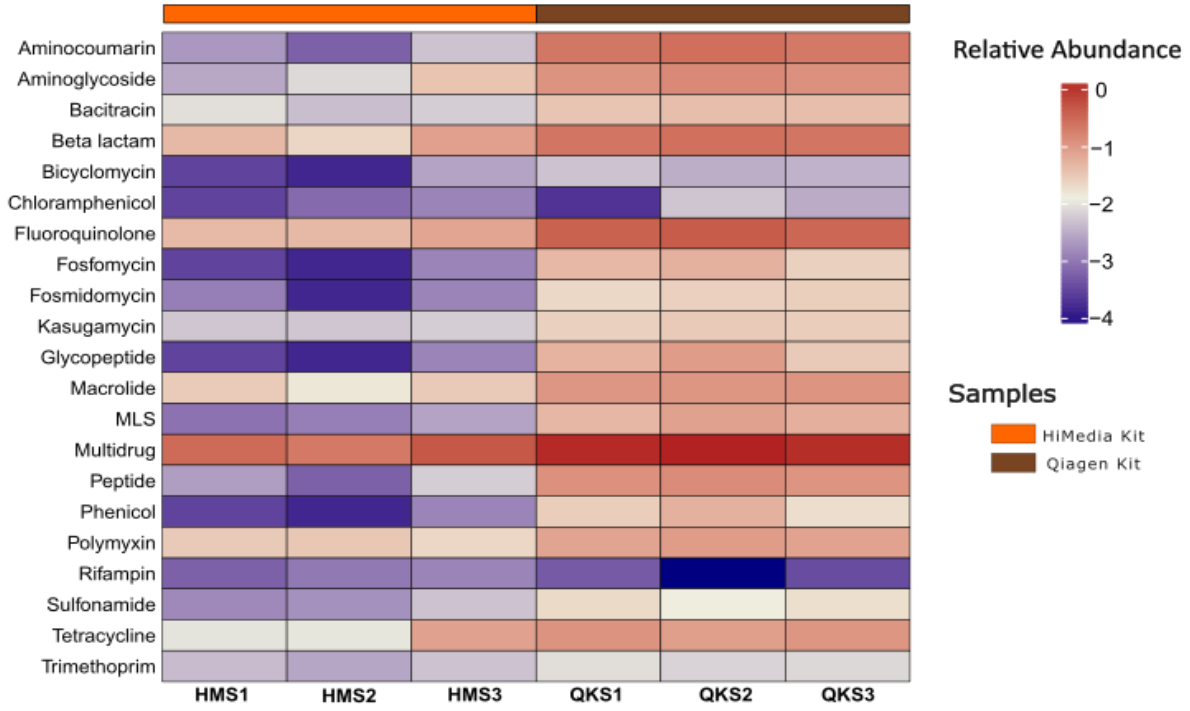

Figure 1. Comparison of ARG family relative abundance detected using the HMS and QKS kits

The Qiagen kit's broader detection suggests a more accurate representation of the environmental resistome, which is crucial for monitoring antibiotic resistance dissemination in natural systems. Given that DNA extraction bias is a well-recognized issue in microbiome and resistome studies, the choice of extraction kit can significantly influences downstream data interpretation, particularly when quantifying functionally important genes such as ARGs, making method selection critical for accurately assessing resistome risk and developing reliable predictive models for antibiotic resistance management in water environments (Gerasimidis et al. 2016; Pollock et al. 2018; Yang et al. 2025). Because ARGs are frequently linked to mobile genetic elements, their underrepresentation in HiMedia-extracted samples could lead to false conclusions regarding the prevalence and diversity of resistance in a given environment. Furthermore, the consistent and high ARG detection in Qiagen-derived samples enhances the reliability of environmental surveillance, especially in contexts where multidrug-resistant pathogens pose significant public health risks (Zhu et al. 2017), providing reassurance in the accuracy of the data.

A notable trend is the consistently higher detection of ARGs in samples processed with the Qiagen kit, suggesting that DNA extraction efficiency significantly affects the resolution of resistome profiles (Figure 2). For instance, multidrug resistance-associated genes such as *mdtA*, *mdtB*, *mdtG*, *mexB*, *acrB*, *emrB*, and *tolC* show strong relative abundance in Qiagen samples compared to HiMedia, indicating minimal detection. These genes are commonly associated with efflux pump systems, which bacteria employ to extrude a broad range of antibiotics and environmental toxins, contributing to multidrug resistance (Huang et al. 2022). Furthermore, several β-lactam resistance genes such as *blaB*, *oxaB*, and *ampH* are prevalent in Qiagen-processed samples, with *blaB* and *oxaB* being particularly abundant in QKS2 and QKS3. These genes encode enzymes that hydrolyze β-lactam antibiotics, making them ineffective, and are often found in environmental and commensal bacterial communities exposed to antibiotic residues (Miao et al. 2022). Notably, genes conferring resistance to glycopeptides (*vanTC*, *vanSC*, *vanRC*), tetracyclines (*tetH*, *tet34*), and macrolides (*ermB*, *mefA*) were also more detectable in Qiagen samples. However, their relative abundances were generally lower than those of efflux-associated genes. The presence of multiple ARG classes indicates a diverse and potentially co-selected resistome within the seahorse skin microbiome, likely shaped by environmental exposure to sub-inhibitory antibiotic concentrations, heavy metals, and other pollutants (Inda-Díaz et al. 2023). HiMedia-processed samples, in contrast, show significantly reduced ARG detection across all gene categories. This may be due to lower DNA yield, suboptimal lysis of resistant bacterial taxa (e.g., Actinobacteria or Gram-positive Firmicutes), or the presence of PCR inhibitors in the final eluate (Knudsen et al. 2016). These limitations are well-documented in metagenomics, where DNA extraction is a critical step that can introduce biases in microbial and functional gene profiles (Zielińska et al. 2017). The superiority of the Qiagen kit in this context may be attributed to its use of optimized enzymatic and mechanical lysis steps and inhibitor removal columns, enhancing the recovery of DNA from a wide range of microbial taxa.

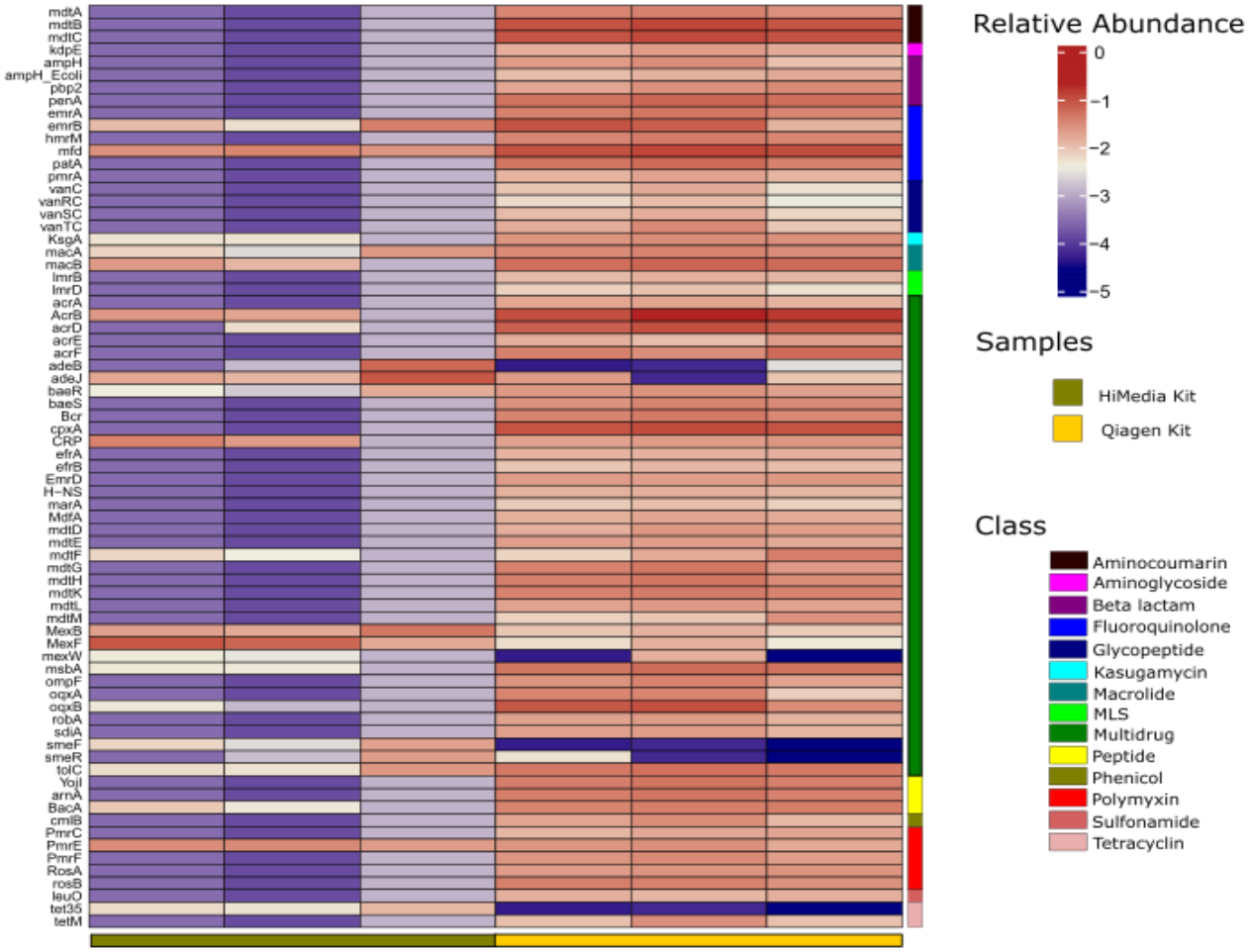

Figure 2. Heatmap showing the distribution and relative abundance of the dominant genes present in the skin of *Hippocampus barbouri* using two different extraction kits

QKS Kit, which was known as DneasyPowersoil Pro Kit, is specifically engineered to handle complex environmental matrices, such as soil, sediment, and biofilms, which are typically rich in PCR inhibitors like humic acids, polysaccharides, and heavy metals (Venturini et al. 2020). In this study, the higher recovery of Antimicrobial Resistance Genes (ARGs) from seahorse skin microbiomes using this kit may be explained by its optimized inhibitor-removal chemistry, which improves DNA purity and yield even from challenging biological samples. In contrast, the HiPurA Water DNA Purification Kit (HMS), although effective for filtering and purifying DNA from aqueous samples, it may be less suitable for processing the viscous, mucosal, and protein-rich nature of host-associated biofilms and skin microbiota (Hwang et al. 2012; Renshaw et al. 2015; Hoorzook and Barnard 2022). This limitation could result in lower DNA recovery or co-extraction of inhibitory substances that interfere with downstream applications like PCR amplification and metagenomic sequencing, ultimately impairing the detection sensitivity for ARGs.

The stark variation in detected ARG profiles between kits highlights a well-documented issue in metagenomic workflows, such as DNA extraction method strongly influences the apparent microbial and functional diversity (Hermans et al. 2018). The overrepresentation of multidrug resistance and macrolide genes in QKS-derived samples suggests that this kit may provide a broader or deeper coverage of the microbial resistome, potentially offering better sensitivity for ARG surveillance. However, it also raises concerns about reproducibility and inter-study comparability when extraction protocols are not standardized. Studies have shown that extraction kit bias can affect not only microbial taxonomy but also quantification of functional genes, including ARGs (Yang et al. 2020). Given the growing importance of monitoring antibiotic resistance in environmental and clinical contexts, the choice of a DNA extraction kit is not merely a technical consideration but a critical factor for analytical validity. As such, researchers must carefully evaluate extraction performance based on target organisms and ARG types, and when possible, adopt standardized protocols to minimize methodological biases (Castaneda and Barbosa 2017). The observed disparities in ARG family detection between the HMS and QKS kits underscore the need for benchmarking studies and comprehensive reporting of DNA extraction methodologies in resistome analyses.

Ecologically, the richness and composition of ARGs detected from the skin microbiota of seahorses underscore

their unique role as passive bio-collectors of environmental resistance genes. Seahorses, as benthic and sedentary fish, inhabit a distinctive environment, maintaining prolonged contact with sediments and biofilm-coated substrates, which are known hotspots of microbial diversity and horizontal gene transfer (Zhao et al. 2023). Their skin mucus, rich in proteins and mucopolysaccharides, provides a moist, nutrient-rich microhabitat conducive to bacterial colonization, potentially harboring both symbiotic and environmental bacteria carrying ARGs (Lira et al. 2021). The detection of ARGs linked to efflux pumps, in particular, reflects environmental selective pressures rather than direct antibiotic use, since such genes also confer resistance to biocides, surfactants, and heavy metals common in polluted aquatic habitats (Yuan et al. 2023). This resistome profile may serve as a sentinel signal of the antimicrobial burden in coastal marine environments, especially in areas adjacent to aquaculture, wastewater discharge, or urban runoff. Additionally, variation in ARG abundance among samples or sites may be attributed to differences in environmental exposure, microbial diversity, or host-associated factors such as immune status, mucus composition, or skin morphology (Kraemer et al. 2022). Overall, sediments contain a higher concentration of aquatic bacterial biomass, resulting in more bacterial density and diversity compared to the mucus layer of fish skin. Nonetheless, mucosal skin provides a less diverse but more specialized bacterial community, favoring taxa adapted to host-associated niches. Confocal laser microscopy revealed denser biofilm population (e.g., *Vibrio*) on fish skins compared to abiotic control surfaces, such as stainless steel, thus highlighting the strong correlation between biofilm formation and the roughness of fish skin surfaces (e.g., seahorses).

Additionally, biofilm imaging suggests that species-specific skin features, such as mucus layers and structural characteristics, may influence bacterial attachment modes (Yu and Rhee 2023). This finding is consistent with the study by Zhou et al. (2022), which demonstrated that captive *Hippocampus trimaculatus* (Leach, 1814) had a different skin microbiota and higher ARG diversity compared to wild individuals, likely due to controlled diet and water conditions in captivity that affect microbial colonization dynamics. This highlights how both anthropogenic factors and husbandry practices can shape the resistome of marine species. From a conservation and public health perspective, seahorses can serve as useful bioindicators of marine resistomes, especially in fragile ecosystems. Given their sensitivity to environmental change and protected status in many regions, monitoring their skin-associated resistomes may provide early warning signs of antimicrobial contamination and resistance gene proliferation in marine systems (Ju et al. 2019; Dželalija et al. 2023). Seahorses offer a sensitive, site-attached indicator of ARG presence and trends. Although they fail to capture the full range of ARG reservoirs within the marine ecosystem, their application as a 'sentinel species' supplements broader resistome assessments and facilitates prioritization for more extensive, multi-species monitoring.

The results of this study underscore the critical importance of methodological compatibility between the sample type and the DNA extraction protocol. In microbial ecology and resistome studies, inadequate DNA extraction can lead to the underrepresentation of specific taxa and genes, thereby skewing diversity estimates and obscuring key functional insights (Brauer and Bengtsson 2022; Le Gall-David et al. 2023). This highlights the need for selecting DNA extraction methods that are specifically tailored to the physical and biochemical properties of the target sample to ensure accurate community profiling and resistome characterization. The findings also support previous studies that emphasize how extraction kits differ significantly in their ability to lyse diverse microbial cells and eliminate PCR inhibitors, which are factors that directly impact the observed microbial diversity and gene abundance (Shi et al. 2022; Wydro 2022). To further enhance analytical depth, shotgun metagenomics sequencing offers comprehensive insights into the diversity and distribution of Antimicrobial Resistance Genes (ARGs), allowing for more precise detection and a deeper understanding of their presence and dynamics in both biological samples and aquatic environments. Nonetheless, the reliability and efficiency of these advanced molecular techniques remain depend on the initial DNA extraction step, underscoring the need for continuous protocol optimization to address potential limitations and maximize data accuracy. Ecological and anthropogenic processes are true driving forces organizing resistomes, but technical heterogeneity in DNA extraction can conceal or pervert those signals. Stringent controls and standardized protocols enable researchers to tease apart genuine environmental drivers from methodological artifacts so that patterns of interest truly represent the environmental resistome.

In conclusion, these results highlight two key insights with critical implications for environmental resistome research and marine conservation. First, they underscore the substantial influence of DNA extraction methodology on the detection and characterization of Antimicrobial Resistance Genes (ARGs) in complex environmental microbiomes. The choice of extraction protocol directly shapes the quality and completeness of metagenomic data, affecting the accuracy of ARG profiling and the interpretation of microbial community structure. Second, the findings emphasize the ecological relevance of seahorses as sentinel or biomonitoring species in marine ecosystems. As site-attached, benthic vertebrates that interact closely with their surrounding microbial milieu, seahorses can serve as effective indicators of localized antimicrobial resistance contamination. These insights highlight the urgent need to standardize DNA extraction protocols for marine ARG studies to ensure comparability, reproducibility, and data integrity across research groups and geographic regions. Such standardization could inform policy frameworks aimed at integrating ARG monitoring into marine biodiversity assessments, fisheries management, and coastal pollution control programs. Incorporating sentinel species like seahorses into regulatory biomonitoring schemes would strengthen early-warning systems, guide mitigation strategies, and support evidence-based decision-making for both environmental and public health protection. At the One Health level, the precise identification and characterization of ARGs are crucial for understanding

patterns of resistance transmission among humans, animals, and the environment. Suboptimal DNA recovery can lead to underrepresentation of specific taxa and genes, as well as biased estimates of diversity and representation, and inaccurate estimation of reservoirs of resistance. These gaps can mask early warning signs of impending resistance threats and may constrain evidence-based interventions. To address this, we recommend the clear standardization of protocol reporting for DNA extraction, sequencing, and bioinformatic pipelines. Developing standardized methodology enhances study transparency, improves comparability, reduces technical variation biases, and enhances the reliability of ARG surveillance data, which is crucial for informing One Health-inspired policies, strategies, and interventions.

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