


# Ancient DNA analysis identifies marine mollusc shells as new metagenomic archives of the past

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## Abstract

Marine mollusc shells enclose a wealth of information on coastal organisms and their environment. Their life history traits as well as (palaeo-) environmental conditions, including temperature, food availability, salinity and pollution, can be traced through the analysis of their shell (micro-) structure and biogeochemical composition. Adding to this list, the DNA entrapped in shell carbonate biominerals potentially offers a novel and complementary proxy both for reconstructing palaeoenvironments and tracking mollusc evolutionary trajectories. Here, we assess this potential by applying DNA extraction, high-throughput shotgun DNA sequencing and metagenomic analyses to marine mollusc shells spanning the last ~7,000 years. We report successful DNA extraction from shells, including a variety of ancient specimens, and find that DNA recovery is highly dependent on their biomineral structure, carbonate layer preservation and disease state. We demonstrate positive taxonomic identification of mollusc species using a combination of mitochondrial DNA genomes, barcodes, genome-scale data and metagenomic approaches. We also find shell biominerals to contain a diversity of microbial DNA from the marine environment. Finally, we reconstruct genomic sequences of organisms closely related to the *Vibrio tapetis* bacteria from Manila clam shells previously diagnosed with Brown Ring Disease. Our results reveal marine mollusc shells as novel genetic archives of the past, which opens new perspectives in ancient DNA research, with the potential to reconstruct the evolutionary history of molluscs, microbial communities and pathogens in the face of environmental changes. Other future applications include conservation of endangered mollusc species and aquaculture management.

## KEYWORDS

ancient DNA, high-throughput DNA sequencing, marine mollusc shells, metagenomics

## 1 | INTRODUCTION

Shells are biomineral exoskeletons offering protection from predation and desiccation to the soft bodies of invertebrates (Marin, Le Roy, & Marie, 2012). Molluscs are prominent producers of shells, which despite microstructure variation among species, all consist of both a calcified and an organic layer (0.01%–5.00% in weight) containing pigments, polysaccharides, lipids and proteins (Marin et al., 2012). Marine mollusc shells survive in the fossil record and provide invaluable information about the past. Their morphology can for instance be used for phylogenetic inference (Ponder & Lindberg, 2008), while their microstructure can reveal important life history traits, including growth rates (Chauvaud, Patry, & Jolivet, 2012) and diseases (Paillard, Roux, & Borrego, 2004; Trinkler, Labonne, & Marin, 2010). Their internal structure is also commonly examined for sclerochronological dating (Gröcke & Gillikin, 2008; Butler, Wanamaker, Scourse, Richardson, & Reynolds, 2013) or uncovering past changes in seawater temperature and salinity (Hiebenthal, Philipp, Eisenhauer, & Wahl, 2012; Reynolds, Scourse, & Halloran, 2016). Another source of information is their biogeochemical composition, with oxygen and carbon stable isotopes providing proxies for past temperatures (Chauvaud, Lorrain, & Dunbar, 2005) and food availability (Lartaud, Emmanuel, de Rafaelis, Pouvreau, & Renard, 2010); trace elements for the presence of pollutants (Pérez-Mayol, Blasco, & Tornero, 2014) and phytoplankton dynamics (Szymanek, 2011); and rates of amino-acid degradation for stratigraphic reconstruction (Penkman, Preece, & Bridgland, 2011).

DNA extracted from marine mollusc shells can potentially offer an extremely informative and complementary proxy. As DNA from both inside and outside the shell could be entrapped during biomineral formation, shell DNA might be used not only for marine mollusc species identification, phylogeographic and demographic reconstructions, but also for bacterial community and environmental DNA profiling. Despite such potential, no work has evaluated the long-term preservation of DNA in marine mollusc shells, and hence, their potential for ancient DNA studies. To the best of our knowledge, only one single study has reported successful PCR amplification of nine nuclear microsatellites and a single mitochondrial (mtDNA) barcode from shells of freshwater pearl mussels *Margaritifera margaritifera* (Geist, Wunderlich, & Kuehn, 2008).

Genome-scale data from ancient specimens, including Middle Pleistocene remains (120–780,000 years Before Present, yBP; Orlando, Ginolhac, & Zhang, 2013; Dabney, Knapp, & Glocke, 2013; Meyer, Fu, & Aximu-Petri, 2014; Meyer, Arsuaga, & de Filippo, 2016), reveal a wealth of information about past organisms, populations and environments (see (Pedersen, Overballe-Petersen, & Ermini, 2015; Ermini, Der Sarkissian, Willerslev, & Orlando, 2015; Slatkin & Racimo, 2016; Leonardi, Librado, & Der Sarkissian, 2016; MacHugh, Larson, & Orlando, 2016) for reviews). This has mainly been made possible through constant improvements in high-throughput DNA sequencing (HTS) technologies that

have recently revolutionized genomics, metagenomics and transcriptomics (Bernstein, Meissner, & Lander, 2007; Metzker, 2010; Segata, Boernigen, & Tickle, 2013; Crosetto, Bienko, & van Oudenaarden, 2015; Orlando, Gilbert, & Willerslev, 2015). Ancient DNA studies have so far mostly relied on the long-term preservation of DNA in teeth and bones (Smith, Chamberlain, Riley, Stringer, & Collins, 2003), which are composed of calcium hydroxyapatite biominerals (Beniash, 2011). Calcium carbonate matrices of subfossilized avian eggshells (Nys, Gautron, Garcia-Ruiz, & Hincke, 2004) have also been shown to occasionally preserve ancient DNA (Oskam, Haile, & McLay, 2010). This work paves the way for the recovery of DNA from ancient marine mollusc shells, also made of calcium carbonate. To assess marine mollusc shell DNA content and investigate its long-term preservation, we extracted, shotgun-sequenced and characterised DNA from marine mollusc shells dated from 5 to ~7,000 yBP. We selected shells from clams (*Ruditapes philippinarum*, *Ruditapes decussatus*, *Venerupis corrugata*), abalones (*Haliotis tuberculata*), oysters (*Crassostrea sp.*), scallops (*Pecten maximus*), mussels (*Mytilus sp.*) and ocean quahogs (*Arctica islandica*) for the economic and ecological importance of these species, their common occurrence in European archaeological sites over long time ranges, and their variability in biomineral microstructure (Table 1). We applied both metagenomic and targeted mapping analyses with particular focus on molluscs, bacteria, chlorophytes and trematodes. This work establishes mollusc shells as (ancient) DNA reservoirs for studying (past) species, populations and communities, as well as host/pathogen co-evolutionary arms races.

## 2 | MATERIAL AND METHODS

### 2.1 | Shell sample-set description

We analysed clean marine mollusc shells, that is, deprived of any soft tissue, from archaeological sites and collections (Tables 1 and S1). The specimens were dated between 5 and ~7,000 yBP based on their archaeological context (radiocarbon dates, ceramics, lithic artefacts (Large & Birocheau, 2004; Andersen, 2008; Gissing, 2011; Champagne, Aoustin, & Dupont, 2012; Le Goff & Dupont, 2015)) or collection records. The sample hav\_Mussel was radiocarbon-dated as part of this study at the Kech Carbon Cycle AMS Facility, University California Irvine, leading to a 95.4% probability calibrated range of 6,809–6,962 yBP (UCIAMS#182492, Marine13 calibration curve; Reimer, Bard, & Bayliss, 2013). Diagnostic macroscopic morphological characters were used for identifying species. A number of shells exhibited damage potentially indicative of microbial or trematode infection (Tables 1 and S1). More specifically, *R. philippinarum* Manila clam shells presented growth lesions and dark conchiolin deposits in their inner surface that are typical of the Brown Ring Disease (BRD) caused by the bacterium *Vibrio tapetis* (Paillard, Percelay, Le Pennec, & Le Picard, 1989; Paillard & Maes, 1995). Other clam shells pre-dating the first characterization of BRD (Paillard et al., 1989) also exhibited weak conchiolin deposits.

**TABLE 1** Sample information and sequencing statistics

Sample name	Species name	Geographical location	Date/Age	Inner shell microstructure	Shell diagnostic	#Sequences	#Retained
MODERN							
POS_Clam	<i>Ruditapes philippinarum</i>	Landéda, France	1988 AD	HA	Recovered after acute BRD	36,371,525	33,530,237
KOR_Clam		Cheju, Korea	2003 AD	HA	Acute BRD	35,083,934	34,501,909
AKKp_Clam		Akkeshi, Japan	2012 AD	HA	Abnormal brown deposit	9,709,393	9,640,133
MATp_Clam		Matsusaka, Japan	2012 AD	HA	Weak BRD	18,091,072	18,001,838
SElp_Clam		Seloy, Norway	2003 AD	HA	Weak BRD	8,455,559	8,373,378
LAN1p_Clam		Landéda, France	1983 AD	HA	Brown deposit	10,499,178	10,402,596
LAN2p_Clam		Landéda, France	1983 AD	HA	Brown deposit	13,812,143	13,667,939
LAN3p_Clam		Landéda, France	1983 AD	HA	Brown deposit	7,981,394	7,925,697
MURp_Clam		Murano, Italy	1990 AD	HA	Weak BRD	9,932,924	9,847,558
NEG_Clam	Landéda, France	1988 AD	HA	Healthy	50,373,801	50,694,338	
BRE1_Abalone	<i>Haliotis tuberculata</i>	Brest, France	2003 AD	NA	Healthy	7,506,486	7,447,143
BRE2_Abalone		Brest, France	2005 AD	NA	Healthy	10,795,266	10,739,804
BRE3m_Abalone		Brest, France	2003 AD	NA	Healthy	9,749,078	9,675,009
BRE3p_Abalone		Brest, France	2003 AD	NA	Acute brown deposit and boring	9,184,721	9,123,253
LEF-B_Scallop	<i>Pecten maximus</i>	Le Faou, France	1960–1970 AD	FC	Healthy	20,565,155	20,104,424
ROS-B_Scallop		Roscanvel, France	1997 AD	FC	Healthy	7,080,764	7,040,427
BRE5m_Oyster	<i>Crassostrea</i> sp.	Brest, France	2005–2007 AD	FC	Healthy	11,484,012	11,430,195
BRE5p_Oyster		Brest, France	2005–2007 AD	FC	Acute brown deposit and boring	7,724,821	7,686,285
ANCIENT							
hav_Mussel	<i>Mytilus</i> sp.	Havnø, Denmark	Late Mesolithic ~7,000 yBP <sup>a</sup>	NA	Healthy	11,183,672	10,515,059
auzay1B_Clam	<i>Ruditapes decussatus</i>	Les Châtelliers du Vieil-Auzay, France	Middle Neolithic 6th mill. BP <sup>b</sup>	NA	Healthy	15,240,748	15,030,940
auzay2B_Clam		Les Châtelliers du Vieil-Auzay, France	Middle Neolithic 6th mill. BP <sup>b</sup>	NA	Healthy	11,095,140	10,913,477
auzay3B_Clam		Les Châtelliers du Vieil-Auzay, France	Middle Neolithic 6th mill. BP <sup>b</sup>	NA	Healthy	13,565,581	13,414,246
auzay4B_Clam		Les Châtelliers du Vieil-Auzay, France	Middle Neolithic 6th mill. BP <sup>b</sup>	NA	Healthy	12,191,311	12,008,593
gripB_Clam		La Gripperie-Saint-Symphorien, France	7th century AD <sup>c,d</sup>	NA	Healthy	10,555,462	10,484,321
lmc1B_Clam		La Maison Champlain, Brouage, France	16–17th century AD <sup>e</sup>	NA	Healthy	12,983,981	12,888,532
lmc2B_Clam		La Maison Champlain, Brouage, France	16–17th century AD <sup>e</sup>	NA	Healthy	10,911,865	10,811,007
lmc3B_Clam		La Maison Champlain, Brouage, France	16–17th century AD <sup>e</sup>	NA	Healthy	17,964,100	17,845,554
nis_Clam	<i>Venerupis corrugata</i>	Nissum Bredning, Denmark	1886 AD	NA	Perforations	8,386,634	8,301,630
thi_Clam		Thisted Bredning, Denmark	1909 AD	NA	Healthy	13,947,789	13,868,126
med_Abalone	<i>Haliotis tuberculata</i>	Mediterranean Sea	Before 1804 AD	NA	Healthy	9,823,899	9,699,275
gue_Abalone		Guernsey Island, UK	1832 AD	NA	Healthy	481,087	475,317

(Continues)

TABLE 1 (Continued)

Sample name	Species name	Geographical location	Date/Age	Inner shell microstructure	Shell diagnostic	#Sequences	#Retained
hel_Quahog	<i>Arctica</i>	Hellebæk, Denmark	1886 AD	HA	Healthy	13,089,376	12,674,074
quahog2_Quahog	<i>islandica</i>	sine loc.	Before 1804 AD	HA	Healthy	18,871,776	18,494,781
ves_Quahog		Vestmanna, Faroe Islands	1901 AD	HA	Healthy	8,264,494	8,153,984
Extraction blank controls for samples							
EBC7_Control	auzay1-4B_Clam, gripB_Clam, lmc1-3B_Clam					8,914,833	8,653,416
EBC8_Control	hav_Mussel, gue_Abalone, med_Abalone, ves_Quahog					7,557,184	3,570,529
EBC9_Control	quahog2_Quahog, hel_Quahog					9,974,348	3,146,625
EBC11_Control	nis_Clam, thi_Clam					9,597,406	8,798,083
EBC12_Control	MATp_Clam and MURp_Clam					106,077	74,324

The suffix “p” alone in the sample name indicates that the shell fragment showed signs of infection; “m” no signs of infection.

“AD,” Anno Domini; “yBP,” years Before Present; “mill.,” millenium; “cent.,” century; “HA,” homogeneous aragonite; “NA,” nacreous aragonite; “FC,” foliated calcite; “BRD,” Brown Ring Disease.

<sup>a</sup>Andersen, 2008)

<sup>b</sup>Large & Birocheau, 2004

<sup>c</sup>Gissinger, 2011

<sup>d</sup>Le Goff & Dupont, 2015

<sup>e</sup>Champagne et al., 2012

## 2.2 | Shell sample preparation

For shells dating from the last 60 years (and hereafter referred to as “modern”), DNA extraction was performed on a fragment of the shells’ ventral margin. For older shells (“ancient”), either a fragment or the whole shell was used.

We first assessed the feasibility of recovering DNA from the “modern” marine mollusc shells by analysing both a healthy (NEG\_Clam) and a BRD-positive (POS\_Clam) Manila clam (~27 yBP; Table 1, Fig. S1). Based on positive preliminary results, the impact of shell pre-extraction bleach decontamination was investigated by comparing shotgun HTS data sets for bleach-treated (KOR\_I<sup>-</sup>B<sup>+</sup>\_Clam, KOR\_I<sup>+</sup>B<sup>+</sup>\_Clam) and nontreated (KOR\_I<sup>-</sup>B<sup>-</sup>\_Clam, KOR\_I<sup>+</sup>B<sup>+</sup>\_Clam) shell fragments of a BRD-positive Manila clam (KOR\_Clam; Table 1, Fig. S1). DNA was analysed both from “diseased” (KOR\_I<sup>+</sup>B<sup>-</sup>\_Clam, KOR\_I<sup>+</sup>B<sup>+</sup>\_Clam) and “healthy” (KOR\_I<sup>-</sup>B<sup>-</sup>\_Clam, KOR\_I<sup>-</sup>B<sup>+</sup>\_Clam) fragments of the same shell. Subsequent to this test, we decontaminated the shells/shell fragments through incubation in one 1% bleach volume for 10 min under constant mixing, followed by three washes in three volumes of distilled water (Fig. S1). After drying, the shells were ground to fine powder using a pestle and mortar.

## 2.3 | DNA extraction

DNA was extracted from the shell powder based on the so-called “Y1” method described in (Yang, Eng, Waye, Dudar, & Saunders, 1998; Gamba, Jones, & Teasdale, 2014; Gamba, Hanghøj, & Gauritz, 2016). In short, powder was incubated overnight at 37°C in 15 ml digestion buffer (0.45 mM EDTA, 0.5% N-laurylsarcosyl, 0.25 mg/ml proteinase K) under constant mixing. After centrifugation (4,000 g; 2 min), the supernatant was filtered through an

Amicon Ultra-15 30 kDa centrifugal filter unit (Merck Millipore; 4,000 g; 50 min). DNA was then purified using the MinElute PCR Purification Kit (QIAGEN) in a final elution volume of 60 µl EB buffer.

## 2.4 | Library construction and sequencing

Extracts were built into blunt-ended DNA libraries for Illumina shotgun sequencing based on the protocol described in Seguin-Orlando, Schubert, and Clary (2013) and Orlando et al. (2013). We used the NEBNext Quick DNA Library Prep Master Mix Set for 454 (New England Biolabs) in 50 µl for end-repair and ligation (0.5 µM Illumina adapter final concentration) and in 25 µl for fill-in. End-repair was carried out at 12°C for 20 min and at 37°C for 15 min, ligation at 20°C for 20 min, fill-in at 37°C for 20 min followed by *Bst* enzyme inactivation at 80°C for 20 min. We used the MinElute kit for purification after end-repair and ligation with EB buffer elution volumes of 30 and 20 µl, respectively.

Prior to Illumina shotgun sequencing, the libraries were amplified by PCR. The number of cycles required to recover DNA library amounts sufficient for sequencing, while minimizing library clonality, was estimated on a per sample basis by qPCR carried out in duplicates in 20 µl of the following reaction mix: 1 µl 1/20 diluted DNA library (in EB buffer), 0.1 units Taq Gold, 1× Gold Buffer, 4 mM MgCl<sub>2</sub> (Life Technologies), 1 mg/ml BSA, 0.25 mM of each dNTP, 0.2 µM of Primer inPE1.0 (5'-AATGATACGGCGACCACCGAGATC TAC-ACTCTTCCCTACACGACGCTCTCCGATCT-3') and 0.2 µM of an Illumina 6 bp-indexed (‘I’) primer (5'-CAAGCAGAAGACGGCA TACGAGATIIIIIGTGACTGGAGTT-CAGACGTGTGCTCTTCCG-3'), and 0.8 µl of 1:4:2,000 ROX:Sybr:DMSO DNA dye mix (Thermo-Fisher Scientific). Thermo-cycling conditions were: activation at 92°C

for 10 min; 40 cycles of: denaturation at 92°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s; final elongation at 72°C for 7 min. qPCRs were performed on a LightCycler 480 Real-Time PCR System instrument (Roche Applied Science). A relative measure of DNA concentration for each library was provided by the Ct value estimated using the second derivative maximum method with the LightCycler Software 4.0. DNA contamination from the laboratory reagents/environment was controlled by analysing blanks at the same time as samples during extraction (Extraction Blank Control, EBC), library building and qPCR. Extraction success rates were calculated considering an extraction successful when a library's qPCR Ct value was inferior to the Ct value minus 7 of the EBC from the corresponding extraction session ( $\geq 128$ -fold concentration compared to EBCs). Only modern shell DNA libraries fulfilling this condition were selected for shotgun sequencing. As this was not satisfied for most ancient shell DNA libraries, those obtained from their corresponding EBCs were independently PCR-amplified and indexed with unique external barcodes, before being pooled and sequenced with the amplified ancient shell DNA libraries (Table S1).

Each library was PCR-amplified using one identifying indexed Illumina primer (Table S1). Library amplification was performed in the same conditions as for qPCR, except that 5  $\mu$ l of undiluted DNA extract was added to 20  $\mu$ l of the qPCR mix without ROX: SybR:DMSO, and that the number of cycles was derived from Ct values for each library (6–21 for shell DNA libraries, 15–29 for EBC libraries; Table S1). After MinElute purification and elution in 25  $\mu$ l EB buffer, the amount of DNA in the amplified indexed libraries was quantified on a 2200 TapeStation instrument (High Sensitivity D1000 ScreenTape, Agilent). DNA amounts in blanks (except EBCs amplified for sequencing) were below the detection threshold of the instrument, in line with low levels of DNA contamination during laboratory work. The indexed DNA libraries were pooled and sequenced in either paired-end or single-end mode on the Illumina HiSeq2000 and MiSeq platforms at the Danish National High-Throughput DNA Sequencing Centre (Table S1).

## 2.5 | DNA sequence read processing and mapping

Postsequencing read processing and mapping were carried out using PALEOMIX (Schubert, Ermini, & Der Sarkissian, 2014), including adapter trimming with ADAPTERREMOVAL2 (Schubert, Lindgreen, & Orlando, 2016), collapse of overlapping pair-end mates as described in (Orlando et al., 2013) and read mapping against reference DNA sequences (see below and Tables S2–S6) with BWA v0.5.9 (Li & Durbin, 2009). Seeding was disabled for mapping, as recommended in Schubert, Ginolhac, and Lindgreen (2012), and only reads showing mapping qualities  $\geq 30$  were kept. PCR duplicates were removed with MARKDUPLICATES in PICARD TOOLS version 1.119 (<http://broadinstitute.github.io/picard/>) for noncollapsed reads, and the PALEOMIX FILTERUNIQUEBAM PYTHON script (Schubert et al., 2014) for collapsed reads.

## 2.6 | Metagenomic analyses using DIAMOND

To characterize the total DNA content of marine mollusc shells, we used the metagenomic program DIAMOND v0.8.17 (Buchfink, Xie, & Huson, 2015) and the BLAST “nr” protein database (downloaded on the 27/07/2016) with default parameters and the “-sensitive” alignment mode (Fig. S1). For POS\_Clam and NEG\_Clam, non-collapsed paired-end reads were disregarded to limit the impact of potential contaminating modern DNA, building on the expectation that these would be longer than damaged endogenous DNA molecules. Taxonomic classification was then performed by the program DAA-MEGANIZER distributed with MEGAN6 (Huson, Auch, Qi, & Schuster, 2007; Huson, Mitra, Ruscheweyh, Weber, & Schuster, 2011) using the weighted lowest-common algorithm (option “-wlca”) and only keeping the top-1% hits (“-top 1.0”) showing sequences identical to database markers (“-mpi 100.0”; Table S7).

## 2.7 | Mollusc DNA reference sequences

The presence of mollusc nuclear DNA was examined by mapping shotgun HTS reads to the genome sequences published for the Pacific oyster *Crassostrea gigas* (Zhang, Fang, & Guo, 2012) and the blue mussel *Mytilus galloprovincialis* (Murgarella, Puiu, & Novoa, 2016), as well as to sets of transcripts for the abalone *H. tuberculata* (Harney, Dubief, & Boudry, 2016), the scallop *P. maximus* (Pauletto, Milan, & Moreira, 2014; Artigaud, Thorne, & Richard, 2014; Artigaud, Richard, & Thorne, 2015) and *M. galloprovincialis* (Moreira, Pereiro, & Canchaya, 2015; Fig. S1, Table S2). For the clam *R. philippinarum*, transcript sequences were collected from the Ruphibase database (<http://compgen.bio.unipd.it/ruphibase/>), the NCBI EST database (<https://www.ncbi.nlm.nih.gov/nucest/>) and previous publications (Milan, Coppe, & Reinhardt, 2011; Moreira, Balseiro, & Planas, 2012; Moreira, Balseiro, & Romero, 2012; Ghiselli, Milani, & Chang, 2012; Allam, Pales Espinosa, & Tanguy, 2014; Table S3).

Mollusc mtDNA content was estimated by mapping shotgun HTS reads to two reference sequence databases. The first database contained 52 mtDNA genomes for the mollusc species represented in our sample-set, or closely related species (Fig. S1, Table S2). To account for the circularity of mtDNA genomes, the first 30 bp in each reference sequence were manually duplicated and added at the end of the corresponding sequence as in (Vilstrup, Seguin-Orlando, & Stiller, 2013). For each sample, DNA reads were mapped to each reference sequence independently in order to control for false-positive species identification. The second mtDNA database contained sequences of the cytochrome *c oxidase subunit I* gene 5'-extremity (COI5P), for which 88,453 barcodes are available for the Mollusca phylum in the Barcode Of Life Database (<http://www.boldsystems.org/>; Fig. S1). Hits to the database were reported as the total read length mapping to all the markers for a given taxon; in cases where this length was <100 bp, the corresponding taxa were excluded.

## 2.8 | *Vibrio* DNA reference sequences

We next assessed the potential of shell DNA for the molecular identification of *V. tapetis* in BRD-positive shells. We mapped shotgun HTS reads to genomic sequences of two *V. tapetis* strains, RP2-3 and HH6087, generated as part of this study (Fig. S1, Table S4) and isolated respectively from a BRD-positive *R. philipinarum* clam in France in 1990 (Paillard, Maes, & Oubella, 1994; Borrego, Castro, & Luque, 1996), and from a healthy Atlantic halibut (*Hippoglossus hippoglossus*) in Scotland in 2002 (Reid, Duncan, Laidler, Hunter, & Birkbeck, 2003). DNA was extracted following (Bidault, Richard, Le Bris, & Paillard, 2015). Shotgun HTS reads were obtained by applying the protocol described above on 1 µg of DNA extract sheared using a diagenode Bioruptor ultrasonicator (four cycles of: 15 s at high intensity; 90 s off), and amplifying libraries for seven cycles before single-end sequencing on an Illumina HiSeq2000 platform at the Danish National High-Throughput DNA Sequencing Centre. Postsequencing, the adapters were removed using ADAPTERREMOVAL v2.1.5 (Schubert et al., 2016). De novo assembly was performed using the assembler SPADES v3.7.0 (Bankevich, Nurk, & Antipov, 2012), using four k-mer sizes (21, 33, 55 and 77), and allowing for mismatch correction. Only contigs ≥300 bp in length were kept for further analyses.

To verify that shell HTS reads mapping to the *V. tapetis* reference sequences belonged to the *Vibrio* genus, they were aligned to the BLAST “nt” nucleotide database (downloaded on 11/10/2016) using the BLAST2 aligner (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho, Coulouris, & Avagyan, 2009), followed by MEGAN6 analyses as described above.

To rule out potential false-positive identification of *Vibrio* species, shell HTS shotgun reads were also mapped to 63 genomic sequences corresponding to one representative sequence per *Vibrio* species so far characterized at the genome level (Table S4). The obtained mapping patterns were compared to those expected when mapping reads deriving from each *Vibrio* genome against every other *Vibrio* reference sequence. This was performed by simulating 101 bp HiSeq2000 single-stranded reads from each *Vibrio* genomic sequence using ART (version Mount-Rainier (Huang, Li, Myers, & Marth, 2012)). The number of reads simulated for each *Vibrio* sequence corresponded to a  $\sim 7 \times$  average depth-of-coverage, the maximal coverage obtained when mapping shell HTS DNA reads to *V. tapetis* reference sequences.

## 2.9 | Chlorophyte, trematode and human DNA reference sequences

We attempted to confirm the results of the DIAMOND metagenomic analyses identifying Chlorophyta green algae in shells, by mapping shotgun HTS reads to (i) the complete mtDNA and chloroplast genomes of *Pseudoclonium akinetum* (GenBank Accession nos AY359242 and AY835431), (ii) the complete chloroplast genomes of *Bryopsis plumosa*, *Phacotus lenticularis* (LN810504, KT625422) and (iii) the complete plastid genome of *Chlorella variabilis* (HQ914635; Table S5). We also investigated the presence of trematode DNA in shells through HTS read mapping to the complete mtDNA sequences

of the candidate trematodes identified by DIAMOND (with the exception of *Opisthorchis viverrini* for which no comparative data are available): *Clonorchis sinensis* (FJ381664), *Schistosoma mansoni* (HE601612), *S. haematobium* (DQ157222), *S. japonicum* (HM120841), *S. mekongi* (NC002529) and *S. spindale* (DQ157223; Table S6). Human contamination was estimated by mapping HTS reads to the *Homo sapiens* complete nuclear and mtDNA reference genomes (assembly hg19 build 37.1).

## 2.10 | Postmortem DNA damage profiles

DNA fragmentation, base misincorporation and fragment size distributions in shell DNA were assessed using MAPDAMAGE v2.0.1 (Jónsson, Ginolhac, Schubert, Johnson, & Orlando, 2013) and 100,000 MCMC iterations. Damage was investigated for mollusc, *V. tapetis*, chlorophyte and trematode DNA, considering both all reads and randomly sampling from bam files the minimum number of sequencing reads mapped across samples for a given marker. For each marker and each sample yielding an accurate taxonomic assignment, we used as input file the bam alignment for which the maximal coverage was obtained (Tables S8–S10). We estimated the following damage parameters: rates of deamination in double-strands ( $\delta_D$ ) and single-strands ( $\delta_S$ ), probability of reads not terminating in overhangs ( $\lambda$ , transformed into  $1/\lambda - 1$ , a proxy for the length of overhanging regions). We then contrasted nuclear and mtDNA damage parameters for each single ancient sample against modern samples. We compared the proportion of MAPDAMAGE iterations belonging to the damage parameter's 97.5% confidence interval in ancient samples that overlapped the 97.5% confidence interval of modern samples, considering each mollusc species individually or the whole set of species altogether. This number was reported as the percentage of overlap between the damage parameter 97.5% confidence interval for the considered ancient sample and modern samples (Table S11).

## 2.11 | Microbial profiling using METABIT

The microbial content of shell DNA extracts was characterized using METABIT; (Louvel, Der Sarkissian, Hanghøj, & Orlando, 2016; Table S7). As in MEGAN analyses, noncollapsed paired-end reads were disregarded for POS\_Clam and NEG\_Clam. Microbial taxon assignment and relative abundance calculation were carried out by first aligning shotgun reads to the METAPHLAN2 database (Segata, Waldron, & Ballarini, 2012; Truong, Franzosa, & Tickle, 2015) using the BOWTIE2 v2.1.0 aligner (Langmead & Salzberg, 2012). PCR duplicates were then removed as described above.

For comparison purposes, shotgun data from 60 seawater samples (deep chlorophyll maximum,  $N = 20$ ; mesopelagic layer,  $N = 14$ ; surface,  $N = 26$ ; Table S12), all published by the Tara Oceans Expedition (Sunagawa, Coelho, & Chaffron, 2015), were profiled using metaBIT as detailed above, except that all reads, including paired-reads, were considered in the analyses. We also used the comparative microbial profiles distributed with metaBIT including 689 human

(Human Microbiome Project Consortium 2012) and 15 soil samples (Fierer, Leff, & Adams, 2012; Louvel et al., 2016).

Statistical analyses of the METAPHLAN2 profiles (Truong et al., 2015) were performed in metaBIT, only considering taxa showing abundances  $\geq 1\%$  in order to reduce noise due to false-positives.

### 3 | RESULTS

#### 3.1 | Preliminary analyses and impact of bleach decontamination

DNA amounts compatible with shotgun HTS could be extracted from the 27-year-old shells of the Manila clams NEG\_Clam and POS\_Clam. For both samples, metagenomic comparison of the shotgun HTS reads with the BLAST *nr* database using DIAMOND and MEGAN6 revealed that most reads were assigned to the Bacteria kingdom (Figure 1a). A fraction (1.3%–4.0%) of these bacterial reads was assigned to the *Vibrio* genus for both shells, and to the *V. tapetis* species for the BRD POS\_Clam only (2.4%; Figure 1a). HTS reads mapped to the genome sequences of the two *V. tapetis* strains HH6087 and RP2-3 with a 29–369-fold enrichment (after sequencing effort normalization) in POS\_Clam compared to NEG\_Clam (Figure 1b). This suggests that DNA assigned as *V. tapetis* can be retrieved from BRD-infected marine mollusc shells, that it does not arise from contamination, and that other *Vibrio* species might represent members of the healthy shell microbiota (Lokmer & Wegner, 2015; Lokmer, Goedknecht, & Thielges, 2016). Within Mollusca, the most assigned eukaryotic phylum (Figure 1a), we identified the expected *R. philippinarum* species by mapping to transcript (0.4–2.0 $\times$  maximal coverage) and mtDNA reference sequences (3.1–29.0 $\times$ ; Figure 1b, Table S8). The data sets contained minimal fractions of DNA arising from contamination during collection, analyses, curation and/or sampling, as 0.17% (NEG\_Clam) and 0.12% (POS\_Clam) of the HTS reads (Figure 1b) mapped to the human reference sequence.

Bleach decontamination prior to DNA extraction of KOR\_Clam reduced the fraction of reads mapping to the human genome (0.03% in bleached vs. 0.05%–0.49% in nonbleached shells; Figure 1b) and being assigned to the Bacteria kingdom (Figure 1a). This, however, preserved mollusc DNA content (Figure 1b) with a sum of 1.39%–3.82% of the reads mapping to the *R. philippinarum* transcripts and mtDNA genomes in bleached shells vs. 2.68%–2.96% in nonbleached shells. This was true even after correcting for the amount of shell powder used (0.05%–0.09% in bleached vs. 0.07%–0.13% in nonbleached samples). For the two BRD-positive fragments, bleach treatment did not result in a loss of reads mapping to the genome of *V. tapetis* RP2-3 (3.22% in bleached vs. 1.83% in nonbleached infected shells; both 0.06% when correcting for the amount of shell powder used for extraction; Figure 1b). Finally, bleach treatment did not increase DNA damage levels (estimated rates of deamination and overhang length) in mollusc and *V. tapetis* DNA (Fig. S2). As bleach treatment only impacted exogenous DNA sources and maintained host and pathogenic DNA content, we

applied pre-extraction bleach decontamination in all subsequent analyses.

#### 3.2 | DNA extraction success rate

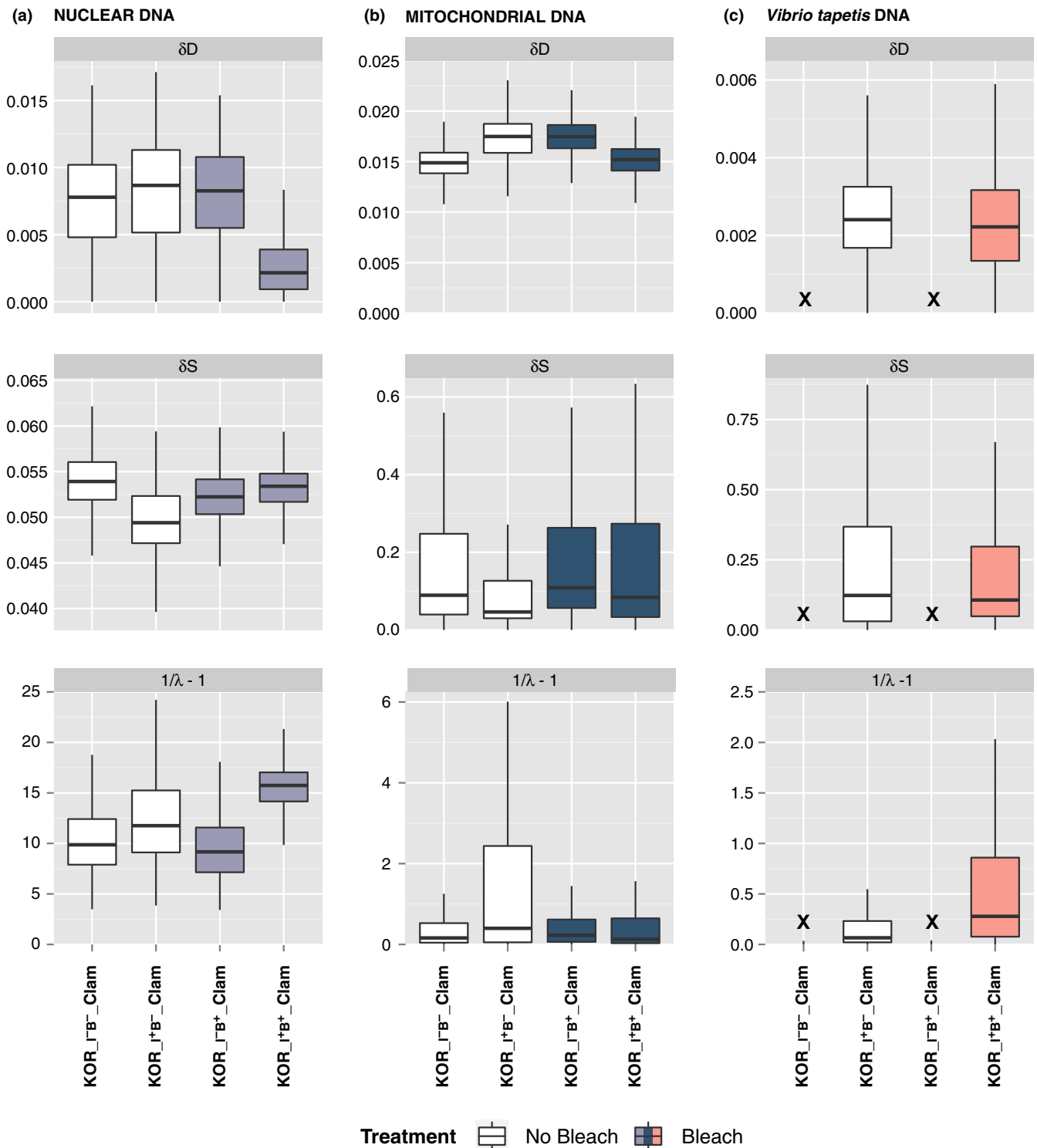
DNA extraction success rates were variable across sample age and species. In modern samples, we could successfully extract DNA from 16 of 21 shells (76%), which included all those for clams (11 of 11) and abalones (three of three), but a limited number of shells for scallops (two of five) and one diseased oyster (one of three). Ancient samples yielded a lower success rate of five of 17 (29%), with successful extraction for two of 10 clams, none of the 2 mussels, 2/2 abalones and 1/3 ocean quahogs.

#### 3.3 | Metagenomic content

Metagenomic analyses revealed that the most abundant kingdom in the majority of HTS data sets was Bacteria followed by Eukaryota, Archaea and viruses (Figure 2a). Mollusca was the most abundant eukaryotic phylum (Figure 2b), except for two samples showing highest abundances for Chlorophyta green algae, Phaeophyceae brown algae, Bacillariophyta diatoms, Basidiomycota fungi and Nematoda. Importantly, Mollusca were virtually absent from all EBCs, suggesting no contamination from exogenous mollusc DNA. Within Mollusca, success rates were variable when attempting to identify, based on maximum number of hits per sample, morphologically defined mollusc genera (Figure 2c): 20%–100% for modern and 0%–100% for ancient shells (Table 2) when considering the totality of samples extracted, including those not sequenced. All misidentified specimens, but no EBCs, were assigned to *Mytilus*, suggesting misidentification likely due to the over-representation of *Mytilus* markers in the *nr* database (i.e., 3,090 vs. 428 for *Ruditapes*, 21 for *Venerupis* and 43 for *Arctica*) rather than to contamination. Within Chlorophyta, only POS\_Clam and BRE5m\_Oyster led to taxonomic identification at the genus level (*Pseudendoclonium*, *Ostreobium*, *Chlorella*, *Bryopsis*, *Phacotus*, *Derbesia* and *Caulerpa*; Figure 2d). We also investigated the presence of DNA from trematodes as possible parasites, and found, in fifteen shells, high-quality hits against *Schistosoma mansoni*, *Clonorchis sinensis* and *Opisthorcis viverrini* (Figure 2e).

#### 3.4 | Microbial profiling

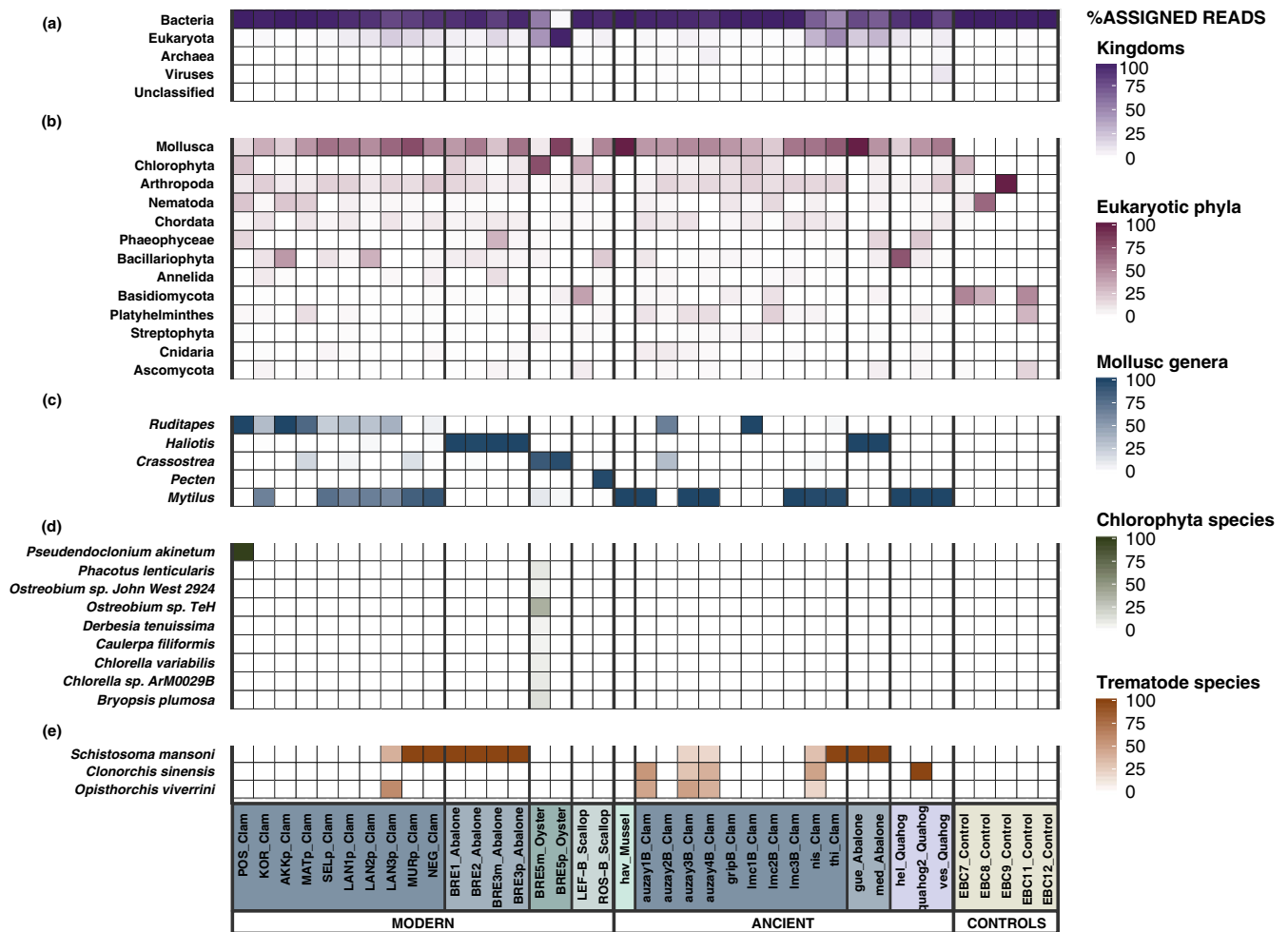
We further characterized the microbial content of shell HTS data sets using METABIT. Principal coordinate analysis (PCoA) of Bray-Curtis distances at the genus level showed that the microbial diversity recovered from the marine mollusc shells fell outside the human microbiome diversity (Figures 3a and S3A). Two samples, hel\_Quahog and NEG\_Clam, however showed affinities for the human skin/nose microbiomes, due to elevated abundances in the skin-associated bacteria *Propionibacterium acnes* (42.8% and 54.6%), suggesting human-derived contamination in these samples that also showed maximal levels of human DNA (0.21% and 0.62%; Fig. S4). Although



**FIGURE 1** Preliminary tests: taxon detection from bleached and nonbleached modern shells. (a) Metagenomic analyses: number of BLAST *nr* database hits, normalized, for each sample, by the total number of reads retained after quality filtering (retained reads). (b) Targeted mapping: coverage is calculated considering the total number of unique high-quality (Mapping Quality,  $MQ \geq 30$ ) reads, normalized, for each sample, by the total number of retained reads. “I<sup>-</sup>B<sup>-</sup>”, noninfected nonbleached fragment; “I<sup>+</sup>B<sup>-</sup>”, infected nonbleached fragment; “I<sup>-</sup>B<sup>+</sup>”, noninfected bleached fragment; “I<sup>+</sup>B<sup>+</sup>”, infected bleached fragment

this result can be explained by the absence of pre-extraction decontamination for NEG\_Clam, it also exemplifies the limit of our bleach decontamination procedure for hel\_Quahog.

Marine mollusc shell microbial profiles showed most affinity with those of soils, and segregated from the seawater microbial diversity (Figures 3b and S3B), probably not representative of the molluscs’



**FIGURE 2** Taxon detection by metagenomic analyses. The colour gradient represents the proportion of the total number of assigned reads that were assigned to taxa for each taxonomic level

mostly coastal environment. METABIT microbial profiling most probably suffers from high false-negative rates due to the limited diversity of environmental microbe markers present in the METAPHLAN database (Louvel et al., 2016). Although bacterial species typical of marine seawaters and sediments could be identified (Fig. S5), our results call for a thorough shotgun HTS-based characterization of the microbiomes in mollusc tissues (Desriac, Le Chevalier, & Brillet, 2014; Lokmer & Wegner, 2015; Meisterhans, Raymond, & Girault, 2015; Lokmer et al., 2016) and in coastal environments (Mustafa, Abd-Elgawad, Ouf, & Siam, 2016). This would improve the resolution power in profiling shell microbial sources, and help understand the interplay between molluscs and climatic changes, pollutants and emerging pathogens.

### 3.5 | Molecular identification of molluscs

After mapping shell shotgun reads against a comparative sequence database for molluscs, taxonomic identification was considered positive when maximal coverage was obtained for a reference sequence representing the expected genus for a given sample.

Based on nuclear data and mtDNA genomes, most modern samples could be taxonomically assigned to the right genus, and sometimes even species, (Figures 4a, b, Tables 2 and S8) with depths-of-coverage of 0.006–2.008 $\times$  and 2.057–54.142 $\times$ , respectively. Standing as exception, MURp\_Clam was misassigned to *Mytilus trossulus* based on mtDNA genome mapping (0.057 $\times$  maximal coverage), as was LEF-B\_Scallop (0.010 $\times$ ) also misassigned to *H. tuberculata* based on nuclear data (0.003 $\times$ ). Both samples might show low mollusc DNA content, probably leading to spurious mapping also observed in EBCs. Genus identification using mtDNA COI5P barcodes was also robust, with genus assignment success for 10 of 10 clams, three of three abalones, one of one oyster and one of two scallops (Figure 4c, Table 2). Of note, MURp\_Clam, initially labelled as *R. philippinarum*, was identified as *R. decussatus* based on COI5P barcodes, which is in line with the pattern of coverage observed when mapping against mtDNA genomes in the absence of reference sequences for *R. decussatus*. Reanalysis of the shell morphology did not dismiss the identification of MURp\_Clam as *R. decussatus*.

Mollusc taxonomic identification success was more variable for ancient shells. Considering nuclear reference sequences (Figure 4a,

Tables 2 and S8), none could be characterized at a coverage  $\geq 0.1\times$  for any ancient sample, except med\_Abalone (0.429 $\times$ ). For ten samples, however, maximal coverage (0.001–0.039 $\times$ ) was obtained for the correct genus. Spurious mapping to the *P. maximus* (0.003 $\times$ ) and *H. tuberculata* (<0.001 $\times$ ) transcripts was observed in EBCs, as well as in samples with low endogenous mollusc DNA content (gripB-Clam and lmc2B-Clam; 0.001 $\times$ ), and/or for which comparative nuclear data from a closely related species were not available (hel\_Quahog, quahog2\_Quahog, and ves\_Quahog; 0.001–0.004 $\times$ ). Mapping shotgun reads to mtDNA genomes, mollusc taxa could be positively identified for all ancient samples (0.223–6.665 $\times$  coverage), except clams. For hav\_Mussel, maximal coverage was obtained for the complete mtDNA genome of *M. galloprovincialis* AY497292 (0.223 $\times$ ), but relatively high coverage (0.145–0.215 $\times$ ) was also recovered for *M. trossulus* (DQ198231, KM192124–28, KM192130–32), *Mytilus edulis* (AY484747) and *M. galloprovincialis* (FJ890849, DQ399833), which all form a monophyletic clade of Baltic Sea taxa (Zbawicka, Wenne, & Burzyński, 2014). The misassigned shells were the *R. decussatus* and *V. corrugata* clams identified as *M. trossulus* or *M. edulis* due to spurious mapping (0.007–0.047 $\times$ ), similar to that

observed in EBCs (0.002–0.007 $\times$ ). This could be explained by low endogenous mollusc content and/or by the lack of comparative mtDNA genome sequences for these species (Figure 4b, Tables 2 and S8). Except for hav\_Mussel, positive genus identification using mitochondrial COI5P barcodes was successful for all ancient samples, when considering *Ruditapes* and *Venerupis* as closely related genera (Figure 4c, Tables 2 and S8).

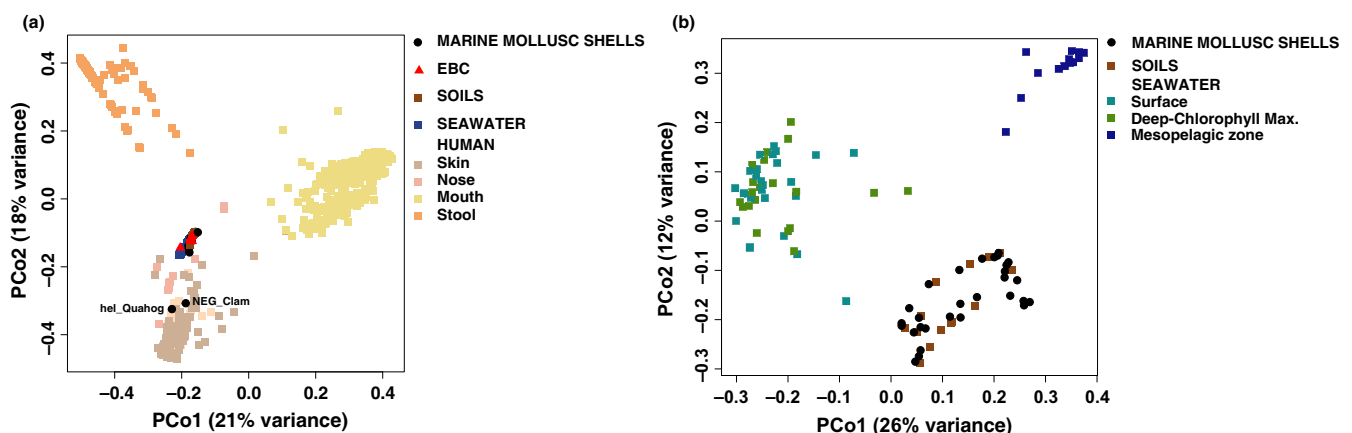
### 3.6 | Molecular identification of *Vibrio tapetis* in shells of infected Manila clams

We next aimed at identifying the *V. tapetis* species in BRD-positive Manila clam shells, using read alignment against the currently known diversity of *Vibrio* genomes (Figures 5 and S6, Table S9). Maximal coverage across all *Vibrio* species was obtained for the *V. tapetis* strain RP2-3 in the shells showing signs of acute BRD: POS\_Clam (7.322 vs. 1.932 $\times$  for *V. tapetis* strain HH6087) and KOR\_Clam (4.195 vs. 1.095 $\times$ ). In Manila clam shells pre-dating the first description of BRD and showing signs of infection, maximal coverage was obtained for the other *V. tapetis* strain HH6087: LAN1p\_Clam

**TABLE 2** Mollusc taxon identification success rates

Mollusc taxa	Groups	Success rates across all samples % (sequenced only)			
		Metagenomics	Nuclear DNA	mtDNA genomes	mtDNA barcodes
Clams	Modern	33 (33)	100 (100)	90 (90)	100 (100)
Abalones		100 (100)	100 (100)	100 (100)	100 (100)
Oysters		33 (100)	33 (100)	33 (100)	33 (100)
Scallops		20 (50)	20 (50)	20 (50)	20 (50)
Mussels	Ancient	100 (100)	100 (100)	100 (100)	0 (0)
Clams		100 (100)	80 (80)	0 (0)	100 (100)
Abalones		20 (10)	100 (100)	100 (100)	100 (100)
Quahogs		100 (100)	0 (0)	100 (100)	100 (100)

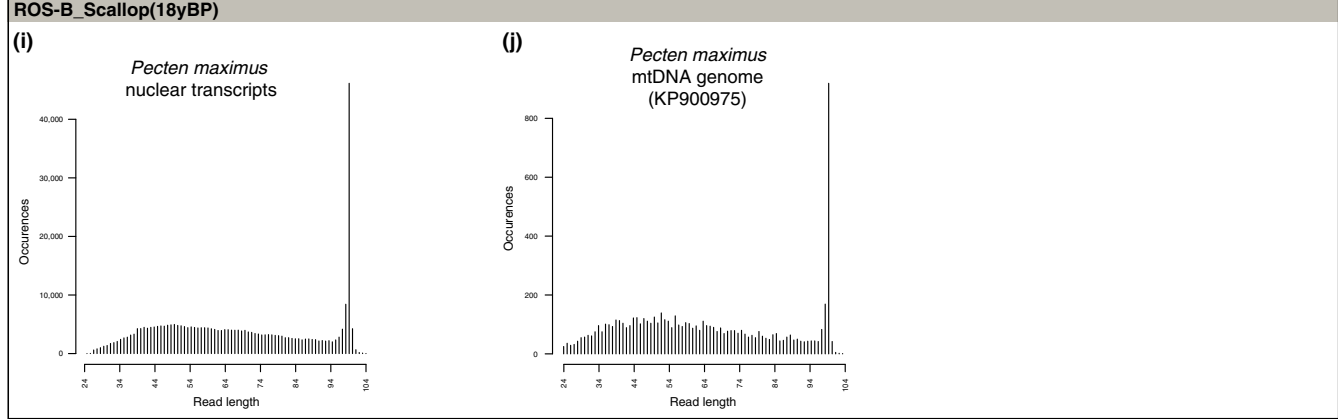
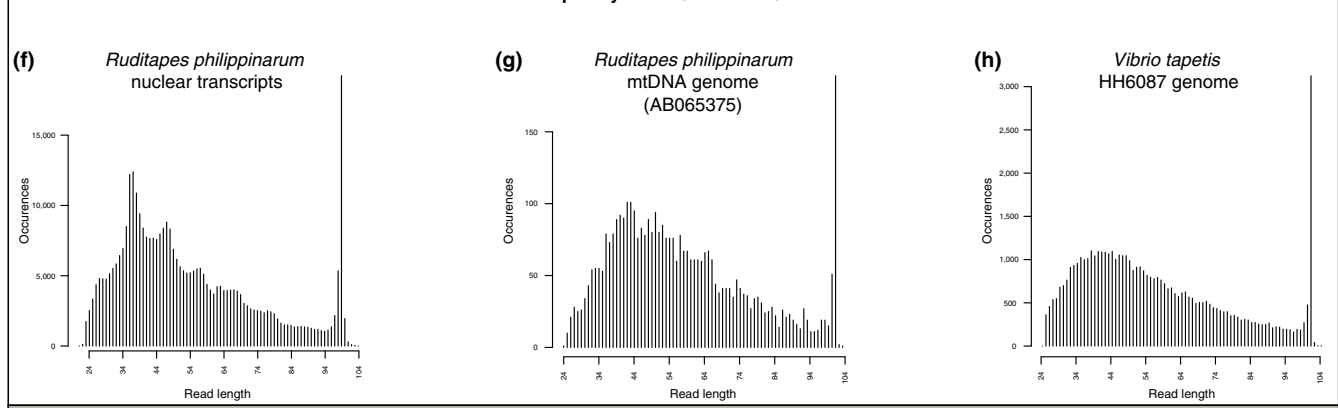
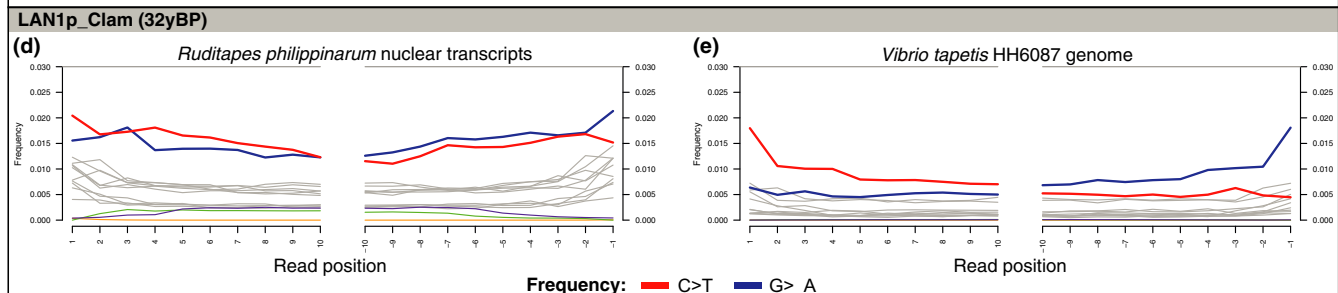
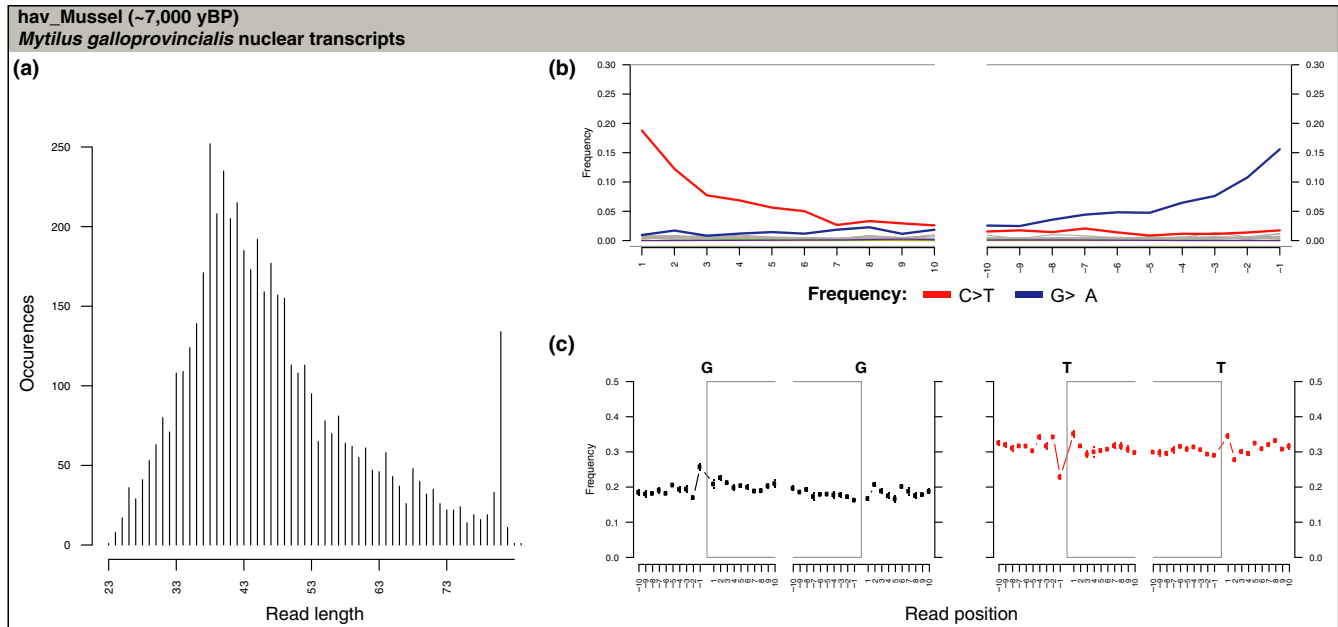
"mtDNA" mitochondrial DNA.



**FIGURE 3** Microbial diversity in shells, seawater, soil and human-associated samples. PCoA of Bray-Curtis distances between genus-level microbial profiles. (a) Considering all taxa. (b) Considering only taxa absent from EBCs and after re-adjusting relative abundances







Meyer, & Paabo, 2013; Figures 6 and S11, Table S10). C → T substitution rates at the 5'-end of ancient nuclear mollusc DNA fragments varied from 2.2% in the 106-year-old thi\_Clam to 16.8% in the ~5,000-year-old auzay1B\_Clam. In modern samples, these rates varied from 1.4% in the 27-year-old NEG\_Clam to 3.0% in the 10-year-old BRE5m\_Oyster. In clams, where sample sizes allow the comparison, 5'-C→T rates were significantly higher than those observed in modern shells (median(ancient) = 10.1%; median(modern) = 1.8%; Wilcoxon test  $p$ -value =  $4.57 \times 10^{-5}$ ; Figures 6, S11 and S12, Table S10).

Some ancient samples showed significant differences with modern samples when considering MAPDAMAGE estimates of deamination in double-strands ( $\delta_D$ ) and single-strands ( $\delta_S$ ) and of the length of overhanging ends ( $1/\lambda - 1$ ). For nuclear mollusc DNA, the range of overlap between the 97.5% confidence interval for all modern samples and the 97.5% confidence interval for each ancient shell was 6.4%–99.4% for  $\delta_D$ , 9.7%–97.7% for  $\delta_S$  and 12.4%–97.9% for  $1/\lambda - 1$  (Table S11; Fig. S13). For mollusc mtDNA, this overlap was increased with ranges of 62.9%–97.7% for  $\delta_D$ , 63.1%–98.8% for  $\delta_S$ , 47.8%–99.3% for  $1/\lambda - 1$  (Table S11; Fig. S14). For clams and abalones, where damage parameter distributions for ancient and modern samples of the same species can be compared, we obtained overlap values similar to those calculated considering all modern samples (Table S11). Compared to  $\delta_D$ ,  $\delta_S$  was 4.1- to 35.3-fold higher in ancient mollusc nuclear DNA and 10.9- to 107.3-fold higher in ancient mollusc mtDNA (Table S10). These damage-driven patterns are characteristic of ancient DNA and reflect the variability in preservation of DNA molecules recovered from shells.

Nucleotide misincorporation typical of ancient DNA was also present in the *V. tapetis* DNA recovered from 12–32 year-old BRD-positive Manila Clam shells, with C → T rates at 5'-ends varying from 0.8% in POS\_Clam to 1.7% in LAN1p\_Clam and LAN2p\_Clam (Figures 6 and S15). In *V. tapetis* DNA,  $\delta_S$  estimates were 9.8- to 42.2-fold higher than  $\delta_D$ , as expected (Table S10 and Fig. S16).

Third, we observed various levels of damage-driven DNA fragmentation across the data set. Ancient shells showed high fragmentation with average 43–50 bp mollusc DNA fragment sizes (hav\_Mussel, auzay1B\_Clam, auzay2B\_Clam, auzay3B\_Clam, auzay4B\_Clam, quahog2\_Quahog, ves\_Quahog), intermediate fragmentation (lmc1B\_Clam, lmc3B\_Clam med\_Abalone, nis\_Clam, ves\_Quahog, thi\_Clam) and long fragment sizes (gue\_Abalone; Figures 6 and S17). In mollusc nuclear DNA from modern shells, low fragmentation was observed in LEF-B\_Scallop, ROS-B\_Scallop, BRE1\_Abalone, BRE3m\_Abalone, KOR\_Clam, BRE2\_Abalone, BRE5m\_Oyster and BRE5p\_Oyster (Figures 6 and S18). Interestingly, mollusc nuclear DNA fragment size distributions in LAN1p\_Clam, LAN2p\_Clam, LAN3p\_Clam, SELp\_Clam, BRE3p\_Clam, AKKp\_Clam and MATp\_Clam showed a ~10 bp periodicity previously proposed to reflect nucleosome protection (Pedersen, Valen, & Velazquez, 2014; Figures 6 and S18). This is supported by this periodicity being absent from mollusc mtDNA and *V. tapetis* DNA (Figures 6; S15 and S19), which are not associated with histones.

### 3.8 | Molecular detection of trematodes

We could not robustly confirm the DIAMOND-based metagenomic identification of trematodes by mapping shell shotgun HTS reads against complete mtDNA sequences. Only one sample, LAN1p\_Clam, showed coverage above 0.10× (0.14× to the *S. mekongi* reference sequence), but no DNA damage signal. No reads from nis\_Clam mapped against the *S. mansoni* reference sequence, despite the fact that the shell exhibited perforation lesions proposed to be caused by trematodes, and that *S. mansoni* was detected in metagenomic analyses. For BRE5m\_Oyster and BRE5p\_Oyster showing signs compatible with trematode attack, no reads mapped to any trematode reference sequence.

### 3.9 | Molecular detection of chlorophytes

The putative presence of chlorophytes indicated by DIAMOND was supported only marginally for *C. variabilis* and *B. plumosa* when mapping shell shotgun HTS reads to their complete plastid and chloroplast reference genome sequences, respectively. For *C. variabilis*, a slight enrichment (after normalizing for sequencing effort) was observed compared to EBCs (<0.001–0.039×) for POS\_Clam (0.707×; 4.6-fold enrichment), BRE5m\_Oyster (0.270×; 5.1-fold enrichment), MATp\_Clam (0.170×; 2.0-fold enrichment), hel\_Quahog (0.124×; 2.1-fold enrichment) and AKKp\_Clam (0.090×; 2.0-fold enrichment). For *B. plumosa*, POS\_Clam (0.486×; 4.2-fold enrichment), BRE5m\_Oyster (0.427×; 10.8-fold enrichment) and hel\_Quahog (0.09×; 2.1-fold enrichment) showed higher depths-of-coverage than in EBCs (<0.001–0.027×). The low number of unique high-quality DNA reads mapping to these chlorophyte reference sequences was not compatible with robust identification of damage authentication signatures in MAPDAMAGE.

## 4 | DISCUSSION

### 4.1 | Long-term DNA preservation in marine mollusc shell biominerals

Here, we demonstrate that authentic endogenous DNA can be extracted from the shell of marine molluscs, including ancient specimens covering the last ~7,000 years. The mollusc species investigated did not show the same DNA extraction success rates, owing to their different biomineral microstructures, preservation and disease state. DNA extraction was more successful for clams, abalones and ocean quahogs, with a shell inner layer composed of homogeneous or columnar nacreous aragonite, than oysters and scallops, both characterized by a foliated calcite inner shell microstructure. Although the presence of nucleic acids in mollusc shells has not been documented to date, differences in DNA content could be due to differences in shell organic content, as nacre and prism microstructures contain a larger organic component (~1% in weight) than crossed-lamellar foliated microstructures (Marin et al., 2012). Additionally, the denser structure of nacreous/prismatic matrices compared to calcite might offer shell DNA a better protection against

postmortem hydrolysis or microbial attack. Interestingly, BRE5\_Oyster, which showed an important brown deposit on the inner layer indicative of a diseased state, was the only oyster providing positive results. We hypothesize that the mollusc defence mechanisms, which involve increased mineralization and cell density and/or the recruitment of hemocytes in the hemolymph and extra-pallial fluid (Paillard, Ashton-Alcox, & Ford, 1996), may have increased DNA content in this particular shell.

We observed that DNA extraction success rates from ancient marine mollusc shells depended on the preservation of the inner layer. Macroscopic examination of clam shells dated ~200–5,000 yBP and yielding low amounts of DNA, revealed a loss of the “porcelaneous” aspect of the inner layer due to the dissolution of aragonite during deposition. We therefore recommend that particular attention be paid to the integrity of the inner layer (preferably aragonitic) when selecting samples for ancient DNA analyses. Further investigation is required to understand the interactions between DNA, proteins and biominerals, especially regarding biomolecular taphonomy in calcium carbonates, and to assess temporal ranges compatible with shell DNA recovery.

Long-term DNA survival within shell calcium carbonate biominerals was supported by degradation patterns characteristic of ancient DNA. These, including increased rates of C → T and G → A mutations at read termini and ~10-bp periodicity in the size distribution of nuclear DNA fragments, also suggests potential for future epigenetics studies in molluscs, as methylation and nucleosome maps can now be reconstructed from patterns of nucleotide misincorporation and depths-of-coverage (Pedersen et al., 2014; Gokhman, Lavi, & Prüfer, 2014; Orlando & Willerslev, 2014; Orlando et al., 2015; Hanghøj, Seguin, & Schubert, 2016).

## 4.2 | Metabarcoding of marine mollusc shells

Among the different computational methods used here, the metagenomic approach based on DIAMOND and MEGAN proved useful to screen for possible candidate taxa. Further confirmation for the presence of chlorophyte, trematode and bacterial DNA would require deeper sequencing or whole-genome in-solution target enrichment for specific DNA markers (Bos, Schuenemann, & Golding, 2011; Schroeder, Ávila-Arcos, & Malaspina, 2015; Ávila-Arcos, Sandoval-Velasco, & Schroeder, 2015; Cruz-Dávalos, Llamas, & Gaunitz, 2016). The metagenomic approach is however computationally heavy and subject to uneven taxon representativeness in comparative databases which, in addition to the absence of normalization for marker sizes, biases the relative taxon abundance estimates, possibly leading to erroneous taxon identification. For that purpose, more specificity and accuracy were achieved when mapping to target reference sequences. This approach however requires prior knowledge regarding mollusc genera/species to target, which is not always available, for example, in cases where shell samples are too fragmentary for robust morphological identification. Targeted mapping is also limited by the amount of data being publicly available, for example, here, for *A. islandica* and *R. decussatus*. Nevertheless, with the increasing

application of HTS to nonmodel organisms, we can be confident that more comparative mollusc data sets will be made available soon. We also show here that mollusc mtDNA barcode sequences can be recovered from shells, making PCR-based metabarcoding applicable to marine mollusc shell DNA (Geist et al., 2008), provided that appropriate comparative databases are available.

## 4.3 | Potential for evolutionary studies

In the future, sequence alignments resulting from targeted mapping against reference nuclear and mtDNA sequences could be used to reconstruct the evolutionary and demographic trajectories of molluscs (Leonardi et al., 2016). Sampling shells in time series would make it possible to investigate the impact of past environmental changes (e.g., temperature, salinity, pollutants, pathogenic outbreaks) that can be tracked from the same specimens through a range of palaeoenvironmental proxies (Chauvaud et al., 2005; Thébault, Chauvaud, & L'Helguen, 2009; Lartaud et al., 2010; Szymanek, 2011; Penkman et al., 2011; Butler et al., 2013; Pérez-Mayol et al., 2014; Reynolds et al., 2016). This information could in turn help predict mollusc demographic and adaptive responses to current and future environmental changes, such as ocean acidification, which, caused by the dissolution of carbon dioxide excess in the atmosphere, can hinder shell formation in molluscs (Parker, Ross, & O'Connor, 2013; Gazeau, Parker, & Comeau, 2013). Additionally, ancient shell DNA could be applied to the conservation of endangered mollusc species. As previously performed with museum material from mammalian endangered species (Der Sarkissian, Ermini, & Schubert, 2015), temporally sampled DNA data could help track changes in genetic diversity through time, assess population genetic viability and identify specific candidate adaptive alleles to inform and improve conservation management. Another interesting application of marine mollusc shell DNA time series is the reconstruction of past human diets from shell midden assemblages at archaeological sites, thereby shedding light on resource usage in ancient human populations and their adaptation to changing environments (Sanger, 1993).

## 4.4 | Potential for the studies of environmental and pathogenic bacteria

Our results, in particular the recovery of DNA likely belonging to *V. tapetis*, the aetiological agent of BRD, also indicate the potential of ancient shell DNA to reconstruct the history of pathogenic diseases. BRD first appeared in Brittany in 1987 and has since caused high mortality in European aquacultures of Manila clams (Paillard et al., 1989, 1994; Borrego et al., 1996; Allam, Paillard, Howard, & Le Penec, 2000; Paillard, Korsnes, & Le Chevalier, 2008). Interestingly, we found that DNA extracted from shells exhibiting small brown conchiolin deposits characteristic of BRD and collected in Brittany 4 years before the first BRD outbreak showed highest genetic affinity for the *V. tapetis* strain HH6087, while affinity for the *V. tapetis* strain RP2-3 was observed in shells postdating the emergence of BRD in Brittany. These results tend to suggest that strains showing lower

virulence than strains responsible for the BRD epizooty were present in Brittany before 1987. Further work, including genome sequencing of additional strains, characterization of virulence genes, and DNA analyses of the whole BRD time series, are however required to refine the history of BRD. This will more generally also help identify the genomic changes underlying the mechanisms of co-evolution between *V. tapetis* and Manila clams, which have developed recovery processes through the deposition of a mineral layer covering brown deposits and, hence, a resistance capacity to BRD (Trinkler, Labonne, et al., 2010; Trinkler, Sinquin, Querne, & Paillard, 2010; Trinkler, Bardeau, & Marin, 2011; Trinkler, Guichard, & Labonne, 2011; Jeffroy, Brulle, & Paillard, 2013). Our results also suggest that DNA analyses of shell material hold the potential for new tools to detect and monitor diseases in aquaculture.

Finally, we detected DNA from various *Vibrio* species in shells. *Vibrio* is a diverse genus of the marine environment (Colwell, 1996), which has become increasingly abundant in the North Atlantic within the last 50 years, following the rise of sea surface temperatures (Vezzulli, Grande, & Reid, 2016; Baker-Austin, Trinanés, Gonzalez-Escalona, & Martínez-Urtaza, 2017). This spread has also been associated with an increase in the prevalence of severe human diseases caused by *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Vezzulli et al., 2016; Baker-Austin et al., 2017). Screening for *Vibrio* species in shell DNA could help detect similar trends in wider temporal and geographical ranges, thus providing invaluable information to predict future human disease (re-) emergence as a consequence of global warming.

## 5 | CONCLUSION

By recovering DNA from ancient marine mollusc shells as old as ~7,000 years, we validated mollusc shells as a new ancient DNA reservoir. This opens to (meta)genomic analyses the important palaeontological and archaeological record of marine mollusc shells, found in for example, sediment cores, shell lags on the seabed, shell middens and museum collections. Future studies thus hold the potential to provide invaluable information to research in archaeology, evolutionary biology, marine community ecology and climate change. DNA analyses of mollusc shells could also be applied to study living communities, including the development of conservation programmes for threatened species, the genetic monitoring of economically important mollusc species, as well as the biological surveillance of aquaculture and early detection of pathogen outbreaks.

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## AUTHOR CONTRIBUTIONS

The study was conceived and coordinated by C.De. and L.O.; molecular and computational analyses were performed by C.De., with input from V.P., M.P. and L.O.; samples and reagents were provided by V.P., C.Du., T.S., P.C.I., C.P. and L.O.; manuscript was written by C.De. and L.O.

## DATA ACCESSIBILITY

All sequencing data generated in this study are available from the European Nucleotide Archive (project PRJEB20113).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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