Sterilization impacts on marine sediment—Are we able to inactivate microorganisms in environmental samples?

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One sentence summary: Different sterilization techniques (autoclaving, gamma-sterilization and addition of NaN₃) of natural sediment samples are compared and are shown to perform quite distinctly in decreasing microbial activities and eliminating signatures of microbial life.

Editor: Tillmann Lueders

ABSTRACT

To distinguish between biotic and abiotic processes in laboratory experiments with environmental samples, an effective sterilization method is required that prevents biological activity but does not change physico-geochemical properties of samples. We compared standard sterilization methods with respect to their impact on microbial abundance and activity. We exposed marine sediment to (i) autoclaving, (ii) gamma-radiation or (iii) sodium azide (NaN₃) and determined how nucleic acids, microbial productivity, colony forming units (CFUs) and community composition of microorganisms, fungi, unicellular protists and protozoa were affected. In autoclaved and gamma-sterilized sediments, only few colonies formed within 16 days. After addition of NaN₃ to the sediment, numerous CFUs (>50) but lower ³H-leucine incorporation rates, i.e. lower protein biosynthesis rates, were found compared to the other two sterilization techniques. Extractable RNA was detected immediately after all sterilization treatments (0.2–17.9 ng/g dry sediment) but decreased substantially by 84%–98% after 16 days of incubation. The total organic carbon content increased from 18 mg L⁻¹ to 220 mg L⁻¹ (autoclaving) and 150 mg L⁻¹ (gamma-radiation) after sterilization. We compare advantages and disadvantages for each tested sterilization method and provide a helpful decision-making resource for choosing the appropriate sterilization technique for environmental studies, particularly for marine sediments.

Keywords: autoclaving; gamma-radiation; sodium azide; ³H-leucine; Mössbauer spectroscopy; T-RFLP; RNA
INTRODUCTION

Biogeochemical cycles, such as the sulfur, nitrogen, carbon and iron cycles, are mediated by abiotic and biotic reactions. In order to quantify the biotic contribution to substrate turnover in experiments with environmental samples, effective abiotic control setups are required (i.e. sterilized samples). Although sterilization is defined as a process that effectively kills, removes, eliminates or inactivates microbial life (Coleman and MacFadyen 1966; Degrange, Lensi and Bardin 1997; McNamara et al. 2003; da Silva Aquino 2012), resilient DNA and RNA and microbial activity often remain and persist in treated natural soil and sediment samples (e.g. Berns et al. (2008)). Sterilization of an environmental sample should not only inactivate microorganisms (examples of different bacterial inactivation states are reviewed in Cangelosi et al. 2014), it should ideally also have minor effects on the physico-geochemical properties of the sample material (Lotrario et al. 1995; McNamara et al. 2003; Herbert et al. 2005).

Commonly used physical sterilization methods comprise the exposure of samples to wet heat, dry heat, gamma-radiation, filtration, pasteurization and UV radiation (Liegel 1986; Trevors 1996; McNamara et al. 2003; Berns et al. 2008). Chemical sterilizing agents include methyl bromide, formaldehyde, mercuric chloride, ethylene oxide and sodium azide (Russell 1990; Trevors 1996).

Thermal sterilization (autoclaving) has been widely used in microbial ecology (Wolf and Skipper 1994; Berns et al. 2008). However, a number of microorganisms are known to survive autoclaving, including spore-forming bacteria (e.g. Bacillus sp. and Clostridium sp.) (Te Giffel et al. 2002; Setlow 2006; Sahlström et al. 2008). It has been demonstrated that multiple autoclaving cycles increase the effectiveness of the inactivation of microbial activity (Wolf et al. 1989; Tuominen, Kairesa and Hartikainen 1994; Lotrario et al. 1995) and that it results in the fragmentation of DNA (Chiter, Forbes and Blair 2000; Berns et al. 2008). Still, it is unknown to what extent RNA and DNA is degraded in such samples. In addition, disadvantages of thermal sterilization are that it leads to changes of mineral phases and affects the geochemistry (e.g. by sulfide release and in particular by carbon mobilization) of soil and sediment samples (Ramsay and Bawden 1983; Lotrario et al. 1995). Specifically, autoclaving causes (i) an increase in Mn(II) concentrations (Wolf et al. 1989; Tuominen, Kairesa and Hartikainen 1994), (ii) heat-induced crystallization of Fe (oxy)hydroxide minerals (Radloff et al. 2008), (iii) a decrease of soil and sediment surface area, (iv) damage of soil structure, (v) release of nutrients and substrates (e.g. sulfide) and (vi) an increase in organic carbon concentrations (Wolf et al. 1989; Lotrario et al. 1995; Trevors 1996; Radloff et al. 2008; Trotsenko and Murrell 2008; Quéméneur et al. 2016).

Sterilization of soil and sediment samples by gamma-radiation has been used for over 60 years (Ramsay and Bawden 1983; Stroetmann, Kämpfer and Dott 1994; McNamara et al. 2003; Herbert et al. 2005; Manning et al. 2006). McNamara et al. (2003) stated that only 10 kGy is required to eliminate Actinomycetes and fungi in most soils. However, the majority of soil bacteria are eliminated by a dose of at least 50 kGy, and 70 kGy is required to kill more resistant bacteria such as Bacillus sp. and Micrococcus radiodurans (McNamara et al. 2003). Regarding the ionizing radiation effects, it is known that only 20% of the cell damage is caused by direct shots of gamma-quanta while the remaining 80% of cell damage is caused by free radicals produced in the cells and surrounding water under gamma-radiation exposure (Halliwell and Guttridge 2015; Cheptsov et al. 2017). Low doses of 0.25 to 5 kGy increase the amount of RNA (Moussa et al. 2005; Kam et al. 2013) due to stimulation of mitochondrial RNA expression (Kam et al. 2013). However, doses higher than 5 kGy were shown to decrease the RNA content and almost completely destroy ATP, RNA and DNA (Novitsky 1986). With respect to the geochemical and mineralogical integrity of the samples, gamma-radiation has disadvantages because it enhances (i) the release of nitrate and ammonium (Lensi et al. 1991; Buchan et al. 2012; Brown et al. 2014), (ii) the release of Mn(II) in soil samples (Wolf et al. 1989), (iii) the transformation of ferrihydrite to hematite (Herbert et al. 2005; Brown et al. 2014), (iv) Fe(III) reduction (Bank et al. 2008; Brown et al. 2015), (v) damage of biomolecules, such as nucleic acids, proteins and lipids by reactive oxygen species at doses higher than 25 kGy (Marschner and Bredow 2002; Brown et al. 2014) and (vi) formation of free hydrogen and hydroxyl radicals (Jackson et al. 1967; Desrosiers 1996).

Chemical sterilization, such as the addition of antibiotics, respiratory inhibitors (e.g. sodium azide; NaN₃) or toxic chemicals (e.g. methyl bromide, formaldehyde) target specific physiological processes. NaN₃ does not kill microbial life, but rather inhibits microbial growth and substrate turnover in soil and sediment samples. Sodium azide inhibits activities of peroxidases, catalases and a few more enzymes that possess heme as prosthetic group. As NaN₃ inhibits the cytochrome c oxidase of the respiratory chain, fermenting bacteria are still able to metabolize and even grow in the presence of NaN₃ (Wolf et al. 1989; Trevors 1996; Dowdle and Oremland 1998; Radloff et al. 2008). It has been reported that NaN₃ inactivates soils and sediments more effectively than antibiotics (Wolf et al. 1989; Dowdle and Oremland 1998), but some disadvantages are that NaN₃ reduces nitrate and nitrite abiotically and triggers changes in pH (Trevors 1996; Marouf-Khelifa et al. 2006). In addition, Bore et al. (2017) showed that soil microorganisms can survive NaN₃ sterilization. More specifically, they can overcome the inhibition of NAD⁺/NADH regeneration. To overcome intracellular inhibition of the electron transport chain induced by NaN₃, microorganisms can couple their intracellular respiration metabolism with extracellular redox processes by using Fe(III), Mn(IV), quinones and humic substances functioning as extracellular electron acceptors (Bore et al. 2017).

In order to clearly distinguish biotic from abiotic processes in studies with environmental samples, an optimal sterilization method needs to be chosen. However, as explained in the previous sections, all methods carry disadvantages and it is necessary to compromise depending on the samples and the research questions to be answered. Here we present a systematic study comparing three commonly applied sterilization methods (autoclaving, gamma-sterilization and NaN₃ addition) for laboratory sediment incubation experiments. We incubated sterilized marine sediment (collected from Norsminde Fjord, Denmark) for up to 16 days, quantified extractable nucleic acids (DNA/RNA) as well as colony forming units (CFUs) and determined the microbial (bacterial and archaeal), fungal, unicellular protists and protozoan community structure, as well as the H⁺-leucine incorporation activity. Finally, we evaluate the advantages and disadvantages of autoclaving, gamma-sterilization and NaN₃ addition for the application to experimental setups containing sediments and to answer research questions in the field of microbial ecology. Based on our data, we provide a decision-making aid for studies with natural soils and sediments that require effective sterilization methods.
MATERIALS AND METHODS

Field site description and sampling procedure

Littoral marine sediments were taken in July 2016 and March 2017 from Aarhus Bay (Denmark). The upper 3 cm of the organic-rich (TOC: 3.1%; DOC: 8 mg L\(^{-1}\)) and muddy bulk sediment from the shallow marine estuary Norsminde Fjord were sampled at 0.5 m water depth near its narrow entrance to Aarhus Bay (N 56°01.171′; E 01°15.390′). Sediment was transported and stored at 4 °C until the start of the experiments. For geochemical characterization of the sediments see Laufer et al. (2016).

Quantification of organic carbon content

The non-purgeable organic carbon (NPOC), which represents the sum of all dissolved organic carbon compounds, was quantified in the supernatant of the microcosms. For NPOC analysis, 2 ml of suspension (water mixed with sediment) was centrifuged (10 min; 7000 g). After centrifugation, the supernatant was filtered through a 0.45 μm filter (MF-Millipore MCE membrane, Merck KGaA, Darmstadt, Germany) and NPOC was quantified with a carbon analyzer (Multi NC 2100, Analytik Jena, Germany).

Sterilization of the sediment

The sediment was homogenized under ambient air. The samples for gamma-sterilization were filled into plastic bags and sent to Synergy Health Allershausen GmbH (Germany) for a routine small amount irradiation. Samples were exposed to a cobalt 60 radiation field and radiated at 52 ± 2.6 kGy in a range as mentioned in McNamara et al. (2003). Sediment for autoclaving was filled into plastic beakers, covered with aluminum foil and autoclaved three times with 2–3 days storage in between at room temperature. For autoclaving the autoclave Systec VE40 and the program for solid material with the setting of 121 °C for 20 min was used. For the chemical sterilization, a 5 M NaN\(_3\) solution was prepared and added to 5 g sediment in 100 ml serum bottles to a final concentration of 160 mM as described in Laufer et al. (2016). The anoxic sediment was slurred to homogenize the sample with NaN\(_3\) for a few minutes by hand.

Preparation of microcosms

The following four different microcosms were each prepared in triplicates: (i) gamma-sterilized sediment, (ii) autoclaved sediment, (iii) sediment amended with NaN\(_3\) and (iv) native untreated sediment as a control. Microcosm incubations were set up in 100 ml serum vials that were wrapped with aluminum foil for dark incubation at 25 °C and incubated over a total period of 16 days. 50 ml of anoxic filtered seawater medium was added to 5 g of homogenized sediment with a N\(_2\)/CO\(_2\) (90:10) headspace. The seawater medium was prepared from native seawater that was flushed with N\(_2\) and subsequently filtered through a 0.22 μm filter (EMD Millipore Steritop\(_{TM}\)). The headspace was replaced by N\(_2\)/CO\(_2\) (90:10). The pH of the medium was adjusted to 7.1 and regularly monitored during incubation. For NaN\(_3\)-microcosm incubations, NaN\(_3\) (sterile and anoxic) was added to the seawater medium (final concentration of 160 mM NaN\(_3\)). The microcosms were sampled four times, i.e. immediately after preparation (t\(_0\)), after 3 days (t\(_1\)), after 7 days (t\(_2\)) only for NPOC and after 16 days of incubation (t\(_{\text{end}}\)).

Colony forming units

For quantifying the CFUs, we used lysogenic broth (LB) plates without any additives: in 500 ml of deionized, distilled water 5 g peptone, 2.5 g yeast extract, 5 g of sodium chloride and 7.5 g agarose were dissolved. 100 μl of microcosm slurry from t\(_0\), t\(_1\) and t\(_{\text{end}}\) were streaked out on plates for anoxic and oxic incubation (8 plates per sterilization method per time point: 4 oxic and 4 anoxic plates). Anoxic incubation was performed in an anoxic container (Anaerocult®, Merck Millipore, Darmstadt, Germany) at room temperature. All LB plates were incubated in light at 35 °C. Colonies were quantified right after sterilization treatments, after 3 days and after 16 days of incubation.

\(^3\)H-leucine incorporation

By quantifying the rate of \(^3\)H-leucine incorporation into microbial biomass, the rate of total microbial biomass production in the sample can be estimated (Kirchman 2001). Here, we used an expanded method suitable for sediment samples (Bååth, Pettersson and Söderberg 2003; Buesing and Marxsen 2005; Demoling and Bååth 2008). Microbial cells were extracted from 6 ml of sediment slurry (sampled at t\(_0\) and t\(_{\text{end}}\)) by shaking on a multitovortex at maximum speed for 3 min. The entire sample was subsequently filtered (0.45 μm; cells are in the filtrate). For each microcosm sample, two technical replicates and one acid-killed control replicate were analyzed. All replicates were incubated with a final concentration of 2.8–3.0 nmol L\(^{-1}\) \(^3\)H-leucine (specific activity of 102.3 Ci mmol\(^{-1}\)) for 1–2 h (t\(_0\): 70 min, t\(_1\): 90 min, t\(_2\): 135 min) at room temperature (Kirchman 2001; Demoling and Bååth 2008). The killed methodological control replicates were inactivated with cold trichloroacetic acid (TCA; final concentration of 6.25%) before tracer addition and all other incubations were terminated the same way after incubation. Subsequently, the samples were pelleted, washed with 5% w/v TCA and then with 80% ethanol, according to Kirchmann (2001) with minor modifications. Finally, the dried pellets were resuspended in 1.5 ml Scintillation Cocktail (Ultima Gold, PerkinElmer, Waltham, USA) and analyzed immediately using a liquid scintillation counter (count time 5 min; Packard TRI-CARB 2500TR, PerkinElmer). Rates of microbial \(^3\)H-leucine incorporation and the corresponding productivity were calculated according to Kirchmann (2001).

RNA and DNA extraction

Samples for RNA and DNA extraction were taken at t\(_0\) and t\(_{\text{end}}\) and stored at −80 °C and −20 °C, respectively. RNA and DNA was extracted using the MoBio, Inc., PowerSoil® RNA and DNA isolation kit as directed by the manufacturer (MO BIO Laboratories, Carlsbad, CA, USA), with the following modifications: 1.5–2.5 g sediment were used from each experiment, 5 minute bead-beating was applied and centrifugation steps were performed at maximum speed (7000 x g) at 4 °C. RNA and DNA were eluted in 100 μl of 10 mM Tris buffer. The extracts were checked for nucleic acid integrity by agarose gel electrophoresis, and concentrations were determined using a Qubit® 2.0 Fluorometer with DNA and RNA HS kits (Life Technologies, Carlsbad, CA, USA). The obtained DNA and RNA concentrations are reported in the supplementary material. The detection limit of Qubit quantification was <0.2 ng/g.
DNA digestion in RNA samples and reverse transcription

RNA extracts were digested with the Ambion Turbo DNA-free™ kit as directed by the manufacturer (Life technologies, Carlsbad, CA, USA). Successful DNA removal was confirmed by PCR using general bacterial primer GM3-8f and 1392R (Stahl et al. 1988; Muyzer et al. 1995). RNA extracts were used for reverse transcription when no PCR products were obtained after 30 PCR cycles (i.e. the DNA had been completely degraded). The reaction mix for the reverse transcription (total volume 20 μl) contained 5 μl DNAase-digested RNA, 6 μl DEPC-treated water, 2 mmol l⁻¹ dNTP mix (New England Biolabs, Ipswich, MA) and 5 ng μl⁻¹ random primer (Invitrogen, Life Technologies, Carlsbad, CA). The reaction mix was incubated for 5 min at 65°C before 1x First Strand buffer (Invitrogen, Life Technologies), 5 mmol l⁻¹ DTT (Invitrogen, Life Technologies), 2 U RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Life Technologies) and 10 U SuperScript® III Reverse Transcriptase (Invitrogen, Life Technologies) were added. The reaction mix was incubated for 5 min at 25°C, 60 min at 50°C and 15 min at 70°C in a S1000 thermal cycler (Bio-Rad Laboratories GmbH, Munich, Germany). cDNA was quantified using a Qubit® 2.0 Fluorometer with the DNA HS kit and stored in −20°C.

T-RFLP

The microbial community composition was analyzed using terminal restriction fragment length polymorphism (T-RFLP) specific for 16S rRNA, 18S rRNA and fungal ITS region (for 16S rRNA genes most PCR products and T-RFs were below the detection limit). 16S rRNA, 18S rRNA and fungal ITS region genes from DNA samples were amplified by PCR on a Bio-Rad (Hercules, CA) C1000 thermal cycler using fluorescently labeled bacterial primers Bac27f(-FAM)/907r (Pilloni et al. 2012), archaeal primers Ar109f/Ar912r(-FAM) (Culman et al. 2008), fungal primers (ITS region sequences were amplified using the fungal ITS primer pair FAM-labelled ITSF1 (Gardes and Bruns 1993) and ITS4R (White et al. 1990)) (Wankel et al. 2017) and distinct eukaryote-targeted (protozoans and protists) primers Euk20f(-FAM)/Euk516r (Liu et al. 1997; Euringer and Lueders 2008). Each 50 μl PCR reaction contained 5 × PCR buffer (MBI Fermentas Taq), 10 μM of each primer, 10 mM dNTPs (Promega, Madison, WI), 25 mM MgCl₂, 5 unit of MBI Taq polymerase (MBI Fermentas Taq) and 1 μl DNA or cDNA template. The reaction conditions for PCR consisted of an initial denaturation at 94°C for 5 min, followed by 25 to 30 cycles of 30 s at 94°C, 30 s at an annealing temperature of 52°C, 60 s at 70°C and a final extension step for 5 min at 70°C. PCR products were visualized using 1% agarose gel to ensure yield of sufficient PCR products. Bacterial 16S rRNA gene, fungal ITS region and eukaryotic 18S rRNA gene amplicons for each individual sample were purified using a PCR Extract column Kit (E.Z.N.A. Cycle pure kit, Omega Biotech). A total of 80 ng of purified PCR products were digested with 0.3 μl 20 U MspI (Bacteria; Promega), TaqI (Archaea; Promega), Hae III (Fungi; Promega) and Bsh1236I (Protozoans; Promega) in a 10 μl reaction system. One microliter digested and desalted DNA and cDNA was subjected to T-RFLP analysis on a ABI 3730xl DNA Analyzer using POP-7 polymer (Applied Biosystems, Foster, CA), using previously described methods (Euringer and Lueders 2008). Raw data was processed using Genemapper V 5.0. Further analysis was performed using T-REX (Culman et al. 2009) with background noise filtering (factor 0.2) and applying a clustering threshold for peak alignment across samples (set to 1.5 bp). The relative terminal restriction fragment (T-RF) abundance was calculated based on peak heights.

Supplementary ferrihydrite experiment

The goal of this experiment was to investigate the effect of the chosen sterilization methods on the mineralogy of ferrihydrite (Fe₂O₃·H₂O), a classical representative for low-crystalline iron(III) minerals in environmental samples (Cornell and Schwertmann 2004). Due to its high abundance in the Earth’s crust, iron (oxy)hydroxide minerals represent an important fraction in sediments and soils (Scheinost 2005; Braunschweig, Bosch and Meckenstock 2013) (e.g. 1–3% in Norsminde Fjord sediment (Laufer et al. 2016)) and they are strongly connected to microbial activity (Colombo et al. 2014). Furthermore, iron minerals provide active surface sites and transformation of the iron mineral can cause mobilization of nutrients (such as phosphate or trace metals) or pollutants (such as toxic metals) (Bonneville, Van Cappellen and Behrends 2004; Kappler and Straub 2005; Gadd 2010). Changes in iron mineralogy therefore do not only affect iron-metabolizing bacteria, they also affect the entire microbial community due to the release or the retention of nutrients. Ferrihydrite was prepared according to Amstaetter, Borch and Kappler (2012). Microcosms (25 ml) were prepared with 40 mM PIPES buffer, sterile ultrapure water and 1 ml ferrihydrite suspension (concentration of 5 mM). Subsamples were exposed to the same sterilization procedures as the sediments. The composition of iron minerals after sterilization was identified using Mössbauer spectroscopy. For this, aqueous mineral suspensions were filtered onto a cellulose filter (diameter of 1 cm, mesh size 0.22 μm) and covered with Kapton tape forming a thin disk. Transmission spectra were collected at 77 K and 5 K using a constant acceleration drive system (WissEL) in transmission mode with a 57Co/Rh source. All spectra were calibrated against a 7 μm thick α₅₇Fe foil that was measured at room temperature. Analysis was carried out using Recoil (University of Ottawa) and the Voigt Based Fitting (VBF) routine (Rancourt and Ping 1991). The half width at half maximum (HWHM) was constrained to 0.131 mm/s during fitting.

RESULTS

For this study, we analyzed the geochemistry (e.g. NPOC), quantified the number of CFUs, detected the microbial productivity, quantified the amount of extractable DNA and RNA, and the number and relative abundance of microbial T-RFs in native sediment directly after collection, directly after sterilization (t₀) and after incubation of the sterilized sediments for 3 (t₃) and 16 days (t₁₆d).

Influence of sterilization methods on geochemistry

The porewater of the collected native sediment from the coastal marine field site had a NPOC content of 18.3 mg L⁻¹ (this study and Laufer 2016)). In the microcosms with natural untreated sediment and filtered seawater, the NPOC value of the porewater remained constant at 18 mg L⁻¹ before sterilization and during incubation of 7 days (Fig. 1, natural untreated sediment). The strongest effect on the NPOC content of the porewater was observed directly after sterilization (t₀) in the microcosms that underwent thermic sterilization (autoclaving) and gamma radiation where the total organic carbon content increased to approximately 220 mg L⁻¹ and 150 mg L⁻¹ compared to the natural untreated sediment (18 mg L⁻¹), respectively. After 3 days (t₃),
a further increase of the total organic carbon of the autoclaved and gamma-radiated sediment in the porewater was observed. After 7 days of incubation (t7), the porewater NPOC of autoclaved and gamma-radiated samples was approximately 280 mg L\(^{-1}\) and 190 mg L\(^{-1}\), respectively. The addition of NaN\(_3\) to the sediment microcosms resulted in an increase of only 10 mg L\(^{-1}\) NPOC compared to the non-treated sediment at t0 (after sterilization), reaching a total of 48 mg L\(^{-1}\) total organic carbon content in NaN\(_3\)-amended microcosms (increase of 30 mg L\(^{-1}\) NPOC when compared with the non-treated sediment) after 7 days of incubation (t7).

Influence of sterilization methods on colony forming units

The suspension of native sediment with porewater collected from the coastal marine field site showed numerous bacterial as well as fungal CFUs (>50) on oxic and anoxic LB plates (Table S1, Supporting Information). Bacterial and fungal colonies were distinguished by their different morphology. The filtered seawater which was used to set up the microcosms did not produce CFUs on LB plates. Sediment slurries from all microcosms after autoclaving, gamma-radiation, ad NaN\(_3\)-sterilization treatments showed bacterial as well as fungal CFUs under oxic and anoxic conditions immediately after sterilization of the sediments (t0) (summary in Fig. 2 and more details in Table S1, Supporting Information). Autoclaved sediment showed around 0–2 bacterial CFUs per 100 \(\mu\)L microcosm slurry on oxic and anoxic plates at t0. After 3 (t3) and 16 days of incubation (tend) of autoclaved sediment, only 0–1 CFUs on oxic and no CFUs on anoxic LB plates were counted. Similar results were obtained for wet gamma-sterilized sediment: at t0 0–1 bacterial CFUs per 100 \(\mu\)L microcosm slurry were counted on oxic as well as on anoxic plates. After 3 and 16 days of incubation of gamma-sterilized sediment, the CFUs on oxic and anoxic LB plates were in a similar range (t3: 0–2; tend: 0–4 CFUs). In dry gamma-sterilized sediment, the lowest CFU numbers were detected (t0: 0–2; t3: 0–1; tend: 0–1) on oxic as well as on anoxic plates. In NaN\(_3\)-amended sediment samples, numerous CFUs (>50) were detected on oxic as well as on anoxic plates. After 16 days of incubation, some NaN\(_3\)-amended samples contained numerous (>20) bacterial and fungal CFUs while other samples contained none on oxic plates. Almost no colonies were found on anoxic plates (only one exception with numerous colonies on one anoxic plate). All controls without sediment (only filtered and sterilized marine water) showed no CFUs at either t0 or after 16 days.

Influence of sterilization methods on microbial activity

For estimating microbial activity and productivity in the treated samples, a \(^{3}H\)-leucine incorporation assay was performed (Fig. 3). Non-sterilized natural marine coastal sediment showed a bacterial productivity of 2.7 nM C incorporation per day per L of sediment.
suspension right after setting up the microcosms (t0). After three days of incubation at room temperature (t3), we observed that the bacterial productivity was lower (1.3 nM C incorporation per day) than at t0 in the natural untreated sediment and remained equal after 7 days of incubation (1.2 nM C incorporation per day). After autoclaving and gamma-radiation (t0), the carbon incorporation rate was 60%–70% lower compared to the natural untreated sediment at t0, further decreased to 0.7–1.0 nM C d\(^{-1}\) after 3 days of incubation and stayed the same after 7 days of incubation. After addition of NaN\(_3\), a slightly higher activity was measured right after sterilization (1.5 nM C d\(^{-1}\)), but it decreased substantially after three days of incubation to 0.2 nM C d\(^{-1}\). After 4 more days of incubation (t7), the microbial activity remained relatively constant in all NaN\(_3\) triplicates at ca. 0.3 nM C incorporation into biomass.

**Influence of sterilization methods on amount of extractable DNA and RNA**

Using fresh untreated marine sediment, we were able to extract around 16–18 ng RNA per g dry sediment and 30–50 ng DNA per g dry sediment (Fig. 2; Table S2, Supporting Information). Following gamma-sterilization, 7–16 ng per g dry sediment RNA (and 40–60 ng per g dry sediment DNA) were extracted from gamma-sterilized wet sediment at t0, with this amount decreasing over time (3–5 ng per g dry sediment RNA at t3 and after 16 days of incubation (tend) 1–3 ng per g dry sediment RNA). Similarly, gamma-sterilized dry sediment showed a decrease from 4–7 ng per g dry sediment RNA at t0 to 0.2 ng per g dry sediment RNA after 16 days of incubation (tend). After autoclaving, about 1.5–2.5 ng per g dry sediment RNA were extracted at t0 and only 0.2 ng per g dry sediment RNA was detectable at tend. A similar trend was found for the NaN\(_3\)-amended sediment (t0: 8–17 ng per g dry sediment RNA; tend: 0.2 ng per g dry sediment RNA). The amount of extractable DNA followed the same trends as RNA in all setups (Summary in Fig. 2 and more details in Table S2, Supporting Information).

**Impact of sterilization on the microbial community composition based on T-RFLP**

The collected native sediment from the coastal marine field site showed around 100 T-RFs for the bacterial, archaeal, fungal and unicellular eukaryote community (Fig. 4 and Fig. S1, Supporting Information). T-RFLP data (based on extracted DNA) of bacteria, archaea, fungi and unicellular eukaryotes indicated that the applied sterilization methods had different impacts on the total number of T-RFs (Fig. S1, Supporting Information), in addition to the impact on community structure (Fig. 4). After gammasterilization of wet sediment (t0), the greatest number of T-RFs was detected in the bacterial group (29 T-RFs), whereas a less complex community was found for archaea, fungi and protozoans (11, 12 and 17 T-RFs, respectively). After 16 days of incubation of gamma-sterilized wet sediment (tend), the microbial community fingerprint had changed substantially with only 1 to 2 T-RFs detected for bacteria, archaea and fungi. The protozoan community changed the least, with 9 T-RFs still detected after two weeks. A different pattern was found for dry gamma-sterilized sediment. At t0, only bacterial T-RFs were detectable (10 in total), whereas after two weeks of incubation, we found 39 bacterial T-RFs, 3 archaeal, 16 fungal and 19 protozoan T-RFs. Directly after autoclaving (t0), only bacteria (19 T-RFs) and archaea (5 T-RFs) were detectable and the number of T-RFs remained constant after 16 days of incubation (tend). The microbial community of sediment with NaN\(_3\) showed similar T-RF patterns compared to the natural untreated sediment directly after NaN\(_3\)-addition (t0). However, more archaeal T-RFs were detected (17 T-RFs in NaN\(_3\) microcosms and only 8 T-RFs in the natural sediment) in NaN\(_3\)-amended sediment related to the untreated sediment. After 16 days of incubation, the number of fungal T-RFs was higher in the NaN\(_3\)-amended sediment than in the untreated sediment (31 and 20, respectively), while the number of protozoan T-RFs was lower than in the natural sediment (24 and 37, respectively).

The T-RF graphs (Fig. 4) show the relative abundance of single T-RFs before and after microcosm incubation. In the case of bacteria, archaea and fungi, it was not possible to obtain 16S rDNA T-RFs for all gamma-sterilized triplicates of wet sediment after incubation. The bacterial T-RFs with the lengths of 89 and 145 base pairs (bp) were the only T-RFs detectable in gamma-sterilized wet sediment after two weeks of incubation. For the archaea in gamma-sterilized wet sediment, T-RFs with lengths of only 54 bp, 67 bp, 93 bp, 178 bp and 784 bp were detected, while for fungi similar T-RF length of 54 bp, 66 bp and 93 bp and for unicellular eukaryotes T-RFs of 65 bp, 67 bp, 95 bp, 557 bp and 558 bp were present. In gamma-sterilized triplicates of dry sediment, numerous bacterial T-RFs were detectable (i.e. 73 bp, 89 bp and 145 bp) but only archaeal T-RFs with the lengths of 54 bp, 93 bp, 178 bp and 784 bp were resistant. The autoclaving process seems to be the most effective method for destroying the DNA of fungal and protozoan microorganisms. No protozoan DNA T-RFs were detectable after two weeks of incubation and only one
Figure 4. Bacterial, archaeal, fungal and protozoan community structure before and after sterilization based on 16S rDNA based T-RFs. Changes in bacterial, archaeal, fungal and protozoan community structure after gamma-radiation (of wet and dry sediment), autoclaving and NaN3-addition as well natural untreated marine sediment at the beginning of the experiment (t0) and after 16 days (tend). * marks low DNA T-RFs and in some cases we were not able to amplify the samples and detect T-RFs (below detection limit).
fungal triplicate showed T-RFs with lengths of 54 bp, 93 bp, 104 bp, 402 bp and 589 bp. NaN₃-addition to the marine sediment showed minor changes compared to the natural untreated sediment after two weeks of incubation. Bacterial T-RFs of a length of 217 bp and 297 bp, archaean T-RFs of 178 bp, 192 bp and 384 bp, fungal T-RFs of 397 bp, 518 bp and 534 bp, and protozoan T-RFs of 65 bp, 66 bp, 67 bp, 224 bp and 558 bp seemed to be prominent after two weeks of NaN₃ incubation.

T-RFLP data of cDNA samples from different sterilization setups were not conclusive enough due to the low encountered cDNA and cDNA-amplicon concentrations.

Impact of sterilization on ferrihydrite (iron mineral) properties

The investigated environmental sediment samples contain 1%–3% iron, of which approximately 70 μmol Fe(III) per g dry sediment in 0.5 M HCl (bioavailable fraction) are denoted as low crystalline (e.g. ferrihydrite) and 200 μmol Fe(II) per g dry sediment in 6 M HCl extractions are denoted as crystalline (Lauffer et al. 2016). The highest extent in mineral transformation was observed for the autoclaved ferrihydrite sample. More than 70% of the initial ferrihydrite material was transformed into goethite and hematite, potentially via dissolution- and re-precipitation (goethite) and solid-state conversion (hematite) mechanisms (Cornell and Schwertmann 2004). Due to changes in mineral surface charges, nutrients being (im-)mobilized can subsequently trigger microbial processes (such as heterotrophic iron(III) reduction) and change the biological dynamics in the iron cycle (Radloff et al. 2008; Trotsenko and Murrell 2008; Quéméneur et al. 2016). The exposure to gamma-rays resulted in a moderately low impact on the transformation of ferrihydrite. Only about 6% of the initial ferrihydrite was transformed to goethite during gamma-ray sterilization, whereas the addition of NaN₃ showed neither significant alteration nor transformation from the initial mineral material (Fig. S2, Table S3, Supporting Information). However, mineral dissolution processes and potential transformations to other iron (oxyhydr)oxides (e.g. akaganéite to hematite) (Cornell and Schwertmann 2004) should be considered in long-term experiments (>two weeks).

DISCUSSION

Sterilization techniques as a prerequisite for control setups in environmental studies

In environmental studies, it is necessary to distinguish between biological and non-biological processes in order to quantify the microbial contribution to substrate turnover. Therefore, it is essential to know how to inactivate biological activities efficiently in environmental samples. While certain life properties of cells such as organized cell structure and microbial growth can be analyzed by e.g. microscopy, viability properties such as energy (ATP) conversion, substrate conversion, changes of protein content and the amount of extractable RNA or DNA need advanced analytical approaches (Table 1). In simple culture experiments, it might be useful to use techniques like microscopy or flow cytometry to determine complete sterility, i.e. the absence of (living) microbial cells. But in complex environmental samples (or similarly a mixture of laboratory cultures) like sediments or soils, most of these analyses have their limitations due to matrix effects caused by the integrity of sedimentary or soil material (e.g. difficulties in extracting DNA, difficulties in distinguishing between DNA from different dead and live cells, heat, UV and radiation shielding and protection of microorganisms by the sedimentary matrix, difficulties in distinguishing between dead or living cells under the microscope, problems with cell counts and quantification of ATP yield) (Bogosian and Bourneuf 2001; Emerson et al. 2017). Nevertheless, a pragmatic strategy is required to quantify the impact of microorganisms on substrate turnover versus abiotic reactions in environmental samples (Melton et al. 2014). In the future, complex environmental studies will likely use, e.g. single-cell techniques, metatranscriptomics, DNA-stable-isotope probing (SIP) or RNA-SIP to distinguish the active and living from the dead microorganisms in microbial ecosystems (Hammes, Berney and Egli 2010; Emerson et al. 2017; Singer, Wagner and Woyke 2017).

Sterilization efficiency with respect to RNA and DNA stability

The natural degradation of nucleic acids is mainly triggered by physicochemical parameters, such as temperature, pH, UV radiation and the presence of radicals (Vincek et al. 2003; Seear and Sweeney 2008; Camacho-Sanchez et al. 2013). For our study, we applied commonly used sterilization treatments that are comparable to or lead directly to such physicochemical impacts. The RNA/DNA ratio is an important indicator of the metabolic status of active microbial communities (Fleming, Sanserverino and Sayer 1993; Fabiano et al. 1995; Hurt et al. 2001). The higher this ratio, the more activity can be expected. Still, we found RNA and DNA in all sediment samples immediately after sterilization (t₀) (Table S2, Supporting Information; Fig. 2). In soils and sediments, the presence of DNA is not coupled to the viability of microorganisms. Moreover, there is no direct correlation between the age of a sample and the preservation of nucleic acids (Poinar et al. 1996; Herrmann and Henke 1999). The majority of DNA in sediments is present in an extracellular form defined as naked, free, ambient or environmental DNA which includes soluble as well as non-soluble and viral DNA (Cornaldesi, Danovaro and Dell’Anno 2005; Nielsen et al. 2007; Corinaldesi, Beolchini and Dell’Anno 2008; Taberlet et al. 2012). Extracellular DNA from dead microorganisms can persist in sediments and soils for weeks to years (Levy-Booth et al. 2007; Nielsen et al. 2007; Pietramellara et al. 2009; Lever et al. 2015; Torti, Lever and Jørgensen 2015; Carini et al. 2017), either sorbed to clay minerals (Pietramellara et al. 2009), bound to humic substances (Levy-Booth et al. 2007) or incorporated in biofilm structures (Alawi, Schneider and Kallmeyer 2014). In addition to that, microorganisms exist in a range of metabolic states (dormant, living, growing and decreasing/non-viable) and their ribosomal RNA (rRNA; 82%–90% of total RNA pool) is frequently employed to identify the ‘potentially active’ fraction of microbes in environmental samples (Lennon and Jones 2011; Blazewicz et al. 2013). Ribosomal RNA is generally more stable than mRNA (Snyder and Champness 2007). Measures of mRNA half-life-times for laboratory cultures were in the range of few minutes (Hambraeus, von Wachenfeldt and Hederstedt 2003; Steglich et al. 2010). Detectable RNA (rRNA and mRNA) in environmental samples likely belongs to living and metabolically active organisms (Edgcomb et al. 2011; Orsi et al. 2013), but for environmental systems, there are indications that RNA persists long after death of the source organisms and that extracellular RNA might be more stable and more widespread in sediments than previously assumed (Fordyce et al. 2013; Torti, Lever and Jørgensen 2015) and may
Table 1. What is life, and how can we determine sterility/cell death? Overview of methods that can be used to determine living cells, more precisely viability (live/dead determination) and activity. In simple culture experiments it might be useful to use these techniques (e.g. microscopy) to determine complete absence of microbial cells. In complex environmental samples it is necessary to analyze RNA and DNA content or/and microbial productivity, respectively. Tools for distinguishing the living from the dead in microbial ecosystems were recently reviewed in Emerson et al. (2017).

<table>
<thead>
<tr>
<th>Life properties of cells</th>
<th>Analyzing method</th>
<th>Useful for determine sterility?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Simple culture experiments</td>
</tr>
<tr>
<td>Organized cell structure and membrane integrity* (viability)</td>
<td>Microscopy with live/dead techniques, including many stains and dyes</td>
<td>yes</td>
</tr>
<tr>
<td>Response to stimuli* (viability)</td>
<td>Stimulation (chemotaxis, phototaxis, etc.)</td>
<td>yes</td>
</tr>
<tr>
<td>Growth and reproduction* (viability)</td>
<td>Culture dependent methods: quantification of cells or biomass by colonies on solid medium, density of liquid culture, cell counts</td>
<td>yes</td>
</tr>
<tr>
<td>Energy (activity/productivity)</td>
<td>Quantiﬁcation of cellular energy (adenosine 5′-triphosphate (ATP))</td>
<td>yes</td>
</tr>
<tr>
<td>Metabolism (activity/productivity)</td>
<td>Culture independent methods: quantification of isotope labelled substrates, RNA or 3H-leucine incorporation, transcriptomics and proteomics, etc.</td>
<td>yes</td>
</tr>
</tbody>
</table>

*Give only limited information about living cells.

used for microbial communication (Tsatsaronis et al. 2018). It has been reported that RNA adsorbs onto clay minerals (Franchi, Ferris and Gallori 2003) and clay-bound RNA is less susceptible to digestion by RNases than free RNA (Franchi and Gallori 2005).

Apart from physically forced degradation, DNA and RNA are naturally degraded by enzymes such as phosphodiesterases including deoxyribonucleases (DNases) and ribonucleases (RNases). The presence of DNA and RNA in sterilized samples is thus not only a function of effective inactivation of microbial cells, but also dependent on the destruction or preservation of degradation enzymes (e.g. DNases, RNases). Heat kills bacteria by destroying lipid membranes and denaturation of proteins, which leads to enzyme denaturation (Chang 1994). However, Miyamoto, Okano and Kasai (2009) reported that when RNase, a thermostable and more robust enzyme than DNase, is heated at 121°C by autoclaving for 20 min, it does not lose its activity (no irreversible theroinactivation). Therefore, RNases will be affected by heat, but not degraded, leading to continuous degradation of RNA after sterilization (Miyamoto, Okano and Kasai 2009). In our experiments we could show that although present right after sterilization, DNA and RNA were clearly degraded during incubation for 16 days after sterilization by heat (Fig. 2; details in Table S2, Supporting Information). As NaN₃ does not kill bacteria or affect non-heme protein structures (Jobelius and Scharff 2000), RNases and DNases remain active and degrade remaining nucleic acids efficiently. In our experiments we could show that sterilization by the addition of NaN₃ which causes inhibition of cytochrome oxidase lead to RNA levels below detection limit enzyme. Gamma-rays kill bacteria by breaking down their nucleic acids and proteins through radical formation. Thus, enzymes become damaged or degraded (Lensi et al. 1991; McNamara et al. 2003). In our gamma-ray radiated samples, low levels of remaining RNA were still present after 16 days of incubation. This most likely resulted from damaged RNases that were not able to degrade the residual free RNA.

Effect of sterilization treatment on microbial productivity

Productivity of microorganisms was measured by 3H-leucine incorporation rates which reflect the protein biosynthesis productivity of bacteria in the marine environment (Fischer and Pusch 1999; Kirchman 2001). In our study, the microbial productivity (2500 pM C d⁻¹) in the untreated sediment samples was in the same order of magnitude as similar samples in the literature (e.g. upper 4 cm of freshwater sediments) (Busing and Gessner 2003; Busing and Marxsen 2005). We found a clear difference in microbial productivity between untreated (active) and sterilized marine sediment for each sterilization treatment (Figs. 2 and 3). Microbial productivity followed a similar trend as RNA concentrations, i.e. both decreased during incubation for 16 days. Based on the 3H-leucine assay, sterilization with NaN₃ had the highest impact on the microbial productivity (Fig. 3, summary in Fig. 2), which might be related to the fact that the 3H-leucine assay mainly targets bacteria (e.g. fermenting bacteria or facultative aerobic bacteria) (Bååth 1994; Medina et al. 2003; Barcenas-Moreno et al. 2009; Rouxk, Brookes and Bååth 2009; Rousk and Bååth 2011) while fungal growth could be monitored with different tracer methods (Bååth 2001). However, numerous CFUs of both microorganisms and fungi (>50) were observed on anoxic and oxic plates with NaN₃-amended sediment. The NaN₃-inhibited cytochrome c oxidase is not required for anaerobic bacteria, e.g. fermenting bacteria (Radloff et al. 2008), and they can overcome intracellular respiration inhibition by extracellular electron transport with other electron acceptors, e.g. Fe(III) and Mn(IV) (Bore et al. 2017). Thus, these microorganisms could grow up to numerous CFUs (>50). Based on the 1H-leucine assay, autoclaving and gamma-radiation had a slightly lower impact on the microbial productivity than NaN₃ but showed a faster effectiveness considering the slower decrease in productivity detected in NaN₃-treated samples (directly after the sterilization process and the incubation time of 16 days). In general, the microbial productivity of autoclaved and gamma-sterilized sediment was very low which is consistent with the low amount of CFUs on anoxic and oxic plates. For quantification of CFUs on
LB plates, it has to be kept in mind that only aerobic microorganisms and fermenters can grow on LB plates and that there are many other metabolisms which need specific electron acceptors, electron donors or organic carbon sources. Nevertheless, the counted CFUs are consistent with the quantified microbial productivity here.

### Microbial survival strategies and microbial community fingerprint of microorganisms

The sedimentary community based on T-RF analysis of bacteria, archaea, fungi, unicellular protists and protozoans showed substantial changes (a) directly after sterilization (t0) compared to the natural untreated sediment, which is in agreement with the fact that NaN3 does not kill microorganisms but inhibits their respiration metabolism. Based on the DNA T-RFs, none of the chosen methods was able to completely destroy resilient DNA in the sediment (Fig. 4). Survival of certain microorganisms after heat, gamma-ray or chemical treatments can be explained by a number of survival strategies, including a reduced metabolism in a dormant state, spore formation and different repair and resistance mechanisms (Table S4, Supporting Information). Spores can be extremely resistant over many decades (Cote et al. 2018). Bacillus spp. and Clostridium spp. are well known spore-forming strains (Cote et al. 2018), but only 0.0005% RNA from a Bacillus spp. was found in Norsminde Fjord sediment (Otte et al. 2018) (see details in Table S4, Supporting Information) and might have survived in heat-sterilized samples. Special heat-resistance mechanisms like complex DNA repair systems, proteins for DNA structure and lipid compositions have been found in Thermococcus spp., Thermotoga spp., Thermus spp. and other thermophilic bacteria (Table S4, Supporting Information). The thermophile Thermoanaerobacter spp. was found at a relative abundance of 0.004% in the untreated marine sediment (Otte et al. 2018), and has also been detected in the sterilized samples (Otte et al., unpublished data).

Based on the obtained data, we can conclude that the T-RFs either belong to non-degraded DNA of dead microorganisms or to bacteria, archaea, fungi or protozoans that were able to survive gamma-radiation of wet sediment (12 T-RFs of the original 90–110 T-RFs), gamma-radiation of dry sediment (69 T-RFs of the original 90–110 T-RFs), autoclaving (26 T-RFs of the original 90–110 T-RFs) or NaN3-amendment (106 T-RFs of the original 90–110 T-RFs) (Fig. S1, Supporting Information). Unfortunately, due to limited sample material (in particular RNA), our analysis does not allow to draw conclusions on whether the detected DNA originates from active microorganisms or from extracellular DNA of dead organisms.

With T-RFLP, it was possible to see that the microbial community differed between the different sterilization treatments, but sequencing data could help to understand which specific clades of bacteria were still present. However, T-RFLP is a cost-efficient fingerprint-method to get an overview of the sterility of an environmental sample.

### Effect of sterilization on the sample geochemistry and mineralogy

Geochemical and mineralogical integrity of sediments is important as it delivers substrates for abundant microorganisms and it determines the geochemical and thermodynamic framework that controls the identity of processes that are occurring. Furthermore, iron minerals provide active surface binding sites and dissolution and formation of iron minerals can cause mobilization or immobilization of nutrients (such as phosphate or trace metals) or pollutants (such as toxic metals), respectively (Bonneville, Van Cappellen and Behrends 2004; Kappler and Straub 2005; Gadd 2010). Consequently, changes in iron mineralogy do not only affect iron-metabolizing bacteria, they also affect the organisms that have survived due to the release or the reten
tion of nutrients and organic carbon (see increase of NPOC following autoclaving and gamma-sterilization procedure). So far it is well known that the sterilization methods affect geochemistry and mineralogy (Table S5, Supporting Information) which we confirmed in a supplementary experiment on the stability of the Fe(III) (oxyhydr-)oxide ferrihydrite (see supplementary material). Specifically, we could show that chemically-sterilized ferrihydrite (with NaN3) showed neither significant alteration nor transformation compared to the initial ferrihydrite (Fig. S2, Table S3, Supporting Information). While gamma-sterilization had a moderately low impact on the transformation of ferrihydrite (only 6% ferrihydrite transformed to goethite), autoclaving showed the highest impact on the mineralogy (more than 70% of the initial ferrihydrite material was transformed into goethite and hematite) (Fig. S2, Table S3, Supporting Information).

### Choice of sterilization method for laboratory experiments

The choice between the different sterilization methods should be based on the research goals at hand, i.e. (i) which (bio)geochemical processes take place and which redox-active compounds are converted; (ii) which microorganisms are involved or responsible for substrate conversion (Table 2). Depending on the parameters that will be measured during an experimental run (i.e. substrate concentration, mineral (trans)formation, cell and activity quantification, RNA and DNA quantification) the interferences of each sterilization method should be considered. In Table S5 (Supporting Information) we provide a comprehensive overview of the advantages and disadvantages of classical sterilization methods. In Table 2 we offer a decision-making aid for the choice between autoclaving, gamma-radiation and the addition for NaN3 as sterilization treatment for complex biogeochemical samples, depending on the research focus.

If one wants to ensure the lowest amount of measurable DNA and/or RNA contamination or microbial productivity, we suggest to apply autoclaving and gamma-radiation, but this comes at the expense of for example the changing the organic matter composition. Autoclaving and gamma-radiation was highly effective in inactivation of microorganisms (based on CFUs, microbial productivity and elimination of fungi and protozoan T-RFs). In order to achieve most efficient results of the
sterilization treatment we recommend a short pre-incubation for at least 7–16 days to for DNA/RNA to degrade. If one wants to ensure the lowest changes of geochemical and mineralogical properties, we recommend the addition of NaN₃ and gamma-radiation for samples that will be analyzed for geochemical and mineralogical properties. Similar to the impact on the iron mineralogy, autoclaving significantly affects the DOC concentrations of the sterilized sediment (increase from 20 mg/L (native sediment) to approximately 220 mg/L (autoclaved sediment)). A draw-back of gamma-radiation and the addition of NaN₃ is the release of nitrate and ammonium (Lenski et al. 1991; Buchan et al. 2012; Brown et al. 2014). The application of NaN₃ for microbial inactivation of experiments that focus e.g. on the quantification of microbial turnover of carbon compounds and other redox-active geochemical compounds such as sulfate, Fe(III) etc., can be recommended (Table 2). Moreover, gamma-sterilization also increases the NPOC content (confirmed in our study: from 20 mg/L to around 150 mg/L). Thus, gamma-radiation can be recommended for pure cultures studies, water, sediment and soil sterilization for microcosm experiments for quantifying substrate turnover. Based on our findings, it is recommended to use dry sample material (sediments and soils) for gamma-sterilization, as the efficiency of RNA removal was higher than for wet sample material. In research approaches with sterilized controls and DNA-/RNA-based methods we would recommend gamma-radiation as the single best method for as-sterile-as-possible conditions. But it should be considered that gamma-radiation is more expensive (around 160 Euro per package; see Synergy Health Care GmbH) than autoclaving and NaN₃ addition, gamma-sterilizing companies work considered that gamma-radiation is more expensive (around 160 Euro per package; see Synergy Health Care GmbH) than autoclaving and NaN₃ addition, gamma-sterilizing companies work

Table 2. Summary of used sterilization methods in this study, their disadvantages and guidance for choice of sterilization method in sediment samples. When designing an experiment it is important to define exactly the research question and, as a consequence, the specific setup of the abiotic controls. Depending on whether the focus is on the analysis of the geochemical conditions, on the identification of the minerals or on quantification of certain metabolic activities, the most suitable sterilization method should be chosen. The last row of this table shows a set of recommendations for which types of studies are best for which method.

<table>
<thead>
<tr>
<th>Research focus</th>
<th>Used sterilization method in this study and their disadvantages</th>
<th>Gamma-radiation (52 kGy radiation)</th>
<th>NaN₃ (final conc.: 160 mM NaN₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolved compound analysis</td>
<td>+ DOC increase (ca. 20 times)</td>
<td>++ DOC increase (ca. 10 times)</td>
<td>+++ Nitrate and nitrite reduction, pH drop (not shown in this study)</td>
</tr>
<tr>
<td>Mineral analysis</td>
<td>+ Mineral transformation (e.g. 70% Ferrihydrite into goethite and hematite), decrease of sediment surface area, damage of sediment structure</td>
<td>++ Less mineral transformation (e.g. 6% Ferrihydrite transformation to goethite)</td>
<td>+++ No immediate mineral transformation (ferrihydrite). Mineral dissolution in long term experiments. Due to acidic pH of NaN₃</td>
</tr>
<tr>
<td>Analysis of microbial productivity</td>
<td>++ Productivity decreased e.g. from 2.5 nM C/d to 1.5 C/d (t₇) and 0.5 nM C/d (t₉)</td>
<td>++ Productivity decreased e.g. from 2.5 nM C/d to 1.0 C/d (t₇) and 0.5–1.3 nM C/d (t₉)</td>
<td>+ → +++(after incubation, at least 7 days)</td>
</tr>
<tr>
<td>Analysis of CFUs/microbial growth on LB plates</td>
<td>++ Few survivors</td>
<td>++ Few survivors</td>
<td>Does not sterilize, only inhibits mitochondrial respiration, does not affect most fungi and fermenters</td>
</tr>
<tr>
<td>Analysis of microbial community (based on DNA) and metabolic response (based on RNA)</td>
<td>+ → +++</td>
<td>+ → ++</td>
<td>+ → ++</td>
</tr>
<tr>
<td>Summary of results and recommended methods for specific research questions</td>
<td>(after incubation) DNA &amp; RNA still present right after treatment, bdl after 16 days of incubation</td>
<td>(after incubation) DNA &amp; RNA still present right after treatment, bdl after 16 days of incubation</td>
<td>(after incubation) DNA &amp; RNA still present right after treatment, decreased DNA &amp; RNA after 16 days</td>
</tr>
<tr>
<td>Microbial studies with maximal sterility</td>
<td>Microbial studies with maximal sterility</td>
<td>Substrate conversion of specific bacteria</td>
<td></td>
</tr>
<tr>
<td>DNA/RNA based studies</td>
<td>Selected mineral studies</td>
<td>Selected mineral studies</td>
<td>Soil/sediment studies</td>
</tr>
</tbody>
</table>

+ less effective method, +++ most effective method; bdl = below detection limit.
gamma-sterilization and NaN₃ addition) have their advantages and disadvantages and should be carefully selected for the corresponding experimental focus of abiotic controls in environmental studies, particularly for marine sediments.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS
We thank Ellen Struve for NPOC measurements and Yuge Bai for the provision of ferrihydrite. We are also grateful to James Byrne for helpful Mössbauer discussions.

FUNDING
This work was supported by the European Research Council under the European Union’s Seventh Framework Program (FP/2007–2013)/ERC Grant, agreement no. 307 320-MICROFOX and by ERC Advanced Grant (Grant No. 294200-MICROENERGY), by an Emmy-Noether fellowship (grant #326028733) from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) to SK and by a Margarete von Wrangell fellowship to CS.

Conflicts of interest. None declared.

REFERENCES


Vincke V, Nassiri M, Nadji M et al. A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. Lab Invest 2003;83:1427–35.


